**ABSTRACT**

Nitric oxide (NO) is a key player in numerous physiological processes. Excessive NO induces DNA damage, but how plants respond to this damage remains unclear. We screened and identified an Arabidopsis NO hypersensitive mutant and found it to be allelic to TEBICHI/POLQ, encoding DNA polymerase θ. The teb mutant plants were preferentially sensitive to NO- and its derivative peroxynitrite-induced DNA damage and subsequent double-strand breaks (DSBs). Inactivation of TEB caused the accumulation of spontaneous DSBs largely attributed to endogenous NO and was synergistic to DSB repair pathway mutations with respect to growth. These effects were manifested in the presence of NO-inducing agents and relieved by NO scavengers. NO induced G2/M cell cycle arrest in the teb mutant, indicative of stalled replication forks. Genetic analyses indicate that POLθ is required for translesion DNA synthesis across NO-induced lesions, but not oxidation-induced lesions. Whole-genome sequencing revealed that POLθ bypasses NO-induced base adducts in an error-free manner and generates mutations characteristic of POLθ-mediated end joining. Our experimental data collectively suggests that POLθ plays dual roles in protecting plants from NO-induced DNA damage. Since POLθ is conserved in higher eukaryotes, mammalian POLθ may also be required for balancing NO physiological signaling and genotoxicity.

**INTRODUCTION**

Nitric oxide is an important signalling molecule involved in many physiological processes in mammals and its effects in biological processes depend on its source, duration and concentration. At low doses, NO exerts cytoprotective effects and triggers carcinogenesis, while at high doses, NO has been shown to produce cytotoxic effects and induce apoptosis (1). Similar effects are observed in plants: at low doses, NO promotes development, while at high doses, it suppresses growth (2–4).

NO and its metabolic derivatives like N2O3, NO2 and ONOO− (peroxynitrite) mainly cause damage to bases (5). For example, N2O3 deaminates guanine to xanthine (6), and ONOO− is a strong oxidant that reacts directly with guanine to produce 8-NO2-deoxyguanine (8-NO2-dG), which is unstable and can be depurinated to abasic (AP) sites (7,8). NO-induced damage to bases can be rescued by base-excision repair, which requires the recognition of the adduct by a specialized DNA glycosylase, followed by a strand break at the AP site and local DNA synthesis (9). If unrepaired, NO-induced adducts may block replication and trigger replication fork collapse, leading to double-strand breaks (DSBs). As one of the most dangerous lesions in living cells, DSB is mainly repaired by non-homologous end-joining (NHEJ) during interphase and by homologous recombination (HR) during S and G2 phases when homologous chromatids are available.

Stalled replication forks may trigger cell cycle checkpoints that slow down S phase progression, arrest cells at G2 and enhance the DNA-damage response (DDR) capacity, for example, by inducing relevant gene expression. During this period, the DNA-damage tolerance (DDT) mechanism, which does not remove but rather facilitates bypass
of the block, is operational at the arrested fork. DDT can be error-free, which utilizes newly synthesized sister chromatids for template switching to bypass the lesion. Alternatively, translesion DNA synthesis (TLS) utilizes specialized TLS polymerases to bypass DNA adducts and avoid replication fork collapse. Because the fidelity of TLS polymerases is lower than that of replicative DNA polymerases, the TLS process often leads to increased mutation (10). In yeast, a lack of Polο or Rev1 decreases the probability of UV-induced mutations at cyclobutane pyrimidine dimers (CTD) (11,12), while a lack of Polη increases the frequency of UV-induced mutation (13,14). Similarly, Arabidopsis Polη, as with its human counterpart, can bypass CTD in vitro (15). When Arabidopsis plants are irradiated with UV, the somatic mutation rates in Atrev3, Atrev7 (defective in Atpolη) and Atrev1 mutants are lower than those in the wild-type, while the mutation rate is higher in Atpolη (defective in Atpolη) plants than wild-type plants (16), indicating that there are at least two TLS polymerases in Arabidopsis that respond to UV damage with different biological consequences.

POLQ (Polο) is an A-family DNA polymerase containing an N-terminal helicase-like domain, an unknown central domain and a C-terminal polymerase domain (17). Polο appears to be able to extend the 3'-OH termini of single- and double-stranded DNA and across AP sites or mismatches in a template-independent manner (18–22). Polο bypasses various adducts (23) in an error-free (24–26) or error-prone (27–29) manner. Recently, the Polο polymerase domain was found to possess DNA-end-trimming activity that is required for microhomology-mediated end-joining (30).

Polο plays an important role in plants. Deletion of POLQ in Chlamydomonas reinhardtii sensitizes the algae to zeocin (31), suggesting that Polο is involved in DSB repair. Similarly, expression of the moss POLQ gene is induced by bleomycin, and POLQ deletion mutants are hypersensitive to bleomycin (32). Deletion of Arabidopsis TEBICHI (TEB)/POLQ leads to reduced root growth, hypersensitivity to DNA-damaging agents, delayed G2/M cell cycle progression and constitutively activated DDR (33). Subsequently, teb was found to be synergistic with mutations involved in replication checkpoints and HR (34). In addition, POLQ deletion in Arabidopsis interferes with the insertion of foreign DNA fragments, such as T-DNA, into the genome (35,36). Polο also participates in the DSB repair induced by CRISPR-Cas9 (37). These observations collectively indicate that, like its mammalian counterpart, the plant Polο plays an important role in processing DSBs produced from various sources. However, whether and how the plant Polο is involved in processing base damage and replication blocks remains unclear.

In the process of characterizing Arabidopsis NO hypersensitive mutants, we found that one of the corresponding genes is allelic to TEB/POLQ. Through forward genetic analyses, it was demonstrated that Arabidopsis Polο indeed protects against spontaneous and NO-induced DSB damage. Furthermore, Polο appears to be required for TLS across lesions caused by NO derivatives like ONOO'. As Polο is highly conserved, the roles Polο plays in protecting against NO toxicity may be common to higher eukaryotes, including plants and animals.

MATERIALS AND METHODS

Plant materials and generation of mutant plants

All the mutants in this study were in the Arabidopsis thaliana ecotype Columbia-0 (Col-0) background. The NO-sensitive mutant sno2-1/teb-6 were isolated from an EMS-induced mutant pool and backcrossed for three times as described previously (38). Another allelic mutant snno2-2/teb-7 were isolated as described (39). The T-DNA-inserted alleles teb-2 (SALK_035610), teb-3 (SALK_001669), teb-4 (SALK_037552), teb-5 (SALK_018851), teb-8 (SALK_009062), rad51d (CS3830262), lig4 (SALK_044027), ku70 (SALK_123114), xrc2 (SALK_029106), rev3 (SALK_029237) and polh (SALK_129731) were obtained from the Arabidopsis Biological Resource Center (https://abrc.osu.edu) as previously described (33,34,40,41). Lines carrying homozygous T-DNA insertion mutations were established, and the presence of T-DNA was confirmed by genomic DNA PCR using the primer sets listed in Supplementary Table S1. The gsnor1-3, cue1-5 and noa1 mutants have been described (42,43). For genetic analyses, the double and triple mutants were generated by standard genetic crosses and were identified in F2 progeny by genomic PCR and phenotypic observation.

In all experiments, plant seeds were surface sterilized with 10% bleach for 10 min and washed five times at least with sterile water before sowing. Plants were grown on plates containing 1/2 Murashige and Skoog (MS) medium, 1% (w/v) sucrose, 0.7% (w/v) agar, 0.05% (w/v) 2-(N-morpholino) ethanesulfonic acid (MES, pH 5.7). After a 3-day incubation at 4°C in dark, the plates were placed vertically in the growth chamber (100 μmol m−2 s−1; 16-h-light/8-h-dark cycle; 22°C) until analysis.

For different chemical treatments, the plants were grown in the 1/2 MS medium supplemented with sodium nitroprusside (SNP, Sigma-Aldrich), old SNP (44), potassium ferricyanide (Sigma), S-nitroso-N-acetylpenicillamine (SNAP, Sigma), 2-phenen-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO, Sigma-Aldrich and Abcam), zeocin (Invitrogen), Hemoglobin (Beyotime), hydrogen peroxide (H2O2), methyl viologen (MV, Sigma), NaCl, mannitol, CdCl2 or Nα-nitro-L-arginine (L-NNa, Sigma-Aldrich) under indicated conditions for 10–14 days, and the lengths of primary roots were determined using ImageJ (http://rsb.info.nih.gov/ij/).

To test the sensitivity of plants to ONOO−-related agents, 5-day-old seedlings preincubated on 1/2 MS plates were transferred to liquid 1/2 MS medium containing various concentrations of SNP, 3-morpholinosydnonimine-N-ethylcarbamide (SIN-1, Cayman), ebselen (Cayman) or c-PTIO, incubated for an additional 2–4 days and then the lengths of the primary roots and chlorophyll content were measured.

