WIDENING OF GENETIC VARIATION
BY TISSUE CULTURE

Report of Symposium
held on
July 19-20, 1984

Institute of Radiation Breeding
NIAR MAFF
Ohmiya-machi, Naka, Ibaraki
Japan
The chairman and speakers in general discussion
From left:
Dr. T. Shiga
Dr. A. Hirai
Dr. H. Uchimiy
Dr. K. Yamamoto
Mr. O. Yatou
Dr. K. Oono
Dr. T. Nagata

Prof. T. Matsuo greets the participants at the reception party

Visit to Gammafield. Irradiation tower in the back
List of Participants

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FOREWORD

Mutation breeding methods have so far produced many valuable mutants which were released directly as new varieties or employed indirectly as parents in cross breeding. These useful mutated genes were obtained by treatment of seeds or plants with physical or chemical mutagens, but application of tissue cultures to mutation breeding may open further a new aspect in this area of research. Although it is known that genetic variability at the levels of genome, chromosome and gene are induced on culture media without mutagenic treatments, it is also reported that application of mutagens on cultured cells do increase mutation rate in the cell populations. Generating of genetic variability through the novel techniques such as tissue culture are widely expected to provide genetic resources that cannot be obtained by conventional means.

Under these circumstances, the committee decided to hold a symposium on tissue culture and other biotechniques in relation to mutation breeding.

The meetings were held in a small town ohmiya and 287 participants attended inspite of remoteness of the place from a big city. The Committee hopes that this Symposium serves as an aid to develop mutation breeding at cellular levels. The Committee wishes to express its hearty thanks to the speakers, chairmen, and to those who undertook the task of preparing the meeting for their contribution to the Symposium.

The Symposium Committee

Kunio Toriyama, Chairman
Etsuo Amano
Taro Fujii
Takane Matsuo
Tetsuo Nakajima
Toranosuke Shichijo
Sachihiko Tanaka
Yasuo Ukai
Hirotada Yamagata
Hikoyuki Yamaguchi
PROGRAM

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Session I
Chairman: T. KAWAI
Prospects of plant improvement by tissue culture ....................... T. NAKAJIMA

Session II
Chairman: T. ADACHI
Chloroplast genomes in hybrid calli derived from cell fusion ............ A. HIRAI
Variance and molecular analysis in somatic hybrid plants .............. H. UCHIMIYA

Session III
Chairman: K. YAMAKAWA
Sexual transfer of a portion of paternal genome by means of
irradiated pollen ......................................................... K. YAMAMOTO

Session IV
Chairman: Y. WATANABE
In vitro mutation breeding in medical plants .................... H. KUKIMURA and O. YATOU
High frequency of somaclonal mutations in callus culture of
rice, Oryza sativa L. ..................................................... K. OONO

Session V
Chairman: S. TANO
Genetic engineering of plant cells ..................................... T. NAGATA

Session VI
Chairman: T. SHIGA
General discussion
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General discussion (in Japanese)
CHLOROPLAST GENOMES IN HYBRID CALLI
DERIVED FROM CELL FUSION

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Introduction

In plant cells, the genetic information is stored in three different organelles, namely the nucleus, the chloroplast and the mitochondrion. The nucleus is fused with another nucleus in the event of sexual hybridization, thus the genetic information in nuclear DNA can be crossed and mixed. Using this principle, the major plant breeding has been performed. On the other hand, organelles in the cytoplasm are solely transferred from maternal parent to the hybrid, thus the genetic information in these organelles is inherited maternally. Most breeding programs, therefore, have concentrated on nuclear genomes with relatively little attention being paid to these organelle genomes, because the appropriate technology to improve the genomes was lacking.

A chloroplast contains 130-160 kb long circular DNAs. Studies in several laboratories showed that this DNA codes many important genes for photosynthesis (Shinozaki and Sugiura 1982; Westhoff 1983). So far, the genes of the large subunit of ribulose 1,5-bisphosphate carboxylase, α, β and ε subunits of chloroplast coupling factor I (CF₁), I and III subunits of CF₀, cytochrome f and b₆, P₇₀₀ chlorophyll and apoprotein and 32KD protein of photosystem II reaction center are located in chloroplast DNA (ctDNA).

Photosynthesis is, in many cases, the limiting factor of plant growth. Therefore the improvement of photosynthetic activity in plants is essential for increase of plant productivity. Since many important genes for photosynthesis are coded in ctDNA, it is essential to improve the DNA for this purpose.

