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Reduction of Ribulose Bisphosphate Carboxylase Activase Levels in Tobacco (*Nicotiana tabacum*) by Antisense RNA Reduces Ribulose Bisphosphate Carboxylase Carbamylation and Impairs Photosynthesis[1]

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The in vivo activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is modulated in response to light intensity by carbamylation of the active site and by the binding of sugar phosphate inhibitors such as 2′-carboxyarabinitol-1-phosphate (CA1P). These changes are influenced by the regulatory protein Rubisco activase, which facilitates the release of sugar phosphates from Rubisco's catalytic site. Activase levels in *Nicotiana tabacum* were reduced by transformation with an antisense gene directed against the mRNA for Rubisco activase. Activase-deficient plants were photosynthetically impaired, and their Rubisco carbamylation levels declined upon illumination. Such plants needed high CO_2 concentrations to sustain reasonable growth rates, but the level of carbamylation was not increased by high CO_2. The antisense plants had, on average, approximately twice as much Rubisco as the control plants. The maximum catalytic turnover rate (k_{cat}) of Rubisco decreases in darkened tobacco leaves because of the binding of CA1P. The dark-to-light increase in k_{cat} that accompanies CA1P release occurred to similar extents in antisense and control plants, indicating that normal levels of activase were not essential for CA1P release from Rubisco in the antisense plants. However, CA1P was released in the antisense plants at less than one-quarter of the rate that it was released in the control plants, indicating a role for activase in accelerating the release of CA1P.

Rubisco (EC 4.1.1.39), the carboxylating enzyme of the photosynthetic carbon reduction cycle, must be activated to become catalytically competent. Activation involves the reversible reaction of a molecule of CO_2 with Lys residue 201 within the active site to form a carbamate, followed by the rapid binding of a Mg^{2+} ion to create an active ternary complex (Lorimer and Miziorko, 1980). Rubisco carbamylation levels change in vivo in response to light intensity (Mächler and Nösberger, 1980; Perchorowicz et al., 1981; Salvucci et al., 1986; von Caemmerer and Edmondson, 1986). Light-regulated changes in carbamylation level match Rubisco activity with the rate of RuBP regeneration (Mott et al., 1984), thereby buffering stromal pH during light fluctuations (Portis, 1990).

Analysis of the kinetics of Rubisco carbamylation raised many questions about how carbamylation was facilitated in vivo. The equilibrium of carbamate formation measured in vitro (Lorimer et al., 1976) is not favorable enough to promote full carbamylation of Rubisco at the pH and concentrations of Mg^{2+} and CO_2 thought to prevail in vivo during illumination (reviewed by Andrews and Lorimer, 1987). Furthermore, when RuBP was present in the in vitro assay, it blocked carbamylation by binding very tightly to the noncarbamylated active site (Jordan and Chollet, 1983). A protein that facilitates carbamylation under in vivo conditions was first identified in a high CO_2-requiring mutant of *Arabidopsis thaliana* (Somerville et al., 1982), which had poorly activated Rubisco in the light (Salvucci et al., 1985). Further studies established that two soluble chloroplast polypeptides encoded by a single *Rca* gene were missing from the *A. thaliana* mutant and that the absence of these polypeptides was responsible for the high CO_2 requirement for growth (Salvucci et al., 1985; Werneke et al., 1989). These polypeptides proved to be the subunits of Rubisco activase—so called because of its role in promoting carbamylation (Salvucci et al., 1985). Studies with chloroplast extracts indicated that Rubisco activase promoted carbamylation of Rubisco at physiological CO_2 concentrations in the presence of RuBP and prevented the deactivation of Rubisco observed in the *rca* mutant with the onset of illumination (Salvucci et al., 1985; Portis et al., 1986). A light requirement for carbamylation was identified and later linked to activase's requirement for ATP hydrolysis (Robinson and Portis, 1988b).

Rubisco activity in vivo can also be affected by the inhibitor CA1P, which binds to the carbamylated active sites of Rub-

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Abbreviations: CA1P, 2′-carboxy-D-arabinitol-1-phosphate; CABP, 2′-carboxy-D-arabinitol-1,5-bisphosphate; CPBP, unresolved mixture of CABP and 2′-carboxy-D-ribitol-1,5-bisphosphate; k_{cat}, moles of substrate converted per mole of enzyme active sites per second; *rca*, gene encoding Rubisco activase; PCR, polymerase chain reaction; ϕPSII, electron flow through PSII per unit of quantum flux; RuBP, D-ribulose-1,5-bisphosphate; T-DNA, DNA transferred from plasmid to plant genome.

isco by virtue of its similarity to the six-carbon intermediate of the carboxylase reaction. It accumulates in the leaves of some plant species in the dark and at low light intensities (Vu et al., 1984; Seemann et al., 1985; Servaites, 1990). Many plants, such as *A. thaliana*, *Spinacea oleracea*, and *Triticum aestivum*, do not accumulate physiologically significant amounts of CA1P (Servaites et al., 1986; Moore et al., 1991). Light-modulated changes in Rubisco activity in these plants appear to occur solely by activase-mediated changes in carbamylation levels (Salvucci et al., 1985; Portis et al., 1986; Salvucci et al., 1986). However, the light-regulated control of Rubisco activity is more complex in CA1P-accumulating plants, such as *Phaseolus vulgaris* and *Nicotiana tabacum*. CA1P binds reversibly to 50 to 100% of the carbamylated active sites in these species in the dark (Servaites et al., 1986; Moore et al., 1991). With the onset of illumination, CA1P is released from the active site and degraded by a specific phosphatase (Seemann et al., 1990), resulting in an increase in carboxylase activity (Vu et al., 1983). In vitro studies indicate that Rubisco activase may have a role in releasing CA1P from Rubisco (Robinson and Portis, 1988a). Therefore, depending on the CA1P content of the plant, Rubisco activase may mediate light-induced changes in Rubisco activity via modulation of carbamylation levels or by inducing CA1P release or a combination of both.

