PLASTIDIAL CARBONIC ANHYDRASE IN COTTON (*GOSSYPIUM HIRSUTUM* L.): CHARACTERIZATION, EXPRESSION AND ROLE IN LIPID BIOSYNTHESIS

Chau Van Hoang, B.A.

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APPROVED:

Kent D. Chapman, Major Professor
Robert M. Pirtle, Committee Member
Robert C. Benjamin, Committee Member
John Knesek, Committee Member
Douglas D. Root, Committee Member
Earl G. Zimmerman, Chair of the Department of Biological Sciences
Warren Burggren, Dean of the College of Arts and Sciences
C. Neal Tate, Dean of the Robert B. Toulouse School of Graduate Studies

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Recently, plastidial carbonic anhydrase (CA, EC 4.2.1.1) cDNA clones encoding functional CA enzymes were isolated from a nonphotosynthetic cotton tissue. The role of CA in photosynthetic tissues have been well characterized, however there is almost no information for the role of CA in nonphotosynthetic tissues. A survey of relative *CA* transcript abundance and enzyme activity in different cotton organs revealed that there was substantial CA expression in cotyledons of seedlings and embryos, both nonphotosynthetic tissues. To gain insight into the role(s) of CA, I examined CA expression in cotyledons of seedlings during post-germinative growth at different environmental conditions. CA expression in cotyledons of seedlings increased from 18 h to 72 h after germination in the dark. Seedlings exposed to light had about a 2-fold increase in CA activities when compared with seedlings kept in the dark, whereas relative CA transcript levels were essentially the same. Manipulation of external CO_2 environments [zero, ambient (350 ppm), or high (1000 ppm)] modulated coordinately the relative transcript abundance of *CA* (and *rbcS*) in cotyledons, but did not affect enzyme activities. On the other hand, regardless of the external CO_2 conditions seedlings exposed to light exhibited increase CA activity, concomitant with Rubisco activity and increased chlorophyll content. Our data revealed that steady-state levels of *CA* and *rbcS* transcripts are regulated at the transcriptional level in response to external CO_2 conditions, while CA and Rubisco activities are modulated at the post-transcriptional

level by light. Thus CA expression in cotyledons during post-germinative growth may be to “prime” cotyledons for the transition at the subcellular level for the transition from plastids to chloroplasts, where it provides CO_2 for Rubisco during photosynthesis. Furthermore, CA expression increased during embryo maturation similar to oil accumulation. Specific sulfonamide inhibitors of CA activity significantly reduced the rate of [^{14}C]-acetate incorporation into total lipids in cotton embryos and tobacco leaves and cell suspensions *in vivo* and *in vitro*. Similar results were obtained in chloroplasts isolated from leaves of transgenic CA antisense-suppressed tobacco plants (5% of wild-type activity). Collectively, these results support the notion that CA plays several physiological roles in nonphotosynthetic tissues.

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GENERAL INTRODUCTION

Assimilation and metabolism of inorganic carbon is essential for all biological organisms. The atmospheric CO_2 concentration is approximately 350 ppm, resulting in μM range of dissolved CO_2 concentrations inside living cells, which is consider to be limiting, since dissolved CO_2 concentration in the mM range is required to meet the demand for most carboxylating enzymes in plants. CO_2 is in equilibrium with HCO_3^-, carbonic acid, and carbonate, however the predominate carbon species are determined by pH conditions. Also, CO_2 is soluble in both aqueous solutions and lipid, but HCO_3^- is only soluble in aqueous solutions. Thus inorganic carbon species are trapped inside living cells mostly in the form of HCO_3^-. The demand for either CO_2 or HCO_3^- is determined for different carboxylating enzymes found in various organisms. The uncatalyzed rate of interconversion is consider to be 10,000 times slower than the required rate in living cells. Thus, enzymatic interconversion of CO_2 and HCO_3^- would not only provide and trap sufficient levels of the required carbon substrates for different carboxylases, but would also help meet the required metabolic flux in overall cellular metabolism.

Structure and mechanism

Carbonic anhydrase (CA, E.C. 4.2.1.1) is a zinc containing metalloenzyme that catalyzes the reversible hydration of CO_2 and HCO_3^- (Silverman, 1991). CA is found in almost all organisms, including algae, bacteria, animals, and plants. Three evolutionary distinct classes of CA have been reported and designated α-, β-, and γ-CA's. Carbonic

anhydrases of the plant kingdom are predominantly grouped into the β family. The β-CA's of dicot plants are reported to be 140 to 250 kDa homooctamers with zinc-binding subunits of 26 to 34 kDa (Reed and Graham, 1981). The structure of β-CA from the dicotyledonous plant *Pisum sativum* at 1.93 angstroms resolution revealed that the active site is located at the interface between two monomers, with Cys160, His220 and Cys223 binding the catalytic zinc ion (Kimber and Pai, 2000). In the hydration reaction, the catalytic mechanism of CA involves the attack of a CO_2 molecule by a zinc-bound OH^- in the active site enzyme to form a metal-bound HCO_3^- enzyme intermediate, which is displaced by a H_2O molecule. The regeneration of the active enzyme (zinc-bound OH^-) involves the transfer of a water proton from the zinc center to His, which delivers the proton to a buffer base in the surrounding medium (Forsman et al., 1996).

Specific inhibitors

Aromatic and certain heterocyclic sulfonamides are specific inhibitors of most CAs. Sulfonamide compounds with an unsubstituted $–SO_2NH_2$ group or with a $–SO_2NH(OH)$ group are efficient inhibitors. These groups bind to the zinc metal ion at the active site of CA as anions, $R\text{-}SO_2NH^-$ (or $R\text{-}SO_2N\text{-}OH^-$) via the nitrogen atom of the sulfonamide group (Lindskog and Wistrand, 1988). The mode of inhibition is the NH group of sulfonamide replaces the zinc-bound water molecule and hydrogen bonds to the adjacent amino acid residue. This prevents the formation of the rate-limiting transfer of a water proton from the metal center to form the active zinc-bound OH^- moiety. Also, there are a variety of other less specific CA inhibitors such as CN^-, F^-, and imidazole that have been reported to inhibit CA activity (Lindskog, 1997).

Gene regulation

The regulation of gene expression in C_3 and C_4 plants have been studied extensively in photosynthetic tissues. The rapid assembly of the photosynthetic apparatus is crucial for the transition from heterotrophic (use of stored lipid reserves for seedling growth and development) to photoautotrophic (photosynthesis) growth in newly germinated seedlings (Furbank and Taylor, 1995). High levels of the cytosolic and chloroplastic mRNAs encoding photosynthetic proteins are required to meet the demand of the rapid assembly of the photosynthetic apparatus. Combinations of environmental and developmental stimuli are required to activate some gene transcription in higher plants. For example, small gene families coding the small subunit (SSU) of ribulose bisphosphate carboxylase/oxygenase (Rubisco; r*bcS* gene) and chlorophyll a/b apoproteins of the photosystem II light-harvesting complex (*Lhcb* gene) have been reported to be up-regulated at the transcriptional level in response to light (Tobin and Silverthorne, 1985; Thompson and White, 1991). Also a cis-acting DNA sequences responsible for light-induced transcription have been identified for the two genes (Gilmartin et al., 1990). *Lhcb* and r*bcS* gene expressions in leaf cells of C_3 plants are developmentally regulated (Muller et al., 1980; Eckes et al., 1985). A signal from the developing chloroplast is required for the activation of these genes even when light signals are fully activated (Oelmuller, 1989). In addition, CA gene expression is differentially regulated under different environmental conditions. There are several reports indicating that plants grown in elevated CO_2 conditions resulted in the down-regulation of CA activity (Peet et al., 1986; Sage et al., 1989; Xu et al., 1994). Thus

different environmental and developmental factors regulate expression of various genes in photosynthetic plants.

Physiological roles

The physiological role of CA has been most extensively studied in green algae and cyanobacteria. At physiological pH, the major dissolved inorganic carbon species are HCO_3^-, which cannot diffuse out of the cells. It has been suggested that chloroplastic CA plays a role in photosynthetic carbon assimilation in green alga by converting accumulated pools of HCO_3^- to CO_2, which is the substrate for Rubisco during photosynthesis (Badger and Price, 1992). Also, periplasmic CA's have been suggested to play a role in the CO_2 concentrating mechanism (CCM) of *C. reinhardtii* (Badger and Price, 1994). High levels of CO_2 concentration at the active site of Rubisco not only elevate the synthesis of photosynthate but also suppressed the oxygenase activity thus inhibit photorespiration, which is considered a wasteful process. Also, an algal mitochondrial carbonic anhydrase was induced when green algae cells grown in 5% CO_2 were transferred to ambient CO_2 levels (Eriksson et al., 1996). Perhaps this enzyme facilitates the diffusion of CO_2 out of the mitochondria matrix (produced by glycine decarboxylation during photorespiration under low CO_2 conditions) for carboxylation by Rubisco in the chloroplast. In addition, CA was determined to play an important role in photosynthesis and CCM in cyanobacteria (Badger and Price, 1994). Therefore, CA plays a role in green algae and cyanobacteria by providing sufficient levels of inorganic carbons for Rubisco during photosynthesis.

In C_4 plants, CA plays an important role in C_4 photosynthesis by indirectly acting as the first enzyme in the pathway catalyzing the fixation of HCO_3^- into C_4 acids (Hatch and Burnell, 1990). Although the initial carboxylation reaction of C_4 photosynthesis is catalyzed by PEP carboxylase, this reaction uses HCO_3^- rather than CO_2. Data clearly show that inhibition of CA activity could effectively reduced photosynthate yields in C_4 plants. Studies with transgenic *Flaveria bidentis* revealed that CA expressed in the bundle sheath cells resulted in the perturbation of the CCM. This decreased the availability of CO_2 for photosynthetic assimilation by Rubisco (Ludwig et al., 1998). Thus a cytosolic CA in the mesophyll cells is required to maintain a functional CCM in C_4 plants.

In C_3 plants, the function of CA is speculated to mediate in the diffusion of CO_2 from the cytosol to the site of carboxylation of Rubisco in the chloroplast stroma (Reed and Gram, 1981). CA in the alkaline chloroplast stroma could aid in trapping inorganic carbon by hydrating CO_2, which diffuses across the chloroplast envelope, to HCO_3^-. Thus the presence of CA in the chloroplast stroma presumably provides for the high demand of CO_2 to Rubisco. In tobacco (*Nicotiana tabacum* L.), chloropastic CA activity co-eluted with other enzymes of the Calvin-Bensen cycle such as Rubisco, phosphoribulokinase (PRK), and ribose-5-phosphate isomerase, in FPLC, gel-filtration experiments (Jebanathirajah and Coleman, 1998). Moreover, the over-expression of chloroplastic CA in tobacco resulted in a concomitant increase in Rubisco activity (Majeau et al., 1994). Despite a number of correlative observations, there is no direct evidence for this hypothesis and the role (s) of CA in C_3 plants remains unclear. Because

antisense suppression of chloroplast CA activity in tobacco plants to levels less than 2% of wild-type did not appear to cause a major limitation of photosynthetic CO_2 assimilation at atmospheric CO_2 levels and did not reveal any detectable morphological changes from wild-type plants (Price et al., 1994). Perhaps in plastids of C_3 plants, CA has additional metabolic roles such as providing inorganic carbon for other carboxylating reactions.

While CA plays a role in C_4 plant systems by providing CO_2 to Rubisco during photosynthesis, in mammalian systems CA provides HCO_3^- to ACCase during lipid biosynthesis (Sly and Hu, 1995). Cao and Rous (1978) reported that inhibition of CA activity at the biochemical level in female mice with acetazolamide, CA specific inhibitor, resulted in a decrease in fatty acid synthesis *in vivo*. In addition, human adipose tissue treated with acetazolamide had significant decrease in lipogensis when compared to untreated tissues (Bray, 1977). Rat hepatocytes incubated with trifluoromethyl-sulphonamide and ethoxyzolamide revealed substantial reduction [^{14}C]-acetate incorporation into total lipids (Lynch et al., 1995). Although, the role of CA in *de novo* lipid biosynthesis by providing HCO_3^- has been well documented and accepted in mammalian systems, it has not been investigated in higher plants.

Fatty acid biosynthesis

De novo plastidial fatty acid biosynthesis consists of two enzyme complexes: acetyl-CoA carbonxylase (ACCase) and Type II fatty acid synthase (FAS). Although the ultimate source of carbon for fatty acid synthesis comes from photosynthesis, the precise way in which photosynthetically fixed CO_2 or its metabolic products are converted to

acetyl-CoA for ACCase is still unresolved (Harwood, 1996). The acetyl-CoA pools may be generated through the action of plastidial pyruvate dehydrogenase (PDH) acting on pyurvate, derived from the metabolism of glucose in the glycolytic pathway (Andrews and Kane, 1991). Also, acetates can be indirectly supplied by the mitochondria pyruvate dehyrogenase and transported to the chloroplasts, subsequently converted to acetyl-CoA by acetyl-CoA synthase (Roughan and Ohlrogge, 1994). Cytosolic malate and glucose-6-phospate have been proposed as precursors of the acetyl-CoA pool in oilseeds (Smith et al., 1992). Hence, several different pathways are implicated in supplying the plastidial acetyl-CoA pool for fatty acid synthesis in plants.

The first reaction of the *de novo* fatty acid biosynthesis pathway is catalyzed by acetyl-CoA carboxylase. This reaction is considered to be the rate limiting and committed step in the fatty acid synthesis pathway (Shintani and Ohlrogge, 1995). Acetyl-CoA carboxylase is a biotin-containing enzyme that catalyses its reaction in two steps and on two different catalytic sites. The first reaction requires an ATP to transfer HCO_3^- to the biotin moiety of biotin carboxyl carrier protein (BCCP). The second partial reaction is catalyzed by carboxyltransferase where the carboxyl group is transferred to acetyl-CoA to form malonyl-CoA.

Once the malonyl-CoA is produced, the acyl-group will be transferred to a protein cofactor, acyl carrier protein (ACP) by malonyl-CoA:ACP transacylase before entering the fatty acid synthesis pathway. Then the malonyl-ACP is condensed with acetyl-CoA catalyzed by β-ketoacyl-ACP synthase III (KAS III) to yield 3-ketobutyryl-ACP with a release of CO_2, which helps to drive the reaction forward, making the reaction

irreversible (Jaworski et al., 1989; Ohlrogge and Browse, 1995). After two reduction steps, using NADPH and a dehydration step, catalyzed by ketoacyl-ACP reductase, enoyl-ACP reductase, and hydroxyacyl-ACP dehydrase, respectively, the ketoacyl-ACP is then fully reduced to a 4-carbon acyl-ACP. This 4-carbon acyl-ACP product then undergoes several rounds of reduction and dehydration steps to further extend the acyl chain up to 14-carbons. Further condensation is catalyzed by KAS I to yield the palmitoyl (16-carbons)-ACP. Finally, elongation of the palmitoyl-ACP to stearoyl (18-carbons)-ACP requires a separate condensing enzyme, KAS II. The free fatty acids (16- and 18-carbons) are released from ACP by either an acyl-ACP thioesterase or an acyltransferase. The fatty acids are transported out of the plastid stroma by simple diffuse across the membrane or by other unknown means of transportation (Ohlrogge and Browse, 1995). Acyl-CoA synthetase in the cytosol is believed to attach the CoA moiety to the free fatty acids for glycerlipid biosynthesis in the endoplasmic reticulum.

The fate of the 16- and 18-carbon acyl chains produced in the plastids are either utilized to make glycerolipids, which are components of all cellular membranes or for the biosynthesis of storage triacylglcerols. The synthesis of glycerolipid involves two acylation reactions that transfer the fatty acids to glycerol-3-phophate to form phosphatidic acid (PA). A phosphatase removes the phosphate group from PA to produce diacylglycerol (DAG). Phosphorylated head groups are added to DAG's to synthesize different phospholipid classes. Furthermore, free fatty acids are used to synthesize TAG's via the Kennedy pathway in the endoplasmic reticulum (Stymne and Stobart, 1987). Fatty acyl-CoA acyltransferases acylate the glycerol-3-phosphate

(derived by the reduction of the dihydrocxyacetone phosphate intermediate of glycolysis) at the sn-1 and sn-2 positions to produced phosphatidic acid. This molecule is hydrolyzed by phosphatidate phosphohydrolase to yield diacylglycerol (DAG), which is the precursor for the final step for storage triacylglycerol synthesis. Then, the DAGs are acylated by diacylglycerol acyltransferase (DAGAT) or phospholipid diacylglycerol acyltransferase (PDAT), by adding the third fatty acid to the vacant sn-3 position of DAG. Finally, TAGs are exported to the cytosol for storage in densely packed lipid bodies that are spherical in shape (Huang, 1992; Murphy, 1993).

Research objectives

The overall goal of this dissertation research was to evaluate the functional activity and expression of a presumed plastidial CA identified in non-photosynthetic tissues of cotton. To address this, my specific objectives were characterized this plastidial CA at the biochemical and molecular levels (Chapter 1), to examine the regulation of CA gene expression in cotyledons of cotton seedlings during post-germinativegrowth under different environmental conditions (Chapter 2), and to investigate the possible involvement of CA in lipid biosynthesis in cotton and tobacco (Chapter 3). Collectively, the data presented in this disseration provided new insight into the metabolic role(s) of CA in nonphotosynthetic tissues.

CHAPTER 1

IDENTIFICATION AND EXPRESSION OF COTTON (GOSSYPIUM HIRSUTUM L.) PLASTIDIAL CARBONIC ANHYDRASE

Portions of this chapter were published in Plant and Cell Physiology (1999) 40: 1262-1270; Hoang, C.V., Wessler, H.G., Local, A., Turley, R.B., Benjamin, R.C. and Chapman K.D. [Hoang et al., 1999]

Abstract

Four carbonic anhydrase (CA) cDNA clones were isolated from a 48 h dark-grown cotton *(Gossypium hirsutum* L.*)* seedling cDNA library (isolated and sequenced by A. Local and H. Wessler of Dr. R. Benjamin's laboratory, UNT). Nucleotide sequence analysis revealed two different CA isoforms designated *GhCA*1 and *GhCA*2. The *GhCA*1 cDNA clone encodes a preprotein of 34.9 kDa containing a stromal-targeting domain at the N-terminus. A single immunoreactive 24 kDa protein was recognized in the plastids isolated from cotton embryos. This confirms the localization of a mature GhCA protein in the plastid stroma. The near full-length *GhCA*1 clone was expressed as as LacZα fusion-protein in *E. coli.* Cell-free extracts from GhCA1 harboring strain produced 9-fold higher levels of CA activity compared to untransformed controls. The CA specific inhibitors, sulfanilamide, acetazolamide, ethoxyzolamide, each at 10 mM, inhibited recombinant CA activity approximately 50%, 65%, and 75%, respectively. This suggests that *GhCA*1 encodes a functional CA enzyme. In plant tissue homogenates, these inhibitors reduced CA activity by 50%, 70%, and 95%, respectively. Although CA

activity was highest in extracts from mature cotton leaves, probing total RNA with GhCA1 revealed *CA* transcript levels to be highest in the cotyledons of dark-grown cotton seedlings. Collectively, our data indicate the presence of a chromosomally encoded, plastid-localized CA in the cotyledons of germinated seeds, suggesting a role for CA in post-germinativegrowth.

Abbreviations

CA, carbonic anhydrase (EC 4.2.1.1); DMSO, dimethyl sulfoxide; DPA, day post-anthesis; IPTG, isopropylthio-β-D-galactopyranoside; MOPS, 3-[N-morpholino]propanesulfonic acid; RT-PCR, reverse transcription-polymerase chain reaction; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); SSC, 150 mM sodium chloride and 15 mM sodium citrate (pH 7.0);

Introduction

Carbonic anhydrase (CA) is a zinc-containing metalloenzyme that catalyzes the interconversion of CO_2 and HCO_3^- (Silverman, 1991). This ubiquitous enzyme is found in different cell types of almost all organisms, including bacteria, green algae, animals, and plants. This enzyme has been implicated in many physiological processes such as pH homeostasis, photosynthesis, calcification, inorganic carbon assimilation and the metabolism of urea, glucose and lipids (Badger and Price, 1994; Bracey et al., 1994; Hewett-Emmett and Tashian, 1996; Sultemeyer et al., 1993; Suzuki et al., 1994; Tashian et al., 1991). A recent study with transgenic *Flaveria* indicated CO_2 assimilation in C_4 plants involves CA (Ludwig et al., 1998). This is consistent with predictions by others based on CA distribution in mesophyll and bundle-sheath cells (Burnell and Hatch, 1988).

Multiple isoforms of CA exist among organisms and are grouped into three distinct families. The α-CA's, the earliest family discovered, were first identified in red blood cells (Tashian et al., 1991). Homologues have been found in bacteria (*Neisseria gonorrhoeae*) (Hewett-Emmett and Tashian, 1996), green algae (*Chlamydomonas reinhardtii)* (Fukuzawa et al., 1990), and seven isoforms (CAI-CAVII) have been identified in higher vertebrates (Tashian, 1989). Members of the β-CA family have been isolated from green algae (*C. reinhardtii)*, prokaryotes such as *Escherichia coli* (*CynT*) and the cyanobacterium *Synechococcus* (*LcfA*), as well as from both monocot and dicot plants (Badger and Price, 1994; Eriksson et al., 1996; Guilloton et al., 1992). Thus far, the only γ-CA isolated and characterized comes from *Methanosarcina thermophila*

(Alber and Ferry, 1994), though some homologous sequences are appearing in EST and genome databases of higher plants (Hewett-Emmett and Tashian, 1996). Individual CA isoforms are expressed in a cell-specific manner and may be localized to specific subcellular compartments. For example, the *C. reinhardtii* α-CA is localized to the periplasmic space (Fukuzawa et al., 1990), whereas the β-CA are found in the mitochondria (Eriksson et al., 1996).

Carbonic anhydrases of the plant kingdom are predominantly grouped into the β family. The β-CA's of C_3 dicot plants are reported to be 140 to 250 kDa homooctamers of 26 to 34 kDa zinc-binding subunits (Reed and Graham, 1981). In contrast, monocot β-CA's are dimers with a reported molecular mass of about 42 kDa (for *Tradescantia* CA) (Atkins et al., 1972).

In this chapter, we report the isolation and characterization of two cDNAs encoding putative plastidial CA isoforms (*GhCA*1 and *GhCA*2) from cotyledons of dark-grown cotton seedlings. The sequence demonstrates CA activity when expressed in *E. coli* cells. *GhCA* expression in cotyledons of dark-grown seedlings and leaves of mature plants was judged by northern and RT-PCR analyses. Total CA activity, although not as high as in leaves, was readily detectable in the cotyledons of dark-grown seedlings. Isolation of CA isoforms from a dark-grown seedling cDNA library and their expression in cotyledons suggests CA may play a presently under-appreciated role in post-germinativegrowth.

Materials and Methods

Plant material

Cottonseeds (*Gossypium hirsutum* L., cv. Paymaster HS26) were kindly provided by Dr. John Burke (USDA-ARS, Lubbock, TX). Plants were grown in a glasshouse with a 14 h photoperiod (supplemented with sodium lamps to extend day length when necessary) and temperatures of approximately 38°C during the day and 25°C at night. Plants were watered daily and fertilized biweekly with a dilute solution of Miracle Gro™. The age of cotton bolls was determined by tagging flowers at anthesis. Ovule and embryo morphology also provided an approximation of embryo age (Choinski and Trelease, 1978). Seeds were imbibed, germinated, and grown as described by Chapman and Trelease (1991). For exposure of seedlings to light, seed coats were removed after 24 h and seedlings were placed on moistened filter paper in petri dishes at 30°C with continuous white light (55 µmol m^{-2} s^{-1}).

Southern blot analyses

Cotton genomic DNA was isolated from nuclei of young leaves according to Paterson et al. (1993). The DNA (15 µg) was digested with 10 units of *Eco*RI for 6 h at 37°C (Promega). The restriction fragments were resolved on 0.8% agarose gels and transferred by alkaline blotting (Reed and Mann, 1985) to Zeta-probe nylon membrane (BioRad). Blots were hybridized at 62°C overnight in 5 x SSC, 5% dextran sulfate, 0.5% SDS, denatured sheared salmon sperm DNA (100 µg ml^{-1}), and nonradioactive DNA probe generated from 0.6 kb cotton *GhCA*1 cDNA fragment by random priming using the Gene Images system from Amersham. After a final rinse with 0.2 x SSC, the hybridizing

fragments were visualized by chemiluminescence according to the manufacturer's protocol (Gene Images CDP-Star detection module, Amersham)

Northern blot analyses

Total cellular RNA was isolated from mature leaves, seedlings, and embryos of cotton plants according to the hot borate procedure described by Wan and Wilkins (1994). RNA yield and quality were evaluated spectrophotometrically and by analytical gel electrophoresis according to Sagerstrom and Sive (1996). Approximately 10 μg of total RNA was electrophoresed in a 1% agarose gel containing 6% formaldehyde and 1 x MOPS Buffer (20 mM MOPS-NaOH (pH 7.0), 5 mM sodium acetate, 0.1 mM EDTA). RNA was transferred to nylon membranes by capillary transfer with 20 x SSC (overnight) and probed with a random prime-labeled (Gene Images random prime-labeling module, Amersham Life Science, Arlington Heights, IL, USA) GhCA1. Hybridization and washing were both carried out at 62°C and with a final wash at 0.2 x SSC. Hybridized bands were identified by an alkaline phosphatase-catalyzed chemiluminescent reaction (Gene Images CDP-Star detection module, Amersham Life Science, Arlington Heights, IL, USA). Autoradiograms were subjected to densitometric analysis using NIH image software and normalized to internal 28S rRNA.

