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Effects of Gamma-ray Irradiation on Cultured Anthers of Tobacco (*Nicotiana tabacum* L.)——Radiosensitivity and Morphological Variants Appearing in the Haploid Plants

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Tobacco anthers which had been cultured for 0, 10 and 20 days were irradiated with 0 to 160 Gy of gamma-ray. Anthers cultured for 10 days before irradiation, which were correspondence to a plantlet initiation stage of microspore, were most radiosensitive and RD_{50} for RACR (defined as dose resulting in 50% reduction in relative anther culture response) was 8 Gy. On the other hand, RD_{50}s for RACR in the anthers precultured for 0 and 20 days were 50 Gy. The highest frequency of morphological variants was also obtained from the plants derived from irradiated anthers precultured for 10 days. These results suggest that gamma-ray irradiation on the anthers precultured for 10 days is most effective in inducing mutation in tobacco. Some of the variants were observed as sectorial chimera, but there was no clear relationship between the size of chimera and the developmental stage of the microspore at irradiation.

Introduction

One of the advantages of haploid breeding is that mutations induced at haploid stage can be detected directly in the plants derived from anther culture. After dihaploidization of the plants derived from anther culture, mutant lines are easily established in the case of both recessive and dominant mutations[1]. Several attempts of mutagenic treatment on cultured anthers have been reported in higher plants[2-4]. However, the protocol of mutation induction at haploid level has not been established.

It has been known that morphological variants were found in dihaploid plants derived from anther culture in some crop plants[5,6]. In tobacco (*Nicotiana tabacum* L.), in which haploid plantlets develop directly from microspore, mutants with disease resistance[7,8] and other agronomic characteristics[9,10] were obtained from dihaploid plants derived from anther culture. However, for enlarging genetic variability by anther culture techniques in tobacco breeding, it is necessary to induce more mutations at the haploid stage by treatment with gamma-ray[11] or a chemical mutagen[12,13] to anthers. In this experiment, the effects of gamma-ray irradiation at different stages of microspore development in tobacco on anther culture response and morphological variants appearing in anther-derived plants were investigated for determining the most effective mutagenesis.

Materials and Methods

1. *Plant material and gamma-ray irradiation*

A tobacco cultivar, Xanthi, was used. Anthers were excised from flower buds of 10 to 12 mm in length, which carried pollens at mid- and late-uninuclear stage. The collected anthers were cultured with modified Nakata's medium[14] in a growth chamber with continuous illumination of fluorescent lamps (3, 800 lx) at 25°C. Anthers, which had been cultured for 0, 10 and 20 days (preculture), were irradiated with 0 to 160 Gy of gamma-ray from ^{60}Co source of the Research Center of Radiation, University of Osaka Prefecture at a dose rate of 20 Gy/h at room temperature. Three stages of microspore examined in this experiment were correspondent to uninuclear, embryoid differentiating and embryoid developing stage, respectively[15]. Immediately after irradiation, anthers were transferred to the new medium with the same nutrient composition as before. Anthers without being irradiated were also transferred when the irradiated ones were done. In the case of irradiation on anthers without preculture, collected flower buds were irradiated directly and then the anther culture was initiated.

2. *Gamma-ray sensitivity*

Anthers were cultured for 50 days after the initiation and the anther culture response (ACR), which was defined as the percentage of anthers producing plantlets per total number of cultured anthers, was scored. Radiosensitivity of anthers was evaluated based on the relative anther culture response (RACR, per cent of non-irradiated control). To compare the effect of gamma-ray irradiation on different developmental stages of anthers, RD_{50} for ACR, which was defined as dose resulting in 50% reduction in RACR, was estimated from the figures showing the dose response. In each treatment, 120 to 425 anthers were used.

3. *Observation of morphological variation*

In each treatment, 50 plantlets derived from the cultured anthers irradiated with 2.5 to 40 Gy of gamma-ray were randomly chosen and were cultivated on a small bed with vermiculite for several weeks and then were transplanted to small pots with sterilized soil in a greenhouse under natural daylight. Twenty-five plants were cultivated as non-irradiated control in each preculture duration of anthers. Variations in morphological characteristics as shown in **Table 1** were scored at 15-leaves-stage and flowering stage.