Antisense RNA and plant transformation techniques enable the production of plants that are deficient in specific proteins. Analysis of the antisense plants provides information about the roles of the deficient protein and its relationship to other physiological processes. We have used an antisense gene directed against the *rca* mRNA to reduce the level of Rubisco activase in a CA1P-synthesizing plant, tobacco, to study the effect on Rubisco activity in vivo and photosynthesis.

MATERIALS AND METHODS

Isolation and Manipulation of Tobacco (*Nicotiana tabacum*) and Spinach (*Spinacea oleracea*) *rca* cDNAs

A partial cDNA for tobacco *rca* was isolated from *N. tabacum* cv W38 RNA by the PCR technique using a specific 5′ primer and a general 3′ primer (Hudson et al., 1992b). The specific primer 5′-GGGAGGCAAGGGTCAAGGTAA-3′ was based on the codons for the amino acid sequence Gly-Gly-Lys-Gly-Gln-Gly-Lys, which is located at the putative ATP-binding site of *S. oleracea* and *A. thaliana* Rubisco activases (Werneke et al., 1988; Werneke and Ogren, 1989). The 1.2-kb cDNA corresponding to the 3′ two-thirds of the *rca* mRNA was cloned into the *Sma*I site of pTZ18R and one clone, pTACT2, was found to have the cDNA inserted in the correct orientation for subcloning. DNA sequencing of the insert revealed an open reading frame with a sequence similar to that of the spinach *rca* cDNA (Werneke et al., 1988). This reading frame was later found to correlate with the nucleotide sequence of another tobacco *rca* cDNA (S. Rodermel and J. Qian, unpublished data, GenBank accession No. Z14980). A *Bam*HI-*Kpn*I fragment from pTACT2 encompassing the cDNA was subcloned into pBIN19(CaMV-nos), which is a derivative of pBI121 (Jefferson et al., 1987) without the *uidA* gene. The resulting plasmid, pαTACT, had the cDNA in the antisense orientation with respect to the cauliflower mosaic virus 35S promoter.

The construction of plasmid pSACTc, which contains a spinach *rca* cDNA encoding only the mature 45-kD polypeptide without the transit peptide, has been described previously (Hudson et al., 1992a). The *Nde*I (filled)-*Eco*RI insert from pSACTc was subcloned into the *Sma*I and *Eco*RI sites of pGEX-2T (AMRAD, Melbourne, Australia). The GSH *S*-transferase-activase fusion protein synthesized from this plasmid in *Escherichia coli* was purified on a GSH-agarose column as described by Smith and Johnson (1981). The purified fusion protein was used to immunize a rabbit, and the resulting antiserum was found to react with two Rubisco activase polypeptides on immunoblots of spinach leaf extracts and a single activase polypeptide on immunoblots of tobacco leaf extracts.

Transformation and Growth of the Antisense Plants

The T-DNAs of pαTACT and pBI121 (as a control) were introduced into tobacco by the leaf disc method and selected on kanamycin-containing medium (Herrera-Estrella and Simpson, 1988). Regenerated plantlets were initially transferred into pots of vermiculite and placed in a growth cabinet. The cabinet temperature was 22°C (day) and 15°C (night), with 60% RH and a 12-h photoperiod. Two series of plantlets were raised in tissue culture; the first series was transferred into the growth cabinets at atmospheric CO_2 and an irradiance of 750 μmol quanta m^{-2} s^{-1}. Approximately 80% of the pαTACT primary transformants died under these conditions. The second series of plants was raised under a higher CO_2 concentration (0.3–0.5%) with an initial irradiance of 400 μmol quanta m^{-2} s^{-1}, which was increased after 7 d to 750 μmol quanta m^{-2} s^{-1}. Under these conditions, the fatality rate was only 7%. The plants were supplied with complete nutrient solution containing 12 mM nitrate three times per week (Hewitt and Smith, 1975). The plants were transferred into 3-L pots containing sterilized garden soil after 3 weeks, and 3 weeks later they were screened by immunoblot and biochemical assays for activase and Rubisco carbamylation levels. The primary transformants were allowed to self-fertilize, and the R_1 seed was collected and stored at 4°C. The R_1 seedlings were raised in a growth cabinet with a 12-h photoperiod at 25°C (day) and 17°C (night), 0.3 to 0.5% CO_2, 60% RH, and 550 μmol quanta m^{-2} s^{-1}. The plants were grown in 5-L pots and analyzed approximately 6 to 8 weeks after germination.

Sampling Procedures

All of the plants were sampled or assayed in the growth cabinets except those used in the gas-exchange analysis. The plants were sampled by taking leaf punches that were collected in liquid nitrogen and stored at −80°C until required. Samples from the primary transformants for immunoblotting were obtained 6 weeks after the plantlets had been removed from tissue culture. Samples for the carbamylation assays were collected in the dark (5 min before the photoperiod commenced) and 90 min after the onset of illumination. After

the 90-min sample was obtained, the CO_2 concentration in the growth cabinets was lowered to 0.03%. The low CO_2 leaf samples were collected the following day.

The R_1 seedlings were initially screened by Chl fluorescence in the growth cabinet. Measurements were taken at high CO_2, after which the CO_2 concentration in the growth cabinet was decreased to 0.03% for approximately 24 h before taking the next fluorescence measurements. The growth cabinet was returned to high CO_2, and the plants were sampled for determination of activase levels the following day.

For the time course experiments, the R_1 seedlings were placed in a darkened growth cabinet at 0.3 to 0.5% CO_2 12 to 15 h before sampling. Immediately before sampling, the plants were removed to a dark room while the growth cabinet lights were switched on. The time-zero samples were taken in the dark room, and then the plants were returned to the growth cabinet when the illumination had reached 1000 μmol quanta m^{-2} s^{-1}.

