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Silencing of CPD Photolyase Makes *Arabidopsis* Hypersensitive and Hypermutable in Response to UV-B Radiation

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Plants are exposed to solar ultraviolet radiation (UV), which has deleterious effects on plant growth, development and physiology. Cyclobutane pyrimidine dimers (CPDs) are a major form of UV-induced DNA damage. It is conceivable that the reversal of CPDs is important for the reduction of lethal and mutagenic effects. Photoreactivation catalyzed by CPD photolyase is an efficient CPD repair system with a mechanism dependent on UV-A/visible light, which is contained in solar radiation. Photoreactivation presumably functions to protect plants from solar UV. We generated a CPD photolyase knock-down in *Arabidopsis thaliana* by RNA interference (RNAi) to investigate the role of CPD photorepair for protection of plants from solar UV. These knock-down lines exhibited hypersensitivity to UV-B and an increased occurrence of mutation induced by UV-B radiation compared with *Arabidopsis* proficient in CPD photolyase. Mutations induced by UV-B were determined by an *rpsL* mutation assay system. G:C to A:T transitions were frequently observed in CPD photolyase knock-down lines at the site of dipyrimidine sequences. A high incidence of frameshifts was observed in irradiated knock-down lines. These results indicate that CPD photoreactivation plays an important role for UV resistance of *Arabidopsis* and suppression of UV-induced mutagenesis.

Key words: Arabidopsis, UV sensitivity, CPD photolyase, UV-induced mutation

Introduction

Ultraviolet radiation (UV) is one of a variety of genotoxic agents to which living organisms are exposed. Plants growing under sunlight are always exposed to solar UV because of their sessile trait. UV induces pyrimidine dimers, a major UV-induced DNA lesion, at dipyrimidine sites on DNA. These lesions have deleterious effects on plant growth, development and physiology. There are two types of pyrimidine dimers, cyclobutane pyrimidine dimers (CPD) and pyrimidine pyrimidone (6–4) photoproducts. Both types block DNA synthesis during replication by DNA polymerase and inhibit the synthesis of mRNA during transcription by RNA polymerase (1,2). Mutations may occur when a DNA polymerase bypasses lesions due to pyrimidine dimers during DNA synthesis in the process known as translesion synthesis (3). DNA polymerases such as *Escherichia coli* DNA polymerase IV (4) and V (5) and eukaryotic Rev1 (6–8), DNA polymerase ζ (9,10), and DNA polymerase η (11,12) have such a capability.

Organisms have evolved various types of DNA repair mechanisms. Pyrimidine dimers are mainly repaired by nucleotide excision repair and photoreactivation (13). Nucleotide excision repair functions for both types of pyrimidine dimers and many other kinds of DNA lesions. Photoreactivation catalyzed by photolyase using UV-A and blue light is specific for pyrimidine dimers. Distinct photolyases specific to CPDs and (6–4) photoproducts are identified from many organisms. *Arabidopsis* has both types of photolyase (14,15). The yield of CPD, which is much higher than that of (6–4) photoproducts, constitutes about 70% of total pyrimidine dimers caused by UV. Reversal of CPDs in DNA is a prerequisite to maintain genetic stability.

The gene for CPD photolyase has been cloned from many organisms such as *Escherichia coli*, *Anacystis nidulans*, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, the goldfish *Carassius auratus* and the South American opossum *Monodelphis domestica* (16–21). The *Arabidopsis* CPD photolyase gene was first identified among plants (14). After a decade, the gene was cloned from cucumber, rice and spinach (22–24). It has been suggested that CPD photolyase plays a role for protection of plants from UV (25–27). In this study we investigated the effect of CPD photolyase on UV sen-

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sitivity and UV-induced mutation in *Arabidopsis*.

Materials and Methods

Plant and bacterial strains: Plant material was *Arabidopsis thaliana* ecotype Columbia. *Arabidopsis* was grown at approximately 25°C under a 14-h light and 10-h dark cycle. The light source was four fluorescent lamps (40 W) with an intensity of 6800 lx. *Arabidopsis* was transformed by *Agrobacterium tumefaciens* GV3101 (pMP90). *Escherichia coli* strain DH10B/pFSE101 was used for detection of mutations occurring in the *Escherichia coli rpsL* gene, which was recovered from *Arabidopsis* chromosome DNA.