Cell fusion between two different types of cells brings about the mixture of two kinds of chloroplasts as well as the fusion of two nuclei. Thus it may be possible to improve ctDNA by cell fusion. In this paper, we will show the results of studies about chloroplast genomes in hybrid calli derived from cell fusion mainly using Fraction I protein as a marker for chloroplast genomes.
Fraction I protein is a major soluble protein in green leaves (Wildman 1976) and has enzymatic activities of ribulose 1,5-bisphosphate carboxylase and oxygenase. It consists of large and small subunits which are coded by chloroplast (Chan and Wildman 1972) and nuclear (Kawashima and Wildman 1972) DNA, respectively. The polypeptide composition of the protein, revealed by isoelectrofocusing in 8 M urea, shows a unique composition for each plant species in many cases, serving as a genetic marker for chloroplast and nuclear genomes (Kung 1976). Analysis by this method has been widely used in *Nicotiana* and other genera.

This protein has been employed to characterize the gene expression in parasexual hybrids produced by protoplast fusion of higher plants (Chen et al. 1977; Melchers et al. 1978). In these cases, it was found that nuclear genes coding for Fraction I protein small subunit (SS) polypeptides from both parents seemed always to be expressed whereas only one or the other of the chloroplast genomes coding for the Fraction I protein large subunit (LS) polypeptides was expressed. Elimination of one of the chloroplast genomes seemed to be on a random basis with an equal chance that one or the other would be missing in the hybrid plants. The question arose, therefore, as to the nature of the mechanism responsible for elimination of one kind of chloroplast genome. Previous results were obtained by analysis of Fraction I protein obtained from leaves of parasexual hybrid plants regenerated from calli. However, it is conceivable that elimination of one of the kinds of chloroplast genomes did not occur until after a callus was induced to differentiate shoots and leaves. The recent development of a method (Hirai 1982) of analyzing the electrofocussing composition of Fraction I protein obtained from a minute amount of green callus made possible a test of whether elimination of one kind of chloroplast genome is an early or late event in the formation of parasexual hybrid plants.

**Materials and Methods**

1) **Plant materials**

Seeds of *Nicotiana glauca* and *N. Langsdorffii* were obtained from Japan Monopoly Corporation. Plants were grown in a greenhouse where the temperature was controlled between 20° and 25°C. Suspension culture cells were initiated from calli which were obtained by a method described previously (Hirai 1982), and cultured in MurashigeSkoog medium (Murashige and Skoog 1962) supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg/l kinetin in the case of *N. glauca* and 3 mg/l naphthaleneacetic acid (NAA) and 0.2 mg/l benzyladenine in the case of *N. langsdorffii*.
2) Cell fusion and selection

Protoplasts were isolated by the method previously reported (Hirai and Wildman 1977) and fused by the aid of polyethylene glycol (Uchimiya 1981). Polyethylene glycol-treated protoplasts were cultured in Murashige-Skoog medium supplemented with 3 mg/l 2,4-D, 0.5 mg/l kinetin and 72 g/l glucose until most of the cells divided 2 or 3 times. The cell clusters were transferred to 0.5% agar containing Murashige-Skoog medium with 63 g/l glucose and without any phytohormones. Only cell clusters derived from interspecific fused cells could grow and become green calli on this medium (Schaeffer and Smith 1963; Carlson et al. 1972).

3) Microscopic observation

Observation was made with an Olympus CKP-type inverted microscope. Photomicrographs were taken with Fujichrome 100 color film and printed in black and white.

4) Analysis of Fraction I protein

The method of Uchimiya et al. (1979) was used with modifications. Half gram of green callus tissue was ground with mortar and pestle in 1 ml of buffer B (Chan et al. 1972, 25 mM Tris-HCl pH 7.4, 200 mM NaCl, 0.5 mM EDTA) containing 100 mM 2-mercaptoethanol and 0.5% sodium ascorbate. The homogenate was centrifuged at 10,000 xg for 4 min and the supernatant was passed through a Sephadex G-25 column equilibrated with buffer B. Boid volume fraction was centrifuged at 20,000 xg for 20 min and the supernatant was incubated at 37°C for 2 hrs. and at 4°C overnight with antiserum prepared against tobacco crystalline Fraction I protein. The antibody-Fraction I protein complex was precipitated by centrifugation and washed twice with buffer B. The complex, in a test tube, was dissolved in 30-70 µl of a buffer containing 0.5 M Tris-HCl (pH 8.5), 1 mM EDTA, 8 M urea and 50 mg/ml of dithiothreitol and sealed with a serum cap. The test tube was evacuated and then filled with N₂ gas using a hypodermic needle through a serum cap, and kept at 25°C for 2 hrs. The protein solution was then passed through Sephadex G-25 equilibrated with 8 M urea in 0.5 M Tris-HCl (pH 8.5), by the centrifugation method (Neal and Florini 1973). The protein fraction at the bottom of the centrifugation tube was immediately isoelectrofocused using the technique of Kung et al. (1974).

