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RESEARCH PAPER

RecA maintains the integrity of chloroplast DNA molecules in *Arabidopsis*

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Abstract

Although our understanding of mechanisms of DNA repair in bacteria and eukaryotic nuclei continues to improve, almost nothing is known about the DNA repair process in plant organelles, especially chloroplasts. Since the RecA protein functions in DNA repair for bacteria, an analogous function may exist for chloroplasts. The effects on chloroplast DNA (cpDNA) structure of two nuclear-encoded, chloroplast-targeted homologues of RecA in *Arabidopsis* were examined. A homozygous T-DNA insertion mutation in one of these genes (*cpRecA*) resulted in altered structural forms of cpDNA molecules and a reduced amount of cpDNA, while a similar mutation in the other gene (*DRT100*) had no effect. Double mutants exhibited a similar phenotype to *cprecA* single mutants. The *cprecA* mutants also exhibited an increased amount of single-stranded cpDNA, consistent with impaired RecA function. After four generations, the *cprecA* mutant plants showed signs of reduced chloroplast function: variegation and necrosis. Double-stranded breaks in cpDNA of wild-type plants caused by ciprofloxacin (an inhibitor of *Escherichia coli* gyrase, a type II topoisomerase) led to an alteration of cpDNA structure that was similar to that seen in *cprecA* mutants. It is concluded that the process by which damaged DNA is repaired in bacteria has been retained in their endosymbiotic descendent, the chloroplast.

Key words: DNA structure, fluorescence microscopy, homologous recombination, leaf variegation, pulsed-field gel electrophoresis.

Introduction

All organisms experience DNA damage, which must be repaired in order to avoid deleterious phenotypic consequences (Thompson and Schild, 2001; Tuteja *et al.*, 2009). Many processes can be used to maintain the integrity of the genome: photoreactivation, base excision repair, nucleotide excision repair, mismatch repair, homologous recombination, and cross-link repair (Sancar and Sancar, 1988; Niedernhofer, 2008). Mechanistic details are becoming increasingly clear in bacteria, eukaryotic nuclei, and the mitochondria of yeast and mammals (Bohr and Anson, 1999; Nowosielska, 2007; Aguilera and Gomez-Gonzalez, 2008). By comparison, however, DNA repair processes in the mitochondria and chloroplasts of plants remain poorly understood (Kimura and Sakaguchi, 2006; Vlcek *et al.*, 2008).

Homologous recombination is important for the repair of double-strand DNA breaks (DSB), DNA gaps, and interstrand cross-links, and occurs by invasion of a homologous DNA strand followed by strand exchange (Li and Heyer, 2008). The *Escherichia coli* RecA/RecBCD pathway of strand invasion and exchange is perhaps the best characterized system for DNA repair by homologous recombination (Cox, 2007; Michel *et al.*, 2007). The RecA protein is highly conserved among diverse bacterial species, and the RecBCD complex has different functional analogues in different bacterial lineages (Rocha *et al.*, 2005).

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; cpDNA, chloroplast DNA; PFGE, pulsed-field gel electrophoresis; DSB, double-strand DNA breaks.

Eukaryotic homologues of RecA (RAD51) and organelle-targeted prokaryotic RecA homologues are encoded by the nuclear genomes of many organisms, including plants (Lin *et al.*, 2006). Repair and recombination of chloroplast DNA (cpDNA) in *Chlamydomonas reinhardtii* is suppressed when a dominant-negative version of *E. coli* RecA is targeted to chloroplasts (Cerutti *et al.*, 1995). The prevalence of chloroplast-targeted *RecA* mRNA increases in response to DNA damage in *C. reinhardtii* (Nakazato *et al.*, 2003), *Physcomitrella patens* (Inouye *et al.*, 2008), pea (Cerutti *et al.*, 1993), and *Arabidopsis* (Cao *et al.*, 1997). Mitochondrial-targeted RecA has been shown to be important for mitochondrial DNA (mtDNA) repair in *P. patens* (Odahara *et al.*, 2007) and for mtDNA recombination and repair in *Arabidopsis* (Khazi *et al.*, 2003; Shedge *et al.*, 2007).

