

## Rapid evaluation of effective linear energy transfer in heavy-ion mutagenesis of *Arabidopsis thaliana*

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**Abstract** Heavy-ion beams are an effective mutagen for use in plant breeding and analyses of gene function. Heavy-ion beams have high linear energy transfer (LET) and thus they effectively induce DNA double-strand breaks. To rapidly evaluate effective LETs for inducing mutations, we investigated mutation frequencies in the M<sub>1</sub> generation using the *Arabidopsis* heterozygous mutant of the *ALBINO PALE GREEN 3* gene. Leaves of heterozygous plants in which intact alleles were mutated showed white sectors. We irradiated heterozygous plants with heavy-ion beams with LETs ranging from 22.5 to 61.5 keV  $\mu\text{m}^{-1}$  and doses ranging from 0 to 450 Gy. The results showed that the effect of LETs could be classified into three types: first, the LET of 22.5 keV  $\mu\text{m}^{-1}$  did not effectively induce mutations, regardless of the dose; second, LETs of 30.0 or 42.5 keV  $\mu\text{m}^{-1}$  were not effective at low doses (100–200 Gy) but were effective at 300 Gy; third, LETs of 50.0 or 61.5 keV  $\mu\text{m}^{-1}$  produced comparatively high mutation frequencies at all doses. Irradiation with 42.5–61.5 keV  $\mu\text{m}^{-1}$  reduced seed productivity at the dose providing the highest mutation frequencies (300 Gy). We concluded that an LET of 30.0 keV  $\mu\text{m}^{-1}$  was optimal for obtaining *Arabidopsis thaliana* mutants in the M<sub>2</sub> generation.

**Key words:** *ALBINO PALE GREEN 3*, *Arabidopsis thaliana*, heavy-ion beam, linear energy transfer (LET), M<sub>1</sub> generation.

Several mutagens, including chemical mutagens and ionizing radiation, are commonly used to induce mutations in higher plants. Inducing mutations is a powerful method for plant breeding and for analyses of gene functions. Forward genetics techniques using mutated plant materials are useful for studying gene networking. For example, many researchers have isolated suppressor mutants from mutants of interest. Reverse genetics techniques also rely on the use of efficient mutagens to induce mutations, and their detection using single-nucleotide polymorphism (SNP) techniques, such as the CEL1 nuclease assay or high-resolution melting curve (HRM) analysis (McCallum et al. 2000; Wittwer et al. 2003). Therefore, efficient techniques to induce mutations are important for several areas of research.

Heavy-ion irradiation is an excellent technology to induce mutations with high frequency. It induces a broad spectrum of phenotypes without affecting other plant characteristics (Tanaka et al. 2010; Abe et al. 2012). Genetic approaches involving the use of heavy-ion irradiation have been used to analyze several biological processes (Hase et al. 2000; Hase et

al. 2006; Shitsukawa et al. 2007; Kazama et al. 2008a; Shimada et al. 2009; Kazama et al. 2012; Fujita et al. 2012; Sasaki et al. 2012; Yasui et al. 2012). The heavy-ion beams comprise accelerated ions produced by an ion accelerator such as a cyclotron or synchrotron. A notable physical characteristic of heavy-ion beams is that the accelerated particles deposit their energy densely in a localized region along the particle path. The degree of locally deposited energy is represented by linear energy transfer (LET; the energy transferred per unit length, keV  $\mu\text{m}^{-1}$ ). Thus, heavy-ion beams are classified as high-LET radiation. Low-LET radiation includes  $\gamma$ -rays and X-rays, which sparsely deposit their energy in a large target volume. The LETs of  $\gamma$ -rays and X-rays are 0.2 and 2.0 keV  $\mu\text{m}^{-1}$ , respectively, whereas the LET of heavy-ion beams can be altered by selecting ion species or controlling their velocity. In the RIKEN RI-beam factory (RIBF), the values of LET can range from 22.5 to 4000 keV  $\mu\text{m}^{-1}$  for use in biological research (Ryuto et al. 2008).

