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Analysis of Chloroplast Ultrastructure, Photosystem Ⅱ Light Harvesting Complexes and Chlorophyll Synthesis in a Chlorophyll-Less Rice Mutant *W2555*

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Abstract: A comparative study on chloroplast ultrastructure and light harvesting complex of photosystem Ⅱ (LHC Ⅱ) was conducted between a new rice mutant (*W2555*) and its wild type (WT). The chloroplasts of *W2555* had less thylakoids and grana stacks compared with the wild type. There was no significant change in the composition of LHC Ⅱ polypeptide in *W2555*, while a decline had been noted in LHC Ⅱ content. Northern blot analysis with a specific *cab* gene probe showed no appreciable difference in the LHC Ⅱ mRNA level between the *W2555* and its wild type. The precursors of chlorophyll synthesis, δ-aminolevulinic acid (ALA) and porphobilinogen (PBG) were over accumulated in *W2555*, but the other precursors were all decreased. These results indicated that the decreased level of LHC Ⅱ in the mutant *W2555* was attributed to the change of *cab* gene transcription, but a blockage in chlorophyll biosynthesis due to the formation of uroporphyrinogen Ⅲ (Urogen Ⅲ).

Key words: chloroplast ultrastructure; photosystem Ⅱ; chlorophyll synthesis precursors; chlorophyll-less mutant; rice

The biosynthesis of chlorophyll comprises a number of challenging topics, while the mutants of higher plants are convenient models to study the biosynthesis of chlorophyll, photosynthetic activity, the spectral, biochemical, ultrastructural and genetic characteristics of chloroplasts. A great diversity of these mutants makes it difficult to classify them by phenotypes, as analogous phenotypes may result from many different mutations.

Mutants can be used as an important tool to elucidate the function of complex systems in normal organisms. A series of chlorophyll-less mutants have been reported in a number of plant species, mainly *Arabidopsis*[1], barley[2-3], wheat[3-4], rice[5-6], pea[7], maize[8] and tobacco[9]. Many of them were considered to be resulted from a partial block at the Mg-insertion step in chlorophyll synthesis, and had been used in studies of developmentally regulated assembly of the photosynthetic apparatus.

Recently, more attentions have been paid on the genetic and biochemical controls of chlorophyll biosynthesis and the assembly of light-harvesting Chl a/b proteins. A new chlorophyll-less rice mutant *W2555* has shown alteration in chloroplast ultrastructure with decrease in photosystem proteins. Therefore, further studies of present mutant maybe helpful to reveal the mechanisms involved in dramatic decrease of chlorophyll content.

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MATERIALS AND METHODS

Plant materials

A chlorophyll-less mutant *W2555*, derived from a nuclear mutation in natural environment was used. For measurement of the contents of chlorophyll synthesis precursors, the seeds of the mutant and its wild type were germinated in complete darkness at 28℃ for 10 days by using an equal fresh weight of leaf tissue.

Assay of pigment composition and protein content

Chlorophyll (Chl) contents were determined according to Arnon[10], with some modification

described by Porra[11]. Carotenoid (Car) content was determined by the method of Lichtenthaler and Wellburn [12], while the protein contents were according to Markwell et al [13].

Electron microscopy observation of chloroplast ultrastructure

Electron microscopy observation of chloroplast ultrastructure was followed by the method of Kech and Dilley [14]. Leaf segments were fixed in buffered 4% glutaraldehyde (pH 7.2) and postfixed in buffered 2% OsO_4 (pH 7.2). Thin sections were obtained with an LKB_5 ultratome. After sectioning, the samples were post-stained with uranyl acetate and lead citrate and examined with a JEM-100 cx electron microscope.

Isolation of thylakoid membranes and pigment-protein complexes

Thylakoid membranes were isolated as described by Dunkley and Anderson [15]. Isolation of chlorophyll-protein complexes was performed according to Dunahay and Staehelin [16] with some modification. Thylakoid polypeptides were separated using the gel system of Laemmli [17], modified by the addition of 6 mol/L urea to the resolving gel.

