Planta (1994) 193:331–340

Planta

Specific reduction of chloroplast carbonic anhydrase activity by antisense RNA in transgenic tobacco plants has a minor effect on photosynthetic CO_2 assimilation

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Received: 16 August 1993 / Accepted: 7 October 1993

Abstract. As an approach to understanding the physiological role of chloroplast carbonic anhydrase (CA), this study reports on the production and preliminary physiological characterisation of transgenic tobacco (*Nicotiana tabacum* L.) plants where chloroplast CA levels have been specifically suppressed with an antisense construct directed against chloroplast CA mRNA. Primary transformants with CA levels as low as 2% of wild-type levels were recovered, together with intermediate plants with CA activities of about 20–50% of wild-type levels. Plants with even the lowest CA levels were not morphologically distinct from the wild-type plants. Segregation analysis of the low-CA character in plants grown from T_1 selfed seed indicated that at least one of the low-CA plants appears to have two active inserts and that at least two of the intermediate-CA plants have one active insert. Analysis of CO_2 gas exchange of a group of low-CA plants with around 2% levels of CA indicated that this large reduction in chloroplastic CA did not appear to cause a measurable alteration in net CO_2 fixation at 350 μbar CO_2 and an irradiance of 1000 μmol quanta $\cdot m^{-2} \cdot s^{-1}$. In addition, no significant differences in Rubisco activity, chlorophyll content, dry weight per unit leaf area, stomatal conductance or the ratio of intercellular to ambient CO_2 partial pressure could be detected. However, the carbon isotope compositions of leaf dry matter were significantly lower (0.85‰) for low-CA plants than for wild-type plants. This corresponds to a 15-μbar reduction in the CO_2 partial pressure at the sites of carboxylation. The difference, which was confirmed by concurrent measurement of discrimination with gas exchange, would reduce the CO_2 assimilation rate by 4.4%, a difference that could not be readily determined by gas-exchange techniques given the inherent variability found in tobacco. A 98% reduction in CA activity dramatically reduced the ^{18}O discrimination in CO_2 passing over the leaf, consistent with a marked reduction in the ratio of hydrations to carboxylations. We conclude that a reduction in chloroplastic CA activity of two orders of magnitude does not produce a major limitation on photosynthesis at atmospheric CO_2 levels, but that normal activities of the enzyme appear to play a role in facilitated transfer of CO_2 within the chloroplast, producing a marginal improvement in the efficiency of photosynthesis in C_3 plants.

Key words: Antisense RNA – Carbon dioxide assimilation – Carbonic anhydrase – *Nicotiana* (transgenic plants)

Introduction

The enzyme, carbonic anhydrase (CA) (EC 4.2.1.1), catalyses the interconversion of the dissolved inorganic carbon species, CO_2 and HCO_3^-, through the following net reaction: $CO_2 + H_2O \Leftrightarrow HCO_3^-$. In the absence of CA, the interconversion is relatively slow, particularly around pH 8.0, but in the presence of sufficient CA activity the reaction is close to being instantaneous. The enzyme plays a vital role in the early events of photosynthesis in many photosynthetic organisms, particularly those which employ a CO_2-concentrating mechanism to improve photosynthetic efficiency. In C_4 plants, CA is localised in the cytosol of the mesophyll cells and is required for the supply of HCO_3^- to phosphoenolpyruvate carboxylase (Hatch and Burnell 1990), and calculations show that photosynthesis would be severely rate-limited without it. In cyanobacteria, CA is specifically localised in the carboxysome (Badger and Price 1992; Price et al. 1992), and is required for the conversion of HCO_3^- to CO_2, so that CO_2 can then be fixed by ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco).

Abbreviations and symbol: CA = carbonic anhydrase; Chl = chlorophyll; p_a = ambient CO_2 partial pressure; $p_c = CO_2$ partial pressure in the chloroplast stroma; $p_i = CO_2$ partial pressure in the intercellular air spaces; Rubisco = ribulose bisphosphate carboxylase-oxygenase; WT = wild type; Δ = carbon isotope discrimination

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An absence of this enzyme results in a severe limitation on photosynthesis (Price et al. 1992). In green microalgae, CA is located in the periplasmic space, the cytosol and the chloroplast, and studies show that its presence in these various locations is required to achieve the highest affinity of photosynthesis for external inorganic carbon (Badger and Price 1992). Despite the obvious role of CA in these photosynthetic organisms, the function of the enzyme in C_3 plants remains enigmatic (see reviews by Graham et al. 1984; Poincelot 1979).

In C_3 plants, most if not all, of the CA activity is located in the chloroplast stroma (Everson 1970; Werden and Heldt 1972; Jacobson et al. 1975; Tsuzuki et al. 1985). The most commonly held view is that chloroplastic CA aids in the passive diffusion and equilibration of inorganic carbon species across the volume of the plastid, thus facilitating the supply of CO_2 to the sites of carboxylation in the stroma (see Poincelot 1979, and refs therein). A number of studies, however, have concluded that photosynthesis is not limited by the transfer of CO_2 into and across the chloroplast, and that chloroplastic CA is, therefore, either not required for efficient photosynthesis or would only enhance CO_2 assimilation by a small percentage (Randall and Bouma 1973; Jacobson et al. 1975). Cowan (1986), however, has argued that even a small enhancement of CO_2 fixation, say less than 5%, would still be of benefit to the plant, particularly in the selective processes evident in population genetics. Jacobson et al. (1975) have suggested that chloroplastic CA, and the chloroplastic HCO_3^- pool, might act to buffer against transient pH changes in the stroma occurring as a result of rapid changes in the light intensity available for photophosphorylation and that CA may play a role in the hydration of compounds other than CO_2. As yet, no clearly defined role for chloroplastic CA in C_3 plants has been proven.

Previous experimental approaches aimed at probing the function of chloroplastic CA by reducing its activity have suffered from a lack of specificity. Reducing CA levels by growing plants under zinc deficiency (Edwards and Mohamed 1973; Randall and Bouma 1973) or the use of CA inhibitors on intact chloroplasts (Swader and Jacobson 1972; Jacobson et al. 1975) result in non-specific inhibition of photosynthesis in addition to effects on CA levels. A highly specific approach, that has only recently become available, is to suppress chloroplastic CA activity by antisense RNA inactivation of mRNA transcribed by the nuclear-encoded gene. We report on the production and preliminary physiological analysis of transgenic tobacco plants where chloroplastic CA levels have been specifically targeted by the stable introduction into the genome of an homologous antisense RNA construct directed against the transcript for chloroplast CA.

