Part I
Induced Mutations

The Handbook of Plant Mutation Screening. Edited by Günter Kahl and Khalid Meksem

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Physically Induced Mutation: Ion Beam Mutagenesis

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Abstract

Ion beams are novel physical mutagens that have been applied to a wide variety of plant species. Unlike other physical mutagens such as X-rays, γ-rays, and electrons, ion beams have high linear energy transfer, leading to high double-strand break yields and the resulting strong mutational effects. Takasaki Ion Accelerators for Advanced Radiation Application (TIARA) in Japan was established as the first ion beam irradiation facility for biological use. In this facility, positively charged ions are accelerated at a high speed and used to irradiate living materials, including plant seeds and tissue cultures. By utilizing this approach, several novel mutants have been successfully isolated even from *Arabidopsis*, in which thousands of mutants have already been obtained using different mutagens. This demonstrates that ion beams are a powerful alternative mutagen with a mutation spectrum different from other chemical, physical, and T-DNA-based mutagens. The application of such an alternative mutagen is of great importance not only to analyze any gene functions through novel mutant isolation, but also to improve global food situations by providing new crop varieties with beneficial traits. In this chapter, we describe the detailed methods of ion beam irradiation and discuss its applications in genetic research as well as plant breeding.

1.1
Introduction

Mutagenesis is one of the most critical steps for genetic studies as well as selective breeding. Successful mutant isolation largely relies on the use of efficient mutagens. In plant research, a chemical mutagen, ethylmethane sulfonate (EMS), has been commonly used for this purpose. Although this mutagen can be handled easily and applied to any plant, it primarily produces single base substitutions, but not drastic mutations such as large genomic deletions. Therefore, application of more powerful mutagens with different mutation spectra is of great significance in some cases. One good technology for this end is ion beam mutagenesis. The ion beam is a physical mutagen

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that has just recently come into use for plants. In this type of mutagenesis, positively charged ions are accelerated at a high speed (around 20–80% of the speed of light) and used to irradiate target cells. As a physical mutagen, ion beams are similar to other forms of radiation such as X-rays, γ-rays, and electrons, but it is different from them in that ion beams have much higher linear energy transfer (LET). This characteristic is important to understand the high biological effectiveness of ion beams.

1.1.1 LET

LET is the energy deposited to target material when an ionizing particle passes through it. Once an accelerated particle encounters any substance, it gradually loses its own energy (i.e., the same amount of energy is transferred to the substance causing "damage") and eventually stops at the point where the maximum energy loss is observed (Figure 1.1). LET is usually expressed in kiloelectronvolts per micrometer

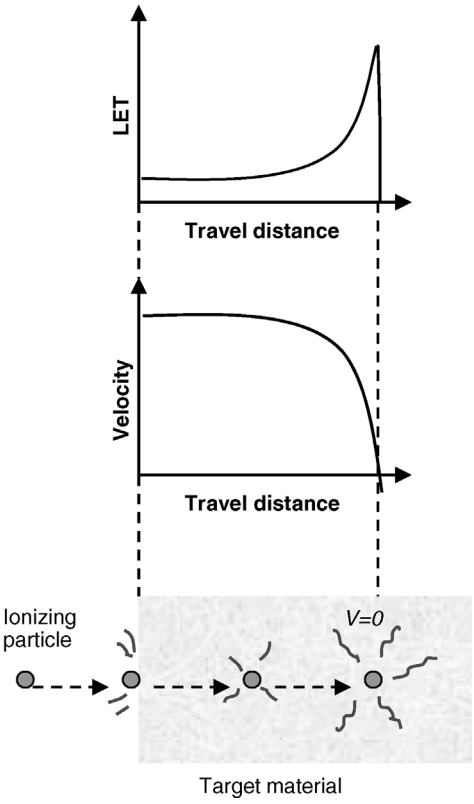

Figure 1.1 Conceptual diagram of LET. An ionizing particle gradually loses its own energy as it slows down in the target material. LET refers to this energy loss, which is deposited to the material. In this cartoon, LET is represented by wavy lines. LET reaches its maximum just before the ionizing particle stops. Immediately after this peak, LET plunges to zero.

