Biochemical and Molecular Inhibition of Plastidial Carbonic Anhydrase Reduces the Incorporation of Acetate into Lipids in Cotton Embryos and Tobacco Cell Suspensions and Leaves

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Two cDNAs encoding functional carbonic anhydrase (CA) enzymes were recently isolated from a non-photosynthetic, cotyledon library of cotton (Gossypium hirsutum) seedlings with putative plastid-targeting sequences (GenBank accession nos. AF132854 and AF132855). Relative CA transcript abundances and enzyme activities 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 [14C]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-coenzyme A carboxylase 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), 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 the CA inhibitor ethoxyzolamide showed a substantial loss of CO2 compared with controls. The rate of [14C]acetate incorporation into lipid in cell suspensions was reduced by limiting external [CO2] (scrubbed air), and this rate was further reduced in the presence of ethoxyzolamide. Together, these results indicate that a reduction of CA activity (biochemical or molecular inhibition) impacts the rate of plant lipid biosynthesis from acetate, perhaps by impairing the ability of CA to efficiently "trap" inorganic carbon inside plastids for utilization by acetyl-coenzyme A carboxylase and the fatty acid synthesis machinery.

Carbonic anhydrase (CA, EC 4.2.1.1) is a zinc-containing metalloenzyme that catalyzes the reversible hydration of CO2 to HCO3−. The widespread abundance of CA isoforms in plants, animals, and microorganisms suggests that this enzyme has many diverse roles in biological processes. CA plays a critical role in biological systems because CO2 gas is the membrane permeable form of inorganic carbon for cells, and, in general, the uncatalyzed interconversion between HCO3− and CO2 is slow when compared with 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 CO2-concentrating mechanism 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 HCO3− to CO2, which is the substrate for 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 CO2-concentrating mechanism 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 sp.). Also, Maeda et al. (2000) identified a gene (CmpA) that encodes a substrate-binding protein that can specifically bind to HCO3− in cyanobacteria, which may further aid in the diffusion of HCO3− and elevation of CO2 in the carboxysome. In C4 plants, CA is localized to the cytosol of mesophyll cells, where it supplies HCO3− to phosphoenolpyruvate carboxylase (Burnell and Hatch, 1988; 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 CO2 in C3 plants (Ludwig et al., 1998). CA in C3 plants is distributed primarily in the stroma of chloroplasts (Poincelot, 1972) and is speculated to mediate in the diffusion of CO2 from the cytosol to the site of carboxylation by Rubisco in the chloroplast stroma during photosynthesis (Reed and Graham, 1981). Thus, the principal function of CA in photosynthetic organ-
isms 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, gluconeogenesis, 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-coenzyme A (CoA) carboxylase (ACCase) was colocalized with CA 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 ACCase (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 plastidial lipid biosynthesis. cDNAs were identified recently in a dark-grown cotton (Gossypium hirsutum) 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; Fig. 1). In developing oilseeds, the majority of fatty acids that are synthesized in plastids are exported to the endoplasmic reticulum 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). Moreover, the ACCase $K_m$ for HCO$_3^-$ is reported to be quite high from several plant species, in the millimolar range (Nikolau and Hawke, 1984). This suggests that in most plant systems, ACCase may be operating far below saturation because in higher plants the dissolved CO$_2$ concentration is in the micromolar range (Badger and Price, 1994). 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 (Nicotiana tabacum), 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)

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Comparison of triacylglycerol content (A), total CA activity (B), and relative CA expression (C) in embryos excised from cotton bolls at 25 and 40 DPA. The relative amounts of TAG in cell-free homogenates were estimated by scanning densitometry (National Institutes of Health Image) of thin-layer chromatography (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 evaluated by northern-blot analyses. The results depicted here are representative of replicate experiments.
in vivo and in vitro. In addition, molecular suppression of CA activity to 5% of wild-type (WT) 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 HCO3−-requiring pathways in plastids) indirectly, serving to “concentrate” CO2 in plastids as HCO3− and reduce the rate of CO2 diffusion out of plastids.

