Running Title: Arabidopsis Tyrosyl-DNA phosphodiesterase

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Identification of tyrosyl-DNA phosphodiesterase as a novel DNA damage repair enzyme in Arabidopsis

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ABSTRACT

Tyrosyl-DNA phosphodiesterase (Tdp1) is a key enzyme which hydrolyzes the phosphodiester bond between tyrosine of topoisomerase and 3′-phosphate of DNA and repairs topoisomerase-mediated DNA damage during chromosome metabolism. However, functional Tdp1 has been only described in yeast and human to date. In human, mutations of Tdp1 gene are involved in the disease spinocerebellar ataxia with axonal neuropathy (SCAN1). In plant, we have first identified the functional nuclear protein, AtTDP, homologue to human Tdp1 from Arabidopsis. The recombinant AtTDP protein certainly hydrolyzes the 3′-phosphotyrosyl DNA substrates related to repairing of in vivo topoisomerase I-DNA induced damages. The loss-of-function AtTDP mutation displays developmental defects and dwarf phenotype in Arabidopsis. And this phenotype is substantially caused by decreased cell numbers without any changes of individual cell sizes. The tdp plants exhibit hypersensitivities to camptothecin (CPT), a potent topoisomerase I inhibitor, and show rigorous cell death in cotyledons and rosette leaves, suggesting the failure of DNA damage repair in tdp mutants. These results indicate that AtTDP plays a clear role for the repair of topoisomerase I-DNA complexes in Arabidopsis.
INTRODUCTION

In all living organisms, a variety of DNA damages are constantly arisen by replication errors, UV, ionizing radiation, DNA damage agents, etc. Once DNA damage is occurred, specific DNA repair proteins, such as AP endonuclease, RAD1, RAD9, RAD51, XRCC2, Ku80, ligase, etc., initiate to act through the repair pathways (Wood et al., 2001). Defects in DNA damage repair have evolved into the cancer or genetic disease in mammals and affect productivity or growth in plants (Tuteja et al., 2001; Wood et al., 2001).

In repair of DNA-protein crosslinks, tyrosyl-DNA phosphodiesterase 1 (Tdp1) is known as unique protein. Tdp1 protein was initially reported as an active enzyme in Saccharomyces cerevisiae that specifically removes tyrosine group from the covalent intermediate between the tyrosine residue and the terminal 3’-phosphate of the oligonucleotide (Yang et al., 1996). Subsequently, yeast TDPI gene was identified and showed highly conserved sequences with other organisms, Caenorhabditis elegans, Drosophila melanogaster, Mus musculus, and Homo sapiens (Pouliot et al., 1999). The Tdp1 homologues of these species are a member of phospholipase D (PLD) superfamily (Pouliot et al., 1999; Interthal et al., 2001). Yeast Tdp1 is mainly studied about topoisomerase I-repair pathway by using double or triple mutants. The deletion mutations of yeast Tdp1 were shown lacking in the repair of DNA damage induced by a topoisomerase inhibitor, anticancer drug camptothecin (CPT) (Pouliot et al., 2001; Liu et al., 2002; Vance and Wilson, 2002). Tdp1 has been further implicated in multiple repair pathways including the damage repair of topoisomerase II-DNA in yeast (Nitiss et al., 2006).

In multicellular eukaryotes, the defect of human Tdp1 has been resulted in neurodisorder disease, spinocerebellar ataxia with axonal neuropathy (SCAN1) (Takashima et al., 2002). SCAN1 is a rare autosomal recessive neurodegenerative disease and the patients present distal muscle weakness and peripheral neuropathy (Interthal et al., 2001; Takashima et al., 2002). SCAN1 was caused by the missense mutation (His493Arg) in the Tdp1 catalytic site. As in yeast, human Tdp1 protein plays a role in the repair of topoisomerase I-DNA complex lesions in SCAN1 cells (El-Khamisy et al., 2005; Miao et al., 2006). SCAN1 cells are hypersensitive to CPT (Interthal et al., 2005; Miao et al., 2006) and accumulate single strand break (SSB) and double strand break (DSB) DNAs by CPT (El-Khamisy et al., 2005).

At present, although the functional analysis of Tdp1 has been widely conducted in yeast and human cell lines, its role in overall growth and development of higher plants remains unknown. Here, we investigate the function of novel Arabidopsis TDP, human and yeast Tdp1 homologues. The AtTDP protein shows the DNA damage repairing activity and substrate specificities in biochemical assay. The dwarf phenotype of Arabidopsis tdp
mutant may be due to the reduced cell number caused by accumulation of DNA damage and progressive cell death during Arabidopsis development.

RESULTS

AtTDP encodes tyrosyl-DNA phosphodiesterase-related protein in Arabidopsis

Molecular analysis of AtTDP gene revealed that the inserted locus of T-DNA was in exon 4 of the At5g15170 gene of Arabidopsis (Genbank accession number. FJ858738) (Fig. 1A). This gene encodes tyrosyl-DNA phosphodiesterase-related protein and consists of 5′- and 3′- UTRs, 15 exons, and 14 introns. The full-length of the AtTDP cDNA was 1,818 bp and it encoded the protein of 605 amino acids. We determined the expression levels of neighboring genes near T-DNA insert site in tdp plants including AtTDP. Only the AtTDP gene was fully suppressed in tdp plants (Fig. 1B). The neighboring genes were not affected by enhancers of T-DNA. At the level of transcript accumulation in wild types, AtTDP was ubiquitously expressed in all examined tissues while its transcript level was relatively low in roots (Fig. 1C).