Knock-down of CPD photolyase in *Arabidopsis*: CPD photolyase of *Arabidopsis* was silenced by RNAi. The region of the 62nd to the 486th base from adenine of AUG codon as a start signal of *Arabidopsis* CPD photolyase cDNA was cloned by PCR. The primer sequences were 5′-AAGCTT GGTACC CAACCGTTGGATCAAACGGT-3′ for the forward direction and 5′-GGATCC CTCGAG CATTGGAACTACATTGTGAG-3′ for the reverse direction. These forward and reverse primers contain *Hin*dIII/*Kpn*I and *Bam*HI/*Xho*I sites at the 5′-end, respectively. PCR products were subcloned into a pGEM-T Easy Vector (Promega) and subsequently excised from the vector at the *Kpn*I and *Xho*I sites, and the *Bam*HI and *Hin*dIII sites. These DNA fragments containing a portion of the gene for CPD photolyase were inserted into the *Kpn*I/*Xho*I site and *Bam*HI/*Hin*dIII sites of pKANNIBAL in the inverted direction on either side of the intron sequence (28). The silencing cassette was excised at the *Not*I site and inserted into pPMA8 carrying the *Escherichia coli rpsL* gene (29) to construct pPMAtCPHRs (Fig. 1). *Arabidopsis thaliana* Columbia was transformed with pPMAtCPHRs mediated by *Agrobacterium tumefaciens* GV3101 (pMP90) by the floral dip method (30). Transformants were screened on B5 agar medium supplemented with 50 μg/mL of kanamycin. Kanamycin-resistant *Arabidopsis* lines were self-pollinated to obtain homozygous strains. Transgenic *Arabidopsis* lines were established referred to as AtCPHRs/rpsL. AtCPHRs/rpsL1-2 and 7-5 were used for investigation of UV sensitivity and mutagenesis.

UV-B sensitivity: *Arabidopsis* was irradiated with UV-B radiation emitted from two 20 W UV-B bulbs (GL20SE; Sankyo Denki Co., Tokyo, Japan) at a dose rate of 1.7 $J \cdot m^{-2} \cdot s^{-1}$. UV-C region was removed by a plastic (polystyrene) dish cover (Nissui Pharmaceutical Co., Tokyo, Japan), of which relative transmittance at 280 nm was less than 1% of visible light region. *Arabidopsis* seeds spread on 1/2 B5 medium were chilled at 4°C for 2 days for vernalization and allowed to germinate at 25°C. After germination, plants were grown for 7 days and subsequently grown with supplemental UV-B for 8 h a day for 14 days under the light cycle above. The dose of UV-B to plants was 49 $kJ \cdot m^{-2}$ a day. Fresh weight of plants was used to evaluate the inhibitory effects of UV-B on growth.

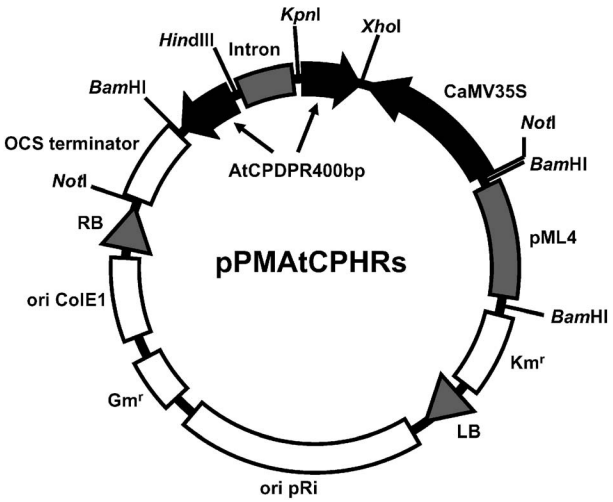

Fig. 1. Physical map of pPMAtCPHRs. pPMAtCPHRs is basically comprised of an *Arabidopsis* CPD photolyase silencing cassette and plasmid pML4 bearing the *Escherichia coli rpsL* gene. The stem loop region including an intron is expressed by the CaMV 35S promoter. Plasmid pML4 was rescued from chromosomal DNA for mutation assay. The region between the right border and left border, referred to as RB and LB, respectively, is incorporated into *Arabidopsis* DNA.