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 before (25 DPA) and during (40 DPA) the maximum period of TAG accumulation (Fig. 1). 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 increase the efficiency of inorganic carbon assimilation for lipid biosynthesis in developing embryos.

To further determine whether CA could play a role in lipid biosynthesis in cotton embryos (32 DPA), CA activity was reduced by pre-incubation of embryo extracts with CA specific inhibitors (at 1 and 10 mM sulfanilamide, acetazolamide, or ethoxyzolamide; Fig. 2). Both ethoxyzolamide and acetazolamide at 1 or 10 mM concentration inhibited more than 50% of CA activity in homogenates of cotton embryos; however, ethoxyzolamide was the most potent CA inhibitor. Embryos at 32 DPA were radiolabeled with [14C]acetate in vivo and the incorporation of [14C]acetate into total cottonseed lipids was quantified (Fig. 3). Cotton embryos (30–38 DPA) incubated with CA inhibitors at 1 and 10 mM ethoxyzolamide showed a significant reduction in the rate of [14C]acetate incorporation into total lipids compared with controls (no inhibitors). A linear rate of [14C]acetate incorporation into lipids was established within 10 min of incubating embryos with [14C]acetate (Fig. 3) and continued up to 30 min. A 50% reduction in the rate of radiolabeled acetate incorporation into total lipids was observed for embryos treated with 10 mM ethoxyzolamide and controls (no inhibitors). Application of inhibitors appeared to be specific for reserve lipid accumulation because storage protein synthesis, measured by [35S]-Met incorporation into total protein, was relatively unaffected (Fig. 4A). We confirmed that CA-specific inhibitors did not inhibit ACCase activity significantly in vitro (Fig. 4B) to reduce lipid synthesis in vivo.

The distribution of radioactivity in major lipid classes after incorporation of [14C]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 phospholipids) and 18% was in the nonpolar lipids (free fatty acids and triacylglycerol) of untreated cotton embryos. Embryos incubated with 10 mM ethoxyzolamide or acetazolamide had a distribution of radioactivity 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 fatty acid 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 free fatty acid product, determined by radiometric scanning of TLC-fractionated lipid classes as above) when compared with controls (Table II). Incorporation of [14C]acetate into plastid lipids was inhibited to a lesser extent by acetazolamide. Hence, results from both in vivo and in vitro [14C]acetate labeling experiments indicated that application of CA inhibitors effectively reduced the rate of lipid synthesis from acetate in cotton embryos.
CA and Lipid Synthesis in Tobacco Cell Suspensions

The influence of CA on lipid biosynthesis was examined with a similar approach in a different plant system (tobacco cell suspensions, Fig. 5). Tobacco cell suspensions (in log phase) were radiolabeled with [14C]acetate in vivo and the incorporation of [14C]acetate into total lipids was quantified (Fig. 5). A linear rate of [14C]acetate incorporation into total lipid was established after 1 h of incubating tobacco cells with radiolabeled acetate. An approximate 65% reduction in the rate of [14C]acetate incorporation was observed with 10 mM ethoxyzolamide (159.4 ± 41 pmol acetate h⁻¹ mg⁻¹ protein) when compared with controls (453.4 ± 42.4 pmol acetate h⁻¹ mg⁻¹ protein). 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 non-polar lipids (fatty acids and triacylglycerols) in control samples. Similar to results with cotton embryos, tobacco cell suspensions incubated with 10 mM ethoxyzolamide showed an overall reduction in radioactivity incorporated into both polar and nonpolar lipids compared with controls (no inhibitors).

Plastids from tobacco cells were isolated and incubated with [14C]acetate in vitro. The rate of [14C]acetate incorporation into lipids was reduced by about 50% in plastids incubated with ethoxyzolamide when compared with controls (no ethoxyzolamide; Table II), consistent with results from in vivo studies with intact tobacco cells (Fig. 5), and also consistent with results of plastids isolated from other plant cell types (embryo plastids and leaf chloroplasts, Table II).

