

## Optimization of ion-beam irradiation for mutagenesis in soybean: effects on plant growth and production of visibly altered mutants

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**Abstract** Ion-beam irradiation is attracting increasing attention as a new mutagen. Here, we describe for the first time the dose response and mutagenic effects of ion-beam irradiation in soybean. We irradiated the hilum side of dried mature soybean seeds with 320-MeV carbon ions within a 0.25–20-Gy range. The growth or seed production of the irradiated plants was profoundly affected. In particular, the number of plants that survived until seed-set decreased with the increase of the irradiation dose and was very low in plants irradiated at doses higher than 5.0 Gy, whereas the frequency distribution of the number of seeds produced by each seed-setting plant was not affected by lower doses of irradiation. Based on these results, we produced plant populations irradiated at 2.5 Gy and 5.0 Gy on a large scale to obtain M<sub>2</sub> seeds. Despite the duplicate composition of the soybean genome, which originated from tetraploids, chlorophyll-deficient mutants were detected with a frequency of 0.47% in the M<sub>2</sub> generation of plants irradiated at 5.0 Gy. These results demonstrate that irradiation of the hilum side of dried soybean seeds with carbon-ion beams at a dose range around 2.5–5.0 Gy induces genetic changes while also allowing the production of a considerable number of seed-setting plants, suggesting that these irradiation conditions are suitable for producing a mutant population potentially useful for breeding and/or identifying gene function.

**Key words:** Chlorophyll deficiency, dose-response relationship, ion-beam irradiation, mutagenesis, soybean.

Soybean is an important crop that provides food, oil, forage, and industrial raw materials. Mutation breeding has been applied to soybean, as it has to other important crops, and mutants have been produced via neutron, X-ray, and  $\gamma$ -ray irradiation, as well as chemical treatments. Irradiation of soybean seeds with X-rays and thermal neutrons has significantly increased the genetic variation in yield, plant height, maturity, seed size (Rawlings et al. 1958), and oil and protein contents (Williams and Hanway 1961). Soybean lines with desirable characters have been produced via mutagenesis, including a line with high linolenic acid content via X-ray irradiation (Rahman et al. 1998; Takagi et al. 1989, 1990), a line lacking all three lipoxygenases (L-1, L-2, and L-3) via  $\gamma$ -ray irradiation (Hajika et al. 1991), lines deficient in subunits of seed-storage protein via  $\gamma$ -ray irradiation (Hayashi et al. 1998; Kitamura 1991; Takahashi et al. 1994), lines that nodulate in the presence of a high nitrate concentration via ethyl methanesulfonate (EMS) treatment (Carroll et al. 1985), a line with resistance to sulfonylurea herbicides via EMS and *N*-nitroso-*N*-methylurea (NMU) treatment (Sebastian et al. 1989), and

a line with reduced palmitic acid content via NMU treatment (Fehr et al. 1991). The number of soybean mutant lines, however, is limited, which is a constraint on the genetic study and breeding of this plant.

Soybean is thought to be derived from an ancestral plant(s) with a tetraploid genome, and as a consequence, large portions of the soybean genome are duplicated (Shoemaker et al. 1996) with nearly 75% of the genes present in multiple copies (Schmutz et al. 2010). In addition, genes in the soybean genome are sometimes duplicated in tandem (e.g., Kong et al. 2010; Matsumura et al. 2005; Schlueter et al. 2008; Yoshino et al. 2002). Our recent studies have indeed shown the functional redundancy of duplicated genes in soybean (Kanazawa et al. 2009; Liu et al. 2008). Such gene duplication can be an obstacle to mutant production by conventional methods of mutagenesis.

Ion-beam irradiation is attracting increasing attention as a new mutagen. The use of ion beams as a mutagen produces a high frequency (Fujii et al. 1966; Mei et al. 1994; Shikazono et al. 2003) and broad spectrum (Okamura et al. 2003; Shikazono et al. 1998) of mutants.

Various novel mutants of a wide variety of plants, which include *Arabidopsis thaliana*, *Lotus japonicus*, tobacco, rice, verbena, rose, carnation, *Torenia*, petunia, and Hinoki cypress, have been obtained via ion-beam irradiation (reviewed by Tanaka 2009). Ion beams comprise a type of high linear energy transfer (LET) radiation that bombards the target with higher energy than can be conferred by neutrons, X-rays, or  $\gamma$ -rays. Systematic analyses in *A. thaliana* revealed that approximately 50% of ion beam-induced mutations are large DNA alterations including deletions ranging from 5 kb to 230 kb; the frequency is twice as high as the frequency of large DNA alterations induced by low LET radiation such as  $\gamma$ -rays (reviewed by Tanaka et al. 2010). These characteristics of ion beams, as well as the successful production of novel mutants in the plants mentioned above, suggest that ion-beam irradiation could be useful for producing novel mutants in soybean, even though the genome is rich in duplicated genes. However, methods and conditions to induce mutations via ion-beam irradiation have not been established in soybean.

