INTRODUCTION

The DNA methylation is methylation at the carbon 5 position of cytosine occurs in CG, CHG, CHH sequence contexts (where H = A, T or C). In plants, 5-methylcytosin is the key epigenetic modification that mediates silencing and transcriptional activation of genes. Recent study showed that the DNA methylation on gene body is positively related to gene expression, while the DNA methylation on promoter and enhancer represses gene expression (Lister et al., 2009; Ball et al., 2009). DNA methylation is also involved in important developmental process such as transposon silencing, gene imprinting and response to biotic and abiotic stress.

The patterns of DNA methylation on genome are delicately regulated by time and cell type specific manner. For example, plant central cell and vegetative cell undergoes extensive changes in DNA methylation before fertilization (Ibarra et al., 2012). To convert cytosine into 5-methylcytosine, two classes of DNA methyltrasferase enzymes transfer the methyl group from S-adenosyl-methionine to 5'-position of cytosine. In plants, DRM2 are the de novo DNA methyltransferase, notably for DNA methylation of CHH sites carrying plant specific DNA methylation mechanism, the RdDM (RNA-directed DNA Methylation) pathway (Mette et al., 2000). To maintain the DNA methylation level while cell division, CG and CHG methylation is catalyzed by MET1 and CMT3, respectively.

Despite its importance in regulating DNA methylation dynamics, few studies have examined the mechanism of DNA demethylation. The 5-methylcytesine DNA glycosylase family proteins catalyze DNA demethylation. DEMETER (DME), a founding member of the DNA demethylase family in

Arabidopsis, functions as a bifunctional DNA glycosylase/lyase that excises a glycosidic bond of 5-mC while generating β-elimination (3' phospho-α,β-unsaturated aldehyde) and δ-elimination (3' phosphate) products (Gehring et al., 2006). To complete DNA demethylation, the remaining β/δ-elimination intermediates need to be processed to generate 3'-OH for subsequent base incorporation by DNA polymerase. However, little is known that which enzymes process further steps of DNA demethylation. It was shown that AtZDP, a plant polynucleotide kinase 3' phosphatase, has phosphatase activity on the 3' phosphate end after DNA demethyltion (Martinez-Macias et al., 2012). However, another enzyme activity may be required to process the other DNA demethylation product, β-elimination, which is likely mediated by apurinic/apyrimidinic (AP) endonucleases.

AP endonuclease (APE) is involved in one of the major DNA repair systems, the base excision repair (BER) pathway (Hegde et al., 2008). The first step of BER is initiated by a repair enzyme termed DNA glycosylase. After recognition of the damaged base, the glycosylase hydrolyzes an N-glycosidic bond between a modified base and deoxyribose, creating the abasic (apurinic/apyrimidinic, AP) site. Subsequently, the phosphodiester bond immediately at 3' end of the AP site or or 3' blocking end is incised by AP endonuclease (APE) or AP lyase to create a single strand break with a 3'-OH. The gap is filled and sealed by the DNA polymerase and the DNA ligase, respectably. In this way, the BER pathway completes the repair of mismatched and damaged bases there by sustain the of genome stability.

Early studies on bacteria and eukaryotic AP endonucleases found that they are conserved in many organisms. AP endonucleases are classified into two

sub-families, exonuclease III (Exo III) and endonuclease IV (Endo IV) families (Demple and Harrison et al., 1994; Demple et al., 1986). In E. coli, Exo III functions as a major AP endonucelase in the DNA repair (Rogers and Weiss, 1980). In human, two AP endonucleases APE1 and APE2 have been identified. Human APE1 enzyme exhibits robust APE endonuclease activity than the APE2 (Hadi et al., 2006). Both also possess 3' phosphodiesterase and 3' exonuclease activities (Demple and Harrison et al., 1994).

Even though many studies have been done on AP endonucleases from bacteria, yeast and human, little is known about plant AP endonuclease. In Arabidopsis, three putative AP endonucleases abasic endonuclease-1-like (APE1L), abasic endonuclease-2 (APE2) and apurinic endonuclease-redox (ARP) are present and their mutants were reported to show developmental defects (Murphy et al., 2009). One of Arabidospsis AP endonucleases, ARP showed AP endonuclease activity when tested with recombinant protein and arp mutant extract (Babiychuk et al., 1994). However, clear evidence for in vitro and in vivo AP endonuclease activity using delicate biochemical experiment has not revealed yet.

In this study, to characterize Arabidopsis AP endonuclease, I cloned and purified the putative three AP endonucleases. Both APE1L and ARP were found to have conventional AP endonuclease activity in vitro. AP endonuclease activity was also verified in the heterologous bacterial system. These findings suggest that AP endonucleases are required for efficient processing of base excision products during the course of the DNA repair. This study will help understand the BER of plants in addition to the DNA demethylation pathway initiated with 5-methylcytosine (5mC) -specific DNA glycosylase.

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LITERATURE REVIEWS

1. Base excision repair (BER) pathway

To repair a damaged, modified or mismatched base, plants have developed diverse repair systems. The base excision repair (BER) is one of the major DNA repair pathways. The BER pathway has ability to fix various of damaged bases, maintaining the genome integrity. BER has several branch pathways to complete the replacement damaged base with a normal base, but the core pathway concise of three major steps. The first step of BER is catalyzed by the repair enzyme termed DNA N-glycosylase. After recognition of the damaged base, the glycosylase hydrolyzes an N-glycosidic bond between the modified base and a deoxyribose, leaving the abasic (apurinic/apyrimidinic, AP) site. Subsequently, the phosphodiester bond immediately at 3' end of the AP site is incised by AP endonuclease (APE) or AP lyase to create a single strand break with a 3'-OH. The gap is filled and sealed by DNA polymerase and DNA ligase, respective, to complete the repair.

The BER pathway has several branch pathways. However, junctions at the branch points are poorly understood. The BER pathway can be divided into the short-patch and long-patch BER (Figure 1). If the BER pathway is initiated with monofunctional glycosylases, the AP endonuclease will cleave the AP site, leaving a 5'-deoxyribosephosphate (5'-dRP). Then, the 5'-dRP and neighboring nucleotides are removed and displaced by DNA polymerase β in a PCNA dependent manner (Fortini et al., 1998; Gary et al., 1999). It results in the removal of a patch of oligonucleotides downstream of the cleaved base with insertion of a

few nucleotides, completing the long-patch BER. By contrast, the short-patch BER replaces one nucleotide at once. It was reported that if the 5'-dRP is removed by dRPase, polymerase β drives the pathway to short-patch BER (Dianov etl al., 1999). Many researchers suggest that 5' lesion that remains after AP endonuclease cleavage would determine the direction toward a short-patch or long-patch BER pathway.

2. Structure and function of AP endonucleases

The first AP endonuclease was discovered in *E. coli* as exonuclease III (xth) (Weiss, 1976). The xth was initially described to have both 3' exonuclease and 3' phosphatase activities that remove 3' blocks to produce the 3'-OH terminus. The AP site endonuclease activity was demonstrated later (Wilson et al., 1995). Another AP endonuclease, endonuclease IV (nfo) was also found in *E. coli*, which does not have the 3' exonuclease activity with no need of Mg^{2+} for the enzyme activity (Demple et al., 1986). These two enzymes belong to the class II exonulcease III family and their homologs are found in many organisms from bacteria to yeast, *C. elegans* and mammals.

This family is interesting in that xth and nfo have significantly different structures but similar functions. Structures of xth and nfo family proteins and as well as nfo were defined by X-ray crystallography. xth has a four layered α,β-sandwich structure (Mol et al., 1995). The human APE1 also has similar structure with two potential Mg^{2+} binding sites. Moreover, core residues and motifs for catalytic activity are conserved in all xth family proteins. Unlike *E. coli*, Yeast and mammals do not have an nfo ortholog. Only orthologs of xth, APE1 and APE2 are found in mammals. They have distinct enzyme activity. APE1 has strong AP

endonuclease activity while APE2 has very weak activity (Burkovics et al., 2006). When compared with xth, APE1 has weak AP endonuclease and 3' block cleaning activities with little 3' phosphatase activity (Wiederhold et al., 2004). However, studies with APE1-null mice which failed to develop in early embryonic stage suggest the essential role of APE1 in mammals (Izumi et al., 2005). In addition, increase of single-strand breaks and AP sites were found in null APE1 mutant fibroblasts (Mitra et al., 2007).

Human APE1 has additional N-terminal residues compared to xth. This region contains nuclear localization signals and may aid redox reactions for transcriptional regulation independent of BER functions (Kelley et al., 2001).