To examine if there was a relationship between the rate of lipid synthesis, CA activity, and the availability of intracellular inorganic carbon for lipid synthesis, several experiments were conducted in which external CO2 concentrations were manipulated. First, the release of CO2 was measured from tobacco cell suspensions incubated with 1,000 μM ethoxyzolamide. The data revealed about a 3-fold increase in the release of CO2 from treated tobacco cell suspensions when compared with controls (Table III), suggesting that the reduction by CA-specific inhibitor of acetate incorporation into lipids may be due to the reduction of intracellular inorganic carbon pools. More directly, tobacco cells grown under limiting CO2 (scrubbed air) conditions had about a 50% reduction in the rate of [14C]acetate incorporation into total lipids compared with cells grown under ambient CO2 conditions (Table IV). The rates of lipid synthesis for tobacco cells grown under scrubbed CO2 conditions were approximately equivalent to the rates of lipid synthesis for cells grown under ambient conditions, but treated with ethoxyzolamide. In both cases, incorporation of [35S]-Met into total protein was similar between treatments and controls (not shown), indicating that like embryos (Fig. 4), inhibition of CA and acetate incorporation in tobacco cell suspensions did not affect other vital cellular processes such as general protein synthesis. Rates of acetate incorporation into total lipids were further reduced in tobacco cells grown under limiting CO2 conditions and treated with ethoxyzolamide (Table IV). Taken together, these results revealed that inhibiting CA with ethoxyzolamide (or acetazolamide) in tobacco cell suspensions or incubating tobacco cells

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**Figure 3.** Time-dependent incorporation of [14C]acetate into cottonseed total lipids. Embryos (30–38 DPA) were pre-incubated with DMSO (control) or different concentrations of CA inhibitors. Both 1 and 10 mM concentrations of ethoxyzolamide were incubated for 30 min before the addition of radiolabeled [14C]acetate. Total lipids were extracted at 10, 15, 20, and 30 min after the addition of the radiolabeled acetate. Incorporation of [14C]acetate into lipids was not linear after 30 min. Aliquots were used to quantify the incorporation of [14C]acetate into embryos by liquid scintillation counting. Data points represent mean and SD of three independent experiments for 10 and 30 min. Lines are plotted from linear regression analyses (Prism version 3.02, GraphPad Software, San Diego) of the data with r² = 0.97 for controls, r² = 0.97 for 1 mM ethoxyzolamide, and r² = 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⁻¹ protein, respectively. For reference, the rate of acetate incorporation into embryos not pre-incubated with DMSO was 12.44 ± 0.62 pmol acetate min⁻¹ mg⁻¹ protein.
WT levels, respectively (Fig. 6B). Price et al. (1994) previously reported that chloroplasts isolated from these antisense plants (αTBOCA 1.10) had 2% of CA activity when compared with WT (on a chlorophyll basis). Rates of [14C]acetate incorporation into lipids, equivalent to 4.2 ± 0.5 nmol acetate h⁻¹ mg⁻¹ protein, were routinely measured in chloroplasts isolated from expanding young tobacco leaves (WT). A linear rate of [14C]acetate incorporation into lipids was established after 5 min of incubating chloroplasts with [14C]acetate and continued up to 4 h. Approximately a 50% reduction in the rate of [14C]acetate incorporation into lipids was observed in chloroplasts from both transgenic (antisense CA-suppressed A and B) plants when compared with WT plants (Fig. 6A). For comparison, a substantial reduction in [14C]acetate incorporation into total lipids was observed in WT chloroplasts incubated with ethoxyzolamide (Table II).

