Nonenzymatic release of N7-methylguanine channels repair of abasic sites into an AP endonuclease-independent pathway in Arabidopsis

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ABSTRACT

Abasic (apurinic/apyrimidinic, AP) sites in DNA arise from spontaneous base loss or by enzymatic removal during base excision repair. It is commonly accepted that both classes of AP site have analogous biochemical properties and are equivalent substrates for AP endonucleases and AP lyases, although the relative roles of these two types of enzymes are not well understood. We provide here genetic and biochemical evidence that, in Arabidopsis, AP sites generated by spontaneous loss of N7-methylguanine (N7-meG) are exclusively repaired through an AP endonuclease-independent pathway initiated by FPG, a bifunctional DNA glycosylase with AP lyase activity. Abasic site incision catalyzed by FPG generates a single-nucleotide gap with a 3′-phosphate terminus that is processed by the DNA 3′-phosphatase ZDP before repair is completed. We further show that the major AP endonuclease in Arabidopsis (ARP) incises AP sites generated by enzymatic N7-meG excision but, unexpectedly, not those resulting from spontaneous N7-meG loss. These findings, which reveal previously undetected differences between products of enzymatic and nonenzymatic base release, may shed light on the evolution and biological roles of AP endonucleases and AP lyases.

Significance

Abasic (apurinic/apyrimidinic, AP) sites in DNA result from spontaneous and repair-mediated base release. They may be processed by AP endonucleases or AP lyases, but the relative roles of both types of enzymes are poorly understood. Our study reveals that the model plant Arabidopsis uses an AP lyase-dependent pathway to repair AP sites generated by spontaneous loss of N7-methylguanine (N7-meG), a major lesion arising from DNA methylation damage. We further show that the main Arabidopsis AP endonuclease is active on AP sites generated by enzymatic excision of N7-meG, but not on those arising from N7-meG loss. Our findings identify an important role for AP lyase activity in plants and challenge the assumption that spontaneous and repair-generated AP sites have identical biochemical properties.

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A basic (apurinic/apyrimidinic, AP) sites are ubiquitous DNA lesions generated by spontaneous hydrolysis of the N-glycosyl bond connecting the base with the deoxyribose moiety of the nucleotide (1). It has been estimated that 2,000–10,000 AP sites arise spontaneously per mammalian cell per generation (2). Abasic sites are also generated as intermediates during the base excision repair (BER) pathway, following excision of damaged bases by monofunctional DNA glycosylases (3, 4). In addition, they may be induced directly by oxygen radical species (5) and indirectly by spontaneous release of alkylated bases such as N7-methylguanine (N7-meG) (5–7). Under physiological conditions, AP sites exist in an equilibrium mixture of α- and β-hemiacetals of the closed furanose form, with ~1% present in the opened aldehyde form (8). The latter is prone to spontaneous hydrolysis by β- and β,δ-elimination, generating single-strand breaks (SSB) (9). AP sites can block DNA replication and transcription and slowly decay to form SSB, therefore causing cytotoxic effects. They are also mutagenic due to erroneous bypass by translesion DNA synthesis (10).

Abasic sites are mainly repaired through BER initiated either by AP endonucleases or by AP lyase activities associated with bifunctional DNA glycosylases (4, 11, 12). AP endonucleases hydrolyze DNA at the 5′-side of the AP site, leaving 3′-hydroxyl (3′-OH) and 5′-deoxyribose phosphate (5′-dRP) termini (13). AP lyases cleave 3′ to the AP site by β-elimination, generating 3′-phosphor-α,β-unsaturated aldehyde (3′-PUA), and 5′-phosphate (5′-P) termini. A subset of AP lyases catalyze β,δ-elimination and generate 3′-phosphate (3′-P) termini (13). Therefore, AP endonucleases and AP lyases generate single-nucleotide gaps with 5′- and 3′-blocked ends, respectively. The processing of such noncanonical termini influences subsequent steps of the repair process, which may continue through insertion of either one (short-patch, SP-BER) or several (long-patch, LP-BER) nucleotides (14, 15).