NO and ONOO− measurement

NO levels were measured in roots using the fluorescent NO indicator dye 3-amino, 4-aminomethyl-2',7'-difluorofluorescein diacetate (DAF-FM DA, Sigma-Aldrich) as described previously (45). Briefly, seeding
roots were incubated in 10 mM Tris–HCl (pH 7.4) for 30 min, followed by staining with 10 μM DAF-FM DA for 30 min in the dark. After washing three times with 10 mM Tris–HCl for 5 min each, the roots were mounted on a microscope slide and analyzed under a microscope (Nikon ECLIPSE Ni) equipped with a charge-coupled device camera (excitation, 495 nm; acquisition, 515 nm). NOONO−levels were detected in roots using a similar method except for using the ONOO−-specific fluorescent dye 2-[6-(4-aminophenoxy)-3-oxo-3H-xanthene-9-y1]-benzoic acid (APF, Cayman). The signal intensity was quantified using ImageJ.

**SNO2 gene mapping**

The NO-sensitive mutants (M3 generation; Col-0 background) were crossed with Landsberg erecta wild-type plants. DNA was extracted from 1,034 NO-sensitive mutant plants selected from the F2 plant population based on their NO-hypersensitive phenotype upon treatment with 50 μM SNP, and analyzed using simple sequence length polymorphism and cleavage-amplified polymorphic sequence markers (46).

**Root morphology analysis**

To observe root tip structures, roots of 6-day-old seedlings grown in medium with or without SNP were stained with 10 μg/ml PI for 1 min, washed with water twice and images were captured using a confocal fluorescence microscope (LSM510; Zeiss) with excitation and emission wavelengths of 559 and 619 nm, respectively. At least 10 plants per line were observed.

**Promoter GUS activity analyses**

To study the TEB promoter with a GUS activity assay, a 0.7-kb TEB promoter sequence was amplified from Col-0 genomic DNA using primers TEB promoter-F and TEB promoter-R (Supplementary Table S2). The amplified DNA fragments were purified with a gel extraction kit (Omega) and subcloned into the pBlunt plasmid (TransGen) in accordance with the manufacturer’s protocol. The cloned genomic DNA fragment was confirmed by sequencing and then subcloned into the destination vector pCambia1301, which harbours the GUS reporter gene. The resulting plasmid was mobilized into Agrobacterium tumefaciens (GV3101), which harbours the GUS reporter gene. The resulting plasmid was transferred into Col-0 plants using the floral dip method. Transformants were selected on 1/2× MS salt plates containing 1% sucrose and 25 μg/ml hygromycin. T3 transformants harbouring homozygous T-DNA inserts were used for the GUS activity assay.

To detect GUS activity, seedlings were stained with a solution containing 100 mM Na2HPO4, pH 7.0, 0.1% Triton X-100, 2 mM K3Fe(CN)6, 2 mM K4Fe(CN)6, and 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid (X-gluc) for 1 h at 37°C in the dark. The GUS-stained seedlings were treated with 70% ethanol and then 96% ethanol followed by embedding in a clearing solution (80% chloral hydrate and 10% glycerol) and analysis by microscopy (Axio Zoom.V16, Zeiss).

**Subcellular localization assay**

For a transient expression assay, the TEB-coding region was amplified using primers TEB CDS-F and TEB CDS-R (Supplementary Table S2) and cloned into plasmid pCambia1300-GFP as a GFP fusion. Then the construct was delivered into the Arabidopsis protoplasts via a method as described previously (47). The GFP fluorescence was observed under a confocal fluorescence microscope (LSM510, Zeiss).

**Chlorophyll content determination**

After various chemical treatments, seedlings were weighed and placed in appropriate amount of 90% (v/v) acetone for extraction. The chlorophyll content was determined by measuring the absorbance at 652, 665 and 750 nm using a spectrophotometer.

**Gene expression analysis**

Total RNA was extracted from various tissues as indicated by a TRIZOL reagent (Invitrogen). 1 μg of the total RNA was used for cDNA synthesis using a PrimeScript Reagent Kit with gDNA eraser (TaKaRa). Quantitative RT-PCR (qRT-PCR) for TEB, DSB-inducible and cell cycle-related genes was performed using SYBR Premix Ex Taq II (TaKaRa) on an Applied Biosystems 7500 Fast real-time PCR system (Applied Biosystems). The genespecific primer sets were listed in Supplementary Table S3 and the housekeeping gene SAND (44) and ACTIN8 were used as internal controls. The program was at 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by 95°C for 15 s and 60°C for 15 s. Three technical replicates were performed with each sample and the expression level was calculated by the 2−ΔΔCt method (48).

**Apurinic/apyrimidinic (AP or abasic) site analysis**

Total genomic DNA was extracted from 7-day-old seedlings incubated on vertical MS plates with or without another day of SNP, MV or SIN-1 treatment using the DNeasy Plant Mini Kit (Qiagen). The AP number was quantified with an OxiSelect Oxidative DNA Damage Quantitation Kit (AP sites, BioCells, STA-324) following manufacturer’s instructions.

**In vitro single-strand DNA break assay**

A previously published protocol (49) was followed to quantify single-strand breaks in close-circular plasmid pET28a DNA.

**Cell ploidy analysis**

The nuclei were extracted from the seedlings and analysed by flow cytometry as previously described (50).

**Histone preparation and γ-H2AX immunoassay**

Histones were extracted from nuclear preparations of seedlings as previously described (51). Protein samples
were subjected to SDS-PAGE, blotted, and immunode-
tected with a rabbit monoclonal anti-γ-H2AX antibody at
1:2500 dilution (Abcam, ab81299) and rabbit polyclonal
anti-Histone H3 antibodies (Abcam, ab1791) at 1:2500 di-
lution. Slide preparation, immunostaining and quantifica-
tion of γ-H2AX foci were performed as previously de-
scribed (52). Briefly, root tips were fixed for 45 min in 4% paraformaldehyde in PME (50 mM PIPES, pH 6.9, 5 mM MgSO4, and 1 mM EGTA) and then washed 3 × 5 min in PME. Tips were digested for 30 min in a 1% (w/v) cellulase solution in PME and then washed 3 times in PME. These roots were gently squashed onto slides, air dried, and stored at -80°C. Each slide was incubated overnight at 4°C with 100 μl anti-γ-H2AX antiserum. Slides were washed 3 times in PBS solution and then incubated for 2 h at room temperature in 100 μl blocking buffer consisting of Alexa 488-conjugated goat anti-rabbit secondary antibod-
ies. Finally, slides were washed 3 times in PBS and mounted in Vectashield mounting medium with 4′,6-diamidino-2-
phenylindole (DAPI).

**Comet assay**

Comet assays were performed as described (50,53). Briefly, the seedlings were chopped into pieces with a razor blade in 500 μl 1 × PBS buffer supplemented with 20 mM EDTA on ice. The nucleus suspension was filtered into a new tube through a 50 μm nylon mesh, combined with low-melting agarose at a ratio of 1:1, and pipetted onto CometSlides. Af-

ter incubation in lysis solution for 1 h at 4°C, the slides were placed in 1 × Tris-acetate electrophoresis buffer for 30 min prior to electrophoresis in the same buffer for 10 min at 4°C. The nuclei were stained with SYBR Green I. Images were captured and quantified with CometScore software (Tritek Co.), and at least 50 nuclei were scored per slide.

**DNA extraction and whole-genome sequencing (WGS)**

The genomic DNA from 14-d-old Col-0, teb-6, teb-5 and rev3 seedlings on medium with or without 15 mM MV or 25 μM SNP was extracted based on the CTAB protocol (54). The tissues were grounded to powder in liquid nitrogen, transferred to a preheated (65°C) CTAB lysis buffer and mixed by vortex. The samples were centrifuged at 10,000 rpm at room temperature (RT) for 5 min after incubating at 65°C for 60 min. The supernatant was extracted with equal volume of phenol/chloroform/isopentanol (25:24:1) fol-

lowed by centrifuging at 10,000 rpm at RT for 10 min. Ap-

proximately 70% volume of precooled (-20°C) isopropanol was added and put at -20°C for more than 2 h to precipi-
tate the DNA, followed by centrifuging at 12,000 rpm for 15 min at RT. 75% ethanol was added to wash the pellet and removed by centrifugation, and the DNA pellet was air-dried for 3–5 min. The pellet was dissolved by 30–200 μl TE buffer for further study. After DNA extraction, 1 μg genomic DNA was randomly fragmented by Covaris, fol-

lowed by fragments selection by Agencourt AMPure XP-

Medium kit to an average size of 200–400 bp. Selected frag-

ments were end repaired and 3′ adenylated, and the adap-
tors were ligated to the ends of these 3′ adenylated frag-

ments. The products were amplified by PCR and purified

by the Agencourt AMPure XP-Medium kit. The purified double-strand PCR products were heat denatured to single strand, and then circularized by the splint oligo sequence. The single-strand circle DNA (ssCir DNA) was formatted as the final library and qualified by QC. The final qualified libraries were sequenced by BGISEQ-500. ssCir DNA molecule formed a DNA nanoball (DNB) containing >300 copies through rolling-cycle replication. The DNBs were loaded into thepatterned nanoarray by using high density DNA nanochip technology. Finally, pair-end 100 bp reads were obtained by combinatorial Probe-Anchor Synthesis (cPAS).