### 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 enzyme activ-

### Table I. Distribution of radioactivity in major lipid classes after incorporation of [14C]acetate in cotton embryos (30 min) and tobacco cell suspensions (4 h)

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Treatments</th>
<th>Polar Lipids</th>
<th>Nonpolar Lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotton</td>
<td>Control (DMSO)</td>
<td>273 ± 22.3</td>
<td>43.8 ± 11.5</td>
</tr>
<tr>
<td></td>
<td>10 mM Ethoxyzolamide</td>
<td>93.6 ± 11.4</td>
<td>14.4 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>10 mM Acetazolamide</td>
<td>130.1 ± 18.0</td>
<td>26.95 ± 3.8</td>
</tr>
<tr>
<td>Tobacco</td>
<td>Control (DMSO)</td>
<td>61.5 ± 5.4</td>
<td>15.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>10 mM Ethoxyzolamide</td>
<td>2.6 ± 0.13</td>
<td>0.7 ± 0.05</td>
</tr>
</tbody>
</table>

Values represent the mean and SD of three independent experiments. Disintegrations per minute × 10⁻³.

### Table II. Radiolabeling of total lipids with [14C]acetate in isolated plasts of cotton embryos (34 DPA) and tobacco cell suspensions and chloroplasts for 1 h

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Embryos</th>
<th>Cell Suspensions</th>
<th>Leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.8 ± 1.8</td>
<td>16.6 ± 2.8</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td>Ethoxyzolamide</td>
<td>3.2 ± 0.5</td>
<td>8.4 ± 2.1</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>Acetazolamide</td>
<td>4.3 ± 0.9</td>
<td>12.2 ± 1.9</td>
<td>NDb</td>
</tr>
</tbody>
</table>

*Measured with DMSO; without DMSO, rates were 9.8 ± 1.8, 16.6 ± 2.8, and 4.2 ± 0.5 for plasts isolated from embryos, cell suspensions, and leaves, respectively. b ND, Not determined.

### CA and Lipid Synthesis in Tobacco Chloroplasts

Inhibition of CA at the molecular level also significantly reduced the rate of lipid synthesis from [14C]acetate (Fig. 6A). Chloroplasts of WT or antisense CA-suppressed transgenic tobacco plants were analyzed for [14C]acetate incorporation in vitro. Transformed plants refer to αTBOCA 1.10 AS-A and AS-B (two separate antisense plants), which had total CA activity levels reduced to less than 5% and 10% of
ities in vitro by 60% to 90% and in turn could effectively reduce the rate of [14C]acetate incorporation into lipids in vivo and in vitro (Figs. 2 and 3; Table II). Also, tobacco cells treated with ethoxyzolamide had a significantly reduced rate of lipid synthesis when compared with controls (no inhibitors; Fig. 5). Likewise, chloroplasts of antisense CA-suppressed transgenic tobacco plants had a 50% reduction in the rate of [14C]acetate incorporation into lipids when compared with WT chloroplasts (Fig. 6). 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 [14C]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 (Figs. 3, 5, and 6; Table II). Low in vivo rates of lipid synthesis observed could be due to a limited uptake of [14C]acetate into plant tissues, 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 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 with dihydroxyacetone phosphate, malate, or acetate as substrates. In any case, we used [14C]acetate as a general radiotracer for lipid synthesis to evaluate the impact of CA activity on the rates of lipid synthesis in cotton or tobacco tissues. Despite the differences between in vitro and in vivo rates of lipid synthesis from [14C]acetate, we consistently found a reduction of acetate incorporation into total lipids in both cotton and tobacco plants when CA was inhibited.

Although 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 in the diffusion of CO2 into the chloroplast as suggested by Badger and Price (1994). Because CO2, but not HCO3−, can exit plastids by simple diffusion, movement of CO2 out of plastids

Table III. Release of CO2 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.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>CO2 Released</th>
<th>ΔCO2 (Treatment – Control) μL L−1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>206.14</td>
<td>0</td>
</tr>
<tr>
<td>10 μM</td>
<td>215.07</td>
<td>8.9</td>
</tr>
<tr>
<td>100 μM</td>
<td>283.34</td>
<td>77.21</td>
</tr>
<tr>
<td>1000 μM</td>
<td>759.41</td>
<td>553.27</td>
</tr>
</tbody>
</table>

* Measured with DMSO; without DMSO, CO2 released after 35 min was 210.5 μL L−1.

