Plant Molecular Biology **25**: 377–385, 1994.

Modification of carbonic anhydrase activity by antisense and over-expression constructs in transgenic tobacco

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Received 29 November 1993; accepted in revised form 18 February 1994

Key words: carbonic anhydrase, antisense, over-expression, tobacco, photosynthesis

Abstract

The activity and location of carbonic anhydrase has been modified by transformation of tobacco with antisense and over-expression constructs. Antisense expression resulted in the inhibition of up to 99% of carbonic anhydrase activity but had no significant impact on net CO_2 assimilation. Stomatal conductance and susceptibility to water stress appeared to increase in response to the decline in carbonic anhydrase activity. An over-expression construct designed to increase cytosolic carbonic anhydrase abundance resulted in a significant increase in net activity, a small increase in stomatal conductance but little impact on CO_2 assimilation. Chloroplastic carbonic anhydrase activity was enhanced by the expression of an additional construct which targeted the polypeptide to the organelle. The increase in chloroplastic carbonic anhydrase appeared to be accompanied by a concomitant increase in Rubisco activity.

Introduction

The enzyme carbonic anhydrase (CA), which catalyses the reversible hydration of CO_2, is present in the chloroplast stroma of all C_3 higher plants that have been examined [17, 20]. It has been suggested that its major role in photosynthesis is to facilitate the equilibration of the inorganic carbon (C_i) species within the stroma [3, 17]. This would result in the maintenance of the CO_2 diffusion gradient across the chloroplast envelope as well as ensuring that the supply of CO_2 to the principal carboxylase, ribulose-1,5-bisphophate carboxylase/oxygenase (Rubisco), is not limited by the rate of dehydration of the large HCO_3^- pool in the alkaline stroma. This presumed role for CA in C_3 plants is based on the fact that the uncatalysed interconversion of CO_2 and HCO_3^- is relatively slow at alkaline pH values. There are data to suggest however that the abundance of CA in the chloroplast (ca. 1–2% of the soluble protein) is well in excess of that required for the equilibration of the C_i pools, and that the transfer of CO_2 across the chloroplast envelope and through the stroma to the carboxylation site is not enhanced by high CA activity [20]. Zinc deprivation studies were able to reduce CA levels to below 10% of wild-type activities and yet growth and photosynthesis were not markedly affected [4, 16]. Photoautotrophic cell suspension cultures, which exhibit photosynthetic characteristics of leaf cells, can have very little CA activity

and yet display affinity constants for CO_2 which are comparable to cell lines expressing abundant CA activity [18]. Attempts to determine a role for CA by using CA inhibitors with intact chloroplasts resulted in inhibition of photosynthesis [5], however a direct effect of these inhibitors on photosynthetic electron transport activity has also been shown [19]. A more specific reduction in CA activity can be achieved with the use of antisense gene expression in transgenic plants. In addition, over-expression constructs may also provide information on the role of CA in photosynthetic carbon acquisition. In this paper we report on the generation and characterization of transgenic tobacco plants expressing CA antisense constructs as well as constructs that result in over-expression of CA in the chloroplast or the cytosol.

Materials and methods

Construction of over-expression and antisense vectors

The plasmid pTOBCA containing a 1223 bp cDNA encoding tobacco chloroplastic CA and cloned into pBluescript SK^- as a *Not* I-*Eco* RI fragment was the source material for the CA gene [14]. *In planta* expression of the full-length gene product including the transit peptide was achieved by cloning a *Hin*d III-*Ssp* I fragment into the *Agrobacterium* binary vector pGA643 [1] such that the gene was positioned between the CaMV 35S promoter and the *nos* 3′ element, utilizing the *Hin*d III and *Hpa* I sites. This construct was designated pSTCA and should result in over-expression of the chloroplast-localized CA. The plasmid pSCCA was designed to result in over-expression of a truncated CA protein that lacks a transit peptide and should be restricted to the cytosol. This was achieved by digestion of the *Hin*d III-*Ssp* I cDNA fragment with *Pvu* II which removed a 300 bp 5′ fragment coding for the transit peptide. A 17 bp linker (5′-AGCTTCTC-GAAAGATGG-3′) which contains a ribosome binding site from potato virus X [9] and an ATG codon in frame with the mature protein sequence, as well as compatible 5′ and 3′ ends, was ligated to the *Pvu* II-*Ssp* I fragment prior to ligation into the *Hin*d III and *Hpa* I sites of the pGA643. The antisense expression construct (designated pATCA) was generated by ligation of a *Cla* I-*Ssp* I fragment (obtained from digestion of pTOBCA) in the antisense orientation into the *Cla* I-*Hpa* I sites of pGA643. The cloning strategies employed for all three vectors are shown in Fig. 1.

