EFFECTS OF ION BEAM IRRADIATION ON MUTATION INDUCTION IN *ARABIDOPSIS THALIANA*

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Abstract

We evaluated the effects of the conditions of heavy-ion beam irradiation on effective mutation induction in *Arabidopsis thaliana*. First, mutation rates were compared between nuclides. Dry seeds were irradiated with $^{12}C^{6+}$, $^{14}N^{7+}$, $^{20}Ne^{10+}$ (135MeV/u), $^{40}Ar^{17+}$ (95 MeV/u), or $^{56}Fe^{24+}$ (90 MeV/u) ions at doses ranging from 5 to 400 Gy. M_2 seeds were harvested from self-pollinated M_1 plants, and the mutation rates were calculated by counting the number of albino mutants in the M_2 generation. Of these ions, $^{14}N^{7+}$ ion (30 keV/μm) was the most effective for inducing albino plants. Second, the effects of linear energy transfer (LET) on mutation induction were examined. The LETs of C, N, Ne, and Ar ion beams were controlled to 30, 61, and 280 keV/μm, 61 and 280 keV/μm, and 280 and 680 keV/μm, respectively. Regardless of ion species, irradiation with the same LET resulted in the same mutation rate. Thus, the LET of ion beams seems to be an important factor for efficient mutagenesis.

INTRODUCTION

Heavy-ion beam mutagenesis is generally accepted as an effective method for producing mutations, as well as for functional studies of genes. Heavy-ion beams have high linear energy transfer (LET). In contrast to electron irradiation, which has a low LET, heavy-ion beams are thought to produce more DNA damage and double-strand breaks (DSBs) [1]. However, there is still little experimental evidence of a correlation between LET and the effect on mutation induction in the plant kingdom.

Arabidopsis thaliana (L.) is a widely used model plant and its entire genome has been sequenced. A gene-tagging method involving mutagenesis is used to identify mutated genes. *A. thaliana* also has advantages for investigating the effects of mutation induction: it is a small plant with a short life cycle that produces small seeds and is highly resistant to sparse ionizing radiation [2, 3]. However, techniques for controlling the size of DNA deletions have not been exploited fully. Gene deletions are considered most valuable for investigating gene function because they can be defined as nulls. Therefore, methods for deletion-controlling mutagenesis are desired. We postulated that control of deletion size may be achieved by using heavy-ion beam irradiation with an appropriate LET.

A previous study showed that the peak of the relative biological effectiveness (RBE) of a carbon-ion beam for survival was around 252 keV/μm in *A. thaliana*. Its value was 11–12 when D_{37} electrons were used as the standard [4]. The mutation spectrum of a carbon-ion beam has also been studied using *A. thaliana* [5]. Half of the mutants induced by the carbon-ion beam had small mutations, such as base changes and deletions of a few base pairs, whereas the other half had large DNA rearrangements, such as inversions, translocations, and large deletions (See Table 1). It has been reported that LET affects the lethality rate after ion beam irradiation in *A. thaliana*: $^{20}Ne^{10+}$ or $^{40}Ar^{17+}$ ions with LETs higher than 350 keV/μm were more effective than 113 keV/μm C ions [6]. However, the nuclide- or LET-dependent effects of a heavy-ion beam have not been investigated fully. A more detailed molecular analysis of the mutations induced by a heavy-ion beam is needed to develop more effective methods of plant mutagenesis. The overall goal of our study is to investigate the correlation between the amount of the DNA damage and LET.

Table 1. DNA damage induced by carbon-ion irradiation*.

Point mutations		Rearrangements	
Type of mutation	No. of mutants	Type of mutation	No. of mutants
Deletion	11	Deletion	6
Insertion	1	Inversion	5
Base substitutions	2	Insertion	2
		Translocation	3
Total	14		15

*This table summarizes results described by Shikazono *et al.*[5].

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Here, we investigated the nuclide- and LET-dependence of the survival and appearance rate of albino mutants, and isolated several morphological mutants, which we will use to examine the LET-dependence of the DNA damage in future studies.

EFFECTS OF NUCLIDE ON MUTATION INDUCTION

Dry seeds of *Arabidopsis* ecotype Columbia (about 250 μm in diameter) were packed with Hybri-Bag Hard (95 μm thickness; Cosmo Bio), which is made of polypropylene, to provide a monolayer of seeds for homogenous irradiation (Fig. 1a). They were irradiated with $^{12}C^{6+}$, $^{14}N^{7+}$, $^{20}Ne^{10+}$, $^{40}Ar^{17+}$, or $^{56}Fe^{24+}$ ions with a dose range of 5 to 500 Gy. These ions were accelerated up to 135, 135, 135, 95, and 90 MeV/nucleon, and their LETs were 23, 30, 61, 280, and 630 keV/μm, respectively. All LETs were calculated at the seed surface.