Immunoblotting

Immunoblotting was carried out as described by Harlow and Lane (1988). Leaf punches (2 cm^2) were extracted in 0.5 mL of buffer (50 mM bis-Tris propane [pH 7.0], 10 mM $MgCl_2$, 1 mM sodium EDTA, 10 mM DTT, 1.5% polyvinylpolypyrrolidone, 1 mM PMSF). Mercaptoethanol and SDS were added to the extract to 4% (v/v) and 2% (w/v), respectively, before boiling for 10 min. The extract was centrifuged, and a sample of the supernatant was separated on a Pharmacia Phastgel (SDS buffer, 12.5% homogenous). The proteins were electroblotted to nitrocellulose then blocked with 5% milk powder in 20 mM Tris-HCl (pH 8.5), 0.5 M NaCl (Tris-buffered saline) and probed with the following antibodies and reagents in 5% milk powder, 0.05% Triton X-100 in Tris-buffered saline: (a) primary antibody, anti-GSH *S*-transferase/spinach activase fusion protein, (b) secondary antibody, biotinylated goat anti-rabbit immunoglobulin (Amersham), and (c) streptavidin-alkaline phosphatase (Bio-Rad). The activase bands were visualized by incubation with the substrates 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium.

Determination of T-DNA Copy Number

Leaf DNA was prepared by the hexadecyltrimethylammonium bromide method (Saghai-Maroof et al., 1984). An inverse PCR technique (Does et al., 1991) with α-thio-^{35}S-dATP (Amersham) included in the amplification reaction was used to determine the number of T-DNA copies inserted into the genomes of individual plants. The segregation of kanamycin resistance and sensitivity in the R_1 generation was also used to determine the T-DNA copy number of the primary transformants. Surface-sterilized seeds were germinated on half-strength Murashige-Skoog media (Herrera-Estrella and Simpson, 1988) containing 200 μg mL^{-1} of kanamycin and 200 μg $^{-1}$mL of cefotaxamine. After 5 weeks in a growth cabinet at 0.5% CO_2, the kanamycin-sensitive seedlings turned white, whereas the resistant seedlings remained green.

Measurements of CO_2 Assimilation Rate

Gas-exchange measurements were made in an open gas-exchange system, as described by Brugnoli et al. (1988) with modifications as outlined by Hudson et al. (1992b). Plants were placed in the gas-exchange system in the dark following 12 h of darkness, and the first measurements were taken. The lights were switched on to give an irradiance of 1000 μmol quanta m^{-2} s^{-1}, and the rate of CO_2 assimilation was measured. The leaf temperature was 25°C, the leaf to air vapor pressure difference was 11 mbar, and the CO_2 concentration was 350 μbar.

Chl fluorescence was measured with a fluorimeter (Pam 101; Waltz, Effeltrich, Germany) under growth conditions approximately 90 min after the onset of illumination following 12 h of darkness. The ϕPSII was calculated according to the method of Genty et al. (1989).

Biochemical Assays

Leaf punches (0.5 cm^2) were rapidly extracted in 1 mL of buffer (50 mM Hepes-KOH [pH 7.1], 5 mM $MgCl_2$, 1 mM sodium EDTA, 10 mM DTT, 20 mM sodium isoascorbate, 1 mM PMSF, and 1.5% polyvinylpolypyrrolidone) on ice. Rubisco carbamylation was measured immediately (within 10–15 s), after which Rubisco content and activity, phosphoribulokinase activity, and soluble protein were measured.

The Rubisco catalytic site concentrations and carbamylation levels were measured by the stoichiometric binding of [^{14}C]CABP (Collatz et al., 1978), based on the method of Butz and Sharkey (1989). [*carboxy*-^{14}C]CPBP was prepared as described by Collatz et al. (1978). The catalytic site concentration was determined by measuring the [^{14}C]CABP-binding capacity of extract in which the Rubisco had been fully carbamylated by saturating CO_2 and Mg^{2+}. The buffer was 50 mM Bicine (pH 7.8), 20 mM $MgCl_2$, 15 mM $NaHCO_3$, 1 mM sodium EDTA, and [^{14}C]CPBP at a 10-fold or greater excess over Rubisco catalytic sites. All buffers used in these experiments were sparged overnight with N_2 to displace any CO_2 present in solution. The extract (50 μL) was incubated with 150 μL of the buffer containing [^{14}C]CPBP for 45 min at 25°C, during which time all Rubisco sites were carbamylated and [^{14}C]CABP was bound irreversibly. Rubisco-[^{14}C]CABP complexes were precipitated with BSA (2.5 mg mL^{-1}) as carrier by adding PEG (4000–6000 kD) and $MgCl_2$ to a final concentration of 20% (w/v) and 25 mM, respectively. Precipitation was carried out on ice for 30 min, followed by centrifugation at 4°C for 15 min. The pellet was washed (but not resuspended) three times using 500 μL of 50 mM Bicine (pH 7.8), 15 mM $MgCl_2$, 1 mM sodium EDTA, and 20% PEG. Each wash was followed by 5 min of centrifugation at 4°C. The pellet was dissolved in 300 μL of water and transferred to a scintillation vial, and 3 mL of Amersham ACS II fluid was added. The ^{14}C retained within the sample was determined by liquid scintillation counting. It was assumed that eight binding sites occur per 550-kD holoenzyme.

The carbamylation level was determined by exchanging loosely bound [^{14}C]CABP at noncarbamylated catalytic sites with an excess of [^{12}C]CABP (Butz and Sharkey, 1989). Carbamylation levels measured this way correlate with the

ratio between initial Rubisco activity after rapid leaf extraction and total Rubisco activity following preincubation with CO_2 and Mg^{2+} (Butz and Sharkey, 1989). First, 50 μL of extract was added to a 150-μL solution containing buffer (50 mM Bicine [pH 7.8], 5 mM $MgCl_2$, 1 mM sodium EDTA) plus [^{14}C]CPBP (at a 10-fold or greater excess over Rubisco catalytic sites) and incubated on ice for 45 min. Then, [^{12}C]CPBP was added at a 200-fold higher concentration than that of [^{14}C]CPBP, and the extract was incubated at 25°C for 5 min. The Rubisco-CABP complexes were precipitated, washed, and counted as described above.