Agrobacterium-*mediated transformation and growth of plant material*

The plasmids were introduced into *Agrobacterium tumefaciens* strain LBA4404 and positive clones were selected by growth on kanamycin-containing plates (50 μg/ml). Leaf disks of *Nicotiana tabacum* cv. Carlson were transformed, and whole plants regenerated essentially as described by Horsch *et al.* [7]. Transformed tissue was selected by culturing callus on Murashige-Skoog (MS) plates containing 1 μg/ml 6-benzylaminopurine, 0.25 μg/ml α-naphthaleneacetic acid, 500 μg ml carbenicillin and 100 μg/ml kanamycin sulphate. Shoots were rooted on MS plates containing 3% sucrose and 300 μg/ml kanamycin sulphate. Plantlets were transplanted into a sterile soil mixture (Promix) and placed into a temperature controlled glasshouse under natural light conditions. Day/night temperature regimes were 25/16 °C and maximum photon flux was approximately 800 $\mu mol\ m^{-2}\ s^{-1}$. Plants were fertilized weekly with a complete nutrient solution. Only the most recent fully expanded leaves were used for biochemical and gas exchange analyses.

Biochemical analysis of tobacco leaf tissue

Leaf punches were collected under full-light conditions from fully expanded leaves of similar age, quickly weighed and then frozen in liquid nitrogen. The frozen samples were ground with a mortar and pestle in cold extraction buffer (50 mM Hepes/KOH pH 7.1 containing 20 mM

