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Growth and Photosynthesis under High and Low Irradiance of *Arabidopsis thaliana* Antisense Mutants with Reduced Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Activase Content[1]

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Photosynthesis and growth to maturity of antisense ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase *Arabidopsis thaliana* with reduced concentrations of activase relative to wild-type (Wt) plants were measured under low (200 μmol m^{-2} s^{-1}) and high (600 μmol m^{-2} s^{-1}) photosynthetic photon flux density growing conditions. Both growth and photosynthesis were significantly reduced in an Arabidopsis clone (R100) with 30 to 40% Wt activase, an effect that was more pronounced in high light. The aboveground biomass of the antisense clone R100 reached 80% of Wt under low light and 65% of Wt under high light. Decreased growth in the antisense plants was attributed to reduced relative rates of growth and leaf area expansion early in development; all plants attained similar values of relative rates of growth and leaf elongation by 21 d after planting. Reductions in photosynthesis were attributed to decreased Rubisco activation in the antisense plants. Rubisco constituted about 40% of total soluble protein in both Wt and clone R100 under both light regimes. Activase content was 5% and 1.4% of total soluble protein in Wt and clone R100, respectively, and also was unaffected by growth irradiance. The stoichiometry of Rubisco to activase was estimated at 20 Rubisco active sites per activase tetramer in Wt Arabidopsis and 60 to 80 in the transgenic clone R100. We conclude that Wt Arabidopsis does not contain Rubisco activase in great excess of the amount required for optimal growth.

Light regulation of photosynthesis is a complex phenomenon that is centered around the activation state of Rubisco, the primary enzyme in CO_2 fixation. The light response of photosynthesis is highly correlated to the Rubisco activation state in vivo (Salvucci et al., 1986). The Rubisco activation state is regulated to a large extent by Rubisco activase, a soluble stromal protein that catalyzes the removal of bound sugar phosphate inhibitors from Rubisco. Rubisco activase does not appear to affect Rubisco activation per se, but it allows uninhibited access to the active site for binding of the activators CO_2 and Mg^{2+} (Portis, 1990, 1992). Rubisco activase is thought to control the release of RuBP and other inhibitors from Rubisco, and thus to regulate its activation state and in vivo activity in response to changing the light intensity (Brooks and Portis, 1988; Portis, 1990). The essential role of Rubisco activase is illustrated by the *rca* mutant of Arabidopsis, which lacks this protein and is incapable of maintaining sufficient activation of Rubisco to grow under atmospheric CO_2 concentrations (Somerville et al., 1982; Salvucci et al., 1985, 1986).

Rubisco activity is one of the principal factors limiting photosynthesis at saturating light and atmospheric CO_2 concentrations. Plant growth is dependent on the carbohydrates that are provided by photosynthesis. Thus, under certain conditions, growth may be directly proportional to Rubisco activity, as recently shown in experiments with antisense *rbc*S tobacco with reduced Rubisco grown under high irradiance and high temperature (Krapp et al., 1994). Under more moderate or controlled environmental conditions, the relationship between Rubisco content and photosynthesis (or biomass production) can be hyperbolic (Lauerer et al., 1993; Masle et al., 1993), reflecting significant limitations by other factors. Nonetheless, Rubisco has been found to exert considerable control over photosynthesis under a variety of growing conditions (Sharkey, 1989; Sage et al., 1990; Hudson et al., 1992; Quick et al., 1992; Masle et al., 1993; Krapp et al., 1994).

Plant growth is affected by many developmental and environmental factors that complicate the concept of a

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Abbreviations: A_{sat}, light-saturated assimilation rate; CABP carboxyarabinitol-1,5-bisphosphate, CA1P, carboxyarabinitol-1-phosphate; HL, high light growth condition (600 μmol m^{-2} s^{-1}); dap, days after planting; LL, low light growth condition (200 μmol m^{-2} s^{-1}); *rbc*S, gene for small subunit of Rubisco; *rca*, gene for Rubisco activase; RGR, relative growth rate; RLE, relative rate of leaf elongation; RuBP, ribulose bisphosphate; Tricine, *N*-Tris(hydroxymethyl)-methylglycine; tsp, total soluble protein; Wt, wild type.

simple, linear relationship between Rubisco activity, photosynthesis, and growth (Gifford and Jenkins, 1982). For example, plants growing under natural conditions are often limited by light (Ort and Baker, 1988), and photosynthetic acclimation to irradiance during growth occurs in many plant species. Acclimation to low light involves changes in protein concentration and chlorophyll *a/b* ratios, which result in a lower light-saturated rate of photosynthesis per unit leaf area compared with growth under high irradiance (Björkman, 1981; Sims et al., 1994). It not is known if Rubisco activase plays a role in the acclimation of photosynthesis to growth irradiance, or if activase protein expression is altered relative to other stromal proteins at different growth irradiances.

Two groups have previously studied transgenic tobacco plants with reduced levels of Rubisco activase protein. Mate et al. (1996) reported that more than a 95% reduction of activase was required in transgenic tobacco before reductions in the CO_2 assimilation rate and Rubisco carbamylation were observed. On the other hand, Jiang et al. (1994) observed measurable reductions in light-saturated photosynthesis and Rubisco carbamylation in antisense tobacco with more moderate reductions in the activase content. Both groups also made some general observations about plant growth, but detailed growth analyses were not reported.

Light activation of Rubisco in tobacco is complicated by the occurrence of the nocturnal carboxylase inhibitor CA1P. Rubisco activase is capable of releasing CA1P from Rubisco in vitro (Robinson and Portis, 1988), but control of CA1P binding in vivo may be shared between activase and CA1P phosphatase (Moore and Seemann, 1994). The study of the photosynthetic characteristics of tobacco may be further complicated by a high light saturation point for photosynthesis. Light saturation of photosynthesis occurs at around 1200 μmol m^{-2} s^{-1} in tobacco at ambient CO_2 concentrations (Jiang et al., 1994), making it difficult to conduct controlled environment experiments under high light intensity. This possibly contributed to the controversy regarding Rubisco control over photosynthesis from studies of transgenic tobacco with reduced Rubisco content (see Hudson et al., 1992; Lauerer et al., 1993; Krapp et al., 1994). In contrast, Arabidopsis does not accumulate significant levels of CA1P, and Rubisco activity appears to be regulated principally by Rubisco activase control of RuBP binding to inactive Rubisco (Brooks and Portis, 1988). The light saturation point of photosynthesis occurs at 600 μmol m^{-2} s^{-1} in Arabidopsis at ambient CO_2 (Salvucci et al., 1986), also making it ideal for conducting controlled experiments under high versus low irradiance. In this paper we report on the growth and photosynthesis of antisense Rubisco activase Arabidopsis grown under high and low irradiance.

