

Role of AtPol ζ , AtRev1, and AtPol η in UV Light-Induced Mutagenesis in Arabidopsis^{1[W]}

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Translesion synthesis (TLS) is a DNA damage tolerance mechanism in which DNA lesions are bypassed by specific polymerases. To investigate the role of TLS activities in ultraviolet light-induced somatic mutations, we analyzed Arabidopsis (*Arabidopsis thaliana*) disruptants of *AtREV3*, *AtREV1*, and/or *AtPOLH* genes that encode TLS-type polymerases. The mutation frequency in *rev3-1* or *rev1-1* mutants decreased compared with that in the wild type, suggesting that AtPol ζ and AtRev1 perform mutagenic bypass events, whereas the mutation frequency in the *polh-1* mutant increased, suggesting that AtPol η performs nonmutagenic bypass events with respect to ultraviolet light-induced lesions. The *rev3-1 rev1-1* double mutant showed almost the same mutation frequency as the *rev1-1* single mutant. The increased mutation frequency found in *polh-1* was completely suppressed in the *rev3-1 polh-1* double mutant, indicating that AtPol ζ is responsible for the increased mutations found in *polh-1*. In summary, these results suggest that AtPol ζ and AtRev1 are involved in the same (error-prone) TLS pathway that is independent from the other (error-free) TLS pathway mediated by AtPol η .

Plants are continuously exposed to various environmental stresses including UV light and other DNA-damaging agents. UV light induces DNA lesions such as the formation of cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts that block DNA replication and transcription (Umlas et al., 1985). To remove the DNA lesions, plants possess various DNA repair mechanisms such as photorepair, excision repair, and recombination repair. Some lesions, however, are not removed by the repair processes (Hidema et al., 1999) and therefore can endanger faithful DNA replication.

Translesion synthesis (TLS) is an important damage tolerance mechanism in which damaged DNA is bypassed by the action of specific polymerases, thus avoiding potential replication arrest (Friedberg et al., 2005). Many organisms ranging from bacteria to humans possess TLS activities (Woodgate, 1999; Baynton and Fuchs, 2000), and loss of TLS often results in survival rate reduction and increased sensitivity to DNA-damaging agents (Lawrence, 2004; Waters et al.,

2009). DNA synthesis during TLS often causes mutations due to the low fidelity of replication that occurs when the DNA lesions are bypassed (Friedberg et al., 2005).

The specific polymerases involved in TLS are conserved among species (Waters et al., 2009). For example, DNA Polymerase ζ (Pol ζ), Rev1, and Pol η from yeast and mammals have been well characterized (Waters et al., 2009) and show high conservation. Genes for REV3 and REV7, the subunits of Pol ζ , were originally isolated from yeast using a screening process based on the reduction of UV light-induced reversions (Lemontt, 1971; Lawrence et al., 1985). Pol ζ belongs to the B-family of DNA polymerases, possessing mismatch-extension activity (Nelson et al., 1996; Johnson et al., 2000a). Rev1 was also isolated from a yeast reversionless mutant (Lawrence and Christensen, 1976) and possesses deoxycitidyl transferase activity (Nelson et al., 1996). Based on several lines of evidence, Pol ζ and Rev1 are thought to promote damage-induced mutations (Kunz et al., 2000). On the other hand, Pol η was originally identified in yeast from its homology with the *Escherichia coli* DinB protein (McDonald et al., 1997). Lack of Pol η in humans causes xeroderma pigmentosum variant (XPV), a disease characterized by high susceptibility to sunlight-induced cancer (Kraemer, 2003). Therefore, Pol η is thought to possess a function that involves the prevention of UV light-induced mutations in humans (Pagès and Fuchs, 2002).