Materials and methods

Engineering of an antisense construct against chloroplastic CA. Figure 1 A shows the steps involved in the engineering of an antisense RNA construct against tobacco chloroplast CA. A cDNA clone for tobacco chloroplast CA was isolated by screening a *Nicotiana tabacum* L. cv. SR1 lambda ZAP cDNA library (leaf tissue cDNA; Strategene, La Jolla, Cal., USA). Plaque screening and in-vivo excision were carried out according to the manufacturer's recommendations. A cDNA clone for spinach chloroplast CA (Burnell et al. 1990) was used as the ^{32}P-labelled probe. The resulting 1.01-kb cDNA *Eco* RI/*Eco* RI clone (in pBluescript) was sequenced using an Applied Biosystems Inc. (Foster City, Cal., USA) *Taq*/dye terminator/cycle sequencing kit with reverse and universal primers. Reactions were sequenced on an ABI automated sequencer. The sequence has been deposited on GenBank as accession No. L19255. The cDNA clone (TOBCA) contains the mature-peptide coding region, the 3′ untranslated region and approx. 40% of the transit-

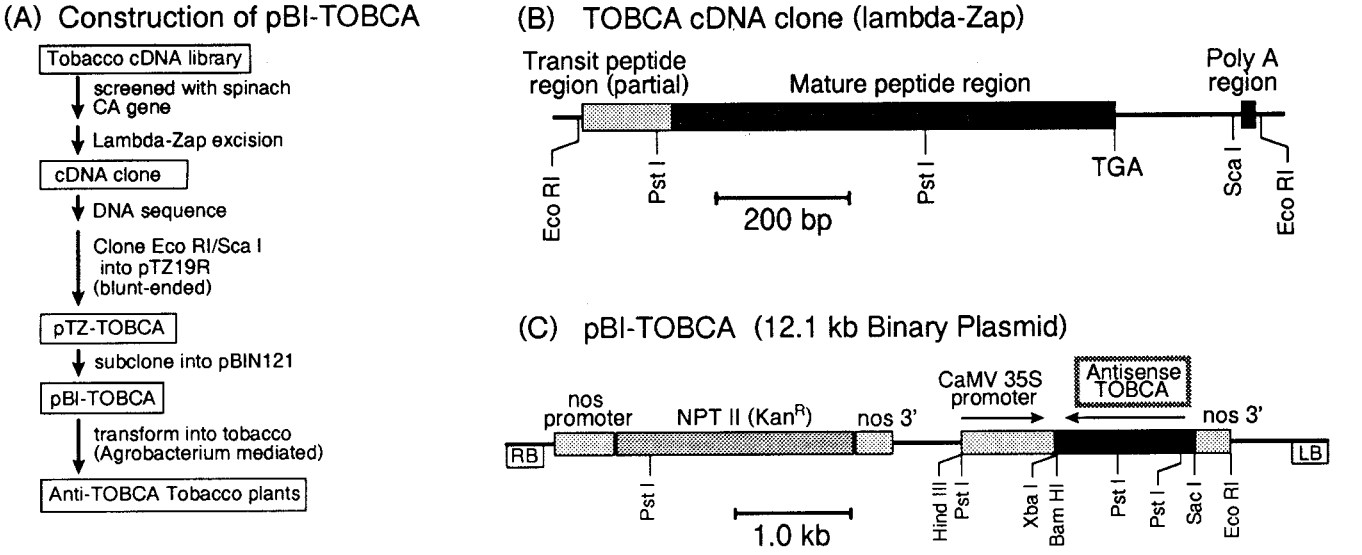

Fig. 1. **A** Steps involved in the construction and introduction of an antisense CA gene directed against chloroplast-located CA. **B** Features of the tobacco cDNA clone showing the location of the regions coding for the transit peptide (partial) and the mature peptide. The locations of key restriction sites are also shown. **C** Locations of the kanamycin-resistance gene and the CaMV/antisense CA element within the left and right borders of the binary plasmid, pBI-TOBCA

peptide coding region (Fig. 1 B). The putative mature peptide bears 77.4% identity (over a 221-amino-acid overlap) with the known mature peptide of the spinach chloroplast CA enzyme (see Burnell et al. 1990). The DNA sequence of this clone is essentially the same as the sequence for *Nicotiana tabacum* (cv. Samson) that was recently published by Majeau and Coleman (1992) except that the nucleotides at positions 617 (G), 855 (C) and 1132 (T) were A, G and C respectively in our clone. This meant that the amino-acid sequence predicted from the Majeau and Coleman (1992) sequence coded for valine-180 and alanine-259 rather than isoleucine and glycine as predicted from our sequence. The former nucleotide sequence also lacks 25 bases at position 1209 and immediately upstream of the poly-A region. This 25-base region contains the *Sca* I restriction site (Fig. 1 B). The mature-peptide region for this CA clone (TOBCA) has been expressed in *Escherichia coli* and the resulting active enzyme has been found to have properties the same as the CA activity in tobacco leaf extracts (data not shown). The DNA cloning methods and southern hybridizations were carried out according to general protocols (Sambrook et al. 1989). Tobacco genomic DNA was isolated essentially as described by Shure et al. (1983).

A 0.97-kb *Eco* RI/*Sca* I fragment of the cDNA clone, that deletes the poly-A region (see Fig. 1 B), was blunt-ended and cloned into the *Sma* I site of the plasmid pTZ19R (Mead et al. 1986), and recovered in both orientations relative to the multiple cloning region. The *Xba* I-*Sac* I fragment, containing the antisense orientation of the CA gene was then cloned into the binary plasmid pBI121 (Jefferson et al. 1987) so as to replace the β-glucuronidase gene. The resulting 12.1-kb plasmid, pBI-TOBCA, contains the antisense CA gene driven from the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 1 C). The *nos* 3′ element is located immediately downstream of the CA antisense gene. The binary plasmid, pBI-TOBCA was transformed into *Agrobacterium tumefaciens* (strain LBA4404) by electroporation (Nagel et al. 1990) and transformants selected for growth on MinA plates (Herrera-Estrella and Simpson 1988) containing 50 $\mu g \cdot ml^{-1}$ kanamycin. Two apparently identical *Agrobacterium* colonies harbouring the pBI-TOBCA plasmid were selected and designated TOBCA 1 and TOBCA 2. *Agrobacterium*-mediated transformation of tobacco (*Nicotiana tabacum* cv. W38) leaf discs was carried out essentially as described by Herrera-Estrella and Simpson (1988). Kanamycin selection was maintained at 200 $\mu g \cdot ml^{-1}$ for callus tissue and 100 $\mu g \cdot ml^{-1}$ for rooting explants. Transformants were grown under glasshouse conditions (peak irradiance around 800 $\mu mol\ quanta \cdot m^{-2} \cdot s^{-1}$) in pots containing vermiculite and fertilised with a complete nutrient solution. Plants grown for gas-exchange analysis were grown in 5-L pots of soil and also fertilised with a complete growth solution. Plants were grown under PH1 containment.

