Photosynthetic Pigments, Photosynthesis and Plastid Ultrastructure in *Rbc*S Antisense DNA Mutants of Tobacco (*Nicotiana tabacum*)

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*Rbc*S antisense DNA mutants of tobacco have reduced amounts of ribulose bisphosphate carboxylase oxygenase (Rubisco). We found that carotenoid and chlorophyll contents decrease in parallel as Rubisco is decreased, however, pigment levels are not significantly altered until Rubisco levels are reduced sharply. The mutants have normal Chl *a* /Chl *b* ratios and normal plastid ultrastructures, suggesting that reductions in Rubisco do not dramatically alter the composition of the thylakoid membranes. Nevertheless, chlorophyll fluorescence measurements, in which developmentally homogenous leaves were sampled, showed that there is reduced photosynthetic capacity of PSII and an enhanced photosensitivity in the mutants, especially in transgenics with severe reductions in Rubisco content. Support for this conclusion comes from several observations: 1) light saturation occurs at a lower light intensity in the mutants, resulting in an earlier closure of PS II (lower photochemical quenching); 2) the mutants have reduced photosynthetic efficiency (lower $\Delta F/Fm'$); and 3) the mutants have a slower recovery of Fv/Fm. We found that acclimation to increasing light intensies in the mutants appears to involve an enhanced inactivation of PSII reaction centers as well as an increased activation of photoprotective mechanisms, notably an engagement of the xanthophyll cycle at lower than normal light intensities. We conclude that the photosensitivity of the antisense mutants is due, in part, to a limitation in Rubisco activation state.

Introduction

To better understand the mechanisms that regulate plastid biogenesis, Rodermel *et al.* (1988) generated Rubisco antisense DNA mutants of tobacco. These mutants have reductions of up to 90% in Rubisco content due to the expression of *Rbc*S antisense RNAs. The antisense mutants have been used for a wide variety of molecular, biochemical and physiological studies to examine the mechanisms that regulate Rubisco biosynthesis, and to investigate the control that Rubisco exerts on photosynthesis, plant growth and development (reviewed by Stitt and Schulze, 1994; Rodermel, 1999).

Investigations of pigment composition and content in the antisense mutants have focused on chlorophyll. These studies revealed that chlorophyll levels are reduced in plants with very low amounts of Rubisco, but that Chl *a*/Chl *b* ratios are only weakly affected in these plants (Quick *et al.,* 1991, 1992; Jiang and Rodermel, 1995). While this suggests that the composition of the thylakoid membrane is relatively refractory to changes in Rubisco content, chlorophyll fluorescence and fluorescence quenching measurements demonstrated that photosynthesis is inhibited in the antisense plants, especially in mutants with sharp reductions in Rubisco (Quick *et al.,* 1991; 1992; Stitt *et al.,* 1991). These experiments further showed that the mutants are photoinhibited at lower light intensities than in the wild-type (WT), although the precise mechanisms were not investigated.

The studies of Stitt and his group (Quick *et al.,* 1991, 1992; Stitt *et al.*, 1991) were conducted on first fully-expanded leaves of the antisense and WT plants. More recent studies however, have shown that photosynthetic rates and other photo-

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synthetic parameters (e.g., chlorophyll levels, Rubisco content and activity) change dramatically during tobacco leaf ontogeny: photosynthetic rates increase during leaf expansion, reach a maximum at full expansion, then undergo a prolonged, senescence decline in the fully-expanded leaf (Miller *et al.,* 1997; 2000). Photosynthetic rates also change markedly as a function of leaf nodal position on the canopy, with rates falling progressively as one moves down the canopy from fully-expanded leaves at the top of the plant to fully-expanded leaves at the base of the plant (Jiang and Rodermel, 1995). Therefore, when one wishes to compare photosynthetic parameters between plants it is necessary to use developmentally-similar leaves at similar stages of their ontogeny.

A long range goal of our experiments is to explore how the photosynthetic apparatus of the antisense plants adapts to different light intensities. Towards this goal, the objective of the present experiments was to examine the carotenoid content and composition of the antisense plants and to determine whether the xanthophyll cycle is more active in the mutants. Given the desirability of using developmentally homogenous samples, it was first necessary to repeat the earlier chlorophyll fluorescence and fluorescence quenching experiments that provided evidence for a higher photosensitivity in the mutants (Quick *et al.,* 1991, 1992; Stitt *et al.,* 1991). Is there a developmental component to photosensitivity? Our final objective was to assess how photosynthetic function and the activity of the xanthophyll cycle correlate with chloroplast ultrastructure in the mutants. Surprisingly, despite the wealth of information on the antisense mutants (reviewed by Stitt and Schulze, 1994; Rodermel, 1999), ultrastructural studies of the mutant plastids have not been reported.