In Arabidopsis (*Arabidopsis thaliana*), we and other groups have isolated several genes encoding TLS-type polymerase homologs: *AtREV3*, *AtREV7*, *AtREV1*, *AtPOLH*, and *AtPOLK* (Sakamoto et al., 2003; García-Ortiz et al., 2004; Takahashi et al., 2005; Santiago et al., 2006). *AtREV3* and *AtREV7* encode a catalytic subunit

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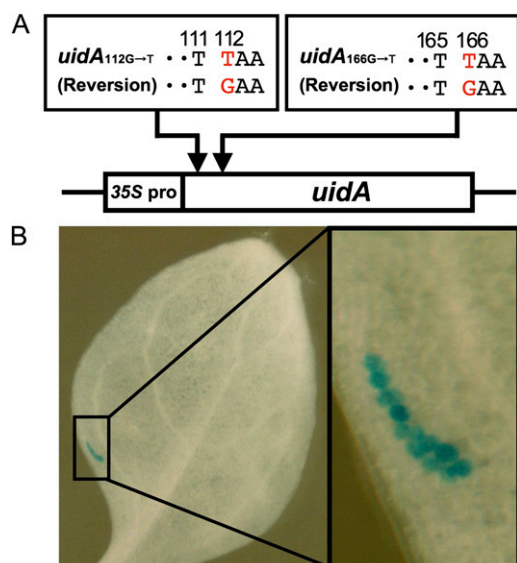


Figure 1. Detection of reversion using nonsense codon-introduced *uidA* genes. A, Design of reporter genes used for the detection of base substitutions. Two reporter genes, *uidA*_{112G-T} and *uidA*_{166G-T} were generated by substituting thymine (T) for guanine (G) at positions 112 or 166 of the *uidA* gene (Kovalchuk et al., 2000). B, Visualization of a GUS reversion sector in Arabidopsis following histochemical staining with 5-bromo-4-chloro-3-indolyl- β -D-GlcUA cyclohexylammonium salt. The box indicates GUS-positive cells with reversion of the *uidA* gene. Left, whole leaf; right, higher magnification image of stained cells.

and a regulatory subunit of AtPol ζ , respectively. AtRev1, encoded by the *AtREV1* gene, possesses deoxynucleotidyl transferase activity with low fidelity and inserts a nucleotide opposite apurinic/aprimidinic sites in vitro (Takahashi et al., 2007). AtPol η , encoded by *AtPOLH*, bypasses cyclobutane pyrimidine dimers with comparable activity to human or yeast Pol η (Anderson et al., 2008; Hoffman et al., 2008). *AtREV3*-, *AtREV7*-, *AtREV1*-, or *AtPOLH*-disrupted plants are more sensitive to UV light exposure compared with wild-type plants, although the levels of sensitivity differ (Sakamoto et al., 2003; Takahashi et al., 2005; Curtis and Hays, 2007; Anderson et al., 2008), suggesting that these genes play a role in conferring tolerance to UV light-induced damage.

In this study, we analyzed UV light-induced somatic mutation frequencies in disruptants of the *AtREV3*, *AtREV1*, and *AtPOLH* genes. Point-mutated, and thus inactivated, *uidA* genes were employed to score UV light-induced mutations. Nucleotide substitutions that may occur by error during TLS restore the *uidA* genes, whose activity can be detected by examination of the blue sectors following GUS staining. We demonstrate that the mutation frequency in the disruptants was significantly altered compared with that of the wild type. This result suggests that AtPol ζ , AtRev1, and AtPol η , encoded by *AtREV3*, *AtREV1*, and *AtPOLH*, respectively, are involved in TLS processes related to UV light mutagenesis.

