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Plastid DNA polymerases from higher plants, *Arabidopsis thaliana*

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Abstract

Previously, we described a novel DNA polymerase, designated as *OsPolI-like*, from rice. The OsPolI-like showed a high degree of sequence homology with the DNA polymerase I of cyanobacteria and was localized in the plastid. Here, we describe two PolI-like polymerases, designated as *AtPolI-like A* and *AtPolI-like B*, from *Arabidopsis thaliana*. In situ hybridization analysis demonstrated expression of both mRNAs in proliferating tissues such as the shoot apical meristem. Analysis of the localizations of GFP fusion proteins showed that AtPolI-like A and AtPolI-like B were localized to plastids. *AtPolI-like B* expression could be induced by exposure to the mutagen H_2O_2. These results suggested that AtPolI-like B has a role in the repair of oxidation-induced DNA damage. Our data indicate that higher plants possess two plastid DNA polymerases that are not found in animals and yeasts.

Keywords: *Arabidopsis thaliana*; Plastid; Chloroplast; *AtPolI-like A*; *AtPolI-like B*; DNA polymerases; DNA repair; DNA replication

As is well known, higher plant cells contain chloroplasts and self-proliferating organelles that are not present in animals. The photosynthetic apparatus in chloroplasts is one of the largest sources of reactive oxygen species produced by UV irradiation. The DNA repair and replication mechanisms of chloroplast DNA and the stabilization of the chloroplast genome are mostly obscure.

We have undertaken a long-term study of DNA polymerases and related factors in higher plants [2–6]. In a previous report, we described a novel DNA polymerase, designated as *OsPolI-like*, from rice [1]. *OsPolI-like* has sequence homology to DNA polymerase I of cyanobacteria. Subcellular fractionation analysis suggested that *OsPolI-like* is localized to plastids (immature chloroplasts) [1]. *OsPolI-like* was considered to be involved in DNA replication itself or in the repair of errors occurring during replication [1]. Further studies of this plastid DNA polymerase may shed light on the mechanisms of chloroplast DNA repair and replication.

To further elucidate the mechanism of chloroplast DNA repair and replication, we have isolated and characterized *Arabidopsis* homologue of *OsPolI-like*. Here, we describe two PolI-like DNA polymerases from *Arabidopsis thaliana*, designated as *AtPolI-like A* and *AtPolI-like B* (*Arabidopsis thaliana DNA polymerase I-like DNA polymerase A and B*). We found that these DNA polymerases were localized in plastids but not in cell nuclei. *AtPolI-like B* was found to show upregulated expression after DNA damage was induced by H_2O_2, suggesting that it may play a role in DNA repair in plastids.

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Materials and methods

cDNA cloning of AtPolI-like A and AtPolI-like B. We searched for the *Arabidopsis* genome sequence to identify the *Arabidopsis* homologue of *OsPolI-like*. We found the two homologues in the genome and designated them as *AtPolI-like A* and *AtPolI-like B*. *AtPolI-like A* cDNA (Accession No. AF462826) was provided by the Riken Bioresource Center (http://www.brc.riken.jp/). *AtPolI-like B* cDNA was isolated using RT-PCR using *Arabidopsis* cDNA produced from purified mRNA with a SuperScript First-strand system for RT-PCR kit (Invitrogen) as template. The nucleotide sequences of the primers used for RT-PCR amplification of *AtPolI-like B* were as follows: *B*, 5′-ATGGGGGTTTCTCTTCGTCA-3′ (forward primer), 5′-TTTGCC AGCATACCAGTTCT-3′ (reverse primer). The amplified cDNA products were purified from a low-melting-point agarose gel and cloned into the pGEM vector (Promega).

The nucleotide sequence data reported in this paper appear in the DDBJ nucleotide sequence database with the Accession Nos. AB211532 and AB211533.