An alignment of AtTDP exhibited the sequence homology to the TDPs of H. sapiens (34% identity), S. cerevisiae (43% identity), C. elegans (28% identity), D. melanogaster (29% identity), M. musculus (32% identity), and O. sativa (54% identity) (Fig. 1, D and E). The encoded AtTDP protein consisted of two conserved domains, SMAD/Forkhead-associated (FHA) domain and Tyrosyl-DNA phosphodiesterase (TDP) domain (Fig. 1E). The FHA domain contained 95 amino acid residues. Recently, FHA containing proteins have been shown the interaction with phosphorylated protein targets (Durocher and Jackson, 2002). TDP proteins show sequence identities as members of phospholipase D (PLD) superfamily. An important feature of their sequences is the presence of HKD signature motif for the active site of TDP domain (Interthal et al., 2001). Even though Arabidopsis TDP shows the low sequence homology to those of human and yeast, AtTDP protein remarkably contains two conserved HKD (H\_K\_D) motifs in tyrosyl-DNA phosphodiesterase (TDP) domain needed for catalytic activity that enables the TDP enzyme to specifically hydrolyze the topoisomerase I-DNA complexes (Fig. 1E). Moreover, AtTDP obviously contained histidine and lysine in the HKD motifs which are in the active site of this enzyme (Pouliot et al., 1999; Interthal et al., 2001).

To further test the nuclear localization of the AtTDP, green fluorescence protein (GFP) was ligated to the 3′ terminal of the AtTDP gene and the construct was bombarded into Arabidopsis mesophyll protoplasts. As a
control, red fluorescent protein (RFP) gene fused with terminal flower (TFL) gene was expressed under the same promoter. Although AtTDP does not possess any nuclear localization sequences (NLSs) or putative NLSs in sequence analysis, while human Tdp1 has two putative NLSs for nucleus localization in the N-terminal regions (Barthelmes et al., 2004), AtTDP was detected to be localized in the nucleus for its enzymatic activity (Fig. 2). Therefore, the further sequence analysis of AtTDP may be necessary to elucidate the mechanism for nucleus-targeting.

**AtTDP has tyrosine-phosphodiesterase activity for topoisomerases-DNA complex**

When DNA replication or transcription is progressing, topoisomerases form complexes with DNA as short-lived catalytic intermediates. The tyrosine residue (Tyr723) of topoisomerase I is attached to the 3'-terminus of single strand DNA (Pommier et al., 1998). To determine the enzyme activity of recombinant AtTDP protein, we generated the recombinant AtTDP protein in E.coli. We added serial dilutions of the proteins and incubated with the 18-Y oligonucleotide, an 18-mer containing a tyrosine 3'-terminus, as a substrate (Fig. 3A). As shown in Fig. 3B (upper), the recombinant AtTDP protein efficiently hydrolyzed the phosphodiester bond between tyrosine and the 3'-end of the DNA, oligonucleotide-tyrosine substrate. It is likely eliminated that the enzymatic activity observed here is caused by a contaminating E.coli protein because E.coli does not possesses a tyrosyl-DNA phosphodiesterase and no activity was detected in the mock-purified preparation when tested at the highest concentration. Furthermore, to show a direct evidence, we were generated the deletion mutation in the coding region for the most conserved amino acids in the HKD motif. The mutant protein (Δ235-244) was expressed in E.coli, purified and tested for activity. As in Fig. 3B (bottom), mutant protein was approximately 1000-fold less active than those of wild type AtTDP protein.

If the topoisomerases-DNA complexes are not cleaved properly, these complexes accumulate in the cells and lead to DNA damages (Svejstrup et al., 1991; Chen and Liu, 1994; Pommier et al., 1998). When topoisomerases I-DNA covalent complexes meet replication forks, various DNA breaks appear before and after collisions, such as single stranded DNA, double strand blunted, double strand nicked, and double strand tailed DNA. But TDP efficiently repair these damages in normal cells. Therefore, to test the substrate specificity of AtTDP, we prepared the potential substrates including nicked, tailed, and blunted duplex DNA (Supplemental Fig. S1). AtTDP protein showed that blunted, tailed, and nicked duplex DNAs were cleaved to the similar extents to single stranded DNA (Fig. 3D).
Loss-of-function of AtTDP exhibits the extreme dwarfism in Arabidopsis

A tdp mutant was isolated from an Arabidopsis activation T-DNA tagging mutant pools screened by abnormal phenotypes. Homozygous tdp mutants exhibited a wide variety of developmental defects, including loss of apical dominance, early flowering, and a dwarf phenotype (Fig. 4; Table 1), whereas heterozygous TDP/tdp plants were morphologically similar to wild type (Supplemental Fig. S2A). When AtTDP open reading frame was overexpressed in sense orientation in tdp background, the wild-type phenotype was restored (Supplemental Fig. S2B). From an early vegetative stage, tdp plants displayed extremely small cotyledons, extremely small juvenile rosette leaves, and short petioles (Fig. 4, A and B). The length of rosette leaves in tdp plant was approximately 10-20% that of wild-type leaves. The length of tdp petioles was about 20% of the wild-type petioles (Table 1). The tdp mutants exhibited uneven leaf surface when compared with that of wild-type (Supplemental Fig. S3). The trichome numbers on the 5th leaves of tdp plants were reduced upto 20%, and the trichomes were abnormally branched (Fig. 4, E and F). One of the most remarkable features of tdp plants was observed in stems. The inflorescence stem was slender, shorter, and twisted. The trichomes on stem showed the reduction in number and length (Fig. 4, G and H). In the transverse section, the diameter of tdp1 stems was much shrunk (Fig. 4, I and J). The central cylinder contained only 4 to 5 vascular bundles in contrast to 8 vascular bundles in wild-type plants. The tdp1 stems did not show distinctive layers in epidermis, cortex, and central cylinder (Fig. 4, K and L).