5) Preparation of chloroplast DNA

The method of chloroplast DNA (ctDNA) isolation was essentially the same as that of Saltz and Beckman (1981). Callus tissue was briefly blended in a homoblender (Nihon Seiki Co. Ltd.) with two volumes of Kool's buffer A (50 mM Tris-HCl, pH 8.0; 0.35 M Sucrose; 7 mM EDTA; 5 mM 2-mercaptoethanol) containing 0.1% bovine serum
albumin. The homogenate was filtered through four layers of gauze. The filtrate was filtered again through two layers of Miracloth and centrifuged for 10 min at 1000 xg. The green pellet was washed once with Kool's buffer A. The pellet suspended in Kool's buffer A was loaded on top of a stepwise 20-45-60% sucrose gradient made in 50 mM Tris-HCl, pH 8.0; 0.3 M sorbitol; 7mM EDTA and centrifuged for 30 min at 2000 xg. The green band at the middle of the tube was collected, diluted 1:3 with Kool's buffer B (50 mM Tris-HCl, pH 8.0; 20 mM EDTA), and centrifuged for 10 min at 3000 xg. The chloroplast pellet was resuspended in Kool's buffer B, and lysed by adding sodium dodecylsarcosinate at a final concentration of 3%. One twentieth volume of 10 mg/ml Pronase E (Kaken Kagaku Co. Ltd.) was added to the solution, and incubated overnight at 37°C. DNA was twice extracted from the lysate with phenol and once with phenol-chloroform (1:1). The DNA was precipitated from the aqueous phase to which 0.1 volume of 3 M sodium acetate was added with 2.5 volumes of ethanol. The precipitate was washed twice with 70% ethanol and dissolved in TE buffer (1 mM Tris-HCl, pH 8.0; 0.1 mM EDTA).

6) Restriction endonuclease analysis

Chloroplast DNAs were digested with Bam HI (Takara Shuzo Co. Ltd.) according to the supplier's instructions. The sizes of fragments were analyzed by electrophoresis on 0.7% agarose gels by the method of Sugiura and Kusuda (1979).

Results

1) Mixture of two kinds of chloroplast genomes in a fused cell (Akada et al. 1983)

To be certain that two different kinds of chloroplast genomes were mixed within the cell arising from fusion of protoplasts derived from two different species of plants, microscopic observations were made. They were facilitated by use of protoplasts obtained from suspension cultures of N. langsdorffii because suspension cells do not contain organelles that are green in appearance. The other protoplasts were obtained directly from N. glauca leaf cells where green chloroplasts are the most conspicuous organelles in the cells after the nucleus. As shown by the photomicrograph in Fig. 1, the green N. glauca chloroplasts were still conspicuous 48 hr after a cell was observed to arise following fusion of a colorless N. langsdorffii protoplast with a green N. glauca protoplast. The chloroplasts appear to be broadly dispersed throughout the parasexual hybrid cell. We also observed that when a fused cell divided, leaf chloroplasts were about equally distributed within the two daughter cells.
Fig. 1. Photomicrographs of a cell observed to have arisen by fusion of a protoplast from an *N. langsdorffii* suspension cell and a protoplast from an *N. glauca* leaf cell. 1: 1 h after protoplast fusion; 2: 24 h; 3: 48 h; 4: 72 h; 5: 96 h. In 1, left side is the *N. glauca* protoplast and right side is the *N. langsdorffii* protoplast; in 3, the large object is a nucleus, the other organelles being *N. glauca* chloroplasts. X 200.

2) Two kinds of chloroplast genomes in unseparated calli (Akada et al. 1983)

Protoplasts were isolated from *N. glauca* and *N. langsdorffii* suspension culture cells and fused. They were then cultured under the conditions where only parasyexual hybrid cells could grow into green callus in hormone free medium. Each callus was transferred without cutting or separation of cells into a separate flask. When the green
callus had grown to about 2 cm in diameter, the callus was used for Fraction I protein analysis, a total of 13 individuals being used in this experiment. The results of isoelectrofocusing of Fraction I protein are shown in Fig. 2 and Table 1. All calli had Fraction I protein with *N. langsdorffii* plus *N. glauca* type SS showing the expression of the two different nuclear genomes. Nine of the 13 calli had Fraction I protein displaying both the *N. langsdorffii* and *N. glauca* type LS showing that more than half of the calli still contained two kinds of chloroplast genomes.

