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Alteration of the regulation of Rubisco via Rubisco activase.

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Introduction

Rubisco activase is regulated in vitro by the ADP/ATP ratio (Kallis et al., 2000) and reduction/oxidation (redox) of the larger isoform by thioredoxin-f (Zhang and Portis, 1999). Redox alters the sensitivity of the Rubisco activation and ATPase activities of the protein to the ADP/ATP ratio. These two factors provide possible explanations for the observed response in plants of Rubisco activation to light intensity (redox) and for reduced activation when there are sink limitations and reduced triose phosphate utilization (ADP/ATP) (Portis, 1992). In vitro studies with recombinant proteins have shown that redox regulation of activase is not possible if only the smaller isoform is present or critical cysteines, present only in the larger isoform, are replaced by alanine. Regulation by the ADP/ATP ratio is greatly altered by replacement of glutamine at position 111 in a nucleotide-binding loop (i.e. the P-loop) by either glutamate or aspartate. The *Arabidopsis rca* mutant does not express either form of activase and cannot survive under atmospheric levels of carbon dioxide. Transformation of this mutant to express altered forms of activase provides a means to determine the role of redox and ADP/ATP regulation of Rubisco via the activase in planta and the possible impact of changes in these regulatory processes on photosynthesis and plant performance.

Materials and methods

Arabidopsis cDNAs of the 43-kD (or 43-kD with Q111 substitutions) and 46-kD isoforms of activase were cloned into the pCGN1547 (Calgene) and pBIN121 (Clonetech) vectors respectively, with a cauliflower mosaic virus 35S promoter and a nopaline synthase terminator. These vectors were then transferred to *Agrobacterium tumefaciens* by either triparental mating (Rogers et al., 1986) or freeze-thaw (Holster, 1978), which were then used to transform the *Arabidopsis rca* mutant by in planta vacuum infiltration (Bechtold and Pelletier, 1998) and selection of the resulting seeds with kanamycin. Plants expressing both the 43- and 46-kD isoforms were obtained by genetically crossing the 43- and 46-kD transformed lines and screening the resulting progeny by SDS gel electrophoresis and western blotting. Activase protein levels and Rubisco activation in leaf extracts of the various plants was measured as previously described (Eckardt et al., 1997). All plants were grown under 10 h of light at 150 $\mu E\ m^{-2}\ s^{-1}$, with 22°C days and 18°C nights at 60% RH, but the *rca* mutant was grown at 3000 ppm CO_2.

Results

To compare Rubisco activation in the transformants with the *rca* mutant and the wildtype, we used a protocol that measured the activation state of Rubisco in leaf extracts after exposure of the plants to high light (600 μE m^{-2} s^{-1}) for one hour, followed by exposure to low light (35 μE m^{-2} s^{-1}) for one hour and then a return to the high light for another hour. In the wildtype plants (WT, Fig. 1), Rubisco was nearly fully activated under high light, whereas after one hour at low light, the activation state had decreased to 40%. In the *rca* plants (rca-), the activation state of Rubisco was very low (about 20%) and was not affected by light intensity.

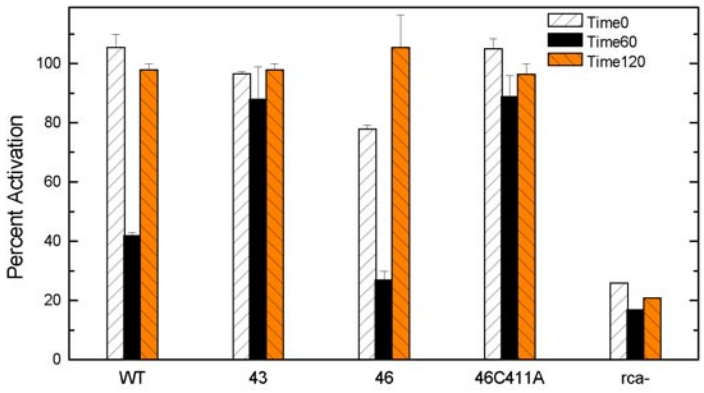

Fig. 1. Rubisco activation state in wildtype (WT), *rca* (rca-) and transgenic plants expressing only one activase isoform (43, 46, or 46C4111A). Activation was measured after exposure to high light for one hour (Time0), followed by one hour at low light (Time60), and finally after a return to high light for one hour (Time120).

These results are consistent with previous reports (Salvucci et al., 1986; Brooks and Portis, 1988). In the transformants of the *rca* mutant that expressed only the shorter, 43-kD isoform (43, Fig. 1), the activation state of Rubisco was as high as the wildtype under high light, but was not decreased by exposure to low light. In contrast, transformants expressing only the larger, 46-kD isoform (46, Fig. 1) exhibited a large decrease in the activation state of Rubisco after exposure to low light. These results indicate that light modulation of Rubisco is dependent on the presence of the larger isoform. Furthermore, if the *rca* mutant was transformed to express a larger isoform in which one of the critical cysteines (Cys-411) required for disulfide bond formation (and thus redox regulation) was replaced by alanine (46C411A, Fig. 1), the activation state of Rubisco did not decrease under low light. Thus we conclude that *Arabidopsis* requires the larger, redox sensitive activase isoform to achieve a low Rubisco activation state under low light.