The nuclear genome of *Arabidopsis* encodes five putative homologues of RecA that are predicted to be localized in mitochondria and chloroplasts (Table 1). Locus AT1G79050 encodes a protein (RECA1; cpRecA) that has been shown experimentally to be located in chloroplasts (Cao *et al.*, 1997; Shedge *et al.*, 2007) and has a 61% identity with cyanobacterial RecA (Cerutti *et al.*, 1992). Locus AT3G12610 encodes a protein (DRT100) that has only weak homology to *E. coli* RecA and has a putative chloroplast-targeting signal peptide (Pang *et al.*, 1992). *DRT100* cDNA can, however, partially restore the growth phenotypes of *recA* mutants of *E. coli* (Pang *et al.*, 1992, 1993). Locus AT2G19490 encodes a protein (RECA2) that localizes to both mitochondria and chloroplasts, and locus AT3G10140 (RECA3) encodes a protein that localizes to mitochondria only (Shedge *et al.*, 2007). Locus AT3G32920 has been annotated as encoding a mitochondrial-targeted RecA protein, but the gene is probably a pseudogene (Shedge *et al.*, 2007).

Previous reports describing the consequences of mutation in a chloroplast-targeted *RecA* homologue for any plant could not be found [other than lethality previously reported for *RECA1/cpRecA* (Shedge *et al.*, 2007)]. In this study, the function of two of the three chloroplast-targeted RecA proteins in *Arabidopsis* is addressed using T-DNA insertion mutants and it is shown that the *cpRecA* gene encodes a protein that functions similarly to bacterial RecA. Pulsed-field gel electrophoresis (PFGE) and fluorescence microscopy of individual ethidium bromide-stained cpDNA molecules were used to analyse cpDNA structure. In wild-type (wt) *Arabidopsis*, as in other plants, in-gel-prepared cpDNA consists of multigenomic, complex forms that do not migrate into the gel, discrete bands of monomeric and oligomeric linear molecules, and a smear of linear molecules. Circular forms are rarely found. Reduction of *cpRecA* mRNA leads to a decrease in the prominence of the bands, an increase in the smear, and leaf abnormalities that probably result from a lack of DNA damage repair in chloroplasts.

Materials and methods

Plant growth conditions

Seeds of *Arabidopsis thaliana* (Columbia or SALK T-DNA insertion lines obtained from the Arabidopsis Biological Resource Center, http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm) were sown on soil (for the experiments shown in Figs 1–4) or on sterile agar plates containing MS salts (Murashige and Skoog, 1962), 1% sucrose, and 0, 0.5, 1, or 2 μM ciprofloxacin (for the experiments shown in Figs 5 and 6). Seeds were held at 4 °C for 3 d to promote uniform germination before transferring them to a greenhouse (soil) or growth room at 20 °C with constant light at 30 μEinsteins m^{-2} s^{-1}.

Obtaining homozygous T-DNA insertion mutants

Wt plants and plants homozygous for a T-DNA insertion near the 3′ end (*drt100-1*) or in the middle (*drt100-2*) of the single exon in the *DRT100* gene were selected from SALK lines 021479 and 102492, respectively, using PCR-based genotyping. Primers to distinguish wt alleles from those containing the T-DNA insertion were designed using the T-DNA express tool at http://signal.salk.edu/cgi-bin/tdnaexpress (see Table 2). The wt plants and plants homozygous for a T-DNA insertion in the third to last exon of the *cpRecA* gene (*cprecA*) were selected similarly from SALK line 072979. Seeds from single individual homozygous plants (wt or *cprecA*; generation 1) were collected and raised to maturity (generation 2). Seeds from two individuals were then collected (generation 3) and used to produce the plants used for chloroplast isolation and PFGE experiments. Two individuals were selected at each generation starting at generation 3 to give rise to generations 4–7.

Measurement of mRNA levels for DRT100 *and* cpRecA *using reverse transcriptase real-time quantitative PCR*

RNA from shoots of 13-day-old seedlings was prepared using a Sigma Spectrum™ Plant Total RNA Kit, treated with DNase (DNA-free™ Kit, Applied Biosystems, http://www3.appliedbiosystems.com/AB_Home/index.htm) and reverse transcribed using the BioRad iScript™ cDNA synthesis kit (www.biorad.com). Amplification of 2 μl of cDNA was carried out using the BioRad iQ™ SYBR Green Supermix (www.biorad.com) with the primer sets described in Table 2. Following an initial denaturation at 94 °C for 3 min 15 s, 45 cycles of 20 s denaturation at 94 °C, 20 s annealing at 57 °C, and 30 s extension at 72 °C were