Table IV. Radiolabeling of total lipids with [14C]acetate in tobacco cell suspensions incubated under ambient or limiting CO2 conditions and with or without 1 mM ethoxylzolamide

Values represent the mean and SD of three experiments. Rates of lipid synthesis are represented as pmol acetate h−1 mg−1 protein.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Rate of Lipid Synthesis pmol acetate h−1 mg−1 protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (ambient CO2 and DMSO)</td>
<td>472.0 ± 38.4</td>
</tr>
<tr>
<td>Limiting CO2</td>
<td>232.1 ± 7.2</td>
</tr>
<tr>
<td>Ambient CO2 and ethoxylzolamide</td>
<td>206.4 ± 16.8</td>
</tr>
<tr>
<td>Limiting CO2 and ethoxylzolamide</td>
<td>166.8 ± 4.8</td>
</tr>
</tbody>
</table>

Figure 5. Incorporation of [14C]acetate into total lipids of tobacco cv xanthi cell suspensions. Cells were pre-incubated with DMSO (control), 1 mM ethoxylzolamide, or 10 mM ethoxylzolamide for 30 min before the addition of radiolabeled [14C]acetate. Total lipids were extracted at 1, 2, and 4 h after the addition of the radiolabeled acetate. Data points represent mean and SD of three independent experiments. Lines are drawn from linear regression analyses of the data with r² = 0.98 for controls, r² = 0.98 for 1 mM ethoxylzolamide, and r² = 0.88 for 10 mM ethoxylzolamide. 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−1 protein, respectively. For reference, the rate of acetate incorporation into tobacco cell suspensions not pre-incubated with DMSO was 461.3 ± 11.5 pmol acetate h−1 mg−1 protein.
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 that is similar to the conservation of CO$_2$ in mesophyll cells of C$_4$ plants by the cooperative action of CA and phosphoenolpyruvate 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.

A substantial increase in CO$_2$ loss was measured from tobacco cell suspensions treated with CA inhibitors (ethoxzolamide), 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, wherein CA improves the efficiency of fatty acid synthesis by rapidly cycling inorganic carbon for ACCase. As such, a long-term physiological consequence of CA reduction likely would be difficult to observe under optimal growth conditions (where CO$_2$ levels were not limited), especially because the hydration of CO$_2$ occurs spontaneously at appreciable rates. We detected no obvious growth differences in tobacco plants with less than 2% of WT CA activity consistent with previous results (Price et al., 1994). The physiological similarities between WT 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, $^{13}\text{C}/^{12}\text{C}$, in leaf dry matter of antisense CA-suppressed plants compared with WT plants. This suggested that there was a greater loss of CO$_2$ in antisense CA-suppressed plants than WT plants (Price et al., 1994), which is consistent with our notion of a “trapping” function for CA.

Besides concentrating inorganic carbon for lipidogenesis 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 (Glycine max) root nodules during early stages of development. The recycling and concentrating of CO$_2$ thus would 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 (Spinacia oleracea) could act to buffer against transient pH changes in the stroma during photosynthesis. This remains a possibility that warrants investigation, given the well-known role of pH regulation by CA in animal cells (Sly and Hu, 1995). Although 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. cDNAs encoding CA proteins have been isolated from non-photosynthetic tissues such as cotton seedlings and alfalfa (Medicago sativa) nodules (Coba de la Pena et al., 1997; Hoang et al., 1999). 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 nitro-
gen metabolism in root nodules (Galvez et al., 2000). Multiple CA and CA-like genes are expressed in Arabidopsis (Arabidopsis Genome Initiative, 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.

