J. RADIAT. RES., 43: SUPPL., S157–S161 (2002)

Chlorophyll-deficient Mutants of Rice Demonstrated the Deletion of a DNA Fragment by Heavy-ion Irradiation

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Rice / Heavy-ion beam / RLGS / Albino / Mutant

Heavy-ion irradiation is a new method of mutation breeding to produce new cultivars. We established the application of this method in rice plants to obtain mutants. Rice seeds were irradiated by C or Ne ions (135MeV/u) with a LET (linear energy transfer) of 22.7 or 64.2 keV/μm, respectively. Chlorophyll-deficient mutants (CDM) segregated in M_2 progeny were albino, pale-green, yellow or striped-leave phenotypes. The highest rate of CDM with C-ion irradiation, 7.31%, was obtained at 40 Gy among the doses examined. Ne-ion irradiation gave the highest rate, 11.6%, at 20 Gy. We used the RLGS (Restriction Landmark Genomic Scanning) method to analyze DNA deletion in an albino mutant genome. *Not* I-landmark RLGS profiles detected about 2000 spots in rice. We found that one of the polymorphic spots was strongly linked to the albino phenotypic mutant derived from deleting of a DNA fragment, and demonstrated the high ability to detect of polymorphic regions by the RLGS method.

INTRODUCTION

Various mutagens can be applied to induce mutations. Most mutant cultivars have been obtained by radiation treatments of these crops with γ-rays and X-rays, because deletion mutations induced by such low-LET radiations show wide range of variation in both size and frequently. Such low-LET radiation also causes large deletions, translocations and various rearrangements in plants[1)]. High-LET radiation, such as heavy-ions, can be controlled so as to deposit high energy at precise positions. Recently, heavy-ion irradiation has become a new method for mutation breeding to produce new cultivars. Heavy-ion irradiation-induced mutations at the molecular level have been most extensively studied in mammalian cells[2,3)]. It is reported that the frequency of deletion is higher for heavy-ion beams than for γ-rays[4,5)]. In the case of Arabidopsis plants, half of the mutants show small mutations, such as base changes and small deletions involving a few bases; the other half show large DNA alterations, such as inversions, translocations and deletions[6)]. From these results, it can be concluded that heavy-ion irradiation-induced mutations show a broad spectrum and a high frequency. We found that irradiation by heavy ion beams is very effective to produce the mutations of seed embryos at a particular stage during fertilization without damaging other plant tissues[7)]. We isolated albino, periclinal chimera, sectorial chimera, herbicide-tolerant and salt-tolerant mutants in tobacco[8,9)]. In this paper, we describe the mutational effect of heavy ions on rice seeds and the deleted regions in an albino-mutant line detected by the RLGS method. The rice plant is one of the most important food cereals. Sequencing of the rice genome has begun, and the genome organization of cereals appears to be very highly conserved; rice, wheat, maize, sorghum, millet and other cereals exhibit a high degree of synteny[10)]. Therefore, new mutants in rice induced by ion-beam irradiation could be important genetic resources for research in plant functional genomics. The RLGS method has the following advantages: (i) It has an informative scanning capacity, allowing the detection of thousands of landmarks in a single profile. (ii) Using different landmark enzymes extends the

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scanning field. (iii) The intensity of spots reflects the copy number[11–13]. We also demonstrate here the results as baseline data for the applications of heavy-ion beams to plant mutation research and genome analysis.

MATERIALS AND METHODS

Plant materials and heavy-ion irradiation

Rice seeds (*Oryza sativa* L. ssp. japonica) were soaked for 3 days in water with 0.8% agar at 30°C in the dark. Imbibition seeds were exposed to Ne-ion or C-ion accelerated to 135 MeV/u by the Riken Ring Cyclotron within a dose range of 5 to 100 Gy or 10 to 160 Gy, respectively. The LET values of the Ne and C ions were 64.2 and 22.7 keV/μm at the surface of the seeds. The ranges in water for these LET values are sufficient to penetrate: 2.3 cm and 4.0 cm for Ne ions and for C ions, respectively. After irradiation, seedlings were transplanted into soil in pots, and grown in a greenhouse at 26°C in the daytime (12 hrs) and 20°C at night (12 hrs). One month after irradiation, the number of surviving plants and the morphologically abnormal plants were counted, and the surviving plants were transplanted to a field. M_2 seeds from self-pollinated M_1 lines were harvested approximately 5 months after irradiation. M_2 seeds were sown on seedbeds, and grown in a greenhouse. One month later, the number of M_2 lines showing a chlorophyll-deficient phenotype was recorded. M_2 plants were transplanted to the field. M_3 seeds from self-pollinated M_2 green plants were harvested. We scanned the *Not*I landmarks by the RLGS method in albino mutants of the 8–17 line. Albino mutants in M_3 progeny were segregated from one M_2 greenplant. Eight albino plants in the M_2 progeny and 23 albino plants in the M_3 progeny underwent an RLGS analysis.