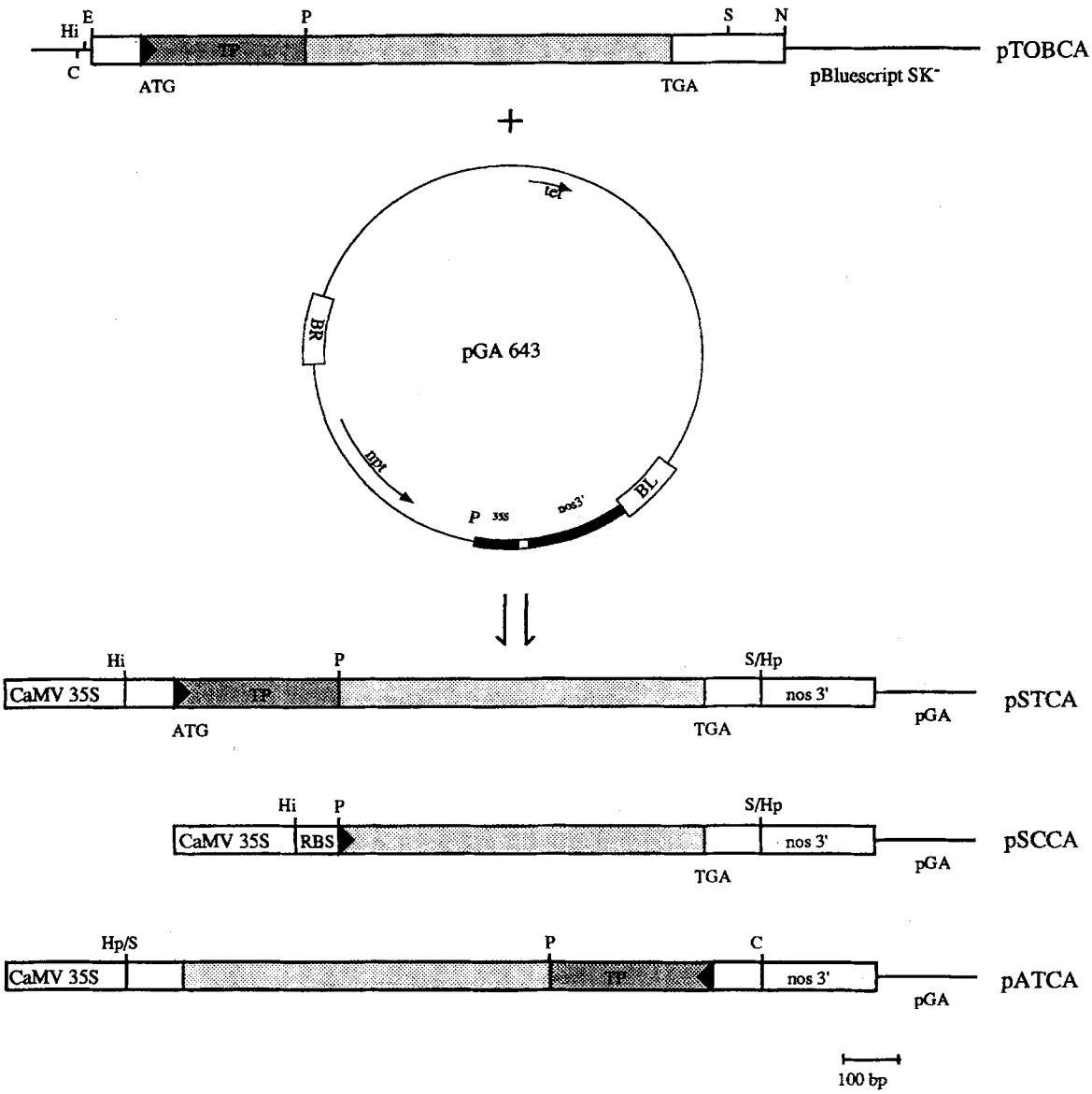

Fig. 1. Construction of CA over-expression and antisense vectors. A 1142 bp cDNA fragment encoding the entire tobacco CA was isolated from the pTOBCA, modified as required and inserted into the binary plasmid pGA643 as described in Materials and methods. Abbreviations used: TP, transit peptide; Hi, *Hin*d III; C, *Cla* I; P, *Pvu* II; S, *Ssp* I; N, *Not* I; Hp, *Hpa* I; BR and BL, right and left border of the T-DNA; npt, neomycin phosphotransferase.

$MgCl_2$, 1 mM Na_2EDTA, 10 mM DTT, 10 mM $NaHCO_3$, 20 mM isoascorbate, and 0.1% w/v insoluble polyvinylpyrrolidone). Sub-samples were removed for chlorophyll analysis [13] and the remaining homogenate clarified by centrifugation at 10000 $\times g$ for 3 min at 4 °C. CA activity in the supernatant was determined using an electrometric assay as described previously [13]. Rubisco activity in the supernatant was determined by measuring the rate of ribulose bisphosphate-dependent incorporation of $^{14}CO_2$ into acid-stable counts at 25 °C, after full activation of the enzyme as described previously [8]. The concentration of soluble protein in the super-

natant was determined as described by Bradford [2].

Western blot analysis of tobacco protein

Frozen tissue samples (both leaf and root) were ground in the extraction buffer as described above and the soluble protein present in the supernatant fraction precipitated by the addition of trichloroacetic acid (final concentration 10% v/v) and incubation on ice for 20 min. Protein samples were resuspended in 0.1 M Na_2CO_3 and 0.1 M DTT, solubilized by the addition of SDS and boiled for 1 min prior to electrophoresis using a 12% polyacrylamide gel. The separated proteins were transferred to nitrocellulose and the CA monomer identified following incubation with a polyclonal antibody directed against pea chloroplastic CA and using a protocol previously described [15].

Gas exchange measurements

Leaf gas exchange values were determined using a LI-COR portable gas exchange system (Model 6200). Fully expanded leaves of similar age were placed into the leaf chamber and rates of CO_2 assimilation and conductance determined at 350 ppm and 260 ppm CO_2. The relative humidity of the chamber was between 40 and 50%, leaf temperature varied from 25 to 27 °C, and the photon flux was 250 μmol m^{-2} s^{-1} for all measurements.