Assay of CA activity. Each plant extract was prepared from a 0.64-cm^2 leaf punch ground in 0.25 mL of WB buffer that contained: 100 mM N-(2-hydroxyethyl)-piperazine-N′-3-propanesulfonic acid (Epps)-NaOH buffer (pH 8.0), 2 mM Na_2EDTA, 10 mM dithiothreitol (DTT), 1% (w/v) polyvinyl-polypyrrolidone (Polyclar), 1% (w/v) casein, 1 mM Na-diethyldithiocarbamate, 20 mM $MgSO_4$, 20 mM $NaHCO_3$ and 20 mM K_2PO_4, 0.1% Triton X-100, 10% glycerol (w/v); 0.1 mM phenylmethylsulfonyl fluoride (PMSF) was added just prior to grinding. Cell extracts were centrifuged at $10000 \cdot g$ in an Eppendorf microfuge (4° C) and the supernatants frozen until they could be used for measurements of CA and Rubisco activity. Initial screening measurements of CA activity were based on the method of Wilbur and Anderson (1948). Each assay contained 3.5 mL of 15 mM Barbitone-NaOH buffer (pH 8.2) and 15–30 µL of cell extract in a cuvette controlled to 0° C. The reaction was started by the addition of 2 mL of CO_2-saturated water (4° C) and the time taken for a pH change between 8.0 and 7.0 was recorded. Carbonic anhydrase activity (as WA units; see Wilbur and Anderson (1948)) was calculated by the equation, $[10\{(t_u/t_c)-1\}]$, where t_u is the time for the uncatalysed rate in s, and t_c is the time for the catalysed rate. Enzyme units were finally expressed as WA units per cm^2 of leaf area. Later measurements for CA activity utilised a mass-spectrometric technique that measures the rate of exchange of ^{18}O label from doubly labelled $^{13}C^{18}O_2$ to $H_2{}^{16}O$. Experimental particulars were as previously described (Badger and Price 1989) except that the assay buffer was 100 mM Epps-NaOH (pH 8.0) and contained 10 mM DTT and 1 mM $NaH^{13}C^{18}O_3$; the cuvette was maintained at a temperature of 25° C. In this assay system, one unit (U) of enzyme activity is equivalent to a 100% stimulation in the first-order rate constant. Chlorophyll (Chl) content was measured in 80% acetone according to Porra et al. (1989).

Assay of Rubisco. The Rubisco assays were carried out in 250 µL of 100 mM Epps-NaOH buffer (pH 8.0) containing 20 mM $MgCl_2$ and 15 mM $NaH^{14}CO_3$ and 20 µL of leaf extract. Following a 3-min activation step at 25° C, each assay was initiated with 20 µL of 20 mM ribulose-1,5-bisphosphate and terminated after 1 min with 200 µL of 10% (v/v) formic acid. The samples were dried down and counted in a liquid scintillation counter.

Segregation tests. Selfed F_1 seed from each of several selected primary transformants were analysed for segregation of the antisense CA character by measuring CA levels in young plants. Plants with CA levels below 50% of wild-type (WT) levels were scored as possessing an antisense CA character(s).

Gas-exchange measurements. Gas-exchange measurements for the rate of CO_2 assimilation and transpiration were made on attached leaves in an open gas-exchange system, as described by Brugnoli et al. (1988) with modifications noted by Hudson et al. (1992). Calculation of gas-exchange parameters was made according to the method of von Caemmerer and Farquhar (1981). Measurements were made at an irradiance of 1000 $\mu mol\ quanta \cdot m^{-2} \cdot s^{-1}$, 350 µbar CO_2 in air, a leaf temperature of 25° C and a leaf-to-air vapour-pressure deficit of 10 mbar. Leaf dry weight and carbon isotope discrimination, Δ, were measured on portions of leaves that had been dried in a oven at 80° C. Carbon isotope discrimination was measured according to the method of Hubick et al. (1986) and is defined as

$$\Delta = (\delta_a - \delta_p)/(1 + \delta_p)$$

where δ_p and δ_a are the carbon isotope compositions of the plant and the air surrounding the leaf, respectively, with respect to the Pee Dee Belemnite (PDB) standard. In our well-ventilated glasshouses, $\delta_a = -8.0 \times 10^{-3}$, thus plant material with a carbon isotope composition of $\delta_p = -27.4 \times 10^{-3}$ has a $\Delta = 20 \times 10^{-3}$. The magnitude of the CO_2 gradient between the substomatal cavities and the sites of carboxylation, $(p_i - p_c)$, was calculated from equation 4 in von Caemmerer and Evans (1991), and as originally derived from Evans et al. (1986), with b = 29‰.

On-line measurements of $\delta^{13}C$ and $\delta^{18}O$. Measurements of CO_2 assimilation and water loss were also made in conjunction with on-line $^{13}CO_2$ and ^{18}O in CO_2 discrimination measurements. Measurements were made with an irradiance of 1000 $\mu mol\ quanta \cdot m^{-2} \cdot s^{-1}$ and with leaf temperature maintained at 27.5 ± 0.7° C and with a leaf-to-air vapour-pressure deficit of around 10 mbar. The isotopic composition of CO_2 in the airstream before and after passing over the leaf was determined as previously described (von Caemmerer and Evans 1991). The parameter ξ [calculated as $C_e/(C_e - C_o)$ where C_e and C_o are CO_2 partial pressures in the dry air entering and leaving the chamber, respectively; Evans et al. (1986)] was maintained at around 8. Leaf internal conductances were calculated according to equation 6 of Lloyd et al. (1992) using f = 8‰ and b = 29‰.

Chloroplast isolation and assay of intactness. Tobacco plants were placed in the dark for 24 h prior to chloroplast isolations in an attempt to reduce starch content. Chloroplasts were isolated by a mechanical disruption method as previously described for spinach (Yu and Woo 1988) except that in the blending medium 4 mM DTT and 10 mM ascorbate and 1% (w/v) polyvinylpyrrolidine

(PVP-40) were included. Chloroplast intactness was determined by comparing the uncoupled rate of ferricyanide-dependent O_2 evolution before and after osmotic shock in H_2O. The assay medium was the same as the resuspension medium used by Yu and Woo (1988) but containing 2.5 mM $K_3Fe(CN)_6$ and 10 mM NH_4Cl.

Rapid gas-exchange transients. Rapid gas-exchange transients were performed at 25° C using a fast gas-exchange system as previously described by Laisk et al. (1989). During the periods at elevated CO_2, care was taken that stomata did not close significantly, so as to avoid the kinetic nature of the CO_2 transients being influenced by alterations in stomatal conductance.

