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Correlation of Carbonic Anhydrase and Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Expression in Pea[1]

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The enzyme carbonic anhydrase (carbonate dehydratase, EC 4.2.1.1) is an abundant soluble protein in the C_3 plant chloroplast; however, its function in photosynthetic carbon assimilation is not well defined. In this study we have examined the relationship between carbonic anhydrase (CA) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) expression during pea (*Pisum sativum*) development as well as in various tissues and cultivars. Although absolute levels of activity and transcript abundance for the two proteins can vary considerably during development, a relatively constant ratio of CA to Rubisco transcript abundance and enzyme activity appears to be initiated during greening and maintained in mature and senescing photosynthetic tissue. Various pea cultivars, although exhibiting differing amounts of Rubisco and CA, also appear to maintain an invariant CA:Rubisco ratio. These data are discussed with respect to gene copy number, regulation of expression, and the proposed role of CA in photosynthetic carbon fixation.

The enzyme CA (carbonate dehydratase, EC 4.2.1.1) is considered to be one of the more abundant soluble proteins in the C_3 plant chloroplast, second only to Rubisco in concentration (Reed and Graham, 1981; Okabe et al., 1984). Its precise role in C_3 photosynthetic carbon assimilation, however, has remained speculative. Functionally, the enzyme is capable of rapidly interconverting the major forms of C_i, and therefore maintaining the supply of CO_2 for Rubisco by speeding the dehydration of HCO_3^- in the stroma. The uncatalyzed rate of CO_2 generation from HCO_3^- is relatively slow and could limit Rubisco catalytic activity at ambient levels of C_i. CA may also facilitate the diffusion of CO_2 across the chloroplast membrane by inducing rapid hydration of dissolved CO_2 as it enters the more alkaline environment of the stroma.

Although a number of studies have reported the isolation and characterization of cDNAs encoding CA, little is known about the regulation of CA expression in C_3 higher plants (Burnell et al., 1990a; Fawcett et al., 1990; Roeske and Ogren, 1990; Majeau and Coleman, 1991, 1992). If one assumes that CA plays a role in C_i acquisition by the leaf, the question arises whether there exists some degree of coordination of CA and Rubisco expression that will result in an optimal ratio of the two proteins. Some stromal proteins (as well as electron transport components) are known to increase in parallel with increases in Rubisco levels; however, measurements of CA activity have not been included in these earlier studies (von Caemmerer and Farquhar, 1981; Makino et al., 1983). Other reports have suggested that plant CA activity is regulated by light intensity and CO_2 concentration, parameters known to influence levels of Rubisco activity (Reed and Graham, 1981). For example, in both cucumber and bean, levels of CA and Rubisco were reduced when plants were grown at elevated levels of CO_2 (Porter and Grodzinski, 1984; Peet et al., 1986). Most recently it was noted that a decrease in Rubisco abundance obtained by *rbc*S antisense expression also resulted in a major decline in CA abundance, whereas other Calvin cycle enzymes were unaffected by the reduced Rubisco levels (Hudson et al., 1992).

In addition, Makino et al. (1992) have shown that the CA:Rubisco activity ratio in rice, pea (*Pisum sativum*), and spinach, but not wheat, was maintained at a constant level with increasing leaf nitrogen. In wheat, CA activity increased more rapidly with increasing leaf nitrogen than did Rubisco activity. The authors suggest that for wheat, a reduction in CO_2 transfer resistance generated by the increased CA:Rubisco ratio may be responsible for the maintenance of the balance between Rubisco and electron transport activities in vivo. The low (perhaps limiting) levels of CA activity in wheat, relative to other C_3 plants, may result in this somewhat different response to nitrogen nutrition; however, all these data suggest a more intimate coordination of Rubisco and CA expression than that previously observed for other stromal enzymes.