Since two kinds of chloroplast genomes existed in about 70% of somatic hybrid calli, we asked whether similar results would be obtained when two different kinds of cells were fused that should have had different copy numbers of ctDNA. It is likely that suspension cells have fewer chloroplasts per cell and hence fewer copy numbers of ctDNA than leaf cells. Therefore, protoplasts from *N. glauca* suspension culture cells were fused with protoplasts from *N. langsdorffii* leaf cells. Ten green calli were obtained and subjected to Fraction I protein analysis. All of the results of this experiment and the reciprocal experiment are also compiled in Table 1. The results were similar to those

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Fig. 2. Photograph of gel after electrofocusing of Fraction I protein obtained from leaves of *N. langsdorffii* (L), *N. glauca* (G) and from five calli (1-5) obtained by fusion of protoplasts from G and L suspension cells.
Table 1. Kind of chloroplast and nuclear genomes in individual somatic hybrid calli

Chloroplast and nuclear genomes were characterized by large and small subunits of Fraction I protein respectively.

<table>
<thead>
<tr>
<th>Type and species of cells used for fusion of protoplasts</th>
<th>Chloroplast genomes (No. of calli)</th>
<th>Nuclear genomes (No. of calli)</th>
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<tr>
<td>N. glauca (G)</td>
<td>N. langsdorffii (L)</td>
<td>G</td>
</tr>
<tr>
<td>Suspension cells</td>
<td>Suspension cells</td>
<td>4</td>
</tr>
<tr>
<td>Suspension cells</td>
<td>Leaf cells</td>
<td>1</td>
</tr>
<tr>
<td>Leaf cells</td>
<td>Suspension cells</td>
<td>5</td>
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</table>

previously shown in that more than 50% of the calli in both types of protoplast fusion contained two kinds of chloroplast genomes. Where separation of the chloroplast genomes occurred, there seemed to be a tendency for genomes from leaf protoplasts to gain ascendency over chloroplast genomes in suspension chloroplasts. However, the results also show that chloroplast genomes in suspension cells can gain ascendency over leaf cell genomes.

3) Separation of chloroplast genomes during callus development (Akada et al. 1983)

Since, in many cases, two kinds of chloroplast genomes remained in hybrid calli, we tried to examine the stability of chloroplast genomes in calli subjected to subdivision followed by further growth of a small piece of the original callus. Calli weighting about 2 g fresh wt. were cut with a knife so that one piece weight about 0.5 g could be transferred to fresh medium and the remainder used for Fraction I protein analysis. In some cases, the small piece was allowed to grow to about 2 g when a second subdivision was made and in one case a single callus was subjected to three successive subdivisions. The results of the Fraction I protein analysis are shown in Fig. 3. Some calli (GL-1-7, for example) lost one of the two chloroplast genomes following the subdivision. Others such as GL-1-2 still retained the two genomes originally present before subdivision. Callus GL-1-2 was subjected to further subdivisions and growth periods in hormone free medium. By the second subdivision, the N. glauca type of Fraction I protein LS clearly predominated over the N. langsdorffii type, densitometric measurements of the LS bands indicating 90% of the N. glauca type to 10% of the N. langsdorffii type. After a third subdivision (GL-1-2-3), no N. langsdorffii type of LS could be detected in
Fig. 3. Photograph of gels after electrofocusing of Fraction I protein from calli subjected to subdivision followed by further growth. GL-1-2(1) and GL-1-7(4) calli underwent one subdivision; GL-1-2-2(2), two successive subdivisions; GL-1-2-3(3), three successive subdivisions.

the Fraction I protein although it still contained SS of both parental type of nuclear genomes.

4) Distribution of chloroplast genomes in hybrid calli (Akada and Hirai 1983)

Unseparated hybrid calli produced by fusions between suspension culture cells in N. glauca and N. langsdorffii were allowed to grow to about 3 g fresh wt. Calli were cut in a horizontal direction in such a way as to separate the upper and the lower parts, and each part was then separated into four pieces by cutting crosswise. Each piece was cultured in a separate flask and was allowed to grow to about 2 g. Fraction I protein from each separated callus was analyzed by isoelectrofocusing and the ratio of N. glauca and N. langsdorffii type of LS was estimated from the data obtained from densitometric measurements of the three LS bands of calli, which consist of a N. glauca specific band, a hybrid band and a N. langsdorffii specific band. The distributions of the two kinds of LS in three original hybrid calli are shown in Fig. 4. The results clearly indicate that two kinds of chloroplast genomes are heterogeneously distributed in parasexual hybrid calli.
5) Chloroplast genomes in single cells contained in hybrid callus (Akada and Hirai 1983)

