Mutational effects of $\gamma$-rays and carbon ion beams on Arabidopsis seedlings

Ryouhei YOSHIHARA$^1$, Shigeki NOZAWA$^2$, Yoshihiro HASE$^2$, Issay NARUMI$^3$, Jun HIDEMA$^4$ and Ayako N. SAKAMOTO$^2,*$

$^1$Research Center for Environmental Genomics, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan
$^2$Ion Beam Mutagenesis Research Group, Medical and Biotechnological Application Division, Quantum Beam Science Directorate, Japan Atomic Energy Agency, 1233 Watanuki, Takasaki, 370-1292, Japan
$^3$Department of Life Sciences, Faculty of Life Sciences, Toyo University, 1-1-1 Izumino, Itakura, 374-0193, Japan
$^4$Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai 980-8577, Japan
*Corresponding author. Ion Beam Mutagenesis Research Group, Medical and Biotechnological Application Division, Quantum Beam Science Directorate, Japan Atomic Energy Agency, 1233 Watanuki, Takasaki, 370-1292, Japan. Tel: +81-27-346-9537; Fax: +81-27-346-9688; Email: sakamoto.ayako@jaea.go.jp

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To assess the mutational effects of radiation on vigorously proliferating plant tissue, the mutation spectrum was analyzed with Arabidopsis seedlings using the plasmid-rescue method. Transgenic plants containing the Escherichia coli rpsL gene were irradiated with $\gamma$-rays and carbon ion beams (320-MeV $^{12}\text{C}^{6+}$), and mutations in the rpsL gene were analyzed. Mutant frequency increased significantly following irradiation by $\gamma$-rays, but not by 320-MeV $^{12}\text{C}^{6+}$. Mutation spectra showed that both radiations increased the frequency of frameshifts and other mutations, including deletions and insertions, but only $\gamma$-rays increased the frequency of total base substitutions. These results suggest that the type of DNA lesions which cause base substitutions were less often induced by 320-MeV $^{12}\text{C}^{6+}$ than by $\gamma$-rays in Arabidopsis seedlings. Furthermore, $\gamma$-rays never increased the frequencies of G:C to T:A or A:T to C:G transversions, which are caused by oxidized guanine; 320-MeV $^{12}\text{C}^{6+}$, however, produced a slight increase in both transversions. Instead, $\gamma$-rays produced a significant increase in the frequency of G:C to A:T transitions. These results suggest that 8-oxoguanine has little effect on mutagenesis in Arabidopsis cells.

Keywords: mutation spectrum; $\gamma$-rays; carbon ion beams; Arabidopsis; rpsL gene

INTRODUCTION

The biological effect of ionizing radiation has been studied in bacteria and animals over many decades. Ion beams and $\gamma$-rays are utilized as models of high-linear energy transfer (LET) and low-LET radiation, respectively, to study the biological effects from various radiations. It is known that high-LET radiations tend to confer greater biological effects (such as cell-killing and mutagenesis) than low-LET radiations [1–3]. It is thought that the greater cell-killing effects of high LET radiation are due to the formation of double-strand breaks (DSBs) and clustered DNA damage, which are deleterious for organisms [4, 5]. Mutations induced by high- and low-LET radiations are also of different types. It has been reported for mammalian cells that ion beams induce large-sized deletions and insertions, and chromosomal rearrangements, whereas $\gamma$-rays induce shorter deletions and insertions, and more frequent base substitutions [3].

Several studies have reported that high-LET radiations confer greater and more distinctive biological effects on plant cells than low-LET radiations. For instance, high-LET radiations produce a more lethal effect on Arabidopsis and tobacco cells [6–9]. As for the types of mutation, irradiation of Arabidopsis dry seeds by carbon ion beams induces point-like mutations (base substitutions and several-base deletions/insertions) and chromosomal rearrangements with similar frequencies, whereas electron beams, one of the low-LET forms of radiation, predominantly induce point-like mutations [10, 11]. Furthermore, genomic in situ hybridization in wheat has shown that ion beams induce chromosomal...
rearrangements more frequently than X-rays [12]. Recently, Hase et al. (2012) showed that high-LET ion beams induce DSBs that are seldom repaired by the non-homologous end-joining (NHEJ) pathway in Arabidopsis dry seed [13]. These results suggest that the significant biological effects of high-LET radiation on plants seem to be due to differences in DNA damage, as reported in other organisms.

