

Comprehensive effects of heavy-ion beam irradiation on sweet potato (*Ipomoea batatas* [L.] Lam.)

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Abstract Sweet potato is a major root crop with nutritious tuberous roots. The mechanism of tuberous root development has not yet been adequately elucidated. Genetic resources are required to develop the molecular understanding of sweet potato. Heavy-ion beams were applied to hexaploid sweet potato for an increase in genetic variation, after which the comprehensive effects of heavy-ion beam irradiation were investigated. In vitro cultured shoots with an axillary bud of 'Beniharuka' were irradiated with Ar-ions at a dose of 1–5 Gy and C-ions at a dose of 5–20 Gy, and three irradiated lines were separated from each irradiated shoot. The shoot regeneration was inhibited at high doses of each ion irradiation. Ar-ion irradiation had an especially high biological effect on shoot regeneration. A total of 335 lines were obtained, consisting of 104 and 231 lines derived from Ar- and C-ion irradiation, respectively. The change in the DNA content of the lines was analyzed by flow cytometry to evaluate the irradiation-induced damage to the DNA. The two lines demonstrated significant differences in the DNA content and changes at the chromosome level. The screening for the morphological mutants was conducted in the field. Some irradiated lines showed inhibited or no tuberous root phenotype as mutant candidates. Additionally, the high-yield mutant candidates were dominated by Ar-ion irradiation. It was indicated that heavy-ion beam mutagenesis is effective in broadening the range of the phenotypes corresponding to tuberous root formation in hexaploid sweet potato.

Key words: heavy-ion beam, linear energy transfer, sweet potato, tuberous root.

Introduction

Sweet potato is one of the most important food crops and plays a critical role in food supply and safety worldwide. It is a nutritious crop rich in carbohydrates, proteins, and minerals (Bovell-Benjamin 2007). Japan is the sixteenth largest sweet potato-producing country, with a yield of approximately 0.7 million tons (FAOSTAT 2020). Although sweet potato has a wide range of applications, such as alcohol production and processing, its table-use accounts for more than 40% in Japan (Katayama et al. 2017). It is known that the formation of tuberous roots comprises a complex process regulated by internal and external factors and can be anatomically hypertrophied by the action of primary and secondary cambium and abnormal cambium (Ravi et al. 2009; Wilson and Lowe 1973). Comprehensive research has been conducted on the development of the tuberous root through

transcriptome analysis. Plant hormones, lignin synthesis, starch synthesis, and transcription factor-related genes have been revealed as regulation factors associated with the tuberous root formation (Dong et al. 2019; Firon et al. 2013; He et al. 2021). A study based on the late-storage root-forming mutant suggests that an increase in root zeatin riboside level, a major cytokinin, is involved in the regulation of the formation of tuberous roots (Nakatani et al. 2002). However, the detailed mechanisms of how tuberous root formation is controlled by these factors are still unknown. Its understanding may therefore be advanced if additional mutants related to tuberous root formation are obtained.

Mutagenesis has been applied to induce genetic variations in plants, where the induced mutations bring about changes in functional genes (Prasanna and Jain 2017). Various mutant pools in crops have been established, and genetic resources have been supplied for

Abbreviations: EMS, ethyl methanesulfonate; FCM, flow cytometry; LET, linear energy transfer.

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breeding materials and functional genomics, for example, *Tos17* retrotransposon insertion lines in rice (Miyao et al. 2003), N-methyl-N-nitrosourea induced mutant pools in rice (Suzuki et al. 2008), and soybean mutant library treated with ethyl methanesulfonate (EMS) (Tsuda et al. 2015). Moreover, in sweet potato, there are mutant pools derived from X-ray, γ -ray, and EMS (Kukimura and Kouyama 1982; Luan et al. 2007; Mashima and Sato 1959; Wang et al. 2007), where several mutants have been screened from the mutant pools.

Heavy-ion beams have also been used for mutation breeding and functional studies as a powerful physical mutagen (Abe et al. 2015; Hirano et al. 2022; Kazama et al. 2018; Tanaka et al. 2010; Yamaguchi 2018). Linear energy transfer (LET; $\text{keV } \mu\text{m}^{-1}$) is the amount of energy that an ionizing particle transfers to the material traversed per unit distance and is used as an index for expressing the radiation quality. Heavy-ion beams have higher and more variable LET values than X-ray and γ -ray (Ryuto et al. 2008); the radiation with a high LET value deposits energy densely on the target. When a heavy-ion beam with a high LET value is irradiated to the cells, it effectively induces DNA double-strand breaks (Alloni et al. 2010; Scholz 2006). Heavy-ion beams with a high LET value can induce a wide range of mutant phenotypes with high frequencies at relatively low absorbed doses (Abe et al. 2015; Tanaka et al. 2010).