Rubisco activity, phosphoribulokinase activity, and total protein were assayed as described by Hudson et al. (1992b) except that all volumes were halved for enzyme assays. The activated Rubisco catalytic rate (k_{cat}) was determined by preincubation of 25 μL of leaf extract for 10 min in the presence of 20 mM [^{14}C]$NaHCO_3$ and 20 mM $MgCl_2$. The reaction was initiated by the addition of RuBP to a final concentration of 0.5 mM and terminated after 1 min by the addition of formic acid. The k_{cat} (s^{-1}) was calculated by dividing the activated Rubisco activity by the total number of catalytic sites determined by CABP binding. A molecular mass of 80 kD and a specific activity of 6800 μmol g^{-1} s^{-1} (Porter et al., 1986) were used to calculate phosphoribulokinase content.

RESULTS

Characterization of the Primary Transformants

Tobacco was transformed with a T-DNA containing the kanamycin resistance gene linked to either the *uidA* gene for β-glucuronidase (control) or the antisense gene (see "Materials and Methods"). Transformed plants were grown in growth cabinets at high CO_2 and were screened for activase protein by immunoblotting of proteins in leaf extracts. Eighteen anti-activase plants were screened using this technique. Several plants had low to very low levels of activase (Fig. 1A). Taking into account the sensitivity of the immunoblotting technique, we compared the control and anti-activase plants and determined that activase levels had been reduced by at least 50% in plants 38 and 50 and by at least 75% in plant 52. All 18 plants were grown to seed.

DNA was prepared from primary transformants 50 and 52, and the number of T-DNA copies inserted into the genome was determined by an inverse PCR method (Fig. 1B). Plant 52 had two inserts. The R_1 progeny of plant 52 had a fatality ratio of 59:850 on kanamycin-containing medium, which is consistent with the stable inheritance of two T-DNA inserts (expected fatality rate 1:15). All subsequent results presented in this paper were from the R_1 progeny of plant 52. Because plant 52 had two inserts, we expected that the R_1 progeny would have a range of zero to four inserts, with activase levels varying inversely with the number of inserts inherited.

Phenotype of the R_1 Antisense Plants

The R_1 anti-activase plants were grown at high and low CO_2. There were two phenotypes at low CO_2 (ambient) (Fig. 2, left). Approximately half of the seedlings were yellow-green, and the other half were dark green. The dark-green

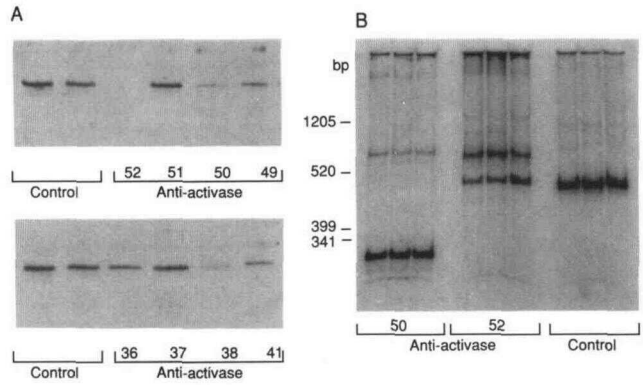

Figure 1. Characterization of the primary transformant anti-activase plants. A, Immunological detection of Rubisco activase in tobacco leaf extracts separated by SDS-PAGE. Leaf extracts were obtained from mature tobacco plants that had been transformed with either pBI121 (control) or pαTACT (anti-activase). The numbers refer to individual anti-activase transformants from which the extracts were obtained. B, Inverse PCR assay for determining T-DNA copy number. This technique is based on the varying distance between *TaqI* sites within the *neo* gene of the T-DNA and the tobacco DNA flanking the right border (Does et al., 1991). Shown is an autoradiograph of the radiolabeled amplification products on a 4% sequencing gel. The samples are, from left to right, antisense transformants 50 and 52 and a control transformant. Before amplification, the samples were cut with a second enzyme, either *Bsu*361, *Sac*II, or *Ssp*I (from left to right), so that three lanes occur for each DNA sample.

phenotype grew more quickly than the yellow-green, but both phenotypes grew slower than the wild-type controls (not shown). Several of the yellow-green phenotype hardly grew at all. At high CO_2, the anti-activase seedlings were initially homogeneous in appearance (Fig. 2, right) and looked similar to the controls (not shown). Later in development (1–2 weeks), some anti-activase plants (approximately 25%) developed veinal chlorosis. All plants assayed by immunoblotting (four of dark-green phenotype and five of yellow-green phenotype) had a greater than 75% reduction in activase content compared to the control plants (results not shown). Differences in the activase content of the R_1 progeny could not be detected because of the limitations of the immunoblot technique. Plants from the high CO_2-grown group were used in the experiments. The control plants (R_1 progeny of plants transformed with pBI121) were also raised at high CO_2.

Photosynthesis

The Chl fluorescence parameter, ϕPSII, measures the quantum efficiency of PSII. ϕPSII reflects the activity of the light reactions and the consumption of their products. Therefore, it provides a quick and useful estimate of light reaction activity and, by inference, photosynthetic rate. Chl fluorescence was measured in the growth cabinets under high CO_2 growth conditions and again after 24 h of equilibration at ambient CO_2. ϕPSII was quite high in both control and anti-activase plants at high CO_2, indicating healthy rates of pho-

Figure 2. Anti-activase plants germinated and grown at high (0.3–0.5%, right) and atmospheric (0.03%, left) CO_2. Growth conditions for both CO_2 concentrations were as follows: irradiance 550 μmol quanta m^{-2} s^{-1}, 25°C day/17°C night, 12-h photoperiod, 60% RH.

tosynthesis. There was no difference between high and low CO_2 measurements for the control plants (Table I), probably because of light limitation of the photosynthetic rate. In contrast, the Chl fluorescence parameter of the anti-activase plants decreased by 40% when transferred from high to low CO_2, reflecting a decrease in the photosynthetic rate.

