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Potato Virus Y-resistant Mutation Induced by the Combination Treatment of Ion beam Exposure and Anther Culture in *Nicotiana tabacum* L.

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Abstract

To develop an efficient procedure for obtaining a desired mutation, we performed ion beam exposure on tobacco anthers and selected Potato Virus Y (PVY)-resistant mutants in the haploid generation.

Anthers were exposed to C ion beams of 5 to 200Gy or He ion beams of 5 to 400Gy, and cultured on modified Nakata's medium. Anther culture response, the percentage of anthers producing plantlets, was markedly reduced by the exposure and, at higher doses, all plantlets were vitrified. RD_{50} based on the anther culture response was about 5Gy for C ion beams and about 10Gy for He ion beams. The frequency of mitotic cells with chromosome aberrations in root tips of plantlets derived from exposed anthers, ranged from 11.9% to 16.4%, compared to 3.1% in the control.

All plants in the non-exposed regimen died within 21 days after inoculation of PVY. On the other hand, 15 of 472 plants in the exposed regimen were viable and continued to grow, with minor disease symptoms observed in some plants after 50 days. Accordingly, these plants were considered to be PVY-resistant. In particular, one plant showed no disease symptoms and grew vigorously.

Abbreviations

PVY, Potato Virus Y; LET, Linear Energy Transfer

Introduction

Potato virus Y (PVY) is highly variable and different strains cause different disease reactions on the host genotypes (Gooding 1985). This virus is distributed worldwide, resulting in economic losses in many solanaceous crops. In tobacco, *Nicotiana tabacum* L., several varieties are resistant to PVY, such as Virgin-A Mutante, Perevi and Havana 307. High resistance to PVY strain N in Virgin-A Mutante is controlled by a single recessive gene derived from a mutation induced by X-ray-irradiation (Koelle 1961).

Haploid plants are useful to detect gene expression, including recessive mutations, and can be genetically fixed by chromosome duplication. Procedures for producing haploid plants have been established for different plant species (Sunderland 1974, Maheshwari *et al.* 1982). To obtain a useful mutant, anther culture combined with radiation and chemical treatment has been used (Mondeil 1974, Medrano *et al.* 1986, Kinoshita *et al.* 1989, Hasegawa *et al.* 1995).

Ion beams have higher Linear Energy Transfer (LET), compared to that of X-rays or gamma-rays, and the penetration range can be controlled in the target material. Accordingly, a lot of energy can be deposited on the focused point of material exposed to ion beams. Therefore, ion beams are considered to cause different biological effects. In *Arabidopsis thaliana*, Tanaka *et al.* (1997) reported that He, C and Ne ion beams more effectively reduced germination and growth than electrons. Nagatomi *et al.* (1996) reported that mutations of flower-color and -shape in chrysanthemum exposed to C ion beams were different from those irradiated with gamma-rays. In *Nicotiana tabacum*, the phenomenon specific for ion beam exposure, "leaky pollen", in which internal substances leak through an opening in the outer wall of the pollen grain, has been reported (Inoue *et al.* 1992).

In the present study, we utilized characteristics of anther culture and ion beams to induce a PVY-

resistant mutation and to select a mutant in *N. tabacum*. The usefulness of the combination treatment was also discussed.