The results shown above suggest the hypothesis that two kinds of cells, each with only one or the other type of chloroplasts, are heterogeneously distributed in hybrid calli. Therefore, we characterized chloroplast genome products in subcloned calli were developed from single cells derived from the hybrid calli. So far, we have obtained five calli which were definitely derived from single cells and which were able to grow in hormone-free medium showing that they are hybrid calli. Fraction I protein in each callus was analyzed by isoelectrofocusing. The results obtained from four of these calli are shown in Fig. 5. Callus 1 had only N. glauca type LS of Fraction I protein and callus 3 and 4 had only N. langsdorffii type LS. However, callus 2 had both types of LS and this result shows that the cell which yielded callus 2 had both chloroplast genomes. The fifth callus also had two kinds of LS, but a densitometric measurement showed that it had about 90% of N. glauca type LS and 10% of N. langsdorffii type.
6) **Analysis of chloroplast DNA in hybrid calli** (Ichikawa et al. 1984)

The above results showed that the hybrid callus is a chimera composed of three kinds of cells: cells with one kind of LS, cells with the other kind of LS, and cells with both kinds of LS. However, it is not certain that the callus in which only one kind of LS is expressed has only that one kind of ctDNA. It is possible that the callus still had both kinds of ctDNA, but the gene for only one kind of LS was expressed. This possibility was examined in the present study by extracting ctDNA and Fraction I protein from the same tissue of above parasexual hybrid calli, and analyzing the DNA by the restriction enzyme pattern and Fraction I protein by isoelectriofocusing.

Leaf ctDNAs were isolated from *N. glauca*, *N. langsdorffii* and their reciprocal sexual hybrids. The four kinds of ctDNA were digested with Bam HI and fragments were analyzed by agarose gel electrophoresis. As shown in Fig. 6A, ctDNA from *N. glauca* contained an 8.8 kb fragment (Bam-4), which is marked by an arrow. In contrast, ctDNA from *N. langsdorffii* contained a smaller, 8.4 kb Bam-4 fragment. The distinct
Fig. 6. Bam-4 fragments in *Nicotiana* chloroplast DNA. A. 0.7% agarose gel electrophoresis of Bam HI digest of ctDNAs from leaves of *N. glauca* (G), *N. glauca* x *N. langsdorffii* (GL), *N. langsdorffii* x *N. glauca* (LG), and *N. langsdorffii* (L). Specific Bam-4 fragments for two species are shown by arrows. B. The location of the Bam-4 fragment and the gene for the large subunit of Fraction 1 protein (LS) in *Nicotiana* ctDNA (Tassopulu and Kung 1984). The extent of the inverted repeats (IR) are indicated by two lines out side of the circle.

difference in sizes of Bam-4 fragments between *N. glauca* and *N. langsdorffii* was also found in restriction fragment patterns of ctDNAs from their reciprocal hybrids. Each hybrid had the same pattern as its female parent. The location of the Bam-4 fragment in relation to the gene for LS in *N. tabacum* ctDNA was reported by Tassopulu and Kung (1984) and is illustrated in Fig. 6B. Since Bam HI restriction patterns of *N. tabacum* and *Nicotiana* species we used are similar except the size of Bam-4 fragment of *N. langsdorffii*, it is likely that a similar degree of separation of Bam-4 fragment and the genes for the LS are present in *Nicotiana* species used in this study. Therefore, it was of interest to use the Bam-4 fragment to test whether two different types of ctDNA could be present in a parasexual hybrid callus, but only one expressed as LS.

Sixteen parasexual hybrid calli between *N. glauca* and *N. langsdorffii* were analyzed for the type of Bam-4 fragment of ctDNA and LS. The calli numbered from 1 through
10 were primary, and they were not subcultured from time of origin from single colonies; the other six calli (Nos.11-16) had been regularly subcultured over three years. The Bam HI cleavage patterns of ctDNAs from calli-1 and -11 were those of *N. glauca* ctDNA, whereas that of callus-12 was the *N. langsdorffii* type (Fig. 7). Restriction patterns of ctDNA from primary calli-2 and -3 contained both the 8.8 and 8.4 kb Bam-4 fragment, indicating that a mixture of *N. glauca* and *N. langsdorffii* ctDNAs were present in these parsexual hybrid calli. The patterns of the remaining types are summarized in Table 2.