Previous studies have revealed LET-dependent effects of heavy-ion beam irradiation on the induction of mutation to plant genome. The frequency of large-scale mutations increases with increasing LET values in *Arabidopsis thaliana* and rice (Abe et al. 2021; Hirano et al. 2015, 2012; Kazama et al. 2011, 2017; Morita et al. 2021). For example, in *Arabidopsis*, carbon-ion beams at 22.5 and 30.0 $\text{keV } \mu\text{m}^{-1}$ primarily induce small mutations, such as base changes, deletions, and insertions (Kazama et al. 2011, 2017), and argon-ion beam at 290 $\text{keV } \mu\text{m}^{-1}$ frequently induce large deletions and chromosomal rearrangements such as inversions and translocations (Hirano et al. 2015, 2012; Kazama et al. 2017). Mutant libraries have been constructed through mutagenic studies in *Arabidopsis* and rice. In addition, mutant library in diploid einkorn wheat (*Triticum monococcum*) is also induced by the heavy-ion beam (Murai and Abe 2021; Murai et al. 2013). The mutants derived from the libraries have contributed to revealing the molecular mechanisms in plant functions.

In this study, Ar- and C-ion beam irradiation were applied with different LET values to in vitro cultured shoots of sweet potato, and mutant lines were established to screen the mutants on tuberous root formation. The effects of the heavy-ion beam irradiations were evaluated on the sweet potato genome using flow cytometry (FCM) analysis and tuberous root formation through field screening. Some irradiated lines on tuberous root

formation were selected by the first screening, suggesting that the heavy-ion beam irradiations expanded the genetic variation of the sweet potato.

Materials and methods

Irradiation of heavy-ion beams and irradiated line construction

Each of the 30 in vitro cultured shoots with an axillary bud of sweet potato (*Ipomoea batatas* 'Beniharuka') was irradiated with $^{12}\text{C}^{6+}$ ions (LET; $30 \text{ keV } \mu\text{m}^{-1}$) at absorbed doses of 5.0, 10.0, and 20.0 Gy, or $^{40}\text{Ar}^{17+}$ ions (LET; $184 \text{ keV } \mu\text{m}^{-1}$) at absorbed doses of 1.0, 2.5, and 5.0 Gy using the E5 beam line in the RIKEN RI-beam factory. Each bud was then incubated on 0.4% gellan gum containing 1/2 Murashige and Skoog medium and 3% sucrose at 25°C under long-day conditions (16 h light, 8 h dark) in a growth chamber (TOMY Cultivation Chamber CFH-405; Tomy Digital Biology Co., Ltd., Tokyo, Japan). The shoot formation rate was then surveyed after approximately one month. The axillary bud was treated as the shoot formation when the regenerated shoot from each irradiated axillary bud formed three or more leaves. The regenerated shoot was cut into nodes, each with an axillary bud, after three months of culture. The third or later nodes from the base were used for constructing irradiated lines, and three lines were created from each shoot.

FCM analysis

As the first screening to detect damage to the DNA, nuclei suspensions were prepared by chopping the young fresh leaf of each plant with a sharp razor blade in 200 μl of the Otto I buffer (Otto 1990) and were filtered through a layered 30 μm nylon mesh. The filtrates were mixed with 1 ml of DAPI staining buffer (Mishiba et al. 2000) and analyzed using a CyFlow Ploidy Analyzer (Sysmex Corporation, Kobe, Japan). The tetraploid *Ipomoea trifida* K233-1 was used as an internal standard to compare changes in the relative DNA content between unirradiated control and the mutant lines. The leaves from the control or mutant lines were chopped with K233-1 leaf in 200 μl of the Otto I buffer, and the suspensions were treated the same as described above. Three replicates of the experiment were performed for each line, after which the means of the relative DNA contents were compared with analysis of variance followed by Tukey's test.

