

# Studies on Biological Effects of Ion Beams on Lethality, Molecular Nature of Mutation, Mutation Rate, and Spectrum of Mutation Phenotype for Mutation Breeding in Higher Plants

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## Ion beam/Mutation/Plant/Mutation breeding/Biological effect/LET.

Recently, heavy ions or ion beams have been used to generate new mutants or varieties, especially in higher plants. It has been found that ion beams show high relative biological effectiveness (RBE) of growth inhibition, lethality, and so on, but the characteristics of ion beams on mutation have not been clearly elucidated. To understand the effect of ion beams on mutation induction, mutation rates were investigated using visible known Arabidopsis mutant phenotypes, indicating that mutation frequencies induced by carbon ions were 20-fold higher than by electrons. In chrysanthemum and carnation, flower-color and flower-form mutants, which are hardly produced by gamma rays or X rays, were induced by ion beams. Novel mutants and their responsible genes, such as UV-B resistant, serrated petals and sepals, anthocyaninless, etc. were induced by ion beams. These results indicated that the characteristics of ion beams for mutation induction are high mutation frequency and broad mutation spectrum and therefore, efficient induction of novel mutants. On the other hand, PCR and sequencing analyses showed that half of all mutants induced by ion beams possessed large DNA alterations, while the rest had point-like mutations. Both mutations induced by ion beams had a common feature that deletion of several bases were predominantly induced. It is plausible that ion beams induce a limited amount of large and irreparable DNA damage, resulting in production of a null mutation that shows a new mutant phenotype.

## INTRODUCTION

Mutants are widely used in plant research, such as plant physiology, genetics, and plant breeding. Mutations are generated by spontaneous mutation, ultraviolet light, chemical mutagens, such as ethyl methane sulfonate (EMS), and ionizing radiation, i.e., X-rays, gamma-rays and so forth. Mutation has also been generated by biological mutagen, such as T-DNA insertions and transposon tagging, in the last 15 years. Each mutagen has particular features for mutation induction; for example, EMS predominantly induces point mutation throughout the genome, whereas T-DNA induces a knock-out phenotype by inserting a DNA fragment into the gene in question.

Since the presentation of mutation induction with X-rays

in *Drosophila* by Muller,<sup>1)</sup> and with X-rays and gamma-rays in maize and barley by Stadler<sup>2)</sup> in 1927, millions of plant mutants have been isolated by using ionizing radiation and used in many fields of plant biology. Although the mutation rate is not much higher than EMS, ionizing radiation could generate many kinds of phenotypes, because it induces DNA damage relatively randomly, and therefore induces a series of mutations, ranging from point mutation to chromosome aberration. Most Arabidopsis mutants first introduced into academic fields were treated by chemical mutagens and ionizing radiations, chiefly X-rays. In plant breeding, more than 3,000 mutant varieties of cereals, beans, flowers, etc. have also been officially registered with the FAO/IAEA organization,<sup>3)</sup> most of which were treated by ionizing radiation, chiefly gamma-rays. As different mutant phenotypes have been realized using gamma- or X-ray mutation, because of their long history of about 80 years, new chemical and physical mutagens are expected to be developed.

Ion beams, including proton, helium and the heavier charged particles, deposit high energy locally compared to gamma-rays or X-rays. The biological effect of ion beams was investigated in detail and it was found that ion beams show higher relative biological effectiveness (RBE) of

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doi:10.1269/jrr.09143

lethality and cell inactivation compared to low LET (linear energy transfer) radiation, such as gamma-rays, X-rays and electrons in the 1960s.<sup>4,5)</sup> It is suggested that ion beams predominantly induce single- or double-strand DNA breaks with damaged end groups whose reparability would be low;<sup>6)</sup> however, the precise characteristics of ion beams on mutation induction have not been clarified by comparing with those of gamma-rays and chemical mutagens. Since then, studies on mutations in plants and plant breeding with ion beams have hardly advanced. On the base of the consultative committee for advanced radiation technology in Japan, the Takasaki Ion Accelerators for Advanced Radiation Application (TIARA) was established and basic research into plant mutation by ion beams was started in around 1991.<sup>7,8)</sup> Over 18 years, the biological effects of ion beams have been greatly elucidated, and novel mutants and varieties of crops have been consistently induced by ion beams. At present, plenty of research activities utilize the ion beam irradiation in several irradiation facilities such as RIKEN RI beam factory,<sup>9)</sup> the Wakasa Wan Energy Research Center Multi-purpose Accelerator System with Synchrotron and Tandem (W-MAST),<sup>10)</sup> and the Heavy Ion Medical Accelerator in Chiba (HIMAC) of National Institute of Radiological Sciences.<sup>11)</sup>

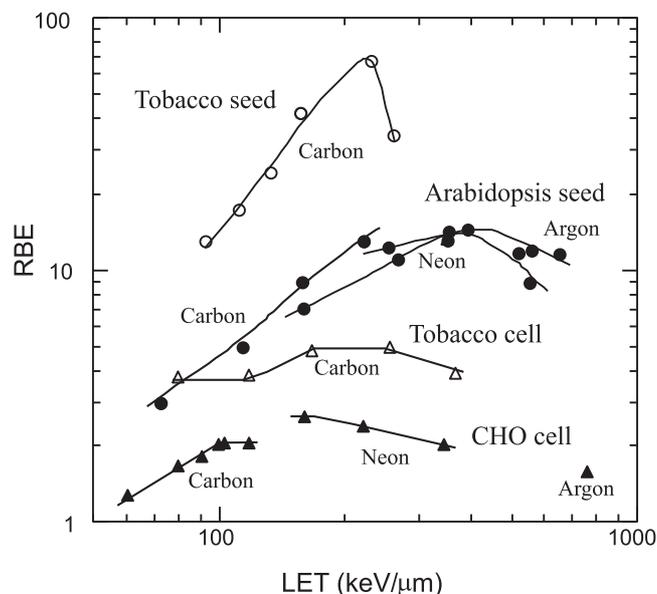
The aim of this review is to describe the effects of ion beams on plant mutation at the molecular level, mutation frequency, and spectrum of mutant phenotype. Finally, the characteristics of ion beam-induced mutation will be discussed.