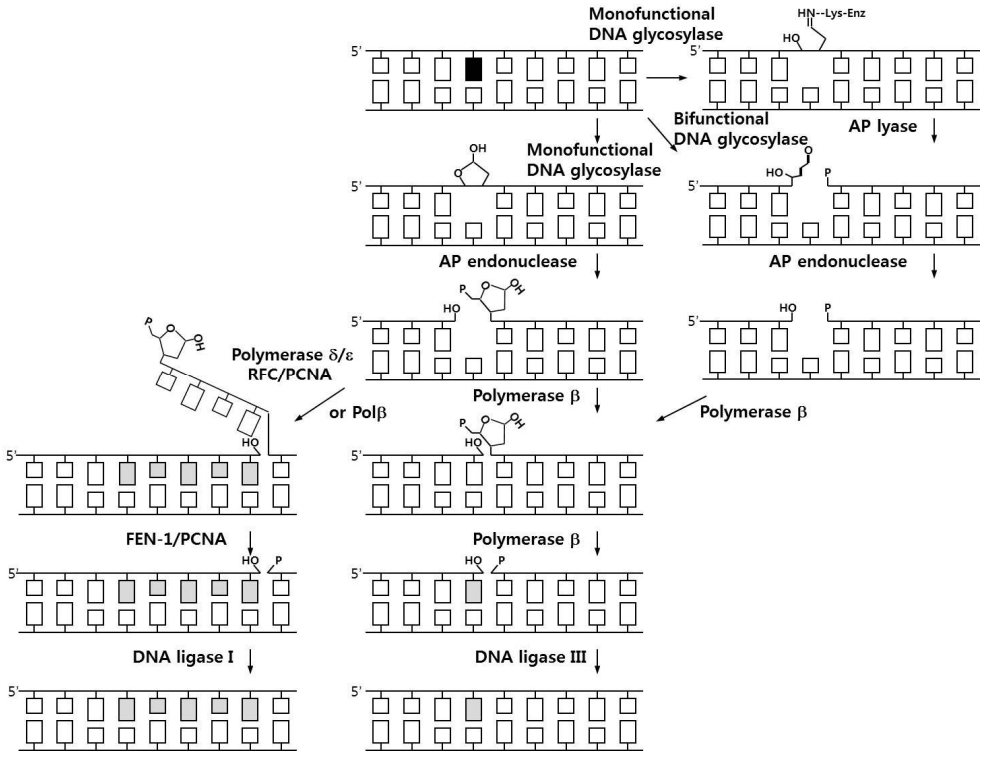

Figure 1. Schematic illustration of branch BER pathways. The damaged base is colored in black. Replaced bases are represented as gray.

MATERIALS AND METHODS

Plant materials

Three putative AP endonuclease T-DNA insertion mutants were obtained from the Arabidopsis Biological Resource Center. They are in a Col-0 ecotype background. The ape1l-1 mutant (SALK_024194) has a T-DNA insertion at the upstream of a translation start site of AtAPE1L gene (At3g48425). The mutant lines were confirmed by PCR amplification using three primers DG326, DG327 and DG535 (LBb1.3). The ape2-2 mutant (SALK_021847) has a T-DNA insertion at the promoter of AtAPE2 gene (At4g36050.1),. The mutant lines were confirmed by PCR amplification with three primers DG328, DG329 and DG535. The arp-1 mutant (SALK_021478) has a T-DNA insertion at the 5th exon of AtARP gene (At2g41460). The mutant lines were confirmed by PCR amplification using three primers DG160, DG161 and DG535.

For sterilization, seeds were treated with bleach solution (30 % bleach, 0.025 % Triton X-100) for 3 min and washed with sterile water. After 2 days at 4 °C, seed were sowed on an MS medium. Seedlings were transported and grown on soil for 10 days after germination. Plants were grown under 16 hours of light, 8 hours of dark at 23°C.

Cloning of AtAPE1L, AtAPE2, AtARP in Arabidopsis for protein expression

The full-length sequences of *AtAPE1L*, *AtAPE2* and *AtARP* were isolated by a RT-PCR. Using *AtAPE1L* RT-PCR products as templates, primers DG326 and DG327 generated at 5' and 3' ends with *Bam* HI and *Sal* I. The pLM302 vector is a

pET27a (EMD Millipore, Germany) derivative that encodes an N-terminal 6xHis + Maltose Binding Protein (MBP) for fusion with a protein of interest, which can be cleaved by PreScission Protease (GE Healthcare, UK). The product was digested with *Bam* H I and *Sal* I and cloned into the PLM302 vector at the corresponding sites creating the pLM302-APE1L. Subsequently, the plasmid was transformed into E. coli Rosetta2 (DE3) strain (EMD Millipore, Germany) for protein expression. pLM302-APE2 and pLM302-ARP also generated in the same way as described above. The primers for amplification were DG328 and DG329 for *AtAPE2* and DG160 and DG161 for *AtARP*.

RNA extraction and gene expression analysis

For RT-PCR analysis, total RNA was extracted from various tissues of *Col-0* plants using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from total RNA using Oligo (dT) and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The primers used to amplify *AtAPE1L* mRNA were DG326 and DG327. The primers used to amplify *AtAPE2* mRNA were DG328 and DG329. The primers used to amplify *AtARP* mRNA were DG160 and DG161. The primers used to amplify control *AtACTIN11* mRNA were DG243 and DG244. The RT-PCR experiment without reverse transcriptase was used as a negative control.

Expression and purification of AtAPE1L, AtAPE2 and AtARP

A single fresh colony was inoculated in 5 mL of LB medium containing kanamycin (50 μg/ml) and chloramphenicol (50 μg/ml). The culture was incubated at 37 °C *overnight with shaking. An aliquot of overnight culture was inoculated*

into 2L LB medium with the same antibiotics and incubated at 30 ℃ until the OD_{600} *reached 0.4. Expression was induced by 0.1 M IPTG at* 16 °C *overnight with shaking. Cells were harvested by centrifugation and the pellet was resuspended in 50 mL of lysis buffer (*50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, 0.1 mM dithiothreitol, 0.1 mM PMSF). *The lysate was sonicated and clarified by centrifugation. The supernatant was collected and loaded onto the HisTrap FF column (GE Healthcare, UK) and eluted with a linear gradient of imidazole (1 mM to 100 mM). Fractions were collected and* loaded onto a 5-mL HiTrap Heparin HP column (GE Healthcare, UK) with a linear gradient of NaCl (0.1 to 1 M). Finally, gel filtration was performed on a HiLoad 16⁄60 Superdex 200-pg column (GE Healthcare, UK). Fractions were collected and stored in a storage buffer (20 mM Tris-HCl, pH 7.4, 40 mM NaCl, 10% glycerol, 0.1 mM dithiothreitol, 50% glycerol) at -80 °C.

AP endonuclease assay and 3' end cleaning assay

Oligonucleotide substrates were synthesized from Integrated DNA Technologies (Integrated DNA Technologies, IA, USA). 35 nt double stranded DNA containing a single tetrahydrofuran, the AP site analog, at position 18 on a top strand (R35AP) was used as substrate for AP endonuclease activity assay. Twenty pmol of oligonucleotide was end-labeled 30 μCi of (γ-32P) ATP (6000 Ci/mmol, Bio-Medical Science, Korea) in a 50 μL reaction using 20 units of T4 polynucleotide kinase at 37 °C for 1 hr. The labeled oligonucleotide was purified using a Qiaquick Nucleotide Removal Kit (Qiagen, Germany) as described by the manufacturer. Labeled oligonucleotides were annealed to appropriate

complementary oligonucleotides in 10 mM Tris-HCl (pH 8.0), 1mM EDTA and 0.1 M NaCl. The mixture was boiled for 10 min and then slowly cooled down to room temperature overnight. The DNA substrate (25 nM) was incubated with or without 5 nM or 25 nM APEs in the 16 μl of reaction buffer (10 mM Tris-HCL pH 7.4, 50 mM NaCl, 200 ug/ml BSA, 10 mM $MgCl_2$, 1 mM DTT) for 30 min at 37 °C. 0.4 unit of hAPE1 (New England Biolabs, Ipswich, MA, USA) was used as a reaction control. AP endonuclease assay differing for magnesium concentrations was conducted with 0-300 mM $MgCl_2$. 25 nM oligonucleotide substrate R35AP was reacted with 5 nM APEs in the reaction buffer supplemented with different concentrations of $MgCl_2$ at 37℃ for 30 min. All reactions were terminated with 16 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) and boiled for 10 min. For 3' end cleaning assay, labeled 30 nt double stranded DNA containing a 5 mC in center of top strand (R30M) was used for substrate of DME enzyme. 25 nM of labeled and annealed oligonucleotides were incubate with 250 nM DME in the 16ul of reaction buffer for 60 min at 37 °C. 20 min of 65 °C heat on reaction tubes were treated for DME inactivation. Used products of DME reaction as substrate, 5 nM of APEs were incubated in 32 μl of reaction buffer for indicated time at 37 °C. All reactions were terminated with 32 ul of stop solution. Products were separated on a 15% polyacrylamide gel containing 7.5 M urea and 1× TBE. Electrophoresis was done at 1200 V for 3 hr. The gel was exposed to X-ray film at −80°C.

