Arabidopsis Uracil DNA Glycosylase (UNG) Is Required for Base Excision Repair of Uracil and Increases Plant Sensitivity to 5-Fluorouracil

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Uracil in DNA arises by misincorporation of dUMP during replication and by hydrolytic deamination of cytosine. This common lesion is actively removed through a base excision repair (BER) pathway initiated by a uracil DNA glycosylase (UDG) activity that excises the damage as a free base. UDGs are classified into different families differentially distributed across bacteria, archaea, yeast, and animals, but remain to be ambiguously identified in plants. We report here the molecular characterization of AtUNG (Arabidopsis thaliana uracil DNA glycosylase), a plant member of the Family-1 of UDGs typified by Escherichia coli Ung. AtUNG exhibits the narrow substrate specificity and single-stranded DNA preference that are characteristic of Ung homologues. Cell extracts from atung−/− mutants are devoid of UDG activity, and lack the capacity to initiate BER on uracil residues. AtUNG-deficient plants do not display any apparent phenotype, but show increased resistance to 5-fluorouracil (5-FU), a cytostatic drug that favors dUMP misincorporation into DNA. The resistance of atung−/− mutants to 5-FU is accompanied by the accumulation of uracil residues in DNA. These results suggest that AtUNG excises uracil in vivo but generates toxic AP sites when processing abundant U:A pairs in dTTP-depleted cells. Altogether, our findings point to AtUNG as the major UDG activity in Arabidopsis.

Uracil arises frequently in DNA, both by misincorporation of dUMP instead of dTMP during replication and by spontaneous deamination of cytosine (1). Misincorporated uracil results in U:A pairs that are non-mutagenic but may be cytotoxic (2), whereas deaminated cytosines generate U:G mispairs that cause C:G to T:A transition mutations if not repaired prior to replication (3). In vertebrates, uracil is also a mutagenic intermediate generated by enzymatic cytosine deamination during production of antibody diversity at the Ig loci (4). Cells minimize dUTP misincorporation by maintaining a low dUTP/dTTP ratio through the enzyme deoxyuridin triphosphate: nucleotidohydrolase (dUTPase), which catalyzes the hydrolysis of dUTP to dUMP (5). Uracil arising in DNA either from misincorporation of dUMP or from deamination of cytosine is actively removed through the multistep base excision repair (BER)3 pathway (6). BER of uracil is initiated by a uracil DNA glycosylase (UDG) activity that cleaves the N-glycosidic bond and excises uracil as a free base, generating an abasic (apurinic/apyrimidinic, AP) site in the DNA. Repair is completed through subsequent steps that include incision at the AP site, gap tailoring, repair synthesis, and ligation (7, 8).

UDGs are distributed across a protein superfamily consisting of five families (6, 9). The first UDG identified was the Ung enzyme from Escherichia coli (10), which typifies the ubiquitous Family-1 of UDGs. This highly conserved family is represented in most species analyzed, with some remarkable exceptions such as Drosophila melanogaster and Archaea (11). Family-1 enzymes, such as human UNG, recognize uracil in an extrahelical conformation and are active both on dsDNA and ssDNA. Family-2 enzymes, represented by E. coli Mug and human TDG, are mismatch specific, show ssDNA dependence, and contact the opposite G in the complementary strand. Family-3 UDGs are represented by SMUG1 from vertebrates, are active both on dsDNA and ssDNA, and have a broader specificity than family-1 enzymes. UDGs from families 4 and 5 have been identified in thermophilic bacteria and archaea. In addition to the five UDG families, at least three families of the HhH-GPD superfamily of DNA glycosylases (named after its hallmark helix-hairpin-helix and Gly/Pro-rich loop followed by a conserved aspartate) include members with UDG activity (6, 9).

In contrast to the wealth of information available for bacteria, yeast, and mammalian systems, our knowledge about uracil excision repair in plants remains very limited. UDG activity has been detected and partially purified from several plant species, including carrot, wheat, onion, and maize (12–16). However, the proteins responsible for uracil excision in plants have not been identified, and their roles in plant physiology remain unknown.

The abbreviations used are: BER, base excision repair; DTT, dithiothreitol; UDG, uracil DNA glycosylase; AtUNG, Arabidopsis thaliana uracil DNA glycosylase; 5-FU, 5-fluorouracil; Ugi, uracil-DNA glycosylase inhibitor.

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Uracil DNA Repair in Plants

In this work we aimed to identify and characterize the molecular basis of uracil excision repair in Arabidopsis through genetic and biochemical analysis. We report that AtUNG, a member of the Family-1 UDGs, is the major UDG activity in Arabidopsis cell extracts and is absolutely required for initiation of BER of uracil in vitro. We also found that Arabidopsis mutants lacking AtUNG activity are phenotypically normal but display increased resistance to a compound that increases dUMP incorporation into DNA.