RESULTS

Detection of Somatic Mutations in Arabidopsis

To examine whether TLS pathways are employed in UV light resistance in higher plants, we analyzed the mutation frequency in Arabidopsis somatic tissues following UV-C irradiation. The reporter genes used for this analysis comprised *uidA* genes containing a nonsense mutation, *uidA*_{112G-T} or *uidA*_{166G-T}, generated by replacing the 112th or 166th guanine with thymine, respectively (Fig. 1A; Kovalchuk et al., 2000). The substituted thymine and the flanking 111th or 165th thymine generate a potential thymine-thymine (TT) dimer target. The reporter gene will become active when a T-to-G reversion occurs at the 112th or 166th thymine position. Transgenic plants carrying the reporter gene were treated with or without UV-C light and then grown for an additional 10 d, which allows cells with an active *uidA* gene to proliferate and produce a detectable blue sector on somatic tissues (Fig. 1B). Therefore, the number of blue sectors present is indicative of the number of reversions that have actually taken place (Kovalchuk et al., 2000).

To investigate the role of TLS-type polymerases in UV light-induced mutation, the mutation frequency in disruptants of the *AtREV3*, *AtREV1*, and *AtPOLH* genes, *rev3-1*, *rev1-1*, and *polh-1*, respectively, was compared with that of the wild type.

The reversion frequencies were initially measured without UV light exposure to determine the level of spontaneous mutations. Twenty-four-day-old nonirradiated plants were examined by counting the active GUS sectors. In the wild type, the number of reversion events per 100 plants was 1.48 ± 0.46 . This result is comparable to that of a previous report (Kovalchuk et al., 2000). We also examined the mutation frequencies in *rev3-1*, *rev1-1*, and *polh-1*. The frequencies in the *rev3-1* and *rev1-1* mutants were slightly lower compared with that of the wild type ($P < 0.05$; Table I). By contrast, the mutation frequency in *polh-1* plants did not change significantly compared with that of the wild type. This result suggests that AtPol ζ and AtRev1 play some role in the generation of spontaneous mutations in Arabidopsis somatic cells.

Table 1. Spontaneous mutation frequency in *AtREV3*-, *AtREV1*-, and *AtPOLH*-disrupted plants

Each experiment was repeated at least three times.

Reporter	Background	Reversion Event per 100 Plants	Fresh Wt per Plant
			mg
<i>uidA</i> _{166G-T}	Wild type	1.48 ± 0.46	14.76 ± 3.44
	<i>rev3-1</i>	0.46 ± 0.05	11.32 ± 3.44
	<i>rev1-1</i>	0.64 ± 0.54	12.93 ± 2.05
	<i>polh-1</i>	1.97 ± 1.58	11.39 ± 3.51

UV-Induced Mutation Frequency in *AtREV3*, *AtREV1*, or *AtPOLH* Disruptants

The mutation frequencies in UV-irradiated *rev3-1*, *rev1-1*, *polh-1*, and wild-type plants were then examined. With the *uidA*_{166G-T} reporter gene, the average reversion in wild-type plants was approximately 11 per 100 plants (Fig. 2; Supplemental Table S1). By contrast, the average reversion in *rev3-1* plants was approximately one-quarter that of wild-type siblings ($P < 0.05$; Fig. 2; Supplemental Table S1). A similar result was obtained with the *uidA*_{112G-T} gene, where the reversion frequency in *rev3-1* was significantly lower than that of the wild type ($P < 0.05$; Fig. 2). These results indicate that AtPolζ plays a role in promoting mutagenesis following UV light exposure. Since two independent reporter genes showed similar results, we concluded that these two inactivated genes were similarly useful in the detection of reversion events, and hereafter only *uidA*_{166G-T} was used for further analysis. The UV light-induced mutation frequency in *rev1-1* plants was less than one-quarter that of wild-type siblings ($P < 0.05$; Fig. 2). This result suggests that AtRev1 also promotes UV light-induced mutagenesis.

By contrast, disruption of the *AtPOLH* gene significantly increased the UV light-induced mutation frequency, where the average reversion in *polh-1* plants was approximately 2.5 times that of wild-type siblings ($P < 0.05$; Fig. 2). This result indicates that AtPolη plays a role in suppressing the induction of mutations following UV light exposure.