MATERIALS AND METHODS

Plant Material

Cotton (Gossypium hirsutum L. cv Paymaster HS26) seeds were provided by Dr. John Burke (U.S. Department of Agriculture-Agricultural Research Service, Lubbock, TX). WT and transgenic tobacco (Nicotiana tabacum L. SR1) seeds (Price et al., 1994) were kindly provided by Dr. G. Dean Price (Australian National University, Canberra). 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 fertilizer (Miracle Gro, Stern Clifton, NJ) for 40 min in hexane:diethylether:acetic acid (80:20:1 [v/v/v]) solvent system. Radiometric scanning was used to quantify the radiolabeled lipids after TLC (System 200 Imaging Scanner, Bioscan, Washington, DC).

Total Lipid Extraction/TLC

Total lipids were isolated from cotton embryos, tobacco cell suspensions, and plastids essentially as described previously (Chapman et al., 1995). Cell suspensions in log phase (72 h after subculture) were used for plastid isolations and [14C]acetate labeling experiments.

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 Suc, 100 mM sodium phosphate (pH 7.2), 10 mM KCl, 1 mM MgCl₂, and 1 mM EDTA. Homogenates were filtered through four layers of cheesecloth and the filtrate was analyzed immediately for CA activity. Also, embryo homogenates were pre-incubated with sulfanilamide, acetazolamide, and ethoxyzolamide inhibitors for 30 min before 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 (Wilbur and Anderson, 1948) as described previously (Hoang et al., 1999). One unit of activity (Wilbur-Anderson unit) was defined as 10[(T₂/T₁) - 1], where T₀ 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 anhydrase assays) with a polytron (PT10/35, speed at 8, Brinkmann Instruments, Westbury, NY). Embryo homogenates were pre-incubated with different inhibitors for 30 min before assaying for ACCase activity. ACCase activity was based on acetyl-CoA-dependent H¹¹⁷CO₃⁻ (0.05 μCi per sample; 8.4 mCi mmol⁻¹) incorporation into acid-stable product (malonyl-CoA; Roesler et al., 1996). The production of [¹⁴C]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 oligo-dT cellulose column chromatography according to Aviv and Leder (1972). Approximately 2 μg of mRNA was electrophoresed in a 1% (w/v) agarose gel containing 6% (v/v) formaldehyde and 1× MOPS buffer (20 mM MOPS-NaOH, pH 7.0; 5 mM sodium acetate, and 0.1 mM EDTA; Sagerström and Sive, 1996). RNA was transferred to nylon membranes by capillary transfer with 20× SSC (overnight) and probed with a random prime-labeled (Gene Images random prime-labeling module, Amersham, Buckinghamshire, UK) 1.16-kb CA 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× SSC. Hybridized bands were identified by an alkaline phosphatase-catalyzed chemiluminescent reaction (Gene Images CDP-Star detection module, Amersham) and quantified by densitometric scanning (National Institutes of Health version 6.1 image software).

Incorporation of [³⁵S]-Met 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 (0.1–10 mM) were dispensed onto each embryo 30 min before the addition of [³⁵S]-Met (2 μCi per embryo; 1.175 mCi μmol⁻¹). After radiolabeling for 1 h with [³⁵S]-Met, the embryos were homogenized in a 1:1 (w/v) buffer (same buffer used above in CA assays) and the total proteins from cell-free homogenates were precip-
itated in TCA (4% [w/v] final concentration). TCA precipitated material was washed twice with 70% (v/v) ethanol, once with diethyl ether, and 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 (LS 3861 counter [Beckman Instruments, Fullerton, CA] and ScintiSafe Plus 50% LSC cocktail, Fisher Scientific, Houston) was used to quantify $^{35}$S]-Met 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-benothiazolesulfonamides (ethoxyzolamide; Sigma Chemical Co., St. Louis). Sulfanilamide was dissolved in water, whereas 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, 100, and 1,000 $\mu$M ethoxyzolamide and subsequently CO$_2$-free air (scrubbed with soda lime) was flushed through the system. CO$_2$ released from tobacco cell suspensions either treated with DMSO (control) or ethoxyzolamide was monitored in line with a plant CO$_2$ analysis system (Qubit Systems Inc., Kingston, Ontario). These same conditions were utilized in combination with $^{14}$C]acetate radiolabeling to evaluate the influence of external CO$_2$ (without and with CA inhibitors) on lipid synthesis in vivo.