Material and Methods

Plant material and growth conditions

Two *Rbc*S antisense DNA mutants of tobacco described by Jiang and Rodermel (1995) containing approx. 40% (AL3 mutant) or 20% (AL5 mutant) of normal Rubisco amounts were used in this study. The plants were maintained in the greenhouse with one plant per pot on a natural soil/perlite mixture (50/50 v/v). They were watered with a 5 mM NH_4NO_3 solution (i.e., abundant nitrogen) as described by Quick *et al.* (1992). The plants were grown under a light/dark regime (16/8 h) during the Spring. Natural sunlight with a daily maximum of about 500 μmol m^{-2} s^{-1} PAR was used for illumination. When necessary, supplemental illumination was provided by 400 W high pressure sodium lamps. WT and antisense leaves from the fourth node (from the base of the plant) that had just attained full expansion were used for analysis; these leaves were at comparable stages of development (Jiang and Rodermel, 1995).

Pigment extraction and HPLC analysis

Pigments were extracted in methanol from a leaf area of 0.5 cm^2 according to Bertrand and Schoefs (1997). The HPLC analyses were performed according to Darko *et al.* (2000) using a reversed-phase column. Standards of the pigments that were used for calibration were prepared according to Schoefs *et al.* (1995). Quantifications were performed by the method of external standards using the extinction coefficients published by Britton (1995) and Porra *et al.* (1989). The resulting calibration curves were linear over the concentration ranges tested with regression coefficients between 0.998 and 0.999. The different pigments were quantified on the basis of their elution peak recorded at 430 nm (Chl *a* and *cis*-violaxanthin), 437 nm (neoxanthin, *trans*-violaxanthin, lutein-5,6-epoxide, *cis*-antheraxanthin and *cis*-lutein), 450 nm (*trans*-antheraxanthin, *trans*-lutein and zeaxanthin) and 458 nm (Chl *b* and β-carotene). Each experiment was repeated at least three times.

Variable fluorescence measurements at room temperature

Chl *a* fluorescence was measured using a PAM-2000 portable Chl fluorometer (Walz, Germany) on the upper side of intact attached leaves (the measuring area was approx. 2 cm^2). After 20 min dark adaptation, the leaves were exposed to a weak red modulated measuring light beam for measurements of initial fluorescence yield (Fo). The leaf samples were then exposed to a white saturating pulse (2000 μmol m^{-2} s^{-1} PAR, 0.9 s duration) to determine the maximum fluorescence level (Fm) of the dark-adapted leaves. After a 90s lag period the red actinic light was switched on

and the quenched level of the maximum fluorescence (Fm') was determined by saturating pulses at the end of the 20 min actinic light illumination. The actinic light intensity was fixed at a given light intensity ranging from 50 to 550 μmol m^{-2} s^{-1} PAR. After the actinic light had been switched off, far red light was applied for determination of Fo'. After a 20 min dark adaptation the measurements were resumed using a higher actinic light intensity than previously.

The Fv/Fm parameter and photochemical quenching coefficients (qP) were calculated as (Fm-Fo)/Fm and (Fm'-F)/(Fm'-Fo') respectively (van Kooten and Snel (1990). The quantum efficiency of photochemistry was calculated as $\Delta F/Fm' = (Fm'-F)/Fm'$, as described by Genty *et al.*, (1989). The Stern-Volmer coefficient *i.e.* nonphotochemical quenching (NPQ) (NPQ = (Fm-Fm')/Fm') (Bilger and Björkman, 1990) was also used.

Electron microscopy

Samples for transmission electron microscopy were fixed in 2.5% glutaraldehyde in phosphate buffer (50 mM, pH 7) for 24 h and postfixed overnight in 1% OsO_4 in the same buffer at 1 °C. The samples were then embedded in Spurr's resin (Spurr, 1969). Thin sections were prepared using a diamond knife mounted on a Leika UCT ultramicrotome, picked up on copper grids and stained with uranyl acetate and lead citrate according to Reynolds (1963). The sections were examined with a Philips 420 transmission electron microscope.