**Variant calling**

The GATK best practices pipeline was used to analyze the 12 Arabidopsis samples. Briefly, the raw read files were quality checked and trimmed with Trimmomatic (v0.39). The trimmed reads were mapped to the A. thaliana TAIR10 reference genome with Burrows-Wheeler Aligner (v0.7.17-r1188). The format conversions and removal of duplicated reads were done with Samtools (v1.10) and Picard (v22.4) (http://broadinstitute.github.io/picard/), respectively. Variants were detected separately with GATK (v 4.1.7.0) and later merged with the function CombineVari-

nants. A known Arabidopsis variation dataset from the 1001 genomics project (55) was introduced to annotate our SNVs at this step. A customized script was implemented to iden-
tify transition or transversion.

**Statistical analysis**

Statistical analyses were performed using software Graph-
Pad Prism version 6.01 and SPSS23. P-value, sample number, and adjusted P-value are included in figures or figure legends.

**RESULTS**

**SNO2 mutant is specifically sensitive to NO**

To elucidate molecular mechanisms of NO signal response in plants, we used SNP, a well-established NO donor, to screen an existing Arabidopsis mutant library (38) and ob-
tained a genetically stable NO-sensitive mutant designated sensitive to nitric oxide 2 (sno2-1). Under normal culture conditions, the root length of the sno2-1 mutant was approx-
imately 70% that of the wild-type (Figure 1A), and sno2-1 plants were hypersensitive to SNP (Figure 1A–C). As the sno2-1 mutant did not display increased sensitiv-
ity to chemicals like potassium ferricyanide [K3Fe(CN)6] or old SNP (containing no NO but nitrate, nitrite and fer-
rocyanide) (44), the inhibitory effect was unlikely due to other components (such as cyanide) being released from SNP (Figure 1D) (56). The application of SNAP, another NO donor, and c-PTIO, a specific scavenger of NO, fur-
ther confirmed that NO was responsible for the NO sensi-
tive phenotype of sno2-1 (Figure 1E). Compared with wild-
type plants, sno2-1 was no more sensitive to H2O2 or MV (Supplementary Figure S1A and B), indicating that the NO-sensitive phenotype of sno2-1 is unrelated to reactive oxy-
gen species (ROS). The sno2-1 mutants also did not display
increased sensitivity to NaCl, osmotic stress or cadmium (Supplementary Figure S1C), demonstrating that sno2-1 is specifically sensitive to NO. Unlike wild-type plants, approximately 67% (12/18) of the sno2-1 mutants contained dead cells in the meristem zone of the root tips, as judged by propidium iodide (PI) staining. This phenomenon was aggravated by SNP treatment (Figure 1F), when the meristem zone of sno2-1 decreased significantly (Figure 1G), indicating that SNO2 protected root tips from exogenous NO-induced cell-cycle arrest and/or cell death in the meristem zone.

**SNO2 alleviates endogenous NO-induced growth inhibition**

To assess whether sno2-1 is also hypersensitive to the accumulation of endogenous NO, sno2-1 was crossed with cue1-5 (3) and gsnor1-3 (43) mutants known to have elevated endogenous NO levels to obtain the corresponding double mutants. There were no significant differences in the NO content between sno2-1 and the wild-type plants; however, the NO content in sno2-1 cue1 was significantly higher than that of wild-type plants but similar to that of cue1 (Figure 2A–D), indicating that the inactivation of SNO2 did not alter endogenous NO levels. However, the growth of the aerial parts and roots of sno2-1 cue1 was significantly inhibited in comparison to that of the cue1 single mutant (Figure 2A and B). c-PTIO and L-NNA (a NOS inhibitor) effectively rescued the phenotype of sno2-1 cue1 (Figure 2E), demonstrating that the retarded growth of sno2-1 cue1 was due to intolerance to the endogenous NO. The inhibitory effect was even more exacerbated in sno2-1 gsnor1 plants than in sno2-1 cue1, as the normal development of the true leaf...
and elongation of the primary root were severely impaired, leading to rapid death of sno2-1 gsnor1 plants (Figure 2A and B). Inactivation of NOA1, a GTPase involved in NOS-like pathway (57), could partially restore the sno2-1 mutant phenotype (Figure 2F–H), indicating that the growth defect in sno2-1 was due to accumulated NO. Together with the effect of c-PTIO treatment (Figure 1E), our observations collectively imply that SNO2 effectively protects plants from endogenous NO toxicity.

**sno2 is allelic to TEB/POLQ**

Using a map-based cloning strategy, we located the sno2-1 mutation to the TEB gene encoding Polθ (33), with a C2971T single-nucleotide substitution on exon 13 resulting in a nonsense mutation (Supplementary Figure S2A and B). The sno2-1 mutation in TEB was further confirmed by BseMI digestion, which cleaved the wild-type sequence but not that of sno2-1 (Supplementary Figure S2B and C). sno2-2 contains a G3204A mutation on exon 14 at the TEB locus (Supplementary Figure S2D), causing a nonsense mutation and increased NO sensitivity comparable to sno2-1 (Supplementary Figure S3). Five available TEB T-DNA insertion lines, SALK_035610, SALK_001669, SALK_037552, SALK_018851 and SALK_200962, designated teb-2, teb-3, teb-4, teb-5 and teb-8, respectively, were confirmed by genomic DNA PCR (Supplementary Figure S2E) and further characterized by qRT-PCR using five pairs of primers (Supplementary Figure S2A and F). teb-5 abolished transcription at all five locations; teb-2 and teb-8 transcripts could only be detected by the N-terminal primer pair; all three mutants displayed increased NO sensitivity reminiscent of sno2-1 and sno2-2 (Supplementary Figure S3). Hence, the above five lines are considered null mutants, and we designated sno2-1 and sno2-2 as teb-6 and teb-7, respectively from herein. As anticipated, teb-4 contains a T-DNA insertion at the very C-terminus and its transcript was detected by all five pairs of primers. To our surprise, the teb-3 transcript was also detected by all primer pairs (Supplementary Figure S2F), which was further confirmed by two 'trans-T-DNA insertion' primer pairs F5 + R5 and F6 + R6 (Supplementary Figure S2G). Consistently, teb-3 displayed no obvious growth defect and very moderate sensitivity to SNP treatment (Supplementary Figure S3).
Based on the assumption that teb-3 contains a T-DNA insertion at the C-terminal polymerase domain, it was previously concluded that the polymerase activity of Polθ is dispensable for normal plant growth (33). To ask whether the N-terminal helicase domain alone is sufficient to support plant growth, we grew plant seedlings in the presence of novobiocin (NVB) that selectively binds to and inhibits the Polθ ATPase activity (58), and found that NVB treatment of teb-3 plants phenocopies teb null mutants under both untreated and SNP-treated conditions (Supplementary Figure S4). Hence, the Polθ helicase activity appears to be essential to protect plants from endogenous and exogenous NO toxicity.

To further explore the relationship between NO and TEB, the expression of TEB in response to SNP treatment was monitored. A dose-response study (Supplementary Figure S5A) found that TEB expression was induced by SNP in a dose-dependent manner, while a time-course study (Supplementary Figure S5B) revealed that TEB expression was induced by 100 μM SNP during a period of 6–24 h, after which its mRNA level plateaued up to 48 h. A high-level TEB expression was observed in the shoot apical meristem, vascular tissue of roots and leaves, trichomes, anthers and stigma in Pro_{TEB}GUS transgenic plants (Supplementary Figure S6A), and its relative expression in different tissues as measured by qRT-PCR showed the similar pattern (Supplementary Figure S6B). Transient expression of Pro_{35S}:TEB-GFP in Arabidopsis protoplasts showed that TEB-GFP was located in the nucleus (Supplementary Figure S6C), consistent with its known activities (59,60).

**Teb/sno2 is hypersensitive to NO-induced DSBs**

*Arabidopsis* TEB is homologous to *Drosophila* MUS308 and mammalian POLQ. Previous studies showed that Polθ is involved in DSB repair in animals and now named Polθ-mediated end joining (TMEJ) (17,61,62). The *Arabidopsis* teb mutant is sensitive to mitomycin C (MMC) and methyl methanesulfonate (MMS) (33). MMC causes inter-strand breaks (63) and found that the teb-6 mutant root growth was indeed potently inhibited by zeocin in a dose-dependent manner (Figure 3A and B). We tested a hypothesis that NO induces DSBs by performing a neutral comet assay to visualize DSBs. SNP treatment induced comet tails; however, more DSBs were found in the teb-6 mutants than in wild-type plants (Figure 3C and D). The cellular histone H2AX phosphorylation (γ-H2AX) level serves as a reliable marker of DSB (51). The number of γ-H2AX foci in teb-6 root tips was much more than wild-type plants after treatment with SNP or zeocin (Supplementary Figure S7). Consistently, immunoblotting data revealed that teb-6 mutants accumulated much more DSBs than wild-type upon SNP and zeocin treatment (Figure 3E). Furthermore, the γ-H2AX band began to appear after 6-h SNP treatment in wild-type plants, while it was detected after 1-h SNP treatment in the teb-6 mutant plants, which were inhibited by the NO scavenger c-PTIO (Supplementary Figure S8A).