Expression of the gene for CPD photolyase: Wild-type and knock-down lines grown for 7 days were irradiated with UV-B for 1 or 3 h at a dose rate of 1.7 $J \cdot m^{-2} \cdot s^{-1}$. Total RNA was isolated from unirradiated and UV-B-irradiated plants with TRIzol Reagent (Invitrogen). We repeated this step three times independently. The relative expression of the gene for CPD photolyase was represented as the ratio of the amount of mRNA for CPD photolyase to that for *Arabidopsis* actin 2 (At3g18780). Primers for synthesis of cDNA of CPD photolyase and actin 2 by RT-PCR were AtCPD-2R (5′-AAACCGGCCTCTCTTTCCAT-3′) and At-ACT2-2R (5′-AACCACCGATCCAGACACTGTA-3′), respectively. The relative quantity of mRNA of target sequence was determined by real-time PCR (GeneAmp 5700 Sequence Detection System, PE Biosystems) using SYBR Green following RT-PCR. Primers for real-time PCR were AtCPD-3F (5′-TGACCCGAGTGGATATGTTGG-3′) and AtCPD-2R for CPD photolyase, or AtACT2-2F (5′-TTGCACCAAGCAGCATGAA-3′) and AtACT2-2R for actin 2.

Detection of cyclobutane pyrimidine dimmers: *Arabidopsis* grown for 2 weeks after germination was irradiated with 7.3 $J \cdot m^{-2} \cdot s^{-1}$ of UV-B for 2 h. Chromosomal DNA was prepared by a MagExtractor-Plant Genome kit (Toyobo Co., Osaka, Japan). The relative amount of CPDs was determined by an ELISA method

(31). The CPD-specific antibody used was MX-thymine dimer-HRP (Kyowa Medex Co., Tokyo, Japan).

UV-B-induced mutation in *Arabidopsis*: Mutational specificity of *Arabidopsis* was examined with a mutation detection system using *Arabidopsis* carrying the *Escherichia coli rpsL* gene as a target for mutation (29). Wild-type lines, Arabidopsis/rpsL3-3 and 5-2, and CPD photolyase knock-down lines, AtCPHRs/rpsL1-2 and 7-5, were grown for 14 days in plastic dishes and were irradiated with 7.3 $J \cdot m^{-2} \cdot s^{-1}$ of UV-B for 1 h and subsequently grown for 5 days. Two UV bulbs as described above were used for UV-B irradiation. A plastic (polystyrene) dish cover was used for removal of the UV-C region. DNA was isolated from UVB-irradiated plants by a CTAB method (32). No difference in mutational spectrum was found between Arabidopsis/rpsL3-3 and 5-2 lines or between AtCPHRs/rpsL1-2 and 7-5 lines. Results obtained from each line were combined. Extracted DNA, 150–200 μg, was digested with *Bam*HI and subjected to a ligation reaction by T4 DNA ligase (Takara Bio Inc., Otsu, Japan) to rescue the *rpsL* gene as plasmid pML4. DNA prepared to the concentration of 1 to 2 $\mu g/\mu L$ was introduced into *Escherichia coli* DH10B/pFSE101 by electroporation. It was spread on LB medium supplemented with streptomycin to detect cells carrying the mutated *rpsL* gene. The region of the *rpsL* gene on the plasmid was sequenced to examine mutational nucleotide sequence changes (ABI PRISM® 310 Genetic Analyzer, Applied Biosystems, USA).

Results

Hypersensitivity of CPD photolyase knock-down *Arabidopsis* to UV radiation: We produced transgenic *Arabidopsis*, AtCPHRs/rpsL, carrying CPD photolyase inactivated by RNAi-mediated gene knock-down. Wild-type *Arabidopsis* and two transgenic lines, AtCPHRs/rpsL1-2 and 7-5, were grown for 14 days under a 14-h light and 10-h dark light cycle with supplemental UV-B for 8 h a day. The total UV-B dose to plants was 49 $kJ \cdot m^{-2}$ a day. Growth of the two transgenic lines was completely inhibited by the UV radiation, although only a slight inhibition was observed in wild-type plants (Fig. 2a and b). Relative expression of CPD photolyase was examined by real-time PCR, demonstrating almost complete suppression of the expression in both lines of AtCPHRs/rpsL. The expression in wild-type *Arabidopsis* increased with the time after UV irradiation (Fig. 3). We also examined the removal of CPDs from DNA in UV-B-irradiated transgenic plants by ELISA, resulting in little or no reduction of CPDs after 12 h illumination by white light in AtCPHRs/rpsL, although about 10% of CPDs were eliminated in wild-type *Arabidopsis* (data not shown). Hypersensitivity of AtCPHRs/rpsL to UV-B may be due to decreased CPD photoreactivation caused by RNAi-induced silencing.