It is interesting to note that coordination of CA and PEPCase expression is also thought to occur in C_4 plants. It is now recognized that CA activity in maize and presumably other C_4 species is required for mesophyll cell-localized hydration of CO_2 and the resultant formation of HCO_3^-, the substrate for PEPCase (Hatch and Burnell, 1990). PEPCase and CA activities exhibit a similar pattern of expression with leaf position, light intensity, and nitrogen availability (Burnell et al., 1990b). In addition, the nitrogen-dependent accumulation of mRNAs encoding both CA and PEPCase exhibit a similar requirement for cytokinin (Sugiharto et al., 1992a), and the expression of both mRNAs is induced by Gln (Sugiharto et al., 1992b). These data all suggest that similar ele-

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Abbreviations: CA, carbonic anhydrase; *ca*, gene coding for carbonic anhydrase; *cab*, gene coding for Chl *a/b* binding protein; C_i, inorganic carbon; PEPCase, phospho*enol*pyruvate carboxylase; *rbc*S, gene coding for the small subunit of Rubisco.

ments are controlling the coordinated expression of CA and PEPCase in C_4 plants.

In the work presented here, we have studied the expression of CA and Rubisco during development of the photosynthetic apparatus in the C_3 plant pea, and in addition, we have determined the abundance of CA and Rubisco in different organs of the mature plant. CA expression was also examined in pea cultivars with widely differing levels of Rubisco activity and photosynthetic capacity. The data show that CA and Rubisco expression are correlated during development and that CA protein levels in mature tissue are modulated with respect to Rubisco abundance.

MATERIALS AND METHODS

Plant Material

Seeds of various pea (*Pisum sativum*) genotypes (Little Marvel, Homesteader, P1244197, P1244253, P1269770, P1269810) were grown in growth rooms for either 4 or 5 weeks in complete soil, with a daylength of 16 h, illumination of 400 μE m^{-2} s^{-1}, and a day/night temperature of 20°C/16°C. Tissues of pea plants were harvested at the appropriate developmental stage, frozen in liquid nitrogen, and stored at −70°C. For the greening experiment, pea seeds were germinated in total darkness for 14 d before transfer sequentially to continuous white light for periods of up to 48 h. The apices were then harvested and rapidly frozen under liquid nitrogen.

Determination of CA and Rubisco Activity

Leaves were excised from fully illuminated plants, quickly weighed, and then frozen in liquid nitrogen. When required, these frozen samples were ground with a mortar and pestle in the extraction buffer (100 mM Bicine, pH 8.2, 20 mM $MgCl_2$, 1 mM EDTA) and the samples were clarified by centrifugation for 10 min at 10,000*g* at 4°C. CA activity in the supernatant was determined using an electrometric assay (Wilbur and Anderson, 1948). Rubisco activity in the supernatant was determined by measuring the rate of ribulose-1,5-bisphosphate-dependent incorporation of $^{14}CO_2$ into acid-stable counts at 25°C following full activation of the enzyme and using the protocol described previously (Hudson et al., 1992). Enzymes were assayed in triplicate and expressed on the basis of leaf fresh weight. The supernatants were also assayed for soluble protein (Bradford, 1976).