(keV/μm), which represents the average amount of energy lost per unit distance. Ion beams have a relatively high LET (around 10–1000 keV/μm or higher), while X-rays, γ-rays, and electrons have low LETs (around 0.2 keV/μm). Therefore, ion beams are able to cause more severe damage to living cells than other forms radiation, resulting in the high relative biological effectiveness [1, 2].

1.1.2 Mutational Effects of Ion Beams on Plants

Biological effects of ion beams have been investigated not only in mammals, but also in plants. For example, studies using *Arabidopsis thaliana* and *Nicotiana tabacum* showed that ion beams were more efficient in decreasing the germination rate and the survival rate than low-LET radiation [3, 4]. More importantly, analysis focusing on *transparent testa* (*tt*) and *glabrous* (*gl*) loci revealed that 113-keV/μm carbon ions induced a 20-fold higher mutation rate per dose than 0.2-keV/μm electrons, thus demonstrating the power of ion beams as a mutagen [5, 6]. The detailed characterization of the carbon ion-induced mutations showed that ion beams can cause large DNA alterations (large deletions, inversions, and translocations) as well as small intragenic mutations and that ion beams frequently, but not always, produce deletions with variable sizes from 1 bp up to 230 kbp, compared to electrons (summarized in Table 1.1) [6]. Since such deletions possibly lead to frameshifts or total gene losses, mutants derived from ion beam mutagenesis can be considered as nulls in many cases. This is a significant difference from the conventional chemical mutagen EMS, which mostly generates point mutations resulting from GC → AT transitions.

These great mutational effects of ion beams are partly due to high double-strand break (DSB) yields induced by ions. The study using tobacco BY-2 protoplasts as a model system showed that initial DSB yields were positively correlated with LET, and that high-LET helium, carbon, and neon ions were more effective in causing DSBs

Table 1.1 Classification of mutations induced by carbon ions and electrons (modified from [6]).

Mutagen (LET)	Intragenic mutation		Large DNA alteration	
Carbon ions (113 keV/μm)	48%		52%	
	deletion	38%	inversion/translocation	21%
	base substitution	7%	total deletion	31%
	insertion	3%		
Electrons (0.2 keV/μm)	75%		25%	
	deletion	33%	inversion/translocation	25%
	base substitution	33%	total deletion	0%
	insertion	8%		

The distributions of the indicated mutation patterns were determined based on the sequence analysis with 29 and 12 mutant alleles produced by carbon ions and electrons, respectively [6]. Note that carbon ions induced large DNA alteration in the tested loci more frequently than electrons. Such large DNA alterations include total deletion, which refers to a complete loss of a gene locus.

than γ-rays [7]. Further, it was found that at least carbon and neon ions produced short DNA fragments more frequently than γ-rays, suggesting that ion particles can act densely and locally on target genomes [7].

It is plausible that DSBs are more difficult for cells to repair than single-strand breaks (i.e., DSB repair can be error-prone), which might partly explain the high mutation rates caused by ion beams. However, the molecular mechanism of ion-mediated mutation induction remains largely unknown. To address this issue, Shikazono *et al.* analyzed the DNA sequences flanking the breakpoints generated by carbon ions and showed that many of the tested sequences contained deletions (1–29 bp), whereas most of the electron-induced breakpoints were flanked by duplications (1–7 bp) [6]. Based on these findings, they hypothesize that unlike electrons, high-LET ions could induce not only DSBs, but also cause severe damage in the broken ends and that such damaged sequences might be eventually excised during the repair processes, resulting in deletion mutations (Figure 1.2) [6].

