Antisense Inhibition of Rubisco Activase Increases Rubisco Content and Alters the Proportion of Rubisco Activase in Stoma and Thylakoids in Chloroplasts of Rice Leaves 1

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Abstract

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase (RCA) is a nuclear-encoded chloroplast protein that modifies the conformation of Rubisco, releases inhibitors from the active sites, and increases enzymatic activity. It appears to have other functions, which are related to its distribution within the chloroplast. The aim of this research was to resolve uncertainty about the localization of RCA, and to determine whether the distributions of Rubisco and RCA were altered when RCA content was reduced. Gas exchange and Rubisco were measured, and the sub-cellular locations of Rubisco and RCA were determined using immunogold-labeling electron microscopy, in wild-type and antisense rca rice plants. Net photosynthetic rate and the initial Rubisco activity in the antisense rca plants decreased much less than RCA content in the antisense plants. Immunocytolocalization showed that Rubisco in

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wild-type and antisense plants was localized in the stroma of chloroplasts. However, the amount of Rubisco in the antisense rca plants was greater than in the wild type plants. RCA was detected in both the stroma and in the thylakoid membranes of wild-type plants. We show that the percentage of RCA labeling in the thylakoid membrane was substantially decreased, while the fraction in the stroma was increased, by the antisense rca treatment. From the changes in RCA distribution and alterations in Rubisco activity, RCA in stroma of chloroplast probably contributes to the activation of Rubisco, and RCA in thylakoids compensates for the reduction of RCA in the stroma, allowing steady-state photosynthesis to be maintained when RCA is depleted. RCA may also have a second role in protecting membranes against environmental stresses as a chaperone.

Keywords: Oryza sativa L., Rubisco, Rubsico activase, Cellular localization

1 Introduction

In green plants, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) catalyzes the irreversible carboxylation of ribulose-1,5-bisphosphate and CO₂ to form two 3-phosphoglyceric acid molecules. However, the rate of the reaction is extremely slow, and Rubisco must be activated and carbamylated to become catalytically competent. Activation is achieved by Rubisco activase (RCA), which can remove inhibitors from Rubisco’s catalytic sites, alter the conformation, and activate Rubisco in vivo [1]. RCA is a nuclear-encoded chloroplast protein, and is essential for plants [2]. Komatsu and coworkers [3] reported that a giberillin-binding protein in rice was homologous to RCA. Sharma et al. [4], using an in-gel protein kinase assay, suggests that RCA was associated with Ca²⁺-dependent protein kinases in gibberillin signaling. These studies suggest some additional role for RCA beyond Rubisco regulation [5]. Possibly, the role of RCA depends on its location within the chloroplast, as protein functions may be related to their cellular localization. Immunogold labeling for electron microscopy have been widely used to localize macromolecules in plant tissues. Therefore, one aim of this study was to establish where RCA is located in higher plants.

A second aim of this study was to determine whether Rubisco and RCA contents were altered, or the proportions in different parts of the chloroplast changed, when RCA content was reduced by use of antisense rca: this is made possible by using genetically modified rice plants with antisense-RNA to RCA [6-7]. The photosynthetic rate of such plants was largely unaffected by RCA concentration until it was reduced below approximately 35% of that of wild-type plants [6]. These results were similar to results obtained in transgenic tobacco [8-10] and Arabidopsis thaliana [11-12]. In these plants, modest reduction (49% – 32%) in Rubisco activation did not mirror the large decrease (0.02 – 0.0025) in RCA/Rubisco ratio that occurred [13]. Although the reduced amount of RCA in the anti-activase plants might be partially compensated by an increase in ATP or in the ATP/ADP ratio in vivo, in no case was compensation by ATP sufficient to explain the relative insensitivity of photosynthesis to loss of RCA. Other factors, such as
increased amount of Rubisco protein, or re-location of sequestered RCA to maintain Rubisco activity might also explain the insensitivity of photosynthesis to loss of RCA. We therefore hypothesize that changes in amount and distribution of RCA and Rubisco are responsible for the discrepancy between changes in RCA and gas exchange and Rubisco activity, and test this by examining the localization of Rubisco and RCA in the wild type and antisense rca rice plants.

