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Biological effects of ion beams in *Nicotiana tabacum* L.

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Abstract The biological effects of ion beams on *Nicotiana tabacum* L., particularly the induction of chromosome aberrations, were investigated. Dry seeds were exposed to $^{12}C^{5+}$, $^{4}He^{2+}$ and $^{1}H^{+}$ beams with linear energy transfer (LET) ranging from 1 to 111 keV/µm and irradiated with gamma-rays. Ion beams were more effective in reducing germination and survival of the seeds than gamma-rays. The LD_{50} for $^{12}C^{5+}$ beams, $^{4}He^{2+}$ beams and gamma-rays were 35, 60 and 500 Gy, respectively. The frequencies of mitotic cells with chromosome aberrations, such as chromosome bridges, acentric fragments and lagging chromosomes in the root tip cells of the exposed seeds, increased linearly with increasing doses. Relative biological effectiveness (RBE) values, based on the doses that induced a survival inhibition of 50% and a 10% frequency of aberrant cells, were 14.3–17.5 for the $^{12}C^{5+}$ beams, 7.0–8.3 for the $^{4}He^{2+}$ beams and 7.8 for the $^{1}H^{+}$ beams. Furthermore, the relative ratios of the chromosome aberration types were significantly different between the ion beam and the gamma-ray regimes: chromosome fragments were more frequent in the former, and chromosome bridges in the latter. Based on these results, we concluded that the repair process of initial lesions induced by ion beams may be different from that induced by low- LET radiation.

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Introduction

Ion beams have a higher linear energy transfer (LET) than do x-rays and gamma-rays, and the range of ion beams in the target materials can be controlled. Accordingly, a significant amount of energy can be deposited on the focal point of the material exposed to an ion beam. Therefore, the biological effects induced by heavy ion beams may be different from those induced by low-LET radiation. To clarify this understanding, various studies on cell inactivation and genetic changes induced by ion beams as well as the relationship between LET and relative biological effectiveness (RBE) have been performed (e.g .[1–3]), although only a limited number of such studies have focussed on plants [4–10].

In mammalian cells, it was shown that heavy ion beams were more effective than gamma-rays in inhibiting growth and inducing mutation, and that the highest RBE for the loss of colony-forming ability, for neoplastic transformation, and for premature chromosome condensation (PCC) breaks were obtained with LET of around 100 keV/µm [3, 11,12]. Kraft [3] reported that although x-ray exposure induced breaks and exchanges at a similar frequency, heavy ion exposure predominantly induced chromosome breaks. In plant cells, ion beams have also been shown to be effective in inhibiting growth [4,8] and in inducing mutations [5–7] and chromosome aberrations [5, 9,10]. In *Pisum sativum* L., Vasilenko and Sidorenko [9] reported that He ion beams with a LET of 0.95 keV/µm produced 6 times more micronuclei than did ^{60}Co gamma-rays. Tanaka et al. [8] analysed the effects of various ion beams on the seeds of *Arabidopsis thaliana* (L.) Heynh, with LET values ranging from 17 to 549 keV/µm. They demonstrated that the peak RBE for the reduction in survival rate (11–12) occurred at 252 keV/µm. These results suggest that the biological action of ion beams is different from that of low-

LET radiation. However, the bioeffects of ion beams on plants, in particular, their effects on several endpoints such as reduction of survival rate and induction of chromosome aberrations, have not been sufficiently examined. An understanding of these effects is important in order to employ ion beams as a novel mutagen in plant breeding.

In this study, the relationship between LET and RBE regarding the reduction of survival rate and chromosome aberration frequency was analysed in the seeds of *Nicotiana tabacum* L. exposed to ion beams with LET values ranging from 1 to 111 keV/μm, with gamma-rays being used as the reference radiation. The chromosome aberration spectrum is also discussed.