EXPERIMENTAL PROCEDURES

Plant Material—Wild-type and mutant Arabidopsis plants (Col-0) were grown in pots in a growth room at 23 °C under long day conditions (16 h light, 8 h darkness). For in vitro germination and culture, seeds were surface sterilized and plated on 10-cm Petri dishes containing 25 ml of 0.44% (w/v) MS medium (Sigma) supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar, pH 5.8. Plates were transferred to the growth chamber and incubated under long day conditions at 23 °C.

An Arabidopsis mutant line (GK-440E07) harboring a T-DNA insertion in the AtUNG gene was obtained from the GABI-Kat collection (17). Plants homozygous and heterozygous for the T-DNA insertion were identified by PCR with primers AtUNG_F4, AtUNG_R3 and 08409 (supplemental Table S1).

Protein Expression and Purification—A cDNA containing the entire open reading frame of AtUNG was amplified by RT-PCR on total RNA using nested primers designed according to genome sequence information (see supplemental Table S1). The AtUNG cDNA was cloned into pGEM-T (Promega), sequenced, and subcloned into pET15b expression vector (Novagen) to add a polyhistidine (His6) tag at the N terminus of AtUNG protein.

Expression of recombinant AtUNG was carried out in E. coli BL21 (DE3) ung-151 cells (18). A fresh single transformant colony was inoculated into 10 ml of LB medium containing ampicillin (100 μg/ml), tetracycline (10 μg/ml), and chloramphenicol (34 μg/ml), and the culture was incubated at 37 °C overnight with shaking. A 2.5-ml aliquot of the overnight culture was inoculated into 250 ml of LB medium containing ampicillin (100 μg/ml), tetracycline (10 μg/ml), and chloramphenicol (34 μg/ml), and incubated at 37 °C, 200 rpm until the A600nm was 0.1. The culture was then placed at 23 °C, and incubation continued at 200 rpm. When A600 reached 0.7, expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside (IPTG) to 1 mM and incubating for 2 h. After induction, cells were collected by centrifugation at 13,000 × g for 30 min, and the pellet frozen at −80 °C. The stored pellet was thawed and resuspended in 3.5 ml of sonication buffer (SB: 50 mM Na2PO4, pH 7.0, 10% glycerol, 50 mM NaCl, 5 mM imidazole). Cells were disrupted by sonication and the lysate was clarified by centrifugation and filtration.

The supernatant was loaded onto a 1-ml HiTrap SP XL column (Amersham Biosciences) preequilibrated with SB buffer. The column was washed with 10 ml of SB and proteins were eluted with a 30-ml gradient of NaCl (50 mM to 1 M) in SB and collected in 0.5-ml fractions. An aliquot of each fraction was analyzed by SDS-PAGE, and those containing the overex-pressed protein were pooled and loaded onto a 1-ml Ni2+-Sepharose column (GE Healthcare) preequilibrated with SB. The column was washed with 10 ml of SB, and proteins were eluted with a 30-ml gradient of imidazole and NaCl (5 mM imidazole, 50 mM NaCl to 1 M imidazole, 500 mM NaCl) in SB and collected in 0.5-ml fractions. An aliquot of each fraction was analyzed by SDS-PAGE, and those containing a single band of the overexpressed protein were pooled. The protein was concentrated and buffer was exchanged to 20 mM Tris-HCl pH 8.0, 500 mM NaCl, using Centricon YM-10 (Millipore). Finally 50% glycerol was added, and protein preparation was divided into aliquots, and stored at −80 °C. All steps were carried out at 4 °C or on ice. Protein concentrations were determined by the Bradford assay. Denatured proteins were analyzed by SDS-PAGE (10%) using broad-range molecular weight standards (Bio-Rad). Final AtUNG concentration was 3.31 μg/ml, and the specific UDG activity was 1200 units/mg. One unit of UDG activity was defined as the amount of enzyme releasing 1 nmol of uracil per min at 30 °C.

Site-directed Mutagenesis—Site-directed mutagenesis of AtUNG was performed using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The D173N mutation was introduced into pET15b-ATUNG by using the oligonucleotides D173-F and D173-R (supplemental Table S1). The mutant sequence was confirmed by DNA sequencing, and the construct was used to transform E. coli strain BL21(DE3) ung-151. Mutant protein was overexpressed and purified as described above.

