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Cyclobutane pyrimidine dimer (CPD) photolyase repairs ultraviolet-B-induced CPDs in rice chloroplast and mitochondrial DNA

Masaaki Takahashi[1], Mika Teranishi[1], Hiroyuki Ishida[2], Junji Kawasaki[1], Atsuko Takeuchi[1], Tomoyuki Yamaya[2], Masao Watanabe[1], Amane Makino[2] and Jun Hidema[1,*]

[1]*Graduate School of Life Sciences, Tohoku University, Sendai 980-8577, Japan, and*

[2]*Graduate School of Agricultural Sciences, Tohoku University, Sendai 981-8555, Japan*

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*For correspondence (fax +81 22 217 5691; e-mail j-hidema@ige.tohoku.ac.jp).

SUMMARY

Plants use sunlight as energy for photosynthesis; however, plant DNA is exposed to the harmful effects of ultraviolet-B (UV-B) radiation (280–320 nm) in the process. UV-B radiation damages nuclear, chloroplast and mitochondrial DNA by the formation of cyclobutane pyrimidine dimers (CPDs), which are the primary UV-B-induced DNA lesions, and are a principal cause of UV-B-induced growth inhibition in plants. Repair of CPDs is therefore essential for plant survival while exposed to UV-B-containing sunlight. Nuclear repair of the UV-B-induced CPDs involves the photoreversal of CPDs, photoreactivation, which is mediated by CPD photolyase that monomerizes the CPDs in DNA by using the energy of near-UV and visible light (300–500 nm). To date, the CPD repair processes in plant chloroplasts and mitochondria remain poorly understood. Here, we report the photoreactivation of CPDs in chloroplast and mitochondrial DNA in rice. Biochemical and subcellular localization analyses using rice strains with different levels of CPD photolyase activity and transgenic rice strains showed that full-length CPD photolyase is encoded by a single gene, not a splice variant, and is expressed and targeted not only to nuclei but also to chloroplasts and mitochondria. The results indicate that rice may have evolved a CPD photolyase that functions in chloroplasts, mitochondria and nuclei, and that contains DNA to protect cells from the harmful effects of UV-B radiation.

Keywords: cyclobutane pyrimidine dimer (CPD), CPD photolyase, DNA repair, mitochondria, chloroplasts, rice plant.

INTRODUCTION

Ultraviolet-B (UV-B; 280–320 nm) radiation in sunlight suppresses photosynthesis and protein biosynthesis, thereby decreasing growth and productivity (Teramura, 1983). UV-B radiation also induces photodamage in DNA. The major UV-B-induced DNA lesions are cyclobutane pyrimidine dimers (CPDs), and pyrimidine–pyrimidone (6–4) photoproducts that are formed between adjacent pyrimidines on the same DNA strand (Sancar and Sancar, 1988). CPDs account for most of these lesions (approximately 75%), whereas the remainder are (6–4) photoproducts (Mitchell and Nairn, 1989). DNA damage can be mutagenic or lethal by impeding replication and transcription (Brash *et al.*, 1991, 1987). We previously demonstrated that UV-B-induced CPDs are a principal cause of UV-B-induced growth inhibition in rice grown under supplementary UV-B radiation (Hidema *et al.*, 2007). Plants possess mechanisms to cope with UV-B-induced CPD in nuclear DNA (ncDNA), such as photoreactivation (photorepair) and nucleotide excision repair (dark repair). Photorepair is mediated by the photolyase, which absorbs blue/UV-A (320–400 nm) radiation, and uses the energy to monomerize dimers (Britt, 1996). In dark repair, the dimers are replaced by way of *de novo* DNA synthesis, where the undamaged complementary strand is employed as the template (Britt, 1996). Photorepair is the primary mechanism for repairing UV-B-induced CPDs in higher plants, as the rate of dark repair is slower than that of photorepair (Hidema *et al.*, 1997; Quaite *et al.*, 1994; Sutherland *et al.*, 1996).

In addition to the nuclear genome, the cells of higher plants contain two additional genomes, one in chloroplasts and another in mitochondria. The chloroplast and mitochondrial genomes contain genes that encode many

proteins important for photosynthesis and respiration, respectively. UV-B radiation also induces the formation of CPDs in these organellar DNAs (Chen *et al.*, 1996). Thus, chloroplasts and mitochondria might also use a pathway(s) that repairs CPDs and efficiently removes the DNA lesions before replication and transcription. However, Chen *et al.* (1996) reported that the multiple copies of the chloroplast and mitochondrial genomes might make repair unnecessary in plants because UV-B-irradiated cells would contain multiple undamaged copies of organelle genomes. Thus, the necessity for CPD repair in chloroplasts and mitochondria is still under debate.

Saccharomyces cerevisiae efficiently photoreactivates CPDs using CPD photolyase in both nuclei and mitochondria (Prakash, 1975; Yasui *et al.*, 1992), and both the plastid and nuclear genomes of *Chlamydomonas* undergo photoreactivation (Small, 1987). *Chlamydomonas* CPD photolyase contains a chloroplast-targeting sequence. However, no genes that encode CPD repair proteins have been identified in the chloroplast or mitochondrial genomes of Arabidopsis or *Oryza sativa* (rice). Several studies examining CPD repair activity in chloroplasts and mitochondria of higher plants under conditions of visible light or darkness have been reported. Photoreactivation activity was not detected in isolated *Spinacia oleracea* (spinach) chloroplasts (Hada *et al.*, 2000), and young Arabidopsis seedlings (5 days old) displayed no photorepair activities in their chloroplasts or mitochondria (Chen *et al.*, 1996). Conversely, CPDs in chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) of *Zea mays* (maize) leaves (Stapleton *et al.*, 1997), or UV-induced polymerase-blocking lesions in the cpDNA of *Glycine max* (soybean) cells (Cannon *et al.*, 1995), are reduced in number in response to visible light. Leaves of Arabidopsis (14 days old) showed blue-light-dependent removal of CPDs in cpDNA and mtDNA (Draper and Hays, 2000); however, it was unclear whether this was mediated by CPD photolyase. We demonstrate here that rice CPD photolyase, encoded by a single gene and not a splice variant, is expressed and targeted not only to nuclei but also to chloroplasts and mitochondria, and functions to repair UV-B-induced CPDs. Thus, a single DNA repair enzyme CPD photolyase is 'triple targeted' in rice cells, functioning in nuclei, chloroplasts and mitochondria. Our results indicate that rice may have evolved a CPD photolyase that protects cells from the harmful effects of UV-B radiation.