SNP treatment also induced the expression of *BRCA1* (Supplementary Figure S8B) and *GR1* (Supplementary Figure S8C), which were also inhibited by c-PTIO, particularly in the teb-6 mutant, suggesting that Polθ prevents NO-induced DSBs accumulation in *Arabidopsis*.

To further ascertain if the teb-6 mutant accumulated spontaneous and NO-induced DSBs, we examined the expression level of a panel of DDR marker genes known to be induced after γ-ray irradiation (64,65). It is apparent from Figure 3F that the teb-6 mutation induced the expression of all DDR marker genes examined; in addition, the expressions of genes involved in DSB sensing and repair, including *RAD51*, *BRCA1*, *GRI* (mammalian CtIP ortholog), *PARP1*, *PARP2* and *ATM*, were further induced by SNP. These results indicate that spontaneous DNA damage was accumulated in teb-6 mutant plants, and that NO treatment further increased the level of DNA damage, most likely in the form of DSBs. Furthermore, the expression of GRI was found to be increased in cue1 and gsnor1 mutants (Figure 3G), but GRI and PARP1 were reduced in noa1 (Figure 3H), indicating that endogenous NO could cause DNA damage. The above data supports a notion that TEB can restrain DNA damage induced by NO.

Deletions at the DSB containing microhomology at the junction are characteristic mutational signature associated with TMEJ repair. In an attempt to understand impacts of plant Polθ on TMEJ, we performed WGS and found more deletions with microhomology in the SNP-induced wild-type genome (17/44) than in teb-6 (7/39) and teb-5 (7/32) (Supplementary Figure S9A and B). Templated insertions (TINs) at the break site are characteristic genomic scars associated with TMEJ repair (62,66,67). SNP induced more direct or inverted repeat TINs in wild-type (15/58) than in teb-6 (3/59) and teb-5 (2/49) mutants (Supplementary Figure S9C and D).

**Genetic interactions between tebs and DSB repair pathway mutations**

To address roles of Polθ in the protection against DSBs, we examined genetic relationships between TEB and major DSB repair genes. DSB is one of the most severe types of DNA damage in animal and plant cells, and NHEJ and HR are considered to be the two major DSB repair pathways (68). The teb-6 mutation moderately affected plant growth (Figures 1A and 4A). Remarkably, while *ku70* and *lig4* mutations defective in the NHEJ pathway, did not alter normal growth, they strongly enhanced the impaired developmental phenotype of teb-6 and teb-5. The teb-6 *ku70*, teb-6 *lig4*, teb-5 *ku70* and teb-5 *lig4* double mutants exhibited severe growth retardation, much shorter roots than teb-6, and more severe morphological defects in the leaves (Figure 4A and B). The roots of teb-6 *lig4* were extremely twisted, and the root tips contained many dead cells (Figure 4C).

The *lig4* *xrc2* double mutation still did not affect normal plant growth (Supplementary Figures S10A–D), but the homozygous teb-6 *lig4* *xrc2* triple mutant appeared to be lethal (Figure 4D). A teb-6+/− *lig4*+/− *xrc2*−/− heterozygote was used to obtain homozygous triple mutants, but they died soon after germination (Figure 4D), indicating that the growth-inhibition effect of teb-6 mutation is
Figure 3. Roles of Pol1 in protecting spontaneous and NO-induced DSBs. (A) Col-0 and teb-6 seedlings grown in medium with or without 10 μM zeocin for 10 days. Scale bars = 1 cm. (B) Effects of zeocin treatment on root elongation in wild-type Col-0 and teb-6 seedlings. Root length data are expressed as means ± SD (n = 15, **P < 0.01, compared with Col-0, two-tailed Student’s t-test). (C) Representative comet assay images for SNP-induced toxicity. The images show the degree of DNA damage inflicted on single nuclei from 5-d-old Col-0 and teb-6 seedlings treated with 0 μM or 100 μM SNP for 48 h. The intensity of the dispersed signal in the tail indicates severity of DNA damage. Scale bars = 20 μm. (D) Quantitative analysis of olive tail moments reflecting the extent of DSBs in the nucleus. Values are relative to Col-0 plants grown in the absence of SNP. Data are expressed as means ± SD from at least 50 comets. Different letters represent significant differences (P < 0.05, one-way ANOVA and Tukey’s HSD). (E) Accumulation of γ-H2AX in Col-0 and teb-6 seedlings after treatments with 100 μM SNP and 10 μM zeocin for 24 h. Histone H3 served as a loading control. Two images were from the same gel. Similar results were obtained in at least two separate experiments. (F) DNA damage-inducible gene expression in response to SNP. Histogram of relative gene expression levels in wild-type and teb-6 plants on qRT-PCR analysis. At least 50 plants were used per replicate. Data were normalized to SAND mRNA levels in the same samples and are expressed as means ± SD (n = 3) relative to untreated Col-0 seedlings. Different letters represent significant differences (P < 0.05, one-way ANOVA and Tukey’s HSD). (G) Relative GR1 and PARP1 expression in Col-0, teb-6, noa1 and teb-6 noa1 seedlings (n = 3, *P < 0.05, **P < 0.01, compared with Col-0, two-tailed Student’s t-test).
Figure 4. Genetic interactions between teb-6 and DSB repair pathway mutations. (A) Representative images of Col-0 and indicated mutant seedlings grown for 10 d. Scale bars = 1 cm. (B) Quantitative analysis of the primary root length of 10-d-old seedlings grown as described in (A) (n = 12–27, means ± SD, **P < 0.01, compared with Col-0, two-tailed Student’s t-test). (C) Representative images of PI staining (red) to visualize the cell walls in root tips of Col-0 and indicated mutant seedlings grown for 10 days. Completely stained cells are dead. Scale bars = 50 μm. (D) Representative image of teb-6 xrcc2 and teb-6 lig4 xrcc2 seedlings grown for 7 days (upper panel, scale bars = 1 cm) and its enlargement (lower panel, scale bars = 1 mm). (E–H) Relative expression of GRI (E and G) and PARP1 (F and H) in teb-6, lig4, teb-6 lig4, ku70 and teb-6 ku70 seedlings (E and F), and xrcc2, teb-6 xrcc2, rad51d and teb-6 rad51d seedlings (G and H) in response to SNP as determined by qRT-PCR analysis. At least 50 plants were used per replicate. Data were normalized to SAND mRNA levels in the same samples and expressed as means ± SD (n = 3) relative to untreated Col-0. Different letters represent significant differences (P < 0.05, one-way ANOVA and Tukey’s HSD).

The expression of GRI and PARP1 was examined in seedlings of various mutants grown with or without SNP treatment. The teb-6 mutation caused a significant elevation in GRI and PARP1 transcript levels. In sharp contrast, mutations affecting NHEJ (e.g. lig4, ku70) and HR (e.g. xrcc2, rad51d) had no effect on the spontaneous GRI and PARP1 expression (Figure 4E–H). Furthermore, NHEJ pathway mutations did not further induce GRI, but mildly elevated PARP1 expression in the teb-6 background (Figure 4E and F); instead, HR pathway deficiency moderately elevated GRI but not PARP1 expression in teb-6 mutants (Figure 4G and H). SNP treatment elevated GRI and PARP1 expression in teb-6 and HR pathway mutants, but not in NHEJ.
pathway mutants (Figure 4E–H). In all cases, SNP treatment induced GRI and PARP1 expression in the double mutants in comparison to the corresponding single mutants (Figure 4E–H). As expected, cPTIO and Hb treatments reduced the BRCA1 and GRI expression in teb-6 lig4 and teb-6 xrc2 mutants (Supplementary Figure S11B and C). Similarly, teb-3 and lig xrc2 mutations slightly increased the basal-level PARP1 but not GRI expression; the corresponding triple mutation strongly induced GRI and PARP1 expression, which was further exacerbated by treatment with SNP (Supplementary Figure S11D). The above observations collectively indicate that tebs and mutations in the DSB repair pathways are synergistic with respect to spontaneous and NO-induced DSB accumulation.

teb-6 is sensitive to NO-derived ONOO−
In animals, NO and O2− can be rapidly converted into ONOO− (69). Within a certain concentration range (e.g. at the μM level), ONOO− is toxic to animal cells but does not have an obvious toxic effect on plant cells (70), implying the presence of a detoxification mechanism in plants that protects against ONOO−-induced cell death. To ask whether ONOO− is indeed an important metabolic intermediate after NO treatment, and whether Pol\(\theta\) protects against ONOO−-induced genotoxicity, we first established a detection system in which SIN-1 (a peroxynitrite donor)-induced ONOO− in root tips could be specifically detected by the fluorescent dye APF (Figure 5A and B). Under the same experimental conditions, SNP also induced a strong APF fluorescent signal that could be eliminated by treatment with an ONOO− scavenger ebselen (Figure 5A and B), indicating that NO produces ONOO−. Treatment with 5 mM SIN-1 had a devastating effect on the growth of teb-6 plants (Figure 5C and D), while the root growth of teb-6 was sensitive to SIN-1 in a dose-dependent manner that was alleviated by ebselen and the NO scavenger c-PTIO (Figure 5E). Because PARP1 expression was induced by SIN-1 and enhanced further by the inactivation of TEB (Figure 5F), and the ebselen treatment partially restored SIN-1 and SNP induced BRCA1 and GRI expression in teb-6 (Supplementary Figure S12), we concluded that Pol\(\theta\) indeed plays a critical role in protecting plants from DNA damage by NO-derived ONOO−.