Results and Discussion

Chlorophyll fluorescence and fluorescence quenching measurements

The first chlorophyll fluorescence characterizations of the Rubisco mutants used first fully-expanded leaves of growth chamber-grown plants (Quick *et al.*, 1991, 1992; Stitt *et al.*, 1991). Leaves were selected irrespective of their nodal position on the plant or their stage of leaf ontogeny (e.g., early versus late fully-expanded). For our experiments we wished to examine leaves at defined stages of leaf development, so it was necessary to revisit the earlier experiments using developmentally homogenous material. As our test material we chose to examine fourth leaves (from the base of the plant) that had just attained full expansion, using mutant plants that have moderate (AL3) and severe (AL5) reductions in Rubisco content (Jiang and Rodermel, 1995). WT tobacco served as controls. The fluorescence quenching analyses were performed at light intensities ranging from 50 to 550 μmol m^{-2} s^{-1} PAR under steady state conditions. In parallel, the efficiency of pigment conversion through the operation of the xanthophyll cycle was studied by HPLC.

Fv/Fm reflects the primary charge separation capacity of PSII and thus the maximum proportion of functioning PSII reaction centers (van Kooten and Snel 1990). The optimal quantum yield of photosystem II, *i.e.*, Fv/Fm, was somewhat lower in AL5 than in either AL3 or WT plants (Fig. 1 A). After some light-dark transitions, the photosynthetic apparatus cannot relax completely within the 20 min of darkness separating the two analyses, and consequently, a slight decrease in the Fv/Fm ratio was also observed. This may be due to an increase in the relaxation time of non-photochemical processes and/or to an increase in inactivation of PSII reaction centers. The latter possibility is supported by an increase in the Fo to Fm ratio (Fig. 1B). This ratio increased by a 18% in AL3 and 30% in AL5.

It can be deduced from the sharp decrease in qP values as a function of light intensity (Fig. 1C) that activation of photosynthesis by continuous illumination results in an increase in the reduction of the primary electron acceptor of PS II – Q_A – and in the closure of PS II reaction centers in the WT and both mutants. Under steady state conditions, qP decreased much more in AL5 than in AL3 or in the WT. The qP values of AL3 and the WT plants were similar at light intensities below 200 μmol m^{-2} s^{-1} PAR, but the decrease in AL3 was greater at higher intensities. In the mutant biotypes, especially AL5, the low values of qP – even at low actinic irradiances – suggest that a fraction of the PS II traps are closed during steady-state illumination. Because they are closed, these reaction centers are unable to undergo stable charge separation and therefore do not participate in linear photosynthetic electron transport. Similar light intensity variations to those in Figure 1C were observed for the effective quantum yield of PS II ($\Delta F/Fm'$, Fig. 1D). Although our observa-

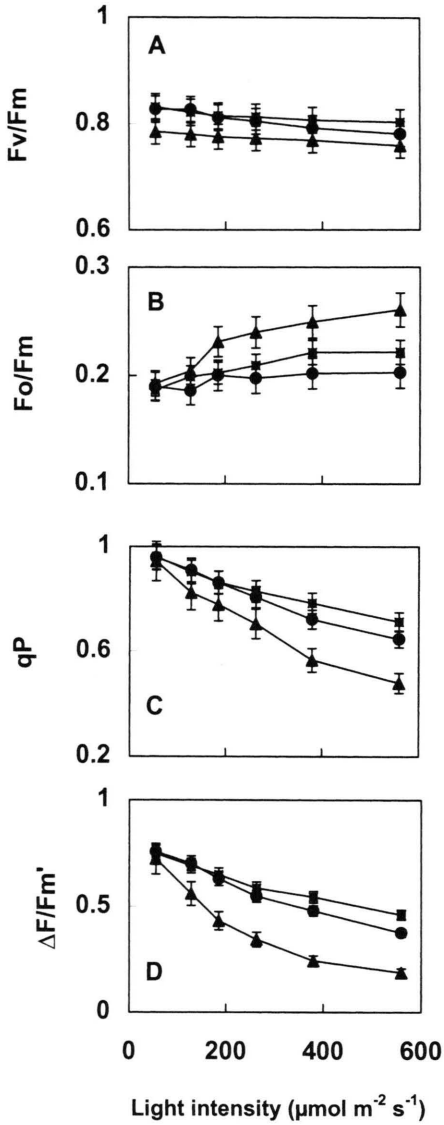

Fig. 1. Impact of reductions in Rubisco content on the adaptation capacity to increasing light intensities. (A) optimum quantum yield of PSII (Fv/Fm), (B) Fo/Fm, (C) photochemical quenching (qP) and (D) effective quantum yield of PSII (△F/Fm'). WT (■), AL3 (•), AL5 (▲). Each point corresponds to the means ± SE of the results from four independent measurements.

tions on Fv/Fm, qP and ΔF/Fm' differ in some details from those published by Quick *et al.* (1991, 1992) and Stitt *et al.* (1991), the same general trends are evident. We therefore conclude that the leaf samples in the present study respond similarly to those in the earlier studies.