## RELATIVE BIOLOGICAL EFFECTIVENESS

Several biological endpoints, such as survival, growth and chromosomal aberration, have been investigated in order to elucidate the effects of ion beams on plants. In previous studies, it was shown that RBE for growth inhibition reached a peak at around 72–174 keV/ $\mu\text{m}$ <sup>12)</sup> or 190 keV/ $\mu\text{m}$ ,<sup>13)</sup> suggesting that the LET dependence of RBE of higher plants is similar to that of mammalian cells; however, in these experiments, the Bragg peak was adjusted on the target and/or only small numbers of LET were investigated. To acquire more detailed data by using penetrating ions with various kinds of LET, the biological effects of ion beams on the survival and germination of Arabidopsis seeds were investigated. Dry seeds of ecotype Columbia and Landsberg erecta were exposed to He, C, Ar and Ne ions with LET in the range of 17–549 keV/ $\mu\text{m}$ .<sup>14)</sup> LD<sub>50</sub> of these dry seeds were ca. 1,300 Gy in the case of electrons and gamma-rays, and all the experiments for ion beam-irradiation were done within three minutes with 0–2,000 Gy. The RBE of the survival of both ecotypes showed similar results with a maximum RBE of 11–12 at 252 keV/ $\mu\text{m}$  of Ar ions. Using only carbon ions with different LET, the RBE for survival increased with increasing LET and showed the highest value at 230 keV/ $\mu\text{m}$

in the case of in tobacco seeds (cv. Tsubu 1), which showed LD<sub>50</sub> of ca. 500 Gy with gamma-rays.<sup>15)</sup> Whereas, the highest LET of the peak RBE for the lethality of Arabidopsis seeds was over 221 keV/ $\mu\text{m}$  for carbon ions and over 350 keV/ $\mu\text{m}$  for neon and argon ions.<sup>16)</sup> For the lethality of single tobacco BY-2 cells, which showed LD<sub>50</sub> of ca. 30 Gy with gamma-rays, the RBE peaked at a LET of 247 keV/ $\mu\text{m}$  by irradiation with carbon ions.<sup>17)</sup> From these results, it is almost certain that LET with a maximum of RBE for lethality is higher in plants than in mammalian systems<sup>18)</sup> (Fig. 1). It is also shown that DNA damages and some mutagenic effectiveness in bacteria and *D. melanogaster* showed LET dependence, but RBE are generally less than 2.<sup>19)</sup> Therefore, it is plausible that RBE values and LET with a maximum of RBE for lethality in plants are relatively higher among living things.

The effect on chromosomal aberration was also investigated in detail by using tobacco cv. tsubu 1. The frequencies of mitotic cells with chromosome aberrations, such as chromosome bridges, acentric fragments and lagging chromosomes, were much higher in ion beams than in gamma-rays. The highest RBE was 52.5 at 230 keV/ $\mu\text{m}$  of LET with carbon ions, at which irradiation caused 29% aberrant cells with 3 Gy.<sup>15)</sup> The relative ratios of initial chromosome aberration types, such as bridges and fragments, were not different between ion beams and gamma-rays, but chromosome aberrations induced by ion beams are likely to remain longer in a cell due to mitotic delay than by gamma-rays.<sup>20)</sup> Furthermore, the frequency of cells with chromosome aberrations did not decrease after fractionated irradiation with carbon ions, although a clear decrease was observed after exposure



**Fig. 1.** The relative biological effectiveness (RBE) of lethality as a function of linear energy transfer (LET). Data are modified from ref. 12, 13, 17 and 18.

to electrons.<sup>21)</sup> These observations indicate that the error-free repair process has not been induced or activated, and/or that DNA damage induced by ion beams is likely irreparable compared to by low LET radiation such as gamma-rays and electrons.

Recently, DNA double-strand breaks (DSBs) of tobacco protoplasts were quantified by pulsed-field gel electrophoresis.<sup>22)</sup> Initial DSB yields depended on LET and highest RBE was obtained at 124 and 241 keV/ $\mu\text{m}$  with carbon ions. It was also observed that ion beams yielded short DNA fragments of several hundred kbp more frequently than gamma-rays. These observations are consistent with the large biological endpoints caused by ion beams.