SIN-1 directly induced DNA single strand breaks (SSBs) in vitro, indicating that ONOO− is capable of causing DNA strand breaks; however, more strand breaks were observed when Fapy glycosylase (FGP) was used to treat plasmid DNA incubated with SNP or SIN-1 (Figure 5G), indicating that the majority of the DNA damage caused by SNP and SIN-1 was in the form of dG adducts that were recognized and processed by FGP. Furthermore, treatment of wild-type and teb-6 plants with SNP or SIN-1 induced the formation of AP sites that were comparable to those seen with oxidative damage, indicative of DNA base damage by NO and its derivative ONOO− that lead to increased depurination (Figure 5H).

NO induces G2/M cell cycle arrest
A variety of genotoxities activate checkpoint systems to arrest the progression of the cell cycle (50,71). We speculated that DNA damage caused by both endogenous and environmental NO could activate this checkpoint. Checkpoint activation was first assessed by the expression of G2/M phase marker genes, including CYCB1;1, CYCA1;1, CYCA2;1, CYCA2;2, CYCA2;3, CYCA2;4, CDKA1 and CDKB2;1. Compared to wild-type plants, the expression of all marker genes was elevated in teb-6 (Figure 6A). SNP treatment alone did not induce the above marker gene but significantly increased their expression in the teb-6 plants, indicating that G2/M arrest can be induced by NO and that Pol\(\theta\) is required to avoid such arrest. Consistently, NO stalled teb-6 mutant root cells at the G2/M phase, as revealed by a CYCB1;1-GUS transgenic reporter (Figure 6B). G2/M phase arrest often induces early cell maturation and promotes endoreduplication (50,72). A flow cytometry assay showed that the proportion of 8C and 16C cells was moderately increased in teb-6 mutants and was further elevated by SNP treatment (Figure 6C). Hence, Pol\(\theta\) appears to function at the G2 phase to prevent NO-induced DNA damage.

Error-free TLS by Pol\(\theta\) protects against NO-induced DNA damage
Our observations that the teb and NO jointly trigger the G2/M phase checkpoint and cell cycle arrest suggest that NO toxicity causes replication fork arrest. As purified mammalian Pol\(\theta\) has TLS activity (26–28,73), and TLS primarily functions at the G2 phase (74,75) through DDT, in which the replication block is bypassed but the adduct is not removed (76), we hypothesized that Pol\(\theta\) plays a critical role in bypassing the NO-induced lesion. To test this hypothesis, we measured the relative sensitivity of teb-6 and two other TLS polymerase mutants polh and rev3 to SNP. Because NO-derivatives such as ONOO− can cause DNA oxidative damage (49,77), we also performed parallel MV treatment for comparison. As previously observed, the teb-6 single mutant grew more slowly than the wild-type plants, while rev3 and polh single mutations or even the rev3 polh double mutation did not affect plant growth (Figure 7A). However, the teb-6 rev3 double and teb-6 rev3 polh triple mutants showed more severe growth defects than their respective single and double mutants (Figure 7A), implying that Pol\(\theta\) plays a major role in tolerating spontaneous DNA damage, while Pol\(\eta\) and Pol\(\gamma\) play backup roles. After SNP treatment, both teb-6 and rev3 mutants showed increased sensitivity, and the two mutations were additive (Figure 7A and B), indicating that Pol\(\theta\) and Pol\(\gamma\) bypassed the NO-induced DNA adduct(s) via different pathways. Interestingly, rev3 polh, teb-6 polh and teb-6 rev3 polh mutants were no more sensitive to SNP than their corresponding single and double mutants (Figure 7A and B), indicating that Pol\(\eta\) is not involved in TLS of NO-induced DNA damage. In sharp contrast, teb-6, either alone or in combination with other TLS mutations, did not increase sensitivity to the MV treatment (Figure 7A and B), suggesting that Pol\(\theta\) is not primarily responsible for TLS of oxidative DNA damage.

Knowing that Pol\(\theta\) plays a critical role in bypassing NO-induced lesions, we attempted to understand its effects on mutagenesis and the mutational spectrum by whole-genome sequencing (WGS) using TAIR10 as a reference genome. Treatment of wild-type plants with SNP induced
Figure 5. Phenotypes of Arabidopsis teb-6 mutants under SIN-1 treatment. (A) Representative images showing fluorescence intensity of peroxynitrite in root tips of 7-day-old Arabidopsis seedlings exposed to 1 mM SIN-1, 50 μM SNP and 50 μM SNP + 20 μM ebselen in liquid medium for 12 h. Scale bars = 50 μm. (B) Quantitative analysis of results shown in (A) expressed as arbitrary units (A.U.) using ImageJ software (n = 23–24, means ± SD; P < 0.05, one-way ANOVA and Tukey’s HSD). (C) Phenotypes of 7-day-old wild-type (Col-0) and teb-6 plants were transplanted to the liquid medium supplemented with or without SIN-1 for another 5 d. Scale bars = 1 cm. (D) Quantitative analysis of chlorophyll contents in wild-type (Col-0) and teb-6 as shown in (C) (n = 3, means ± SD; P < 0.05, one-way ANOVA and Tukey’s HSD). FW, fresh weight. (E) Relative root length of wild-type (Col-0) and teb-6 plants grown in liquid medium supplemented with 1, 2 and 5 mM SIN-1, 500 μM c-PTIO and 20 μM ebselen as described in (C) (n = 12, average ± SD; **P < 0.01, compared with Col-0, Student’s t-test; ns no significant difference). (F) Relative PARP1 expression in 10-day-old wild-type (Col-0) and teb-6 seedlings grown in liquid medium supplemented with 3 mM SIN-1 for different time periods (n = 3, **P < 0.01, untreated Col-0 served as a control, two-tailed Student’s t-test). (G) DNA damage of pET28a plasmid was induced by 50 μM SNP or 10 μM SIN-1 in phosphate buffer (pH 7.4) with or without 8 units E. coli Fapy glycosylase (FPG) for 1 h (n = 3, average ± SD; P < 0.05, one-way ANOVA and Tukey’s HSD). (H) AP sites in 5-d-old wild-type and teb-6 seedlings grown in liquid medium supplemented with 50 μM SNP, 30 nM MV or 2 mM SIN-1 for another 2 days. Results are expressed as mean number of AP sites per Mbp genomic DNA ± SD (n = 3; P < 0.05, one-way ANOVA and Tukey’s HSD).
DISCUSSION

Our previous studies showed that excessive NO inhibits Arabidopsis root growth, most likely by reducing the number of meristem cells (3,80), but the underlying mechanisms were obscure. Through the isolation and characterization of an NO hypersensitive mutant teb-6, the current study has led us to conclude that excessive NO inhibits plant growth by primarily inducing DNA damage. Firstly, the comet assay and γ-H2AX assays revealed that NO induces DNA DSBs. Secondly, the expression of a panel of known DNA-damage responsive genes was induced upon the treatment of plants with NO. Thirdly, NO induced cell death in the stem cell niche around the quiescent root centre, which is characteristic of DNA damage (81). Fourthly, NO treatment arrested cell-cycle progression at the G2/M phase and induced cycle-related gene expression in the teb-6 mutant. Fifthly, expression of the TEB was induced by NO at the transcriptional level. Finally, TEB was found to encode DNA Polθ, whose known functions to date are exclusively involved in DNA metabolism (17,82).

Since both teb-6 and teb-7 produced wild-type level full-length transcripts and their premature translation termination occurs between the N-terminal helicase domain and C-terminal polymerase domain, yet they displayed null mutant phenotypes, we infer that the polymerase activity of plant Polθ is absolutely required to protect NO-induced DNA damage. On the other hand, teb-1, a helicase-defective but polymerase-intact mutant, also displayed growth defects (33). Together with our observation that the chemical inhibition of the Polθ ATPase activity displayed the teb null mutant phenotype, we cautiously conclude that both polymerase and helicase activities of Polθ are required for the protection against NO-induced DNA damage, although this conclusion is subject to further examination.