Results and Discussion

1. *Radiosensitivity in tobacco anther*

In each preculture duration of anthers examined in this study, ACRs and number of plantlets per responded-anther decreased with the increase in gamma-ray dose (**Table 1**). In Experiment I, anthers were irradiated with 0, 2.5, 5, 10, 20 and 40 Gy of gamma-ray. In the anthers precultured for 10 days, ACR decreased with increase in gamma-ray dose at low level and RACR decreased to 19.8% at 40 Gy. From **Table 1**, RD_{50} for ACR in the anthers precultured for 10 days was estimated to be approximately 8 Gy. On the other hand, RACRs in the anthers precultured for 0 and 20 days did not decrease to under 50% at less than 40 Gy. Number of plantlets per responded-anther was not affected by irradiation (**Table 1**). In order to determine RD_{50} for ACR in the anthers precultured for 0 and 20 days, anthers were also irradiated with 0, 20, 40, 80 and 160 Gy of gamma-ray (Experiment II). From **Table 1**, anthers precultured for 0 and 20 days seemed to have similar levels of radiosensitivity and RD_{50}s for ACR were estimated to be approximately 50 Gy in spite of the fluctuations of the data. ACR in 20 Gy-irradiated anthers without preculture and ACR in 160

Table 1. Anther culture response (ACR) after gamma-ray irradiation to anthers containing different developmental stages of microspores.

Days of preculture	Dose (Gy)	No. of anthers cultured	No. of anthers responded	ACR (%)	No. of plantlets obtained
Experiment I					
0	0	120	39	32.5 (100*)	219 (5.6**)
	2.5	172	50	29.1 (89.5)	213 (4.3)
	5.0	178	51	28.7 (88.3)	179 (3.5)
	10	239	72	30.1 (92.6)	335 (4.7)
	20	195	52	26.7 (82.1)	284 (5.5)
	40	289	82	28.4 (87.4)	200 (2.4)
10	0	207	113	54.6 (100)	388 (3.4)
	2.5	204	68	33.3 (61.0)	207 (3.0)
	5.0	195	74	37.9 (69.4)	205 (2.8)
	10	277	62	22.4 (41.0)	171 (2.8)
	20	345	57	16.5 (30.2)	103 (1.8)
	40	425	46	10.8 (19.8)	127 (2.8)
20	0	186	98	52.7 (100)	280 (2.9)
	2.5	208	108	51.9 (98.5)	313 (2.9)
	5.0	190	75	39.5 (75.0)	182 (2.4)
	10	180	58	32.2 (61.1)	122 (2.1)
	20	251	125	49.8 (94.5)	253 (2.0)
	40	337	120	35.6 (67.6)	162 (1.4)
Experiment II					
0	0	159	39	24.5 (100)	263 (6.7)
	20	145	30	20.7 (84.5)	142 (4.7)
	40	169	23	13.6 (55.5)	94 (4.1)
	80	172	18	10.5 (42.9)	13 (0.7)
	160	176	19	10.8 (44.1)	6 (0.3)
20	0	160	88	55.0 (100)	473 (5.4)
	20	119	38	31.9 (58.0)	216 (5.7)
	40	128	42	32.8 (59.6)	176 (4.2)
	80	149	30	20.1 (36.5)	193 (6.3)
	160	171	10	5.8 (10.5)	8 (0.8)

* Relative anther culture response (RACR)

** No. of plantlets per anther responded

Gy-irradiated anthers precultured for 20 days seemed to be under- and over-estimated, respectively. These results indicated that, among the three stages of microspore development examined in this experiment, anthers precultured for 10 days were most radiosensitive. The high radiosensitivity is possibly due to the fact that the anthers precultured for 10 days have the microspores at the plantlet initiation stage.

It was interesting that ACRs in the non-irradiated anthers precultured for 10 and 20 days were higher than the anthers without preculture. ACRs of precultured anthers were 52.7 to 55.0%, while ACRs of non-precultured anthers were 24.5 to 32.5%. The higher ACR might be explained as stimulus effect of exchanging medium during the anther culture[16].