RESULTS

UV-B-induced CPDs in chloroplasts, mitochondria and nuclei are reduced under blue-light conditions

To ascertain the existence of pathways that repair UV-B-induced CPDs in cpDNA and mtDNA, we used two different DNA repair assays to examine the fourth leaf of 20-day-old rice seedlings. The first assay was a site-specific assay for

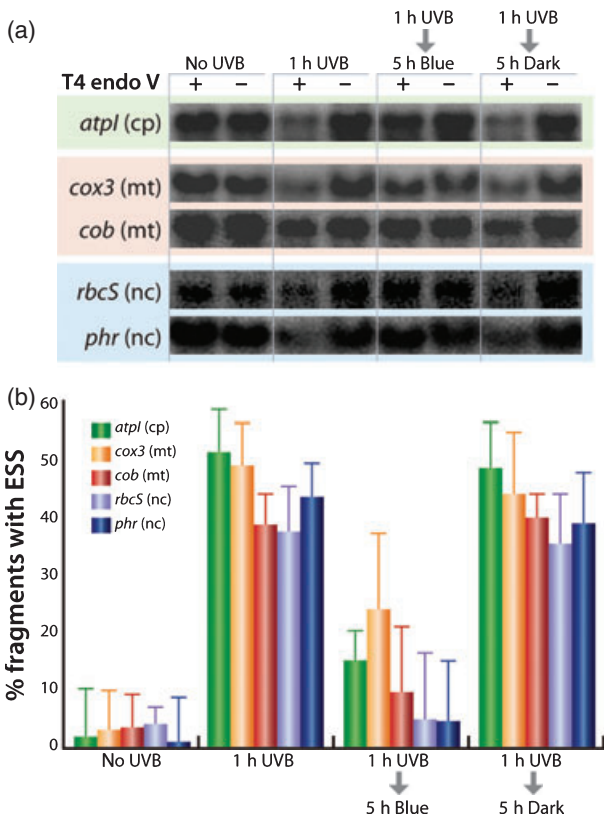

Figure 1. Repair of UV-B-induced DNA damage in rice genomic DNA.
(a) Detached rice leaves were harvested (no UV-B) or exposed to UV-B radiation at a dose of 5 W m^{-2} for 1 h. Leaves were then harvested immediately (1 h UV-B) and exposed to blue light for 5 h (5 h Blue) or kept in a light-tight box for 5 h (5 h Dark). Genomic DNA was isolated and digested with restriction enzymes, and then the digested DNA was incubated with (+) or without (–) T4 endo V. The membrane was incubated with ^{32}P-labeled probes for the chloroplast-encoded gene *atpI*, the mitochondrial-encoded genes *cox3* and *cob*, and the nuclear-encoded genes *rbcS* and *phr*.
(b) The percentage (%) fragments with ESS was calculated as the ratio of the intensity of T4 endo V-treated to the intensity of untreated DNA. Data represent the means ± SDs of at least three samples.

detecting CPDs by using T4 endonuclease V (T4 endo V) digestion and Southern blot analysis (Chen *et al.*, 1996; Hanawalt, 1989). Restriction enzyme-digested genomic DNA was either subjected to T4 endo V digestion or not. The DNA fragments were separated by alkaline gel electrophoresis and analysed by Southern blot using radiolabeled probes specific for chloroplast (ATPase a subunit, *atpI*, 11.0 kb), mitochondrial (cytochrome *c* oxidase subunit 3, *cox3*, 4.5 kb; apocytochrome *b*, *cob*, 4.0 kb) and nuclear (small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase, *rbcS*, 6.0 kb; CPD photolyase, *phr*, 4.4 kb) genes. Figure 1a shows the T4 endo V-digested and undigested DNA detected by Southern blot analysis. The intensities of the DNA bands were quantified, and the percentage of fragments with enzyme-sensitive sites (ESSs) was calculated as the ratio of the intensity of T4 endo V-treated DNA

to the intensity of untreated DNA (Figure 1b). The percentage of fragments with ESS induced by exposure to UV-B radiation at a dose of 5 W m^{-2} for 1 h increased in each of the targeted genes. Notably, the rates of fragments with ESS in chloroplasts and mitochondria, as well as in nuclei, decreased following a 5-h exposure to blue radiation. There was no apparent change in the percentage of fragments with ESS in any targeted gene in response to 5 h of darkness. These results suggest that, as for rice nuclei, rice chloroplasts and mitochondria can photoreactivate CPDs.

Similar results were obtained using quantitative PCR (qPCR) with genome-specific primers to measure polymerase-blocking lesions (Figure 2). Quantitative PCR is inhibited when *Taq* DNA polymerase encounters UV-induced photoproducts or other lesions in the template DNA. Thus, the use of this assay assumes that PCR-blocking photoproducts are randomly distributed along the DNA template (Kalinowski *et al.*, 1992; McCarthy *et al.*, 1996). The frequency with which lesions are encountered is a measure of the percentage of DNA fragments with one or more lesions. The percentage of DNA fragments with one or more lesions from chloroplasts, mitochondria and nuclei increased in response to increasing doses of UV-B radiation (Figure 2a).

Next, leaves were exposed to UV-B radiation at a dose of 5 W m^{-2} for 1 h to produce an initial level of approximately 30% of DNA fragments with one or more lesions. Immediately after exposure to UV-B, each sample was irradiated with blue light for various times and the percentage of fragments with lesions remaining was determined. The percentages of DNA with lesions in chloroplasts, mitochondria or nuclei decreased under blue light conditions (Figure 2b).