Table 1. Organelle-targeted homologues of bacterial RecA encoded by the *Arabidopsis* nuclear genome

Locus	Name	Compartment of gene product	Reference
AT1G79050	*RECA1; cpRecA*	Chloroplast	Cerutti *et al.* (1992); Cao *et al.* (1997); Shedge *et al.* (2007)
AT3G12610	*DRT100*	Chloroplast	Pang *et al.* (1992, 1993)
AT2G19490	*RECA2*	Chloroplast; mitochondria	Shedge *et al.* (2007)
AT3G10140	*RECA3*	Mitochondria	Khazi *et al.* (2003); Shedge *et al.* (2007)
AT3G32920	None	Mitochondria[a]	Shedge *et al.* (2007)

[a] Mitochondrial targeting based on NBCI GenBank annotation.

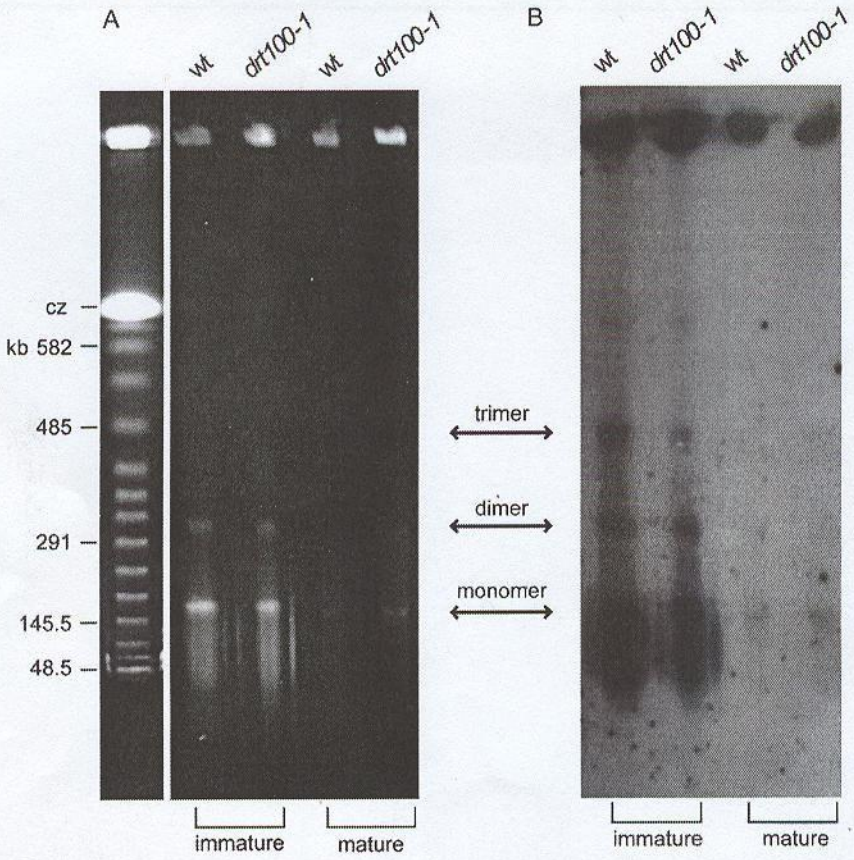

Fig. 1. The effect of a T-DNA insertion in *DRT100* on cpDNA amount and structure. (A) PFGE of cpDNA obtained from an equal volume of pelleted chloroplasts from wt and *drt100-1* mutant plants after staining with ethidium bromide. (B) Blot hybridization of the gel in (A) with an 854 bp cpDNA-specific probe that contains a portion of the *petA* gene. Similar results were obtained with a different T-DNA insertion allele (*drt100-2*; data not shown). Immature, entire shoots of plants grown for 16 d post germination; mature, third rosette leaves of plants grown for 30 d post-germination. The ratio of the hybridization signals for each of the lanes is 2.5:2.2:1:1.2 for wt immature:*drt100-1* immature:wt mature:*drt100-1* mature, respectively. Linear DNA sizes (in kb) are indicated. cz, compression zone.

performed, and amplification of the reactions was monitored using the Chromo 4 real-time detection system (Bio-Rad Laboratories, www.biorad.com). A melting curve from 65 °C to 95 °C was used to confirm the presence of single products. Data were analysed using the Opticon Monitor 3 software (Bio-Rad Laboratories, www.biorad.com) and the amount of each transcript was determined relative to the actin gene *ACT2* using the $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001). The highest amount was assigned a value of 1 and all other values are expressed relative to 1.