RNA Isolation and Northern Blot Hybridization

Previously frozen tissue, harvested at the appropriate time, was ground in liquid nitrogen prior to extraction of total RNA in phenol:chloroform using the buffer system described previously (Cashmore, 1982). RNA was precipitated using 2 M LiCl, resuspended in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and then reprecipitated with 0.2 M Na-acetate and 2 volumes of ethanol. Equal aliquots of RNA (5 μg sample^{-1} as determined spectrophotometrically) were denatured with formaldehyde:formamide prior to blotting on nitrocellulose using a slot-blot apparatus (Fourney et al., 1988; Sambrook et al., 1989). Prehybridization and hybridization of the blots was performed at 42°C in 5× SSPE (1× SSPE is 0.15 M NaCl, 10 mM Na-phosphate, 1 mM EDTA, pH 7.4) containing 50% deionized formamide, 1% (w/v) nonfat dried milk powder, and 0.1% (w/v) SDS. Probe DNA was labeled using random primers as previously described (Feinberg and Vogelstein, 1984). Gene probes used included a 0.95-kb pea *ca* cDNA (Majeau and Coleman, 1991), a 0.68-kb *rbc*S cDNA clone (Coruzzi et al., 1983), and the 18S rRNA sequence isolated from soybean. Equal concentrations of probe DNA were labeled under uniform conditions and allowed to hybridize to the RNA for 16 h. Hybridization was followed by two 15-min washes in 2× SSPE containing 0.1% SDS and an additional two 15-min washes in 0.1× SSPE containing 0.1% SDS. All washes were performed with agitation at room temperature. The extent of hybridization to the pea RNA was determined by phosphorimaging (model 400S, Molecular Dynamics, Sunnyvale, CA).

Genomic DNA Extraction and Southern Blot Hybridization

Genomic DNA was extracted from fully greened material using a method described previously (Doyle and Dickson, 1987) and digested overnight with the restriction endonuclease *Dra*I. Genomic DNA fragment electrophoresis on a 0.7% agarose gel, transfer to nylon membranes, prehybridization, and hybridization conditions were as previously described (Sambrook et al., 1989).

RESULTS

The exposure to light of dark-grown pea seedlings resulted in the rapid synthesis of Rubisco and CA (Fig. 1). The time course of expression of activity of the two proteins was similar and a constant ratio of CA to Rubisco was established very early in the greening process. As expected, the change in

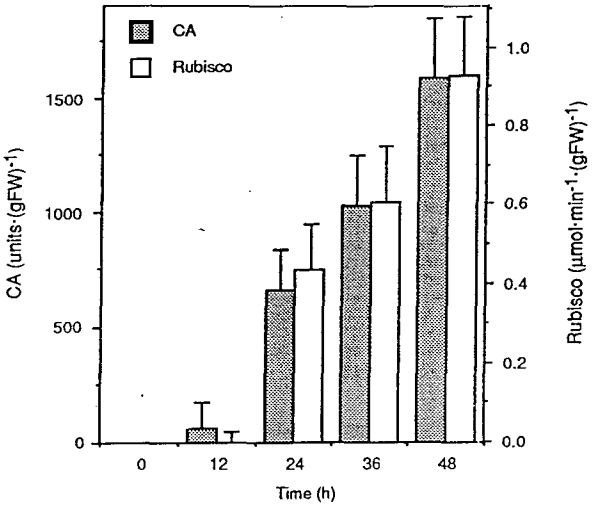

Figure 1. Effect of illumination of dark-grown pea seedlings on CA and Rubisco activity. Seeds of *P. sativum* var Little Marvel were germinated in the dark for 14 d prior to sequential transfer to continuous white light for periods up to 48 h. CA and Rubisco activity are expressed per g fresh weight, and values are means ± SE of four plant replicates.