In SP-BER, the 5′-dRP group generated by AP endonucleases is converted to 5′-P by a deoxyribonucleophilic activity that in mammalian cells is associated to DNA polymerase β (16). However, such step is rate-limiting (17) and the 5′-dRP may be also removed as part of an oligonucleotide excised by FEN1 nuclease after strand displacement during LP-BER (14). Since AP lyases produce canonical 5′-P termini, it has been proposed that they usually initiate SP-BER (18). In this case, processing of 3′-PUA generated by β-elimination is carried out by a 3′-phosphodiesterase activity associated to AP endonucleases (19, 20) and 3′-P produced by β,δ-elimination is removed by a DNA 3′-phosphatase, such a mammalian PKN (21) or plant ZDP (22, 23). Therefore, AP site incision by either AP endonucleases or AP lyases determines downstream BER steps requiring different subsets of proteins. However, the factors influencing the choice between both types of enzymes are unknown.

BER has been extensively studied in bacterial, yeast, and mammalian systems, but knowledge about this crucial repair pathway has been gained in plants only recently. Results obtained so far, mostly in Arabidopsis, indicate that plants share many BER components with other organisms but possess some distinctive features and combinations. In Arabidopsis, repair of uracil is initiated by the AP endonuclease ARP (see supplemental material for a complete list of abbreviations).


The authors declare no conflict of interest.

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monofunctional uracil DNA glycosylase UNG (24), and the ensuing AP sites may be processed by both AP lyase and AP endonuclease activities detectable in cell extracts (15, 25). Interestingly, and despite the lack of plant homologs of Pol β and LigIII, repair proceeds not only by LP-BER but also through SP-BER, and in both cases the final ligation step is catalyzed by LIGI (15, 25). Arabidopsis also possesses an MBD4-like protein active on U-G and T-G mismatches but, unlike its mammalian homolog, it lacks a methyl-CpG-binding domain (26). Removal of oxidized pyrimidines is carried out by an NTHI homolog (27), whereas repair of oxidized guanine (8-oxoG) involves both FPG and OGG1 homologs (22, 28–30), a distinctive combination of bacterial-like and eukaryotic-like 8-oxoG DNA glycosylases characteristic of plants and some fungi (31). Repair intermediates generated by the bifunctional DNA glycosylases FPG and OGG1 are processed by the DNA 3′-phosphatase ZDP and ARP, the major AP endonuclease detectable in Arabidopsis cell extracts (22). In addition to removing damaged bases, plants use BER for epigenetic reprogramming initiated by 5-methylC DNA glycosylases/lyases of the ROS1/DME family (32, 33). These enzymes generate single-nucleotide gaps with either 3′-P or 3′-P ends (32, 33) that are processed by the 3′-phosphodiesterase activity of AP endonuclease APE11 (20) and the DNA 3′-phosphatase ZDP (23), respectively. Although Arabidopsis possesses several DNA glycosylases/lyases (27–29, 32, 33) and three different AP endonucleases (34), their relative roles in the repair of AP sites have not been established so far.

It is generally accepted that AP site repair in vivo is predominantly initiated by AP endonucleases, but there is evidence that AP lyases play a prominent role in yeast. Thus, AP sites in Schizosaccharomyces pombe are primarily incised by the DNA glycosylase/AP lyase Nth1, which generates 3′-P and 3′-P ends that are further processed by the phosphodiesterase activity of Apn2, the major AP endonuclease in fission yeast (35, 36). Rather than AP incision, the main function of Apn2 appears to be the removal of 3′-blocked ends generated by the AP lyase activity of Nth1. A similar mechanism operates in Saccharomyces cerevisiae (37).

The biological relevance of AP lyases in the processing of abasic sites is poorly understood, and it has been suggested that it may be a yeast-specific feature (38). In this paper we report biochemical and genetic evidence that the AP lyase activity of Arabidopsis DNA glycosylase FPG plays a major role in the repair of AP sites generated by either modified DNA glycosylases/lyases (39) or by triplet AP endonuclease N7-meg (25). FPG functions upstream ZDP, enabling DNA polymerase and ligase activities to complete repair in an AP endonuclease-independent pathway. Importantly, we found that ARP, the major AP endonuclease in Arabidopsis, is active on AP sites generated by enzymatic excision of N7-meg but not on products of spontaneous N7-meg depurination. Our results indicate that hitherto unknown differences between the products of enzymatic and nonenzymatic base release dictate AP site DNA repair choice in Arabidopsis.

Results

Preparation and Characterization of Substrates to Monitor Repair of DNA Methylation Damage in Arabidopsis Cell-Free Extracts. ZDP is the major, if not the only, DNA 3′-phosphatase activity detected in Arabidopsis cells (23). We have previously reported that ZDP is required to process 3′-P termini generated by FPG during 8-oxoG repair (22) or by ROS1 during active DNA demethylation (23). Intriguingly, 3′-P−mutants are hypersensitive to methyl methanesulfonate (MMS) (23), suggesting that ZDP also plays an important role in the repair of alkylation DNA damage.