Gas-exchange measurements were made on three anti-activase and two control plants. The plants were illuminated at 1000 μmol quanta m^{-2} s^{-1} in the gas-exchange system after 12 to 15 h at ambient CO_2 in the dark. An increase in the rate of carbon fixation with the onset of illumination was observed in both control and anti-activase plants (Fig. 3A). However, the assimilation rate of the anti-activase plants plateaued at a rate that was approximately half that of the control. The intercellular partial pressure of CO_2 in the anti-activase plants was similar to or higher than that of the control plants (Fig. 3B). Therefore, diffusional resistance did not contribute to the disparity in photosynthetic rate. The difference in CO_2 assimilation rate was also not due to differences in Rubisco content because assays showed that the anti-activase plants contained, on average, approximately twice as much Rubisco protein as the control plants. The assimilation rate of the control plants plateaued and stabilized after about 60 min of illumination. In contrast, there was a continual decline in the assimilation rate of the anti-activase plant for the duration of measurement. The decline was approximately 30% during 2 h (Fig. 3A).

Table I. *Chl fluorescence parameter (φPSII) of control and R_1 anti-activase plants at high and atmospheric CO_2*

Measurements were taken under the growth conditions according to the procedure of Hudson et al. (1992b) approximately 90 min after the onset of illumination following 12 h of darkness. The Chl fluorescence parameter, which represents the quantum efficiency of PS II, was measured on the adaxial surface and calculated according to the method of Genty et al. (1989).

	φPSII	
	High CO_2 (0.35%)	Low CO_2 (0.03%)
Control	0.66 ± 0.001 (n = 3)	0.652 ± 0.001 (n = 3)
Anti-activase	0.58 ± 0.002 (n = 7)	0.327 ± 0.010 (n = 9)

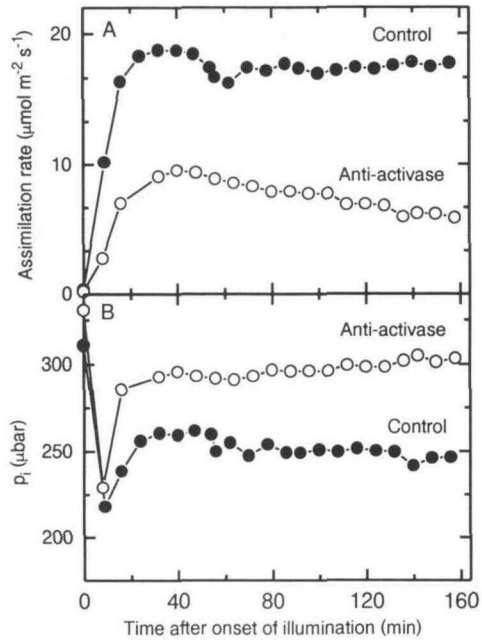

Figure 3. Time courses of CO_2 assimilation rate and intercellular CO_2 partial pressure (p_i) after the onset of illumination for control and R_1 antisense plants. The irradiance was 1000 μmol quanta m^{-2} s^{-1}, except for the zero-time measurement, which was in darkness. The leaf temperature was 25°C, the leaf to air vapor pressure difference was 11 mbar, and the CO_2 partial pressure was 350 μbar.

Rubisco Carbamylation Levels

Both the primary transformants and the R_1 seedlings were grown at high CO_2 and were sampled for carbamylation assays. The primary transformants were also sampled at low CO_2. Preliminary results with control and anti-activase (plants 52 and 50) primary transformants showed little difference in Rubisco carbamylation levels between high and low CO_2 (Table II). Therefore, subsequent leaf samples were taken at high CO_2 in the dark and after 90 min of illumination at 1000 μmol quanta m^{-2} s^{-1}. The Rubisco carbamylation level in the primary transformant controls was 75% after 90 min of illumination compared to 30 to 36% in the anti-activase plants. The 90-min leaf samples of the R_1 progeny of plant 52, taken at the same light intensity as the primary transformants, had very similar levels of Rubisco carbamylation.

The R_1 progeny were also sampled for Rubisco carbamylation after 3 h of illumination at 550 μmol quanta m^{-2} s^{-1} following a 12-h night (Table II). The carbamylation level of the control plants was only 55%, probably because of the low light intensity. It is interesting that there was very little variation in the carbamylation levels among the nine R_1 progeny, which were all approximately 20%. This result indicates that the R_1 progeny are physiologically similar despite their expected range of genotypes.

The change in Rubisco carbamylation level in control and anti-activase plants was followed after the onset of illumination at 1000 μmol quanta m^{-2} s^{-1} (Fig. 4). The carbamylation level in the control plants was about 40% in the dark

Table II. *Carbamylation level of Rubisco (%) in control and anti-activase tobacco plants in the light and dark at two CO_2 concentrations*

Carbamylation levels in primary transformants (plants 52 and 50) and a selection of R_1 seedings were measured as described in "Materials and Methods." Carbamylation level was measured by CABP binding.

	Carbamylation Level (%)			
	Primary transformants		R_1 generation	
	Dark	Light[a]	Dark	Light[b, c]
Control				
0.3–0.5% CO_2	55.5 ± 5.5 (n = 2)	74.5 ± 3.5 (n = 2)	41 ± 9 (n = 2)	77.5 ± 3.5[b] (n = 2) 55.3 ± 0.9[c] (n = 3)
0.03% CO_2	61 ± 5 (n = 2)	74.5 ± 5.5 (n = 2)		
Anti-activase				
0.3–0.5% CO_2	49 ± 1.4 (n = 2)	30.5 ± 0.5 (n = 2)	66.3 ± 1.5 (n = 3)	34.3 ± 1.7[b] (n = 3) 21.7 ± 1.2[c] (n = 9)
0.03% CO_2	58.5 ± 6.5 (n = 2)	36.5 ± 4.5 (n = 2)		

[a] Leaves sampled after 90 min at 750 μmol quanta $m^{-2}s^{-1}$. [b] Leaves sampled after 90 min at 1000 μmol quanta $m^{-2}s^{-1}$. [c] Leaves sampled after 3 h at 550 μmol quanta $m^{-2}s^{-1}$.

compared to 65% in the anti-activase plants. With the onset of illumination, the Rubisco carbamylation level in the control increased sharply within the first 5 min to 75 to 80%. The Rubisco carbamylation levels in the anti-activase plants decreased by approximately 20% in the first 5 min of illumination and then continued to decline at a slower rate for the remainder of the time course.

