A Thesis for the Degree of Master of Science

AP endonuclease promotes DNA demethylation

by facilitating the turnover of DEMETER

reaction intermediates in Arabidopsis

애기장대에서의 AP 엔도뉴클레아제에 의한
DEMETER 반응 산물의 처리 속도 증가에 따른
DNA 탈메틸화의 촉진

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ABSTRACT

DNA methylation is one of the major epigenetic modifications that control chromatin structure and gene expression. DNA methylation generally refers to the conversion of a cytosine into 5-methylcytosine (5mC) by DNA methyltransferase and plants have a distinct DNA demethylation system, in which 5mC is directly excised by DNA demethylases such as DEMETER (DME). Base excision repair (BER) pathway especially plays a critical role to process harmful lesions generated during the course of DNA demethylation by DME family proteins. Apurinic/apyrimidinic (AP) endonucleases are necessary for processing base excision intermediates in the BER pathway, but the detailed mechanisms and their contribution to DNA demethylation are largely unknown in plants. Here, it was demonstrated that one of Arabidopsis AP endonucleases APE2 has three isoforms resulted from alternative splicing, with differential activities to process an
AP site and 5mC excision intermediates to generate a 3’-OH for subsequent polymerization. In particular, the APE2.3 isoform was found to increase the 5mC excision reaction rate of DME, suggesting that APE2.3 is not only required for processing of harmful lesions, but also promotes DNA demethylation probably by increasing the turnover rate of 5mC excision intermediates.

Key Words: AP endonuclease; APE2; DME; DNA demethylation; Arabidopsis thaliana; DNA repair; Base excision repair.

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CONTENTS

ABSTRACT ....................................................... i

CONTENTS ....................................................... iii

LIST OF TABLES ............................................... v

LIST OF FIGURES ............................................... vi

LIST OF ABBREVIATIONS ................................... viii

INTRODUCTION .................................................... 1

LITERATURE REVIEWS ......................................... 6

1. The BER pathway
2. DNA methylation
3. DNA demethylation mediated by DNA glycosylases and DME
4. AP endonucleases

MATERIAL AND METHODS ................................... 14

Plant materials and growth conditions

RNA isolation and gene expression level analysis for APE2 isoforms
Cloning of APE2.2 and APE2.3 for protein expression
Expression and purification of the proteins
*In vitro* assay for AP endonuclease and DME
Electrophoretic mobility shift assay
Molecular cloning of APE2.2 and APE2.2 into pYOON01
Bacterial cell toxicity assay

**RESULTS** ................................................................. 23

APE2 has three isoforms because of alternative splicing
APE2.2 and APE2.3 has AP endonuclease activity on the
AP sites and DME excision intermediates
APE2.3 stimulates DNA demethylase activity
APE2.2 and APE2.3 expression could not reduce
cytotoxicity resulted from DME expression in *E. coli*

**DISCUSSION** ............................................................. 42

**REFERENCES** ............................................................ 47

**ABSTRACT IN KOREAN** ................................................. 58
LIST OF TABLES

Table 1. List of primers for PCR amplification

Table 2. Oligonucleotides for in vitro activity test
LIST OF FIGURES

Figure 1. Schematic illustration of the BER pathway

Figure 2. Analysis of the purified proteins of five *Arabidopsis* AP endonucleases and DME

Figure 3. Three isoforms of *APE2* in *Arabidopsis*

Figure 4. Alternative splicing of *APE2* isoforms

Figure 5. AP endonuclease activity on the AP site

Figure 6. AP endonuclease activity on the DME products

Figure 7. AP endonuclease assay for 3’ phosphatase activity

Figure 8. Electrophoretic mobility shift assay of *APE2* isomers

Figure 9. DME reaction with various metal cation conditions
Figure 10. Co-incubation tests

Figure 11. Complementation of AP endonuclease activity in *E. coli* with plant APEs

Figure 12. Schematic illustration of DNA demethylation pathway in *Arabidopsis*
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>5mC</td>
<td>5-methylcytosine</td>
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<tr>
<td>DME</td>
<td>DEMETER</td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>AP</td>
<td>Abasic, apurinic/apyrimidinic</td>
</tr>
<tr>
<td>DRM2</td>
<td>DOMAINS REARRANGED METHYLTRANSFERASE 2</td>
</tr>
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<td>RdDM</td>
<td>RNA-directed DNA methylation</td>
</tr>
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<td>DNA METHYLTRANSFERASE 1</td>
</tr>
<tr>
<td>CMT3</td>
<td>CHROMO METHYLTRANSFERASE 3</td>
</tr>
<tr>
<td>APE</td>
<td>Abasic endonuclease</td>
</tr>
<tr>
<td>3’-PUA</td>
<td>3’-Phosphor-α,β-unsaturated aldehyde</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>Magnesium ion</td>
</tr>
<tr>
<td>hOGG1</td>
<td>8-OXOGUANINE-DNA GLYCOSYLASE 1</td>
</tr>
<tr>
<td>MPG</td>
<td>N-METHYLPURINE-DNA GLYCOSYLASE</td>
</tr>
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<td>Exo III</td>
<td>EXONUCLEASE III</td>
</tr>
<tr>
<td>Endo IV</td>
<td>ENDONUCLEASE IV</td>
</tr>
<tr>
<td>hMYH</td>
<td>human MutY homolog</td>
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<tr>
<td>APE1L</td>
<td>AP ENDONUCLEASES ABASIC ENDONUCLEASE-1-LIKE</td>
</tr>
<tr>
<td>ARP</td>
<td>APURINIC ENDONUCLEASE-REDOX</td>
</tr>
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<td>Description</td>
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<tr>
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<tr>
<td>ZDP</td>
<td>ZINC FINGER DNA 3’-PHOSPHATASE</td>
</tr>
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<td>DNMT1</td>
<td>DNA METHYLTRANSFERASE 1</td>
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<td>5-Hydroxymethylcytosine</td>
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<td>THYMINE DNA GLYCOSYLASE</td>
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<td>REPRESSOR OF SILENCING</td>
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<td>DME-LIKE 2</td>
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<td>AP ENDONUCLEASE 1</td>
</tr>
<tr>
<td>MMS</td>
<td>Methyl-methanesulfonate</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
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<tr>
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<td><em>Zea mays</em> DIPHOSPHONUCLEOTIDE PHOSPHATASE 2</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription PCR</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>CD-search</td>
<td>Conserved domain-search</td>
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\[\beta \quad \beta\text{-elimination products} \]
\[\delta \quad \delta\text{-elimination products} \]
INTRODUCTION

In eukaryotes, DNA methylation often refers to the addition of a methyl group at the fifth carbon of cytosine in sequence contexts of CG, CHG, and CHH (H = A, T or C) in plants. 5mC is the major epigenetic modification, and mediates transcriptional activation and silencing. Previous studies showed that DNA methylation on gene body upregulates gene expression. In contrast, DNA methylation located in the promoter or enhancer region represses gene expression (Ball et al., 2009; Lister et al., 2009). DNA methylation also plays the essential roles in developmental processes, such as transposon silencing and gene imprinting, and in biotic and abiotic stress responses.

DNA methylation pattern is delicately regulated according to the specific cell type and time. For example, the central cell and vegetative cell in the plant female and male gametophytes display extensive changes in DNA methylation pattern before fertilization (Ibarra et al., 2012). There are two categories of DNA methyltransferase enzymes which can mediate transfer of methyl group from S-adenosyl-methionine to cytosine generating 5mC. In plants, DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), the de novo DNA methyltransferase, mediates DNA methylation in the CHH sequence context using the RNA-directed DNA methylation (RdDM) pathway which is a plant specific DNA methylation mechanism (Mette et al., 2000). For maintenance of DNA methylation during the cell division, DNA methylation at CG and CHG sequence contexts is mediated by DNA METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLTRANSFERASE 3 (CMT3), respectively.
DNA demethylation is a reverse process of DNA methylation. There are two kinds of DNA demethylation mechanisms, the passive and active DNA demethylation. Inactivation of maintenance DNA methyltransferase, such as MET1 in plants, is involved in passive DNA demethylation, and DNA methylation level gradually decreases in a replication-dependent manner. However, active DNA demethylation is enzymatically induced by specific DNA demethylases in a replication-independent manner (Wu and Zhang, 2010).