The most abundant lesion caused by MMS is N7-meg, which accounts for 80–85% of total DNA methylation (39). This lesion is neither cytotoxic nor mutagenic but under physiological conditions undergoes spontaneous depurination, exhibiting half-lives ranging from 69 to 192 h at neutral pH and 37 °C (7). In addition, N7-meg may suffer opening of its imidazole ring to yield 5-N-methyl-2,6-diamino-4-hydroxyformamidopyrimidine (me-FAPy-G), in a reaction favored by basic conditions (7).

To monitor repair of MMS-induced DNA damage in Arabidopsis cell extracts we prepared a DNA duplex containing a single N7-meg residue using an enzymatic method (40) (Fig. S1A). We also generated an analogous substrate with me-FAPy-G by incubating the N7-meg-containing DNA at 37 °C for 5 h at pH 11. N7-meg is known to be excised by the monofunctional human alkyladenine DNA glycosylase (hAAG) (41), whereas Escherichia coli Fpg excises me-FAPy-G very efficiently (42). As shown in Fig. S1B, the oligonucleotide containing N7-meg was completely cleaved upon incubation with hAAG and human AP endonuclease 1 (hAPE1), while no product was observed for the me-FAPy-G substrate. Conversely, the oligonucleotide containing me-FAPy-G was fully cleaved by E. coli Fpg but was resistant to treatment with hAAG and hAPE1. As expected, reaction products generated by hAPE1 and E. coli Fpg contained 3′-OH and 3′-P termini, respectively (Fig. S1B).

The DNA Phosphatase ZDP Functions Downstream of FPG During Repair of DNA Containing N7-meg. We next incubated cell extracts from WT and zdp−/− or fpg−/− mutant plants with DNA substrates containing either N7-meg or me-FAPy-G (Fig. 1A–C). No incision products were detected in reactions with the DNA substrate containing me-FAPy-G (Fig. 1A). However, when incubated with the duplex containing N7-meg in the absence of Mg2+, WT extracts generated a product with a 3′-P terminus that was converted into a 3′-OH end upon Mg2+ addition (Fig. 1B, lanes 2 and 6). Similar conversion was undetectable in zdp−/− mutant extracts (Fig. 1B, lanes 3 and 7, but was restored when purified recombinant ZDP protein was added to the repair reaction (Fig. 1C, lanes 3 and 4). These results indicate that ZDP phosphatase activity is required to process a 3′-P intermediate generated during the repair of DNA containing N7-meg.

We have previously shown that ZDP processes 3′-P termini generated by FPG during 8-oxoG repair (22). Therefore, we hypothesized that this DNA glycosylase/lyase might be also responsible for the generation of such intermediates during N7-meg repair. We found that cell extracts from mutant fpg−/− plants do not generate detectable repair incision products, either in the absence or the presence of Mg2+ (Fig. 1B, lanes 4 and 8). These results indicate that FPG functions in the repair of DNA containing N7-meg, performing a β,δ-elimination and generating a single-nucleotide gap with a 3′-P terminus that is converted to 3′-OH by the DNA 3′-phosphatase activity of ZDP.

Since ZDP is required to process FPG products, we tested for a direct interaction between both proteins using pull-down assays (Fig. 1D). We found His-FPG bound to MBP-ZDP, but not to MBP alone, immobilized in an amylose column (Fig. 1D, Upper). Conversely, MBP-ZDP, but not MBP alone, bound to His-FPG immobilized in a nickel-agarose column (Fig. 1D, Lower). These results suggest that FPG and ZDP directly interact in vitro.