Our observations that spontaneous and NO-induced DSBs accumulated in the teb-6 mutant indicate that Polθ protects Arabidopsis from this type of DNA damage. However, it remains unclear whether Polθ prevents DSB formation or if it is required for the repair of DSBs, or both. As a matter of fact, Polθ has been reported to function both ways in mammalian cells. Polθ is a TLS polymerase required for bypassing various DNA adducts (24–28,73), many of which are replication-blocking lesions. Failure to bypass such lesions at the stalled replication fork causes fork collapse, resulting in DSBs. However, Polθ can also extend single-strand and double-strand termini or process damaged termini, priming them for DSB repair. Loss of any of the above activities would be synergistic with defects in NHEJ and HR with respect to plant growth and viability, concurring with the striking phenotype observed in this study. Results obtained from this study provide evidence that Polθ plays a critical role in protecting plant cells from NO-induced endogenous DNA damage, since teb plant displayed moderate growth retardation, while mutants in c-NHEJ, HR and other TLS pathways did not. When facing excessive endogenous (e.g. in cuel or gsnor1 mutants) and/or exogenous NO-induced DNA adducts, Polθ is required to prevent the formation of DSBs. Polθ, as a TLS polymerase, can use damaged DNA, including DNA adducts, AP sites or even strand termini, as a template to prime replication.
This study demonstrated that Polθ is preferentially involved in the TLS of NO-induced lesions, while in the absence of Polθ, other TLS polymerases, such as Polζ, could serve backup roles. Our observation of plant rev3 sensitivity to NO is consistent with a report that chicken DT40 cells deficient in Rev3 are hypersensitive to NO (83). Our conclusion on the involvement of Arabidopsis Polθ in TLS relief of replication stress agrees with a very recent report (84). Although this study revealed that Polθ is required to prevent oxidation-induced signature G:C > T:A transversions, we could not rule out the possibility that it is also required for TLS bypassing other types of DNA damage like its mammalian counterparts (24–28,73). It has been reported that mammalian Polθ possesses a 5′dRP lyase activity within its C-terminal polymerase domain (85), and chicken DT40 cells lacking Polθ and Polβ are sensitive to MMS due to reduced base excision repair (BER) activity (86). Our observations cannot rule out the possibility that Polθ protects plants from NO-induced DNA damage through BER, particularly since the Arabidopsis genome lacks an ortholog en-
Figure 8. A working model of AtPolβ-mediated responses to NO-induced DNA damage. Polβ plays multiple roles in dealing with NO-induced DNA lesions. Firstly, Polβ and Polε jointly bypass NO-induced and stalled replication blocks through largely error-free TLS; lack of such activities may result in replication fork collapse and DSBs. Secondly, Polβ may have a 5′dRP lyase activity and participate in base excision repair (BER). Thirdly, Polβ is directly involved in processing spontaneous and NO-induced DSBs; lack of such an activity causes plants to rely heavily on HR and NHEJ for growth or even survival. Hence, Polβ plays a crucial role in maintaining genomic stability, particularly in response to NO-induced lesions. Thin and dash lines represent reduced and lost activities, respectively.

coding Polβ (87), making Polβ an attractive candidate for the source of dRPase activity.

It remains unclear how excessive NO induces DNA damage in plants. In mammals, NO and its derivatives, e.g. N₂O₃ and ONOO⁻, induce DNA adducts, AP sites and strand breaks (77). In vitro, NO-derived ONOO⁻ induces the formation of 8-oxo-dG and 8-NO₂-dG; the former is rapidly depurinated (88). This study revealed that, although in vitro treatment of plasmid DNA with the ONOO⁻ donor SIN-1 induced nicks, the vast majority of nicks were generated by combined treatment with FPG and SNP or SIN-1, indicating that the adduct(s) are recognized and processed by FPG to produce single-strand breaks, reminiscent of the previously reported induction of DNA oxidative adducts by ONOO⁻ (89,90). The Arabidopsis genome contains FPG and OGG1 orthologs (91) capable of processing the above ONOO⁻-induced adducts. This study also revealed that SNP and SIN-1 induced AP sites in vivo in Arabidopsis, suggesting that, similar to in mammals, NO-mediated DNA damage and toxicity in plants depend at least partly on the NO metabolite ONOO⁻.

NO and reactive nitrogen species (RNS) produces more complex base modifications than ROS and leads to a variety of base substitutions. Although NO gas and NO donor-induced mutational spectra differ, as judged from the supF reporter assays in bacterial and human cells, G:C > T:A, A:T > G:C and G:C > A:T are always the most frequent mutations (92). While such an assay is currently unavailable for use in plants, we took a next-generation sequencing approach and found that NO mainly induced G:C > T:A, A:T > G:C, A:T > T:A and G:C > A:T base substitutions in wild-type plants. In addition, our WGS data revealed both reduced TMEJ signature mutations and increased G:C > T:A and G:C > A:T mutations in teb lines, further supporting dual roles of plant Polβ in DSB repair and TLS in response to NO-induced lesions. It is interesting to note that mutations accumulate in A. thaliana lineages grown at elevated temperatures and in high-salinity soil (93), two environmental stresses known to cause NO and ROS accumulation (94,95), implying that NO contributes to such processes. Indeed, teb mutants displayed certain degrees of salt sensitivity (84), reminiscent of their increased NO sensitivity as revealed in this study, suggesting that Polβ also plays a critical role in the protection against high-salinity stress.

In conclusion, we propose a working model (Figure 8) for the involvement of Polβ and other DDR pathways in response to NO toxicity. Under normal growth conditions, endogenous NO-induced toxicity is mainly processed by Polβ, lack of which results in moderate growth retardation, while mutations in other relevant pathways do not. When excessive NO is accumulated either due to NO metabolic mutations (e.g. cue1 or gsnor1) or by treatment with NO-inducing agents, Polβ becomes pivotal for plant growth and survival, while other pathways may also contribute to the detoxification. The dual defects of Polβ and other TLS polymerases, particularly Polε, result in increased sensitivity to endogenous NO stress, which can be further exacerbated...
by exogenous sources of NO. Inactivation of Polθ results in DSB accumulation, either due to NO-induced replication fork collapse or an inability to process strand termini, which requires NHEJ during G1/S phase and HR during G2/M phase to maintain telomere viability. Although not experimentally addressed in this study, plant Polθ may also possess a 5’dRP lyase activity like mammalian Polθ, which can be used to process NO-induced base damage through BER for detoxification. As NO is also an important signalling molecule in other organisms, particularly mammals, the findings in this study may shed light on ways to investigate the novel roles of Polθ in other organisms.

DATA AVAILABILITY
The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (96) in National Genomics Data Center (97), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences, under accession number CRA004785 that are publicly accessible at https://ngdc.cncb.ac.cn/gsa. Shared URL: https://ngdc.cncb.ac.cn/gsa/s/5e1Y111k.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS
We would like to thank the Arabidopsis Biological Resource Center for the T-DNA inserted mutants. We thank Dr Hailong Wang and Xiangxi He from Capital Normal University for supplying technical support. We also thank Dr Min Zhang from Capital Normal University, for supplying pCambia1300-GFP vector.

FUNDING
National Natural Science Foundation of China [31530006, 31970658]. Funding for open access charge: National Natural Science Foundation of China [31530006, 31970658]. Conflict of interest statement. None declared.

