Original Article

The role of carotenoid isomerase in maintenance of photosynthetic oxygen evolution in rice plant

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Carotenoid isomerase (CRTISO) has been suggested to protect photosystem II (PS II) from photodamage, probably through its product lutein. However, the mechanism of the photoprotection still remains to be further elucidated. In this work, we cloned a point mutated gene reported to encode a CRTISO which is responsible for the accumulation of lutein in rice mutant zel1 by a map-based cloning approach. The mutant phenotype was rescued by transformation with the corresponding gene of the wild type (WT). The activity of photosynthetic oxygen evolution was evidently suppressed in zel1. The amount of the core protein of PS II CP47 was much lower in all the PS II complexes especially in the LHClI-PS II supercomplexes and CP43-free PS II of zel1 than that of WT. On the other hand, the amount of another core protein of PS II CP43 of zel1 was decreased in the higher supercomplexes, whereas it was increased in the lower ones and PS II monomer. The immunodetection displayed that CP43, CP47, and the oxygen-evolving extrinsic proteins PsbO and PsbP were reduced, but the amount of reaction center protein D1 did not show significant change in zel1. Northern blot analysis showed that the transcriptional level of CP43 was down-regulated but not that of CP47 or D1 in zel1. In addition, the plastoquinone (PQ) QA was in a reduced state in zel1. On the basis of the results, we suggest that CRTISO might function in regulating the transcription of CP43 and the translation of CP47 by affecting the redox state of the PQ to stabilize the extrinsic proteins of oxygen evolution complexes in the rice plant.

Keywords: carotenoid isomerase; lutein; PS II supercomplexes; oxygen evolution

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Introduction

In higher plants, photosynthesis takes place with two photosystems, photosystem I (PS I) and photosystem II (PS II). PS II is involved in the oxidation of water and consequent evolution of oxygen. PS II core complexes are composed of D1, D2, CP43, CP47, α- and β-subunits of cytochrome b559, and PsbI protein [1]. The D1 and D2 proteins are the reaction center (RC) proteins in the heart of PS II where the charge separation occurs. CP43 and CP47 binding to the opposite side of RC are light-harvesting proteins like the inner antennae. CP43 is next to D1, whereas CP47 closely associates with D2 [2–5]. CP43 and CP47 accept the excited energy harvested by peripheral antennae and then transfer it to the RC [6]. However, when the ability to utilize light in photosynthesis is limited under stressed conditions, excessive photon flux would lead to the generation of reactive oxygen species (ROS), which would damage the photosynthetic apparatus [7]. The absorbed excess light energy can be quenched directly or indirectly by xanthophylls, thereby protecting the PS II from oxidative damage [8].

Lutein is the most abundant pigment of xanthophylls and has been demonstrated to function in photoprotection [8–11]. The analysis of the pigments complexes in maize has indicated that lutein is bound to PS II core proteins CP43 and CP47 [12]. Lutein is also suggested to be involved in non-photochemical quenching by the study of the lutein-deficient Arabidopsis mutant lut2 [13]. In addition, carotenoid isomerase (CRTISO) catalyzes the isomerization of poly-cis-carotenoids to all trans-carotenoids in higher plants. Knockout of CRTISO leads to the accumulation of poly-cis-carotenoids and results in reduced lutein content in light conditions in Arabidopsis ccr2 (carotenoid and chloroplast regulation) and tomato tangerin...
Rice carotenoid isomerase functions in photosynthetic oxygen evolution

Mutants [14,15]. The *Arabidopsis* CRTISO has been demonstrated to be required for carotenoid biosynthesis to form the prolamellar bodies which are the lattice structures with tubular membranes [14] and for the regulation of carotenoid composition and shoot branching in *Arabidopsis* by a chromatin-modifying histone methyltransferase [16]. The rice carotenoid isomerase (OsCRTISO) is essential for the biosynthesis of carotenoid precursor of abscisic acid and mutation of this gene by T-DNA insertion lead to pre-harvest sprouting and photo-oxidation, resulting in a decrease in the maximal quantum efficiency of PS II ($F_\text{m} / F_\text{m}$) and PS II core proteins CP43 and CP47 [17]. The results indicated that OsCRTISO plays a protective role for PS II core proteins from photo-oxidation probably through its product lutein. However, the mechanism of the photoprotection of lutein for the core proteins of PS II is still unclear.

