

AtREV1, a Y-Family DNA Polymerase in Arabidopsis, Has Deoxynucleotidyl Transferase Activity in Vitro^{1[W]}

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To clarify the functions of the *Arabidopsis thaliana* REV1 (AtREV1) protein, we expressed it in *Escherichia coli* and purified it to near homogeneity. The deoxynucleotidyl transferase activity of the recombinant AtREV1 was examined in vitro using a primer extension assay. The recombinant AtREV1 transferred one or two nucleotides to the primer end. It efficiently inserted dCMP regardless of the opposite base. AtREV1 also inserted a dCMP opposite an apurinic/aprimidinic site, which is physiologically generated or induced by various DNA-damaging agents. In contrast, AtREV1 had no insertion activities against UV-inducible DNA lesions as reported in yeast or mammalian system. Although the substrate specificity of AtREV1 was rather narrow in the presence of magnesium ion, it widened in the presence of manganese ion. These results suggest that AtREV1 serves as a deoxycytidyl transferase in plant cells.

Plants are continuously exposed to harmful UV-B (290–320 nm) as well as the photosynthetic light (400–700 nm). Therefore, an increase of UV-B is predicted to inhibit plant growth in a large area, causing the disruption of ecosystems. UV-B radiation induces various lesions on plant DNA. The vast majority of UV-B-induced damage is cyclobutane pyrimidine dimer (CPD), which corresponds to 75% to 78% of total damage (Mitchell, 1995; Cadet et al., 2005). The 6-4 photoproducts (6-4PP) and Dewar isomer, a derivative of 6-4PP, correspond to most of the remainder (20%–24%; Mitchell, 1995; Cadet et al., 2005). Other types of DNA damage, including 8-oxoG, pyrimidine hydrate, thymine glycol, DNA single-strand break, etc., are generated by UV-B radiation at very low yield (<0.1%–2%; Mitchell, 1995; Gao and Murphy, 2001; Song et al., 2002; Cadet et al., 2005; Friedberg et al., 2006). On the other hand, apurine/aprimidine (AP) sites are among the most abundant DNA lesions generated spontaneously (Boiteux and Guillet, 2004). For example, approximately 10,000 AP sites arise spontaneously in a mammalian cell per day (Nakamura et al.,

1998). The AP site also is generated when exposed to the various DNA-damaging agents, such as ionizing radiation and alkylating chemicals, through the reaction of DNA *N*-glycosylases that remove the damaged base (Friedberg et al., 2006). The AP sites are rarely formed after UV irradiation directly (Song et al., 2002) or as a consequence of a cellular process removing the damaged base (Friedberg et al., 2006). Such lesions can lead to incorporation of the wrong base or can inhibit DNA replication and transcription, resulting in mutations and cell death (Britt, 1999). To prevent the effects of UV and induced DNA lesions, plants have many DNA repair mechanisms, such as photorepair by the CPD and 6-4 photolyases (Landry et al., 1997; Nakajima et al., 1998). In addition, they have nucleotide excision repair (Gallego et al., 2000; Liu et al., 2001, 2003), base excision repair (García-Ortiz et al., 2001), and recombination repair (Osakabe et al., 2006).

Translesion synthesis (TLS) is one of the damage-tolerant mechanisms prevalent in both prokaryotes and eukaryotes (Prakash et al., 2005). In TLS, a DNA lesion is bypassed by the action of specialized DNA polymerases (Broomfield et al., 2001; Friedberg et al., 2005). DNA synthesis during TLS often results in mutations because of the intrinsic nature of TLS-type polymerases (Friedberg et al., 2000). The TLS-type polymerases include DNA polymerases (Pols) ζ , ι , η , REV1, and other specialized polymerases (Prakash et al., 2005). The *REV1* gene was first identified in yeast through a screening for reversionless mutants (Lemontt, 1971). Subsequent analyses showed that REV1 is a Y-family DNA polymerase. In vitro analyses showed that yeast Rev1 inserts a C opposite the AP site (Nelson et al., 1996a), and then the mismatched end is efficiently extended by Pol ζ (Nelson et al., 1996b; Lin et al., 1999). Thus, Rev1 and DNA Pol ζ are thought to be major components of the error-prone TLS (Nelson et al., 1996a, 1996b).

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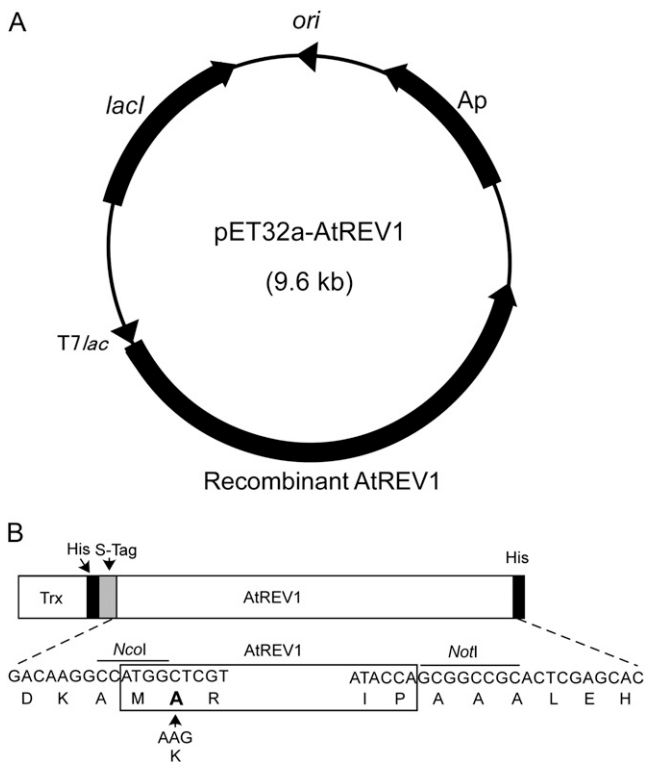


Figure 1. Structure of the recombinant AtREV1 expression plasmid. *A*, AtREV1 cDNA was modified to include restriction sites on both sides and inserted into the pET32a(+) vector. The pET32a(+) vector includes a T7lac promoter (T7lac), an ampicillin-resistant gene (Ap), a lacI coding sequence (lacI), and pBR322 origin (ori). *B*, Schematic representation of the recombinant AtREV1 protein. The recombinant protein contains a thioredoxin tag (Trx), a His tag (His), and an S protein-binding tag (S-tag) at the N terminus, and another His tag at the C terminus. The bottom DNA and amino acid sequences represent the restriction sites (NcoI and NotI) induced for subcloning. The K (Lys) of the native AtREV1 amino acid sequence was changed to A (Ala; in boldface) in the recombinant AtREV1.