Kinetic study of AP endonuclease

For multiple turnover kinetics of APE1L and APE2, the same reaction condition described above was used with various substrate concentrations. Reactions were carried out in 16 μl of reaction buffer (10 mM Tris-HCL pH 7.4, 50 mM NaCl, 200 μg/ml BSA, 1 mM DTT). Reactions were terminated with 16 μl of stop solution and boiled for 10 min. Products were loaded on a 15% polyacrylamide gel with 7.5 M urea and 1× TBE. Electrophoresis was done at 1200V for 4 hr. The gel was exposed to a phosphor imager screen (Fujifilm, Tokyo, Japan) and the radioactivity was measured using the Fujifilm BAS-5000 (Fujifilm, Tokyo, Japan). The signal intensity of products relative to the substrate was measured using the Multi Gauge V2.2 (Fujifilm, Tokyo, Japan). The gel was exposed to X-ray film at −80 °C.

Construction of pYOON01 and sub-cloning of AP endonucleases

For co-expression of two different proteins in *E. coli*, a new vector was generated. The pYOON01 vector is a pMAL (New England Biolabs, Ipswich, MA, USA) and a pACYC184 (New England Biolabs, Ipswich, MA, USA) derivative that contains a p15a origin, a gene encoding resistance to chloramphenicol and a tac promoter for expression of protein of interest. It encodes N-terminal 6xHis + MBP for fusion with a protein of interest (Figure 10). The pLM302-APE1L was digested with *Bam* HI and *Sal* I and the APE1L fragment was cloned into the pYOON01 vector. pYOON01-APE2 and pYOON01-ARP were generated with the same strategy. The plasmids were transformed into *E. coli* strain RPC501. RPC501 is an isogenic strain of AB1157 (F−thr-1 ara-14 leuB6(Am) lacY1 (gpt-proA2)62 tsx-33 supE44(Am) galK2 rac hisG4(Oc) rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3(Oc) thi-1) that carries mutants for xth and nfo. Competent cells

were prepared for co-transformation with the calcium chloride method (Dagert et al., 1979).

Bacterial cell toxicity assay

The pMAL-c2X or pMAL-c2X-DMEΔN677ΔIDR1::lnk was transform into RPC501 that carried pYOON01, pYOON01-APE1L, pYOON01-APE2 or pYOON01-ARP. Fresh colonies were picked and grown in liquid LB/Glu/Amp media (LB supplemented with 0.2% glucose and 100 μg/mL of ampicillin) at 37 °C overnight. The culture was diluted to an O.D. 0.004 and spotted on LB/Amp plates with 0-125 μM of IPTG (isopropyl-β-Dthiogalactopyranoside; Sigma, St.Louis, MO, USA). The plates were incubated at 26 °C for 20-28 hr.

Table 1. List of primers for PCR amplification

Primer name	sequence (5'→3')	use
DG326	AATTGGATCCATGAAGCGATTCTTCAAGC	Cloning, RT-PCR
DG327	AATTGTCGACTTAGTTTGACACCTGGTTCTGTTC	Cloning, RT-PCR
DG328	AATTGGATCCATGGATCGATGTGAAGCTG	Cloning, RT-PCR
DG329	AATTGTCGACTTACTTGTCTCTGAATTTTGATG	Cloning, RT-PCR
DG160	AATTGGATCCATGAACAACGTTCTTCAGTTTGG	Cloning, RT-PCR
DG161	AATTGTCGACTCAGAGCTTGAGAATAAGGCCAATGG	Cloning, RT-PCR
DG539	GGCCATCTACAACTTAGCAGC	Genotyping
DG540	AAGCACATTTACCCACATTGC	Genotyping
DG543	GTTTTTCTCAAAATCAGGCCC	Genotyping
DG544	CGAACGAACTACAGTGGGAAG	Genotyping
DG545	GCTGCAGGACCAGAAACTATG	Genotyping
DG546	CGAGAAAAAGGCATGAACTTG	Genotyping
DG535	TAGCATCTGAATTTCATAACCAATCTCGATACAC	Genotyping
DG244	AACTTTCAACACTCCTGCCATG	RT-PCR
DG245	CTGCAAGGTCCAAACGCAGA	RT-PCR

Table 2. List of oligonucleotides for biochemical assay

oligo name	sequence (5'→3')	
R35AP	CTGTGTGATACTATFGAATTCAGTATGATC	F, Tetrahydrofuran
R35_reverse	GATCATACTGAATTCGATAGTATCACACAG	
R30M	GTGTGATACTATMAATTCAGTATG	M, 5-methylcytosine
R30_reverse	CATACTGAATTCGATAGTATCACAC	

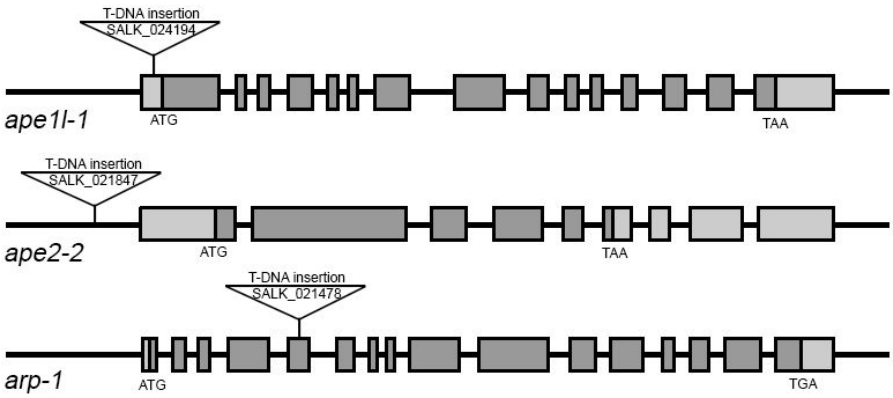

Figure 2. Schematic diagram of AP endonuclease T-DNA mutant lines, *ape1l-1*, *ape2-2*, *arp-1*. Dark gray and gray bars indicate exons and UTR, respectively. Insertion of T-DNA is shown as a triange.

RESULTS

AtAPE1L, AtAPE2 and AtARP encodes AP endonucleases

To date, three putative AP endonucleases in Arabidopsis, APE1L, APE2 and ARP were reported (Murphy et al., 2009; Cordoba-Canero et al., 2011). They are AP endonuclease genes (xth/exodeoxyribonuclease III family). The Conserved Domain-search (CD-search) revealed that all three putative AP endonucleases have a common EEP domain for endonuclease, exonuclease and phosphatase activities (Figure 3A). The EEP domain comprises most protein structure of APE1L. APE2 and ARP have additional domains, a zinc-finger domain and sap domain for DNA binding, respectively. All three enzymes were classified into xth family. AtAPE1L and AtARP have a high degree of homology to human APE1 while AtAPE2 shares a homology with human APE2.

AP endonucleases are highly conserved among species (Robertson et al., 2009). Human APE1 was the most studied. The crystal structure of hAPE1 was solved and the catalytic mechanism was proposed (Mol et al., 2000). According to the structural study, hAPE1 contains five regions which contact with DNA strand and AP site. And a few core residues are essential for AP site binding and catalytic activity. The primary structures of Arabidopsis APEs were compared with E. coli xth and human APE. The amino acid comparison indicates that the residues for AP site binding and catalytic activity are conserved in all three enzymes (Figure 3B). However, some important residues (i.e., Asp^{100} and Asn^{102}) are missing in APE2. RT-PCR analysis revealed that all APEs were expressed in almost all tissue types investigated (Figure 3C). APE2 and ARP were constantly expressed in all

vegetative and reproductive tissues. Notably, the transcription level of APE1L was significantly higher in developing silique than in other tissues and no expression of APE1L was detected in roots. This suggests that, unlike APE2 and ARP, APE1L exists in specific cell types for unknown functions.