RT-PCR analysis

Equivalent amounts of total RNA (200 ng) were used as template for relative RT-PCR experiments (RT-PCR access kit, Promega, Madison, WI, USA). RT-PCR of total RNA from leaves and tissues of 48 h grown seedlings (cotyledons, hypocotyls, and roots) was conducted in a Perkin Elmer Model 2400 thermal cycler (Perkin Elmer, Norwalk,

CT, USA). First strand cDNA synthesis was carried out at 48°C for 45 min. Subsequent amplification of a targeted region within the *CA* cDNA was achieved through 35 cycles of 94°C for 30 sec, 54°C for 1 min, and 68°C for 2 min followed by a final polymerization step at 68°C for 7 min. CA forward (5'-AGGTTTCCAAGGGATACA-3') and reverse (5'TGAGAGACTGAAAGAAGG-3') primers (Biosynthesis Inc., Lewisville, TX, USA) were designed from the cDNA clone *GhCA*1 using DNAsis® software (Hitachi Software, San Francisco, CA, USA) and used to amplify a predicted fragment of 490 bp. The two horizontal arrows in Figure 1 denote the locations of these primer sequences and the amplified fragment. The actin forward (5'-TGCAGGTCGTGATCTAACCG-3') and reverse (5'-CCTTGGAAATCCACATCTGC-3') primers were selected from the partial cDNA sequence (GenBank Accession D88414) (Shimizu et al. 1997) and used to amplify a fragment of 539 bp. RT-PCR products were visualized by ethidium bromide staining following electrophoresis in 3% agarose gels.

Antibody production and immunoblots

Polyclonal anti-peptide antibodies directed against amino acid residues 252-270 (AEHGGEPLGVQCTHCEKEAVN) were produced by Alpha Diagnostics. Analysis in Dr. Benjamin's laboratory indicated that these residues encode a portion of the mature GhCA1 protein.

Proteins from isolated plastids were separated by Tris-SDS-PAGE (12%T/0.3%C resolving gel and 3.9% T/0.3% C stacking gel) in a Mini-Protean II Cell (Bio-Rad) according to the method of Laemmli (1970). Plastids were isolated from cotton embryos by centrifugation through a 10% Percoll gradient (Trimming and Emes, 1993; Sparace

and Mudd, 1982). Plastid protein contents were estimated according to Bradford (1976). Cast gels were pre-electrophoresed in 25 mM Tris (pH 8.3), 200 mM glycine, 1% (w/v) SDS, and 1 mM thioglycolate for 45-60 min at 50 volts (Microprocessor Controlled Electrophoresis FB703, FisherBiotech). The same buffer without thioglycolate was replaced prior to loading of protein samples. Protein samples dissolved in SDS-treatment buffer (above) were electrophoresed at 30 volts until the samples were fully within the stacking gel (30-45 min). The voltage was then gradually increased by maintaining constant current (75 mA) until the dye front reached the bottom of the gel (~2 h). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Biorad) via semi-dry electroblotting (Schagger, 1994) in a Bio-Rad Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell with the current limited to 100 mA / gel (5.5 mA / cm^{-2}). Transfer time was typically 1 h and proteins immobilized on the PVDF membrane were incubated in 20 mM Tris, pH 7.5, 500 mM NaCl, 0.1% Tween-20 and 3% (w/v) defatted BSA prior to antibody incubations. Polyclonal antisera directed toward a 20-mer peptide of the cotton protein was diluted 5,000-fold and antigen recognition was visualized by Alkaline Phosphatase Color Development Reagents (Bio-Rad), BCIP (5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt) and NBT (*p*-nitro blue tetrazolium chloride), with goat anti-rabbit IgG / alkaline phosphatase conjugate diluted 1:5000 (New England Biolabs).

Carbonic anhydrase assay

Freshly harvested plant tissues (leaves, seedlings, and embryos) were weighed and then frozen in liquid nitrogen. Frozen samples were ground with a mortar and pestle

with 1:1 (w/v) homogenization buffer containing 400 mM sucrose, 100 mM Na phosphate (pH 7.2), 10 mM KCl, 1 mM $MgCl_2$, and 1 mM EDTA. Homogenates were filtered through 4 layers of cheesecloth presoaked in homogenization buffer and the samples were analyzed immediately for CA activity. Bacterial lysates were prepared essentially as described by Kunz and Chapman (1981). Protein contents were determined according to Bradford (1976).

Total CA activity was determined electrometrically (Wilbur and Anderson, 1948). CA assay reaction mixtures contained 100 µl aliquots of homogenate (bacterial lysate or plant tissue homogenate) and 3.5 ml of pre-chilled 50 mM Tricine-HCl buffer, pH 8.2. Assays were initiated by addition of 2.4 ml cold, CO_2-saturated water with constant stirring. Reactions were monitored by recording the rate of pH change from 8.2 to 6.6 with a glass combination electrode (Ag / AgCl) fitted to a Fisher Scientific Accumet model 15 pH meter (Fisher Scientific, Pittsburgh, PA, USA). One unit of activity (Wilbur Anderson, WA unit) was defined as $10[(T_0/T)-1]$, where T_0 and T are equal to the rate of pH change of the reaction without (control) and with cell homogenate, respectively. Enzymatic assays were validated by the addition of CA specific inhibitors (e.g., ethoxyzolamide) or use of boiled homogenate aliquot. Assays were also verified utilizing erythrocyte CA, (Sigma Chemical Co., St. Louis, MO, USA) with and without inhibitors (not shown) to evaluate the relative activity of cotton CA.

Inhibitors

Classical specific inhibitors of CA (Maren, 1992) were utilized to demonstrate that the in vitro activity measured in extracts was indeed attributable to a CA-type

enzyme. 4-Aminobenzenesulfonamide (sulfanilamide), 5-acetamido-1,3,4-thiadiazole-2-sulfonamide, (acetazolamide), and 6-ethoxy-2-benzothiazolesulfonamides (ethoxyzolamide) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Sulfanilamide was dissolved in water, while acetazolamide and ethoxyzolamide inhibitors were dissolved in 1:2 v/v DMSO:water.

Results

Isolation of *GhCA*1 and *GhCA*2 cDNA clones

Four putative *CA* cDNAs were isolated in a library prepared from the mRNA of cotyledons of 48 h dark-grown seedlings (Dr. R. Benjamin's laboratory). Nucleotide sequence analysis revealed that these clones represent two each of two different CA isoforms, designated *GhCA*1 and *GhCA*2. The nucleotide and the deduced amino acid sequence of the GhCA1 and GhCA2 isoforms are shown in Figure 1 (completed by Dr. R. Benjamin's laboratory). The *GhCA*1 sequence encodes a nearly complete preprotein of 34.9 kDa with a N-terminal stromal-targeting domain. Nucleotide 297 indicates the putative cleavage site of the preprotein, yielding a predicted mature protein of 24.3 kDa.

The figure 2 shows alignment includes the deduced amino acids sequences of GhCA1 and GhCA2 and of other plant CAs (completed by Dr. R. Benjamin's laboratory). Selected regions of predicted polypeptides had substantial identity to other plant plastidial β-CAs (e.g. 93% to *Arabidopsis*, 96% to spinach, and 86% to pea). The predicted mature proteins of GhCA1 and GhCA2 indicated high conservation to other mature plant β-CAs.

Genomic blot hybridization analysis

Analysis of cotton genomic DNA by Southern alkaline blot and hybridization with a 0.6 kb cotton *CA* cDNA probe (Fig 3) revealed several major hybridizing fragments following cleavage with *Eco*RI. Since there are several hybridizing genomic fragments under relatively stringent hybridization conditions

```
                                                                        A   T
5' TCC ATC AAC GGT TGG TGC CTT ACC TCC TCT TCT TCC TCC ACC ACC ACC TCC TCT TTT TCC GCT CGT   66
    S   I   N   G   W   C   L   T   S   S   S   S   S   T   T   T   S   S   F   S   A   R

                                A
   CGA CGA CCT ACA TTG CGG CCT TCG GTC GTC GCT AGC CTT AAC TCT TCT CCT TCT CCT CCG ACT CTT  132
    R   R   P   T   L   R   P   S   V   V   A   S   L   N   S   S   P   S   P   P   T   L

                            T           A
   ATC CAA GAC CGC CCG GTT TTC GCT GCC CCT GTT CCT TTG CTC ACC CCG AGA GAA GAG ATG GGA AAC  198
    I   Q   D   R   P   V   F   A   A   P   V   P   L   L   T   P   R   E   E   M   G   N

   AAG TCC TAC GAC GAA GCC ATT GAA GCT CTC AAG AAA CTT CTC AGT GAG AAA GGA GAA CTG AAA GCT  264
    K   S   Y   D   E   A   I   E   A   L   K   K   L   L   S   E   K   G   E   L   K   A

                                                            G           T
   GAA GCA GCT GCA AGG GTA GAT CAA ATA ACA GCA GAG TTA AAC ACA ACA TCA GCT GAC GGC AAA CCA  330
    E   A   A   A   R   V   D   Q   I   T   A   E   L   N   T   T   S   A   D   G   K   P

   TCT GAC TCT TCT GTT GAG AGA CTG AAA GAA GGC TTC GTT TAC TTC AAG AAA GAA AAA TAT GAA AAG  396
    S   D   S   S   V   E   R   L   K   E   G   F   V   Y   F   K   K   E   K   Y   E   K

   AAT CCT GCT CTG TAT GGT GAG CTT GCC AAG GGT CAA AGC CCT AAG TAT ATG ATT GTT GCC TGC TCG  462
    N   P   A   L   Y   G   E   L   A   K   G   Q   S   P   K   Y   M   I   V   A  *C*  S

   GAC TCT AGG GTC TGC CCA TCT CAT GTG CTG GAC ATG CAA CCT GGT GAA GCT TTT GTG GTC CGT AAT  528
   *D*  S   R   V   C   P   S   H   V   L   D   M   Q   P   G   E   A   F   V   V   R   N

   GTT GCT AAC ATG GTG CCA CCA TAT GAC CAG ATT AAA TAT GCT GGC ATT GGA TCT GCT ATT GAA TAT  594
    V   A   N   M   V   P   P   Y   D   Q   I   K   Y   A   G   I   G   S   A   I  *E*  Y

                                                                            T
   GCA GTT TTG CAT CTC AAG GTA CAA GAA ATT GTG GTG ATT GGA CAC AGT GCC TGT GGA GGA ATC AAG  660
    A   V   L   H   L   K   V   Q   E   I   V   V   I   G  *H*  S   A  *C*  G   G   I   K

                            T
   GGG CTT ATG TCT TTC CCA TTA GAT GGA AAC AAC TCA ACT GAT TTC ATA GAG GAT TGG GTT AAG ATT  726
    G   L   M   S   F   P   L   D   G   N   N   S   T   D   F   I   E   D   W   V   K   I

                        A
   GGA ATC CCT GCT AAG GCC AAG GTG CTA GCT GAA CAT GGT GGT GAG CCT TTG GGA GTC CAA TGT ACA  792
    G   I   P   A   K   A   K   V   L   A   E   H   G   G   E   P   L   G   V   Q   C   T

   CAC TGC GAG AAG GAA GCA GTG AAT GTA TCC CTT GGA AAC CTG CTG AGT TAT CCA TTT GTG AGA GAT  858
    H   C  *E*  K   E   A   V   N   V   S   L   G   N   L   L   S   Y   P   F   V   R   D

   GGA TTG GTG AAG AAA ACC CTG GGA ATC AAG GGC GGT TAC TAT GAC TTC GTT AAA GGA AGT TTC GAG  924
    G   L   V   K   K   T   L   G   I   K   G   G   Y   Y   D   F   V   K   G   S   F   E

                                        T
   CTA TGG AGT CTT CAG TTC CAA CTT TCA AGC TCT CTC TCT GTA TGA AAACACACCAAACCATCACCATTGACA  996
    L   W   S   L   Q   F   Q   L   S   S   S   L   S   V   *

                                           A                                     G
   CCATCTTTGTTCCTTAACTACCAATCCTTTTTCTTA-CAATGTACTATGTTCATGTCTTTATCGTCTATTACAATAAATATAGATGA  1082

              T  A        A                     C                 -------------
   TAGAGATCACCTGCCGCCCGCCGTCTGCGGTGGTGGCTGCGGTGGCTCTTTCCATCTATAAATTCTCCTTTGAAGAGGTGTTCA_n 3'  1166
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Figure 1. Nucleotide and deduced amino acid sequence of an 1,164 bp cDNA clone (*GhCA*1; GeneBank Accession #AF132854; isolated, sequenced and analyzed by A. Local and H. Wessler of Dr. R. Benjamin's laboratory) isolated from a cotton cDNA library (R. N. Trelease, Arizonia State Univ.) prepared from cotyledon mRNA of 2-day-old-seedlings. Differences in the nucleotide sequence of *GhCA*2 (GeneBank Accession #AF132855) are shown above the *GhCA*1 sequence. Differences in the GhCA2 amino acid sequence are shown in parenthesis. The double-headed arrow indicates the putative cleavage site of the preprotein. Single-headed arrows denote the forward and reverse CA primers used for RT-PCR experiments. The putative polyadenylation signal is underlined. Amino acid residues likely to be involved in Zn^{++} binding and catalysis are marked by asterisks.

```
                    *        20         *        40         *        60         *
GhCA1      : ----SINCWCLTSSSSSTTTTSSFSARRR---------PTLRPSVVASLNSSPSPPTLIQDRPVFAAPVPLLTP--REE :  63
GhCA2      : -------CWCLTSSSSTTTTSSFSARRR---------PTLRPSVVASLNSSPSPPTLIQDRPVFAAPIPLLTP--REE :  60
S.oleracea : MST--INCCLTS-ISPSRTQLKNTSTLR---------PTFIANSRVNPSSS-VPPSLIRKQPVFAAPAPIITPTLKED :  65
N.tabacum  : MSTASINSCLT--ISPAQASLKKPT--R---------PVAFARLSNSSSST-SVPSLIRNEPVFAAPTPIINPILREE :  64
P.sativum  : MSTSSINCFSLSSLSPAKTSTKRTT-LRP------FVFASLNTSSSSSSSS-TFPSLIQDKPVFASSSPIITPVLREE :  70
A.thaliana : MSTAPLSCFFLTSLSPSQSSLQKL-SLRTSSTVACLPPASSSSSSSSSSSRSVPTLIRNEPVFAAPAPIIAPYWSEE :  77
H.vulgare  : MS-LQI-ERTERARSPVFVFAHKRQLLHGRCSTIDNANCSTCSMKINS-ICTLTALPIAALRGPRTTSHYSIAAANWC :  75
O.sativa   : MSTA-----------------------------------------AAAAAAQSWCFAT----VTPRSRA- :  24

            80         *       100         *       120         *       140         *
GhCA1      : VGNKSYDEAIEALKKLLSEKGELKAEAAA---------RVDQITAEL----NTTSADGKP--SDSSVERLKEGFVYFK : 126
GhCA2      : VGNKSYDEAIEALKKLLSEKGELKAEAAA---------RVDQITAEL----NTASADGKP--SDSSVERLKEGFVYFK : 123
S.oleracea : VA---YEEAIAALKKLLSEKGELENEAAS---------KVAQITSEL--ADGGTPSASYP------VQRIKEGFIKFK : 123
N.tabacum  : VAKESYEQAIAALEKLLSEKGELGPIAAA---------RVDQITAEL--QSSDGSKPFDP------VEHMKAGFIHFK : 125
P.sativum  : VG-KGYDEAIEELQKLLREKTELKATAAE---------KVEQITAQL---GTTSSSDGIP--KSEASERIKTGFLHFK : 133
A.thaliana : VGTEAYDEAIEALKKLLIEKEELKTVAAA---------KVEQITAALQTGTSSDKKAFDP------VETIKQGFIKFK : 140
H.vulgare  : YATVAPRARSSTIAASLGTPAPSSSAS----------FRPKLIRTT-----PVQAAPVAPALMDAAVERLKTGFEKFK : 138
O.sativa   : -----------TVVASLASPSPSSSSSSSNSSNLPAPFRPRLIRNT-----PVFAAPVAPAAMDAAVDRLKDGFAKFK :  86

             160         *       180         *       200         *       220         *
GhCA1      : KEKYEKNPALYGELAKGQSPKYMIVACSDSRVCPSHVLD-MQPGEAFVVRNVANMVPPYDQIKYAGIGSAIEYAVLHL : 203
GhCA2      : KEKYEKNPALYGELAKGQSPKYMIVACSDSRVCPSHVLD-MQPGEAFVVRNVANMVPPYDQIKYAGIGSAIEYAVLHL : 200
S.oleracea : KEKYEKNPALYGELSKGQAPKFMVFACSDSRVCPSHVLD-FQPGEAFMVRNIANMVPVFDKDKYAGVGAAIEYAVLHL : 200
N.tabacum  : TEKYEKNPALYGELSKGQSPKFMVFACSDSRVCPSHVLN-FQPGEAFVVRNIANMVPAYDKTRYSGVGAAIEYAVLHL : 202
P.sativum  : KEKYDKNPALYGELAKGQSPPFMVFACSDSRVCPSHVLD-FQPGKAFVVRNVANLVPPYDQAKYAGTGAAIEYAVLHL : 210
A.thaliana : KEKYETNPALYGELAKGQSPKYMVFACSDSRVCPSHVLDFFQPDGAFVVRNIANMVPPFDKVKHGGVGAAIEYAVLHL : 218
H.vulgare  : TEVYDKKPDFFEPLKAGQAPKYMVFACADSRVCPSVTLG-LEPGEAFTIRNIANMVPAYCKNKYAGVGSAIEYAVCAL : 215
O.sativa   : TEFYDKKPELFEPLKAGQAPKYMVFSCADSRVCPSVTMG-LEPGEAFTVRNIANMVPAYCKIKHAGVGSAIEYAVCAL : 163

              240         *       260         *       280         *       300         *
GhCA1      : KVQEIVVIGHSACGGIKGLMSFPLDGNNST-DFIEDWVKIGIPAKAKVLAEHGGEPLGVQCTHCEKEAVNVSLGNLLS : 280
GhCA2      : KVQEIVVIGHSACGGIKGLMSFPFDGNNST-DFIEDWVKIGIPAKTKVLAEHGGEPLGVQCTHCEKEAVNVSLGNLLS : 277
S.oleracea : KVENIVVIGHSACGGIKGLMSFPDAGPTTT-DFIEDWVKICLPAKHKVLAEHGNATFAEQCTHCEKEAVNVSLGNLLT : 277
N.tabacum  : KVENIVVIGHSACGGIKGLMSLPADGSEST-AFIEDWVKIGLPAKAKVQGEHVDKCFADQCTACEKEAVNVSLGNLLT : 279
P.sativum  : KVSNIVVIGHSACGGIKGLLSFPFDGTYST-DFIEEWVKIGLPAKAKVKAQHGDAPFAELCTHCEKEAVNASLGNLLT : 287
A.thaliana : KVENIVVIGHSACGGIKGLMSFPLDGNNST-DFIEDWVKICLPAKSKVISELGDSAFEDQCGRCEREAVNVSLANLLT : 295
H.vulgare  : KVEVIVVIGHSRCGGIKALLSL-KDGADDSFHFVEDWVRIGFPAKKKVQTECASMPFDDQCTVLEKEAVNVSLQNLLT : 292
O.sativa   : KVELIVVIGHSRCGGIKALLSL-KDGAPDSFHFVEDWVRTGFPAKKKVQTEHASLPFDDQCAILEKEAVNQSLENLKT : 240

               320         *       340         *
GhCA1      : YPFVRDGLVKKTLGIKGGYYDFVKGSFELWSLQFQLSSSLSV- : 322
GhCA2      : YPFVRDGLVKKTLGIKGGYYDFVKGSFELWSLQFQLSSSLSV- : 319
S.oleracea : YPFVRDGLVKKTLALQGGYYDFVNGSFELWGLEYGLSPSQSV- : 319
N.tabacum  : YPFVREGLVKKTLALKGGHYDFVNGGFELWGLEFGLSPSLSV- : 321
P.sativum  : YPFVREGLVNKTLALKGGYYDFVKGSFELWGLEFGLSSTFSV- : 329
A.thaliana : YPFVREGLVKGTLALKGGYYDFVKGAFELWGLEFGLSETSSV- : 337
H.vulgare  : YPFVKEGVTNGTLKLVGGHYDFVSGKFETWEQ----------- : 324
O.sativa   : YPFVKEGIANGTLKLVGGHYDFVSGNLDLWEP----------- : 272
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Figure 2. Amino acid sequence alignment of two putative cotton (*Gossypium hirsutum*) CA isoforms with CAs from other plants including*, S. oleracea* (Genebank Accession #AI822159)*, N. tabacum* (Genebank Accession #AB009887)*, P. sativum* (Genebank Accession #M63627)*, A. thaliana* (Genebank Accession #AF326863)*, H. vulgare* (Genebank Accession #L36959)*,* and *O. sativa* (Genebank Accession #AF182806; assembled by H. Wessler in Dr. R. Benjamin's laboratory). Shaded areas represent conserved regions.

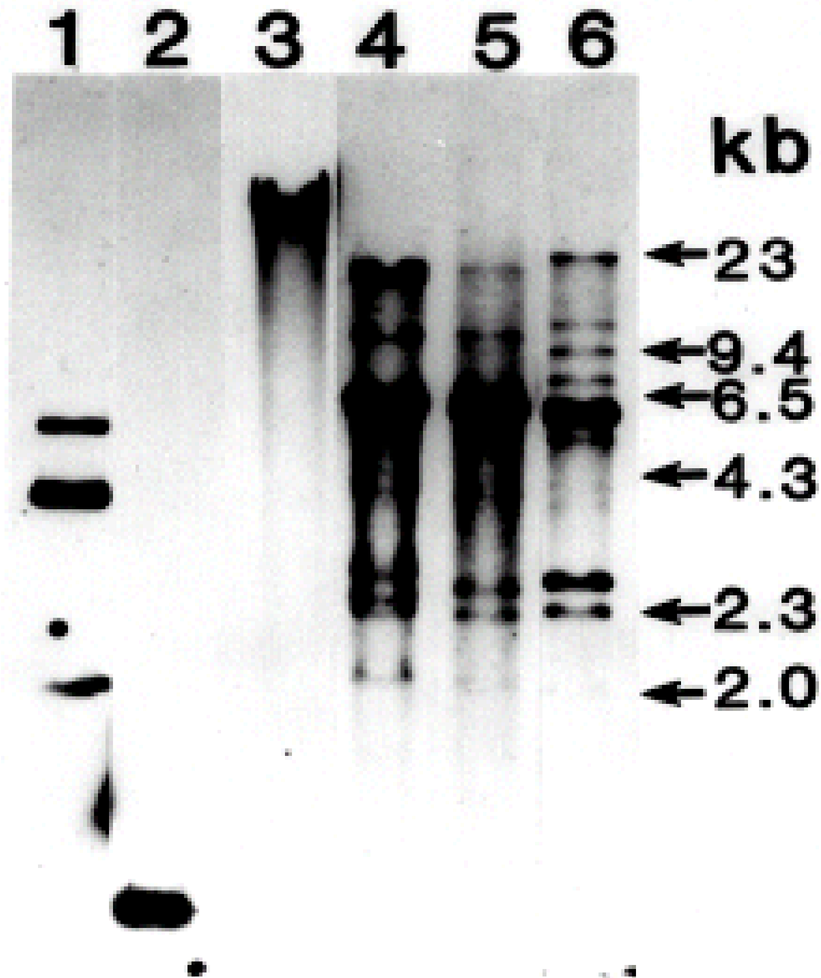


Figure 3. Genomic blot hybridization of cotton DNA from different cotton species. The cotton DNAs were digested with *Eco*RI for 6 h and the blot was hybridized with probe derived from a 0.6 kb cotton *CA* cDNA. Lane 1, pGem vector DNA containing CA insert was linearized with *Hin*dIII for 1 h; Lane 2, 0.6 kb CA fragment; Lane 3, uncut G. *hirsutum* DNA; Lane 4, digested G. *hirsutum* DNA; Lane 5, digested G. *tomentasum* DNA; Lane 6, digested G. *harknessil* DNA.

(0.2 x SSC / 62°C, it is likely that there are multiple copies of *GhCA* genes in the allotetraploid cotton genome.

Detection of GhCA1 mature protein in plastids

Polyclonal CA anti-peptide (AEHGGEPLGVQCTHCEKEAVN; amino acid residues #252-270, selection of polypeptide fragment by H. Wessler of Dr. R. Benjamin's laboratory) antibodies were used to detect the GhCA1 mature proteins in isolated plastids of cotton embryos (Fig 4). Pellets 1 and 2 containing the plastids of cotton embryos, the anti-CA antibodies recognized a 24 kDa protein not observed in the blot probed with preimmune serum. This apparent molecular weight was estimated from plots of M_r in comparison with known protein molecular weight standards, and is consistent with a predicted molecular weight of the mature protein of 24 kDa after cleavage at the putative transient peptide of the CA preprotein. Immunoblots revealed that the mature CA proteins are enriched in the plastid fractions.

