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NtPolI-like1 and *NtPolI-like2*, Bacterial DNA Polymerase I Homologs Isolated from BY-2 Cultured Tobacco Cells, Encode DNA Polymerases Engaged in DNA Replication in Both Plastids and Mitochondria

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Two cDNAs encoding homologs of bacterial DNA polymerase I were isolated from cultured tobacco (*Nicotiana tabacum*) BY-2 cells, and the corresponding genes were named *NtPolI-like1* and *NtPolI-like2*. High sequence similarity suggested that they are orthologous genes each derived from respective parental species of *N. tabacum*, an allotetraploid plant. Each of the *NtPolI-like1/2* gene products had a putative transit peptide for plastid localization at the N-terminus, followed by a 3′-5′ exonuclease domain in the internal region, and a DNA polymerase domain in the C-terminal region. Among family A DNA polymerases, NtPolI-like proteins formed, together with other plant DNA polymerase I homologs, a phylogenetic group distinct from mitochondrial DNA polymerase γ in animals and fungi, as well as eukaryotic cell nuclear-localized repair enzymes. In contrast to computer predictions, experiments with green fluorescent protein (GFP) fusion protein and Western blotting analysis suggested dual targeting of the gene products to both plastids and mitochondria. The recombinant NtPolI-like2 protein exhibited DNA polymerase activity in vitro. Their biochemical character roughly coincided with those of the 116 kDa DNA polymerases found in the plastid and mitochondrial nuclei (nucleoids) isolated from BY-2 cells. Pre-treatment of the organelle nuclear extracts with anti-NtPolI-like antibody removed most of the DNA polymerase activity. Reverse transcription–PCR (RT–PCR) and Western blotting analyses demonstrated transient activation of *NtPolI-like* gene expression in the initial phase of cell proliferation, exactly when the 116 kDa DNA polymerases in the isolated organelle nuclei were activated and preferential synthesis of organelle DNAs occurred. Taken together, our results suggest that *NtPolI-like1/2* genes encode DNA polymerases engaged in DNA replication in both plastids and mitochondria.

Keywords: BY-2 — DNA polymerase — Dual targeting — Mitochondria — Plastids — Tobacco.

Abbreviations: CaMV, cauliflower mosaic virus; ddNTP, dideoxyribonucleoside triphosphate; DNAP, DNA polymerase; DTT, dithiothreitol; EST, expressed sequence tag; GFP, green fluorescent protein; IPTG, isopropyl-β-D-thiogalactopyranoside; NEM, N-ethylmaleimide; PolI, DNA polymerase I of E. coli; RT–PCR, reverse transcription–PCR; RACE, rapid amplification of cDNA ends.

The nucleotide sequences reported in this paper have been submitted to DDBJ/EMBL/GenBank under the accession numbers of AB174897 for the BY-2 EST clone, and, AB174898 and AB174899 for *NtPolI-like1* and *2*, respectively.

Introduction

Plant cells have two DNA-containing cytoplasmic organelles, plastids and mitochondria. According to the endosymbiont hypothesis (Margulis and Burmudes 1985), they are descendants of free-living bacteria engulfed by a host cell. Control of endosymbiont DNA replication by the host cell should have been one of the most important steps during the conversion of free-living bacteria to subordinate organelles. In contemporary eukaryotic plant cells, DNA replication within plastids and mitochondria should be coordinately controlled, in accordance with the proliferation and differentiation of the cells in which these organelles are contained. Therefore, regulation of organelle DNA replication represents a key subject, from both evolutionary and physiological viewpoints, towards understanding the nature of eukaryotic plant cells. Nonetheless, our knowledge of the plant organelle DNA replication machinery is quite limited.

In animals and fungi, DNA polymerase (DNAP) γ has been identified as the enzyme responsible for both replication and repair of mitochondrial DNA (Wang 1991, but see also Torri and Englund 1995, Klingbeil et al. 2002). DNAP γ belongs to the family A DNAPs. The DNAP family A includes, in addition to DNAP γ, bacterial DNAP I

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(a gap-filling/repair enzyme) and some eukaryotic cell nuclear repair enzymes such as Mus308, DNAP θ and DNAP ν (Marini et al. 2003). Among them, bacterial DNAP I is regarded as the prototype of the family members, from which eukaryotic (mitochondrial and cell nuclear) enzymes are derived.

In 2000, the genome sequence of *Arabidopsis thaliana* was determined (Arabidopsis Genome Initiative 2000). In the nuclear genome of *Arabidopsis*, however, no gene that shows high similarity to DNAP γ could be found (Burgers et al. 2001, Sakai 2001). Instead, there are two genes encoding proteins homologous to bacterial DNAP I (At1g50840 and At3g20540) whose products are predicted to localize in plastids and mitochondria, respectively. This information from the *Arabidopsis* genome project suggested that, in higher plants, DNAP γ is absent and DNAP I homologs might be involved in DNA synthesis in both plastids and mitochondria.

In 2002, a DNAP I homolog (designated as *OsPolI-like*) was isolated in rice and reported as a plastid-localized DNAP (Kimura et al. 2002). The same research group also cloned the two DNAP I homologs in *Arabidopsis* (At1g50840 and At3g20540) and named them *AtPolI-like A* and *AtPolI-like B*, respectively (Mori et al. 2005). The same genes were also analyzed independently by another research group and named *POL γ2* and *POL γ1* (Elo et al. 2003, Christiansen et al. 2005). For these *Arabidopsis* genes, experiments with green fluorescent protein (GFP) fusion proteins suggested plastid localization of the former gene product and dual targeting to both plastids and mitochondria for the latter gene product. These studies further support the opinion that DNAP I homologs are responsible for DNA replication in both plastids and mitochondria in higher plants, although DNAP activity of these gene products has not yet been demonstrated.

Biochemical data have also suggested that DNAP I homologs, rather than DNAP γ homologs, are involved in DNA replication in plant organelles (Sakai et al. 1999, Sakai 2001, Sakai et al. 2004). Both plastid nuclei and mitochondrial nuclei (DNA–protein complexes of plastids and mitochondria; Kuroiwa 1982, Kuroiwa et al. 1991, Sakai et al. 2004) isolated from cultured tobacco BY-2 cells contained DNAPs of approximately 116 kDa with quite similar biochemical characteristics (Sakai et al. 1999). Among all the plastid and mitochondrial 116 kDa DNAPs were relatively insensitive to ddCTP. As mitochondrial DNAP γ is characterized by its high sensitivity to dideoxyribonucleoside triphosphate (ddNTP; Sala et al. 1980), plastid and mitochondrial DNAPs are biochemically distinct from DNAP γ (Sakai et al. 1999). Taking these facts into consideration, we considered the DNAP I homologs the most probable candidates for the 116 kDa DNAPs (Sakai et al. 2004). For further analysis, however, purification of the 116 kDa DNAPs or isolation of tobacco DNAP I homologs was necessary.

In the present study, we isolated two cDNAs encoding DNAP I homologs (designated *NtPolI-like1* and *NtPolI-like2*) from cultured tobacco (*Nicotiana tabacum*) BY-2 cells, and examined whether they could be the DNAPs involved in organelle DNA replication. The molecular size, biochemical properties and subcellular localization of the gene products, as well as their patterns of gene expression, strongly suggest their involvement in organelle DNA replication.

Results

Isolation of two cDNAs encoding DNAP I homologs from tobacco

To identify DNAP I-like DNAP(s) in cultured tobacco BY-2 cells, the BY-2 expressed sequence tag (EST) database (developed for the TAB project of RIKEN) was searched with the tBLASTN program using the *OsPolI-like* (Kimura et al. 2002) sequence as a query. One EST clone, BY245, exhibited significant homology with *OsPolI-like*. In the 978 bp BY245 clone, however, only the 5′ region (612 bp from the 5′ end) showed high similarity to *OsPolI-like*; the remaining 3′ region (366 bp) exhibited no homology, suggesting a chimeric nature for this clone. The original 3′ and 5′ ends of cDNA for the *OsPolI-like* homolog were obtained by 3′- and 5′-RACE (rapid amplification of cDNA ends). They were cloned, sequenced and used to design primers to amplify the full-length coding region. Finally, two types of cDNA sequences (3,935 bp) encoding *OsPolI-like* homologs were obtained, and the corresponding genes were named *NtPolI-like 1* (for the clone with a nucleotide sequence coinciding with that of BY245) and *NtPolI-like 2*. Both of these cDNAs harbored an open reading frame encoding a putative polypeptide of 1,153 amino acid residues (Fig. 1). The coding regions of the two cDNA sequences were 98.3% identical at the nucleotide level. Deduced amino acid sequences of the gene products were 97.2% identical.