Materials and methods

Dry seeds of *Nicotiana tabacum* L.cv.Tukuba 1 were exposed to $^{12}C^{5+}$, $^{4}He^{2+}$ and $^{1}H^{+}$ beams from an AVF cyclotron (JAERI, Takasaki, Japan). The properties of the ion beams used in this experiment are shown in Table 1. The mean LET for $^{12}C^{5+}$, $^{4}He^{2+}$ and $^{1}H^{+}$ was 111, 15 and 1 keV/μm, respectively, and the ranges were 1.0, 1.5 and 31.0 mm, respectively. Since the seed length was about 0.5 mm in the exposure direction, all ion beams used in this study could pass through the seeds, and the Bragg peak was not included in the track of ion beams in the exposed seeds. The mean LET and ranges of the ion beams in the seed were calculated by ELOSS code, a modified OSCAR code [13], taking the present exposure conditions into consideration. Ion beams were scanned to expose an area of more than 50×50 mm on the sample plate. The beams passed through a 30-μm-thick titanium window into the exposure chamber, which was kept at atmospheric pressure. The distance between the beam window and sample was 10 cm, and this space was filled with He gas to reduce energy loss of the ion beams. For homogeneous exposure of the seeds to the ion beams, the seeds were placed in a monolayer between kapton films (7.5 μm in thickness, 45 mm square in size, Toray-Dupon, Japan) and exposed within 3 min for all doses at room temperature. Particle fluences were determined by a harzlas TNF-1 track detector (Nagase Landauer, Japan). Simultaneously, dry seeds were irradiated with ^{60}Co gamma-rays with a LET of 0.2 keV/μm, at a dose rate of $1–6\times10^{2}$ Gy/h for 1 h at room temperature. These procedures were separately replicated 3 times with 50 seeds each in all exposure regimes.

The exposed seeds were allowed to germinate on a moistened filter paper in a growth chamber at 25°C under light conditions. Seeds that produced enlarged cotyledons were identified as germinating seeds. After germination, the seeds were transplanted to soil and grown under the same culture conditions. Seedlings that developed more than 3 leaves were scored as surviving plants. Unexposed seeds germinated 5–6 days after sowing and the 3rd leaves were observed 4–5 weeks after sowing.

For the cytological study, roots were collected when their length reached 1 cm. Roots of unexposed seeds were 1 cm about 5 days after sowing. Root elongation of ion beam-exposed seeds was delayed for 1–3 days because of mitotic delay. The collected roots were fixed in a solution of ethanol:acetic acid (3:1, v/v) for 24 h, hydrolysed in 1N-HCl at 60°C for 6.5 min and stained in Schiff's reagent for 1–2 h. After additional staining by aceto-carmine, they were squashed for microscopic observation. Chromosome aberrations were observed in mitotic cells of 9–18 root tips for each exposure. The mitotic index was also determined. The ratio of fragments to bridges was determined for each exposure. The difference in the ratios obtained with the gamma-rays and each of the ion beams was tested with a chi-square analysis.

Results and discussion

Figure 1 shows the dose-response curves for germination and survival rate following exposure of the seeds to the ion beams and gamma-rays. Although gamma-rays up to 600 Gy had a slight effect on the germination rate, the ion beams had a marked effect: the germination rate was 18.1% for seeds exposed to 80 Gy of $^{12}C^{5+}$, 7.1% for seeds exposed to 160 Gy of $^{4}He^{2+}$ and 91.7% for seeds exposed to 600 Gy of gamma-rays (Fig. 1a). Shoulders were observed on the dose-response curves for the $^{12}C^{5+}$ and $^{4}He^{2+}$ beams at around 60 and 120 Gy, respectively. In all exposure regimes, the survival rate was reduced in a similar fashion, but the extent of reduction was greater than that observed in the germination rate (Fig. 1b). The LD_{50} for the $^{12}C^{5+}$ and $^{4}He^{2+}$ beams and the gamma-rays were 35, 60 and 500 Gy, respectively. These values corresponded to the LET of each type of radiation.

The mitotic activity in the root tips of the exposed seeds was reduced. For example, the mitotic index was 3.8% in the control, 2.3% in the root tip cells exposed to 40 Gy $^{12}C^{5+}$ beams, and 2.9% in the cells exposed to 40 Gy $^{4}He^{2+}$ beams. As for the cells exposed to gamma-irradiation, the mitotic index was reduced to 3.5%, 2.2% and 1.7% in the 200, 400 and 600 Gy regimes, respectively.