In general, ionizing radiation predominantly induces the formation of radicals by water radiolysis, and these then attack DNA and produce oxidative damage. The formation of 8-oxoguanine is one of the major forms of radiation-induced oxidative damage in animal and bacterial cells, and it is believed to induce major base substitutions involving G:C to T:A and A:T to C:G transversions [14]. The oxidative DNA damage causes mispairings during DNA replication [15].

In a prior study, we irradiated Arabidopsis dry seeds with carbon ion beams and γ-rays to analyze radiation-induced mutation in Arabidopsis somatic cells. Our results showed no significant increase of G:C to T:A or A:T to C:G transversions with either type of radiation, suggesting the low effect of 8-oxoguanine in Arabidopsis dry seeds [16]. This may be because the low water content and/or low cell proliferation activity in dry seed help to avoid the effect of 8-oxoguanine and other oxidative damage. In this study, we analyzed the mutations induced by carbon ion beams (320-MeV 12C6+) and γ-rays in Arabidopsis seedlings. This is the first report detecting radiation-induced mutations in vigorous plant cells with high water content and high cell proliferation activity. Our results provide an important insight for evaluation of the mutational effects of ionizing radiation on plant systems.

MATERIALS AND METHODS

Plant and bacterial strains

For mutation spectrum analysis, we used transgenic Arabidopsis containing the E. coli rpsL gene integrated into the chromosomal DNA [17]. Mutations occurring in the rpsL gene were detected by means of the plasmid-rescue technique. E. coli strain DH10B (F− mcrA Δ(mrr-hsdRMS-mcrBC) 80d lacZΔM15 ΔlacY74 deoR recA1 endA1 araD139 Δ(ara leu)7697 galU galK rpsL nupG λ−) carrying the supF-containing plasmid pFSE101 (DH10B/pFSE101) was used for the screening of rpsL mutant clones [18].

Ionizing radiation

Transgenic Arabidopsis seedlings were irradiated with carbon ion (12C6+) beams generated using an azimuthally varying field (AVF) cyclotron and 60Co γ-rays (Japan Atomic Energy Agency, Takasaki, Gunma, Japan). The energy of a carbon ion beam was 320-MeV (26.7 MeV/u) and the average LET was 86.2 keV/μm, which was calculated using the ELOSSM code, a program used to calculate the energy loss of heavy ions under practical irradiation conditions [19].

Plant cultivation and irradiation

The rpsL-transgenic plants were sown on 1/2 B5 medium (1/2 × Gamborg’s B5 salts, 1/1000× hyponex, 1% sucrose, 0.7% agar) aseptically. After a three-day 4°C treatment, plants were grown at 23°C under a 16-h light and 8-h dark cycle for 7 days. For mutation spectrum analysis, 7-day-old plants were irradiated with carbon ions or γ-rays. Irradiated plants were grown under the same conditions for another 7 days and used for mutation analysis.

Sensitivity to ionizing radiation

Sensitivity to ionizing radiation was evaluated by measuring the area of the fifth leaf of irradiated 14-day-old plants. The leaves were mounted on a paper and the leaf area was measured using Adobe Photoshop Elements 4.0 software (Adobe systems Inc., San Jose, CA, USA). Radiation sensitivity was determined as the relative leaf size of irradiated leaves to that of non-irradiated control leaves. Sensitivity curves fitted using a least-square method were defined by the following equation:

\[ \text{Sensitivity rate} = 1 - \left(1 - e^{-D/D_0}\right)^m, \]

where \( D, D_0 \) and \( m \) indicate the dose, the dose conferring a 37% sensitivity rate (mean lethal dose), and the extrapolated number (the number of targets), respectively.

Mutation analysis

We used ~270 plants per experimental plot. The experiment was repeated at least five times. Chromosomal DNA was isolated by the CTAB method [20]. Approximately 80 μg of DNA was digested with 20 units of BamHI (Takara Bio, Otsu, Shiga, Japan) in a 400-μl reaction mixture. Digested DNA was purified by phenol/chloroform extraction and ethanol precipitation. The DNA was self-ligated in an 800-μl reaction mixture containing 11 Weiss units of T4 DNA ligase (Takara Bio). Reconstructed plasmid purified by phenol/chloroform extraction and ethanol precipitation was electroporated into E. coli DH10B/pFSE101. To select clones carrying a mutated rpsL gene, this E. coli was screened on agar medium containing 100 μg/ml of kanamycin (Km) and 60 μg/ml of streptomycin (Sm). The number of total clones was determined by plating a portion of electroporated cells on the medium containing 100 μg/ml of Km. Mutant frequency was calculated as the ratio of mutant clone to total clone. Sequence analysis was performed using a GenomeLab GeXP Genetic Analysis System (Beckman Coulter Inc., Brea, CA, USA).