Chloroplast isolation and preparation for PFGE and fluorescence microscopy

Tissue collected from plants grown in a greenhouse was washed for 3–5 min in 0.5% sarkosyl and rinsed exhaustively four times in tap water and four times in distilled water before isolating chloroplasts using the high-salt method that avoids using DNase (Shaver *et al.*, 2006; Rowan *et al.*, 2007). For PFGE and analysis of cpDNA molecules from individual chloroplasts, the isolated chloroplasts were embedded in agarose and lysed overnight at 48 °C in 1 M NaCl, 5 mM EDTA, 1% sarkosyl, and 200 μg ml^{-1} proteinase K. Agarose plugs were washed extensively in many plug volumes of 10 mM TRIS, 1 mM EDTA (TE) before PFGE with 1.5% (w/v) agarose at 5 V cm^{-1}, in 45 mM TRIS, 45 mM boric acid, 1 mM EDTA, and a pulse time of 50 s. Prior to mung bean nuclease treatment, washed cpDNA–agarose plugs were treated with 2 mM phenylmethylsulphonyl fluoride (PMSF) for 3 h, then treated with an additional 2 mM PMSF for 1 h. PMSF-treated cpDNA–agarose plugs were washed extensively in many plug volumes of TE before soaking in 30 mM sodium acetate, pH 7.5, 50 mM NaCl, 1 mM $ZnCl_2$, 5% glycerol for 1 h on ice and treating with or without 2.5 U of mung bean nuclease for 15 min or 30 min at 37 °C in a total volume (liquid plus gel) of 120 μl. Digestion was stopped by adding 0.1% sarkosyl, 10 mM EDTA, pH 9. For analysis of cpDNA from individual chloroplasts, agarose-embedded cpDNA was prepared at a concentration 100- to 500-fold less than was used for PFGE, stained with 0.1 μg ml^{-1} ethidium bromide, and visualized as described (Oldenburg and Bendich, 2004*b*). For estimation of genome equivalents per plastid, isolated chloroplasts were stained with 4′,6-diamidino-2-phenylindole (DAPI) and the relative fluorescence intensity (Rfl) was measured as described (Rowan *et al.*, 2004, 2007). Rfl was determined similarly for glutaraldehyde-fixed, DAPI-stained vaccinia virus particles. The number of chloroplast genome equivalents per plastid was calculated using the equation: chloroplast genome equivalents=1.33V (where V=the DAPI–DNA Rfl of the plastid divided by the mean Rfl of vaccinia virus particles). The value 1.33 is a constant that accounts for the differences between the size and base composition between the *Arabidopsis* chloroplast genome and the vaccinia virus genome, and was determined as (%AT content of vaccinia virus genome/%AT content of *Arabidopsis* chloroplast genome)×(number of bp of vaccinia virus

