Expression and Complementation Analyses of a Chloroplast-Localized Homolog of Bacterial RecA in the Moss Physcomitrella patens

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RecA protein is widespread in bacteria, and it plays a crucial role in homologous recombination. We have identified two bacterial-type recA gene homologs (PprecA1, PprecA2) in the cDNA library of the moss Physcomitrella patens. N-terminal fusion of the putative organellar targeting sequence of PpRecA2 to the green fluorescent protein (GFP) caused a targeting of PpRecA2 to the chloroplasts. Mutational analysis showed that the first AUG codon acts as initiation codon. Fusion of the full-length PpRecA2 to GFP caused the formation of foci that were colocalized with chloroplast nucleoids. The amounts of PprecA2 mRNA and protein in the cells were increased by treatment with DNA damaging agents. PprecA2 partially complemented the recA mutation in Escherichia coli. These results suggest the involvement of PpRecA2 in the repair of chloroplast DNA.

Key words: Physcomitrella patens; homologous recombination; DNA repair; chloroplast; RecA

Chloroplasts, which have evolved from endosymbiosis of ancient cyanobacteria, have their own genetic systems, replication, transcription, and translation, resembling those of bacteria. Although not extensively studied yet, it is reasonable that bacteria-type recombinational repair systems function in chloroplasts. In fact, homologous recombination has been observed in a chloroplast genome.1–3) Homologous recombination is a process that occurs in all organisms. It is responsible for genetic diversity, the maintenance of genome integrity, and the proper segregation of chromosomes.5) The functional relationships between DNA replication and recombinational processes have recently been recognized.5–8) RecA protein, a crucial component in homologous recombination and the related repair system in bacteria, binds to DNA to form a nucleoprotein filament that then aligns with a homologous duplex to promote single-strand exchange.9,10) Recent study suggests that RecA and other recombination proteins also participate in the nonmutagenic repair of stalled or collapsed replication forks.9)

Homologs of RecA have been identified in plant species,11) and functions of them in chloroplasts have been suggested. A plant Arabidopsis thaliana cDNA encoding for a bacterial RecA homolog, AtcpRecA (At1g79050), has been isolated,12) and was found to be targeted to chloroplasts.13) In pea chloroplasts, strand transfer activity resembling that of bacterial RecA was detected14) and a protein cross-reacting with the Escherichia coli RecA antibody was identified.15) A RecA dependent recombination system has also been suggested for the chloroplast of Chlamydomonas reinhardtii, unicellular green algae,16) and the C. reinhardtii chloroplast RecA was identified.17) Thus it is likely that an RecA-dependent recombination and repair system is ubiquitous and well conserved among chloroplast genetic systems, but little is known about the biological significance of homologous recombination in chloroplasts.

A moss, Physcomitrella patens, has become a model plant in the study of gene function by targeted gene disruption, which occurs with efficiency similar to that observed in yeast.18) We have reported that two P. patens RecA homologs, PpRecA1 and PpRecA2, have been identified, and that PpRecA1 was specifically

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Abbreviations: cpDNA, chloroplast DNA; DAPI, 4′,6-diamidino-2-phenylindole; DSBs, double strand breaks; GFP, green fluorescent protein; MMS, methyl methanesulfonate; RT-PCR, reverse transcriptase-polymerase chain reaction; TBS, Tris-buffered saline
localized to mitochondria. Here we report the analysis of a PprecA2 cDNA encoding a putative chloroplast RecA protein. Analysis of the RecA in this model plant cells should open ways to understand the chloroplast DNA maintenance system in plant cells.

Materials and Methods

Plant materials, growth conditions, and DNA damage treatments. Protonemata of Physcomitrella patens Bruch and Schimp subsp. patens were cultured on cellophane-overlaid BCDAT medium (1 mM MgSO₄, 10 mM KNO₃, 45 μM FeSO₄, 1.8 mM KH₂SO₄, 1 mM CaCl₂, 5 mM ammonium tartrate, and 0.8% agar). All plants were grown at 25°C under constant light conditions (70 μmol photons m⁻² s⁻¹). For semi-quantitative RT-PCR or Western blot analysis of PprecA2, methyl methanesulfonate (MMS) or bleomycin treatments were performed, as described previously.