Expression of a plastidal cotton CA cDNA in *E. coli*

*GhCA*1 cDNAs were expressed as N-terminal *LacZ* α- fusion proteins in *E. coli* SOLR cells (Transformed *E. coli* cells and cell-free extracts were prepared by H. Wessler of Dr. R. Benjamin's laboratory). Transformed cells induce with IPTG revealed an approximate 9-fold increase in measurable CA over untransformed cells (Fig 5a). CA activity was measured by following the rate of pH change in the assay medium containing CO_2-saturated buffer solution (Fig 5b). CA activity in GhCA1-fusion lysates was inhibited by ethoxyzolamide (Fig 5b) as well as acetazolamide and sulfanilamide (Fig 5c). Ability of these compounds to inhibit recombinant CA activity supports the

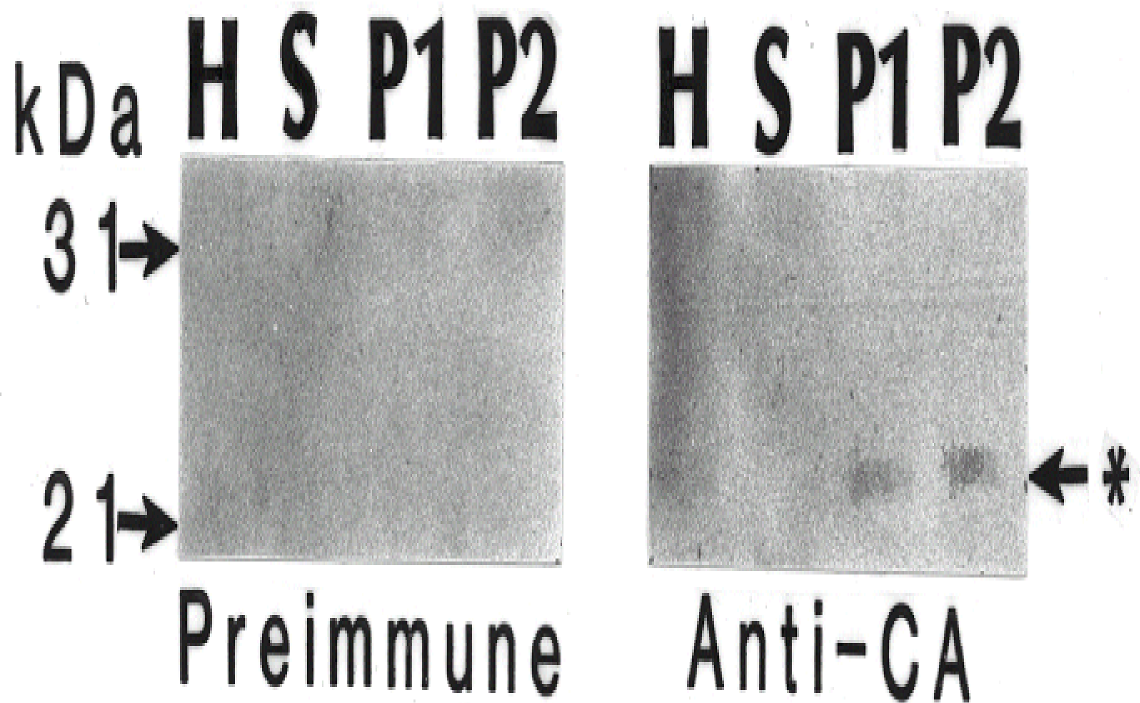
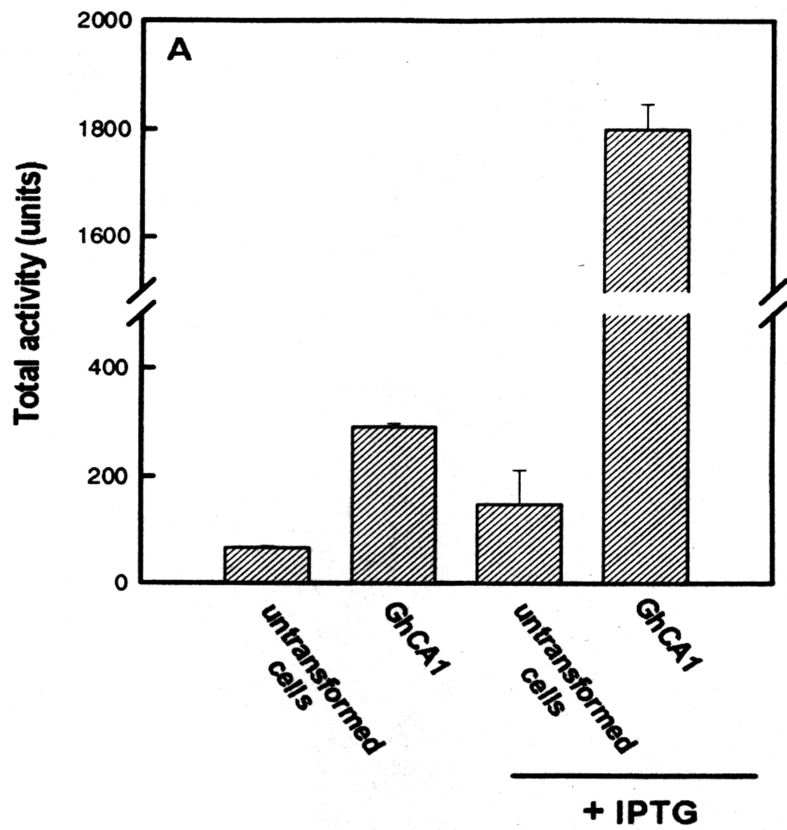
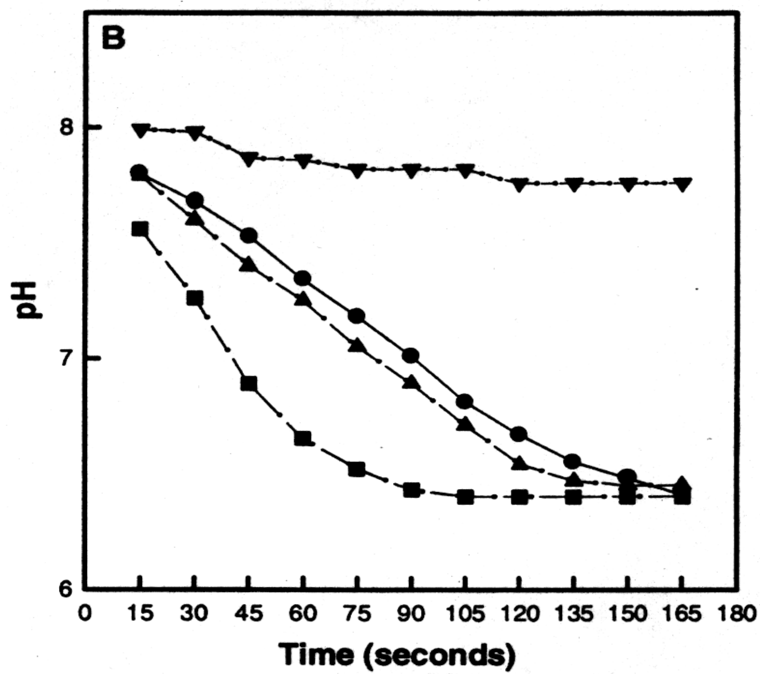


Figure 4. Detection of the mature CA protein in embryo plastids. Immunoblots were probed with 1:5000 preimmune serum and 1:5000 anti-GhCA1 antibodies. Cotton embryo homogenates (H) were centrifuged at a low speed to obtain the supernatant fractions (S). The resuspended pellet was then layered over a 10% percoll gradient and centrifuged again to give the P1 pellet (containing plastids). P1 was further purified to give the P2 pellet. The asterisk represents the 24 kDa mature CA protein.



A
Total activity (units)
2000
1800
1600
400
200
0
untransformed cells
GhCA1
untransformed cells
GhCA1
+ IPTG



B
pH
8
7
6
0
15
30
45
60
75
90
105
120
135
150
165
180
Time (seconds)

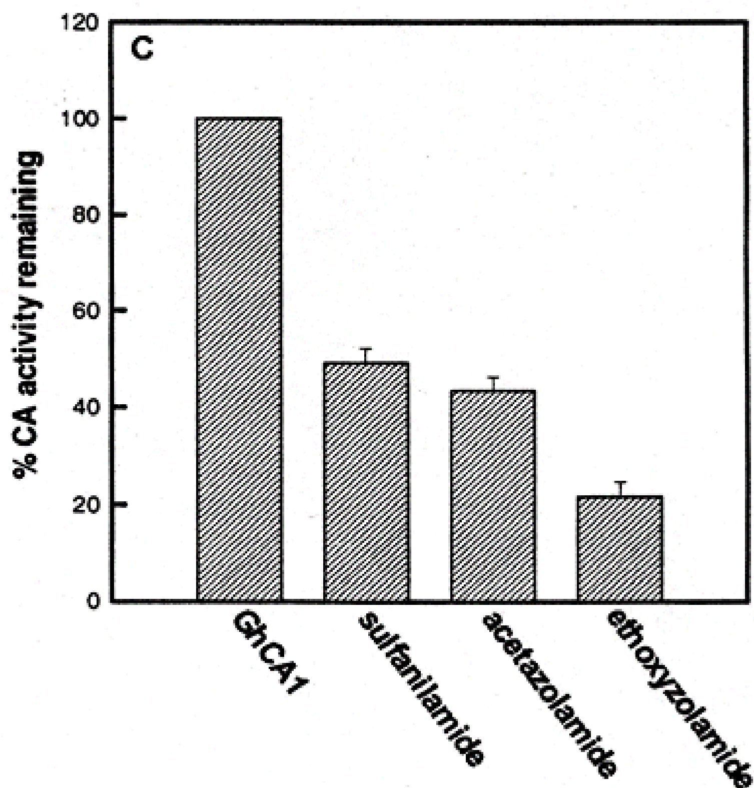


Figure 5. Recombinant carbonic anhydrase activity assay (Transformed *E. coli* cells and cell-free extracts were prepared by H. Wessler of Dr. R. Benjamin's laboratory). **A**. Carbonic anhydrase activity in *E. coli* cells expressing the two putative CA cDNA clones (1,164 bp *GhCA*1 and 1,152 bp *GhCA*2). Expression of the recombinant protein was induced by the addition of 0.4 mM IPTG for 90 min before lysis and assaying for CA activity. **B**. Characterization of recombinant CA activity in *E. coli* cells (■). Control assays were performed in reaction mixture with no enzyme (●), cold H_2O not saturated

with CO_2 (▼), and 10 mM ethoxyzolamide (▲). **C**. Effects of different carbonic anhdyrase inhibitors in extracts of transformed *E. coli* cells. The graph shows the percent of remaining CA activity when inhibited with 10 mM of sulfanilamide, acetazolamide, and ethoxyzolamide. Standard bars represent the standard deviation of three independent experiments

notion that the isolated cDNAs encode functional CA enzymes. Although *E. coli* possesses its own CA enzyme (*CynT*) gene (Guilloton et al., 1992), IPTG-induced expression of strains containing the *GhCA*1 construct is clearly not attributable to the endogenous *E. coli* CA enzyme (see Fig 5a).

Cotton carbonic anhydrase activity

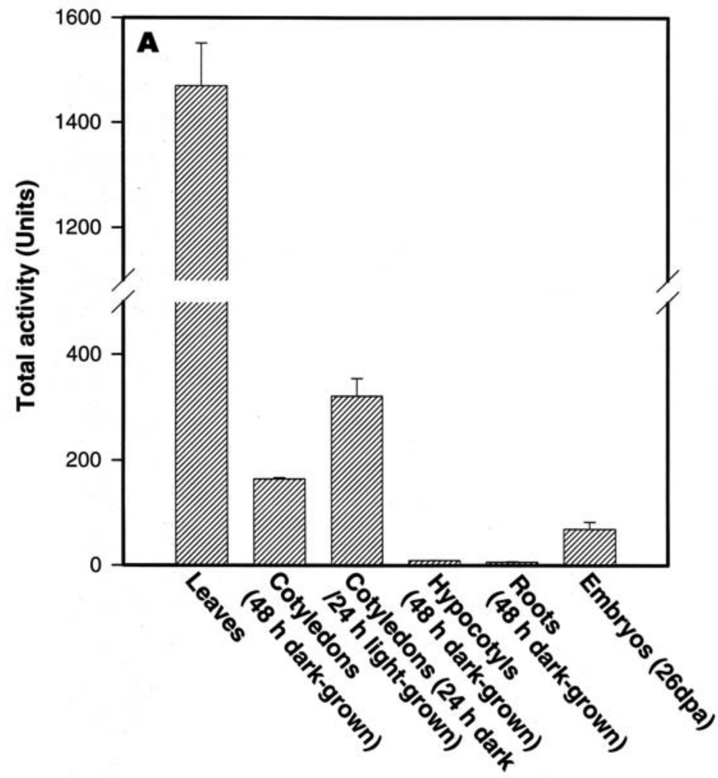
CA activity also was measured in cotton cell-free extracts under standard assay conditions (Fig 6). Negative controls (no enzyme, ▲) exhibited a decrease in pH from 8.2 to 6.6 over 2.5 min due to H^+ release from the spontaneous hydration of CO_2 to HCO_3^- (Fig 6). The rate of pH change was discernibly more rapid in the presence of erythrocyte CA (◆) or cotton tissue extracts from, for example, 48 h dark-grown cotyledons (▼). CA activity in crude extracts was standardized to the activity of erythrocyte CA. When cotyledon homogenates were boiled for 15 min (●), the rate of pH change was similar to that of negative controls (no enzyme). Assay mixtures underwent no pH change when the buffer was not saturated with CO_2 (■). Ethoxyzolamide (10 mM) inhibited both erythrocyte CA and CA activity in cotton homogenates by about 95% (data not shown). It is clear that CA catalyzed the activity measured in cotton cell-free homogenates. A survey of CA activity in different cotton organs and embryos revealed that total CA activity was about 8-fold higher in mature leaves versus 48 h dark-grown cotyledons (Fig 6a, b), about 2-fold higher in 24 h dark / 24 h light-grown versus 48 h dark-grown cotyledons, and barely detectable in hypocotyls and roots of 48 h dark-grown seedlings. Significant CA activity was detectable in 26 d post-anthesis (DPA) cotton embryos.

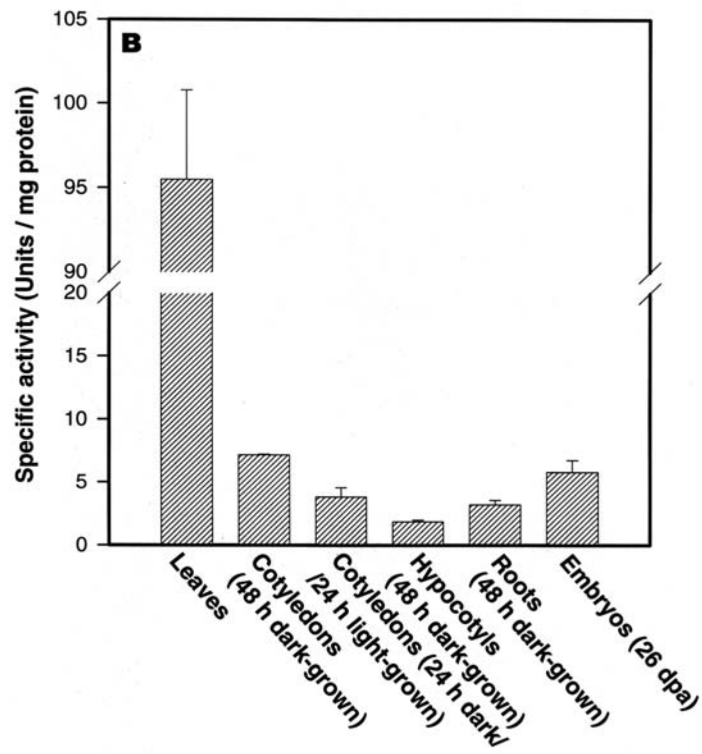
β-CAs of dicot plants, such as spinach (*Spinacia oleracea*) and pea (*Pisum sativum*) (Rickli et al., 1964), are sensitive to the same types of sulfonamide inhibitors as α-CA of mammalian cells, including the membrane permeable (ethoxyzolamide) and two membrane impermeable (acetazolamide and sulfanilamide) sulfonamides (Fig 6d). Ethoxyzolamide (10 mM) inhibited 95±5% of CA activity from 48 h dark grown-cotyledons (Fig 6d), whereas acetazolamide and sulfanilamide only inhibited 70±10% and 50±5%, respectively. This suggests that some CA activity in our cotton homogenates may be compartmentalized, which is of course, consistent with the demonstrated expression of plastid-targeted CA isoforms in cotton. Alternatively, these data may simply reflect that ethoxyzolamide is a more potent inhibitor of cotton CA activity than either acetazolamide or sulfanilamide

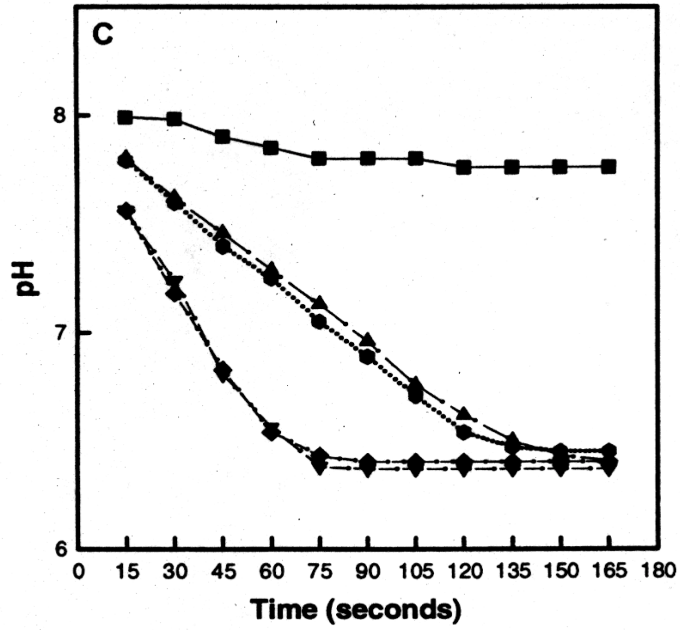
Relative expression of GhCA

Relative expression of *CA* transcripts was evaluated in different cotton organs and embryos (Fig 7). The level of 1.2 kb transcripts detected by *GhCA*1 was approximately 30-fold higher in 48 h dark-grown cotyledons and 23-fold higher in young leaves (mature plants) than in hypocotyls (48 h dark-grown), roots (48 h dark-grown), or 26 DPA embryos as determined by northern analysis of total cellular RNA (Fig 7). Previous work by Bracey et al. (1994) indicated CA is involved in providing CO_2 to the active site of Rubisco or HCO_3^- to phosphoenolpyruvate carboxylase in the photosynthetic tissues of plants. We expected, then, that the levels of 1.2 kb transcripts in 24 h dark / 24 h light-grown cotyledons would be comparable to those in leaves. However, relative levels of these transcripts in 24 h dark / 24 h light-grown cotyledons were about 5-fold less than

those in leaves. Intriguingly, 1.2 kb transcript levels were highest at a developmental stage prior to the synthesis of photosynthetic machinery (the cotyledons of 48 h dark-grown seedlings). RT-PCR experiments (Fig 7c) were consistent with the northern analyses and confirmed that 48 h dark-grown cotyledons and leaves of mature plants had higher relative *CA* transcript levels than hypocotyls and roots.


A
Total activity (Units)
1600
1400
1200
400
200
0
Leaves
Cotyledons (48 h dark-grown)
Cotyledons (24 h dark /24 h light-grown)
Hypocotyls (48 h dark-grown)
Roots (48 h dark-grown)
Embryos (26dpa)


B
Specific activity (Units / mg protein)
105
100
95
90
20
15
10
5
0
Leaves
Cotyledons (48 h dark-grown)
Cotyledons (24 h dark/ /24 h light-grown)
Hypocotyls (48 h dark-grown)
Roots (48 h dark-grown)
Embryos (26 dpa)


C
pH
8
7
6
0
15
30
45
60
75
90
105
120
135
150
165
180
Time (seconds)

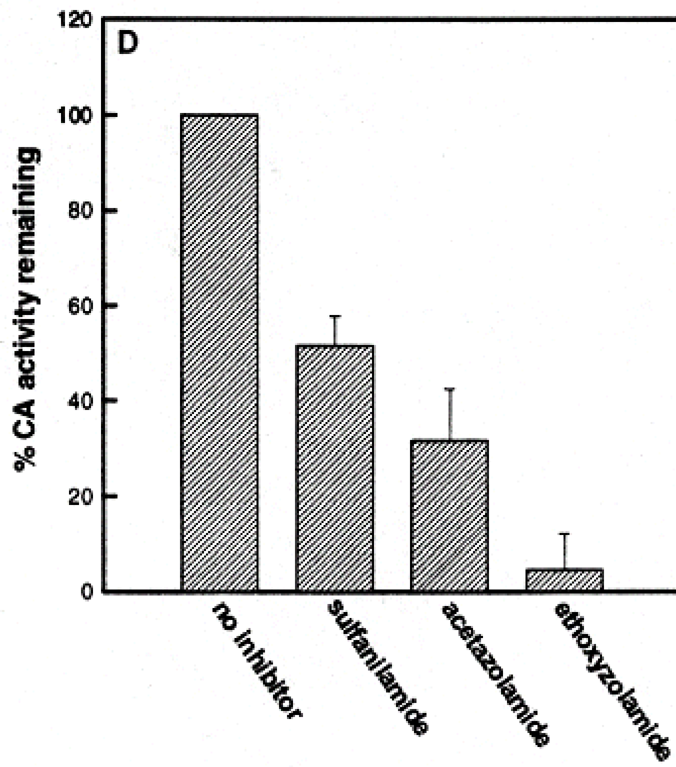
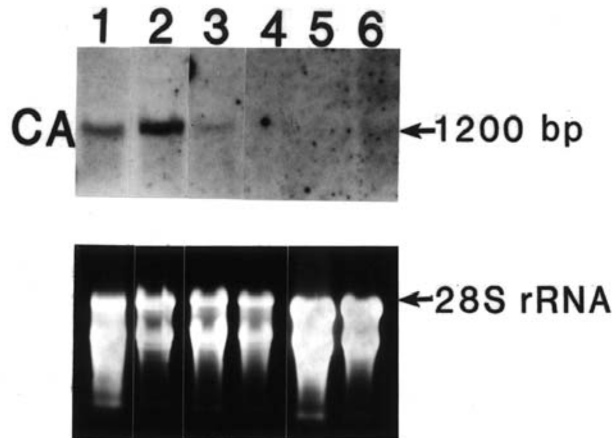
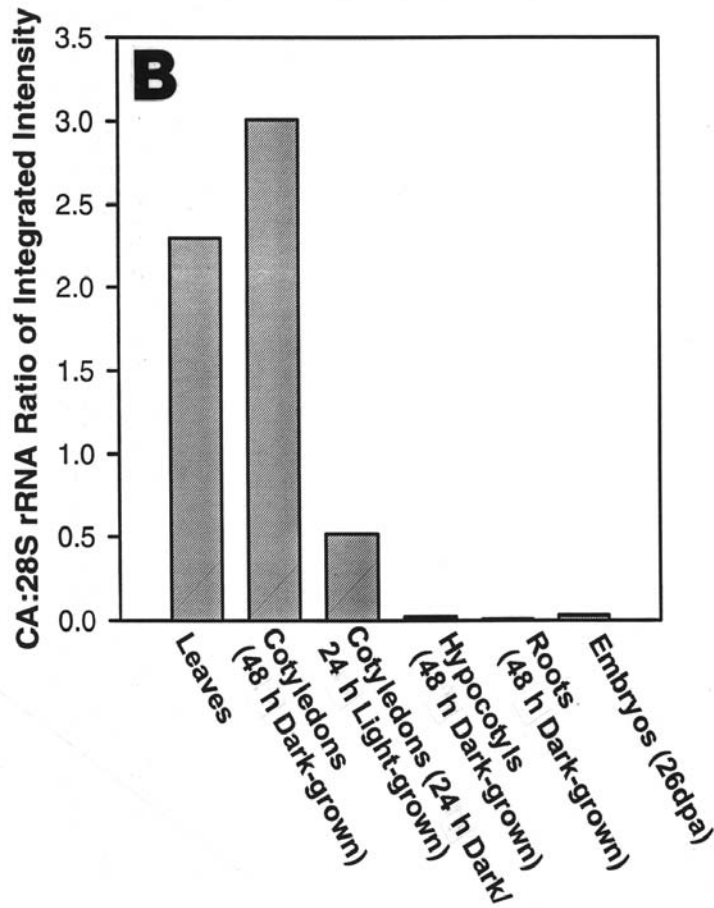

D
% CA activity remaining
120
100
80
60
40
20
0
no inhibitor
sulfanilamide
acetazolamide
ethoxyzolamide

Figure 6. Carbonic anhydrase activity assay of cotton organs. Electrometric assay for total **(A)** and specific **(B)** CA activity in total protein extracts from leaves, cotyledons (48 h dark-grown), cotyledons (24 h dark / 24 h light-grown), hypocotyls 48 h dark-grown), roots (48 h dark-grown), and embryos (26 dpa). **C**. Characterization of CA activity assay in 48 h dark-grown cotyledons (▼) and erythrocytes (◆). Control assays were performed in reaction mixture with no enzyme (▲), cold H_2O not saturated with CO_2 (■), and boiled 48 h dark grown cotyledons (●). **D**. Effects of different carbonic anhydrase inhibitors on CA activity in homogenate of 48 h dark-grown cotyledons. The graph shows the percent remaining CA activity when inhibited with 10 mM of sulfanilamide, acetazoamide, and ethoxyzolamide. Standard bars represent the standard deviation of three independent experiments.