We next wished to examine the non-radiative energy dissipation processes, expressed as NPQ. In the AL5 mutant, the NPQ parameter was higher than normal even at low light intensities (100 μmol m^{-2} s^{-1} PAR). In fact, NPQ saturated at approximately 400 μmol m^{-2} s^{-1} PAR in these plants (Fig. 2A). As shown in Figure 2B, an extremely high proportion of violaxanthin is transformed to zeaxanthin at this intensity. In contrast to AL5, NPQ in the AL3 plants diverged from the WT only at light intensities higher than 200 μmol m^{-2} s^{-1} PAR (Fig. 2A); the de-epoxidation ratios were similar in the two plants, regardless of light intensity (Fig. 2B). These results imply that the accessibility of violaxanthin for violaxanthin deepoxidase is normal in both mutants and, further, that non-radiative energy dissipation processes are activated and saturated at lower light intensities in AL5 than in either AL3 or the WT.

Taken together, our data indicate that there is reduced photosynthetic capacity of PSII in the

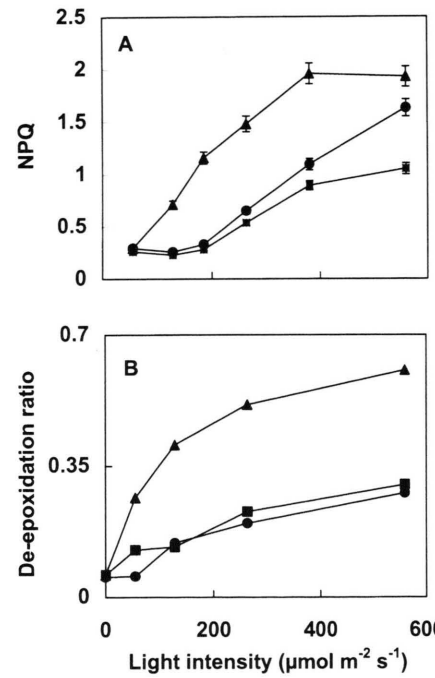

Fig. 2. Impact of reductions in Rubisco content on non-photochemical quenching processes calculated as NPQ and on xanthophyll cycle capacity in the wild-type and the two mutant lines. Xanthophyll cycle capacity was calculated as (A + Z)/(V + A + Z) where A, Z and V represent concentrations of antheraxanthin, zeaxanthin and violaxanthin, respectively. The plant symbols are the same as in figure 1.

antisense mutants, especially in the AL5 line. Support for this conclusion comes from several observations: 1) light saturation occurs at a lower light intensity in the mutants, resulting in an earlier closure of PS II (lower qP); 2) the mutants have reduced photosynthetic efficiency (lower ΔF/Fm'); and 3) the mutants have a slower recovery of Fv/Fm. The increase in the relaxation time (i.e., the slower recovery of Fv/Fm) can have two causes– an increased activation of photoprotective mechanisms and/or an enhanced inactivation of PSII reaction centers. Both of these mechanisms appear to be operative in the antisense mutants (Figs. 1 and 2).