Plant Cell Extract Preparation—Seedlings extracts were prepared from snap-frozen 15-day old seedlings grown in Petri dishes as described above. All steps were performed at 0–4 °C. Frozen plant material was ground in a handle mortar with liquid N2, and the resulting powder was resuspended in 2–3 volumes (w/v) of homogenization buffer containing 25 mM HEPES-KOH pH 7.8, 100 mM KCl, 5 mM MgCl2, 250 mM sucrose, 10% glycerol, 1 mM DTT, and 1 μl ml−1 protease inhibitor mixture (Sigma). The homogenate was incubated for 30 min at 4 °C and centrifuged at 13,000 × g for 1 h. The supernatant was filtered through a 20-μm nylon mesh and dialyzed overnight against 25 mM HEPES-KOH pH 7.8, 100 mM KCl, 17% glycerol, and 2 mM DTT. Protein concentration was determined by the Bradford assay, and the extract was stored in small aliquots at −80 °C.

Reagents and Enzymes—E. coli Ung, and uracil DNA glycosylase inhibitor (Ugi) were obtained from New England BioLabs. HpaII was purchased from Roche.

DNA Substrates—Oligonucleotides used to prepare DNA substrates (see supplemental Table S2) were synthesized by Operon and purified by PAGE before use. Double-stranded DNA substrates were prepared by mixing a 5 μM solution of the upper strand oligonucleotide (labeled at the 5′-end with fluorescein, when indicated) with a 10-μM solution of the lower strand oligonucleotide (labeled at the 5′-end with Alexa Fluor 647, when indicated), heating to 95 °C for 5 min, and slowly cooling at room temperature. DNA containing a natural AP site opposite guanine was prepared by incubating a DNA duplex containing a U·G mispair, prepared as above, with E. coli Ung (0.5 units) at 30 °C for 5 min.
DNA Glycosylase Assay—Double-stranded oligodeoxynucleotides (40 nM) were incubated at the indicated temperatures and times in a reaction mixture containing 125 mM Hepes KOH, pH 7.8, 500 mM KCl, 10 mM DTT, 2 mM EDTA, 17% glycerol, and the indicated amount of purified protein or cell extract in a total volume of 50 μl. Reactions were stopped by adding 20 mM EDTA, 0.6% sodium dodecyl sulfate, and 0.5 mg/ml proteinase K, and the mixtures were incubated at 37 °C for 30 min. Samples were treated with 100 mM NaOH, heated at 95 °C for 10 min, and then neutralized by adding 30 mM Tris-HCl, pH 7.6. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol precipitated at 20 °C in the presence of 0.3 mM NaCl and 16 μg/ml glycerol. DNA was resuspended in 10 μl of SURE/Cut Buffer L containing 5 units of HpaII (Roche) and incubated at 37 °C for 1 h. Reactions were then stopped by adding 5 μl of 90% formamide and heated at 95 °C for 5 min. Reaction products were separated in a 12% denaturing polyacrylamide gel containing 7M urea. Alexa-labeled DNA was visualized in an FLA-5100 imager and analyzed using Multigauge software (Fujifilm).

Base Excision Repair Assay—Repair reactions (50 μl) contained 45 mM HEPES-KOH pH 7.8, 70 mM KCl, 5 mM MgCl2, 1 mM DTT, 0.4 mM EDTA, 2 mM ATP, 36 μg of bovine serum albumin, 1 mM NAD, 2% glycerol, 20 μM each deoxynucleotide (dCTP, dGTP, dATP, and dTTP, except when indicated), 2 mM ATP, 22 mM phosphocreatine, 0.25 ng of creatine phosphokinase, substrate DNA (40 nM), and 30 μg of cell extract. After incubation at 30 °C for 3 h, reactions were stopped by adding 20 mM EDTA, 0.6% SDS, and 0.5 mg ml−1 proteinase K, and the mixtures were incubated at 37 °C for 30 min. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol-precipitated at −20 °C in the presence of 0.3 mM NaCl and 16 μg/ml glycerol. DNA was resuspended in 5 μl of SuRE/Cut Buffer L containing 5 units of HpaII (Roche) and incubated at 37 °C for 1 h. Reactions were then stopped by adding 5 μl of 90% formamide and heating at 95 °C for 5 min. Reaction products were separated in a 12% denaturing polyacrylamide gel (20 × 20 cm or 40 × 20 cm) containing 7 mM urea. Alexa-labeled DNA was visualized in an FLA-5100 imager and analyzed using Multigauge software (Fujifilm).

