Plant Biotechnology 25, 113–117 (2008)

Original Paper

LET-dependent effects of heavy-ion beam irradiation in *Arabidopsis thaliana*

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Received November 5, 2007; accepted January 15, 2008 (Edited by T. Kameya)

Abstract We irradiated *Arabidopsis thaliana* with several kinds of heavy-ion beams to investigate the linear energy transfer (LET)-dependent effects of heavy-ion beam irradiation. First, dry seeds were irradiated with C, N, Ne, Ar, or Fe ions at doses ranging from 5 to 400 Gy to compare the flowering and mutation rates among the ion species. The sensitivity of the flowering and mutation rates to irradiation differed markedly among the ion species. Of the ion species, N (30 keV μm^{-1}) was the most effective at inducing albino plants. Second, we examined the effects of LET on mutation induction. The LET of C, N, Ne, and Ar ion beams was controlled at 30–640 keV μm^{-1}. Regardless of ion species, irradiation with the same LET value resulted in the same flowering rate and mutation rate. Thus, the LET of ion beams seems to be an important factor affecting mutagenesis. We found a 440-bp deletion in the *hy* (elongated hypocotyl) mutant that was isolated from M_2 progeny. These fundamental data on LET-dependence can be used to develop advanced technologies for plant mutagenesis.

Key words: *Arabidopsis thaliana*, heavy-ion beam, LET.

Heavy-ion beam mutagenesis, in addition to its utility in functional studies of genes, is generally accepted to be an effective method of producing mutations. Heavy-ion beams have high linear energy transfer (LET), and based on radiobiological considerations, high-LET irradiation is postulated to produce double-strand breaks whose damaged ends are difficult to be repair (Hagen 1994; Ward 1994; Goodhead 1995). Therefore, large structural rearrangements are likely caused by high-LET irradiation more frequently than by low-LET irradiation, but little experimental evidence exists of a correlation between LET and mutation induction in plants.

High-LET irradiation can induce stable knockout mutants through large structural rearrangements. Producing knockout mutants is useful in functional studies of genes (Bouche and Bouchez 2001) because the function of a gene can be determined if a knockout alters the phenotype. Recently, we reported that the deletion size of mutated genes is 985 bp in *Arabidopsis thaliana* irradiated with C ions at 22.5 keV μm^{-1} (Kazama et al. 2007) and 43–203 bp in *Mesorhizobium loti* irradiated with C ions at 22.5–40 keV μm^{-1} (Ichida et al. 2007). Such deletions are valuable for investigating gene function because they can be defined as nulls. Moreover, the technique for producing gene deletions can be used to study the functions of tandemly arrayed genes. At least 14% of genes form tandem arrays in the sequenced plant genomes (Arabidopsis Genome Initiative 2000; International Rice Genome Sequencing Project 2005). If a method for deletion-size-controlled mutagenesis is created, studying gene function of tandem arrays will be possible.

The effects of heavy ions and other kinds of radiation have been well studied in *A. thaliana* because its seeds and plants are small, it produces a large number of seeds, and its life cycle is short (Bork et al. 1989). LET affects the lethality rate after ion-beam irradiation in *A. thaliana*: Ne or Ar ions (LET>350 keV μm^{-1}) are more effective than C ions (LET=153.5 keV μm^{-1}; Shikazono et al. 2002). These results raise the possibility that LET is a critical factor influencing the biological effects of radiation. However, the ion species- or LET-dependent effects of heavy-ion beams have not been fully investigated. A more detailed molecular analysis of the mutations induced by heavy-ion beams is needed to develop more effective methods of plant mutagenesis.

As a first step toward determining LET-dependent effects in plants, we investigated the ion species- and

Abbreviations: LET, linear energy transfer; *hy*, elongated hypocotyl; RIBF, RI-beam factory; MS, Murashige-Skoog

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LET-dependence of flowering rate and the appearance of albino mutants. Both the flowering rate and appearance of albino mutants showed marked dependency on ion species and LET. In addition, we identified mutations in the elongated hypocotyl (*hy*) gene that were isolated from M_2 progeny. These mutants provided a means to perform gene analysis to identify the deletions caused by ion-beam irradiation. These fundamental data will be valuable in determining the appropriate LET for effective mutagenesis and in developing a method to control the deletion size of mutants. The overall goal of our study was to investigate the correlation between deletion size and LET.