Mutation induced by UV in *Arabidopsis*: We investigated various types of UV-induced mutation to examine the mutational specificity of higher plants and the effects of CPD photolyase on UV-induced mutation in CPD photolyase proficient Arabidopsis/rpsL and a CPD photolyase knock-down *Arabidopsis* line. Mutation was assayed by the *rpsL* system, which we previously developed to detect various types of mutation in *Arabidopsis* (29). The mutant frequency of irradiated Arabidopsis/rpsL, 8.1×10^{-5}, was approximately 3.2-times higher than that of unirradiated plants, 2.5×10^{-5}. The UV-B-irradiated knock-down *Arabidopsis* line, AtCPHRs/rpsL, was about 2.5 times more mutable than UVB-irradiated Arabidopsis/rpsL (data not shown). Although we analyzed more than 10^5 clones from unirradiated knock-down *Arabidopsis* lines, no mutation was detected. Then background mutant frequency of knock-down lines might be similar to that of Arabidopsis/rpsL. These results indicate that UV-B induces mutation in *Arabidopsis* and CPD photolyase efficiently suppresses UV-B-induced mutation.

G:C to A:T transition induced by UV-B irradiation: Mutational nucleotide sequence changes were found within the *rpsL* gene without any marked mutational hotspots (Fig. 4). G:C to A:T transitions were frequently detected in irradiated plants and were a major type of mutation in CPD photolyase knock-down lines (Table 1). G:C to A:T transition is also major type of mutation induced by UV irradiation in other organisms (33–35). Dipyrimidine sequences are the site of dimerization of pyrimidine bases by UV irradiation and the target for G:C to A:T events. Three of four base changes found in unirradiated Arabidopsis/rpsL occurred at sites other than dipyrimidine sequences (Fig. 4). G:C to A:T transitions in UV-irradiated knock-down lines occurred at dipyrimidine sites 3.8 times more frequently than in irradiated Arabidopsis/rpsL (Fig. 5a). Then it is conceivable that G:C to A:T transitions occurred in this study is caused by CPD formed on Arabidopsis chromosomal DNA by UV-B radiation.

Frameshift mutation occurring in UVB-irradiated AtCPHRs/rpsL: The frequency of frameshifts occurring in UV-B-irradiated AtCPHRs/rpsL was 4.4 times higher than that of Arabidopsis/rpsL (Fig. 5b). Six of eight frameshift events in UV-B-irradiated AtCPHRs/rpsL were found at dipyrimidine sequences. The increased number of frameshifts occurring in UV-B-irradiated AtCPHRs/rpsL was likely due to CPDs. CPD photorepair is presumably important for suppression of this type of mutation.