### MUTATION RATE AND FREQUENCY

The mutation rate, i.e. ratio of mutation per gene locus or cell, was first investigated on a molecular basis in plants by using the Arabidopsis visible mutation phenotypes caused by two kinds of locus groups: transparent testa (*tt*), in which the seed coat is transparent because it is pigmentless and glabrous (*gl*), having no hair on the leaves and stems.<sup>23)</sup> About 35,000 M<sub>1</sub> (Mutanized 1st generation) seeds treated with carbon ion (LET = 113 keV/ $\mu\text{m}$ , dose = 150 Gy) and their offspring were used to calculate mutation rate. The average mutation rate per dose by carbon ions was  $1.9 \times 10^{-6}$ , which is 20-fold higher than that of 2 MV electrons (LET = 0.2 keV/ $\mu\text{m}$ ) as representative low LET radiation (Table 1).<sup>24)</sup> The mutation rate, or mutation frequency, i.e. number of mutant plant per M<sub>2</sub> lines (Mutanized 2nd generation) or tissue lines, is generally calculated per dose and compared which radiation or mutagen would be better from the aspect of the Radiation Biology. But, it is also important to compare mutation rate or mutation frequency without 'per dose', from the aspect of the actual usage of mutants, or agricultural usage. Carbon ions with the LET of 113 keV/ $\mu\text{m}$  could induce four times more Arabidopsis mutants than 2 MV electrons, because carbon ions need one fifth of the dose (i.e. RBE = 5) to induce the same biological effects, such as lethality, of electrons.<sup>14)</sup> From this point of view, Data of Table 1 also indicates that carbon ions had a frequency of

$0.07 \times 10^{-3}$  mutants/locus (No. of mutants/No. of M<sub>2</sub> plants/locus). Koornneef *et al.*<sup>25)</sup> investigated mutations induced by EMS (10 mM for 24 hrs) with a frequency of about  $0.20 \times 10^{-3}$  mutants/locus. Thus, in Arabidopsis, carbon ions seemed to be about three-fold less mutagenic than EMS treatment. It is noteworthy that the high mutation rate or frequency by carbon ion irradiation was observed at a relatively low dose of 150 Gy, at which virtually all plants survive.

Mutation frequency was investigated from the point of view of phenotype. For the flower colors of chrysanthemum, the mutation frequencies of floral petals and leaves as irradiation samples induced by carbon ions (LET = 122 keV/ $\mu\text{m}$ , dose = 5–20 Gy) were ca. 16% and 7%, respectively, which are approximately half of rates induced by gamma-rays.<sup>26)</sup> The mutation frequencies of flower colors and shapes in carnation were 2.8%, 2.3% and 1.3% by carbon ions (LET = 122 keV/ $\mu\text{m}$ ), gamma-rays and X-rays, respectively.<sup>27)</sup> A high frequency (11.6%) of chlorophyll-deficient mutants, including albino, was obtained by irradiating rice with 20 Gy neon ions (LET: 64 keV/ $\mu\text{m}$ ).<sup>28)</sup> Mutation frequencies induced by ion beams were also investigated in rose for flower mutations,<sup>29)</sup> buckwheat for chlorophyll and morphological mutations,<sup>30)</sup> and hinoki cypress for chlorophyll and wax mutations.<sup>31)</sup>

It is very important to decide how the LET of ions will effect mutation induction. Recently, Kazama *et al.* found that a LET of 30 keV/ $\mu\text{m}$  (N and C ions) was the most effective for inducing Arabidopsis albino plants, although LETs of 22.5 keV/ $\mu\text{m}$  (C ions) and 61.5 keV/ $\mu\text{m}$  (C, N and Ne ions) were not so effective.<sup>9)</sup> On the other hand, Yamaguchi *et al.* investigated the survival, fertility and chlorophyll mutations of rice induced by gamma-rays and two different carbon ions (LET of 76 and 107 keV/ $\mu\text{m}$ ) and helium ions (LET: 9 keV/ $\mu\text{m}$ ).<sup>32)</sup> The mutation frequency of ion beams was higher than that of gamma-rays, especially in the case of carbon ions. It is very interesting that the mutation yield, i.e. the number of M<sub>1</sub> plants that produced chlorophyll mutants in their progeny divided by the number of sown M<sub>1</sub> seeds sown after irradiation, had a peak at around the shoulder dose of the survival curve in all radiation tested. Although it has long been accepted that LD<sub>50</sub> would be the

**Table 1.** Mutation rate induced by carbon ions and electrons.

Mutagen (dose)	No. of M1 plants	No. of M2 plants	No. of loci (mutant phenotype)	No. of mutants	Mutation rate ( $\times 10^{-6}$ ) (/locus/diploid cell/dose (Gy))
Carbon ions (150 Gy)	26,200	104,088	12 loci ( <i>tt&amp;gl</i> phenotypes)	88	1.9 (20 times)
Electrons (750 Gy)	ca.15,600	80,827	12 loci ( <i>tt&amp;gl</i> phenotypes)	18	0.097

\*Compared to the frequency of electrons

best dose for inducing mutants, the shoulder dose, which hardly affected survival, would be sufficient to efficiently obtain mutants using any radiation.