Construction of GFP-fusion plasmids. A DNA fragment containing the green fluorescent protein (GFP) gene, in which the initiation codon ATG is converted to TTG, was obtained by KpnI-EcoRI digestion of pGFPmutNPTII (T. Fujita, unpublished), and was then cloned into KpnI-cleaved p7133-PprecA1-sGFP, a derivative of p7133TP-sGFP (21) carrying the coding segment of the second ATG, yielding pINO42. Next, the first 500 bp amplified from pINO41 was cloned into PprecA2-F001 and PprecA2-R04. The PCR product pINO41 and pINO42, was amplified using primers PprecA1 cleaved p7133-sGFP (TTG), yielding pINO44. The PCR product amplified from pINO42 was cloned into KpnI-ClaI cleaved p7133-sGFP (TTG), yielding pINO47.

The full-length PprecA2 coding region was amplified by PCR using cloned PprecA2 cDNA as template and primers 5'-CCCGGTACGGCCGCCGGGGGCATGGACGAAATAGCTGTC-3' and 5'-CCCGAGTCGACTGGCCCAGGAGTCAAGTCCTCCAAATTGTCC-3'. The PCR product was cloned into KpnI-Neol cleaved p7133-PprecA1-sGFP, (19) yielding pMAK125.

Transient expression of GFP-fusion constructs in P. patens and fluorescent microscopy observation. The generation of P. patens protoplasts and the subsequent transformation were performed as described previously. Two days after transformation, the GFP, chlorophyll, and 4', 6-diamidino-2-phenylindole (DAPI) fluorescence of the protoplasts was observed using an Olympus AX80 microscope (Olympus, Tokyo). For DAPI staining, protoplasts were fixed with 1% glutaraldehyde (Wako, Osaka, Japan) dissolved in TAN buffer (20 mM Tris–HCl, pH 7.65, 0.5 mM EDTA, 7 mM 2-mercaptoethanol, 0.4 mM phenylmethylsulfonyl fluoride, and 1.2 mM spermidine) and were stained with 0.2 μg/ml of DAPI for 10 min, followed by fluorescent microscopy observation.

Semi-quantitative reverse transcriptase-PCR (RT-PCR) for expression analysis. Total RNA was isolated from wild-type protonemata with the RNase Plant Mini Kit (Qiagen, Valencia, CA). Reverse transcription and subsequent PCR were performed as described previously. To amplify a segment of PprecA2, primers 5'-CCCTCTAGACGGGTTTAATGAGTCCA-3' and 5'-GAAATCCCTACAACACACAACC-3' were used. PCR amplification was performed in the exponential amplification phase. The PCR products were analyzed by electrophoresis in an agarose gel, and the gel was stained with SYBR Green I (Takara Bio Inc., Otsu, Japan). The integrated intensities of the product bands were determined using Typhoon 9210 and Image Quant (GE Healthcare, Little Chalfont, UK).

Antisera generation. For PCR amplification of the coding segment of the PpRecA2 C-terminal region (amino acids 321-460), cloned PprecA2 cDNA as template and primers 5'-CCCTCTAGACGGGTTTAATGAGTCCA-3' and 5'-GAAATCCCTACAACACACAACC-3' were used. The PCR product was cloned into NdeI-SacI cleaved with pET-22b (Novagen, Inc., Madison, WI, USA) to produce C-terminal 6 x His-tagged recombinant protein, yielding pINO21. The recombinant protein was purified with a Ni-NTA agarose (Qiagen) according to the manufacturer’s instructions. The protein was further purified by SDS-PAGE, and the excised protein in the gel slice was used to immunize the rabbits.
Western blot analysis. Protonema were flash-frozen in liquid nitrogen and disrupted with a Multi-beads shocker (MB400U, Yasui-kikai, Osaka, Japan). Soluble plant proteins were extracted in 50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 10 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. Samples were separated into supernatant and pellet fractions by centrifugation at 20,000 × g for 20 min. All procedures were performed on ice or at 4 °C. Protein concentrations were measured with Coomassie plus protein assay reagent (Pierce Chemical, Rockford, IL, USA). For Western blot analysis, 50 µg of soluble proteins was separated in 12% polyacrylamide gels and electroblotted onto polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were blocked in 5% (w/v) skimmed milk powder in Tris-buffered saline (TBS; 20 mM Tris–HCl, 150 mM NaCl, pH 7.5) for 1 h, and incubated with anti-PpRecA2 antiserum at a dilution of 1:1,000 in TBS as primary antibody reaction, then in goat anti-Rabbit antibody conjugated with alkaline phosphatase at a dilution of 1:5,000 in TBS. Washes were performed with three changes of TBS. The alkaline phosphatase reaction was visualized using the Amplex Red Fluorescent AP substrate system (Promega, Madison, WI, USA). The integrated intensities of the signal were determined by Typhoon 9210 and Image Quant.