Results

Screening transformants for reduced CA activity. Table 1 displays the characteristics of a number of primary transformants with low or intermediate levels of CA activity. The transgenic plants referred to in Table 1 were selected from two independent transformation experiments. In both cases the plants were screened over a five-week period commencing two weeks after the plants had been hardened and transferred to soil and glasshouse conditions. In each weekly screen, the third expanding leaf, or the second and third expanding leaves, from the shoot apex (as indicated in Table 1) was assayed for CA activity. This meant that each week, leaves of a comparable developmental age were assayed. The second and third expanding leaves were chosen because they usually had the highest CA activity (or Rubisco activity), whether measured on a surface-area basis or on a Chl basis (see below). Plants that were clearly of the WT phenotype were discarded after the first weekly screen. In the case of transformation 2, plants with intermediate CA levels were also discarded. In both transformations, plants with reduced CA levels fell into two broad classes, those with intermediate CA levels of 20–50% of WT plants, but usually clustered around 30–40%, and those with less than 11% of WT levels, with a clustering around 4–5% (Table 1). This is more clearly seen in Fig. 2 which shows the results of a third transformation where the plants were clearly clustered into an intermediate- and a low-CA grouping. The differences between the TOBCA 1 and TOBCA 2 constructs appear to be due to small differences in transformation conditions since in other transformations either construct produced groups of plants with both low and intermediate CA levels.

Several low-CA plants from the first transformation (αTOBCA 1.10, 1.15 and 2.4; αTOBCA refers to antisense plants for tobacco chloroplast CA) were selected and analysed for CA and Rubisco activities and Chl content in a top-to-bottom profile experiment (Fig. 3). These measurements show that the antisense suppression of leaf CA activities did not reach a maximal reduction until a leaf developmental age corresponding to the third or fourth expanding leaf from the apex was reached. However, even in the first expanding leaves of αTOBCA plants CA levels were reduced to less than 20% of WT levels. In WT plants, CA levels (on a surface-area or Chl basis) reached a peak around the second or third leaf (Fig. 3) whereas leaves approached full expansion at the fourth or fifth leaf. Older leaves tend to show

Table 1. Transformed tobacco plants with low CA activity screened from two transformations. Transformation #1: A total of 30 transformants was screened over five weeks after the plants reached an age of two weeks; samples were taken from the third expanding leaf from the shoot apex ($n=5$). Transformation #2: A total of 32 transformants was screened for five weeks after the plants reached an age of two weeks; samples were taken from the third expanding leaf from the shoot apex for the first three weeks and the second and third expanding leaf for the last two weeks ($n=7$)

Plant	CA activity (WA units·cm^{-2})	SD	% of WT
Transformation #1			
Bin121 Controls (11 plants)	501	132	100
αTOBCA 1.7	113	22	23
αTOBCA 1.8	105	15	21
αTOBCA 1.10	56	26	11[a]
αTOBCA 1.12	243	64	49
αTOBCA 1.13	175	81	35
αTOBCA 1.15	55	18	11[a]
αTOBCA 2.1	27	11	5[a]
αTOBCA 2.4	222	36	44
αTOBCA 2.9	188	109	38
αTOBCA 2.11	216	92	43
Transformation #2			
Bin121 Controls (8 plants)	409	68	100
αTOBCA 1D	8	5	2
αTOBCA 1L	20	13	5
αTOBCA 1M	19	13	5
αTOBCA 1O	8	6	2
αTOBCA 2C	15	8	4
αTOBCA 2L	19	15	5
αTOBCA 2N	16	8	4

[a] Plants used in profile experiment (Fig. 3)

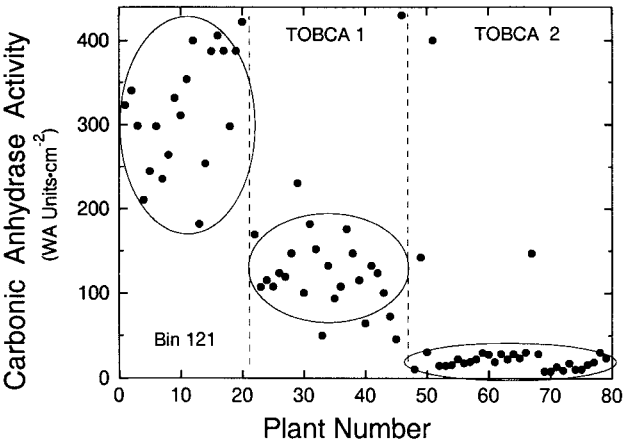

Fig. 2. Carbonic anhydrase activity in leaves from primary tobacco transformants recovered from a third transformation. Plants with reduced CA levels fall into two broad categories, those with low CA levels (<12% of WT levels) and those with intermediate CA levels around 40% of WT levels. Bin121 plants are control plants transformed with *Agrobacterium* containing the plasmid pBI121. Plants had been in glasshouse conditions for three weeks

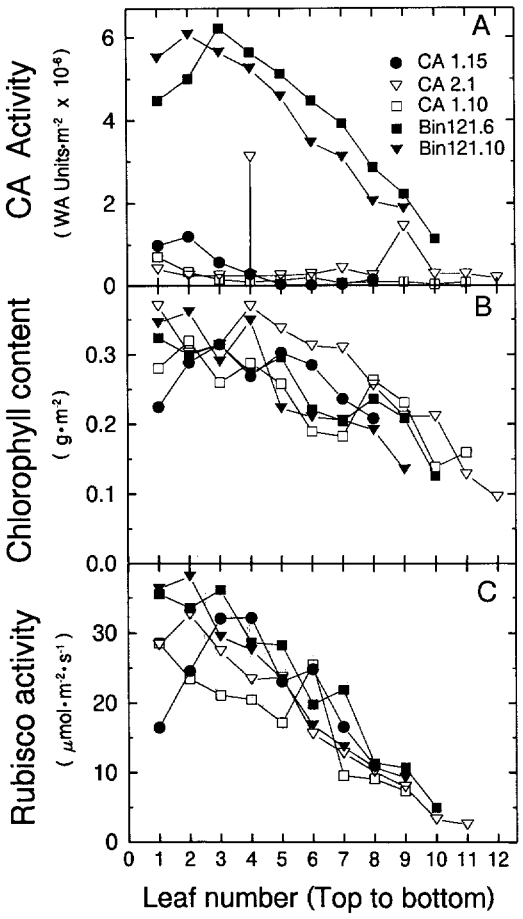

Fig. 3A–C. Carbonic anhydrase activity in WA units (**A**), chlorophyll content (**B**) and Rubisco activity (**C**) in leaves from three low-CA tobacco plants measured from the top to the bottom of each plant and compared to two Bin121 control plants

a steady decline in CA and Rubisco levels in response to a normal, slow senescence response, particularly in plants of the age used for these experiments, i.e. eight weeks. Presumably the antisense suppression is most effective as the leaf approaches maturity and where transcription levels for non-light-harvesting genes tend to decline. One of the three αTOBCA plants tested, αTOBCA 2.1, showed signs of instability with the fourth expanding leaf having normal CA levels on one side of the leaf and low activity on the other; leaf 9 also showed CA activity that was higher than expected (Fig. 3). The instability in this plant may have resulted from some type of somatic gene inactivation.