In this study, we irradiated the hilum side of dried soybean seeds with carbon ions and investigated the dose–response relationship of both greenhouse- and field-grown plants to determine the irradiation dose suitable for producing a population harboring mutants. Based on the results, we produced irradiated plant populations on a large scale and consequently detected mutants with visibly altered phenotypes, which provides evidence for mutagenic effects of ion-beam irradiation in soybean.

Soybean [*Glycine max* (L.) Merr.] line Nourin No. 2 was used for irradiation. Seeds were dried for at least 4 months at room temperature. Dried soybean seeds were placed on 60-mm-diameter Petri dishes and exposed to 320-MeV carbon ions (LET  $76 \text{ keV } \mu\text{m}^{-1}$ ) generated by an AVF-cyclotron (Japan Atomic Energy Agency, Takasaki, Japan). To ensure that meristem tissues were irradiated with equal doses, we fixed the seeds to plastic dishes with the hilum facing the irradiation source (Figure 1A, B).

Plants were grown either in a greenhouse or the experimental field at Hokkaido University, Sapporo, Japan, in the years from 2005 to 2010. The irradiated seeds of greenhouse-grown plants were germinated on wet paper towels for 4 days, after which the germinated seeds were potted in soil and grown in the greenhouse. In the field, irradiated seeds were sown directly onto the soil. Differences in the effects of different irradiation doses on plant growth were statistically analyzed using Tukey-Kramer test.  $M_2$  population was used for detecting phenotypic changes after half a month of growth in the field.

Chlorophyll contents were analyzed according to the

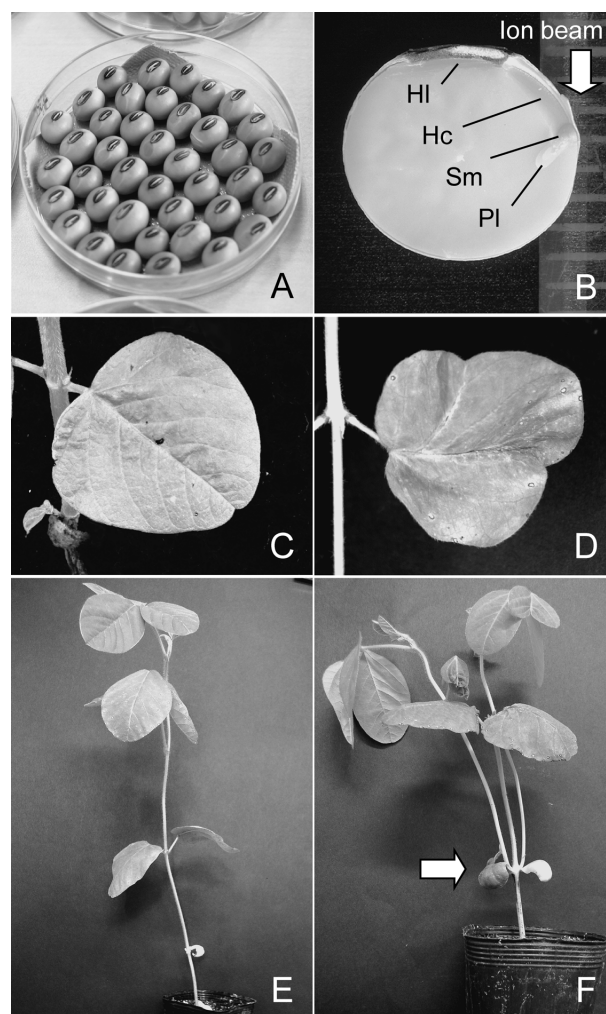


Figure 1. Soybean seeds irradiated with ion beams and morphological changes in irradiated plants. (A–B) Irradiated soybean seeds. (A) Soybean seeds fixed to a Petri dish for irradiation, with the hilum facing the irradiation source to ensure irradiation at an equal dose and onto meristem tissues. (B) Cross section of a soybean seed. HI, hilum; Hc, hypocotyls; Sm, shoot meristem; PI, primary leaves. The arrow indicates the direction of the ion-beam irradiation. Scale divisions = 1 mm. (C–F) Morphological changes. (C) Primary leaf of a control plant. (D) Altered primary leaf of irradiated plant, with a narrow portion in the central part. (E) Control plant with two sets of trifoliate leaves. (F) Irradiated plant showing unscheduled generation (branching) of stems from the node where cotyledons are formed. Arrow indicates the branching position.

method of Porra et al. (1989). Chlorophylls were extracted from leaf discs (10–30 mg) using 1 ml *N, N'*-dimethylformamide at 4°C for overnight. Absorbance at 663.8 nm and 646.8 nm was measured by spectrophotometer (U-2800S, Hitachi, Tokyo, Japan). Concentrations ( $\mu\text{g ml}^{-1}$ ) of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and total chlorophylls *a* and *b* (Chl *a+b*) were calculated using the equations as follows: Chl *a* =  $12.00 A^{663.8} - 3.11 A^{646.8}$ ; Chl *b* =  $20.78 A^{646.8} - 4.88 A^{663.8}$ ; Chl *a+b* =  $17.67 A^{646.8} + 7.12 A^{663.8}$ .