CPD photoreactivation activity in chloroplasts, mitochondria and nuclei of rice strains with different levels of CPD photolyase activity

To determine whether the photoreactivation activity in chloroplasts or mitochondria was mediated by CPD photolyase, we measured the CPD photoreactivation rate in chloroplasts and mitochondria of the UV-resistant Sasanishiki and UV-sensitive Surjamkhi rice strains using Southern blot analysis. The Surjamkhi strain is deficient in CPD photorepair

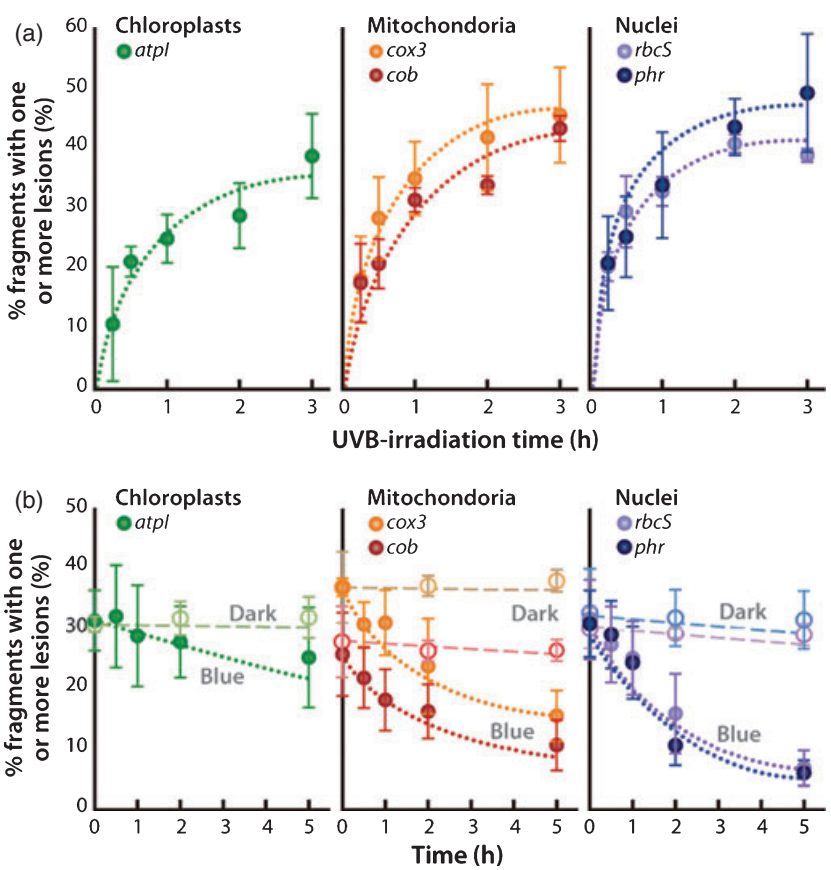

Figure 2. Repair of UV-B-induced DNA damage in rice genomic DNA, as determined by quantitative PCR analysis.
(a) Detached leaves of Sasanishiki rice plants were exposed to UV-B radiation at a dose of 5 W m^{-2} for various lengths of time. Genomic DNA was isolated from the leaves and amplified using primers specific for a chloroplast-encoded gene (*atpI*), mitochondrial-encoded genes (*cox3* and *cob*) or nuclear-encoded genes (*rbcS* and *phr*). The percentage of fragments with one or more lesions was calculated as described in the Experimental procedures.
(b) Detached leaves of Sasanishiki rice plants were exposed to UV-B radiation at a dose of 5 W m^{-2} for 1 h. Leaves were then exposed to blue light (closed circles) or were kept in a light-tight box (open circles) for various lengths of time. The data represent the means ± SDs of at least five samples.

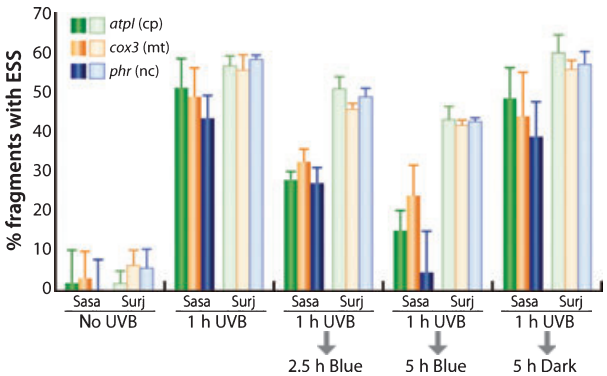

Figure 3. Repair of UV-B-induced DNA damage in rice genomic DNA in the UV-resistant Sasanishiki and UV-sensitive Surjamkhi strains.
Detached leaves of Sasanishiki (Sasa) or Surjamkhi (Surj) rice plants were analyzed using the probes for the chloroplast-encoded gene *atpI*, the mitochondrial-encoded gene *cox3* and the nuclear-encoded gene *phr*, as described in Figure 1. The data represent the means ± SDs of at least three samples.

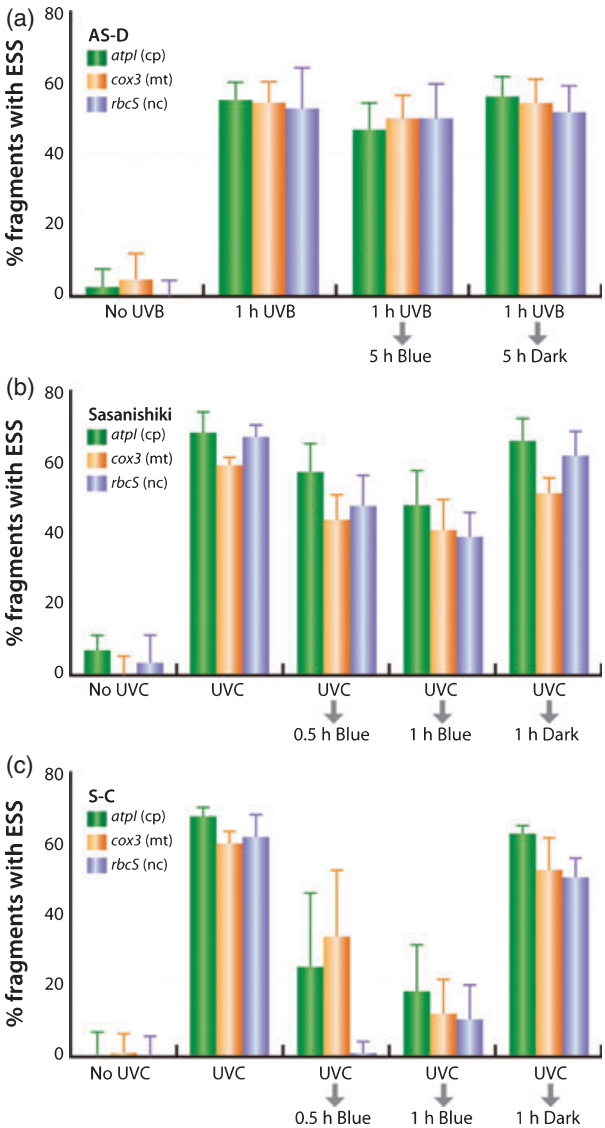

Figure 4. UV-B-induced DNA damage repair in rice genomic DNA.
(a) Detached rice leaves of transgenic rice (AS-D) expressing antisense RNA for cyclobutane pyrimidine dimer (CPD) photolyase were harvested (no UV-B) or exposed to UV-B radiation at a dose of 5 W m^{-2} for 1 h. The leaves were harvested immediately after radiation (1 h UVB) and then exposed to blue light for 5 h (5 h Blue), or were kept in a light-tight box for 5 h (5 h Dark). The percentage of fragments with enzyme-sensitive sites (ESSs) was measured using the probes for the chloroplast-encoded gene *atpI*, the mitochondrial-encoded gene *cox3* and the nuclear-encoded gene *phr*, as described in Figure 1.
(b, c) Detached rice leaves of Sasanishiki (b) and transgenic S-C (c) plants were treated as described in (a), except that leaves were exposed to UV-C radiation instead of UV-B radiation. The data represent means ± SDs of at least three samples.