Mutant screening

Each in vitro cultured irradiated lines was transplanted into a small black pot filled with culture soil and acclimatized in the greenhouse. The survived lines were maintained in the greenhouse for approximately two months until transplanting at the field. A total of 116 lines were cultivated in the field at Kushima, Miyazaki, Japan from June 9, 2020, to October 24, 2020, and 211 lines at Tano, Miyazaki, Japan from May 29, 2021, to October 16, 2021. Six control plants and one plant of each irradiated line were cultivated. In the field, the rows were

made 54 cm wide and mulched with black polyethylene film. The distance between rows was 30 cm, and the interval between transplanted plants was 35 cm. The total weight and number of the tuberous roots were measured only for those weighing 50 g or more. The fresh color of the tuberous roots was also investigated. A scatter plot of the relative weight and number of tuberous roots in each line was drawn using ggplot2, an open-source R package.

Results and discussion

Shoot formation rate and line construction

The shoot formation rate was surveyed from each axillary bud after a month of ion-beam irradiation. A decrease in the shoot formation was observed at high-dose irradiation of both ion beams (Figure 1). Shoulder doses on the shoot formation curve were considered 10 Gy for the C-ion beam and 2.5 Gy for the Ar-ion beam. Heavy-ion beams with different LET values provide different characteristics on mutation induction or degree of lethal effects on plants (Abe et al. 2015; Tanaka et al. 2010). In this study, the Ar-ion beam, which has a LET value six times higher than the C-ion beam, demonstrated higher biological effects to shoot formation. The LET-dependent effect was also observed in the hexaploid sweet potato. In heavy-ion beam breeding, the shoulder dose on the survival curve or regeneration rate curve is practically used for mutant screening and is thought to be sufficient for mutant induction due to the high mutation frequency

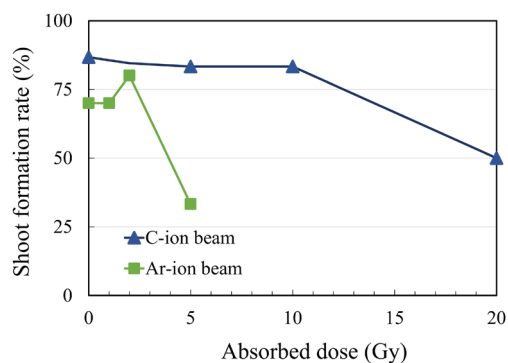


Figure 1. The shoot formation rate of the irradiated axillary buds. The shoot formation rate was surveyed after a month of heavy-ion beam irradiation. A total of 30 axillary buds were applied to each irradiation.

Table 1. The total number of mutant lines derived from heavy-ion irradiation.

Ion species	Dose (Gy)	Number of lines
Carbon ($^{12}\text{C}^{6+}$)	5.0	78
	10.0	79
	20.0	74
Argon ($^{40}\text{Ar}^{17+}$)	1.0	18
	2.5	77
	5.0	9

of the heavy-ion beam. The mutation frequency is high around the shoulder dose in the M_1 generation (Kazama et al. 2012). Therefore, 10 Gy and 2.5 Gy are suggested to be appropriate for C- and Ar-ion irradiation in ‘Beniharuka,’ respectively, considering the practical use for breeding.

The regenerated shoot from each irradiated axillary bud was cut into nodes, and a total of 335 irradiated lines were obtained. This consisted of 104 and 231 lines derived from Ar and C ion irradiation, respectively (Table 1).

Changes in nuclear DNA content

To evaluate the effects of the irradiation on the sweet potato genome, changes in the nuclear DNA content of all irradiated lines were analyzed using FCM. Two lines (C20-8-1 and C20-8-3) demonstrated significant differences in the DNA content (Table 2). The relative DNA contents of C20-8-1 and C20-8-3 were decreased compared to the unirradiated control. When the changes in the DNA content were converted to changes in the chromosome number ($2n=6x=90$), the changes in the chromosome numbers in C20-8-1 and C20-8-3 were estimated as -3.1 and -2.7 , respectively. Three lines were created from the shoot regenerated from each irradiated axillary bud, assuming that chimeras would be observed in the shoots. In the case of C20-8-1 and C20-8-3, they are considered to harbor common mutations because they were originally derived from the same shoot.

It has been revealed that heavy-ion beams, particularly with high LET values, induce intra- and inter-chromosomal rearrangements, the rearrangements causing duplications or deletions of chromosomal regions (Hirano et al. 2015; Kazama et al. 2017; Morita et al. 2021). Dicentric and acentric chromosomes are also formed by heavy-ion beam irradiation, and abnormalities of chromosome separation have been observed (Hirano et al. 2022; Kikuchi et al. 2009). Therefore, the DNA content changes were detected in sweet potatoes, which are interpreted to originate from duplications or deletions of chromosomal regions and abnormalities of chromosome separation. A decrease in the nuclear DNA content was induced at a fairly high rate by irradiation with ion beams and γ -rays in

Table 2. DNA content analysis in heavy-ion irradiated lines by flow cytometry.