A
1 2 3 4 5 6
CA
←1200 bp
←28S rRNA


B
CA:28S rRNA Ratio of Integrated Intensity
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Leaves
Cotyledons (48 h Dark-grown)
Cotyledons (24 h Dark/ 24 h Light-grown)
Hypocotyls (48 h Dark-grown)
Roots (48 h Dark-grown)
Embryos (26dpa)

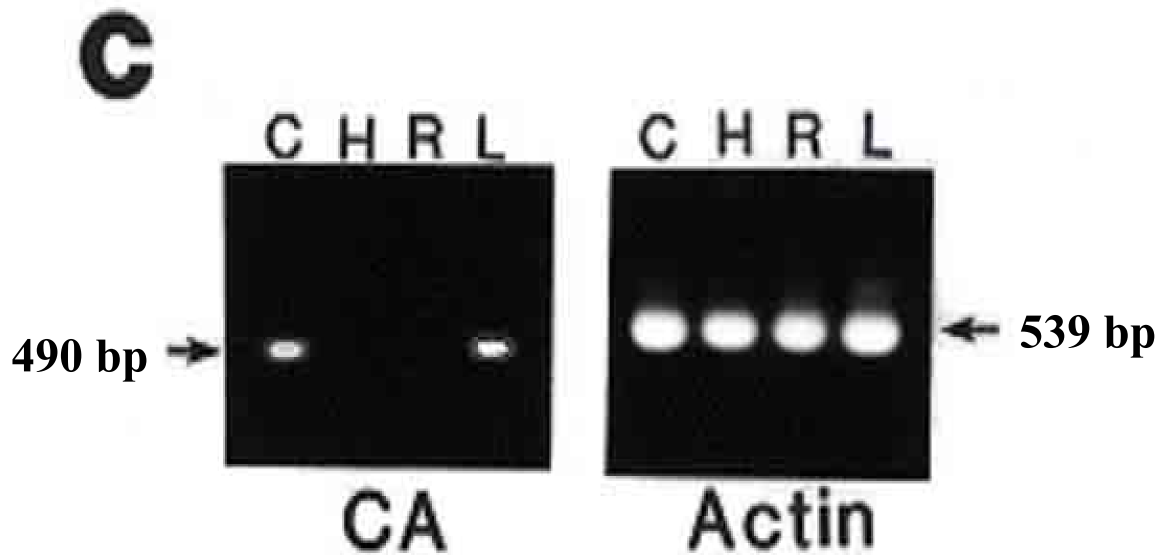


Figure 7. Relative transcript abundance in different tissues of cotton plant. **A.** Northern analyses of total RNA from various cotton organs. In each lane, approximately 10 µg of total RNA was electrophoresed in a 1% agarose gel and hybridized with probe derived from a CA cDNA. Lane 1, leaves; Lane 2, 48 h dark-grown cotyledons; Lane 3, 24 h dark / 24 h light-grown cotyledons; Lane 4, hypocotyls (48 h dark-grown); Lane 5, roots (48 h dark-grown); Lane 6, embryos (26 dpa). The gel was stained with ethidium bromide and the intensity of the 28S rRNA band was used to normalize the amount loaded in each lane. **B.** A histogram quantifying CA:28S rRNA ratios from integrated band intensity from northern blots. The ratios were formulated using NIH Image software and are from a single experiment. Similar trends were observed in replicate

experiments. **C.** The relative transcript abundance of *CA* and actin in C (cotyledons), H (hypocotyls), and (R) roots of 48 h old seedlings and L (leaves) of mature plants were evaluated by RT-PCR. Arrows indicate the predicted size of each product.

Discussion

We have isolated and characterized two *CA* cDNAs (*GhCA*1 and *GhCA*2) from cotyledons of 48 h dark-grown cotton (*Gossypium hirsutum* L.) seedlings. Alignment of the deduced GhCA1 / GhCA2 amino acid sequences to other plant CAs provides evidence these belong to the β-CA family. Immunoblot studies revealed that mature CA proteins are localized in the plastid stroma of cotton embryos. Expression of *GhCA*1 in *E. coli* clearly indicates that this cDNA isoform encodes a functional CA enzyme, even though the cDNA may not encode a full-length preprotein. Activity of the recombinant enzyme is sensitive to sulfonamide inhibitors (Fig 5), as is CA activity measured in different cotton tissues (Fig 6) and other plants (Jacobson et al., 1975; Majeau and Coleman, 1996; Tobin, 1970). The putative stromal targeting sequence encoded by the *GhCA*1 and *GhCA*2 cDNAs predict that these CAs are localized to plastids. Collectively, these data indicate that GhCA1 encodes a functional, plastid-localized CA.

Cotton CA expression and enzyme activity were not directly correlated. The total CA activity measured in leaves of mature cotton plants was about 8-fold higher than that in cotyledons of 48 h dark-grown seedlings (Fig 6), while the relative expression of *CA* (using the *GhCA*1 probe) appeared somewhat higher in cotyledons (Fig 7) of dark-grown seedlings than in leaves. Certainly comparisons of GhCA expression in these two different plant organs should be made with caution as often the regulation of plant gene expression is substantially variable in different plant tissues, and this is especially true for plastidial enzymes (Gallie, 1993). Differences may be due to mRNA stability, translational regulation, enzyme regulation, or other factors. Nevertheless it is clear that

GhCA1 is expressed and is functional in cotyledons of cotton seedlings during post-germinativegrowth.

Although no clearly defined roles for CA in C_3 photosynthesis exist, two possible functions have been proposed. HCO_3^- cannot readily diffuse across the chloroplast envelope. Thus, CA in the alkaline chloroplast stroma could aid in trapping inorganic carbon by hydrating CO_2, which diffuses across the chloroplast envelope, to HCO_3^-. Badger and Price (1994) also have proposed a unique role for CA based upon recent evidence indicating that there is a close association of CA with Rubisco in the carboxysome compartment of the cyanobacterium S*ynechococcus* (Price et al., 1992). This association would allow CO_2 to be selectively elevated at the active site of Rubisco. However, no direct experimental evidence of this association has been reported in C_3 plants, and antisense suppression of chloroplastic CA activity in tobacco plants to levels less than 1% of wild-type did not reveal any detectable changes in photosynthesis per unit leaf area at ambient CO_2 levels. In this study, we provide evidence indicating that GhCA1 transcript levels were about 30% higher in cotyledons of 48 h dark-grown seedlings than in leaves of mature plants. This suggests that plastidial CAs may be involved in nonphotosynthetic, physiological roles in cotton seedlings and that GhCA1 encodes a plastidial CA with an as-yet undiscovered role in post-germinativegrowth. This role may be a metabolic one, providing HCO_3^- to plastidial carboxylases, or more subtle, such as pH regulation of the nonphotosynthetic proplastid. Further research is required to address these or other possibilities.

Acknowledgements

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CHAPTER 2

REGULATION OF CARBONIC ANHYDRASE GENE EXPRESSION IN COTYLEDONS OF COTTON (*GOSSYPIUM HIRSUTUM* L.) SEEDLINGS DURING POST-GERMINATIVE GROWTH

(Submitted to Plant Molecular Biology, 2001)

Abstract

Recently, plastidial carbonic anhydrase (*CA*) cDNA clones encoding functional carbonic anhydrase enzymes were isolated from a 48 h dark-grown cotton seedling (cotyledons) cDNA library (Hoang et al., 1999). Here we examined the levels of relative transcript abundance and enzyme activities in cotyledons at different developmental stages and under different environmental conditions (i.e. altering CO_2 and light conditions), during post-germinative seedling growth. Relative *CA* transcript levels and total CA activity in cotyledons of cotton seedlings increased from 18 h to 72 h of post-germinative growth in the dark, although somewhat later than the glyoxylate cycle enzyme, malate synthase. When 24 h old seedlings were exposed to light for an addition 24 h, CA activity in greening cotyledons increased about 2-fold (compared with controls kept in the dark), whereas relative *CA* transcript levels were essentially the same. Removal of seed coats from cotyledons of 24 h old seedlings dramatically increased relative *CA* transcript abundance (measured 24 h later) in the dark, but did not influence CA activity. Manipulation of external CO_2 environments (zero, ambient, or high) modulated coordinately the relative transcript abundance of *CA* (and *rbcS*) in cotyledons,

but did not affect enzyme activity. On the other hand, regardless of the external CO_2 conditions cotyledons of seedlings exposed to light exhibited increased CA activity, concomitant with increased Rubisco activity and increased chlorophyll content. Collectively, our data suggest that steady-state levels of *CA* and *rbcS* transcripts are increased in response to environmental CO_2 conditions, while CA (and Rubisco) activities are modulated at the post-transcriptional level following exposure of seedlings to light, and in parallel with development of functional chloroplasts.

Abbreviations

Carbonic anhydrase, CA; malate synthase, MS; photosystem II 10 kDa protein (*psbR*), PSII; polymerase chain reaction, PCR; Reverse Transcription-PCR, RT-PCR; ribulose-1,5-bisphosphate carboxylase/oxygenase, Rubisco; and Rubisco small subunit gene product, *rbcS*

Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) is a zinc-containing enzyme that catalyzes the reversible hydration of CO_2 to HCO_3^- (Silverman, 1991). This ubiquitous enzyme is found in all animals and photosynthesizing organisms, such as green algae (e.g., *Chlamydomonas reinhardtii*) and plants, and in some non-photosynthetic bacteria (Lindskog, 1997). One physiological role of CA is to provide adequate levels of inorganic carbon for carboxylases such as ribulose-1,5-bisphospate carboxylase/oxygenase (Rubisco) (Badger and Price, 1994) and phosphoenolpyruvate carboxylase (PEP carboxylase) (Hatch and Burnell, 1990), in photosynthetic organs of plants. CA expression may change under different CO_2 conditions to regulate the supply of carbon for a variety of metabolic processes. Several proteins are induced and required for higher plants to grow at low CO_2 conditions. These proteins include Rubisco activase, the enzymes of the C_2 or photorespiratory cycle, and enzymes involved in nitrogen assimilation (Rawat and Moroney, 1995). In addition to these proteins, polypeptides such as Ala aminotransferase (Chen et al., 1996), chloroplast envelope carrier protein (Chen et al., 1997), mitochondrial CA (Eriksson et al., 1996) and periplasmic CA (Fujiwara et al., 1990) are induced under low CO_2 conditions in *C. reinhardtii*.

The regulation of CA expression in microalgae has been well characterized. For example, *C. reinhardtii* grown under low CO_2 conditions revealed that CA plays a role in a CO_2 concentrating mechanism (CCM), which concentrates high levels of CO_2 at the active site of Rubisco (Badger and Price, 1992). CA localized to the periplasmic space of

green algae was identified as a component of the CCM (Coleman et al., 1984). Periplasmic CA proteins encoded by *CAH*1 and *CAH*2 genes are regulated differently in response to changes in CO_2 concentrations (Fujiwara et al., 1990). Algal cells grown under low- CO_2 and continuous light conditions revealed an up-regulation of *CAH*1 transcript levels. However, CAH2 is only expressed under high- CO_2 and dark conditions. Also, Fujiwara et al. (1990) reported that light had a negative effect on the expression of *CAH*2. Furthermore, the increase in *CAH*1 transcript levels was accompanied by an increase in CA activity in *C. reinhardtii* grown at low CO_2 conditions (Coleman and Grossman, 1984). Toguri et al. (1984) and Fukuzawa et al. (1990) have suggested that *CAH*1 is regulated at the transcriptional level in low CO_2 conditions. In addition, β-CA1 and β-CA2 encoding mitochondrial CA isozymes in green algae were reported by Villand et al. (1997) to be up-regulated under low CO_2 conditions. While considerable evidence in green algae points to regulation of CA expression by low CO_2 conditions, this has not been thoroughly investigated in higher plants.

There are several studies indicating that there is a coordinate regulation of CA and Rubisco expression in leaves of higher plants and this expression is modulated by high external CO_2. Majeau et al. (1994) showed that in tobacco transgenic plants over-expressing a chloroplastic *CA* cDNA, there was a concomitant increase in Rubisco activity. Also Majeau and Coleman (1994) reported that a constant level of *CA* and Rubisco transcript abundance and enzyme activity were maintained during leaf development of pea (*Pisum sativum* L.). Long-term exposure of plants, such as cucumber (*Cucumis sativus* L.), pea and soybean (*Glycine max* L.), to elevated CO_2 conditions

resulted in a decrease in Rubisco activity and protein content (Peet et al., 1986; Sage et al., 1989; Xu et al., 1994). In addition, studies with mature leaves of higher plants indicated that plants grown at elevated CO_2 levels had a substantial loss of CA activity (Porter et al., 1984). *Arabidposis thaliana* plants exposed to elevated CO_2 (1000 ppm) conditions resulted in a dramatic decrease in Rubisco protein content, due to an approximately 35-40% decrease in *rbcL* mRNA levels, and a greater decline in *rbcS* mRNA levels (approximately 60%)(Cheng et al., 1998). Also, Nie et al. (1995) and van Oosten and Besford (1995) reported that a decrease in transcript levels of *rbcS* and *rbcL* mRNA was observed when wheat (*Triticum aestivum* L.) and tomato (*Lcopersicon esculentum* L.) plants were grown at elevated CO_2 partial pressures. Thus these studies demonstrated that CA and Rubisco expression are down-regulated under elevated CO_2 conditions.

Previously, we reported the isolation and characterization of cDNAs encoding plastidial CA enzymes from cotyledons of dark-grown cotton seedlings (Hoang et al., 1999). To begin to elucidate the role of CA in cotyledons of seedlings, we focused initially, on how CA expression is regulated under different environmental conditions during post-germinative growth. Parenchyma cells in cotyledons of cotton seedlings mobilize triacylglycerols during heterotrophic seedling growth. Upon exposure to light these cells differentiate and this organ becomes photoautotrophic, persisting for about 30 days (Fig 1). In this paper, relative transcript levels and enzyme activities of CA (and Rubisco) were measured in cotyledons during post-germinative growth (18 h to 72 h) of

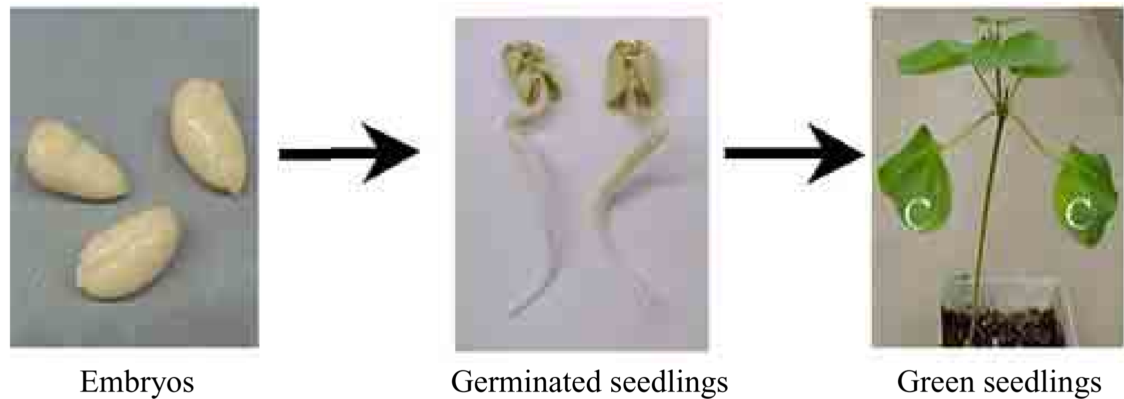


Figure 1. Cotyledons at different developmental stages. Cotyledons of the developing embryos are involved in storage lipid synthesis and accumulation. Lipids are mobilized during seed germination in cotyledons of cotton seedlings. At this stage the seedlings are involved in heterotrophic growth using the storage lipids for seedling growth and development. Upon exposure to light, these cotyledons serve as the primary organs for photosynthesis, which convert light energy into chemical energy for photoautotrophic growth (cotyledons of green seedlings are labeled with the letter C).

cotton seedlings. Also the impact of light and external CO_2 on CA and Rubisco expression was evaluated. Our results suggest that CA is expressed to function in chloroplasts of cotyledons of seedlings as these organs undergo a transition from metabolism aimed at reserve mobilization to metabolism aimed at photosynthesis. Interestingly, the increase in CA transcript levels was responsive to external CO_2, while the development of functional enzyme activity was modulated by light. Under normal post-germinative growth conditions, these environmental parameters likely coordinate the expression of CA and other chloroplast proteins, but by manipulating the two parameters independently, mRNA accumulation was uncoupled from acquisition of enzyme function in this epigeous, leaf-type cotyledon system.

Materials and Methods

Plant material

Cotton (*Gossypium hirsutum* L., cv. Paymaster HS26) seeds were provided by Dr. John Burke (USDA-ARS, Lubbock, TX). For post-germination time course studies, seeds were surfaced sterilized, imbibed, germinated, and grown as described by Chapman and Trelease (1991). For exposure of seedlings to different environmental conditions, seed coats were removed from 24 h old seedlings and the seedlings wee placed on moistened filter paper in petri dishes at 30°C for an additional 12 h to 48 h either in the dark or light (~300 µmol m^{-2} s^{-1}) or altering CO_2 (high CO_2, 1000 ppm balance with nitrogen and 21% oxygen; ambient CO_2, 350 ppm; or zero CO_2, air was scrubbed with soda lime). CO_2 concentrations were monitored in line with a plant CO_2 analysis system (Qubit Systems Inc., Canada). Cotyledons were harvested at various post-germinative growth times and under different environmental conditions for measuring enzyme activity and relative transcript levels of CA and Rubisco (or several other gene products).

Enzyme assays

Cell free extracts were prepared from freshly-harvested cotyledons. Plant materials were powdered in liquid N_2 with a mortar and pestle in 1:1 (w/v) homogenization buffer containing 400 mM sucrose, 100 mM sodium phosphate (pH 7.2), 10 mM KCl, 1 mM $MgCl_2$, and 1 mM ethylenediamine tetracetic acid (EDTA). Homogenates were filtered through 4 layers of cheesecloth and the filtrates were analyzed immediately for CA activity. Total CA activity was determined electrometrically (Wilber and Anderson, 1948) as described previously (Hoang et al.,

1999). One unit of activity (Wilbur-Anderson, WA unit) was defined as $10[(T_o/T)-1]$, where T_o and T are equal to the rate of pH change of the reaction without (control) and with cell homogenates, respectively. The method used for preparing cotyledon extracts for measuring Rubisco activity was described by Vu et al. (1997). Briefly, about 400 mg of cotyledons were homogenized in 1 mL of extraction medium consisting of 50 mM CO_2-free Tricine-NaOH, pH 8.0, 10 mM $MgCl_2$, 5 mM dithiothretiol (DTT), 10 mM isoascorbic acid, 0.1 mM EDTA, and 2% (w/v) polyvinylpoly pyrrolidone (PVP) 40. The homogenates were centrifuged at 13,000 x *g* for 45 s at 4°C and 100 µl aliquots of the supernatant were immediately assayed for Rubisco activity. Rubisco activity was based on $NaH^{14}CO_3^-$ incorporation into an acid stable product. Radioactivity in the acid-stable fraction was determined by liquid scintillation counting (Beckman LS Model 3801 Liquid Scintillation Counter).

Total chlorophyll amounts in lipid extracts were determined spectrophotometrically in 80% acetone at 664 nm and 647 nm according to Lichtenthaler (1987). Protein content in cell-free extracts was determined according to Bradford (1976) using bovine serum albumin for standard curve calibration.

Northern blot analyses

Total RNA was isolated from cotyledons of cotton seedlings (approximately 1 g fresh weight; 20 cotyledons) by a hot borate method developed by Wan and Wilkins (1994). RNA yield and quality were evaluated spectrophotometrically and by analytical gel electrophoresis according to Sagerström and Sive (1996). Approximately 10 µg of total RNA was electrophoresed in a 1% agarose gel containing 6% formaldehyde and

MOPS buffer solution (20 mM MOPS (3-[N-morpholino] propanesulfonic acid)-NaOH, pH 7.0, 5 mM sodium acetate, 0.1 mM EDTA). RNA was transferred to nylon membranes by capillary transfer in 20 x SSC (150 mM NaCl and 15 mM sodium citrate; pH 7.0) (overnight) and probed with a random prime-labeled (Gene Images random prime-labeling module, Amersham) 1.16 kb cotton *CA* cDNA (*GhCA*1; GenBank Accession AF132854), malate synthase cDNA (*MS*; 964 bp), photosystem II 10 kDa protein (*psbR*) cDNA (456 bp), Rubisco small subunit cDNA (*rbcS*; 855 bp), and actin cDNA (539 bp) probes. PCR or RT-PCR reactions were used to amplify the cDNA sequences for probe labeling. The probe for cotton MS was generated by PCR amplification of pBluescript® SK(-) phagemids containing the cotton *MS* cDNA clone using MS forward (5'-AGTTACGGTTACACTGCACC-73') and reverse (5'-TGAAGTGTTGGGTCATACG-3') primers (GenBank Accession X52305; Turley et al., 1990). A photosystem II (PSII) 10 kDa protein (*psbR*) cDNA probe was generated by PCR amplification from pGEM plasmid containing a partial PSII (*psbR*) cDNA clone using forward (5'-TGTTCAGTTACCCTCAAGCC-3') and reverse (5'-ACAATTACATCGGACAGCC-3') primers (partial cDNA clone isolated by Dr. Rick Turley; Stoneville, MS). The cDNA probe for Rubisco small subunit (*rbcS*) was generated by PCR amplification from pGEM plasmid containing *rbcS* cDNA clone using forward (5'-ATAGGGGATTGCACCAAGGC-3') and reverse (5'-GCACTTGACGAACGAACGTTGTCG-3') primers (GenBank Accession X54091; Sagliocco et al., 1991). The actin cDNA probe was generated by RT-PCR from approximately 200 ng of total cotyledon RNA with forward (5'-

TGCAGGTCGTGATCTAACCG-3’) and reverse (5’-CCTTGGAAATCCACATCTGC-3’) primers (GenBank Accession D88414; Shimizu et al., 1997).

Hybridization and washing were both carried out at 62°C and with a final wash at 0.2 x SSC. Hybridized bands were identified by an alkaline phosphatase-catalyzed chemiluminescent reaction (Gene Images CDP-Star detection module, Amersham) and quantified by densitometric scanning (NIH v 6.1 image software). Increasing concentrations of fluorescien-labeled probe (Gene Images labeling module kit) were used to create the calibration curve. Exposures of blots used for densitometric scanning were in the linear range of the calibration curve.

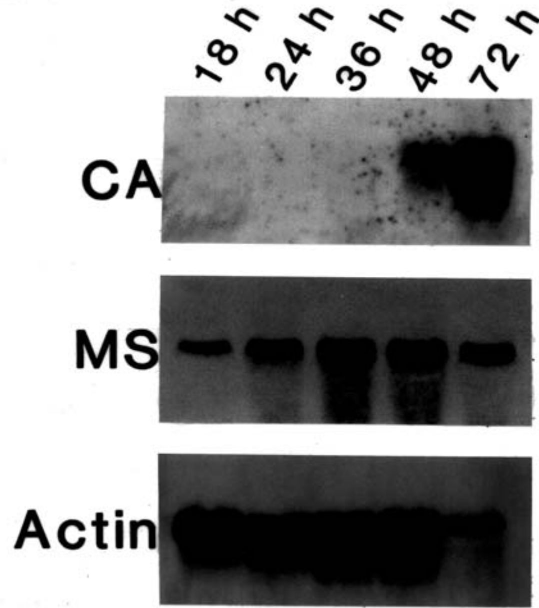
Reverse transcription - polymerase chain reaction (RT-PCR) analyses

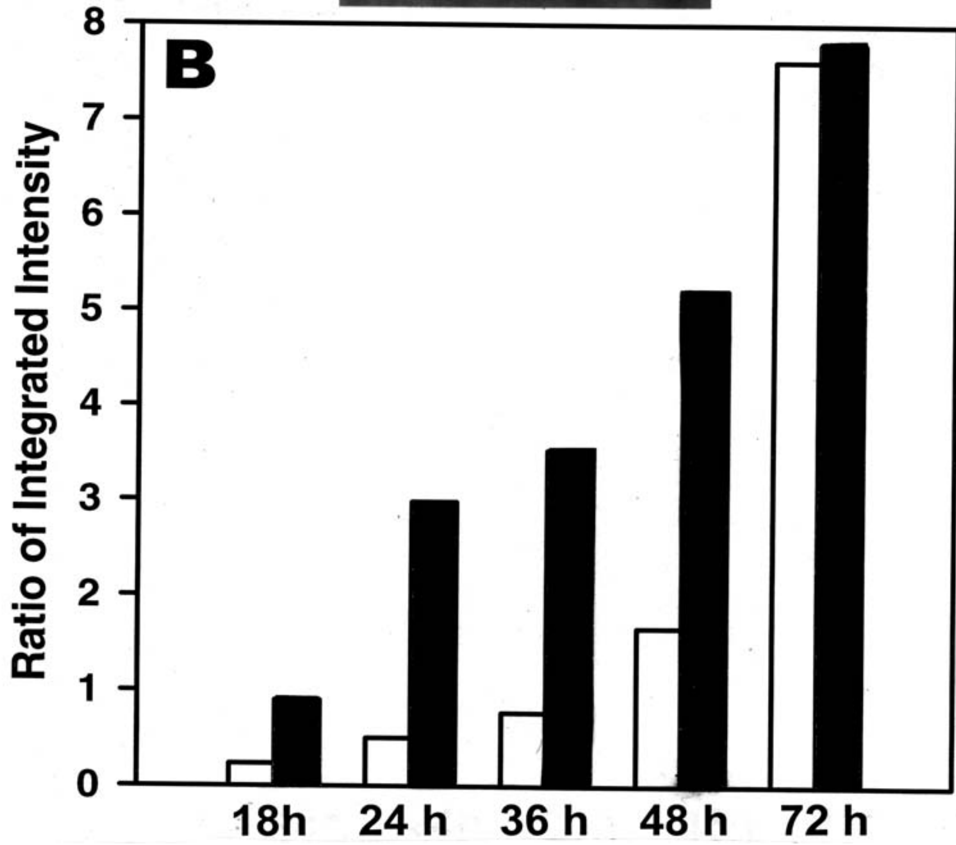
RT-PCR of total RNA (approximately 200 ng) from cotyledons at different developmental stages (18 h to 72 h) was conducted with a Promega Access RT-PCR system in a Perkin Elmer 2400 thermal cycler. First stand cDNA synthesis was carried out at 48°C for 45 min. Subsequent amplification of a targeted region within the RNA sequence was achieved through 35 cycles of 94°C for 30 s, 54°C for 1 min, and 68°C for 2 min followed by a final polymerization step at 68°C for 7 min. CA (forward; 5’-AGGTTTCCAAGGGATACA-3’ and reverse; 5’-TGAGAGACTGAAAGAAGG-3’) and actin (forward and reverse, above) primers were used to amplify the 490 bp and 539 bp fragment, respectively. RT-PCR products were visualized by ethidium bromide staining following electrophoresis in 3% agarose gels, and as expected, yielded results similar to those observed in Northern blot analyses.