Segregation analysis. The analysis of young T_1 plants for kanamycin resistance using an agar-plate assay was not found to be a reliable measure of segregation patterns. A considerable degree of variability existed between experiments and the segregation ratios obtained could not be easily equated with typical Mendellian ratios for one or two alleles. As an alternative, segregation ratios were determined for the inheritance of the antisense CA character. A disadvantage of this approach is that it is not feasible to test large numbers of T_1 plants for CA activity, but the results so obtained do conform more readily with Mendellian genetics. On this basis, it can be seen that αTOBCA 1–10, a plant with low CA levels, had a segregation ratio of 1:13.3 (WT:low-CA phenotype; 43 plants tested) that conforms well to the theoretical ratio of 1:15 for the segregation of two independent alleles. Plants with intermediate CA levels, such as αTOBCA 1–8 and αTOBCA 2–4, had segregation ratios that were close to 1:3 (15 and 14 plants tested, respectively) indicating that the antisense character in these plants tended to act as a single independent allele. Southern-blot analysis of genomic DNA isolated from the primary transformant, αTOBCA 1–10, and probed with DNA for the tobacco CA gene, revealed the presence of two new hybridising bands when compared to DNA from WT plants, i.e. new 1.3- and 3.8-kb bands arose when cut with *Bam* HI and new 1.5- and 9.7-kb bands arose when cut with *Eco* RI (blots not shown). This is consistent with the view that the genome of αTOBCA 1–10 has two active insertions for antisense RNA. Several intermediate-CA plants were also analysed by southern blotting and these generally had a single new hybridising band present (data not shown).

Activity of CA in isolated chloroplasts. In C_3 plants, most if not all, of the CA activity is located in the chloroplast stroma (Everson 1970; Werden and Heldt 1972; Jacobson et al. 1975; Tsuzuki et al. 1985). However, as a precaution, the level of CA activity in chloroplasts isolated from a WT and a αTOBCA 1–10 plant was determined in an attempt to test the assumption that antisense treatment did produce a suppression of CA levels in the chloroplast compartment. Intact chloroplasts were isolated from a WT plant and also from an antisense plant with 1.4% of WT levels of the enzyme (on a Chl basis). The results in Table 2 show that chloroplasts from the antisense plant had the same low CA levels, i.e. 1% of the level in WT chloroplasts (on a Chl basis), as that evident in the leaf extract prior to chloroplast isolation. Considerable loss in the yield of CA activity in chloroplasts was found in both antisense and WT preparations. This may have occurred in the Percoll centrifugation step. Here, a partial loss of stromal contents can occur as starch granules tear through the envelope at the onset of centrifugation, with the envelope membranes then re-

Table 2. Carbonic anhydrase activities in intact chloroplasts from a WT and an antisense tobacco plant

	μg Chl/ml	CA (U/ml)	CA (U/mg Chl)	% (mut/WT)
1–10 homogenate	24.3	4.6	189	1.4%
WT homogenate	46.3	618.4	13356	
1–10 chloroplasts (82% intact)[a]	596.4	32.2	54	1.0%
WT chloroplasts (72% intact)	805.1	4367.3	5425	

[a] Chloroplast intactness (see *Materials and methods*)

sealing. Chloroplast intactness was determined to be 70–80% following isolation. Provided that this loss of stromal contents was similar for WT and antisense chloroplasts, which appears to be likely, these experiments do indicate that antisense treatment does in fact alter CA levels in the chloroplast.

Gas-exchange analysis. T_1 progeny from the primary transformant, αTOBCA 1–10, were screened to identify plants with low and intermediate levels of CA activity. Although αTOBCA 1–10 had CA levels that were about 11% of WT levels (Table 1), T_1 progeny with CA levels as low as 1% of WT levels were detected. This would appear to indicate that the two independent insertions of the antisense construct in αTOBCA 1–10 can act in a gene dosage response as they approach a homozygous condition; the response would appear to be asymptotic in nature. Low-CA plants and WT plants were subjected to gas-exchange analysis to ascertain if low CA levels had effects on various photosynthetic parameters. Leaf CA activities as low as 1–3% of WT levels (Fig. 4B) did not result in a significant reduction in the rate of CO_2 assimilation at 1000 μmol quanta·m^{-2}·s^{-1}, 350 μbar CO_2 and 25° C (Fig. 4A) compared to WT plants. Likewise it was not possible to detect any statistical alteration in leaf conductance (Fig. 4C), although there was a tendency for low-CA plants to have a higher conductance than WT plants. There were no significant alterations in Rubisco activity (Fig. 5A), the ratio of intercellular to ambient CO_2 partial pressure, p_i/p_a (Fig. 5B), Chl content (Fig. 5C), or dry weight per unit leaf area (Fig. 6A). There was, however, a slight tendency for the low-CA plants to be slightly smaller in stature following a ten-week growth period (observations not shown).

The carbon isotopic composition, $^{13}C/^{12}C$, in leaf dry matter (Δ; Fig. 6B) was significantly lower (0.83‰; $P<0.05$ level; *t*-test) in low-CA antisense plants compared to WT plants. The intermediate-CA group, with a mean CA activity of 10% of WT levels, showed a Δ value that was 0.58‰ lower than for the WT group; this was also significant at the $P<0.05$ level. Given that p_i/p_a was the same in the low-CA and WT plants, this indicates that the partial pressure of CO_2 at the sites of carboxylation for the antisense plants was below that in the WT plants, consistent with the role of CA in facilitating CO_2 diffusion within the chloroplast. The magnitude of the CO_2 gradient from the substomatal cavities to the sites of carboxylation for the low-CA plants can be calculated to be about 15 μbar larger than that in WT plants for the period over which the leaf was laid down (Fig. 6C).