Little is known about the proteins that are involved in TLS in plants. We recently described an Arabidopsis (*Arabidopsis thaliana*) homolog of Pol ζ , which is a heterodimer of family-B DNA polymerase REV3 (Sakamoto et al., 2003) and the regulatory subunit REV7 (Takahashi et al., 2005). The Arabidopsis genome has three Y-family polymerases, which are most similar to REV1, DinB1, and Rad30, respectively (Ohmori et al., 2001). The homolog of *DinB1* (*AtPolK*) was recently characterized and its polymerase activity was analyzed in vitro (García-Ortiz et al., 2004). The homolog of *Rad30* (*AtPolH*) was shown to suppress the UV sensitivity of the *Saccharomyces cerevisiae rad30* strain (Santiago et al., 2006). However, it is still unknown whether these Y-family polymerases perform TLS and whether they are involved in damage tolerance in plants. We recently showed that a mutant with disrupted *AtREV1* had increased sensitivity to UV-B and other DNA-damaging agents (Takahashi et al., 2005), suggesting that *AtREV1* has a role in the damage tolerance in Arabidopsis.

Here, we show that recombinant AtREV1 has deoxynucleotidyl transferase activity. The recombinant AtREV1 clearly inserted a nucleotide opposite AP sites in vitro. Our results suggest that AtREV1 has a role in providing tolerance to DNA damage through the TLS pathway.

RESULTS

Overproduction and Purification of Recombinant AtREV1 Protein

To prepare soluble and active AtREV1 protein, the *AtREV1* cDNA was inserted into the pET32a vector that carries a thioredoxin-tag fusion protein, which increases the solubility of the expressed protein (Fig. 1). Under optimized growth conditions of *Escherichia coli* cells (see "Materials and Methods"), the recombinant AtREV1 protein was successfully expressed on a large scale. SDS-PAGE analysis showed the presence of a thick band that migrated between the 116-kD and 194-kD size markers after induction with isopropyl β -D-1-thiogalactopyranoside (IPTG; Fig. 2, lane 3). Since the size of the band (approximately 140 kD) was consistent with the predicted molecular mass of the recombinant AtREV1 fusion protein and since the band cross-reacted with the anti-His tag antibody (data not shown), we concluded that the band corresponded to

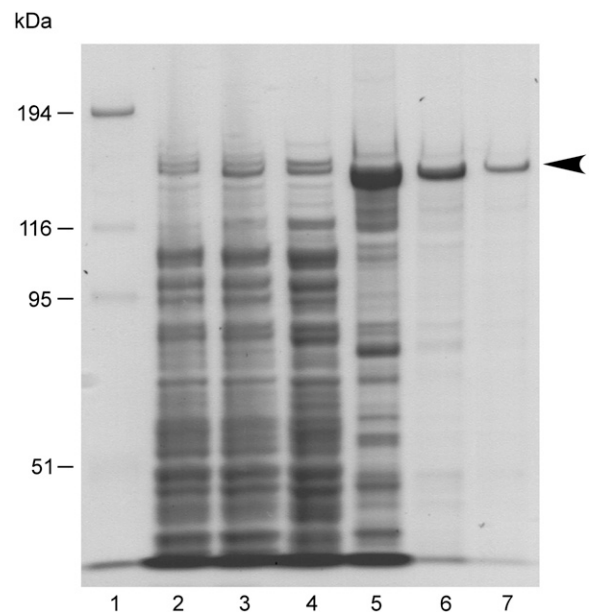


Figure 2. Purification of recombinant AtREV1 proteins. Overproduction and purification of recombinant AtREV1 protein in *E. coli*. Fractions in each purification step were separated on a 7.5% SDS-polyacrylamide gel, then stained with Coomassie Brilliant Blue. Lane 1, M_r marker (Invitrogen); lanes 2 and 3, total *E. coli* proteins with (lane 3) or without (lane 2) IPTG induction; lane 4, clear lysate; lane 5, fraction eluted from Ni-affinity column; lane 6, fraction from a heparin affinity column; lane 7, purified fraction from HiTrap Q HP column. Molecular weight is shown on the left.

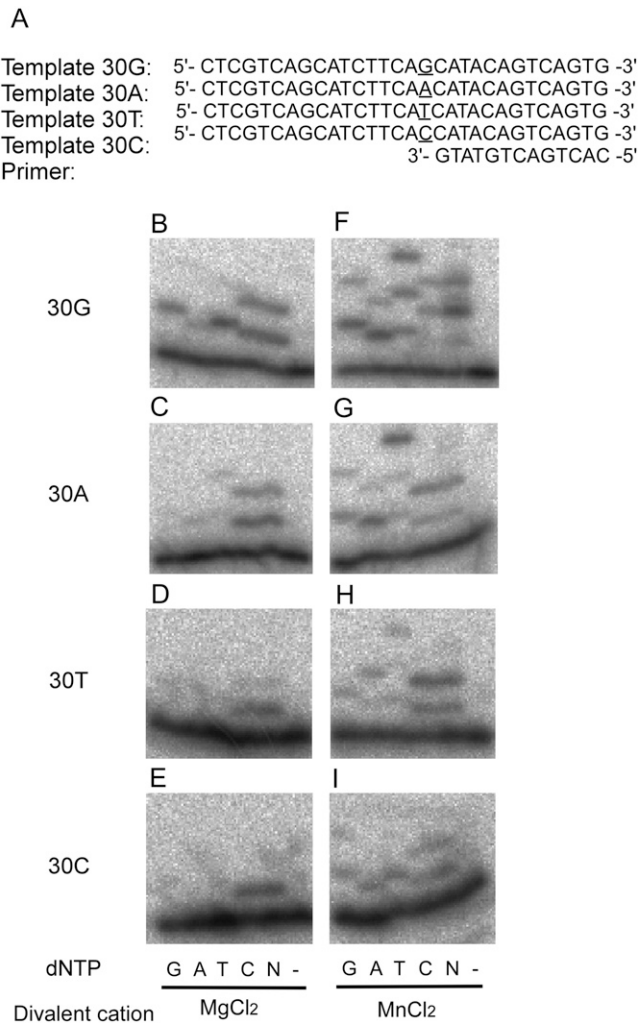


Figure 3. Deoxynucleotidyl transferase activity of the recombinant AtREV1 protein. The primer P13 was ³²P-labeled at the 5' end and annealed with each of the templates, preparing four template/primer pairs: 30G (B and F), 30A (C and G), 30T (D and H), and 30C (E and I). The nucleotide sequences around the primer terminus are shown in A. The underlined sequences represent the variation of templates. One hundred and sixty nanograms of AtREV1 recombinant protein and the indicated template/primer were incubated with 0.1 mM of a single dNTP (G, A, T, or C), 0.1 mM each of all four dNTPs (N), or no dNTP (–) under standard reaction conditions with 2 mM magnesium chloride (B–E) or 1 mM manganese chloride (F–I) at 30°C for 10 min. The reaction products were resolved in 20% polyacrylamide gels containing 8 M urea and visualized by autoradiography.