The mutation frequency in the wild type was 7.7 times higher with UV light exposure than without UV light exposure. Similarly, the frequency in *rev3-1*,

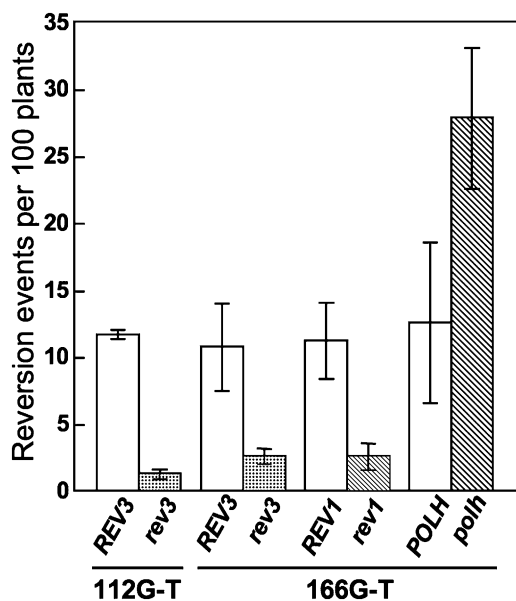


Figure 2. UV light-induced mutation frequencies in *AtREV3*-, *AtREV1*-, and *AtPOLH*-disrupted plants. Bars represent average frequencies per 100 plants derived from multiple experiments. Error bars indicate SD.

rev1-1, or *polh-1* was 5.6, 4.0, or 14 times higher with UV light exposure. This result indicates that mutations are greatly induced by UV-C light treatment. Under these assay conditions, the average fresh weights of mutant plants and wild-type siblings grown side by side under the same conditions were not significantly different (Supplemental Table S1). Therefore, the decreased or increased reversion frequencies in the mutants are not likely due to the reduced or enhanced growth of these plants.

Mutation Frequency in *rev3 polh* Double Mutants

We and other groups previously reported that the *AtREV3* and *AtPOLH* double disruptant was more sensitive to UV irradiation than either of the single mutants (Curtis and Hays, 2007; Anderson et al., 2008). This observation suggests that AtPolζ and AtPolη are involved in independent pathways pertaining to UV light tolerance. On the other hand, analysis of the UV light-induced mutation frequency in *rev3-1* and *polh-1* mutants showed that AtPolζ and AtPolη seem to possess opposite functions with respect to UV light-induced mutagenesis. To explore the relationship between AtPolζ and AtPolη with respect to UV light-induced mutagenesis, we prepared the *rev3-1 polh-1* double mutant and compared the UV light-induced mutation frequency in the double mutant with that of the single mutant siblings *rev3-1* and *polh-1*. The high reversion frequency in the *polh-1* mutant was significantly reduced in the *rev3-1 polh-1* double mutant, and the frequency in *rev3-1 polh-1* was almost similar to that of the *rev3-1* mutant (Fig. 3A; Supplemental Table S2). This result indicates that the mutations observed in *polh-1* are mainly induced in an AtPolζ-dependent manner.

Mutation Frequency in *rev1 rev3* Double Mutants

In a previous report, we showed the possibility that AtPolζ and AtRev1 might cooperate to bypass certain types of DNA damage (Takahashi et al., 2007). When the *rev3-1* and *rev1-1* mutants were grown under long-term UV-B light exposure, their fresh weights were similarly inhibited (Takahashi et al., 2005). Furthermore, the reversion frequencies in both *rev3-1* and *rev1-1* mutants were lower compared with that of the wild type (Fig. 2). Based on these data, we further hypothesized that AtPolζ and AtRev1 may operate in the same pathway that confers tolerance to UV damage. In an effort to examine this possibility, we prepared the *rev3-1 rev1-1* double mutant and compared its UV light-induced mutation frequency with that of the single mutants *rev3-1* and *rev1-1*. It was found that the reversion frequency in the *rev3-1 rev1-1* mutant was slightly lower than that in the *rev3-1* mutant ($P < 0.05$) and similar to that in the *rev1-1* mutant (Fig. 3B; Supplemental Table S2). This result is consistent with the notion that AtPolζ and AtRev1 are involved in the same pathway and work cooperatively