### SPECTRUM OF MUTATION PHENOTYPE

The spectrum of mutation phenotype by ion beams was first investigated for the flower color of chrysanthemum cv. Taihei by Nagatomi *et al.* (Table 2).<sup>26)</sup> Explants of leaves and petals incubated in agar medium were irradiated with 18.3 MeV/n carbon ions (LET: 122 keV/ $\mu\text{m}$ ). After irradiation, the mutation induction of the regenerated plants was investigated, comparing the results with gamma-ray irradiation. Most flower color mutants induced by gamma-rays were light pink and a few were dark pink. On the other hand, flower mutants induced by ion beams were not only light and dark pink but also orange, white, yellow, complexes of these individual colors, and stripes of white and yellow. The complex-type flower color mutants increased as the dose of ion beams rose. These specific mutants, such as complex and striped color types, have never been obtained by gamma-ray irradiation in this cultivar. The mutation spectrum of flower color and shape was also investigated in carnation by Okamura *et al.* (Table 2).<sup>27)</sup> When the cv. Vital, characterized by spray type and cherry pink flowers with frilly petals, was used as an original variety, flower color mutants, such as pink, red or striped, were obtained by gamma-rays, X-ray irradiation, or ethyl methane sulfonate (EMS), whereas the color spectrum of the mutants obtained by carbon ion (LET: 122 keV/ $\mu\text{m}$ ) was as wide as pink, light pink, salmon, red, yellow, complex and striped types. Furthermore, many different round shapes of petals were induced by carbon ion irradiation

in addition to flower colors, indicating that ion beams can induce novel flower colors and shapes with high frequency.

In Arabidopsis, more than 100,000 M<sub>2</sub> plants irradiated with carbon ions and about 81,000 M<sub>2</sub> seeds irradiated with electrons were screened for *tt* and *gl* mutants. The clear difference in the spectrum of these mutant phenotypes between carbon ions (LET: 113 keV/ $\mu\text{m}$ ) and 2 MV electrons was not observed on these loci statistically, although new mutant loci, *tt18* and *tt19* were first found by carbon ions.<sup>23,24)</sup> No distinct difference in the spectrum of visible mutants has been found in rose<sup>29)</sup> and rice.<sup>32)</sup> It is not apparent whether this difference in spectrum by ion beams is due to the type of plants, phenotypes or genes investigated. Further examples and their analyses are necessary to identify the common features of ion beams in the mutation spectrum of phenotype.

Recently, the chimerical structure of axillary buds of chrysanthemum has been investigated.<sup>33)</sup> No significant difference has been found in mutation frequency between gamma-rays and ion beams (carbon ions with LET of 122 keV/ $\mu\text{m}$ , and helium ions with LET of 9 keV/ $\mu\text{m}$ ), but all flower color mutants induced by gamma-rays were periclinal chimeras, whereas solid mutants were frequently induced by ion beams. This result suggests that not only mutation induction in a cell but also mutation selection in apical meristem should be considered to understand the mutation induction in multicellular system.

The control of mutation frequency for a specific phenotype was studied by using carbon ions (LET: 76 keV/ $\mu\text{m}$ ). Among stresses, such as UV light, cold temperature and high sucrose content, pre-treatment of petunia seedlings with

**Table 2.** Mutation spectrum of flower color.

Chrysanthemum: Original variety 'Taihei' with pink petals was used. <sup>26)</sup>										
Mutagen	Mutation frequency (%)									
	White	Light pink	Dark pink	Orange	Yellow	Complex/Stripe				
Not irradiated	0	0.3	0	0	0	0				
Gamma-rays	0	27.7	2.1	0	0	0				
Carbon ions	0.3	4.6	0.3	0.3	0.2	10.2				

Carnation: Original variety 'Vital' with cherry color and serrated petals was used. <sup>27)</sup>										
Mutagen	Mutation frequency ( $\times 10^{-1}\%$ )									
	Light pink	Pink	Dark pink	Red	Salmon	Cream	Yellow	Minute striped	Complex	Stripe
EMS	0	5.2	0	1.0	0	0	0	0	0	3.1
Soft X-rays	1.7	8.4	0	3.4	0	0	0	0	0	0
Gamma-rays	1.7	2.6	0	1.7	0	0	0	11.3	0	0
Carbon ions	2.4	4.7	2.4	3.5	2.4	1.2	2.4	0	2.4	3.5

3% sucrose caused more than two times higher mutation frequency for flower color mutation but not for chlorophyll mutation.<sup>34</sup> This report suggests that sucrose pretreatment could regulate the spectrum of mutation phenotype of flower color following ion beam irradiation.

## MOLECULAR NATURE OF MUTATION

Although few studies have been carried out on the molecular analysis of mutation with gamma-rays, X-rays or neutrons,<sup>35,36</sup> systematic analysis have never been carried out in mutation induced by any ionizing radiation at molecular level in higher plants. To understand the nature of mutation induced by ion beams, DNA alterations were investigated using *Arabidopsis tt* and *gl* mutants induced by carbon ions (LET: 113 keV/ $\mu$ m) (Table 3).<sup>23,24,37,38</sup> It has been revealed that of the 29 carbon-ion-induced alleles, 14 had intragenic point-like mutations and 15 had intergenic rearrangements such as deletions, inversions, translocations and insertions. In the case of electrons as representatives of low LET radiation, nine alleles had point-like mutations and three had rearrangements. For the 14 point-like mutations induced by carbon ions, 11 mutations were 1–100 bp deletions, one was a 1 bp insertion, and two had base substitutions. For the nine point-like mutations induced by electrons, four were 1–8 bp deletions, four were base substitution, and one had a 1 bp insertion. For the 15 rearrangements induced by carbon ions, 14 were analyzed successfully on a molecular basis. Carbon-ion-induced rearrangements consisted of deletions, inversions, translocations, insertions, and their combination. Six deletions were in the range of 5 kbp to 230 kbp. Electron-induced rearrangements consisted of inversions and translocations. The difference in mutations between carbon ions

