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Gold Immunolocalization of Rubisco and Rubisco Activase in Pyrenoid of *Chlamydomonas reinhardtii*

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The pyrenoid ultrastructure and distribution of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and Rubisco activase in the chloroplasts of *Chlamydomonas reinhardtii* was studied using the immunogold localization technology with electron microscopy. There were several tubular thylakoids invading in the pyrenoid matrix to form several spokewise channels. The connections between pyrenoid matrix and stroma of chloroplast were the partial of channels. The starch sheath surrounding the pyrenoid was separated into several parts by the connections in transection. Some thylakoids were packed together near the connections in one side of the pyrenoid. Those special structures might be used to transport substance between pyrenoid and stroma of chloroplasts. With the antibody raised against the large subunits of Rubsico from *C. prototheco*ides, the result of the gold immunolocalization of Rubisco in *Chlamydomonas reinhardtii* showed most of the gold particles heavily labeled the pyrenoid matrix, as well as the starch sheath matrix, and very few in the stroma of chloroplasts. The gold particle density was 880.00 ± 164.32, 190.00 ± 152.39 and 9.60 ± 5.37 μm^{-2} in pyrenoid matrix, starch sheath and stroma region of chloroplast respectively (background: 5.67 ± 1.53 μm^{-2}). 99.59% of the total Rubiscos was calculated to be concentrated in the pyrenoid matrix and starch sheath by spatial densities. The gold immunolocalization of Rubisco activase also showed that Rubisco activase was mainly concentrated in the periphery of the pyrenoid and the starch sheath (the density was as high as 229.69 ± 96.96 μm^{-2}). There were very few gold particles located in the stroma of chloroplasts. These results indicated that pyrenoid surface and starch sheath was the site for Rubisco activation and CO_2 fixation, which supported the suggestion that pyrenoids perform photosynthesis function.

Key Words: *Chlamydomonas reinhardtii*, gold immunolocalization, pyrenoid, Rubisco, Rubisco activase, ultrastructure

INTRODUCTION

Pyrenoids are proteinaceous structures located in chloroplasts in many photosynthetic algae (Griffiths 1970; Dodge 1973; Okada 1992) and hornworts (Vaughn *et al.* 1990). With biochemistry and immunochemistry technology, it was demonstrated that the pyrenoid was mainly composed of ribulose-1, 5-bisphosphate carboxylase (Rubisco, EC 4.1.1.39), the first key enzyme of the photosynthetic CO_2 reduction pathway in many pyrenoid-containing algae (Holdsworth 1971; Salisbury and Floyd 1978; Kerby and Evans 1981; Satoch *et al.* 1984; Caers *et al.* 1987; Vladimirova *et al.* 1982; Lacoste-Royal and Gibbs 1987; Kuchitsu 1988; Kajikawa *et al.* 1988; Mackay and Gibbs 1990; Osafune *et al.* 1990; McKay *et al.* 1991,1992; Nozaki *et al.* 1994; Ascaso *et al.* 1995; Suss *et al.* 1995; Miyamura *et al.* 1996; Lin and Carpenter 1997; Morita *et al.* 1997, 1999; He *et al.* 2001, 2002). The activity of Rubisco is localized in isolated pyrenoids (Okabe and Okada 1988; Kuchitsu *et al.* 1991; Okada 1992), suggesting that the pyrenoid might be an active site of CO_2 fixation and an important component of CO_2 concentration mechanism (CCM) in pyrenoid-containing algae (Pronina and Semenenko 1992; Ramazanov *et al.* 1994; Badger and Price 1994; Graham and Wilcox 2000). Quantifying the Rubisco in the pyrenoid and the stroma with immunogold localization technology might be a very important way to verify the photosynthetic function of pyrenoids (Morita *et al.* 1997). However the ratio of Rubisco in pyrenoid and stroma in chloroplasts of pyrenoid-containing algae with immunogold localiza-