Assay for complementation of E. coli recA deficient strain. The PprecA2 gene was amplified by PCR with primers 5′-GGGCTGCGATGGCAAAGCCAAAGGAAAA-GA-3′ and PprecA2-3′PBS, and the PCR product was cloned between the NcoI and PstI sites of pBAD24,23 yielding pINO1. E. coli CSR603 [recA1, uvrA6, phrJ-1]24 cells bearing pINO1, PMAK13219 carrying E. coli recA or pBAD24 were cultured to OD_{660} of 0.5 in M9 medium25 containing 0.2% glucose and then transferred to M9 medium25 containing 0.2% arabinose and incubated for 30 min at 37 °C for gene induction. Cells were treated with different concentrations of mitomycin C and incubated for 30 min at 37 °C for gene induction. Cells were treated with different concentrations of mitomycin C and incubated for 30 min at 37 °C. After washing, appropriate dilutions of treated cells were spread on LB plates and incubated overnight at 37 °C. Colony-forming units were counted, and the surviving fraction of cells was calculated as described previously.19

Results

Identification of Physcomitrella patens recA (Pprec-A2)

By means of a BLAST search of full-length enriched cDNA libraries of P. patens,25 we have identified two distinct recA homologs which we named PprecA1 and PprecA2.19 The PprecA2 cDNA had two putative translation initiation sites in its 5’ region and encoded a 460-residue or a 417-residue protein. Except for an N-terminal peptide, which was predicted to be a chloroplast transit peptide, the putative gene product shared high similarity with E. coli RecA protein and chloroplast RecA proteins from A. thaliana and C. reichardtii (Fig. 1A). We determined the genomic DNA sequence encompassing PprecA2 and compared it with the cDNA sequence. The PprecA2 gene was composed of 13 exons and 12 introns, and the locations of the introns corresponded well with those of AttcpRecA (At1g79050) (Fig. 1B).

Subcellular localization of PpRecA2-GFP

To determine the subcellular localization of PpRecA2, we prepared GFP fusion constructs containing the 5′-upstream sequence. The first 500 bp of the PprecA2 cDNA sequence, encompassing the coding region for the putative signal peptide, was fused to the GFP gene under the control of a modified cauliflower mosaic virus 35S promoter, E7133,26. The construct was introduced into P. patens protoplasts by polyethylene glycol-mediated transformation. The subcellular distributions of PpRecA2-GFP were completely included in the areas of red chlorophyll fluorescence, indicating that PpRecA2 was targeted to the chloroplasts (Fig. 2A, top). Using this construct, we did not detect the fluorescence of the PpRecA2-GFP in the mitochondria.

There exist two in-frame ATG codons in the 5′ region of PprecA2. Both of the translated products were predicted to be targeted to chloroplasts according to TargetP27 (the scores for the larger and smaller ones were 0.957 and 0.620 respectively). The nucleotide sequence context of the AUG plays a role in the efficiency of translation initiation, but it was difficult to estimate the functional initiation codon by a comparison of plant consensus sequences around AUG.28 To examine the translation initiation site, we prepared GFP fusion constructs in which one of the ATG codons was mutated, and introduced it into P. patens protoplasts. Using the construct with a mutation in the first ATG codon, faint green fluorescence was detected (Fig. 2A, middle). On the other hand, using the construct with a mutation in the second ATG codon, strong fluorescence was detected in the chloroplasts (Fig. 2A, bottom), similarly, to the result of the construct without the mutation. These results indicate that despite the presence of two in-frame ATGs in the 5′ region of PprecA2, the first ATG is recognized as the initiation site, resulting in the production of a protein that is targeted to chloroplasts.