The BER pathway plays vital roles in active DNA demethylation pathway. DNA demethylation in plants is likely to involve direct removal of 5mC by specific DNA glycosylases, and the BER pathway mediates cytosine replacement (Agius et al., 2006; Gehring et al., 2006; Gehring et al., 2009; Morales-Ruiz et al., 2006; Zhu, 2009). The DNA glycosylase, which is the first enzyme of the BER pathway, recognizes a modified or damaged base and hydrolyzes a N-glycosyl bond between deoxyribose and the base, generating an abasic (apurinic/apyrimidinic, AP) site. The phosphodiester bond is immediately incised by abasic endonuclease (APE) creating a single strand break with a 3’-OH. Subsequently, DNA polymerase and the DNA ligase fill the gap. In this way, the BER pathway repairs DNA bases with modification, and also mediates DNA demethylation.

The 5mC DNA glycosylase family proteins in Arabidopsis catalyze DNA demethylation. DME is a founding DNA demethylase family member in Arabidopsis and displays activity of a bifunctional DNA glycosylase/AP lyase. Bifunctional DNA glycosylase/lyase excises the N-glycosyl bond of 5mC by β-elimination and δ-elimination, generating 3’-phosphor-α,β-unsaturated aldehyde (3’-PUA) and 3’-phosphate, respectively (Gehring et al., 2006). To complete DNA demethylation,
DME excision intermediates should be processed to 3’-OH for subsequent polymerization, and these processes are likely mediated by AP endonucleases.

Notably, intranuclear concentration of magnesium ion (Mg\(^{2+}\)) is highly variable, and the role of Mg\(^{2+}\) on DNA glycosylases is not well understood in *Arabidopsis*. However, some researches in human suggest that Mg\(^{2+}\) lowers the efficiency of the base excision or strand incision activity of 8-OXOGUANINE-DNA GLYCOSYLASE 1 (hOGG1), the bifunctional DNA glycosylase with AP lyase activity in human, on DNA containing 8-oxoG (Morland et al., 2005). In addition, Mg\(^{2+}\) is not required for the activity of N-METHYLPURINE-DNA GLYCOSYLASE (MPG), the monofunctional DNA glycosylase in human, but Mg\(^{2+}\) can significantly inhibit activity of MPG at physiologically relevant concentrations by eliminating its substrate binding ability without any influence on its catalytic chemistry. However, Mg\(^{2+}\) contribution to *Arabidopsis* DNA glycosylase activity should be further investigated.

AP endonuclease is conserved in many organisms from bacteria to eukaryotes. There are two types of class II AP endonucleases; EXONUCLEASE III (Exo III) and ENDONUCLEASE IV (Endo IV) families (Demple et al., 1986; Demple and Harrison et al., 1994). In *Escherichia coli*, Exo III is the major AP endonuclease for the DNA repair (Rogers and Weiss, 1980). In human, there are two AP endonucleases, hAPE1 and hAPE2. hAPE1 displays robust AP endonuclease activity (Hadi et al., 2002). Both of hAPE1 and hAPE2 possess 3’-phosphodiesterase and 3’-5’ exonuclease activities (Demple and Harrison et al., 1994).

Mg\(^{2+}\) is the essential requirement for activities of the major BER enzymes (Izumi et al., 2003), so it may function as a BER pathway regulator for the effective
and balanced repair of damaged bases that are often less toxic or mutagenic than their subsequent products of repair intermediates. Also, earlier researches of DNA glycosylases/AP lyases in bacteria and most eukaryotes show that their AP lyase activity is comparable with the base excision activity, in order that no free AP sites could be generated during the removal course of damaged base (Rabow and Kow, 1997). In previous study, hOGG1 is significantly stimulated by hAPE1 which is the downstream enzyme of hOGG1 in BER pathway (Hill et al., 2001). Also, it appears that hAPE1 stimulates activity of human MutY homolog (hMYH) DNA glycosylase by increasing formation of the hMYH-DNA complex. This stimulation is independent from the hAPE1 catalytic activity (Yang et al., 2001). These previous studies suggest coordinated functions of DNA glycosylase with AP endonuclease and possibly other enzymes in the BER pathway.

In *Arabidopsis*, three putative AP endonucleases, *AP ENDONUCLEASES ABASIC ENDONUCLEASE-1-LIKE (APE1L)*, *APE2* and *APURINIC ENDONUCLEASE-REDOX (ARP)*, have been identified as homologs of *hAPE1*. Each single mutant does not display any developmental defects. However, seed abortion occurs in the double homozygous *ape1l/ape2* mutant (Murphy et al., 2009). Therefore, APE1L and APE2 are crucial in seed development and active DNA demethylation procedures.

In one study for *in vitro* biochemical activity of three AP endonucleases, only ARP can process analogs of the AP sites. ARP and APE1L harbor the 3’-phosphodiesterase activity, but APE2 display no repair activity (Lee et al., 2014). In contrast, another study shows that all three proteins in *Arabidopsis* harbor the processing activity about the AP sites. In addition, only APE1L can repair 3’-PUA
and 3’-phosphate, generating 3’-OH (Li et al., 2015). ZINC FINGER DNA 3’-
PHOSPHATASE (ZDP) is able to remove 3’-phosphate, but not 3’-PUA, generating 
3’-OH (Martinez-Macias et al., 2012). Moreover, double homozygous mutants of 
apel/zdp show seed abortion, but not arp/zdp nor ape2/zdp (Lee et al., 2014; Li et 
al., 2015).

In this study, it was demonstrated that APE2, which is one of Arabidopsis 
AP endonuclease homologs, has three types of isoforms. These isoforms have 
different and distinct functions to repair the AP site and DME excision intermediates 
to produce 3’-OH for subsequent polymerization. They were named APE2.1, APE2.2, 
and APE2.3, respectively. In particular, when APE2.3 isoform and DME were co-
incubated, they facilitate activities of each other, producing 3’-OH. This result 
suggests that APE2.3 is the most proper form of APE2 gene to facilitate DME 
turnover rate, and that contributes significantly in active DNA demethylation 
pathway in Arabidopsis.
LITERATURE REVIEWS

1. The BER pathway

Because of the susceptibility of DNA to various chemical modifications delivered by endogenous or exogenous agents, the genetic integrity is under constant threat (Lindahl, 1993). DNA repair systems found in every domain of life resist to threat on genetic information (Lindahl and Wood, 1999; Scharer, 2003). The BER pathways is one of crucial protective systems, and deals with damage appearing at the nitrogenous bases of DNA (Krokan et al., 2013; Kim and Wilson, 2012; Lindahl, 1974; Wallace, 2014).

Three kinds of chemical modification, alkylation mediated by endogenous or exogenous electrophiles, hydrolytic deamination resulting from exocyclic amino groups, and oxidation caused by various reactive oxygen species, can occur to the nucleobases of DNA (David et al., 2007; Lindahl, 1993). For example, there are 3-methyladenine by methylation of adenine, uracil by deamination of cytosine, and 8-oxoguanine by oxidation of guanine. These examples and other plenty of harmful base lesions arising in DNA are repaired by the BER pathway. If the BER pathway does not happen, lesions caused by DNA damages can lead to mutations, hinder critical DNA operations such as replication or transcription, and trigger apoptosis (Wilson and Bohr, 2007). Uncorrected base damage also results in premature aging and diseases such as cancer (Wilson and Bohr, 2007).

The basic steps of the BER pathway are conserved in prokaryotes and higher eukaryotes while they differ in minute chemical reactions and the enzymes. The steps of BER involve (i) incision of the modified base mediated by DNA
glycosylase, leaving an AP site; (ii) excision of the phosphodiester bond of the abasic sugar at one or both sides; (iii) trimming of the termini to produce the 3’-OH which is essential for the following DNA synthesis and ligation steps; (iv) DNA synthesis to take place of the removed nucleotide; and (v) ligation of the remaining nick (Figure 1).