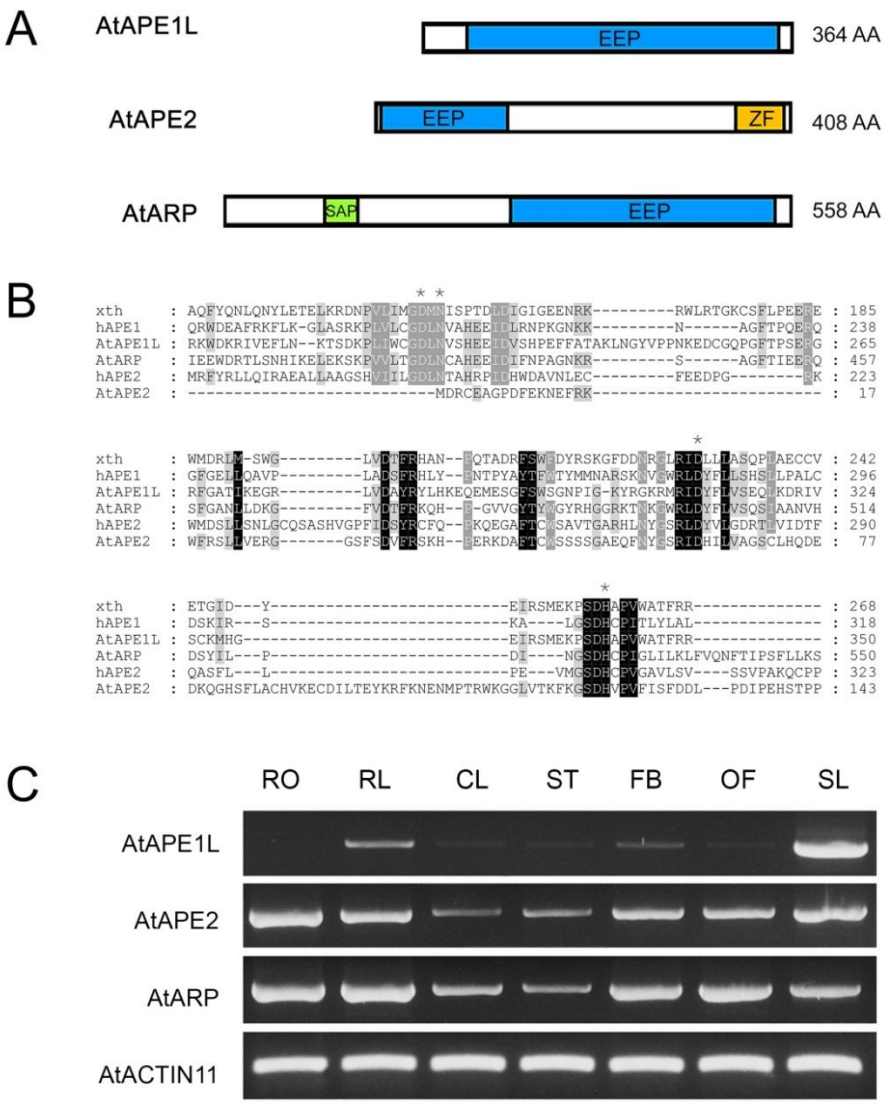

Figure 3. Three putative AP endonucleases in Arabidopsis. (A) Schematic representation of APE1L, APE2 and ARP proteins. EEP, endonuclease-exonuclease-phosphatase; SAP, DNA binding; ZF, GRF-type zinc-finger protein. (B) Sequence alignment of E. coli exonuclease III (xth) with human hAPE1 and hAPE2, and Arabidopsis AtAPE1L, AtAPE2 and AtARP. Highly conserved residues are colored in black and moderately conserved ones are in light grey. Residues that are directly involved in catalysis are shaded in grey star. (C)

Expression pattern of AtAPE1L, AtAPE2 and AtARP in various tissue types is assessed by RT-PCR. ACTIN11 is a loading control. RO, root; RL, rosette leaves; CL, cauline leaves; ST, stem; FB, flower buds; OF, open flower; SL, Silique.

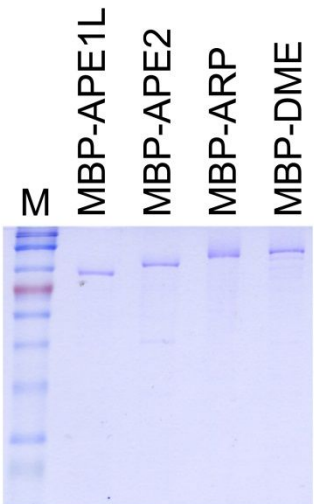

Figure 4. Analysis of purified three Arabidopsis AP endonucleases and DME protein. 200 ng of protein sample was electrophoresed on 10% polyacrylamide gel and visualized by coomassie brilliant blue staining.

APE1L and ARP but not APE2 has AP endonuclease activity

An AP site is often generated by attack of free radical species to DNA. Cleavage of damaged base by DNA glycosylase also produces AP sites in DNA. AP endonuclease processes AP sites that are produced by monofunctional glycosylase lacking in AP lyase activity (Fromme et al., 2004). In an effort to identify intrinsic AP endonuclease activity on AP site, purified AP endonuclease enzymes were incubated with 35 mer oligonucleotides containing an AP site analog (Tetrahydrofuran) at position 18 (R35AP). Consistent with previous studies using plant extracts and recombinant protein (Cordoba-Canero et al., 2011; Babiychuk et al., 1994), ARP processed AP site to generate 3'-OH (Figure 5A). APE1L also catalyzed the incision of the AP site, leaving a 3'-OH group, however its enzyme activity seems to weak. The rest enzyme, APE2 showed no AP endonuclease activity on an AP site (Chou and Cheng, 2002; Burkovics et al., 2006). Human APE1 was used as a reaction control for incision specificity of Arabidopsis AP endonucleases.

Reaction rates were examined with increasing amounts of APEs. Products were made proportional to the enzyme concentrations. Interestingly, I observed gradual degradation of the substrate by APE1 and APE2 at high enzyme concentrations, which suggests a 3'-5' exonuclease activity. The 3'-5' exonuclease activity of APE1L and ARP was also exemplified by low molecular weight products. The 3'-5' exonuclease activity of human APE1 and human APE2 was previously reported (Burkovics et al., 2006). To assess catalytic efficiency of ARP, I conducted kinetic analysis (Figure 5B). Determined kinetic parameters using Michaelis-Menten equation showed differences in their activity. As shown in Figure 3, ARP has about eight-fold higher k_{cat} values, with K_m values less than 3

fold, which indicate ARP has stronger catalytic activity than APE1L. Altogether, these results indicate that APE1L and ARP, but not APE2, has AP endonuclease activity following the conventional AP endonuclease reaction mechanism. APE2 appears to be devoid of canonical AP endonuclease activity.

Next, effects of magnesium (Mg^{2+}) and salt on AP endonuclease activity were investigated. Previous report on endonucleolytic activity, Exonuclease III activity was stimulated by magnesium but endonuclease IV was not (Fromme et al., 2004). According to the crystal structure of human APE1, Mg^{2+} is predicted to be located in a catalytic pocket. This magnesium ion binds the P-O3 group after phosphodiester bond cleavage (Mol et al., 2000). Salt was also known to affect enzyme activity (Warren and Cheatum et al., 1966). Three AP endonucleases were tested under low and high Mg^{2+} concentrations. I found that each AP endonuclease require Mg^{2+} to incise AP site (Figure 6). Degradation of DNA strand at low Mg^{2+} concentrations was observed in APE1L and ARP.

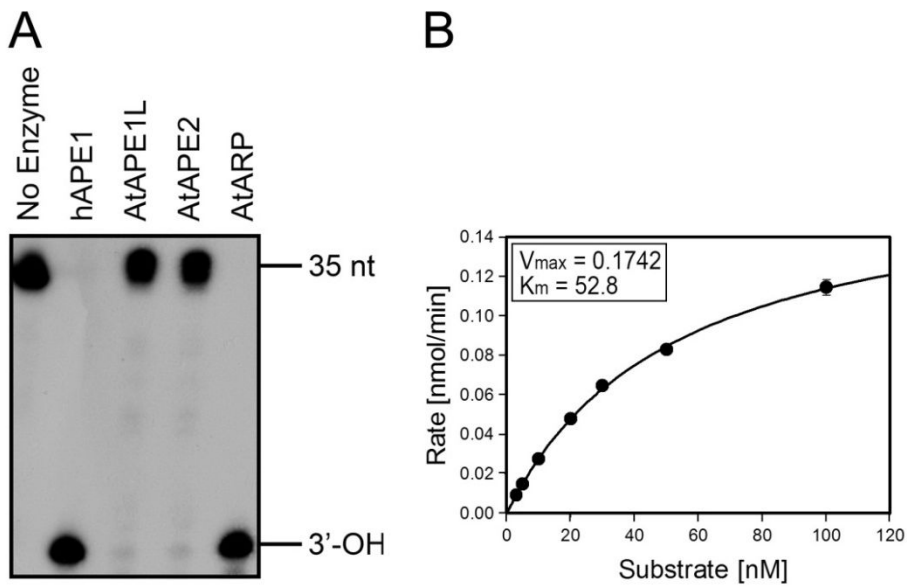

Figure 5. AP endonuclease activity of AtAPE1L, AtAPE2 and AtARP. (A) The AP endonuclease activity on AP sites. 35 nt double stranded DNA containing a single tetrahydrofuran, the AP site analog, at position 18 (R35AP) was used for substrate in AP endonuclease activity assay. Reaction were done with 5 nM each AtAPEs at 37 ℃ for 15 min. 0.5 unit of hAPE1 was used as a reaction control. APE reaction products (17 nt with 3'-OH) are indicated at the right of the panel. (B) Kinetic analysis of Arabidopsis AP endonucleases. Multiple conditions (substrates, R35AP, are 3 to 100 fold to enzyme concentration) were applied for kinetics study. The incision activity of APR on AP site analog with various substrate concentrations (1-120 nM). Reaction were done by 5 nM ARP with indicated substrate concentrations at 37 ℃ for 4 min.