Pigment content and composition

Carotenoids have several major functions in the photosynthetic apparatus. These include light-harvesting and photoprotection. Photoprotection is largely carried out by the xanthophyll cycle pigments (in the light-harvesting complex, reviewed by Demmig-Adams and Adams, 1996; Eskling *et al.,* 1997; Rmiki *et al.,* 1999) and by β-carotene (in the reaction centers, De Las Rivas *et al.,* 1993, Telfer *et al.,* 1994). The data in Fig. 2 indicated that the xanthophyll cycle is engaged at lower light intensities in AL5 than in AL3 or WT plants. To examine carotenoid pigment composition in greater detail in the mutant plants, we performed quantitative analyses of the pigment contents of just fully-expanded fourth leaves of the mutant and WT plants by HPLC. We found that total chlorophyll and carotenoid contents are lower in the two mutants, with the largest decreases in those plants having the greatest reductions in Rubisco (Fig. 3, top panel). However, when pigment concentrations are expressed relative to Rubisco content, a linear relationship is not observed with either chlorophylls or carotenoids (Fig. 3, bottom panel). In fact, reductions of up to 60% in Rubisco do not strongly affect photosynthetic pigment content. Figure 3 further reveals that the reductions in chlorophylls and carotenoids are coordinately regulated. This is consistent with observations showing that chlorophyll and carotenoid accumulation are frequently coordinated, for example, during greening (Bartley and Scolnik, 1995; Hartel and Grimm, 1998; Schoefs *et al.,* 1998). However, decreases in Rubisco do not affect individual carotenoids similarly. In fact, there are significant decreases in β-carotene levels relative to the other carotenoids in plants with decreased Rubisco. These decreases appear to be compensated for, at least in part, by increases in the proportion of violaxanthin cycle pigments, viz., violaxanthin, antheraxanthin and zeathanthin (Fig. 4).

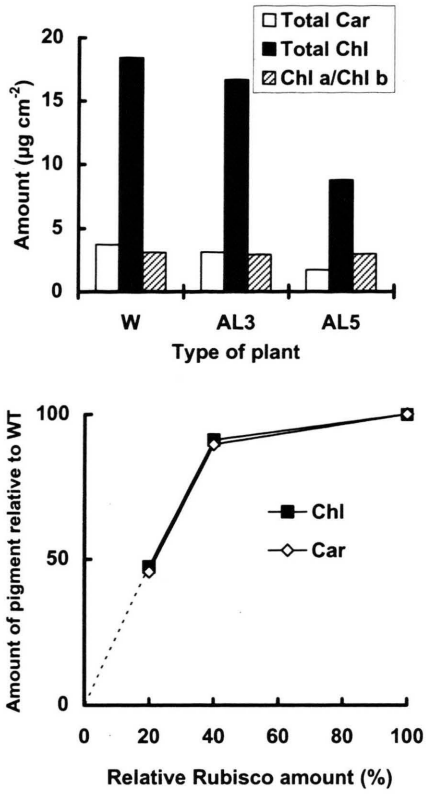

Fig. 3. Impact of reductions in Rubisco content on photosynthetic pigment content in the antisense and wild-type plants. Top panel: Total chlorophyll and carotenoid amounts (in $\mu g\ cm^{-2}$), and Chl *a* to Chl *b* ratios. Each point corresponds to the means ± SE of the results from four independent measurements. Bottom panel: Effect of reductions in leaf Rubisco content on total chlorophyll and carotenoid amounts as expressed relative to maximum pigment amounts.

The pigment reductions in the antisense plants do not seem to have an appreciable influence on Chl *a* to Chl *b* ratios (Fig. 3, top panel). This suggests that there is not a significant change in the organization of the light-harvesting antenna of photosystem II in the antisense plants. This conclusion is supported by ultrastructural analyses on chloroplasts from just fully-expanded fourth

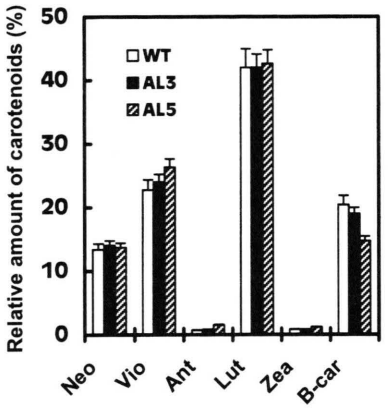

Fig. 4. Impact of reductions in Rubisco content on the relative amount of individual carotenoids. Each pigment is plotted as a fraction of the total carotenoid pool present in the mutant and wild-type plants. Abbreviations: Ant: antheraxanthin, B-car: β-carotene, Lut: lutein, Neo: neoxanthin, Vio: violaxanthin, Zea: zeaxanthin.

leaves of the WT and mutant plants. As illustrated in Figure 5B, the structures of AL3 chloroplasts are similar to those of the WT (Fig. 5A), with typical starch grains and granal stacking. The chloroplasts in the AL5 mutants also appear to have a normal structure, despite the severe reductions in pigment content (Fig. 5C). On the other hand, the light staining of the granal stacks in the AL5 plastids may indicate that there are subtle structural differences in the thylakoid membranes of these plastids. This light staining is unlikely an artifact of preparation because it was observed consistently in different samples and because other membranes like those of mitochondria are well contrasted (Fig. 5D). Our conclusion that the composition of the thylakoid membranes is not drastically impaired in the antisense mutants is consistent with previous measurements showing that the two mutants used in this study have normal levels of various thylakoid membrane proteins (Jiang and Rodermel, 1995). It is also in line with the observation that under ambient conditions, the rate of photosynthesis remains constant when Calvin cycle enzyme activities are decreased sharply in amount (*e.g.*, Rubisco, chloroplast fructose bisphosphatase) (Quick *et al.*, 1991; Stitt and Sonnewald, 1995; Bilger *et al.*, 1995).