In our previous study on the effects of LETs on inducing mutation, we investigated the incidence of

Abbreviations: ABRC, *Arabidopsis* Biological Resource Center; APG3: *Albino Pale Green 3*; HRM, high-resolution melting curve; LET, linear energy transfer; RIBF, RIKEN RI-beam factory; SNP, single-nucleotide polymorphism.

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albino mutants in the  $M_2$  generation of *Arabidopsis thaliana* after irradiation with LETs with values of 22.5, 30.0, 61.5, 290, and 640  $\text{keV } \mu\text{m}^{-1}$  (Kazama et al. 2008b). We found that irradiation with a LET of 30.0  $\text{keV } \mu\text{m}^{-1}$  was the most effective for inducing mutations in the  $M_2$  generation. This treatment showed similar mutagenic efficiency to that of ethyl methanesulfonate (Koornneef et al. 1982; Kazama et al. 2011). This highly efficient LET was designated as LETmax. Molecular characterization of the mutated DNA revealed that 80% of the total mutations caused by heavy-ion beams at LETmax were deletions with a size range of 1–53 bp (Kazama et al. 2011). Such small changes can be determined by SNP detection systems. Therefore, heavy ions with LETmax are useful as a highly efficient reverse genetic system in conjunction with SNP detection systems, as well as a forward genetics.

To determine the most effective conditions to induce mutations, a more detailed examination of LET values was required. However, analysis of mutation frequency in the  $M_2$  generation is expensive in terms of time and space requirements. Thus, a rapid method to detect the effects of LET on inducing mutations was required. Detection of mutagenic effects in the  $M_1$  generation is one possible method for rapid analysis. Yoshiyama et al. reported a system to detect mutagenic effects on the  $M_1$  generation using a heterozygous mutant of the *Albino Pale Green 3* (APG3) gene (Yoshiyama et al. 2009). The *Arabidopsis* APG3 gene is involved in chloroplast development (Motohashi et al. 2007). Homozygous mutants of APG3 ( $APG3^{-/-}$ ) show albino or pale-green leaves, whereas heterozygous mutants ( $APG3^{+/-}$ ) and wild-type plants ( $APG3^{+/+}$ ) have green leaves. In heterozygous plants in which intact alleles are mutated by mutagens, leaves show white sectors (Figure 1). This system allowed us to investigate the effect of LETs on heavy-ion irradiation in the  $M_1$  generation.

In this study, we investigated the effect of LETs on inducing mutations in the  $M_1$  generation using the heterozygous APG3 mutant. The values of LETs ranged from 22.5 to 61.5  $\text{keV } \mu\text{m}^{-1}$ . The results showed a clear LET effect on inducing mutations in the  $M_1$  generation. By considering the proportion of mutations transmissible to the next generation, we determined the most effective LET conditions for heavy-ion mutagenesis of *Arabidopsis*.

## Materials and methods

### Plant material

Seeds of APG3 heterozygous plants (CS16118) were obtained from the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University). This allele carries BASTA resistance, and uniformly heterozygous populations can be selected as photosynthetic, BASTA-resistant seedlings.

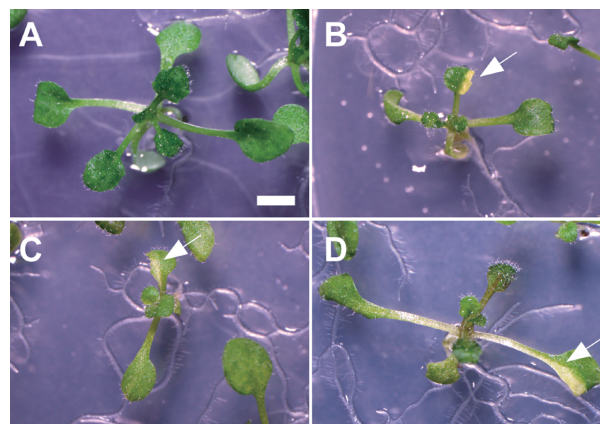


Figure 1. Photograph of heterozygous APG3 mutant showing a white sector after irradiation treatment. (A) Heterozygous APG3 mutant with no irradiation treatment. (B–D) Irradiated heterozygous APG3 mutant with white sector (arrows). Bar = 2.0 mm.