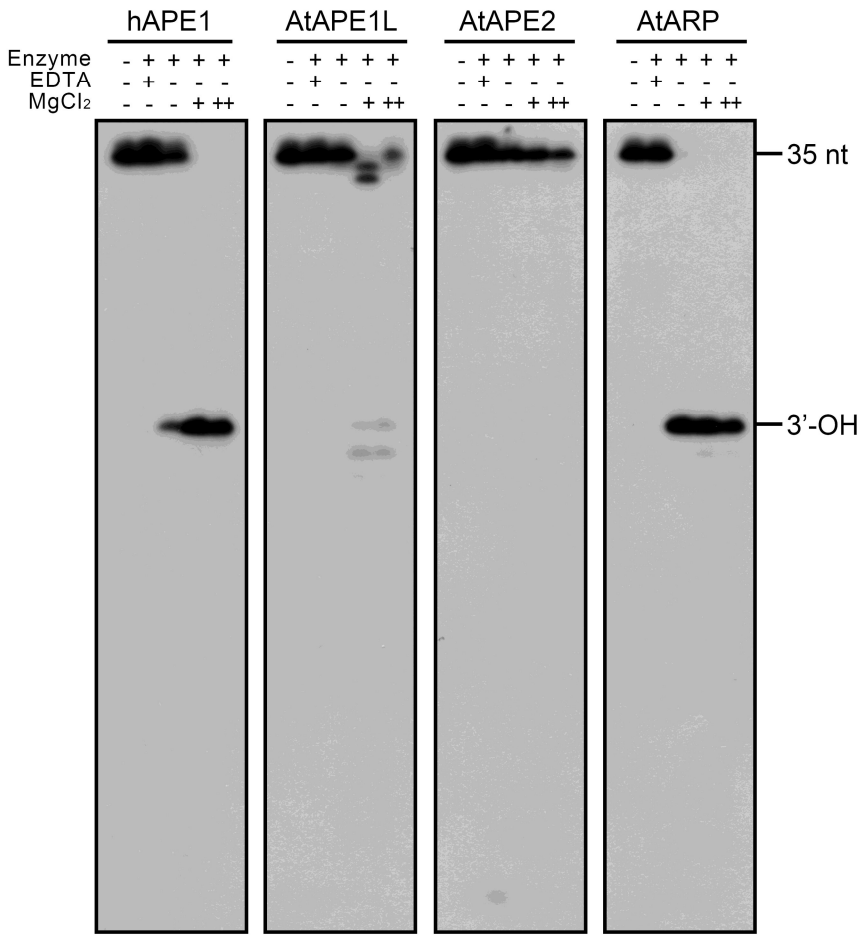

Figure 6. Effect of magnesium concentrations on AP endonuclease activity. Three AP endonucleases were tested under 2 mM EDTA and 3, 10 mM or in absence of Mg2+ concentrations. 5 nM (hAPE1L, AtAPE2 and AtARP) or 25 nM (AtAPE1L) AtAPEs were incubated with R35AP at 37 ℃ for 15 min. 0.5 unit of hAPE1 was used as a reaction control. APE reaction products (17 nt with 3'-OH) are indicated at the right of the panel.

APE1L and ARP process 3'end block generated by DNA demethylase

The first step in DNA demethyltion is initiated by 5mC DNA glycosylase that is regarded as a DNA demethylase. Glycosylase domain of DNA demethylase excises a glycolsidic bond between 5-methylcytosine and a ribose, generating two intermediates 3'-phospho-α-β-unsaturated aldehyde (3'-dRP) and 3'-phosphate. These products are also generated by oxidized base-specific mammalian bifunctional DNA glycosylase/AP lyases, NTH1 and OGG1 (Ikeda et al., 1998). Such intermediates are further processed by the AP endonuclease. A current model postulates the participation of BER enzymes immediately downstream of the 5mC excision step by DNA demethylase such as DME which is a founding member of the 5mC DNA glycosylase (Gehring et al., 2009). This led me to test whether Arabidopsis AP endonucleases play an essential role in DNA demethylation. The substrate (stated as DME products in Figure 7A) generated by DME treatment was reacted with putative AP endonucleases. I detected 3' end processing activity from APE1L and ARP (Figure 7A). Though APE1L found to have weak AP endonuclease activity , APE1L displayed robust 3'-dRP nuclease activity than ARP. ARP was able to process DME-catalyzed β-elimination product, with significant exonuclease activity leading to DNA degradation (data not shown). It is worth that all Arabidopsis AP endonucleases seem to have different substrate preference. These findings suggest that APE1L is a major player in BER which has essential function to process β-elimination products that are produced by DME family 5mC DNA glycosylses during the course of DNA demethylation. In time dependent manner, APE1L and ARP showed generation of β-elimination products but not δ-elimination products.

AP endonuclease does not stimulate DNA demethylase

It has been controversial that constitution of BER complex by BER enzymes. It is reported that some glycosylase interact with replication related enzymes like PCNA and RPA (Parker et al., 2001). However in initial BER step, there was no strong evidence of interaction of DNA glycosylase and APE. When concern in APE-independent BER pathway, it is wasteful that intimate interaction of APE and glycosylase. So mechanism of DNA glycosylase and AP endonuclease seems to act on separate way. But some researchers suggest stimulation of glycosylase activity by APE (Sidorenko et al., 2007; Vidal et al., 2001). To verify that the observed excision activity on DME product also acts and interacts on co-incubation with DME I co-incubate DME on 30 nt oligonucleotide, labeled double strand containing 5mC in middle, with increasing amount of each APE. As expected, co-incubation of DME and hAPE1 or atAPE1L showed DME products and APE1L in same gel (Figure 7B). And as APE ratio to DME is increased, DME products decreased and turn over product of APE products, 3'-OH, were increased significantly. Contrastively co-incubation of DME and APE2 or ARP did not generate DME products when both APE are enough high to DME amount. Comparing the resulting excision products to treated amount of APE2 or ARP, a significant interruption on DME activity could be observed. ARP act on DME product when DME exists five times as much to ARP amount. Which confirming that APE2 and ARP is not compatible to DME in vitro. Therefore this result suggests that DNA demethylase and APE might be interact but would not stimulate their activity.

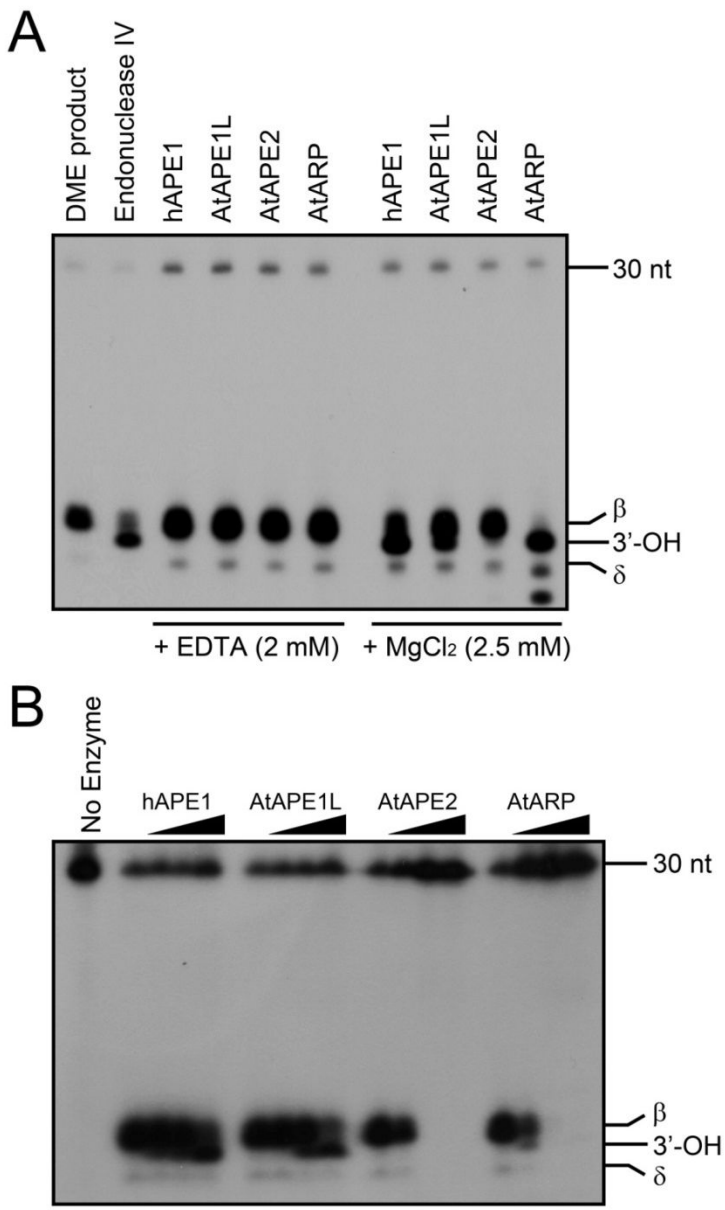

Figure 7. The 3' end cleaning activity of ARP and APE1L. (A) The 3' end processing activity of AtAPE1L, AtAPE2 and AtARP on DME products. The R30M dsDNA oligonucleotide was incubated with DME, and subsequently reacted with APEs. The DNA substrate (25 nM) was incubated with 5 nM APEs in 2 mM EDTA, 2.5 or $MgCl_2$ at 37 ℃ for 20 min. 0.5 unit of hAPE1 was used as a

reaction control. APE reaction products (14 nt with 3'-OH) are indicated at the right of the panel. (B) Co-incubation of APEs with DME. 25 nM of oligonucleotide R35M was incubated with increasing amounts of APEs (1, 5, 25, 125 nM) were co-incubated with DME at 37 ℃ for 30 min. DME products (β, δ) and APEs product (14 nt 3'-OH) are indicated at the right of the panel. Fist lane is for no enzyme control.