The results described above suggest that FPG functions up-steam ZDP during repair of DNA containing N7-meg. We therefore hypothesized that the hypersensitivity of zdp−/− mutants to MMS could be due to the accumulation of unprocessed SSB intermediates containing 3′-P ends generated by FPG. To test this idea, we generated a double fpg−/− zdp−/− mutant and assessed its resistance to MMS in comparison with WT and single fpg−/− or zdp−/− mutants (Fig. 1E). The results show that inactivation of FPG activity in zdp−/− mutant plants restores MMS resistance to nearly WT levels. Altogether, these results indicate that FPG functions upstream ZDP during repair of DNA containing N7-meg. We also found that, while single fpg−/− mutants are not sensitive to MMS, the combined deficiency of FPG and ARP, the major Arabidopsis AP endonuclease (25), causes an MMS sensitivity similar to that of zdp−/− mutants. These results suggest that ARP also plays a role in the repair of MMS-induced DNA damage.
FPG Incises AP Sites Generated by Spontaneous Depurination of N7-meG. We next examined in detail the role of FPG during repair of DNA containing N7-meG. As indicated above, N7-meG may undergo either spontaneous depurination to generate an AP site or imidazole-ring opening to yield me-FAPy-G. We therefore tested the activity of Arabidopsis FPG protein against N7-meG and its two derivatives (Fig. 2). Control reactions confirmed that N7-meG was only incised by the simultaneous addition of hAAG and hAPE1, me-FAPy-G was processed by E. coli Fpg, and the AP site was incised by hAPE1 (Fig. 2A, lanes 5, 8, and 11, respectively). We found that Arabidopsis FPG did not display detectable incision activity against either N7-meG or me-FAPy-G (Fig. 2A, lanes 4 and 7). However, it efficiently incised the AP site, generating as a product a DNA repair intermediate with a 3'-P terminus (Fig. 2A, lane 10). The robust AP lyase activity of Arabidopsis FPG has been previously reported (31). It has been described that a truncated form of Arabidopsis FPG processes me-FAPy-G with low efficiency (43), but we could not detect such activity, either with the full-length enzyme or with cell extracts, at least under our experimental conditions.

We therefore hypothesized that, during DNA repair reactions with cell extracts, spontaneous depurination of N7-meG generates AP sites that are substrates for FPG. To test this idea, we preincubated a DNA duplex with a single N7-meG residue during different time periods in the absence of cell extract and then submitted DNA either to an alkali treatment at 70 °C or to
incubation with hAPE1, *E. coli* Fpg, or *Arabidopsis* FPG. We found that alkali/heat-labile sites sensitive to all three enzymes accumulated in DNA at position 28 (Fig. 2B). The observed accumulation rate is compatible with that reported for depurination of N7-meG in dsDNA at neutral pH and 37 °C (7). As expected for AP sites, incision by hAPE1 generated 3′-OH termini whereas incision by *Arabidopsis* FPG or heat/alkali treatment generated 3′-P termini (Fig. 2B). Altogether, these results suggest that FPG enzyme present in *Arabidopsis* cell extracts efficiently processes AP sites generated by spontaneous depurination of N7-meG, but it is not active either on N7-meG itself or its me-FAPy-G derivative.

**ARP, the Major Arabidopsis AP Endonuclease, Plays a Negligible Role in the Repair of Depurinated N7-meG.** We next examined the relative roles of FPG and ARP in the repair of AP sites generated by spontaneous depurination of N7-meG. We first analyzed the level of AP endonuclease and AP lyase activity in cell extracts from WT, fpg<sup>−/−</sup>, arp<sup>−/−</sup>, and double fpg<sup>−/−</sup> arp<sup>−/−</sup> mutant plants. Equivalent cell extract quality and DNA repair competence were previously verified by measuring UDG activity on a DNA duplex containing a U:C mismatch (Fig. S2). We then incubated cell extracts with a DNA substrate containing an AP site opposite C generated by uracil excision (Fig. 3, Left). To mimic the partial depurination of DNA containing N7-meG (discussed below), heteroduplex DNA with the enzymatically generated AP:C was mixed with homoduplex G:C at a 1:9 ratio before initiating repair reactions. We found that arp<sup>−/−</sup> extracts catalyzed AP incision with efficiency similar to WT extracts, either in the absence or the presence of Mg<sup>2+</sup> (Fig. 3, lanes 2 and 3 and 7 and 8). This result indicates that arp<sup>−/−</sup> extracts only exhibit AP lyase activity, which is Mg<sup>2+</sup>-independent. However, fpg<sup>−/−</sup> extracts also catalyzed AP incision at levels comparable to those of WT extracts, but only in the presence of Mg<sup>2+</sup> (Fig. 3, lanes 4 and 9). This result indicates that they only exhibit AP endonuclease activity, which is Mg<sup>2+</sup>-dependent. No AP incision activity was detected in fpg<sup>−/−</sup> arp<sup>−/−</sup> mutant extracts, although limited spontaneous hydrolysis was detected in the form of β-elimination products (Fig. 3, lanes 5 and 10). These results indicate that the only AP lyase and AP endonuclease activities detectable in *Arabidopsis* cell extracts under the experimental conditions used are FPG and ARP, respectively.