Results

Expression of CA vectors in transgenic tobacco

The expression of each vector in kanamycin-resistant transgenic plants was confirmed by SDS-gel electrophoresis and western blot analysis of tissue extracts. The western blot profile of leaf extracts from control plants transformed with the vector pGA643 exhibits two bands of 27.7 and 24 kDa (Fig. 2A, lane 1). The 24 kDa polypeptide is located in the chloroplast whereas the larger polypeptide is found in the cytosol ([18], unpublished data). These proteins could not be detected in leaf extracts from two representative plants containing the antisense vector pATCA (Fig. 2A, lanes 2 and 3). Northern blot analysis of RNA isolated from these plants also showed a significant reduction in *ca* mRNA abundance (data not shown). Plants containing the overexpression vectors pSCCA (cytoplasm targeted) and pSTCA (chloroplast targeted) were expected to produce polypeptides that when processed would be of similar size to that found in wild-type plants. As such, it was difficult to confirm the presence of these vector products by western blot analysis of leaf extracts, although the intensity of the immuno signal is perhaps increased over that

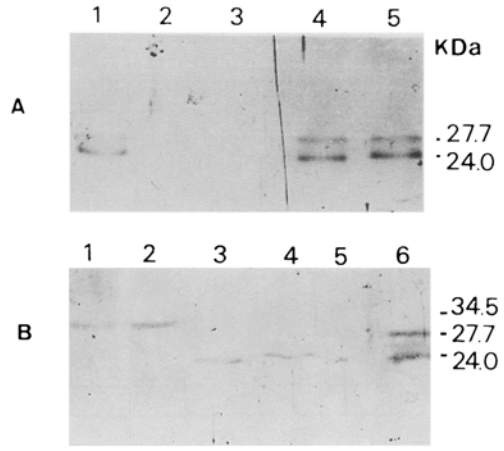

Fig. 2. Analysis of CA proteins present in leaves (A) and roots (B) of transgenic tobacco. A. 3.5 μg of total soluble protein, isolated from fully expanded leaves of tobacco plants transformed with the unmodified vector pGA643 (lane 1), constructs pATCA (lanes 2 and 3), pSTCA (lane 4) and pSCCA (lane 5), were separated by SDS-PAGE and transferred to nitrocellulose. The location of the CA monomer was identified using polyclonal antibodies directed against pea CA. B. Soluble root proteins were obtained from tobacco plants transformed with pSTCA (lanes 1 and 2), pSSCA (lanes 3 and 4), pGA643 (lane 5). For comparison, soluble proteins from leaves of plants transformed with pGA643 is shown in lane 6. The identity of the various CA monomeric species following SDS-PAGE was determined as described in A. Lanes 1 and 2 contain 20 μg of total soluble protein whereas other lanes contain 2 μg of protein. For both A and B, the molecular mass of the recombinant and leaf CA monomers are indicated.

Table 1. CA activity in root tissue of transgenic tobacco.

Plant type	CA activity (units per g soluble protein $\times 10^{-5}$)
1. STCA	0.17
2. STCA	0.15
3. SCCA	1.58
4. SCCA	1.25
5. pGA643	0.0

Data were obtained from 5 different transgenic plants. A western blot of these root proteins, and for comparison, a leaf extract is shown in Fig. 2B.

exhibited by control plants (Fig. 2A, lanes 4 and 5 compared with lane 1). Western blot analysis of non-green tissue (root protein preparations) was used to confirm the expression of a 34.5 kDa unprocessed, polypeptide product of pSTCA (Fig. 2B, lanes 1 and 2) and a 24.0 kD product of pSCCA (Fig. 2B, lanes 3 and 4). Root extracts from control plants (transformed with the vector alone) contain no CA protein (Fig. 2B, lane 5) and a western blot profile of a leaf extract is shown for comparison (Fig. 2B, lane 6). Table 1 shows the CA activity found in root tissue extracts of plants transformed with pSTCA, pSCCA, as well as the vector pGA643.

Biochemical analysis of leaf tissue

Fully expanded leaves of a similar age obtained from control plants and plants expressing the various CA constructs were analyzed for CA and Rubisco enzymatic activity, soluble protein and chlorophyll content. The mean values for each group of plants are shown in Table 2. The leaves of the 10 antisense plants examined exhibit a large reduction in CA activity to a mean level of 30% of the control. Individual plants however, were found to have extremely low levels of CA activity, less than 1% of the control values. The mean CA activity of plants containing the over-expression vectors STCA and SCCA was ca. 60% greater than the control plants with some individuals exhibiting up to 200% of control plant activity. A few plants containing the over-expression vectors were also found to have relatively low levels of CA, well below that found in wild-type plants. Presumably, CA expression in these plants is inhibited by co-suppression, and these plants were discarded. Rubisco levels in CA antisense plants were unaffected by the large reduction in CA activity. In contrast, Rubisco activity appeared to be significantly increased in plants expressing the chloroplast CA vector when compared with control plants. This result was

Table 2. Characteristics of fully expanded leaves of transgenic tobacco plants.