There are two general classes of DNA glycosylases according to the reaction mechanisms: monofunctional and bifunctional DNA glycosylases (Figure 1) (Drohat and Maiti, 2014; Stivers and Jiang, 2003). Both categories of DNA glycosylases initiate the BER pathway. Monofunctional DNA glycosylases catalyze hydrolytic excision of the N-glycosyl bond, generating the AP site. On the contrary, bifunctional DNA glycosylases generally use an amine nucleophile to cleave the N-glycosyl bond, yielding imine intermediate (Prakash et al. 2012). This intermediate is used to initiate the AP lyase activity. Moreover, some bifunctional DNA glycosylases can catalyze hydrolysis of the N-glycosyl bond generating the AP site.

AP endonucleases in the BER system act directly downstream of DNA glycosylases, and they are expectedly essential to cleanup harmful lesions. In the BER pathway, class II AP endonucleases play the crucial roles in processing the AP sites and blocking termini to produce 3’-OH which is accessible to DNA synthesis enzymes (Mitra et al., 1997; Mitra et al., 2001; Suh et al., 1997).

2. DNA methylation

DNA methylation is one of the major epigenetic modifications that controls chromatin structure and gene expression (Jones, 2012; Law and Jacobsen, 2010; Smith and Meissner, 2013). DNA methylation is the conversion of cytosine to
Figure 1. Schematic illustration of the BER pathway. The damaged base or 5mC is colored in black. Replaced bases are represented in gray.
5mC, and is catalyzed by DNA methyltransferases (Law and Jacobsen, 2010). DNA methylation often occurs within the context of CG in animals, but is also observed in CHH and CHG contexts in plants (Bauer and Fischer, 2011; Cokus et al., 2008; Zhang and Zhu, 2012). Repressed state of chromatin is associated with DNA methylation, and tight regulation of DNA methylation is critical in organisms because it is important for various developmental processes such as gene imprinting and transposon silencing (Huh et al., 2008; Law and Jacobsen, 2010; Nabel et al., 2012).

DNA methylation has reversibility similar to other most epigenetic modifications, and DNA demethylation is the reverse process of DNA methylation. DNA demethylation can be categorized into two mechanisms. The level of 5mC can gradually reduce in a replication-dependent manner by passive DNA demethylation because of inactivation of maintenance activity of DNA methyltransferases like DNA METHYLTRANSFERASE 1 (DNMT1) and MET1, in mammals and plants, respectively. On the contrary, active DNA demethylation occurs in a replication-independent manner, and the BER pathway especially plays the critical roles in active DNA demethylation pathway (Wu and Zhang, 2010).

3. DNA demethylation mediated by DNA glycosylases and DME

One of the most important roles for the BER pathway is active DNA demethylation, a multiple enzymatic pathway which converts 5mC into cytosine (Bellacosa and Drohat, 2015; Kohli and Zhang, 2013). DNA glycosylases play the critical roles in finding and excising damaged or modified bases to initiate the BER pathway. In plants, specific DNA glycosylases directly recognize and excise 5mC,
but DNA demethylation in mammals does not involve direct elimination of 5mC. Instead, chemical modifications of 5mC are initiated to generate thymine by oxidative deamination or 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) by oxidation processes. After modification, mismatch DNA glycosylases like THYMINE DNA GLYCOSYLASE (TDG) excise altered bases (Cortellino et al., 2011; Guo et al., 2011; Song and He, 2013; Tahiliani et al., 2009; Williams et al., 2011; Xu et al., 2011).

DME is one of the plant specific DNA demethylases, and DME is the first identified DNA demethylase in Arabidopsis (Choi et al. 2002). Wild-type Arabidopsis rarely aborts their seeds, but DME heterozygous mutant plants present 50% seed abortion. Therefore, DME activities are essential for seed viability. DME, REPRESSOR OF SILENCING (ROS1), DME-LIKE 2 (DML2), and DML3, which are the DME family proteins in Arabidopsis, display bifunctional 5mC DNA glycosylase activity in vitro (Agius et al., 2006; Gehring et al. 2006; Morales-Ruiz et al., 2006; Ortega-Galisteo et al., 2008; Penterman et al., 2007). They can catalyze 5mC excision and the cleavage of a sugar-phosphate backbone through β- and δ-elimination reactions, yielding 3’-PUA and 3’-phosphate, respectively. For subsequent polymerization, these harmful lesions must be processed to 3’-OH, and further demethylation steps need machineries of the BER pathway.

4. AP endonucleases

AP endonucleases have been classified into four types according to their reaction mechanisms and incision sites. Class I AP endonucleases, which are known as AP lyases, cleave 3’ to the AP sites by β-elimination reactions mechanism,
generating 3’-(4-hydroxyl-5-phospho-2-pentenal) residues and 5’-phosphate (Bailly and Verly, 1987; Manoharan et al., 1988; Kim and Linn, 1988). All class I AP endonucleases possess associated DNA glycosylase activity that can catalyze the incision of modified bases (Wallace, 1988; Boiteux et al., 1990). By contrast, class II AP endonucleases do not have associated glycosylase activity, and incise 5’ to the AP sites, leaving 3’-OH and 5’-phosphate residues (Weiss et al., 1978; Warner et al., 1980; Mosbaugh and Linn, 1980). Class III and IV enzymes incise 3’ and 5’ to the AP sites, respectively and produce 3’-phosphate and 5’-OH. Most predominant AP endonucleases in organisms are in class II (Myles and Sancar, 1989).

Class II AP endonucleases have been categorized into two families according to their homology to the two enzymes in Escherichia coli, the Exo III and Endo IV families. Exo III is the main AP endonuclease in E. coli, and accounts for 90% of the total AP endonuclease activity. By contrast, Endo IV represents 10% of the cellular AP endonuclease activity (Seeberg et al, 1995). The AP ENDONUCLEASE 1 (APN1) and APN2 proteins in Saccharomyces cerevisiae are homologous to the Endo IV and the Exo III family proteins, respectively (Johnson et al., 1998; Unk et al., 2000). APN1 displays a strong AP endonuclease activity in yeast cells representing more than 90% of the total cellular AP endonuclease activity (Popoff et al., 1990; Seeberg et al, 1995). On the contrary, the APN2 protein exhibits very weak AP endonuclease activity. APN2 also has strong 3’-exonuclease-phosphodiesterase activities (Unk et al., 2000; Unk et al., 2001; Unk et al., 2002). In addition, apn1Δ strain shows much higher sensitivity to methyl-methanesulfonate (MMS), an alkylating agent, than the apn2Δ strain. Neither of the single mutant strains display sensitivity to hydrogen peroxide (H₂O₂), an oxidative agent, but the
apn1Δapn2Δ double mutant does (Unk et al., 2001). APN1 plays a more prominent role in the AP sites processing than the APN2 in *S. cerevisiae*, while APN1 and APN2 identically repair for 3’ oxidatively damaged DNA termini. By contrast, APN2 displays the prominent AP endonuclease activity, whereas the APN1 shows a back-up activity in *Schizosaccharomyces pombe*, which is phylogenetically closer to mammalians than *S. cerevisiae* (Ribar et al., 2004).

In case of humans, Endo IV homologs are unknown, but two Exo III family proteins, hAPE1 and hAPE2, have been identified. hAPE1 displays strong AP endonuclease activity representing more than 95% of the total cellular AP endonuclease activity. Therefore, hAPE1 is believed to be the notable AP endonuclease in human cells. In addition, it also shows phosphodiesterase and exonuclease activities (Suh et al., 1997; Wilson et al., 1995). hAPE2 is relatively unknown, but hAPE2-null mice show the growth retardation and dyshematopoiesis with G2/M arrest phenotype. hAPE2 also displays very weak AP endonuclease activity, but strong 3’-5’ exonuclease and 3’-phosphodiesterase activities (Burkovics et al., 2006).