To determine the optimum irradiation dose that induced genetic changes without seriously affecting plant

growth or yield, we irradiated soybean seeds with a range of doses and analyzed their effect on greenhouse- and field-grown plants. Prior to irradiation, we examined whether ion beam could reach the meristem tissues of soybean seeds, where the DNA might undergo a heritable mutagenic effect, because soybean seeds are much larger than the penetration distance of ion beam. The penetration distance of ion beams is 2.2 mm in water (Tanaka and Hase 2010), whose shielding effect is expected to be greater than that of dried plant tissues. We dissected a dried soybean seed and found that the meristem tissues were 1.0–1.5 mm distant from the seed surface (Figure 1B), suggesting that ion beams could definitely reach the meristem tissues, if the hilum side of the seeds was exposed to the ion beams. Accordingly, we fixed soybean seeds onto Petri dishes with the hilum facing the irradiation source upon irradiation (Figure 1A). Exposing the hilum side of the seeds to the irradiation source also ensured an equal dose of irradiation to the meristem tissues of all of the seeds.

Dried soybean seeds were exposed to 320-MeV carbon ions at doses ranging from 0.25 to 20 Gy. The irradiated seeds were germinated, and plant growth was analyzed in the greenhouse. All of the seeds germinated, except for one of 20, which were irradiated at either 10 Gy or 20 Gy. Plant growth was conspicuously inhibited when the seeds were irradiated at a dose higher than 2.5 Gy (Figure 2A), but not at 1.0 Gy (Figure 2A) or doses lower than 1.0 Gy (data not shown). Of the plants grown from irradiated seeds, 11/20 and 9/20 irradiated at 10 Gy and 20 Gy, respectively, did not produce primary leaves even after growing for 3 weeks. On average, plant height was reduced 14%, 30%, 62%, and 66% relative to nonirradiated plants from seeds irradiated at 2.5 Gy, 5.0 Gy, 10 Gy, and 20 Gy, respectively, after 3 weeks of growth (Figure 2A).

In addition to inhibiting plant growth, irradiation also induced morphological changes. One prominent morphological change occurred in primary leaves, which produced a narrow portion in the central part of a leaf (Figure 1C, D). This change was detected in 1 or 2 of every 20 plants irradiated at doses of 2.5 Gy or higher. The other prominent morphological change was the unscheduled generation of stems from the node where cotyledons were formed (Figure 1E, F). The frequency of this change, together with its heritability, was examined using numerous field-grown plants (see below).

To produce a plant population suitable for screening mutants, we next examined the effects of irradiation on plant growth in the field. The growth of plants irradiated at doses higher than 5.0 Gy was inhibited (Figure 2B), as it was in the greenhouse, after 1.5 months of growth in the field. The effect of irradiation on seed yield was analyzed after further field growth. The ratio of the number of plants that survived until seed-set to the