As shown in Fig. 2, three types of chromosome aberrations chromosome bridge (a), acentric fragment (b) and lagging chromosome (c) were observed in the metaphase and telophase cells of the root tips. Furthermore, micronuclei were observed in some interphase cells.

The frequencies of mitotic cells with chromosome aberrations tended to increase as the dose increased (Fig. 3). With an exposure of 40 Gy, the frequency of aberrant cells in the $^{12}C^{5+}$, $^{4}He^{2+}$ and $^{1}H^{+}$ beams was 14.0%, 8.7% and 6.2%, respectively. With gamma-ray exposures of 200, 400 and 600 Gy, the frequency was 8.2%, 9.5% and 14.9%, respectively. The highest frequency was 15.8% in the 20 Gy $^{12}C^{5+}$ beams. Furthermore, the doses of the $^{12}C^{5+}$, $^{4}He^{2+}$ and $^{1}H^{+}$ beams and gamma-rays that were

Table 1 Properties of ion beams used in this study

Radiation	Total energy (MeV)	Mean LET[a] in seed (keV/μm)	Range of LET[a] in seed (keV/μm)	Range[a] (mm)
$^{12}C^{5+}$	220	111	101–124	1.0
$^{4}He^{2+}$	50	15	14–17	1.5
$^{1}H^{+}$	60	1	1	31.0
Gamma-rays		0.2	0.2	

[a] Calculated as water equivalent

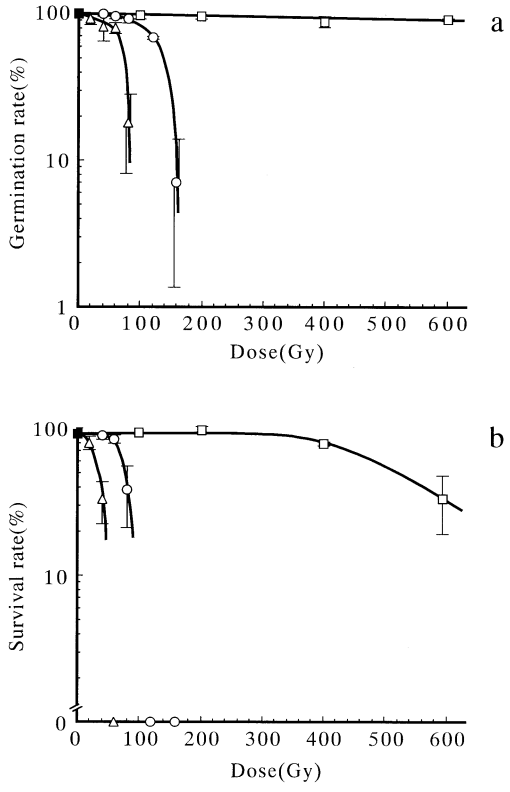

Fig. 1 Changes in the germination rate (**a**) and survival rate (**b**) of tobacco seeds exposed to ion beams:. ■ control, △ 220 MeV $^{12}C^{5+}$, ○ 50 MeV $^{4}He^{2+}$, □ gamma-rays. Each point is the average of three replications, and *vertical bars* represent one SD on either side of the average

needed to induce a frequency of 10% aberrant cells were 20, 50, 45 and 350 Gy, respectively (Fig. 3).

The RBE values for the respective ion beams based on the LD_{50} and the dose required to induce a frequency of 10% aberrant cells were determined (Fig. 4). For example, the RBE values for the induction of chromosome aberrations were 7.8 for $^{1}H^{+}$ beams of 1 keV/μm, 7.0 for $^{4}He^{2+}$ beams of 15 keV/μm, and 17.5 for $^{12}C^{5+}$ beams of

Fig. 2 Chromosome aberrations observed in the root tip cells of tobacco seeds exposed to ion beams: chromosome bridge (**a**), acentric fragment (**b**), lagging chromosome (**c**)