MATERIALS AND METHODS

Arabidopsis Transformation

Genetic transformation was performed using a modification of the methods developed by Valvekens et al. (1988) and Marton and Browse (1991). Seeds of *Arabidopsis thaliana* (RLD ecotype) were grown on GM media (10 g L^{-1} Suc in a MSVB base Murashige and Skoog salts; Murashige and Skoog [1962]), B5 vitamins, 1.0 mg L^{-1} Gly, 0.5 mg L^{-1} biotin, 0.5 g L^{-1} Mes, pH 5.7, and solidified with 5.0 g L^{-1} Agargel]) for 2 weeks, followed by treatment at 4°C at a PPFD of <25 μmol m^{-2} s^{-1} for a minimum of 2 weeks. The roots were removed at the base of the hypocotyl and placed onto the MSVB base, 20 g L^{-1} Glc, 5.0 mg L^{-1} IAA, 0.5 mg L^{-1} 2,4-D, and 0.5 mg L^{-1} kinetin (CIM medium) in the dark at room temperature for 3 d. They were then cut into 2-cm sections, immersed in 30 mL of *Agrobacterium tumefaciens* culture: sterile liquid CIM (1:1, v/v) for 5 to 15 min, blotted dry, and returned to the CIM medium in the dark at room temperature for another 3 d. Root sections were washed for 5 to 15 min in 30 mL of liquid SIM (MSVB base, 20 g L^{-1} Glc, 0.05 mg L^{-1} IAA, 2.0 mg L^{-1} *N*-6-[δ-2-isopentyl] adenine, and 1.0 mg L^{-1} zeatin riboside) containing 1.0 mg mL^{-1} vancomycin, blotted dry, and placed onto SIM50/750 medium (SIM plus 50 mg L^{-1} kanamycin and 750 mg L^{-1} vancomycin). The explants were incubated in a growth chamber at 24°C under a PPFD of 50 to 100 μmol m^{-2} s^{-1} (16 h d^{-1}) for 2 weeks, and then transferred to fresh medium every 2 weeks until shoots formed. Shoots were isolated and placed on GM medium supplanted with 2.0 mg L^{-1} indole-3-butyric acid, 100 mg L^{-1} kanamycin, and 200 mg L^{-1} cefotoxamine, and incubated in the growth chamber as above until seeds were produced.

Cloning and Bacterial Culture

The cDNA encoding the Arabidopsis 42-kD isoform of Rubisco activase (Werneke and Ogren, 1989) was cloned in the antisense orientation 3′ to either the spinach *rca* promoter (RAAt) or the enhanced 35S cauliflower mosaic virus promoter (EAAt). These cassettes were then spliced into pCGN1547 (McBride and Summerfelt, 1990) and transformed into a "disarmed" strain of *A. tumefaciens* PC2760::pAL4404 Ti (An et al., 1985). The *A. tumefaciens* was used to inoculate 2 mL of Luria Bertania broth containing 200 mg L^{-1} streptamycin and 25 m L^{-1} gentamycin 2 d prior to transformation and was grown overnight at 30°C. A 0.1-mL inoculum from the overnight culture was transferred to 10 mL of liquid medium containing AB salts and AB buffer (An et al., 1985), 50 g L^{-1} Glc, 5.0 mM octopine, and 100 μM acetosyringone, pH 5.5 (S. Gelvin, personal communication), and grown 12 to 24 h at 30°C. The culture was diluted and used to inoculate Arabidopsis root segments as described above.

Plant Culture

Seeds from primary transformants (T_0) were surface-sterilized in 70% ethanol for 2 min, followed by 10% Clorox, 1.0% Tween 20 for 20 min, and 5 changes of double-distilled, sterile H_2O. The sterilized seeds were placed onto GM media containing 100 mg L^{-1} kanamycin, incubated at 4°C for 1 week, and placed into the growth chamber under the conditions listed above for 1 week. Seedlings were transplanted to 2-L pots and kept in the growth chamber at

24°C, 16-h days at a PPFD of 300 μmol m^{-2} s^{-1}. Plants were either under ambient or 3000 μL CO_2 L^{-1} as noted in "Results." Seeds were collected from self-pollinated transformants for further experiments. T_3 seedlings were grown as described above for the plants that were used to generate light-response curves (Fig. 4). For all other experiments, T_3 or T_4 seed was grown in Sunshine Mixture[5] (SunGro Horticulture Inc., Bellevue, WA) in 32-cell flats and transplanted to 2-L pots at 21 dap. Plants were maintained in growth chambers at 60% RH, 18°/24°C (12/12 h, night/day) with a PPFD at a plant height of 200 (LL) or 600 (HL) μmol m^{-2} s^{-1}, which deviated not more than 25 μmol m^{-2} s^{-1}. Plants were watered as necessary with a nutrient solution (Somerville and Ogren, 1982).

DNA and RNA Analysis of Transformed Arabidopsis

Genomic DNA was isolated as described by Dellaporta et al. (1983), cut with the restriction enzymes *Xba*I and *Pst*I, separated on a 1.0% agarose gel, and transferred to a nylon membrane. Total RNA was extracted from whole plants with hot (60°C) phenol 0.7:1 in an extraction buffer (0.1 M Tris, pH 8.0, 0.02 M EDTA, 0.5 M NaCl, 0.5% SDS, and 0.5% β-mercaptoethanol). RNA was purified on a Qiagen q-100 tip (Qiagen, Chatsworth, CA) following the manufacturer's instructions. Approximately 10 μg of total RNA was denatured using the glyoxal method (Maniatis et al., 1982), separated on a 0.8% agarose gel, and transferred to a nylon membrane. The blots containing RNA or DNA were probed using the cDNA encoding the 42-kD Rubisco activase protein (Werneke and Ogren, 1989), generated using asymmetric PCR (Bednarczuk et al., 1991), and labeled with biotin (Tropix, Bedford, MA) or digoxygenin (Boehringer Mannheim) following the manufacturer's instructions.

Biochemical Assays

Rubisco activity was measured either by incorporation of $^{14}CO_2$ into acid-stable products (Chastain and Ogren, 1985) or by a spectrophotometric assay (Esau et al., 1996). Approximately 2 cm^2 leaf area was quick-frozen and ground in liquid N_2 in a microtube with a mini-pestle and homogenized in 0.5 mL 100 mM Tricine, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, 10 μM leupeptin sulfate, and 1 mM PMSF. The homogenate was centrifuged for 15 s in a microcentrifuge. For the ^{14}C assay, 50-μL aliquots were immediately injected into 450 μL of Rubisco activity assay buffer containing 100 mM Tricine, pH 8.0, 10 mM $NaH^{14}CO_3$ (1.0 μCi $assay^{-1}$), 10 mM $MgCl_2$, and 0.5 mM RuBP. After 30 s at 25°C, the assay was stopped by injection of 0.1 mL of 4N formic acid/1N HCl. Another aliquot was assayed for total activity following a 12-min incubation at room temperature in 10 mM $NaHCO_3$, 10 mM $MgCl_2$, and 0.05 mM 6-phosphogluconate to allow for complete activation of Rubisco. Samples were dried, redissolved in 0.5 mL of 0.1 N HCl, mixed with 4 mL of Bio-Safe II scintillation fluid (Research Products Industrial Corp., Mount Prospect, IL), and counted in a scintillation counter.

For the spectrophotometric method, 10-μL aliquots were injected into the assay mixture containing 0.1 M Tricine, pH 8.0, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 2 mM DTT, 2 mM ATP, 2 mM phosphoenol pyruvate, 0.3 mM NADH, 15 units of pyruvate kinase, 20 units of 3-phosphoglyceric phosphokinase, 100 units of triose phosphate isomerase, and 10 units each of glyceraldehydephosphate dehydrogenase and glycerophosphate dehydrogenase. Total activity was measured in the same manner following the activation of Rubisco as described above. RuBP was synthesized as described by Edmonson et al. (1990). The RuBP content of crude leaf extracts was determined as in Campbell and Ogren (1990).