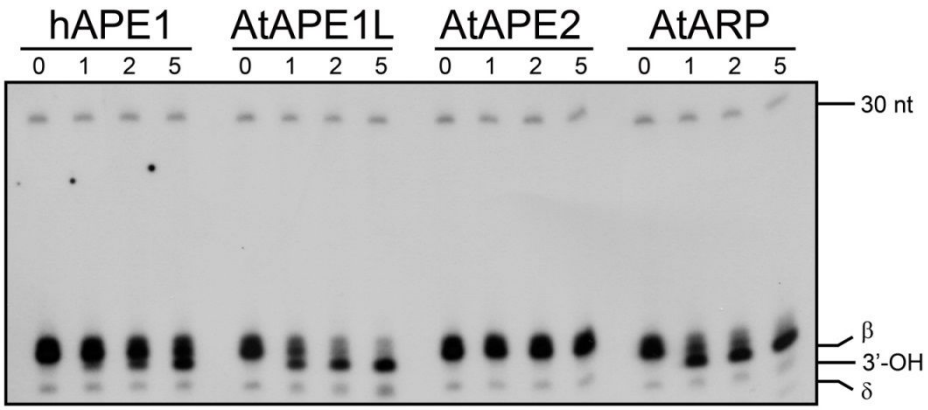

Figure 8. 3' end cleaning activity of APEs in time dependent manner. 5 nM of APEs were incubated for the indicated times on 25 nM DME products in presence of 2.5 mM $MgCl_2$ at 37 ℃ for 20 min. Lane DME products and endonuclease IV is size control of β-, δ-elimination (β, δ) and APE product (14 nt 3'-OH), respectably.

APE1L and ARP expression reduced cytotoxicity in E. coli resulted from DME expression

It was reported that expression of DME, was toxic to E. coli cells (Mok et al., 2010). Susceptibility to DME expression is due probably to the production of abasic sites and single strand breaks by DME. Such harmful lesions may compromise bacterial genome integrity. The lethal effect of DME overexpression was more severe in a strain bearing two AP endonuclease gene mutations (*xth* and *nfo*) (Gehring et al., 2006). I performed a complementation test with Arabidopsis AP endonucleases to see whether exogenous AP endonucleases would compensate for AP endonuclease mutations in E. coli (Figure 9B). I developed the expression vector to deliver AP endonuclease gene into E. coli strain RPC501 (*xth* and *nfo*) (Figure 9B). Two plasmids which have different origins and resistant genes were simultaneously transformed into bacteria. AP endonuclease and DME were expressed from vectors pYOON01 and pMAL-c2X, respectively. DME expression was induced with IPTG. And I verified colony formation for each combination of DME and AP endonuclease expression. Cell growth was affected by DME expression as the IPTG concentration increased, which was consistent with previous study (Figure 10A) (Gehring et al., 2006). By contrast, I found that expression of APE1L or ARP significantly reduced cytotoxicity caused by DME expression (Figure 10B). The expression of APE1L or ARP itself was not toxic to E. coli cells (data not shown). Such compensation was not observed for APE2 expression, suggesting no AP endonuclease activity. These results well support the in vitro activities. I therefore conclude that APE1L and ARP expression reduced cytotoxicity in E. coli by efficiently processing 3'-blocks to provide 3'-OH for subsequent BER process.

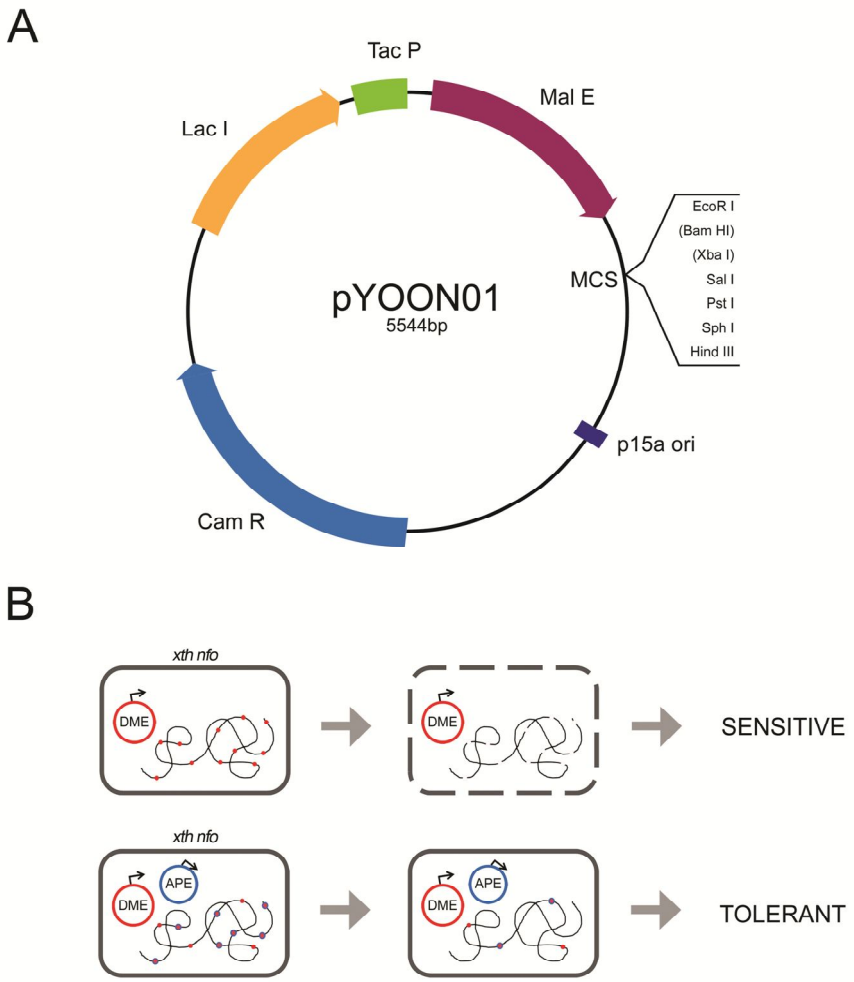

Figure 9. Complementation analysis strategy. (A) The pYOON01 vector for co-expression with pMAL-c2X expression vector. The vector is derived from the pMAL-c2X and pSTV28. (B) Scheme of toxicity test in *E. coli* strain RPC501 with AP endonuclease mutations xth- and nfo-. DME expression in the presence of IPTG is cytotoxic to E. coli probably due to excessive 3' blocks (red) generated by DME. Simultaneous expression of APE with DME reduces cytotoxicity by effectively processing 3' blocks (blue) for subsequent base incorporation.

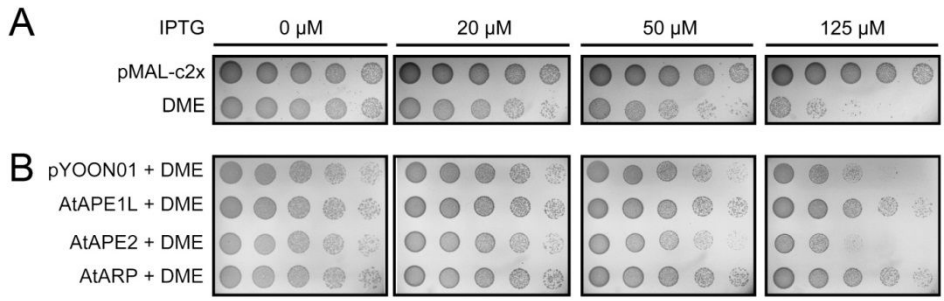

Figure 10. Complementation of AP endonuclease activity in E. coli with plant APEs. Reduced DME toxicity by exogenous APE expression. DME and/or APEs were expressed with increasing amounts of IPTG (0-125 μM) in E. coli strain RPC501. Bacterial culture was spotted on an LB agar plate with serial dilution from left to right.

A mutator phenotype in *arp* mutant

Previous study with APEs in Arabidopsis showed that single mutants were normal (Murphy et al., 2009). A double mutant of APE1L and APE2 was lethal. To clarify the function of AP endonucleases in plant development, I examined each single mutant. All single mutants were normal except for arp-1, where I found an albino phenotype with sepal and silique in an individual (Figure 11). Flower buds of the *arp-1* mutant were white from the early development (Figure 11F, 11H, 11J), and silique had green sectors on surface (Figure 11I). This albino phenotype is often variegated, when green and white sectors being mixed. Interestingly, this albino phenotype was observed single individual, whereas other siblings were normal. It is possible that a second site mutation related to chloroplast function was induced by poor DNA repair system of *arp-1* mutants. This suggests that ARP has an essential function for prevention of mutagenesis, and that its mutation resulted in the mutator phenotype due to a defect in BER.