Sequence alignment (Fig. 1) shows that *NtPolI-like1/2* genes are homologous to known genes for DNAP I-like DNAPs in higher plants. Similarly to these plant homologs, each of the NtPolI-like1/2 proteins had a putative transit peptide at their N-terminus (61 amino acids from the first methionine), followed by a 3′-5′ exonuclease domain in the internal region (amino acids 362–592) and a DNAP domain in the C-terminal region (amino acids 818–1,153), but did not contain a 5′-3′ exonuclease domain, which is present in the N-terminal region of bacterial DNAP I. The putative transit peptides (61 amino acids) of *NtPolI-like1* and *NtPolI-like2* gene products were 100% identical. The TargetP program (Emanuelsson et al. 2000) suggested

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Fig. 1 Similarity among plant organelle DNAPs. Sequence alignment of the deduced amino acid sequences of *NtPolI-like 1/2* with those of *OsPolI-like*, *At1g50840* (*AtPolI-likeA* or *POL γ2*) and *At3g20540* (*AtPolI-likeB* or *POL γ1*) genes. Gaps introduced to maximize similarity are shown by a dash. Amino acids identical to NtPolI-like 1 are shown as periods. Sequence numbering is shown on the right. The overall consensus sequence is indicated with asterisks on the bottom line. Predicted cutting sites for transit peptides are shown by arrowheads. Putative 3′-5′ exonuclease and DNAP domains are boxed. Lines below the sequences indicate conserved motifs A, B and C. Amino acid residues conserved among all family A members and implicated in polymerase functions are: Asp899 and Glu904 in motif A; Arg974, Lys978, Tyr986 and Gly987 in motif B; and His1101 and Asp1102 in motif C.

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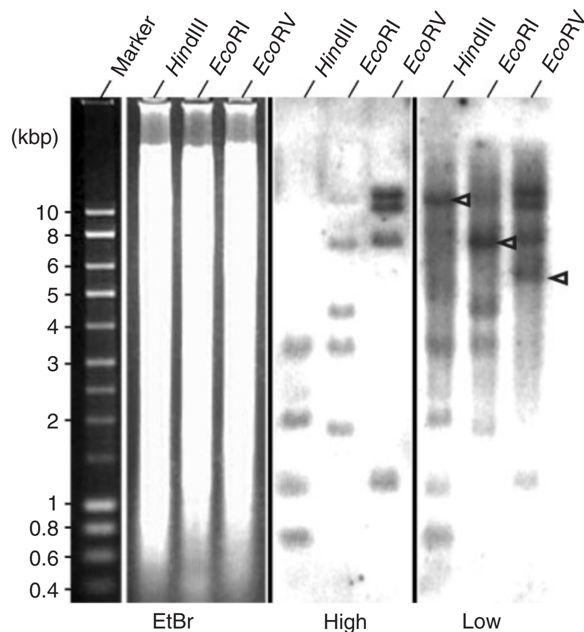

Fig. 2 Genomic Southern hybridization analysis. Purified cell nuclear DNA was digested with *Hin*dIII, *Eco*RI or *Eco*RV, electrophoresed in an agarose gel (10 µg per lane) and stained with ethidium bromide (EtBr). The DNA fragments were transferred to a nylon membrane and hybridized with NtPolI-like2 full-length cDNA under high- and low-stringency conditions. The bands detected only under low-stringency conditions are marked. The sizes of DNA markers are shown to the left of the panel in kbp.

plastid localization of the gene products with a high score (0.907). The Predotar (Small et al. 2004) and PCLR (Schein et al. 2001) programs also predicted a plastid localization. The predicted molecular mass of the primary *NtPolI-like1/2* gene products was 128 kDa, while that of the mature ones was 122 kDa.

The similarity between NtPolI-like1/2 proteins and other plant homologs was low in the N-terminal region, but increased towards the C-terminal region. In particular, the DNAP domain in the C-terminal region exhibited the highest similarity. Within the DNAP domains of NtPolI-like1/2 proteins, the sequence motifs A, B and C, conserved among functional family A DNAPs (Sousa 1996), were retained. Within the motifs, amino acid residues essential for polymerase function (Steiz 1998, Patel et al. 2001, Astatke et al. 1998) were also well conserved.

Genomic Southern hybridization analysis (Fig. 2), in which full-length *NtPolI-like1* cDNA was used as a probe, detected multiple hybridization signals for *Hin*dIII, *Eco*RI and *Eco*RV digests of tobacco cell nuclear DNA. This result suggests that *N. tabacum* harbors several genes homologous to *NtPolI-like1*, which is consistent with the presence of two almost identical genes, i.e., *NtPolI-like1* and *NtPolI-like2*. When hybridization was performed at low stringency, additional hybridization signals appeared, suggesting the presence of additional gene(s) homologous to *NtPolI-like 1/2* with lower sequence similarity (see Discussion).

Phylogenetic analysis

Family A DNAPs include bacterial DNAP I (gap-filling/repair enzyme) as the prototype, together with some eukaryotic cell nuclear repair enzymes (Mus308, DNAP θ and DNAP ν) and mitochondrial DNAP γ (replicative enzyme) of animals and fungi as distant homologs (Burgers et al. 2001). To examine the evolutionary relationship between NtPolI-like1/2 proteins and other family A DNAPs, a phylogenetic analysis was carried out. As the N-terminal sequences were quite divergent among the members of family A DNAPs, we used only the well-conserved DNAP domains for construction of the phylogenetic tree. The tree (Fig. 3) revealed four major branches, namely (i) bacterial DNAP I (gap-filling/repair enzyme); (ii) nuclear DNAPs such as Mus308, DNAP θ and DNAP ν (repair enzyme); (iii) mitochondrial DNAP γ (replicative enzyme); and (iv) plant DNAP I homologs. Interestingly, a mitochondrial DNAP of slime mold (*Dictyosterium discoideum*), a non-plant eukaryote, was clustered not with DNAP γ but with plant DNAP I homologs.

Subcellular localization of NtPolI-like proteins

We then examined the subcellular localization of NtPolI-like proteins. The N-terminal amino acid sequences of the two NtPolI-like proteins were 100% identical over 93 amino acid residues, in which the TargetP program predicted the presence of a plastid-targeting sequence (61 amino acids in length; see Fig. 1). The *NtPolI-like* sequence corresponding to the N-terminal 93 amino acids was translationally fused to the GFP sequence, and the fusion protein was transiently expressed in *N. tabacum* leaf cells. Fig. 4 shows the localization of GFP fluorescence in the guard cell. In contrast to the computer predictions, fluorescence from the fusion protein was observed in both plastids and mitochondria, demonstrating dual-targeting properties of the *NtPolI-like1/2* gene products. Fluorescence from unfused GFP (control) was observed in the cytoplasm and cell nucleus.

We then performed Western blotting analysis to confirm the localization of NtPolI-like proteins in both plastids and mitochondria (Fig. 5). Polyclonal antibody was raised against the internal region (between the 3′-5′ exonuclease domain and the polymerase domain, amino acid residues 594–812) of NtPolI-like2 protein (Fig. 5A), where amino acid identity between the two NtPolI-like proteins was 96.8%. The antibody reacted with proteins of approximately 110 kDa in both the plastid and mitochondrial nuclei isolated from BY-2 cells (Fig. 5B). Treatment of the isolated organelle nuclei with 2 M NaCl resulted in