We next performed analogous repair reactions with a DNA substrate containing depurinated N7-meG (Fig. 3, Right). Depurination was achieved by preincubating a DNA duplex containing N7-meG in the absence of cell extracts for 16 h (Fig. 2B and Methods). As previously observed (Fig. 1B), we found that in the absence of Mg<sup>2+</sup>, WT extracts generated a product with a 3′-P terminus that was converted into a 3′-OH end upon Mg<sup>2+</sup> addition (Fig. 3, lanes 14 and 19), whereas fpg<sup>−/−</sup> extracts did not catalyze any incision, either in the absence or the presence of Mg<sup>2+</sup> (Fig. 3, lanes 16 and 21). In contrast, arp<sup>−/−</sup> mutant extracts displayed an incision pattern very similar to that of WT plants (Fig. 3, lanes 15 and 20). Extracts from double fpg<sup>−/−</sup> arp<sup>−/−</sup> mutant plants did not display any detectable incision activity (Fig. 3, lanes 17 and 22). Altogether, these results indicate that incision of depurinated N7-meG in *Arabidopsis* cell extracts is ARP-independent and it is carried out exclusively by FPG.

**Depurinated N7-meG Is Repaired Through FPG-Dependent SP-BER.** It has been previously suggested that BER initiated by monofunctional DNA glycosylases continues via both SP- and LP-BER, whereas that initiated by bifunctional DNA glycosylases/lyases
continues primarily via SP-BER (18). Since repair of depurinated N7-meG is AP lyase-dependent and AP endonuclease-independent we hypothesized that it should mainly involve SP-BER. To test this idea, we performed gap-filling DNA repair reactions either in the presence of dGTP or all four dNTPs (Fig. 4).

When gap-filling DNA repair reactions were performed with DNA containing an AP site generated by uracil excision, WT, arp-/-, and fpg-/- extracts catalyzed the insertion of up to three deoxynucleotides when all four dNTPs were present in the repair reaction (Fig. 4, lanes 6, 9, and 12), suggesting the operation of an LP-BER. As expected, no DNA repair intermediates were detected with double mutant fpg-/- larp-/- extracts (Fig. 4, lanes 13–15). Since only FPG and ARP activities are detectable in arpa/- and fpg-/- extracts, respectively (Fig. 3), these results suggest that the nature of the enzyme performing AP incision is not the only factor influencing the choice between SP- and LP-BER.

When reactions catalyzed by WT extracts were performed with DNA containing depurinated N7-meG the insertion of just one
deoxynucleotide was detected, regardless of the presence of only dGTP or all four dNTPs in the repair reaction (Fig. 4, lanes 20 and 21). The same insertion pattern was observed with *arp*−/− mutant cell extracts, which only exhibit FPG incision activity (Fig. 4, lanes 23 and 24). As expected, no DNA repair intermediates were detected with either single mutant *fpg*−/− or double mutant *fpg*−/−*arp*−/− extracts (Fig. 4, lanes 25–30). These results suggest that depurinated N7-meG is repaired through FPG-dependent SP-BER.

**ARP Discriminates Between AP Sites Generated by Enzymatic and Nonenzymatic Release of N7-meG.** The results described above suggested that nonenzymatic hydrolysis of N7-meG channels repair into an AP endonuclease-independent pathway. To test this idea, we compared the incision activity of recombinant and native FPG and ARP on DNA substrates containing AP sites generated either by enzymatic or nonenzymatic release of N7-meG (Fig. 5). We found that recombinant FPG incised both types of DNA substrates with similar efficiency (Fig. 5A). Native FPG enzyme present in *arp*−/− mutant extracts, which, as previously shown (Fig. 3) only display FPG-dependent AP incision activity, was also active on both types of AP sites (Fig. 5B). When tested on the same DNA substrates recombinant ARP-incised AP sites generated by enzymatic release of N7-meG but, unexpectedly, did not display detectable activity on AP sites generated by spontaneous depurination of N7-meG (Fig. 5C). Mutant *fpg*−/− extracts, which only show ARP-dependent AP incision activity (Fig. 3), were also active on enzymatic AP sites but lacked activity on nonenzymatic AP sites (Fig. 5D). Importantly, recombinant hAPE1 did not exhibit such differential activity (Fig. S3). Altogether, these results indicate that ARP discriminates between AP sites generated by enzymatic and nonenzymatic release of N7-meG.