Green gel and SDS-PAGE electrophoresis

Green gel analysis was performed as described by Allen and Staehelin [18]. Thylakoids were washed twice in ice-cold 2 mmol/L Tris-maleate (pH 7.0) and then solubilized for 30 min on ice in a solution consisting of 0.45% (*W*/*V*) n-octyl-ß-$_D$- glucopyranoside (OG), 0.45% (*W*/*V*) n-dodecyl-ß-$_D$-maltoside (DM), 0.1% (*W*/*V*) sodium dodecyl sulphate (SDS), 10% (*W*/*V*) glycerol and 2 mmol/L Tris-maleate (pH 7.0). Unsolubilized material was removed by centrifugation at 20 000×*g* for 10 min and the supernatant was loaded onto 10% disc polyacrylamide gels (30 μg Chl/tube).

Western blot analysis

Electrophoresed proteins without staining were immediately electro-transferred onto nitrocellulose films according to Sambrook et al [19]. The antisera used for LHC II were kind gifts from Dr. Naoki Yamamoto. Alkaline phosphatase-conjugated antibodies were used as the secondary antibodies. The blots were visualized with a substrate (BCIP/NBT) for 20-min reaction.

RNA preparation and Northern blot analysis

Preparation of total RNA from fresh rice leaves was carried out according to the method described by Chomczynski and Sacchi [20]. For Northern blotting, 10 μg of total RNA were separated on a 1.2% formaldehyde-denaturing agarose gel and transferred onto a nylon membrane. *cab* gene (provided by Dr. Zhu Yu-sheng) were labeled with [α]-^{32}P and used as probes for RNA blot analysis. All procedures were carried out according to the standard methods of Sambrock et al [19].

Measurement of the contents of chlorophyll synthesis precursors

The leaves selected for analysis was weighed. The δ-aminolevulinic acid (ALA) was extracted as described by Del [21]. The isolation of porphobilinogen (PBG) was performed using the method of Wang et al [22] with some modification. Coproporphyrinogen III (Coprogen III), protoporphyrin Ⅸ (Proto Ⅸ), Mg-protoporphyrin Ⅸ (Mg-Proto) and proto-chlorophyllide (Pchlide) were determined according to the methods described by Rebeiz and Smith [23].

RESULTS

Alteration in leaf pigment composition and chloroplast ultrastructure

The result showed that total leaf proteins content in the mutant was almost the same as in the wild type, but the chlorophyll contents were lower than those in the wild type, while the *W2555* leaves contained nearly a half of chlorophyll a and one-third of chlorophyll b in the wild type (Table 1). Moreover, the Chl a/b ratio in *W2555* leaf was higher than that in the wild type, but the Chl/Car ratio and Chl/protein ratio in *W2555* were lower than those in the wild type (Table 1).

The predominant ultrastructural characteristic of thylakoid membranes from higher plants is due to the presence of granum stacks. As shown in Fig. 1, the thylakoids and grana stacking were lower in the

Table 1. Pigment composition and protein contents (based on fresh weight) of the mutant *W2555* and its wild type (WT).

Content	WT	*W2555*
Chl a (μg/g)	1428±55	770±45
Chl b (μg/g)	572±32	192±20
Car (μg/g)	375±10	275±15
Chl a/b	2.8±0.2	4.0±0.2
Chl/Car	5.3±0.3	3.5±0.2
Protein (μg/g)	3762±30	3386±30
Chl/Protein	0.53±1.00	0.28±1.00

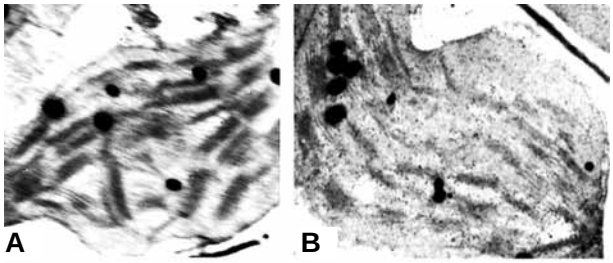

Fig. 1. Electron micrographs of chloroplasts from the wild type (A) and the mutant *W2555* (B).

mutant chloroplast than in the wild type. This finding is similar to the report for a chlorophyll-less barley *NYB* [24].