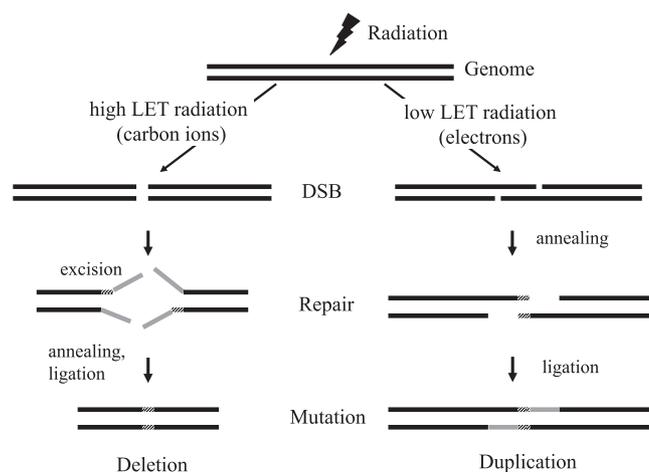
and electrons is found in the breakpoints of these rearrangements. Of the 17 carbon-ion-induced breakpoints, 11 had 1–29 bp deletions and four had duplications of 1–5 bp (Remaining 2 breakpoints were hard to be sequenced). On the other hand, of the eight electron-induced breakpoints, six had duplications of 1–7 bp and one had 1 bp deletion. Most of these break and rejoined sites for rearrangements have microhomologies, suggesting that non-homologous end-joining (NHEJ) plays an important role in the rejoining of double-strand breaks or clustered damage induced by carbon ions.<sup>39,40</sup> Thus, rearrangements preferably induced by carbon ions have different molecular types of rearrangements induced by electrons as low LET radiation (Fig. 2). Recent analysis using a transgenic *Arabidopsis* line also indicated that the NHEJ pathway is involved in the rejoining of DNA damage caused by ion beams.<sup>41</sup>

The pollen-irradiation method was attempted to understand transmissible and nontransmissible mutation induced by radiation.<sup>42</sup> Most mutants carried very large deletions (> 80 kbp) at various irradiation doses (40–600 Gy) with both gamma-rays and carbon ions (LET: 113 keV/ $\mu$ m), and the majority of mutants were not transmitted normally. Even transmissible mutants had large deletions, which are thought to be generated by NHEJ.

On the other hand, mutation in vegetative tissues of ion-beam-irradiated plants has been investigated using an *Arabidopsis* transgenic line.<sup>43</sup> Carbon ions with LET of 113 keV/ $\mu$ m had a greater tendency to induce more than 2 bp deletions/insertion, but 1 or 2 bp deletions (frameshift) was higher only in the gamma-ray-irradiated group. It was interesting that G:C to T:A and A:T to C:G transversions, which are frequently induced by oxidative DNA damage in bacterial and animal cells, were low in plant seeds irradiated with both gamma-rays and carbon ions.

**Table 3.** Classification of mutations induced by carbon ions and electrons (modified from ref. 23 and 24). The distributions of the indicated mutation patterns were determined based on sequence analysis with 29 and 12 mutant alleles produced by carbon ions and electrons, respectively.

<i>tt, gl</i> loci	Carbon ions (113 keV/ $\mu$ m)		Electrons (0.2 keV/ $\mu$ m)	
	Intragenic mutation	Large DNA alteration	Intragenic mutation	Large DNA alteration
<b>Mutation</b>	48%	52%	75%	25%
	(as 100%)		(as 100%)	
Deletion	79%		44%	
Base substitution	14%		44%	
Insertion	7%		11%	
<b>Breakpoint</b>		(as 100%)		(as 100%)
Deletion		65%		13%
Duplication		24%		75%



**Fig. 2.** A model of dsb rejoining after irradiation with high and low LET radiation.

## CHARACTERISTICS OF NOVEL MUTANTS AND THEIR MUTATIONS

Until now, many mutants have been isolated by using ion beams in higher plants. Here we will introduce these characteristics of mutations induced by ion beams.

### *Model plants*

*Arabidopsis* UV-B-resistant mutants were first isolated in higher plants by irradiation with carbon ions with LET of 113 keV/μm.<sup>44)</sup> Four UV-B-resistant mutants (*uvi1-4*) that show 1.5–2-fold growth under increased UV-B radiation compared to the wild type, have been isolated in 5,100 M<sub>2</sub> families derived from 1,280 M<sub>1</sub> seeds (Fig. 3). The *uvi4* gene, which stimulated endoreduplication, i.e. duplication of the genome without cell division, and therefore became

resistant to UV-B, has been isolated and found to possess 123 bp deletion.<sup>45)</sup> One of six UV-B-sensitive mutants induced by carbon ions, *rev3-1*, was first found to have loss of function in the translesion synthesis mechanism in plants.<sup>46)</sup> Chromosome 1 of *rev3-1* was broken at a minimum of three points, causing chromosome inversion and translocation. Another UV-B-sensitive mutant, *svv2-1*, which is defective in cell-cycle arrest in response to DNA damage, has chromosome rearrangement with an insertion.<sup>47)</sup> On the other hand, new anthocyanin-accumulated or -defective mutants were isolated through the mutation frequency analysis described above. One mutant, *ast*, which has spotted pigmentation on the seed coat, has been isolated from 11,960 M<sub>2</sub> plants derived from 1,488 self-pollinated M<sub>1</sub> (Fig. 3).<sup>48)</sup> The *ast* has 49 bp deletion in the *Banyuls* gene that encodes anthocyanidin reductase,<sup>49)</sup> whereas two new loci of the anthocyanin-defective phenotype have been found. Both