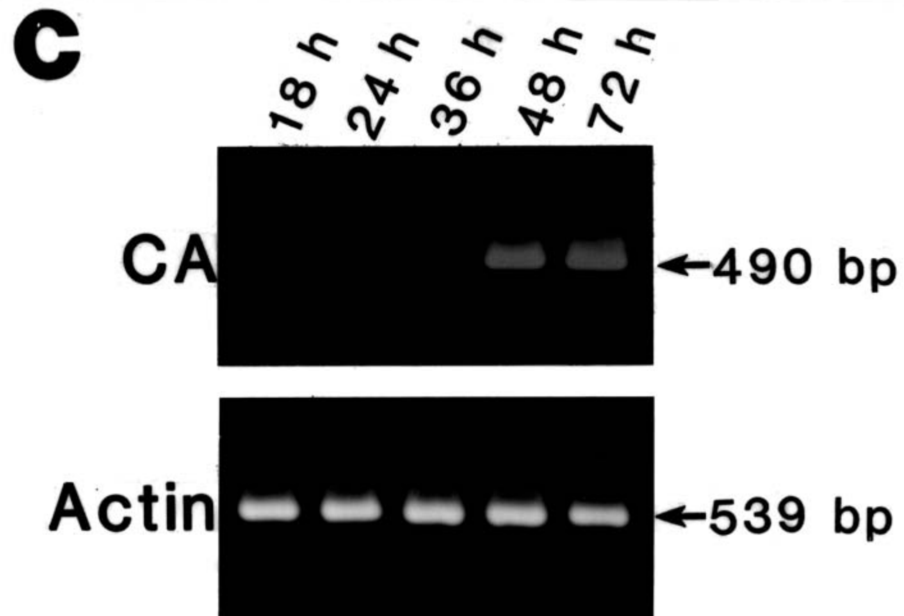
Results

Expression of CA in cotton seedlings during post-germinative growth

During the first 24 h to 48 h of post-germinative growth of cotton seedlings in the dark, most cellular efforts in cotyledons are directed toward mobilization of stored reserves (Trelease and Doman, 1984; Huang et al., 1983). During this period, Northern blot analyses revealed that *CA* mRNA levels were highest at 48 h and 72 h with minimal expression earlier (Fig 2A, B, open bars). Relative malate synthase (*MS*) transcript levels (glyoxysomal protein marking acquisition of lipid mobilization capacity) increased during the 18 h to 72 h post-germinative growth period, with transcripts appearing somewhat earlier than CA (Fig 2B, solid bars). RT-PCR of total RNA with cotton CA-specific primers revealed a product of 490 bp, confirming that *CA* transcript levels were highest at 48 h and 72 h and minimal at prior stages (Fig 2C). Total CA activity increased during post-germinative growth from 18 h to 72 h, but the dramatic increase in transcript levels, particularly from 48 h and 72 h, was not reflected proportionally in expression of functional protein (Fig 2D). Although not shown here, the relative trend plotted as specific enzyme activity was indistinguishable from that shown for total enzyme activity.


A
18 h
24 h
36 h
48 h
72 h
CA
MS
Actin


B
Ratio of Integrated Intensity
0
1
2
3
4
5
6
7
8
18h
24 h
36 h
48 h
72 h


C
18 h
24 h
36 h
48 h
72 h
CA
490 bp
Actin
539 bp

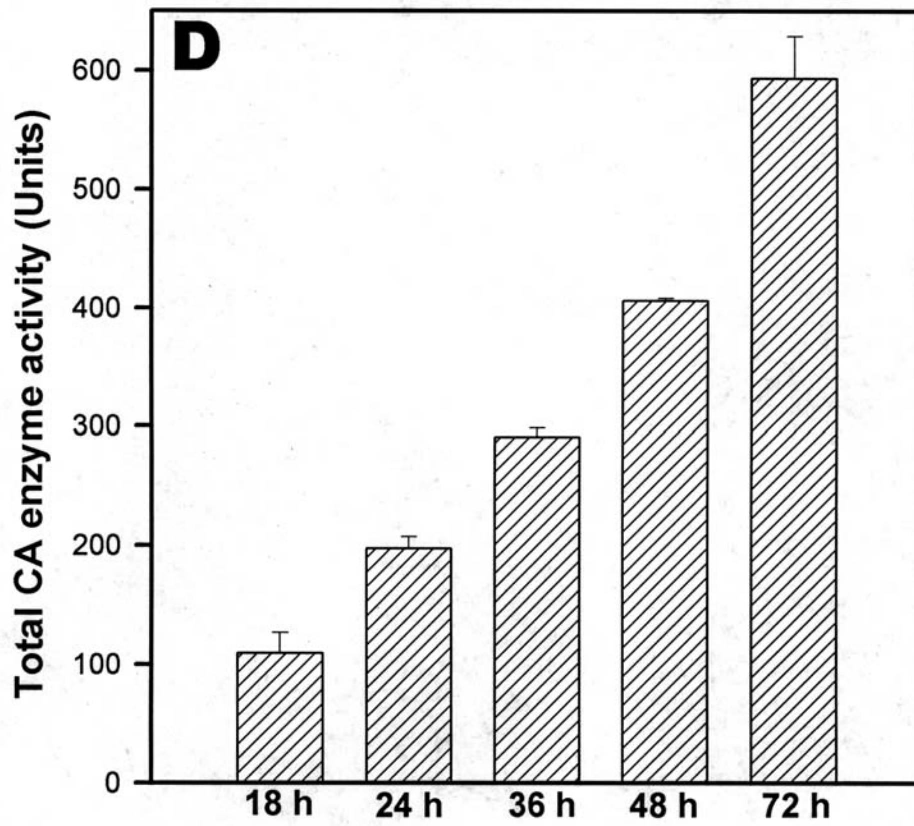
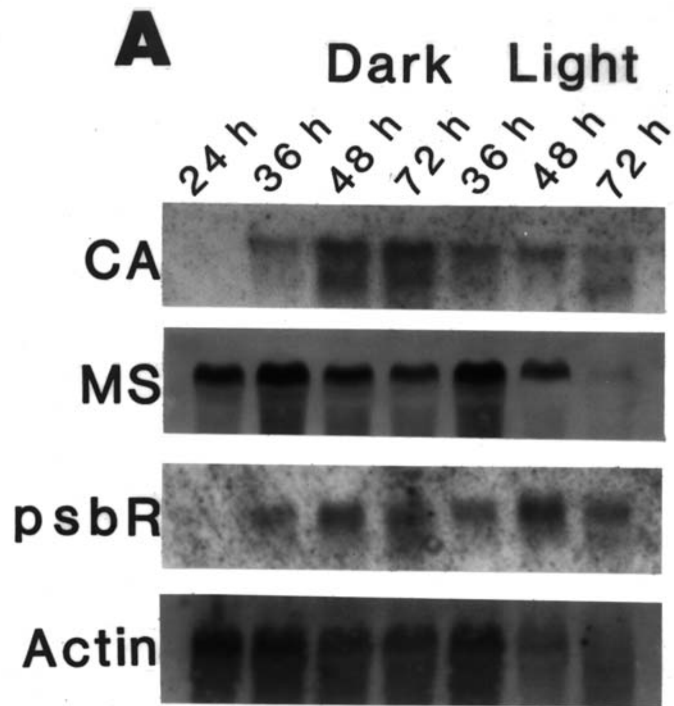

D
Total CA enzyme activity (Units)
0
100
200
300
400
500
600
18 h
24 h
36 h
48 h
72 h

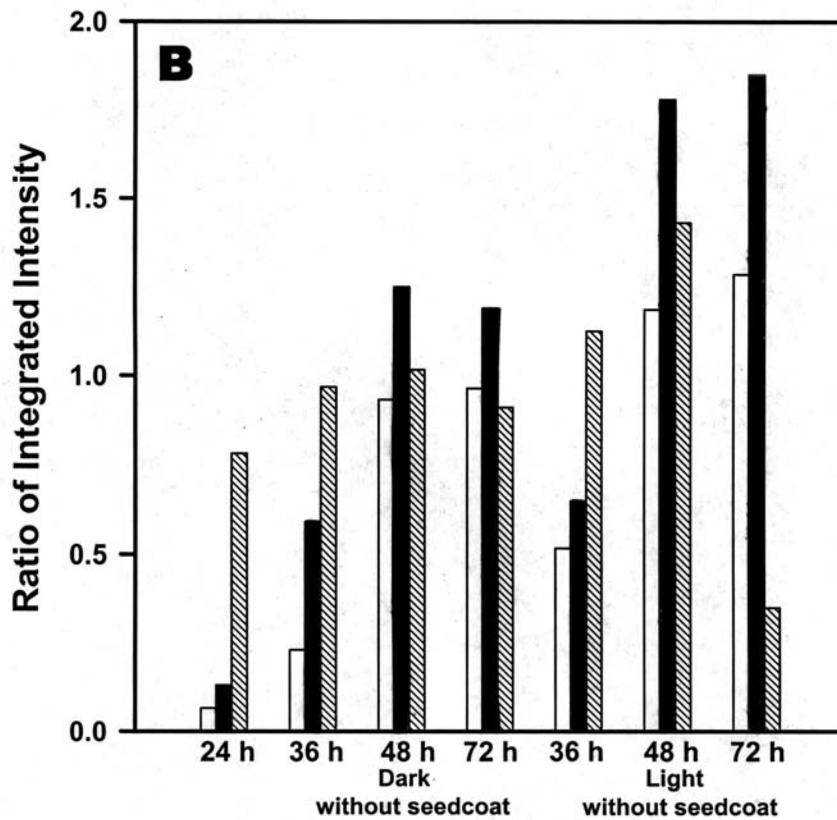
Figure 2. CA expression levels in cotyledons of cotton seedlings during post-germinative growth (18 h to 72 h after imbibition). (A) Northern blot analyses of total RNA (10 μg) from cotyledons of cotton seedlings during post-germinative growth periods. Blots were hybridized with carbonic anhydrase (CA), malate synthase (MS), and actin cDNA probes that were generated by random priming. The predicted sizes of CA, MS and actin transcripts are 1200 bp, 2000 bp and 1600 bp, respectively. (B) A histogram quantifying CA:actin (open bars) and MS:actin (solid bars) ratios of integrated band intensity from Northern blots. The ratios were formulated by using NIH Image software and are from a single experiment. Similar trends were observed in replicate experiments. (C) Relative transcript abundance of *CA* judged by RT-PCR. CA and actin primers (forward and reverse) were used to RT-PCR amplify a 490 bp and 539 bp product, respectively from total RNA. (D) Total CA activity in protein extracts from cotyledons of seedlings during 18 h to 72 h post-germinative growth periods. Bars represent the mean and standard deviation of three independent experiments.

CA expression in seedlings under different environmental conditions

Several studies have reported that different environmental conditions can regulate CA expression (Porter et al., 1984; Fujiwara et al., 1990; Coleman and Grossman, 1984). Hence, we compared *CA* steady-state transcript levels and enzyme activities in cotyledons of cotton seedlings exposed to light or kept in the dark. Seedlings were germinated and grown in the dark for 24 h then seed coats were removed from cotyledons and seedlings were incubated in ambient CO_2 conditions in the dark or light for an additional 12 h to 48 h. Northern blot analyses revealed that *CA* transcript levels of cotyledons (36 h-, 48 h- or 72 h-old seedlings) were not increased by exposure to light (Fig 3A, B). However, there was approximately a 2-fold increase in CA enzyme activity in cotyledons of seedlings exposed to light compared with cotyledons of dark-grown seedlings (Fig 3C). As expected, relative transcript levels of a nuclear-encoded PSII (*psbR*) gene (encodes a 10 kD protein) increased in cotyledons of seedlings exposed to light (Fig 3A, B, hatched bars). A steady decrease in MS transcript levels was observed from the 36 h to 72 h post-germinative growth period in cotyledons exposed to light (Fig 3A, B, solid bars), consistent with the glyoxysome - to - peroxisome transition that occurs as these cotyledons become photoautotrophic (Trelease and Doman, 1984; Huang et al., 1983).

In these experiments, seed coats were removed for exposure to light and relative CA transcript levels appeared to increase somewhat earlier in the dark (rapid increase from 36 h to 48 h without seed coats; Fig 3A, B), compared with previous time courses (with seed coats; Fig 2A, B). Since light did not seem to increase relative *CA* transcript


A
Dark Light
24 h
36 h
48 h
72 h
36 h
48 h
72 h
CA
MS
psbR
Actin


B
Ratio of Integrated Intensity
2.0
1.5
1.0
0.5
0.0
24 h
36 h
48 h
72 h
36 h
48 h
72 h
Dark
without seedcoat
Light
without seedcoat

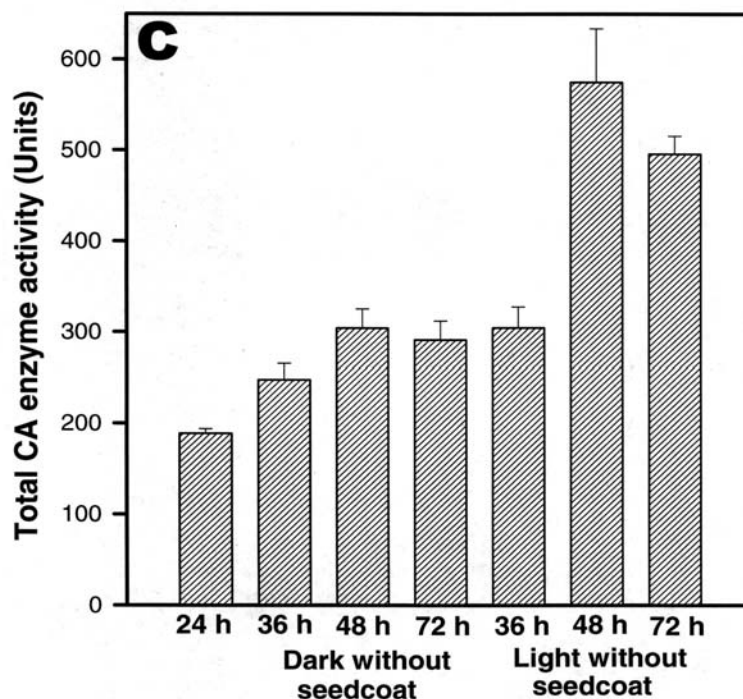
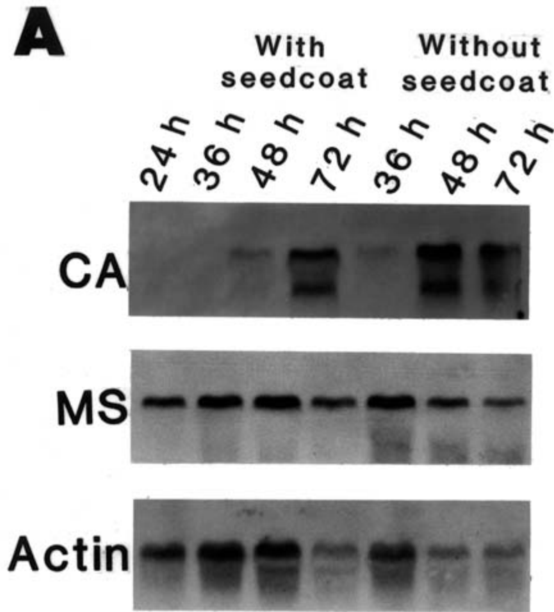


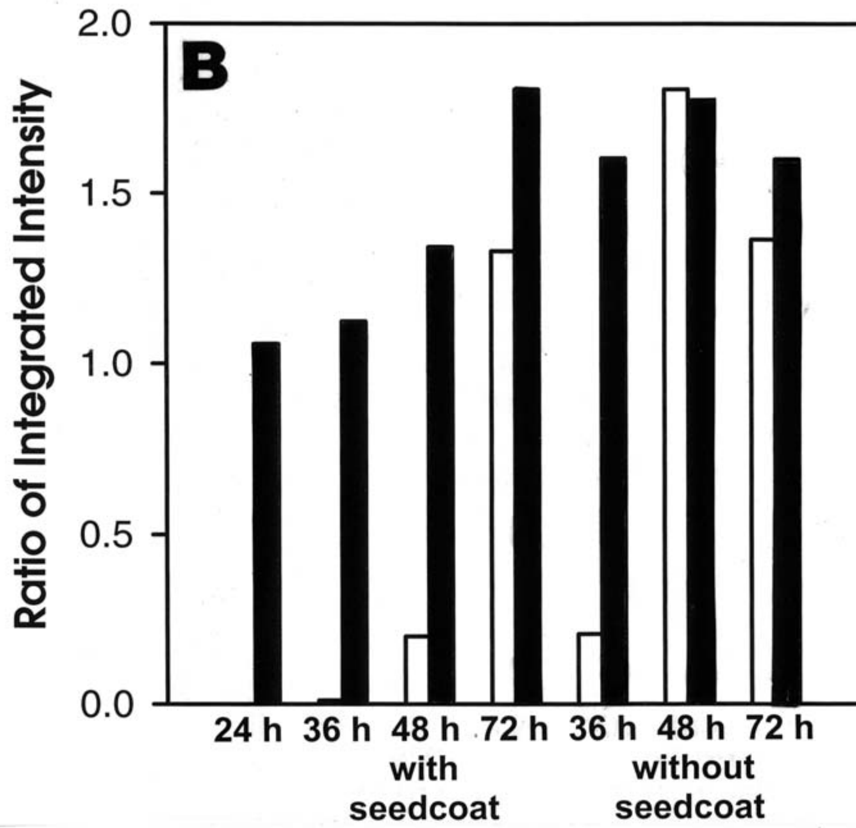
Figure 3. Influence of light on CA expression in cotyledons of cotton seedlings. (A) Northern blot analyses of *CA*, *MS*, PSII 10 kDa protein (*psbR*), and actin transcripts in cotyledons of 36 h-, 48 h-, or 72 h-old seedlings. Seedlings were germinated and grown in the dark for 24 h then seed coats were removed from cotyledons and seedlings were incubated in ambient CO_2 conditions in the dark or light for an additional 12 h to 48 h (for a total of 36 h to 72 h periods). The predicted size of PSII 10 kDa protein (*psbR*) transcripts is 600 bp. (B) A histogram quantifying CA:actin (open bars), MS:actin (solid bars), PSII:actin (hatched bars) ratios of integrated band intensity from Northern blots. The ratios were calculated by using NIH Image software and are from a single experiment. Similar trends were observed in replicate experiments. (C) Total CA activity in protein extracts of cotyledons exposed to dark or light. Bars represent the mean and standard deviation of three independent experiments.

levels, CA expression was evaluated in cotyledons of dark-grown seedlings (36 h to 72 h) with or without seed coats (Fig 4). Northern blot analyses revealed that relative *CA* transcript levels in cotyledons of 48 h dark-grown seedlings were approximately 8-fold higher when seed coats were removed at 24 h (Fig 4A, B, open bars). However, surprisingly removing the seed coats did not substantially increase CA activity (Fig 4C). Relative MS transcript levels were unaffected by removing the seed coats of germinated cotyledons when compared to seedlings with seed coats (Fig 4A, B, hatched bars). It was possible that relative *CA* transcript levels were responsive to changes in internal CO_2 brought about by removal of the seed coats (more rapid diffusion of respiratory CO_2 out of cotyledons).

Expression of CA at different external CO_2 levels

To directly examine the influence of CO_2 on CA gene expression, seeds were germinated and grown for 24 h at ambient CO_2 conditions, seed coats were removed, and seedlings were grown for an additional 24 h at zero CO_2 (scrubbed atmospheric air), ambient CO_2 (350 ppm), or high CO_2 (1000 ppm). Figure 5 shows a dramatic increase in *CA* relative transcript levels, in cotyledons of 48 h dark-grown seedlings at zero external CO_2 conditions, when compared to seedlings exposed to ambient or high CO_2 conditions. *MS* mRNA levels were not influenced by altering CO_2 conditions (Fig 5B, solid bars). Despite the approximate 8-fold differences in relative *CA* and *rbcS* transcript abundance at low external CO_2 conditions (compared with those at ambient CO_2), enzyme activities of CA (Fig 6A) or Rubisco (Fig 6B) in 48 h cotyledons (open bars) were not substantially


A
With seedcoat
Without seedcoat
24 h
36 h
48 h
72 h
36 h
48 h
72 h
CA
MS
Actin


B
Ratio of Integrated Intensity
2.0
1.5
1.0
0.5
0.0
24 h
36 h
48 h
72 h
36 h
48 h
72 h
with seedcoat
without seedcoat

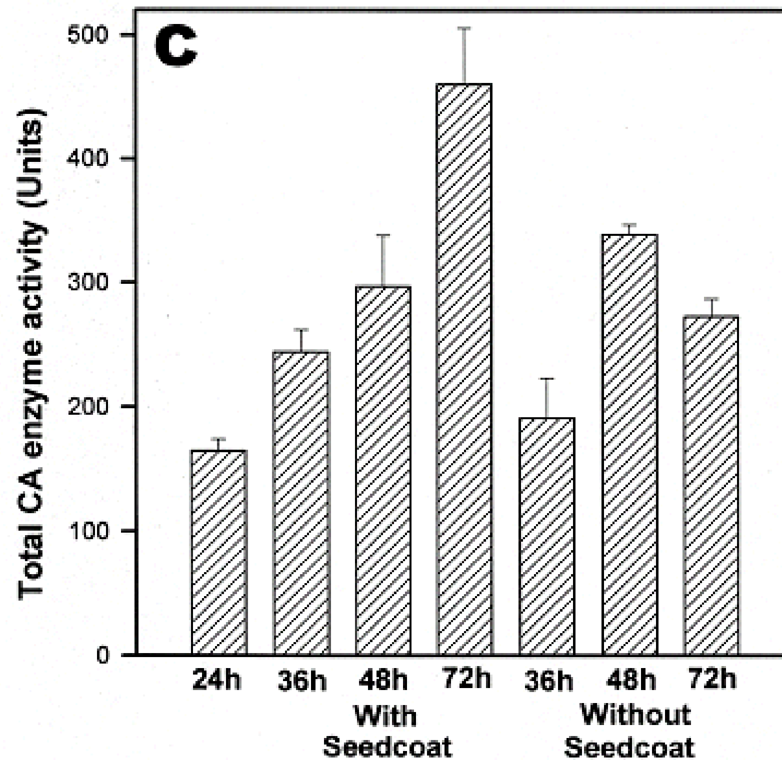
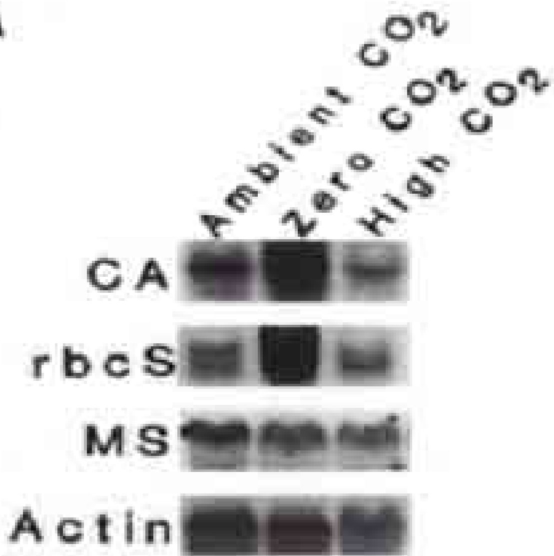


Figure 4. Influence of seed coat on CA expression in cotyledons of cotton seedlings (A) Northern blot analyses of *CA*, *MS*, and actin transcripts in cotyledons of cotton seedlings. Seed coats were removed from 24 h dark-grown seedlings and incubated for an additional 12 h to 48 h (for a total of 36 h to 72 h periods) in the dark under ambient CO_2 conditions. (B) A histogram quantifying CA:actin (open bars) and MS:actin (solid bars) ratios of integrated band intensity from Northern blots. (C) Total CA activity in protein extracts of cotyledons with or without seed coats. Bars represent the mean and standard deviation of three independent experiments.

affected by the changes in CO_2 conditions. Nonetheless, regardless of CO_2 conditions, cotyledons exposed to light (hatched bars) showed substantial increase in CA and Rubisco activities (Fig 6A, B). Exposure of cotyledons to light, resulted in a measurable increase in total chlorophyll content at all CO_2 conditions (Fig 6C), indicating proplastid to chloroplast transition was not particularly sensitive to [CO_2] but mostly influenced by light. These results suggest that steady-state levels of *CA* transcripts (and *rbcS*) are up-regulated in response to low CO_2 conditions, however, light is required for increases in CA and (Rubisco) activities probably in parallel with development of a functional chloroplasts.