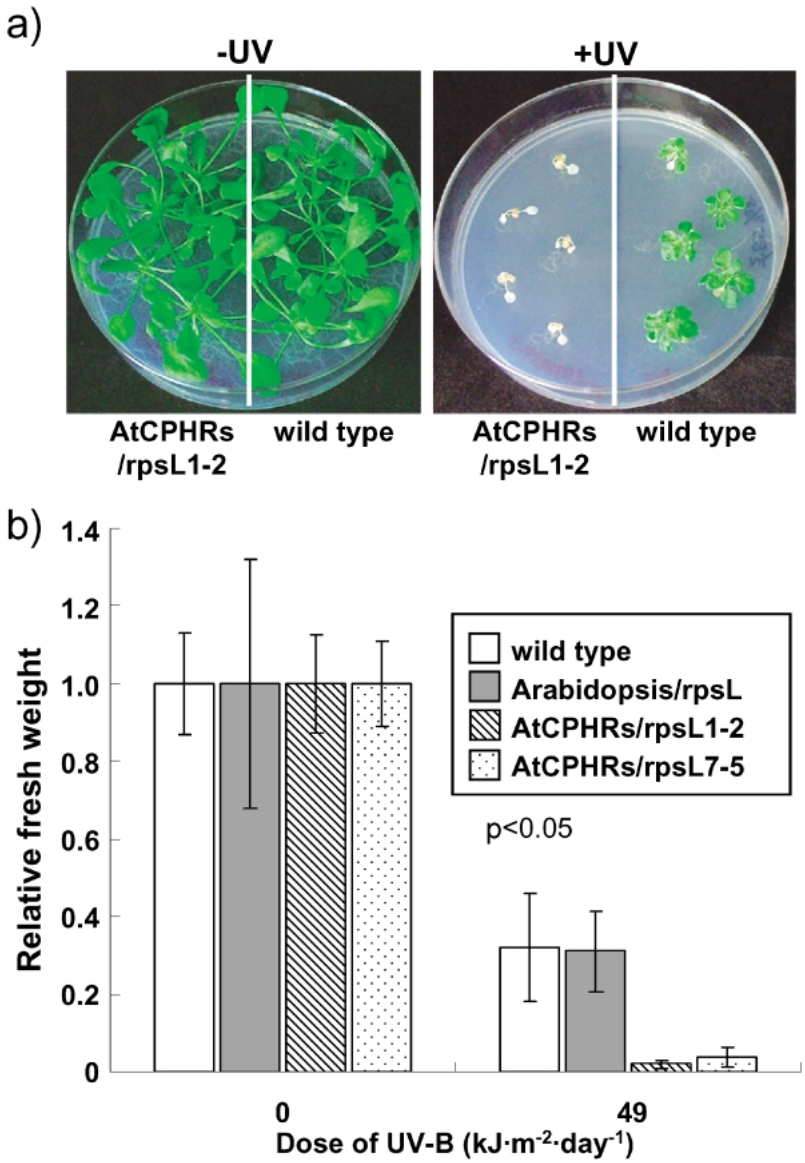

Fig. 2. UV-B sensitivity of CPD photolyase knock-down *Arabidopsis*. (a) Growth inhibition of wild type and AtCPHRs/rpsL1-2 by UV-B radiation. Plants about 7 days after germination were irradiated with UV-B at a dose rate of 1.7 $J \cdot m^{-2} \cdot s^{-1}$ for 8 h a day ($= 49\ kJ \cdot m^{-2} \cdot day^{-1}$) throughout 14 days under the light conditions previously described. (b) Relative fresh weight of irradiated to unirradiated wild-type, Arabidopsis/rpsL and AtCPHRs/rpsL1-2 and 7-5 plants. Inhibitory effect is indicated as the ratio of irradiated to unirradiated plant. Fresh weight of unirradiated plant is calibrated to one. Growth and irradiation conditions were the same as (a). Error bars represent standard deviation. The p value of t-test was less than 0.05.

Discussion

An *Arabidopsis* mutant defective in photorepair of CPD is hypersensitive to UV-B (14,25). Here we demonstrated that defect in photorepair of CPD, due only to CPD photolyase suppression by RNAi, caused severe damage in *Arabidopsis*. In our study mRNA of CPD photolyase gene was not completely suppressed in *Arabidopsis*. Then lower expression of CPD photolyase gene is not sufficient and induction of the gene is necessary for UV defense mechanism in *Arabidopsis*. CPD photorepair activity in AtCPHRs/rpsL was lower than that of wild-type *Arabidopsis* in our study. The decrease of CPD photorepair activity might be caused by the lower expression of CPD photolyase gene. Hidema *et al.* (36) showed that the rice cultivar Sasanishiki bearing the gene for CPD photolyase from Sasanishiki showed increased resistance to UV-B causing growth inhibition and a lower level of CPDs in leaves. Our preliminary study suggested that *Arabidopsis* transformed with the gene for spinach CPD photolyase shows slightly increased tolerance to UV-B in growth and enhanced removal of CPDs (data not shown). Then it is conceiva-