RT-PCR analysis. Total RNA was isolated from cultured cells using the RNeasy Plant Mini Kit (Qiagen). RT-PCR analysis was performed using SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen). RNA isolation and RT-PCR were performed according to the manufacturer's protocols. The following primers were used for RT-PCR amplification: *AtPolI-like A*, forward primer: 5′-ATGGCC ATGGGGGTTTCTCT-3′; *AtPolI-like A*, reverse primer: 5′-TTT CGGCTACCTCCGTCTGA-3′; *AtPolI-like B*, forward primer: 5′-AT GGGGGTTTCTCTTCGTCA-3′; *AtPolI-like B*, reverse primer: 5′-GCTTGTTTCACAGTTGGCGG-3′; and *AtActin*, forward primer: 5′-ATGGCTGAGGCTGATGATAT-3′; *AtActin*, reverse primer: 5′-ATTGGCACAGTGTGAGACAC-3′.

In situ hybridization analysis. Riboprobes for in situ hybridization were labeled with digoxigenin-11-rUTP using a DIG RNA Labeling Kit (Boehringer–Mannheim) according to the manufacturer's protocol. Antisense and sense probes were subjected to mild alkaline hydrolysis by heating at 60 °C in carbonate buffer and used at a concentration of 2 μg/ml. Plant tissues from 10-day-old rice seedlings were fixed for 16 h at 4 °C with a mixture of 4% (w/v) paraformaldehyde and 0.25% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.2). The fixed tissues were dehydrated through a xylene and ethanol series and embedded in paraffin (HISTPREP 568, Wako). Embedded tissues were sectioned at a thickness of 10 μm and placed on