2. *Morphological variants in haploid plants derived from anther culture*

In tobacco, several useful variants have been selected from the dihaploid plants derived from anther culture[7,17-19]. This experiment shows the effectiveness of gamma-ray irradiation to anthers for increasing the frequency of variants. A considerable number of variants (10.0 to 28.6%) were obtained in the anthers irradiated with the lowest dose, 2.5 Gy. In the anthers precultured for 10

Table 2. Number of morphological variations appearing in plants derived from gamma-ray irradiated anthers.

Days of preculture		No. of plants	No. of variants	No. of plants showing variant trait*						
				Plant type	Leaf shape	Leaf color	Leaf spot	Flower shape	Flower color	Diploid like
0	Control	25	0	0	0	0	0	0	0	0
	Irradiated	247	61	20	15	14	31	7	2	0
10	Control	25	2	0	0	0	1	1	0	1(1**)
	Irradiated	245	93	45	31	9	27	22	3	7(6)
20	Control	25	1	0	0	0	1	0	0	0
	Irradiated	240	64	26	8	9	23	9	3	3(1)

* Total number of the plants showing a variant trait is not equal to the number of variants, because some variants had more than two variant traits.

** No. of plants producing seeds.

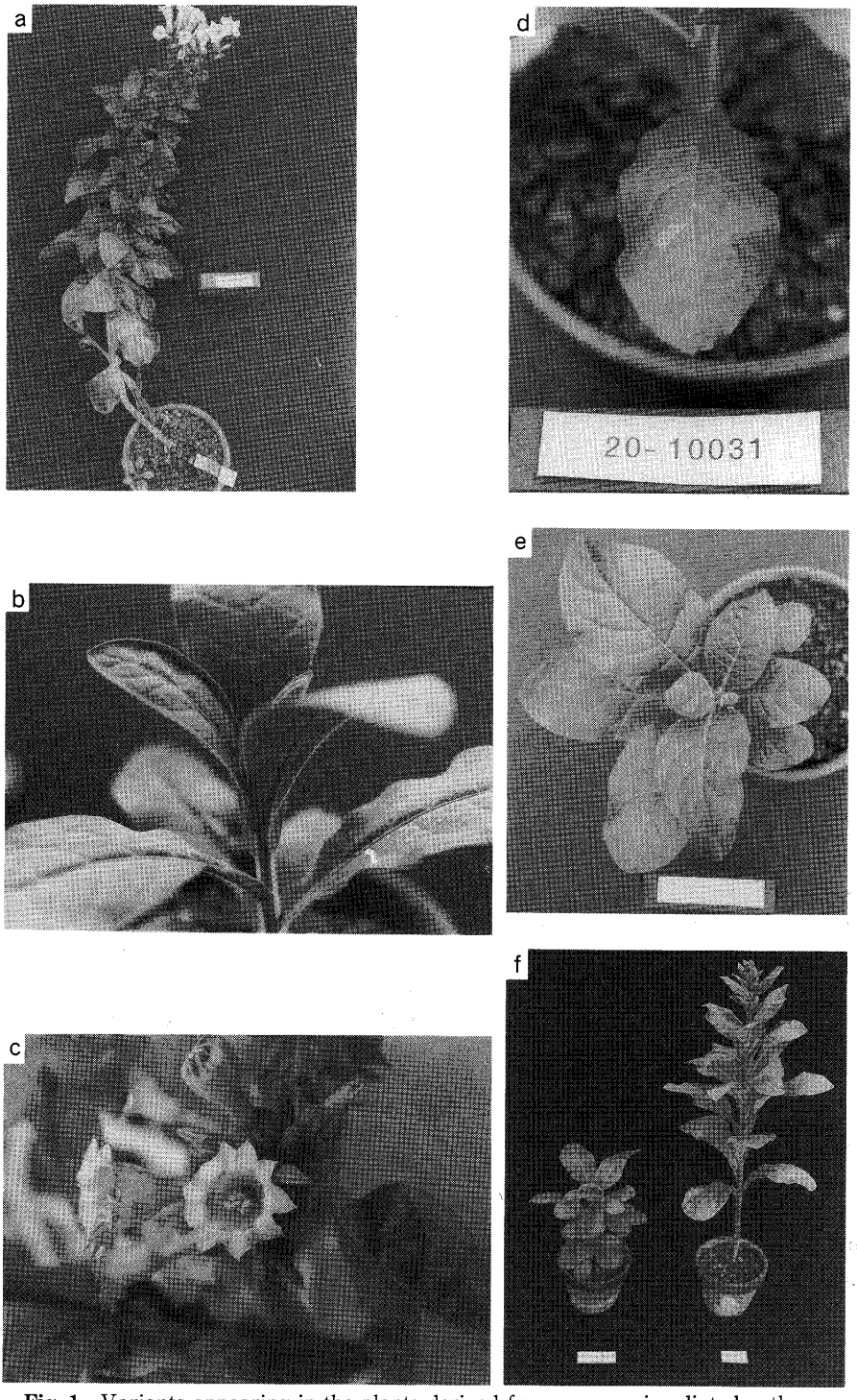

Fig. 1 Variants appearing in the plants derived from gamma-irradiated anthers.
a : Fasciation variant described as a genetic trait.
b : Curly leaf variant described as a genetic trait.
c : Aberrant flowers.
d : Pale green leaf spot.
e : Small leaf variant appearing as a sectorial chimera.
f : Dwarf (left) and normal haploid plant (right).

days, the frequencies of variants (26.5 to 56.0%) were highest among the stage examined in this experiment. In the anthers precultured for 0 and 20 days, the frequencies ranged from 18.0 to 28.6% and 10.0 to 38.3%, respectively. Three variants were obtained from non-irradiated anthers (**Table 2**).

As for the spectrum of variants, morphological changes in plant type, leaf and flower characters were frequently observed (**Table 2**). Some plants had more than two kinds of variant traits. **Fig. 1a-f** show the typical variants observed in this experiment. At present, it has not been concluded whether the variants obtained in this experiment