Although further analysis is necessary to elucidate its precise mode of action, ion beam mutagenesis appears to be a good alternative that can accomplish high mutational effects and a mutation spectrum presumably different from other mutagens such as EMS and low-LET radiation. To date, ion beam mutagenesis has

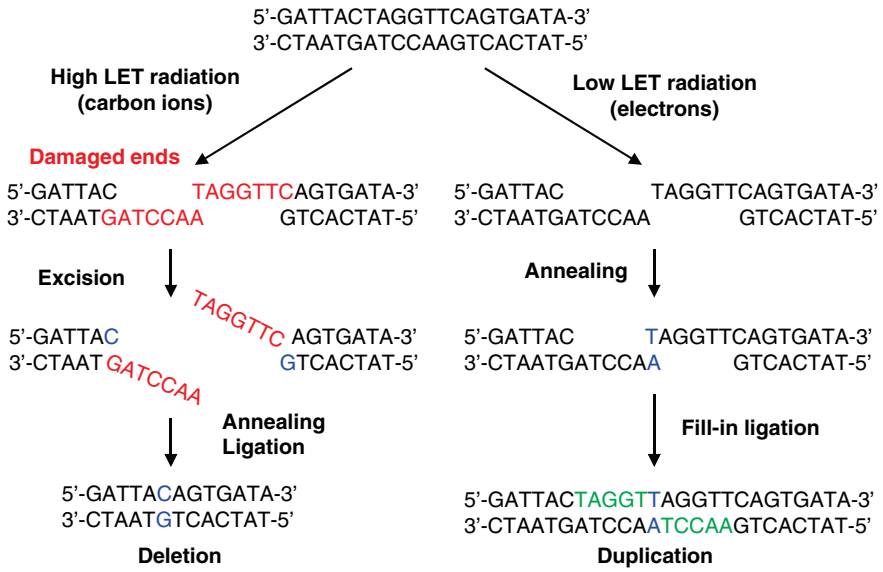

Figure 1.2 Model of mechanisms by which high-LET and low-LET radiation induce mutations (originally proposed by N. Shikazono). High-LET radiation such as carbon ions produce damaged ends of DSBs, which are excised before annealing and ligation of the broken fragments. On the other hand, low-LET radiation such as electrons cause intact ends, which are repaired without any removal of the end sequences. This difference in DSB repair leads to deletions and duplications generated by high-LET and low-LET radiation, respectively. Red letters: bases to be excised; blue letters: bases used for religation; green letters: bases filled in during DSB repair.

been applied to a wide variety of plant species, including *Arabidopsis thaliana*, *Lotus japonicus*, carnations, chrysanthemums, and so on. It is noteworthy that this approach has been successful in the isolation of novel mutants, making a great contribution to plant genetics and breeding (see Section 1.3).

1.2 Methods and Protocols

Currently, there are four facilities available for plant ion beam mutagenesis: Takasaki Ion Accelerators for Advanced Radiation Application (TIARA) of the Japan Atomic Energy Agency (JAEA), RIKEN RI Beam Factory (RIBF), the Wakasa Wan Energy Research Center Multi-purpose Accelerator with Synchrotron and Tandem (W-MAST), and the Heavy Ion Medical Accelerator in Chiba (HIMAC) of National Institute of Radiological Sciences (NIRS). Table 1.2 shows the physical properties of

Table 1.2 Ion beam irradiation facilities and the physical properties of the radiations [modified from the list in The Ion Beam Breeding Society web site (http://wwwsoc.nii.ac.jp/ibbs/)].

Facility	Radiation	Energy (MeV/u)	LET (keV/μm)	Range (mm)
TIARA, JAEA (http://www.taka.jaea.go.jp/index_e.html)	He	12.5	19	1.6
	He	25.0	9	6.2
	C	18.3	122	1.1
	C	26.7	86	2.2
	Ne	17.5	441	0.6
RIBF, RIKEN Nishina Center (http://www.rarf.riken.go.jp/Eng/index.html)	C	135	23	43
	N	135	31	37
	Ne	135	62	26
	Ar	95	280	9
	Fe	90	624	6
W-MAST, The Wakasa Wan Energy Research Center (http://www.werc.or.jp/english/index.htm)	H	200	0.5	256
	C	41.7	52	5.3
HIMAC, National Institute of Radiological Sciences (http://www.nirs.go.jp/ENG/index.html)	C	290	13	163
	Ne	400	30	165
	Si	490	54	163
	Ar	500	89	145
	Fe	500	185	97

Listed are representative ion radiations that have been used in each facility. The energy, LET, and effective range for each ion species are shown.

the ion beams frequently used in these facilities. Here, we describe the protocol of ion beam irradiation in TIARA, which was originally described elsewhere [3, 8].