Inhibitor Release

The k_{cat} of Rubisco is calculated by dividing the fully activated in vitro activity by the total concentration of catalytic sites. Inhibitors that bind to the active site with a greater affinity than RuBP are not displaced during the in vitro assay; therefore, k_{cat} reflects the in vivo level of Rubisco active sites that are not complexed to inhibitors such as CA1P (Kobza and Seemann, 1988). In the dark (time zero), the control plants had a lower k_{cat} than did the anti-activase plants (Table III). After 90 min of illumination, the k_{cat} had increased to 2.7 to 2.8 s^{-1} in both control and antisense plants, indicating the release of inhibitor (presumably CA1P). The kinetics of inhibitor release were followed more closely by monitoring the preincubated activity of Rubisco after the onset of illumination (Fig. 5A). The inhibitor was released from Rubisco more slowly in the antisense plants than in the control plants. The observation is made clearer by calculating the concentration of Rubisco-bound inhibitor in the leaves following illumination, assuming that bound inhibitor was the sole cause for reductions in k_{cat} below the maximal value observed at 90 min (Fig. 5B). Although data at shorter illumination times would be required to measure accurately the rate of decline in inhibitor concentration in the control plants, it is clear that bound inhibitor disappears at least four times faster in the control plants than it does in the antisense plants. It is also apparent that, although the antisense leaves in this experiment contained 3.5 times as much Rubisco as the control leaves, the concentration of bound inhibitor during darkness was only 1.5 times greater in the antisense leaves (Table III and Fig. 5)

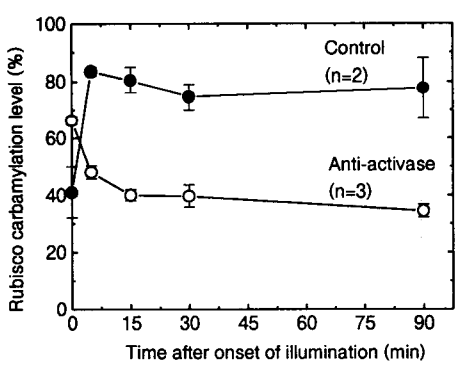

Figure 4. Time course of Rubisco carbamylation level after the onset of illumination, following 12 h of darkness in control and antisense R_1 generation plants. The irradiance was 1000 μmol quanta m^{-2} s^{-1}, and the CO_2 concentration was 0.35%. Carbamylation levels were determined by CABP binding.

Table III. *Catalytic rate of fully carbamylated Rubisco (k_{cat}) in control and R_1 anti-activase plants in the dark and after 90 min of illumination at 1000 μmol quanta $m^{-2}s^{-1}$*

	k_{cat} (s^{-1})	
	0 min	90 min
Control	1.22 ± 0.26 (n = 2)	2.73 ± 0.21 (n = 2)
Anti-activase	2.07 ± 0.14 (n = 3)	2.69 ± 0.28 (n = 3)

Relationship between Rubisco and Soluble Protein

The anti-activase plants had more soluble protein in their leaves than the control plants (Fig. 6). A constant relationship between phosphoribulokinase and protein content was observed for both control and anti-activase plants. All points fell on the same line of constant slope drawn through the origin (Fig. 6A), indicating that a constant proportion of the soluble protein was partitioned into phosphoribulokinase. However, the anti-activase points fell on a line of steeper slope than did the control plants when Rubisco content was plotted against protein (Fig. 6B). This indicates that a greater amount of protein was partitioned into Rubisco in the anti-activase plants (0.33 g of Rubisco g^{-1} of soluble protein) than in the control plants (0.2 g of Rubisco g^{-1} of soluble protein). The anti-activase plants' soluble protein content was approximately 1.0 to 1.5 g m^{-2} higher than that of the control plants. Rubisco accounted for most of this increase.

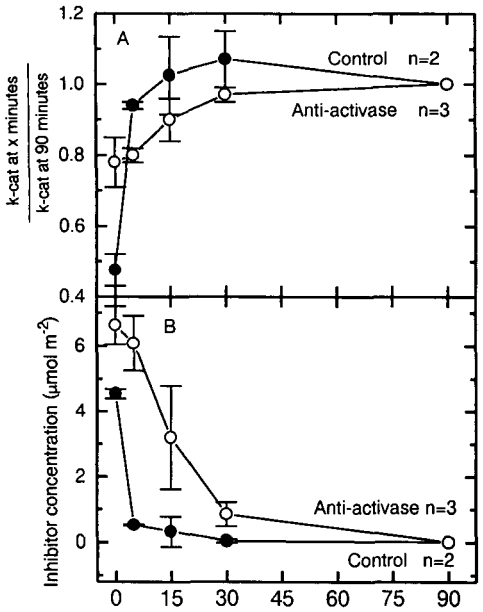

Figure 5. Release of Rubisco from CA1P inhibition with the onset of illumination. A, k_{cat} is proportional to the amount of Rubisco free from inhibitor. The k_{cat} at 90 min was 2.7 s^{-1} in both control and anti-activase leaves. In this experiment, the anti-activase leaves contained 3.5 times as much Rubisco as the control leaves. B, The concentration of inhibitor bound to Rubisco within plant extract was estimated, assuming that all reductions in k_{cat} below that observed at 90 min were due to presence of inhibitor. The inhibitor concentration (I) was calculated according to the following equation:

$$I = R\left(1 - \frac{k_{cat}^{x}}{k_{cat}^{90}}\right)$$

where k_{cat}^{x} and k_{cat}^{90} are the k_{cat} values at x and 90 min, respectively, and R is the mean Rubisco site concentration (μmol m^{-2}) of all leaf discs sampled during the time course.