Materials and Methods

Plants of *Nicotiana tabacum* L. cv. Bright Yellow 4 (2n = 48, susceptible to PVY) were grown in a greenhouse at 25-30 ℃ under natural daylight conditions. Anthers with pollen at the mid- to late-uninuclear stage were collected from flower buds, 10-15 mm in length. They were cultured on modified Nakata's medium (Nakata and Kurihara 1972), in petri dishes (35 mm in diameter) set in a growth chamber continuously illuminated with fluorescent lamps (2500 lux) at 26 ℃. The medium contained 25mg l^{-1} Fe-EDTA in stead of 37.35mg l^{-1} Na_2 EDTA and 27.85mg l^{-1} $FeSO_4$-$7H_2O$, 1.5mg l^{-1} Indole-acetic acid in stead of 1.5mg l^{-1} kinetine, 3g l^{-1} gellan gum in stead of 6g l^{-1} agar, and 3g l^{-1} activated carbon. After 2 days, the anthers were covered with sterilized kapton film (7.5 μm thickness, Toray-Dupont Co., Ltd., Japan), and exposed to 220 MeV C ion beams of 5 to 200Gy or to 50 MeV He ion beams of 5 to 400Gy from AVF Cyclotron Accelerator (Takasaki Ion Accelerators for Advanced Radiation Application, JAERI). About 200 anthers were used in each dose. Properties of ion beams are shown in **Table 1**. Four days after exposure, anthers exposed to ion beams were transferred to fresh medium in glass bottles (60 × 110 mm) and cultured in a growth chamber under the same conditions mentioned above. Fifty days after the initiation of culture, the anther culture response, percentage of anthers producing plantlets, was determined. Considering RD_{50}, the dose resulting in 50% reduction in the control ratio of anther culture response, about 350 anthers were exposed to C ion beams of 5 and 10Gy or to He ion beams of 5, 7 and 10Gy, and cultured under the same conditions described above. Plantlets obtained were transferred to plastic pots (7 × 7 × 16 cm) with vermiculite and cultured in a growth chamber at 26 ℃ under light condition.

During the course of plantlet growth, the frequency of mitotic cells with chromosome aberrations in root tips was determined. In each dose regimen, root tips were collected from about 15 plantlets, fixed in 3 ethanol : 1 acetic acid solution for 1-2 days at 4 ℃ and stored in 70% ethanol at 0 ℃. They were washed in distilled water for 3 min, hydrolyzed in 1 N HCl for 7 min at 60 ℃ and stained with Feulgen reagent (5g l^{-1} Fuchsine Basic). More than 10 cells were observed in each root.

When plants developed 7-8 leaves, 40-50 mm in length, a young leaf near the top of each plant was dusted with carborundum (600 mesh) and inoculated with a suspension of PVY strain T. The suspension was prepared by grinding 1g fresh leaf tissue of PVY-infected "Bright Yellow 4" in 50 m*l* buffer (0.05 M Na_2HPO_4-KH_2PO_4, pH 7.0). Inoculated plants were kept in a greenhouse controlled at 25 ℃ . PVY-resistance was judged, based on the degree and time of symptom appearance, and plant survival.

Roots of PVY-resistant mutants were collected and pretreated in distilled water at 0 ℃ for 24 h. After preparing chromosome spreads using the procedure mentioned above, the chromosome number of each mutant was ascertained.

Table 1. Properties of ion beams used in this experiment

Ion	Energy (MeV)	LET (keV/μm)	Penetration depth (mm)
$^{12}C^{5+}$	220	111	1.0
$^{4}He^{2+}$	50	15	1.5

Results and Discussion

As shown in **Fig. 1**, the anther culture response was markedly reduced by ion beam exposure as follows: 78.3% in the control; 25.0%, 16.3% and 5.8% in C ion beams of 5, 25 and 200Gy; 66.7%, 51.7% and 0% in He ion beams of 5, 7 and 50Gy, respectively. One to 5 plantlets were obtained from each anther in both the exposure and non-exposure

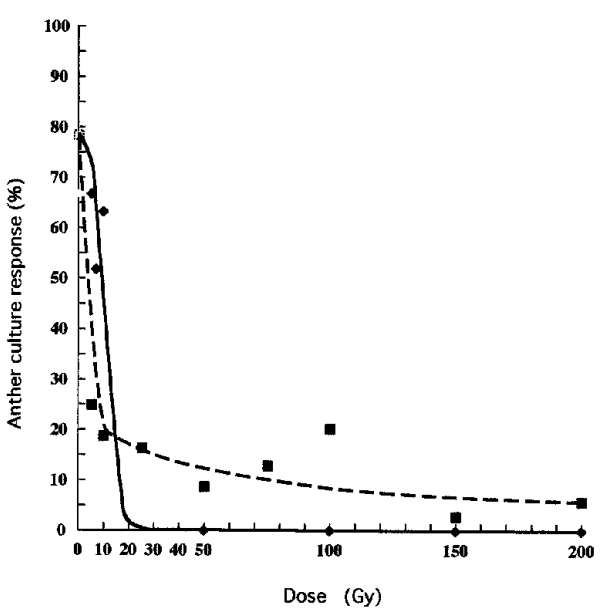

Fig. 1 Anther culture response following exposure to ion beams.
◆, He ; ■, C.