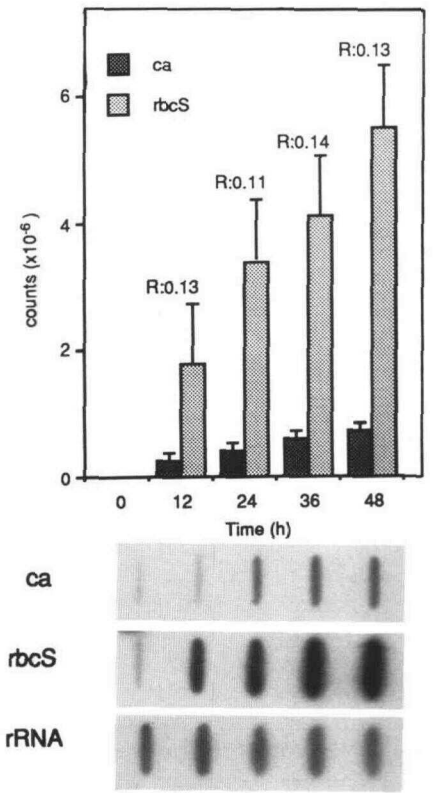

Figure 2. Effect of illumination on *ca* and *rbc*S transcript abundance. Slot blots (each containing 5 μg of total RNA obtained from tissue grown as described in Fig. 1) were probed with [^{35}S]dCTP-labeled cDNA encoding pea *ca* and *rbc*S. A radiolabeled soybean rDNA probe was used to ensure that each slot contained equal aliquots of pea RNA. The extent of probe hybridization was determined by phosphorimaging. R represents the ratio of the *ca*:*rbc*S hybridization signals, and cpm values represent the means ± SE of three sample replicates from two separate experiments.

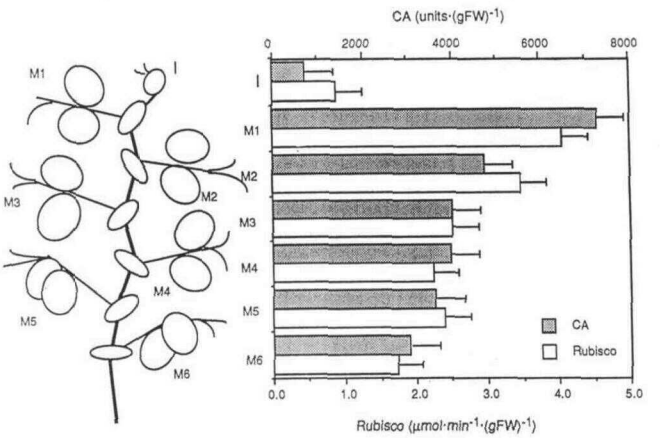

Figure 3. Determination of CA and Rubisco activity in pea leaves of differing age. Leaves of 5-week-old plants were harvested and the activity of CA and Rubisco was determined. I represents the immature leaves and apex; M1 designates the first fully expanded leaf pair below the apex; M2, M3, M4, M5, and M6 designate the second, third, fourth, fifth, and sixth pairs of mature leaves, respectively. Values represent means ± SE of four plant replicates.

enzyme activity with time of light exposure was correlated with an increase in the abundance of *ca* and *rbc*S transcripts (Fig. 2). Although the quantity of the *rbc*S mRNA was much higher than the *ca* transcript level, the ratio of the two mRNA species remained relatively constant over the time course.

A study of CA and Rubisco activity in mature pea plants showed a similar pattern of co-expression of the two proteins (Fig. 3). Activities of both enzymes were highest in the youngest, fully expanded leaves (M1 leaf, proximal to the apex) and exhibited a significant decline in the older, second set of fully expanded leaves (M2 leaf pair, Fig. 3). A slower rate of decrease in enzymic capacity occurred in the older leaves (M3–M6, Fig. 3). A relatively constant CA:Rubisco activity ratio was maintained over this time period (Fig. 3). The total amount of soluble protein per gram of fresh weight was higher in the immature tissue and decreased with increasing age of the leaves (data not shown). CA and Rubisco activity declined in the mature leaves as the soluble protein content was reduced, with activity per unit of soluble protein for both enzymes remaining constant (data not shown). Transcript levels for *ca* and *rbc*S showed a similar pattern of