2 Materials and Methods

2.1 Plant materials and growth

Transgenic rice with reduced amounts of RCA was grown from seed collected from selfed R1 progeny of rice (Oryza sativa L. cv. ZhongHua 11) transformed with an antisense gene directed against RCA by the CaMV35S promoter using the Agrobacterium tumefaciens system [7]. The R1 seeds of the transformant with 30% of wild-type RCA were used for this study to test the effects of a large but not damaging change in RCA. Untransformed cv. ZhongHua11 rice plants were used as controls. About 50 R1 seeds and 30 wild type seeds were germinated, and their seedlings were grown in paddy soil in 15 L pots in a shaded greenhouse with natural sunlight during the day (maximum of 800 µmol photons m⁻² s⁻¹) at the Huajiachi Campus of the Zhejiang University. The greenhouse temperature was 28±3°C during the day and 25±2°C at night.

2.2 Gas exchange measurements

The gas exchange was determined with a portable photosynthesis system (LiCor-6400; LiCor Inc. Lincoln, Nebraska, USA) and a LED light source, 6400-02. This experiment was conducted at a light intensity of 1500 µmol m⁻² s⁻¹, a leaf temperature of 28°C, and CO₂ of 380±5 µmolCO₂ mol⁻¹ in the sample chamber. Measurements were made on fully expanded uppermost leaves of the main stem of 50 day-old plants of antisense rca and wild-type rice plants, and were repeated at least six times on each. After measurements, the leaves were excised, frozen in liquid N₂ and stored at –80 °C for Rubisco and RCA assays. There were at least six replications for each plant-type.

2.3 Measurements of Rubisco content and activity

About 6-7 cm² (0.10 g) of frozen rice leaves were ground to a powder using a chilled mortar and pestle with liquid N₂, a small amount of quartz sand and insoluble polyvinylpolypyrrolidone (PVP), then homogenized with 1.9 mL cooled extraction buffer containing 50 mM Tris-HCl (pH 7.5), 1mM EDTA, 10mM MgCl₂, 12% (v/v) glycerol, 0.1% (v/v) β-mercaptoethanol and 1% (w/v) PVP-40 (soluble PVP) at 0-4°C. The homogenate was centrifuged at 15,000 × g for 15 min at 4°C. The supernatant was used to determine the concentration and activity of Rubisco. The Rubisco concentrations were measured with the single radial immunodiffusion method as described [14].

The Rubisco activity in the supernatant was assayed according to Sawada et al. [15] with minor
The initial Rubisco activity was measured at 30°C by adding 100 µl of supernatant into 900 µl of assay buffer containing 50 mM HEPES-KOH (pH 8.0), 1 mM EDTA-2Na, 20 mM MgCl₂, 2.5 mM dithioerythritol (DTT), 10 mM NaHCO₃, 5 mM ATP, 0.15 mM NADH, 5 mM creatine phosphate, 0.6 mM RuBP, 10 units of phosphocreatine kinase, 10 units of glyceraldehydes-3-phosphate dehydrogenase and 10 units of phosphoglycerate kinase. The total Rubisco activity was assayed by adding 100 µl of the Rubisco-containing supernatant into 200 µl of an activation medium containing 33 mM Tris-HCl (pH 7.5), 0.67 mM EDTA-2Na, 33 mM MgCl₂ and 10 mM NaHCO₃, and then incubating the sample at 30°C temperature for 10 min prior to measurements.

2.4 Quantification of RCA content

The amount of RCA was quantified by the immuno-diffusion method using rabbit serum antibody and the purified RCA from rice leaves as a standard [16].