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tion technology was not consistent among different authors. For example, in *Chlamydomonas reinhardtii*, Süss *et al*. (1995) showed 50-60% of Rubisco located in pyrenoid with antibodies of Rubisco from spinach, while Morita *et al.* (1997) showed about 99% of Rubisco was heavily concentrated in pyrenoid with the antibodies raised against Rubsico from *Chlamydomonas reinhardtii*. It indicated that the antibody from different Rubisco resources might result in different ratio of Rubsico in pyrenoid and stroma of chloroplasts. On the other hand, Lin and Carpenter (1997) thought the pyrenoid might be the activation site of Rubisco according to their research results of Rubisco immunofluorescence localization. Since 1980, it was found that Rubsico needed to be activated with light and CO_2 *in vitro*, while with the Rubisco activase *in vivo* (Portis 2002). Therefore, Rubisco activase immunogold localization might be a better protocol to identify the site of the Rubisco activation and pyrenoid photosynthetic function. In this research, the pyrenoid ultrastructure and the molecular organization of Rubisco as well as Rubisco activase by the immunogold technology were studied in *Chlamydomonas reinhardtii*.

MATERIALS AND METHODS

Algal culture

The alga *Chlamydomonas reinhardtii* were kindly provided by Prof. Chunhe Xe (Shanghai Institute of Plant Physiology, Chinese Academy of Science). They were grown in glass flasks with the HSM medium (Sueoka *et al.* 1967) and cultured in a photo-incubator at 25°C. The cultures were mixed with aeration. Illumination was provided by cool-white fluorescent lamps, with light intensity of 40-50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and with a photoperiod of 12:12 h light:dark.

Preparation of Rubisco antibody

The Rubisco for antibody preparation was extracted from *Chlorella protothecoides* according to Zhang *et al*. (1994). The cultures of *Chlorella protothecoides* were collected and grinded with liquid nitrogen. The Rubisco was purified by the precipitation with 35-55% ammonium sulfate fractionation and chromatograph with Sephacryl S-300 and DEAE DE-52 column. The activity of purified Rubisco was analyzed, and large and small subunits were isolated by the urea method (Li 1998). The mixture of 2 mg purified large subunit of Rubisco and 1ml of whole adjuvant was injected into the rabbit with hypodermic injection, and was enhanced 3 times. After 40 days, the antiserum against Rubsico was prepared from the rabbit blood.

Preparation of Rubisco activase antibody

Rubisco activase was prepared from spinach following the method described by Tang *et al.* (1997). Firstly the supernatant protein of spinach leaves was obtained with centrifugation after grinding, and was precipitated by 35% ammonium sulfate fractionation. The Rubisco activase was purified with chromatograph of DEAE Sephacel, MonoQ and FPLC system (Pharmacia product), respectively. With the purified Rubisco activase, the antiserum against Rubsico activase was prepared following the method described above. The specificity of the polyclonal antibody was verified by western blotting analysis with tobacco Rubisco antibody.

SDS-PAGE and western blotting

The SDS-PAGE and western blotting was carried out using the methods described by Sambrook *et al.* (1987). The specificity of the polyclonal antibody raised against large subunit of Rubsico prepared from *C. protothecoides* was verified using western blotting with *Chlamydomonas reinhardtii* crude protein extract. The primary antiserum was diluted by 1:500 with PBS (phosphate-buffered saline). The secondary antibody, goat anti-rabbit IgG labeled with phosphoesterase was used at a dilution of 1:100.

Gold immunolocalization

The Immunogold electronic microscopy was carried out according to Monita *et al.* (1997) and He *et al.* (2001). The samples growing in the exponential phase were collected with centrifugation and fixed in the cold 3% glutaraldehyde with PBS (pH 7.4) for 2 days at 4°C.

For avoiding the decrease of the affinity between antigens and antibody, the 2% osmic acid solution was not used for post-fixation. The cells then were centrifuged and embedded by 2% agar and the sections for electronic microscope was prepared following the standard method described previously (He *et al.* 2001). Sections of samples embedded in Epon 812 resin (Tousimis Research Co., Japan) were mounted on formvar-coated nickel slot grids. And the immunogold labeling of sectional cells by anti-Rubisco and anti-Rubisco activase was performed as the method described by Nozaki *et al.* (1994). The primary antibodies raised against Rubisco large unit from *Chlorella protothecoides* and Rubisco activase from spinach were diluted 1:300 and 1:500 with PBS

containing 1% BSA (Bovine serum albumin), respectively. Sections on the grids were incubated with the primary antibody at room temperature for 30 min, and rinsed with the PBS 6 times, and then incubated in the second antibody, 10 nm gold-conjugated goat anti-rabbit IgG (Sigma) was diluted 1:20 with PBS containing 1% BSA for 30 min. After the samples were washed with distilled water, the sectioned cells were counterstained with 4% uranyl acetate and 2% lead citrate.