when compared with the Sasanishiki strain because of two amino acid differences in the CPD photolyase (Hidema *et al.*, 2005). The CPD photoreactivation rate in chloroplasts, mitochondria or nuclei was higher in Sasanishiki leaves than in Surjamkhi leaves (Figure 3). Exposure to blue radiation for 5 h after UV-B radiation reduced the percentages of Sasanishiki DNA fragments with ESSs of *atpI*, *cox3* and *phr* by approximately 70, 51 and 89%, respectively, whereas in Surjamkhi DNA fragments, these percentages were reduced by approximately 24, 25 and 27%, respectively. The CPD photoreactivation rate was undetectable in chloroplasts, mitochondria or nuclei in transgenic rice (AS-D) engineered to express antisense RNA targeting CPD photolyase in wild-type Sasanishiki rice cultivar with low levels of CPD photolyase activity (Hidema *et al.*, 2007) (Figure 4a). We also examined the CPD photoreactivation rate in transgenic rice (S-C) overexpressing sense RNA targeting CPD photolyase in a wild-type Sasanishiki rice cultivar that had higher CPD photolyase activity than the UV-resistant Sasanishiki plants (Hidema *et al.*, 2007). The CPD photoreactivation rate in chloroplasts and mitochondria was markedly higher in S-C plants than in Sasanishiki plants (Figure 4b,c). These results indicate that the photoreactivation activity in chloroplasts and mitochondria is mediated by CPD photolyase. Furthermore, as the transgenic rice (S-C) was constructed by transformation with the cDNA sequence of rice CPD photolyase, the full-length CPD photolyase, encoded by a single gene and not a splice variant, is expressed and targeted not only to nuclei but also to chloroplasts and mitochondria in rice cells.

The subcellular location of CPD photolyase in rice

To confirm the location of CPD photolyase within cells, rice plants were examined by immunogold electron microscopy (IEM) using an anti-rice CPD photolyase antibody. Twice affinity-purified anti-rice CPD photolyase IgG cross-reacted mono-specifically with denatured CPD photolyase in extracts prepared from transgenic S-C leaves when denatured proteins were separated by SDS-PAGE (Figure 5a).

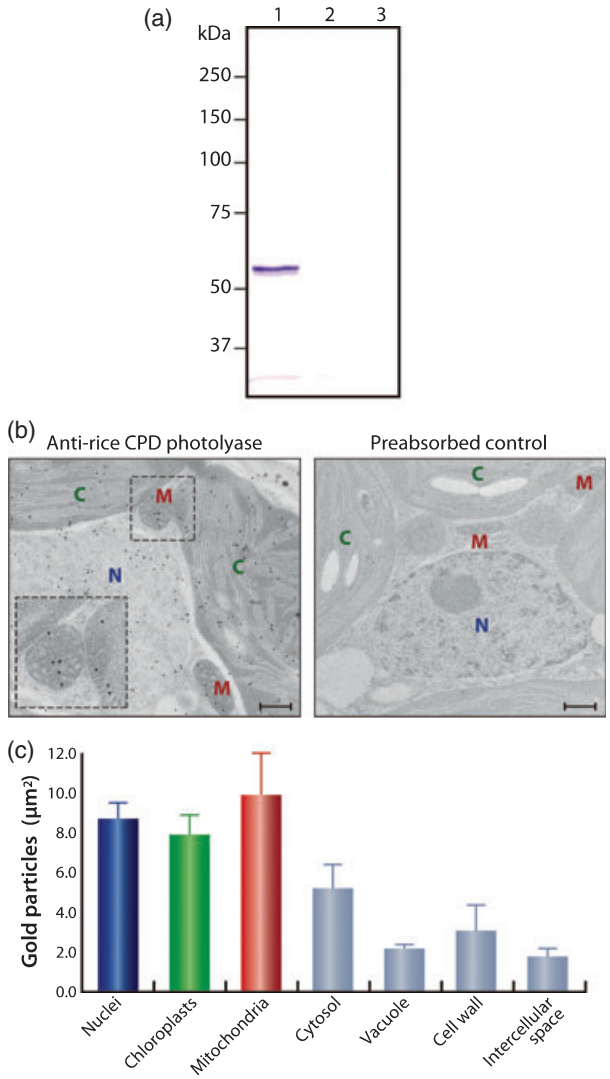

Figure 5. Subcellular location of cyclobutane pyrimidine dimer (CPD) photolyase in rice.
(a) Monospecificity of twice affinity-purified anti-rice CPD photolyase IgG. Lane 1, transgenic rice (S-C); lane 2, wild-type Sasanishiki; lane 3, transgenic rice (AS-D). The positions of the size markers are indicated on the left.
(b) Immunogold labeling of CPD photolyase protein in 20-day-old fourth leaves from S-C transgenic rice with a twice affinity-purified anti-rice CPD photolyase IgG (left) or an anti-rice CPD photolyase antibody that was pre-absorbed with excess recombinant rice CPD photolyase as a control (right). The inset shows an increased magnification of the boxed section. Key: C, chloroplasts; M, mitochondria; N, nuclei. Scale bar: 1 μm.
(c) Immunogold labeling was quantified by counting gold particles per unit area (gold particles μm^{-2}) of each cellular compartment. Values represent the immunolabeling density of each cellular compartment. The data represent the means ± SDs of at least three samples.