The Rubisco content in the leaf extracts was determined by the binding of ^{14}CABP to fully activated Rubisco. ^{14}CABP was added to a 300-μL aliquot of leaf extract (brought to 10 mM each $NaHCO_3$ and $MgCl_2$) at an approximate 3:1 ratio of the CABP:Rubisco sites (assuming 14 nmol sites mg^{-1} Rubisco). ^{14}CABP was synthesized according to Butz and Sharkey (1989). The ^{14}CABP was separated from carboxyribitol biphosphate as described by Gutteridge et al. (1989) and had a specific activity of 12,000 dpm $nmol^{-1}$. The mixture was incubated on ice for 1.5 h, then mixed with 150 μL of 60% PEG and 8 μL of 1.0 M $MgCl_2$. The mixture was vortex-mixed, incubated on ice for 30 min, and microcentrifuged for 10 min. The supernatant was discarded and the pellet was resuspended in 300 μL of 100 mM Tricine, pH 8.0, 10 mM $NaHCO_3$, and 10 mM $MgCl_2$. The protein was precipitated a second time with PEG/$MgCl_2$, incubated 30 min on ice, and centrifuged as before. The supernatant was again discarded and the pellet resuspended in 550 μL of 0.1 N NaOH. Following a complete resuspension of the pellet, 500 μL was transferred to a scintillation vial and ^{14}C counts were determined in 5 mL of scintillation fluid. The extract to which purified Rubisco was added indicated that the recovery of this method was >90%. Rubisco was purified from spinach according to Wang et al. (1992).

Rubisco activase content was measured by enhanced chemiluminesence detection of immunoblots as described by Eckardt and Portis (1995). Rubisco activase was purified from Arabidopsis according to Robinson et al. (1988) and used as a standard. Purified activase and tsp were quantified with Coomassie Plus Protein Assay Reagent (Pierce) using BSA as a standard.

Gas-Exchange Measurements

Photosynthesis was measured in an open gas-exchange system adapted from Brooks and Portis (1988). Calculations were made according to von Caemmerer and Farquhar (1981). The air supply was fully saturated at a dew point of 14°C and the leaf temperature was maintained at or near 25°C to maintain a leaf-to-air vapor pressure deficit of ≤12 mbar. CO_2 was measured with an IR gas analyzer (model 225 MK3, Analytical Development Co., Hoddesdon, UK) fitted with ice traps. Measurements were con-

[5] Names are necessary to report factually on available data; however, the U.S. Department of Agriculture (USDA) neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

ducted at ambient CO_2 of 350 $\mu L\ L^{-1}$. Water vapor leaving the chamber and leaf temperature were measured with a digital hygrometer (Fisher Scientific) and copper-constantin thermocouple (Cole Parmer, Chicago, IL), respectively. The plant chamber had a temperature-controlled water jacket, and a water bath and an IR filter were placed between the light source and chamber. Photosynthesis was measured on whole plants that were removed from the soil under water and placed into water-filled microtubes before sealing into the gas-exchange cuvette, or plants that were grown in 50-mL polypropylene centrifuge tubes with holes drilled in the bottoms and the tops cut off at the 40-mL mark. In the latter case, the tubes in which plants were grown were sealed into the chamber with modeling clay. The top of the tube and around the plant stem were sealed with 0.15% agarose.

Growth Analyses

Growth analyses were conducted in two experiments with HL and LL treatments. For each experiment, three plants per clone per treatment were harvested weekly for 5 weeks beginning at 14 or 21 dap. Total leaf area was measured with an area meter (Li-Cor Inc., Lincoln, NE). Leaves and stems were separated, the fresh weight was recorded immediately, and the dry weight was recorded following drying for 3 to 5 d at 65 to 70°C. The effect of reduced Rubisco activase content on biomass and leaf area accumulation was assessed by analysis of variance using Minitab for Windows Version 10.51 Xtra (Minitab Inc., State College, PA). Significance was accepted as $P \leq 0.05$.

RESULTS

Transformation with Antisense *rca* Reduced Activase Content in Arabidopsis Leaves

Evidence of *rca* down-regulation was first noted in the T_1 plants generated from seed that was collected from primary transformants and plated onto GM media containing kanamycin. Plants that were resistant to kanamycin fell into three classes: dark green (nearly indistinguishable from Wt), light green, and yellow (both smaller than Wt). Generally, plants transformed with the RAAt gene had the near-Wt phenotype, whereas plants expressing EAAt produced lighter green to chlorotic phenotypes, the latter requiring elevated CO_2 for growth. A preliminary screen of CO_2 fixation among T_3 plants led to the choice of six clones for further analysis: R100, R155, R156, E170, E177, and E011 (R and E denoting RAAt and EAAt cassettes, respectively).

Chemiluminescent detection of the immunoblots allowed quantitation of activase down to 5 to 10% of the Wt concentration of the protein in crude leaf extracts. The immunoblot analysis showed that the antisense plants expressed activase protein at levels substantially reduced from the Wt (Fig. 1A). Standard curves with purified Arabidopsis activase were linear between 10 and 100 ng (Fig. 1B). We routinely loaded 1.0 to 1.5 μg of tsp of Wt extract per lane for SDS-PAGE, which was subsequently transferred to PVDF membrane for immunoblotting. Because Rubisco activase averaged about 5% of tsp in Wt leaf extracts, this produced a signal that fell in the middle of the standard curve. No more than 4 μg of tsp per lane could be loaded without leading to an incomplete transfer of the proteins to the membrane. Therefore, quantitation of the antisense plants was limited to 10 ng of activase protein in 4 μg of tsp, or roughly 5% of the Wt activase concentration.

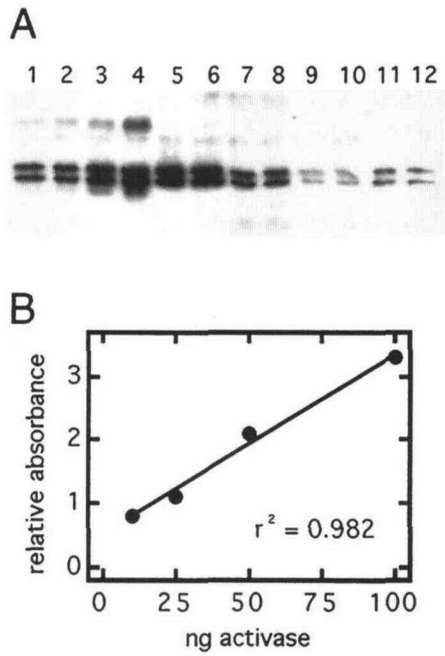

Figure 1. Quantitation of Rubisco activase in leaf extracts. A, Proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Rubisco activase was detected by incubation with a primary antibody to spinach Rubisco activase and a horseradish peroxidase-linked secondary antibody, developed with Amersham ECL reagents, and exposed to XAR film (Kodak) for 5 to 15 s. Lanes 1 to 4 contain 10, 25, 50, and 100 ng, respectively, of activase purified from Arabidopsis. Lanes 5 to 12 contain 1.5 μg of tsp from Wt (5 and 6) and antisense clones R100 (7 and 8), E170y (9 and 10), and E170g (11 and 12). B, Relative absorbances of standards obtained from laser densitometry of lanes 1 to 4 in A.