In the reproductive stage, the organs of tdp flower were drastically small (Fig. 4C). Stamen and pistil were significantly less than for wild type. Furthermore the stamen length was too short to release the pollen onto the stigmatic surface (Fig. 4, M-R). The tdp anther was hardly dehiscent and dried up (Fig. 4, O and P). The tdp mutants significantly displayed low fertility (<0.01%). The silique length in tdp plants was about 50% less than that of wild type (Fig. 4S); however, seed size was indistinguishable from wild type (Fig. 4, T and U).

The tdp dwarf mutant is caused by reduced cell numbers

Since tdp plants displayed decreased organ size, tdp plants were further analyzed on a cellular basis. In general, organ size is affected by cell number or cell size. Therefore, we took these parameters into account to analyze the reduced size or length of tdp organs. Scanning electron microscopy (SEM) showed the epidermal tissues with similar cell size in these leaves and petals (Fig. 5, A-L). As shown in Fig. 5M, cell sizes in adaxial
and abaxial epidermis of tdp rosette leaf and petal epidermis were not changed. However, the organ sizes of leaf and petal in the tdp mutants exhibited approximately 90% and 75% reduction, respectively (Fig. 5, N and O).

Therefore, we further examined the causes of reduced cell number. Firstly, we analyzed whether the expression of mitotic cell cycle-related genes, such as G2/M specific genes (CYCA and CYCB genes), G1/S specific genes (CYCD genes), S-specific genes (histone H4 and WEE1), and CDKs, are changed. Although the expression of CYCA1;1, CYCB2;3, CDKB2;2 was slightly increased in tdp mutants (≤ 120%), the expression levels of the other genes did not show significant changes between wild-type and tdp plants (Fig. 6A; primers in Supplemental Table S1). Moreover, we examined the ploidy in tdp mutant. The cytometric data revealed that cells in 10-day-old wild-type and tdp cauline leaves contained nuclei up to 2C, 4C, 8C, and 16C (Fig. 6B). The ploidy in leaf cells of wild-type and tdp plants showed the highest peak at 4C. The overall peak pattern was similar between wild-type and tdp plants (Fig. 6B).

The tdp mutant is hypersensitive to CPT and caused by progressive cell death

Camptothecin (CPT) specifically binds to both topoisomerase I and DNA by hydrogen bonding and stabilizes these complexes. This prevents DNA religation, and thus causes DNA damage (Vance and Wilson, 2002). When we examined the effect of CPT treatment in Arabidopsis, tdp mutants were significantly hypersensitive to CPT. The tdp plants showed severe growth retardation and chlorophyll contents were remarkably reduced at 0.1 µM CPT (Fig. 7, A and B). In addition, when treated with 0.1 µM CPT and stained with trypan blue, tdp leaves were strongly stained throughout the entire leaf while only a few little blue spots were observed in a wild-type leaves (Fig. 7C), suggesting the failures of DNA damage repair results in progressive cell death in the tdp mutant. Excessive accumulation of DNA damage often results in apoptosis, senescence, tumor, or cell death. We further examined the possibility that decreased cell number in tdp mutants is caused by progressive cell death during Arabidopsis development. To investigate whether the dwarf phenotypes of the mutants are generated by cell death, we directly stained cotyledons and rosette leaves of wild-type and tdp plants with trypan blue. Trypan blue is used to selectively stain dead tissues or cells with a blue color (Koch and Slusarenko, 1990). Cell death in tdp plants began to be visible from early developmental stages. The cotyledons of tdp plants were strongly stained in the marginal regions and juvenile rosette leaves were also intensively stained all over, while the staining of those in wild type was insignificant. In addition, whole tdp seedling also showed intensive staining (Fig. 7C).
DISCUSSION

We have identified Arabidopsis TDP gene, human and yeast tyrosine-DNA phosphodiesterase (Tdp1) homologues, related to the repair of covalent protein-DNA adducts. Arabidopsis TDP has 34% sequence identity with *H. sapiens* and 43% identity with *S. cerevisiae*. Tdp1 proteins in yeast and human, as a member of PLD superfamily, show the HKD signature motif which is in an active site of Tdp1 enzyme (Interthal et al., 2001). Even though Arabidopsis TDP shows the low sequence homology to those of human and yeast, AtTDP protein remarkably contains two conserved HKD motifs needed for enzyme activity of TDP that specifically hydrolyzes the topoisomerase I-DNA complexes. And the protein is efficiently localized in nucleus. Therefore, we supposed the possibility that AtTDP protein may show the enzyme activity in a similar manner as yeast and human Tdp1 proteins. In biochemical and functional analysis, the recombinant AtTDP protein was certainly active on single stranded, paired, and blunted duplex DNAs, catalyzing the hydrolysis of 3′-phosphotyrosine bond and the substrate preferences of AtTDP are well consistent with those of yeast and human Tdp1s.