The plane densities of gold particles ($n \cdot \mu m^{-2}$) were calculated basing on the counting of the number of gold particles in some squares of cell components on photographs (n = 25). And the plane densities can be converted to the spatial densities ($N \cdot \mu m^{-2}$) by the formula $N = n^{3/2}$ as Morita *et al.* (1997) described. For the volume of pyrenoid almost is equal to the volume of starch sheath and is 1/15 of the stroma of chloroplast, the relative and spatial amounts of gold particles in pyrenoid matrix, starch sheath and stroma of chloroplast were calculated by multiplying N by 1, 1, 15 respectively (see Lacoste-Royal and Gibbs 1987; Morita *et al.* 1997).

RESULTS

Ultrastructure of pyrenoids in *Chlamydomonas reinhardtii*

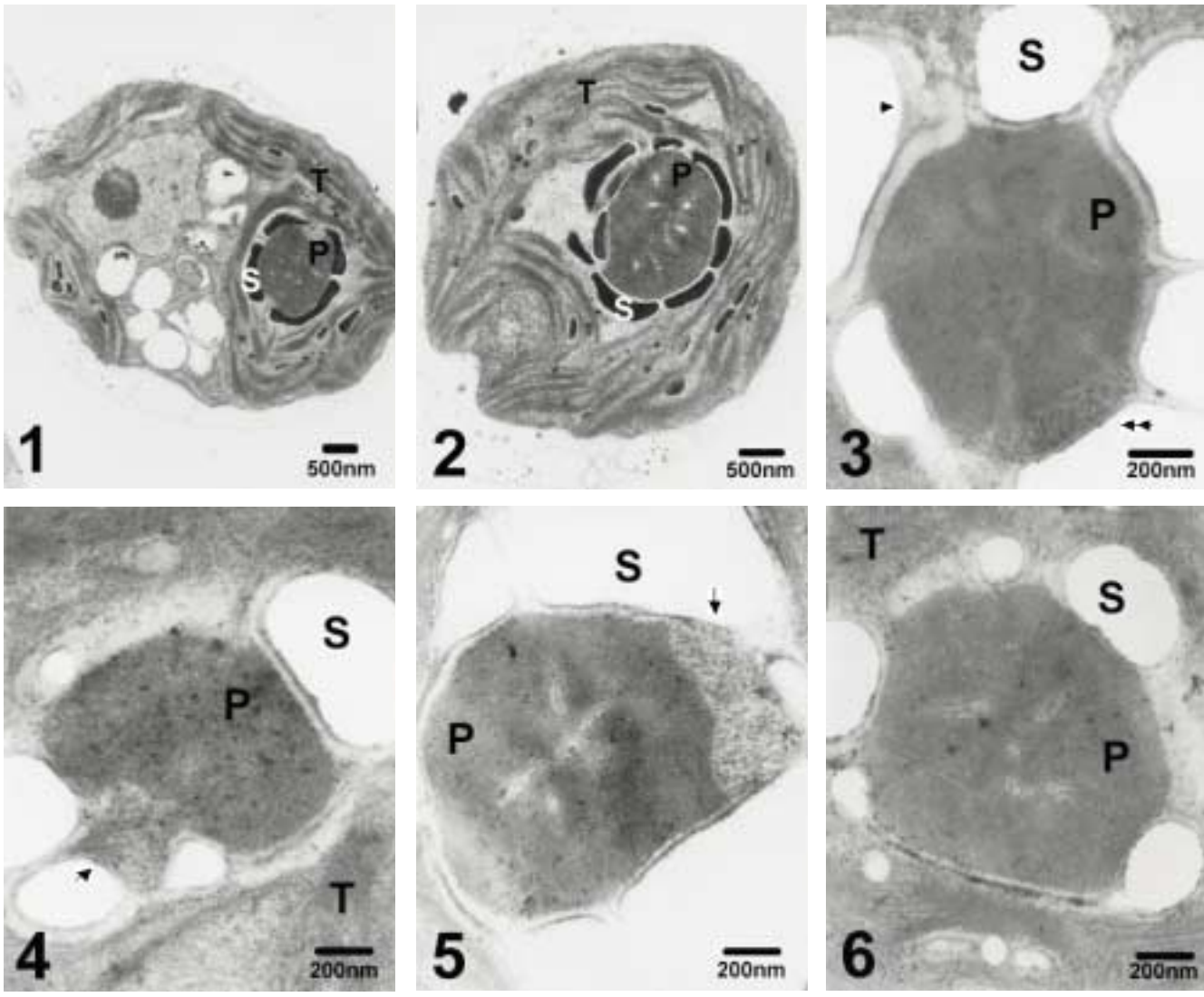
The pyrenoid was found localized among the thylakoids at the center of chloroplast at the base of a cell. Its shape was round or multi-angular with 1,000-1,200 nm in size. There was one pyrenoid in each cell. A layer of starch was embedded in the pyrenoid, which was called starch sheath with higher electron density (Figs 1, 2). The starch sheath was composed of starch that was easy to be solubilized and become lower electron density during gold immunolocalization preparation (Figs 3, 4, 5, 6). In transverse section, there were several tubular channels distributed in the pyrenoid matrix in various directions, usually in spokewise arrangement (Figs 2, 3, 5, 6). The tubular channels were the thylakoids that invaded pyrenoid matrix. The channels were hollow and the wall was consisted of two layers of membranes (Figs 3, 5, 6). As the starch sheath was soluble and disappeared after immersing in the antiserum solution, the connections between pyrenoid matrix and stroma of chloroplast become clear and obvious (Figs 3, 4, 5, 6). Those connections seemed to be the partial of thylakoids, which had invaded into pyrenoid matrix and formed channels (Figs 3, 4). The starch sheath was not intact and there were so many holes in the starch sheath. In every section, the starch sheath was separated into several parts by the connections or channels (Figs 1, 2). And there were many thylakoids packed together near the connections in the matrix of pyrenoid (Figs 3, 4, 5, arrow).