REFERENCES
1. Choudhari,S.K., Chaudhary,M., Bagde,S., Gadball,A.R. and Joshi,V. (2013) Nitric oxide and cancer: a review. World J. Surg. Oncol., 11, 118.
2. Beligni,M. and Lamattina,L. (1999) Is nitric oxide toxic or protective? Trends Plant Sci., 4, 299–300.
3. He,Y., Tang,R.H., Hao,Y., Stevens,R.D., Cook,C.W., Ahn,S.M., Jing,L., Yang,Z., Chen,L., Guo,F. et al. (2004) Nitric oxide represses the Arabidopsis floral transition. Science, 305, 1968–1971.
4. Liu,W.Z., Kong,D.D., Gu,X.X., Gao,H.B., Wang,J.Z., Xia,M., Gao,Q., Tian,L.L., Xu,Z.H., Bao,F. et al. (2013) Cytokinins can act as suppressors of nitric oxide in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A., 110, 1548–1553.
5. Thomas,S., Lowe,J.E., Knowles,R.G., Green,J.C. and Green,M.H. (1998) Factors affecting the DNA damage activating superoxide and nitric oxide. Mutat. Res., 402, 77–84.
6. Nguyen,T., Brunson,D., Crespi,C.L., Penman,B.W., Wishnok,J.S. and Tannenbaum,S.R. (1992) DNA damage and mutation in human cells exposed to nitric oxide in vitro. Proc. Natl. Acad. Sci. U.S.A., 89, 3030–3034.
7. Yermilov,V., Rubio,J. and Ohshima,H. (1995) Formation of 8-nitroguanine in DNA treated with peroxynitrite in vitro and its rapid removal from DNA by depurination. FEBS Lett., 376, 207–210.
8. Tuo,J., Liu,L., PoulSEN,H.E., Weimann,A., Svendsen,O. and LofS, (2000) Importance of guanine nitration and hydroxylation in DNA in vitro and in vivo. Free Radiic Biol Med, 29, 147–155.
9. Friedberg,E.C., Walker,G.C., Siede,W., Wood,R.D. and Ellenberger,T. (2005) In: DNA Repair and Mutagenesis. ASM Press.
10. Friedberg,E.C. (2005) Suffering in silence: the tolerance of DNA damage. Nat. Rev. Mol. Cell Biol., 6, 943–953.
11. Lawrence,C.W. and Christensen,R.B. (1978) Ulitraviolet-induced reversion of cycl alleles in radiation-sensitive strains of yeast. I. rev mutation. J. Mol. Biol., 122, 1–21.
12. Lawrence,C.W. and Christensen,R.B. (1979) Ulitraviolet-induced reversion of cycl alleles in radiation-sensitive strains of yeast. III. rev3 mutant strains. Genetics, 92, 397–408.
13. Yu,S.L., Johnson,R.E., Prakash,S. and Prakash,L. (2001) Requirement of DNA polymerase eta for error-free bypass of UV-induced CC and TC photoprodcts. Mol. Cell. Biol., 21, 185–188.
14. Kozmin,S.G., Pavlov,Y.I., Kunkel,T.A. and Sage,E. (2003) Roles of Saccharomyces cerevisiae DNA polymerases pola and polzeta in response to irradiation by simulated sunlight. Nucleic Acids Res., 31, 4541–4552.
15. Sakamoto,A.N. (2019) Translesion synthesis in plants: ultraviolet resistance and beyond. Front. Plant Sci., 10, 1208.
16. Nakagawa,M., Takahashi,S., Tanaka,A., Narumi,I. and Sakamoto,A.N. (2011) Role of Atpolzeta, Atrev1, and Atpoleta in UV light-induced mutagenesis in Arabidopsis. Plant Physiol., 155, 414–420.
17. Yousefzadeh,M.I. and Wood,R.D. (2013) DNA polymerase POLQ and cellular defense against DNA damage. DNA Repair (Anst.), 12, 1–9.
18. Seki,M., Masutani,C., Yang,L.W., Schuffert,A., Iwai,S., Bahar,I. and Wood,R.D. (2004) High-efficiency bypass of DNA damage by human DNA polymerase Q. EMBO J., 23, 4484–4494.
19. Hogg,M., Seki,M., Wood,R.D., Doubleb,S. and Wallace,S.S. (2011) Lesion bypass activity of DNA polymerase theta (POLQ) is an intrinsic property of the pol domain and depends on unique sequence inserts. J. Mol. Biol., 405, 642–652.
20. Hogg,M., Sauer-Eriksson,A.E. and Johansson,E. (2012) Promiscuous DNA synthesis by human DNA polymerase theta. Nucleic Acids Res., 40, 2611–2622.
21. Laverty,D.J., Averill,A.M., Doubleb,S. and Greenberg,M.M. (2017) The A-rule and deletion formation during abasic and oxidized abasic site bypass by DNA polymerase theta. ACS Chem. Biol., 12, 1584–1592.
22. Laverty,D.J. and Greenberg,M.M. (2017) In vitro bypass of thymidine glycol by DNA polymerase theta forms sequence-dependent frameshift mutations. Biochemistry, 56, 6726–6733.
23. Sale,J.E. (2013) Translesion DNA synthesis and mutagenesis in eukaryotes. Cold Spring Harb. Perspect. Biol., 5, a012708.
24. Conde,J., Yoon,J.H., Roy,C.J., Prakash,L. and Prakash,S. (2015) Genetic control of replication through N1-methyladenine in human cells. J. Biol. Chem., 290, 29794–29800.
25. Yoon,J.H., Hodge,R.P., Hackfeld,L.C., Park,J., Roy,C.J., Prakash,S. and Prakash,L. (2018) Genetic control of prominently error-free replication through an acrolein-derived minor-groove DNA adduct. J. Biol. Chem., 293, 2949–2958.
26. Yoon,J.H., Johnson,R.E., Prakash,L. and Prakash,S. (2019) DNA polymerase theta accomplishes translesion synthesis opposite 1,N(6)-ethenodeoxyadenosine with a remarkably high fidelity in human cells. Genes Dev., 33, 282–287.
27. Yoon,J.H., Roy,C.J., Park,J., Prakash,S. and Prakash,L. (2014) A role for DNA polymerase theta in promoting replication through oxidative DNA lesion, thymine glycol, in human cells. J. Biol. Chem., 289, 13177–13185.
28. Yoon,J.H., McArthur,M.J., Park,J., Basu,D., Wakamiya,M., Prakash,L. and Prakash,S. (2019) Error-prone replication through UV lesions by DNA polymerase theta protects against skin cancers. Cell, 176, 1295–1309.
29. Kohzaki,M., Nishihara,K., Hirota,K., Soneda,E., Yoshimura,M., Ekino,S., Butler,J.E., Watanabe,M., Halazonetis,T.D. and Takeda,S. (2010) DNA polymerases and theta are required for efficient
immunoglobulin V gene diversification in chicken. J. Cell Biol., 189, 1117–1127.

30. Zahn,K.E., Jensen,R.B., Wood,R.D. and Doublet,S.C. (2021) Human DNA polymerase theta harbors DNA end-trimming activity critical for DNA repair. Mol. Cell, 81, 1534–1547.

31. Pleceniakova,A., Slaninova,M. and Riha,K. (2014) Characterization of DNA repair deficient strains of Chlamydomonas reinhardtii generated by insertional mutagenesis. PLoS One, 9, e105482.

32. Kamisugi,Y., Whitaker,J.W. and Cuming,A.C. (2016) The transcriptional response to DNA-Double-Strand breaks in Physcomitrella patens. PLoS One, 11, e0161204.

33. Inagaki,S., Suzuki,T., Ohto,M.A., Urawa,H., Horiiuchii,T., Nakamura,K. and Morikami,A. (2006) Arabidopsis TEBICHI, with helicase and DNA polymerase domains, is required for regulated cell division and differentiation in meristems. Plant Cell, 18, 879–892.

34. Inagaki,S., Nakamura,K. and Morikami,A. (2009) A link among DNA replication, recombination, and gene expression revealed by genetic and genomic analysis of TEBICHI gene of Arabidopsis thaliana. PLoS Genet., 5, e1000613.

35. van Kregten,M., de Pater,S., Romeijn,R., van Schendel,R., Hooykaas,P.J. and Tijsterman,M. (2016) T-DNA integration in plants results from polymerase-theta-mediated DNA repair. Nat Plants, 2, 16164.

36. Nishizawa-Yokoi,A., Saika,H., Hara,N., Lee,L.Y., Toki,S. and Nakamura,K. (2009) Peroxisomes are required for in vivo nitric oxide accumulation during salinity stress of Arabidopsis plants. J. Exp. Bot., 60, 2533–2546.

37. Mara,K., Charlot,F., Guyon-Debast,A., Schaefer,D.G., Gutierrez,L. and Bellini,C. (2012) A collection of INDEL markers for DNA repair. Nucleic Acids Res., 2022, Vol.50, No.12

38. Liu,C.H., Finke,A., Diaz,M., Rozhon,W., Poppenberger,B., Huang,C.H., Mirabelli,C.K., Jan,Y. and Crooke,S.T. (1981) Huang etal. (2015) A first-in-class polymerase theta inhibitor selectively targets homologous-recombination-deficient tumors. Nat. Cancer, 2, 598–610.

39. Ceccaldi,R., Liu,J.C., Amunugama,R., Hajdu,I., Primack,B., Petalcorn,M.I., O’Connor,K.W., Konstantinopulos,P.A., Elledge,S.J., Boulton,S.K. et al. (2015) Homologous-recombination-deficient tumours are dependent on Polhetheta-mediated repair. Nature, 518, 258–262.

40. Mateos-Gomez,P.A., Gong,F., Nair,N., Miller,K.M., Lazzner-Denchi,E. and Sfeir,A. (2015) Mammalian polymerase theta promotes alternative NHEJ and suppresses recombination. Nature, 518, 254–257.

41. Schimmel,J., van Schendel,R., den Dunnen,J.T. and Tijsterman,M. (2019) Templated insertions are associated specifically with BRCA2 deficiency and overall survival in advanced ovarian cancer. Mol. Cancer Res., 17, 1012.

42. Ramsden,D.A., Carvajal-Garcia,J. and Gupta,G.P. (2022) Mechanism, cellular functions and cancer roles of polymerase-theta-mediated DNA end joining. Nat. Rev. Mol. Cell Biol., 23, 125–140.

43. Huang,C.H., Mirabelli,C.K., Jan,Y. and Cooke,S.T. (1981) Single-strand and double-strand deoxyribonucleic acid breaks produced by several bleomycin analogues. Biochemistry, 20, 233–238.

44. Liu,C.H., Finke,A., Diaz,M., Rozhon,W., Poppenberger,B., Baubec,T. and Pecinka,A. (2015) Repair of DNA damage induced by the cytidine analog 5-azacytosine depends on the Mre11-Rad50-Nbs1 complex in the maintenance of chromosomal stability in Arabidopsis. Plant Cell, 27, 1788–1800.

45. G.,C. (2016) 1,135 Genomes reveal the global pattern of polymorphism in Arabidopsis thaliana. Cell, 166, 481–491.

46. Bethke,P.C., Libourel,I.G., Reinhol, V. and Jones,R.L. (2006) Sodium nitroprusside, cyanide, nitrite, and nitrate break Arabidopsis seed dormancy in a nitric oxide-dependent manner. Planta, 223, 805–812.

47. Guo,F.Q., Okamoto,M. and Crawford,N.M. (2003) Identification of a plant nitric oxide synthase gene involved in hormonal signaling. Science, 302, 100–103.

48. Zhou,J., Gelot,C., Pantelidou,C., Li,A., Yueli,H., Davis,R.E., Farkkila,A., Kochupurakkal,B., Syed,A., Shapiro,G.I. et al. (2021) A first-in-class polymerase theta inhibitor selectively targets homologous-recombination-deficient tumors. Nat. Cancer, 2, 598–610.