The irradiated M_1 seeds were incubated on 1/2 MS agar medium at 4°C in the dark for 3 days for vernalization and then at 22°C under continuous illumination (Fig. 1c). The germination rate (ratio of the number of germinated seeds to the total number of incubated M_1 seeds) was determined 2 to 3 weeks after the initiation of incubation. Plants producing true leaves were scored as surviving. The survival rate was calculated as the ratio of surviving plants to sown seeds. Seedlings that developed true leaves were transplanted into plastic trays (13×9 cm) containing soil. Eleven seedlings were planted in each tray and cultured at 22°C under continuous illumination in a greenhouse, as shown in Figure 1d. Similarly, M_2 seeds were collected and incubated under the same conditions as used for germinating M_1 seeds. The frequency of albino plants (Fig. 1b) was calculated as the ratio of albino plants to the total number of germinated M_2 seedlings after most seedlings had expanded their cotyledons.

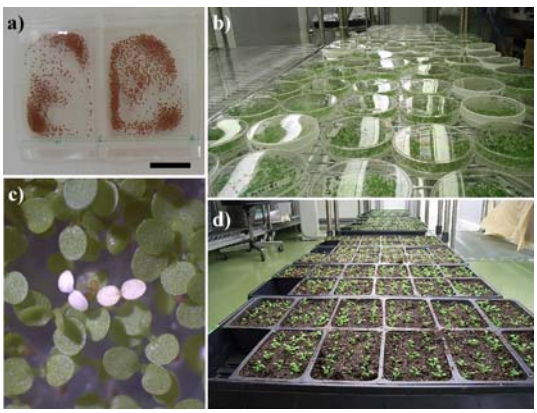

Figure 1. Photographs of *Arabidopsis thaliana*. (a) Dry seeds prepared for irradiation. (b) Germinating plants after heavy-ion beam irradiation. (c) Albino mutants induced by heavy-ion beam irradiation. (d) Plants transplanted to plastic trays. Bar = 1 cm.

For the control non-irradiated seeds, the germination rate was 97.1% and all seedlings flowered within one month after transfer to soil. The effects of heavy-ion beam irradiation on mutagenesis in *Arabidopsis* are shown in Figure 2. The germination of M_1 plants was unaffected by irradiation with any of the ions at the doses tested. However, the survival rate of M_1 plants decreased as the dose increased. The survival curves differed markedly according to the type of ion used. After high-dose irradiation, most M_1 plants that showed decreased growth produced few seeds. For $^{14}N^{7+}$ ions under irradiation conditions using different energies, a LET of 30 keV/μm was more effective for inducing albino plants.

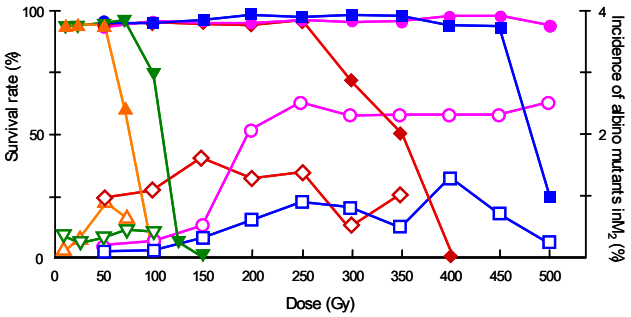

Figure 2. Effects of heavy-ion beam irradiation on survival and the incidence of albino mutants. The filled and opened symbols indicate the survival rate in M_1 and the incidence of albino mutants in M_2, respectively. Circles, rectangles, diamonds, triangles, and inverted triangles indicate $^{12}C^{6+}$, $^{14}N^{7+}$, $^{20}Ne^{10+}$, $^{40}Ar^{17+}$, and $^{56}Fe^{24+}$, respectively.