Chemiluminescent detection of the immunoblots from T_3 plants showed that clones R100 and R155 (not shown) contained 30 to 40% of the Wt activase concentration and that clone R156 showed no reduction in the activase content relative to Wt (not shown). Clones E170 and E177 continued to segregate for the green and yellow plants (designated by the suffix "g" or "y," respectively) when grown at ambient CO_2, with the yellow plants having almost undetectable levels of activase, and the green plants having about 20% of Wt. Clone E011 had undetectable levels of activase (not shown) and produced only severely chlorotic plants in the air.

Southern hybridization (Fig. 2A) shows the antisense construct at 2.8 kb (RAAt) and 2.6 kb (EAAt) and the endogenous *rca* gene at about 8 kb. Figure 2B, with DNA digested with *Bam*HI, an enzyme with only one site in the tDNA region, reveals the copy number of the inserted DNA. The control DNA (lane 1, RLD) has a single band representing the endogenous *rca* gene, with the remaining

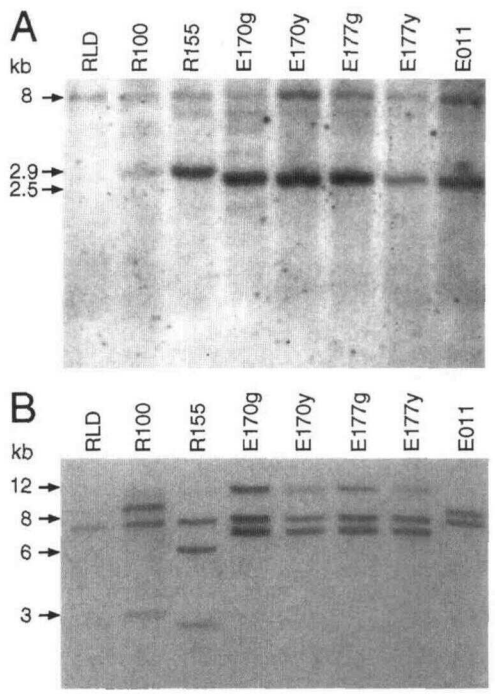

Figure 2. Southern hybridization of genomic DNA from antisense *rca* Arabidopsis. Genomic DNA was separated by electrophoresis, transferred to a nylon membrane, digested with *Xba*I and *Pst*I, and hybridized with biotin (A), or digested with *Bam*HI and hybridized with a digoxygenin-labeled probe from the cDNA encoding the 42-kD Rubisco activase protein (B).

lanes showing a corresponding band at approximately 7.5 kb. Two and three copies of the antisense insert were detected in R155 and R100, respectively, whereas one insert was seen in E011. E170 and E177 showed two inserts each, with identical banding patterns, indicating that these clones probably arose from the same transformation event. The band intensity suggests that these plants are homozygous at all three loci, unresolving the cause for the segregation pattern seen in these plants. The difference in the activase protein levels in E170y compared with E170g may be because of a genomic modification such as methylation in some of the plants, preventing transcription from either the endogenous or inserted genes.

Northern analysis (Fig. 3) shows reduced *rca* message levels generally corresponding to the reduction of activase protein in the plants, with the exception of R155, which shows slightly lower mRNA levels than E170g, a plant with

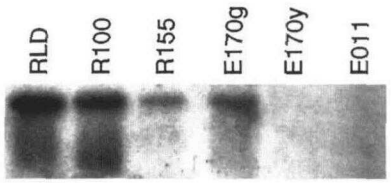

Figure 3. Northern hybridization of total RNA. Total leaf mRNA was separated by electrophoresis, transferred to a nylon membrane, and hybridized with a biotin-labeled probe from the cDNA encoding the 42-kD Rubisco activase protein.

about 30% less activase protein. This may be because of the difference in promoters, position effects, and/or posttranscriptional modification.

Photosynthesis in Antisense *rca* Arabidopsis Depends on Rubisco Activase Content and Light Intensity during Growth

Figure 4 shows light-response curves for a number of T_3 transformants grown under a PPFD of 300 μmol m^{-2} s^{-1} at ambient CO_2 for 2 weeks before measurements were taken. The light response for clone R100 was similar to the Wt even though it contained at least 60% less activase protein. The light response of E170g, which expressed about 20% of the Wt protein, was intermediate to the Wt and *rca* mutant. Clones E170y and E011, each with less than 5% Wt activase, showed light-response curves similar to the *rca* mutant. The latter two clones grew poorly in air and required elevated levels of CO_2 (e.g. 3000 μL L^{-1}) to reproduce seed.

Percent activation of Rubisco and RuBP content was determined on Wt plants and a number of T_3 transformants held under saturating light (600 μmol m^{-2} s^{-1}) for 15 min prior to quick-freezing of leaves. A positive correlation was observed between light-saturated photosynthesis at ambient CO_2 (A_{sat}) of the various plants and the Rubisco activation state (Fig. 5A). Rubisco in R100 and R155 was near full activation under saturating light, whereas the activation state declined in the clones with reduced A_{sat}. A negative correlation was observed between the RuBP content and the Rubisco activation state in the various plants (Fig. 5B).

Based on these results, further experiments were conducted under HL and LL growing conditions at ambient CO_2 to test the hypothesis that activase was not limiting to photosynthesis or to growth in plants with moderate reductions in the activase content (e.g. up to 70% reduction relative to Wt). Figure 6 shows A_{sat} measured at 24 dap as a function of the activase content for HL and LL plants. The

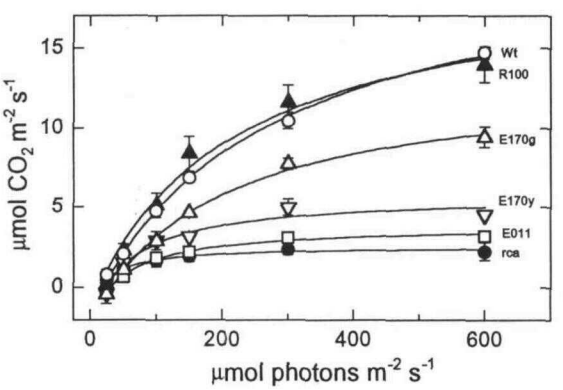

Figure 4. Light response of photosynthesis under ambient CO_2 in T_3 antisense *rca* and Wt Arabidopsis. Plants were grown under a PPFD of 300 μmol m^{-2} s^{-1} for 3 weeks and were dark-adapted for 1 h prior to exposure to the light levels that are indicated. CO_2 assimilation was averaged over a 5-min exposure following a 5-min pretreatment, allowing the plant to reach steady-state photosynthesis at each light level. ○, Wt; ▲, R100; △, E170g; ▽, E170y; □, E011; and ●, *rca* mutant.