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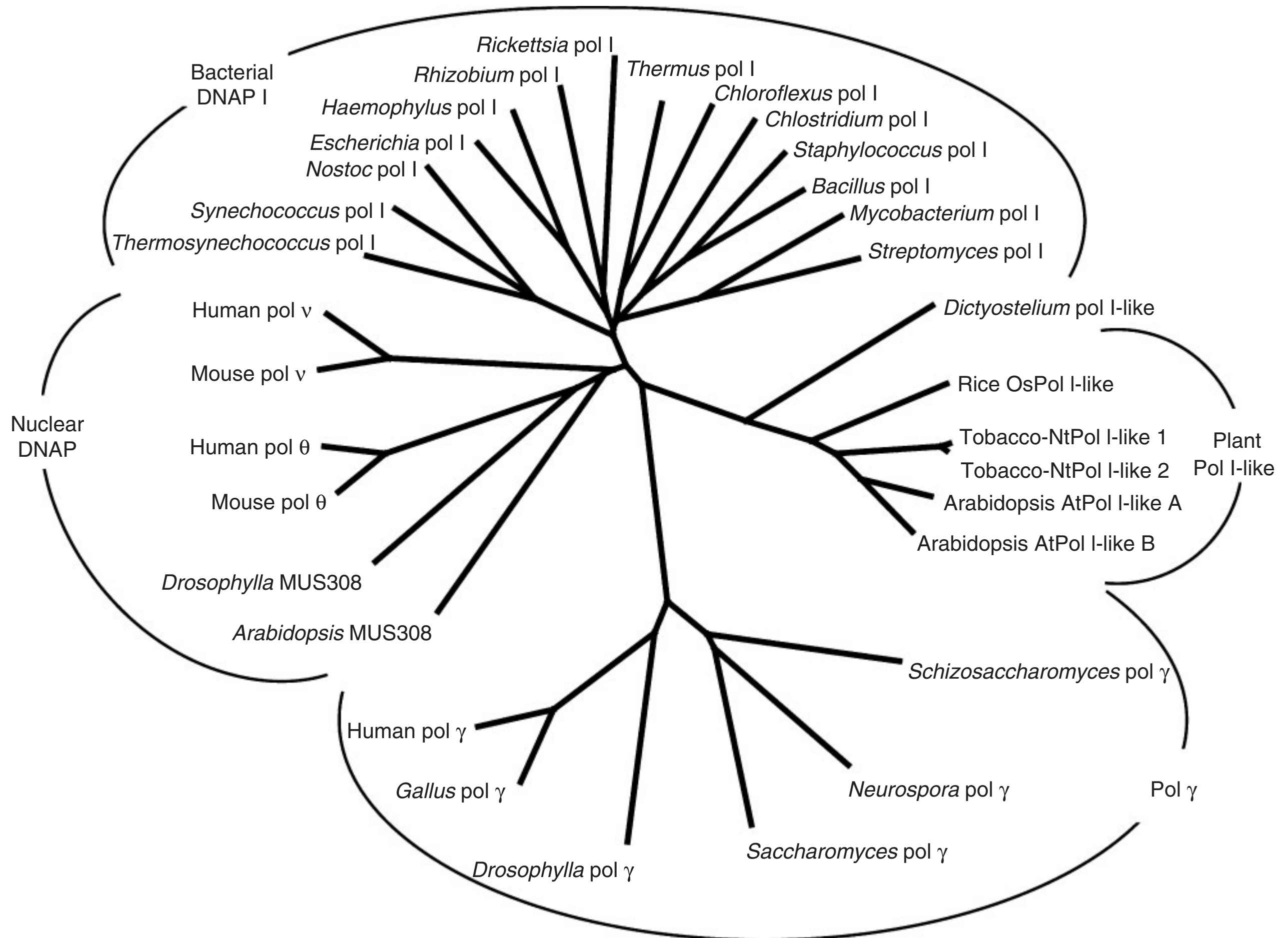

Fig. 3 Phylogenetic analysis based on family A DNAP amino acid sequences. A phylogenetic tree was constructed using the neighbor-joining method in Clustal X software.

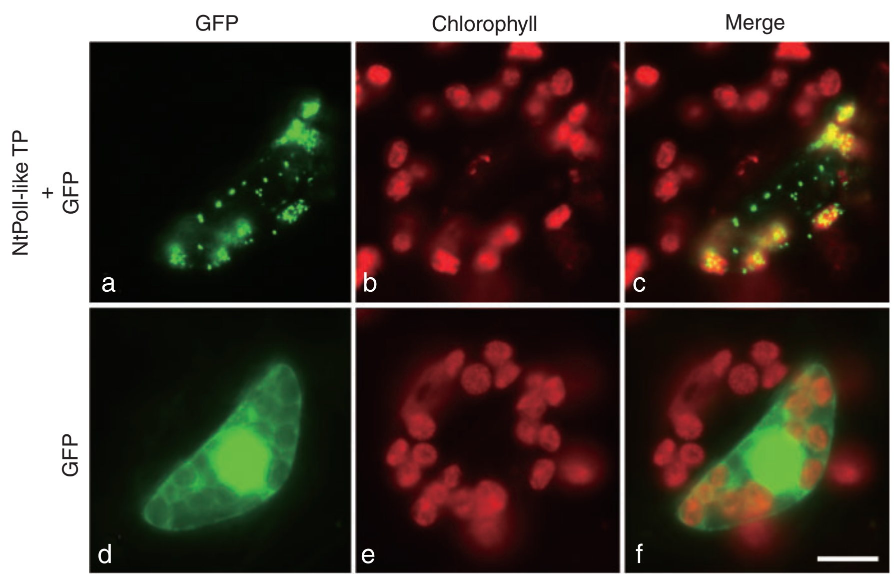

Fig. 4 Subcellular localization of the NtPolI-like–GFP fusion protein. Fluorescent microscopic images of a pair of guard cells in the *N. tabacum* leaves transformed with NtPolI-like–GFP fusion plasmids (a–c) or sGFP control plasmids (d–f). (a, d) Fluorescence of GFP. (b, e) Autofluorescence of chlorophyll. (c, f) Merged images. In both (a–c) and (d–f), one guard cell is transformed but the other is not. The scale bar represents 50 µm.

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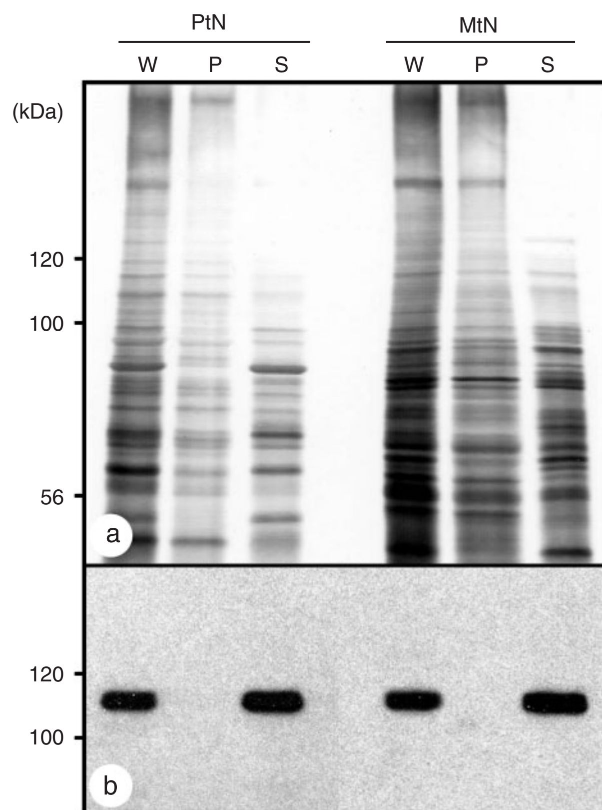

Fig. 5 Localization of *NtPolI-like* gene products in the plastid and mitochondrial nuclei as determined by Western blotting. Plastid and mitochondrial nuclei isolated from log-phase BY-2 cells were treated with 2 M NaCl, and the proteins were fractionated into pellet and soluble fractions. Whole proteins (W lanes), as well as the pellet (P lanes) and soluble (S lanes) proteins were separated by SDS–PAGE, blotted onto a PVDF membrane, and labeled with antiserum raised against the NtPolI-like2 internal region. (a) Silver-stained gel. (b) Result of Western blotting analysis. Approximate molecular size markers are indicated on the left.

complete solubilization of the proteins. These features (apparent molecular mass, solubilization with NaCl treatment, and presence in both plastid and mitochondrial nuclei) were consistent with those reported for the 116 kDa DNAPs present in plastid and mitochondrial nuclei isolated from BY-2 cells (Sakai et al. 1999, Sakai et al. 2004).

DNAP activity of NtPolI-like protein

To confirm the DNAP activity of NtPolI-like proteins, the C-terminal half of NtPolI-like2 protein (amino acid residues 594–1,151), which included the entire DNAP domain, was overexpressed in *Escherichia coli*, purified and assayed. Within the expressed domain, amino acid identity between the two NtPolI-like proteins was 98%. The recombinant NtPolI-like2 protein exhibited considerable DNAP activity (Table 1), continuing DNA polymerization over a 60 min incubation in a dose-dependent manner

Table 1 DNAP activity of recombinant NtPolI-like2 protein.

Sample	Activity	
	(dpm per reaction)	(pmol μg^{-1} protein)
Recombinant NtPolI-like2	$2{,}210 \pm 80$	3.40 ± 0.12
pQE30 vector control	10 ± 10	–

DNAP activity was measured as described in Materials and Methods. Each reaction contained 1 µg of the Ni-affinity-purified polymerase domain of NtPolI-like2 protein (recombinant NtPolI-like2), or Ni-column-bound protein derived from *E. coli* harboring empty pQE30 vector (pQE30 vector control), obtained by parallel preparations starting with equal amounts of *E. coli* culture. Values represent average ± SD of three measurements.