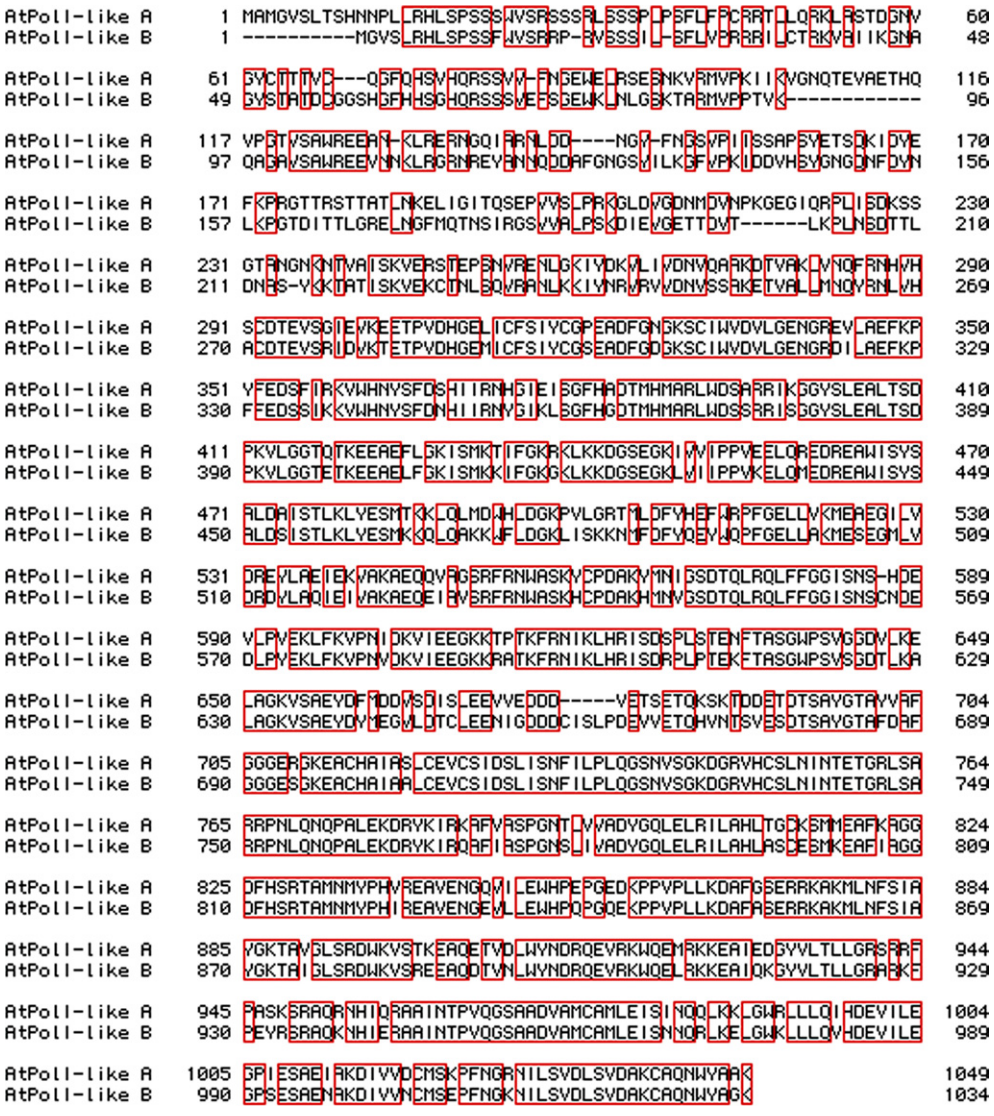

Fig. 1. Alignment of the deduced amino acid sequences of *AtPolI-like A* and *AtPolI-like B*.

microscope slides precoated with 3-aminopropyltriethoxysilane (APS). Sections were deparaffinized with xylene and rehydrated through a graded ethanol series. They were subsequently pretreated with proteinase K in 100 mM Tris–HCl, pH 7.5, and 50 mM EDTA at 37 °C for 30 min, dehydrated, and dried under vacuum for 2 h. The hybridization and detection of hybridized riboprobes were performed as described by Sato et al. [3].

Subcellular localization of AtPolI-like A and AtPolI-like B proteins. The subcellular localization of AtPolI-like A and AtPolI-like B proteins were determined by transient expression and visualization of AtPolI-like A-GFP and AtPolI-like B-GFP fusion proteins in BY2 cells. The N-terminal region of AtPolI-like A and AtPolI-like B was fused to the 5′ ends of the coding region of 35S-GFP (sGFP). AtPolI-like A-GFP and AtPolI-like B-GFP chimeric constructs were introduced into suspension-cultured tobacco BY-2 cells using a particle gun (Biolistic PSD-1000/He Particle Delivery System, Bio-Rad, Hercules, CA) according to the manufacturer's recommendations. In brief, plasmid DNAs were constructed using PCR amplification and standard cloning techniques. Gold particles with a diameter of 1.0 μm were coated with plasmid DNA. The cell suspensions were plated on filter paper placed on BY-2 and bombarded. The conditions of bombardment were: vacuum of 27 in. of Hg, helium pressure of 1100 psi, and a 6 cm target distance. After bombardment, tissues were incubated at 28 °C for 24 h. GFP fusion protein in suspension-cultured tobacco BY2 cells were screened using a Laser Scanning Confocal Microscope System (Radiacce 2100).

3D models of protein structures. We generated models of the structures of AtPolI-like A and AtPolI-like B proteins using the Swiss-Model automated protein structure homology-modeling server (http://www.expasy.ch/swissmod/SWISS-MODEL.html) [11,12,14]. We aligned amino acid sequences using ClustalW and the Swiss-Model Alignment Interface mode.

Other methods. DNA sequence analysis was carried out using GENETYX MAC var. 12.3 (Software Development). Phylogenetic analysis was performed based on the amino acid sequence by the neighbor-joining method using GENETYX MAC. The reliability of the tree was evaluated by bootstrap analysis.

Results and discussion

Identification and molecular cloning of AtPolI-like A and AtPolI-like B

We found that *Arabidopsis* had the two homologues of plastid DNA polymerase and isolated the cDNAs as described in Materials and methods. We named the homologues as *AtPolI-like A* and *AtPolI-like B*. The length of the *AtPolI-like A* and *AtPolI-like B* cDNA was 3150 and 3105 bp, respectively, which are slightly shorter than that of *OsPolI-like* (3776 bp). The open reading frames of *AtPolI-like A* and*AtPolI-like B* encoded predicted products of 1049 amino acids (molecular mass of 117 kDa) and of 1034 amino acids (molecular mass of 115 kDa), respectively (Fig. 1).