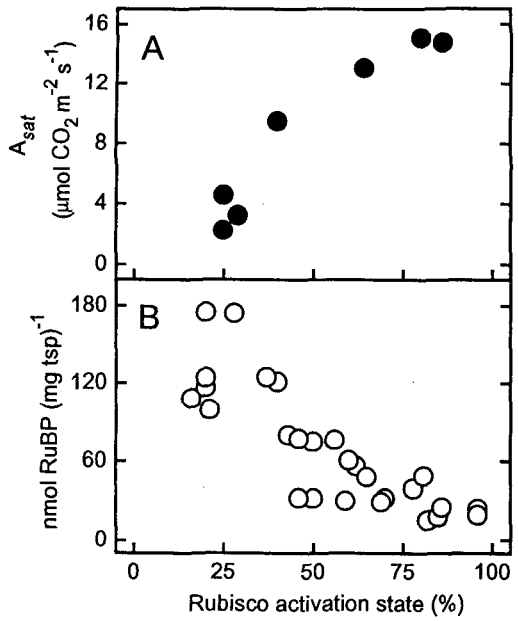

Figure 5. Relationships between Rubisco activation state and A_{sat} (A) or RuBP content (B) among a number of *rca* antisense Arabidopsis transformants and Wt. Plants grown under a PPFD of 300 μmol m^{-2} s^{-1} were exposed to a PPFD of 600 μmol m^{-2} s^{-1} at ambient CO_2 for 15 min and then immediately frozen in liquid N_2. Leaf samples were split in half to obtain Rubisco activation state and RuBP concentration from the same sample.

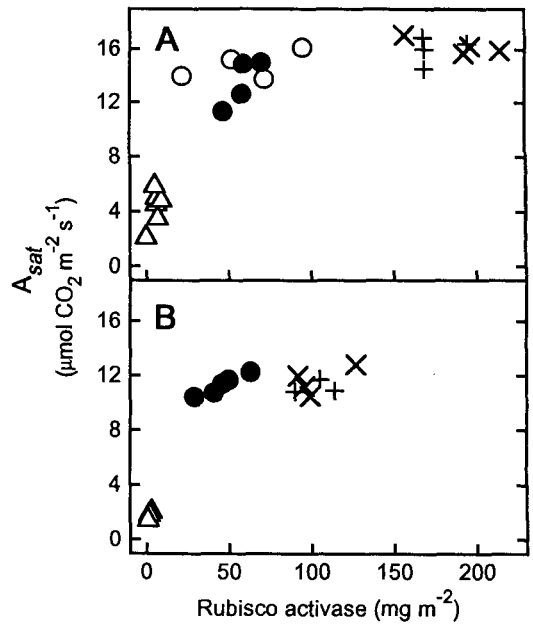

Figure 6. Dependence of A_{sat} on Rubisco activase content in plants grown under HL (A) or LL (B) at 24 dap. ×, Wt; +, R156; ○, R155; ●, R100; and △, E177.

data show a near-hyperbolic response for plants under both irradiance treatments.

Photosynthesis and Rubisco activity were examined in more detail in clone R100, one of two clones with a 60 to 70% reduction in activase relative to Wt. A_{sat} measured on 24 dap was significantly lower in clone R100 relative to Wt in HL plants but not in LL plants (Table I). A_{sat} on a leaf area basis was higher overall in HL plants than in LL plants, but expressed per milligram of tsp it was lower in HL relative to LL (Table I). Correspondingly, the HL plants had nearly twice the tsp content per unit leaf area of the LL plants. Percent activation of Rubisco was measured in extracts of mature leaves that were sampled directly from the growth chambers at HL or LL on 19, 24, and 28 dap (Table II). The activation state was lower (overall) in LL relative to HL. The R100 activation state was significantly lower than Wt in HL on 5 of the 6 d measured in the two experiments, and in LL on 3 of the 6 d measured in two experiments. The total k_{cat} of Rubisco (maximum turnover rate of fully activated Rubisco in mol CO_2 mol Rubisco^{-1} s^{-1}) was 20.2 ± 0.3 (mean ± SE of all measurements), and was not altered by the growth light regime or antisense *rca* expression (data not shown).

Moderate Reductions of Rubisco Activase in Antisense Arabidopsis Negatively Affects Plant Growth

Total leaf area and aboveground biomass were monitored at ambient CO_2 in Wt and clones R156, R155, and R100 grown under HL, and in Wt and R100 grown in LL. Leaf area and aboveground biomass accumulation over the course of development for two experiments are shown in Figure 7. Analysis of variance results showed that reduced Rubisco activase concentration in clone R100 had a significant effect on aboveground biomass and leaf area in HL, and on aboveground biomass in LL (the effect on leaf area in LL was significant at $P = 0.08$). On the last sampling days (49 and 42 dap for LL and HL, respectively), aboveground biomass of R100 reached 80% of Wt in LL, but only 65% of Wt in the HL (Fig. 7, C and D). Total leaf area of R100 reached 92% of Wt in LL and about 69% of Wt in HL (Fig. 7, A and B). The growth characteristics in HL of clone R156 were not significantly different from Wt, and clones R100 and R155 were not significantly different from each other (data from R156 and R155 not shown). Similar results to those reported above for growth of the antisense clones R156, R155, and R100 relative to Wt were recorded in another experiment with the HL and LL growth conditions (data not shown). In the

Table I. *Net photosynthesis and protein content at 24 dap*

Plant	Photosynthesis[a] on an Area Basis	Photosynthesis[a] on a Protein Basis	Protein Content[b]
	μmol m^{-2} s^{-1}	*nmol mg^{-1} s^{-1}*	*g tsp^{-1} m^{-2}*
HL Wt	15.8 ± 0.5[d]	4.0 ± 0.1[d]	4.0
HL R100	13.8 ± 0.7[e]	3.4 ± 0.2[e]	4.1
LL Wt	11.8 ± 0.4[d]	5.0 ± 0.2[d]	2.3
LL R100	11.3 ± 0.3[d]	4.8 ± 0.2[d]	2.3

[a] Values represent light-saturated photosynthesis measured on eight plants per line for HL-grown and five plants per line for LL-grown plants ± SE. [b] SE < 0.05 for all values. [c] mg indicates mg tsp^{-1}. [d,e] Values from the same growth irradiance followed by different letters are significantly different (Student's *t* test) at $P < 0.05$.

Table II. *Rubisco activation state*

Values represent percent activation of Rubisco ± SE of two plants per treatment sampled under the growing conditions.

dap	Line	HL-Grown		LL-Grown	
		Experiment 1	Experiment 2	Experiment 1	Experiment 2
19	Wt	77 ± 1[a]	82 ± 7[a]	67 ± 5[a]	65 ± 3[a]
	R100	68 ± 3[b]	73 ± 3[a]	61 ± 2[a]	61 ± 2[a]
24	Wt	90 ± 6[a]	90 ± 3[a]	65 ± 6[a]	61 ± 4[a]
	R100	71 ± 6[b]	76 ± 6[b]	50 ± 1[b]	56 ± 2[a]
28	Wt	94 ± 5[a]	93 ± 6[a]	62 ± 2[a]	71 ± 2[a]
	R100	81 ± 2[b]	82 ± 5[b]	54 ± 3[b]	56 ± 3[b]

[a,b] Within each experiment, values from the same growth irradiance and same dap followed by different letters are significantly different (Student's *t* test) at $P < 0.05$.

latter experiment the absolute growth of plants was less than that shown in Figure 7, possibly because the plants were grown in 32-cell flats and not transplanted to larger pots.