(data not shown). In contrast, little DNAP activity was detected in the parallel preparation from *E. coli* that harbored pQE 30 vector only.

Characterization of DNAP activity of recombinant NtPolI-like2 protein was performed in parallel with that of the DNAPs present in the crude extracts prepared from plastid and mitochondrial nuclei isolated from BY-2 cells (Fig. 6). The recombinant NtPolI-like2 protein preferred a relatively high temperature (50°C) and weak alkaline pH (8.0–8.5). The optimal concentration for KCl was 150 mM. Divalent cation was absolutely required for activity, with the maximum activity achieved at 2.5 mM Mg^{2+}. Manganese ion at low concentrations ($\sim 10^{-4}$ M) could also support DNAP activity, but it was not as effective as Mg^{2+} (data not shown). These characteristics of recombinant NtPolI-like2 protein roughly coincided with those of DNAPs present in the isolated organelle nuclei. The organelle nuclear DNAPs also had higher activity at relatively high temperature (50°C) and weak alkaline pH (7.5–8.0), with an optimal concentration for KCl of 200 mM. They also required Mg^{2+} for activity, although the optimal Mg^{2+} concentration was higher than that of recombinant NtPolI-like2 protein.

A test for template preferences (Table 2) revealed that the preferred template for the recombinant NtPolI-like2 protein was poly(dA)–oligo(dT_{12-18}), although activated DNA was routinely used as template under our standard assay conditions. Native DNA was also used as template with low efficiency, probably because spontaneous nicks and gaps in the molecules provide primers necessary for DNAP activity. Poly(rA)–oligo(dT_{12-18}) was also used as a template, demonstrating reverse transcriptase activity of the recombinant NtPolI-like2 protein. Primer-less templates, poly(dA) and poly(rA), were not utilized as effective templates. These template preferences of recombinant

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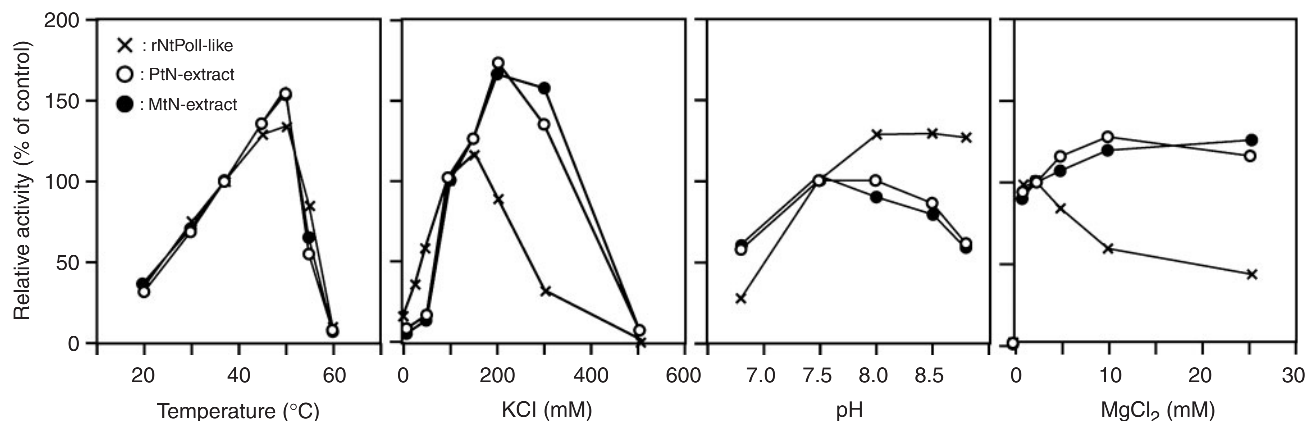

Fig. 6 Influence of temperature, pH, ionic strength and divalent cations on DNAP activities. Crosses, recombinant NtPolI-like2 protein; open circles, protein extract prepared from isolated plastid nuclei; filled circles, protein extract prepared from isolated mitochondrial nuclei. The activity under standard assay conditions (i.e. 30 min incubation, 37°C, pH 7.5, 2.5 mM $MgCl_2$, 100 mM KCl) was set at 100%. Each data point represents the average of three measurements.

Table 2 Effect of template on DNAP activity of recombinant NtPolI-like2 protein and DNAPs present in the isolated plastid and mitochondrial nuclei

Template	Relative activity (%)		
	rNtPolI-like2	PtN extract	MtN extract
Activated DNA	100	100	100
Native DNA	10	11	14
Poly(dA)–oligo(dT$_{12–18}$)	540	1,440	1,678
Poly(rA)–oligo(dT$_{12–18}$)	84	30	22
Poly(dA)	1	13	18
Poly(rA)	0	12	17

DNAP activity was measured as described in Materials and Methods, except that [methyl-^{3}H]dTTP was used instead of [5-^{3}H]dCTP to radiolabel product DNA. The activity under standard assay conditions, in which activated calf thymus DNA was used as template, was set at 100%. Values represent the average of three measurements. rNtPolI-like2, recombinant NtPolI-like2 DNAP domain; PtN extract, protein extract prepared from isolated plastid nuclei; MtN extract, protein extract prepared from isolated mitochondrial nuclei.

Table 3 Effects of inhibitors on DNAP activity of recombinant NtPolI-like2 protein and DNAPs present in the isolated plastid and mitochondrial nuclei

Inhibitor/treatment	Relative activity (%)		
	rNtPolI -like2	PtN extract	MtN extract
Control	100	100	100
Heat (70°C 15 min)	0	0	0
Actinomycin D (25 μg ml^{-1})	35	62	62
Ethidium bromide (25 μg ml^{-1})	34	18	17
Aphidicolin (25 μg ml^{-1})	100	99	97
ddCTP (ddCTP/dCTP = 1)	94	104	120
ddCTP (ddCTP/dCTP = 10)	58	80	92
ddCTP (ddCTP/dCTP = 100)	4	28	21
N-Ethylmaleimide (2.5 mM)	96	94	92

DNAP activity was measured as described in Materials and Methods, with the additions indicated. For heat inactivation, samples were pre-incubated at 70°C for 15 min, chilled on ice and then subjected to DNAP assay. The activity under standard assay conditions (in the absence of inhibitors) was set at 100%. Values represent the average of three measurements. Abbreviations as for Table 2.

NtPolI-like2 protein were in good agreement with those of DNAPs present in the organelle nuclear extracts.

DNAP activity of the recombinant NtPolI-like2 protein was destroyed by pre-incubation of the protein for 15 min at 70°C (Table 3). The activity was sensitive to intercalating agents (actinomycin D or ethidium bromide) but was insensitive to aphidicolin, a potent inhibitor of eukaryotic DNAP α. A high ddCTP/dCTP ratio (>10) was necessary to reduce the DNAP activity to <60% of the control value, demonstrating rather low sensitivity to dideoxyribonucleotide. *N*-Ethylmaleimide, a sulfhydryl group-blocking agent, did not inhibit the activity. These characteristics of the recombinant NtPolI-like2 DNAP activity were in basic agreement with those of DNAP activities present in the organelle nuclear extract. Moreover, when protein extracts prepared from isolated organelle nuclei were pre-treated with antibody raised against NtPolI-like proteins, the DNAP activity in the extract was

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Table 4 Recognition of DNAPs present in organelle nuclear extracts by antibody raised against NtPolI-like protein

Treatment	Activity (dpm per reaction)	
	PtN extract	MtN extract
Pre-immune serum	$1,430 \pm 80$	$2,690 \pm 170$
Anti-NtPolI-like antiserum	160 ± 20	440 ± 40

Samples were incubated for 1 h at 4°C with protein A–agarose beads that had been mixed for 1 h at 4°C with either pre-immune serum or anti-NtPolI-like antiserum. Then, protein A–agarose beads were removed by centrifugation and the supernatant was assayed for DNAP activity as described in Materials and Methods. Values represent the average of three measurements. PtN extract, protein extract prepared from isolated plastid nuclei; MtN extract, protein extract prepared from isolated mitochondrial-nuclei.

significantly reduced (Table 4). This result suggests that most of the DNAP activity present in the isolated organelle nuclei is due to NtPolI-like proteins.

Expression analysis

To follow the changes in *NtPolI-like1/2* gene expression during proliferation of BY-2 cells, reverse transcription–PCR (RT–PCR) analysis was carried out (Fig. 7). As the primers were designed for the sequences identical between the two *NtPolI-like* cDNA sequences, the result reflects the summation of the changes in the transcript abundance of both genes.