The novel AtTDP protein is required for normal growth and development in Arabidopsis. Plant homozygous for loss-of-function *AtTDP* mutation resulted in dwarf phenotype with developmental defects in Arabidopsis. The abnormalities of *tdp* plants were observed in vegetative and flowering development, showing reduced fertility. From the early vegetative stage, growth was retarded and organ size was very small in *tdp* mutants. Moreover, stems of *tdp* plants were very slender. The flowers were a quite small and displayed very low fertility.

To understand the causes of decreased organ size in *tdp* plants, we analyzed some parameters in detail. As in results, while the organ sizes of leaves and petals in *tdp* mutants were largely reduced as compared with those of wild-type plants, the epidermal cell sizes in leaves and petals were unchanged in *tdp* mutants. Therefore, we analyzed whether the mitotic cell cycle genes were expressed in *tdp* mutants. CYCD3:1 and CDKA have an important role in G1-to-S phase transition (Menges et al., 2006). A nonmitotic cyclin, *CYCD3:1* gene plays the integration of cell division in Arabidopsis leaf. At a DNA damage checkpoint, CDKA stops the progression of the cell cycle when DNA is damaged. Moreover, CDKA was reported as a target of WEE1 kinase in Arabidopsis. The Arabidopsis *wee1* gene decreases cell division related to cell cycle arrest on the DNA integrity checkpoints (De Schutter et al., 2007). The expression of mitotic cell cycle related genes did not differ significantly between wild-type and *tdp* plants. These results indicate that AtTDP did not affect genes that are involved in the control of cell cycle. Therefore, we further examined the possibility that decreased cell number...
in *tdp* plants is caused by progressive cell death during Arabidopsis development. Indeed, our sensitivity test to DNA damage agents revealed that *tdp* plants were hypersensitive to CPT. In addition, CPT-induced cell death was intensively observed in rosette leaves of *tdp* plants than in wild-type, suggesting the failure of DNA damage repair and progressive cell death in *tdp* mutant. To determine whether mutant plants are generated by cell death, we directly stained cotyledons and rosette leaves of wild-type and *tdp* plants with trypan blue. The *tdp* plants strongly showed progressive cell death from early developmental stage. These results indicate that accumulation of DNA damage caused by the loss-of *AtTDP* function induced cell death in Arabidopsis. CPT-induced cell death was also observed in yeast and human *tdp1* mutants. The Human *Tdp1* gene in SCAN1 cells is hypersensitive to CPT and induces the cell death in treated cells (El-Khamisy et al., 2005; Interthal et al., 2005). Yeast *tdp1* mutants, when coupled with mutation of the other DNA repair genes, show a significant effect on survival of CPT-treated cells (Pouliot et al., 2001; Liu et al., 2002; Vance and Wilson, 2002; Liu et al., 2004).

All together our data demonstrate that the correlation between the *tdp* mutant phenotype and biochemical function of *AtTDP* protein is well consistent with Tdp1’s role in related to repairs of topoisomerase I-induced damages. Therefore, we conclude that failure in repair of topoisomerases-mediated DNA damage could be accumulation of these products in the cells and to cell death that results in the dwarf phenotype during Arabidopsis development. This finding provides a better understanding of role of *AtTDP* during Arabidopsis development.

**MATERIALS AND METHODS**

**Identification and characterization of the mutant**

A population of *Arabidopsis thaliana* ecotype Col-0 plants was transformed with pSKI015 (Weigel et al., 2000) by using the floral-dip method (Clough and Bent, 1998) and screened for mutants with abnormal phenotypes. T-DNA tagged plants were selected with 0.1% BASTA (Duchefa) by spraying twice a week for 3 weeks. The genomic DNA flanking the T-DNA insertion was identified by the modified thermal asymmetric interlaced (TAIL)-PCR (Liu et al., 1995). T-DNA border–specific primers (AtLB1, AtLB2, and AtLB3) and a pool of two arbitrary degenerate primers (DEG1, DEG2) were used for three rounds of TAIL-PCR cycling (Supplemental Table S2). Homozygous *tdp* was selected by BASTA segregation analysis and verified by PCR. For complementation, the cDNA of *AtTDP* was amplified using RT-PCR with specific primers (AtTDP-F and...
AtTDP-R) (Supplemental Table S2). The cDNA fragment containing AtTDP open reading frame was cloned into pGEM-T Easy vector (Promega). The positive plasmid was subcloned into the binary vector pBI121 (CLONTECH). An identified positive clone was used for transformation of Agrobacterium tumefaciens C58C1 by the heat-shock method. And the plasmids were transformed into Arabidopsis tdp plants using floral dip method (Clough and Bent, 1998). We obtained the independent transgenic lines with kanamycin and BASTA resistances.

All Arabidopsis plants were grown in long days (16h light/8h dark) under fluorescent lights at 22°C with 70% humidity.

Nuclear localization of AtTDP-GFP fusion protein

To make a AtTDP-GFP fusion protein, the AtTDP cDNA sequence was amplified by PCR using the G-F and G-R primers containing a BamHI site and fused to the GFP (Supplemental Table S1). Rosette leaves of wild-type plants grown for 2 weeks were used for the isolation and the transformation of protoplasts. Ten μg of plasmid DNAs containing AtTDP-GFP fusion constructs was transfected into protoplasts. Then, protoplasts were incubated in a dark condition at 24°C for 24 h. Images were obtained using a confocal microscope (BIO-RAD, Radiance 2000/MP).