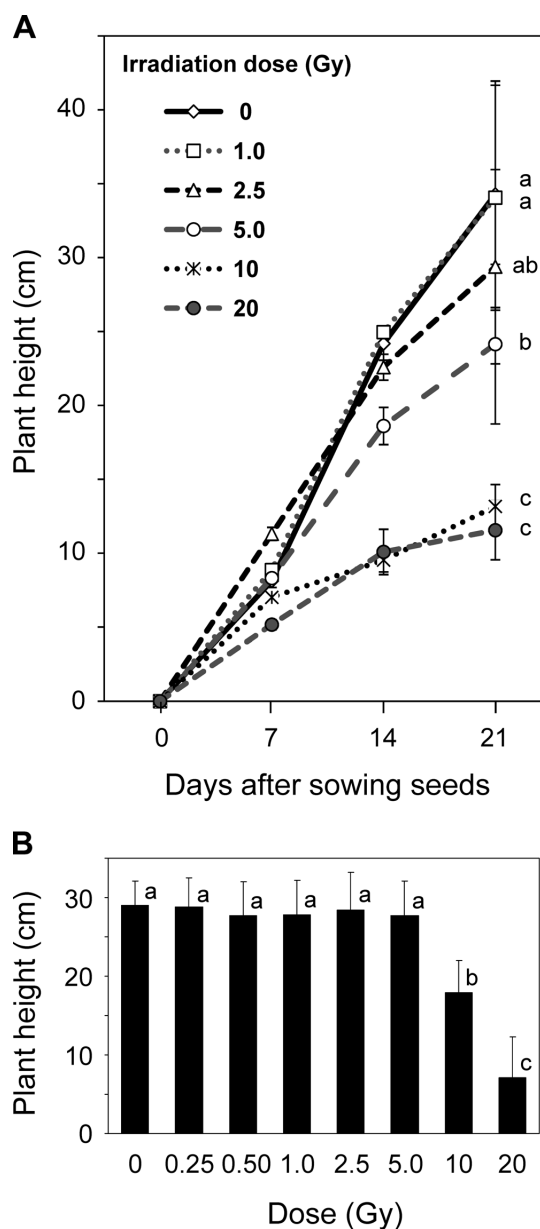


Figure 2. Effect of irradiation on the growth of plants. (A) Effect of irradiation on the early stages of plant growth in a greenhouse. Plant height was measured once a week for 3 weeks after the seeds were sown. Data are means  $\pm$  SE ( $n=20$ ). Tukey–Kramer test was used to analyze the data 3 weeks after the seeds were sown. Means with different letters are significantly different ( $P<0.05$ ). Studentized range LSD=6.27. (B) Effect of irradiation on the growth of plants in the field. Plant height was measured 1.5 months after sowing. Data are means  $\pm$  SD;  $n=83, 54, 51, 50, 43, 52, 24,$  and 14 for plants irradiated at 0 Gy, 0.25 Gy, 0.50 Gy, 1.0 Gy, 2.5 Gy, 5.0 Gy, 10 Gy, and 20 Gy, respectively. Tukey–Kramer test was used for the statistical analysis. Means indicated by different letters are significantly different ( $P<0.05$ ). Studentized range LSD=2.97. The smaller number of samples at 10-Gy and 20-Gy irradiation reflects the lower rate of survival of these plants.

number of seeds sown in the field clearly decreased as the irradiation dose increased, with 79%, 49%, 31%, and 5% of the number of nonirradiated plants surviving until seed-set when irradiated at 2.5 Gy, 5.0 Gy, 10 Gy, and 20

Gy, respectively. In contrast, the frequency distribution of the number of seeds produced by each seed-setting plant was not affected by irradiation at 2.5 Gy and 5.0 Gy (Figure 3). Similarly, no difference was detected in either the total or mean weight of seeds per seed-setting plant (data not shown). From these results, we inferred that irradiation at a dose range around 2.5–5.0 Gy induces genetic changes while also allowing the production of a considerable number of seed-setting plants; this condition is presumably suitable for producing a plant population harboring mutants.

We grew a large number of plants from irradiated

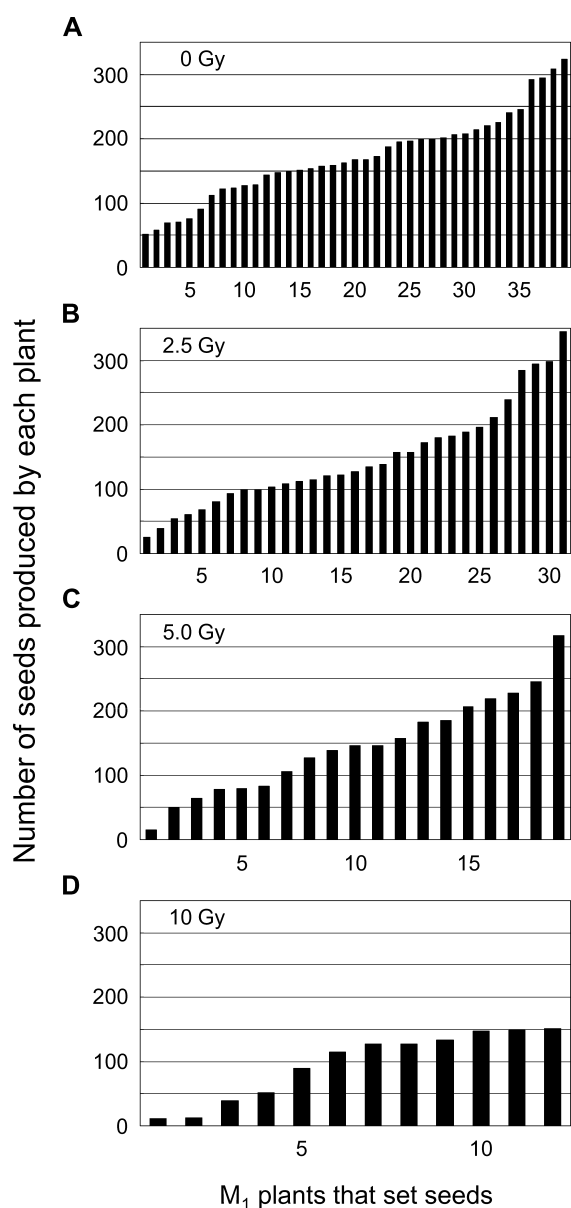


Figure 3. Effect of irradiation on seed production. The number of seeds produced by each plant that survived until seed-set from 80 seeds field-sown after irradiation at doses of 0 Gy (A), 2.5 Gy (B), 5.0 Gy (C), and 10 Gy (D). Plants were numbered according to the number of seeds produced. Note that the number of plants that produced seeds decreased as the irradiation dose increased.

seeds to examine whether irradiation under the conditions described above could induce mutations. We irradiated 3,320 and 3,200 seeds at 2.5 Gy and 5.0 Gy, respectively, and grew plants for seed harvest. The survival ratio until seed-set was higher in plants irradiated at 2.5 Gy than at 5.0 Gy. Seeds were harvested from approximately 1,400 plants irradiated at 2.5 Gy, which was twice the number of 5.0-Gy-irradiated plants that survived until seed-set. The harvested M<sub>2</sub> seeds were field-grown the next year, and this generation of individuals was examined for visibly altered phenotypes.