### Irradiation treatment

Seeds of CS16118 were irradiated with  $^{12}\text{C}^{6+}$  ions with a dose range of 100 to 450 Gy using the E5 beam line in the RIBF. The ions were accelerated up to 1.62 GeV, at which the LET value of the  $^{12}\text{C}^{6+}$  ions was 22.5  $\text{keV } \mu\text{m}^{-1}$ . The LET value of the  $^{12}\text{C}^{6+}$  ions was adjusted to 30.0, 42.5, 50.0, or 61.5  $\text{keV } \mu\text{m}^{-1}$  by reducing the ion velocity. As a negative control, seeds of wild-type plants were irradiated with  $^{12}\text{C}^{6+}$  ions with a LET of 30.0  $\text{keV } \mu\text{m}^{-1}$  at a dose of 300 Gy. To reduce ion velocity, the ions were passed through a combination of absorbers (Ryuto et al. 2006). The LET values were measured behind the seeds using an ionization chamber.

### Analysis of LET effects on inducing mutation in $M_1$ generation

The irradiated seeds were incubated on 1/2 MS agar medium (Murashige and Skoog 1962) containing 2% (w/v) sucrose and BASTA (2  $\mu\text{g/mL}$ ) at 4°C in the dark for 3 days for vernalization, and then at 23°C under long-day conditions (16-h light/8-h dark) for 5 days. Then, the selected  $APG3^{+/-}$  plants were transplanted onto BASTA-free 1/2 MS agar medium and grown at 23°C under long-day conditions. At 8 days after transplantation, plants were examined for the presence of white sectors. We counted the number of heterozygous plants with a white sector on the true leaf. The mutation frequency was calculated as the proportion of plants with a white sector out of the total number of heterozygous plants. For each LET value, at least three independent experiments for each irradiation dose were conducted. At least 1,000 seeds were used for each independent experiment.

### Effect of LET on plant survival

After measurement of mutation frequency, 110  $M_1$  plants were randomly selected and transplanted into pots. The percentage survival of the transplanted  $M_1$  plants was determined as described previously (Kazama et al. 2008b). For each LET value, at least three independent experiments at each dose of

irradiation were conducted.

### Analysis of LET-dependent effects on seed production

We measured the length of mature siliques of irradiated  $M_1$  plants, then dissected the siliques and counted the number of normal seeds under a stereomicroscope. At least six siliques per plant were evaluated ( $n=12$  plants per treatment). The means were compared using the least significant difference (LSD) test.

## Results and discussion

### LET-dependent effect on survival rate in $M_1$ generation

Dry seeds of progenies of CS16118 plants were irradiated with C-ion beams with LETs of 22.5, 30.0, 42.5, 50.0, and  $61.5 \text{ keV } \mu\text{m}^{-1}$ . These seeds were a mixture of segregated  $APG3^{+/+}$ ,  $APG3^{+/-}$ , and  $APG3^{-/-}$  seeds. After irradiation, the heterozygous  $APG3^{+/-}$  plants were selected on BASTA-containing MS medium. Some irradiated heterozygous plants showed a white sector in the true leaf (Figure 1). Such sectors were never observed in non-irradiated heterozygous plants (1009 plants observed) or in wild-type plants irradiated with C-ion beams with LET of  $30.0 \text{ keV } \mu\text{m}^{-1}$  at a dose of 300 Gy (708 plants observed). This result indicated that these sectors formed because of mutations in the *APG3* gene. These heterozygous plants were used to investigate survival rates and mutation rates.