First, we compared the expression of *NtPolI-like* genes in BY-2 cells cultured in two different culture media, D medium and B medium, to examine the relationship between expression of *NtPolI-like* genes and organelle DNA replication. Normally, BY-2 cells are maintained in D medium, which contains auxin (0.2 mg l^{-1} 2,4-D). When BY-2 cells at stationary phase are transferred to fresh D medium, they proliferate rapidly and organelle DNA replication is transiently activated in the initial phase of cell proliferation (within 12 h post-transfer). In contrast, when BY-2 cells are transferred to B medium, which contains cytokinin [1 mg l^{-1} benzyladenine (BA)] instead of auxin, they proliferate little and no transient activation of organelle DNA replication occurs (Sakai et al. 1992, Sakai et al. 1996, Okamura et al. 2002).

When BY-2 cells were grown in D medium, the level of *NtPolI-like* transcripts was low at the start of subculturing, but increased rapidly, peaked 12 h after transfer and decreased thereafter. Throughout the culture period, the actin gene control exhibited no detectable changes in transcript level. This result demonstrated that *NtPolI-like1/2* gene expression is transiently activated in the initial

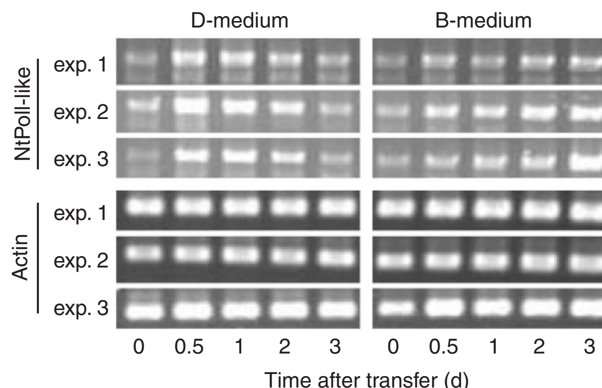

Fig. 7 RT–PCR analyses of *NtPolI-like* gene expression under different culture conditions. RT–PCR analyses were carried out to follow accumulation of *NtPolI-like* transcripts after transfer of BY-2 cells to D and B medium, which activate and do not activate, respectively, organelle DNA synthesis immediately after medium renewal. Primers were designed to amplify both *NtPolI-like1* and *NtPolI-like2* transcripts simultaneously. Time after transfer to fresh medium (d) is shown below the lanes. As a control, bands of RT–PCR products amplified from actin transcripts are also shown. Results of three experiments run in parallel (exp. 1, exp. 2 and exp. 3) are shown.

phase of proliferation of BY-2 cells in D medium, exactly when transient activation of organelle DNA replication takes place (Yasuda et al. 1988, Suzuki et al. 1992, Okamura et al. 2002). In contrast, when BY-2 cells were grown in B medium, a transient increase in the *NtPolI-like* transcript level was not observed, although the transcript level increased gradually during the later phase of the culture period.

The relationship between the transient activation of *NtPolI-like* gene expression and organelle DNA replication was further examined by RT–PCR, Western blotting and in situ DNAP activity assay following SDS–PAGE (Fig. 8). The results demonstrated that not only the *NtPolI-like* transcript level but also NtPolI-like protein levels and DNAP activity in the isolated organelle nuclei increased transiently during the initial phase of cell proliferation. This result strengthened the possibility that *NtPolI-like* genes are involved in the active replication of organelle DNAs in the initial phase of cell proliferation in D medium.

Discussion

Evolution of PolI-like DNAPs in higher plants

DNAPs are classified into four families (A, B, C and X) based on their sequence similarities and drug sensitivities (Brown and Doolittle 1997). Family A includes bacterial DNAP I as well as several eukaryotic cell nuclear (Mus308, DNAP θ and DNAP ν) and organellar DNAPs.

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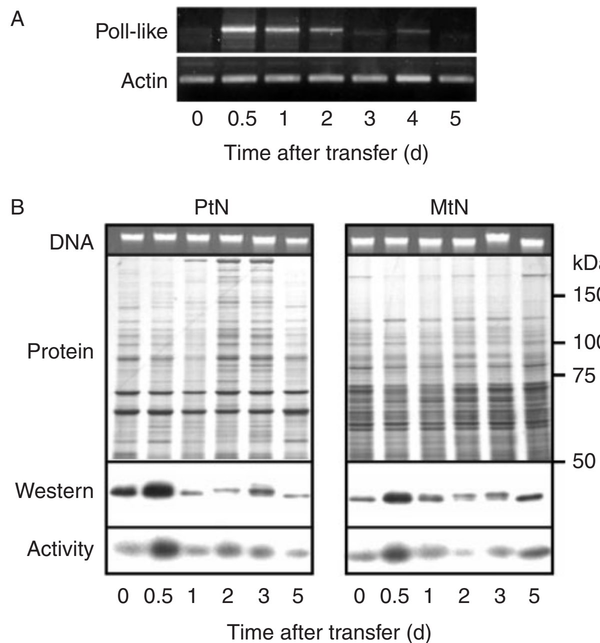

Fig. 8 Changes in the levels of *NtPolI-like* transcripts, NtPolI-like proteins and the 116 kDa DNAP present in the isolated plastid and mitochondrial nuclei during proliferation of BY-2 cells cultured in D medium. (A) Changes in transcript abundance examined by RT–PCR. (B) Changes during culture in the amount of NtPolI-like proteins and DNAP activities present in isolated plastid and mitochondrial nuclei. Top row: DNA extracted from respective samples was electrophoresed in a 0.8% agarose gel and visualized by ethidium bromide staining. Second row: total proteins extracted from respective samples were electrophoresed in a 7.5% SDS–polyacrylamide gel and visualized by silver staining. Third row: NtPolI-like proteins present in respective samples were detected by Western blotting analysis. Bottom row: DNAP activity present in respective samples was detected by an in situ DNAP activity assay following SDS–PAGE. PtN, plastid nuclei. MtN, mitochondrial nuclei. Numbers below panels indicate time after transfer (d). For visualization of DNA and total protein, each lane was loaded with sample prepared from isolated organelle nuclei containing 200 ng of DNA. For detection of NtPolI-like proteins and DNAPs, each lane was loaded with sample prepared from isolated organelle nuclei containing 1 μg of DNA.

Because archaebacteria do not harbor family A DNAP, eukaryotic family A DNAPs were presumably introduced by bacteria, i.e. the ancestors of mitochondria and plastids. Phylogenetic analysis of family A DNAPs has suggested that a single gene duplication event occurred early during eukaryotic evolution (soon after mitochondrial endosymbiosis), giving rise to cell nuclear repair enzymes and the mitochondrial DNAP γ (Klingbeil et al. 2002). Our expanded phylogenetic tree (Fig. 3), which includes plant DNAP I homologs, basically agrees with this scenario.

In our phylogenetic tree (Fig. 3), all known plant DNAP I homologs were clustered in a small branch rather distantly related to that of mitochondrial DNAP γ in animals and fungi. This result, together with their relative insensitivity to ddNTPs (Table 3), indicates that plant organelle DNAPs should be distinguished from DNAP γ in animals and fungi. Therefore, we prefer the gene name *PolI-like* (Kimura et al. 2002, Mori et al. 2005) to *POL*γ (Elo et al. 2003, Christensen et al. 2005).

Presently, the origin and evolutionary process of plant DNAP I homologs remain unclear. We previously proposed that the plant DNAP I homologs might have been introduced by the plastid ancestor, based on their plant specificity and close relationship with prototypic bacterial DNAP I (Sakai et al. 2004). However, this hypothesis may need re-examination. One of the problems with the hypothesis of a cyanobacterial origin of plant DNAP I homologs is that plant-specific DNAP I homologs do not cluster with cyanobacterial DNAP I. However, this apparent lack of monophyly may be explained if different physiological roles for the two subfamilies (while bacterial DNAP I is a gap-filling/repair enzyme, the plant DNAP I homolog is presumably engaged in DNA replication) are considered, because such a difference in the physiological roles may result in different rates and different bias for molecular evolution. Another observation that contradicts the cyanobacterial origin hypothesis more seriously is the presence of a plant-type DNAP I homolog in a slime mold (*DdmtpolA*; Fig. 3), which has no plastids. To explain this situation in accordance with the cyanobacterial origin hypothesis, we need to assume horizontal gene transfer. Clearly, more information on mitochondrial DNAP in primitive eukaryotes will be necessary to follow the evolution of plant organellar DNAP. If ‘plant-specific’ DNAP I homologs are widely found among relatively primitive non-plant eukaryotes, they may simply represent a more primitive state of organellar DNAP during evolution from DNAP I to DNAP γ.