Production of recombinant AtTDP protein

Full length of AtTDP cDNA was prepared from the leaves of 2-week old plants by RT-PCR. RT-PCR was performed using the AtTDP-specific primers, TDPBamHI-F and TDPSacI-R primers (Supplemental Table S2) and the full length coding sequence was PCR-amplified. And PCR products were cloned into pGEM-T easy vector (Promega). The deletion protein (Δ235-244) was produced by Mutagenex (Poscataway, NJ, USA) and mutation was confirmed by sequencing. The mutant proteins were expressed in E.coli BL21 (DE3) cells by using the pET expression system (Novagen). The recombinant AtTDP protein was purified by a His-Bind Resin and His-Bind Kit (Novagen) as described by manufacturer.

Enzyme assays of recombinant AtTDP protein

The enzymatic activity of AtTDP as a phosphodiesterase to cleave tyrosyl residue was examined as described by Yang (Yang et al., 1996). The oligonucleotide sequence of the 18-Y substrate used for the enzyme
assay was 5'-TCCGTTGAAGCCTGCTTT-3'. Purified AtTDP proteins and 18-Y substrates were incubated in
the reaction mixture (50 mM Tris-HCl, pH 8.0, 80 mM KCl, 2 mM EDTA, 1 mM dithiothreitol, 40 μg/ml BSA,
and 5% glycerol) at 28°C. After 10 minutes of incubation, reactions were quenched by the addition of 10 μl
formamide sequencing buffer. Six μl aliquots were electrophoresed on a 20% sequencing gel. In addition, blunt
substrate 18-mer (5'-AAAGCAGGCTTCAACGGA-3'), tail substrate 43-mer (5'-ACCGTTTCGCYCAAGGTTAATGCTCAAAGCAGGCTTCAACGGA-3'), and nick substrate 25-mer (5'-
GACATACTAAGCGGAAACGTT-3') were prepared as DNA substrates. Duplex substrates were
prepared by mixing 10 pmol of the 18-Y substrate with 18-mer, 43-mer, and 25-mer followed by heat
denaturation and slow cooling at room temperature. Duplex substrate ratios were as follow: blunted duplex, 18-
Y:18-mer = 1:1; tailed duplex, 18-Y:43-mer = 1:1; nicked duplex, 18-Y:43-mer:25-mer = 1:1:1 (Supplemental
Fig. S1). The substrate-change assay was examined in the same manner as the enzyme activity assay.

Histology and Microscopy

To obtain the cross-sections of stems, samples were placed in a fixation solution containing 4%
paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) under vacuum conditions for 2 days at 4°C. Fixed
samples were rinsed with phosphate buffer twice and dehydrated through a series of graded ethanol. After
infiltration with xylene, samples were embedded in paraplasts. Eight μm thick sections were stained in toluidine
blue solution (0.1% toluidine blue and 0.1 M sodium phosphate buffer, pH 5.0) for 1 min. After destaining in
running water, sections were dried and mounted (Cerri and Sasso-Cerri, 2003). Images were taken on a Zeiss
Stemi SV11 microscope with a Nikon COOLPIX 4500 digital camera.

For scanning microscope images, all fixed plant samples were rinsed twice with 0.1 M phosphate buffer (pH
7.0) and transferred to 2% osmium tetroxide solution in 0.1 M phosphate buffer (pH 7.0) at 4°C for at least one
week. After osmium treatment, samples were dehydrated through a graded ethanol series (Bowman et al., 1989).
Dehydrated samples were critical-point-dried in liquid CO2 and coated with platinum.

Cell number and cell size measurement

SEM images of leaves and petals of wild-type and tdp plants were taken, and the number of cells in the
middle of leaf and petal surface was determined. For the quantitative comparison between wild-type and tdp
plants in terms of individual cell size, the projected areas of abaxial and adaxial leaves, and petal were measured.
First, the cell perimeter was defined manually using corresponding SEM images. Using MATLAB (Mathworks, Natick, MA), the segmented images were further processed and individual cell sizes were measured. A minimum of 3 nonoverlapping fields with 20–60 cells were analyzed for each experimental group, and two independent experiments were conducted per condition.

**RT- PCR**

Total RNA extracted from rosette leaves of wild-type and *tdp* mutant plants isolated using a RNAeasy kit (QUIAGEN) and reverse transcribed using a RT-PCR kit (Takara). RT was performed at 42°C for 60 min in a PCR system 9700 (Perkin Elmer, USA). The denatured cDNA was amplified by using designed specific primers (Supplemental Table S1). The PCR was performed using the PCR system 9700 for 30 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. PCR products were analyzed on 0.7% (w/v) agarose gel. *TUB2* gene was as a control.

**Flow cytometry**

The ploidy levels of leaf cell nuclei from 10- and 28-day-old wild-type and *tdp* plants were determined by flow cytometry using FACS canto (BD Biosciences). Leaves were chopped with a razor blade in Galbraith buffer (pH 7.0, 45 mM MgCl₂, 30 mM sodium citrate, 20 mM 4-morpholinepropane sulfonate) with 0.1 % Triton X-100 (Galbraith, 1983), filtered through a 30 μm-pore nylon (BD Biosciences), and stained with propidium iodide solution (50 μg/ml; Sigma). Data was collected for ~ 5,000 nuclei per run and were presented on a logarithmic scale.