A
Ambient CO2
Zero CO2
High CO2
CA
rbcS
MS
Actin

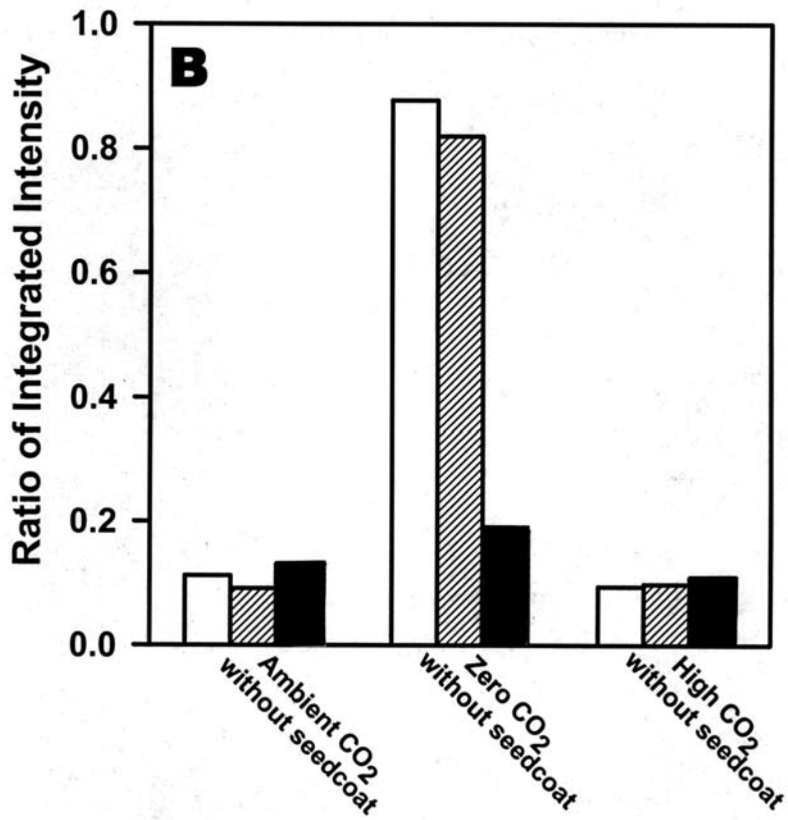
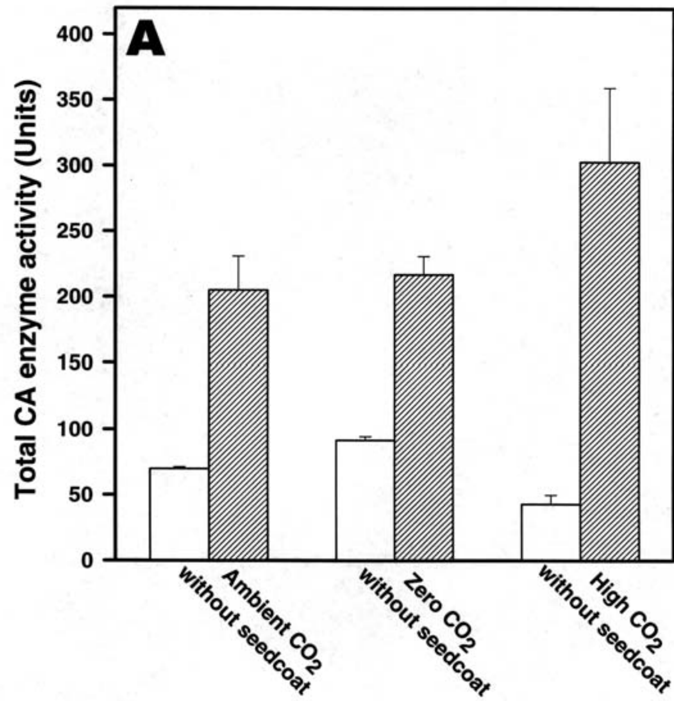
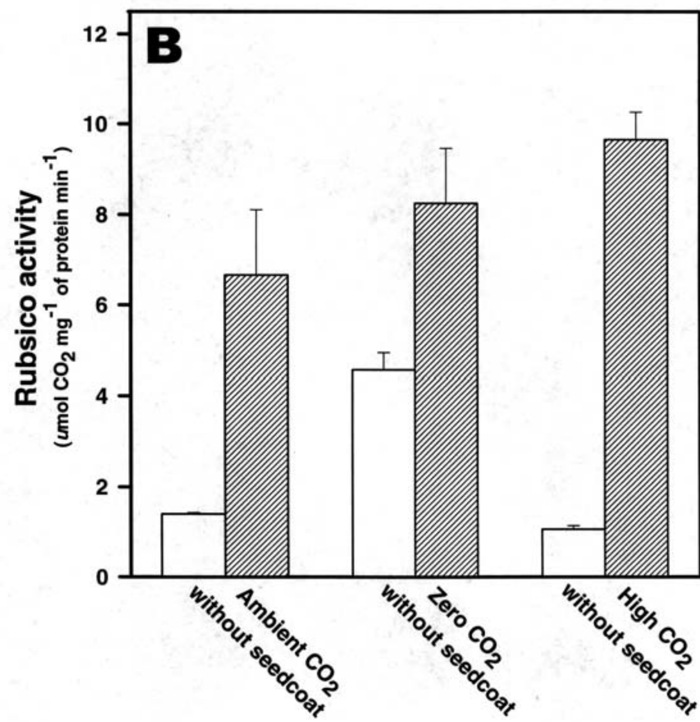

B
Ratio of Integrated Intensity
1.0
0.8
0.6
0.4
0.2
0.0
Ambient CO2 without seedcoat
Zero CO2 without seedcoat
High CO2 without seedcoat

Figure 5. Effect of CO_2 level on CA (*GhCA*1) and Rubisco small subunit (*rbcS*) relative transcript levels in cotyledons of 48 h dark-grown seedlings. (A) Northern blot analyses of total RNA extracted from cotyledons of seedlings germinated in the dark without seed coats under high (1000 ppm), zero or ambient (350 ppm) CO_2 conditions. The predicted size of Rubisco (*rbcS*) transcripts is 1400 bp. (B) A histogram quantifying CA:actin (open bars), Rubisco:actin (hatcted bars), and MS:actin (solid bars) ratios of integrated band intensity from Northern blots. The ratios were calculated by using NIH Image software and are from a single experiment. Similar trends were observed in replicate experiments.


A
Total CA enzyme activity (Units)
0
50
100
150
200
250
300
350
400
Ambient CO_2
without seedcoat
Zero CO_2
without seedcoat
High CO_2
without seedcoat


B
Rubsico activity
(μmol CO_2 mg^{-1} of protein min^{-1})
0
2
4
6
8
10
12
Ambient CO_2
without seedcoat
Zero CO_2
without seedcoat
High CO_2
without seedcoat

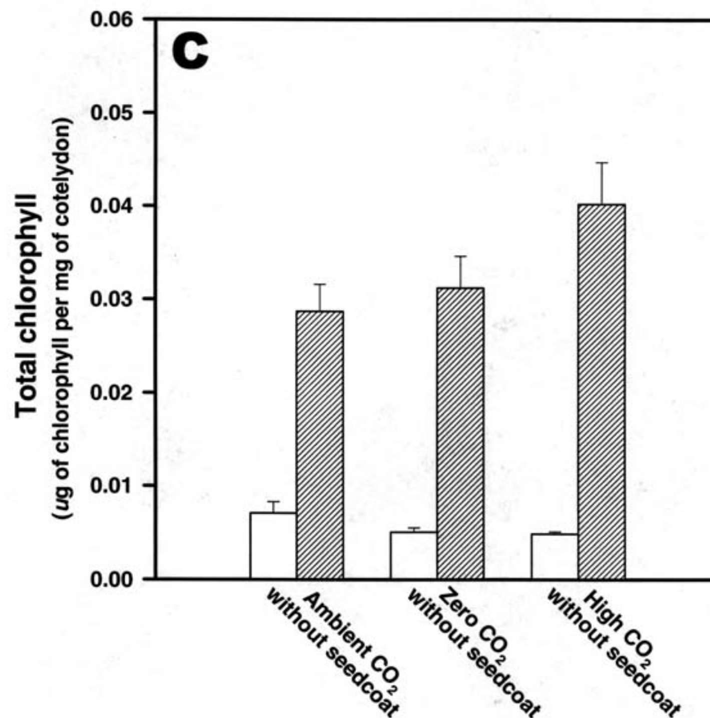


Figure 6. Total CA and Rubisco activities in cotyledons of 48 h seedlings under different environmental conditions. (A) Total CA activities were measured in protein extracts of seedlings (cotyledons) grown without seed coats in the dark (open bars) or light (hatched bars) under different CO_2 conditions. (B) Total Rubisco activities were measured in the same samples. (C) Total chlorophyll amount in cotyledons of 48 h seedlings exposed to the dark (open bars) or light (hatched bars) under different CO_2 conditions. Bars represent the mean and standard deviation of three independent experiments.

Discussion

We report here that the regulation of *CA* (and *rbcS*) transcript levels and enzyme activity in cotyledons is modulated by different environmental factors during post-germinative growth of cotton seedlings. The data revealed that CA and *rbcS* transcript levels are regulated in response to external [CO_2] in cotton seedlings. Exposure of seedlings to light did not affect *CA* (or *rbcS*) transcript levels (Fig 3A), however, both CA (and Rubisco) activities (Fig 3C, 6B) were increased in the light when compared to dark-grown seedlings. Collectively, these results indicated that external CO_2 regulates *CA* and *rbcS* transcript levels and light modulates functional CA and Rubisco activities.

When cotton seedlings were exposed to zero CO_2 conditions, *CA* (and *rbcS*) relative transcript levels in cotyledons increased significantly when compared to cotyledons of seedlings grown under high (1000 ppm) or ambient (350 ppm) CO_2 conditions (Fig 5A). This suggests that cotton seedlings may have a sensory mechanism that perceives the changes in the level of environmental CO_2. Specifically which mechanism is (are) responsible to activate the expression of *CA* (and *rbcS*) under zero CO_2 conditions is unknown. In cyanobacterium, *Synechococcus* sp. PCC7942, Ronen-Tarazi et al. (1995) reported the existence of a low- CO_2-inducible promoter, but the detailed sequence(s) of the CO_2-responsive element was not identified. Also, Villand et al. (1997) showed that a CO_2-reponsive element controls the regulation of a mitochondrial *CA* (β-CA) gene expression in *C. reinhardtii* under low CO_2 conditions. Furthermore, Kucho et al. (1999) indicated that up-stream of the periplasmic *CA* (*CAH*1) gene there are enhancer and silencer regions responsive to external CO_2 concentrations.

These regions control the transcriptional regulation of *CAH*1 gene expressions in *C. reinhardtii* under changing CO_2 conditions. Perhaps, the *CA* (and *rbcS*) genes in cotton seedlings also contain CO_2-responsive elements in its 5'- upstream region to regulate the transcript levels in response to external CO_2 conditions. At present, it is not known whether *CA* (and *rbcS*) gene expression in cotyledons of cotton seedlings are induced under low- CO_2 conditions or are repressed under high and ambient CO_2 conditions.

The results presented here revealed that *CA* and *rbcS* transcript levels did not correlate proportionally with enzyme activity under different CO_2 conditions in dark-grown cotton seedlings (Fig 4, 5, 6). This suggests that the regulation of CA (and Rubisco) activity is not due to external CO_2 conditions. Regardless of the different CO_2 conditions, seedlings exposed to light showed significant increases in CA and Rubisco activities when compared to dark-grown seedlings (Fig 6A, B). It is possible that the regulation of CA and Rubisco activities are due to different factors such as structural or metabolic changes, which are induced when proplastids are converted to chloroplasts in these cotyledons. A stimulation of CA activity in pea thylakoids was observed when plants were grown under high light (Moskvin et al., 2000), but the mechanism for activation is unknown. Non-photosynthetic proplastids have a dense stroma and lack the inner membrane found in chloroplasts (Whatley, 1991). The proplastid to chloroplast transition in greening cotyledons (Reiter et al., 1994; McCormac and Greenberg, 1992) likely allows for a more efficient synthesis, import, and assembly of active CA and Rubisco enzymes. Consequently, we conclude that the increase in CA and Rubisco enzyme activities observed in cotyledons exposed to light, likely results from an increase

in cellular machinery for chloroplast biogenesis, although other factors may contribute as well (such as alkalinization of the stroma of greening cotyledons).

Alternatively, we cannot rule out that increased CA activity in the light is a result from increased expression of an alternative, "light dependent" CA isoform with a substantially different nucleotide sequence so as to not cross hybridize on Northern blots. This possibility seems remote, since *rbcS* and *CA* expression in this case appears to be coordinately regulated. Also, we isolated four cDNA clones encoding CA from cotyledons of 48 h cotton seedlings which fell into two nearly identical groups (*GhCA*1 and *GhCA*2) with just a few nucleotide differences in the ORF (Hoang et al., 1999). Our probe does not distinguish between these two nearly identical cDNA's and consequently signals on Northern blots represent combined expression of these cotyledon cDNAs. Moreover, expression of a "light-dependent" isoform would exaggerate further the uncoupling of *CA* mRNA accumulation and acquisition of functional enzyme activity.

In conclusion, we propose that CA expression is transcriptionally regulated by CO_2 concentration and post-transcriptionally regulated by light. Physiologically, in the epigeous cotyledon system of cotton seedlings, these two factors likely cooperate to coordinate the development of photoautotrophic organs as the seed coat is shed during seedling emergence.

Acknowledgements

The authors thank Dr. Rick Turley (USDA-ARS; Stoneville, MS) for providing vectors containing *MS* and *psbR* and Dr. Rebecca Dickstein (UNT-Biochemistry) for critically reviewing this manuscript. This research was supported by a grant from the Herman Frasch Foundation (#427-HF97) for Agricultural Chemistry.

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CHAPTER 3

BIOCHEMICAL AND MOLECULAR INHIBITION OF PLASTIDIAL CARBONIC ANHYDRASE REDUCES LIPID SYNTHESIS IN COTTON (*GOSSYPIUM HIRSUTUM* L.) EMBRYOS AND TOBACCO (*NICOTIANA TABACUM* L.) CELL SUSPENSIONS AND LEAVES

(In preparation for submission to Plant Physiology)

Abstract

Two cDNAs encoding functional carbonic anhydrase (CA) enzymes were recently isolated from a non-photosynthetic, cotyledon library of cotton (*Gossypium hirsutum* L.) seedlings with putative plastid targeting sequences (Hoang et al., 1999). Relative *CA* transcript abundance and enzyme activity increased 9- and 15- times respectively in cotton embryos during the maximum period of reserve oil accumulation. Specific sulfonamide inhibitors of CA activity significantly reduced the rate of [^{14}C]-acetate incorporation into total lipids in cotton embryos *in vivo*, and in embryo plastids *in vitro*, suggesting a role for CA in plastid lipid biosynthesis. CA-inhibitors did not affect acetyl-CoA carboxylase (ACCase) activity or total storage protein synthesis. Similar results were obtained for two other plant systems - - cell suspensions (and isolated plastids therefrom) of tobacco (*Nicotiana tabacum* L.), and chloroplasts isolated from

leaves of transgenic CA antisense-suppressed tobacco plants (5% of wild-type CA activity). In addition, tobacco cell suspensions treated with 1 mM ethoxyzolamide had a significant amount of CO_2 loss when compared with controls (DMSO). Together, these results indicate that a reduction of CA activity (biochemical or molecular inhibition) impacts the rate of plant lipid biosynthesis, by impairing the ability of CA to efficiently "trap" inorganic carbon inside plastids for utilization by ACCase and the fatty acid synthesis machinery.

Abbreviations

ACCase, acetyl-CoA carboxylase; CA, carbonic anhydrase; CCM, CO_2 concentrating mechanism; DMSO, dimethyl sulfoxide; PEP carboxylase, phosphoenolpyruvate carboxylase; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SSC, 150 mM sodium chloride and 15 mM sodium citrate (pH 7.0); TAG, triacylglycerol; TCA, trichloroacetic acid; TLC, thin layer chromagraphy; WT, wild-type

Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) is a zinc-containing metalloenzyme that catalyzes the reversible hydration of CO_2 to HCO_3^-. The widespread abundance of CA isoforms in plants, animals and microorganisms suggest that this enzyme has many diverse roles in biological processes. CA plays a critical role in biological systems because CO_2 gas is the membrane permeable form of inorganic carbon for cells, and, in general, the uncatalyzed interconversion between HCO_3^- and CO_2 is slow when compared to the required rate in living cells (Badger and Price, 1994).

In photosynthetic organisms, one generally accepted physiological role of CA is to provide sufficient levels of inorganic carbon as part of a CO_2 concentrating mechanism (CCM) for improved photosynthetic efficiency. In *Chlamydomonas reinhardtii*, Badger and Price (1992) suggested that chloroplastic CA plays a role in photosynthetic carbon assimilation by converting accumulated pools of HCO_3^- to CO_2, which is the substrate for ribulose-1,5-bisphosphate carboxylase/oxyenase (Rubisco). Moroney et al. (1985) revealed that the reduction of periplasmic CA activity by using CA specific inhibitors significantly reduced the efficiency of external inorganic carbon utilization for photosynthesis. Like green algae, CA in cyanobacteria plays an important role in the CCM and in photosynthesis (Badger and Price, 1994). Price et al. (1992) reported that there was an association of CA with Rubisco in the carboxysome of cyanobacteria (*Synechococcus*). Also, Maeda et al. (2000) identified a gene (*cmpA*), which encodes a substrate-binding protein that can specifically bind to HCO_3^- in cyanobacteria, which may further aid in the diffusion of HCO_3^- and elevation of CO_2 in the carboxysome. In C_4

plants, CA is localized to the cytosol of mesophyll cells where it supplies HCO_3^- to phosphoenolpyruvate carboxylase (Hatch and Burnell, 1990), and calculations show that without CA the rate of photosynthesis is reduced. In fact, a recent study with transgenic *Flaveria bidentis* clearly supported a role for CA in the assimilation of CO_2 in C_4 plants (Ludwig et al., 1998). Carbonic anhydrase in C_3 plants is distributed primarily in the stroma of chloroplasts (Poincelot, 1972) and is speculated to mediate in the diffusion of CO_2 from the cytosol to the site of carboxylation by Rubisco in the chloroplast stroma during photosynthesis (Reed and Gram, 1981). Thus the principal function of CA in photosynthetic organisms is to support the efficient assimilation of inorganic carbon for the primary carboxylation reactions.

In animals, CAs have been shown to provide inorganic carbon to other important metabolic pathways such as pyrimidine biosynthesis, gluconeogensis, and lipogenesis (Sly and Hu, 1995), all requiring HCO_3^- as the inorganic carbon substrate for initial carboxylation reactions. Cytological and biochemical evidence point to a metabolic role for CA in lipogenesis. For example, acetyl-CoA carboxylase (ACCase) was co-localized with carbonic anhydrase in oligodendrocytes and fatty acid synthase was localized by immunostaining to be in the same cell type (Cammer, 1991). Lipogenesis was inhibited by acetazolamide, a CA specific inhibitor, in human adipose tissue (Bray, 1977). The administration *in vivo* of acetazolamide in female mice resulted in decreased fatty acid synthesis (Cao and Rous, 1978). In addition, Herbert and Coulson (1983) demonstrated that *de novo* fatty acid synthesis (measured by [^{14}C]-acetate incorporation into total lipid) in liver of American chameleons (*Anolis carolinensis*) was inhibited by CA- specific

inhibitors (ethoxyzolamide and acetazolamide). CA was suggested to play a role in *de novo* lipogenesis in hepatocytes by increasing the rate of CO_2 hydration to bicarbonate for acetyl-CoA carboxylase (Dodgson et al., 1984), and Lynch et al. (1995), reported a reduction of [^{14}C]-acetate incorporation into total lipid in rat hepatocytes incubated with CA inhibitors trifluoromethylsulphonamide and ethoxyzolamide. Together these results support the notion that CA assists in providing HCO_3^- for lipid biosynthesis in animal systems.

Evidence in the literature from other eukaryotes and some preliminary results from our laboratory lead us to formulate a working hypothesis that plastidial CA in C_3 plants plays a role in lipid biosynthesis. Recently cDNAs were identified in a dark-grown cotton seedling library that encoded functional CA enzymes with putative plastid targeting sequences (Hoang et al., 1999). The expression of CA increased (estimated by relative mRNA abundance and specific enzyme activity) during the period of storage lipid accumulation in maturing embryos of cotton (Hoang et al., 1998 and Fig 1). In developing oilseeds, the majority of fatty acids that are synthesized in plastids are exported to the ER and converted to storage lipids (Ohlrogge and Browse, 1995). ACCase requires HCO_3^- as a substrate and is considered to be the rate-limiting and committed step in fatty acid biosynthesis (Ohlrogge and Jaworski, 1997) (Fig 1). Here we provide evidence to support our hypothesis in three different plant systems a) maturing embryos of developing cotton seeds, b) cell suspensions of tobacco, and c) leaves of transgenic tobacco plants. Our results indicate that treatment of plant cells with CA-specific inhibitors reduced the rate of lipid synthesis (from [^{14}C]-acetate) *in vivo* and

in vitro. In addition, molecular suppression of CA activity to 2% of wild-type levels (antisense-suppressed plants, Price et al., 1994) reduced lipid biosynthesis in chloroplasts from transgenic plants compared with chloroplasts from WT plants. We propose that CA is involved in lipid synthesis (and perhaps other HCO_3^- requiring pathways in plastids) indirectly, serving to "concentrate" CO_2 in plastids as HCO_3^- and reduce the rate of CO_2 diffusion out of plastids.

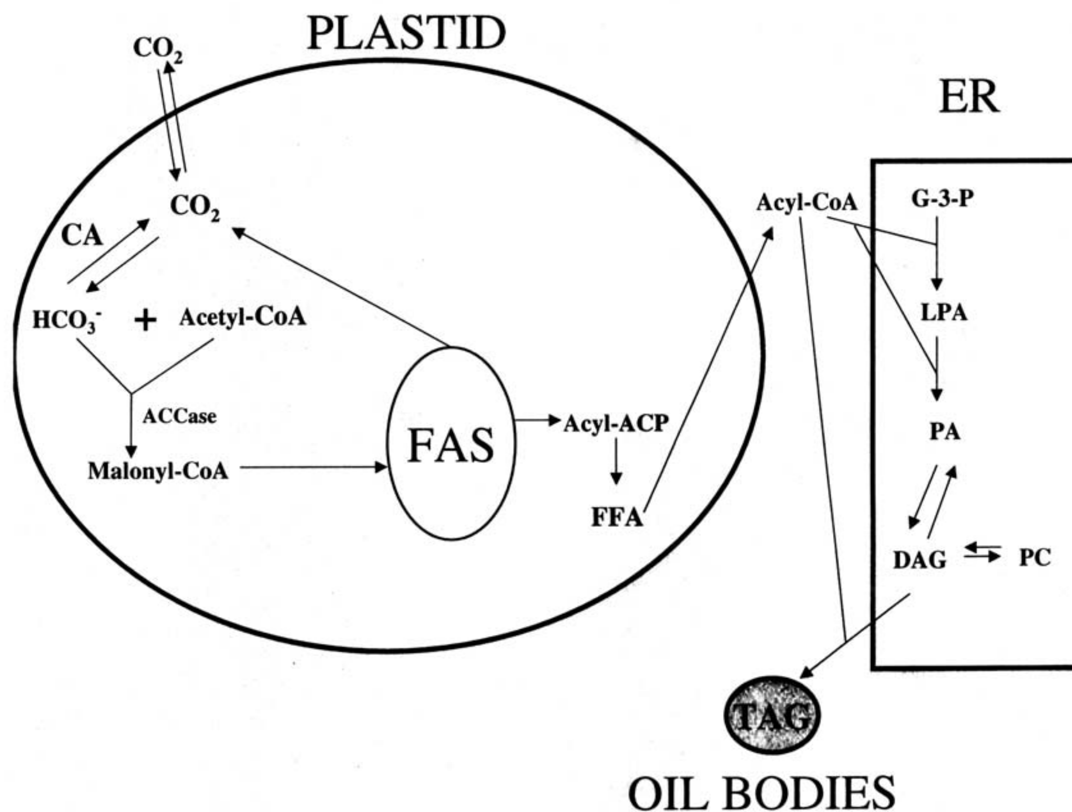


Figure 1: Schematic diagram depicting the flow of carbon to the fatty acid synthase complex and the synthesis of storage lipid in plants (carbonic anhydrase, CA; acetyl-CoA carboxylase, ACCase; fatty acid synthase, FAS; acyl carrier protein, ACP; free fatty acid, FFA; glycerol-3-phosphate, G-3-P; lysophosphatidic acid, LPA; phosphatidic acid, PA; diacylglycerol, DAG; phosphatidylcholine, PC; triacylglycerol, TAG; endoplasmic reticulum, ER).

Materials and Methods

Plant Material

Cottonseeds (*Gossypium hirsutum* L., cv. Paymaster HS26) were provided by Dr. John Burke (USDA-ARS, Lubbock, TX). Wild-type and transgenic tobacco (*Nicotiana tabacum* L., SR1) seeds (Price et al., 1994) were kindly provided by Dr. G. Dean Price (Australian National University, Canberra, Australia). Cotton and tobacco plants were grown in a greenhouse or growth room with 14 h photoperiod (supplemented with sodium lamps to extend day length when necessary) and temperatures of approximately 38°C during the day and 25°C at night. Plants were watered daily and fertilized biweekly with a dilute solution of Miracle Gro™. The age of cotton bolls and developing embryos was determined by tagging flowers at anthesis and by ovule morphology (Choinski and Trelease, 1978).

Tobacco leaves of similar sizes and developmental stages were harvested for chloroplast isolation. Tobacco (*Nicotiana tabacum* cv., *xanthi*) cell suspensions were grown and maintained as described previously (Chapman et al., 1995). Cell suspensions in log phase (72 h after subculture) were used for CO_2 measurements, plastid isolations and [^{14}C]-acetate labeling experiments.

Total lipid extraction / Thin layer chromatography

Total lipids were isolated from cotton embryos, tobacco cell suspensions, and plastids essentially as described previously (Chapman and Moore, 1993). The chloroform fraction was washed three times with 1 M KCL and then the chloroform/lipid

mixture was evaporated to dryness with N_2. Total lipids were resuspended in 50 μl of chloroform and analyzed by fractionation on TLC. Total lipids were fractionated on TLC plates (Whatman silica gel G-60, μm) for 40 min in hexane:diethylether:acetic acid (80/20/1, v/v) solvent system. Radiometric scanning (Bioscan system 200 Imaging Scanner, Bioscan, Washington D.C.) was used to quantify the radiolabeled lipids after TLC separation (Chapman and Sprinkle, 1996).