EFFECTS OF LET ON MUTATION INDUCTION

The effects of several ions with similar LETs on mutation induction in *A. thaliana* were examined. Dry seeds of *A. thaliana* were irradiated with $^{12}C^{6+}$, $^{14}N^{7+}$, $^{20}Ne^{10+}$, and $^{40}Ar^{17+}$ ions with a dose range of 50 to 250 Gy. These ions were accelerated up to 135, 135, 135, and 95 MeV/nucleon and their LETs were 23, 30, 61, and 280 keV/μm, respectively. To determine the effects of the LET of heavy ions on mutation induction, the LETs of $^{12}C^{6+}$, $^{14}N^{7+}$, and $^{20}Ne^{10+}$ ion beams were controlled to *ca.* 30, 61, and 280 keV/μm, 61 and 280 keV/μm, and 280 keV/μm, respectively, by passing the beam through a set of absorbers in the range shifter. At least 416 M_1 seeds were germinated and cultured. The frequencies of albino plants were scored after most of the seedlings had expanded their cotyledons.

The effects of heavy-ion beam irradiation at different LETs on the incidence of albino mutants are shown in Table 2. Similar frequencies of albino plants were observed for irradiation treatments at the same LETs. In addition, the same doses were required for higher frequencies. A LET of 30 keV/μm was the most effective for inducing albino plants, as also shown in Figure 2. It has been reported that the flowering rate (FR: number of

flowering plants to the total number of incubated M_1 seeds) decreased as the LET increased, and that similar FRs were observed for treatments with several ions at the same LETs [7]. Therefore, we concluded that the LET of ion beams is a critical factor for the efficient mutagenesis of plants.

Table 2. Effects of LET on the incidence of albino mutants

LET (keV/μm)	Ion	Dose (Gy)	No. of M_1 plants	Incidence of albino mutants in M_2
23	$^{12}C^{6+}$	250	594	0.83 ± 0.2
30	$^{12}C^{6+}$	250	513	2.20 ± 0.3
	$^{14}N^{7+}$	250	416	2.57 ± 0.4
61	$^{12}C^{6+}$	150	573	1.35 ± 0.3
	$^{14}N^{7+}$	150	461	1.41 ± 0.2
	$^{20}Ne^{10+}$	150	585	1.67 ± 0.7
280	$^{12}C^{6+}$	50	425	1.34 ± 0.7
	$^{14}N^{7+}$	50	516	1.21 ± 0.3
	$^{20}Ne^{10+}$	50	462	1.10 ± 0.2
	$^{40}Ar^{17+}$	50	518	1.01 ± 0.4

ISOLATION OF MORPHOLOGICAL MUTANTS

We screened the morphological mutants to examine the LET dependence of DNA damage, focusing on *hy*, *gl*, and *pg* mutants, as their phenotypes can be distinguished easily on plate cultures (Fig. 3). The *gl* mutant is characterized as having no or reduced trichomes on the leaves. The *hy* mutant has an elongated hypocotyl when grown continuously under weak light. The *pg* mutant has pale green leaves. We screened 3,470 M_2 plants irradiated with a $^{12}C^{6+}$-ion beam at a dose of 200 Gy and 3,501 M_2 plants irradiated with an $^{40}Ar^{17+}$-ion beam at a dose of 50 Gy. These ions were accelerated up to 135 and 95 MeV/nucleon, and their LETs were 23 and 280 keV/μm, respectively.

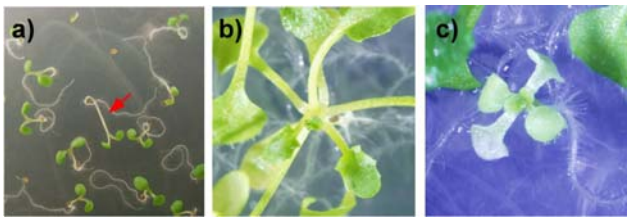

Figure 3. Morphological mutants screened in this study: (a) *hy* (indicated by the arrow); (b) *gl*; and (c) *pg* mutants.

The results of this screen are shown in Table 3. We isolated 77 mutants. The numbers of mutants isolated differed according to phenotype, which was probably due to the number of genes responsible for each mutant. In addition, the mutation rate may be biased by the chromosomal loci of the responsible genes. The genes responsible for these mutant phenotypes have been identified [8–10]. Consequently, the mutated genes producing these mutants can be determined by sequencing the candidate genes. Molecular analyses of the mutated genes will clarify the reasons for the differences in incidence rate of mutants and the effects of ions and their LETs.

Table 3. Number of mutants isolated.

Mutant	No. of mutants isolated		No. of genes responsible
	$^{12}C^{6+}$	$^{40}Ar^{17+}$	
hy	5	9	5
gl	1	0	5
pg	46	16	11
Total	52	25	21

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