Alkaline Agarose Gel Electrophoresis—Genomic DNA aliquots (20 μg) were incubated at 37 °C overnight in a reaction buffer containing 100 mM NaCl, 100 mM Tris-HCl, pH 7.5, 10 mM EDTA, 10 mM DTT, 1 mg/ml bovine serum albumin, and 10% glycerol (final volume 25 μl) either in the absence or the presence of 40 units of E. coli Ung protein (New England Bio-Labs). Prior to loading on a 0.6% alkaline agarose gel, samples were denatured for 2 h at room temperature by adding 5 μl of 100 mM NaOH, 4 mM EDTA, and 5 μl of denaturing gel-loading buffer (1M NaOH, 50% glycerol, and 0.05% bromocresol green). Electrophoresis was performed at 70 V for 3 h at 4 °C. Later, the gel was neutralized with Tris-HCl, pH 8, 0.5 mM during 30 min, stained with ethidium bromide at 1 mg/ml, and washed with water. Finally, fluorescence was measured with a Gel Doc EQ system (Bio-Rad), and images were analyzed using Quantity One software (Bio-Rad).

RESULTS

AtUNG Encodes a Family-1 Uracil DNA Glycosylase—The Arabidopsis genome contains two putative genes (At3g18630 and At2g10550) encoding polypeptides with sequence similarity to uracil DNA glycosylases. Based on the results described below, we have designated the gene At3g18630 as AtUNG. A
full-length cDNA containing the entire open reading frame of *AtUNG* was cloned. Analysis of the cDNA sequence revealed that the *AtUNG* gene contains 7 exons and encodes a protein of 330 amino acids (Fig. 1). This structure agrees with the gene-model prediction on the *Arabidopsis Information Resource* (TAIR) website and matches two full-length cDNA clones currently available. At2g10550, on the other hand, is located in a chromosomal region-rich in repetitive DNA sequences, and it is most likely an inactive paralog of *AtUNG* generated by a gene duplication event and subsequently disrupted by at least two transposon insertions (supplemental Fig. S1).

The *AtUNG* protein sequence shows the active site motifs A and B characteristic of the five UDG families (Fig. 1), but exhibits the highest degree of sequence similarity to members of the Family-1 of UDGs (supplemental Fig. S2). *AtUNG* conserves critical residues involved in substrate recognition and catalysis in Family-1 enzymes (1). Amino acids Tyr-175 and Asn-231 correspond to residues that contribute to selectivity against other pyrimidines, including thymine, in human UNG (19). The conserved Leu-299 is involved in nucleotide flipping by penetrating in the minor groove and expelling uracil (19), and Asp-173 acts as a general base that activates water for attack of the N-glycosyl bond (20).

We expressed a recombinant full-length *AtUNG* in *E. coli* ung− cells (Fig. 2A), and investigated whether the purified protein exhibited UDG activity on oligonucleotide incision assays (Fig. 2, B and C). As a substrate we used a 51-mer DNA duplex in which the 5′-end-labeled upper strand contained a single U residue mispaired with a G on the complementary strand (supplemental Table S2). We found that *AtUNG* efficiently excised U and generated an abasic site that was cleaved upon alkaline treatment of the reaction products (Fig. 2B). The enzymatic activity was higher at 30 °C compared with 25 or 20 °C (Fig. 2B), and 2 nM *AtUNG* efficiently processed 40 nM of DNA substrate after 10 min of incubation (Fig. 2C).

Although *AtUNG* was expressed in *E. coli* ung− cells, we wished to rule out the possibility that trace amounts of the Family-2 bacterial *Mug* UDG might be responsible for the activity detected. We therefore examined the effect of Ugi on the reaction. This small peptide is a specific inhibitor of Family-1 UDGs, but does not affect Family-2 bacterial *Mug* UDG might be responsible for the activity detected. We therefore examined the effect of Ugi on the reaction. This small peptide is a specific inhibitor of Family-1 UDGs, but does not affect Family-2 bacterial *Mug* UDG activity (21). Uracil excision by *AtUNG* was completely inhibited in the presence of Ugi (Fig. 3A), thus confirming that the activity detected is associated to a Family-1 UDG protein. To verify that the observed DNA glycosylase is intrinsic to *AtUNG*, we generated a mutant recombinant protein in which the conserved aspartic acid residue (Asp-173) in Motif A (Fig. 1) was changed to asparagine. The mutant D173N protein, purified by the same procedure as the wild-type protein described above, showed a greatly reduced but detectable DNA glycosylase activity. A 25-fold higher amount of D173N *AtUNG* generated ~16-fold less product than the wild-type protein (Fig. 3B). This low level of residual activity is similar to that found in the analogous D145N *E. coli* Ung mutant protein (22). Altogether, these results indicate that *AtUNG* encodes a Family-1 uracil DNA glycosylase.