On-line $\delta^{13}C$ and $\delta^{18}O$ discriminations. In an attempt to verify the preceding result, on-line discrimination measurements for ^{13}C and ^{18}O were undertaken. We obtained mean (±SE; $n=4$) values for the internal conductance for CO_2 diffusion from the stomatal cavity to the sites of carboxylation of 0.22±0.03 and 0.16±0.02 mol·m^{-2}·s^{-1} bar^{-1} for leaves of WT plants and

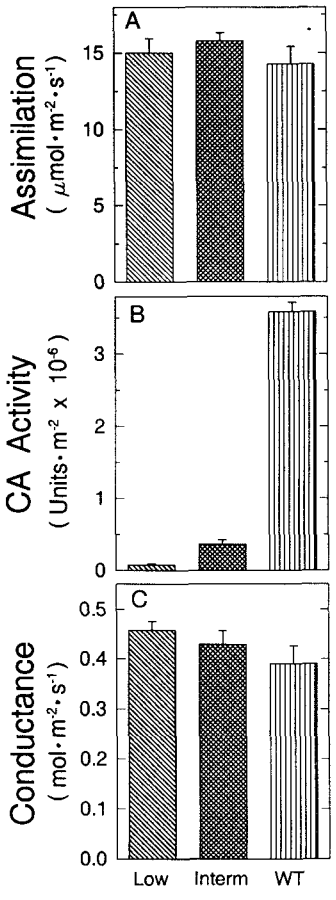

Fig. 4A–C. Leaf characteristics for the three groups of tobacco plants, namely low-CA plants (*Low*; leaf CA levels of approx. 1–3% of WT levels; mean value of 2.1%), intermediate-CA plants (*Interm*; leaf CA levels of approx. 7–15% of WT levels; mean value of 10%) and WT plants (*WT*). Data are presented in box plots as the mean±SE (error bars), where $n=4$, 5 and 9 for Low, Interm and WT plants, respectively. Leaf CO_2-assimilation rate (**A**), CA activity measured using the ^{18}O CA assay method (**B**), and leaf conductance (**C**) are shown. Leaf gas-exchange parameters were measured at 1000 μmol quanta·m^{-2}·s^{-1} and 350 μbar CO_2, and a leaf temperature of 25° C. Plants had been in glasshouse conditions for four weeks

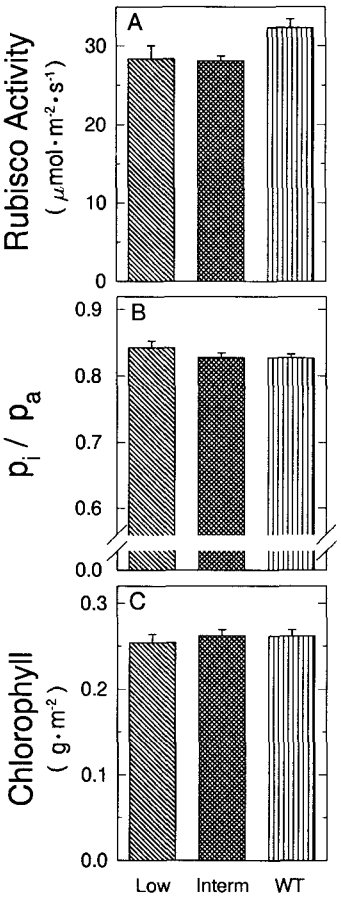

Fig. 5A–C. Leaf characteristics for the three groups of tobacco plants (see caption to Fig. 4) showing Rubisco activity (**A**), the ratio of intercellular to ambient partial pressure of CO_2, p_i/p_a (**B**) and Chl content (**C**)

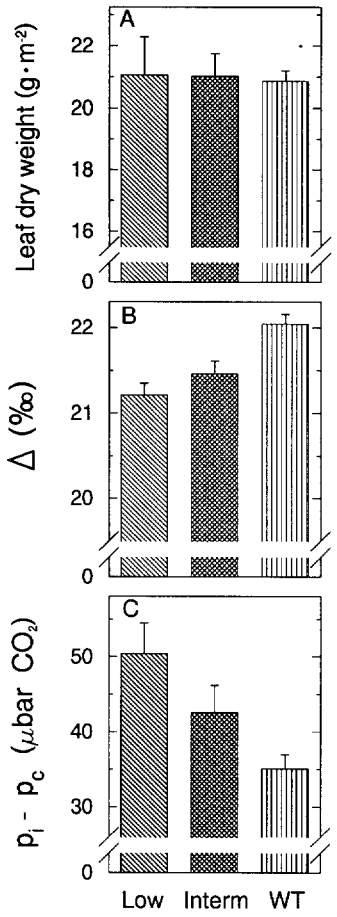

Fig. 6A–C. Leaf characteristics for the three groups of tobacco plants (see caption to Fig. 4) showing leaf dry weight per unit leaf area (**A**) and leaf Δ value for ^{13}C discrimination (**B**) and the calculated CO_2 gradient from the substomatal cavities to the sites of carboxylation, $p_i - p_c$ (**C**)

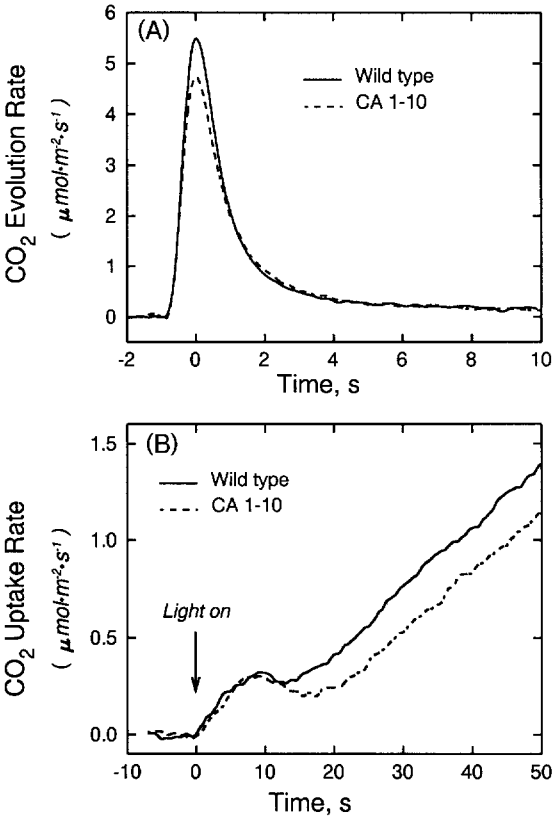

Fig. 7A, B. Kinetics of CO_2 uptake and evolution for both WT and antisense CA 1–10 plants. **A** Leaves were maintained in the dark in CO_2-free air for 30 min and then exposed to 3800 $\mu L \cdot L^{-1}$ CO_2 in air for 5 min to pre-load them with inorganic carbon. After this period, the gas phase passing over the leaf was rapidly switched back to zero CO_2 in air and the evolution of CO_2 into the CO_2-free gas stream was recorded (see traces). **B** Leaves were maintained in the dark in CO_2-free air for 30 min and then exposed to 3800 $\mu L \cdot L^{-1}$ CO_2 in air. After 5 min at elevated CO_2 the light (1000 μmol quanta $\cdot m^{-2} \cdot s^{-1}$) was switched on and the CO_2 uptake from the high-CO_2 gas stream was recorded (see trace)

the lowest-CA antisense plants (mean CA level of 2.1% of WT plants), respectively. The two plant groups had CO_2 assimilation rates of 16.9 ± 1.8 and 15.6 ± 1.9 $\mu mol \cdot m^{-2} \cdot s^{-1}$, respectively. Values for internal conductance to CO_2 diffusion represent a CO_2 gradient ($p_i - p_c$) of 78 ± 11 μbar for the WT plants and 98 ± 18 μbar for the low-CA plants. The 20-μbar difference between the two plant types is consistent with the values determined from leaf dry matter (Fig. 6B). Even more dramatic were the differences in discrimination against ^{18}O in CO_2; values averaging 46 ± 6‰ for WT and 24 ± 12‰ for the lowest-CA antisense plants (mean $\pm$ SE; $n = 4$). This is consistent with the proposed mechanism of ^{18}O discrimination in CO_2 by plants being, in part, due to carbonic anhydrase mediating the isotopic exchange of oxygen atoms with those of chloroplast water (Farquhar et al. 1993). The differences in ^{18}O discrimination also provide an independent method of verifying that CA activity relative to Rubisco carboxylations in the chloroplasts of antisense-CA plants have been reduced by at least 10-fold.