2.5 Fixation and Immunolocalization

Sub-cellular protein distribution was analysed by electron microscopy, on sections from the middle portions (about 10 mm taken at 100 mm away from the leaf tip) of fully expanded uppermost leaves of the main stem, similar to those used for gas exchange. Three leaves from different plants of each type were collected, and two pieces (1×3 mm) of each were taken and used for analysis. Small pieces of the leaves were fixed with 0.1M phosphate buffer (pH 7.2) containing 3% (v/v) paraformaldehyde and 1% (v/v) glutaraldehyde for 2 h at 4°C and washed in the buffer. The segments were dehydrated in an ethanol series and embedded in Lowicryl K₄M according to the following protocol: 100% ethanol/resin 1:1 (v/v) for 1 h, 100% ethanol/resin 1:2 (v/v) for 1 h, pure resin for 12 h at -20°C. The embedded samples were transferred to 0.5 ml of tubes filled with resin and polymerized completely under UV-radiation at -20°C for 72 h, followed by 24 h at room temperature. Ultrathin sections (70-90 nm) were cut with a diamond knife and placed on nickel grids. Two sections from each small piece were analysed. The sections were washed with distilled water for 15 min, and then incubated in blocking buffer (0.05M phosphate-buffered saline (PBS) with 1% (w/v) BSA, 0.02% PEG20000, 0.1M NaCl, 1% (w/v) NaN₃) for 1 h at room temperature. The sections were then incubated for 1 h at room temperature with anti-Rubisco, or anti-RCA serum applied at dilutions of 1:1000 and 1:200, respectively, in blocking buffer. For control sections, antiserum was replaced with non-immune serum. After washing with blocking buffer, the sections were incubated in blocking buffer containing protein A conjugated with 15-nm colloidal gold particles for 1 h, and then were washed in PBS and in deionized distilled water. Finally, the sections were stained with uranyl acetate and lead citrate, observed and photographed with an electron microscope (JEM-1200EX, JEOL, Japan) at 80 kV.

The labeling density was determined by counting the gold particles on electron micrographs at 20 000× magnification and calculating the number per unit area (µm²). Between 7 and 10 individual cells from different immunolabelled sections for each cell type were examined. The areas occupied by starch grains
were omitted from the calculation of chloroplast area. No significant labeling for Rubisco or RCA was present in the vacuole, cell wall, mitochondria or cytosol, and these labeling densities were taken as the background value. To obtain the proportions of Rubisco and RCA in the stroma and thylakoids, the average density of immunogold particles in the background was subtracted from the average density within the stroma and thylakoids.

2.6 Antibodies

Rabbit antibodies to whole molecule of rice Rubisco and to rice RCA, which recognized both forms of RCA \cite{17}, were used in this work.

2.7 Statistical methods

Data were analysed statistically by ANOVA, using the Student’s t-test for comparison of means. For these analyses we used SPSS 10.0 software (SPSS Inc., Chicago, IL, USA), and set the statistical significance level at $P < 0.05$.

3 Results

The RCA content of the antisense plants was about 30% of that of the wild-type. However, the antisense plants possessed much more (1.8-fold) Rubisco in their leaves (Table 1). The net photosynthetic rate (Pn) and the initial activity of Rubisco in the antisense plants were reduced by about 50%, as compared to those in the wild type plants (Table 1). Thus, the magnitude of the decrease in the initial Rubisco activity and Pn was much less than that in RCA content. Nevertheless, the total activity of Rubisco in antisense plants exposed to light was significantly higher than that in the wild type ($P < 0.05$), correlating with the measured Rubisco concentrations (Table 1). The intercellular CO$_2$ concentrations (Ci) of the antisense rca rice were higher than that of controls ($P < 0.05$), while there were no changes in stomatal conductance ($g_s$) between the antisense and wild-type plants (Table 1), indicating that the reductions of photosynthetic rate of the selected antisense plants were not due to stomatal conductance.