To examine further the subcellular localization of PpRecA2 as a whole protein, we prepared a GFP construct containing full-length PprecA2, which encompasses the entire coding region, and introduced it into P. patens protoplasts. The green fluorescence of PpRecA2-GFP formed small foci in chloroplasts (Fig. 2B). By staining with DAPI, the foci were colocalized with chloroplast nucleoids (Fig. 2B). This result indicates that PpRecA2 in the chloroplasts is associated with chloroplast nucleoids.
Enhanced expression of \textit{PprecA2} after DNA damage

Expression of the mitochondrial \textit{PprecA1} has been shown to be enhanced after DNA damage. Moreover, the \textit{recA} homologs of pea, \textit{A. thaliana}, and \textit{C. reinhardtii} chloroplasts perhaps increase after DNA damage. To examine \textit{PprecA2} expression in DNA-damaged cells of \textit{P. patens}, we analyzed the level of \textit{PprecA2} expression in MMS- and bleomycin-treated cells by semi-quantitative RT-PCR and immunoblot analysis using anti-PpRecA2 antiserum. MMS, a DNA methylating agent, perhaps causes double strand breaks (DSBs) by forming methylated bases in DNA, while bleomycin, a radiomimetic DNA-damaging agent, causes DSBs directly. Total RNA and total soluble proteins were extracted from protonemata treated with MMS or bleomycin for 6 h, and semi-quantitative RT-PCR or immunoblot was performed. \textit{PprecA2} expression was strongly induced by treatment with MMS and bleomycin.

Fig. 1. \textbf{A} Bacterial RecA Homologs.
A, Alignment of the amino acid sequence of two RecA homologs from \textit{P. patens} (PpRecA1, PpRecA2), chloroplast RecA homologs from \textit{A. thaliana} (AtcpRecA, At1g79050) and \textit{C. reinhardtii} (CrRecA), and \textit{E. coli} RecA. The accession numbers are PpRecA1 (AB284534), PpRecA2 (AB284535), AtcpRecA (NP_001077844), CrRecA (AB048829), and \textit{E. coli} RecA (AP_003266). Sequence identities are inverted. The conserved functional domains of RecA are indicated under the sequences: Walker A and B are involved in ATP hydrolysis, and loop 1 and loop 2 are important for DNA binding. The box represents the second methionine in the putative transit peptide of PpRecA2, which was mutated to leucine as in Fig. 2. B, Gene organizations of \textit{PprecA2} and AtcpRecA (At1g79050). The filled boxes and the lines between the boxes represent exons and introns respectively. The corresponding exons are joined to each other by lines.
with bleomycin (Fig. 3A). Correspondingly, the amount of PpRecA2 protein in MMS- or bleomycin-treated cells increased by up to about 3-fold as compared with non-treated cells (Fig. 3B). These results suggest that PpRecA2 is involved in DNA repair.

Complementation of an E. coli recA deficient strain by PprecA2

To determine the functional similarities between chloroplast RecAs and their bacterial counterpart, complementation analysis was performed with an E. coli recA deficient strain. The cDNA fragment encoding the PpRecA2 protein without the putative signal peptide was placed under the control of the arabinose promoter, and the construct was transformed into an E. coli recA deficient strain. Survival rates were measured after treating the cells with the DNA cross-linking agent mitomycin C. Upon the addition of arabinose, the survival fraction of E. coli recA deficient cells bearing the PprecA2 plasmid increased by up to 100-fold as compared to that of the strain bearing a plasmid lacking PprecA2, whereas no increase was observed when PprecA2 expression was repressed by the addition of glucose (Fig. 4). These results suggest that PpRecA2 partially complements the function of E. coli RecA.

Discussion

In this study, we identified a Physcomitrella homolog of the bacterial-type RecA gene. The gene product shares extensive sequence similarity with E. coli RecA protein and chloroplast RecA from A. thaliana [12] and C. reinhardtii [17] except for the amino-terminal region. The PprecA2 transcript possesses two in-frame AUG codons in the 5’ terminal region. It has been reported that 5’ sequences of phage-type RNA polymerase (PpRPOT1 and PpRPOT2) genes in P. patens also contained two in-frame AUG codons, and that both of proteins were translated from the second AUG codon and targeted to mitochondria. [31] It has also been reported that an A. thaliana RecA homolog, At2g19490, is dual-targeted to mitochondria and chloroplasts. [32] In vivo experiments with constructs containing the mutated first AUG codon or the mutated second one revealed that PpRecA2 was translated from the first ATG codon, and...
that this protein was targeted only to chloroplasts (see Fig. 2A). Using these constructs, we did not detect the fluorescence of PpRecA2-GFP in the mitochondria. On the other hand, fluorescence of PpRecA1-GFP was detected only in the mitochondria. On the basis of these lines of evidence, we concluded that PprecA2 gene encodes a bacterial-type RecA homolog that functions in the chloroplasts. This is consistent with a phylogenetic analysis suggesting that PpRecA2 is closely related to chloroplast RecA and cyanobacterial RecA.