To further examine the ability of the larger isoform to regulate the activity of the shorter isoform and thus down-regulate the activity of Rubisco under low light in planta, we introduced the 46-kD isoform into various 43 lines by genetic crossing. We then determined the effect of light on the Rubisco activation state of the progeny and the parents in another series of experiments. Fig. 2 shows that plants expressing both isoforms as a result of this genetic cross exhibited a partial restoration of low light Rubisco deactivation compared to the 43 plants. These results again demonstrate that the presence of the native larger isoform is necessary for the light modulation of Rubisco.

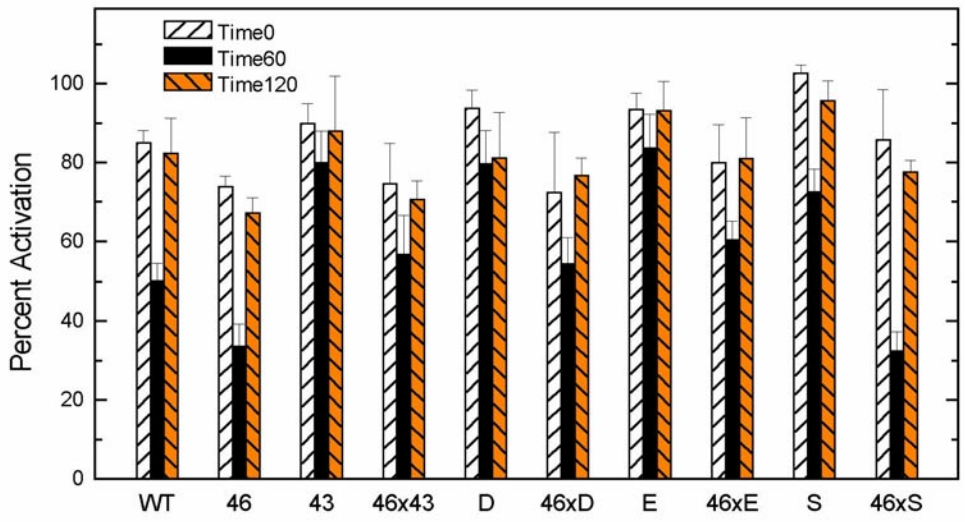

Fig. 2. Co-presence of the larger activase isoform results in a deactivation of Rubisco under low light in all 43-kD transformants, including those with Q111 mutations. Samples are identified and were taken using the same protocol as described in Fig. 1. Transformants are identified as follows: wildtype (WT); 46-kD isoform (46); 43-kD isoform (43); 43-kD with Q111D replacement (D); 43-kD with Q111E replacement (E); 43-kD with Q111S replacement (S); crosses of 46 transgenic lines with 43, D, E and S lines respectively (46x43, 46xD, 46xE, 46xS).

In previous work, we were able to modify the in vitro response of activase to the ADP/ATP ratio by replacement of Q111 in the activase protein sequence with D and E whereas a replacement with S resulted in a response similar to the WT (Kallis, 2000). Transgenic plants were then obtained that expressed the smaller isoform with these substitutions, with the expectation that they would be useful to examine the role of ADP/ATP regulation of activase in planta. However, we did not know about the redox regulation of the larger isoform when these plants were first examined. As shown in Fig. 2, there is little deactivation of Rubisco under low light in the D and E transgenic plants and we initially thought that this might be due to the alteration in the ADP/ATP response of the activase observed in vitro with the recombinant enzymes. To clarify the role of redox regulation in the low light response of the lines with Q111 replacements, we examined the progeny of crosses of these transformants with the 46 transformants. As shown in Fig. 2, a more extensive deactivation of Rubisco occurred with exposure to low light in each case. Thus the larger activase isoform is able to significantly down-regulate the activity of the shorter isoform even when its ADP/ATP response has been substantially altered. At present we do not completely understand why the Q111S plants exhibit some Rubisco deactivation under low light.

Evidence from activase antisense transgenic *Arabidopsis* plants indicates that the activation state of Rubisco rapidly saturates in response to the amount of activase and that the amount of activase is affected by growth conditions (Eckardt et al, 1997). Thus it is essential to determine the amount of activase expression in the various transgenic plants under similar growth conditions. This can be easily done by quantitative analysis of leaf extracts separated by gel electrophoresis, blotted and detected with activase antibodies and chemiluminescence as shown in Fig. 3.