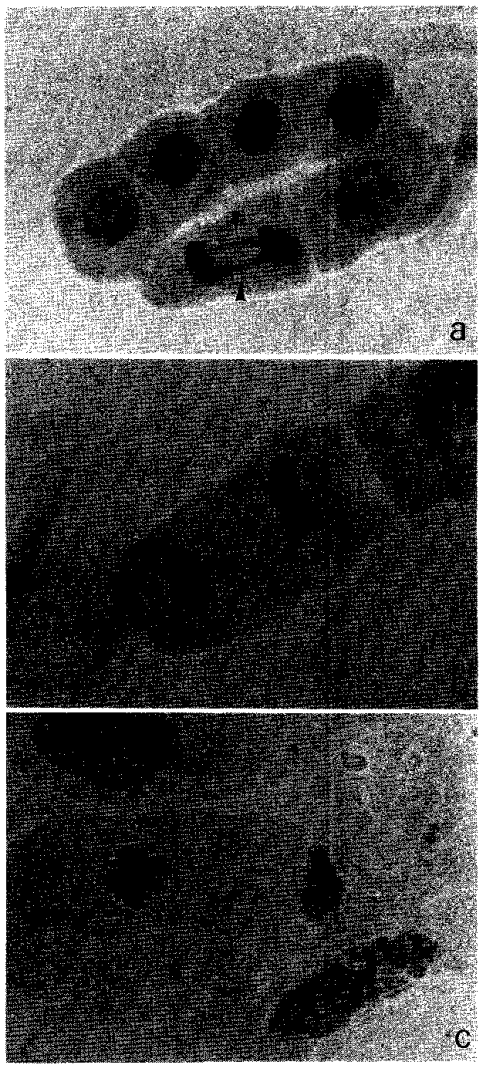

Fig. 2 Chromosome aberrations observed in root tip cells of plantlets
a : bridge. b : fragment. c : lagging.

regimens, but the relationship between the number of plantlets per anther and exposure doses were unclear. Although several plantlets were obtained with doses over 150Gy of C ion beams, all of these plantlets were vitrified and could not survive. In addition, no callus formation was observed in this experiment. Considering that the penetration depth of the C ions used here was 1 mm (**Table 1**) and that the average length of anthers was 1.2 mm along the exposure direction, some microspores at the bottom part of anthers may not have been irradiated sufficiently. In wheat, Ling *et al.* (1991) demonstrated that anther culture response was enhanced by low dose of gamma-rays-irradiation. However, no such effect was observed in this experiment.

Based on the dose-response curve of the anther culture response, RD_{50} was about 5Gy for C ion beams and about 10Gy for He ion beams. In *N. tabacum*, Hasegawa *et al.* (1995) reported that RD_{50} of the anther culture response following gamma-rays-irradiation was 40Gy. Combined with our data on RD_{50}, the findings suggest that ion beams can induce more biological effects than gamma-rays.

Considering RD_{50}, anthers were exposed to C ion beams of 5 and 10Gy or He ion beams of 5, 7 and 10Gy, and cultured. Three types of chromosome aberrations, including bridge, fragment and lagging (**Fig. 2**), were observed in the root tip cells of plantlets. The frequencies of mitotic cells with chromosome aberrations were significantly enhanced by ion beam exposure, although the dose-effect was not recognized (**Table 2**). Mei *et al.* (1994) demonstrated that high-LET ions induced chromosome deletion and rearrangement. In the present experiment, chromosome fragments were frequent at all doses of C and He ion beams. However, there was no difference in the aberration spectrum between the exposure regimens.