DNA preparation and the RLGS method

The total DNA was obtained from sterile plants grown in vitro to prevent contamination by parasites, such as endophytes and bacteria. DNA was prepared as described previously[13,14]. After the confirmation of intact DNA extraction by agarose-gel electrophoresis, 300 ng of DNA was used for each RLGS analysis. In the labeling step, DNA was digested with a landmark enzyme *Not*I. The cohesive ends of the fragments were filled in using Sequenase Ver. 2.0 (Amersham) in the presence of a radioisotope, such as [α-^{32}P]-dGTP (3000 Ci/mmol) or [α-^{32}P]dCTP (6000 Ci/mmol). The labeled DNA was electrophoresed in 0.8% agarose gels with a first-dimension buffer (100 mM Tris-HCl, 40 mM sodium acetate trihydrate, 35 mM NaCl, 3 mM EDTA, pH 8.0) and 5% sucrose at 4 V/cm for 22 hrs for 1D-electrophoresis. In-gel digestion was performed using the restriction enzyme *Mbo*I. 2D-electrophoresis was then performed in 5% polyacrylamide gel with the TBE buffer (50mM Tris-HCl, 60mM boric acid, 1mM EDTANa_2) containing 6M Urea at 3 V/cm for 22 hrs. Finally, the gels were dried and exposed to X-ray films (Kodak, XAX5) for 7 to 21 days at –80°C.

RESULTS AND PERSPECTIVE

The rates of survival and abnormality in the M_1 progeny observed after high-LET irradiations are shown in Fig. 1. The morphologically abnormal plants demonstrated retarded growth, dwarfism, white stripes or split leaves. The LD_{50} values were 56.2 Gy with Ne ion and 84.8 Gy with C ion. Thirty-seven M_2 progeny of 730 treated-lines and 42 M_2 progeny of 984 treated-lines were segregated into green plant and CDM showing albino, pale green-, yellow- or striped-leave phenotypes (Table 1). Higher frequencies of CDM of 11.6 and 7.31% were obtained after the irradiation of M_1 seeds with 20 Gy Ne-ion and 40 Gy C-ion, respectively, whereas the frequencies were 2.18% with γ-ray, 1.58% with thermal neutron and 1.16% with N-ion irradiation of dry seeds on LD_{50}[15]. The above results indicate that C and Ne-ion irradiation of imbibition seeds can induce a higher mutation rate compared to γ-rays, thermal neutrons or the N-ion exposure to dry seeds. Many CDM have been subjected to gene analysis, and more than 50 genes affecting chlorophyll, carotenoids pigments, have been identified. Also ten recessive alleles causing albinism, *al-1 – al-10*, were idendified[16]. The percentage of M_2 plants showing the phenotype of CDM in the 5–16 and the 8–17 lines was 6.7 to 6.8; this is assumed to be consistent with the value of 6.25% expected from the segregation ratio (15:1 hypothesis) for two recessive genes (Table 2). The segregation ratio in M_2 was considered to provide some information on the chimeric nature of the M_1 spikes. When the M_1 spikes are genetically uniform, the M_2 segregation ratios are normally expected to be equal to the inherent segregation ratios of induced mutations. The results of mutation studies have established the fact that M_1 spikes are chimerical for induced mutation in radiation treatments and chemical treatments of dry rice seed[17,18]. The observed low M_2 segregation ratios, as compared with the M_3 segregation ratios, suggested that the M_1 spikes were chimeric for induced mutations. To examine this point, we should further compare the segregation ratios of M_2 mutants and the segregation ratios of mutated M_3 strains.