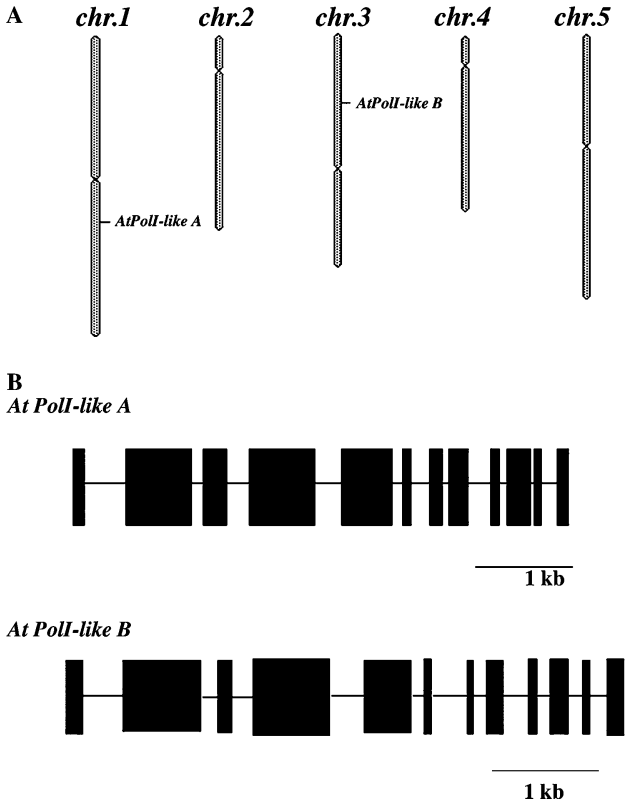

Fig. 2. Chromosomal locations of the *AtPolI-like A* and *AtpolI-like B* loci and the intron/exon patterns of the genes. The approximate positions of the loci are shown in the karyotype on the left. Gene structures are shown in the drawings to the right: boxed areas are exons and lines are introns.

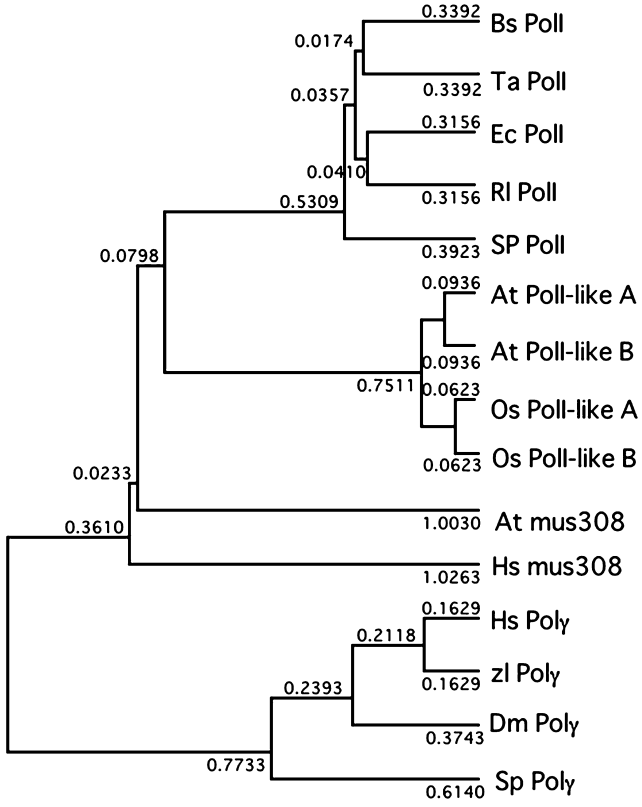

Fig. 3. Phylogenetic analysis. A phylogenetic tree was constructed by the UPGMA method, based on the amino acid sequences of the DNA polymerase domains of the *AtPolI-like* and the other family A DNA polymerases. Horizontal distances are proportional to evolutionary divergence expressed as substitutions per site.

AtPolI-like A and AtPolI-like B had significant homology with *Escherichia coli* DNA polymerase I (PolI) (overall amino acid identities of 34.7% and 32.9%), and were very similar to each other (overall amino acid identity of 72.3%). As described later, we excluded the possibility that *AtPolI-like B* is a pseudo-gene as it is efficiently transcribed in proliferating tissues (Fig. 5). Both *AtPolI-like* proteins showed a high degree of conservation in the C-terminal DNA polymerase domain (Fig. 1), implying that they have DNA polymerase activity.