When plants developed 7-8 leaves, they were inoculated with PVY. In plants derived from the

Table 2. Frequency of mitotic cells with chromosome aberrations in root tips of plantlets obtained from culture of anthers exposed to ion beams

Ion	Dose	Mitotic index (%)	Number of mitotic cells observed	Number of mitotic cells with chromosome aberrations			Frequency of mitotic cells with chromosome aberrations (%)
				Bridge	Fragment	Lagging	
Control		6.5	258	4	3	1	3.1
C	5Gy	5.8	549	11	42	25	14.2***
	10Gy	6.7	217	7	13	6	12.0***
He	5Gy	6.5	390	10	32	22	16.4***
	7Gy	6.0	162	3	14	6	14.2***
	10Gy	6.3	522	13	28	21	11.9***

*** Significantly different from control at 0.1% level by the chi-square test.

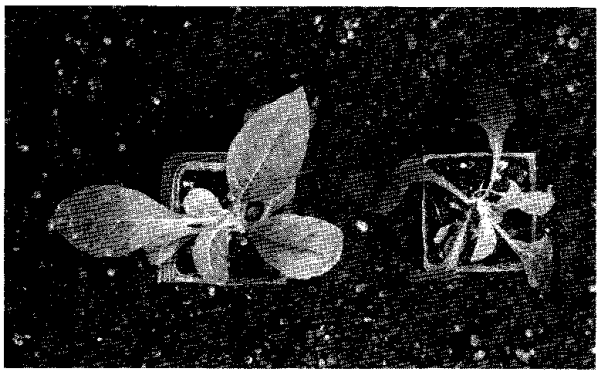

Fig. 3 Haploid plant resistant (right) and susceptible (left) to potato virus Y race T, 21 days after inoculation. Severe symptoms such as veinal necrosis, mottling puckered leaves and stunting were observed in susceptible plant.

non-exposed anthers, veins in the leaf inoculated turned brown 10 days after inoculation, vein banding extended to the upper leaves and leaves turned yellow. Then, necrotic symptoms spread over the whole area of the leaves and stem (**Fig. 3**). Finally, all plants died within to 21 days after inoculation (**Table 3**). On the other hand, some plants survived in the exposure regimen (**Fig. 3**). Among the 472 plants derived from anthers exposed to C and He ion beams, 15 plants had no disease symptoms at 40 days after inoculation. Thereafter, they continued to grow in a greenhouse, with minor symptoms, such as necrotic symptoms in some leaves, observed in some plants at various times after 50 days. One plant obtained in He ion beams of 10Gy, showed no disease symptom during the observation period, and grew vigorously. These 15 plants were considered to be resistant to PVY. Mutant frequencies in C ion beams of 10Gy, He ion beams of 5 and 10Gy were 2.9, 3.9 and 3.4%, respectively (**Table 3**). All mutants had 24 chromosomes (**Fig. 4**) and sticky leaves as seen in plants obtained from the non-exposed anthers.

Fig. 4 Twenty-four chromosomes observed in PVY-resistant mutant.

We concluded that the combination treatment of anther culture and ion beam exposure is efficient to obtain a disease-resistant mutant. With regard to other characteristics associated with PVY-resistance, low yield, poor quality of leaves and the inability to produce leaf surface exudates were reported (Burk and Chaplin 1980, Nielsen *et al.* 1982, Komari *et al.* 1986). These features are considered to result from the tight linkage between the gene(s) controlling PVY-resistance and other genes. Since PVY-resistant mutants obtained here showed sticky leaves, they likely produce leaf surface exudates. If so, they may be useful as new sources to produce PVY-resistant tobacco.

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Table 3. Frequency of mutants resistant to PVY

Ion	Dose	Number of plants inoculated	Number of plants		Frequency of resistant mutants (%)
			susceptible	resistant	
Control		91	91	0	0
C	5Gy	42	42	0	0
	10Gy	104	101	3	2.9
He	5Gy	255	245	10	3.9
	7Gy	13	13	0	0
	10Gy	58	56	2	3.4

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