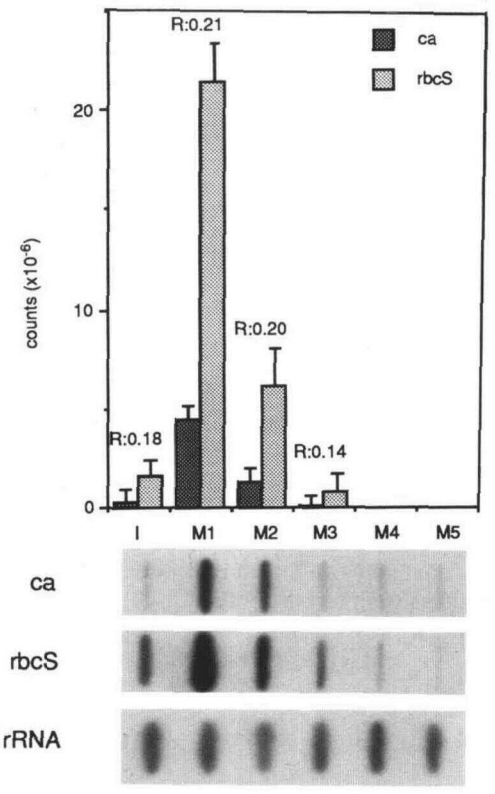

Figure 4. *ca* and *rbc*S transcript abundance in leaves of differing age. Total RNA was extracted from leaves of the appropriate developmental stage (as described in Fig. 3) and probed with labeled cDNA encoding pea *ca* and *rbc*S as well as a soybean rDNA probe as described in Figure 2. R represents the ratio of the *ca*:*rbc*S hybridization signals, and cpm values represent the means ± SE of three sample replicates from two separate experiments.

Table I. *Tissue-specific expression of CA and Rubisco in pea (var Little Marvel)*

Tissue[a]	CA[b]	Rubisco[b]	Activity Ratio[c]	mRNA Ratio[d]
Leaf	5670 ± 410	2.1 ± 0.3	2700	0.139
Stems	188 ± 35	0.11 ± 0.01	1709	0.196
Roots	nd[e]	nd		
Pods	73 ± 12	0.03 ± 0.01	2192	0.10

[a] Plants used were 4 to 5 weeks old with M1 leaf pairs used for leaf tissue. Stem tissue was obtained between M2 and M3 leaf pairs. Pod length was approximately 15 cm, and whole, intact pods were extracted for activity and mRNA analysis. [b] CA expressed as Wilbur-Anderson units g^{-1} fresh weight and Rubisco expressed as μmol C fixed min^{-1} g^{-1} fresh weight. Values represent means ± SE of a minimum of four plant replicates. [c] Ratio of CA to Rubisco activity. [d] Ratio of *ca* to *rbc*S transcript abundance. Data were obtained from slot-blot analysis of 5 μg of total RNA probed with [^{35}S]dCTP-labeled *ca* and *rbc*S cDNA. [e] Not detected.

expression in leaves of different age (Fig. 4). Although total RNA levels were highest in the immature leaves, *ca* and *rbc*S transcript abundance was greatest in the youngest, fully expanded leaves.

A quantitative comparison of Rubisco and CA expression was made in the different organs of the plant (Table I). As expected, the activity of both enzymes when expressed on a fresh weight basis was highest in the leaves, followed by stems and whole juvenile pods. CA and Rubisco activity were not present in root tissue. The relative abundance of CA to Rubisco activity was conserved for all photosynthetic tissues. When equal amounts of total RNA from the different organs were used to quantify *rbc*S and *ca* transcript abundance, a constant ratio of the two transcripts was also observed (Table I).

Six genetically diverse pea cultivars were also examined with respect to development of CA and Rubisco activity. After a 48-h period of light exposure, the greening seedlings expressed very different levels of CA and Rubisco activity (Fig. 5) and transcript abundance (Fig. 6); however, the ratios of activity and mRNA levels for the two enzymes were similar for all cultivars. Levels of CA activity in mature leaves were also found to correlate with the amount of Rubisco present (Fig. 7). Even though Rubisco levels exhibited almost a 3-fold difference across the range of pea genotypes, CA levels were found to vary in a similar fashion. The CA:Rubisco activity ratio was relatively constant for all genotypes.