The genome of *Arabidopsis* includes three genes that encode homologs of *hAPE1, APE1L, APE2*, and *ARP*. In previous genetic research, *Arabidopsis* mutant plants that are deficient for single AP endonuclease genes display no obvious defective phenotype, but double knock-out of *APE1L* and *APE2* genes shows seed abortion (Murphy et al., 2009). Therefore, APE1L and APE2 are essential for repair of endogenous DNA damage and for the harmful 3’-end processing procedure during active DNA demethylation in seed development.

In one study for *in vitro* assay of *Arabidopsis* AP endonucleases, only ARP
can repair the AP sites, and both of ARP and APE1L contain a 3’-phosphodiesterase activity, while APE2 showed no repair activity (Lee et al., 2014). However, an other study reports that all three proteins contain repair activity for the AP site, but only APE1L efficiently repairs the 3’-PUA and possesses 3’-phosphatase activity producing 3’-OH (Li et al., 2015).

The first plant enzyme which acts on 3’-phosphate was identified in maize and termed Zea mays DIPHOSPHONUCLEOTIDE PHOSPHATASE 2 (ZmDP2) (Betti et al., 2001). Its homolog in Arabidopsis is ZDP containing a C-terminal 3’-phosphatase domain and an N-terminal DNA binding domain (Petrucco et al., 2002). ZDP is 3’ DNA phosphatase and catalyzes the conversion of 3’-phosphate, but not 3’-PUA, to a 3’-OH. ZDP can also remove the AP sites and 5mC by combined action with ROS1 in the absence of ARP (Martinez-Macias et al., 2012). Previous reports agree that double homozygous mutants of ape1l/zdp, but not arp/zdp nor ape2/zdp, display seed abortion (Lee et al., 2014; Li et al., 2015). Therefore, APE1L and ZDP are crucial in seed development and active DNA demethylation procedures.
MATERIALS AND METHODS

Plant materials and growth conditions

*Arabidopsis thaliana* Col-0 seeds were sterilized with bleach solution (30% bleach, 0.025% Triton X-100) for 3 min, and were washed with sterile water. After 2 days at 4°C, seeds were sowed on an MS medium with 2% sucrose and 0.8% agar at pH 5.7. For 10 days after germination, seedlings were transferred to soil, and were grown under 16 h of light and 8 h of dark cycles at 23°C.

RNA isolation and gene expression level analysis for *APE2* isoforms

Total RNA was extracted from various tissues of Col-0 plants using the TRIZOL reagent (Ambion, Austin, TX, USA). cDNA was synthesized using a QuantiTect reverse transcription kit (Qiagen, Hilden, Germany) as described by the manufacturer. The expression levels of *APE2* isoforms were examined by reverse transcription PCR (RT-PCR) using isoform specific primers. The primers used to amplify *APE2.1* mRNA were DG2637 and DG2638. The primers used to amplify *APE2.2* mRNA were DG2639 and DG2640. The primers used to amplify *APE2.3* mRNA were DG2638 and DG2639. The primers used to amplify control *ACTIN11* mRNA were DG243 and DG244. The primer information is listed in Table 1.

Cloning of *APE2.2* and *APE2.3* for protein expression

The full-length sequences of *APE2.2* and *APE2.3* were isolated by RT-PCR using primers DG2624 and DG2625 to generate enzyme site at 5’ and 3’ ends with *Bam* HI and *Sal* I, respectively. The primers for amplification of *APE2.2* and *APE2.3*
Table 1. List of primers for PCR amplification

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<th>primer</th>
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<td>DG2637</td>
<td>CCATGATAAGAGTATTACTCTGTTGAATCTAGG</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>DG2638</td>
<td>TGTCTTCTGGATTAGAGGAAGGTCC</td>
<td>RT-PCR</td>
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<tr>
<td>DG2639</td>
<td>ATCATTTGCTTCCAGGAGACG</td>
<td>RT-PCR</td>
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<tr>
<td>DG2640</td>
<td>TAACCACAGTTGTGCTTTCAG</td>
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</tr>
<tr>
<td>DG2624</td>
<td>AATTTGATCCATGAGATAGTCTAAACAGG</td>
<td>cloning PCR</td>
</tr>
<tr>
<td>DG2625</td>
<td>AATGTCGACTCATTTTGATCTCATCCTCTTTTA</td>
<td>cloning PCR</td>
</tr>
<tr>
<td>DG210</td>
<td>CTGATGGGAAAGGAGATGAAAAAGAAGGG</td>
<td>colony PCR</td>
</tr>
<tr>
<td>DG083</td>
<td>CAGCCCTCCTCGGAAGACTC</td>
<td>colony PCR</td>
</tr>
<tr>
<td>DG218</td>
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<tr>
<td>DG482</td>
<td>CTGCAAGGCGATTAAGTTGGGTAAC</td>
<td>colony PCR</td>
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were same, and they were distinguished by sequencing. pLM302 vector was taken from the previous study (Lee et al., 2014). The products were digested with Bam HI and Sal I, and were cloned into the pLM302 vector at the corresponding restriction sites creating the pLM302-APE2.2 and pLM302-APE2.3. Subsequently, the plasmids were transformed into E. coli Rosetta2 (DE3) strain (EMD Millipore, Darmstadt, Germany) for protein expression. The constructs for pLM302-APE1L, pLM302-ARP and pLM302-APE2.1 described in the previous study (Lee et al., 2014) were transformed into the same E. coli Rosetta2.

**Expression and purification of the proteins**

A single colony was inoculated in 5 mL of LB medium containing kanamycin (50 µg mL⁻¹) and chloramphenicol (50 µg mL⁻¹) at 37°C overnight with shaking. An aliquot of culture was inoculated into 1 L LB medium with kanamycin and chloramphenicol, and was incubated at 28°C until the OD₆₀₀ reached 0.7. Expression was induced by adding 0.1 M of isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Sigma, St.Louis, MO, USA), and the culture was shaken at 18°C for 4 h. Cells were harvested by centrifugation at 7,000 rpm at 4°C for 20 min. The pellet was resuspended in 23 mL of lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, 0.1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF)). The lysate was sonicated, and was clarified by centrifugation at 12,000 rpm at 4°C for 1 h. The filtered supernatant was loaded onto the 5 mL HisTrap FF column (GE Healthcare, NJ, USA), and was eluted with a linear gradient of imidazole (1 mM to 100 mM). Fractions were collected, and were loaded onto the 5 mL HisTrap Heparin HP column (GE Healthcare, NJ, USA). These were eluted with
a linear gradient of NaCl (0.1 M to 1 M). Collected fractions were concentrated, and were stored in a storage buffer (20 mM Tris-HCl, pH 7.4, 40 mM NaCl, 0.1 mM dithiothreitol, 50% glycerol) at -80°C. The same method was used for protein purification of all APE homologs in *Arabidopsis* (Figure 2).

**In vitro assay for AP endonuclease and DME**

Oligonucleotides used in this study are listed in Table 2. Oligonucleotide substrates were synthesized from Integrated DNA Technologies (IA, USA). Twenty pmol of 35-nt double stranded DNA containing a single tetrahydrofuran (THF), the AP site analog, at position 18 on a top strand (F35[AP]) was end-labeled with 30 μCi of [γ-32P] ATP (6,000 Ci mmol⁻¹, Bio-Medical Science, Korea) in a 50 μL reaction using 20 units of T4 polynucleotide kinase (Takara, Tokyo, Japan) at 37°C for 1.5 h. The labeled oligonucleotide was purified using a Qiaquick Nucleotide Removal Kit (Qiagen, Hilden, Germany) as described by the manufacturer’s protocol. Labeled oligonucleotides were boiled for 10 min to anneal with complementary oligonucleotides (R35), and then cooled down to room temperature slowly. The DNA substrate (25 nM) was incubated with or without APEs in 16 μL of APE reaction buffer (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 200 μg mL⁻¹ bovine serum albumin (BSA), 2.5 mM MgCl₂, 0.5 mM DTT) for 2 hr at 37°C. 0.4 unit of hAPE1 (New England Biolabs, Ipswich, MA, USA) was used as a reaction control. Reactions were terminated with adding 16 μL of stop solution (95% formamide, 20 mM ethylenediaminetetraacetic acid (EDTA), 0.05% bromophenol blue, 0.05% xylene cyanol FF) and boiling for 10 min.