In the wild-type and antisense rca rice plants, when the thin sections were treated with antibody directed against Rubisco of rice, almost all of the immunogold was in the stroma of chloroplasts (Fig 1A,B). However, the labeling density in the stroma was related to the RCA content; in the antisense plants small RCA content resulted in a higher densities of particles in the stroma (Table 2), consistent with the increased Rubisco content measured \textit{in vitro} (Table 1). There were only a few particles over the thylakoid in both types of plant (Fig 1A, B; Table 2).
Table 1. The net photosynthetic rate (Pn), intercellular CO₂ concentrations (Cᵢ), stomatal conductance (gₛ), RCA contents, Rubisco contents, initial and total Rubisco activity of the leaves of the wild type and antisense rca plants.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild type</th>
<th>Antisense</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pn (µmol m⁻²s⁻¹)</td>
<td>18.9±0.7 a</td>
<td>9.68±0.5 b</td>
<td>-48.8</td>
</tr>
<tr>
<td>Cᵢ (µmol mol⁻¹)</td>
<td>311±8 b</td>
<td>369±5 a</td>
<td>+18.6</td>
</tr>
<tr>
<td>gₛ (mol m⁻²s⁻¹)</td>
<td>0.46±0.09 a</td>
<td>0.44±0.07 a</td>
<td>-4.0</td>
</tr>
<tr>
<td>RCA (mg m⁻²)</td>
<td>20.1±0.6 a</td>
<td>6.11±0.9 b</td>
<td>-69.2</td>
</tr>
<tr>
<td>Rubisco (g m⁻²)</td>
<td>1.68±0.20 b</td>
<td>2.97±0.57 a</td>
<td>+76.8</td>
</tr>
<tr>
<td>Initial activity (µmol m⁻²s⁻¹)</td>
<td>32.7±3.3 a</td>
<td>17.2±2.58 b</td>
<td>-47.4</td>
</tr>
<tr>
<td>Total activity (µmol m⁻²s⁻¹)</td>
<td>42.4±6.4 b</td>
<td>70.8±13.84 a</td>
<td>+67.0</td>
</tr>
</tbody>
</table>

Fig. 1 Immunogold labeling of Rubisco (A,B) and RCA (C,D) in mesophyll cell chloroplasts of leaves of wild type (A,C) and rca1 (B,D) rice plants. T – thylakoid; S – stroma. Bars = 0.1 µm.

When sections of rice leaves were treated with antibody directed against rice RCA, most of the
immunogold particles in the wild type were heavily concentrated in the stroma (dark part), and some in the thylakoid membranes (white part) (Fig 1C). Gold particle densities were 389±62 µm\(^{-2}\) in the stroma, and 132±36 µm\(^{-2}\) in the thylakoid membranes (Table 2). In contrast, we observed less RCA labeling in the chloroplasts of antisense \textit{rca} plants than in the wild-type, which correlates with the RCA concentrations previously observed \textit{in vivo} (Table 1). The densities of gold particles in the antisense plants were 161±47 and 12±4 µm\(^{-2}\) in the stroma and thylakoid membrane of chloroplasts (Table 2), respectively. Interestingly, the percentage of the immunogold RCA labeling in the chloroplast stroma depended on the RCA concentration. There were a significantly higher percentage of immunogold particles in the \textit{rca1} chloroplast stroma than in the wild type (93 compared to 74\%) \((P < 0.05)\) (Fig 1D, Table 2). It is clear that almost all of the RCA was in the chloroplast stroma of antisense plants. When sections were incubated with non-immune serum, they showed only non-specific and negligible labeling with gold particles (data not shown).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Label density of Rubisco (µm(^{-2}))</th>
<th>Rubisco in stroma (%)</th>
<th>Label density of RCA (µm(^{-2}))</th>
<th>RCA in stroma (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stroma</td>
<td>Thylakoid</td>
<td></td>
<td>Stroma</td>
</tr>
<tr>
<td>Wild type</td>
<td>736±82</td>
<td>29±7</td>
<td>96.2</td>
<td>389±62</td>
</tr>
<tr>
<td>Anti-rca</td>
<td>867±97</td>
<td>33±11</td>
<td>96.3</td>
<td>161±47</td>
</tr>
</tbody>
</table>