Fraction I protein was also extracted from each hybrid callus and analyzed by isoelectric focusing. Typical results are presented in Fig. 8. All calli contained a mixture of *N. glauca* and *N. langsdorffii* types of SS of Fraction I protein confirming that each callus was composed of two kinds of nuclear genomes. Calli-2 and -3 contained mixtures of two parental types of LS, while calli-1 and -11 contained only *N. glauca* type LS and callus -12 contained only *N. langsdorffii* type LS. The LS results are thus in complete

Fig. 7. Restriction fragment patterns of ctDNA from *N. glauca* (G), *N. langsdorffii* (L), and parsexual hybrid calli (1,2,3,11 and 12).
Table 2. Chloroplast and nuclear genomes in parasexual hybrid calli

<table>
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<tr>
<td></td>
<td>Large subunit of</td>
<td>Small subunit of</td>
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<td></td>
<td>Fraction I protein</td>
<td>Fraction I protein</td>
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<tr>
<td>1</td>
<td>G</td>
<td>G + L</td>
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<td>16</td>
<td>L</td>
<td>G + L</td>
</tr>
</tbody>
</table>

G: *Nicotiana glauca*;  L: *langsdorffii*.

Callus No.1-10: primary;  No.11-16: Subcultured.

Fig. 8. Isoelectrofocusing analysis of Fraction I proteins obtained from leaves of *N. glauca* (G), *N. langsdorffii* (L), and parasexual hybrid calli (1,2,3,11 and 12).
correspondence with those showing the remaining 11 calli.

The results of analyzing sixteen parosexual hybrid callus are summarized in Table 2. There was complete agreement between the type of Bam-4 fragment and the type of LS as indicators of whether the callus contained *N. glauca*, or *N. langsdorffii* ctDNA, or a mixture of both. Moreover, in callus-2, which had two types of chloroplasts, the bands of *N. glauca* type LS stained more intensively than those of *N. langsdorffii* type LS, and *N. glauca* type Bam-4 fragment of ctDNA was also greater in amount than *N. langsdorffii* type. Callus-3 also had two types of chloroplasts; however, the amounts of *N. langsdorffii* type LS and Bam-4 fragment were more than those of the other one. These results indicate that the correlation between the type of Bam-4 fragment in ctDNA and that of LS is not only qualitative but quantitative.

**Discussion**

The preceding results appear to eliminate some explanations that were advanced to explain the mechanism whereby one of the two chloroplast genomes become separated in an apparently indiscriminate manner in the time between protoplast fusion and differentiation of an intact plant from the products of protoplast fusion. One idea that two kinds of chloroplasts do not actually mix in the fused cell seems to be eliminated by the photomicrographic evidence in Fig. 1. A second idea that elimination of one of the chloroplast genomes might occur shortly after the fused cell divides is not supported by the finding that 70% of the calli that developed as a consequence of many cell divisions still retained the two types of chloroplast genomes.

Glimelius *et al.* (1981) and Melchers *et al.* (1978) suggested that separation of the chloroplast genomes resulted from fusions involving suspension cell protoplasts and leaf cell protoplasts with the latter containing many more chloroplasts per cell which gradually overwhelmed the former during successive divisions and unequal partitioning of the two kinds of chloroplasts into daughter cells. However, the reciprocal experiments whose results are shown in Table 1 do not support this view.

The successive subdivision experiments indicate that a callus is a three dimension mosaic of cells containing different ratios of two kinds of chloroplast genomes. Elimination of one of the chloroplast genomes occurred after successive selection of a small fraction of a callus which was allowed to continue growth (Fig. 3). The results shown in Fig. 4 more clearly indicate that the ratios of the two kinds of chloroplast products are different in different positions in a hybrid callus, even though the entire callus contains almost equal numbers of the two kinds of chloroplast products.

According to our calculations, there are 30 cell doublings from the original fused
protoplasts to the callus that we used for the isolation of protoplasts. This means that two out of five cells retained two kinds of chloroplast genomes after about 30 rounds of cell division. Although the sample number is very small, our results agree with the probability of random fixation of genotypes in a small mixed population (Wright 1952) by assuming that there are 10-20 chloroplasts per green callus cell. If we assume that about 20-40% of cells in a bud-forming callus retain two kinds of chloroplasts, the idea that cells which have two kinds of chloroplasts have less chance to differentiate into a shoot than cells with only one kind can be proposed since the evidence indicates that parasexual hybrid plants usually have only one kind of chloroplast (Chen et al. 1977; Melchers et al. 1978; Iwai et al. 1980; Douglas et al. 1981).