For DME reaction test and 3’ end cleaning assay using intermediates synthe
Figure 2. Analysis of the purified proteins of five *Arabidopsis* AP endonucleases and DME. 200 ng of protein samples were running on 10% polyacrylamide gel using electrophoresis, and were visualized by coomassie brilliant blue staining.
Table 2. Oligonucleotides for *in vitro* activity test

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
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<tr>
<td>F35[AP]</td>
<td>5’-GTACTGTGTGATACTAT[THF]GAATTCA GTATGATCTG</td>
</tr>
<tr>
<td>F35[5mC]</td>
<td>5’-GTACTGTGTGATACTAT[5mC]GAATTCA GTATGATCTG</td>
</tr>
<tr>
<td>F17F[3P]</td>
<td>5’-GTACTGTGTGATACTAT[3Phos]</td>
</tr>
<tr>
<td>[5P]F17B</td>
<td>5’-[5Phos]GAATTCA GTATGATCTG</td>
</tr>
<tr>
<td>R35</td>
<td>5’-CAGATCATACATGAATTGATACACACAGTAC</td>
</tr>
</tbody>
</table>

*THF, tetrahydrofuran; 5mC, 5’-methylacytosine; Phos, phosphorylation*
-sized after DME reaction, radiolabeled and purified 35-nt double stranded DNA containing a 5mC in the center of the top strand (F35[5mC]) was prepared in the same manner to be used for DME substrates. 25 nM of labeled oligonucleotides were incubated with 100 nM DME in the 16 µL of DNA glycosylase reaction buffer (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 200 µg mL⁻¹ BSA, 0.5 mM DTT) for 1 h at 37°C. For DME reaction, reactions were terminated with adding 16 µL of stop solution. For 3’ end cleaning assay, reaction tubes were heated to 65°C for 15 min for DME inactivation. Then, APEs were added forming 20 µL APE reaction buffer (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 200 µg mL⁻¹ BSA, 2.5 mM MgCl₂, 0.5 mM DTT), and were inoculated for 2 h at 37°C. All reactions were terminated with adding 20 µL of stop solution.

For preincubation of DME with Mg²⁺, Mg²⁺ was added to DME in storage buffer, and the concentration of MgCl₂ reached to 2.5 mM. Then, DME incubated at 37°C for 30 min. Next, 25 nM of labeled oligonucleotides were incubated with 100 nM DME in the 16 µL of DNA glycosylase reaction buffer for 1 h at 37°C. Reaction was terminated with adding 16 µL of stop solution.

For co-incubation assay, oligonucleotides containing 5mC were prepared for substrates in same way. End-labeled oligonucleotides were incubated with 100 nM DME and 100 nM APEs in the 16 µL of APE reaction buffer (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 200 µg mL⁻¹ BSA, 2.5 mM MgCl₂, 0.5 mM, DTT) for 1 h at 37°C. All reactions were terminated with adding 16 µL of stop solution.

The 17-mer oligonucleotides with a 3’-phosphate (F15F[3P]) were 5’-end-labeled with [γ-³²P] ATP using T4 Polynucleotide Kinase (3’phosphatase minus) (NEB). After purification, these radiolabeled upstream oligonucleotides were
annealed together to the 35-mer complementary strand (R35) and another 17-mer non-labeled upstream oligonucleotide ([5P]F18B) in order to produce DNA substrate with a single nucleotide gap. 25 nM DNA substrate was incubated with APEs at 37°C for 2 h in the 16 µL of APE reaction buffer (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 200 µg mL^-1 BSA, 2.5 mM MgCl₂, 0.5 mM DTT). Reactions were terminated with adding 16 µL of stop solution and boiling for 10 min.

All products were separated on a 15% polyacrylamide gel containing 7.5 M urea and 1× TBE. Electrophoresis was done at 1,200 V for 4 h. The gel was exposed to an X-ray film at −80°C.

**Electrophoretic mobility shift assay**

50 nM radiolabeled 35-mer oligonucleotide duplexes containing the THF, 5mC, or 3’-phosphate at the middle were incubated with each APE2 isoform proteins on the ice for 10 min and on room temperature for 15 min. Increasing amounts of each proteins (0, 100, and 200nM) were incubated in the DNA binding buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Triton X-100, 1.0 mg mL^-1 BSA, 10% glycerol, and 10 mM DTT). The reaction samples were separated on the 4% native polyacrylamide gel for 2 h at 40 V. The gel was exposed to an X-ray film at −80°C.

**Molecular cloning of APE2.2 and APE2.2 into pYOON01**

For co-expression of two different proteins in *E. coli*, pYOON01 vector was taken from the previous study, and was used to generate pYOON01-APE2.2 and pYOON01-APE2.3 (Lee et al., 2014). The pLM302-APE2.2 was digested with Bam HI and Sal I, and the APE2.2 fragment was cloned into the pYOON01 vector.
pYOON01-APE2.3 was generated in the same way. The plasmids were transformed into *E. coli* strain *RPC501* that carries mutation in *Exo III* and *Endo IV* genes. Competent cells were generated for co-transformation with the calcium chloride method (Dagert et al., 1979). The constructs for pYOON01-APE1L, pYOON01-ARP, and pYOON01-APE2.1 described in previous study were identically used (Lee et al., 2014).

**Bacterial cell toxicity assay**

The pMAL-c2X or pMAL-c2X-DMEΔN677Δ IDR1::lnk was transform into *RPC501* that carried pYOON01, pYOON01-APE1L, pYOON01-ARP, pYOON01-APE2.1, pYOON01-APE2.2, or pYOON01-APE2.3, respectively. Colony PCR was performed to confirm plasmid combinations using primers DG210 and DG083 for DME, and DG218 and DG482 for pYOON constructs. Fresh colony was picked, and was grown in liquid LB/Amp/Chl media (LB supplemented with 100 µg mL⁻¹ of ampicillin and 50 µg mL⁻¹ of chloramphenicol) at 37°C overnight. The culture was diluted to an OD₆₀₀ 0.8, and was spotted on LB/Amp/Chl plates with 0-100 µM of IPTG with serial dilution. The plates were incubated at 28°C for 20-28 h.
RESULTS

APE2 has three isoforms because of alternative splicing

Three Arabidopsis AP endonucleases APE1L, APE2, and ARP have been identified, and their activities were tested in vitro (Cordoba-Canero et al., 2011; Lee et al., 2014; Murphy et al., 2009). All of them are considered as homologs of hAPE1, but APE2 did not convert the harmful lesions such as 3’-PUA and 3’-phosphate formed by DNA glycosylase into 3’-OH (Lee et al., 2014). Two isoforms are found to be produced by alternative splicing which are different from the APE2 isoform (APE2.1) used in the previous study (Lee et al., 2014). They were named APE2.2 and APE2.3, respectively (Figure 3A). The detailed information about alternative splicing of APE2 isoforms is presented in Figure 4. The conserved domain-search (CD-search) revealed that APE2.1 has an incomplete EEP domain for endonuclease, exonuclease, and phosphatase activities, whereas APE2.2 and APE2.3 have a more conserved and complete EEP domain (Figure 3B). Also, all of them have an additional zinc-finger domain for DNA binding, and APE2.1 and APE2.3 have more conserved and longer zinc-finger domain than APE2.2. RT-PCR analysis revealed that APE2.1 was expressed mainly in the cauline leaf, the floral bud, and the open flower (Figure 3C). APE2.2 was expressed in almost all tissue types. The leaf and the floral bud were main expression tissues of APE2.2. In the case of APE2.3, it was also expressed in all tissue types, but APE2.3 expression in the open flower was relatively lower than in the floral bud. DME expression is primarily detected in immature flower buds and in the central cell (Choi et al., 2002). Interestingly, APE2.3 expression pattern overlaps with that of DME expression in the floral bud.
**Figure 3.** Three isoforms of *APE2* in *Arabidopsis*. (A) Schematic representation of alternative splicing for three isoforms of *APE2* gene. The translated regions are colored in black and untranslated 5’ or 3’ regions are represented in white boxes. The primers used for amplifying respective *APE2* isoform are presented as arrows. The dotted regions of arrows indicate skipped intron regions. (B) Schematic illustration of proteins for *APE2* isoforms. EEP, endonuclease-exonuclease-phosphatase; ZF, GRF-type zinc finger motif. (C) Expression patterns of *APE2* isoforms in various tissues by RT-PCR. Seedling is a sample of 2-week-old wild-type *Arabidopsis*, and the others are samples of 6-week-old. *ACTIN11* is a loading control. SE, seedling; RL, rosette leaf; CL, cauline leaf; ST, stem; FB, flower bud; OF, open flower.
Figure 4. Alternative splicing of APE2 isoforms. Genomic sequences of APE2 isoforms are presented. The exons and introns are presented as upper and lower cases, respectively. The translation start and stop sites are colored in red and blue, respectively. The splice donor and acceptor dinucleotides used for alternative splicing are represented in gray and black backgrounds, respectively.
APE2.2 and APE2.3 have AP endonuclease activity on the AP sites and DME excision intermediates