Because transcript abundance can be influenced by gene copy, genomic DNA isolated from each of the pea cultivars used in this study was probed with the cDNA encoding chloroplastic CA. Southern blot analysis of the various pea cultivars suggested the presence of a single *ca* gene encoding chloroplastic CA (Fig. 8). Genomic DNA from cultivars Homesteader, P1244253, P1269770, and Little Marvel appeared to generate the same hybridization pattern when probed with the *ca* cDNA. Cultivars P1244197 and P1269810 appeared to generate two additional hybridization patterns.

DISCUSSION

During light induction, CA and Rubisco showed similar patterns of expression and accumulation. Both transcript abundance and enzymic activity increased at a relatively constant rate during the 48-h period of light exposure. This is in contrast to the transient increase in transcription rate exhibited by another group of light-regulated genes, which include the *cab* gene family (Wehmeyer et al., 1990). In etiolated seedlings, *cab* transcripts encoding polypeptides associated with both PSI and PSII exhibited maximal abundance levels approximately 4 h after the onset of continuous white light. This was followed by a decline in the mRNA levels of up to 50% after 16 h. That both CA and Rubisco display an identical pattern in the accumulation of light-induced transcripts may indicate that they share a common mechanism for light induction of gene expression during the development of the photosynthetic apparatus.

The decline in enzyme activity and transcript abundance with increasing leaf age was again similar for both proteins. The decrease in transcript abundance was more precipitous and resulted in the virtual elimination of both *ca* and *rbc*S transcripts in the M4 leaves. In contrast, levels of enzyme activity were reduced by only approximately 50% in leaves of the same age. A similar pattern of decline with increasing leaf age has been described for Rubisco activity and *rbc*S transcript abundance in bean (Bate et al., 1991). Studies in soybean, however, have shown that the decline in Rubisco abundance is accompanied by a coordinate reduction in the levels of both *rbc*L and *rbc*S transcripts, suggesting that transcriptional control is the primary determinant of Rubisco abundance during leaf aging (Jiang et al., 1993). The differences noted may reflect variation within plant systems, in terms of the relative importance of transcript abundance versus protein turnover in the determination of enzyme activity during leaf aging.

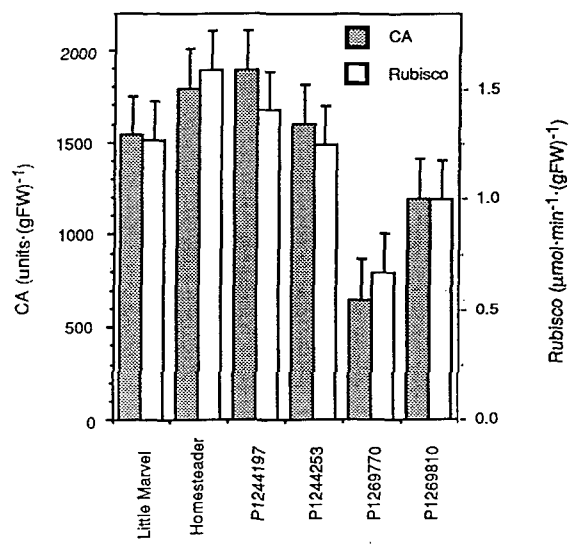

Figure 5. Determination of CA and Rubisco activity in greening tissue of six different pea genotypes. Seeds of different cultivars of pea were germinated in the dark for 14 d and then transferred to continuous white light for 48 h prior to harvesting and enzyme assays. CA and Rubisco activity are expressed per g fresh weight, and values are means ± SE of four plant replicates.