**DNA damage agent treatment and measurement of total chlorophyll contents**

Wild-type and *tdp* plants were used to evaluate their sensitivities to CPT. Five-day old seedlings were transferred into 0.5% MS media supplemented with the varying concentrations of CPT. Seedlings in CPT-treated medium were allowed to grow for about 2 weeks. The total chlorophyll contents of whole plants treated by DNA damage agents were measured, following the method outlined by Porra (Porra, 2002). The absorbance of the supernatants was measured by the spectrophotometer (Pharmacia) at 646.6 nm and 663.6 nm. The chlorophyll contents were calculated by using the following equation: \( \text{Chl}_{a+b} = 17.76A_{646.6} + 7.34A_{663.6} \).
Cell death measurement

The leaves and cotyledons of wild-type and tdp plants were boiled for 1.5 min in the solution prepared as follow: phenol: lactic acid: glycerol: water: 95% ethanol at the ratio of 1:1:1:4 with 0.4% (w/v) trypan blue (Invitrogen). Samples were destained using 0.1% chloral hydrate (Sigma-Aldrich) for 2 days and then mounted in 50% glycerol (Koch and Slusarenko, 1990). The treated plants were imaged with a Nikon COOLPIX 4500 digital camera.

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Supplemental data

Figure S1. A variety of substrates

Figure S2. A. Morphologies of wild-type, heterozygous TDP/hdp, and homozygous tdp plants (45-day olds). B. Morphology of transgenic plant that AtTDP cDNA was overexpressed in tdp background.

Figure S3. Scanning electron micrographs of leaves. A. Adaxial side of wild-type. B. Adaxial side of tdp plants. Bar=1 mm.

Table 2. Oligonucleotides used in this study.

Table 1. Primers of cell cycle related genes.

REFERENCES

Bartheim HU, Habermeyer M, Christensen MO, Mielke C, Interthal H, Pouliot JJ, Boege F, Marko D (2004) TDP1 overexpression in human cells counteracts DNA damage mediated by topoisomerases I and II. J Biol Chem 279: 55618-55625

Bowman JL, Smyth DR, Meyerowitz EM (1989) Genes directing flower development in Arabidopsis. Plant Cell 1: 37-52

Cerri PS, Sasso-Cerri E (2003) Staining methods applied to glycol methacrylate embedded tissue sections. Micron 34: 365-372
Chen AY, Liu LF (1994) DNA topoisomerases: essential enzymes and lethal targets. Annu Rev Pharmacol Toxicol 34: 191-218

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735-743

De Schutter K, Joubes J, Cools T, Verkest A, Corelou F, Babychuk E, Van Der Schueren E, Beeckman T, Kushnir S, Inze D, De Veylder L (2007) Arabidopsis WEE1 kinase controls cell cycle arrest in response to activation of the DNA integrity checkpoint. Plant Cell 19: 211-225

Durocher D, Jackson SP (2002) The FHA domain. FEBS Lett 513: 58-66

El-Khamisy SF, Saifi GM, Weinfield M, Johansson F, Heleday T, Luski JR, Caldecott KW (2005) Defective DNA single-strand break repair in spinocerebellar ataxia with axonal neuropathy-1. Nature 434: 108-113

Galbraith H (1983) Response of cattle and sheep to hormonal anabolic compounds. Vet Res Commun 7: 27-34

Interthal H, Chen HJ, Keil-Fie TE, Zotzmann J, Leppard JB, Champoux JJ (2005) SCAN1 mutant Tdp1 accumulates the enzyme–DNA intermediate and causes camptothecin hypersensitivity. EMBO J 24: 2224-2233

Interthal H, Pouliot JJ, Champoux JJ (2001) The tyrosyl-DNA phosphodiesterase Tdp1 is a member of the phospholipase D superfamily. Proc Natl Acad Sci U S A 98: 12009-12014

Koch E, Sliusarenko A (1990) Arabidopsis is susceptible to infection by a downy mildew fungus. Plant Cell 2: 437-445

Liu C, Pouliot JJ, Nash HA (2002) Repair of topoisomerase I covalent complexes in the absence of the tyrosyl-DNA phosphodiesterase Tdp1. Proc Natl Acad Sci U S A 99: 14970-14975

Liu C, Pouliot JJ, Nash HA (2004) The role of TDP1 from budding yeast in the repair of DNA damage. DNA Repair (Amst) 3: 593-601

Liu YG, Mitsukawa N, Oosumi T, Whittier RF (1995) Efficient isolation and mapping of Arabidopsis thaliana T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J 8: 457-463

Menges M, Samiland AK, Planchais S, Murray JA (2006) The D-type cyclin CYCD3;1 is limiting for the G1-to-S-phase transition in Arabidopsis. Plant Cell 18: 893-906

Miao ZH, Agama K, Sordet O, Povirk L, Kohn KW, Pommier Y (2006) Hereditary ataxia SCAN1 cells are defective for the repair of transcription-dependent topoisomerase I cleavage complexes. DNA Repair (Amst) 5: 1489-1494

Nitiss KC, Malik M, He X, White SW, Nitiss JL (2006) Tyrosyl-DNA phosphodiesterase (Tdp1) participates in the repair of Top2-mediated DNA damage. Proc Natl Acad Sci U S A 103: 8953-8958