We analyzed a total of 3.1–20 × 10^4 clones in each rescue experiment and repeated experiments at least five times.
Plural mutations in one clone, i.e. multiple base changes, a base change combined with a frameshift, or a deletion accompanied with an insertion, were scored as a 'complex mutation'. All mutations from independent clones were counted as independent mutations.

RESULTS

Sensitivity of Arabidopsis seedlings to γ-rays and 320-MeV 12C6+

To compare the effects of γ-rays and 320-MeV 12C6+ ion beams on plant tissues, the growth inhibition on young plant tissues was quantified. Seven-day-old seedlings, on which 1–2 true leaves have emerged, were irradiated with γ-rays and 320-MeV 12C6+ ion beams. The plants were grown for another 7 days, by which time the plants had developed 4–6 true leaves. We cut the fifth leaf from each plant and measured the area of the leaf using graphics software. Results showed that 320-MeV 12C6+ more severely inhibited the growth of plant leaves than the same dose of γ-rays (Fig. 1). This is due to the deleterious effect of 320-MeV 12C6+ on cell proliferation. To standardize the effect of both radiations, we adopted the Multi-Target Single-Hit model (Fig. 1) that has been shown to work well in plants [7, 9, 16]. Based on these sensitivity curves, 40–100 Gy of γ-rays and 5–35 Gy of 320-MeV 12C6+ reduced the leaf size dose-dependently. We expected that these ranges of radiations might induce mutations effectively in the plant. Based on this prediction, we chose the dose of 64 Gy and 20 Gy for γ-rays and 320-MeV 12C6+, respectively, which reduced the leaf size to ~50%, and then conducted mutation spectrum analysis.

Mutagenicity of γ-rays and 320-MeV 12C6+ ion beams

To investigate the mutational effect of γ-rays and 320-MeV 12C6+ ion beams on Arabidopsis seedlings, the mutation frequencies were determined using the plasmid-rescue method (Table 1). The mutant frequency of unirradiated seedlings was 3.4 × 10^-3, as found in our previous report [16]. By irradiating with 64 Gy of γ-rays, the mutant frequency increased significantly (4.1-fold, P < 0.01). In contrast, 20 Gy of carbon ions increased the mutant frequency by 2.9-fold, but this increase was not statistically significant. Therefore, these results suggest that a 320-MeV 12C6+ ion beam is less effective than γ-rays in inducing detectable mutations in this assay system.

Table 1. Mutant frequencies induced by γ-rays and 320-MeV 12C6+ ion beams

<table>
<thead>
<tr>
<th></th>
<th>Mutant clone</th>
<th>Total clone</th>
<th>Mutant frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background</td>
<td>33</td>
<td>9.2</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td>γ-rays</td>
<td>44</td>
<td>3.6</td>
<td>14 ± 7*a</td>
</tr>
<tr>
<td>320-MeV 12C6+ ion beams</td>
<td>27</td>
<td>3.3</td>
<td>10 ± 12</td>
</tr>
</tbody>
</table>

*aSum of 5–10 independent experiments. bAverage of 5–10 independent experiments with SD. *Statistically significant compared to unirradiated ('background') plants (P < 0.01).

Mutation induced by γ-rays and a 320-MeV 12C6+ ion beam in Arabidopsis seedlings

We analyzed mutation spectra induced by γ-rays and a 320-MeV 12C6+ ion beam, as well as unirradiated ('background') seedlings. All mutations were classified into three groups: base substitutions, frameshifts, and 'other' (Fig. 2). In comparison with unirradiated plants, both radiations increased the frequency of frameshifts and ‘other’ mutations, but only γ-rays resulted in a significant increase in the frequency of total base substitutions.