AtREV1. After sonicating the *E. coli* cells, the recombinant AtREV1 as well as some degradation products were detected in the soluble fraction (clear lysate; Fig. 2, lane 4). The recombinant AtREV1 proteins were then fractionated by their affinity for nickel (Ni)-chelated Sepharose and successfully concentrated to a major band (Fig. 2, lane 5). To exclude the other remaining proteins derived from *E. coli*, the fraction was purified by heparin column and anion-ion-exchange column chromatography (Fig. 2, lanes 6 and 7). The activity of

AtREV1 was monitored in each purification step. The fractions eluted from the heparin column and the anion-exchange column-purified fraction were examined by a primer extension assay using a primer template containing G and dCTP (template 30G; see “Materials and Methods”). As a result, the dCMP insertion activity was detected in both fractions (data not shown). Thus, the anion-exchange column-purified recombinant AtREV1 protein, represented as a single band (Fig. 2, lanes 6 and 7), was used to assay deoxynucleotidyl transferase activity.

In Vitro Assay of Deoxynucleotidyl Transferase Activity of Recombinant AtREV1

To examine the substrate specificity of the recombinant AtREV1 protein, we performed a primer extension assay using four different primer templates (Fig. 3A) in the presence of dGTP, dATP, dTTP, or dCTP individually, or all four dNTPs together. At first, the activity was examined in the presence of 2 mM magnesium ion, based on previous observations (Masuda and Kamiya, 2002). The recombinant AtREV1 protein efficiently inserted a dCMP regardless of the opposite base (Fig. 3, B–E). The protein sometimes inserted additional dCMPs, for example, when the first template base was G, A, or T (Fig. 3, B–D). AtREV1 also inserted dTMP or dGMP when the opposite base was G (Fig. 3B). Although the efficiency was low, we detected dGMP insertions opposite G, T, and C, dAMP insertions opposite G, A, and T, and dTMP insertion opposite A (Fig. 3, B–E). These results indicate that the recombinant AtREV1 has an ability to transfer a deoxynucleotide at the end of the primer. Comparing the nucleotide transferase activities to the four templates, we found that the recombinant AtREV1 seems to prefer template G to other templates. This is consistent with the character of yeast and human REV1s (Haracska et al., 2002; Zhang et al., 2002). Although the recombinant AtREV1 incorporated two to four kinds of nucleotides depending on the template base, a robust dCMP incorporation activity was detected at all four templates we examined. Thus, we concluded that the recombinant AtREV1 has deoxycytidyl transferase activity.

Effects of Divalent Cations on Activity of Recombinant AtREV1

To examine the effects of divalent cations on the transferase activity of AtREV1, the assay was performed in the presence of 1 mM manganese chloride (Fig. 3, F–I). The substrate specificity of the recombinant AtREV1 was weaker in the manganese buffer than in the magnesium buffer. Namely, in the manganese buffer, the recombinant AtREV1 transferred almost all four dNTPs regardless of the opposite base (Fig. 3, F–I). Also, up to three nucleotides were inserted in the manganese buffer, while a maximum of two nucleotides were inserted in the magnesium buffer.

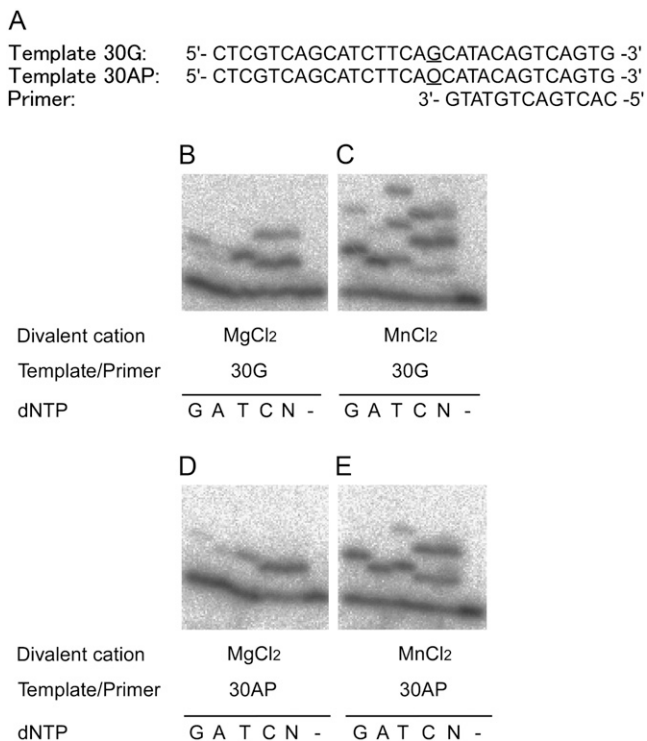


Figure 4. Deoxynucleotidyl transferase activity of the recombinant AtREV1 protein opposite the AP site. The primer P13 was ³²P-labeled at the 5' end and annealed with the template 30G or 30AP. The nucleotide sequences are shown in A. The underlined bases represent the variation of templates; Q indicates the AP site in template 30AP. One hundred and sixty nanograms of AtREV1 recombinant protein and the indicated template/primer were incubated with 0.1 mM of a single dNTP (G, A, T, or C), 0.1 mM each of all four dNTPs (N), or no dNTP (–) under standard reaction conditions with 2 mM magnesium chloride (B and D) or 1 mM manganese chloride (C and D) at 30°C for 10 min. The reaction products were resolved in 20% polyacrylamide gels containing 8 M urea, and then visualized by autoradiography.

These results suggest that the cations chelated in the active site of AtREV1 affect the substrate specificity of this protein, as reported in other polymerases (Doublet et al., 1998; Steitz, 1998).

Insertion of Nucleotide Opposite the AP Site by Recombinant AtREV1

To examine whether AtREV1 recombinant protein can insert a nucleotide opposite an AP site, we performed the *in vitro* transferase assay with template 30AP (Fig. 4A). In the presence of magnesium ion, the recombinant AtREV1 protein efficiently inserted only one nucleotide opposite the AP site (Fig. 4D). Among four deoxynucleotides, one dCMP was preferentially incorporated opposite the AP site when compared to the other three deoxynucleotides. Other nucleotides, dTMP and dAMP, were also inserted opposite AP, but activity of incorporation was lower than that of the dCMP insertion. In the presence of manganese chlo-

ride, the recombinant AtREV1 protein inserted one or two deoxynucleotides opposite the AP site (Fig. 4E).