The RLGS profile against rice genomic DNA using *Not*I

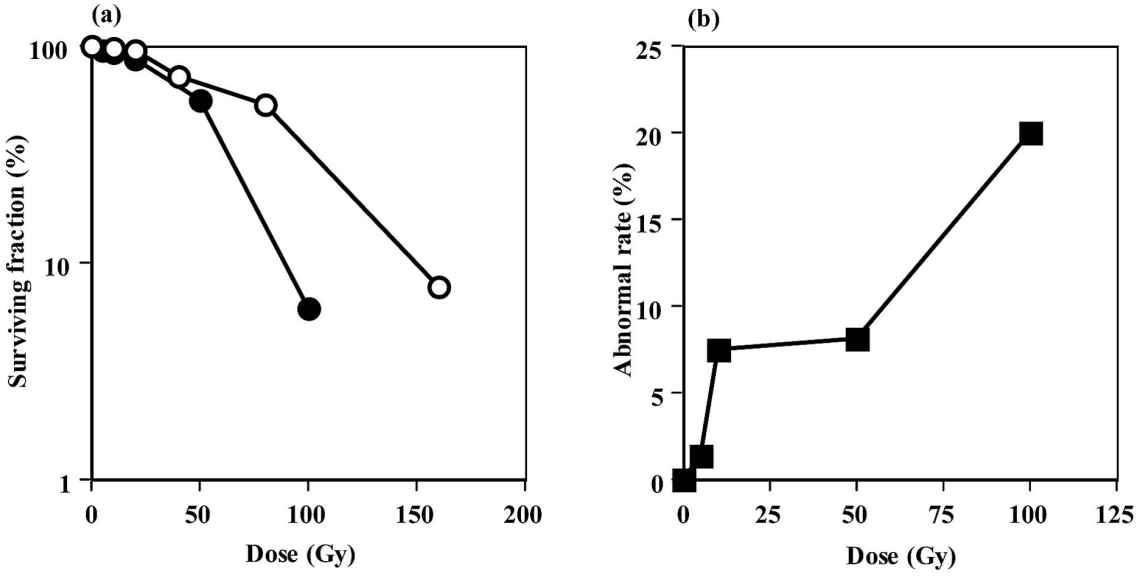

Fig. 1. Effects of irradiation on both the viability (a) and abnormality (b) of imbibition seeds.
(a) The surviving fraction after Ne-ion (●) or with C-ion (○) irradiation is expressed as percentage. (b) The rate of morphologically abnormal M_1 plants with Ne-ion (■) is also expressed as percentage.

Table 1. Frequency of chlorophyll deficient mutants (CDM) induced by heavy-ion irradiation

Ion	Dose (Gy)	Fertile M_1 lines	M_2 lines		Total CDM	Frequency of CDM (%)
			Albino	Others		
Ne	5	225	2	0	2	0.89
	10	196	11	1	12	6.12
	20	181	21	0	21	11.60
	50	115	0	2	2	1.74
	100	13	0	0	0	0
	Total	730	34	3	37	5.07
C	10	238	5	0	5	2.10
	20	228	11	0	11	4.82
	40	301	19	3	22	7.31
	80	200	3	0	3	1.50
	160	17	1	0	1	5.88
	Total	984	39	3	42	4.27

Table 2. Segregation of albino mutants in M_2 progeny after Ne-ion irradiation

line	Phenotype		Total
	Green	Albino	
5–16	139	10	149
6–19	273	14	287
8–17	273	20	293

5–16 and 6–19 lines induced by 10 Gy, 8–17 line induced by 20 Gy Ne-ion irradiation.

landmark can detect about 2,000 spots in a single experiment. An Albino mutant analysis demonstrated two polymorphic spots in RLGS profiles. These located spots at 2.2 kb in a 1D profile, and were present in all green plants, but not present in either profile of albino plants in the M_2 and M_3 progeny. One of the polymorphic spots is shown in Fig. 2. These spots may be derived from the same fragment possessing *Not*I sites at both end sides. This spot strongly links to the albino phenotypic variation, and this DNA fragment may contain genes related to the greening of plants. Recently, whole genome sequences of arabidopsis and rice have been published[19–21], and the accumulation of mutants as novel genetic resources will be developed (The Arabidopsis Information Resources: http://www.arabidopsis.org/, Arabidopsis Biological Resource Center: http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm, Ministry of Agriculture, Forestry and Fisheries Genebank: http://www.gene.affrc.go.jp/index.html, Rice Genome Program: http://rgp.dna.affrc.go.jp/, Rice GD: http://210.83.138.53/rice/reference.php,). The methods used in the present study, the induction of mutation by heavy-ion irradiation and genome analysis using RLGS, are expected to be powerful tools for not only post-genome plant research, but also the plant breeding field.