Lines	Relative DNA contents (%)
Control	100.0 \pm 0.1 ^a
C20-8-1	96.8 \pm 0.6 ^b
C20-8-3	97.3 \pm 0.2 ^b

Each value is expressed as the mean \pm SE relative to the value of unirradiated control, which was set at 100. Values with different letters are significantly different according to Tukey’s test ($n=3$, $p<0.01$).

Chrysanthemum × *morifolium* (Yamaguchi et al. 2009, 2010). However, in this study, the changes in the DNA content were detected in two lines only. These results possibly suggest that the chromosomal stability of sweet potato is higher than that of chrysanthemum ($2n=6x=54$). It would be interesting to compare the responses to DNA damage immediately after the irradiation between sweet potato and chrysanthemum.

Mutant screening

For the mutant screening, 116 and 211 irradiated lines were grown, and their phenotype was investigated in the fields in 2020 and 2021, respectively. The irradiated lines demonstrated a wide range of phenotype variations in the tuberous roots. The relative values were calculated based on the control and shown on a scatter plot for the weight and number of the tuberous roots (Figure 2). Generally, the total weight and number of the tuberous roots tend to decrease in the C- and Ar-ion irradiated

lines. The tendency according to each ion or absorbed dose could not be confirmed. Some irradiated lines could be identified as having traits that looked superior in the total weight or the number of tuberous roots compared to the control. In particular, high-yield lines, with an increase of more than 180%, were all derived from Ar-ion irradiation (Figure 3A, B). Considerable inhibition of tuberous root formation was observed in the line derived from C- and Ar-ion irradiation (Figure 3A, C). One of the inhibited lines, C20-8-1, detected a decrease in the DNA content (Table 2). In addition, C20-8-3, the line with the decreased DNA content, demonstrated a low yield (33.3%). These results suggested that gene deletions associated with the DNA content decrease cause an inhibition in the formation of tuberous roots. No fresh-color mutant in the tuberous roots was observed. In the shoots of the lines, a dwarf phenotype was observed in C5-24-1 (Figure 4). The stem elongation in C5-24-1 was severely inhibited compared to unirradiated control.

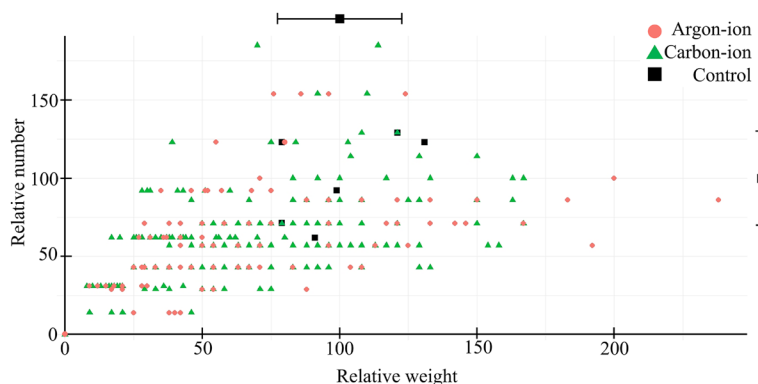


Figure 2. Scatter plot showing the relative weight and number of tuberous roots in each irradiated line. Each value is relative to the respective unirradiated control value, the mean value of which was set at 100. Black bars indicate a 95% confidence interval of the control values on each side.