Enzyme assays

Freshly harvested embryos were weighed and then frozen in liquid nitrogen. The frozen samples were ground with a mortar and pestle in a 1:1 (w/v) homogenization buffer containing 400 mM sucrose, 100 mM Na phosphate (pH 7.2), 10 mM KCl, 1 mM $MgCl_2$, and 1 mM EDTA. Homogenates were filtered through 4 layers of cheesecloth and the filtrate was analyzed immediately for CA activity. Also, embryo homogenates were preincubated with sulfanilamide, acetazolamide and ethoxyzolamide inhibitors for 30 min prior to assaying for CA activity. Protein content in cell-free extracts was determined according to Bradford (1976) using bovine serum albumin for standard curve calibration. Total CA activity was determined electrometrically (Wilber and Anderson, 1948) as described previously (Hoang et al., 1999). One unit of activity (Wilbur-Anderson, WA unit) was defined as $10[(T_o/T)-1]$, where T_o and T are equal to the rate of pH change of the reaction without (control) and with cell homogenates, respectively.

Embryos were homogenized in 1:1 (w/v) buffer (same buffer used as in preparation of fractions for carbonic anhyrase assays) with a polytron (Brinkmann Instruments, PT10/35, speed at 8). Embryo homogenates were preincubated with

different inhibitors for 30 min prior to assaying for ACCase activity. ACCase activity was based on acetyl-CoA-dependent $H^{14}CO_3^-$ (.05 μCi per sample)(8.4 mCi/mmol) incorporation into acid-stable product (malonyl-CoA)(Roesler et al., 1996). The production of [^{14}C]-malonyl-CoA was quantified by liquid scintillation counting (same conditions as above).

mRNA isolation and Northern blot analyses

Total cellular RNA was isolated from embryos of cotton plants by a hot borate procedure developed by Wan and Wilkins (1994). RNA yield and quality were evaluated spectrophotometrically and by analytical gel electrophoresis according to Sagerström and Sive (1996). Poly A^+RNA from cotton embryos was isolated by oglio-dT cellulose column chromatography according to Aviv and Leder (1972). Approximately 2 μg of mRNA was electrophoresed in a 1% agarose gel containing 6% formaldehyde and 1 x MOPS Buffer (20 mM MOPS-NaOH, pH 7.0, 5 mM sodium acetate, 0.1 mM EDTA) (Sagerström and Sive, 1996). RNA was transferred to nylon membranes by capillary transfer with 20 x SSC (overnight) and probed with a random prime-labeled (Gene Images™ random prime-labeling module, Amersham) 1.16 kb carbonic anhydrase and 539 bp actin probe (Hoang et al., 1999). Hybridization and washing were both carried out at 62°C and with a final wash at 0.2 x SSC. Hybridized bands were identified by an alkaline phosphatase-catalyzed chemiluminescent reaction (Gene Images CDP-Star detection module, Amersham) and quantified by densitometric scanning (NIH v. 6.1 image software).

Incorporation of [^{35}S] methionine into proteins *in vivo*

Cotton embryos were excised from bolls and placed on moist filter paper in petri dishes. CA inhibitors in 5 μl at different concentrations (0.1 to 10 mM) were dispensed onto each embryo 30 min prior to the addition of [^{35}S]-methionine (2 μCi per embryo)(1.175 mCi/μmol). After radiolabeling for 1 h with [^{35}S]-methionine the embryos were homogenzied in a 1:1 (w/v) buffer (same buffer used above in carbonic anhydrase assays) and the total proteins from cell-free homogenate were precipitated in TCA (4% w/v final concentration). TCA precipitated was washed twice with 70% ethanol, once with diethyl ether then resuspended in 0.4 M NaOH. Insoluble material was removed by centrifugation and supernatants were air-dried overnight (Coligan et al., 1983). Liquid scintillation counting (Beckman LS 3861 counter and ScintiSafe Plus 50% LSC cocktail) was used to quantify [^{35}S]-methionine incorporation into total protein.

Enzyme-specific inhibitors

CA-specific sulfonamide inhibitors (Maren 1967) were utilized for *in vivo* and *in vitro* [^{14}C]-acetate incorporation experiments into total lipids. These were 4-aminobenzene-sulfonamide (sulfanilamide), 5-acetamido-1,3,4-thiadiazole-2-sulfonamide (acetazolamide) and 6-ethoxy-2-benzothiazolesulfonamides (ethoxyzolamide) (Sigma Chemical Co). Sulfanilamide was dissolved in water, while acetazolamide and ethoxyzolamide inhibitors were dissolved in 1:2 v/v DMSO:water.

Measurements of CO_2 released from tobacco cell suspensions

Tobacco cell suspensions in log phase were incubated with 10 μM, 100 μM, and 1000 μM ethoxyzolamide and subsequently CO_2 free air (scrubbed with soda lime) was

flush through the system. Thus only CO_2 released from tobacco cell suspensions either treated with control (DMSO) or ethoxyzolamide were monitored in line with a plant CO_2 analysis system (Qubit Systems Inc., Canada).

Incorporation of [^{14}C]-acetate into total lipids of cotton embryos and tobacco cells *in vivo*

Cotton bolls were harvested and immediately placed on ice. The embryos were excised from the ovules and then placed on wet filter paper. CA inhibitors in 5 μl at different concentrations (0.1 to 10 mM) were dispensed onto each embryo and then incubated for 30 min prior to the addition of radiolabeled [2-^{14}C]-acetate (54 mCi/mmol). Total lipids were extracted at different time points after the addition of the 0.5 μCi of radiolabeled acetate (Chapman and Moore, 1993). Aliquots of total lipids dissolved in chloroform were evaporated to dryness then the incorporation of [^{14}C] acetate was quantified by liquid scintillation counting (Beckman LS 3861) or by radiometric scanning following TLC.

Tobacco cell suspensions in log phase were used for the [^{14}C]-acetate incorporation experiments *in vivo*. Tobacco cells were collected, washed and suspended in fresh medium. Two mL of the suspended cells were transferred to 15 mL tube and preincubated with CA inhibitors for 30 min prior to the addition of radiolabeled acetate. Aliquots of the total lipids were analyzed and quantified as described above.

Plastid isolation

Tobacco plants were taken out of the growth room and placed in the dark for 48 h in preparation for chloroplast isolation to reduce the amount of starch and allow for

higher yields of intact chloroplasts. Chloroplasts were isolated according to Yu and Woo (1988), but with the following modifications. The pellet at 800 x g for 5 min was layered over a Percoll gradient composed of 3 mL of a 90%, 15 mL of 35% and 10 mL of 15% percoll. Intact chloroplasts were recovered and the chlorophyll content was estimated according to Bruinsma (1961) in 80% acetone. Plastids were isolated from tobacco cell suspensions (in log phase) and cotton embryos by centrifugation through a 10% Percoll gradient (Trimming and Emes, 1993; Sparace and Mudd, 1982). Plastid protein contents were estimated according to Bradford (1976).

Incorporation of [^{14}C]-acetate into total plastid lipids *in vitro*

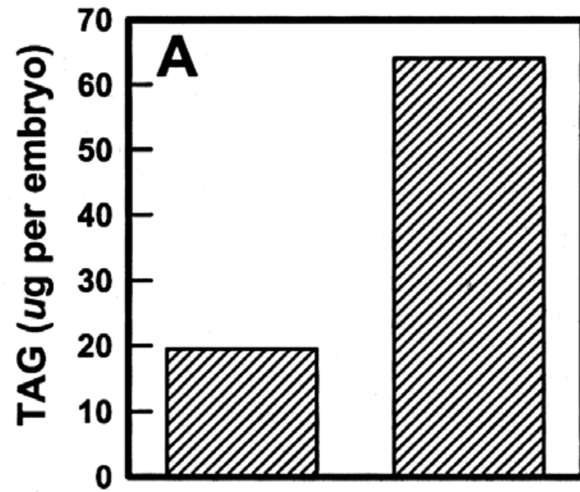
The conditions used for assaying the incorporation of the [2-^{14}C]-acetate into fatty acids in isolated plastids are described in Stahl and Sparace (1991). The reaction mixture contained 0.02 µM Na-[2-^{14}C]acetate, 0.04 mM cold Na-acetate, and 3 mM each of $MgCl_2$ and ATP and 50 µg to 100 µg of plastid protein and 0.167 mg of chlorophyll.

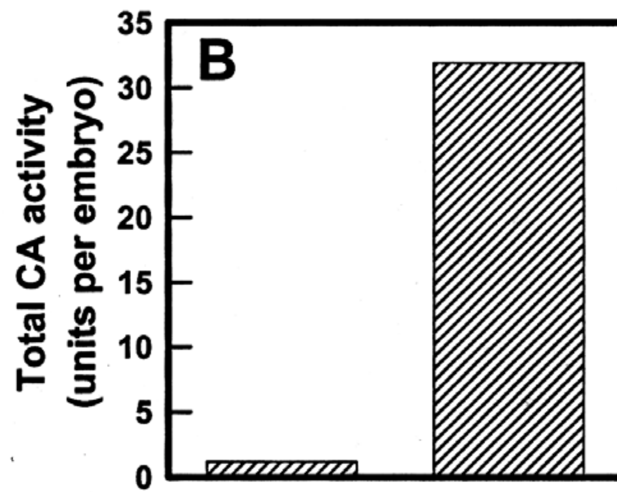
Results

CA and lipid synthesis in embryos

To examine whether CA could be involved in lipid biosynthesis in developing cotton embryos, we compared CA activities and expression prior to (25 DPA) and during (40 DPA) the maximum period of TAG accumulation (Fig. 2). Total CA activity and steady-state *CA* mRNA levels increased approximately 15- and 9- fold, respectively, during embryo maturation and oil accumulation. It is possible that this substantial increase in CA activity is required to supply the intense demand for inorganic carbon for lipid biosynthesis in developing embryos.

To further determine whether CA could play a role lipid biosynthesis in cotton embryos (32 DPA), CA activity was reduced by preincubation of embryo extracts with CA specific inhibitors (at 1 mM and 10 mM sulfanilamide, acetazolamide or ethoxyzolamide) (Fig 3). Both ethoxyzolamide and acetazolamide at 1 mM or 10 mM concentration inhibited more than 50% of CA activity in homogenates cotton embryos, however ethoxyzolamide was the most potent CA inhibitor. Embryos at 32 DPA were radiolabeled with [^{14}C]-acetate *in vivo* and the incorporation of [^{14}C]-acetate into total cottonseed lipids was quantified (Fig 4). Cotton embryos (30-38 DPA) incubated with CA inhibitors at 1 mM and 10 mM ethoxyzolamide showed a significant reduction in the rate of [^{14}C]-acetate incorporation into total lipids compared with controls (no inhibitors). A linear rate of lipid synthesis was established within 10 min of incubating embryos with [^{14}C]-acetate (Fig 4) and continued up to 30 min. A 50% reduction in the rate of


A
TAG (ug per embryo)
70
60
50
40
30
20
10
0


B
Total CA activity
(units per embryo)
35
30
25
20
15
10
5
0

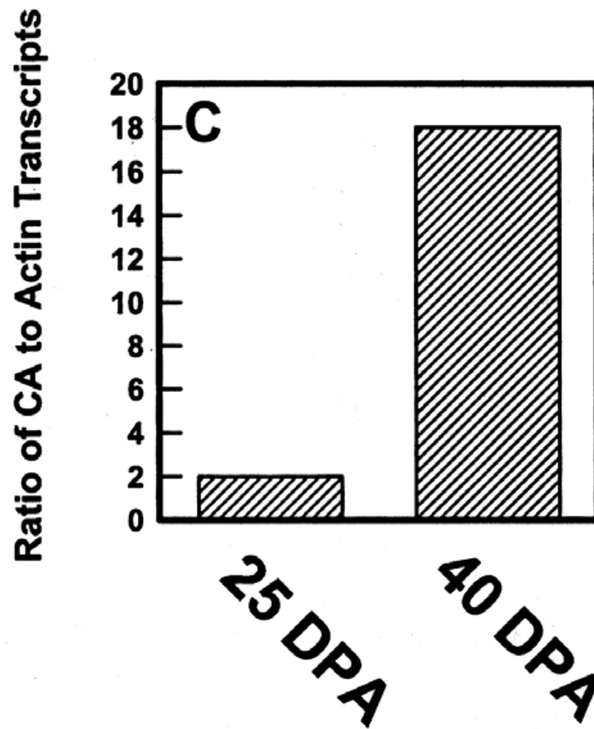


Figure 2. Comparison of triacylglycerol content (A), total CA activity (B), and relative CA expression (C) in embryos excised from cotton bolls at 25 DPA and 40 DPA. The relative amounts of TAG in lipid extracts were estimated by scanning densitometry (NIH Image) of TLC fractionated lipid classes in comparison with triolein standards. Total CA activity was determined electrometrically in cell free homogenates. Poly A^{+}RNA was isolated from cotton embryos and the ratios of CA to actin transcripts were determined by Northern blot analyses. The results depicted here are representative of replicate experiments.

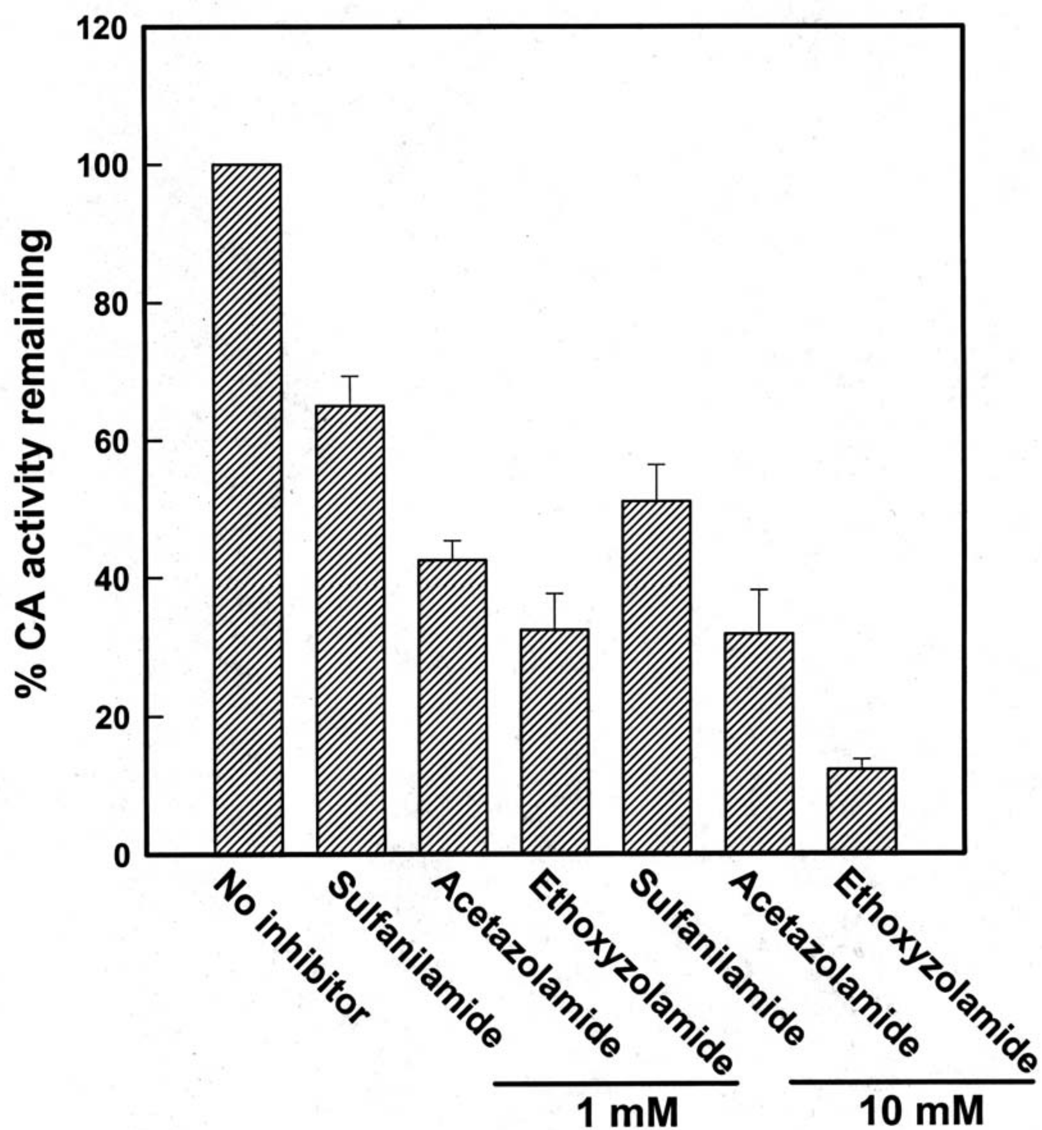


Figure 3. Effects of different carbonic anhydrase inhibitors in homogenates of cotton embryos (34 DPA). The graph shows the percent remaining CA activity when inhibited with 1 mM and 10 mM sulfanilamide, acetazolamide, and ethoxyzolamide. Values shown represent the means and standard deviations from three separate experiments.

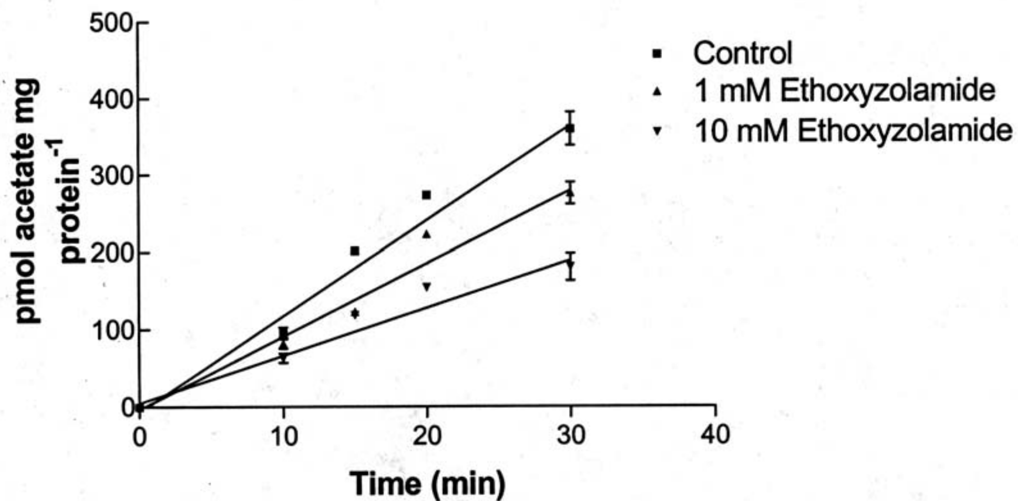


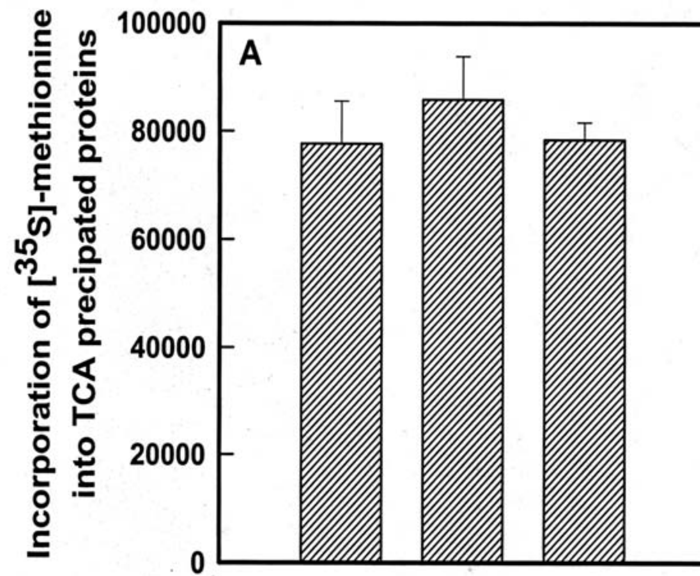
Figure 4. Time-dependent incorporation of [^{14}C]-acetate into cottonseed total lipids. Embryos (30-38 DPA) were preincubated with dimethyl sulfoxide (control) or different concentrations of CA inhibitors. Both 1 mM and 10 mM concentrations of ethoxyzolamide were incubated for 30 min prior to the addition of radiolabeled [^{14}C]-acetate. Total lipids were extracted at 10 min, 15 min, 20 min and 30 min after the addition of the radiolabeled acetate. Incorporation of [^{14}C]-acetate into lipids was not linear after 30 min. Aliquots were used to quantify the incorporation of [^{14}C]-acetate into embryos by liquid scintillation counting. Data points represent mean and standard deviation of three independent experiments for 10 min and 30 min. Lines are plotted from linear regression analyses (Graphpad Prism V. 3.02) of the data with r^2=0.97 for controls, r^2=0.97 for 1 mM ethoxyzolamide and r^2=0.94 for 10 mM ethoxyzolamide.

Rates estimated from linear regression analyses were 12.36±0.71, 9.42±0.52, and 6.15±0.53 pmol acetate min^{-1} mg protein $^{-1}$, respectively.

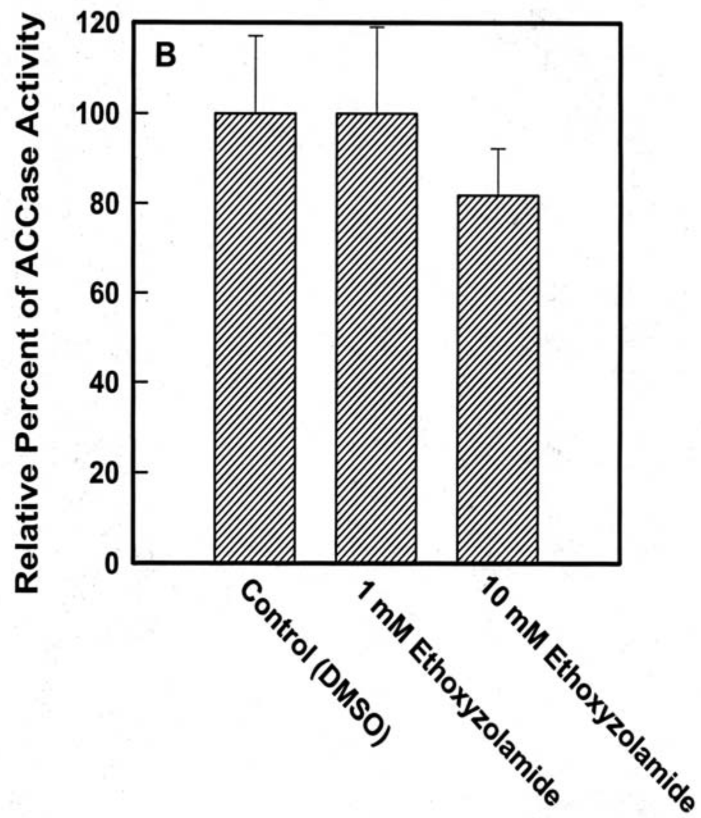
radiolabeled acetate incorporation into total lipids was observed for embryos treated with 10mM ethoxyzolamide when compared to controls (no inhibitors). Application of inhibitors appeared to be specific for reserve lipid accumulation, since storage protein synthesis, measured by [^{35}S]-methionine incorporation into total protein, was relatively unaffected (Fig 5A). We confirmed that CA-specific inhibitors did not inhibit ACCase activity significantly *in vitro* (Fig 5B) to reduce lipid synthesis *in vivo*.

The distribution of radioactivity in major lipid classes after incorporation of [^{14}C]-acetate for 30 min in cotton embryos was evaluated by TLC (summarized in Table I). About 82% of the radioactivity was in polar lipids (mostly phosopholipids) and 18% was in the nonpolar lipids (free fatty acids and triacylglyercol) of untreated cotton embryos. Embryos incubated with 10 mM ethoxyzolamide or acetazolamide had a distribution of [^{14}C]-acetate in polar and nonpolar lipids (albeit lower overall) similar to DMSO controls, suggesting that lipid synthesis overall was reduced and that inhibition was not selective for extraplastidial FA elongation.

To examine specifically whether *de novo* fatty acid synthesis in cotton embryos was influenced by CA inhibitors, we isolated plastids from cotton embryos (34 DPA). Acetate is commonly used as a radioactive tracer in *in vitro* studies of fatty acid biosynthesis because it can be incorporated efficiently into fatty acids (Qi et al., 1995; Roughan and Ohlrogge, 1996). Plastids treated with 10 mM ethoxyzolamide revealed an approximately 67% reduction of the rate of lipid synthesis (mostly FFA) when compared to controls (Table II). Incorporation of [^{14}C]-acetate into plastid lipids was inhibited to a lesser extent by acetazolamide. Hence, results from both *in vivo* and *in vitro* [^{14}C]-acetate



A
Incorporation of [35S]-methionine into TCA precipated proteins
100000
80000
60000
40000
20000
0



B
Relative Percent of ACCase Activity
120
100
80
60
40
20
0
Control (DMSO)
1 mM Ethoxyzolamide
10 mM Ethoxyzolamide

Figure 5. Comparison of ACCase activities *in vitro* (A) and in embryo protein synthesis *in vivo* (B) in absence or presence of CA inhibitors. Embryos (34 DPA) were preincubated with ethoxyzolamide and acetazolamide 30 min prior to the addition of [^{35}S]-methionine. After 1 h of incubation , the incorporation of [^{35}S]-methionine into TCA precipitated protein was quantified by liquid scintillation counting. ACCase activity (34 DPA) was assayed. The embryo homogenates were preincubated with DMSO (control), 1 mM ethoxyzolamide, 10 mM ethoxyzolamide, or 10 mM acetazolamide for 30 min prior to homogenization. Values shown represent the mean and standard deviation from three independent experiments.

Table I. Distribution of radioactivity in major lipid classes after incorporation of [^{14}C]-acetate in cotton embryos (30 min) and tobacco cell suspensions (4 h). Values represent the mean and standard deviation of three independent experiments.