Chromosomal locations and intron/exon structures of the AtPolI-like genes

The chromosomal locations and the intron/exon structures of the genes are given in Fig. 2. *AtPolI-like A* mapped to the long arm of chromosome 1, and *AtPolI-like B* to the long arm of chromosome 3 (Fig. 2A). *AtPolI-like A* contains 12, and *AtPolI-like B* has 12 exons (Fig. 2B). These results suggested that either of the *AtPolI-like* genes was the genomic coded, single copy gene of *A. thaliana*.

Phylogenetic analysis

To determine the phylogenetic relationship between *AtPolI-like A, AtPolI-like B*, and other DNA polymerases of family A, a phylogenetic tree was constructed using alignment by the UPGMA method (Fig. 3). Like *OsPolI-like*, neither *AtPolI-like A* nor *AtPolI-like B* was closely related to pol γ or pol θ, but was most closely related to cyanobacterial PolI, implying that AtPolI-like A and AtPolI-like B also belong to a chloroplast or plastid DNA polymerase. As shown in Fig. 3, we found that rice also has two homologues of *PolI-like DNA polymerase* (*OsPolI-like A* and *OsPolI-like B*).

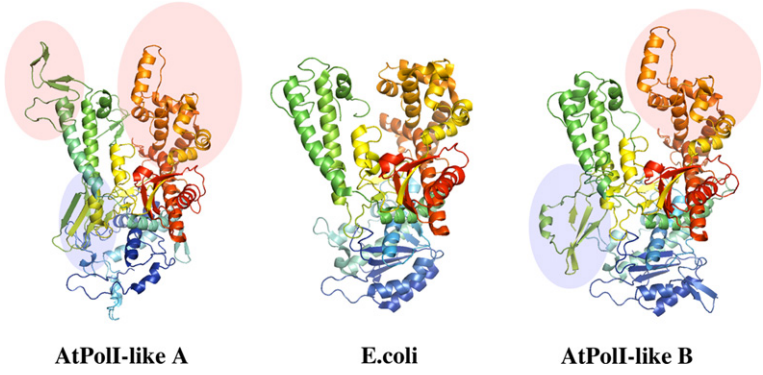

Fig. 4. Modeled 3D-structures for *AtPolI-like A* (left), the X-ray structure of the Klenow fragment of *E. coli* PolI (middle), and *AtPolI-like B* (right). The Klenow fragment was used as a template for modeling.

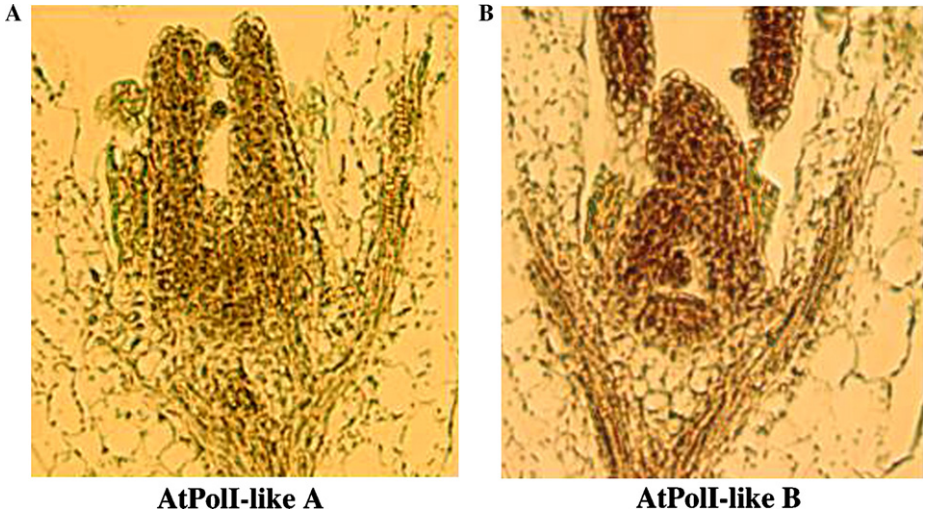

Fig. 5. Spatial expression patterns of *AtPolI-like A* and *AtPolI-like B* by in situ hybridization. Longitudinal sections from the shoot apex regions of 7-day-old *Arabidopsis* seedlings. (A) *AtPolI-like A* and (B) *AtPolI-like B*.