Gold immunolocalization of Rubisco

With the antibody raised against the large subunits of Rubsico from *Chlorella protothecoides*, the gold immunolocalization of Rubisco in *Chlamydomonas reinhardtii* was carried out. The distribution of gold particles labeled with large subunit of Rubisco in chloroplasts was shown in Figs 3, 4 and 5. The results revealed that most of the gold particles were heavily concentrated in the pyrenoid matrix, some in the starch sheath matrix, and very few in the thylakoid stroma. According to the calculation of the gold particle numbers per unit area, the gold particle density (n) was 880.00 ± 164.32 μm^{-2} in pyrenoid, 190.00 ± 152.39 μm^{-2} in starch sheath, 9.60 ± 5.37 μm^{-2} in stroma region, and 5.67 ± 1.53 μm^{-2} in background, respectively. According to the formula $N = n^{3/2}$ (Morita *et al.* 1997), the spatial density (N) were calculated to be 25853.12, 2502.61and 7.79 μm^{-3}. By the planar density (eliminating the background density), the relative ratio of gold particles in the pyrenoid, starch sheath and thylakoid stroma region were 82.28%, 17.35% and 0.37%, respectively. According to the spatial density, the relative ratio of Rubisco in pyrenoid, starch sheath and stroma of chloroplast were calculated to be 90.80%, 8.79% and 0.041%, respectively (see Table 1).