1.2.1 Ion Beam Irradiation

In general, a variety of ion species, from protons to uranium ions, can be utilized for ion beam applications. In the case of carbon ions, they are produced by an electron cyclotron resonance ion source and accelerated by an azimuthally varying field (AVF) cyclotron to obtain 18.3 MeV/u $^{12}C^{5+}$ ions. At the target surface, the energy of the carbon ions slightly decreases to 17.4 MeV/u, resulting in the estimated 122 keV/μm mean LET in the target material (0.25 mm thick) as water equivalent. In this case, the effective range of the carbon ions is about 1.1 mm. These physical properties can be predicted by the ELOSSM code program [8]. ELOSSM requires the elemental composition and density of the specified substance to determine the potential LET of ion beams. As shown in Figure 1.3, ion beams scan a field of more than $60 \times 60\,mm^2$ in a vacuum chamber and exit it through a 30-μm titanium foil in the beam window. The samples to be irradiated are placed in the air at a distance of 10 cm below the beam window. In the case of *Arabidopsis* or tobacco seeds, for example, 100–3000 seeds are sandwiched between two Kapton films (7.5 μm in thickness; Toray-Dupont) to make a monolayer of seeds for homogeneous irradiation. As for rice or barley seeds, the embryo sides should be kept facing toward the beam window. On the other hand, when calli or explants cultured in a Petri dish need to be irradiated, the lid of the Petri dish should be replaced by a Kapton film cover to

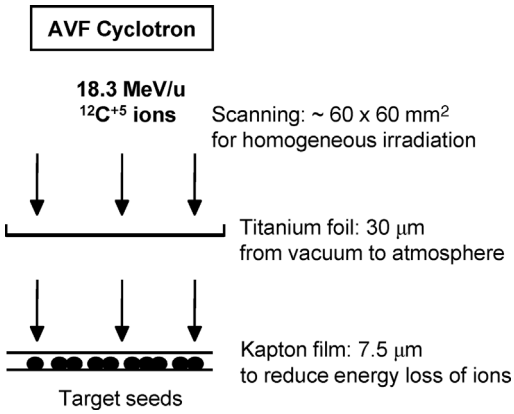

Figure 1.3 Schematic diagram of ion beam irradiation. Ion beams such as carbon ions accelerated by the AVF cyclotron first scan the irradiation field (greater than $60 \times 60\,mm^2$) in a vacuum chamber. Then, the accelerated ion beams exit through a titanium foil into the atmospheric conditions. Finally, the ion particles attack thinly prepared target samples. Here, plant seeds kept between two Kapton films are shown as an example of target biological materials.

minimize the energy loss of ion beams. The target samples are irradiated for less than 3 min for any dose.

1.2.2
Dose Determination for Ion Beam Irradiation

Determining an optimal irradiation dose of ion beams is the most important and laborious step before irradiating your samples. In principal, the ideal irradiation dose would be a dose at which ion beams show the highest mutation rate at any loci of interest; therefore, you might want to figure out your own favorite irradiation doses by testing different doses at a time and screening all of the resulting samples for your desired mutants. However, such an approach is not practical because plenty of time and effort need to be taken. Alternatively, survival rate, growth rate, chlorophyll mutation, and so on, can be the good indicators to determine appropriate doses for mutation induction.

Figure 1.4 shows the survival curves of *Arabidopsis* dry seeds against several ion beams in comparison with low-LET electrons. The effect of ion beams on the survival rate is higher than that of electrons, but it varies by energy and species of ions. Until now, 18.3 MeV/u carbon ions have been widely used, leading to high mutation rates and efficient novel mutant isolations. However, it has not been fully understood which kind of ions with how high energy would be the most effective for mutation induction. Supposedly, the optimal ion radiation might depend on plant species and materials as well as genome size, ploidy, water content, and also what kind of mutation a researcher wants to produce. Based on several results up to date, it has been suggested that the effectiveness of ion beams as a mutagen might not be