The twice affinity-purified anti-rice CPD photolyase IgG cross-reacted specifically with two polypeptides of approximately 54- and 56-kDa following SDS-PAGE and immunobloting (Figure 5a, lane 1). We recently demonstrated that CPD photolyase is phosphorylated, and that both phosphorylated and non-phosphorylated CPD photolyase are present in rice *in vivo*. Furthermore, the 54- and 56-kDa polypeptides correspond to the non-phosphorylated and phosphorylated CPD photolyase, respectively (Teranishi *et al.*, 2008). The 54- and the 56-kDa CPD photolyase were not detected in extracts prepared from Sasanishiki or transgenic rice, AS-D (Figure 5a, lanes 2 and 3), because the expression levels in Sasanishiki and AS-D were low (Hidema *et al.*, 2007). Sasanishiki rice leaves were examined by IEM using the twice affinity-purified anti-rice CPD photolyase IgG. However, gold particles were not detected in Sasanishiki rice cells because the level of CPD photolyase in Sasanishiki rice leaves is below the limit of detection (data not shown). Next, we attempted the IEM assay using transgenic S-C leaves. Immunolabeling with the twice affinity-purified anti-rice CPD photolyase IgG was specific for nuclei, chloroplasts and mitochondria (Figure 5b). With the exception of cytosol, background labeling of other cellular compartments such as the vacuole, cell wall and intercellular space was low or negligible. The control, which was labeled with anti-rice CPD photolyase IgG that had been pre-absorbed with excess quantities of recombinant rice CPD photolyase, showed little immunogold labeling (Figure 5b). Immunogold labeling was quantified by counting gold particles per unit of area of the cell compartment and a mean immunolabeling density for each cellular compartment in the section is shown in Figure 5c. Nuclei, chloroplasts or mitochondria contained approximately 8.7, 7.9 or 9.9 gold particles μm^{-2}, respectively. These densities were higher than the densities found for vacuoles, cell walls or intercellular space. Labeling of the cytosol was lower than that of the nuclei, chloroplasts or mitochondria, but it was somewhat higher than that of the other cellular compartments. However, the ratio of gold particle number detected in nucleus, chloroplasts and mitochondria per cell to total gold particle number detected in a cell was approximately 76.1% (n = 6), whereas the ratio of that in cytosol was approximately 7.4% (n = 6). Thus, the gold particles detected in the cytosol could be the CPD photolyase that was being transferred into nuclei, chloroplasts or mitochondria, because a great number of CPD photolyase was expressed in a cell of the transgenic rice S-C line using the cauliflower mosaic virus *35S* promoter; the CPD photolyase transcript level in the S-C line is 150-fold higher than in the wild-type plant (Hidema *et al.*, 2007).

We also prepared chloroplasts, mitochondria or nuclei-enriched fractions from the leaves or calli of S-C plants and analyzed the crude extracts by Western blot using twice affinity-purified anti-rice CPD photolyase antiserum (Figure 6a). The 54- and 56-kDa polypeptide bands were detected in chloroplasts, mitochondria or nuclei-enriched fractions. As described above, both phosphorylated and non-phosphorylated CPD photolyase are present in rice *in vivo*. To confirm whether the 54- and 56-kDa polypeptides shown in Figure 6b were phosphorylated and non-phosphorylated CPD photolyase, we used λ-protein phosphatase

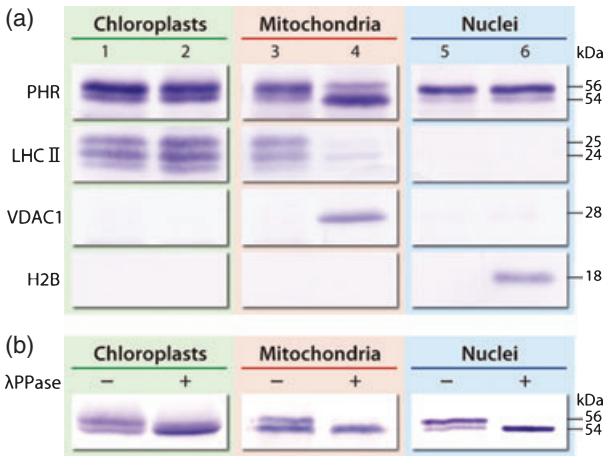

Figure 6. Western-blot analysis of cyclobutane pyrimidine dimer (CPD) photolyase contained in organelle-enriched fractions from the leaves of transgenic S-C plants.

(a) Crude extracts prepared from S-C plants were subjected to SDS-PAGE followed by western-blot analysis using twice affinity-purified anti-rice CPD photolyase IgG (PHR), anti-light-harvesting chlorophyll *a*/*b* protein of PSII antiserum (LHCII; chloroplast marker), anti-voltage-dependent anion-selective channel protein 1 antibody (VDAC1; mitochondrial marker) or anti-histone H2B antibody (H2B; nuclear marker). Lane 1, total protein before fractionation of chloroplasts; lane 2, chloroplast-enriched fraction; lane 3, total protein before fractionation of mitochondria; lane 4, mitochondria-enriched fraction; lane 5, total protein before fractionation of nuclei; lane 6, nuclei-enriched fraction.

(b) The organelle-enriched fractions treated without (−) or with (+) λ-PPase were separated with SDS-PAGE, and then western-blot analysis was performed using twice affinity-purified anti-rice CPD photolyase IgG.

(λ-PPase), an enzyme that removes a phosphate from phosphorylated Ser, Thr and Tyr residues in substrate proteins. After treatment with λ-PPase and subsequent SDS-PAGE, the 56-kDa polypeptide was not detected and the intensity of the 54-kDa polypeptide was increased in the chloroplasts, mitochondria or nuclei-enriched fraction.

We conclude that these two polypeptides represent the phosphorylated and non-phosphorylated forms of the CPD photolyase, and that CPD photolyase translocates to the chloroplasts, mitochondria and nuclei of rice, where it functions in the repair of UV-B-induced CPDs. There was a difference in the quantity of phosphorylated CPD photolyase in chloroplasts, mitochondria and nuclei *in vivo.* Mitochondria showed a high proportion of non-phosphorylated CPD photolyase (Figure 6a, lane 4; 54 kDa), whereas nuclei and chloroplasts had a relatively high proportion of the phosphorylated form (Figure 6a, lanes 2 and 6; the slower migrating band at 56 kDa). Although the locations of the phosphorylation sites and the biochemical and physiological functions of phosphorylated CPD photolyase in rice are unclear, our results raise the possibility that the phosphorylation state of CPD photolyase might be associated with its targeting to different organelles. The molecular mechanism of import, particularly the potential role of phosphorylation, with respect to CPD photolyase in organelles will be the subject of future studies.

DISCUSSION

Rice CPD photolyase, which is encoded by a single-copy gene in the nuclear genome, translocates to chloroplasts, mitochondria and nuclei, and repairs UV-B-induced CPDs in all three genomes.