In order to identify and compare the activities of APE2.2 and APE2.3 with other homologs in Arabidopsis on the AP site, purified AP endonuclease enzymes were incubated with oligonucleotide containing THF in the middle as an AP analog (R35AP) (Figure 5A). Consistent with the previous studies, ARP processed the AP site generating 3’-OH, whereas APE1L and APE2.1 displayed weak or no activity (Figure 5B) (Lee et al., 2014). However, APE2.2 and APE2.3 showed AP endonuclease activity on the AP site and produced 3’-OH. In particular, as the concentration of APE2.2 and APE2.3 increased, they generated 3’-OH in a particular range of concentration (Figure 5C). DNA ladder formation was observed in APE2.2 and APE2.3 reactions. These results suggest that they might have 3’-5’ exonuclease activity similar to hAPE2 (Burkovics et al., 2006). Next, the effects of metal ion on APE2.2 and APE2.3 activity on the AP site were investigated. It was previously shown that Mg$^{2+}$ can stimulate Exo III activity, but not Endo IV (Fromme et al., 2004). According to previous research on the crystal structure of hAPE1, Mg$^{2+}$ is assumed to locate in a catalytic pocket and binds to the PO$_3$ group after cleavage of phosphodiester bond (Mol et al., 2000). APE2.2 and APE2.3 was tested under diverse Mg$^{2+}$ concentrations, and EDTA was used as a chelating agent that sequesters metal ions (Figure 5D). With 2.5 mM EDTA, APE2.2 and APE2.3 did not display incision activity. However, with 2.5mM EDTA and 2.5 mM MgCl$_2$, APE2.2 and APE2.3 processed the AP sites. DNA ladder formation can be also observed in this condition, so 3’-5’ exonuclease activity is not due to exonucleases in E. coli, but APE2.2 and APE2.3. Also, when MgCl$_2$ concentration increased, APE2.2 and
**Figure 5.** AP endonuclease activity on the AP site. (A) Structure of 35-mer oligonucleotide duplex containing THF in the middle as an analog of the AP site. (B) The AP endonuclease activity on the AP sites of APE1L, ARP, APE2.1, APE2.2 and APE2.3. 5 nM APEs were used and reacted with oligonucleotide duplex in (A) in the APE reaction buffer for 2 h. (C) Activity test of APE2.2 and APE2.3 on the AP site with increasing concentration. 5, 25, 50, and 100 nM of APEs were reacted with oligonucleotide duplex in (A) in the APE reaction buffer for 2 h. (D) The AP site incision activity of APE2.2 and APE2.3 under various magnesium concentration. Concentrations of APE2.2 and APE2.3 are 50 nM and 25nM, respectively. EDTA concentrations in lane 3, 4, 7, and 8 are 2.5 mM. MgCl$_2$ concentrations in lane 5 and 9 are 0.5mM, and MgCl$_2$ concentrations in lane 4, 6, 8, and 10 are 2.5mM. (E) Trimming of the AP site by APE2.2 in time course. 50 nM APE2.2 was used for the time course reaction. (F) Product accumulation of APE2.2. Using the phosphorimager, the amounts of products from the experiment in (E) were measured and plotted over time. Standard deviations from three independent experiments are represented by error bars. hAPE1 was used as a positive reaction control in all reactions.
APE2.3 displayed increased incision activity and generated 3’-OH. Thus, APE2.2 and APE2.3 require Mg\(^{2+}\) to incise the AP site. This suggests that Mg\(^{2+}\) is an essential cation for the AP site incision activity of APE2. Time-course reactions of APE2.2 were performed, and products of APE2.2 on the AP site were quantitated and plotted over time (Figure 5E and 5F). The kinetics study showed that the product formation rate of 50 nM APE2.2 was comparable to 5 nM ARP because product formation of ARP in previous study was similar with APE2.2 (Lee et al., 2014).

APE2.2 and APE2.3 were also tested whether they can play the essential roles in DNA demethylation. First, oligonucleotide duplex containing 5mC in the middle was reacted with DME (Figure 6A). Then, DME generated 3’-PUA and 3’-phosphate which were used as APE substrates. After DME inactivation, these 5mC excision intermediates were incubated with Arabidopsis APEs. Consistent with the previous studies, APE1L and ARP processed the substrates and generated 3’-OH, but APE2.1 displayed no activity (Figure 6B) (Lee et al., 2014; Li et al., 2015). 5 nM APE2.2 and APE2.3 also displayed no discernable activity. However, when the concentration of APE2.2 and APE2.3 was increased, they processed DME products, and produced 3’-OH in a particular range of concentration (Figure 6C). DNA ladder formation was also observed in APE2.2 and APE2.3 processing of 5mC excision intermediates, suggesting again that they might have 3’-5’ exonuclease activity. Activity of APE2.2 and APE2.3 on DME products was also tested under diverse EDTA and Mg\(^{2+}\) concentrations (Figure 6D). In 2.5 mM EDTA condition, APE2.2 and APE2.3 did not generate 3’-OH. However, under the conditions with each of 2.5 mM EDTA and MgCl\(_2\), APE2.2 and APE2.3 processed the AP sites. In addition, APE2.2 and APE2.3 displayed increased incision activity in MgCl\(_2\) condition than
Figure 6. AP endonuclease activity on the DME products. (A) Structure of 35-mer oligonucleotide duplex containing 5mC for 3’ end cleaning assay. The radiolabeled 35-mer oligonucleotide duplex was reacted with DME, generating 35-mer oligonucleotide duplexes that mimic DME-catalyzed β- and δ-elimination products (β and δ, respectively). (B) The 3’-end processing activity on DME products of APE1L, ARP, APE2.1, APE2.2, and APE2.3. DME excision intermediates (β and δ) were reacted with 5 nM APEs in the APE reaction buffer for 2 h. (C) Activity test of APE2.2 and APE2.3 on DME products with increasing concentration. 5, 25, 50, and 100 nM of APEs were reacted with DME excision intermediates (β and δ) in the APE reaction buffer for 2 h. (D) The incision activity of APE2.2 and APE2.3 for DME products under various magnesium concentration. Concentrations of APE2.2 and APE2.3 are 50 nM and 25nM, respectively. All EDTA and MgCl₂ concentrations are 2.5mM. (E) Trimming of DME products by APE2.2 in time course. 50 nM APE2.2 was used for the time course reaction. (F) Product accumulation of APE2.2. Using the phosphorimager, the amounts of products from the experiment in (E) were measured and plotted over time. Standard deviations from three independent experiments are represented by error bars. Endo IV was used as a positive reaction control in all reactions.
in MgCl$_2$ and EDTA condition. Collectively, APE2.2 and APE2.3 require Mg$^{2+}$ to process DME products. This suggests that Mg$^{2+}$ is essential cation for DME products trimming activity of APE2. Moreover, time course reactions of APE2.2 were performed, and the products of APE2.2 on DME products were quantitated and plotted over time (Figure 6E and 6F). The kinetics study showed that the product formation of APE2.2 on DME products was different to the product formation on the AP site. APE2.2 reached its plateau in the former case.