The lethality effect increased with increasing LET values (Figure 2), when the number of flowering plants was counted as described previously (Kazama et al. 2008b). At doses up to 300 Gy, none of the LETs affected plant survival. At doses exceeding 300 Gy, survival rates decreased in a LET-dependent manner. This trend was consistent with previous results (Kazama et al. 2008b; Kazama et al. 2011). Plants irradiated at doses greater than 300 Gy with LET of  $61.5 \text{ keV } \mu\text{m}^{-1}$  showed severe growth inhibition, as did plants irradiated at doses greater than 350 Gy with LETs of 42.5 and  $50.0 \text{ keV } \mu\text{m}^{-1}$ . Some of the plants in those treatments did not survive. The white sectors were indistinguishable in plants showing severe growth inhibition. Consequently, the mutation frequencies could not be calculated for these plants.

### Effect of LET on mutation rate in $M_1$ generation

There was a clear effect of LET on the incidence of albino sectors (Figure 3). The LET of  $22.5 \text{ keV } \mu\text{m}^{-1}$  was the least effective for inducing mutations, resulting in a frequency of only 3.08% white sectors, even at the most effective dose (450 Gy). In contrast, C ions with greater LET values produced approximately double the frequency of white sectors, when compared with that induced by C ions with  $22.5 \text{ keV } \mu\text{m}^{-1}$  at a dose of 300 Gy. The low efficiency of C

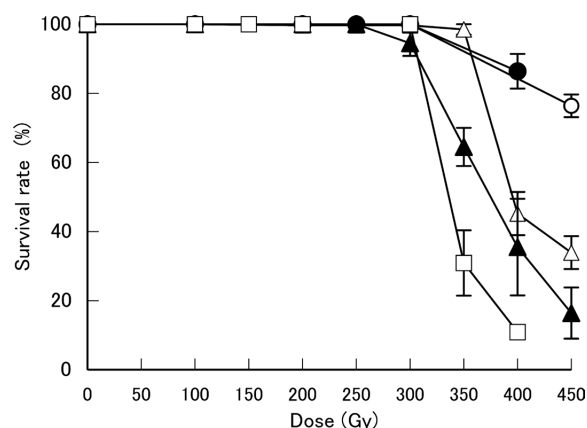


Figure 2. Effect of LET on plant survival. Heterozygous *APG3* mutants were irradiated with C ion beams with various LET values. 110 plants were randomly replanted in pots, and survival rate was calculated by counting number of flowering plants as described previously (Kazama et al. 2008b). Error bars indicate SE. (○)  $22.5 \text{ keV } \mu\text{m}^{-1}$ ; (●)  $30.0 \text{ keV } \mu\text{m}^{-1}$ ; (△)  $42.5 \text{ keV } \mu\text{m}^{-1}$ ; (▲)  $50.0 \text{ keV } \mu\text{m}^{-1}$ ; (□)  $61.5 \text{ keV } \mu\text{m}^{-1}$ .

ions with  $22.5 \text{ keV } \mu\text{m}^{-1}$  was consistent with the results of previous studies on mutations in the  $M_2$  generation (Kazama et al. 2008b; Kazama et al. 2011). We also observed differences in mutation frequencies among the other LETs. At low doses (100–200 Gy), LETs of 50.0 and  $61.5 \text{ keV } \mu\text{m}^{-1}$  produced greater mutation frequencies than did LETs of 30.0 and  $42.5 \text{ keV } \mu\text{m}^{-1}$ . At these doses, C ions with LETs of  $30.0 \text{ keV } \mu\text{m}^{-1}$  and  $42.5 \text{ keV } \mu\text{m}^{-1}$  produced similar mutation frequencies to that of the LET of  $22.5 \text{ keV } \mu\text{m}^{-1}$ . At the 300 Gy dose, C ions with LETs of 30.0 or  $42.5 \text{ keV } \mu\text{m}^{-1}$  showed similar mutation frequencies to those produced by LETs of 50.0 and  $61.5 \text{ keV } \mu\text{m}^{-1}$ ; these irradiation conditions (30.0, 42.5, 50.0, and  $61.5 \text{ keV } \mu\text{m}^{-1}$  at a dose of 300 Gy) were the most effective on producing high mutation frequencies in the  $M_1$  generation.