In this work, we cloned a point mutated gene reported to encode OsCRTISO which is responsible for accumulation of lutein in rice mutant zell (zebra-leaf1) by a map-based cloning approach. The mutation leads to the decrease in the activity of photosynthetic oxygen evolution accompanied by the lack of LHCCI trimers and reduced amount of the CP43-free PS II complexes and the PS II supercomplexes. Further investigation showed that the core proteins of PS II CP43 and CP47 and the oxygen-evolving extrinsic proteins PsbO and PsbP were reduced but not RC protein D1. The transcript level of CP43 was down-regulated but not that of PsbO and PsbP were reduced but not RC protein D1. The activity of photosynthetic oxygen evolution accompanied by the lack of LHCCI trimers and reduced amount of the CP43-free PS II complexes and the PS II supercomplexes. Further investigation showed that the core proteins of PS II CP43 and CP47 and the oxygen-evolving extrinsic proteins PsbO and PsbP were reduced but not RC protein D1. The transcript level of CP43 was down-regulated but not that of D1 and CP47. In addition, the plastoquinone (PQ) $Q_\Lambda$ was in a reduced state in *zell*. On the basis of the results, we suggest that OsCRTISO might function in the regulation of the transcription of CP43 and the translation of CP47 by affecting the redox state of the PQ.

Materials and Methods

Plants materials

A rice mutant *zell* was screened from a mutagenic *japonica* line 9522 with γ-ray [18–21]. The plants for experiments were planted in a phytotron (about 400 m², 12-h light at 28°C and 12-h dark at 20°C, 50% relative humidity).

Map-based cloning of *ZEL1*

By using a 96 F2 population, the *ZEL1* locus was first mapped to a region between InDel marker CH1137 and LH11-2. Afterwards, *ZEL1* was fine mapped to a region about 200 kb between InDel marker WJL11-52 and WJL11-16 in which there are about 50 putative genes predicted by TIGR rice database (http://rice.plantbiology.msu.edu/). Considering the alteration of pigments composition, a putative gene named carotenoid isomerase included in this region was strongly suggested to be the candidate gene. We designed a primer based on the rice genome sequence to amplify the genomic DNA of the gene from the *zell* and 9522 cultivar. Then, we identified the mutation locus by comparing the two sequences by bi2seq. The cDNA of *ZEL1* was assembled by several expressed sequence tags (ESTs), including the EST TA25549_4530 and EST CK056723 from the TIGR database (http://planutta.tigr.org/) and the EST 012-M019F-C07 based on KOME (http://cdna01.dna.afrc.go.jp/cDNA/), which was the 5′-terminal EST sequence of *ZEL1*. These three fragments almost cover the full cDNA of *ZEL1*, only two gaps totally about 400 bp exited. To cover the two gaps, we amplified the total RNAs purified from young leaves by reverse transcription—polymerase chain reaction (RT–PCR) by using the *zell*-specific primers with the annealing temperature of 55°C, 5′-GAGGGTGCTGTGCTGGAG-3′ (forward) and 5′-AACATGTCATGATAGAACAGAATTTTCAGGA T-3′ (reverse); 5′-AGGACAGTGAAATACGCTACA-3′ (forward) and 5′-ATCCCTTCTGGGACAGACCTTT-3′ (reverse). The gene structure was deduced by comparing the sequence of cDNA and genomic DNA.

Measurements and analysis of chlorophyll fluorescence

The parameters of chlorophyll fluorescence were measured by using a PAM emitter-detector unit 101 ED according to the previous description [22–24]. The minimum chlorophyll fluorescence at the open PS II center ($F_\text{m}$) and at a steady state during actinic light illumination ($F_\text{m}$). $F_\alpha$ is the average of the pre-trigger fluorescence signal for 0.2 after a saturation pulse is triggered. Chlorophyll parameter $q_\text{L}$ was calculated as $(F_\text{m} - F_\alpha) / (F_\text{m} - F_0) \times F_0/F_\alpha$ [25]. The leaves of both wild type (WT) and mutant applied to these experiments were grown in the phytotron and dark adapted overnight before the measurements.