Fast gas-exchange analysis. When CO_2 is taken up by the leaf, it is accumulated into both CO_2 and HCO_3^- pools. The HCO_3^- pool is largely restricted to the chloroplast, where the pH of the stroma in the light is more alkaline than that of the surrounding cytosol (see Graham et al. 1984). Thus the rate at which CO_2 is absorbed and desorbed by the leaf is dependent upon the rate at which CO_2 is converted to HCO_3^- within the chloroplast. Considering this, there was at least some expectation that plants with low-CA levels may show minor alterations in the kinetics of CO_2 uptake and efflux under various conditions.

To check for such changes, fast gas-exchange measurements were made for WT and low-CA plants. Typical responses for a WT plant and a CA 1–10 plant with a 1% level of chloroplastic CA activity are shown in Fig. 7. Both the transients for CO_2 evolution after a switch from high CO_2 (3800 $\mu L \cdot L^{-1}$) to a CO_2-free condition (Fig. 7A) and the rate of CO_2 uptake after the onset of illumination (Fig. 7B) were found to be similar in both plant types. This suggests that the level of CA in the low-CA plants does not significantly limit the uptake and efflux of CO_2 on the time scale measured here.

Discussion

The precise role of chloroplastic CA activity in C_3 plants, and the reason for its relatively high abundance in the chloroplast, have occupied a considerable amount

of attention in the past. The results of this study clearly show that normal levels of chloroplast CA are not required for photosynthesis. Through the use of antisense-RNA technology we have been able to specifically suppress the levels of chloroplast CA to activities as low as 1% of WT levels. Within the experimental limitations, even the most severe reduction in CA levels caused no detectable reduction in the rate of CO_2 assimilation at 350 μbar CO_2 (Fig. 4A). Neither was it possible to detect any statistical alteration in leaf conductance (Fig. 4C), p_i/p_a (Fig. 4B), Rubisco activity (Fig. 5A), Chl content (Fig. 5C), or dry weight per leaf area (Fig. 6A). Low-CA plants exhibited no gross morphological differences when compared to WT plants, but there was a tendency for the lowest-CA antisense plants to exhibit a slightly smaller stature at the completion of a normal ten-week growth period (results not shown).

Analysis of leaf dry matter for ^{13}C discrimination (Fig. 6B) did, however, reveal the existence of a 0.83‰ lower Δ value in low-CA antisense plants compared to WT plants. From the difference between the two groups of plants, the CO_2 gradient from the substomatal cavities to the sites of CO_2 fixation in the chloroplast stroma ($p_i - p_c$) was calculated to be about 15 μbar greater in the low-CA plants for the period over which the leaf carbon was laid down (Fig. 6C). For the group of plants with around 10% of WT CA levels the calculated difference in CO_2 gradient was only 8 μbar. The difference was confirmed by concurrent measurements of ^{13}C discrimination and gas exchange. A 20-μbar difference between the two plants types was found at high irradiance, although this was only significant at the $P = 0.80$ level.

Using kinetic constants of Rubisco estimated from tobacco [namely, $\Gamma_* = 38.6$ μbar, $K_c = 258$ μbar, $K_o = 171$ mbar, K_mCO_2 at 21% $O_2 = 560$ μbar (data not shown), $p_i = 290$ μbar (this study) and using equations of Farquhar and von Caemmerer (1982)], and assuming identical Rubisco activities, it is possible to calculate for the low-CA plants (around 2% of WT levels) that a 15-μbar difference in $p_i - p_c$ relative to the WT will cause a 4.4% decline in the rate of CO_2 assimilation. Similarly for low-CA plants with 10% of WT levels of CA it can be calculated that a 2.2% reduction in the rate of CO_2 assimilation would be expected. To detect even the largest difference in assimilation rate with gas-exchange techniques would require sample sizes of more than 70, especially given the plant-to-plant variation normally experienced with tobacco. Nevertheless, although a reduction in CA levels of two orders of magnitude does not produce any easily detectable reduction in CO_2-assimilation rates, it can be argued that the seeming over-abundance of CA in the chloroplast could function to produce a small but desirable increase in the efficiency of CO_2 assimilation. This would be consistent with the model of Cowan (1986) in which it is predicted that an increase in chloroplast CA levels will lead to a small improvement in the rate of CO_2 assimilation. The model also assumes that the investment in CA protein occurs at the expense of Rubisco, but that facilitation of CO_2 diffusion more than offsets the reduction in Rubisco activity, resulting in an increase in plant competitiveness. By using the specific activity of spinach CA (Pocker and Ng 1973) and the fact that 1.0 unit of CA activity is equivalent to approx. 2.34 WA units, for the enzyme assay conditions presented in this paper, and assuming that the WT plants used here had Rubisco protein levels of around 33% of soluble leaf protein (von Caemmerer et al. 1992) it is possible to calculate that chloroplast CA protein represents about 0.6% of soluble leaf protein. In a nitrogen-sufficient growth environment this level of nitrogen investment would not seem to be expensive in terms of the apparent benefits to net CO_2 assimilation and possible benefits to competitiveness.

The measurement of CA activity using the ^{18}O method makes it possible to calculate the catalysis factor which is likely to be operative in the chloroplast volume of WT as well as low-CA plants. The conditions of the assay, 25° C, 10 μM CO_2 (1 mM HCO_3^-), pH 8.0 and chemical equilibrium for CO_2 and HCO_3^-, are quite similar to the conditions in the chloroplast stroma. If the volume/chlorophyll characteristics of tobacco chloroplasts is similar to other C_3 plants, namely 30 μL of chloroplast space per mg chlorophyll (Oja et al. 1986), then it is possible to calculate from the data in Fig. 4B that low-CA plants, intermediate-CA plants and WT plants have CA catalysis factors for the chloroplast volume of around 9600, 46300 and 453000 times the uncatalysed rate, respectively. If the half-time for chemical equilibrium between CO_2 and HCO_3^- is taken to be approx. 15 s at pH 8.0 and 25° C, then it follows that the half-times for chemical equilibrium in the low-CA plant and the WT plant would be in the vicinity of 2 ms and 30 μs, respectively. Clearly this type of calculation is simplistic since it does not take into account the effect of CA in facilitating transfer of CO_2 across the chloroplast envelope and the precise kinetics of CA under non-equilibrium conditions. This calculation would, however, help to explain why rapid gas exchange on plants with even the lowest CA levels failed to detect a difference in the period required to reach chemical equilibrium as CO_2 is absorbed by the chloroplast in a low-to-high CO_2 transition (Fig. 7). Only with a null mutation for CA would a clearly detectable difference be expected using fast gas-exchange techniques.