We analyzed the type of each mutation (Table 2). Regarding the types of base substitutions, γ-rays resulted in a significant increase in the frequency of G:C to A:T transitions (P < 0.01). Furthermore, γ-rays increased the frequencies of −1 and −2 frameshifts, deletions/insertions, and complex mutations (P < 0.01). In contrast, 320-MeV 12C6+ increased G:C to T:A and A:T to C:G transversions, −1 frameshifts and complex mutations (P < 0.01). There was no increase in both transversions associated with γ-rays, which was consistent with our previous mutation analysis in Arabidopsis dry seeds [16].

![Fig. 1. Radiation sensitivity of rpsL-transgenic Arabidopsis. The effect of γ-rays (open circles) and 320-MeV 12C6+ ion beams (filled circles) on the growth of Arabidopsis leaves. Radiation sensitivity is shown as the relative leaf size of irradiated plants to that of the unirradiated control plants. Error bars represent the standard deviation of the mean.](https://academic.oup.com/jrr/article-abstract/54/6/1050/1135644)


**DISCUSSION**

In this study, we irradiated *Arabidopsis* seedlings with 64 Gy of γ-rays and 20 Gy of 320-MeV $^{12}$C$_6^+$ ion beams, which reduced leaf size to ~50%, then analyzed the mutation spectrum. In the previous study, we irradiated *Arabidopsis* dry seeds with 740 Gy of γ-rays and 140 Gy of 208-MeV $^{12}$C$_5^+$, each of which correspond to 0.8 Dq of survivals and provide similar mutation frequencies (Yoshihara et al., 2010). Here, we used the reduction of leaf size as an index and expected the doses causing a similar degree of reduction to also cause a similar induction of mutation frequency. However, results revealed that the mutant frequency increased following irradiation by γ-rays (64 Gy), but did not increase significantly following irradiation by 320-MeV $^{12}$C$_6^+$ (20 Gy), although both conditions reduced leaf size to ~50%. Since the growth of leaves depends on multiple factors, such as cell death, cell-cycle arrest, or cell-expansion, it did not simply correlate with the DNA damage and/or mutations. Namely, the reason that the 320-MeV $^{12}$C$_6^+$ ion beam did not elevate mutant frequency could be because the dose was too low to induce mutations. It would be necessary to analyze the dose response of mutations in order to elucidate all the characteristics of these radiations. Nevertheless, though limited, the data obtained in this work shed light on the understanding of mutations by γ-rays and a 320-MeV $^{12}$C$_6^+$ ion beam, as discussed below.

The mutation spectrum analysis showed an increase in the total number of base substitutions after γ-ray irradiation, but not after radiation from the 320-MeV $^{12}$C$_6^+$ ion beam (Fig. 2). Oxidative damage to DNA, which is mainly caused by radicals from water radiolysis as an indirect effect of ionizing radiation, is often involved in the induction of base substitutions [21]. Therefore, it is possible that more radicals were induced in plant cells by γ-rays than by the 320-MeV $^{12}$C$_6^+$ ion beam in these assay conditions. This difference might have caused the difference in mutant frequencies.

In contrast, both types of radiations increased the frequency of deletions/insertions and complex mutations compared with that observed in unirradiated (‘background’) plants (Fig. 2). It is known that these mutations occur during the process of DSB repair by non-homologous end-joining (NHEJ) [22]. It is reasonable to claim that both radiations induced DSBs in *Arabidopsis* plants. In our previous work, *Arabidopsis* plants with a disrupted DNA Ligase IV gene, a key component of the NHEJ pathway, showed a markedly higher sensitivity than the wild-type to ion beams [13]. These data also support the idea that DSBs formed by the radiation exposure were mostly repaired by the NHEJ pathway in *Arabidopsis*.

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**Table 2. Mutation spectra of *Arabidopsis* seedlings following ionizing radiation**