**Fig. 3.** Novel mutants and varieties induced by ion beams. From top left to right: 1 month old plants of *Arabidopsis* wild type (upper rank) and UV-B resistant mutants (lower rank) under high UV-B condition;<sup>44)</sup> *Arabidopsis* mutant, *ast* (lower rank), which accumulates anthocyanin in immature seeds;<sup>48)</sup> *Arabidopsis* frilled flower mutant, *frl1*;<sup>50)</sup> From middle left to right: New chrysanthemum complex-color variety, “Ion-no-Seiko”;<sup>26)</sup> New rose-flower type carnation variety;<sup>27)</sup> New cyclamen flower with delphinidin that is the novel pigment in the genus of cyclamen.<sup>61)</sup> From bottom left to right: New chrysanthemum variety ‘Aladdin’, which has reduced axillary flower buds;<sup>56)</sup> New variety ‘KNOX’ of *Ficus thumbergii*, which has a high capability for the uptake and assimilation of atmospheric nitrogen dioxide;<sup>71)</sup> New cultivar candidate of rice ‘Akinouta’ that is grown well even in low fertilizer.<sup>74)</sup>

genes, *TT18* and *TT19*, were found to encode proteins related to the anthocyanin biosynthetic pathway. Overall, four mutants were isolated from 104,088 M<sub>2</sub> plants derived from 26,200 self-pollinated M<sub>1</sub> mutanized with carbon ions. Two *tt18* mutants had 2 bases and 5 bases deletion in their *tt18* coding regions, respectively.<sup>23)</sup> As for two *tt19* mutants, a large inversion (ca. 1000 kb) was found in *tt19-1*, whereas translocation to the upstream region was found in *tt19-2*.<sup>49)</sup> A novel Arabidopsis flower mutant, *fr11*, which has serrated petals and sepals, was obtained from 1488 M<sub>1</sub> seeds exposed to carbon ions (Fig. 3).<sup>50)</sup> The *fr11* with one base deletion in the *FRL1* gene, which encoded sterol methyltransferase 2, caused ectopic endoreduplication in petal tips.<sup>51)</sup> A novel auxin mutant, *aar1-1*, was obtained from approximately 30,000 M<sub>2</sub> seedlings derived from ca. 6,700 irradiated M<sub>1</sub> seeds.<sup>52)</sup> The *aar1-1* mutation, which is specifically resistant to phytohormone 2,4-D, had a 43,895 bp deletion on chromosome 4.

*Lotus japonicus* is used as a leguminous model plant. A novel hypernodulation mutant, named *klavier* (*klv*), was isolated by irradiation with helium ions with LET of 19 keV/ $\mu\text{m}$ .<sup>53)</sup> The *klv* had several characteristics, such as being densely covered with small nodules, a wider nodulation zone, aberrant leaf vein formation, delayed flowering, and dwarfism, all the phenotypes of which would be caused by a single recessive mutation. Another novel mutant, named *too much love* (*tml*), which produced many more nodules, was obtained from approximately 35,000 M<sub>2</sub> seedlings derived from ca. 3,300 carbon ion-irradiated M<sub>1</sub> seeds.<sup>54)</sup>

### Ornamental flowers

As described above, complex and striped flower colors have been obtained in chrysanthemum (Fig. 3). A higher mutation frequency of complex flower color mutants was derived from floral petal irradiation than from leaf irradiation.<sup>26)</sup> Morphological mutant phenotypes are well observed in chrysanthemum,<sup>55)</sup> and one of these mutations, a few axillary flower bud mutants, was first induced by carbon ions.<sup>56)</sup> These mutant phenotypes have been selected as only one characteristic changed without detrimental characteristics, such as malformed leaves and decreasing DNA contents in a cell by reducing the irradiation dose as little as possible. This indicates that ion beams can induce a mutant variety with a one-point changed characteristic, which is called 'one-point breeding', which was previously very difficult by mutagenesis.<sup>56,57)</sup> In carnation, many flower colors, such as pink, light pink, salmon, red, yellow, complex and striped types, and various round petals were induced from carnation variety Vital (Fig. 3).<sup>27)</sup> Petunia also showed flower color mutation varieties by ion beams.<sup>58)</sup> In rose, a more intense flower color than the parent cultivar (red or orange) or mutants in the number of petals, flower size and shape were obtained, but marked changes, such as yellow or white, have not been obtained by ion beams.<sup>29)</sup> In the analysis of Torenia,

flower color mutants induced by 135 MeV nitrogen or neon ions were divided into two groups: one group that changed from blue to pink involved the deletion of the color gene, but the other group with decreased or highly increased pigment content was related to pigment production gene expression.<sup>59)</sup> In cyclamen, ion beam irradiation of the tuber was found to be more useful for changing flower characteristics than irradiation of other areas, such as the callus, somatic embryo, and so on.<sup>60)</sup> New anthocyanin pigment, delphinidin, was created in the genus of cyclamen (Fig. 3).<sup>61)</sup> Sterile mutants of verbena were also isolated at high frequency without alterations in other important traits by nitrogen ions (LET: 31 keV/ $\mu\text{m}$ ).<sup>62)</sup>