Relationship between NtPolI-like1 and NtPolI-like2 genes

We isolated two cDNAs for DNAP I homologs, *NtPolI-like1* and *NtPolI-like2*, from *N. tabacum*. Although we have not isolated genomic DNA clones corresponding to the respective *NtPolI-like* cDNAs, the result of Southern hybridization analysis after high-stringency washing (Fig. 2) supported the presence of at least two highly homologous genes within the *N. tabacum* cell nuclear genome.

Nictotiana tabacum is an amphidiploid plant originating from a hybridization event between *N. sylvestris* and *N. tomentosiformis* (Hedtke et al. 2002). A non-amphidiploid plant *Arabidopsis* contains two DNAP I homologs (*Arabidopsis* Genome Initiative 2000, Elo et al. 2003, Christensen et al. 2005, Mori et al. 2005). Provided that the situation with *Arabidopsis* represents those with a majority of so-called ‘diploid’ higher plants, each *N. tabacum* parent species should harbor two DNAP I

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homologs, and *N. tabacum* should harbor four homologs. Then, there are two possibilities for the relationship between *NtPolI-like1* and *NtPolI-like2*. First, they may be orthologous genes derived respectively from each parental species. Secondly, they may represent paralogous genes that had already been present in the respective parents. To establish the relationship between *NtPolI-like1* and *NtPolI-like2*, isolation and characterization of the *PolI-like* homologs in the two parental species is necessary. However, based on the high similarity between the *NtPolI-like1* and *NtPolI-like2* genes (97% identical at the amino acid level), we tentatively consider the first possibility more likely, since orthologous gene pairs often exhibit higher similarity than paralogous gene pairs. For example, among six genes for phage-type organelle RNA polymerase in *N. tabacum* (Hedtke et al. 2002), orthologous gene pairs exhibit higher (~97%) similarity than paralogous gene pairs (58–68%). Moreover, the similarity between the two paralogous DNAP I homologs in *Arabidopsis* is relatively low (74%, Mori et al. 2005).

Provided that *N. tabacum* harbors four *PolI-like* homologs and that *NtPolI-like1* and *NtPolI-like2* represent one orthologous gene pair, then the *N. tabacum* genome should harbor the other orthologous gene pair, each of which is paralogous to *NtPolI-like1/2*. The presence of additional Southern hybridization signals detected only after low-stringency washing (Fig. 2) suggests the existence of such additional homologs with lower sequence homology. Thus, the possible presence of additional DNAP I homologs and their expression should be examined carefully in future work. However, we think that the expression level of an unidentified paralogous gene pair in BY-2 cells, if any, should not greatly exceed that of *NtPolI-like1/2*, because we could not find additional sequences corresponding to such additional DNAP I homologs in the BY-2 EST database.

Dual targeting of the NtPolI-like1/2 gene products

The N-termini of *NtPolI-like1/2* gene products were 100% identical over 93 amino acid residues, including the 61 amino acid putative transit peptide sequences (Fig. 1). Thus, the subcellular localization of the two gene products should be identical. Although computer programs predicted plastid localization of the gene products, the NtPolI-like leader sequence directed GFP fusions to both mitochondria and plastids (Fig. 4). According to Peeters and Small (2001), there are two types of mechanisms for dual targeting. One is the twin pre-sequences mechanism (two types of pre-sequence are formed by an alternative transcription start, alternative splicing or an alternative translation start), and the other is the ambiguous pre-sequence mechanism (a single pre-sequence has the potential to target proteins to both plastids and mitochondria). An alternative translation start reportedly plays an important role in switching of the targeting sites of phage-type organelle RNA polymerases in plants (Hedtke et al. 2000, Hedtke et al. 2002, Kabeya et al. 2002, Richter et al. 2002, Sato et al. 2003). The same is true for one of the DNAP I homologs in *Arabidopsis* (*POL γ2/AtPolI-like A*, AGI code At1g50840, Christensen et al. 2005). *Arabidopsis POL γ2/AtPolI-like A* gene product was exclusively localized to plastids when translation was initiated at the annotated ATG start codon, but was dually targeted to plastids and mitochondria when translation initiation was also allowed at a CTG codon located seven codons upstream of the annotated start codon. In contrast, the other *Arabidopsis* DNAP I homolog (*POL γ1/AtPolI-like B*) exhibited dual-targeting capacity when translation was initiated at the annotated ATG codon (Christensen et al. 2005), suggesting the involvement of the ambiguous pre-sequence mechanism.

As shown in Fig. 4, the *NtPolI-like* pre-sequence downstream of the annotated initiation ATG codon (which included no untranslated region and no additional potential initiation codon) could direct GFP fusions to both plastids and mitochondria. This result indicates that the ambiguous pre-sequence mechanism is sufficient for their dual-targeting property. However, because the CTG codon that probably acts in *Arabidopsis POL γ2* as the additional non-ATG initiation codon is also conserved in *NtPolI-like 1/2* (data not shown), an alternative translation start may also influence their targeting capability. Christensen et al. (2005) suggested that the relative efficiency of the ATG site vs. the upstream non-ATG site for translation initiation was influenced by trans-acting cellular or developmental cues. Such a possibility should be examined in future work.

NtPolI-like1/2 gene products are functional DNAPs

Because the *NtPolI-like1/2* sequences were obtained through cDNA-based techniques, it is clear that they are active genes. Their gene products retained domain structures similar to bacterial DNAP I (except a 5′-3′ exonuclease domain), A–C sequence motifs conserved among family A DNAPs and amino acid residues essential for polymerase function (Fig. 1). These all suggest that they are functional DNAPs. For *NtPolI-like2*, DNAP activity of the gene product was directly demonstrated by a DNAP assay using recombinant protein (Table 1). Although we have not yet directly demonstrated the polymerase activity of NtPolI-like1 protein, its high similarity to NtPolI-like2 suggests that it is also a functional DNAP.

Relationship between NtPolI-like1/2 gene products and DNAPs biochemically detected in the plastid and mitochondrial nuclei

The plastid and mitochondrial nuclei isolated from BY-2 cells contain 116 kDa DNAPs that were biochemically

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quite similar (Sakai et al. 1999). Recombinant NtPolI-like2 protein and the organelle nuclear DNAPs exhibited similar biochemical properties (Tables 1–3 and Fig. 6), such as apparent molecular mass (~110 kDa), stimulation by KCl, requirement for a divalent metal cation (preferring Mg^{2+} to Mn^{2+}), insensitivity to aphidicolin and, above all, relatively low sensitivity to ddCTP, an important feature that distinguishes them from DNAP γ (Sakai 2001, Sakai et al. 2004). These similarities suggest that dually targeted *NtPolI-like* gene products might be the 116 kDa DNAPs that had been biochemically detected in the isolated plastid and mitochondrial nuclei. If so, the biochemical similarity between the plastid and mitochondrial DNAPs (Sakai et al. 1999, Fig. 6 and Tables 2, 3) is quite reasonable.

However, several biochemical properties (e.g. optimal KCl or $MgCl_2$ concentrations and sensitivity to ddCTP) did not fully coincide between the recombinant NtPolI-like2 protein and the organelle nuclear DNAPs (Fig. 6 and Table 3). One simple explanation is that different protein configurations caused such differences; while recombinant protein was truncated and did not contain an exonuclease domain, DNAPs present in organelle nuclear extracts were full-length proteins that may be complexed with accessory proteins. Another possibility involves the presence of DNAP(s) other than NtPolI-like1/2 proteins. Although involvement of DNAP(s) unrelated to DNAP I homologs seems unlikely (Table 4), *N. tabacum* may harbor two unidentified DNAP I homologs in addition to *NtPolI-like1/2* (see discussion above). Their possible involvement should be examined carefully.

In the present study, we demonstrated correlation between *NtPolI-like* gene expression and organelle DNA replication in two ways. First, when BY-2 cells were grown under different culture conditions, the presence or absence of transient activation of *NtPolI-like* gene expression was well correlated with the presence or absence of transient activation of organelle DNA replication (Fig. 7). Secondly, the transient increase in the *NtPolI-like* transcript level during the initial phase of cell proliferation was accompanied by corresponding changes in the NtPolI-like protein level and the 116 kDa DNAP activity level present in the isolated plastid and mitochondrial nuclei (Fig. 8). These results strongly suggest that activation of *NtPolI-like* gene expression is involved in the active replication of organelle DNAs in the initial phase of cell proliferation, by way of an increase in the levels of NtPolI-like proteins and DNAP activities in the organelle nuclei.