Characteristic	Plant type			
	pGA643 ($n = 9$)	pATCA ($n = 10$)	pSTCA ($n = 4$)	pSCCA ($n = 9$)
CA activity (units/m^2 $\times 10^{-6}$)	1.96 ± 0.48	0.58 ± 0.25	3.16 ± 0.47	3.16 ± 0.35
Rubisco activity (μmol m^{-2} s^{-1})	39 ± 18	34 ± 15	78 ± 15	56 ± 13
Soluble protein (g/m^2)	4.7 ± 1.1	4.4 ± 0.9	7.6 ± 0.7	6.1 ± 1.2
Chlorophyll (g/m^2)	0.21 ± 0.02	0.21 ± 0.04	0.24 ± 0.04	0.23 ± 0.04
Leaf fresh weight (g/m^2)	183 ± 23	187 ± 28	184 ± 12	191 ± 15

Tobacco plants transformed with the vector pGA643 were used as control plants. Data represent the mean $\pm$ SD of n number of plants with a single leaf assayed from each plant. The most recent, fully expanded leaf was used for each assay to minimize developmental differences.

consistent with the measured increase in leaf soluble protein content of the pSTCA-transformed tissue. Plants expressing the cytoplasm-targeting CA construct did not exhibit any major change in Rubisco activity or leaf soluble protein levels. Modification of CA expression in trans-