The oligonucleotide containing 3’-phosphate was also reacted (Figure 7A). However, when the concentrations of APE2.2 and APE2.3 were increased, they displayed no activity and cannot produce 3’-OH at 5 nM concentration (Figure 7B). Therefore, APE2.2 and APE2.3 do not have 3’-phosphatase activity.

All of APE2 isoforms were tested whether they can form the DNA-protein complex with oligonucleotides containing the AP site analog THF, 5mC, and 3’-phosphate. All isoforms can make the DNA-protein complex, and these results suggest that enzymes can bind to the AP site, 5mC, and 3’-phosphate (Figure 8). APE2.1 displayed relatively higher binding efficiency than APE2.2 and APE2.3. These results are thought to be due to conformational change of APE2.1 which is influenced by its truncated EEP domain form.

**APE2.3 stimulates DNA demethylase activity**

DME reaction was performed with Mg$^{2+}$ and other various metal ions such as Mn$^{2+}$, and Co$^{2+}$ to investigate whether DME is affected by metal cations, especially Mg$^{2+}$. Surprisingly, DME activity with Mg$^{2+}$ decreased in a concentration-dependent manner (Figure 9A). In DME reactions with Mn$^{2+}$ and
**Figure 7.** AP endonuclease assay for 3’ phosphatase activity. (A) Structure of 35-mer oligonucleotide duplex that imitates DME-catalyzed δ-elimination product. To produce DNA substrate containing the 1-nt gap in the middle, the radiolabeled upstream 17-mer oligonucleotide including a 3’-phosphate (F17F[3P]) and the downstream 17-mer containing a 5’-phosphate ([5P]F17B) were annealed with the complementary 35-mer strand (R35) together. (B) 3’ phosphatase activity of APE1L, ARP, APE2.1, APE2.2, and APE2.3. 5 nM APEs are used. (C) Activity test of APE2.2 and APE2.3 on 3’-phosphate with increasing concentration. 5, 25, 50, and 100 nM of APEs were reacted with one of DME excision intermediates, 3’-phosphate (δ), in the APE reaction buffer for 2 h. Endo IV was used as a positive reaction control in all reactions.
Figure 8. Electrophoretic mobility shift assay of APE2 isoforms. Standard electrophoretic mobility shift assay was performed following the method described in previous research (Mok et al., 2010). (A) Analysis of purified proteins of three APE2 isoforms. 0, 100, and 200 ng of respective protein samples were running on 10% polyacrylamide gel, and were visualized by coomassie brilliant blue staining. (B) AP site substrate, (C) 5mC, and (D) 3’ phosphate were using for substrates. The protein-DNA complex and free DNA are indicated on the left.
Figure 9. DME reaction with various metal cation conditions. (A) DME reaction with Mg$^{2+}$, Mn$^{2+}$, and Co$^{2+}$ in diverse concentration conditions. DME was reacted in the DNA glycosylase reaction buffer for 1 h. (B) Preincubated DME reaction. DME was preincubated with Mg$^{2+}$ at 37°C for 30 min. After preincubation, DME was reacted without Mg$^{2+}$ in the DNA glycosylase reaction buffer for 1 h.
Co$^{2+}$, DME activities were also reduced, but they displayed weak excision activity. Because Mg$^{2+}$, Mn$^{2+}$, and Co$^{2+}$ are cations, and DNA have phosphate backbone which has negative charge, there are possibilities that these cations affect DNA oligonucleotide solidity, not DME protein, and these effects result in reduction of DME activity under cation conditions. To confirm that cation hinders DME protein itself and does not affect the DNA oligonucleotide solidity, DME was preincubated with Mg$^{2+}$ before reaction with DNA oligonucleotide containing 5mC (Figure 9B). Preincubated DME did not generate 3’-PUA and 3’-phosphate, whereas reaction buffer did not contain Mg$^{2+}$. Therefore, Mg$^{2+}$ does not affect DNA oligonucleotide, but can inhibit DME activity. These results suggest that Mg$^{2+}$ may also act as a regulator for the BER pathway in *Arabidopsis*, and some partners in the BER pathway are required for DME activity under metal ion conditions. Therefore, DME was co-incubated with APEs to verify that the excision activity of DME on 5mC can be increased under Mg$^{2+}$ condition. Surprisingly, co-incubation of DME and APE2.3 generated 3’-OH in time-dependent manner (Figure 10A). This result suggests that DNA demethylase and APE2.3 might interact and stimulate activities of each other.

*ape1l* and *ape2* double homozygous mutants displayed seed abortion phenotype (Murphy et al., 2009). There are possibilities that interaction between DME and APE2 can be facilitated by APE1L or other AP endonucleases. Therefore, co-incubation of DME with double APEs was performed (Figure 10B). However, addition of APE1L or ARP could not increase generation of 3’-OH. Also, Endo IV could not generate 3’-OH when co-incubated with DME. These results might be explained by metal ions requirement of Endo IV. In contrast to APE2.3, Endo IV does not need metal ions for reaction (data not shown). Mg$^{2+}$ inhibited DME reaction.
Figure 10. Co-incubation tests. (A) Co-incubation of DME with various APEs. First and second panels were reactions performed in DME reaction buffer with DME. Reactions in the other panels were performed in APE reaction buffer with DME and APEs. DME products (β and δ) and 3’-OH are indicated at the right as a size marker. First lane is for no enzyme control. (B) Co-incubation of DME with double APEs or Endo IV. Reactions were performed in APE reaction buffer for 1h. 3’-OH is indicated at the right as a size marker.
and was essential for APE2.3 reaction. Mg\textsuperscript{2+} concentration might be reduced around the DME when APE2.3 binds to DME, and this interaction might facilitate reaction turnover rate of each other.

APE2.2 and APE2.3 expression could not reduce cytotoxicity resulted from DME expression in *E. coli*

It was reported that DME expression in *E. coli* cells was toxic and caused cell death especially in the strain with mutation in two AP endonucleases genes (Exo III and Endo IV). Susceptibility to DME expression is due to the incision activity of DME generating abasic sites and single strand breaks, and these harmful lesions can be a threat of bacterial genome integrity. In addition, expression of APE1L or ARP but not APE2.1 significantly reduced cytotoxicity caused by DME expression. (Gehring et al.; 2006, Lee et al., 2014; Mok et al., 2010). Complementation tests with APE2.2 and APE2.3 were performed to see whether they would compensate for mutations of *E. coli* AP endonucleases. Two plasmids having different origins and antibiotic genes were sequentially transformed into *E. coli* strain RPC501 (exo III/endo IV) used in previous research (Lee et al., 2014). APE2.2 and APE2.3 were expressed from the vector pYOON01, and DME was expressed from vector pMAL-c2X, respectively. Combination of DME and AP endonuclease was verified by colony PCR (Figure 11A), and protein expression was induced with IPTG. Consistent with previous study, cytotoxicity induced by DME in an IPTG-dependent manner was reduced by expression of APE1L or ARP (Figure 11B) (Gehring et al., 2006; Lee et al., 2014). However, such compensation was not observed in *E. coli* that have *APE2* isoforms.
Figure 11. Complementation of AP endonuclease activity in *E. coli* with plant APEs.

(A) Colony PCR results for confirmation of plasmid combinations in respective *E. coli* strains. (B) DME toxicity and complementation affected by APE expression. DME and/or APEs were expressed with increasing amounts of IPTG (0-100 μM) in *E. coli*. Bacterial culture was spotted on the LB agar plate with serial dilution from left to right.
APE2.2 and APE2.3 have intact EEP domains essential for activities, and are expressed in almost all tissue types. However, APE2.3 expression in the open flower was relatively low than expression the floral bud. DME expression is generally detected in immature flower buds and in the central cell (Choi et al., 2002). These observations suggest that APE2.3 expression pattern may be correlated to DME expression in the floral bud. Moreover, ape1/lape2 seeds display abortion phenotype (Murphy et al., 2009). Therefore, to exactly understand in vivo APE2 functions, expression of APE2 isoforms in the central cells or tissues in seed such as endosperm and embryo should be investigated.