Figure 8 shows the natural log of aboveground biomass (A) and total leaf area (B) plotted against dap; the instantaneous slope of these graphs reflects the RGR (g g^{-1} d^{-1}) and RLE (cm cm^{-2} d^{-1}), respectively. The maximum steady-state rates of RGR and RLE achieved during the periods measured were similar for all plants in HL and LL. Attainment of the steady-state rate for LL plants lagged behind HL plants by 7 to 10 d. Antisense R100 showed lower RGR and RLE than Wt prior to 21 dap in HL, whereas there was no difference between R100 and Wt in LL. However, growth measurements were not made in LL prior to 21 dap. When RGR and RLE were calculated over the entire experimental period (14–42 dap in HL and 21–49 dap in LL), the values for Wt and antisense R100 were identical. Overall, RGR (g g^{-1} d^{-1}) in HL was 0.0472 and 0.0471 for Wt and R100, respectively, whereas in LL it was 0.0472 and 0.0470 for Wt and R100, respectively. Overall, RLE (cm cm^{-2} d^{-1}) was 0.0472 and 0.0471 in Wt and R100, respectively, and in LL, 0.0466 and 0.0464, respectively.

Figure 9 shows Rubisco activase (A) and Rubisco (B) concentrations throughout development for both Wt and R100 grown in HL or LL. Mean activase concentrations were 95 ± 9 mg m^{-2} in Wt and 30 ± 4 mg m^{-2} in R100 in LL, and 161 ± 34 mg m^{-2} in Wt and 47 ± 10 mg m^{-2} in R100 in HL (means ± SE of average values measured on all four sampling days from 14–35 dap). The differences between HL and LL reflected differences in tsp per unit leaf area. Activase as a percent of tsp was the same under both light regimes, at 5.3 ± 0.5% (LL) versus 5.0 ± 0.4% (HL) for Wt, and 1.4 ± 0.2% (LL) versus 1.4 ± 0.1% (HL) for R100. Rubisco content was also the same percentage of tsp in HL or LL, with an overall mean of 40 ± 2% in LL and 42 ± 1% in HL. Although antisense plants appeared to have a Rubisco content greater than Wt on 1 d in LL overall there was no significant difference in the Rubisco content between the Wt and R100 (Fig. 9B). Figure 10 shows the stoichiometry of Rubisco to activase on each sampling day. The ratio was independent of growth irradiance and was estimated at 20 Rubisco active sites per activase tetramer in Wt Arabidopsis and 50 to 80 in transgenic clone R100. Except for the last sampling day (35 dap) when the ratio in clone R100 was >100, the ratio was relatively constant

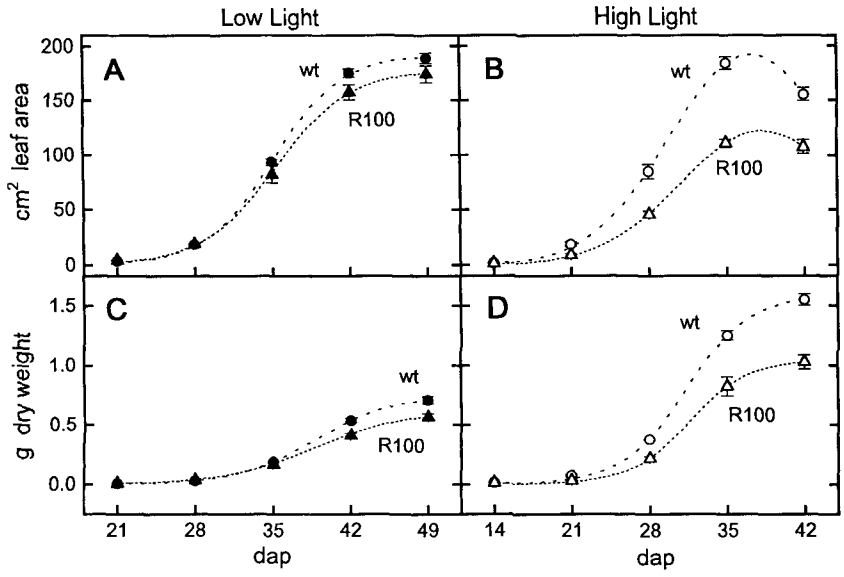

Figure 7. Leaf area and aboveground biomass (leaves plus stems) under LL (A and C) and HL (B and D). Values represent the mean ± SE of three plants. ○, Wt HL; △, R100 HL; ●, Wt LL; and ▲, R100 LL.

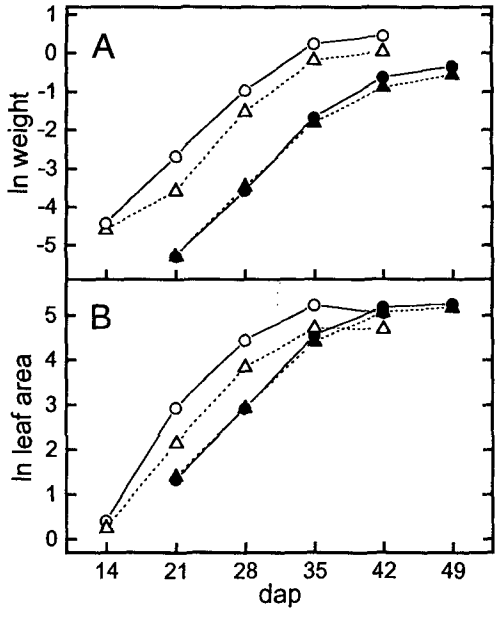

Figure 8. Natural log of aboveground biomass (A) and total leaf area (B) versus dap for LL- and HL-grown Wt and R100 plants. ○, Wt HL; △, R100 HL; ●, Wt LL; and ▲, R100 LL.

throughout the sampling period for both Wt and R100 plants.

DISCUSSION

Quantitation of Rubisco Activase in Wt and Antisense Arabidopsis

We estimated that Rubisco activase made up 5% of tsp in Wt Arabidopsis. Rubisco activase concentration has been estimated at 1% of tsp in tobacco (Mate et al., 1993, 1996) and 2% in spinach (Robinson et al., 1988). Values of around 0.25 mg m^{-2} in tobacco (equal to about 0.01% tsp) reported by Jiang et al. (1994) were determined using an ELISA assay with antibody raised against spinach activase and purified spinach activase as a standard. We found that antibody to spinach activase produced a significantly greater signal against spinach activase compared with an equal quantity of tobacco activase (not shown). We also found the ELISA to be an unreliable method for quantitation of activase in Arabidopsis leaf extracts containing less than the Wt activase concentration. When the extract was diluted in accord with the binding capacity of the ELISA plates, an extract with activase concentrations of about 1% tsp or less (20–25% Wt) did not produce a color change above the background (not shown). SDS-PAGE immunoblot analysis of protein concentration avoids potential problems with nonspecific binding (which can easily occur with the ELISA and dot-blot immunoblot techniques), because the target protein is separated from other proteins in the extract. These observations may help to explain the abnormally low estimates reported by Jiang et al. (1994). In agreement with Mate et al. (1993, 1996), we measured an activase concentration of 1.5 to 2% tsp in 6- to 8-week-old tobacco using the procedure described above with purified tobacco activase as a standard (not shown). It may be significant that Arabidopsis, a plant that normally grows under low light, contains considerably more Rubisco activase.