Pommier Y, Pourquier P, Fan Y, Strumberg D (1998) Mechanism of action of eukaryotic DNA topoisomerase I and drugs targeted to the enzyme. Biochim Biophys Acta 1400: 83-105

Porra RJ (2002) The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b. Photosynth Res 73: 149-156
Pouliot JJ, Robertson CA, Nash HA (2001) Pathways for repair of topoisomerase I covalent complexes in Saccharomyces cerevisiae. Genes Cells 6: 677-687

Pouliot JJ, Yao KC, Robertson CA, Nash HA (1999) Yeast gene for a Tyr-DNA phosphodiesterase that repairs topoisomerase I complexes. Science 286: 552-555

Svejstrup JQ, Christiansen K, Gromova, II, Andersen AH, Westergaard O (1991) New technique for uncoupling the cleavage and religation reactions of eukaryotic topoisomerase I. The mode of action of camptothecin at a specific recognition site. J Mol Biol 222: 669-678

Takashima H, Boerkool CF, John J, Saifi GM, Saleh MA, Armstrong D, Mao Y, Quirocho FA, Roa BB, Nakagawa M, Stockton DW, Lupski JR (2002) Mutation of TDP1, encoding a topoisomerase I-dependent DNA damage repair enzyme, in spinocerebellar ataxia with axonal neuropathy. Nat Genet 32: 267-272

Tuteja N, Singh MB, Misra MK, Bhalla PL, Tuteja R (2001) Molecular mechanisms of DNA damage and repair: progress in plants. Crit Rev Biochem Mol Biol 36: 337-397

Vance JR, Wilson TE (2002) Yeast Tdp1 and Rad1-Rad10 function as redundant pathways for repairing Top1 replicative damage. Proc Natl Acad Sci U S A 99: 13669-13674

Weigel D, Ahn JH, Blazquez MA, Borevitz JO, Christensen SK, Fankhauser C, Fernandez C, Kardailsky I, Malancharuvil EJ, Neff MM, Nguyen JT, Sato S, Wang ZY, Xia Y, Dixon RA, Harrison MJ, Lamb CJ, Yanofsky MF, Chory J (2000) Activation tagging in Arabidopsis. Plant Physiol 122: 1003-1013

Wood RD, Mitchell M, Sgouros J, Lindahl T (2001) Human DNA repair genes. Science 291: 1284-1289

Yang SW, Burgin AB, Jr., Huizenga BN, Robertson CA, Yao KC, Nash HA (1996) A eukaryotic enzyme that can disjoin dead-end covalent complexes between DNA and type I topoisomerases. Proc Natl Acad Sci U S A 93: 11534-11539
Legends

**Figure 1.** Molecular characterization of the *AtTDP* gene. A, Genomic structure of *tdp* T-DNA insertion mutant. T-DNA was inserted in *AtTDP* on chromosome 5. The *AtTDP* gene contains 15 exons, 14 introns, and 5′- and 3′-UTRs. Black boxes represent exons and lines between boxes represent introns. White boxes represent 5′- and 3′-UTRs. B, RT-PCR analyses of genes in wild-type and *tdp* plants. *At5g15150*: homeobox-leucine zipper protein 7 (*HAT7*), *At5g15160*: bHLH protein family, *At5g15170*: tyrosyl-DNA phosphodiesterase-related (*AtTDP1*), *At5g15180*: similar to peroxidase ATP12a, *At5g15190*: unknown protein. C, Expression of *AtTDP* in various tissues of wild-type plants using RT-PCR, *TUB2*: tubulin 2 as control. F, flowers; S, stems; L, leaves; R, roots. D, The phylogenetic relationships among *AtTDP* and other TDPs. E, Alignment analyses of *AtTDP* and TDP proteins from other organisms. Sequence alignments of the TDP proteins were analyzed by the program CLUSTALW. Abbreviations and accession numbers are as followed: sc (*Saccharomyces cerevisiae*, NCBI RefSeq accession no. NP_009782), ce (*Caenorhabditis elegans*, NCBI RefSeq accession no. NP_500149), dm (*Drosophila melanogaster*, NCBI RefSeq accession no. NP_523465), mm (*Mus musculus*, NCBI RefSeq accession no. NP_082630), hs (*Homo sapiens*, NCBI RefSeq accession no. NP_060789), os (*Oryza sativa*, NCBI RefSeq accession no. NP_001059844), at (*Arabidopsis thaliana*, NCBI RefSeq accession no. NP_197021). Amino acid sequence identity (%) with *AtTDP*; sc=43%, ce=28%, dm=29%, mm=35%, os=54%, hs=34%, *- single, fully conserved residue, -: conservation of strong groups, ·- conservation of weak groups, - no consensus. *AtTDP* protein has two conserved domains, a SMAD/FHA domain of gray dashes and a TDP domain of black line. The two HKD motifs in the TDP domain are indicated by gray boxes and conserved among eukaryotes as well as yeast.

**Figure 2.** Nuclear localization of *AtTDP* protein. A, *AtTDP-GFP* constructs. B, Light microscope image. C, *AtTDP-GFP* localization. D, Merged image of *AtTDP-GFP* localization. E, A positive control used *TFL* (*terminal flower*) gene. *TFL-RFP* expression; nucleus control image.