Recombination of two kinds of chloroplast DNA after cell fusion is of doubtful occurrence (Belliard et al. 1978; Glimelius et al. 1981; Schiller et al. 1982; Scowcroft and Larkin 1981). However, it should be possible to detect the recombination, if it occurs, by selecting only calli grown from cells which retain two kinds of chloroplasts for 30-50 cell-doubling times and analyzing them using two or more chloroplast DNA markers.

Summary

The mode of chloroplast genome separation after cell fusion between Nicotiana glauca and N. langsdorffii protoplasts was studied by microscopic observation, isoelectrofocusing analysis of Fraction I protein and restriction enzyme analysis of chloroplast DNA (ctDNA) obtained from parasexual hybrid calli. Chloroplasts from one species of plant were observed to spread all over the fused cell at 48 h after cell fusion indicating that two kinds of chloroplasts were mixed together within the same fused cell. Analysis of Fraction I protein showed that 70% of the calli contained two kinds of chloroplast genomes and all contained two kinds of nuclear genomes.

Selection of a small piece of the callus followed by further growth and further subdivisions caused separation of one of the kinds of chloroplast genomes within the callus. Unseparated hybrid callus was also cut into eight pieces and Fraction I protein in each piece was analyzed. The results showed that the ratios of two kinds of chloroplast genomes are completely different in different positions in the callus. This evidence demonstrates that a hybrid callus is a three dimensional mosaic of cells containing different ratios of two kinds of chloroplasts.

The Fraction I protein of five sub-cloned calli grown from single cells isolated from the hybrid callus was analyzed. Three calli had only one or the other kind of chloroplast genome. However, two calli contained two types of chloroplast genomes, showing that
some cells in the original hybrid callus contained chloroplasts from both sources.

Restrictionendonuclease patterns of ctDNA were also used as a chloroplast marker. Chloroplast DNA and Fraction I protein were isolated from 16 hybrid calli and analyzed. Results showed that the expression of one kind of large subunit of Fraction I protein was correlated with presence of the kind of ctDNA. Whenever two kinds of ctDNA were present in a callus, both types of large subunit were expressed. This evidence shows that Fraction I protein is a reliable marker for chloroplasts.

Acknowledgements

We are greatly indebted to Drs. S.G. Wildman and H. Uchimiya for helpful advice and criticism. This research was partly supported by a Grant-in-aid for Scientific Research from The Ministry of Education, Science and Culture.

References


細胞融合に由来する雑種細胞の葉緑体ゲノム

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光合成に必要な遺伝子を多数コードしている葉緑体DNAを改変するための基礎データとするためにタバコ属植物のNicotiana glauca（G）とN. langsdorffii（L）の細胞融合を行い、雑種カルス内の2種の葉緑体の分布についてFraction 1 テンパク質の大サブニュート（LS）と葉緑体DNAの制限酵素パターンをマーカーにして調べた。細胞融合による雑種植物体ではほとんどの場合どちらか一方の親の葉緑体のみが存在することがすでに報告されている。そこで最初に、融合した新しい細胞で2種の葉緑体が混ざり合うかを光学顕微鏡で調べたところ、融合後48時間で混ざり合うことが確認された（図1）。

さらに細胞融合後末分裂のカルスの葉緑体をLSをマーカーにして調べると、植物体での結果と反し多くの場合G、L両種の葉緑体が存在していた（図2、表1）。だがこれらのカルスの一部を細胞培養し続けると、Gの葉緑体のみを持つカルスとLの親の葉緑体のみを持つカルスに分離した（図3）。そこでさらに雑種カルスを2分割し、それらの小片に含まれる両種のLSの量比を調べたところ、カルス内の場所により両種の比が大きく異なっていた（図4）。これは分割前の雑種カルスが、すでに葉緑体に関してキメラであったことを示している。このように細胞融合による雑種カルスにおいては2種の葉緑体が急速に分離することが明らかになったので、細胞レベルでの両種の葉緑体の分布を調べることが必要になった。

このためカルスからプロトプラストを再び分離し、カルスに再生させた。その中から単細胞由来のカルスのみを選抜しLSを等電点電気泳動で調べた（図5）。これによると、1個のカルスがG種のLSのみを、また2個のカルスがL種のLSのみを持っていたが、2個のカルスはG、L 2種のLSを保有していた。但しこの内1個はG：Lの比が9：1であった。この結果はカルスを構成する細胞の20－40％は2種類のLSを保有しており、2種の葉緑体を依然として保有している細胞がかなりあることを示唆している。