Biochemical tests revealed the properties of APE2 isoforms in plants. First, the incision activity was checked, and APE2.2 and APE2.3 displayed the endonuclease activity on the AP site and DME incision intermediates unlike APE2.1. Their activities were weaker than that of ARP or APE1L. Although APE2.2 and APE2.3 would not be major enzymes that contribute to repair of the AP site and DME incision intermediates, they can generate 3′-OH clearly in increased concentration conditions. They might contribute to some part of processing in the BER pathway for DNA demethylation.

In cellular systems, Mg$^{2+}$ at physiologically relevant concentrations is required to maintain genome stability. Mg$^{2+}$ can stabilize DNA and chromatin structure, and Mg$^{2+}$ is also an essential cofactor in most enzymatic system, mediating DNA replication and DNA repair. Mg$^{2+}$-mediated enzyme inhibition is not common, and for most cases, Mg$^{2+}$ functions as a cofactor. In addition, as the essential cofactor
in major three repair pathways, nucleotide excision repair, mismatch repair pathways, and the BER pathway, Mg\(^{2+}\) is required for the removal of DNA damage produced by environmental mutagens, endogenous processes, or DNA replication. In addition, Mg\(^{2+}\) is important for endonuclease activity of Exo III family (Demple et al., 1986). *In vitro* and *in vivo* activities of hAPE1 are regulated by the concentration of Mg\(^{2+}\) (Wilson, 2005). Mg\(^{2+}\) is predicted to place at a catalytic pocket of hAPE1 according to its crystal structure, and has roles in binding with PO\(_3\) group right after cleavage of phosphodiester bond (Mol et al., 2000). In reactions with Mg\(^{2+}\), APE2.3 exhibits 3’ blocking end cleaning activity with Mg\(^{2+}\) at the AP site like other Exo III family enzymes.

In this study, it has also been demonstrated that Mg\(^{2+}\) can significantly inhibit DME activity. Moreover, the cell nucleus contains a lot of metals, which might affect DME activity as well, but in the BER viewpoint, it was intriguing to explain that Mg\(^{2+}\) is an essential cofactor for most of the downstream BER enzymes. Unlike BER enzymes, DME does not require Mg\(^{2+}\) or any other cation cofactor for damage recognition and excision. Surprisingly, Mg\(^{2+}\) is a strong inhibitor of DME, and is required for APE2 processing activity. One possible explanation is that in DNA demethylation pathway, a glycosylase/AP lyase reaction by DME would create single-strand breaks in the genome which could result in lethality to cells. Thus, Mg\(^{2+}\) that can inhibit DME activity may provide protective functions until APE2 and other BER pathway components required for subsequent steps are recruited. The delayed effect of Mg\(^{2+}\) may allow APE to bind substrates and protect cells from the deleterious effect of substrates. Therefore, the Mg\(^{2+}\) concentration might function as a regulator for the BER pathway ensuring efficient and balanced DNA demethylation
and prevent genomic instability. How such modulation of Mg\textsuperscript{2+} might regulate the BER pathway remains to be investigated.

There are several researches showing that APEs can stimulate DNA glycosylase activity (Sidorenko et al., 2007; Vidal et al., 2001; Hill et al., 2001). Structurally unrelated hAPE1 and bacterial Endo IV did not stimulate AP lyase activity, but stimulate DNA glycosylase activity of hOGG1. In addition, occupying the AP site by hAPE1 allows the recycling of hOGG1, which stimulates DNA glycosylase activity (Vidal et al., 2001). DME is sensitive to Mg\textsuperscript{2+}, and its activity results in degradation of substrate in presence of Mg\textsuperscript{2+} (data not shown). However, that robust degradation was reduced when DME was co-incubated with APE2.3. Also, the addition of APE2.3 with Mg\textsuperscript{2+} results in highly generated DME products, producing 3’-OH. These results are highly surprising because DME with Mg\textsuperscript{2+} cannot operate well and APE2.3 alone cannot process DME products well. These results suggest the coupling of activities of DNA demethylase and AP endonuclease in Arabidopsis. Relationship between DME and APE2.3 may be a case of DNA glycosylase recycling. Also, there are possibilities that DME and APE2.3 make complex. After complex formation, conformational change may happen, and facilitate turnover rate of APE2.3 and DME, respectively. APE2.2 is very similar with APE2.3, but cannot generate 3’-OH in enhanced reaction rate. There are different regions in zinc finger domain between APE2.2 and APE2.3. Therefore, interaction between DME and APE2 isoforms can be influenced by zinc finger, and how they can form complex to facilitate each other activity should be studied further. In addition, APE2.3 should be investigated more because APE2.3 may largely contribute to the seed abortion phenotype in ape11 ape2 double homozygous mutants.
Moreover, there are possibilities of optimization of APE2.3 activity on the AP site by co-incubation with monofunctional DNA glycosylase that has the AP site for substrate. The downstream enzymes of APE2 should also be clearly identified to completely understand the whole DNA demethylation pathways.

The reason why APE2.2 and APE2.3 cannot complement DME activity in *E. coli* may be that they, especially APE2.3, cannot combine or interact properly with DME in *E. coli*. Also, growth condition of *E. coli* can affect the results. That system is considered to be suitable for enzymes that can effectively process DME products in low concentration because APE1L and ARP have complementation effects in *E. coli*.

Taken together, roles of APE2 in *Arabidopsis* can be proposed (Figure 12). In this research, the functional APE2 isoform and its AP endonuclease activity were demonstrated. These findings suggest new possibilities about complex formation and interaction between DME and APE2 in DNA demethylation pathway. After removing mismatched or damaged bases by monofunctional glycosylases, the AP site is generated. APE2.2 and APE2.3 do not seem to be the major AP endonuclease that processes the AP site or DME products in the genome, but have weak activity and clearly function. Moreover, APE2.3 can facilitate the turnover rate of DME, the bifunctional DNA glycosylase. Therefore, APE2 might be mainly involved in DNA demethylation. These studies on APE2 functions on the BER pathway will provide advanced insights into mechanism of DNA repair and the DNA demethylation pathway.
Figure 12. Schematic illustration of DNA demethylation pathway in *Arabidopsis*. The damaged base is colored in black, and replaced bases are represented as gray.
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초 록

DNA 메틸화는 크로마틴 구조와 유전자 발현을 조절하는 주요한 후생유전학적 기작 중 하나이다. DNA 메틸화는 일반적으로 DNA 메틸전달효소에 의해 이루어지는 시토신에서 5-메틸시토신으로의 전환을 의미한다. 식물은 DEMETER (DME)와 같은 DNA 탈메틸효소에 의해 5mC가 직접적으로 절단되는 독특한 DNA 탈메틸화 시스템을 갖추고 있다. 특히 염기 절제 수선은 DME 군 단백질에 의한 DNA 탈메틸화 과정 중에 형성되는 해로운 말단을 처리하는 두 역할이 있지만 그 자세한 작용기작과 DNA 탈메틸화 과정에서의 기여에 대하여 식물에서 잘 구명되어 있지 않다. 본 논문에서는 애기장대의 AP 엔도뉴클레이즈인 APE2 에 선택적 스플라이싱에 의한 세 종류의 동형단백질이 존재하고 각각의 동형단백질들이 AP 부착자리와 5mC 절단에 의한 중간산물을 이후의 중합과정에 필수적인 3'-OH 로 바꾸는 기능을 차등적으로 지니고 있음을 구명하였다. 특히 APE2.3 동형단백질의 경우 DME에 의한 5mC 전달 반응 속도를 높일 수 있음을 확인되었고 이를 통해 APE2.3가 해로운 말단을 처리할 뿐만 아니라 5mC 절단 중간산물을 형성하는 속도를 높임으로써 DNA 탈메틸화를 촉진한다는 가능성을 제시할 수 있다.
주요어: AP 엔도뉴클레이즈, APE2, DME, DNA 탈메틸화, 애기장대, DNA 수신, 염기 절제 수신.

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