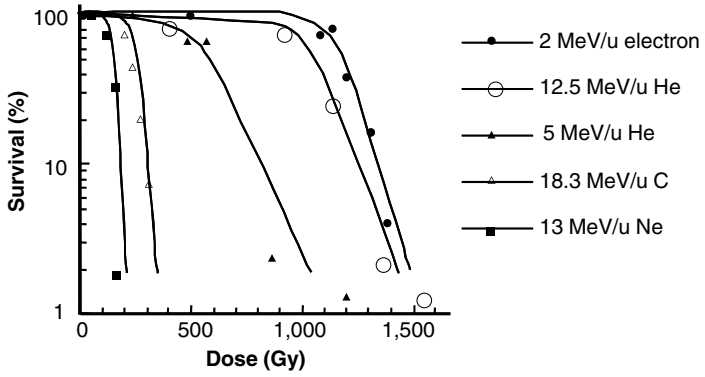

Figure 1.4 Survival curves of *Arabidopsis* dry seeds after irradiation of ion beams (modified from [3]). Dry seeds of the Columbia ecotype of *Arabidopsis* were irradiated with different kinds of ion beams as well as electrons for a low-LET radiation control. Survival responses are shown as a function of irradiation dose. A dose at the shoulder end of each survival curve (e.g., 200 Gy for carbon ions) or less than this dose is supposed to be the most efficient for mutation induction.

determined by the species of ions, but mostly by the LET of ions. So far, ion beams with LET of around 10–500 keV/u appear to be suitable.

As for doses, the median lethal dose (i.e., LD_{50}) has been thought to be the best dose for mutation induction using X-ray or γ-ray irradiation. Recent studies have shown that the dose at the shoulder end of the survival curves (200 and 1000 Gy for carbon ions and electrons, respectively, in Figure 1.4) or less than these doses is more efficient for ion beams as well as low-LET radiation (unpublished data). In fact, we are currently using 150 Gy with 18.3 MeV/u carbon ions for *Arabidopsis* dry seeds. In the case of plantlets, we usually irradiate ion beams at such doses that show 100–80% growth rate (around the shoulder end of the growth curve). Also, when tissue culture is concerned, we favor doses that lead to more than around 80% regeneration or growth rate of calli compared to unirradiated controls.

1.2.3
Plant Radiation Sensitivity

In order to determine irradiation doses, it is very useful to understand general radiation sensitivities of plants against radiation. Radiation sensitivities of plants differ greatly among not only plant species, but also plant materials (seeds, plantlets, tissues, etc.). Table 1.3 shows a comparison of the D_{50}s of representative plant materials. Basically, the radiation sensitivity of living cells depends on the genome size (i.e., the nuclear contents per cell). With increasing genome size of plant species,

Table 1.3 Effective irradiation dose on plant materials.

Plant material	Radiation		
	18.3 MeV/u C	12.5 MeV/u He	Low-LET radiations
(a) Dry seeds (genome size)			
Arabidopsis (130 Mb)	300	1100	1200 (electrons)
Rice (430 Mb)	40–50	200	350
Tomato (950 Mb)	70	240	—
Barley (4.8 Gb)	10–20	—	—
Wheat (16 Gb)	25	—	—
(b) Tissue culture			
Chrysanthemum var. Taihei	15	10–20	∼60–80
Chrysanthemum var. Jimba	3	2–3	∼10
Carnation	15	40	60

Listed are D_{50}s (Gy), the irradiation doses that lead to 50% lethality (a. dry seeds) or growth rate (b. tissue culture). D_{50} is a good indicator to know general sensitivity of plants against radiations. Here, carbon and helium ions with the indicated energy were used for high-LET radiation. For low-LET radiation comparison, γ-rays were used, unless otherwise noted. Note that D_{50} decreases as genome size increases (a) (i.e., plant species with larger genomes are more sensitive to radiation). In addition, even in the same species, D_{50} varies among different varieties (b). Data were extracted from experiments performed at TIARA, the electron beam facilities in JAEA, and the γ-ray irradiation facilities in Institute of Radiation Breeding (unpublished data).