The CA catalysis factors can also be converted to enzyme activity units for the chloroplast volume and then compared with the observed rates of CO_2 assimilation. This can be done by using published data for the uncatalysed rate for CO_2 hydration, namely $3 \cdot 10^{-4}$ mmol·s^{-1}·L^{-1} (Magid and Turbeck 1968). On this basis the ratio of CO_2 hydrations to CO_2 fixations within the chloroplast volume can be calculated as 69.2 in a typical WT plant and 0.98 for a low-CA plant (1% of WT levels). This can be compared to a hypothetical null mutant for CA that would be expected to have a ratio of around 0.00016 CO_2 hydrations per CO_2 fixed. The values for WT and low-CA antisense plants are consistent with the recently developed theory on ^{18}O isotope effects on CO_2 assimilation (Farquhar and Lloyd 1993) and would be equivalent to ρ values (percent carboxylations to hydrations) of approx. 1% and 100%, respectively. The theory of Farquhar and Lloyd (1993) provides an independent means of determining the ratio of carboxylations to hydrations. The results of subsequent experiments with CA antisense plants and the application of this theory will be presented at a later date.

A number of studies have reported a considerable variation in the CA levels in leaves from various species, e.g. Poincelot (1979), Tsuzuki et al. (1985), Hatch and Burnell (1990) and Makino et al. (1992). Wheat is an example of a plant that appears to operate with CA levels some 10- to 15-fold lower than species such as spinach, pea (Makino et al. 1992), though in one study wheat had CA levels that were only 2-fold lower than for pea and spinach (Hatch and Burnell 1990). By comparison with these published data, tobacco (Fig. 3A) tends to have CA levels that are up to twofold lower than for spinach and pea. The results of this study, using stable-isotope techniques, indicate that plants with a reduction in chloroplastic CA levels of two orders of magnitude may have rates of CO_2 assimilation that are as much as 4.4% lower than WT plants. In the group of plants with a reduction in CA levels of one order of magnitude the calculated disadvantage to net CO_2 assimilation has already reduced to as little as 2.2%. Clearly it would be possible for plants to show considerable variability in the upper-range CA activities without suffering a significant trade-off in photosynthetic performance.

Although most recent data have suggested that leaf CA is localised within the chloroplast of C_3 plants (Jacobsen et al. 1975; Tsuzuki et al. 1985), there were some early reports of multiple isoenzymes of CA (Kachru and Anderson 1974; Poincelot 1979; Graham et al. 1984), some of which may be cytosolic forms of the enzyme. However, recent identification of a second CA cDNA in *Arabidopsis thaliana* (Fett and Coleman 1993), which may code for a cytosolic CA, raises some interest in this matter. It is difficult to argue a role for a cytosolic CA in improving diffusion of CO_2 to the chloroplast. This is because diffusion distances from the cell membrane to the chloroplast envelope are small and the HCO_3^- level is low due to the more-neutral pH of the cytoplasm (Cowan 1986). The data reported here for tobacco show that antisense to the chloroplast cDNA clone can reduce total leaf CA activity by up to 99%. This can be used as evidence to argue for a lack of any other significant form of CA in the leaf. However, it is feasible that the chloroplastic and cytosolic CA isoforms are coded by the same or very similar genes and that the antisense effect reported here is reducing expression of both forms. The data reported in Table 2 really only show that chloroplastic CA is suppressed but give no evidence as to what may be happening to any other CA isoform. If cytosolic CA is present in tobacco and is suppressed in the antisense plants, then the data can be used to argue for a relatively small role for cytosolic CA in increasing the efficiency of photosynthesis.

Some studies of CA levels in intact leaves have suggested that there may be some co-ordinate regulation of CA and Rubisco expression under various growth conditions such as variations in nitrogen supply (Makino et al. 1992) and CO_2 level (Porter and Grodzinski 1984; Peet et al. 1986). This has been more closely examined recently in pea cultivars (Majeau and Coleman 1993), where both transcript abundance and protein activity for both CA and Rubisco were regulated in almost identical fashions during plant development and a constant ratio of Rubisco to CA appeared to be maintained, even between cultivars with different photosynthetic capacities. Such observations can be used to suggest some importance in this apparent co-ordination. The model predictions of Cowan (1986) would argue that such a constant balance may be necessary to achieve the optimal use of nitrogen in the leaf, but this remains to be proved. The data presented here for tobacco clearly show that the co-ordination between CA and Rubisco expression is not obligatory and can be easily broken by the antisense effect. Other studies with both spinach (Randall and Bouma 1973) and *Phaseolus* (Edwards and Mohamed 1973) also show that growth at low zinc levels can specifically reduce CA without large effects on Rubisco and photosynthesis. Thus the importance of and mechanistic basis for the apparent co-ordination between CA and Rubisco remains to be fully explained.

The results of this paper can be used to argue that the major role for CA in the C_3 chloroplast is to facilitate the passive diffusion of CO_2 within the stroma and thus reduce the CO_2 gradient from the cell wall to the active site of Rubisco. Such a conclusion would seem to rule out other roles for CA which might have been suggested based on studies with other photosynthetic systems. There appears to be no special association between Rubisco and CA which may lead to a localised elevation of CO_2 at the active site of Rubisco, as has been found for the carboxysomal association in cyanobacteria and may be the case for the pyrenoid of many algae (Badger and Price 1992). In addition, this reinforces the already well-accepted notion that the C_3 chloroplast possesses no mechanism for active accumulation of inorganic carbon, as appears to be the case in microalgal chloroplasts (Badger and Price 1992). However, it should be noted that the antisense effects produced here still resulted in significant activities of CA within the chloroplast that may be playing some as yet unexplained role. The final resolution of this somewhat remote possibility can probably only be achieved by the isolation and analysis of a true null genetic mutant.

We thank Dr. John R. Coleman (Department of Botany, University of Toronto, Canada) for making his manuscript available to us prior to acceptance and Barbara Setchell for help with plant maintenance. G.D.P. acknowledges support from a QE II Research Fellowship (Australian Research Council).

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