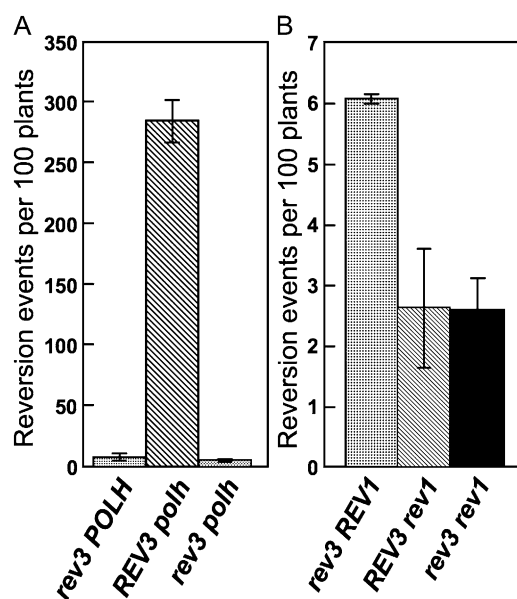


Figure 3. UV light-induced mutation frequencies in *AtREV3*, *AtREV1*, and *AtPOLH* double disruptions. A, Mutation frequencies in *rev3*, *polh*, and *rev3 polh* mutants. B, Mutation frequencies in *rev3*, *rev1*, and *rev3 rev1* mutants. Bars represent average frequencies per 100 plants derived from multiple experiments. Error bars indicate sd.

in mutagenesis. The almost identical mutation frequencies of the *rev1-1* and *rev3-1 rev1-1* mutants suggest that the pathway requires the presence of AtRev1. By contrast, the slight difference in mutation frequency between the *rev3-1* and *rev3-1 rev1-1* mutants may suggest that AtRev1 could function, at least partly, in an AtPol ζ -independent manner.

DISCUSSION

AtPol ζ , AtRev1, and AtPol η Play Important Roles in UV Light-Induced Mutagenesis

TLS is an important damage tolerance mechanism that is conserved in many organisms. It is thought that two TLS pathways exist for the bypass of UV light-induced DNA damage: an error-prone TLS pathway and an error-free TLS pathway. In yeast, where disruption of *REV3* or *REV1* suppresses the formation of UV light-induced reversions (Lemontt, 1971; Lawrence and Christensen, 1976) and both *REV3* and *REV1* encode proteins containing DNA polymerase domains (Larimer et al., 1989; Morrison et al., 1989), UV damage is believed to be bypassed in a mutagenic manner (error-prone TLS). In this study, disruption of *AtREV3* or *AtREV1* greatly reduced the generation of UV-induced mutations (Fig. 2). Thus, the results suggest that *AtREV3* and *AtREV1* play a similar role in UV-induced mutagenesis as with yeast *REV3* and *REV1*. Additionally, we have shown that the mutation frequency of the *rev3-1 rev1-1* double mutant was almost identical to that of the *rev1-1* single mutant (Fig. 3B).

This result supports the notion that AtPol ζ and AtRev1 work cooperatively to bypass UV damage, which is required for UV light tolerance and UV light-induced mutagenesis.