No Nucleotide Insertion Activity of AtREV1 Opposite the UV Damage

To examine whether the AtREV1 recombinant protein can insert a nucleotide opposite the typical UV-induced damage, we performed an *in vitro* transferase assay with a template containing CPD or 6-4PP (Fig. 5A). The template CPD or 6-4PP and a primer (P16) were annealed and transferase activity was measured in the presence of all four dNTPs. As a result, no insertion activity was detected even in a prolonged (up to 30 min) incubation (Fig. 5B). In contrast, the presence of ladder bands over the primer band with template 30T indicates that the recombinant AtREV1 can replicate this template unless UV damage is present. When the templates were annealed with P17A primer that possesses a correct nucleotide (A) opposite the 3' T of CPD or 6-4PP, AtREV1 did not extend this primer end (Fig. 5B). Naturally, no nucleotide insertion activity was observed when the templates were annealed with P17G that possesses an incorrect nucleotide (G) opposite the 3' T of CPD or 6-4PP, although AtREV1 extended this mismatch end if there was no UV damage. The absence of nucleotide insertion activity of AtREV1 against the UV damage is consistent with several reports about human or yeast REV1s (Haracska et al., 2002; Zhang et al., 2002). However, the absence of nucleotide insertion activity conflicts with the finding that an *AtREV1*-disrupted plant is sensitive to UV-B (Takahashi et al., 2005).

DISCUSSION

REV1 is known as a DNA template-dependent dCMP transferase in yeast, human, and mouse (Nelson et al., 1996a; Masuda et al., 2002; Zhang et al., 2002). We previously identified *AtREV1*, an Arabidopsis homolog of the *REV1* gene (Takahashi et al., 2005), but did not investigate its biochemical characteristics. In this study, we successfully purified an active recombinant AtREV1 protein and examined its deoxynucleotidyl transferase activity using a primer extension assay. The recombinant AtREV1 protein incorporated nucleotides, especially cytosines and thymines, onto the primer end, regardless of the opposite template residue. In addition, the AtREV1 incorporated dCTP or dTTP opposite of the AP sites. The substrate specificity of AtREV1 is similar to the substrate specificities of yeast, human, and mouse REV1 proteins (Haracska et al., 2002; Masuda and Kamiya, 2002; Masuda et al., 2002). These results indicate that AtREV1 is a deoxycytidyl transferase.

Nair et al. (2005) determined the crystal structure of yeast Rev1 protein when it is bound to the template G and ready for an incoming dCTP. In the template/REV1 complex, an incoming dCTP binds to Arg-324 in

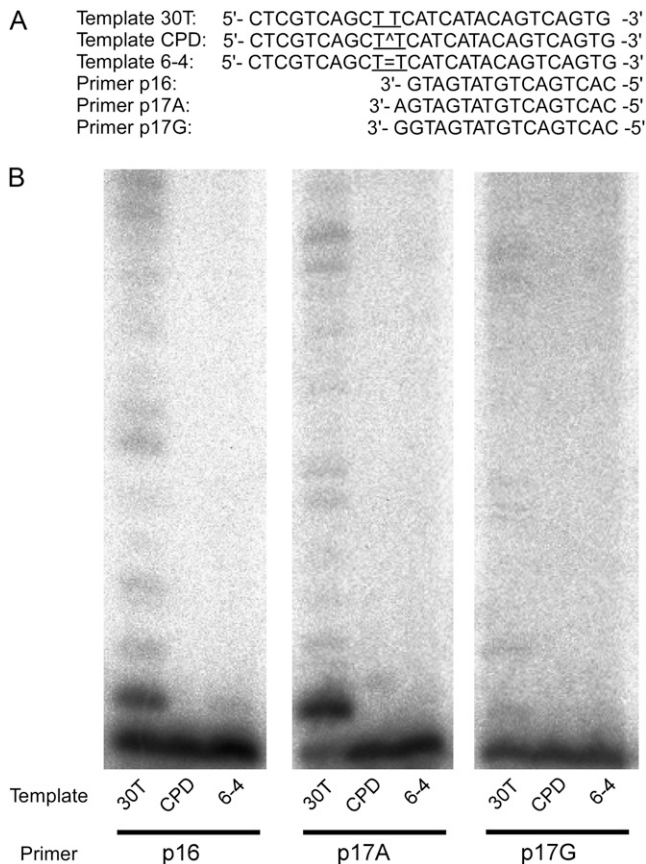


Figure 5. Deoxynucleotidyl transferase activity of the recombinant AtREV1 protein opposite sites of UV damage. The primer P16, P17A, or P17G was 32 P-labeled at the 5' end and annealed with template 30T, CPD, or 6-4 (A). The nucleotide sequences around the primer terminus and position of UV damage (T Δ T for CPD, T = T for 6-4PP) are shown in A. Four hundred nanograms of AtREV1 recombinant protein and the indicated template/primer were incubated with 0.1 mM of each dNTPs under standard reaction conditions with 2 mM magnesium chloride at 30°C for 30 min. The reaction products were resolved in 20% polyacrylamide gels containing 8 M urea and visualized by autoradiography.

the N-digit, a unique subdomain in REV1, rather than to the G in the template (Nair et al., 2005). At the same time, the G residue in the template is evicted from the DNA helix and makes a hydrogen bond with Leu-325 in the N-digit. This hydrogen bond is not made if the G is replaced with an A, T, or C residue in the template. This result supports the previous observation that human REV1 has a high k_{cat}/K_m value for the template G. The Arg and the Leu residues in the N-digit of yeast, mouse, and human REV1s (Masuda et al., 2001, 2002) are conserved in AtREV1 (Takahashi et al., 2005). In addition, AtREV1 more efficiently incorporated nucleotides into template G than into template A, T, or C. These results suggest that AtREV1 transfers a dCMP opposite G in a REV1-specific manner, and not through Watson-Crick pairing between dG and dCTP.

The substrate specificity of AtREV1 was reduced by replacing the magnesium ion in the buffer with man-

gane ion. The specificity of human REV1 was also reduced by replacing magnesium ions with manganese ions (Masuda and Kamiya, 2002). Vaisman et al. (2005) analyzed the effect of metal ions on the activity of a Y-family polymerase from *Sulfolobus solfataricus* Dpo4 based on its crystal structure and kinetics. They suggested that four conditions are required to form an active catalytic center in Dpo4: (1) 3'-OH of the primer end, (2) matching of the template base and the incoming nucleotide, (3) three catalytic carboxylates, and (4) two metal ions in correct coordination. Replacing the magnesium ion with manganese ion, which has a relaxed coordination requirement, dramatically increased the catalytic efficiency of misincorporation by Dpo4. Therefore, magnesium ions are probably essential for limiting misincorporations by AtREV1.

The recombinant AtREV1 protein inserted one or two nucleotides opposite the AP site, but did not extend from the mispaired termini. It is generally believed that the mispaired termini caused by the action of REV1 are extended by another polymerase (Nelson et al., 1996a; Zhang et al., 2002; Guo et al., 2004). In fact, an oligonucleotide including an AP site was efficiently replicated in vitro if the reaction mixture included both REV1 and Pol ζ (Haracska et al., 2001; Acharya et al., 2006). Does such a two-step replication involving AtREV3 occur in Arabidopsis? We do not know the properties of AtREV3 because it is too large to express as a recombinant protein. However, recent evidence strongly suggests that AtREV1 and AtREV3 play roles in a common pathway (Takahashi et al., 2005). First, the sensitivities of double-knockout *rev1rev3* plants to UV-B, cisplatin, and γ -rays were similar to those of *AtREV3*-disrupted plants (Takahashi et al., 2005). Second, when we measured the UV-induced reversion frequencies by using the point-mutated GUS (*uidA*) reporter system (Kovalchuk et al., 2000), the *AtREV1*- and *AtREV3*-disrupted plants showed a similar reversion frequency on somatic cells (M. Nakagawa, S. Takahashi, and A.N. Sakamoto, unpublished data). These facts suggest that the DNA Pol ζ and AtREV1 might cooperate to bypass some classes of DNA damage in Arabidopsis.