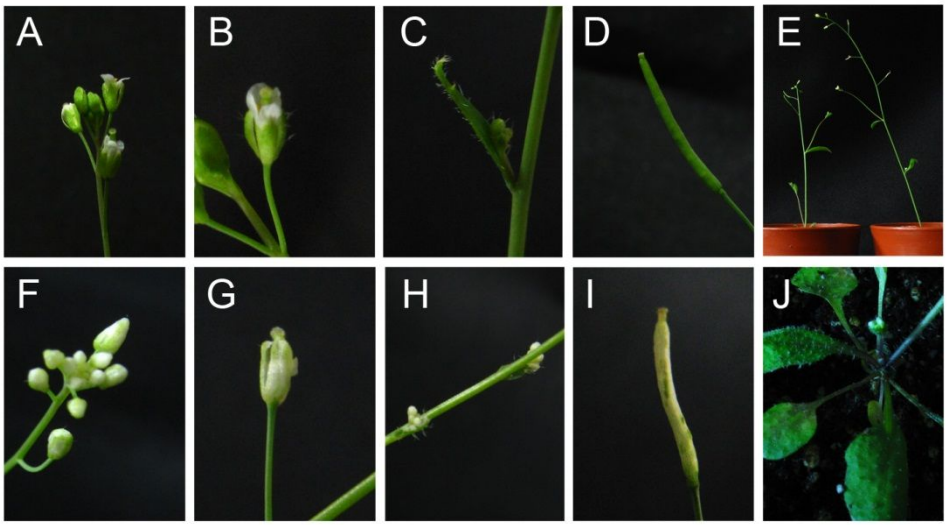

Figure 11. Albino phenotypes in a arp-1 plant. Albino sectors were found in a arp-1 plant. (A-D) Wild-type Col-0 ecotype plant. (A) Flower buds. (B) A inflorescence flower. (C) Axillary buds. (D) A silique (E) The growth of wild-type (left) and *arp-1* (right) plants. (F-J) A *arp* mutant showing variegated albino phenotype. (F) White flower buds. (G) A inflorescence flower. Spears are white. (H) Axillary buds are developed without leaves. (I) A silique showing green sectors. (J) The Somatic growth is identical to wild-type plants.

DISCUSSION

From bacteria to mammal, 2 types of class II exonuclease III (xth and nfo) have been identified. Another name of exonuclease III, AP endonuclease was initially described to have both 3' exonuclease and 3' phosphatase activities that remove 3' blocks to produce the 3'-OH terminus. A decade ago, as the Arabidopsis genome is unraveled, two Arabidopsis AP endonucleases, ARP and APE2 are predicted (Britt, 2002). The APE1L was also reported but its single mutants was normal (Murphy et al., 2009). The Arabidopsis genome does not appear to contain any nfo homologous. Among three enzymes, only ARP has shown the AP endonuclease activity for the DNA repair (Babiychuk et al., 1994; Cordoba-Canero et al., 2011). However, the difficulty in biochemical analysis makes direct evidence for the AP endonuclease activity unclear. To clarify functions of Arabidopsis AP endonuclease, I cloned and purified all three putative AP endonucleases. In this study, I provide the first detailed analysis of biochemical activity of Arabidopsis AP endonucleases. Three AP endonucleases showed distinct nuclease activity *in vitro*. I also demonstrate that *in vivo* AP endonuclease activity in the heterologous bacterial system. The findings suggest that AP endonucleases are required for efficient processing of base excision products during the course of DNA repair and DNE demethylation.

Biochemical works revealed the properties of AP endonucleases in plants. First, I checked the incision activity of Arabidopsis APEs. APE1L and ARP had the endonuclease activity on AP site. But unlike ARP, APE1L does not show strong incision activity on AP site. Their kinetic analysis of two enzymes also revealed difference in kinetic values, indicating weaker activity of APE1L than ARP. Which

implies ARP would be major enzymes contribute to repair of AP site in genome. Recently, Ariza group showed that the extract of arp mutant could not repair the U/G mismatch in vitro (Cordoba-Canero et al., 2011). This work supports propose, role of ARP as major AP endonuclease in plants. In the other hand, APE2 showed the most distinct nuclease activity among three Arabidopsis APEs consistent with previous study. The conventional AP endonuclease activity was not detected from Arabidopsis APE2, though APE2 enzymes in other organisms are known to have weak AP endonuclease activity (Burkovics et al., 2006). In addition, APE2 was not able to compensate for bacteria AP endonuclease mutants. Importantly, I found the absence of core catalytic residues in APE2 which strongly suggests the lack of activity. Remarkably, two absent residues of the APE2 are found in human APE2 and yeast APN2 (data not shown), as well as in APE1 homologs. This implies that the APE2 might have lost incision activity on AP site for the DNA repair during plant evolution for unknown reason.

Roles of APE2 are different in many species. For example, *S. pombe* APN2, ortholog of APE2, seemed to be major AP endonuclease while APN2 was found to have weak AP endonuclease activity in *S. cerevisiae* (Unk et al., 2000; Ribar et al., 2004). Role of APE2 is hard to discern because APE2 showed non-conventional AP endonuclease activity. Arabidopsis APE2 possesses an unconventional 3'-5' exonuclease activity that excises the double strands containing the AP site, which is not previously reported. Although AtAPE2 mutant was normal (Murphy et al., 2009), it seems that there will be something important functions in plant physiology in consideration of expression pattern of Arabidopsis APE2 in all tissue type examined. APE2-null mice show abnormal development in

haemopoietic organs, defect in lymphopoiesis and delayed S-phase and G_2/M-phase arrest (Ide et al., 2004). Recent study found that PCNA strongly stimulate the exonucleotic activity of human APE2 (Burkovics et al., 2009). It is possible that potential coenzyme could enhance Arabidopsis APE2 enzyme activity. Therefore more intensive study for Arabidopsis APE2 is need.

Why does Arabidopsis have two APE1 homologues while other species have only one? Two APE1 homologues, APE1L and ARP showed distinct activity of AP endonucleases. Moreover, the transcription of APE1L was relatively restricted to some many tissue types. Given that many species possess only one APE1 while Arabidopsis has two APE1 homologs, which indicates that APE1L might be responsible for other specialized role in DNA repair. So I performed the 3' end cleaning assay to figure out the function of APE1L in plants. One of possible specialized role of APE1L is catalysis of products generated by the bifunctional glycosylase. In Arabidopsis, AtOGG1, AtFPG and 5mC DNA glycosylase are identified as bifunctional glycosylase (García-Ortiz et al., 2001; Murphy et al., 2001; Gehring et al., 2006). The latter enzyme, plant specific 5-mC glycosylase recognizes and removes 5-mC, leaving 3'-dRP and 3'-phosphate (Gehring et al., 2006). The 3'-dRP is one of the substrates of APE (Agnez et al., 1996). So, AP endonucleases have been proposed to involve in DNA demethylation. APE1L indeed showed robust 3'-dRP activity which is powerful as ARP and reduced cytotoxicity generated by DME, DNA demethylase in bacteria cells. While the *arp* mutant extract fully repaired the β-elimination products generated by DNA demethylase, like wild-type extract (Martinez-Macias et al., 2012). I reasoned that APE1L in *arp* mutant extract might repair the intermediate products in demethylase

pathway. Consequently I conclude that APE1L might have main catalytic activity on the 3'-dRP generated by the plant specific DNA demethylation pathway. It is not certain that APE1L is major 3'dRPase or partially redundant with ARP. Further studies would discover the respective roles of two APE1 homologs in Arabidopsis.

It is interesting that DNA demethylation pathway might have branched pathway. The junction at the first branch points is thought to be product of DME family enzyme. DME enzymes break the phosphodiester linkage on the 3' side of 5 mC and generate β-elimination products (Gehring et al., 2006). The cleavage of portion of β-elimination products on 5' side yields δ-elimination products in many bifunctional DNA glycosylases (Bhagwat and Gerlt, 1996). My results accentuate that each β-,δ-elimination products require different enzyme to get a 3'-OH terminus for DNA demethylation. In my work, APE1L and ARP efficiently catalyze β-elimination products of DME, but not δ-elimination products. δ-elimination products were catalyzed by a plant polynucleotide kinase 3' phosphatase, ZDP, containing phosphatase activity on the 3' phosphate (Martinez-Macias et al., 2012). In a most recent study of plant XRCC was shown to stimulate enzyme activity of ZDP (Martinez-Macias et al., 2013). In mammal system, XRCC also is known to involve in interaction with the disordered N-terminal domain of APE1 and DNA polymerase β. But this coordination seemed not to be critical in DNA repair (Vidal et al., 2001). Importantly, plant LIG I had shown the genetic interaction acts on downstream of DME (Andreuzza et al., 2010). It is therefore likely that several BER enzymes would be coordinate for stimulation or regulation of DNA demethylation initiated by DME and APE.