Oxygen evolution of PS II

Thylakoid membranes were extracted according to previous methods [26]. The rice leaves were homogenized in cold STN medium (0.4 M sucrose, 50 mM Tris–HCl, pH 7.6, and 10 mM NaCl), and the homogenate was centrifuged at 200 g for 5 min at 4°C, then the supernatant was centrifuged at 6000 g for 10 min at 4°C. Finally, the pellet was suspended in STN medium. The chlorophyll content was determined according to the methods of Porra et al. [23]. Total protein content was determined with the Bio-Rad protein assay kit after the thylakoid membranes were treated with 5% Triton X-100 shaking on ice for 1 h.
The thylakoid membranes (5 μg Chl/ml) were stirred in the thermostated glass vessel of a Clark-type oxygen electrode in the reaction buffer [0.4 M sucrose, 50 mM Tris–HCl, pH 7.6, 10 mM NaCl, 2 mM MgCl₂, 2 mM EDTA, 0.5 mM p-benzoquinone (p-BQ), and 2 mM NH₄Cl] at 25°C. O₂ evolution was normally detected 2 min after the start of illumination (800 μmole photons/m² s). The solubility of O₂ in water is 253 μM at 25°C.

**Electrophoresis and immunoblotting assay**

Blue native-polyacrylamide gel electrophoresis (BN-PAGE) was carried out as described [26–28]. The prepared thylakoid membranes were washed in the buffer (330 mM sorbitol and 50 mM BisTris–HCl, pH 7.0). After centrifugation at 12,000 g for 1 min, the thylakoid membranes were resuspended in suspension buffer (20% glycerol and 25 mM BisTris–HCl, pH 7.0) containing 1.0 mg Chl/ml. The 2% n-dodecyl-β-D-maltoside (DM) solution was added to the thylakoid suspension gently with equal volume. The mixture was centrifuged at 12,000 g for 10 min after shaking on rice for 1 h. The sample buffer (5% Serva blue G in 100 mM BisTris–HCl, pH 7.0, 0.5 M 6-amino-n-caproic acid, and 30% (w/v) glycerol) was added to the supernatant with 1/10th volume of the supernatant. The sample was loaded (each lane containing 30 μg proteins) and separated by a gradient gel from 5% to 13% at 4°C.

For total protein isolation, the leaves of WT and mutant were ground with liquid nitrogen then dissolved in TN buffer [50 mM Tris–HCl, pH 7.6, 10 mM NaCl, and 2% sodium dodecyl sulfate (SDS)] and denatured in a boiling water bath for 5 min. Each sample with 20 μg proteins was separated by SDS–PAGE in a 15% polyacrylamide gel with 6 M urea as described by Laemmli [29]. For the immunoblotting of two dimension gel, each lane containing several separated pigments complexes was cut from the BN-PAGE gel and treated with 1% SDS for 1 h, then subjected to the second dimension SDS–PAGE. Proteins separated in the SDS–PAGE were electrically transferred into a PVDF membrane for western blot analysis. After blocking with 5% skimmed milk in TBST buffer (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20), the membrane was incubated with the first antibodies and second antibody in TBST buffer, respectively. The signals were detected by X-ray films placed on the antibodies containing membranes after treatment with an ECL Plus Western Blotting Detection Kit (GE Healthcare, Little Chalfont, UK). The antibodies of D1, CP43, and CP47 were bought from the Agrisera Company (Vännäs, Sweden). The second antibody against rabbit IgG was purchased from GE Healthcare.

**Northern blot analysis**

Total RNA was extracted using the RNAagents total RNA isolation system (Promega, Madison, USA). Northern blot analysis was performed as described [30]. The digoxigenin (DIG)-labeled probes were obtained by PCR with Roche digoxigenin gel shift kit (Mannheim, Germany). The hybridization signals in the nylon membrane were detected with the DIG nucleic acid detection kit and CDP-STAR kit from Roche Diagnostics (Mannheim, Germany). The primers for the amplification of the probe for detecting PsbA, PsbB, and PsbC were 5′-CCCTCATTAGCAGA TTCGTTT-3′ and 5′-GTATTATATTACGCCCTTCATCG-3′; 5′-TTCAGGAGAAGCTAAGCAA-3′ and 5′-AGGTG GACGGACACTAAGATG-3′; and 5′-CATCTAAATTTCCT CCGCAGTC-3′ and 5′-TACCTAAACCTGTGGGTGCCTT-3′, respectively.