This result indicated that the plants have two homologues of *PolI-like DNA polymerase*.

A modeled 3D-structure for AtPolI-like proteins

The 3D models of the structures of AtPolI-like A and AtPolI-like B proteins are shown in Fig. 4. As described above, the C-terminal regions of the AtPolI-like proteins share substantial identity and similarity with PolI. This degree of sequence identity is sufficient to permit homology modeling on the known crystal structure of the PolI Klenow fragment from *E. coli* [15,16]. Modeling suggested substantial conservation of structure between the Klenow fragment and the last 490 amino acids of the AtPolI-like A protein and the last 735 amino acids of the AtPolI-like B protein. According to this model, the C-terminal region of the two types of AtPolI-like protein would be organized in four different subdomains corresponding to thumb, palm, finger, and 3′–5′ exonuclease domains. The polymerase domain structures of *E. coli* PolI, *AtPolI-like A*, and *AtPolI-like B* are similar. There was a difference between AtPolI-like A and AtPolI-like B and the area is shown by blue circles in Fig. 4. Insertion sequences, which are not found in *E. coli* PolI, are also evident in AtPolI-like A and AtPolI-like B as shown by red circles in Fig. 4. As judged by the method of Luthy et al. [17], the modeled structure has no seriously misfolded regions within the major secondary-structure elements that form the DNA-binding cleft (Fig. 4). These results suggested that the C-terminal domains of the AtPolI-like proteins are homologous to prokaryotic PolI, but that the N-terminal regions may have some other roles.

Spatial expression patterns of AtPolI-like A and AtPolI-like B

We examined expression patterns of the *AtPolI-like* polymerases by in situ hybridization using digoxigenin-labeled sense and antisense *AtPolI-like A and AtPolI-like B* RNAs as probes. When the sense probes were applied, no hybridization signals were detected (data not shown). The antisense probes for *AtPolI-like A* and *AtPolI-like B* gave strong hybridization signals in the shoot apical meristem (Fig. 5). This result suggests that *AtPolI-like A* and *AtPolI-like B* are mainly expressed in actively proliferating tissue. One possibility is that *AtPolI-like A* and *AtPolI-like B* might be required for plastid DNA replication in plant meristem to accompany active cell proliferation.

Subcellular localization of AtPolI-like A and AtPolI-like B

Transiently expressed AtPolI-like A-GFP and AtPolI-like B-GFP fusion proteins were used to deter-