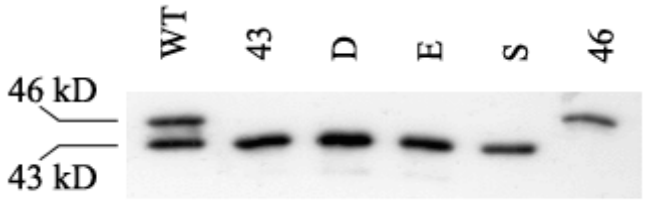

Fig 3. Western analysis of activase expression using total soluble protein from leaf extracts. Two µg total soluble protein from each leaf sample was run on SDS-PAGE, blotted to PVDF membrane and activase detected with activase antibodies and chemiluminescence.

In the wild-type plants (WT), two bands corresponding to the 46- and 43-kD isoforms are present at nearly equal intensity. In the other lanes, which are from transformants of the *rca* mutant with cDNAs of either the smaller (43, D, E, S) or larger isoform (46), only one band corresponding to expected size can be detected.

The results of chemiluminescent detection and quantitation of activase in the various transgenic lines is presented in Table I. Although expression was driven by the 35S promoter, the levels of activase in the transgenic lines was somewhat less than in the wildtype. The lowest level of expression, about 50%, was observed in the 43-kD Q111S and 46-kD transgenic plants.

Table I. Quantitation of Rubisco activase. The relative amounts of Rubisco activase were determined by densitometry measurements of the western blots described in Fig. 3. Amounts are normalized to the quantity of activase in the wildtype line, which was 110 mg activase m^{-2}.

Line	43	D	E	S	46
Percent of WT	74 ± 22	70 ± 12	71 ± 17	56 ± 10	46 ± 8

The low expression of the larger activase isoform in the 46 line also extended to the genetic crosses with this line. Whereas in the wildtype plants the amounts of the two isoforms are about equal, in the 46x43 plants the small to large isoform ratio is almost 3:1 and in the 46xD, 46xE and 46xS lines, the ratio is almost 2:1 (data not shown).

Discussion

Complementation of the *Arabidopsis rca* mutant with the 43, 46, 46C411A, 43Q111D, -E, or -S forms of the activase gene restored the ability of the plants to grow under normal levels of carbon dioxide and resulted an increased Rubisco activation state in the light. The activation state of Rubisco under high and low light intensity in the transgenic lines was generally in line with expectations from previous in vitro experiments with the corresponding activase isoforms.

Exposure to low light decreased the Rubisco activation state of all plants expressing the larger activase isoform. However, the same light treatment did not lower the activation state of Rubisco in plants that expressed only the smaller isoform or in plants expressing the larger isoform without a key cysteine residue (Fig. 1, 2). Thus redox regulation, which is conferred by the larger isoform, is primarily responsible for the light modulation of Rubisco activity. The metabolic and physiological consequences of expressing activase isoforms, which are not redox regulated and exhibit an inability to down-regulate Rubisco under low light conditions are presently being examined.

The activity of Rubisco not only responds to light intensity, but also is down-regulated in response to sink limitations (triose phosphate utilization). Experimentally, the response is most readily observed when plants are subjected to rapid increases in CO_2 or decreases in O_2 concentrations, especially in conjuction with lowered temperature (Sharkey, 1990). Corresponding changes in Rubisco activation and ADP/ATP levels are consistent with regulation by the ADP/ATP response of activase. Experiments measuring these parameters under conditions inducing a triose phosphate limitation have not been conducted with *Arabidopsis*.

In vitro studies with the recombinant isoforms indicate that the redox regulation and ADP/ATP regulation of activase are interlinked and this may complicate a separation of the roles they play in vivo. Oxidation/reduction of the larger isoform alters it sensitivity to inhibition by ADP, which can affect the sensitivity of both isoforms if they are mixed (Zhang and Portis, 1999). However effective down-regulation of the larger isoform requires nearly equal amounts of the two isoforms (Zhang et al., 2001).

The sensitivity to ADP inhibition can be altered independent of redox in the small isoform by replacement of glutamine at position 111 with glutamate or aspartate. Plants expressing only the shorter isoform with these substitutions should exhibit an altered response to ADP/ATP, but they also lack redox regulation of Rubisco activase as shown in our experiments. We appear to have restored the redox regulation component by crossing the 43, 43-Q111D, 43-Q111E, and 43-Q111S plants with the 46 transgenics. However the extent of deactivation under low light was often not as great in the progeny as compared to the wildtype plants. This may be due to the unequal expression of the two isoforms in the plants used in these experiments.

In the future we plan to compare the properties of these plants with the 43, 43-Q111E and 43-Q111D lines under conditions favoring the development of a triose phosphate limitation. These experiments should enable us to further delineate the roles played by the redox state of activase and ADP/ATP levels in regulating Rubisco activity in planta.

Acknowledgements

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