Cryptochrome *Drosophila*, Arabidopsis, *Synechocystis* and human (Cry-DASH), which identify a new branch of photolyase/cryptochrome family, are primarily found in bacteria, fungi, aquatic vertebrates and in plants, e.g. *Arabidopsis thaliana* (Cry3) (Brudler *et al.*, 2003). The Cry-DASH subfamily of cryptochromes has photolyase activity exclusively for single-stranded CPD-containing DNA substrates (Selby and Sancar, 2006). Recently, Kleine *et al.* (2003) reported that AtCry3, a Cry-DASH subfamily of cryptochromes, carries an N-terminal sequence that mediates import into chloroplasts and mitochondria, although it is not clear if the Cry-DASH cryptochrome has a repair function in chloroplasts and mitochondria *in vivo*. Rice also has a putative Cry-DASH cryptochrome (OsCry-DASH, BAD45850). There are some conserved protein sequences between class-II rice CPD photolyase (506 amino acid residues) and OsCry-DASH (582 amino acid residues), and the identity of amino acid sequences between both proteins is 17%. The anti-rice CPD photolyase antibody used in this study was a polyclonal antibody raised against purified *Escherichia coli*-expressed rice CPD photolyase (Teranishi *et al.*, 2008) that had been purified with the antigen twice (twice affinity-purified anti-rice CPD photolyase IgG), as described in the Experimental procedures. The twice affinity-purified anti-rice CPD photolyase IgG cross-reacts monospecifically with denatured CPD photolyase, and does not cross react with the putative Cry-DASH cryptochrome of transgenic rices, S-C, AS-D and wild-type Sasanishiki used in this study (Figure 5a). The subcellular localization analyses (Figures 5 and 6) demonstrated that CPD photolyase translocates to the chloroplasts, mitochondria and nuclei of the rice plant; however, we did not perform tests to measure the enzymatic repair activity of CPD in single-stranded DNA of chloroplasts, mitochondria or nuclei. In the future, such studies will help understand the CPD repair mechanism *in vivo* in plants.

Our results indicate that rice has an efficient DNA repair system and uses CPD photolyase to repair the UV-B-induced CPDs in mitochondria and chloroplasts, so as to maintain the integrity of each organellar genome. Thus, rice may have evolved a CPD photolyase that functions in chloroplasts, mitochondria and nuclei that contain DNA to protect cells from the harmful effects of UV-B radiation. The photoreactivation rate was not detected in isolated spinach chloroplasts (Hada *et al.*, 2000), and young Arabidopsis seedlings (5 days old) displayed no photorepair in their

chloroplasts or mitochondria (Chen *et al.*, 1996), whereas the leaves of Arabidopsis (14 days old) showed the blue-light-dependent removal of CPDs in cpDNA and mtDNA (Draper and Hays, 2000). Recently, Kaiser *et al.* (2009) transiently transfected a GFP fusion of Arabidopsis-CPD photolyase gene (At-PHR1) (*2x35S::At-PHR1::GFP*) into green protoplasts from an Arabidopsis mesophyll cell culture to investigate the subcellular localization of the At-PHR1 protein. They demonstrated that the At-PHR1::GFP fusion protein was found exclusively in the nuclei, and was not transported into the chloroplasts and mitochondria. Therefore, it is unclear whether the light-dependent removal of CPDs in cpDNA and mtDNA in the leaves of 14-day-old Arabidopsis is mediated by CPD photolyase. Therefore, it is important to determine if there are differences in the subcellular localization of CPD photolyase among different plant species, and how plants survive while growing in sunlight containing harmful UV-B radiation.

A single DNA repair enzyme, CPD photolyase, functions in chloroplasts, mitochondria and nuclei, and is subjected to 'triple targeting' in rice cells. Dual targeting to chloroplasts and mitochondria has been described for several proteins (Peeters and Small, 2001). In general, most of these proteins have an organelle-targeting sequence located at the amino (N) terminus, although there are several examples of proteins that have internal or carboxy (C)-terminal targeting signals (Peeters and Small, 2001). The yeast photolyase is transported into both nuclei and mitochondria by signal sequences present in the N-terminus regions (Yasui *et al.*, 1992). The Arabidopsis Cry-DASH, AtCRY3, also has a targeting sequence for import into chloroplasts and mitochondria within N-terminal 63 amino acids (Kleine *et al.*, 2003). We identified a putative chloroplast targeting sequence in the N-terminal 80 amino acids of rice CPD photolyase using TargetP (http://www.cbs.dtu.dk/services/TargetP) (Emanuelsson *et al.*, 2007), which is a subcellular localization prediction program. The predictor failed to identify either a nuclear or a mitochondrial targeting sequence in the deduced amino acid sequence of rice CPD photolyase. The organellar target sequences of rice CPD photolyase and molecular mechanisms of import to each organelle in rice are not understood. This information should still help for the elucidation of the repair pathway in the cpDNA and mtDNA of higher plants. These studies should be conducted in the future.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Rice plant materials, including transgenic rice plants (Hidema *et al.*, 2007), were grown under visible radiation in a growth cabinet (Koito Ind. Ltd Co., http://www.koito-ind.co.jp) (12-h photoperiod, day/night temperatures of 27°C/17°C) as described previously (Hidema *et al.*, 2007).

UV-B irradiation and photoreactivation

To assess susceptibility to UV-B-induced DNA damage in each organelle, detached fully expanded fourth leaves were placed on wet filter paper and irradiated with unfiltered UV-B radiation from a UV-B fluorescent tube (FL20SE; Toshiba Electronic Ltd Co., http://www.toshiba.co.jp) at a dose of 5 W m^{-2} for 0–3 h. To assay repair activity in each organelle, detached fully expanded fourth leaves were irradiated with UV-B radiation at a dose of 5 W m^{-2} for 1 h to induce the formation of CPDs. The leaves were then exposed to blue irradiation from blue fluorescent tubes (20B-F; Toshiba Electronic Ltd Co.), or were immediately placed in a light-tight box for 0–5 h after UV-B exposure. The intensity of blue irradiation was adjusted to approximately 60 μmol m^{-2} s^{-1}, which provided saturating light levels for CPD photorepair. After exposure to blue radiation, the leaves were immediately harvested and stored in liquid nitrogen until analysis. We measured CPD photolyase activities *in vitro* in crude extracts prepared from the 18-kJ m^{-2} UV-B irradiated or non-irradiated rice leaves by alkaline gel electrophoresis methods using UV endonuclease (Hidema *et al.*, 2000). We confirmed that the high dose of 18 kJ m^{-2} UV-B radiation did not affect the CPD photolyase activity in rice cells (data not shown). All subsequent manipulations were carried out under red light to minimize uncontrolled photoreactivation. To analyze photorepair capability, at least three independent experiments were performed and the results were averaged.