To summarize, our pigment analyses showed that a reduction in Rubisco content in *Rbc*S antisense DNA mutants by approximately 60% (AL3 mutant) or 80% (AL5 mutant) affects similarly the total amount of chlorophylls and carotenoids. The fact that Chl *a* to Chl *b* ratios are not strongly modified in the mutants, together with the observation that the mutants have normal chloroplast morphologies, suggest that the organization of the pigment-protein complexes of the photosynthetic

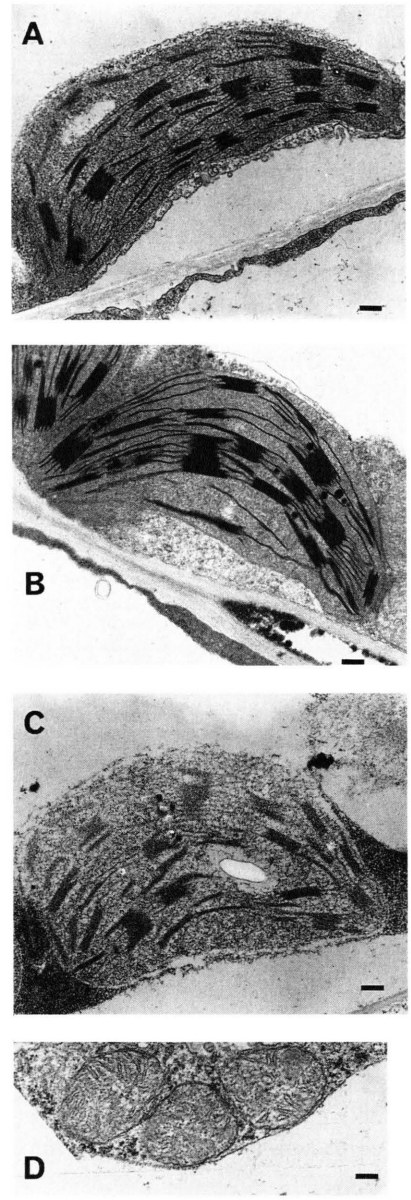

Fig. 5. Impact of reductions in Rubisco content on chloroplast ultrastructure. (A) WT (bar = 0.3 μm), (B) AL3 mutant (bar = 0.28 μm), (C) AL5 mutant (bar = 0.27 μm) and (D) mitochondria (bar = 0.43 μm).

membranes is not strongly affected by drastic reductions in pigment content.

Our analyses of fluorescence quenching parameters and of the efficiency of the xanthophyll cycle in leaves adapted to increasing light intensities revealed that the AL5 mutant is more photosensitive than AL3 or WT plants. We suggest that the antisense mutants, especially AL5, acclimate to increasing light intensities by engaging the xanthophyll cycle at lower light intensities than the WT (Figs. 1D, 2 and 4) and by inactivating PSII reaction centers (Figs 1A and B). Although the mechanisms are obscure, this interpretation would be consistent with observations made by Bilger *et al.*, (1995) and Härtel and Grimm (1998) on chloroplastic fructose bisphosphate and glutamate 1-semialdehyde aminotransferase antisense mutants.

We do not know why the antisense mutants are more photosensitive than normal. One hypothesis is that under the conditions of our experiments the activation state of Rubisco is higher in AL3 and AL5 than in the WT, as observed previously (Quick *et al.*, 1991; Jiang and Rodermel, 1995). Therefore, we suggest that the higher Rubisco activity can partially compensate for the moderate reductions in Rubisco content in the AL3 mutant, allowing normal rates of photosynthesis and light dissipation to occur. Consistent with this interpretation, in nearly all measurements, the AL3 mutant responded similarly to the WT. However, the attainment of a higher activation state of Rubisco may be limited and hence not enough to compensate for the sharp reductions in Rubisco that are observed in the AL5 mutant. This could lead to over-reduction of the thylakoids and photoinhibition in the absence of adequate quenching mechanisms.

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