### Crops

In crops, an important objective in breeding is to establish disease-resistant lines. For this purpose, ion beams were used to induce resistant mutants against the main diseases of the respective crops. Rice mutants resistant to bacterial leaf blight or blast disease were firstly investigated in the world by comparing with gamma-rays, thermal neutrons and ion beams.<sup>63)</sup> The highest mutation frequency was found in helium ions (LET: 19 keV/ $\mu\text{m}$ ) compared to gamma-rays or thermal neutrons. Potato virus Y-resistant mutants have been selected by exposure to the anthers of tobacco.<sup>64)</sup> A high frequency (2.9–3.9%) of resistant mutants was obtained by irradiation with carbon (LET: 111 keV/ $\mu\text{m}$ ) and helium ions (LET: 15 keV/ $\mu\text{m}$ ) at a dose of 5–10 Gy. In banana, plants resistant to black Sigatoka were selected from carbon-ion irradiated *in vitro* plantlets.<sup>65)</sup>

In addition to disease resistance, chlorophyll mutants were frequently observed for these crops. An albino mutant was isolated from an M<sub>2</sub> population of tobacco plants which had been irradiated with nitrogen ions (LET: 28.5 keV/ $\mu\text{m}$ ) at an early stage of embryonic development.<sup>66)</sup> The frequency of chlorophyll mutations and their types were examined in rice.<sup>28,32)</sup> The variegated yellow leaf mutant of rice, which was induced by activation of the endogenous transposable element, was observed by carbon ion irradiation (LET: 122 keV/ $\mu\text{m}$ ).<sup>67)</sup>

### Trees

Ion beams have been used for breeding trees. For the purpose of establishing agrochemical-sensitive/resistant varieties in forest trees, Hinoki cypress shoot primordia were irradiated with helium (LET: 19 keV/ $\mu\text{m}$ ) or carbon ions (LET: 122 keV/ $\mu\text{m}$ ). Wax mutants and chlorophyll mutants, such as Xanta and Albino, were obtained.<sup>31)</sup> Shoot explants of *Ficus thunbergii* were irradiated with several ion beams for the purpose of increasing the capability of plants to assimilate atmospheric nitrogen dioxide, and inheritable variegated plants have been induced.<sup>68)</sup> It is interesting that these recessive-like mutants of both trees are induced in the M<sub>1</sub> generation from tissue culture, and are genetically stable.

## ION BEAM BREEDING

As described above, many mutants have been induced by ion beams. Some of them can be used for commercial use. Furthermore, there are some flowers and crops, which were created as practical use but not published in international journals like aforesaid. Here we will describe together with these new varieties commercialized or nearly put to practical use.

New varieties of verbena,<sup>62)</sup> carnation<sup>27)</sup> and chrysanthemum<sup>69)</sup> were commercialized as the first ion-beam-induced varieties in 2002. Subsequently, many varieties with new flower color and shape, etc. have been created in Petunia, Chrysanthemum, *Trenia*, Pink, *Osteospermum* and so on. In order to accelerate these research and development, Ion Beam Breeding Society (IBBS) was inaugurated in 2004.<sup>70)</sup> The most commercially successful flower is the 'Aladdin' and 'Aladdin 2' of chrysanthemum with a few axillary flower bud (Fig. 3).<sup>56,57)</sup> At present, more than 30 million cut flowers have been sold annually. In 2009, cultivar 'KNOX' of *Ficus thunbergii* that has a high capability to assimilate atmospheric nitrogen dioxide was firstly put to practical use as a new variety of tree by carbon ions (LET = 122 MeV/mm)

(Fig. 3).<sup>71)</sup> Recently, 'Nishina Otome' a new breed of cherry blossom tree that blooms in all four seasons has been created by carbon ions (LET = 22.5 MeV/mm).<sup>72)</sup> In crops, there have not been put to practical use, but salt-tolerant mutant varieties of rice was first isolated in 2006 by carbon ions (LET = 22.6 MeV/mm) with the dose of 20–40 Gy.<sup>73)</sup> New candidate varieties of rice that could be well grown even in low nitrogen fertilizer were generated for the purpose of solution to eutrophication by carbon ions (LET = 86 MeV/mm) in 2008 (Fig. 3).<sup>74)</sup> Nowadays, so many kinds of flowers, vegetables like spinach, fruits like mask melon, citrus fruits tree, coniferous tree, etc. have been exposed to ion beams to produce new varieties.

## SUMMARY AND PERSPECTIVE: CHARACTERISTICS OF ION BEAM-INDUCED MUTATION

From the number of investigations indicated above, it is plausible that the characteristics of ion beams for mutation induction induce mutants with high frequency, show a broad spectrum of mutation phenotype, and therefore produce novel mutants. Another characteristic of ion beams for mutation induction would be to induce a minimum number of mutations compared to other mutagens. As the main reason to