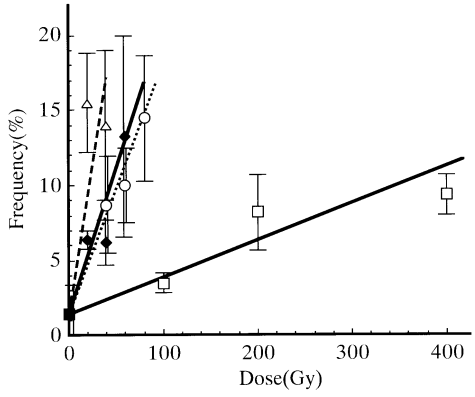

Fig. 3 Frequency of mitotic cells with chromosome aberrations in root tips of tobacco seeds exposed to ion beams: ■ control, △ 220 MeV $^{12}C^{5+}$, ○ 50 MeV $^{4}He^{2+}$, ◆ 60 MeV $^{1}H^{+}$, □ gamma-rays. Each point is the average of three replications, and *vertical bars* represent one SD on either side of the average

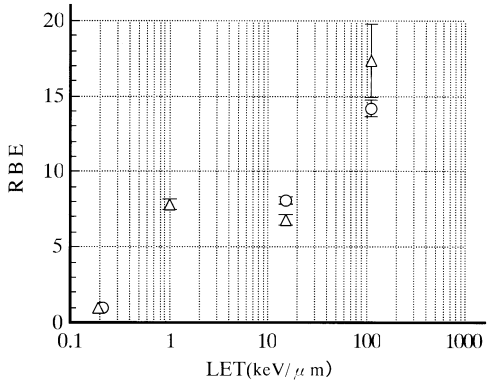

Fig. 4 Relationship between linear energy transfer (LET) and relative biological effectiveness (RBE) based on the survival reduction and the induction of chromosome aberrations: ○ LD_{50}, △ frequency of 10% aberrant cells. Each point is the average of three replications, and *vertical bars* represent one SD on either side of the average

111 keV/μm. These values support the results of other studies [3,4,8], indicating that the biological effects of ion beams depend on LET. However, our RBE values were markedly higher than those reported for other plants. Mei et al. [5] investigated micronuclei formation

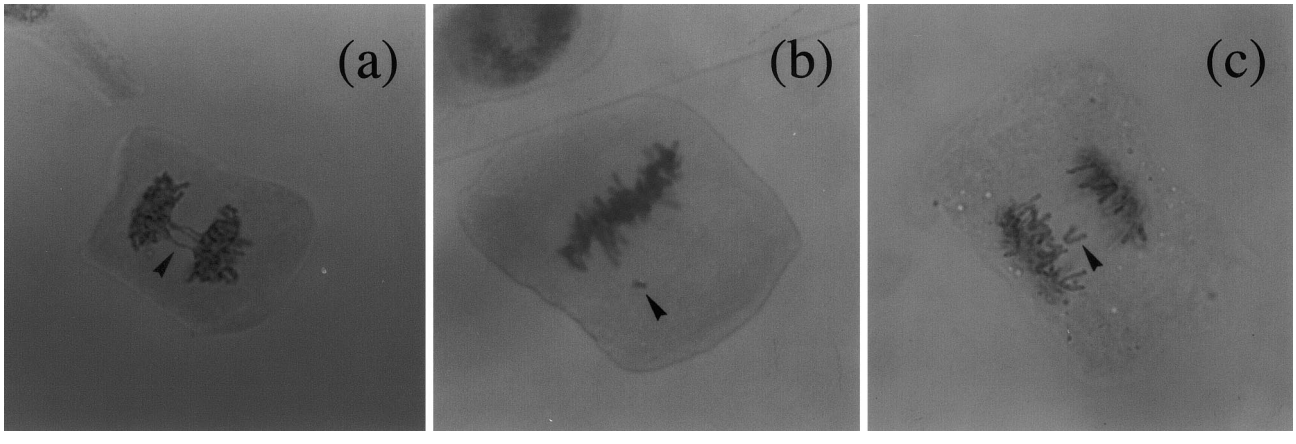

Table 2 Chromosome aberrations observed in the root tip cells of the seeds exposed to ion beams and gamma-rays