Mild denaturing electrophoresis of chlorophyll-protein complexes

The chlorophyll-protein complexes of thylakoid membranes were separated by mild electrophoresis. During the experiment, six chlorophyll-protein complexes were resolved from the wild type and the mutant thylakoid membrane separately, including Core PSI complex (CPI), LHC II trimers (LHC II*), CP47/CP43, LHC II monomers (LHC II) and Free pigments (FP) (Fig. 2). It has been noted that the two types of rice have some differences in individual chlorophyll-protein complexes contents. Moreover, *W2555* had shown less LHC II* and LHC II, while the levels of the others were similar to the wild type.

SDS-PAGE and Western blot analysis

In order to determine the alteration of the PSII proteins more precisely, the thylakoid membranes were analyzed by SDS-PAGE. As shown in Fig. 3-A, compared to the wild type, *W2555* had shown a slight decrease in polypeptides at the high molecular mass range (above 30 kD) and an obvious decrease in proteins content at the low molecular mass range (18-29 kD), which was mainly the LHC II proteins. The results were similar to those of mild denaturing electrophoresis.

To further examine the change in the amount of LHC II in the mutant, immunoblotting was performed by application of antibodies against LHC II. No difference has been noted in LHC II types between the mutant and the wild type, but the quantitative difference was obvious (Fig. 3-B). Moreover, the LHC

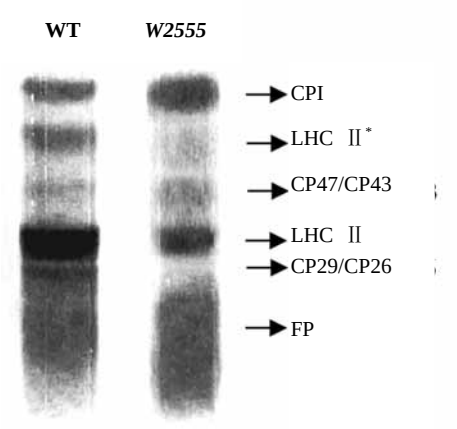

Fig. 2. Chlorophyll-protein complexes from the wild type and the mutant thylakoid membranes separated by mild denaturing electrophoresis.

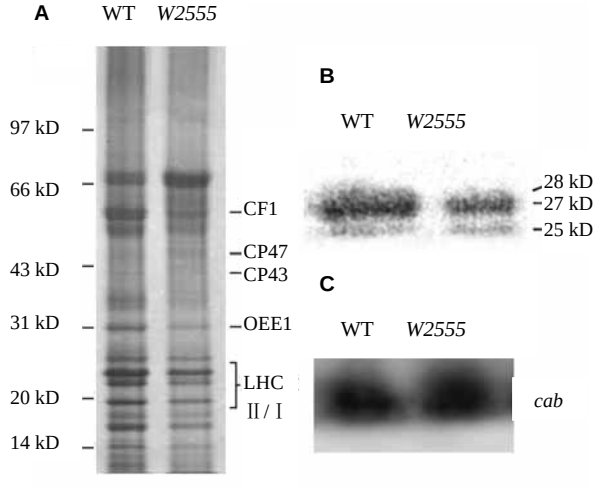

Fig. 3. Fully denaturing SDS-PAGE of thylakoid polypeptides isolated from the wild type and the mutant (A), Western blot analysis of LHC Ⅱ polypeptides (B), and Northern blot analysis of *cab* gene transcript levels (C).