Materials and methods

Plant material

Arabidopsis thaliana (L.) Heynh. ecotype Columbia was used throughout this study. After irradiation, plant seeds were surface-sterilized and sown in soil or on 0.7% (w/v) agar-containing Murashige-Skoog (MS) medium (Wako-Junyaku, Osaka, Japan) supplemented with MS vitamins (Sigma aldrich Japan, Tokyo, Japan) and 1.5% (w/v) sucrose. The germinated seedlings were cultivated according to general methods described previously (Fujiwara et al. 2004).

Ion-beam irradiation

Dry seeds were packed with Hybri-Bag Hard (95 μm thickness; Cosmo Bio, Carlsbad, CA) to provide a monolayer of seeds. 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 at E5 beam line in a RIKEN RI-beam factory (RIBF). These ions were accelerated up to 1.62, 1.89, 2.70, 3.80, and 5.04 GeV, and the LET was 22.5, 30.0, 61.5, 290, and 640 keV μm^{-1}, respectively. All LET values were calculated behind seeds. To determine the effects of heavy ion LET, the LET of $^{12}C^{6+}$, $^{14}N^{7+}$, $^{20}Ne^{10+}$, and $^{40}Ar^{17+}$ ion beams were controlled to approximately 30.0, 61.5, and 290 keV μm^{-1}; 61.5 and 290 keV μm^{-1}; 290 keV μm^{-1}; and 640 keV μm^{-1}, respectively, by passing the ions through a combination of absorbers.

Observation of germination, flowering, and albino mutants

Irradiated M_1 seeds were surface-sterilized, incubated on 0.7% agar-containing MS medium supplemented with 1.5% sucrose at 4°C in the dark for 3 days to achieve vernalization. Then they were grown at 22°C under continuous illumination. The germination rate (ratio of the number of germinated seeds to the total number of incubated M_1 seeds) was determined 2–3 weeks after the initial incubation. 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. Flowering rate (number of flowering plants per total number of incubated M_1 seeds) was determined 1 month after the transfer to soil. Similarly, M_2 seeds were collected and incubated under the same conditions as used for germinating M_1 seeds. The frequency of albino plants was calculated as the ratio of albino plants to the total number of germinated M_2 seedlings after most seedlings had expanded their cotyledons. At least three independent experiments at different doses of irradiation were performed with each ion species.

Table 1. List of gene-specific primers used in this study.

Primer	Sequence (5′–3′)
HY1f1	GTC GCC GTC TTT AGT GGT GGT T
HY1r1	GTA TCC GCT CTG CCA CCT TTC T
HY2f1	GAC TTT GCG GGT TTC ATG GAG C
HY2r1	GTG CTC GCC ATG TCA GGT ACT T
HY3f1	TAA GCA GAA CCG TGT CCG AAT G
HY3r1	CGA GTC ACG CAG AAG CAT ATC A
HY4f1	TTT GCC AGA GAG GCA CTC AG
HY4r1	CAC CGG AGT TAC AGC CCT TA
HY5f1	GAG AAT CTG GAT CGG CGA CC
HY5r1	CAC CAC CAC CTC CTC TCT TGT T
HY2df3	TTC TTG CCG AAT TGA ACC GTT GGT C
HY2dr4	TCA TCT TCT TCC TAC AGG CAT GGC

Screening of hy mutants

The *hy* mutants were screened by growing M_2 seeds on MS agar medium under weak light. Plants showing an elongated hypocotyl were isolated as *hy* mutants. M_3 seeds of *hy* mutants were harvested and the phenotype of the M_3 plants was observed following growth on MS agar medium under weak light.