the sensitivity against radiation increases. Occasionally, radiation sensitivities vary significantly even among different varieties of the same plant species. In the case of "Jimba," which is a major variety of chrysanthemum in Japan, its sensitivity is more than 5 times higher than that of a variety "Taihei," of which the sensitivity is considered as a standard level in chrysanthemum. Radiation sensitivities also differ among plant organs. This difference is thought to be due to DNA content, water content, and so on. Cells in S phase of the cell cycle are the most sensitive to radiation because in this stage, the DNA content increases and the chromosomal DNA molecules are unpacked, leading to a cell status that is readily attacked by radiation and the secondary radical products. Radicals such as hydroxyl radicals are a major cause of DNA damage. It is well known that these radicals are generated by reactions between water and radiation. Therefore, plant materials such as dry seeds, in which the water content is very low, tend to show high resistance to radiations.

In conclusion, irradiation dose should be carefully determined according to the kinds of ion species and energies, plant species, plant varieties, plant state of materials such as cell cycle, and water content.

1.2.4 Population Size of the M1 Generation

Apparently, it is preferable to prepare as much of the target samples as possible because mutations basically happen at random and therefore under the laws of probability. When the mutation frequency of a particular locus is known, the minimum size of irradiation treatment samples can be roughly estimated. In the case of 18.3 MeV/u carbon ions, the mutation rate at *tt* and *gl* loci is 1.9×10^{-6} per locus per dose [5]. As the irradiation was performed with a dose of 150 Gy, the mutation rate was about 2.85×10^{-4} (roughly 1/3500) per locus, indicating that about 3500 seeds are necessary on average to obtain at least one mutant for a certain locus.

In practice, the minimum population size to isolate one phenotypic mutation (not one gene) is likely to be around 2000–5000 M1 seeds for *Arabidopsis* [9–11], rice, and other crops (unpublished results). However, it is not fully understood how many seeds will be required for plants with different genome sizes, gene numbers, and ploidies. On the other hand, it seems that a smaller population size would be sufficient for mutation induction from explants or tissue cultures. Moreover, several phenotypes, such as flower colors and shapes, chlorophyll mutations, waxes, and so on, have been obtained even in the M1 generation, although the mutation mechanisms are still unclear [12–15].

1.3 Applications

Considering its high mutation rate and its mutation spectrum that potentially differs from other chemical and physical mutagens, ion beam mutagenesis can be a powerful and useful technique to induce novel mutants. In fact, ion beam muta-

genesis has been employed in many plant species and several novel mutants have been produced. Identification of such novel mutants will bring about a better understanding of any biological process of interest, and also a dramatic improvement in agriculture and horticulture. Here, we describe the effectiveness of ion beams by citing recent studies using ion beam radiation.

1.3.1 Ion Beams for Forward Genetics

In forward genetics, isolation of mutants is merely the first step, yet it is a very critical procedure that enables us to analyze any relevant gene functions and gain a new insight into any developmental/physiological event. The new technique of ion beam mutagenesis has contributed significantly to plant research in this respect. For example, a novel mutant, *antiauxin resistant1-1* (*aar1-1*), was identified by screening the M2 progeny of carbon-ion-irradiated *Arabidopsis* seeds for plants resistant to *p*-chlorophenoxyisobutyric acid – a chemical that inhibits the auxin signaling pathway [16]. Further characterization of *aar1-1* showed that this mutant exhibits attenuated response specifically to a synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), but not to the native auxin, indole-3-acetic acid (IAA) [16]. This finding is quite surprising because it has been believed that 2,4-D and IAA have similar effects on auxin signaling despite differences in their stability. It was revealed that the *aar1-1* mutation is a 44-kb deletion encompassing eight annotated genes [16]. Among them, a gene encoding a small acidic protein (SMAP1) was shown to be solely responsible for the *aar1* phenotype [16]. Further molecular analysis of SMAP1 is necessary to dissect the previously underestimated 2,4-D-specific auxin signaling pathway.