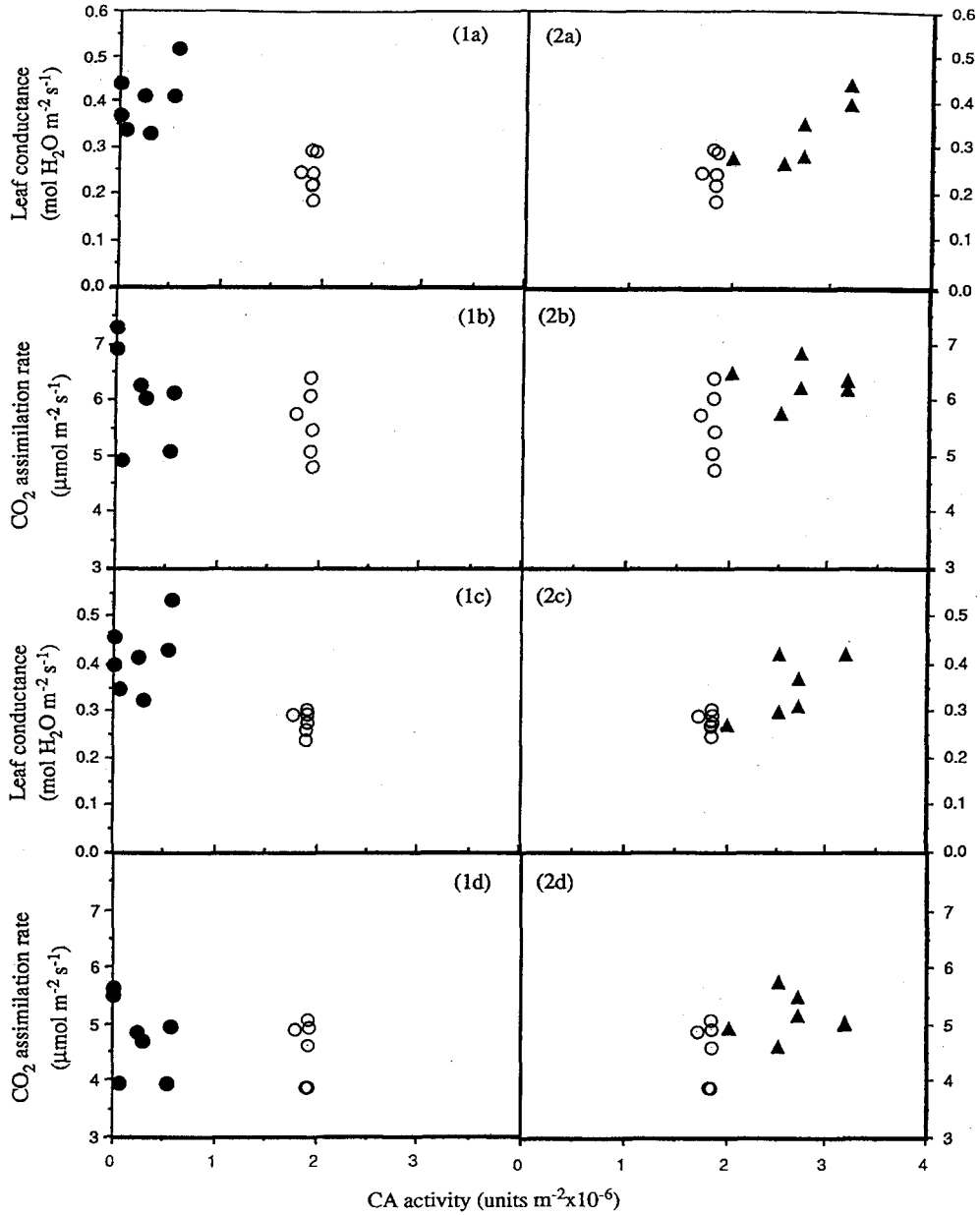

Fig. 3. Stomatal conductance and CO_2 assimilation in control and transgenic tobacco plants. The photosynthetic characteristics of new, fully expanded tobacco leaves were determined using a Li-Cor portable photosynthesis system and plotted as a function of total CA activity in each assayed leaf. Measurements were performed at 25 to 27 °C, 40 to 50% relative humidity and a photon flux of 250 μmol m^{-2} s^{-1}. Panels a and c show stomatal conductance and panels b and d show CO_2 assimilation data at external CO_2 concentrations of 350 ppm (panels a and b) and 260 ppm (panels c and d). Data were obtained from plants transformed with pATCA (●), the unmodified vector pGA643 (○), and pSCCA (▲). Each point represents one measurement from one leaf and no more than two leaves from each individual plant were used.

genic plants did not affect leaf chlorophyll content nor leaf fresh weight. Microscopic analysis of transgenic leaf sections did not reveal any major differences in cell morphology, cell thickness or number of stomata (data not shown). Cellulose nitrate imprints of stomata indicated that when examined under normal growth conditions, plants expressing the pATCA vector had more open stomata than did control plants (data not shown).

Gas exchange analysis

Stomatal conductance and rate of CO_2 assimilation were measured in fully expanded leaves of transgenic plants which exhibited the lowest CA activity (ATCA plants), the highest cytoplasmic CA content (SCCA), as well as those transformed with the vector alone. To reduce variation due to development, the leaves chosen for gas exchange analysis were of similar age for all plants. Measurements were made at external CO_2 concentrations of 350 ppm and 260 ppm and the data are shown in Fig. 3. Plants expressing the antisense construct pATCA generally displayed an increase in conductance when compared with control plants (Fig. 3, panels 1a and 1c). The difference in conductance values between antisense and control plants was apparent at both CO_2 concentrations. A comparison of the rates of CO_2 assimilation for antisense and control plants did not reveal any significant differences at either CO_2 concentration (Fig. 3, panels 1b and 1d). Gas exchange analysis of leaves expressing CA in the cytosol (SCCA plants) also suggested some increase in conductance values relative to control plants, however the difference was less than that found with the antisense plants (Fig. 3, panels 2a and 2c). Again, no significant differences in rates of CO_2 assimilation at either external CO_2 concentration were observed (Fig. 3, panels 2b and 2d).