### Effect of LET on seed productivity

The C ions with  $61.5 \text{ keV } \mu\text{m}^{-1}$  showed a similar mutational effect to that of  $30.0 \text{ keV } \mu\text{m}^{-1}$  in the  $M_1$  generation at the most effective dose (300 Gy). In contrast, our previous study showed that the LET of  $61.5 \text{ keV } \mu\text{m}^{-1}$  was about half as effective as  $30.0 \text{ keV } \mu\text{m}^{-1}$  in inducing mutations in the  $M_2$  generation (Kazama et al. 2008b). This difference implies that the transmissibility of the mutations should be considered. Naito et al. (2005) reported that large deletions (greater than 1 Mbp) were frequently induced by radiation treatment, and that they could not be transmitted to the next generation because they included genes essential for gamete development or viability. Therefore, seed productivity in the  $M_1$  generation should be investigated to determine the most effective irradiation conditions in the  $M_2$  generation. We investigated silique

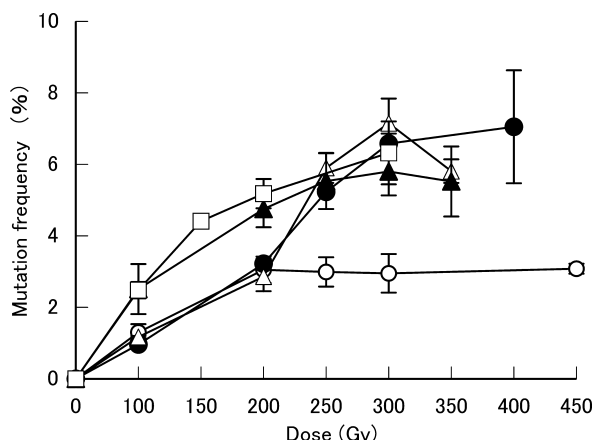


Figure 3. Effect of LET on mutation induction in  $M_1$  generation. Heterozygous APG3 mutants were irradiated with C ion beams with various LET values. Number of white sectors was counted and mutation frequency was calculated.

length and seed productivity of the  $M_1$  generation after irradiation with LETs of different values at 300 Gy—the effective dose for almost all LETs (30.0, 42.5, 50.0, and 61.5  $\text{keV } \mu\text{m}^{-1}$ ) (Figure 3). Both silique length and seed productivity was affected by the values of LETs (Figure 4). Seed productivity was strikingly affected in plants irradiated with higher-LETs (Figure 4B). C ions with LETs of 22.5 and 30.0  $\text{keV } \mu\text{m}^{-1}$  resulted in similar seed productivity (approx. 55–60% of control seeds), while those with LETs of 42.5–61.5  $\text{keV } \mu\text{m}^{-1}$  resulted in lower seed productivities (approx. 30% of control seeds). This result suggested that LETs greater than 30.0  $\text{keV } \mu\text{m}^{-1}$  cause greater defects in seed production than 22.5 and 30.0  $\text{keV } \mu\text{m}^{-1}$ . Considering that heavy-ion beams randomly caused DNA lesions in the genome, approximately half of the mutations induced by irradiation with LET of 61.5  $\text{keV } \mu\text{m}^{-1}$  were not transmitted to the next generation, compared with seeds irradiated with an LET of 30.0  $\text{keV } \mu\text{m}^{-1}$ . We suggest that an LET of 30.0  $\text{keV } \mu\text{m}^{-1}$  is appropriate for generating mutants that can produce an  $M_2$  generation efficiently.