By contrast, Pol η was originally identified from analysis of the yeast *rad30* mutant and its homology with the bacterial UmuC or DinB protein (McDonald et al., 1997; Roush et al., 1998). Pol η deficiency in humans is associated with the inherited disorder XPV, a disease characterized by hypersensitivity to UV light and an increased incidence of skin cancer (Johnson et al., 1999a; Masutani et al., 1999). Based on these facts and in vitro bypass activities (Johnson et al., 1999b), Pol η is thought to be involved in the error-free TLS pathway. Pol η -deficient yeast strains showed elevated UV light-induced mutation frequencies (Yu et al., 2001; Kozmin et al., 2003), and the high UV light-induced mutation frequency in XPV cells was suppressed by the presence of an intact *POLH* gene (Stary et al., 2003). In Arabidopsis, disruption of *AtPOLH* resulted in an increase in the mutation frequency (Fig. 2). This result suggests that AtPol η , encoded by *AtPOLH*, is also involved in the bypass of UV light-induced damage in an error-free manner.

In a previous study, we found that disruption of both *AtREV3* and *AtPOLH* resulted in an additive inhibitory effect on root growth (Anderson et al., 2008), suggesting that AtPol ζ and AtPol η act via two independent pathways. Furthermore, disruption of *AtREV3* decreased the mutation frequency while disruption of *AtPOLH* increased the mutation frequency in this study (Fig. 2). These results suggest that two pathways, an error-prone pathway involving AtPol ζ and an error-free pathway involving AtPol η , compete in part for the bypass of UV light-induced DNA damage. The high mutation frequency of the *polh-1* mutant was completely suppressed by the *rev3-1* mutation (Fig. 3A). This result could be accounted for if the error-prone pathway involving AtPol ζ predominates in the bypass of UV damage when AtPol η is unavailable. By contrast, the mutation frequency of the *rev3-1* mutant was not lower than that of the *rev3-1 polh-1* mutant (Fig. 3A). This might indicate that the error-free pathway involving AtPol η does not complement the error-prone pathway even when AtPol ζ is unavailable.

Template Preference, Replication Fidelity, and Mutagenesis

Results of mutational analyses suggested the mutagenic roles of AtPol ζ and AtRev1 and the antimutagenic role of AtPol η . However, a series of biochemical analyses of Arabidopsis and other organisms do not support such roles in mutagenesis. For example, yeast and human Pol η have lower replication fidelity than yeast Pol ζ when replicating undamaged DNA (McCulloch and Kunkel, 2008). This contradiction is mostly due to differences in template preference and assay conditions. Since our reporter genes are

designed to detect a base substitution at 3'T of TT, we focused here on the bypass activity and fidelity for cyclobutane TT dimers (CTDs) and (6-4) TT photoproducts (6-4TPs). Analysis of yeast and mammals revealed that Pol η bypasses CTDs efficiently, although the fidelity is quite low (Johnson et al., 2000b; Washington et al., 2001). Any misincorporation by Pol η , however, is corrected by other polymerases (Bebenek et al., 2001; Washington et al., 2001) to effect bypass in an error-free manner. In Arabidopsis, AtPol η bypassed CTDs efficiently (Anderson et al., 2008) and disruption of *AtPOLH* increased the mutation frequency (Fig. 2). Therefore, we hypothesize that the bypass of CTDs by AtPol η is still more efficient and accurate compared with other polymerases and that AtPol η "prevents" mutagenesis by other polymerases (Fig. 4A). By contrast, in vitro replication analysis using recombinant polymerases revealed that human, yeast, and Arabidopsis Pol η poorly bypass 6-4TPs (Hoffman et al., 2008).

On the other hand, although several reports have detailed the biochemical activities of Pol ζ and Rev1, most of these suggest that neither Pol ζ nor Rev1 can efficiently bypass CTDs and 6-4TPs (Johnson et al., 2000a; Guo et al., 2001; Zhang et al., 2002). Recombinant AtRev1 showed no insertion activity against templates with this type of DNA damage (Takahashi et al., 2007). However, in vivo replication assays in other organisms revealed that although Pol ζ and Rev1 are required for the bypass of 6-4TPs (Otsuka et al., 2005), they are dispensable for the bypass of CTDs (Nelson et al., 2000; Gibbs et al., 2005). If this were also true in Arabidopsis, most of the mutations observed in the wild-type background would be caused by the

mutagenic bypass of 6-4TPs by AtPol ζ and AtRev1 (Fig. 4A). It is conceivable that AtRev1 plays a role in recruiting other TLS-type polymerase(s) such as AtPol ζ , AtPol κ , and AtPol η , thereby allowing these enzymes to perform the bypass of damaged DNA, as suggested in our previous report (Takahashi et al., 2007).