are real mutants or not. However, the characteristics described as genetic traits such as fasciation and curly leaf[20,21] were found in the variants. This fact suggests the practical usefulness of gamma-ray irradiation to anthers in tobacco breeding, although no variants with agronomically useful traits were obtained in this experiment.

Two kinds of interesting variants were found. Firstly, eight plants showing seed fertility were classified as diploid-like variants. Six of eight were found in the plants derived from gamma-ray -irradiated anthers precultured for 10 days. Secondly, small pale green spots (**Fig. 1-d**) in the leaves were observed in the plants derived from gamma-irradiated anthers with the frequencies of 9.6 to 12.6% on a plant basis. The spots appeared most frequently around 6th leaf but there was no clear relationship between the frequency of plants showing the leaf spots and gamma-ray dose or developmental stage of microspore at irradiation, except for the disappearance of the spot in the upper (more than 18th) leaves.

Of the variants obtained in this experiment, some characteristics appeared as sectorial chimera (**Fig. 1-e**). The size of chimera appearing after gamma-irradiation can be useful trait for speculating the developmental stage of microspore at irradiation. However, no clear relationship was obtained between the size of the chimera and the preculture duration. It was curious that sectorial chimera was observed in the plants derived from anthers irradiated before culture initiation, because primordium of a generated plant was considered to be a single microspore at the time of irradiation. It is necessary to investigate chimera formation in the plants derived from irradiated anthers.

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《和文要約》

タバコの葯培養へのガンマ線照射効果
——放射線感受性ならびに半数体植物にみられる形態形質の変異体——

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葯培養を利用したタバコの突然変異育種の可能性を調査するため，葯を0，10および20日間培養した後にガンマ線照射を行った．葯反応率に関する RD_{50}(葯反応率を対照区の50%に低下させる線量)は10日間培養区で最も低く8 Gyであり，0および20日間培養区ではともに50 Gyであった．半数体植物にみられる変異体の出現頻度も10日間培養区で最も高くなった．ガンマ線照射を行った葯培養由来の半数体植物から多くの形態変異体が得られたが，そのなかには明らかに突然変異によると考えられる形質も含まれており，本法の有効性が示唆された．