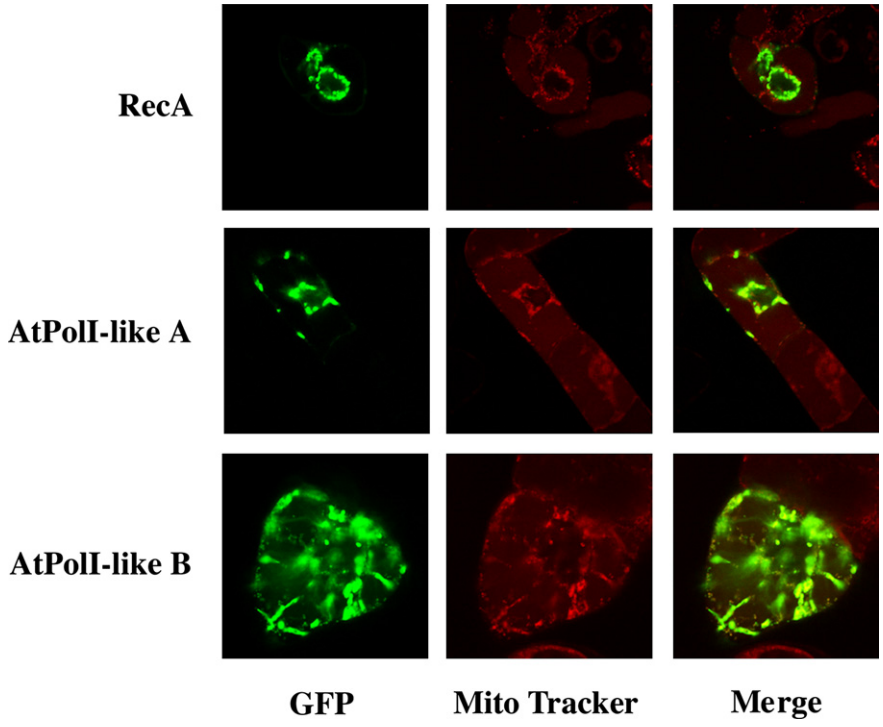

Fig. 6. Subcellular localization of AtPolI-like A and AtPolI-like B. GFP fusion proteins in suspension-cultured tobacco BY2 cells were viewed with a Laser Scanning Confocal Microscope System. Fluorescence microscopic images of RecA, AtPolI-like A-GFP and AtPolI-like B-GFP (left), the mitochondrial marker, Mito tracker (middle), and the merged images (right).

mine the subcellular localizations of the AtPolI-like A and AtPolI-like B. The fluorescence was homogeneously distributed throughout the cytoplasm and nucleus (data not shown). However, in cells expressing the AtPolI-like A and AtPolI-like B fusion proteins, fluorescence was clearly extranuclear for both proteins (Fig. 6). The position of the nucleus is marked in Fig. 6 (see arrow). A homolog of RecA has been shown to be present in chloroplasts of higher plant species, suggesting that it has a function in these plastids [18–20]. In Fig. 6, we also found that the RecA-GFP fusion protein was restricted to the chloroplasts. Moreover, the distribution pattern of RecA coincided with that of the AtPolI-like A and AtPolI-like B fusion proteins, although the AtPolI-like B fusion protein seemed to be also present in the mitochondria (see the Mito Tracker in Fig. 6). Our observations suggest that both AtPolI-like A and AtPolI-like B are plastid polymerases, with the latter also present in the mitochondria.

Induced expression of AtPolI-like A and AtPolI-like B by DNA damage

To determine whether *AtPolI-like A* or *AtPolI-like B* is involved in DNA repair, we tested the effects of DNA-damaging treatments, such as UV, MMS, mitomycin C (MMC), and H_2O_2, on the expression levels of *AtPolI-like A* and *AtPolI-like B* (Fig. 7A–D). We found an increased level of expression of *AtPolI-like A* following exposure to H_2O_2, but not after treatment by any of the other mutagens tested (Fig. 7A–D). The induced expression was at its maximum at 4 h after treatment with H_2O_2 and fell later (Fig. 7D). These data suggest that the *AtPolI-like B* gene product has a role in the repair of oxidation-induced DNA damage, and that the *AtPolI-like A* gene product is involved in some other DNA synthetic event.

The chloroplasts in mature leaves are severely bombarded by sunlight, and continually exposed to high levels of UV. The observation that neither *AtPolI-like A* nor *AtPolI-like B* was induced by UV and that they were not detected in mature leaves is of interest. The *AtPolI-like A* and *AtPolI-like B* appear to be unnecessary for repairing UV-damaged DNA in leaf plastids.

To date, at least 14 classes of DNA polymerase have been identified in mammalians (Pol α, β, γ, δ, ε, ζ, η, θ, ι, κ, λ, μ, σ, and ν) [13,21,22]. However, little is known about DNA polymerases in higher plants in comparison with mammalians [2,23–26,28]. Although nine classes of DNA polymerase (α, δ, ε, ζ, η, θ, κ, λ, and *PolI-like*) have been identified in the genome sequences of higher plants (Table 1), to date only five DNA polymerases have been isolated and characterized: catalytic subunit DNA polymerase α [7], catalytic and small subunits of DNA polymerase δ [8], *OsPolI-like* DNA polymerase [1], DNA polymerase λ [9], and DNA polymerase κ [10], AtRev3 [27]. Table 1 summarizes plant DNA polymerases along with other eukaryotic DNA polymerases.