DNA extraction and molecular analysis

Genomic DNA was extracted from the M_3 mutants with a Nucleon PhytoPure Genomic DNA Extraction Kit (Amersham Biosciences, Piscataway, NJ). Genes responsible for the *hy* phenotype (*HY1*/*HY6*, AT2G26670; *HY2*, AT3G09150; *HY3*/*PHYB*, AT2G18790; *HY4*, AT4G08920; *HY5*, AT5G11260) were isolated by PCR-based cloning using gene-specific primers (see Table 1). Amplification was carried out at 94°C for 3 min followed by 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. At the end of the 30 cycles, the samples were incubated at 72°C for another 7 min to complete extension. The amplified fragments were sequenced, cloned into the pCR4-TOPO TA cloning vector (Invitrogen, Carlsbad, CA), and used for Southern blot analysis as probes. Southern blot analysis of *hy* mutants and the wild type was performed as previously described (Kazama et al. 2006). A probe that shows different band patterns between the wild type and the *hy* mutant in the Southern blot analysis was determined. Then its neighboring region was further amplified from the wild type and *hy* mutant genomes by PCR using two primers, HY2df3 and HY2dr4 (see Table 1). The amplified DNA fragments were analyzed by 1.5% agarose gel electrophoresis and direct sequencing. Mutation regions were determined using the Arabidopsis Genome Database (http://www.arabidopsis.org/Blast/) and GENETYX program version 8 (GENETYX Co., Ltd., Tokyo, Japan).

Results

Effects of ion species on mutation induction

The effects of heavy-ion beam irradiation on mutagenesis

Table 2. Effects of heavy-ion beams on mutation induction.

Ion	LET (keV/μm)	Dose (Gy)	Number of M_1 seeds*	Germination rate of M_1 plants (%)	Flowering rate of M_1 plants (%)	% albino in M_2 plants
Control	–	–	680	97.1±2.5	97.1±2.5	0.07±0.07
$^{12}C^{6+}$	22.5	50	371	93.5±4.0	93.5±4.0	0.16±0.0
		100	384	86.0±2.5	83.1±0.8	0.15±0.0
		150	315	89.3±3.9	87.3±4.3	0.31±0.1
		200	418	94.2±1.0	81.2±0.7	0.51±0.2
		400	337	91.1±4.2	79.2±11.6	0.83±0.3
$^{14}N^{7+}$	30.0	50	339	94.8±2.4	93.5±2.2	0.29±0.0
		100	418	98.4±0.4	97.8±0.8	0.25±0.0
		150	491	96.7±1.3	94.4±2.5	0.47±0.1
		200	476	98.9±0.3	97.5±0.7	2.02±0.3
		400	555	99.7±0.3	98.4±0.6	2.65±0.1
$^{20}Ne^{10+}$	61.5	50	515	97.6±0.4	94.9±1.9	1.02±0.1
		100	412	92.9±3.9	91.8±3.5	1.03±0.4
		150	390	91.9±5.4	90.3±4.4	1.67±0.7
		200	393	88.5±7.9	87.3±8.9	1.39±0.1
		400	452	86.7±11.0	3.0±1.9	nd**
$^{40}Ar^{17+}$	290	5	580	97.9±1.1	89.5±2.1	0.20±0.1
		10	517	94.3±5.3	93.2±4.8	0.25±0.1
		20	479	94.6±4.4	94.4±4.3	0.38±0.1
		50	499	97.6±2.1	97.3±2.0	1.01±0.4
		100	445	93.9±4.0	2.14±1.6	0.8±0.4
		150	452	92.6±5.9	0	nd**
		200	341	95.5±3.7	0	nd**
$^{56}Fe^{24+}$	640	5	410	97.0±0.3	96.6±0.2	0.10±0.1
		10	408	93.8±1.9	93.5±2.0	0.51±0.2
		20	341	92.9±4.2	92.9±4.2	0.32±0.1
		50	324	95.6±3.0	94.4±3.1	0.65±0.0
		100	339	94.1±1.8	74.9±4.7	0.51±0.4
		150	305	91.4±3.4	5.9±5.9	nd**
		200	303	86.9±6.4	0	nd**

* Total number of irradiated M_1 seeds from three independent experiments.