### 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 $\mu$L at different concentrations (0.1–10 mM) were dispensed onto each embryo and then incubated for 30 min before the addition of radiolabeled [2-$^{14}$C]acetate (54 mCi mmol$^{-1}$). Total lipids were extracted at different time points after the addition of the 0.5 $\mu$Ci 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 after 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 milliliters of the suspended cells were transferred to a 15-mL tube and pre-incubated with CA inhibitors for 30 min before the addition of radiolabeled acetate. Also, tobacco cell suspensions were incubated under zero CO$_2$ conditions and subsequently analyzed for the incorporation of $^{14}$C]acetate into total lipids. 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 800g for 5 min was layered over a Percoll gradient composed of 3 mL of 90% (w/v), 15 mL of 35% (w/v), and 10 mL of 15% (w/v) Percoll. Intact chloroplasts were recovered and the chlorophyll content was estimated according to Bruinsma (1961) in 80% (v/v) acetone.

Plastids were isolated from tobacco cell suspensions (in log phase) and cotton embryos by centrifugation through a 10% (w/v) Percoll gradient (Sparace and Mudd, 1982; Trimming and Emes, 1993). Plastid protein content was 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 by Stahl and Sparace (1991). The reaction mixture contained 0.02 $\mu$M Na-[2-$^{14}$C]acetate, 0.04 mM cold sodium acetate, and 3 mM each of MgCl$_2$ and ATP and 50 to 100 $\mu$g of plastid protein or 50 to 200 $\mu$g of chlorophyll.