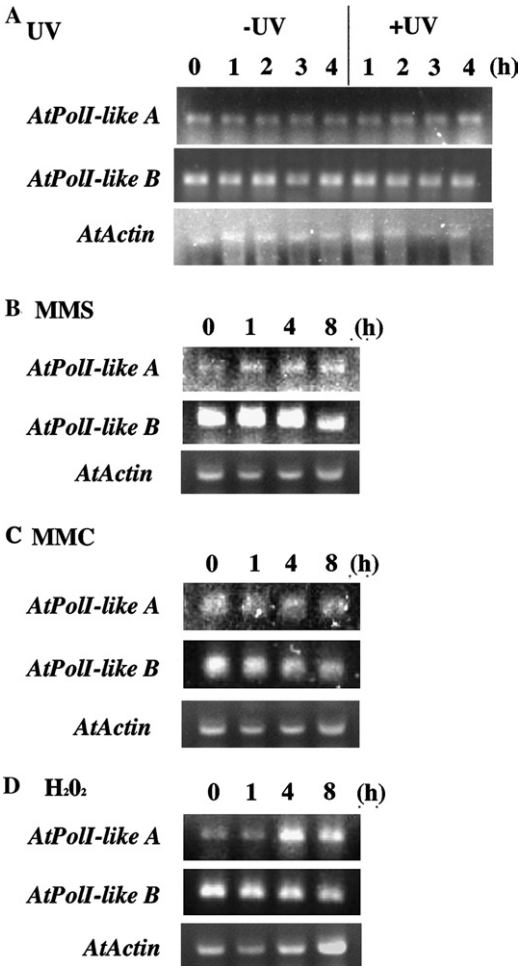

Fig. 7. Induction of expression of *AtPolI-like A* and *AtPolI-like B* by DNA-damaging reagents. *Arabidopsis* cell suspension cultures were either irradiated or had DNA-damaging reagents were added to them (UV, 25 J/m^2; MMS, 4 ppm; MMC, 1 μg/ml; H_2O_2, 0.2 mM).

Chloroplasts have their own genetic system, in which DNA replication, transcription, and translation resemble those of extant eubacteria, especially of cyanobacteria. However, the mechanism of DNA repair in plastids is largely unknown. The present report describes the molecular cloning and characterization of two DNA polymerases from *A. thaliana*, showing a high degree of sequence homology with the *OsPolI-like*. *AtPolI-like A* and *AtPolI-like B* are localized in plastids. The fact that the expression of *AtPol I-like B* increases after exposure to the mutagen H_2O_2 suggested that OsPolI-like B was involved in DNA repair of plastid DNA. Further studies of PolI-like DNA polymerases would reveal the mechanisms of chloroplast DNA repair and replication.

Table 1
Summary of plant DNA polymerases

DNA polymerases	Family	Function/remarks	Rice homolog Accession No.	*Arabidopsis* homolog Accession No.[a]	Publication
α	B	Primer function replication; DSBR[b]; Telomere length regulation	AB004461	AB02072	Yokoi et al. [7]
β	X	BER[c]; meiosis			
γ	A	Mitochondrial DNA replication and repair			
δ	B	Replication; NER[d]; BER	AB037899	AB019227	Uchiyama et al. [8]
ε	B	Replication; NER[d]; BER; cell cycle regulation	XM_465943	AC005623	
ζ	B	TLS[e] (error-prone)	AAC18785	AC011020	Sakamoto et al. [27]
η	UmnC/DinB	TLS (error-prone); Rad30; XPV	AP003409	AC002342	
θ	A	DNA repair of crosslinks; MUS3O8			
ι	UmnC/DinB	TLS (error-prone); MUS308			
κ	UmnC/DinB	TLS (error-prone)	AC135557	AC015445	Garcia-Ortiz et al. [10]
λ	X	Contains a BBRC motif	AB099525	AJ289628	Uchiyama et al. [9]
μ	X	Somatic hyper mutation			
σ	X	Cohesion	AP003264	AB017066	
ν	A	DNA repair of crosslinks; MUS308			
PolI-like A	A	Plant DNA replication and repair	AB047689	AB211532	Kimura et al. [1]
PolI-like B	A	Plant DNA replication and repair	XM_474048	AB211533	

[a] Arabidopsis homologs were identified by searching the GenBank database.
[b] DSBS, double-stranded break repair.
[c] BER, base excision repair.
[d] NER, nucleotide excision repair.
[e] TLS, translesion DNA synthesis.

Acknowledgments

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