**Substrate Specificity of *AtUNG***—It has been described that the activity of UDGs may be influenced both by the bases flanking uracil and by the base opposite the lesion (23, 24). We therefore evaluated the effect of the opposite base and adjacent sequence on uracil excision catalyzed by *AtUNG*. The enzyme activity was compared using various DNA duplexes containing a single U residue opposite to each of the four common bases (G, A, T, or C) in two different sequence contexts (Fig. 4A). The pattern of *AtUNG* activity in a G:C-rich region was similar to that observed in a context containing A:T base pairs. In both sequence contexts, *AtUNG* was significantly more active on U:G mispairs than on U:A pairs. The activity on U:T and U:C mispairs was slightly lower than on U:G, but higher than on U:A pairs (Fig. 4A). These results suggest that excision by *AtUNG* is usually facilitated when the U residue is mismatched.
*E. coli* Ung and human UNG prefer single-stranded DNA as substrate, but also remove U from double-stranded DNA (1). We compared the activity of AtUNG on both types of DNA substrates (Fig. 4B) and found that in the two different sequence contexts examined the protein excised U residues with a slightly higher efficiency in single-stranded DNA compared with double-stranded DNA.

UDGs from Family-1 appear to be rather selective, and display narrow substrate specificity (6). We examined the target base preference of AtUNG on 51-mer duplex substrates that contained different 5-substituted uracil and cytosine derivatives opposite G (Fig. 4, C and D), using *E. coli* Ung as a reference. We found that AtUNG only exhibited detectable activity on U, whereas *E. coli* Ung efficiently excised both U and 5-FU.

**AtUNG Is the Major UDG Activity Detected in Arabidopsis Cell Extracts**—We next investigated whether a UDG activity similar to that of AtUNG could be detected in plant tissues. For this purpose, we performed oligonucleotide incision assays in cell-free extracts derived from Arabidopsis seedlings (25) (Fig. 5). We found that Arabidopsis

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**FIGURE 3. UDG activity is intrinsic to AtUNG.** A, effect of Ugi on AtUNG activity. A DNA duplex (40 nM) containing an U:G mispair was incubated with AtUNG (2 nM) or *E. coli* Ung (1 unit) during 30 min at 30 °C in the absence or the presence of the peptide inhibitor Ugi (2 units). Lane 1, control reaction without enzyme. Lane 6, 28-nt marker. B, effect of D173N mutation on AtUNG activity. A DNA duplex (40 nM) containing an U:G mispair was incubated with wild-type AtUNG (2 nM) or with mutant AtUNG-D173N (2 or 50 nM) during 30 min at 30 °C in the absence or the presence of the peptide inhibitor Ugi (2 units), as indicated. Reaction products were separated in a 12% denaturing polyacrylamide gel, and detected by fluorescence scanning. Lane 1, 28-nt marker.

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**FIGURE 4. Substrate specificity of AtUNG.** A, effect of opposite base and sequence context on UDG activity of AtUNG. AtUNG (1 nM) was incubated during 5 min (white bars) or 60 min (gray bars) at 30 °C with DNA duplexes (40 nM) containing U opposite each of the four common bases (G, A, T, or C) either in a TCUGG or a TAUTA context, as indicated. Data are the mean with S.E. for the percentage of processed substrate in two independent experiments. B, uracil glycosylase activity of AtUNG on single- and double-stranded DNA. Increasing amounts of AtUNG were incubated for 30 min at 30 °C with single-stranded (black lines) or double-stranded (gray lines) DNA substrates (40 nM) containing U either in a TCUGG (left panel) or a TAUTA (right panel) sequence context. Data are the mean with S.E. for the percentage of processed substrate in two independent experiments. C, chemical structures of substrate DNA bases tested in this study. D, AtUNG activity on DNA substrates containing different modified bases. Purified AtUNG (1 nM, lanes 2–8) or *E. coli* Ung (1 unit, lanes 9–15) was incubated at 30 °C during 30 min with 51-mer double-stranded substrates (40 nM) containing at position 29 of the labeled upper-strand different target DNA bases paired with guanine. Reaction products were separated in a 12% denaturing polyacrylamide gel and detected by fluorescence scanning. Lane 1, control reaction without enzyme, using DNA duplex (40 nM) containing a U:G mispair. Lane 16, 28-nt marker.
extracts contain an enzymatic activity that excises U residues from a DNA substrate containing a single U residue opposite G. The AP sites formed after U excision are processed by AP lyases in the absence of Mg\(^{2+}\) (25).

We next set up to determine whether the UDG activity detected in the *Arabidopsis* cell extracts is encoded by the *AtUNG* gene. We searched for mutants in public T-DNA insertion line collections and found one *Arabidopsis* mutant line with an insertion in the *AtUNG* coding sequence (GK-440E07). The T-DNA is inserted in exon 6 (Fig. 5E), and the mutant *AtUNG* transcript would presumably encode a truncated protein lacking sequence motif B, which is conserved in all members of the Family-1 of UDGs (6, 9). Plants homozygous for the T-DNA insertion were identified by PCR. Neither the heterozygous nor the homozygous mutant plants showed any obvious phenotypic alterations under the standard growth conditions.