One noteworthy change was the unscheduled generation of stem(s) from the node where cotyledons formed (Figure 1F). This branching trait is an obstacle to the machine harvest of soybean seeds. Although this phenomenon was highly frequent in M<sub>1</sub> plants irradiated at 2.5 Gy (23.1%;  $n=654$ ), its frequency in the M<sub>2</sub> population (7.2%;  $n=710$ ) was as low as that in nonirradiated plants (4.4%;  $n=641$ ). Furthermore, no stable inheritance of this trait was detected in the progeny of M<sub>2</sub> plants with this abnormality (data not shown). Thus, branching in irradiated plants is not likely due to a heritable change, but is possibly caused by a physical stress from irradiation, although differences in the frequency of this phenomenon between soybean lines suggest that certain genetic mechanism(s) may be involved (N. Yamaguchi, personal communication).

The other observed change was a chlorophyll deficiency, as evidenced by pale-green leaves (Figure 4A, B), albino plants (Figure 4C), or variegated leaves (Figure 4D). We further produced M<sub>2</sub> population on a larger scale to analyze the frequency of these mutations. The pale-green-leaf mutants, the albino mutants, and variegated-leaf mutants were generated at the frequency of 0.28%, 0.17%, and 0.02%, respectively, of M<sub>2</sub> plants irradiated at 5.0 Gy ( $n=5724$ ). None of more than 1500 nonirradiated plants had such an altered phenotype. All the albino mutants died at the primary leaf stage. In contrast, the pale-green-leaf mutants survived (Figure 4E) and set seeds. We grew the progeny of four pale-green M<sub>2</sub> plants in the greenhouse and found that the M<sub>3</sub> progeny of M<sub>2</sub> plants maintained the pale-green phenotype (Figure 4F; the progeny of ‘D-2,’ ‘B-1,’ ‘B-2,’ and ‘C-2’ plants). Thus, these M<sub>2</sub> plants had stable heritable change(s) that caused the altered phenotype, demonstrating that ion-beam irradiation at 2.5–5.0 Gy can induce mutation. Chlorophyll contents in the leaf tissues of the M<sub>3</sub> progeny were analyzed (Figure 5). Plants of the all pale-green lines had lower levels of both chlorophylls *a* and *b* than the control plants as expected from the outward phenotype. In particular, B-1 and B-2 plants had no detectable level of chlorophyll *b*.

To our knowledge, no previous study has examined ion-beam irradiation doses to induce mutations in soybean. Thus, our results may prove useful in applying

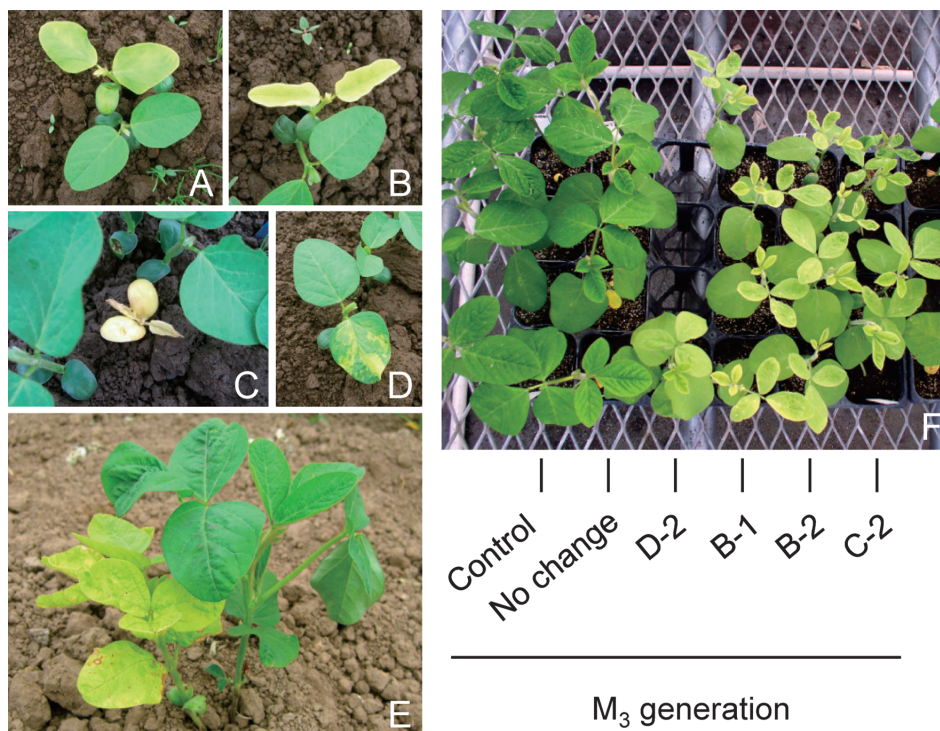


Figure 4. Chlorophyll-deficient mutation caused by ion-beam irradiation. (A–D) Chlorophyll-deficient mutants in the  $M_2$  population after 16 days of growth in the field. (A) A pale-green mutant. (B) A pale-green mutant with morphologically altered primary leaves. (C) An albino mutant. (D) A variegated mutant. Visibly unaltered plants from the same population are also shown (Figure 4A, below the mutant; Figure 4B, below the mutant; Figure 4C, left and right of the mutant; Figure 4D, above the mutant). (E) A pale-green mutant after 35 days of growth in the field. Left, pale-green mutant; right, visibly unaltered plant from the same population. (F) Inheritance of the pale-green-leaf phenotype. Four progeny plants from each  $M_2$  plant are shown (except D-2, for which one progeny plant is shown): from left to right, nonirradiated control (“control”), progeny of  $M_2$  plant with no visible change (“no change”), progeny of D-2 plant, progeny of B-1 plant, progeny of B-2 plant, progeny of C-2 plant. Note that all of the progeny of D-2, B-1, B-2, and C-2 show the pale-green-leaf phenotype. B-1, B-2, and D-2 plants, and C-2 plant were obtained via irradiation at 2.5 Gy and 5.0 Gy, respectively.