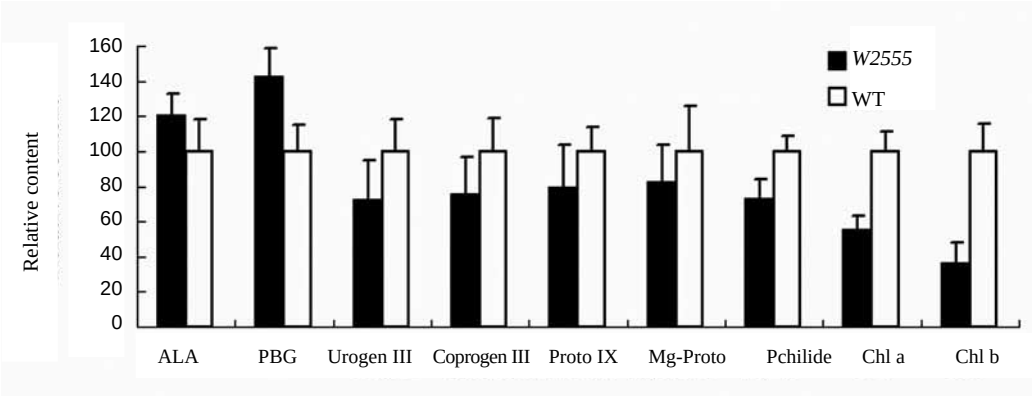

Fig. 4. Comparison of chlorophyll synthesis precursors between *W2555* and the wild type (supposed the every precursor content of wild rice was 100%).

II polypeptide of 25 kD in the mutant was similar to that of the wild type, while 27 kD and 28 kD proteins in the mutant were much lower (about 1/3) than those of the wild type.

Northern blot analysis

The reduction in the level of LHC II in *W2555* may be attributed to the decreased mRNA levels in antenna proteins. On basis of this, the total RNA was isolated from both wild-type and mutant seedlings, and the transcript level of the nuclear gene *cab* coding LHC II was determined by Northern blotting. It can be observed from the blot signals that the amount of *cab* mRNA was consistent in the mutant and wild type (Fig. 3-C), suggesting that the difference in LHC II apoproteins is probably not attributed to the difference in gene transcript levels.

Conversion of 5-aminolevulinic acid (ALA) to chlorophyll

In order to evaluate the potentiality of the enzyme systems for transformation of ALA to chlorophyll, the series of precursors of chlorophyll must be examined. As a small amount of chlorophyll precursors could accumulate in light, therefore the seedlings of the mutant and its wild type were all grown in darkness for the measurement. Results showed that the ALA and PBG contents were higher in *W2555* than the wild type, while there was a significant decrease in Urogen III, Coprogen III, Proto IX, Mg-Proto, Pchilide, Chl a, and Chl b in the mutant (Fig. 4), suggesting that the enzyme complex converting PBG to Urogen III was hampered in *W2555*.

DISSCUSSION

The shortage of chlorophyll can affect availability of certain proteins and pigment-protein complexes. Moreover, the lack of certain proteins can also affect the abundances of chlorophyll and pigment-protein complexes [2]. The analysis of chloroplast ultrastructure and light-harvesting complexes showed that the rice mutant *W2555* possessed less thylakoids and grana stacks and decreased LHC II. These characteristics may suggest that LHC II is crucial for thylakoid stacking.

It is well known that LHC proteins are encoded by a nuclear gene family and synthesized on cytoplasmic polyribosomes as protein precursors [25]. The accumulation of LHC proteins within the thylakoid membranes depends on the light-induced accumulation of LHC protein mRNAs and on chlorophyll accumulation [26]. The previous studies on *chlorina-f_2* barley mutant have established that chlorophyll b plays a key role in stabilizing the binding proteins. In this study, we found that the chlorophyll-less mutant *W2555* contains similar levels of LHC II mRNAs compared to the wild type (Fig. 3). Thus, the decreased accumulation of LHC II should not be attributed to a decreased gene transcription. It could be suggested that the *W2555* mutant has decreased chlorophyll levels, which affects the stabilization of chlorophyll b containing complexes in the membranes, such as some types of LHC II.

To find the reason of chlorophyll deficiency, we

conducted an experiment to detect the chlorophyll precursors. It has been reported that chlorophyll is assembled in chloroplast from eight molecules of ALA, the mainly next-to-last precursors in angiosperms are PBG, Urogen III, Coprogen III, Proto Ⅸ, Mg-Proto, Pchlide and Chlide [27]. The blockage in any step of chlorophyll biosynthesis would lead to the accumulation of the former precursors, and the decrease of the later precursors. During this experiment we noted that in *W2555*, the ALA and PBG contents are higher than those in wild type, but the Coprogen III Proto Ⅸ, Mg-Proto and Pchlide contents, are lower than those in wild type. It has been preliminary proved that there was a blockage in chlorophyll biosynthesis in Urogen III formation.

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