**Results**

Isolation of a lutein-reduced mutant **zel1**

To identify rice genes that are important for photosynthesis in rice, a lutein-reduced mutant **zel1** (zebra-leaf1) of *Oryza sativa* with variegated leaves was isolated from a γ-ray-irradiated *japonica* line (Fig. 1). The M₂ progeny of heterozygote yields a segregation of 370 normal and 108 mutant plants (χ²[3:1] = 1.40; 0.25 > P > 0.1), indicating monofactorial recessive inheritance of the mutant characteristic [18–21]. Different from the greenish leaves of the WT rice plants [Fig. 1(B)], the leaves of **zel1** showed yellowish at the first week after germination (data not shown) and became obviously variegated yellowish after about 1 month [Fig. 1(B)]. The height of a WT plant grown in the green house for 3 months was 660 ± 12 mm, whereas that of the mutant was 486 ± 21 mm (n = 6), and **zel1** had the normal tillering capacity [Fig. 1(A)]. Moreover, the flowering time of **zel1** was delayed about 1 month, and its yield was evidently decreased.

Pigment analysis showed that the total chlorophyll content in the leaves of **zel1** was about 40% lower than that of the WT under normal growth conditions and the ratio of chlorophyll a/b was higher (3.0 ± 0.05 in WT; 3.5 ± 0.04 in **zel1**). Especially, the lutein content was about 30% in **zel1** (about 36 ± 3 mmole/ mole chlorophyll) of that in the WT (123 ± 19), zeaxanthin was hardly detectable, whereas the violaxanthin and antheraxanthin increased nearly 3-fold in the mutant.

**Molecular cloning of **ZEL1** gene**

The gene that controls the phenotype of **zel1** was cloned using a map-based cloning approach combined with pigment analysis. The gene was predicted to encode a carotenoid isomerase, which has been named *OsCRTISO* by a T-DNA insertion mutant of the rice *OsCRTISO/phs3-2* [17],...
indicating that *zell* is a new allele of *Oscrtiso/phs3-2*. RT–PCR analysis using *OsCRTISO*-specific primers and comparison of the sequence similarity with that in the TIGR plant transcript database (www.tigr.org) revealed that the *OsCRTISO* gene consists of 13 exons and 12 introns and encodes 586 amino acid residues. *OsCRTISO* was constitutionally expressed in the leaf, stem, and root from early to later stages of growth. The alignment analysis of these carotenoid isomerases indicated that the *OsCRTISO* protein had 83% identity with the CCR2 of *Arabidopsis*. The complement line recovered the phenotype of the WT (Fig. 1), confirming *ZEL1* is an *OsCRTISO* gene.

**Activity of photosynthetic oxygen evolution in *zell***

Previous study has indicated that the inactivation of *OsCRTISO* caused the decrease in the maximal quantum efficiency of PS II (*Fv/Fm*) and PS II core proteins CP43 and CP47 in rice [17]. To further investigate the mechanism of photoprotection of PS II, the activity of photosynthetic oxygen evolution in thylakoid membranes was compared between the WT and *zell* in the presence of an artificial electron acceptor *p*-BQ. The activity of oxygen evolution was lowered by about 18% in *zell* (Fig. 2), suggesting that the function of PS II core complexes was impaired in *zell*. Although the activity of oxygen was increased in the complement line, there is no significant difference with that in WT. The results indicated that the inactivation of *OsCRTISO* influences the activity of PS II in the mutant.

**PS II core proteins in different PS II complexes of *zell***

To further investigate the amount of the PS II core proteins CP43 and CP47 and D1 in the PS II complexes, immuno-detection analysis was carried out with the antibodies raised against these proteins after the pigment–protein bands separated by the BN gel were subjected to second dimension SDS–PAGE. The amount of CP47 was much lower in all the PS II complexes especially in the LHCII-PS II supercomplexes and CP43-free PS II of *zell* than that of WT (Fig. 3). On the other hand, the amount of CP43 in *zell* was decreased in the top two supercomplexes, whereas it was increased in the lower ones (band I) and PS II monomer (band III).

**The amount of PS II core proteins and oxygen-evolving extrinsic proteins in *zell***

It is generally accepted that the PS II core proteins play an important role in maintaining oxygen-evolving extrinsic proteins. Therefore, the total amount of the PS II core proteins CP43 and CP47 and the oxygen-evolving extrinsic proteins PsbO and PsbP in chloroplasts was compared between *zell* and the WT. As shown in Fig. 4(A), either the amount of the PS II core proteins CP43 and CP47 or the oxygen-evolving extrinsic proteins PsbO and PsbP was lower in *zell* than those in WT, but not that of D1 protein,
indicating that a lower amount of the PS II core proteins affected the accumulation of the oxygen-evolving extrinsic proteins in \textit{zel1}.