** Data were not determined.

are shown in Table 2. For the control nonirradiated seeds, the germination rate was 97.1% and all seedlings flowered within 1 month after transfer to soil. Germination of M_1 plants was unaffected by irradiation with any of the ion species at the doses tested. Irradiation with Ne, Ar, and Fe ions decreased the flowering rate as the irradiation dose increased. In the C and N ion treatments, the flowering rate was not reduced at doses lower than 400 Gy. The sensitivity of flowering rate to irradiation differed among ion species; it increased as the LET value of the ion species increased. However, flowering rates of 2.14 and 74.9% were obtained after irradiation with 100 Gy of Ar (290 keV μm^{-1}) and Fe (640 keV μm^{-1}) ions, respectively. The sensitivity of flowering was lower with Fe irradiation than with Ar irradiation. The percentage of albino mutants in the M_2 generation also differed among ion species. A peak in the frequency of albino mutants was observed for each ion species. The irradiation dose required for the most effective induction of albino mutants decreased as the LET increased (C, 400 Gy; N, 400 Gy; Ne, 150 Gy; Ar, 50 Gy; Fe, 50 Gy). Among the ion species tested, N was the most effective for inducing albino mutants. The frequency of albino

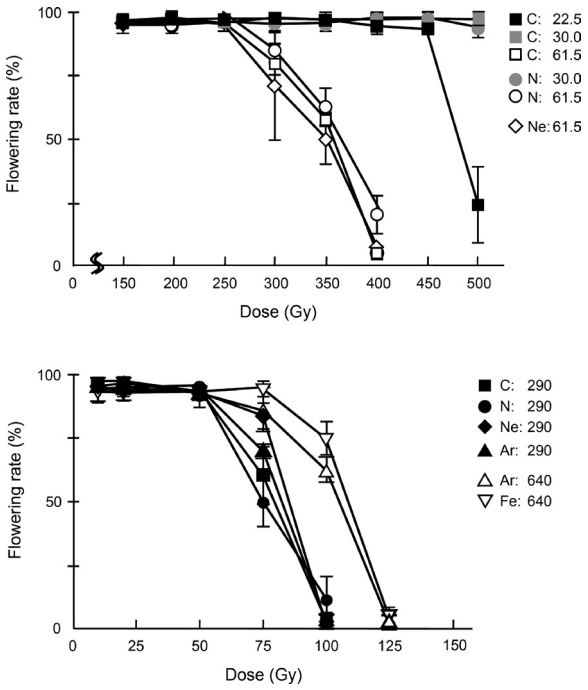

Figure 1. LET-dependent effects of heavy-ion beams on flowering rate. Rectangles, circles, diamonds, triangles, and inverted triangles indicate C, N, Ne, Ar, and Fe ion beams, respectively.

mutants was 2.02 and 2.65% at 200 and 400 Gy, respectively.

Effects of LET on mutation induction

We examined the flowering rate and percent of albino mutants produced by different ion species with the same LET value via adjustments with the range shifter. The effects of heavy-ion beam irradiation at different LET levels on the flowering rate are shown in Figure 1. Similar flowering rates were observed at the same LET value, regardless of ion species. A LET of 290 keV μm^{-1} was the most effective in decreasing the flowering rate. Conversely, irradiation at a LET of 30 keV μm^{-1} did not decrease the flowering rate. The effects of heavy-ion beam irradiation at different LET values on the percent of albino mutants are shown in Table 3. Similar frequencies of albino mutants were observed for irradiation treatments at the same LET value, A LET of 30 keV μm^{-1} was the most effective for inducing albino plants.