#### Usefulness of the rapid evaluation of LET effects on $M_1$ generation

The current study clearly showed effect of LETs on mutation frequency in the  $M_1$  generation. We found that LETs of 30  $\text{keV } \mu\text{m}^{-1}$  or more were effective to induce mutations in the  $M_1$  generation. Especially at low doses (100 Gy), LETs of 50.0  $\text{keV } \mu\text{m}^{-1}$  and 61.5  $\text{keV } \mu\text{m}^{-1}$  were more efficient than the other LETs. The current data will contribute to determining the most efficient LET conditions to induce mutations in *A. thaliana*. In addition, the current data could provide valuable insights for breeding of other plants. In the case of horticultural plants, new cultivars are often bred by vegetative propagation of plantlets after irradiation ( $M_1$  generation)

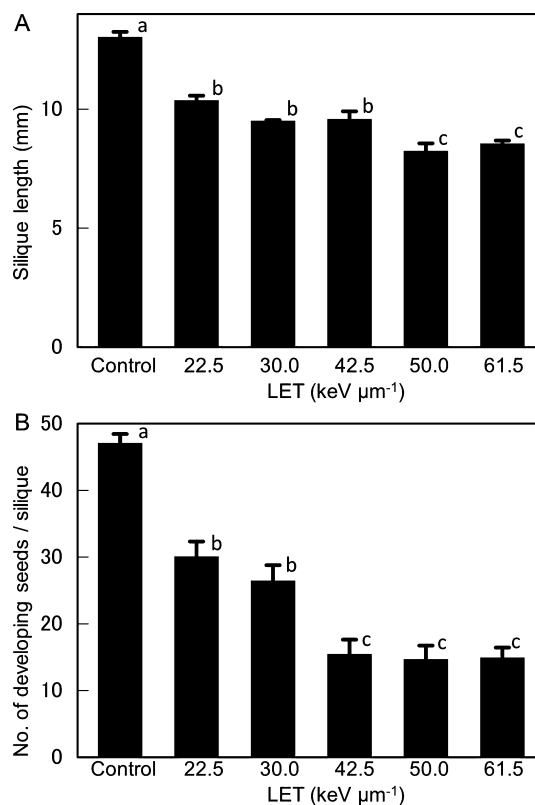


Figure 4. Effect of LET on seed productivity. Mature siliques were opened, and silique length and number of seeds were counted. Analyses were conducted using at least 12 plants; at least six siliques per plant were evaluated. Different letters after values indicate significant difference (LSD test,  $p < 0.05$ ).

(Miyazaki et al. 2006; Kanaya et al. 2008). Based on these results, it would be reasonable to test C ions with LETs of 30  $\text{keV } \mu\text{m}^{-1}$  or over in irradiation experiments on horticultural plants.

The mechanism behind the effects of LET on the seed productivity is unknown. One possible explanation is that C ions with higher LET might induce larger deletions, disrupting genes that are essential for gamete development or viability. The results of previous studies support this idea, since LETs with increasing value resulted in greater sizes of deletions in the  $M_2$  generation (Shikazono et al. 2005; Kazama et al. 2011; Hirano et al. 2012). A cytological analysis in the  $M_1$  generation also revealed that Ne ions with a LET of 63.0  $\text{keV } \mu\text{m}^{-1}$  induced chromosomal rearrangements more frequently than those with an LET of 30.0  $\text{keV } \mu\text{m}^{-1}$  (Kikuchi et al. 2009). These chromosomal rearrangements may also result in seed abortion. In the current experiments, we postulated that the extent of such large alterations may differ among LETs of different values.

In conclusion, we investigated the effects of LET on mutation induction, seed productivity, and survival rate in the  $M_1$  generation. From the data obtained, we concluded that the LET of 30.0  $\text{keV } \mu\text{m}^{-1}$  was the



most effective to induce mutations, because of its high mutation frequency and low inhibitory effect on seed production.

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