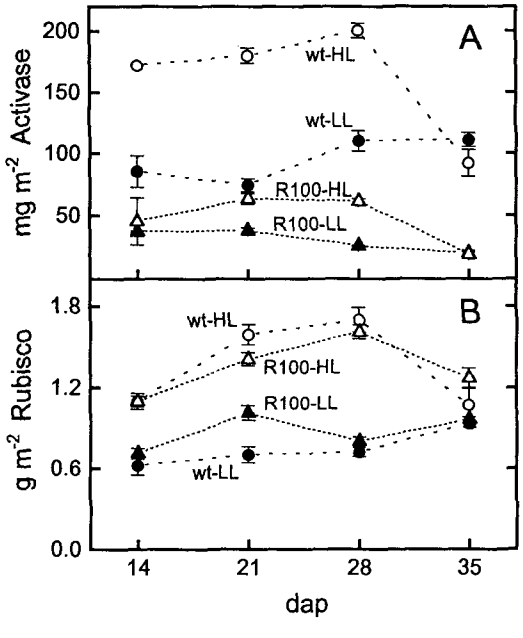

Figure 9. Activase (A) and Rubisco (B) content under HL and LL. Values represent the means ± SE of four measurements. ○, Wt HL; △, R100 HL; ●, Wt LL; and ▲, R100 LL.

Effect of Antisense Activase Expression on Activase and Rubisco Content

Expression of antisense cDNA directed against Rubisco activase mRNA effectively reduced the concentration of Rubisco activase in Arabidopsis. Three phenotypes were observed among a series of independently transformed lines found to have reductions in activase content. One set was severely stunted and chlorotic, and did not set viable seed when grown in air. This phenotype was similar to the *rca* mutant of Arabidopsis, which completely lacks the activase protein. Plants exhibiting this phenotype were

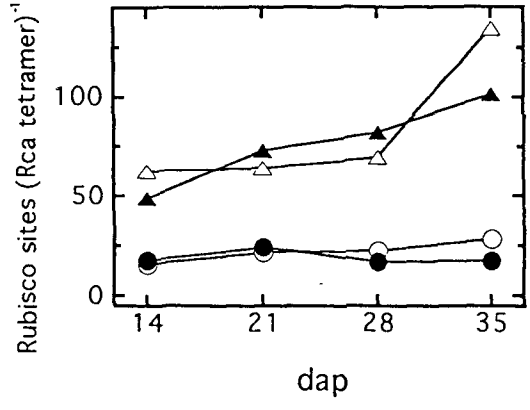

Figure 10. Stoichiometry of Rubisco active sites to activase tetramers. The functional size of activase is unknown, but it is presumably more than tetrameric (Salvucci and Ogren, 1996). ○, Wt HL; ●, Wt LL; △, R100 HL; and ▲, R100 LL.

estimated to have less than 5 to 10% of the Wt activase concentration. Another set of plants, with about 20% Wt activase content, were light green and stunted relative to Wt, but were able to reproduce seed in air. The third group, with more moderate reductions in activase content, were somewhat smaller than Wt but exhibited little or no symptoms of chlorosis and were able to grow and set seed under normal atmospheric conditions. The correlation of activase concentration with the phenotype in independently transformed antisense lines strongly suggests that the observed reductions in growth and photosynthetic parameters were due to reduced activase content and not to a nonspecific effect of the transformation procedure.

Plants grown in HL had almost twice as much soluble protein per square meter as LL-grown plants (Table I), a commonly observed effect (Björkman, 1981). However, both Rubisco activase and Rubisco were maintained as a constant percentage of tsp in HL versus LL. Lauerer et al. (1993) reported an increase of Rubisco as a percentage of soluble protein from 36 to 47% in Wt and from 20 to 26% in antisense *rbc*S tobacco grown at a PPFD of 100 relative to 750 μmol m^{-2} s^{-1} (although absolute Rubisco and tsp concentration decreased). A more extreme difference between high and low light regimes may also induce this effect in Arabidopsis, and we do not know if the ratio of Rubisco activase to Rubisco would be altered under these conditions.

We did not find a significant difference in Rubisco concentration between Wt Arabidopsis and transformants with a moderately reduced activase content. Unlike our results, Mate et al. (1993) reported that antisense activase tobacco plants contained about twice as much Rubisco as the Wt, apparently due to an increased percentage of Rubisco. In their experiments, initial observations of phenotype were made on plants grown in air, but all subsequent experiments were conducted on plants grown at high (1%) CO_2. No correlation was made between the phenotype in air (dark-green versus yellow-green) and activase content, and it was not possible to distinguish between the two phenotypes in plants grown in high CO_2 (Mate et al., 1993). Mate et al. (1996) reported similar concentrations of Rubisco in Wt compared with antisense activase tobacco grown in air, whereas from 9 to 13 weeks after germination, Rubisco stayed roughly the same in Wt (32 to 29% tsp) while increasing somewhat in antisense plants from 31 to 37% tsp. Mate et al. (1995) reported that older anti-activase tobacco accumulated increasing amounts of Rubisco relative to Wt, but they were unable to carbamylate it. The data of Jiang et al. (1994) showed no significant difference in total activity of Rubisco in Wt and anti-activase tobacco, with a range of activase concentrations from about 10 to 100% Wt, although the authors did not examine the characteristics of older plants. It is possible that antisense plants with severely reduced activase have a higher concentration of Rubisco than Wt, and plants with more moderate reductions in activase do not, and/or that Rubisco content is higher than Wt only in older tissue.

Effect of Reduced Activase Content on Photosynthesis and Growth

The Rubisco activation state was positively correlated with photosynthesis and negatively correlated with the RuBP content across the population of Wt and antisense *rca* Arabidopsis (Fig. 5). Accumulation of RuBP is a predicted consequence of reduced Rubisco activation, because the Calvin cycle would continue to regenerate RuBP. High steady-state levels of RuBP have previously been measured in the *rca* mutant of Arabidopsis (Salvucci et al., 1986). Severe repression of Rubisco activase in the antisense plants (<10% Wt amount) had the predicted effect of dramatically reducing the Rubisco activation state, photosynthesis, and plant growth, similar to what occurs in the *rca* mutant. Antisense plants with approximately 30% of the Wt Rubisco activase content showed a near-Wt light response to photosynthesis (Fig. 4). We conducted further experiments with plants grown at HL and LL to test the hypothesis that photosynthesis and growth of the clone R100, which contains 30 to 40% of the Wt activase content, were not limited by activase content.