Identification of the mutated gene in hy mutants induced by heavy-ion beam irradiation

Three mutants were successfully isolated from the M_2 generation after irradiation with Ne ions at a dose of 150 Gy. We determined the mutated region of one of the mutants (*hy1-1*). Five genes (*HY1*-5) responsible for elongated hypocotyl phenotypes have been identified through extensive genetic studies (Koornneef et al. 1980; Reed et al. 1993; Ahmad and Cashmore 1994; Oyama et

al. 1997; Muramoto et al. 1999). We performed a Southern blot analysis of *hy1-1* using these five genes as probes. When a *HY2* probe was hybridized, different band patterns were seen in the wild type and *hy 1-1* (data not shown). To investigate if this plant had DNA damage in the *HY2* gene, PCR analysis was performed on total genomic DNA of the mutant using gene-specific primers (Table 1). However, no mutation was identified in the coding region of *HY2*. We performed PCR using other primers (*HY2*df3 and *HY2*dr4), which were downstream of the *HY2* gene (Figure 2A). A single DNA product (3,203 bp) was obtained for the wild-type plant, while a shorter DNA fragment (2,763 bp) was amplified from *hy 1-1*. Sequence analysis revealed that the 2,763-bp PCR fragment contained a 440-bp deletion (Figure 2B). Four base changes were also found upstream and downstream of the deletion site. Half of the base changes were transitions and the others were transversions. Although whether the identified mutations were truly responsible for the elongated hypocotyl phenotype was unclear, we characterized a sample of the mutation induced by the Ne ion beam. Characterization of the mutations in the other *hy* mutants is now in progress.

Table 3. Effects of LET on the incidence of albino mutants.

LET (keV/μm)	Ion	Dose (Gy)	Number of M_1 plants	Incidence of albino mutants in M_2
22.5	$^{12}C^{6+}$	250	594	0.83±0.2
30.0	$^{12}C^{6+}$	400	353	3.28±0.2
	$^{14}N^{7+}$	400	555	2.65±0.1
61.5	$^{12}C^{6+}$	150	523	1.35±0.3
	$^{14}N^{7+}$	150	416	1.41±0.2
	$^{20}Ne^{10+}$	150	390	1.67±0.7
290	$^{12}C^{6+}$	50	496	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	618	1.01±0.4
640	$^{40}Ar^{17+}$	50	488	1.08±0.1
	$^{56}Fe^{24+}$	50	324	0.65±0.0

Discussion

In this study, we demonstrated the LET-dependent effects of heavy-ion beam irradiation on the flowering rate and induction of albino mutants in *A. thaliana*. The peak relative biological effectiveness (RBE) for lethality of *Arabidopsis* seeds occurs at a LET of around 350 or 400 keV μm^{-1} for Ne or Ar ions, respectively (Shikazono et al. 2002). Accordingly, we found that Ne and Ar ions were more effective at decreasing the flowering rate than C and N ions. However, in terms of the induction of albino mutants, N ions (30 keV μm^{-1}) were more effective than Ne or Ar ions (Table 2). Moreover, irradiation with C ions whose LET was controlled at 30 keV μm^{-1} produced a similar frequency of the albino mutant as N ions. These results indicate that the LET of heavy-ion beams is an important factor influencing the effects of heavy-ion beam irradiation on plants, and that the most effective LET for changing the flowering rate in the M_1 generation differs from that for inducing albino