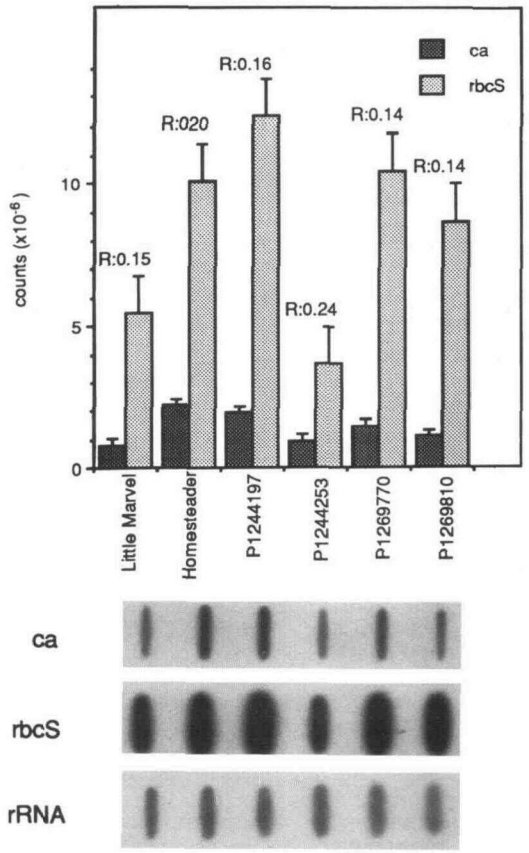

Figure 6. *ca* and *rbc*S transcript abundance in greening tissue of six different pea genotypes. Plants were grown and tissue harvested as described in Figure 5. Total RNA was extracted, transferred to nitrocellulose, and probed as described in Figure 2. *R* represents the ratio of the *ca*:*rbc*S hybridization signals, and cpm values represent the means ± SE of three sample replicates from two separate experiments.

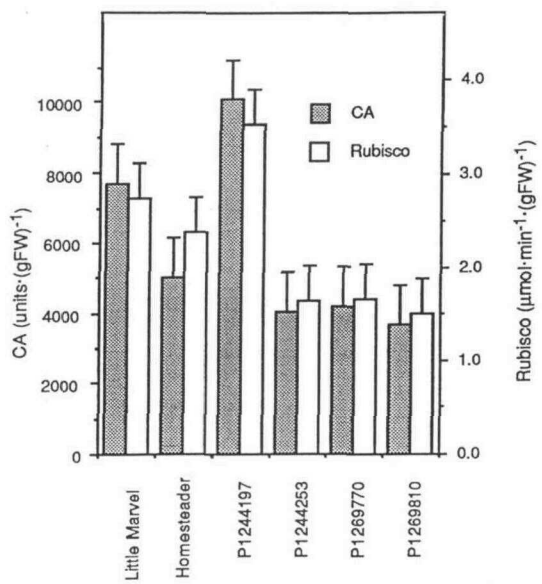

Figure 7. Determination of CA and Rubisco activity in mature leaf tissue of six pea genotypes. The first fully expanded leaf pair below the apex of 4-week-old pea cultivars (M1 leaf pairs) were analyzed for CA and Rubisco activity. CA and Rubisco activity are expressed per g fresh weight and values are means ± SE of four plant replicates.

The examination of CA and Rubisco levels in different tissues showed that activities varied widely but maintained a relatively constant ratio for both *ca*:*rbc*S transcript abundance and activity. The association of this isoform of CA with Rubisco and photosynthesis is further strengthened by our inability to detect CA activity in nongreen tissue. There are earlier reports on the presence of CA in etiolated leaves and root (Kachru and Anderson, 1974; Goustiana et al., 1988); however, in our study northern blot analysis did not reveal the presence of transcripts homologous to the chloroplastic *ca* in these specific tissues. It is certainly possible that other isoforms of CA exist and that the electrometric method used to monitor CA activity in our study was less sensitive than the polyacrylamide electrophoresis method that detected the nongreen CA isoforms. Species variation, the expression of a root nodulation-specific CA (Atkins, 1974), or low-light contamination of etiolated growth conditions may also account for the differences.