The transcript level of the PS II core proteins of \textit{zel1}

Northern blot analysis was carried out to investigate whether the decrease in both CP43 and CP47 proteins in the mutant was influenced at the transcriptional level. Figure 4(B) showed that the transcriptional level of \textit{PsbA} gene (encoding D1 protein) was similar between \textit{zel1} and the WT, which is coincident with the expression level of D1 protein. However, the transcriptional level of \textit{PsbC} gene encoding CP43 protein was remarkably lowered, whereas \textit{PsbB} gene encoding CP47 protein was slightly high in \textit{zel1}. The results indicated that the transcript of \textit{PsbC} gene is down-regulated but that of \textit{PsbB} is slightly up-regulated when the \textit{OsCRTISO} gene is defective.

The redox state of the PQ pool

To study whether the down-regulation of the PS II core proteins is related to the redox state of the electron transport chain, the redox state of the PQ pool was compared between WT and \textit{zel1} with the parameter of chlorophyll fluorescence $1 - qL$ which reflects the reduction state of PQ pool $Q_A$ of PS II. Figure 5 showed that there was not much difference in reduction state of $Q_A$ between WT and mutant at low light $<50 \mu$mole photons/m$^2$s, but significantly increased in \textit{zel1} at high light $>50 \mu$mole.
photons/m² s, indicating that Qₐ was in a much more reduced state in zeal even under the growth light condition (400 μmole photons/m² s).

**Discussion**

In higher plants and cyanobacteria, at least six intrinsic proteins (D1, D2, CP43, CP47, and the two subunits of cytochrome b559) are required for oxygen-evolving activity [31]. In the present work, we investigated the role of OsCRTISO in the expression of the PS II core proteins CP43 and CP47 using a lutein-reduced mutant zeal. Reduced accumulation of lutein by mutation of the OsCRTISO gene caused the suppression of accumulation of PS II core proteins CP43 and CP47 [Fig. 3(B)], consistent with the result from its allele of Oscrtiso/phs3-2 [17]. Our study further suggested that the lowered amount of the PS II core protein CP47 might result from the suppression of LHCII-PS II supercomplexes due to the defect in LHCII trimer in zeal [Fig. 3(A)].

The lower level of CP43 is caused by the lower level of its transcript level but that of CP47 is not [Fig. 4(B)], indicating that OsCRTISO might regulate the transcript of CP43 and the translation of CP47. On the other hand, the lack of CP47 in the supercomplexes has no effect on the position of the smaller supercomplex bands in band I [Fig. 3(A)]. It might be the compensation by the increased amount of CP43 in the smaller supercomplex [Fig. 3(B)]. To acclimate to the changing environmental conditions, photosynthesis functions as a sensor of external signals to regulate photosynthesis genes expression [32]. It has been known that some photosynthesis and stress-responsive genes are transcriptional and translational regulated by the redox state of the intersystem electron carriers in both cyanobacteria and higher plants [32–37]. Appropriately oxidized state of PQ induced expression of ndh genes in translation level [38]. The mutation in the OsCRTISO gene caused the PQ pool in a reduced state (Fig. 5), which might suppress the expression of the PS II core proteins CP43 and CP47 (Fig. 4).

CP43 is suggested to play a role in the binding of PsbO (33 kDa protein) protein, whereas PsbP (23 kDa protein) is bound to PsbO [4]. The decrease in PsbO and PsbP in the mutant might be the result of the lower amount of CP43 [Fig. 4(A)]. Since PsbO and PsbP function in stabilizing the Mn cluster of the water oxidizing complex [39], the decrease in these proteins would result in the suppression of the activity of photosynthetic oxygen evolution in the lutein-reduced mutant (Fig. 2). It is commonly accepted that photosynthesis is the basis of plant yield. The restrain of the growth of zeal mutant might be attributed to the suppression of the photosynthetic oxygen evolution (Fig. 2). In addition, the significant increase in xanthophyll cycle pigments, violaxanthin and antheraxanthin, while zeaxanthin is suppressed in zeal suggests that the xanthophyll cycle was inhibited, which would cause lowering in the ability of dissipation of excess excitation energy and increase in ROS production. In this case, photoinhibition would occur under the strong light condition in zeal.

In conclusion, the above results suggested that OsCRTISO might be involved in the regulation of the expression of the PS II core proteins, which are essential for the stabilization of oxygen-evolving extrinsic proteins, thereby maintaining the activity of photosynthetic oxygen evolution in rice.

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