We previously showed that the *AtREV1*-disrupted plants are more sensitive to UV-B and γ -ray irradiation than wild-type plants (Takahashi et al., 2005). It is known that AP sites are generated by various DNA-damaging agents, including ionizing radiation (Boiteux and Guillet, 2004). Thus, it is reasonable that the γ -ray sensitivity of *AtREV1*-disrupted plants is due to the loss of the dCMP insertion activity against the AP site (Takahashi et al., 2005). On the other hand, the majority of UV-induced DNA damage consists of CPDs, which are approximately 75% to 78%, while the 6-4PPs and Dewar isomers account for most of the remainder (Mitchell, 1995; Cadet et al., 2005). The monomeric damage, such as 8-oxoG or cytosine hydrate, which could be turned to AP sites by the action of DNA N-glycosylase, is generated at very low level (Cadet et al., 2005; Friedberg et al., 2006). Based on these facts,

it is presumable that the UV sensitivity of *AtREV1*-disrupted plants is caused by loss of activity to bypass CPDs and/or 6-4PPs rather than the AP site. However, in this study, the recombinant AtREV1 protein showed no insertion or extension activity either on CPD- or 6-4PP-containing oligonucleotides *in vitro*. There are at least two possible explanations for this contradictory result. One of the possibilities is that the presence of the AP site is quite toxic for plants, even though the yield after UV irradiation is low, and that the inability of bypassing the AP site caused inhibition of growth. However, this hypothesis is unlikely because the AP site is abundantly generated even under no exogenous agent (Nakamura et al., 1998; Friedberg et al., 2006). Thus, if the inability of bypassing the AP site were essential for plants, the *REV1*-disrupted plant would show the severe growth defect even under natural growth condition. The indistinguishable growth of *rev1-1* plants from wild-type plants without UV treatment (Takahashi et al., 2005) suggests that this hypothesis is not appropriate to explain the UV sensitivity of *REV1*-deficient plants.

The alternate possibility is that AtREV1 has another function(s) independent of dCMP transferase activity, which contributes the UV tolerance. Ross et al. (2005) showed that the human REV1 protein with point mutations in its polymerase domain completely suppresses the UV sensitivity of *REV1*-disrupted chicken cells. This result indicates that transferase activity is not directly required for damage tolerance at least in vertebrates. Moreover, the yeast strain involving the *rev1-1* mutation, which was disrupted in the BRCT domain, could not bypass 6-4PPs on the plasmid (Nelson et al., 2000), although the *REV1*-1p protein with the same mutation showed normal dCMP transferase activity *in vitro*. These results support the idea that the BRCT domain of REV1s has a distinct function in addition to its dCMP transferase activity. Thus, by analogy, it is conceivable that the BRCT domain of AtREV1 plays some important roles in UV tolerance in Arabidopsis.

Recently, it was reported that the proliferating cell nuclear antigen (PCNA) plays a major role in polymerase switching, which is regulated by the posttranslational modification (Stelter and Ulrich, 2003; Lehmann et al., 2007). Especially, the monoubiquitination of the Lys-164 residue increases the affinity of PCNA to TLS-type polymerases, promoting TLS at the replication fork (Parker et al., 2007). Consistently, all the Y-family polymerases have conserved ubiquitin-binding sequences (UBM or UBZ), which are thought to provide an interface interacting with monoubiquitinated PCNA (Bienko et al., 2005). In yeast, the damage-bypass activity of REV1 is enhanced in the presence of ubiquitinated PCNA *in vitro* (Garg and Burgers, 2005), and this enhancement needs one of two UBM motifs in the C-terminal region of REV1 (Wood et al., 2007). A mutation in the UBM motif diminished the tolerance of yeast against UV, methylmethane sulfate, and 4-nitroquinoline-1-oxide to the same level as the *REV1*-knockout mutant (Guo et al., 2006; Wood et al., 2007). Similarly, the mouse *REV1* gene that has a mutation in the UBM failed to suppress the UV or cisplatin tolerance of *REV1*-deficient chicken cells (Guo et al., 2006). These results indicate that the interaction of REV1 to the monoubiquitinated PCNA is commonly important for the function of REV1. The presence of putative UBMs in the C terminus of AtREV1 (Supplemental Fig. S1) strongly suggests the involvement of AtREV1 with monoubiquitinated PCNA in Arabidopsis.

Murakumo et al. showed that human REV1 interacts with the REV7 subunit of Pol ζ through its C-terminal domain (Murakumo et al., 2001). Also, the C-terminal region of mouse REV1 interacts with Pol η and Pol ι , and Pol κ (Guo et al., 2003). From these facts, it is further proposed that the REV1 protein bound to the monoubiquitinated PCNA serves as a platform of polymerase switching to mediate recruitment of the appropriate polymerase to the damage site (Tissier et al., 2004; Lehmann et al., 2007). Taken together, the protein-protein interactions through the BRCT domain, UBMs, and/or C-terminal domain of REV1 seem

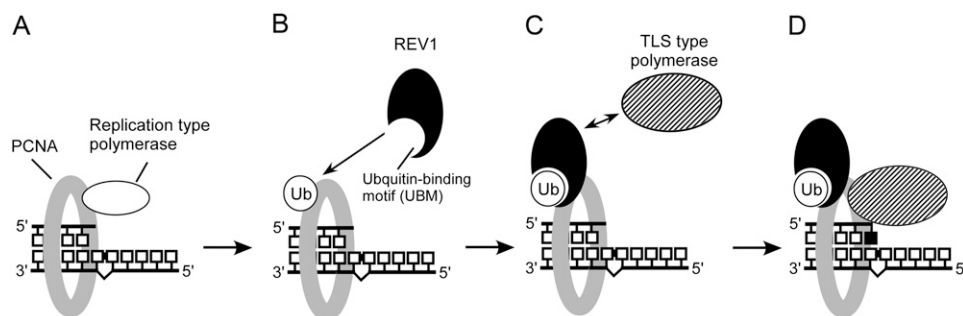


Figure 6. Possible role of AtREV1 in polymerase switching. A, Replication-type polymerase (white oval) bound to PCNA (gray ring) is stalled at the damage. B, PCNA is monoubiquitinated (Ub), which increases its affinity for AtREV1 (black oval with pocket that represents the UBM). C, TLS-type polymerase (hatched oval) interacts with the PCNA-bound AtREV1. D, A nucleotide (black square) is inserted by the action of the TLS-type polymerase. See the text for more detail.

to be indispensable for polymerase switching and for damage-tolerance activity. The AtREV1 protein putatively contains both a BRCT domain (Takahashi et al., 2005) and UBMs (Supplemental Fig. S1). Thus, it is conceivable that these multiple protein-protein interactions through the distinct domains of AtREV1 play an important role in UV tolerance in plant, although the exact position of the C-terminal polymerase-interacting domain remains to be identified. Based on this hypothesis, we propose a model for a possible role of AtREV1 and polymerase switching at the replication fork (Fig. 6). The replication fork is stalled when it encounters DNA damage (Fig. 6A). A signal from stalled replication triggers ubiquitination of PCNA, which leads to dissociation of the replication-type polymerase and attracts AtREV1 (Fig. 6B). TLS-type polymerases are recruited to the DNA damage through interaction with AtREV1 (Fig. 6C). TLS is accomplished by the action of one or more specialized polymerases (Fig. 6D).