**The Identity of the Base Opposite an Enzymatically Generated AP Site Influences Both AP Endonuclease and AP Lyase Activities.** The results described above indicate that both ARP and FPG are active on enzymatically generated AP sites, regardless of whether the excised base is either N7-meG or uracil. It is important to emphasize that all previous experiments were performed using C as the opposite base in the complementary strand. However, the relevant in vivo lesions arising from enzymatic excision of N7-meG and uracil (deaminated cytosine) are expected to be AP sites opposite C and G, respectively. To further explore the substrate preferences of the major AP incision activities in Arabidopsis we tested the effect of the base identity, either C or G, opposite an enzymatically generated AP site. We compared the incision activity of purified recombinant ARP and FPG proteins on DNA substrates containing AP sites generated by excision of uracil opposite either C or G (Fig. 6). Whereas FPG displayed similar activity on both DNA substrates (Fig. 6A), ARP activity was higher on AP sites opposite G (Fig. 6C). Native ARP in *fpg*−/− extracts (Fig. 6D) and recombinant hAPE1 (Fig. S4) also exhibited a similar preference for G as the orphan base. Interestingly, and unlike recombinant FPG, native FPG activity detected in *arp*−/− extracts was higher on AP sites opposite C (Fig. 6B). Altogether, these results suggest that the identity of the base opposite an enzymatically generated AP site influences the choice between AP endonuclease- and AP lyase-dependent repair.

**Discussion**

The initial motivation of this study was to understand why *zdp*−/− mutant plants are hypersensitive to MMS. Our biochemical and genetic analysis strongly suggests that the nonenzymatic release of MMS-induced N7-meG channels repair into an AP endonuclease-independent pathway in which FPG and ZDP perform consecutive steps (Fig. 7). The fact that FPG inactivation in *zdp*−/− mutants partially restores MMS resistance (Fig. 1E) suggests that the SSB intermediates with blocked 3′-P ends generated by FPG are more cytotoxic than AP sites. A similar observation has been previously reported in *S. cerevisiae*, where deletion of its two Nth homologs (NTG1 and NTG2) partially rescues the MMS hypersensitivity of a double *apn1* *apn2* mutant, presumably by avoiding accumulation of 3′-PUA ends (37). In *S. pombe*, the deletion of *nth1* also relieves the MMS sensitivity of the single *apn2* mutant (35). Our results suggest that it would be interesting to examine the relative activities of yeast AP lyases and AP endonucleases on AP sites of enzymatic and nonenzymatic origin. Unlike the yeast pathway, where AP endonucleases are required to process the 3′-PUA blocking ends generated by Nth homologs, the *Arabidopsis* pathway described here is AP endonuclease-independent, since 3′-P ends generated by FPG are processed by ZDP DNA 3′-phosphatase. This scenario is reminiscent of the AP endonuclease-independent pathway reported for oxidatively damaged bases in mammalian cells (21), in which NEIL1 and NEIL2 DNA glycosylases generate DNA strand breaks with 3′-P termini that are processed by the ZDP homolog polynucleotide kinase (PNK). Interestingly, PNK-deficient cells are sensitive to MMS (44), thus raising the possibility that the mammalian NEIL/PNK pathway may also operate in the repair of depurinated N7-meG. It has also been suggested that a PNK-dependent pathway functions as a backup mechanism for AP site repair in *S. pombe* (45, 46).

An unexpected observation arising from our work is that, unlike its mammalian homolog APE1, *Arabidopsis* ARP endonuclease does not exhibit detectable activity on abasic sites arising from N7-meG depurination (Fig. 5 C and D). We propose that such incapacity underlies the critical function of the FPG/ZDP pathway in protection of *Arabidopsis* against MMS. However, it is likely that ARP plays a role in the repair of AP sites generated by enzymatic
The identity of the base opposite the abasic site influences the choice and function of AP endonuclease activity. DNA substrates (2 nM) contained either a single AP:G (purple circles) or AP:C (green triangles), both generated by uracil excision. Substrates were incubated either with purified proteins (A) FPG: 0.5 nM; (B) ARP: 10 nM or (C) and (D) were supplemented with 2 mM MgCl2. After stabilization with NaBH4, reaction products were separated by denaturing PAGE and detected by fluorescence scanning. Values are means with SEs from two independent experiments.