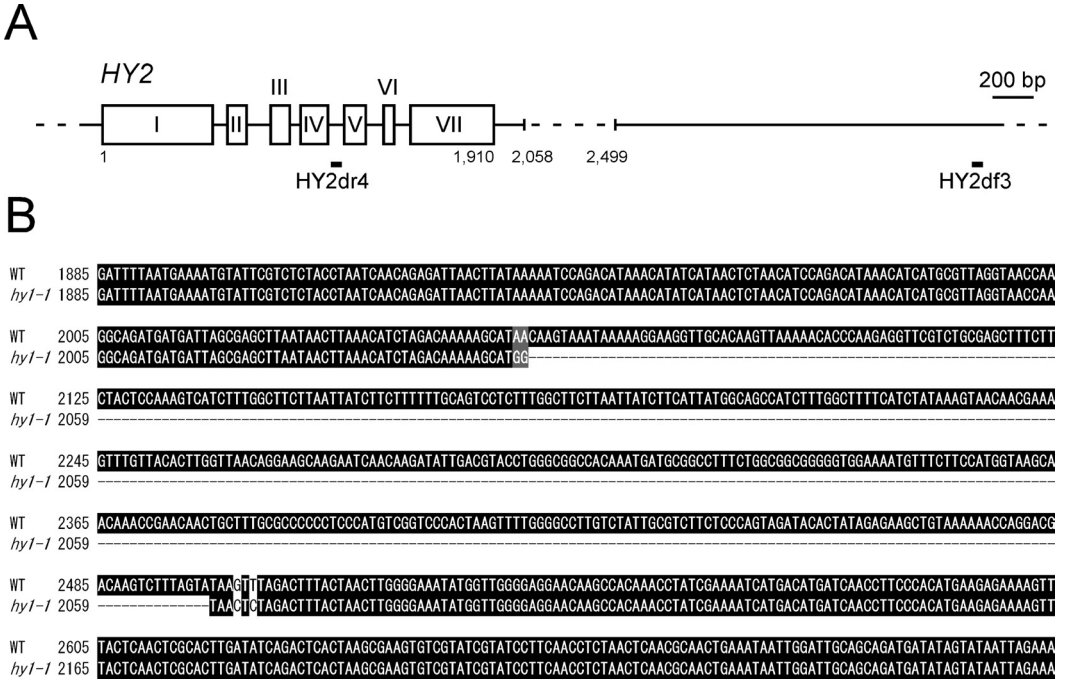

Figure 2. Identification of the deletion in a *hy1-1* mutant. (A) Schematic representation of the *HY2* gene and its flanking regions. Numbers indicate positions from the initiation codon of *HY2*. Bars indicate relative sites of PCR primers. (B) Mutations in the *hy1-1* mutant. Nucleotide sequences of a fragment were aligned with non-mutated genomic sequences using CLUSTAL W ver. 1.8. The sites of base changes caused by transitions were shaded using BOXSHADE ver. 3.21. Numbers on the left sides indicate positions from the initiation codon of *HY2*.

mutants in the M_2 generation.

Fe ion irradiation had less of an effect than Ar ion irradiation for both flowering rate and the frequency of albino mutants (Table 2). Similar results were observed for the flowering rate when several ion beams with LET controlled to 290 and 640 keV μm^{-1} were used (Figure 1). This outcome was likely due to differences in the number of ion particles. Because the dose is proportional to the product of LET multiplied by the number of ion particles, the number of Fe particles is 29/64 that of Ar particles at the same dose. Thus, irradiation with Fe ions requires a higher dose than irradiation with Ar ions to obtain the same effects.

We found that a 440-bp deletion occurred in the *HY2* gene after irradiation with Ne ions (61.5 keV μm^{-1}). A 985-bp deletion was produced in a mutant induced by C ion irradiation (22.5 keV μm^{-1}; Kazama et al. 2007). These results highlight LET effects on DNA damage in plants. Further studies of mutants induced by heavy-ion beams in plants are needed to address this issue. LET-dependent effects on the size of DNA deletions have been observed in transgenic *Rhizobium* (Ichida et al. 2007). Our results indicate that ion-induced mutations are most likely nulls and are useful bioresources for functional genomics.

In conclusion, our results demonstrate that the LET of ion beams is a critical factor for efficient mutagenesis in plants. In dry seeds of *Arabidopsis*, A LET of 30 keV μm^{-1} was the most effective for inducing mutants in the M_2 generation. This finding can be utilized for more effective mutagenesis in *A. thaliana*.

Acknowledgements

We thank Ms. S. Ohbu for technical assistance, and the Research Resources Center of RIKEN BSI for DNA sequencing. This study was supported by a grant from the research project Utilizing Advanced Technologies in Agriculture, Forestry and Fisheries from the Ministry of Agriculture, Forestry and Fisheries of Japan.

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