Structure-function analysis of AtREV1, in which the BRCT, UBM, or C-terminal region is mutated, will be necessary to evaluate the function(s) of AtREV1 protein in plant damage tolerance.

MATERIALS AND METHODS

Construction of a Recombinant AtREV1 Expression Plasmid

To express the His-tagged AtREV1 protein, the AtREV1 coding fragment, prepared previously (Takahashi et al., 2005), was amplified by PCR using the REV1-pET-1B (5'-GCCCATGGCTCGTAGCTGGGTCAAATTC-3') and REV1-pET-2B (5'-GCGCGGCCGCTGGTATACTCAAGCTTCCTC-3') primers, which contain *NcoI* and *NotI* restriction sites (underlined), respectively. To insert the *NcoI* site at the N terminus of AtREV1, the second codon was changed from K to A (Fig. 1B). For sequencing, the amplified fragment was ligated into the pGEM-T easy vector (Promega). The fragment contained two base substitutions that were introduced by PCR, but neither of them caused an amino acid change. The plasmid was digested with *NcoI* and *NotI*, and inserted into the corresponding sites of the pET32a(+) vector (Novagen), which is located downstream of the *T7lac* promoter under the control of *lacI*. The resulting plasmid was named pET32a-AtREV1 (Fig. 1A).

Overexpression and Purification of Recombinant AtREV1

Escherichia coli BL21(DE3) cells (Novagen) carrying pET32a-AtREV1 were inoculated into 3 mL of Luria-Bertani (LB) broth containing 100 $\mu\text{g mL}^{-1}$ carbenicillin and were grown at 37°C for 18 h. One milliliter of this preculture was added into 50 mL of fresh LB broth containing 100 $\mu\text{g mL}^{-1}$ carbenicillin and further grown at the same temperature for 6 h. All of the culture was transferred to 0.9 L of fresh LB broth containing 100 $\mu\text{g mL}^{-1}$ carbenicillin and grown at 37°C until OD_{600} reached 0.6. To optimize the recovery of recombinant protein, expression was induced with IPTG for 3 h at 30°C or 22°C, or overnight at 15°C. At 30°C and 22°C, recombinant AtREV1 was predominantly accumulated in inclusion bodies. However, the recombinant protein was detected in the soluble fraction when the expression was induced at 15°C (data not shown). Therefore, the cells were grown at 37°C until OD_{600} reached 0.6, transferred to 15°C, induced with 0.8 mM IPTG (final concentration), and cultured for additional 20 h. One liter of cell culture was centrifuged at 4°C, and the cell paste was washed once with 100 mL of buffer A (20 mM Na_2HPO_4 , pH 7.4, 0.5 M NaCl, 10% glycerol, 1 mM dithiothreitol) and then stored at -80°C. Cell paste from 3 L of culture (approximately 11.5 g wet weight) was resuspended in 100 mL of buffer A containing lysozyme and the Protease inhibitor cocktail (Sigma-Aldrich), and lysed by sonication. The cell lysate was incubated at 4°C in 0.1% Triton X-100 for 30 min, centrifuged at 12,000g for 30 min, and then the supernatant (clear lysate) was recovered. The His-tagged

recombinant AtREV1 was separated with a Ni-affinity column. First, 10 mM imidazole was added to the clear lysate, then the lysate was mixed with 3 mL of Ni^{2+} -charged Chelating Sepharose Fast Flow (GE Healthcare) equilibrated in buffer B (20 mM Na_2HPO_4 , pH 7.4, 0.5 M NaCl, 10% glycerol, 10 mM 2-mercaptoethanol) containing 10 mM imidazole. The mixture was centrifuged at 500g, washed with 5 volumes of buffer B, and then packed in a PD-10 empty column (GE Healthcare). Bound proteins were eluted with 3 mL of buffer B containing 30, 60, 100, and 300 mM imidazole and collected in 3-mL fractions. The 300 mM imidazole fraction, containing AtREV1 proteins, was collected (Ni fraction). Six milliliters of the Ni fraction was desalted by 5-mL HiTrap Desalting column (GE Healthcare), in which the buffer was replaced with buffer C (20 mM Na_2HPO_4 , pH 7.4, 300 mM NaCl, 10% glycerol, 1 mM dithiothreitol). The desalted Ni fraction was then applied onto a 1-mL HiTrap heparin HP column (GE Healthcare) equilibrated with buffer C. After washing the column with 10 mL of buffer C, proteins were eluted with 3.5 mL of 20 mM Na_2HPO_4 buffer, pH 7.4, supplemented with 400, 450, and 500 mM NaCl. The fractions with 450 and 500 mM NaCl were collected (heparin fraction).

The heparin fraction (7 mL) was desalted as described above; the buffer was replaced with buffer D (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10% glycerol, 1 mM dithiothreitol). The fraction was then applied to a 1-mL HiTrap Q HP column (GE Healthcare) equilibrated with buffer D. After the column was washed with 10 mL of buffer D, proteins were eluted with 3.5 mL of buffer D supplemented with 350 or 400 mM NaCl. The purified AtREV1 protein, eluted in 350 mM NaCl, was collected. The total volume was approximately 1.5 mL and concentration was 80 $\mu\text{g mL}^{-1}$. Aliquots of the purified AtREV1 were dialyzed against buffer E (5 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 50% glycerol, 1 mM dithiothreitol) overnight and stored at -20°C.