DISCUSSION

Reduced Rubisco Carbamylation and Photosynthesis in Anti-Activase Plants

Introduction of the anti-activase gene into the tobacco genome led to a reduction in Rubisco activase content. The primary transformants had a range of activase levels, varying from slight decreases to large decreases in activase content (Fig. 1). We examined only those plants with relatively low activase levels, i.e. less than 25% of wild-type levels. The Rubisco carbamylation levels of two anti-activase primary transformants, plants 52 and 50, were not very different after 90 min of illumination even though they had different activase levels. Rubisco was only 35% carbamylated in both plants compared to 75% in the controls. All subsequent experiments were carried out using the R_1 progeny of plant 52. The activase content was less than 25% of the wild type in all of the R_1 plants. However, there may have been variation in the activase content of the R_1 plants, in the 0 to 25% range, which is below the sensitivity of the immunoblot method. Under high light, the R_1 plants were found to have a mean of 34% carbamylated Rubisco after 90 min of illumination, which is similar to the anti-activase primary transformants (Table II).

The reduction in Rubisco activase was accompanied by a reduction in photosynthesis at low CO_2 as determined by Chl fluorescence (Table I). These results are consistent with a limitation of photosynthetic rate by Rubisco (von Caemmerer and Farquhar, 1981). At high CO_2, the anti-activase plants had a quantum efficiency similar to that of the control plants, which indicates that the light reactions and other photosynthetic carbon reduction cycle enzymes had not been adversely affected by the presence of the antisense genes.

Fluorescence and gas-exchange measurements at atmospheric CO_2 showed that the steady-state photosynthetic rate of the anti-activase plants was approximately half that of the controls (Table I, Fig. 4). Taking into consideration the similar or higher intercellular CO_2 partial pressures and the higher Rubisco content of the leaves of the anti-activase plants compared to the control plants (Fig. 6B), we conclude that diffusional limitations to CO_2 uptake or differences in Rubisco content did not contribute to the disparity in the CO_2 fixation rate. Therefore, the lower Rubisco activity in the anti-activase plants must be due in part to the lower Rubisco carbamylation levels.

High CO_2 concentrations almost completely restored photosynthetic activity in the anti-activase plants, as indicated by the ϕPSII values (Table I), and enabled the majority of R_1 antisense plants to grow (Fig. 2). Significantly, the similarity between the carbamylation levels of Rubisco in the anti-activase plants at low and high CO_2 (Table II) indicated that

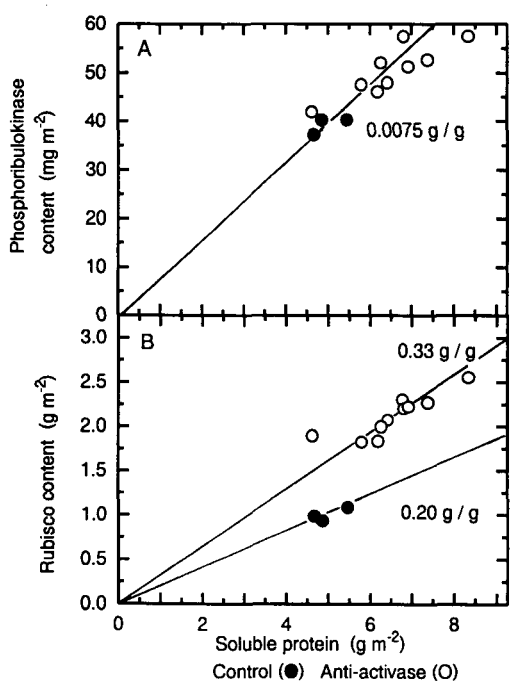

Figure 6. Protein partitioning in transgenic tobacco plants. Shown are the relationships between soluble protein content and phosphoribulokinase (A) or Rubisco content (B).

the increase in photosynthetic rate was not due to an increase in Rubisco carbamylation but to an increase in catalytic rate at the few carbamylated active sites. A similar high CO_2 requirement was observed for the *rca* mutant of *A. thaliana* (Somerville et al., 1982). The lower photosynthetic rate of the *rca* mutant correlated with a decrease in Rubisco carbamylation (inferred from the ratio between initial and total activities) in the light (Somerville et al., 1982; Salvucci et al., 1986).

Our results seem inconsistent with in vitro studies in which spontaneous activation proceeded faster at high CO_2 concentrations (Lorimer et al., 1976). However, these in vitro experiments were carried out in the absence of ligands such as RuBP and CA1P. Results of in vivo studies indicate that the relationship between Rubisco carbamylation and CO_2 concentration is more complex than it appears in vitro. In *Raphanus sativus*, carbamylation in the light was proportional to CO_2 concentration at concentrations below atmospheric but not above (von Caemmerer and Edmondson, 1986), whereas carbamylation was sensitive to CO_2 in the dark but not in the light in *T. aestivum* and *A. thaliana* (Mächler and Nösberger, 1980; Perchorowicz et al., 1981; Salvucci et al., 1986). The Rubisco carbamylation levels of tobacco leaves were insensitive to CO_2 both in the light and in the dark (Table II).

Role of Rubisco Activase in Maintaining Rubisco Carbamylation

Rubisco activase facilitates and maintains Rubisco carbamylation in the light. This is evident in the time courses of assimilation rate (Fig. 3A) and carbamylation levels (Fig. 4). In the dark, the Rubisco carbamylation levels of the control and anti-activase plants were approximately 40 and 60%, respectively (Fig. 4). With the onset of illumination, there was a doubling of Rubisco carbamylation in the control plants in the first 5 min, whereas the carbamylation level in the anti-activase plants declined.