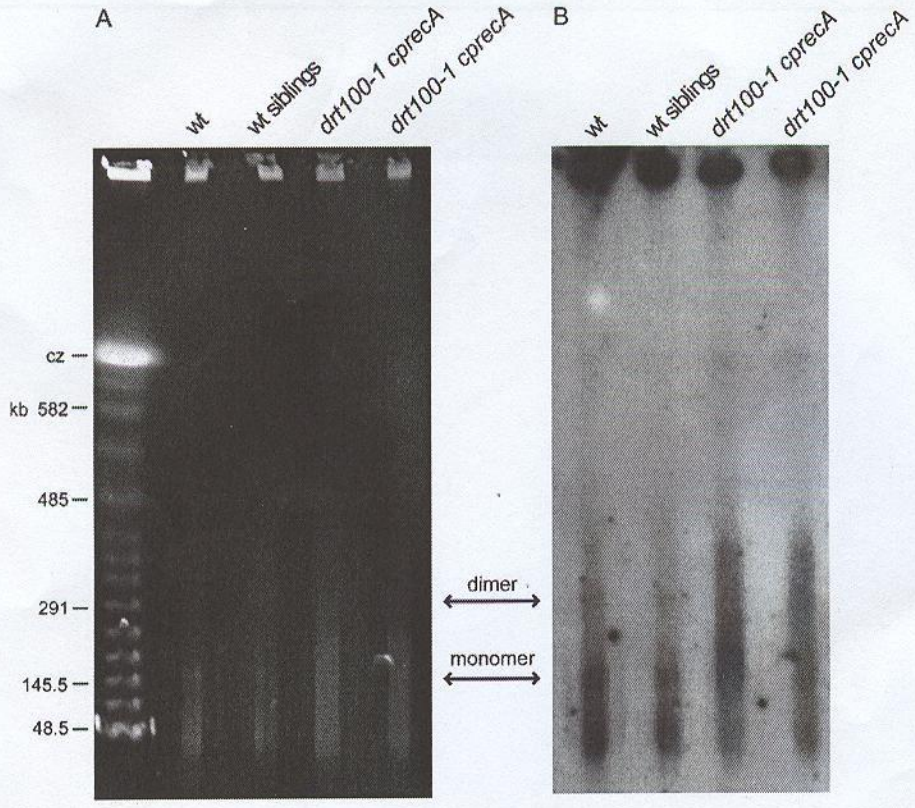

Fig. 3. Structure of cpDNA in *drt100-1 cprecA* double mutants. (A) PFGE of cpDNA obtained from an equal volume of pelleted chloroplasts after staining with ethidium bromide and (B) blot hybridization of the gel in (A) with a *petA* probe. The cpDNA from wt, wt siblings (F_3 wt siblings obtained from the F_2 segregating population), and *cprecA drt100-1* double mutants (two independent F_3 lines obtained from the F_2 segregating population) is indicated. As with *cprecA* single mutants (Fig. 2), the double mutants show no prominent bands of genomic monomers and dimers, and more of the DNA migrates as a smear compared with their wt siblings and unrelated wt (Col) plants. DNA was obtained from chloroplasts isolated from 13-day-old seedlings. Linear DNA sizes (in kb) are indicated. cz, compression zone.

genome/number of bp of *Arabidopsis* chloroplast genome), where %AT for vaccinia virus (Copenhagen strain) is 66.6, %AT for *Arabidopsis* cpDNA is 64%, number of bp for vaccinia virus DNA is 197361 and number of bp for *Arabidopsis* cpDNA is 154361. Brightfield images of the chloroplasts were recorded and used to measure plastid area.

Blot hybridization of cpDNA from PFGE

PFGE-separated cpDNA was alkali-denatured, transferred onto a nylon membrane, and neutralized. Procedural details were described previously (Oldenburg and Bendich, 2004*b*, 2006). An 854 bp fragment of the *Arabidopsis petA* gene was labelled with alkaline phosphatase using AlkPhos Direct Labeling Reagents, and hybridization was detected using the CDP-Star Detection Reagent (GE Healthcare). The hybridization signals were quantified using NIH Image J software (http://rsb.info.nih.gov/ij/). Lanes on the image of the blot were selected and the software plotted the intensity of the signal down the lane. The area under the curves generated by the software was calculated to determine the strength of the signal from the corresponding regions of the lanes.

Protein alignment, phylogenetic analysis, and targeting sequence prediction

Protein sequences were obtained from NCBI GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nuccore), aligned using MacVector™ 8.0 (http://www.macvector.com/), and analysed by the Predotar v. 1.03 (http://urgi.versailles.inra.fr/predotar.html) and TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP) targeting sequence prediction algorithms. The coding sequences, also obtained from NCBI GenBank, were aligned using MacClade 4.0.8. (http://macclade.org/macclade.html), and a Neighbor–Joining tree was created with these aligned sequences using MEGA 4 (http://www.megasoftware.net/) with the default settings.

Results

Organelle-targeted homologues of RecA encoded by the Arabidopsis *nuclear genome*

For two of the three genes encoding chloroplast-targeted RecA proteins of *Arabidopsis*, homozygous T-DNA insertion mutants were obtained by PCR-based genotyping of seeds from SALK T-DNA insertion lines (see Materials and methods). The seeds produced by an individual plant of each genotype (homozygous mutants and their wt counterparts) were collected and the resulting plants were raised to maturity in order to obtain enough seeds to generate sufficient plant tissue for the analysis of cpDNA by PFGE and blot hybridization.