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### LITERATURE CITED


Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing
Bray GA (1977) The Zucker-fatty rat: a review. Federation
Proc 36: 148–153
Bruinsma J (1961) A comment on the spectrophotometric
Burnell JN, Hatch MD (1988) Low bundle sheath carbonic
anhydrase is apparently essential for effective C 4 path-
way operation. Plant Physiol 86: 1252–1256
Cammer W (1991) Immunostaining of carbamoylphos-
phate synthase II and fatty acid synthase in glial cells in
rat, mouse, and hamster brains suggests roles for car-
bonic anhydrase in biosynthetic processes. Neurosci Lett
129: 247–250
Cao TP, Rous S (1978) The Zucker-fatty rat: a review. Federation
Proc 24: 516–520
Chapman KD, Conyers-Jackson A, Moreau A, Tripathy S
Coba de la Pena T, Frugier F, McKhann HI, Bauer P,
Choinski JS, Trelease RN
Chapman KD, Sprinkle WB (1996) Developmental, tissue-
specific, and environmental factors regulate the biosyn-
thesis of plastidial carbonic anhydrase in de novo lipogenesis. Mol Plant-
Microbe Interact 13: 14–22
Cao TP, Rous S (1978) Inhibitory effect of acetazolamide on
the activity of acetyl-CoA carboxylase of mouse liver. Life Sci 22: 2067–2072
Chapman KD, Conyers-Jackson A, Moreau A, Tripathy S
(1995) Increased N-acylphosphatidylethanolamine bio-
synthesis in elicitor treated tobacco cells. Physiol Plant
95: 120–126
Chapman KD, Sprinkle WB (1996) Developmental, tissue-
specific, and environmental factors regulate the biosyn-
Choinski JS, Trelease RN (1978) Control of enzyme activities in cotton cotyledons during maturation and germi-
Coba de la Pena T, Frugier F, McKhann HI, Bauer P,
Coligan JE, Gates FT III, Kimball ES, Maloy WL (1983) Radiochemical sequence analysis of metabolically la-
beled proteins. Methods Enzymol 91: 413–434
Dodgson SJ, Forster RE, Storey BT (1984) The role of
carbonic anhydrase in hepatocyte metabolism. Ann NY
Acad Sci 429: 516–525
Eastmond PJ, Rawsthorne S (2000) Coordinate changes in
carbon partitioning and plastidial metabolism during the
Galvez S, Hirsch AM, Wycoff KL, Hunt S, Layzell DB,
Hatch MD, Burnell JN (1990) Carbonic anhydrase activity
in leaves and its role in the first step of C4 photosynthe-
Herbert JD, Coulson RA (1983) A role for carbonic anhy-
drase in de novo fatty acid synthesis in liver. Ann NY
Acad Sci 429: 425–427
Hoang CV, Wessler HG, Local A, Turley RB, Benjamin
RC, Chapman KD (1999) Identification and expression of cotton (Gossypium hirsutum L.) plastidial carbonic an-
hydase. Plant Cell Physiol 40: 1262–1270
Hoang CV, Turley RB, Chapman KD (1998) Molecular
identification and expression of a plastidial carbonic anhy-
rase in cotton (Gossypium hirsutum L.) (abstract no. 51).
Plant Physiology Meeting, August, 1998. American Soci-
ety of Plant Physiologists, Rockville, MD
Jacobson BS, Fong F, Heath RL (1975) Carbonic anhydrase
of spinach: studies on its location, inhibition and physi-
Kavroulakis N, Fletematakis E, Aivalakis G, Katinakis P
nodules: the role of carbonic anhydrase. Mol Plant-
Microbe Interact 13: 14–22
Ludwig M, van Caemmerer S, Price DG, Badger MR,
Furbank RT (1998) Expression of tobacco carbonic anhy-
drase in the C 4 dicot Flaveria bidentis leads to increased
leakiness of the bundle sheath and a defective CO 2-
Lynch CJ, Fox H, Hazen SA, Stanley BA, Dodgson S,
Lanke KE (1995) Role of hepatic carbonic anhydrase in
Maeda S, Price GD, Badger MR, Enomoto C, Omata T
(2000) Bicarbonate binding activity of the CmpA protein of the cyanobacterium Synechococcus sp. strain PCC 7942
involved in active transport of bicarbonate. J Biol Chem
275: 20551–20555
Maren TH (1967) Carbonic anhydrase: chemistry, physiol-
yogy and inhibition. Physiol Rev 47: 595–781
Moroney JV, Husic HD, Tolbert NE (1985) Effects of car-
bonic anhydrase inhibitors on inorganic carbon accumu-
lation by Chlamydomonas reinhardtii. Plant Physiol 83:
460–463
Nara T, Hshimoto T, Aoki T (2000) Evolutionary implica-
tions of the mosaic pyrimidine-biosynthetic pathway in
eukaryotes. Gene 257: 209–222
Nikolau BJ, Hawke JC (1984) Purification and character-
ization of maize leaf acetyl-coenzyme A carboxylase. Arch Biochem Biophys 228: 86–96
7: 957–970
Ohroge J, Jaworski JG (1997) Regulation of fatty acid
synthesis. Annu Rev Plant Physiol Plant Mol Biol 48:
109–136
Poincelot RP (1972) Intracellular distribution of carbonic
anhydrase in spinach leaves. Biochim Biophys Acta 258:
637–642
Price GD, Coleman JR, Badger MR (1992) Association of
carbonic anhydrase activity with carboxysomes isolated from the cyanobacterium Synechococcus PCC 7942. Plant
Physiol 100: 784–793
Price GD, von Caemmerer S, Evans JR, Yu JW, Lloyd J,
Oja V, Kell P, Harrison K, Gallagher A, Badger MR
(1994) Specific reduction of chloroplast carbonic anhy-
drase activity by antisense RNA in transgenic tobacco
plants has a minor effect on photosynthetic CO 2 assimil-