Despite the rapidity of light-initiated carbamylation in the control plants, the CO_2 assimilation rate did not plateau until after 30 min of illumination (Fig. 3A). Studies by Kirschbaum and Pearcy (1988) showed that the slowest step of photosynthetic induction after illumination is stomatal opening. The slow increase in intercellular CO_2 partial pressure during the initial 30 min of illumination is consistent with a diffusional limitation of CO_2 assimilation rate during this phase (Fig. 3B).

The CO_2 assimilation rate of the anti-activase leaf stabilized at a rate that was approximately half of the control rate and showed a gradual decrease over time (Fig. 3A). This correlates with the declining carbamylation level observed in the carbamylation time course during 30 to 90 min (Fig. 4) and may be associated with increases in the RuBP level. RuBP binds more tightly to the inactive catalytic site than it does to the carbamylated site of Rubisco, thereby sequestering Rubisco in the inactive form in the absence of activase. Analysis of the leaves of the *A. thaliana rca* mutant grown at 1% CO_2 indicated that the RuBP concentration was 4 times higher than in control plants (Salvucci et al., 1986). These high RuBP concentrations, in the absence of activase, would promote decarbamylation of Rubisco. It seems likely that this is also the cause of Rubisco decarbamylation in the activase-deficient antisense plants. In both the *A. thaliana* mutant and tobacco anti-activase plants, there was a recovery in Rubisco carbamylation in the dark to levels equal to or greater than those observed in the control leaves. This recovery in Rubisco carbamylation was accompanied by depletion of the RuBP pool in the *rca* mutant of *A. thaliana* (Salvucci et al., 1986).

The lower Rubisco carbamylation level of the anti-activase plants was partially offset by the increase in Rubisco content (Fig. 6). If the relative Rubisco content and carbamylation levels are considered, the photosynthetic rate of the anti-activase plants after full-light induction should be 80% of that of the control rate rather than 50%. Therefore, there must be another limitation to RuBP carboxylase activity other than carbamylation level. Possibilities include indirect inhibition of the enzyme by high RuBP concentrations, perhaps as a consequence of high stromal pH, or a localized high resistance to CO_2 uptake within the anti-activase plant chloroplasts, which have an unusually high Rubisco content.

Finally, comparison of the k_{cat} and carbamylation time courses highlights the important role of activase in preventing decarbamylation following a transition from dark to light (Figs. 4 and 5). A large decrease in carbamylation levels accompanied the release of CA1P from the active site in the anti-activase plants.

Release of CA1P

The inhibitor CA1P bound to at least half of the active sites of tobacco Rubisco in the dark. The release of this inhibitor from Rubisco was compared in control and anti-activase plants by determining the k_{cat} of Rubisco in leaves in the dark and at different times following the onset of illumination (Fig. 5A). The fraction of Rubisco active sites sequestered by CA1P in the dark was greater in the control than in the antisense plants as shown by the k_{cat} values, despite a 50% larger bound-CA1P concentration in the antisense plants (Fig. 5B). This was because the antisense leaves contained 3.5 times as much Rubisco as the control leaves in this experiment. Therefore, the ratio of CA1P to active sites was lower in the antisense leaves and, consequently, k_{cat} was higher.

The dark-to-light increase in k_{cat} that accompanies CA1P release occurred to similar extents in antisense and control plants, indicating that the light-initiated removal of CA1P proceeded despite low activase concentrations in the antisense plants. This result is consistent with in vitro studies carried out by Seemann et al. (1985), in which CA1P was released from the active site and completely degraded by alkaline phosphatase in the absence of activase. The release of CA1P in the antisense plants might be assisted by accumulation of high concentrations of RuBP, which would tend to displace CA1P (Robinson and Portis, 1988a). The displaced CA1P would then be degraded by a CA1P phosphohydrolase located in the chloroplast stroma (Seemann et al., 1990), which would further enhance dissociation.

Although the k_{cat} of Rubisco in the antisense plants eventually attained the same level when illuminated as in the control plants, it did so more slowly (Fig. 5A). Calculations based on the assumption that all reductions in k_{cat} below the

maximal value observed at 90 min were due to CA1P bound to Rubisco showed that CA1P was released at least 4-fold faster in the control plants than in the antisense plants (Fig. 5B). These results indicate a role for activase in accelerating CA1P release.

High Rubisco Content in the Anti-Activase Plants

The anti-activase plants contained approximately twice as much Rubisco as the control plants. This caused both the total soluble protein and the fraction of it partitioned to Rubisco to be greater in the anti-activase plants (Fig. 6). This may be a long-term compensating response to the shortage of photosynthate experienced by these plants. However, the possibility that Rubisco content may vary with the developmental status of the plant needs to be considered. The control plants grew very rapidly at high CO_2 and may have reached a later developmental stage (with lower leaf protein and Rubisco levels) than the anti-activase plants. Confidence that this is not the case is engendered by results obtained by J. Masle (personal communication), who measured Rubisco levels in the youngest, fully expanded leaves of control tobacco and showed that levels varied only slightly with plant age and CO_2 concentration (over the range 0.035–0.1%) during growth. This raises the interesting possibility that Rubisco levels may be governed in the long term according to photosynthate supply or the balance between photosynthate supply and demand.

CONCLUSIONS

This engineering of a Rubisco activase-deficient form of tobacco provides insight into the roles of activase in vivo. The activase-deficient plants had a lower CO_2 assimilation rate than the control plants, which correlated with their inability to maintain Rubisco in its carbamylated form with the onset of illumination. The nocturnal inhibitor, CA1P, was still released from Rubisco's active sites when the anti-activase plants were illuminated and, therefore did not contribute to the difference in photosynthetic rate after light induction. However, CA1P was released more slowly in the anti-activase plants. Further analysis of these plants will assist understanding of the interrelated roles of RuBP, CA1P, and activase in Rubisco's regulation.

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