Fig. 6. Identity of the base opposite the abasic site influences the choice between AP endonuclease- and AP lyase-mediated repair. DNA substrates (2 nM) contained either a single AP:G (purple circles) or AP:C (green triangles), both generated by uracil excision. Substrates were incubated either with purified proteins (A) FPG: 0.5 nM; (B) ARP: 10 nM or (C) and (D) were supplemented with 2 mM MgCl2. After stabilization with NaBH4, reaction products were separated by denaturing PAGE and detected by fluorescence scanning. Values are means with SEs from two independent experiments.

Fig. 7. A model for repair of AP sites arising from enzymatic and nonenzymatic release of N7-meG. FPG incises AP sites generated by spontaneous release of N7-meG, generating a single-nucleotide gap with a 3′-P terminus that is processed by ZDP to generate a 3′-OH terminus. Repair is continued through SP-BER. AP sites generated by enzymatic release of N7-meG may be incised by both ARP and FPG, and repair may be completed by either SP- or LP-BER. See text for details.
Arabidopsis FPG

Methods

Plant Material and Growth Conditions. The Arabidopsis mutant line SALK_076932, harboring a T-DNA insertion in the FPG gene was obtained from the Arabidopsis Biological Resource Center. Homozygous plants for the T-DNA insertion were identified by PCR using primers FPG_F1, FPG_R5, and Lba3 (Table S1). Arabidopsis arp-2 (SALK_021478) and zdp-1 (SALK_60_C08) lines were previously described (23, 25). All T-DNA insertion mutants were in the Col-0 background. Double fpg-arp-2 and fpg-zdp-1 mutant lines were obtained by crossing homozygous fpg-, zdp-, and arp-2 plants and self-crossing the progeny. Plants and self-crossing the progeny.

Codon Plus cells (Stratagene) were obtained from the University of Córdoba. We thank S. D. Kathe and S. S. Wallace (University of Vermont, Burlington, VT (31), was subcloned into pET30b expression vector (Novagen) using Xhol and XbaI sites. Expression was carried out in E. coli BL21 (DE3) dcm Codon Plus cells (Stratagene) induced during 2 h by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Protein was purified by affinity chromatography on a Ni2+-NTA column (HisTrap HP; GE Healthcare). Protein was eluted with a 60 mM to 1 M gradient of imidazole and analyzed by SDS/PAGE (10%) using broad-range molecular weight standards (Bio-Rad). Protein concentration was determined by the Bradford assay. His-ZDP, MBP-ZDP, and His-ARP were expressed and purified as previously described (23, 25).

DNA Substrates. Oligonucleotides used as DNA substrates (Table S2) were synthesized by IDT and purified by PAGE before use. Double-stranded DNA substrates were prepared by mixing a 5 μM solution of a 5'-fluorescein (F1)–labeled oligonucleotide with a 10 μM solution of an unlabeled complementary oligonucleotide. Annealing reactions were carried out by heating at 95 °C for 5 min followed by slow cooling to room temperature. DNA duplex containing a single N7-meG was synthesized by the primer extension method using a 5'-fluorescein-labeled oligonucleotide annealed to a 51-nt oligonucleotide (Table S2). DNA synthesis was performed in a reaction mixture containing 0.1 μL µL−1 Klenow Fragment (3′–5′ exo−), NEB, 20 μM dCTP, 20 μM dATP, 20 μM dTTP, and 200 μM 7-methyl-dGTP (Jena Bioscience) at 37 °C for 1 h in NEBuffer 2. A control DNA substrate was synthesized in the presence of 20 μM dGTP instead of 7-methyl-dGTP. Reactions were stopped by adding 10 mM EDTA, and DNA was ethanol-precipitated at −20 °C in the presence of 0.3 mM NaCl and 16 mg·mL−1 glycogen. Samples were resuspended in deionized water and stored at −20 °C. The N7-meG was converted into its ring-opened me-FAPy-G form by incubating in 50 mM phosphate buffer (pH 11) at 37 °C for 5 h (65). DNA was ethanol-precipitated as described above.