Several lines of evidence indicate that the mutagenic bypass of CTDs occur in a Pol ζ -dependent manner if Pol η is unavailable (Gibbs et al., 2005; Shachar et al., 2009). We suppose that the increased mutation frequency in *polh-1* was caused by AtPol ζ (and AtRev1), which complements AtPol η for the bypass of CTDs (Fig. 4B).

Spontaneous and Other Mutation Sources

Employment of the GUS assay without UV-C light exposure revealed that Arabidopsis plants carried somatic mutations even under normal growth conditions (Table I). Reduction of the spontaneous mutation frequency in *rev3-1* and *rev1-1* indicates that AtPol ζ and AtRev1 are involved in spontaneous mutagenesis, as reported for yeast (Kalinowski et al., 1995; Harfe and Jinks-Robertson, 2000). In our previous report, we suggested that AtPol ζ and AtRev1 are involved in the bypass of apurinic/apyrimidinic sites that are generated under normal physiological conditions (Takahashi et al., 2007). Additionally, our preliminary results suggested the involvement of AtPol ζ and AtRev1 in γ -ray-induced mutations (M. Nakagawa, S. Takahashi, A. Tanaka, I. Narumi, and A.N. Sakamoto, unpublished data). These lines of evidence indicate that TLS-type polymerases are required to overcome various types of DNA damage under a variety of plant life conditions.

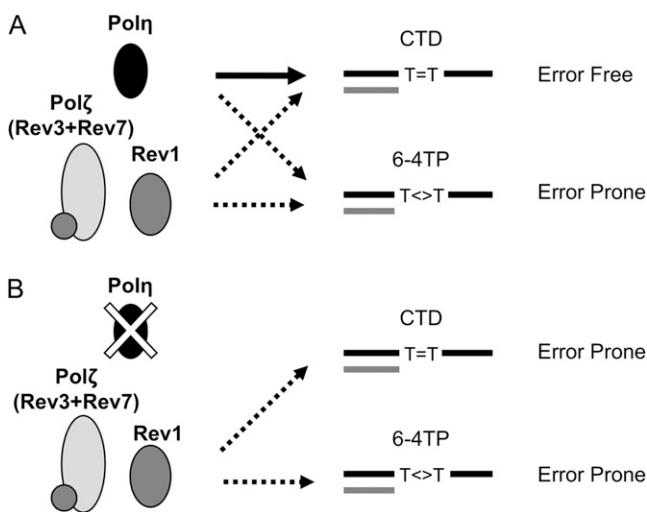


Figure 4. Schematic representation of error-free and error-prone TLS pathways in Arabidopsis. Black and gray lines indicate template and newly synthesized DNA strands, respectively. The solid arrow indicates accurate, efficient bypass activity, whereas the broken arrows indicate inaccurate, inefficient bypass activity. A, TLS activities in wild-type plants. B, TLS activities in *AtPOLH*-disrupted plants.