**Figure 3.** Enzyme activity of recombinant *AtTDP* protein. A, Enzymatic reaction mechanism of *AtTDP*. As substrate for *AtTDP*, synthesized single-strand 18-mer oligonucleotide containing tyrosine residue, 18-Y, was
used. AtTDP hydrolyzes the tyrosine residue from 18-Y and chemically produces 3′-phosphate DNA (18-P) and free tyrosine residue. B, Enzyme assay of recombinant AtTDP protein. The indicated amounts of AtTDP (upper) and deletion protein (Δ235-244, bottom) were incubated with 10 pmol substrate for 1 min. at 28°C. C, Analysis of activities of AtTDP on various substrates. S, single-strand DNA; B, blunted duplex DNA; T, 5′-tailed duplex DNA; N, nicked duplex DNA. The 1 μg AtTDP and each 10 pmol substrate were used.

**Figure 4.** Growth and morphology of tdp mutant. A, 5-day-old cotyledons, B, 20-day-old rosette leaves, and C, Mature flowers of wild-type (left) and tdp (right). D, 45-day-old mature wild-type (left) and tdp (right) plants. E and F, Scanning electron microscopic (SEM) analyses of mature trichomes on rosette leaves of wild-type (E) and tdp plants (F). G and H, SEM analyses of stems of wild-type (G) and tdp plants (H). I and J, Light microscopy of transverse section of stems. K and L, Magnification images of (J) and (L). M and N, SEM analyses of stamen and stigma between wild-type (M) and tdp plants (N). O and P, Anther of wild-type (O) and tdp (P) plants. Q and R, Pistils of wild-type (Q) and tdp (R) plants. S, Comparison of mature siliques between wild type (right) and tdp (left) using light microscopy. T and U, Mature seed of wild type (T) and tdp (U) plants. X, Xylem; P, Phloem; C, Cortex; E, Epidermis. Scale bar for A and C, 1 mm. Scale bar for B, 5 mm. Scale bar for D, 3 cm. Scale bar for E-R and T-U, 100 μm. Scale bar for S, 5 mm.

**Figure 5.** Reduced organ size and cell number in tdp mutant. A-F, SEM analyses of abaxial (A, B, G, H), adaxial (C, D, I, J) leaves and petal (E, F, K, L) epidermis of wild type (A, C, E) and of tdp (B, D, F). G-L, Drawing images of wild type (G, I, K) and tdp (H, J, L). M, Comparison of cell sizes of abaxial and adaxial epidermis of 5th leaves and petal epidermis. The cell perimeter was defined manually using corresponding SEM images (A) to (F). White boxes represent wild type and gray boxes represent tdp mutant. The values are given as mean ± SD relative to the respective wild-type values. N, Comparison of organ size between wild-type and tdp leaves. Measurement was carried out using the 5th leaves of mature plants. O, Comparison of petal size between wild-type and tdp plants. White boxes represent wild type and gray boxes represent tdp mutant. The values (M, N, O) represent averages of 5 independent replicates ± SD. Scale bars = 10 μm (A-D, G-J); 10 μm (E, F, K, L).

**Figure 6.** RT-PCR analysis and flow cytometry. A, Quantitative expression analyses of several mitotic cell cycle genes in wild-type and tdp plants. Total RNAs from rosette leaves from wild type and tdp were reverse
transcribed and amplified by PCR. The individual columns represent the relative expression of each gene in \textit{tdp} mutant compared with wild-type gene expression levels. The experiment was carried out five times and the intensities of RT-PCR bands were measured by densitometer. The values showed the average of independent replicates ± SD. B, Flow cytometry analysis of leaves from 10-day-old Arabidopsis wild-type (left) and \textit{tdp} (right) plants. Aggregate data from individual histograms of independent measurements are presented with ±SD (n=5) (bottom).

**Figure 7.** The sensitivity of \textit{tdp} plants to camptothecin (CPT). A, Twenty-day-old wild-type and \textit{tdp} plants treated with various concentrations of CPT. B, Chlorophyll contents of sample (A) seedlings. The values represent averages of 5 independent replicates ± SD. C, Cell death in wild-type (upper) and \textit{tdp} plants (bottom). The cotyledons, rosette leaves of various growth stages, and seedlings (12-day-old) of wild-type and \textit{tdp} plants were stained with trypan blue. The rosette leaves of wild-type and \textit{tdp} plants were treated by 0.1 μM CPT and stained with trypan blue (right panels). Scale bars = 1 mm

**Table 1.** Development of wild-type and \textit{tdp} plants
Table 1. Development of wild-type and *tdp* plants

|                           | Rosette leaf No. | Time of bolting (DAG) | Leaf No. at the time of bolting | Length (mm) | Inflorescence No. |
|---------------------------|------------------|------------------------|--------------------------------|-------------|-------------------|
|                           |                  |                        |                                | rosette leaf | petiole            |
| **WT**                    | 6.1 ± 0.1        | 22.5 ± 0.61            | 12.6 ± 0.6                     | 41 ± 2.8    | 10 ± 3.1          | 3.72 ± 0.8        |
| **tdp**                   | 5.5 ± 0.1        | 15.3 ± 0.4             | 6.1 ± 0.6                      | 6.3 ± 0.5   | 2 ± 0.1           | 13.33 ± 2.7       |

Data are shown as mean values ± SD. Values were collected at 15 DAG.

*a* Measured the fifth leaves after bolting.