We observed full-length PpRecA2-GFP focus formation in P. patens chloroplasts (Fig. 2B). The foci were colocalized with chloroplast nucleoids, suggesting that PpRecA2 is bound to chloroplast DNA (cpDNA). In E. coli and Bacillus subtilis, RecA-GFP fusion proteins have been visualized as foci in response to DNA damage and replication of damaged DNA. The PpRecA2-GFP foci may form in response to cpDNA damage, which might be caused in the course of the procedure for protoplast preparation and subsequent transformation.

As presented in this study, PprecA2 partially complemented an E. coli recA deficient strain, and this, to our knowledge, is the first time chloroplast RecA has been observed to be involved in DNA repair through complementation of a recA-deficient strain. The strain harboring pINO1 was grown in a medium containing 0.2% arabinose (closed triangle) or 0.2% glucose (open triangle). The other strains were grown in a medium containing 0.2% arabinose. Data are expressed as mean ± S.D. (n = 3).

**Fig. 3.** Expression of PprecA2 in MMS- and Bleomycin-Treated Cells. A, Total RNA was extracted from protonemata cultivated in the presence (+) or absence (−) of DNA damaging agents, MMS (20 mM) or bleomycin (25 μg/ml). The GAPDH gene was amplified as an internal control. PCR was performed for 28 cycles for amplification of PprecA2 and for 22 cycles for the amplification of GAPDH. B, Western blot analysis of PpRecA2 in MMS- and bleomycin-treated cells. Equal amounts of soluble proteins, which were extracted from protonemata cultivated in the presence (+) or absence (−) of MMS (20 mM) or bleomycin (25 μg/ml) were used.

**Fig. 4.** Partial Complementation of an E. coli recA Deficient Strain by PprecA2. The surviving fraction after mitomycin C treatment of the CSR603 strain (recA−) harboring pMAK132 with E. coli recA (square), pINO1 with PprecA2 (triangle), or pBAD24 (circle) alone was calculated as described. The strain harboring pINO1 was grown in a medium containing 0.2% arabinose (closed triangle) or 0.2% glucose (open triangle). The other strains were grown in a medium containing 0.2% arabinose. Data are expressed as mean ± S.D. (n = 3).
been found to substitute for *E. coli* RecA in vivo. This suggests that PpRecA2 is a functional homolog of *E. coli* RecA. Recombinational repair processes can alleviate blocks to DNA replication imposed by genotoxic agents.5–9) Lesions in DNA, such as interstrand cross links produced by mitomycin C, can block DNA polymerases during replication, leading to the accumulation of single-strand DNA gaps or DSBs. It is possible that PpRecA2 does act in repair of stalled or collapsed replication forks. The degree of complementation (up to 100-fold) was high as compared to that (up to 10-fold) of PpRecA1.19) This suggests that PpRecA2 can readily interact with *E. coli* recombinational factors such as RecBCD and/or RecFOR, since the amino acid sequence similarity between PpRecA2 and *E. coli* RecA was higher than that between PpRecA1 and *E. coli* RecA (Fig. 1A). However, the degree of complementation was low as compared to that of *E. coli* RecA (see Fig. 4). *E. coli* RecA, which plays a central role in recombinational repair pathways, is also involved in the SOS response, which also contributes to DNA repair.10) This suggests that the greater survival rate of the *E. coli* recA deficient strain transformed with the *E. coli* rec A expression plasmid might be due to the SOS response. The presence of an SOS response in chloroplast is unclear, and cyanobacteria do not have an *E. coli*-type SOS regulon,53) suggesting that PpRecA2 is unable to induce the SOS response in *E. coli*.

Disruption of *PprecA1* by targeted replacement resulted in lower rates of recovery of mitochondrial DNA from MMS damage, suggesting the involvement of PpRecA1 in the repair of mitochondrial DNA.19) To elucidate a possible role of PpRecA2 in the maintenance of cpDNA in *P. patens*, studies with disrupting transplants are currently in progress.

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