<table>
<thead>
<tr>
<th></th>
<th>Background</th>
<th>γ-rays</th>
<th>320-MeV $^{12}$C$_6^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NM</strong> ($\times 10^{-5}$)</td>
<td><strong>MF</strong> ($\times 10^{-7}$)</td>
<td><strong>MF</strong> ($\times 10^{-7}$)</td>
<td><strong>MF</strong> ($\times 10^{-5}$)</td>
</tr>
<tr>
<td>Transition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G → A</td>
<td>9 (1.0)</td>
<td>11 (3.1)*</td>
<td>2 (0.6)</td>
</tr>
<tr>
<td>A → G</td>
<td>1 (0.1)</td>
<td>0 (&lt;0.3)*</td>
<td>0 (&lt;0.3)</td>
</tr>
<tr>
<td>Transversion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G → T</td>
<td>1 (0.1)</td>
<td>2 (0.6)</td>
<td>3 (0.9)*</td>
</tr>
<tr>
<td>G → C</td>
<td>3 (0.3)</td>
<td>2 (0.6)</td>
<td>2 (0.6)</td>
</tr>
<tr>
<td>A → T</td>
<td>3 (0.3)</td>
<td>4 (1.1)**</td>
<td>0 (&lt;0.3)</td>
</tr>
<tr>
<td>A → C</td>
<td>0 (&lt;0.1)</td>
<td>1 (0.3)</td>
<td>2 (0.6)*</td>
</tr>
<tr>
<td>Frameshifts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+1</td>
<td>0 (&lt;0.1)</td>
<td>1 (0.3)</td>
<td>1 (0.3)</td>
</tr>
<tr>
<td>−1</td>
<td>8 (0.9)</td>
<td>9 (2.5)*</td>
<td>8 (2.4)*</td>
</tr>
<tr>
<td>−2</td>
<td>0 (&lt;0.1)</td>
<td>2 (0.6)*</td>
<td>0 (&lt;0.3)</td>
</tr>
<tr>
<td>Deletion</td>
<td>8 (0.9)</td>
<td>9 (2.5)*</td>
<td>6 (1.8)**</td>
</tr>
<tr>
<td>Complex</td>
<td>0 (&lt;0.1)</td>
<td>3 (0.8)*</td>
<td>3 (0.9)*</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>33 (3.6)</td>
<td>44 (12.4)</td>
<td>27 (8.2)</td>
</tr>
</tbody>
</table>

Mutant frequency is derived from the ratio of mutant clone numbers to predicted total analyzed clone numbers. NM = number of mutants, MF = mutant frequency. The statistical significance of differences between the irradiated group and the unirradiated (‘background’) group was examined using the Poisson test (*$P<0.01$, **$P<0.05$).
seedlings. The simplest interpretation is that the γ-rays and 
320-MeV $^{12}$C***+ carbon beam induced a comparable number of 
DSBs, which led to deletions/insertions or complex 
mutations with similar frequencies (3.4 and 2.7 x 10**−3, 
respectively) (Fig. 2).

In this study, plural base substitutions, base substitution 
accompanied by a frameshift, and a deletion combined 
with an insertion, were categorized as ‘complex mutations’. These 
mutations are known to be induced by repair or replication of 
damaged DNA. For example, in the NHEJ repair process, 
1–2 bp of microhomology near the DNA end is often utilized 
to rejoin the DSB. After annealing of the homologous base 
(s), the flanking mismatched base(s) is sometimes deleted or 
inserted by fill-in activity [23, 24]. Some DNA polymerase 
can fill the strand gap even when the template has a gap, a 
damaged base, or a mismatched base [25–27]. Such error-
prone repair may lead to a deletion combined with an inser-
tion, or a base substitution accompanied by a frameshift, which 
is likely to occur when the DSB end is not comple-
mentary. It is known that a template involving a damaged 
base causes primer–template misalignment, which may 
induce plural base substitutions or a base substitution accom-
panied by a frameshift [28, 29]. Such error-prone replications 
are likely to occur when DNA damage is not completely 
removed before replication. The mutation spectra showed 
that both γ-rays and 320-MeV $^{12}$C***+ ion beams significantly 
induced complex mutations (Fig. 2). We therefore speculate 
that some DSBs, or other base damage induced by both 
radiations, are difficult to repair, and that the plant cells are 
subject to error-prone repair or replication.

Both γ-rays and 320-MeV $^{12}$C***+ ion beams increased the 
frequency of −1 frameshift mutations (Table 2). It is thought 
that +1 and −1 frameshifts are mainly caused by primer–
template misalignment at or near the damaged base during 
DNA replication [30–32]. In addition, it has been shown that 
some +1 and −1 frameshifts may be induced in the process of 
DSB repair by NHEJ [33, 34]. In yeast, it was estimated that 
~50% of +1 and −1 frameshifts are induced by the NHEJ 
pathway [34]. Considering the few base substitutions 
induced by the 320-MeV $^{12}$C***+ ion beams (Fig. 2), it is 
possible that the formation of base lesions was not elevated by 
320-MeV $^{12}$C***+ ion beams compared with that of unirra-
diated plants. In contrast, the frameshifts were significantly 
higher in 320-MeV $^{12}$C***+ ion beam-irradiated plants than in 
inunirradiated plants (Fig. 2). Therefore, it is likely that the 
increase in −1 frameshifts in irradiated seedlings was due to 
failed DSB repair by the NHEJ pathway, at least in part.