DNA substrates containing an enzymatic AP site were generated by incubating a DNA duplex containing either a U:G or a U:U mismatch, prepared as described above, with E. coli UDG (1.5 μl; New England Biolabs, NEB) at 37 °C for 1 h. Enzymatic AP sites were also generated by incubating a DNA duplex containing a N7-meG:pair with HAAG2 (2 μl, NEB) at 37 °C for 8 h in NEBuffer 1. DNA substrates containing an AP site generated by spontaneous depurination were generated by incubating a DNA duplex with a single N7-meG:pair for 16 h at 37 °C in DNA incision assay buffer (45 mM Hepes-KOH, pH 7.8, 70 mM KCl, 1 mM DTT, 0.4 mM EDTA, 36 μg·mL−1 BSA, 0.2% glycerol) incubated at 37 °C for 30 min. When indicated, reaction products were stabilized by the addition of freshly prepared sodium borohydride (NaBH₄, Sigma-Aldrich) to a final concentration of 230 mM and incubated at 0 °C for 30 min. Finally, samples were buffered with 100 mM Tris, pH 8, and DNA was extracted as described above. Reaction products were separated and visualized as previously described.

For assays comparing enzymatic and nonenzymatic AP sites, DNA substrates (20 nM) were a 9:1 mixture of homoduplex G:C and heteroduplex AP:C. Nonenzymatic AP:C was generated by spontaneous N7-meG depurination for 16 h, whereas enzymatic AP:C was generated either by N7-meG excision by hAAG or unassisted excision by E. coli UDG (discussed above). Protein (percent) was calculated as the incision fragments detected relative to those generated by hAPE1 (10 U).

Gap-Filling Assay. Reactions (50 μl) contained 45 mM Hepes-KOH, pH 7.8, 70 mM KCl, 1 mM DTT, 0.4 mM EDTA, 36 μg·mL−1 BSA, 1 mM NAD, 0.2% glycerol, 10 mM ATP, 110 mM phosphocreatine, 0.25 μg·mL−1 creatine phosphokinase, 2 mM MgCl₂, and 20 μM of the indicated deoxyribonucleotides. After incubation at 37 °C for 3 h, reactions were stopped, DNA was extracted, and samples were processed as described above.

Protein Expression and Purification. Arabidopsis FPG cDNA, a gift from Scott Kathe and Susan Wallace, University of Vermont, Burlington, VT (31), was subcloned into pET30b expression vector (Novagen) using Xhol and XbaI sites. Expression was carried out in E. coli BL21 (DE3) dcm Codon Plus cells (Stratagene) induced during 2 h by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Protein was purified by affinity chromatography on a Ni²⁺-NTA column (HisTrap HP; GE Healthcare). Protein was eluted with a 60 mM to 1 M gradient of imidazole and analyzed by SDS/PAGE (10%) using broad-range molecular weight standards (Bio-Rad). Protein concentration was determined by the Bradford assay. His-ZDP, MBP-ZDP, and His-ARP were expressed and purified as previously described (23, 25).

Pull-Down Assays. For His-FPG pull-down, 30 pmol of purified MBP or MBP-ZDP in 30 μl of column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, and 10 mM β-mercaptoethanol) was added to 100 μl of amylose resin (NEB) and incubated for 1 h at 4 °C. The resin was washed three times with 1 ml of binding buffer (20 mM Tris, pH 7.4, 200 mM NaCl, 1 mM EDTA, and 10 mM β-mercaptoethanol). Purified His-FPG (5 pmol) was incubated at 25 °C for 30 min at 450 rpm with either MBP or MBP-ZDP bound to resin. The resin was washed three times with 1 ml of washing buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 10 mM β-mercaptoethanol, 1.5% Triton X-100, and 250 mM NaCl). Bound proteins were analyzed by Western blot using antibodies against His-tag (Santa Cruz).

For MBP-ZDP pull-down, 30 pmol of purified His-FPG in 30 μl of dialysis buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 1 mM DTT) was added to 100 μl of Ni²⁺-Sepharose resin (NEB) and incubated for 1 h at 4 °C. The resin was washed three times with 1 ml of binding buffer 2 (10 mM Tris, pH 8, 1 mM DTT, 0.01 mg·mL−1 BSA, and 60 mM Imidazole). Purified MBP or MBP-ZDP (5 pmol) was incubated at 25 °C for 30 min at 450 rpm with His-FPG-bound resin to the bound. The resin was washed three times with 1 ml of washing buffer 2 (20 mM Tris, pH 7.4, 1 mM EDTA, 10 mM β-mercaptoethanol, 1.5% Triton X-100, 100 mM NaCl, and 60 mM imidazole). Bound proteins were analyzed by Western blot using antibodies against MBP tag (Santa Cruz).

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