49. Epe,B., Ballmaier,D., Roussyn,J., Briviba,K. and Sics,H. (1996) DNA damage by peroxynitrite characterized with DNA repair enzymes. Nucleic Acids Res., 24, 4105–4110.

50. Sakamoto,T., Inui,Y.T., Uraguchi,S., Yoshizumi,T., Matsunaga,S., Mstui,M., Umeda,M., Fukui,K. and Fujwara,T. (2011) Condensin II alleviates DNA damage and is essential for tolerance of boron overload stress in Arabidopsis. Plant Cell, 23, 3533–3546.

51. Friesner,J.D., Liu,B., Culligan,K. and Brit,B.A. (2005) Ionizing radiation-dependent gamma-H2AX focus formation requires ataxia telangiectasia mutated and ataxia telangiectasia mutated and Rad3-related. Mol. Biol. Cell, 16, 2566–2576.

52. Corpas,F.J., Hayashi,M., Mano,S., Nishimura,M. and Barroso,J.B. (2009) Peroxisomes are required for in vivo nitric oxide accumulation during salinity stress of Arabidopsis plants. Plant Physiol., 151, 2083–2094.

53. Acar,D.I., Acar,M.L., Street,N., Bussell,J.D., Pop,T.I., Gutierrez,L. and Bellini,C. (2012) A collection of INDEL markers for map-based cloning in seven Arabidopsis accessions. J. Exp. Bot., 63, 2491–2501.

54. Yoo,S.D., Cho,Y.H. and Shen,J. (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat. Protoc., 2, 1565–1572.

55. Schmittgen,T.D. and Livak,K.J. (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc., 3, 1101–1108.
69. Huie, R. E. and Padmaja, S. (1993) The reaction of NO with superoxide. Free Radic. Res. Commun., 18, 195–199.

70. Delledonne, M., Zeier, J., Marocco, A. and Lamb, C. (2001) Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. Proc. Natl. Acad. Sci. U.S.A., 98, 13454–13459.

71. Menges, M., de Jager, S. M., Gruissem, W. and Murray, J. A. (2005) Global analysis of the core cell cycle regulators of Arabidopsis identifies novel genes, reveals multiple and highly specific profiles of expression and provides a coherent model for plant cell cycle control. Plant J., 41, 546–566.

72. Yoshizumi, T., Tsumoto, Y., Takiguchi, T., Nagata, N., Yamamoto, Y. Y., Kawashima, M., Ichikawa, T., Nakazawa, M., Yamamoto, N. and Matsui, M. (2006) Increased level of poly(ADP-ribose)1- a conserved repressor of CYCLIN A2 transcription, controls endoreduplication in Arabidopsis. Plant Cell, 18, 2452–2468.

73. Yoon, J. H., Roy, C. J., Park, J., Prakash, S. and Prakash, L. (2017) Translesion synthesis DNA polymerases promote error-free replication through the minor-groove DNA adduct 3-deaza-3-methyladenine. J. Biol. Chem., 292, 18682–18688.

74. Daigaku, Y., Davies, A. A. and Ulrich, H. D. (2010) Ubiquitin-dependent DNA damage bypass is separable from genome replication. Nature, 465, 951–955.

75. Karras, G. I. and Jentsch, S. (2010) The RAD6 DNA damage tolerance pathway operates uncoupled from the replication fork and is functional beyond S phase. Cell, 141, 255–267.

76. Fan, L., Bi, T., Wang, L. and Xiao, W. (2020) DNA-damage tolerance through PCNA ubiquitination and sumoylation. Biochem. J., 477, 2655–2677.

77. Burney, S., Caulfield, J. J., Niles, J. C., Wishnok, J. S. and Tannenbaum, S. R. (1999) The chemistry of DNA damage from nitric oxide and peroxynitrite. Mutat. Res., 424, 37–49.

78. Khan, F. H., Dervan, E., Bhattacharyya, D. D., McAuliffe, J. D., Miranda, K. M. and Glynn, S. A. (2020) The role of nitric oxide in cancer: master regulator or NO? Int. J. Mol. Sci., 21, 9393.

79. Lee, D. H. and Pfeifer, G. P. (2007) Mutagenesis induced by the nitric oxide donor sodium nitroprusside in mouse cells. Mutagenesis, 22, 63–67.

80. Bai, S., Li, M., Yao, T., Wang, H., Zhang, Y., Xiao, L., Wang, J., Zhang, Z., Hu, Y., Liu, W. et al. (2012) Nitric oxide represses root growth by DNA damage induced cell cycle arrest in Arabidopsis thaliana. Nitric Oxide, 26, 54–60.

81. Fulcher, N. and Sablowski, R. (2009) Hypersensitivity to DNA damage in plant stem cell niches. Proc. Natl. Acad. Sci. U.S.A., 106, 20984–20989.

82. Schrempf, A., Slysokova, J. and Loizou, J. J. (2021) Targeting the DNA repair enzyme polymerase theta in cancer therapy. Trends Cancer, 7, 98–111.

83. Wu, X., Takenaka, K., Sonoda, E., Hochegger, H., Kawanishi, S., Kawamoto, T., Takeda, S. and Yamazoe, M. (2006) Critical roles for polymerase zeta in cellular tolerance to nitric oxide-induced DNA damage. Cancer Res., 66, 748–754.

84. Nisa, M., Bergis, C., Pedroza-Garcia, J. A., Drouin-Wahbi, J., Mazubert, C., Bergouinouix, C., Benhamed, M. and Raynaud, C. (2021) The plant DNA polymerase theta is essential for the repair of replication-associated DNA damage. Plant J., 106, 1197–1207.

85. Prasad, R., Longley, M. J., Sharief, F. S., Hou, E. W., Copeland, W. C. and Wilson, S. H. (2009) Human DNA polymerase theta possesses 5’-dRP lyase activity and functions in single-nucleotide base excision repair in vitro. Nucleic Acids Res., 37, 1868–1877.

86. Yoshimura, M., Kohzaki, M., Nakamura, J., Asagoshi, K., Sonoda, E., Hou, E., Prasad, R., Wilson, S. H., Tano, K., Yasui, A. et al. (2006) Vertebrate POLQ and POLbeta cooperate in base excision repair of oxidative DNA damage. Mol. Cell, 24, 115–125.

87. Roldan-Arjona, T., Ariza, R. R. and Cordoba-Canero, D. (2019) DNA base excision repair in plants: an unfolding story with familiar and novel characters. Front. Plant Sci., 10, 1055.

88. Ohshima, H., Sawa, T. and Akaike, T. (2006) 8-nitroguanine, a product of nitrosative DNA damage caused by reactive nitrogen species: formation, occurrence, and implications in inflammation and carcinogenesis. Antioxid. Redox. Signal., 8, 1033–1045.

89. Tretjakova, N. Y., Burney, S., Pamir, B., Wishnok, J. S., Dedon, P. C., Wogan, G. N. and Tannenbaum, S. R. (2000) Peroxynitrite-induced DNA damage in the supF gene: correlation with the mutational spectrum. Mutat. Res., 447, 287–303.

90. Tretjakova, N. Y., Wishnok, J. S. and Tannenbaum, S. R. (2000) Peroxynitrite-induced secondary oxidative lesions at guanine nucleobases: chemical stability and recognition by the Fpg DNA repair enzyme. Chem. Res. Toxicol., 13, 658–664.

91. Cordoba-Canero, D., Roldan-Arjona, T. and Ariza, R. R. (2014) Arabidopsis ZDP DNA 3’-phosphatase and ARP endonuclease function in 8-oxoG repair initiated by FPG and OGG1 DNA glycosylases. Plant J., 79, 824–834.

92. Routledge, M. N. (2000) Mutations induced by reactive nitrogen oxide species in the supF forward mutation assay. Mutat. Res., 450, 95–105.

93. Jiang, C., Mithani, A., Bellfield, E. J., Mott, R., Hurst, L. D. and Harberd, N. P. (2014) Environmentally responsive genome-wide accumulation of de novo Arabidopsis thaliana mutations and epimutations. Genome Res., 24, 1821–1829.

94. Goyal, V., Jhanghel, D. and Mehrotra, S. (2021) Emerging warriors against salinity in plants: nitric oxide and hydrogen sulphide. Physiol. Plant., 171, 896–908.

95. Iqbal, N., Umar, S., Khan, N. A. and Corpus, F. J. (2021) Crosstalk between abscisic acid and nitric oxide under heat stress: exploring new vantage points. Plant Cell Rep., 40, 1429–1450.

96. Chen, T., Chen, X., Zhang, S., Zhu, J., Tang, B., Wang, A., Dong, L., Zhang, Z., Yu, C., Sun, Y. et al. (2021) The genome sequence archive family: toward explosive data growth and diverse data types. Genomics Proteomics Bioinformatics, 19, 578–583.

97. CNCB-NGDC Members and Partners (2021) Database resources of the national genomics data center, china national center for bioinformation in 2021. Nucleic Acids Res., 49, D18–D28.