Primer Extension Assay

Deoxynucleotidyl transferase activity of the purified recombinant AtREV1 was assayed according to Masuda and Kamiya (2002). Oligonucleotide template 5'-CTCGTCAGCATCTTCA $\underline{\text{X}}$ CATACAGTCAGTG-3' ($\underline{\text{X}}$ = G, 30G; A, 30A; T, 30T; C, 30C) and the primer 5'-CACTGACTGTATG-3' (P13) were purchased from Sigma Genosys Japan. The template with AP sites (same sequence as above template except for $\underline{\text{X}}$ = AP) was purchased from Midland Certified Reagent Company. Templates with UV damage, 5'-CTCGTCAGC-TTCATCATACAGTCAGTG-3' (TT = CPD or 6-4PP), were kindly provided by Dr. Shigenori Iwai, Osaka University. The primers 5'-CACTGACTGTATCATG-3' (P16), 5'-CACTGACTGTATCATGA-3' (P17A), and 5'-CACTGACTGTATCATGG-3' (P17G) were purchased from Sigma Genosys. To prepare primer templates, the primers were labeled using T4 polynucleotide kinase (Takara BIO) and [γ - ^{32}P]ATP (GE Healthcare) and annealed to templates. The reaction mixture (20 μL) contained 40 mM Tris-HCl buffer, pH 8.0, 10% glycerol, 0.1 mg mL^{-1} bovine serum albumin, 5 mM dithiothreitol, 0.1 mM of a single dNTP or 0.1 mM each of all four dNTPs, 25 nM primer template, 2 mM MgCl_2 or 1 mM MnCl_2 as divalent cations, and 2 μL (approximately 160 ng) of AtREV1. After incubation at 30°C for 10 min, the reactions were terminated with 10 μL of stop solution (30 mM EDTA, 94% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol) and the products were resolved on a 20% polyacrylamide gel containing 8 M urea. The gel was dried and used to expose an imaging plate (Fuji Photo Film) for about 40 h. The radioactivity was visualized using Bio-Imaging Analyzer BAS1800II (Fuji Photo Film).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AB187523.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Conserved UBM sequences in the REV1 proteins.

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LITERATURE CITED

- Acharya N, Johnson RE, Prakash S, Prakash L (2006) Complex formation with Rev1 enhances the proficiency of *Saccharomyces cerevisiae* DNA polymerase ζ for mismatch extension and for extension opposite from DNA lesions. *Mol Cell Biol* **26**: 9555–9563
- Bienko M, Green CM, Crosetto N, Rudolf F, Zapart G, Coull B, Kannouche P, Wider G, Peter M, Lehmann AR, et al (2005) Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis. *Science* **310**: 1821–1824
- Boiteux S, Guillet M (2004) Abasic sites in DNA: repair and biological consequences in *Saccharomyces cerevisiae*. *DNA Repair (Amst)* **3**: 1–12
- Britt AB (1999) Molecular genetics of DNA repair in higher plants. *Trends Plant Sci* **4**: 20–25
- Broomfield S, Hryciw T, Xiao W (2001) DNA postreplication repair and mutagenesis in *Saccharomyces cerevisiae*. *Mutat Res* **486**: 167–184
- Cadet J, Douki T, Pouget J-P, Ravanat J-L (2005) UVB and UVA induced formation of photoproducts within cellular DNA. In E Sage, R Drouin, M Rouabhia, eds, *From DNA Photolesions to Mutations, Skin Cancer and Cell Death*. RSC Publishing, London, pp 1–14
- Doublet S, Tabor S, Long AM, Richardson CC, Ellenberger T (1998) Crystal structure of a bacteriophage T7 DNA replication complex at 2.2 Å resolution. *Nature* **391**: 251–257
- Friedberg EC, Feaver WJ, Gerlach VL (2000) The many faces of DNA polymerases: strategies for mutagenesis and for mutational avoidance. *Proc Natl Acad Sci USA* **97**: 5681–5683
- Friedberg EC, Lehmann AR, Fuchs RPP (2005) Trading places: How do DNA polymerases switch during translesion DNA synthesis? *Mol Cell* **18**: 499–505
- Friedberg EC, Walker GC, Siede W, Wood RD, Schultz RA, Ellenberger T (2006) *DNA Repair and Mutagenesis*, Ed 2. American Society of Microbiology Press, Washington, DC
- Gallego F, Fleck O, Li A, Wyrzykowska J, Tinland B (2000) AtRAD1, a plant homologue of human and yeast nucleotide excision repair endonucleases, is involved in dark repair of UV damages and recombination. *Plant J* **21**: 507–518
- Gao MJ, Murphy TM (2001) Alternative forms of formamidopyrimidine-DNA glycosylase from *Arabidopsis thaliana*. *Photochem Photobiol* **73**: 128–134
- García-Ortiz MV, Ariza RR, Hoffman PD, Hays JB, Roldán-Arjona TR (2004) *Arabidopsis thaliana* AtPOLK encodes a DinB-like DNA polymerase that extends mispaired primer termini and is highly expressed in a variety of tissues. *Plant J* **39**: 84–97
- García-Ortiz MV, Ariza RR, Roldán-Arjona T (2001) An OGG1 orthologue encoding a functional 8-oxoguanine DNA glycosylase/lyase in *Arabidopsis thaliana*. *Plant Mol Biol* **47**: 795–804
- Garg P, Burgers PM (2005) Ubiquitinated proliferating cell nuclear antigen activates translesion DNA polymerases η and REV1. *Proc Natl Acad Sci USA* **102**: 18361–18366
- Guo C, Fischhaber PL, Luk-Paszyc MJ, Masuda Y, Zhou J, Kamiya K, Kisker C, Friedberg EC (2003) Mouse Rev1 protein interacts with multiple DNA polymerases involved in translesion DNA synthesis. *EMBO J* **22**: 6621–6630
- Guo C, Tang TS, Bienko M, Parker JL, Bielen AB, Sonoda E, Takeda S, Ulrich HD, Dikic I, Friedberg EC (2006) Ubiquitin-binding motifs in REV1 protein are required for its role in the tolerance of DNA damage. *Mol Cell Biol* **26**: 8892–8900
- Guo D, Xie Z, Shen H, Zhao B, Wang Z (2004) Translesion synthesis of acetylaminofluorene-dG adducts by DNA polymerase ζ is stimulated by yeast Rev1 protein. *Nucleic Acids Res* **32**: 1122–1130
- Haracska L, Unk I, Johnson RE, Johansson E, Burgers PMJ, Prakash S, Prakash L (2001) Roles of yeast DNA polymerases δ and ζ and of Rev1 in the bypass of abasic sites. *Genes Dev* **15**: 945–954
- Haracska L, Prakash S, Prakash L (2002) Yeast Rev1 protein is G template-specific DNA polymerase. *J Biol Chem* **277**: 15546–15551
- Kovalchuk I, Kovalchuk O, Hohn B (2000) Genome-wide variation of the somatic mutation frequency in transgenic plants. *EMBO J* **19**: 4431–4438
- Landry LG, Stapleton AE, Lim J, Hoffman P, Hays J, Walbot V, Last R (1997) An *Arabidopsis* photolyase mutant is hypersensitive to ultraviolet-B radiation. *Proc Natl Acad Sci USA* **94**: 328–332
- Lehmann AR, Niimi A, Ogi T, Brown S, Sabbioneda S, Wing JF, Kannouche PL, Green CM (2007) Translesion synthesis: Y-family polymerases and the polymerase switch. *DNA Repair (Amst)* **6**: 891–899
- Lemontt JF (1971) Mutants of yeast defective in mutation induced by ultraviolet light. *Genetics* **68**: 21–33
- Lin W, Xu H, Zhang Y, Wu X, Yuan F, Wang Z (1999) The human REV1 gene codes for a DNA template-dependent dCMP transferase. *Nucleic Acids Res* **27**: 4468–4475
- Liu J, Hall JD, Mount DW (2001) *Arabidopsis UVH3* gene is a homolog of the *Saccharomyces cerevisiae* RAD2 and human XPG DNA repair genes. *Plant J* **26**: 329–338
- Liu J, Hong SW, Escobar M, Vierling E, Mitchell D, Mount DW, Hall JD (2003) *Arabidopsis UVH6*, a homolog of human XPD and yeast RAD3 DNA repair genes, functions in DNA repair and is essential for plant growth. *Plant Physiol* **132**: 1405–1414
- Masuda Y, Kamiya K (2002) Biochemical properties of the human REV1 protein. *FEBS Lett* **520**: 88–92
- Masuda Y, Takahashi M, Fukuda S, Sumii M, Kamiya K (2002) Mechanisms of dCMP transferase reactions catalyzed by mouse Rev1 protein. *J Biol Chem* **277**: 3040–3046
- Masuda Y, Takahashi M, Tsunekuni N, Minami T, Sumii M, Miyagawa K, Kamiya K (2001) Deoxycytidyl transferase activity of the human REV1 protein is closely associated with the conserved polymerase domain. *J Biol Chem* **276**: 15051–15058
- Mitchell DL (1995) DNA damage and repair. In WM Horspool, P-S Song, eds, *CRC Handbook of Organic Photochemistry and Photobiology*. CRC Press, New York, pp 1326–1331
- Murakumo Y, Ogura Y, Ishii H, Numata S, Ichihara M, Croce CM, Fishel R, Takahashi M (2001) Interaction in the error-prone postreplication repair proteins hREV1, hREV3, and hREV7. *J Biol Chem* **276**: 35644–35651
- Nair DT, Johnson RE, Prakash L, Prakash S, Aggarwal AK (2005) Rev1 employs a novel mechanism of DNA synthesis using a protein template. *Science* **309**: 2219–2222
- Nakajima S, Sugiyama M, Iwai S, Hitomi K, Otoshi E, Kim ST, Jiang CZ, Todo T, Britt A, Yamamoto K (1998) Cloning and characterization of a gene (*UVR3*) required for photorepair of 6-4 photoproducts in *Arabidopsis thaliana*. *Nucleic Acids Res* **26**: 638–644
- Nakamura J, Walker VE, Upton PB, Chiang SY, Kow YW, Swenberg JA (1998) Highly sensitive apurinic/aprimidinic site assay can detect spontaneous and chemically induced depurination under physiological condition. *Cancer Res* **58**: 222–225
- Nelson JR, Gibbs PEM, Nowicka AM, Hinkle DC, Lawrence CW (2000) Evidence for a second function for *Saccharomyces cerevisiae* Rev1p. *Mol Microbiol* **37**: 549–554
- Nelson JR, Lawrence CW, Hinkle DC (1996a) Deoxycytidyl transferase activity of yeast REV1 protein. *Nature* **382**: 729–731
- Nelson JR, Lawrence CW, Hinkle DC (1996b) Thymine-thymine dimer bypass by yeast DNA polymerase ζ . *Science* **272**: 1646–1649
- Ohmori H, Friedberg EC, Fuchs RPP, Goodman ME, Hanaoka F, Hinkle D, Kunkel TA, Lawrence CW, Livneh Z, Nohmi T, et al (2001) The Y-family of DNA polymerases. *Mol Cell* **8**: 7–8
- Osakabe K, Abe K, Yoshioka T, Osakabe Y, Todoriki S, Ichikawa H, Hohn B, Toki S (2006) Isolation and characterization of the *RAD54* gene from *Arabidopsis thaliana*. *Plant J* **48**: 827–842
- Parker JL, Bielen AB, Dikic I, Ulrich HD (2007) Contributions of ubiquitin- and PCNA-binding domains to the activity of Polymerase η in *Saccharomyces cerevisiae*. *Nucleic Acids Res* **35**: 881–889
- Prakash S, Johnson RE, Prakash L (2005) Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function. *Annu Rev Biochem* **74**: 317–353
- Ross A-L, Simpson LJ, Sale JE (2005) Vertebrate DNA damage tolerance requires the C-terminus but not BRCT or transferase domains of REV1. *Nucleic Acids Res* **33**: 1280–1289
- Sakamoto A, Lan VTT, Hase Y, Shikazono N, Matsunaga T, Tanaka A (2003) Disruption of the *AtREV3* gene causes hypersensitivity to ultraviolet B light and γ -rays in *Arabidopsis*: implication of the presence of a translesion synthesis mechanism in plants. *Plant Cell* **15**: 2042–2057