Gold immunolocalization of Rubisco activase

With the antibodies raised against Rubisco activase from spinach, the gold immunolocalization of Rubisco activase in chloroplast of *Chlamydomonas reinhardtii* was carried out and the distribution of gold particles was shown in Fig 6. The densities of gold particles for Rubisco activases were 229.69 ± 96.96, 54.76 ± 14.66, 3.50 ± 4.69 μm^{-2} in starch sheath (including the periphery of the pyrenoid), the middle of the pyrenoid and thylakoid (after eliminating 22.09 ± 22.63 μm^{-2} of the background density), respectively. The result also showed that gold particles were mainly concentrated in the periphery of the pyrenoid and the starch sheath. There were very few gold particles located in the thylakoid stroma of chloroplasts.



Figs 1-6. Ultrastructure of pyrenoids and the gold immunolocalization of Rubisco and Rubisco activase. **Figs 1-2.** The ultrastructure of a *Chlamydomonas reinhardtii* cell. The pyrenoid (P) located in the cup-shaped chloroplast and was surrounded by the starch sheath. There were some tuber thylakoids in the matrix of pyrenoid. The starch sheath, with high light electron density, was separated by connections between pyrenoid and stroma of chloroplasts. **Figs 3-5.** Gold particles of large subunits of Rubisco were heavily concentrated in the matrix of pyrenoid (P) and starch sheath (S), while few gold particles labeling in the thylakoid stroma (T) of chloroplasts. The starch sheath became low electron density after serum treatment. There were some spoke tube channels distributed in the matrix of pyrenoids. There were many thylakoids packed together near connections (double arrow and single arrows). The channels were arranged in spokewise style. **Fig. 6.** The gold particles of Rubisco activase heavily labeled the peripheral of pyrenoid and starch sheath, and very few gold particles labeled the stroma of chloroplast.

DISCUSSION

In the early stage, the pyrenoid was once considered as a special structure in chloroplasts for storage of protein or nitrogen resource (Osafune *et al.* 1990). And the appearance and number of the pyrenoids are important index for the morphology and taxonomy in the pyrenoid-containing algae.

Resent studies suggest that the pyrenoid may be an important competent of CO_2 concentration mechanism because it contains Rubisco which is the first key enzyme of CO_2 fixation. The fine structure of pyrenoids has attracted more attention in recent years (Miyaji 1999; Hirayama *et al.* 2001; Thoms *et al.* 2001; Zacrys *et al.* 2001; Nagasato and Motomura 2002; Konstantinova and Boldina 2002). Usually 1-2 thylakoids penetrates into pyrenoid and form a portrait channel in *Chlorella* (He *et*

Table 1. Densities of gold particles of the anti-Rubisco LS in cell components of *Chlamydomonas reinhardtii*

Authors	Pyrenoid	Starch sheath	Stroma of chloroplast	Relative[a] ratio of Rubisco in pyrenoid (per unit) (%)	Relative[b] ratio of Rubisco in pyrenoid (total) (%)
			Planar densities ($n \cdot \mu m^{-2}$)		
Süss *et al.* (1995)	74.1	-	10.0	88.11	
Morita *et al.* (1997)	902.0	-	2.4	99.73	
Present study	874.33	184.33	3.93	99.63	
			Spatial densities ($N \cdot \mu m^{-2}$)[c]		
Suss *et al.* (1995)	637.8	-	31.6	59.43	62.97
Morita *et al.* (1997)	27090.0	-	3.7	99.98	99.78
Present study	25853.12	2502.61	7.79	99.97	99.59

[a] Including the amounts of Rubisco in starch sheath;

[b] Including the amounts of Rubisco in starch sheath. The relative ratio of Rubisco in pyrenoid, starch sheath and stroma of chloroplast were calculated by multiplying N by 1, 1, 15 respectively (Lacoste-Royal and Gibbs 1987; Morita *et al.* 1997)

[c] Calculating by the formula: $N = n^{3/2}$ (Morita *et al.* 1997)

al. 2001). *Chlamydomonas reinhardtii* was known to contain several channels in the matrix of pyrenoid with different directions. As starch sheath was soluble, it showed lighter electronic density from deep electronic density (Figs 1, 2) after treating with antiserum. Without the starch covering, the structure of connections between pyrenoid and stroma was clearer. The connections were localized in the same direction with some spoke channels, indicating that the connections also were the parts of the channels.