4 Discussion

Although RCA is an important enzyme activating Rubisco \textit{in vivo} \cite{5,18}, the cellular localization of RCA is not well established. Anderson et al. \cite{19} found that RCA was in the stroma of chloroplasts in pea plants. Recently, Hong et al. \cite{20} demonstrated that RCA was in the chloroplast of the bundle sheath and mesophyll cells in the C\(_4\) plant, \textit{Amaranthus tricolor}. We now show that RCA is mainly in the stroma, and to a smaller extent in the thylakoid membranes, of chloroplasts (Fig 1C). Rokka et al \cite{21} reported that most RCA in spinach was sequestered in the thylakoid membranes region during heat treatment, and that the amount of RCA associated with the thylakoid membrane increased with the temperature and duration of the heat treatment. Interestingly, a reduction of RCA in tobacco plants increased sensitivity of photosynthesis to heat \cite{22}. Therefore, RCA appears to be is a multifunction enzyme, regulating Rubisco activity and photosynthetic rate, and also involved in protection against heat damage in chloroplasts. The RCA in stroma of chloroplasts contributes to the activation of Rubisco, a process dependent on ATP hydrolysis \cite{5,18} and it is possible that the RCA in thylakoid membranes has a second, protective role there.
RCA has been likened to a molecular chaperone [23]. Neuwald et al. [24] reported that RCA is related to an AAA family of proteins, a class of chaperone-like ATPases associated with a variety of cellular activities. They are a novel type of molecular chaperone, typically acting as disruptors of molecular or macromolecular structures [25]. This describes the role of RCA in disrupting Rubisco-inhibitor complexes. AAA+ modules are also often linked covalently to other protein domains that mediate transport and position in cellular membranes. AAA+ modules are also often linked covalently to other protein domains that mediate transporting to, and positioning in, cellular membranes. It is also possible that RCA sequestered to the thylakoid membrane is due to its redox regulation, since the activity of the large RCA isoform is regulated by redox changes via the ferredoxin/thioredoxin system at physiological ATP/ADP ratios [12, 26]. Reduction of RCA by thioredoxin could lead to inactivation and sequestration in thylakoid membranes.

RCA facilitates the dissociation of inhibitors from Rubisco, so it must bind to Rubisco and induce a conformational change at the active site. RCA was chemically cross-linked to the Rubisco [27] and co-immunoprecipitation of the two proteins was reported [23, 28]. Portis [5] suggested that RCA encircles the Rubisco molecule. However, no RCA-Rubisco complex has been isolated. A double immunogold labeling study suggested that most of RCA is associated with Rubisco, forming complexes in the chloroplast, while a large part of the Rubisco is not associated [29]. In our study, approximately 75% of RCA was in the stroma, and 25% in the thylakoids (Table 2). In contrast, 96% or more of the Rubisco was in the stroma (Table 2). These results imply that 75% or less of the RCA interacts with Rubisco.

Rubisco occurs in the chloroplasts of higher plants [30-32] with most immunogold particles from anti-Rubisco antibodies in the stroma. In the antisense rca rices, we found that the Rubisco content was substantially increased (Table 1), although its location was unaltered (Fig 1 and Table 2). This effect of reduced RCA content upon Rubisco concentration is consistent with work using tobacco plants [8-9]. The observed increase in Rubisco concentration tempts us to speculate that Rubisco accumulation counteracts the RCA deficit. Therefore, an increased requirement for RCA in the stroma was required to activate the increased Rubisco. We demonstrated that labeling for RCA decreased, as did the percentage of RCA labeling in thylakoid membranes (Fig 1D, Table 2). Mate et al. [8], Eckardt et al. [11] and Hammond et al. [10] showed that RCA was largely saturating for the steady-state concentration of active Rubisco. This suggests that the changes in subcellular RCA distribution in the antisense plants might be related to compensation for the loss of RCA in the stroma and to the increase in Rubisco. This process may be linked to the redox regulation of large RCA isoform. This may explain why steady-state photosynthesis is largely unaffected until RCA concentration is reduced substantially. RCA in the antisense plants may then be transferred from the thylakoid membranes to the stroma to compensate for the reduced RCA, which activates Rubisco. However, transgenic plants with reduced RCA [10, 33] have a slower rate of photosynthetic induction following a rapid increase in light intensity, suggesting that RCA was not present in excess under those conditions; whether RCA in thylakoid membrane is transferred to the stroma is unclear. Changes in protein distribution following a stress have already been reported. For instance, mechanical stimulation induces a
change in the location of GPXle-1 protein from the wall to the cytosol in collenchyma \cite{34}. To our knowledge, a change of protein location induced by reduction of protein content has not previously been reported. However, we did not rule out a decrease in the binding affinity of the protein for the thylakoid membrane in the antisense \textit{rca} plants: more detailed biochemical characterization of these changes will be required to establish if this occurs.

References


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