Another putative role of APE1L and ARP is that they might have the 3'-5'

exonuclease activity processing nicks generated by the DNA demethylase. They showed 3'-5' exonuclease activity when high magnesium concentrate was treated. Interestingly, APE2 also was shown the activity like other APEs. Unlike other DNA modification, the DNA methylation exists in higher frequency in the genome (Lister et al., 2009). The distribution of the 5-mC concerned the genome to have excess nicks generated by DNA demethylase. Moreover nicks could be generated on either strand nearby, which leads double strand breaks. If these nicks are repaired one by one, universal DNA repair enzymes like DNA polymerase and ligase would waste energy to recognize numerous nicks and repair them. Especially, APE1L and APE2 have unconventional exonuclease activity on double strand containing AP site. They might increase the efficiency of DNA demethylation process if APE1L and APE2 recognize the gap in the genome and remove nucleotides from neighboring gaps. Further biochemical and mechanical studies are needed to understand the DNA repair and the plant specific DNA demethylation pathway.

Further analyses on interaction of APE and DME present some unexpected differences between APE1L and ARP compared with DME co-incubation results. Although 3' end cleaning activity on DME product was shown in both enzymes, a significant reduction of DME products and APE products was apparent in APE2 and ARP incubation with DME. The cause of decreased DME reaction products is unclear. There are several reports that APE stimulates glycosylase activity (Sidorenko et al., 2007; Vidal et al., 2001; Hill et al., 2001). Nevertheless human APE1 and bacterial nfo are structurally unrelated both stimulate only glycosylase activity (not lyase activity) of hOGG1 in similar levels.

And the stimulation of the glycosylase activity arises from permit recycling of hOGG1 by occupying the AP site by hAPE1 (Vidal et al., 2001). However APE2 and ARP seems to disrupt the binding of DME to double strand DNA containing 5mC not β-,δ-elimination products. Perhaps interference by the C-terminal zinc-finger domain of APE2 and N-terminal sap domain of ARP would randomly bind double strand DNA and prevent DNA binding of DME. My results were obtained in suspect magnesium concentration condition because DME was sensitive to Mg2+ results degradation of substrate. Even so, regardless of Mg2+, based on results present here, I believe that APE1L is the most compatible with DME among three APEs. But APE1L was not appeared to stimulate DME activity. The results suggest the uncoupling of DNA demethylase and AP endonuclease activity.

It is known that Mg2+ is essential for endonuclease activity of xth family (Demple et al., 1986). Furthermore APE1 activities were shown are regulated by the relative concentration of Mg2+ in vitro and in vivo (Wilson, 2005). According to the crystal structure of human APE1, Mg2+ is predicted to be located in a catalytic pocket. This magnesium ion appears to have role in binding of the P-O3 group after phosphodiester bond cleavage (Mol et al., 2000). In reactions with Mg2+, AtAPE1L and AtARP exhibits AP endonuclease activity and 3' block end cleaning activity with Mg2+ like that of the xth family enzymes. What is surprising is that addition of enough Mg2+ results APE2 to catalyze DME products, β-,δ-elimination generating δ-elimination product and 3'-OH end. Future studies of APE2 would present a critical insight into the role of APE in DNA demethylation

I also studied APE mutant plants to understand the function of AP endonucleases. APE1-null mice showed early embryonic lethality (Xanthoudakis et

al., 1996), but previous study with APEs in Arabidopsis showed functional redundancy (Murphy et al., 2009). Double mutant for APE1L and APE2 was lethal. In my study, *ape1l-1*, *ape2-2* and *arp-1* mutants were found to normal. However, an individual *arp-1* plant showed an albino phenotype that is spontaneous and variegated. It seemed that the white *arp-1* mutant might be caused by a second site mutation and the *arp-1* leads increase of the mutagenesis frequency of the genome. Similar base substitution in AP endonuclease mutant was reported in yeast. Deletion of yeast APN1, major AP endonuclease, resulted in a mutator phenotype inducing high frequency of base substitutions (Kunz et al., 1994; Glassner et al., 1998). The yeast APN1 is strong AP endonuclease but have weak 3' block processing activity like ARP. This implies that this mutator phenotype is generated by the AP site that is not repaired by ARP protein. And it also supports my hypothesis in which ARP is a major player in the BER pathway.

Taken together, I propose roles of each AP endonuclease in Arabidopsis. Monofunctional glycosylases produce an AP site after removing mismatched or damaged bases. The ARP seems to be major AP endonuclease that cleaves an AP site in the genome. APE1L that has weak AP endonuclease activity might be mainly involved in processing β-eliminate products generated by bifunctional glycosylases such as DNA demethylase. APE2 does not show clear evidence like described above, but it may have unknown functions. These three enzymes coordinate the BER, which is an essential component for genome stability. Future studies on further BER pathway will provide a whole insight into mechanism of DNA repair, plus the DNE demethylation pathway.

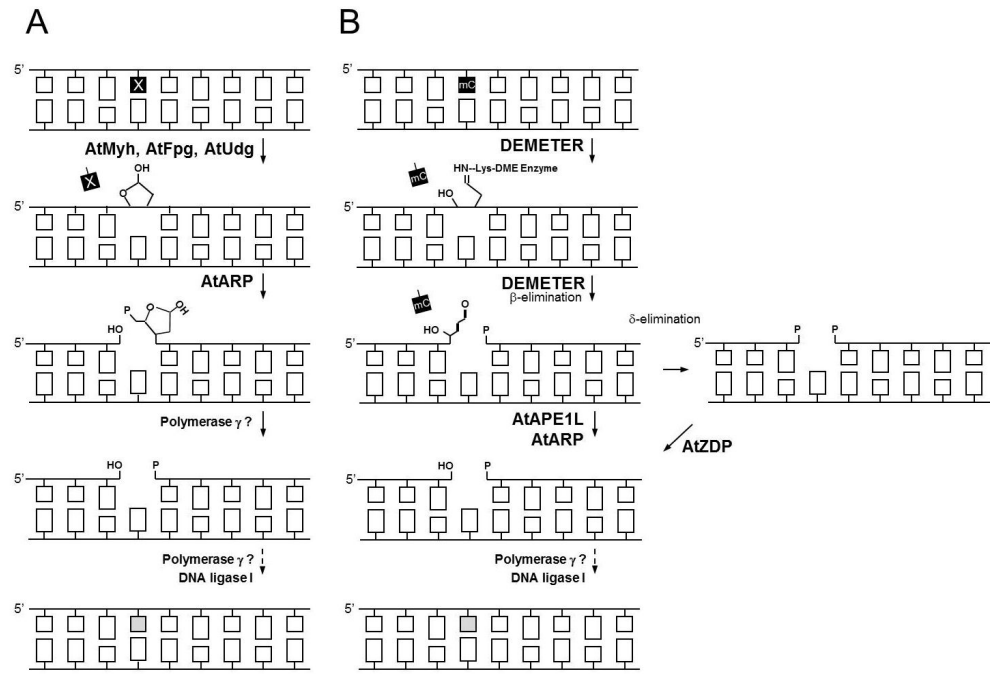

Figure 12. A model for BER pathways in Arabidopsis. Two BER subpathways (A, B) defined by the type and mechanism of DNA glycosylases are described. (A) Monofunctional glycosylases like AtMyh, AtFpg, AtUdg generate AP sites which are cleaved by AtARP. Leaved 5' dedoxyribosephosphate tuminus might be removed by pol γ resulting single nucleotide gap for nucleotide incorporation. (B) When DEMETER catalyzes β elimination, AtAPE1L and AtARP remove remained 3' dRP generating single nucleotide gap. With δ elimination product of DEMETER, a 3' phosphate terminus is produced which is removed by AtZDP.

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초 록

DNA 손상은 외부적 내부적 스트레스에 의해 화학적으로 일어나며, 돌연변이 및 노화를 일으키는 주된 원인이다. 식물은 염기서열 정보를 보존하기 위해 다양한 DNA 수리 기작을 발달시켜 왔으며, 그 중 염기 절단 수리 기작은 염기 한 개 단위로 DNA 를 수리하는 정교한 기작이라고 할 수 있다. 애기장대에는 APE1L 과 AP2, 그리고 ARP 유전자가 염기 절단 수리 과정에 참여하는 AP 엔도뉴클라제로 추정되었다. 박테리아에서 과발현한 애기장대의 AP 엔도뉴클라제들을 정제하여 생화학적 실험을 수행한 결과 3 개의 효소 중 APE1L 과 ARP 가 AP 엔도튜클라제 생화학적 활성을 보였으며 박테리아 시스템에서도 박테리아의 AP 엔도뉴클라제를 상보할 수 있는 활성을 지닌다는 것을 확인하였다. ARP 가 가장 활성이 높은 것으로 확인되어 염기 절단 수리 기작에서 ARP 유전자가 가장 주요 참여자로 작용할 것이라 추정되었다. 또한 ARP 유전자의 돌연변이체 중 한 개체가 생식기관인 꽃받침과 꼬투리에서 백화 현상이 나타남을 확인하면서 AP 엔도튜클라제가 돌연변이화 확률을 낮춘다는 것을 예상할 수 있었다. 이를 통해 애기장대의 AP 엔도뉴클라제는 DNA 염기 수리 기작에 참여하여 식물의 지놈의 안정성을 높이고 종 보존에 공헌한다는 것을 확인하였다.