We found that whole-plant A_{sat} declined in a near-hyperbolic response to decreasing activase content (Fig. 6), as predicted by the results of the first set of experiments (Fig. 4 and 5). Under HL there was a small but significant difference in 24 dap whole plant A_{sat} between the clone R100 and Wt, whereas under LL there was no significant difference. However, the overall growth of moderately reduced activase Arabidopsis (e.g. clone R100) was significantly reduced relative to Wt, even under LL conditions, despite the hyperbolic nature of the relationship of net photosynthesis at 24 dap and activase content. It is possible that important differences in photosynthesis of mature leaves were masked by measurements of whole-plant photosynthesis. The small size and rosette habit of Arabidopsis made it difficult to conduct single leaf photosynthesis measurements, but it was relatively easy to conduct whole-plant measurements (in these measurements, all leaves presented a flat surface to the light source and no leaves were shaded by others). The measurements of the Rubisco activation state (Table II), reflecting in vivo Rubisco activity, suggest that photosynthesis was likely reduced in the mature leaves of clone R100 and to some extent even in LL.

Mate et al. (1996) and Andrews et al. (1995) stated that severe repression of activase to less than 5% Wt was required before the effects on photosynthesis and growth were observed in antisense *rca* tobacco. In an earlier report Mate et al. (1993) reported that both of the observed phenotypes of antisense *rca* tobacco (i.e. green and yellow-green, presumably reflecting moderate and more severe reductions in activase, respectively) grew more slowly than Wt tobacco in air. Mate et al. (1996) also stated that some of the antisense activase tobacco "grew quite slowly" in air. However, detailed growth analyses of antisense *rca* tobacco are lacking in the published reports. We observed significant reductions in the absolute growth of antisense *rca* Arabidopsis with 30% Wt activase, despite a relatively small decline in whole-plant photosynthesis. The effect was dependent on light conditions during growth, with the

greatest reduction occurring under HL. Results from experiments conducted with antisense *rbc*S tobacco with reduced Rubisco also suggest that Rubisco activity does not limit photosynthesis or growth in LL to the same extent that it does under HL growing conditions (Hudson et al., 1992; Masle et al., 1993; Stitt and Schulze, 1994). Thus, the antisense *rca* plants have a phenotype very similar to antisense *rbc*S tobacco with a decreased Rubisco content, which also exhibited greater reductions in growth and photosynthesis under high light relative to low light (Hudson et al., 1992; Masle et al., 1993). In antisense *rbc*S plants in vivo Rubisco activity was limited by Rubisco quantity, whereas in antisense *rca* plants it was limited by the Rubisco activation state.

Photosynthesis is thought to be limited by Rubisco capacity at light saturation and limiting CO_2, while at subsaturating light or high light and above-normal CO_2, it is limited by the rate of RuBP regeneration (i.e. rates of light harvesting and electron transport at low light, and at high light and high CO_2, the rate of triose-phosphate use by starch and Suc synthesis) (Sage, 1990; Sage et al., 1990). Thus, nonlimiting components are supposed to be down-regulated to maintain a balance with the limiting processes. For example, in low light Rubisco should be down-regulated to balance the limitation in the rate of RuBP regeneration, whereas in high light rates of electron transport or triose phosphate use will presumably be down-regulated to balance the limitation by Rubisco activity. Our results provide additional support for this view.

Under HL when Rubisco is hypothesized to exert major control over photosynthesis and growth, there was a large difference between the growth of Wt and antisense Rubisco activase Arabidopsis. Under LL growth conditions, there was a much smaller effect, reflecting the major limitation to growth by RuBP regeneration rather than Rubisco. Under both HL and LL, growth appears to have been affected by the inability of Rubisco to maintain sufficient activation. It is interesting to note, however, that Rubisco still exerted measurable control over growth in LL, although the extent of this control was indeed less than under HL. The results further suggest that Rubisco activase does not play a critical role in the down-regulation of Rubisco activity under LL growing conditions, but is more important in maintaining high Rubisco activity under HL conditions. This hypothesis is consistent with models suggesting that Rubisco activase only recognizes Rubisco that is bound to sugar phosphate inhibitors, and its sole function is in facilitating the release of bound inhibitors from the Rubisco active site (Portis, 1990, 1992; Mate et al., 1996).

Plants grown in LL reached the same steady-state rates of RGR and RLE as those in HL following an additional lag period of 7 to 10 d (Fig. 8). It is possible that total RGR (i.e. including root mass) was significantly different in HL versus LL plants. Sims et al. (1994) measured higher total RGR in high light-grown relative to low light-grown *Alocasia macrorrhiza*. However, similar to our results, the relative difference in RGR between high light and low light was considerably less than the difference in net photosynthesis; low light-grown plants had 43% of the RGR but only 15% of the area-based net photosynthetic rate of high light-grown plants. As in the current study, the higher leaf area ratio of low light-grown plants compensated for the lower area-based net assimilation rate (Sims et al., 1994).

Despite the differences in absolute growth, we observed no differences in overall RGR of aboveground biomass or RLE in antisense R100 compared with Wt Arabidopsis. Masle et al. (1993) found that significant differences in total biomass accumulation between Wt and antisense *rbc*S tobacco were due to the early differences in RLE or RGR; the RGR of all plants reached a similar steady-state value within 2 weeks after germination. Our data similarly indicate that RGR in Wt and antisense *rca* Arabidopsis clone R100 achieved similar maximal values within 2 to 3 weeks after planting, and differences in aboveground biomass accumulation appear to be a result of early differences in RGR and RLE. Figure 8 shows that RGR and RLE were lower in R100 compared with Wt prior to 21 dap in HL; measurements were not taken prior to 21 dap in LL.

Krapp et al. (1994) found that a 35% decrease in Rubisco content in antisense *rbc*S tobacco had relatively little effect on RGR calculated over the entire period of growth. However, their data show a linear relationship between Rubisco activity and absolute biomass accumulation within the same range of Rubisco concentrations, which would seem to indicate that a difference in RGR occurred at some point in development. Thus, measurements of RGR do not necessarily reflect significant differences in overall plant growth (biomass accumulation), because RGR can change dramatically over the course of plant development (Evans, 1972) and compensation often affects a plant's response to different environmental conditions.

Our calculations show that in Wt Arabidopsis activase as a tetramer would be available to service 20 to 30 Rubisco active sites. This estimate is somewhat lower than that of Salvucci and Ogren (1996), who calculated roughly 32 to 96 Rubisco active sites per activase tetramer in normal leaves. Andrews et al. (1995) and Mate et al. (1996) found that photosynthesis and growth were not affected in antisense activase tobacco until activase was reduced to <5% Wt, thus they estimated that one Rubisco activase tetramer may be able to service as many as 1600 Rubisco sites. On the other hand, Jiang et al. (1994) reported reductions in A_{sat} (and Rubisco activity) in antisense *rca* tobacco with an approximate 30% Wt activase content. Our data also indicate a much lower number for Arabidopsis, with activase limiting photosynthesis and growth when there are more than 60 Rubisco sites per activase tetramer.

In conclusion, leaf area and biomass accumulation of transgenic Arabidopsis with moderate reductions in Rubisco activase content were significantly reduced relative to Wt to a greater extent under high compared with low irradiance during growth. Estimates of RGR and RLE were not useful indicators of overall differences in total biomass accumulation, except for HL-grown plants when measured early in plant development; all plants reached similar maximum rates and overall rates calculated between 21 to 42 dap were identical for Wt compared with antisense plants. Rubisco activation state was limiting to

plant growth, even under optimal conditions, in an Arabidopsis clone with 30 to 40% Wt activase. Thus, Wt Arabidopsis does not appear to contain Rubisco activase in great excess of the amount required for optimal growth.

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