- Santiago MJ, Alejandre-Durán E, Ruiz-Rubio M** (2006) Analysis of UV-induced mutation spectra in *Escherichia coli* by DNA polymerase from *Arabidopsis thaliana*. *Mutat Res* **601**: 51–60
- Song JM, Milligan JR, Sutherland BM** (2002) Bistranded oxidized purine damage clusters: induced in DNA by long-wavelength ultraviolet (290–400 nm) radiation? *Biochemistry* **41**: 8683–8688
- Steitz TA** (1998) DNA polymerases: structural diversity and common mechanisms. *J Biol Chem* **274**: 17395–17398
- Stelter P, Ulrich HD** (2003) Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation. *Nature* **425**: 188–191
- Takahashi S, Sakamoto A, Sato S, Kato T, Tabata S, Tanaka A** (2005) Roles of *Arabidopsis AtREV1* and *AtREV7* in translesion synthesis. *Plant Physiol* **138**: 870–881
- Tissier A, Kannouche P, Reck M-P, Lehmann AR, Fuchs RPP, Cordonnier A** (2004) Co-localization in replication foci and interaction of human Y-family members, DNA polymerase pol η and REV1 protein. *DNA Repair (Amst)* **3**: 1503–1514
- Vaisman A, Ling H, Woodgate R, Yang W** (2005) Fidelity of Dpo4: effect of metal ions, nucleotide selection and pyrophospholysis. *EMBO J* **24**: 2957–2967
- Wood A, Garg P, Burgers MJ** (2007) A ubiquitin-binding motif in the translesion DNA polymerase Rev1 mediates its essential function interaction with ubiquitinated proliferating cell nuclear antigen in response to DNA damage. *J Biol Chem* **282**: 20256–20263
- Zhang Y, Wu X, Rechkoblit O, Geacintov E, Taylor JS, Wang Z** (2002) Response of human REV1 to different DNA damage: preferential dCMP insertion opposite the lesion. *Nucleic Acids Res* **30**: 1630–1638