Exposure	No. of miotic cells observed	No. of mitotic cells with chromosome aberrations	Frequency of mitotic cells with chromosome aberrations (%)	Number of aberrations		
				Bridge	Fragment	Lagging
Control	346	4	1.2	0	4	0
$^{12}C^{5+}$						
20Gy	273	43	15.8	23	17	4
40Gy	286	40	14.0	27	13	2
Total				50 (0.58)[a]	30 (0.35)	6 (0.07)
$^{4}He^{2+}$						
40Gy	438	38	8.7	19	17	5
60Gy	422	43	10.2	24	16	4
80Gy	309	46	14.9	30	18	4
Total				73 (0.53)	51 (0.37)	13 (0.09)
$^{1}H^{+}$						
20Gy	218	14	6.4	2	10	3
40Gy	406	25	6.2	11	11	3
60Gy	143	17	11.9	8	4	5
Total				21 (0.36)	25 (0.42)	13 (0.22)
γ-rays						
100Gy	198	7	3.5	3	4	0
200Gy	208	17	8.2	10	3	5
400Gy	169	16	9.5	10	6	1
600Gy	154	23	14.9	18	2	3
Total				41 (0.63)	15 (0.23)	9 (0.14)

[a] Frequency of each aberration types for respective radiation

in rice root tip cells derived from dry seeds exposed to Ar ion beams of 117 keV/μm and reported that the RBE (relative to gamma-rays of 0.27 keV/μm) was 3.88. In *Arabidopsis*, Tanaka et al. [8] showed that C ion beams with a LET of 113 keV/μm caused 4.1–4.6 times greater reduction in survival than electrons of 0.2 keV/μm. This discrepancy in RBE may be caused by differences in the materials analysed and/or the exposure conditions used.

Table 2 shows the types of chromosome aberrations that were observed in mitotic cells. In most of the aberrant cells, only one aberration-type per cell was found. Since the spectrum of aberrations was the same for all exposure regimes, the frequencies of each aberration types, taking all the doses of respective radiations together, were compared. As a result, fewer bridges and more fragments were observed in the root tip cells exposed to the ion beams than in those irradiated with gamma-rays. For example, the frequencies of bridges in the root tip cells exposed to $^{4}He^{2+}$ beams and gamma-rays were 0.53 and 0.63, respectively, and the frequencies of fragments were 0.37 and 0.23, respectively. The ratio of fragments to bridges was significantly higher in the root tip cells exposed to ion beams (0.60, 0.70 and 1.17 for $^{12}C^{5+}$, $^{4}He^{2+}$ and $^{1}H^{+}$ beams, respectively) than in those irradiated with gamma-rays (0.37).

Suzuki et al. [12] reported that in mammalian cells, only 35%–45% of the PCC breaks in Syrian golden hamster embryo (SHE) cells were rejoined 8 h after heavy ion exposure, although 95% of them were rejoined after gamma-irradiation. Using human lymphocytes, Durante et al. [14] investigated the kinetics of rejoining chromosomal breaks induced by high-LET radiation and gamma-rays and reported that the residual level of unrejoined breaks after prolonged incubation was higher with high-LET radiation, although little difference was observed in the yield of initial PCC breaks. These studies suggest that initial lesions induced by high-LET radiation are less reparable than those induced by gamma-rays. Track structure modelling analysis showed that reduced reparability after high-LET radiation could be attributed to more complex and clustered lesions [15,16]. In this study, chromosome aberrations were examined in roots 1 cm long. Some cells in these roots had passed through the first mitosis after exposure because micronuclei were observed in several interphase cells. Simultaneously, the mitotic index was found to be 3.8% in the control cells and to be reduced in the exposed cells. Therefore, the root samples used here were considered to contain cells that had gone through different numbers of cell cycles after exposure. However, the number of mitoses that occurred before the observations should not differ markedly in the respective radiation regimes, since only roots that were 1 cm long were collected. Thus, the chromosome aberrations observed here are considered to be at a similar stage and to reflect the repair process of the initial lesions. This implies that ion beams induce more unrejoined breaks than do gamma-rays.

Our findings suggest that ion beams induce LET-dependent bioeffects and that the repair process of initial lesions induced by ion beams is different from that induced by low-LET radiation.

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