Taken together, our results strongly suggest that (i) *NtPolI-like1/2* genes encode DNAPs engaged in DNA replication in both plastids and mitochondria; (ii) their gene products probably correspond to the 116 kDa DNAPs detected in the isolated plastid and mitochondrial nuclei (Sakai et al. 1999); and (iii) transient activation of expression of *NtPolI-like* genes, together with dual-targeting properties of the gene products, may be responsible for the transient and synchronous activation of organelle DNA replication in the initial phase of BY-2 cell proliferation.

Materials and Methods

Plant materials and growth conditions

The tobacco (*N. tabacum* L.) cell line BY-2 was cultured according to Yasuda et al. (1988). Tobacco plants (*N. tabacum* L. cv. Bright Yellow-2) were grown in a regulated room under a 12 h light/12 h dark regimen at 25°C for 1–2 months.

Isolation of cDNAs encoding DNAP I-like protein from cultured tobacco BY-2 cells

A tobacco BY-2 EST clone for a putative PolI-like DNAP (BY245), identified in the RIKEN TAB (transcriptome analysis of BY-2) project, was obtained from The Laboratory for Structural Construction (Professor Matsuoka) at RIKEN Plant Science Center through the RIKEN Bioresource Center. The total sequence of the BY245 clone was determined with a CEQ2000XL system (Beckman-Coulter Inc., Fullerton, CA, USA). The 5′ and 3′ends of the transcript were obtained by 5′- and 3′-RACE, using the SMART RACE cDNA amplification kit (BD Biosciences Clontech, Palo Alto, CA, USA). A 1 μg aliquot of total RNA, which was isolated from 12-h-old BY-2 cells according to Miyazawa et al. (1999), was converted to cDNA using the CDS primer in the kit and was then used as template in the following 5′- and 3′-RACE reactions. For 5′-RACE, the first PCR was done using gene-specific primer 1 (5′-CGAACTCCATCGAATGTCCAGATCCGT-3′) and the universal primer mix A in the kit for 25 cycles of 5 s at 94°C, 10 s at 68°C and 3 min at 72°C, followed by the second nested PCR using nested gene-specific primer 1 (5′-CCCTCAGTTCCGTCTTTCTTCAGC TT-3′) and the nested universal primer A in the kit for 20 cycles. For 3′-RACE, the first PCR was done using gene-specific primer 2 (5′-GGCTAAACGGATCTGGACATTCGATGG-3′) and the universal primer mix A for 25 cycles of 5 s at 94°C, 10 s at 68°C and 3 min at 72°C, followed by the second nested PCR using the nested gene-specific primer 2 (5′-GCTGGTTGACCGTGCCTATCTTG CT-3′) and the nested universal primer A for 30 cycles. Amplified PCR products were isolated, cloned and sequenced.

For cloning of cDNAs encompassing the total coding region of the *NtPolI-like* gene, single-stranded cDNA was generated from 2 μg of total RNA using PowerScript reverse transcriptase (Clontech) and gene-specific primer 3 (5′-TAGTCGACGGCA AATACCAGTTTTGGC-3′). The whole coding region of the *NtPolI-like* gene was amplified from the single-stranded cDNA by PCR using gene-specific primers 3 and 4 (5′-ATGGCATTCTT GGGGTTCTCAGTTC-3′). After heating at 94°C for 2 min, PCR was performed for 25 cycles of 30 s at 94°C, 30 s at 58°C and 3 min at 72°C, with the time at 72°C on the last cycle extended to 10 min. The PCR products were isolated, cloned and sequenced. We identified two types of cDNAs and named the corresponding genes *NtPolI-like1* and *NtPolI-like2*.

Southern hybridization analysis

DNA was extracted from cell nuclei isolated from BY-2 cells as described previously (Sakai et al. 1998), purified on an anion-exchange column (Qiagen tip-500, Qiagen) and digested with restriction enzymes (*Hin*dIII, *Eco*RI and *Eco*RV, TAKARA BIO INC., Ohtsu, Japan) according to the manufacturer's instructions.

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The restriction fragments (10 μg per lane) were electrophoresed in a 0.8% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 25 V for 13 h, and transferred to a nylon membrane (Biodine B, Pall) according to standard capillary blotting procedures. A cloned full-length *NtPolI-like1* cDNA fragment was labeled with [α-^{32}P]dCTP using a Megaprime random-primed DNA labeling kit (GE Healthcare), hybridized to the Southern blots for 12 h in hybridization buffer (5× SSPE, 5× Denhardt's solution, 0.5% SDS and 20 mg ml^{-1} heat-denatured calf thymus DNA), and washed in 1× SSC, 0.1% SDS four times for 15 min each. Hybridization and washing were performed at 65°C for high-stringency and 55°C for low-stringency hybridization experiments. The hybridization signals were recorded on X-ray film.

Phylogenetic analysis

Amino acid sequences of various family A DNAPs were obtained from the DDBJ, EMBL and GenBank databases, and the DNAP domains corresponding to the 547–927 amino acid sequence of *E. coli* DNAP I (Marini et al. 2003) were aligned using Clustal X software (Jeanmougin et al. 2000); a phylogenetic tree was constructed using the neighbor-joining method in Clustal X. Plant DNAP I-like proteins used were rice *OsPolI-like* protein (BAB40805) and *Arabidopsis* AtPolI-like A (At1g50840) and AtPolI-like B (At3g20540) proteins. Accession numbers of other DNAPs used for this study are: Q55971 (*Synechocystis* sp. PCC6803 DNAP I), NP_682129 (*Thermosynechococcus elongatus* DNAP I), NP_562910 (*Clostridium perfringens* DNAP I), NP_485297 (*Nostoc* sp. PCC7120 DNAP I), NP_418300 (*E. coli* DNAP I), P43741 (*Haemophilus influenzae* DNAP I), Q9S1G2 (*Rhizobium leguminosarum* DNAP I), O05949 (*Rickettsia prowazekii* DNAP I), JX0359 (*Thermus aquaticus* DNAP I), O08307 (*Chloroflexus aurantiacus* DNAP I), AAR00931 (*Bacillus subtilis* DNAP I), NP_374801 (*Staphylococcus aureus* DNAP I), P0A550 (*Mycobacterium tuberculosis* DNAP I), T35748 (*Streptomyces coelicolor* DNAP I), Q9Y767 (*Neurospora crassa* DNAP γ), Q12704 (*Schizosaccharomyces pombe* DNAP γ), P15801 (*Saccharomyces cerevisiae* DNAP γ), U62547 (*Drosophila melanogaster* DNAP γ), Q92076 (*Gallus gallus* DNAP γ), P54098 (*Homo sapiens* DNAP γ), AY170439 (*Dictyosterium discoideum* DNAP I-like), NP_524333 (*D. melanogaster* Mus308), BAD93700 (*A. thaliana* Mus308, At4g32700), NP_084253 (*Mus musculus* DNAP θ), NP_955452 (*H. sapiens* DNAP θ), NP_862905 (*M. musculus* DNAP ν) and NP_861524 (*H. sapiens* DNAP ν).

Localization of a GFP fusion protein of NtPolI-like1

An *NtPolI-like1/2* gene fragment corresponding to the N-terminal 93 amino acids was PCR amplified from a 5′-RACE clone with primers PolI1F1-Sal (5′-gggtcgacCATGGCATTCTTG GGGTTCTCAGTT-3′) and PolI1-R1-Bsp (5′-cctcatgaCGATCC ATCCGTAAGTACTGTCTTCC-3′) (additional nucleotides for the restriction sites in the primers are shown as lowercase letters). This fragment was digested with *Sal*I and *Bsp*HI and cloned into the *Sal*I/*Nco*I restriction site of the cauliflower mosaic virus (CaMV) 35S-sGFP (S65T)-nos3′ vector (a generous gift from Dr. Y. Niwa, University of Shizuoka, Japan; Niwa et al. 1999) so that NtPolI-like polypeptides fused in-frame to the N-terminus of sGFP (S65T) could be constitutively expressed in plant cells under the control of the CaMV 35S promoter. This construct was introduced into *N. tabacum* leaf cells with a PDS-1000/He particle delivery system (Bio-Rad Laboratories, Hercules, CA, USA). The epidermis of leaves was torn off and the fluorescence of transiently expressed NtPolI-like:GFP in cells was observed using an Axioskop2 plus microscope (Zeiss, Germany).