The comparison of the genetically diverse pea cultivars provides additional evidence for coordination of CA and Rubisco expression. Both greening and mature leaf tissue exhibit a wide range of Rubisco and CA expression, and yet activity and transcript abundance ratios for the two proteins remain relatively constant. Previous studies using numerous pea genotypes have shown that the amount of Rubisco activity and *rbc*S transcript levels are positively correlated (Hobbs et al., 1990) and that genotypic variability in leaf CO_2 exchange rates is a consequence of Rubisco abundance (Mahon et al., 1983). The comparison of CA levels in these same genotypes shows both the same degree of variation and a similar correlation between *ca* transcript abundance and activity. The genomic DNA analysis for *ca* and that described previously for *rbc*S (Hobbs et al., 1990) indicate that all pea

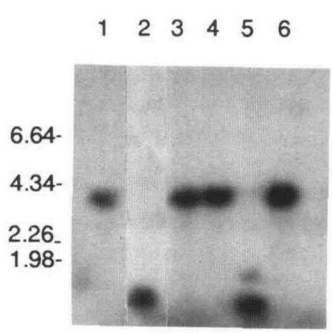

Figure 8. Southern blot hybridization of genomic DNA from pea genotypes. Genomic DNA was digested with restriction endonuclease *Dra*I, electrophoresed, transferred to nitrocellulose, and then probed with [^{35}S]dCTP-labeled *ca* cDNA. DNA was extracted from *P. sativum* varieties Homesteader (lane 1), P1244197 (lane 2) P1244253 (lane 3), P1269770 (lane 4), P1269810 (lane 5), and Little Marvel (lane 6).

genotypes used in this study have one copy of *ca* and five copies of *rbc*S. Therefore, it would appear that the cultivar-specific levels of *ca* and *rbc*S transcripts observed in greening tissue after 48 h of light exposure is not a product of gene copy differences but is the result of unequal rates of mRNA accumulation during greening.

The *rbc*S DNA fragment used to evaluate transcript abundance hybridizes to all mRNA products of the gene family. It has been shown that the five different *rbc*S transcripts in pea are differentially expressed during greening of etiolated pea seedlings (Fluhr et al., 1986). The transcripts from genes 3A and 3C that represent more than 80% of the total *rbc*S expression in leaf tissue accumulate after 48 h of continuous illumination at a level near the steady-state levels detected in mature, green leaves. Expression of the other *rbc*S genes in pea is very low after 48 h of continuous light and then increases somewhat later in leaf development. The kinetics of *ca* transcript accumulation during greening followed the pattern observed for the more abundant *rbc*S genes and may indicate similar controlling elements. The significantly higher levels of *rbc*S transcript, when compared with *ca* transcript abundance, may be a function of copy number and promoter strength as well as transcript stability.

In the earlier study by Hobbs et al. (1990), Southern analysis of pea genomic DNA identified restriction fragment-length polymorphisms of *rbc*S genes among the various pea genotypes examined. These restriction fragment-length polymorphisms were used as a criterion for identifying subgroups within the collection of cultivars examined. Pea varieties Homesteader, P1244253, and P1269770 were placed in the same group; P1269810 and P1244197 were placed in two different categories. Interestingly, restriction fragment-length polymorphic analysis using *ca* as a probe places each of the cultivars tested in exactly the same subgroups. Whether there is a genetic or functional significance to this finding is unknown; however, these data do support the proposed grouping of the various pea cultivars.

In conclusion, our data are consistent with the idea that coordinated expression of CA and Rubisco occurs in pea and, presumably, in other C_3 higher plants. These observations are in agreement with patterns of expression of CA and PEPCase in C_4 plants, where expression of CA is required for catalysis of CO_2 hydration in mesophyll cells. The link between CA and Rubisco expression in C_3 plants may be required for the maintenance of a CA:Rubisco ratio that is optimal for efficient photosynthetic carbon fixation and resource allocation. More specific information on how regulation is coordinated might be obtained by identification and comparison of promoter elements for *ca* and *rbc*S. In addition, it would be interesting to determine if transgenic plants expressing *ca* antisense or overexpression constructs result in the modulation of Rubisco expression.

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