(DPM x 10^3)

Tissues	Treatments	Polar lipids	Nonpolar lipids
Cotton	Control (DMSO)	273±22.3 DPM	48.8 ±11.5
	10 mM Ethoxyzolamide	93.6±11.4	14.4±3.1
	10 mM Acetazolamide	130.1±18.0	26.95±3.8
Tobacco	Control (DMSO)	61.5±5.4	15.6±0.6
	10 mM Ethoxyzolamide	2.6±0.13	0.7±0.05

Table II. Radiolabeling of total lipids with [^{14}C]-acetate in isolated plastids of cotton embryos (34 DPA) and tobacco cell suspensions and chloroplasts for 1 h. Approximately 75 µg of embryo or tobacco plastid proteins or 0.167 mg of tobacco chloroplast chlorophylls were preincubated with 10 mM of ethoxyzolamide or acetazolamide for 30 min prior to the addition of [^{14}C]-acetate. Values represent the mean and standard deviation of three independent experiments. Rates of lipid synthesis in embryo or tobacco cell plastids are represented as nmol acetate h^{-1} mg $protein^{-1}$ and for tobacco leaf chloroplasts as nmol acetate h^{-1} mg chl^{-1}.

Treatment	Embryos	Cell Suspensions	Leaves
Control	9.8±1.8	16.6±2.8	4.2±0.5
Ethoxyozlamide	3.2±0.5	8.4±2.1	1.8±0.6
Acetazolamide	4.3±0.9	12.2±1.9	ND

labeling experiments demonstrated that CA inhibitors effectively reduced the rate of lipid synthesis in cotton embryos.

CA and lipid synthesis in tobacco cell suspensions

The influence of carbonic anhydrase on lipid biosynthesis was examined in a different plant system (tobacco cell suspensions, Fig 6). Tobacco cell suspensions (in log phase) were radiolabeled with [^{14}C]-acetate *in vivo* and the incorporation of [^{14}C]-acetate into total lipids was quantified (Fig 6). A linear rate of [^{14}C]-acetate incorporation into total lipid was established after 1 h of incubating tobacco cells with radiolabeled acetate. Approximately a 65% reduction in the rate of [^{14}C]-acetate incorporation was observed with 10 mM ethoxyzolamide (159.4±41 pmol acetate h^{-1} mg protein $^{-1}$) when compared with controls (453.4±42.4 pmol acetate h^{-1} mg protein $^{-1}$). Tobacco cell suspensions treated with 1 mM ethoxyzolamide had more than a 3-fold increase in CO_2 realeased when compared with controls (Table III). The distribution of radioactivity in the major lipid classes of tobacco cell suspensions after 4 h was analyzed with TLC (Table I). Approximately 75% of radiolabeled acetate was incorporated into polar lipids (phospholipids and glycolipids) and 25% into nonpolar lipids (fatty acids and triacylglyercols) in control samples. Tobacco cell suspensions incubated with 10 mM ethoxyzolamide had a 96% reduction of radiolabeled total polar lipids after 4 h when compared with polar lipids of controls (no inhibitors).

Plastids from tobacco cells were isolated and incubated with [^{14}C]-acetate *in vitro*. The rate of [^{14}C]-acetate incorporation into lipids was reduced by 58% in plastids incubated with ethoxyzolamide when compared with controls (no ethoxyzolamide)

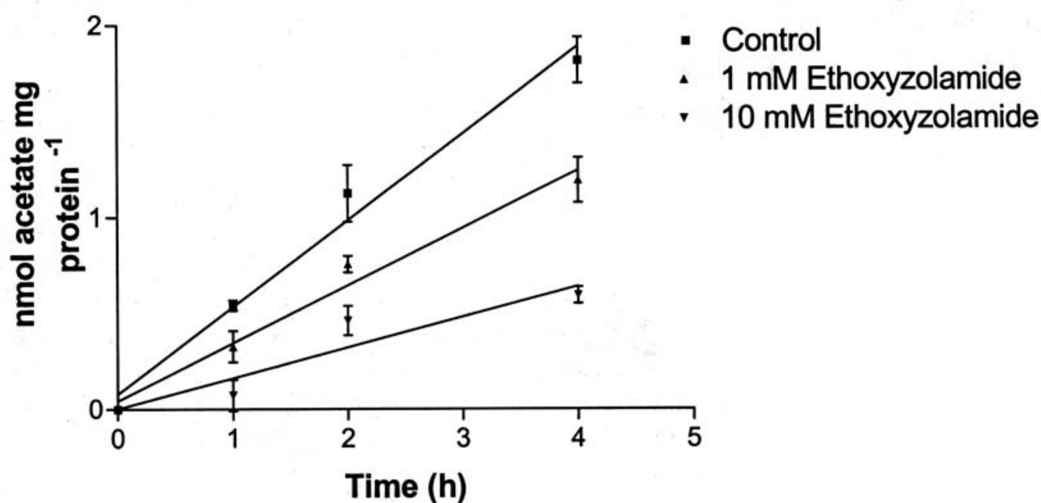


Figure 6. Incorporation of [^{14}C]-acetate into total lipids of tobacco (*Nicotiana tabacum* L., *cv xanthi*) cell suspensions *in vivo*. Cells were preincubated with DMSO (control), 1 mM ethoxyzolamide or 10 mM ethoxyzolamide for 30 min prior to the addition of radiolabeled [^{14}C]-acetate. Total lipids were extracted at 1 h, 2 h, and 4 h after the addition of the radiolabeled acetate. Data points represent mean and standard deviation of three independent experiments. Lines are drawn from linear regression analyses of the data with r^2=0.98 for controls, r^2=0.98 for 1 mM ethoxyzolamide and r^2=0.88 for 10 mM ethoxyzolamide. Rates estimated from linear regression analyses were 453.4±42.4, 300.7±31.4, and 159.4±41.3 pmol acetate h^{-1} mg protein $^{-1}$, respectively.

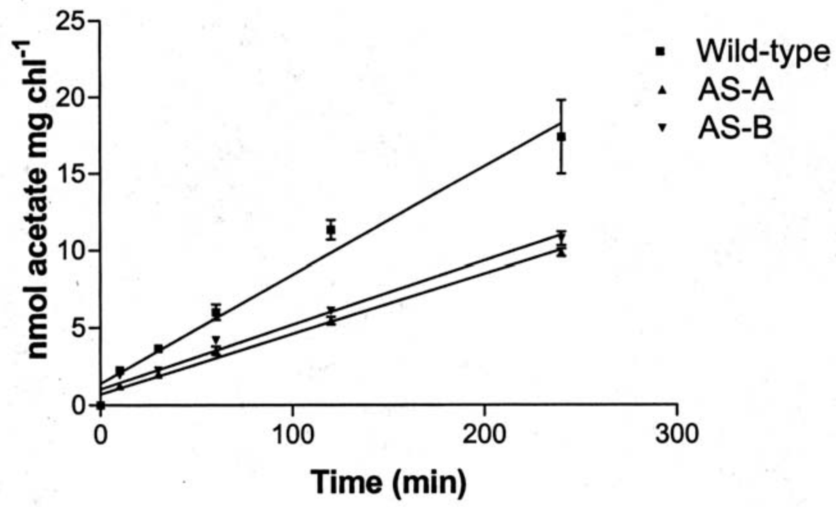
Table III. Release of CO_2 from tobacco cell suspensions incubated at different ethoxyzolamide concentrations after 35 min. Values are representative of a single experiment. Similar trends were observed in replicate experiments.

Treatments	CO_2 released (ppm)	ΔCO_2 (treatment – control, ppm)
Control	206.14	0
10 μM	215.07	8.9
100 μM	283.34	77.21
1000 μM	759.41	553.27

(Table II). These results revealed that inhibiting CA with ethoxyzolamide or acetazolamide in tobacco cell suspensions could effectively reduce lipid synthesis from [^{14}C]-acetate.

CA and lipid synthesis in tobacco chloroplasts

Inhibition of CA at the molecular level also significantly reduced the rate of lipid synthesis [^{14}C]-acetate (Fig 7A). Chloroplasts of wild-type or antisense-CA-suppressed transgenic tobacco plants were analyzed for [^{14}C]-acetate incorporation *in vitro*. Transformed plants refer to αTOBCA 1.10 AS-A and AS-B, which had total CA activity levels reduced to less than 5% and 10% of WT levels, respectively (Fig 7B). Price et al., (1994) previously reported that chloroplasts isolated from these antisense plants (αTOBCA 1.10) had only 2% of CA activity when compared to WT (on a Chl basis). Rates of [^{14}C]-acetate incorporation into lipids, equivalent to 4.2±0.5 nmol acetate h^{-1} mg protein $^{-1}$, were routinely measured in chloroplasts isolated from expanding young tobacco leaves (wild-type). A linear rate of [^{14}C]-acetate incorporation lipid into lipids was established after 5 min of incubating chloroplasts with [^{14}C]-acetate and continued up to 4 h. Approximately a 50% reduction in the rate of [^{14}C]-acetate incorporation into lipids was observed in chloroplasts from both transgenic (antisense CA suppressed A and B) plants when compared to WT plants (Fig 7A). In addition, a significant reduction in lipid synthesis was observed in wild-type chloroplasts incubated with ethoxyzolamide (Table II).


25
20
15
10
5
0
0
100
200
300
nmol acetate mg chl^{-1}
Time (min)
Wild-type
AS-A
AS-B

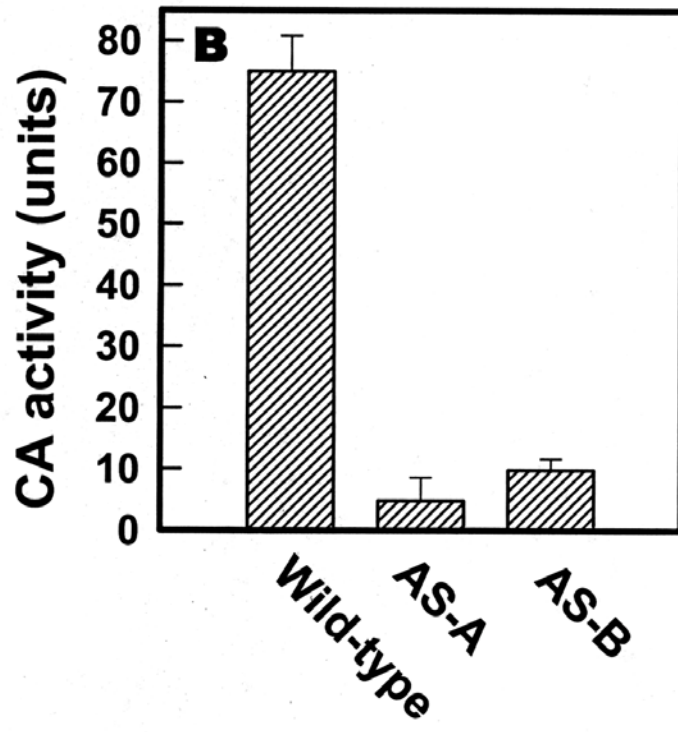

B
80
70
60
50
40
30
20
10
0
CA activity (units)
Wild-type
AS-A
AS-B

Figure 7. Incorporation of [^{14}C]-acetate into total lipids of chloroplasts isolated from tobacco leaves. Total lipids were extracted at 5 min, 10 min, 30 min, 1 h, 2 h, and 4 h after the addition of radiolabeled acetate (A). CA activity determined in leaves of anti-sense CA (A and B) suppressed transgenic and wild-type plants (B). Data points represent mean and range of duplicate samples within a single experiment. Similar trends were observed in replicate experiments. Lines represent linear regression analyses of the data with r^2=0.95 for controls, r^2=0.98 for AS-A and r^2=0.97 for AS-B. Rates estimated from linear regression analyses were 70.3±35.3, 39.9±1.6, and 41.5±2.3 pmol acetate min^{-1} mg chl^{-1}, respectively.

Discussion

We report here that biochemical and molecular inhibition of CA effectively reduced the rate of lipid synthesis in both cotton and tobacco. The data revealed that cotton embryos incubated with ethoxyzolamide or acetazolamide reduced CA activities *in vitro* by 60-90% and in turn could effectively reduced the rate of [^{14}C]-acetate incorporation into lipids *in vivo* and *in vitro* (Fig 3, 4, and Table II). Also, tobacco cells treated with ethoxyzolamide significantly reduced the rate of lipid synthesis when compared with controls (no inhibitors) (Fig 6). Likewise, chloroplasts of antisense-CA suppressed transgenic tobacco plants had a 50% reduction in the rate of [^{14}C]-acetate incorporation into lipids when compared with wild-type chloroplasts (Fig 7). Furthermore, tobacco cell suspensions treated with 1 mM ethoxyzolamide *in vivo* had a substantial loss of CO_2 when compared with controls (Table III). Collectively, the results presented in this paper clearly indicate that a reduction in plastidial CA activity leads to a reduction in the rate of plastidial lipid synthesis (from [^{14}C]-acetate) in developing cotton embryos, cell suspensions of tobacco, and leaves of tobacco plants.

Values of *in vitro* rates of fatty acid synthesis by isolated plastids and chloroplasts were significantly higher than *in vivo* rates (Fig 4, 6, 7 and Table II). Low *in vivo* rates of lipid synthesis observed could be due to membrane permeability of [^{14}C]-acetate into cells, thus limiting the availability of radiolabeled acetate for optimum rate of incorporation into total lipids. In addition, there has been much debate over the actual metabolic source of acetyl-CoA for *de novo* plant fatty acid synthesis and it appears to vary considerably depending upon cell type and physiological demand (Eastmond and

Rawsthorne, 2000). Others showed that exogenous pyruvate and glucose-6-phosphate (Glc-6-P) supplied to oilseed rape embryos during the maximum period of lipid synthesis had the highest rate of incorporation into fatty acid, when compared to dihydroxyacetone phosphate, malate, or acetate as substrates. In any case, we used [^{14}C]-acetate as a radiotracer to compare the impact of CA activity on the rate of lipid synthesis in cotton or tobacco tissues. Despite the inconsistency between *in vitro* and *in vivo* rates of lipid synthesis from [^{14}C]-acetate, we consistently found a reduction of acetate incorporation into total lipids in both cotton and tobacco plants when CA was inhibited.

While the precise mechanism of CA involvement in plant lipid synthesis is unclear at this point, there are a number of possibilities. One is that CA may be present to aid diffusion of CO_2 into the chloroplast as suggested by Badger and Price (1994). Since CO_2, but not HCO_3^-, can exit plastids by simple diffusion, movement of CO_2 out of plastids could result in a substantial loss of inorganic carbon and require additional energy for re-incorporation. The HCO_3^- that is utilized by ACCase to form malonyl-CoA is released as CO_2 by subsequent reactions of the fatty acid synthase complex. It is possible that an enzymatic hydration of CO_2 at this point increases the efficiency of CO_2 utilization in plastids, a concept which is similar to the conservation of CO_2 in mesophyll cells of C_4 plants by the cooperative action of CA and PEP carboxylase (Hatch and Burnell, 1990). In fact, CA in plastids may interact specifically with ACCase and enzymes of the fatty acid synthase complex to efficiently "channel" carbon into fatty acid (Roughan and Ohlrogge, 1996), although a direct interaction remains to be shown.

Interestingly, we have measured an increase in CO_2 loss from tobacco cell suspensions treated with CA inhibitors, compared with untreated cells (Table III), which supports a "trapping" role for plastidial CA. In this case the metabolic role for CA would be an indirect one, and a physiological consequence of CA reduction likely would be difficult to observe over the long term under optimal growth conditions, especially since the hydration of CO_2 occurs spontaneously. Indeed we detected no obvious growth differences in tobacco plants with less than 2% of wild-type CA activity consistent with previous results (Price et al., 1994). The physiological similarities between wild-type and antisense-CA suppressed tobacco plants were noted previously by Price and coworkers (1994). Rates of Rubisco activity and CO_2 assimilation were not different when CA was suppressed. However, there was a lower carbon isotope composition, $^{13}C/^{12}C$, in leaf dry matter of antisense-CA suppressed plants than wild-type plants. This suggested that there was a greater loss of CO_2 in antisense-CA suppressed plants than wild-type plants, which is consistent with our notion of a "trapping" function for CA.

Besides concentrating inorganic carbon for lipogenesis in plastids, CA may participate in other plastidial carboxylation reactions, such as carbamoyl phosphate synthetase (a plastid-localized enzyme in higher plants; Nara et al., 2000), which synthesizes the precursor for pyrimidine biosynthesis. Also Kavroulakis et al. (2000) have suggested that CA facilitates the recycling of CO_2 in developing soybean root nodules during early stages of development. The recycling and concentrating of CO_2 would thus provide adequate availability of inorganic carbon for lipid synthesis and other carboxylation reactions.

Another possible mechanism by which CA might act indirectly to influence fatty acid synthesis in plastids is to modulate plastidial pH. The optimal rate of fatty acid synthesis by fatty acid synthase complex could be influenced by changes in stromal pH. Jacobson et al., (1975) suggested that chloroplastic CA in spinach could act to buffer against transient pH changes in the stroma during photosynthesis. This possibility seems remote, however, since in our work, we observed a reduction in lipid synthesis in a variety of different types of plastids, which most likely have different stromal pH environments. Nonetheless this remains a possibility that warrants investigation, given the well-known role of pH regulation by CA in animal cells (Sly and Hu, 1995).

While most research attention for plastidial CA has focused on its putative role in photosynthetic carbon fixation, it is becoming increasingly likely that CA has a variety of additional functions in non-photosynthetic tissues. Indeed cDNAs encoding CA proteins have been isolated from non-photosynthetic tissues such as cotton seedlings and alfalfa (*Medicago sativa*) nodules (Hoang et al., 1999; Coba de la Pena et al., 1997). In fact, it may be that non-photosynthetic plant systems, lacking the complicating carboxylating activity of Rubisco, are particularly well-suited for evaluating various physiological roles of plastidial CA. In addition to our work here, others have implicated CA isoforms in two distinctly different roles in nitrogen metabolism in root nodules (Galvez et al., 2000). It seems likely that as more CA isoforms are found, so will increase the number of physiological functions attributed to this evolutionarily-conserved enzyme in plants.

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SUMMARY AND SIGNIFICANCE

Meldrum and Roughton, at Cambridge, discovered the first mammalian carbonic anhydrase enzyme in 1932. CAs in mammalian systems are produced in a variety of tissues where they participate in a broad range of physiological processes such as acid-base balance, carbon dioxide, and ion transport, respiration, body fluid generation, ureagenesis, gluconeogenesis, and lipogenesis (Sly and Hu, 1995). The roles of CA in mammalian systems have been well characterized. It is widely accepted that CA plays a role in photosynthesis by providing inorganic carbon for Rubisco. Thus far, there is almost no information regarding the role of CA in nonphotosynthetic plant tissues. The overall goal of this research dissertation is to investigate the possible(s) physiological roles of CA in nonphotosynthetic plant tissues. To gain insights into the role of CA in nonphotosynthetic tissues, my objectives were to characterize a plastidial carbonic anhydrase at the biochemical and molecular levels and to understand CA gene expression in cotyledons of cotton seedlings during post-germinative growth or in cotyledons of cotton embryos at different stages. I also extended the investigation by examining the involvement of CA in lipid biosynthesis in cotton and tobacco plants.

Cotton cotyledon systems are involved in several metabolic processes during different developmental growth stages. Cotyledons of cotton embryos synthesize fatty acids for the conversion of lipids in the endoplasmic reticulum for storage oil bodies. As the seeds germinate, these same cotyledon organs change their metabolism, to mobilize the lipid for seedlings growth and development (heterotrophic growth). Upon exposure

to light, these same cotyledon organs serve as the primary tissues for photosynthesis where light energy is converted to chemical energy. This energy is used to fix CO_2 and to synthesize the reduced carbon compounds for seedlings growth (photoautotrophic growth) when the seedlings naturally shed their seedcoats and the cotyledons are exposed to light (Trelease and Doman, 1984; Huang et al., 1983). This plant system allowed us to study the different metabolic roles of CA in cotyledons of one organ/cell type but at different developmental stages.

This research project originated from the discovery of an expressed sequence tag (EST) corresponding to a β-CA from a non-photosynthetic tissue (e.g. cotyledons of dark-grown cotton seedlings). This partial length cDNA clone was used to screen a cDNA library prepared from mRNA from cotyledons of 2-day old cotton seedlings to isolate cDNA clones, designated *GhCA*1 and *GhCA*2 (isolated and sequenced by A. Local and H. Wessler in Dr. R. Benjamin's laboratory). Chapter 1 described the identification and characterization of *CA* cDNA clones encoding a functional CA enzyme in nonphotosynthetic tissues of cotton. In addition, a survey of the endogenous CA expression in various plant tissues revealed that there were substantial CA activity and transcript abundance in cotyledons of dark-grown cotton seedlings and cotton embryos. Furthermore, *CA* cDNA clones encoding functional CA proteins have also been isolated from non-photosynthetic tissues of alfalfa (*Medicago sativa* L.) nodules (Coba de la Pena et al., 1997). The identification of CA expression in dark-grown cotton seedlings and alfalfa root nodules suggested that it is possible for CA to play a physiological role in nonphotosynthetic plant tissues, perhaps by supplying inorganic carbon to plastidial

carboxylases. These two studies could provide the basis for future research to investigate additional roles of CA in other nonphotosynthetic tissues.

In chapter 2, we investigated the regulation of *CA* gene expression in cotyledons of cotton seedlings during post-germinative growth under different environmental conditions such as CO_2 or light. This detailed characterization of *CA* gene expression could provide insights into its role in cotyledons of cotton seedlings during post-germinative growth. The data revealed that CA expression increased in cotyledons of cotton seedlings during post-germinative growth from 18 h to 72 h, the period of lipid mobilization. In addition, steady-state levels of *CA* and *rbcS* transcripts appeared to be regulated at the transcriptional level in response to environmental CO_2 conditions, whereas enzyme activities appeared to be controlled at the post-transcriptional level following exposure of seedlings to light. The regulation of different plant genes at the transcriptional level, such as Rubisco small subuint (*rbcS*), photosystem II light-harvesting comlex (*Lhcb*), phosphenolpyruvate carboxylase (*PEPC*), and pyruvate orthrophosphosphate dikinase (*PPDK*) have also been reported to be either induced or suppressed by light or atmospheric CO_2 (Tobin and Silverthorne, 1985; Thompson and White, 1991; Hudspeth et al., 1986; Sheen and Bogorad, 1987). However the precise mechanism(s) that regulates these enzyme activities at other levels such as the post-transcriptional, translational or post-translational levels are still unclear. Here we provided evidence for a *CA* gene expression in cotton that is differentially regulated at transcriptional and post-transcripitional levels under different environmental conditions. Though this research may have contributed to a better understanding of *CA* gene

regulation in cotton, more research is required to identify additional regulatory steps, beyond the transcriptional and post-transcriptional levels. Furthermore data presented in Chapter 2 collectively suggested that CA expression in cotyledons during post-germinative growth may be to “prime” cotyledons for the transition at the subcellular level from plastids (heterotrophic growth) to chloroplasts (photoautotrophic growth), which occurs naturally as seedlings shed their seed coats. Perhaps, CA can play a role in photoautotrophic growth in cotton seedlings by providing and trapping CO_2 in the chloroplast stroma for Rubisco during photosynthesis in green seedlings. Furthermore, photosynthetic organisms contain a CO_2 sensing mechanism that could monitor the change of internal carbon concentrations. Fukuzawa et al. (2001) have reported that aquatic photosynthetic organisms grown under limiting CO_2 conditions induce a set of genes for a carbon concentrating mechanism. The induction of *ccm*1 gene product is to regulate the CCM by sensing CO_2 availability in *Chlamydomonas* cells. Thus, the availability of inorganic carbon concentration in cells is tightly monitored by a CO_2 sensing mechanism.

In chapter 3, we explored the role of CA during lipid biosynthesis in cotton embryos. Although the role of CA in lipid biosynthesis in mammalian systems has been investigated, there is almost nothing known about its role in plant lipid synthesis. Cotyledons of cotton embryos are involved in lipid synthesis for storage as TAG in oil bodies. ACCase is a regulatory enzyme in plastids that has been considered to control the rate of fatty acid synthesis in plants. Because this enzyme utilizes HCO_3^- as a substrate, we speculated that CA might play a role in lipid synthesis in plants by providing HCO_3^-

to ACCase. The results indicated that the rate of [^{14}C]-acetate incorporation into total lipids *in vivo* and *in vitro* were substantial reduced in cotton and tobacco treated with the CA specific inhibitor, ethoxyzolamide. Also, the rate of [^{14}C]-acetate incorporation into total lipids was also reduced by 50% in transgenic CA antisense – suppressed tobacco plants when compared with wild-type plants. Thus inhibition of CA activity at the both biochemical and molecular levels could effectively reduce the rate of lipid synthesis in cotton and tobacco plants. Collectively, we provided evidence for a novel metabolic role for plastidial CA in plants.

Cottonseed oil is in great demand in the cooking oil industry and packaged foods industry because of its stability and enhanced taste factors. The market value for cottonseed oil is about $295 million per year in 1993 (Agriculture Statistics), however this number has declined according to Oil Crops Outlook (2001). A number of researchers have focused on elucidating the precise mechanism(s) that regulates the fatty acid biosynthetic pathway. With the current understanding of fatty acid biosynthesis in plant systems, it is possible to assume that ACCase controls the flux of fatty acid synthesis. In animals, ACCase is regulated by phosphorylation, activation by citrate, and feedback inhibition by acyl-CoA (Ohlrogge and Browse, 1995). None of these mechanisms have been reported to occur in plants. It is possible that high concentrations of HCO_3^- are maintained by CA to optimize ACCase activity during fatty acid synthesis in plants. Thus there might be a sensing mechanism of HCO_3^- by ACCase during fatty acid synthesis. The discovery of such a mechanism would provide new insight into the regulation of fatty acid synthesis. In addition, this could be of great value for the oilseed

industry. Manipulation of CA and ACCase expression could alter the overall amount of oil produced in seeds. These enzymes may be potential metabolic targets to genetically increase oil production in crop seeds.

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