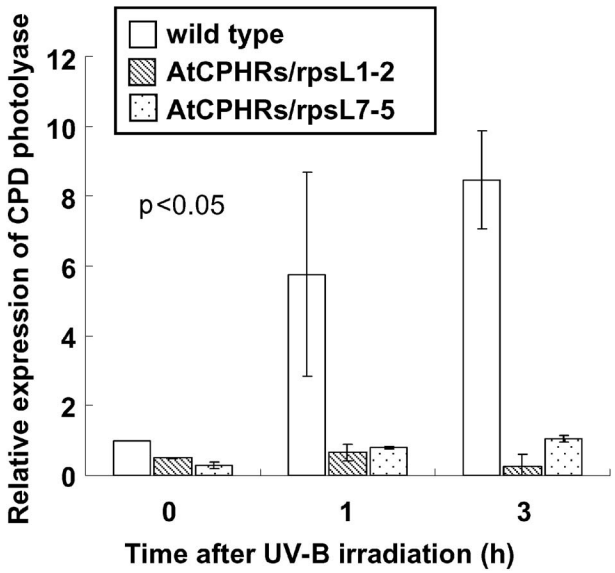

Fig. 3. Expression of CPD photolyase in wild-type and CPD photolyase knock-down *Arabidopsis* lines, AtCPHRs/rpsL1-2 and 7-5. Error bars represent deviation among three independent determinations. The expression level of CPD photolyase gene in unirradiated wild type is calibrated to one. Others are indicated as the ratio to that of unirradiated wild type. Statistical significance is expressed as the p value of a t-test ($p < 0.05$).

ble that expression level of CPD photolyase gene is closely related to CPD photorepair activity and UV resistance. In wild-type plant 90% of CPDs produced by 2h-UV irradiation were still remained after 12 h-illumination for photorepair although toxicity of UV-B was relatively moderate. Growth inhibition of UV-B was examined by taking a prolonged period. The investigation of toxicity and CPD content was carried out under quite different experimental conditions between them.

UV-B radiation was mutagenic for higher plants as well as for microorganisms and animals. In the reversion system of the *uidA* gene in *Arabidopsis*, UV-C-induced mutation was 56 times higher than unirradiated controls (37), indicating severe genotoxicity of UV-C for higher plants as well as for other organisms. The higher mutant frequency of the UV-B-irradiated CPD photolyase knock-down *Arabidopsis* line indicates that CPD photolyase functions to suppress UV-induced mutation in *Arabidopsis* by means of repair of CPDs. UV-induced mutation has been well studied in other organisms. The major type of mutation in *Escherichia coli* is base substitution (33); G:C to A:T transitions occurring at the site of adjacent pyrimidines are the most frequent mutation. Base changes are also a major type of UV-induced mutation in mammalian cells (34,35). In the UV-induced mutation of *Arabidopsis* obtained here, the majority of mutations were G:C to A:T transitions, in-