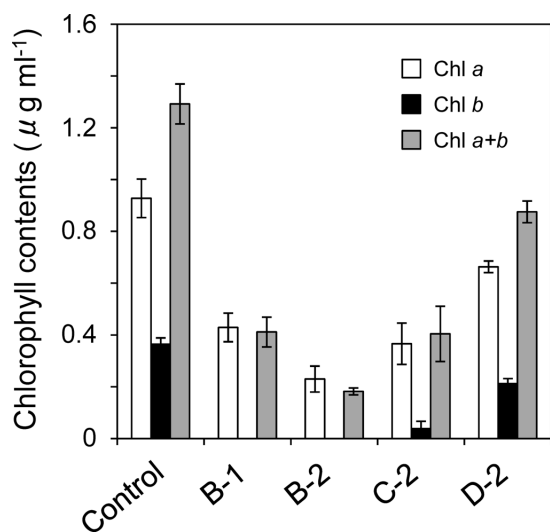


Figure 5. Chlorophyll contents in the leaf tissues of the  $M_3$  progeny. The contents of chlorophyll *a* (open bars), chlorophyll *b* (black bars), and total chlorophylls *a* and *b* (grey bars) in non-irradiated control and four  $M_3$  progeny plants with pale-green leaves are shown. Data represent mean  $\pm$  SD obtained from three replicates.

this technology to produce breeding materials of this plant. We found that a dose range around 2.5–5.0 Gy induced genetic changes while also allowing the production of a considerable number of seed-setting plants, although the survival rate was slightly affected even by irradiation at 2.5 Gy. Recently, Yamaguchi et al. investigated the survival rate, fertility and chlorophyll-mutation frequency as a consequence of irradiation of gamma-rays, two different carbon ions (LET of 76 and 107 keV  $\mu\text{m}^{-1}$ , respectively) and helium ions (LET of 9 keV  $\mu\text{m}^{-1}$ ) in rice (Yamaguchi et al. 2009). They found that the mutation frequency of ion beams, especially carbon ions, was higher than that of gamma-rays. Furthermore, they also found that the mutation yield, i.e., the number of  $M_1$  plants that produced chlorophyll mutants in their progeny divided by the number of  $M_1$  seeds sown after irradiation, had a peak at around the shoulder dose of the survival curve in all radiations tested. These results suggest that the shoulder dose, rather than 50% lethal dose, would be the best dose for inducing mutants, although 50% lethal dose has been considered a promising criterion for gamma-ray irradiation (Yamaguchi et al. 2009). The shoulder dose

was also most effective in obtaining flower mutants in carnation, in which leaf cultures were irradiated with carbon ions (LET of  $107 \text{ keV } \mu\text{m}^{-1}$ ) (Okamura et al. 2003). Accordingly, the dose range of 2.5–5.0 Gy, which we applied for soybean seeds to produce the  $M_2$  populations, is likely sufficient for efficient production of mutants. The shoulder doses of the survival curve of dried seeds irradiated with carbon ions are 60–80 Gy in rice (LET of  $76 \text{ keV } \mu\text{m}^{-1}$ ) (Yamaguchi et al. 2009), 50 Gy in tobacco (LET of  $92 \text{ keV } \mu\text{m}^{-1}$ ) (Hase et al. 2002), and around 220 Gy and 350 Gy (LET of 113 and  $72 \text{ keV } \mu\text{m}^{-1}$ , respectively) in *A. thaliana* (Shikazono et al. 2002). Taken together with these facts, the present results suggest that soybean, at least cv. Nourin No. 2, is slightly more sensitive to ionizing radiations onto dried seeds than these plants.

The presence of genes responsible for sensitivity of soybean plants to  $\gamma$ -ray irradiation have been reported (Takagi 1969), and differential sensitivity to  $\gamma$ -ray irradiation between varieties can be explained mostly by the presence of one major gene,  $rs_1$  (Takagi 1974; Ukai and Yamashita 1969). It is of interest to know whether  $rs_1$  may also confer sensitivity to ion-beam irradiation: this possibility can be examined in future by analyzing the sensitivity of various soybean varieties to ion-beam irradiation. Although a direct comparison of mutagenic effects between ion beams and other mutagens on soybean is yet to be examined, the present results suggest that the irradiation conditions, including doses, are suitable for the production of mutants. Our ongoing analysis of the  $M_2$  populations to find mutants that have desired characters, e.g., an advantage in plant development and/or modified seed components, will reveal the applicability of ion-beam irradiation in soybean breeding. So far, we have been able to screen novel mutants with an early maturing phenotype (our unpublished data). The data of the effects of irradiation on plant growth, seed yield, and phenotypic changes obtained in this study will be useful for producing breeding material.