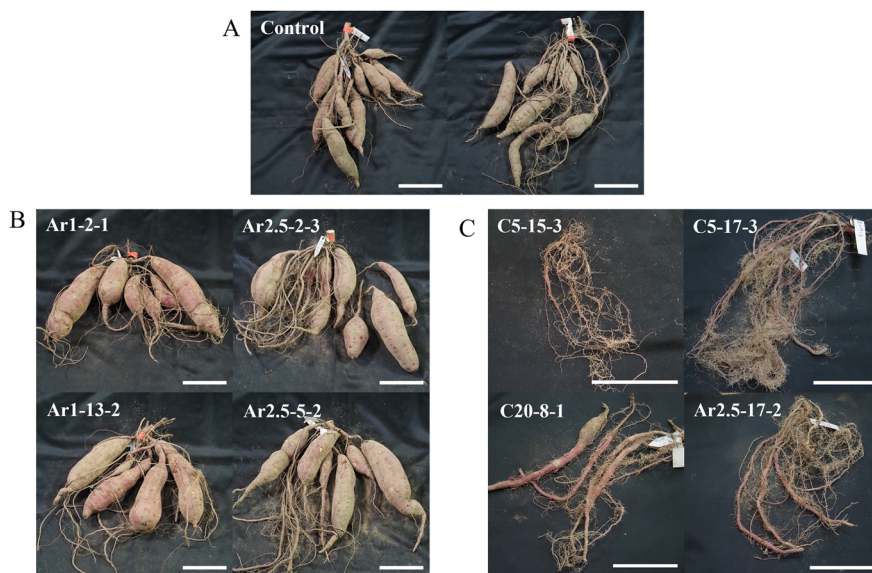


Figure 3. Phenotypes of tuberous roots in the irradiated lines. (A) Unirradiated control of 'Beniharuka', (B) the irradiated lines with high yield, and (C) the irradiated lines that do not form tuber or are inhibited. Scale bars = 10 cm.

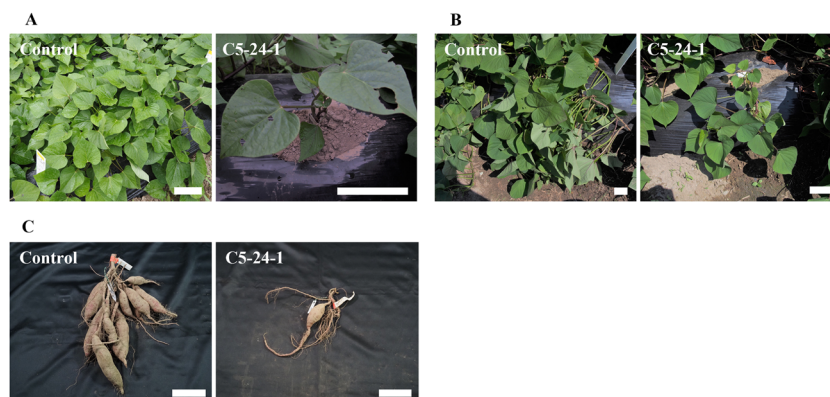


Figure 4. Morphology of C5-24-1 line with dwarf phenotype. Shoots of control and C5-24-1 after 55 days (A) and 75 days (B) of culture. Tuberous roots after harvesting of the control and C5-24-1 (C). Scale bars=(A, B) 12 cm and (C) 10 cm.

Moreover, a remarkable decrease in the production of tuberous roots was observed.

In hexaploid plants, a broad spectrum of mutant phenotypes has been confirmed to be induced by high-LET heavy-ion irradiation in chrysanthemum (Tanokashira et al. 2014). Gene deletions and chromosomal rearrangements were also observed in the irradiated lines of the common wheat genome (Fitzgerald et al. 2010; Kikuchi et al. 2009). It was confirmed that the phenotypes corresponding to tuberous root formation in ‘Beniharuka’ were broadened by the heavy-ion beam irradiations. When considered together, it is suggested that heavy-ion beam mutagenesis is effective for hexaploid plants, including sweet potato. Although no obvious LET-dependent effects on the phenotypic variation of tuberous roots were not observed in this study, the molecular nature of mutations is possibly different between C- and Ar-ion irradiated lines. Further research is required to elucidate the details of the induced mutations in the sweet potato genome, in addition to the flow cytometry analysis.

Mutant candidates with the phenotypes of high yield and inhibition of tuberous root formation were successfully selected (Figure 2) and the stability of the phenotypes is currently being confirmed. In particular, the mutants on tuberous root formation become valuable genetic resources for the functional study of the mechanism of tuberous root formation in sweet potato, in addition to its utilization in breeding. Since irradiated lines potentially harbor broad mutations, it would be possible to obtain novel mutants by application of novel screening methods.

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Author contribution

Conceptualization, T.H., Y.N., and H.K.; methodology, T.H., H.P., T.A., H.K.; investigation, H.P., T.H., H.K., Y.N., T.A.; writing-

original draft preparation, H.P., and T.H.; writing-review and editing, T.H., Y.N., T.A., and H.K.

Conflict of interest

The authors declare that there are no conflicts of interest.

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