Measurement of ESS by Southern blot analysis

Southern blot analysis of site-specific DNA repair was based on the quantitative comparison of the intensity, using densitometry analysis, of T4 endo V-treated (EPICENTRE Biotechnologies, http://www.epibio.com/main.asp) and untreated DNA (Chen *et al.*, 1996; Hanawalt, 1989; Stapleton *et al.*, 1997). T4 endo V specifically cleaves single DNA strands at sites of CPDs. Genomic DNA was isolated from UV-B-irradiated or non-irradiated detached leaves as described previously (Teranishi *et al.*, 2004), and was then digested with restriction enzymes as follows: *Apa*I and *Xho*I for *atpI* and *cob*; and *Xba*I and *Bam*HI for *cox3*, *rbcS* and *phr*. Digested DNA was divided into two equal aliquots, and the DNA solutions were adjusted to 10 mM Tris–HCl (pH 8.0), 10 mM EDTA and 100 mM NaCl. One aliquot was treated with T4 endo V (10 U) and the other was left untreated. The reaction was allowed to proceed at 37°C for 3 h, and was stopped by the addition of alkaline loading solution [100 mM NaOH, 5% (w/v) glycerol and 0.025% (w/v) bromocresol green]. The samples were resolved by alkaline agarose gel [0.5% (w/v)] electrophoresis using static-field electrophoresis and biased sinusoidal field gel electrophoresis (Genofield; ATTO Co., http://www.atto.co.jp) (Hidema and Kumagai, 1998). After electrophoresis, the DNA was blotted onto a nylon membrane (Biodyne B; Pall Co., http://www.pall.com).

To generate probes for Southern blot analysis, chloroplast genomic DNA (including the 3′ end of *atpI*), two mitochondrial-specific genes (*cox3* and *cob*) and two nuclear-specific genes (*rbcS* and *phr*) were amplified using the following primers: *atpI* (forward, 5′-ATTGGCGGATGAATTAGTCG-3′; reverse, 5′-TGCATCGAATTGGATATGGA-3′); *cox3* (forward, 5′-CGCGAGTATAGCATGATGAGCCC-3′; reverse, 5′-CCAAGTCCATGGCCTATTTCGGG-3′); *cob* (forward, 5′-TAGGCGCCAGTCTTCTTCAC-3′; reverse, 5′-GTGCTATTGCGGCTACACCT-3′); *rbcS* (forward, 5′-TTGAGCCAGCCAACGCC-3′; reverse, 5′-GCACCTGATCCTGCCGCCAT-3′); and *phr* (forward, 5′-GGGAACAGCGCTTGAGAATGCCAA-3′; reverse, 5′-CTAGAATGTGCCATGGGTACAG-3′). The amplification program consisted of an initial denaturation of 5 min at 95°C, followed by 40 cycles of 1 min at 95°C, 0.5 min at 58°C (*atpI*, *cob* and *phr*), 63°C (*cox3*) or 66.5°C

(*rbcS*), and 0.5 min at 72°C. Amplified fragments were then labeled with [α-^{32}P]dCTP (14.8 TBq mmol^{-1}; Institute of Isotopes Co., Ltd; http://www.izotop.hu) using the random priming method (Feinberg and Vogelstein, 1983). The hybridization patterns of each probe were as follows: the chloroplast probe for *atpI* hybridized to an 11-kb fragment; the mitochondrial probes for *cox3* and *cob* hybridized to 4.5- and 4.0-kb fragments, respectively; and the nuclear probes for *rbcS* and *phr* hybridized to 6- and 4.4-kb fragments, respectively. Prehybridization for 1 h and overnight hybridization were performed in 0.5 M phosphate buffer (pH 7.0) containing 7% (w/v) SDS and 1 mM EDTA at 65°C. Membranes were then washed in 2 × SSC (300 mM NaCl, 30 mM sodium citrate) containing 0.1% (w/v) SDS at 65°C. The membrane was exposed to an Imaging Plate (BAS-IP MS; Fuji Photo Film Co., Ltd., http://fujifilm.jp), and the signal intensity of each band was measured using a Fluoro-Imaging Analyzer (FLA-2000; Fuji Photo Film Co., Ltd). Data are presented as the percentage (%) of fragments with ESS, which was calculated as the ratio of (the intensity of untreated DNA – the intensity of T4 endo V-treated DNA) to the intensity of untreated DNA.

Measurement of UV-induced DNA lesions by qPCR

Quantitative PCR (qPCR) analysis of UV-B-induced DNA lesions was based on the blockade of PCR polymerases by UV-induced photoproducts (Kalinowski *et al.*, 1992; McCarthy *et al.*, 1996). Specific fragments of the genes were amplified from rice genomic DNA as described above: the size of each PCR product is 571 kb for *atpI*, 430 kb for *cox3*, 318 kb for *cob*, 337 kb for *rbcS* and 298 kb for *phr*. Because targeted sequences that contained UV-induced polymerase-blocking lesions could not be amplified, the quantity of undamaged PCR-template DNA was calculated. The percentage of fragments with one or more UV-induced polymerase-blocking lesions was calculated as $(A_0 - A_D)/A_0 \times 100$ (%) (Mellon *et al.*, 1987), where A_0 was the quantity of PCR-template DNA extracted from non-irradiated leaves and A_D was the quantity of PCR-template DNA extracted from irradiated leaves. Quantitative real-time PCR was performed with the DNA Engine Opticon™ System (Bio-Rad, http://www.bio-rad.com).

Twice affinity-purified anti-rice CPD photolyase IgG

The anti-rice CPD photolyase antibody was polyclonal antibody, which was raised against purified *E. coli*-expressed rice CPD photolyase, and was further affinity purified with the antigen as a ligand twice (twice affinity-purified anti-rice CPD photolyase IgG), as described in Yamaya *et al.* (1992).