We next examined whether the insertional disruption of the *AtUNG* gene exerted any effect on the UDG activity detected in *Arabidopsis* cell extracts. An oligonucleotide incision assay was performed by incubating cell extracts prepared from wild-type plants or *atung\(^{-/-}\)* mutants with a DNA duplex containing either a single U residue (Fig. 5F) or an AP site (supplemental Fig. S3) opposite G. Extracts prepared from wild type and *atung\(^{-/-}\)* mutants exhibited a similar incision activity on the DNA substrate containing an AP site (supplemental Fig. S3). However, excision of U was undetectable with AtUNG-deficient cell extracts (Fig. 5F). We also found that extracts from *atung\(^{-/-}\)* plants did not excise any of the cytosine or uracil derivatives showed in Fig. 4C (data not shown). Therefore, we conclude that UDG activity detected in wild-type *Arabidopsis* cell extracts is encoded by the *AtUNG* gene.

We also analyzed the influence of the opposite base on the efficiency of U excision in two different sequence contexts (Fig. 5B). We found that, in both contexts, the plant UDG activity showed a marked preference for mismatched uracil residues compared with U:A pairs. To examine the substrate specificity of the UDG activity detected in plants, cell extracts were incubated with duplex oligo substrates that contained different 5-substituted uracil and cytosine derivatives (Fig. 5C). We found that U was the only base modification processed by the cell extracts. We therefore conclude that the substrate specificity of the UDG activity detected in *Arabidopsis* cell extracts resembles that of AtUNG.
We performed the BER assay by incubating cell extracts prepared from wild type or atung−/− plants with DNA substrates containing either a single U residue or a synthetic AP site analogue (tetrahydrofuran, THF) opposite G (Fig. 6B). Consistent with previously reported observations (25), wild-type Arabidopsis extracts are capable of performing full BER of the DNA containing U:G or THF:G, in the presence of either dCTP or of all four dNTPs (Fig. 6B). We found that extracts from atung−/− plants repaired the AP-site containing DNA with comparable efficiency to wild-type plants. Although atung−/− extracts displayed a lower repair with all four dNTPs compared with wild-type plants, this difference was not consistently observed in other experiments (data not shown). However, the mutant extracts lacked any detectable BER activity on the uracil-containing DNA (Fig. 6B). These results indicate that the repair deficiency in mutant extracts specifically affects uracil excision, and that atung−/− plants retain the capacity to process the AP site intermediate generated after base removal. We therefore conclude that BER of uracil in Arabidopsis cell extracts is entirely dependent on the UDG activity of AtUNG.

Arabidopsis atung−/− Plants Are Resistant to 5-Fluorouracil—The results above suggest that AtUNG is the major UDG responsible for processing uracil residues in Arabidopsis, at least in vitro. We next explored the consequences exerted by elevated uracil levels on Arabidopsis plants lacking AtUNG activity. We decided to alter the dUTP/dTTP ratio by growing plants in the presence of 5-FU, an uracil analog widely used as a chemotherapeutic agent in the treatment of solid tumors (26). This anticancer agent inhibits thymidylate synthase and depletes thymidine nucleotides for DNA synthesis. As a result, it leads to a dUTP/dTTP imbalance that in turn favors misincorporation of dUMP into DNA (26). Wild-type and atung−/− plants were germinated in plates containing different concentrations of 5-FU (Fig. 7A). We found that after 17 days, growth on medium supplemented with 100 μM 5-FU, wild-type plants showed a significant developmental retardation, and that higher doses severely arrested plant growth. Mutant atung−/− plants were as affected as wild-type plants when grown at the higher dose tested (400 μM), but they showed a significantly reduced sensitivity to intermediate doses of 5-FU (100–300 μM) compared with wild-type plants. We conclude that a deficiency in AtUNG increases Arabidopsis resistance to 5-FU cytotoxicity.

We next tested whether a deficiency in AtUNG exerts a significant impact on the steady levels of uracil in the genome of untreated and 5-FU-treated plants. Purified DNA from wild-type or atung−/− plants germinated either in the absence or the presence of 100 μM 5-FU was treated with an excess of E. coli Ung and incubated under alkaline conditions to break the resulting AP sites. The level of single-strand breaks was then analyzed through alkaline gel electrophoresis (Fig. 7, B and C). Although the sensitivity of this assay is limited, it has been successfully used to detect accumulation of uracil and other types of DNA damage (5, 27). We found a significant increase in the amount of lower molecular weight products in 5-FU-treated atung−/− mutants, but not in 5-FU-treated wild-type plants (Fig. 7B, lanes 6 and 10). Thus, 5-FU causes an increase in the steady-state levels of uracil in mutant, but not wild-type, plants.