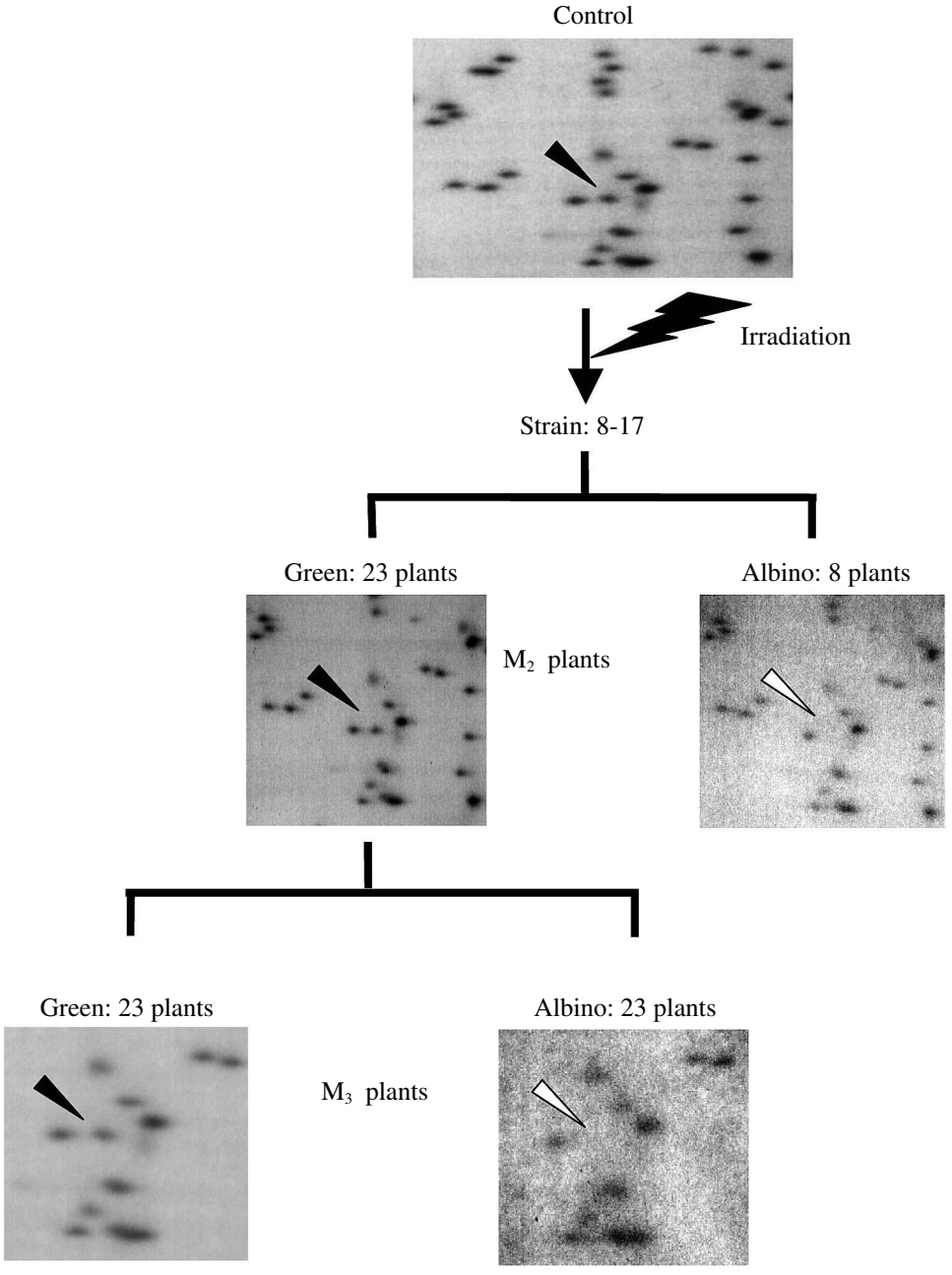

Fig. 2. RLGS analysis of an albino mutant line.
The results of an RLGS analysis for albino mutants in the 8–17 line are given here. These spots, indicated by black arrowheads (+) and white arrowheads (–), were generated in *Not*I-*Mbo*I profiles.

ACKNOWLEDGEMENTS

This work was supported by grants from the Research Project for the study of Biological Cross-talk Functions funded by the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government. The authors are grateful to Mr. Hideo Tokairin, Tohoku University, for his assistance in cultivating plants.

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Received on May 29, 2002
Revision on December 3, 2002
Accepted on December 6, 2002