Such an irradiated plant population may also be useful for identifying gene function, supplementing the recent development of a gene tagging system using the Ds transposon (Mathieu et al. 2009) and our virus-induced gene silencing system (Nagamatsu et al. 2007) in soybean. We here isolated chlorophyll-deficient mutants. Although at least 19 loci responsible for this phenotype have been identified in soybean (Zou et al. 2003), the causative genes of these loci have not been identified. Because ion-beam irradiation frequently induces DNA rearrangements (Shikazono et al. 2005) that can be detected efficiently using a polymerase chain reaction-based method (e.g., the amplified fragment length polymorphism method), mutants obtained by ion-beam irradiation may be suitable for isolating the causative

genes of phenotypic changes, which include the chlorophyll-deficient phenotype and others that will be characterized in our ongoing study. In this regard, the plant material used in this study, cv. Nourin No. 2, may have an advantage because a full-length-enriched cDNA library comprising 40,000 cDNAs has been constructed using this material (Umezawa et al. 2008). Analysis of mutants in combination with the cDNA sequence information may facilitate gene discovery.

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## References

- Carroll BJ, McNeil DL, Gresshoff PM (1985) Isolation and properties of soybean [*Glycine max* (L.) Merr.] mutant that nodulate in the presence of high nitrate concentrations. *Proc Natl Acad Sci USA* 82: 4162–4166
- Fehr WR, Welke GA, Hammond EG, Duvick DN, Cianzio SR (1991) Inheritance of reduced palmitic acid content in seed oil of soybean. *Crop Sci* 31: 88–89
- Fujii T, Ikenaga M, Lyman JT (1966) Radiation effects on *Arabidopsis thaliana*-II. Killing and mutagenic efficiencies of heavy ionizing particles. *Radiat Bot* 6: 297–306
- Hajika M, Igita K, Kitamura K (1991) A line lacking all the seed lipoxigenase isozymes in soybean [*Glycine max* (L.) Merr.] induced by gamma-ray irradiation. *Jpn J Breed* 41: 507–509
- Hase Y, Yamaguchi M, Inoue M, Tanaka A (2002) Reduction of survival and induction of chromosome aberrations in tobacco irradiated by carbon ions with different energy transfers. *Int J Radiat Biol* 78: 799–806
- Hayashi M, Harada K, Fujiwara T, Kitamura K (1998) Characterization of a 7S globulin-deficient mutant of soybean (*Glycine max* (L.) Merrill). *Mol Gen Genet* 258: 208–214
- Kanazawa A, Liu B, Kong F, Arase S, Abe J (2009) Adaptive evolution involving gene duplication and insertion of a novel *Ty1/copia*-like retrotransposon in soybean. *J Mol Evol* 69: 164–175
- Kitamura K (1991) Spontaneous and induced mutations of seed proteins in soybean (*Glycine max* L. Merrill). *Gamma Field Symposia* 30: 61–69
- Kong F, Liu B, Xia Z, Sato S, Kim B, Watanabe S, Yamada T, Tabata S, Kanazawa A, Harada K, et al. (2010) Two coordinately regulated homologs of *FLOWERING LOCUS T* are involved in the control of photoperiodic flowering in soybean. *Plant Physiol* 154: 1220–1231
- Liu B, Kanazawa A, Matsumura H, Takahashi R, Harada K, Abe J (2008) Genetic redundancy in soybean photoreponses associated with duplication of the phytochrome A gene. *Genetics* 180: 995–1007
- Mathieu M, Winters EK, Kong F, Wan J, Wang S, Eckert H, Luth