Ion beam mutagenesis has also been applied to the model legume *Lotus japonicus*. Leguminous plants develop symbiotic root nodules to confine soil bacteria called rhizobia, which provide the host plants with ammonia produced through bacterial nitrogen fixation. Since this organogenesis is energetically expensive, the host plants should tightly regulate the development and number of nodules. For this purpose, legumes have evolved a long-distance signaling pathway that inhibits unfavorable overproduction of nodules. This systemic regulation requires at least a *CLAVATA1*-like receptor kinase gene and the mutations of this gene lead to the hypernodulation phenotype [17–20]. However, the precise molecular mechanism have been unclear, partly due to the absence of any other hypernodulating mutants, in spite of many attempts to isolate such plants from *L. japonicus* using EMS or T-DNA mutagenesis ([18, 21, 22] and N. Suganuma, personal communication). To circumvent this problem, helium ions were utilized as an alternative mutagen and a novel *Lotus* hypernodulating mutant, *klavier* (*klv*), was readily produced [23]. Grafting experiment using *klv* mutants showed that *KLV* is necessary in the shoots rather than in the roots, indicating that *KLV*, together with a *CLV1*-like receptor kinase gene, constitutes a long-distance signaling control of the nodule number control [23]. This successful identification of the *klv* mutant indicates that ion beams can be a relatively efficient mutagen, possibly having a different mutation spectrum from EMS and T-DNA.

1.3.2
Ion Beams for Plant Breeding

The problem of food shortages is one of the most crucial global challenges that we have ever faced. For this concern, production of new crop varieties with beneficial traits such as drought tolerance is important to fulfill a stable food supply. Moreover, industrialization of these induced varieties could have a great economical impact on societies.

Kirin Agribio in collaboration with the JAEA has generated many varieties of ornamental plants including carnations, chrysanthemums, and petunias by utilizing ion beams [12, 24, 25]. In the case of carnations, the parental leaf tissues were irradiated with carbon ions and then the plants were regenerated from them [24]. Using this approach, a great number of flower mutants including unprecedented round-petal carnations were obtained and some of the new varieties have been commercialized as "Ion Series" varieties (Figure 1.5) [12, 25].

1.3.3
Limitations of Ion Beams

We have shown that ion beam mutagenesis has been applied to a wide variety of plant species in many research fields and it has been successful for novel mutant production. The effectiveness of ion beams can be attributed largely to their high-LET characteristics, which lead to high DSB yields, strong mutational effects, and a

Figure 1.5 Carnation varieties codeveloped by Kirin Agribio and the JAEA using ion beams. The flower on the upper-left corner is the parent carnation flower (var. "Vital") and the others are mutant flowers produced by carbon ions. Note that ion beams successfully induced many flower color and shape mutants.

unique mutation spectrum compared to other chemical and physical mutagens. However, some limitations of ion beams also need to be taken into consideration. For example, ion beam-induced mutations are mostly deletions that can cause frame-shifts or total gene losses; therefore, ion beams may not be favorable for hypomorphic mutant isolation. In addition, ion beam irradiation results in various kinds of mutations such as small intragenic deletions, large deletions (greater than 100 kb), translocations, inversions, and chromosomal aberrations. Although this broad mutational effect of ion beams is advantageous with respect to novel mutant induction, the unpredictability of the mutation patterns could potentially hinder the subsequent molecular cloning of the relevant genes in some cases.

1.4 Perspectives

A mutagenesis technique – ion beam irradiation – has been exerting a huge impact on plant basic and applied research. Given that only a small fraction of the annotated genes have been analyzed for their functions even in *Arabidopsis*, the presence of such an alternative mutagen will become increasingly important. Further, application of ion beams in plant biotechnology will be more and more valuable to tackle global issues like food and environmental problems. However, some improvements are still necessary to make this mutagen a more reliable tool. For example, at present, the size of deletions generated by ion beams is variable from 1 bp to over 6 Mbp [26]. In this regard, development of techniques that enable us to control the deletion size will provide us with more efficient gene knockout approaches that can delete only a single gene at a time or sometimes tandem-duplicated multiple genes altogether if necessary. To achieve such an improvement, the precise molecular mechanism by which ion beams induce mutations needs to be elucidated.

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