**BER of Uracil in Arabidopsis Cell Extracts Is Entirely Dependent on the UDG Activity of AtUNG—**We also aimed to determine to what extent BER of uracil in Arabidopsis, cells is dependent on the UDG activity of AtUNG. We have recently developed an in vitro assay to monitor BER of damaged bases in Arabidopsis (25). In this assay, plant cell extracts are incubated with a DNA duplex that contains a single base modification within an HpaI recognition site on the upper strand and is labeled at the 5′-end of the lower strand (Fig. 6A). Repair is detected as conversion of the HpaI-resistant DNA substrate to a form susceptible to digestion, and fully repaired products are visualized by the emergence of a 21-nt labeled fragment following polyacrylamide gel electrophoresis (25).
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These results strongly suggest that AtUNG functions in vivo to remove uracil residues from the plant genome. On the other hand, the genomic DNA from untreated wild-type or mutant atung<sup>−/−</sup> plants did not exhibit significant differences in their migration profiles (Fig. 7B, lanes 4 and 8). These results indicate that a deficiency in AtUNG does not cause a detectable change in the steady-state level of uracil in DNA, at least within the detection limits of the experimental approach used here.

DISCUSSION

We report here the isolation and functional characterization of AtUNG, a plant DNA repair enzyme belonging to the Family-1 of UDGs. AtUNG displays a high degree of sequence similarity to E. coli Ung and its homologues. As other eukaryotic UDGs, AtUNG is longer on its N terminus than its prokaryotic counterparts. Extra N- and/or C-terminal regions are common in the eukaryotic homologues of many DNA repair proteins, and they are frequently involved in interactions with other proteins, as well as in targeting to subcellular compartments (28, 29).

In human cells, alternative transcription initiation and splicing generate different N-terminal sequences that mediate targeting of the nuclear (UNG2) and mitochondrial (UNG1) isoforms of the protein (30). Because we have not found evidence of multiple AtUNG transcripts diverging in the 5′ region (data not shown), isoforms analogous to UNG1 or UNG2 may not exist in Arabidopsis. The N-terminal sequence of AtUNG is more similar to human UNG2 than to UNG1 and also contains a putative PCNA-binding motif (PKTLYSFF, present at amino acids 6–13 of the AtUNG polypeptide). This suggests that, similarly to the human nuclear isoform, AtUNG might have a role in rapid postreplicative removal of misincorporated dUMP at the replication fork in the nucleus (31).

However, the possibility that AtUNG is also targeted to mitochondria and/or chloroplasts cannot be ruled out. Protein localization studies will be needed to assess whether AtUNG is targeted to more than one subcellular compartment.

Like other members of the Family-1 of UDGs, AtUNG has a rather narrow substrate specificity. Out of seven different pyrimidine derivatives, we only observed detectable excision on uracil. In contrast, E. coli Ung excised both U and 5-FU, a property shared with human UNG (1). The lack of AtUNG activity on 5-FU, when compared with bacterial and human enzymes, suggests that the steric constraints that impose selectivity and specificity for uracil against other pyrimidines may be more rigid in the plant enzyme than in other homologs.

We have found evidence indicating that AtUNG encodes the UDG activity detected in Arabidopsis cell extracts. Thus, UDG activity from extracts displays the same narrow substrate specificity as purified AtUNG, is also completely inhibited by Ugi, and is not present in extracts obtained from atung<sup>−/−</sup> plants. In addition, we have found that atung<sup>−/−</sup> extracts retain full capacity to repair AP sites but lack the ability to initiate BER of U. Altogether, these results suggest that AtUNG may be the major UDG activity responsible for initiating BER of uracil residues in Arabidopsis. A survey of the Arabidopsis genome does not reveal any other identifiable member of some UDG family. However, we cannot rule out the possibility that additional plant proteins with UDG activity exist, and that they are not represented in our cell extracts and/or not detectable under our assay conditions.

Despite the absence of detectable UDG activity and the lack of BER of uracil residues, no phenotypic effects were evident in AtUNG-deficient mutant plants. A similar absence of pheno-

FIGURE 7. atung<sup>−/−</sup> mutants exhibit increased resistance to 5-FU with the concomitant accumulation of uracil in DNA. A, 17-day-old seedlings from Arabidopsis wild-type (left) or atung<sup>−/−</sup> plants (right) grown on plates with MS medium supplemented with increasing concentrations of 5-FU (scale bar: 10 mm). B, genomic DNA from wild-type or atung<sup>−/−</sup> plants germinated either in the absence or the presence of 100 μM 5-FU was treated with E. coli Ung, resolved by alkaline agarose gel electrophoresis and stained with ethidium bromide. Sizes in kb of molecular weight markers are shown on the left. C, estimation of the length of the DNA fragments shown in B by densitometry scanning. The intensity in lanes 3-10 is indicated as pink, orange, green, light blue, black, gray, dark blue, and red curves, respectively.