Production of polyclonal antibody

For antibody production, truncated *NtPolI-like* protein was overproduced in *E. coli* and purified as follows. The internal region of the *NtPolI-like2* gene (corresponding to amino acid residues 594–812) was PCR amplified from plasmid DNA containing the entire coding region, with primers 5′-taggatccCGGATCTGAC ATTCGATGGA-3′ and 5′-tactgcagAATTCCTTTCTGCCCAC CTCC-3′ (lowercase letters in each primer indicate additional nucleotides for *Bam*HI and *Pst*I sites, respectively). PCR products were digested with *Bam*HI and *Pst*I and subcloned into the pQE30 cloning vector to introduce an in-frame 6× histidine tag at the N-terminus. The histidine-tagged protein was overexpressed in *E. coli* by culturing the transformed cells with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 h at 25°C. After induction, the bacteria were collected and solubilized in buffer B (8 M urea, 0.1 M NaH_2PO_4) with sonication. The histidine-tagged protein was purified using Ni-NTA agarose (Qiagen), followed by SDS–PAGE fractionation. The gel slices that contained the recombinant protein were excised and injected into rabbits to raise antiserum.

Western analysis

For Western blotting analysis, plastid and mitochondrial nuclei were isolated from BY-2 cells according to Sakai et al. (1998) and the protein components were fractionated into NaCl-soluble and -insoluble fractions as described previously (Sakai et al. 1999). These samples were then separated in 7.5% SDS–polyacrylamide gels using a Mini-PROTEAN II cell (Bio-Rad). For visualization of total protein, the gel was silver stained according to Westermeier (1997). For Western blotting analysis, the separated proteins were blotted onto a PVDF membrane (Hybond-P, Amersham, Picataway, NJ, USA) using a Mini Trans-Blot electro-blotting apparatus (Bio-Rad), and labeled with rabbit polyclonal antibody raised against the internal region of NtPolI-like2 protein. The protein bands were detected using horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit IgG) and the ECL Plus Western blotting detection kit (Amersham), according to the manufacturer's instructions.

Expression and purification of histidine-tagged NtPolI-like2 polymerase domain

To assay DNAP activity, the DNAP domain of NtPolI-like2 protein was overproduced in *E. coli* and purified as follows. A portion of the *NtPolI-like2* gene (corresponding to amino acid residues 594–1,151) was PCR amplified with primers 5′-taggatcc CGGATCTGACATTCGATGGA-3′ and 5′-tagtcgacGGCAGAA TACCAGTTTTTGG-3 (lowercase letters in each primer indicate additional nucleotides for *Bam*HI and *Sal*I restriction sites, respectively) using plasmid DNA containing the entire coding region of *NtPolI-like2* as a template. PCR products were digested with *Bam*HI and *Sal*I, and subcloned into the *Bam*HI/*Sal*I site of the pQE30 cloning vector (Qiagen) to introduce an in-frame 6× histidine tag at the N-terminus. The histidine-tagged protein was overexpressed in *E. coli* by culturing the transformed cells with 0.3 mM IPTG for 5 h at 25°C. The cells were harvested, suspended in a binding buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 10 mM imidazole) and disrupted by sonication. The cell lysate was centrifuged, and the supernatant was mixed with equilibrated Ni-NTA agarose (Qiagen) at 4°C for 1.5 h for binding. The Ni-NTA agarose, which had bound the histidine-tagged protein, was loaded

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into a column and washed twice with wash buffer (50 mM NaH_2PO_4, 0.3 M NaCl, 20 mM imidazole). Finally, the histidine-tagged, truncated *NtPolI-like* protein was eluted with elution buffer (50 mM NaH_2PO_4, 0.3 M NaCl, 0.25 M imidazole). The eluted protein was dialyzed against buffer A (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol, 10% glycerol) and used for the DNAP assay. The protein content of the sample was determined by Bradford's method using the Bio-Rad protein assay kit (Bio-Rad).

DNAP activity assay of the recombinant NtPolI-like protein

For a standard assay, 10 μl of the purified recombinant NtPolI-like protein (10 μg) was mixed with 15 μl of concentrated assay mixture to give a final concentration of 50 mM Tris–HCl (pH 7.5), 0.4 mM EDTA, 2 mM 2-mercaptoethanol, 4% glycerol, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.4 mg ml^{-1} bovine serum albumin (BSA), 60 μg ml^{-1} activated calf thymus DNA (Amersham), 10 μM dATP, 10 μM dGTP, 10 μM dTTP and 10 μM [5-^{3}H]dCTP (adjusted to approximately 150 GBq $mmol^{-1}$). Departures from the standard assay conditions are indicated in the text and figure legends. The reaction mixtures were incubated for 30 min at 37°C, spotted onto DE-81 paper (Whatman), dried and washed four times in 5% (w/v) Na_2HPO_4, twice in water and twice in ethanol. The incorporation of radioactivity from [5-^{3}H]dCTP into DNA was determined by liquid scintillation counting.

Preparation of protein extract from isolated plastid and mitochondrial nuclei, and DNAP activity assay of the extract

To prepare protein extract, plastid and mitochondrial nuclei isolated from BY-2 cells according to Sakai et al. (1998, 1999) were treated with 2 M NaCl for 2 min at 26°C, and the NaCl-soluble fraction was recovered after centrifugation for 30 min at 87,000 × *g* and 4°C. After desalting and buffer exchange (50 mM Tris–HCl pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol, 10% glycerol and 25 mM KCl) using a syringe column (Hi-Trap desalting column, GE Healthcare), the samples were dispensed into aliquots, quickly frozen in liquid nitrogen, and stored at −80°C until use. Conditions for an assay of DNAP activity of the extracts were essentially the same as described above for recombinant NtPolI-like protein.

To remove NtPolI-like proteins from the extracts, the organelle nuclear extracts were incubated for 1 h at 4°C with protein A–agarose beads (GE Healthcare) that had been pre-incubated for 1 h at 4°C with anti-NtPolI-like antiserum. In the control experiment, protein A–agarose beads that had been pre-incubated with pre-immune serum were used. After removal of the agarose beads–protein A–antibody–antigen complexes by centrifugation, the supernatant was used for DNAP assay as described above.

RT–PCR analysis

Expression of *NtPolI-like1/2* genes was examined by RT–PCR analysis. Total RNA was isolated from BY-2 cells at different times (0, 12, 24, 48, 72, 96 and 120 h) after transfer of the cells to fresh medium. A 2 μg aliquot of the total RNA was used as template for cDNA synthesis for 1 h at 42°C using PowerScript reverse transcriptase (Clontech) and oligo(dT)-adaptor primer (TAKARA SHUZO CO., LTD, Kyoto, Japan). After heat inactivation for 15 min at 70°C, the cDNAs were subjected to PCR amplification of *NtPolI-like1/2* sequences, by use of Ex Taq (TAKARA SHUZO CO., LTD) and gene-specific primers (5′-AT GGCATTCTTGGGGTTCTCAGTTC-3′ and 5′-TCCATCATCT ACTTGGCAGA-3′). After heating at 94°C for 2 min, PCR was performed for 20 cycles of 30 s at 94°C, 30 s at 55°C and 60 s at 72°C, with the time at 72°C on the last cycle extended to 10 min.

In situ DNAP assay following SDS–PAGE

DNAP activity was analyzed in situ following SDS–PAGE according to Sakai et al. (1999). Protein components of plastid and mitochondrial nuclei isolated from BY-2 cells were fractionated into NaCl-soluble and -insoluble fractions. These samples were then separated in a 7.5% SDS–polyacrylamide gel containing 2 mM EDTA and 100 μg ml^{-1} heat-denatured salmon testes DNA, using a Mini-PROTEAN II electrophoresis apparatus (Bio-Rad). After electrophoresis for 1.5 h at 100 V, the gel was washed in renaturation buffer (50 mM Tris–HCl, pH 7.6, 1 mM EDTA, 5 mM 2-mercaptoethanol) to remove SDS and to renature proteins separated by SDS–PAGE. For the DNAP assay, the gel was then incubated for 24 h at 26°C in a reaction mixture composed of 40 mM Tris–HCl, pH 7.6, 9 mM $MgCl_2$, 24 μM $(NH_4)_2SO_4$, 0.01% NP-40, 0.2 mM EDTA, 0.5 mM spermidine, 5.8 mM 2-mercaptoethanol, 0.16 mM phenylmethylsulfonyl fluoride (PMSF), 180 μM dATP, 180 μM dGTP, 180 μM dTTP and 1 μM [α-^{32}P]dCTP (∼0.2 TBq $mmol^{-1}$). After washing for 48 h in washing solution (5% trichloroacetic acid, 1% sodium pyrophosphate), the gel was dried for autoradiography.

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