The G:C to T:A and A:T to C:G transversions are mainly 
induced by guanine oxidation and subsequent 8-oxoguanine 
and adenine mispairing during DNA replication [15]. Since 
Arabidopsis seedlings have a high water content and cell pro-
flleration activity, we predicted that the mutations in seed-
lings might reflect the increased 8-oxoguanine and other 
oxidative damage caused by the irradiation. Contrary to our 
prediction, however, γ-rays did not increase the G:C to T:A 
and A:T to C:G transversions in Arabidopsis seedlings 
(Table 2). In contrast, 320-MeV $^{12}$C***+ ion beams increased 
both transversions by 8.4 and >5.6 times, which is statistically-
significant compared with control seedlings. However, the 
total numbers of G:C to T:A and A:T to C:G transversions 
were only 3 and 2, respectively, which were fewer than those 
of other mutations such as −1 frameshifts and deletions/ 
insertions in 320-MeV $^{12}$C***+ ion beam-irradiated plants. It is 
preamature to conclude that 320-MeV $^{12}$C***+ ion beams 
induced more 8-oxoguanine than γ-rays in plant cells.

Instead, as mentioned below, it is possible that various forms 
of oxidative damage other than 8-oxoguanine are major 
 sources of mutations in γ-ray-irradiated plants. It is reason-
able to expect that 8-oxoguanine has a limited effect on mu-
tageneasis in Arabidopsis cells.

The important repair enzymes for 8-oxoguanine in E. coli 
and humans are mutT/MTH1, mutY/MYH and mutM/OGG1 
[35, 36]. The Arabidopsis genome also has the homologs of 
these genes [37]. However, mutant plants deficient in the 
mutT/MTH1- or mutM/OGG1-homolog showed indistin-
guishable sensitivity to oxidative stress compared with wild-
type plants [38, 39]. Our preliminary experiment showed that 
the γ-ray sensitivity of Arabidopsis mth1 and myh1 mutant 
plants was the same as that of wild-type plants (data not 
shown). On the basis of these findings, it is possible that (i) 
ionizing radiation causes only a modest increase in the 
amount of 8-oxoguanine in Arabidopsis cells, or (ii) 
Arabidopsis has another system(s) to suppress the mutagenic 
effect of 8-oxoguanine.

The frequency of G:C to A:T transitions increased signifi-
cantly following γ-ray exposure (Table 2). The G:C to A:T 
transition is known to be induced by various types of base 
lesions. Cytosine glycol, a form of oxidized cytosine, is a 
possible candidate lesion that induced G:C to A:T transitions 
in Arabidopsis seedlings. Cytosine glycol often changes to 
5-hydroxy-uracil by dehydration and deamination, which is 
mispaired with adenine and induces the G:C to A:T transition [14].

Uracil DNA glycosylase (Ung) and single-strand selective 
monofunctional uracil DNA glycosylase (Smug1) 
excise oxidized cytosine derivatives from DNA [14]. An 
et al. (2005) showed that these enzymes have redundant 
roles in resisting the effects of γ-rays and preventing G:C 
 to A:T transitions in mammalian cells [40]. In addition, 
mammalian cells have thymine DNA glycosylase (TDG), 
endonuclease III (Nth) and endonuclease VIII (Nei), and 
E. coli cells have Ung, Nth, Nei and mismatch-specific 
uracil DNA-glycosylase (MUG) as glycosylases for oxidative 
cytosine derivatives [14]. Only Ung and Nth are found 
in Arabidopsis [41, 42]. Therefore, it is possible that 
Arabidopsis has a weak repair ability for oxidative cytosine 
derivatives and cannot prevent G:C to A:T transitions after 
 exposure to γ-rays.
CONCLUSION

In conclusion, our mutation spectrum analysis suggested that plants might have a unique mechanism for maintaining genomic stability against radiation-induced oxidative damage. Further studies, including dose–response analysis of mutagenesis for both radiations, are needed to elucidate the details of the DNA repair pathways and radiation mutagenesis in higher plants.

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