Plant Development and Mutations

In experiments employing the GUS assay, 2-week-old plants possessing two to four true leaves were irradiated with UV-C light and then subjected to GUS staining 10 d after irradiation, by which time plants possessed six to nine true leaves. Interestingly, active GUS sectors were mainly detected on relatively mature leaves or leaf stalks but never on meristems or first to third leaves from inside (data not shown). Furthermore, almost all blue sectors were relatively small and consisted of several cells resulting from a few cell divisions (Fig. 1B). If the mutation had occurred at the initial stage of leaf development, the blue sector should have covered the large area of the leaf. However, such large GUS sectors were never observed. This observation suggests that reversion events do not arise in undifferentiated cells at the time of irradiation. That is, mutagenic TLS activities might be avoided in undifferentiated tissues that could lead to germline cells in plants. In rice, several excision repair genes are expressed predominantly in shoot and root meristems but not in mature leaves (Kimura et al., 2004). It is known that homologous recombinations, an error-free repair pathway, take place more frequently at younger

stages during plant development in Arabidopsis (Boyko et al., 2006). It is possible that higher plants employ error-free pathways to maintain an intact genome in undifferentiated tissue, whereas mutagenic bypass is utilized in differentiated tissues notwithstanding the risk of alterations to the genetic information. Further analyses investigating the possible temporal- and tissue-specific activity of TLS are required in an effort to delineate plant strategies that operate to maintain genome integrity.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) Columbia accession was used as the wild type in this study. The *rev3-1* (Sakamoto et al., 2003), *rev1-1* (SALK_011334; Takahashi et al., 2005), *polh-1* (SALK_129731; Curtis and Hays, 2007; Anderson et al., 2008), and GUS reporter transgenic lines *uidA_{112G-T}* and *uidA_{166G-T}* (Kovalchuk et al., 2000) were in the Columbia background. The *rev1-1* and *polh-1* lines were provided by the Salk Institute Genomic Analysis Laboratory (<http://signal.salk.edu>). The *uidA* transgenic lines were crossed with *rev3-1*, *rev1-1*, *polh-1*, *rev3-1 rev1-1*, and *rev3-1 polh-1*, and F2 plants homozygous for the mutant *rev3-1*, *rev1-1*, *polh-1*, *rev3-1 rev1-1*, and *rev3-1 polh-1* were selected by PCR. F2 lines homozygous for the reporter gene were selected by BASTA resistance. From the same sibling of the cross, homozygous *REV3*, *REV1*, *POLH*, *REV3 rev1-1*, *rev3-1 REV1*, *REV3 polh-1*, and *rev3-1 POLH* plants harboring the *uidA* gene homozygously were obtained and used as controls.

Plants were grown on Rockwool (Nichias) with 0.1% (v/v) commercial nutrient under a 16-h-light/8-h-dark photoperiod at 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 21°C in a growth chamber. To minimize experimental errors caused by uneven growth or physiological conditions, the wild type and mutant derived from a single F1 plant were grown in the same tray and examined side by side.

Reversion Assay

Two-week-old seedlings carrying the reporter gene were irradiated with 1 kJ m^{-2} UV-C light supplied by a UV lamp (CSL-30C; COSMO BIO) and then incubated in the dark for 1 d. Ten days following UV-C irradiation, plants were vacuum infiltrated twice for 10 min with 100 mM sodium phosphate buffer (pH 7.2) containing 0.1% (v/v) Triton X-100, 0.05% (w/v) sodium azide, 0.5 mM each of potassium hexacyanoferrate (III) and potassium hexacyanoferrate (II) trihydrate, and 0.95 M 5-bromo-4-chloro-3-indolyl- β -D-GlcUA cyclohexylammonium salt. Following vacuum infiltration, plants were incubated at 37°C for 2 d in the dark and then bleached with 70% (v/v) ethanol.

The number of blue sectors on each plant was determined visually using a stereomicroscope. For each experiment, 300 to 500 plants were analyzed. All experiments were repeated multiple times to obtain average numbers with SD . The significance level was calculated by the *t* test.

Supplemental Data

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

Supplemental Table S1. UV-induced mutation frequency in *AtREV3*-, *AtREV1*-, or *AtPOLH*-disrupted plants.

Supplemental Table S2. UV-induced mutation frequency in double disruptant in comparison with its siblings.

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