- D, Paz M, Donovan C, Zhang Z, et al. (2009) Establishment of a soybean (*Glycine max* Merr. L.) transposon-based mutagenesis repository. *Planta* 229: 279–289
- Matsumura H, Watanabe S, Harada K, Senda M, Akada S, Kawasaki S, Dubouzet EG, Minaka N, Takahashi R (2005) Molecular linkage mapping and phylogeny of the chalcone synthase multigene family in soybean. *Theor Appl Genet* 110: 1203–1209
- Mei M, Deng H, Lu Y, Zhuang C, Liu Z, Qiu Q, Qiu Y, Yang TC (1994) Mutagenic effects of heavy ion radiation in plants. *Adv Space Res* 14: 363–372
- Nagamatsu A, Masuta C, Senda M, Matsuura H, Kasai A, Hong J-S, Kitamura K, Abe J, Kanazawa A (2007) Functional analysis of soybean genes involved in flavonoid biosynthesis by virus-induced gene silencing. *Plant Biotechnol J* 5: 778–790
- Okamura M, Yasuno N, Ohtsuka M, Tanaka A, Shikazono N, Hase Y (2003) Wide variety of flower-color and -shape mutants regenerated from leaf cultures irradiated with ion beams. *Nucl Instr Meth Phys Res B* 206: 574–578
- Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochim Biophys Acta* 975: 384–394
- Rahman SM, Kinoshita T, Anai T, Arima S, Takagi Y (1998) Genetic relationships of soybean mutants for different linolenic acid contents. *Crop Sci* 38: 702–706
- Rawlings JO, Hanway DG, Gardner CO (1958) Variation in quantitative characters of soybeans after seed irradiation. *Agron J* 50: 524–528
- Schlueter JA, Scheffler BE, Jackson S, Shoemaker RC (2008) Fractionation of synteny in a genomic region containing tandemly duplicated genes across *Glycine max*, *Medicago truncatula*, and *Arabidopsis thaliana*. *J Hered* 99: 390–395
- Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W, Hyten DL, Song Q, Thelen JJ, Cheng J, et al. (2010) Genome sequence of the palaeopolyploid soybean. *Nature* 463: 178–183
- Sebastian SA, Fader GM, Ulrich JF, Forney DR, Chaleff RS (1989) Semidominant soybean mutation for resistance to sulfonylurea herbicides. *Crop Sci* 29: 1403–1408
- Shikazono N, Yokota Y, Tanaka A, Watanabe H, Tano S (1998) Molecular analysis of carbon ion-induced mutations in *Arabidopsis thaliana*. *Genes Genet Syst* 73: 173–179
- Shikazono N, Tanaka A, Kitayama S, Watanabe H, Tano S (2002) LET dependence of lethality in *Arabidopsis thaliana* irradiated by heavy ions. *Radiat Environ Biophys* 41: 159–162
- Shikazono N, Yokota Y, Kitamura S, Suzuki C, Watanabe H, Tano S, Tanaka A (2003) Mutation rate and novel *tt* mutants of *Arabidopsis thaliana* induced by carbon ions. *Genetics* 163: 1449–1455
- Shikazono N, Suzuki C, Kitamura S, Watanabe H, Tano S, Tanaka A (2005) Analysis of mutations induced by carbon ions in *Arabidopsis thaliana*. *J Exp Bot* 56: 587–596
- Shoemaker RC, Polzin K, Labate J, Specht J, Brummer EC, Olson T, Young N, Concibido V, Wilcox J, Tamlonis JP, et al. (1996) Genome duplication in soybean (*Glycine* subgenus *soja*). *Genetics* 144: 329–338
- Takagi Y (1969) The second type of gamma-ray sensitive gene *rs*<sub>2</sub> in soybean *Glycine max* (L.) Merrill. *Gamma Field Symposia* 8: 83–94
- Takagi Y (1974) Studies on varietal differences of radiosensitivity in soybean. *Acta Radiobotanica et Genetica* 3: 45–87
- Takagi Y, Hossain ABMM, Yanagita T, Kusaba S (1989) High linolenic acid mutant in soybean induced by X-ray irradiation. *Jpn J Breed* 39: 403–409
- Takagi Y, Hossain ABMM, Yanagita T, Matsueda T, Murayama A (1990) Linolenic acid content in soybean improved by X-ray irradiation. *Agric Biol Chem* 54: 1735–1738
- Takahashi K, Banba H, Kikuchi A, Ito M, Nakamura S (1994) An induced mutant line lacking the  $\alpha$ -subunit of  $\beta$ -conglycinin in soybean [*Glycine max* (L.) Merrill]. *Breed Sci* 44: 65–66
- Tanaka A (2009) Establishment of ion beam technology for breeding. In: Shu QY (ed) *Induced Plant Mutations in the Genomics Era*. Food and Agriculture Organization of the United Nations, pp 243–246
- Tanaka A, Hase Y (2010) Applications to biotechnology: ion-beam breeding of plants. In: Harano Y, Katsumura Y, Mozumder A (eds) *Charged Particle and Photon Interactions with Matter—Recent Advances, Applications, and Interfaces*—. Taylor & Francis, Florida, pp 943–957
- Tanaka A, Shikazono N, Hase Y (2010) 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. *J Radiat Res* 51: 223–233
- Ukai Y, Yamashita A (1969) Varietal differences in radiosensitivity with special reference to different aspects with different crops. *Gamma Field Symposia* 8: 69–81
- Umezawa T, Sakurai T, Totoki Y, Toyoda A, Seki M, Ishiwata A, Akiyama K, Kurotani A, Yoshida T, Mochida K, et al. (2008) Sequencing and analysis of approximately 40000 soybean cDNA clones from a full-length-enriched cDNA library. *DNA Res* 15: 333–346
- Williams JH, Hanway DG (1961) Genetic variation in oil and protein content of soybeans induced by seed irradiation. *Crop Sci* 1: 34–36
- Yamaguchi H, Hase Y, Tanaka A, Shikazono N, Degi K, Shimizu A, Morishita T (2009) Mutagenic effects of ion beam irradiation on rice. *Breed Sci* 59: 169–177
- Yoshino M, Kanazawa A, Tsutsumi K, Nakamura I, Takahashi K, Shimamoto Y (2002) Structural variation around the gene encoding the  $\alpha$  subunit of soybean  $\beta$ -conglycinin and correlation with the expression of the  $\alpha$  subunit. *Breed Sci* 52: 285–292
- Zou JJ, Singh RJ, Hymowitz T (2003) Association of the yellow leaf (*y10*) mutant to soybean chromosome 3. *J Hered* 94: 352–354