These results strongly suggest that AtUNG functions in vivo to remove uracil residues from the plant genome. On the other hand, the genomic DNA from untreated wild-type or mutant atung<sup>−/−</sup> plants did not exhibit significant differences in their migration profiles (Fig. 7B, lanes 4 and 8). These results indicate that a deficiency in AtUNG does not cause a detectable change in the steady-state level of uracil in DNA, at least within the detection limits of the experimental approach used here.

DISCUSSION

We report here the isolation and functional characterization of AtUNG, a plant DNA repair enzyme belonging to the Family-1 of UDGs. AtUNG displays a high degree of sequence similarity to E. coli Ung and its homologues. As other eukaryotic UDGs, AtUNG is longer on its N terminus than its prokaryotic counterparts. Extra N- and/or C-terminal regions are common in the eukaryotic homologues of many DNA repair proteins, and they are frequently involved in interactions with other proteins, as well as in targeting to subcellular compartments (28, 29).

In human cells, alternative transcription initiation and splicing generate different N-terminal sequences that mediate targeting of the nuclear (UNG2) and mitochondrial (UNG1) isoforms of the protein (30). Because we have not found evidence of multiple AtUNG transcripts diverging in the 5′ region (data not shown), isoforms analogous to UNG1 or UNG2 may not exist in Arabidopsis. The N-terminal sequence of AtUNG is more similar to human UNG2 than to UNG1 and also contains a putative PCNA-binding motif (PKTLYSFF, present at amino acids 6–13 of the AtUNG polypeptide). This suggests that, similarly to the human nuclear isoform, AtUNG might have a role in rapid postreplicative removal of misincorporated dUMP at the replication fork in the nucleus (31).

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We have found evidence indicating that AtUNG encodes the UDG activity detected in Arabidopsis cell extracts. Thus, UDG activity from extracts displays the same narrow substrate specificity as purified AtUNG, is also completely inhibited by Ugi, and is not present in extracts obtained from atung<sup>−/−</sup> plants. In addition, we have found that atung<sup>−/−</sup> extracts retain full capacity to repair AP sites but lack the ability to initiate BER of U. Altogether, these results suggest that AtUNG may be the major UDG activity responsible for initiating BER of uracil residues in Arabidopsis. A survey of the Arabidopsis genome does not reveal any other identifiable member of some UDG family. However, we cannot rule out the possibility that additional plant proteins with UDG activity exist, and that they are not represented in our cell extracts and/or not detectable under our assay conditions.

Despite the absence of detectable UDG activity and the lack of BER of uracil residues, no phenotypic effects were evident in AtUNG-deficient mutant plants. A similar absence of pheno-
typic alterations has been reported for Ung-null mutants from other multicellular organisms. For instance, mice with targeted disruption of the UNG gene are developmentally normal and fertile, and have no obvious phenotype (32). Development, fertility and lifespan are also unaffected in ung-1-null mutants from C. elegans (33). Cell extracts from Ung-deficient mice retain a significant UDG activity (32), later identified as SMUG1 (34). In contrast, no residual uracil excision is detected in C. elegans ung-1 mutants and there are no obvious candidates for any possible backup enzymes encoded in the worm genome (33).

We have found that atung−/− mutants are less sensitive to the cytotoxic effects of 5-FU than wild-type plants, but at the expense of uracil accumulation in their genome. These results strongly suggest that AtUNG excises uracil in vivo, but also indicate that this activity is detrimental for the plant when the dUTP/dTTP ratio is artificially increased by 5-FU treatment. It has been reported that deletion of the Ung gene protects yeast cells against 5-FU (35). These results support the hypothesis that futile cycles of uracil BER burdened by a depleted dTTP pool, with the ensuing accumulation of toxic DNA repair intermediates, may explain part of the cytotoxic effects of 5-FU, at least in plants and yeasts.

We did not detect increased levels of uracil in the genome of untreated atung−/− mutants. It has been reported that dividing cells from UNG-deficient mice and there are no increased levels of uracil (32). However, the amounts of accumulated uracil are low in tissues with low turnover rates, thus suggesting that spontaneous uracil residues in the genome originate predominantly from dUMP misincorporation during replication, rather than cytosine deamination (36). It is possible that uracil accumulation in untreated atung−/− plants is efficiently prevented by mechanisms that counteract dUMP misincorporation, and/or that the steady-state level reached is below the detection limit of the assay method used here.

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