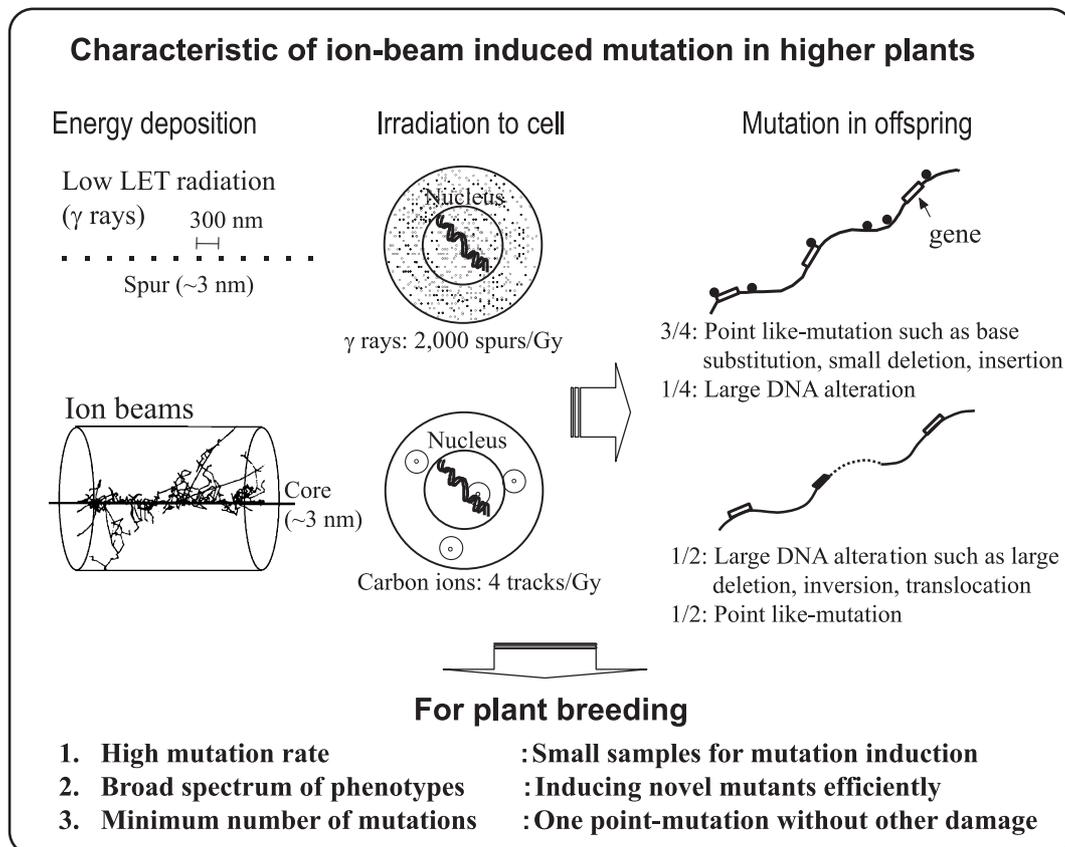


Fig. 4. Characteristics of ion-beam induced mutation in higher plants.

explain these characteristics of ion beams, we hypothesize that chemical mutagens, such as EMS, and low LET ionizing radiation, such as gamma-rays and electrons, will predominantly induce many but small modifications or DNA damage to DNA strands, resulting in several point-like mutations on the genome. In contrast, ion beams as high LET ionizing radiation will efficiently cause not so many but large and irreparable DNA damage locally, resulting in a limited number of null mutations (Fig. 4). Therefore, novel mutants were efficiently obtained, and most showed distinct characteristics without detrimental characteristics, and mendelian traits without backcrossing. This inference for the characteristics of ion beams should be confirmed in the future by numerous experiments at the molecular level with both low and high LET radiation.

Although ion beams have become a new mutagen, several points of the characteristics of ion beam-induced mutation remain unknown and to be resolved.  $M_1$  plants often show a recessive-like phenotype, such as albino or chlorophyll deficiency. These phenomena were seldom observed in  $M_1$  plants grown from ion-beam-irradiated seeds but  $M_1$  plants were generated from irradiated shoot primordia, tissue culture, or cultured cells that seemed to have homozygous dominant gene sets of diploid cells. It is well known that somatic crossing over or mitotic recombination is generally induced by ionizing radiation.<sup>75,76</sup> Molecular analysis will be necessary to elucidate whether the recessive-like phenotype is caused by mitotic recombination enhanced by ion beams or by other factors, such as epigenetic modifications. Secondly, the present characterization of ion-beam induced mutation was acquired from very limited ion types with specific energy and LET. Although certain tendencies of ion beams for biological effects on plants have been identified, no common mutation features by ion beams have been elucidated. In several experiments, small or large deletion has frequently occurred by ion beam irradiation. It is therefore necessary to elucidate whether the size and frequency of DNA alteration, such as deletion, might change depending on the kind of ion and energy. Lastly, as a general question regarding mutation, it is still unknown whether directed or adaptive mutation exist.<sup>77</sup> Nagatomi *et al.* showed that the mutation rates of flower color of plants regenerated from petals were higher than from leaves with both ion beams and gamma-rays.<sup>26</sup> This is considered to be because the genes for flower color in petal cells are more active than in leaf cells, which this may lead to a higher mutation rate of regenerated plants from petals.<sup>69</sup> Recently, the increase of flower color mutation was shown by combining ion beams and high sucrose content as pre-treatment, suggesting the possibility of directed mutation.<sup>34</sup> If directed mutation could be controlled in higher plants, combination of the best ion type for energy and the best plant organ or tissue will enable the induction of objective mutation.

Unlike mammalian or bacteria systems, it is difficult in

the plant kingdom to apply a gene-knockout system; therefore, ion beams as a new mutagen will be indispensable for the next step of plant biology and breeding to make thousands of novel mutants that inform the responsive gene function or are directly applied for practical use.

## ACKNOWLEDGEMENTS

We respectfully thank the technical staff who established the ion beam irradiation systems and thank all the researchers who undertook plant mutagenesis and breeding using ion beams. We greatly appreciate our pioneers, Drs. Watanabe H., Nakai H., Inoue M, Nagatomi S., Yoshida S., and Dr. Tano S., who passed away in July 2006, because ion beam-induced mutation in higher plants could not have been achieved without them.

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Received on December 1, 2009

Revision received on February 19, 2010

Accepted on March 12, 2010