Immunoelectron microscopy

Immunoelectron microscopy (IEM) was performed as described by Chiba *et al.* (2003) using 1-cm sections of the fourth leaves. The sections were vacuum infiltrated with a fixative composed of 4% (w/v) paraformaldehyde and 1% (v/v) glutaraldehyde in 50 mM Na-cacodylate buffer (pH 7.4). The sections were cut into small pieces (1 × 1 mm) and incubated in fixative overnight. After being washed with Na-cacodylate buffer, the samples were dehydrated in a graded series of dimethylformamide at −20°C, and then embedded in LR-White resin (London Resin Co. Ltd., http://www.applegate.co.uk). The LR-White resin was polymerized in an ultraviolet polymerizer (TUV-200; Dosaka EM, http://www.dosaka-em.jp/) at −20°C for 24 h. Ultrathin sections were prepared using an ultramicrotome and then mounted on nickel grids. The sections were incubated in a blocking solution composed of phosphate-buffered saline containing 1% (w/v) bovine serum albumin (BSA) and 0.1% (w/v) sodium azide for 30 min at 20°C, followed by primary antibody in blocking solution at 4°C overnight. The twice affinity-purified anti-rice CPD photolyase IgG was used as the primary antibody. As a control, sections were incubated with the same dilution of antibody that had been pre-absorbed with excess recombinant rice CPD photolyase. Sections were washed and then incubated with secondary antibodies (goat anti-rabbit IgG 15-nm gold conjugate; British BioCell International, http://www.britishbiocell.co.uk) in blocking solution at 20°C for 30 min. The sections were washed, stained with 4% (w/v) uranyl acetate and 0.4% (w/v) lead citrate, and then examined under a transmission electron microscope (H-8100; Hitachi Ltd, http://www.hitachi.co.jp) operated at 100 kV.

Quantification of immunogold labeling

Random micrographs were taken at a magnification of 34 000×. Gold particles were counted by eye (Peat and Tobin, 1996). The area of the cell compartment was calculated using computerized image analysis. Data are presented as number of gold particles per square micrometer.

Isolation of chloroplasts

Ten-day-old seedlings of S-C plants were cut into 3–5-mm pieces and homogenized with an UltraTurrax (IKA-Werke, http://www.ika.net) in an isolation solution consisting of 0.35 M sorbitol, 2 mM EDTA (pH 8.0) and 25 mM HEPES/NaOH (pH 7.0). The homogenate was passed through four layers of gauze and centrifuged at 1200 ***g*** for 5 min at 4°C. The pellet was gently resuspended in isolation solution with a natural bristle paintbrush, and then layered on a discontinuous density gradient consisting of 10, 30 and 80% Percoll in isolation solution. The gradient was centrifuged at 2600 ***g*** for 40 min at 4°C. Intact chloroplasts were recovered from the 30–80% Percoll interface, resuspended in isolation solution and centrifuged at 1800 ***g*** for 5 min at 4°C. The pellet was resuspended in isolation solution.

Isolation of mitochondria

Ten-day-old seedlings of S-C plants were cut into 3–5-mm pieces and homogenized with an UltraTurrax using two 10-s bursts in homogenization solution [0.3 M mannitol, 5 mM EDTA, 30 mM 3-(*N*-morpholino)propanesulphonic acid (MOPS), 1% (w/v) BSA, 5 mM DTT, 1% polyvinylpyrrolidone (PVP), pH 7.3]. The homogenate was strained through nylon cloth (120-μm mesh) and then centrifuged at 1000 ***g*** for 5 min at 4°C. Following centrifugation of the supernatant at 20 000 ***g*** for 25 min at 4°C, the pellet (crude mitochondria) was resuspended in wash solution (0.3 M mannitol, 1 mM EDTA, 10 mM MOPS, pH 7.2). Crude mitochondria were layered on top of a Percoll step gradient consisting of 20, 28 and 40% Percoll in Percoll buffer (0.4 M mannitol, 5 mM MOPS, 1 mM EDTA, 0.4% PVP, pH 7.2) and centrifuged at 40 000 ***g*** for 30 min at 4°C. Mitochondria were recovered from the 28–40% Percoll interface, diluted more than 10 times in wash solution, and then pelleted by centrifugation at 20 000 ***g*** for 20 min. The pellet was resuspended in wash solution.

Isolation of nuclei

Nuclei were isolated from calli derived from mature seeds of S-C plants, as described by Hirose and Yamaya (1999). Briefly, frozen calli were pulverized under liquid nitrogen and the powdered cells were then suspended in NIB [1 M hexylene glycol, 10 mM PIPES-KOH (pH 7.0), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol and 0.5% (v/v) Triton X-100]. The resultant slurry was filtered through two layers of gauze, followed by centrifugation at 1000 ***g*** for 10 min. Crude nuclear pellets were washed twice with NIB and once with NIB without Triton X-100, and then suspended in NIB without Triton X-100. The suspended nuclei were layered on a discontinuous

Percoll density gradient [80% (w/v) sucrose cushion, and then 80 and 30% Percoll solutions containing 0.8% (w/v) sucrose, 5 mM PIPES-KOH (pH 7.0) and 5 mM $MgCl_2$], and then centrifuged at 3000 ***g*** for 30 min at 4°C. The nuclei were recovered from the 30–80% Percoll interface, resuspended in NIB and centrifuged at 1000 ***g*** for 5 min at 4°C. The pellet was resuspended in NIB.

Western-blot analysis of CPD photolyase in each organelle

Western-blot analysis was performed as follows. Proteins in the crude extracts or organelle-enriched fractions were separated with SDS-PAGE [7.5 or 12.5% (w/v)], transferred to a polyvinylidene difluoride membrane (Bio-Rad) and probed with the twice affinity-purified anti-rice CPD photolyase IgG, as described above, anti-rice light-harvesting chlorophyll *a*/*b* protein of PSII (LHCII) antiserum (Hidema *et al.*, 1992), as a chloroplast marker, anti-voltage-dependent anion-selective channel protein 1 (VDAC1) antiserum (anti-VDAC1; Agrisera, http://www.agrisera.com/en/info/home.html) as a mitochondrial marker, and anti-histone H2B (H2B) antiserum (Anti-H2B; Santa Cruz Biotechnology Inc., http://www.scbt.com) as a nuclear marker. The immune complex was detected by an alkaline phosphatase-conjugated anti-rabbit IgG (Sigma-Aldrich, http://www.sigmaaldrich.com), and developed using premixed 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution (Bio-Rad).

Treatment with λ-PPase

Approximately 100 ng of the organelle-enriched fractions was treated with 400 U of λ-PPase (New England Biolabs, http://www.neb.com) according to the instruction manual for 30 min at 30°C, as described in Teranishi *et al.* (2008). After treatment, the organelle-enriched fractions treated with λ-PPase were separated with SDS-PAGE (7.5%), and western-blot analysis was performed using twice affinity-purified anti-rice CPD photolyase IgG, as described above.

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