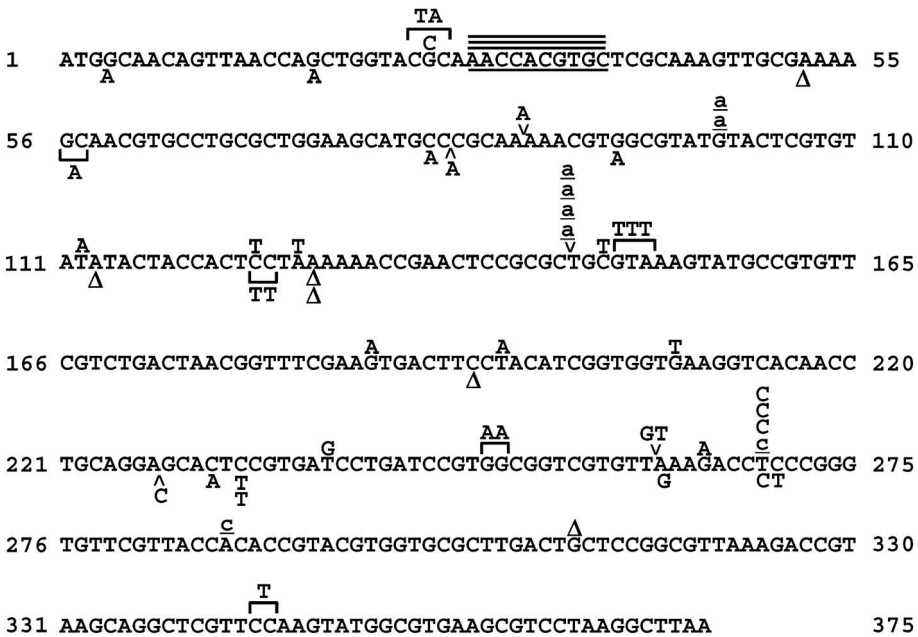

Fig. 4. Mutations occurring in the *rpsL* gene in unirradiated Arabidopsis/rpsL (background) and irradiated Arabidopsis/rpsL and AtCPHRs/rpsL. Mutations of background (small underlined letter) and irradiated Arabidopsis/rpsL are shown above the sequence and AtCPHRs/rpsL below. Δ, V and bars above and below the sequence denote deletion of a base, insertion of bases and deleted sequences, respectively. Complex mutations were found at sites indicted by clamp shape above or below the sequence. At base numbers 25–27, 56–57, 124–125 and 344–345, base deletions and multiple base changes occurred at the same site. Multiple base changes were found at the sites of base numbers 149–151and 250–251.

Table 1. *rpsL* Mutation induced by UV-B radiation

	Number of mutations detected (%)*			
	Background	Arabidopsis/rpsL	AtCPHRs/rpsL	*rpsL* transgenic mouse†
Transition				
G:C to A:T	2 (25)	4 (17)	6 (30)	21 (45.7)
A:T to G:C	1 (12.5)	3 (13)	2 (10)	3 (6.5)
Transversion				
G:C to T:A	0	1 (4.5)	2 (10)	1 (2.2)
G:C to C:G	0	1 (4.5)	0	0
A:T to C:G	1 (12.5)	1 (4.5)	0	3 (6.5)
A:T to T:A	0	3 (13)	0	3 (6.5)
Frameshift				
+2 Frameshift	0	1 (4.5)	0	0
+1 Frameshift	4 (50)	1 (4.5)	2 (10)	3 (6.5)
−2 Frameshift	0	0	0	1 (2.2)
−1 Frameshift	0	1 (4.5)	5 (25)	9 (19.6)
Deletion	0	3 (13)	1 (5)	0
Complex mutation	0	4 (17)	2 (10)	2 (4.3)
Total	8 (100)	23 (100)	20 (100)	46 (100)

*Percentage to the total number of mutation. Background mutation was from unirradiated Arabidopsis/rpsL.
†UVB mutation spectrum in XPA(+/+) *rpsL* transgenic mouse irradiated at 900 J/m^2 [modified from Murai *et al.* (57)].