Even if the Rubisco activity was detected in isolated pyrenoids (Okabe and Okada 1988; Kuchitsu *et al.* 1991; Okada 1992), the Rubisco from the isolated pyrenoids was suspected of being contaminated with the Rubisco from stroma, because the starch sheath that surrounds pyrenoids was solubilized during the extraction procedure. So the immunogold localization of Rubisco was considered as the best way for demonstrating pyrenoid photosynthesis function depends, which could be quantified and show the ratio of Rubisco distributing in pyrenoid and stroma of chloroplasts.

To avoid the influence of the antibody of Rubsico extracted from distantly related plant (Süss *et al.* 1995) or the same species (Morita *et al.* 1997), we used the antibody of Rubisco extracted from closely related alga, *Chlorella*. Before carrying out the gold immunolocalization, the specificity of this polyclonal antibody raised against large subunit of Rubsico from *Chlorella prototheco*ide was verified using western blotting with Chlamydomonas reinhardtii. There was only one band in the Rubisco western blotting map, which indicated that the Rubisco from *Chlorella prototheco*ide and *Chlamydomanas reinhardtii* was homologous and the relationship between them was very close. SDS-PAGE further indicated that the large subunit of Rubisco was about 55kDa in size. According to the amounts of gold particles in cell components, the relative ratio of Rubisco in pyrenoid matrix, starch sheath and stroma of chloroplast could be estimated to 90.80%, 8.79% and 0.041%, respectively by spatial density. This indicated 99.59% of Rubisco was concentrated in the pyrenoid as well as starch sheath. The results strongly support recent studies and suggest that pyrenoid may be functional in CO_2 fixation of photosynthesis. For comparing the data from different authors, Table 1 showed the results of plane densities and spatial densities, relative and planar or spatial ratio of Rubiscos in pyrenoid matrix (including starch sheath) on the basis of the conversion formula between plane density and spatial density, as well as the volume ratio between pyrenoid, starch sheath and stroma of chloroplast. Our results were consistent to the results of Morita *et al.* (1997).

The large subunits, which are encoded by chloroplast genome and synthesized in chloroplasts, and the small subunits, which are encoded by nuclear genome and synthesized in cytoplasm, were assembled to form whole enzymes in the stroma of chloroplasts. The Rubiscos existed in starch sheath might indicate that they were transported from stroma to pyrenoids, as new assembled Rubiscos would be concentrated in pyrenoids from stroma of chloroplasts. In recent years, it has been further shown that the pyrenoid formation or Rubsico

redistribution responded to the diurnal rhythm or light/shade and CO_2 concentration (Lin and Carpenter 1997; Seo and Fritz 2002; Xia and Gao 2002). Until now it is unclear why and how Rubsico proteins aggregate in the pyrenoid (Nagasato and Motomura 2002). There were two difference types of *rbcL* gene sequences having been found in pyrenoid-containing algae and pyrenoid-less algae and it indicated the *rbcL* sequence might determine whether Rubsico molecules would aggregate to form a visible pyrenoid (Nozaki *et al.* 2002).

Rubisco activase is a nuclear-encoding chloroplasts enzyme that is required for Rubsico activation by facilitating the ATP-dependent removal of inhibitory sugar phosphate from Rubsico in plant and green algae (Portis 2002). Especially *in vivo*, Rubisco must be activated by Rubisco activase. In this way, activase is a molecular chaperone, controlling the switching of Rubsico conformation from inactive to active form (Spreitzer and Salvucci 2002). The Rubisco activase also was shown to be co-localized in the pyrenoid with Rubsico (Mckay *et al.* 1991). In this study, the Rubisco activase was shown to be concentrated at the surface of pyrenoid and starch sheath area of *Chlamydomonas reinhardtii*. It indicated the Rubsico distributed at the surface of the pyrenoid and starch sheath could be activated by the Rubsico activases locally and possessed the activity of carboxylation for fixing CO_2. As Rubiscos are light-dependent, the research of Lin and Carpenter (1997) also indicated that pyrenoids probably were the active site of Rubsico activation according to the Rubsico redistribution between pyrenoid and stroma response to light and shade. Rubsico activase distributing in pyrenoids and starch sheath further demonstrated that the pyrenoids possessed the photosynthetic function.

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