Discussion

The expression of the various CA constructs in tobacco that have been described in this paper results in a wide variance in total CA activity in the leaf. We have used this technology to evaluate the requirement and role of CA in C_3 plant carbon assimilation. The most immediate observation of these studies is that significant changes in CA activity however seem to have had little impact on the photosynthetic capacity of the plant under normal growth conditions. In some plants, the expression of the CA antisense construct was able to reduce CA levels to below 1% of wild-type values and yet assimilation rates did not seem to be significantly impaired. There is some evidence to suggest that the plants have compensated for the reduction in CA by increasing stomatal conductance with the resultant increase in intercellular CO_2 levels. The photosynthesis data are somewhat variable and a much more sophisticated analysis of gas exchange capacity will be needed to confirm these observations. It was interesting to note however that the CA antisense plants seemed to be more susceptible to water stress than control plants, and required more frequent watering. This anecdotal evidence would support the observation of increased conductance values. How the reduction in CA activity results in an increase in conductance is unknown but it is possible that reduced CA activity in guard cells may directly affect stomatal opening by mimicking low intercellular C_i levels.

It is important to note however, that apart from the relatively minor changes in conductance, a significant reduction in CA activity had very little impact on photosynthetic capacity and growth form of the plant. It would seem that in the wild-type plant, CA levels in the chloroplast are well in excess of that required for photosynthesis, or that a major reduction in CA activity does not have an easily measured impact on carbon assimilation capacity. Although levels of CA in some plants were reduced to below 1% of wild-type values there may still be enough CA to catalyse C_i interconversion at a sufficiently high rate for facilitation of CO_2 movement into and through the chloroplast, as well as generation of CO_2 at the site of carboxylation. As such, it is possible that only a null mutant in CA activity would exhibit the expected impaired assimilation capacity.

The over-expression of chloroplastic or cytosolic CA did result in significantly higher levels of enzyme activity in the appropriate cell compartment. Products generated by the two over-expression vectors were identified in western blots of root and leaf extracts and by activity measurements. The 34.5 kDa polypeptide encoded by pSTCA, and identified in root extracts, represents the complete, unprocessed CA primary product with an intact transit peptide. It is still able to assemble and is active although the level of expression is lower than that generated by pSCCA which encodes the processed form of the enzyme. This result was not unexpected as an earlier study was able to show that the unprocessed CA monomer when expressed in *Escherichea coli* was capable of assembly and activity [11]. When expressed in leaf tissue, the nascent, full-length polypeptide is directed to the chloroplast and is processed to generate the expected 24 kDa stroma-localized product. The enhanced chloroplastic CA levels appear to have resulted in a concomitant increase in Rubisco activity in the chloroplast. In addition, leaf soluble protein levels were also higher, perhaps suggesting an increase in Rubisco abundance. In an earlier study we have shown that across a number of pea cultivars, and during pea leaf development, a constant ratio of CA to Rubisco is maintained [13] and it was suggested that there is some coordination of expression of the two enzymes. It is possible that the elevated levels of chloroplastic CA in the STCA plants have also induced the enhanced expression of Rubisco. This hypothesis is somewhat difficult to reconcile with the wild-type levels of Rubisco activity found in the CA antisense plants, a situation where Rubisco expression should have been reduced to match the lower levels of CA. We are currently examining the patterns of CA and Rubisco expression in the F_1 generation plants transformed with pSTCA.

Over-expression of the cytosolic CA did not generate a photosynthetic phenotype that was markedly different from wild-type plants although gas exchange analysis did suggest that stomatal conductance was somewhat higher relative to controls. The reasons for this observations are unclear. It is possible that elevated levels of cytosolic CA are perhaps an impediment to efficient CO_2 diffusion to the site of carboxylation, or result in a modified guard cell response to external CO_2 concentrations. This is somewhat surprising as cytosolic isoforms of CA in C_3 plants have been reported although a role for these enzymes has not been shown [12, 17]. The presence of a 27.7 kDa polypeptide in wild-type (and control transformed) plants certainly suggests that a cytosolic form of the enzyme is a normal occurrence. In wild-type plants, low endogenous levels of cytosolic CA would be useful in the generation of HCO_3^- as substrate for cytosolic phosphoenolpyruvate (PEP) carboxylase, a similar role as that proposed for the mesophyll cell-localized C_4 CA [6]. An evaluation of the role of cytosolic CA in guard cell function is currently under study.

Acknowledgements

This research was funded by a grant to J.R.C. Coleman from the Natural Science and Engineering Research Council of Canada (NSERC). N.M. is a recipient of an NSERC post-graduate scholarship. The authors acknowledge Mr. Quishi Liu, Faculty of Forestry, University of Toronto for assistance with the gas exchange system and Dr D. Price, Research School of Biological Science, Australian National University, for helpful discussion of the data.

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