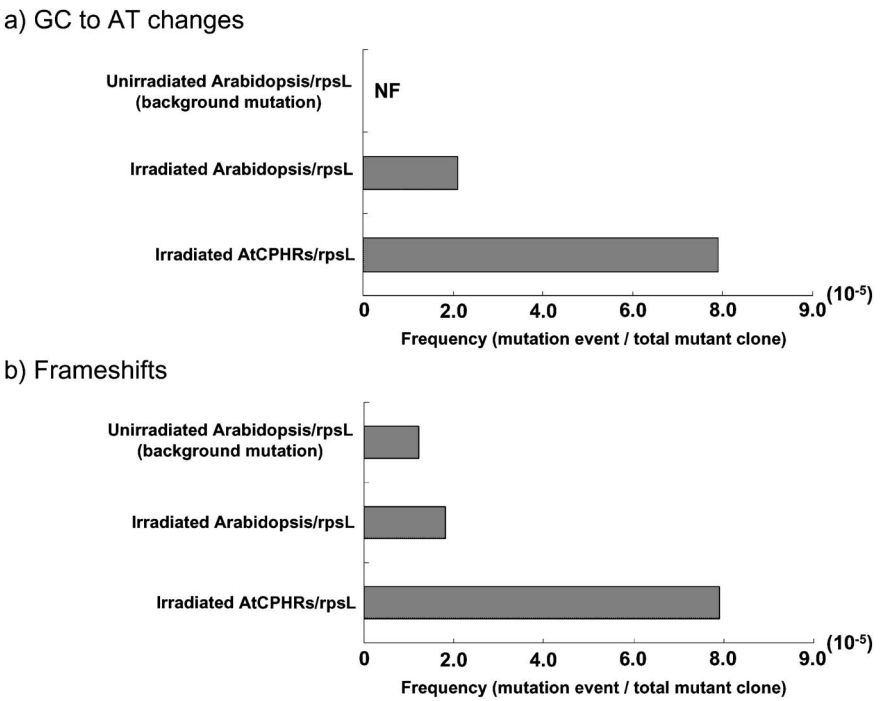

Fig. 5. Frequency of G:C to A:T transitions occurring at dipyrimidine sequences (a) and frameshift mutations (b) in unirradiated and irradiated Arabidopsis/rpsL, and irradiated AtCPHRs/rpsL. Frequencies are represented by the fraction of each mutation among kanamycin-resistant clones carrying rescued plasmid pML4. The type of base change found in unirradiated Arabidopsis/rpsL (background mutation) occurred at sites other than the dipyrimidine sequence. 'NF' denotes 'not found'.

dicating that mutational specificity in UV-B-irradiated higher plants is similar to that of other organisms.

Although the mechanism causing G:C to A:T transitions is not evident, two mechanisms are possible. One is due to misincorporation of an incorrect base by an error-prone DNA polymerase. Pyrimidine dimers are known to block DNA replication because DNA polymerase halts before DNA lesions. Specific DNA polymerases that proceed beyond DNA lesions in a process referred to as translesion synthesis have been found in

both prokaryotes and eukaryotes (3). These polymerases are thought to function by allowing DNA synthesis to be completed. Translesion synthesis presumably provides some tolerance to DNA damage for organisms. DNA polymerase IV (4) and V (5,38) in *Escherichia coli* are well known as polymerases with translesion synthesis capability. In eukaryotes, DNA polymerase η (11,12,39), Rev1 (6–8) and DNA polymerase ζ (9,10) have been extensively studied. These DNA polymerases possess low fidelity for DNA synthesis and readily incorporate incorrect bases. Especially *E. coli* DNA polymerase V has TLS activity but tends to incorporate adenine opposite to DNA lesion (5). Then G:C to A:T transition can be occurred by the incorporation of adenine opposite cytosine in CPD. In *Arabidopsis*, several genes known to be involved in translesion synthesis have been identified, such as the *Arabidopsis* homologs of *REV1, REV3 and REV7* (*AtREV1, AtREV3 and AtREV7*) (40,41), etc. *AtREV3 and AtREV7* are putative catalytic subunits of *Arabidopsis* DNA polymerase ζ and it is presumed that *AtREV1* and *AtREV7* also have TLS acitivity to UV induced DNA damage (40–42). The other possible mechanism causing G:C to A:T transitions is the deamination of cytosine or 5-methylcytosine in dipyrimidines (43). When cytosine or 5-methylcytosine in CPD is deaminated to uracil or thymine, respectively, adenine is incorporated during DNA synthesis by error-free translesion synthesis catalyzed by DNA polymerase η (12,44–49). *Arabidopsis* also has homolog of DNA pholymerase η (*AtPOLH*) and Santiago *et al.* (50) reported that *AtPOLH* complement Polη deficient *Saccharomyces cerevisiae* strain. The mechanism of UV-induced G:C to A:T transitions in *Arabidopsis* is presumably similar to that in bacteria or animals although further investigation is required. The frequency of frameshift mutation occurred at dipyrimidine site was increased in UVB-irradiated AtCPHRs/rpsL. Streisinger slippage model is one of the plausible model for frameshift mutation occurring on repetitive base sequence (51). Other two models, direct misincorporation misalignment (52,53) and dNTP-stabilized misalignment (54,55), are proposed for frameshifts on non-repetitive base sequence. In these models one base frameshift is caused by one base located out of helical plane during DNA synthesis and subsequent misalignment. The mechanism of induction of frameshift mutation found in this study is still unknown. But our findings indicate that CPD is one of the causative factors inducing frameshifts in *Arabidopsis* and that CPD photolyase might suppress the occurrence of this type of mutation.

Our results indicated that CPDs are toxic for higher plants because of the hypersensitivity to and hypermutability in response to UV-B in CPD photolyase knock-down *Arabidopsis*. CPD photoreactivation is an effective defense mechanism to protect DNA from UV and possibly plays an important role for protecting plants from solar UV and maintaining genome stability. Plants have been reported to possess dark repair (excision repair) in addition to light repair (photoreactivation). Kimura *et al.* (56) reported that each repair system works in different tissues in plants. Further investigation of DNA repair systems in higher plants should provide further understanding of their contribution to UV tolerance.

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