以上の結果はタンパク質をマーカーにした解析で、マーカーが検出できない時に遺伝子がないのか、遺伝子が発現していないかが不明である。そこでDNAレベルでの他のマーカーが必要となる。このため、G、L、および両種の雑種から葉緑体DNAを抽出し、制限酵素Bam HIで処理し、アガロース電気泳動を行なった。これによると、Bam-H1ディスクの長さが両種で異なっており、これが両種の葉緑体DNAを識別するマーカーになることが示された。Bam-H1ディスクはLSの遺伝子と葉緑体DNA上で約30kb離れており、葉緑体の第二のマーカーとして使用できる（図6）。そこで16個のカルスのBam-H1ディスクとLSを同時にしきらせた（表2）。図7、図8にも示す通り、すべてのカルスにおいて2種のマーカーによる結果は完全に一致した。すなわち1種のLSのみが発現しているカルスで
はその種のBam-4断片のみが検出され，2種の葉緑体DNAを持つカルスでは2種のLSが発現していた。この結果LSの遺伝子はあれば必ず発現することから，マーカーとして有用であることが明らかにされた。

これらの結果は30回近く細胞分裂を繰り返した雑種カルスにおいて，20〜40%の細胞は依然2種の葉緑体を保持していることを明らかにしている。これは1細胞当たり10〜20個の葉緑体が存在すると仮定した場合，2種の葉緑体が細胞分裂において無作為的に分配されることを示唆している。

質疑応答

武田（岡山大・農研）：細胞質の分裂がランダムかどうかは大変興味のある問題ですが，雑種細胞ではなくて既存の例えば縞の突然変異体などを見ていると，縞の発現が分けつによって非常に異なり，同じ個体の中でもある分けつはほとんどアルビノになるし，ある分けつは殆ど縞を発現しないという現象を，しばしばイネでもオオムギでも見ますので，どうも細胞質というランダムには分裂しないのではないかという印象を持っていたのですが，その辺についてなにかコメントをいただけませんか？

平井：私はそのレベルのことのはあまり知らないのですが，ある突然変異体が出てきますとそれが突然変異体として維持され易いものであるかどうかによって存在形態が変わってくると思います。私が今説明いたしました細胞融合の場合は，ふたつとも正常な葉緑体ですので，その存在しやすさは平等ではないかと考えており，と言われた場合とは少し違うのですねが，是以上のことはちょっと判りません。

西尾（野菜試）：融合したものから最後に植物体ができて，その時に一方の葉緑体が残るということですが，一方が残るということについて何かお考えをお持ちでしょうか？ふたつとも残るものがないということの意義ですが。

平井：我々の立場からはふたつともあったほうがいいのですが，一方が残るというのは，私にもよく判りませんが，自然界では一方だったわけです，なかにそのほうが都合がよいことがあるのではないかと思います。お答えになっていないようですが，私はお答えになるような資料をもっておりませんので。
VARIANCE AND MOLECULAR ANALYSIS IN SOMATIC HYBRID PLANTS

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Introduction

Recent advances in the study of somatic hybridization of higher plants have made it possible to manipulate genetic information encoded by nuclear as well as cytoplasmic DNA (7). The analysis of gene markers in somatic hybrid plants is crucial for the identification of their genome constitutions. Study of the inheritance of the characters of hybrids to their progeny is necessary in the application of somatic hybridization to practical plant breeding.

In this presentation, analysis of several molecular markers including Fraction I protein, ribosomal RNA genes (rDNA) and chloroplast DNA in relation to several variance of hybrids will be discussed. Most of these works have been reported elsewhere (11, 12, 14, 15).

Experiment 1. Ribosomal RNA genes in somatic hybrids

Frequency of somatic hybrids

Protoplasts of Nicotiana glauca and N. langsdorffii were prepared from suspension cultured cells. Protoplasts of two tobacco species were fused with an aid of polyethylene glycol (PEG)-Ca++ . The selection medium (−H medium) lacked 2,4-D and kinetin, whereas non-selection medium (+H medium) contained these phytohormones.

The statistical analysis of the frequency of hybrid formation under selective or non-selective media was carried out. After the fusion treatment, protoplast population was cultured in the medium containing phytohormone for 1 week to sustain cell division, then equal numbers of colonies were plated in either +H or −H medium solidified with 0.8% agar. After 2 weeks, 150 colonies were recorded in +H medium, whereas 8 colonies developed in −H medium. This indicated that about 5% of total protoplast populations after PEG-Ca++ treatment developed to hybrid colonies. Furthermore, 38 clones which had been grown in +H medium was subcultured in the same medium for another 1 month to allow the callus growth. Then each clones were subjected to −H medium by
two successive subculturing (1-month period per each) to minimize the possible carry-over of phytohormones. Only 3 clones survived in -H medium, and one of which was chimera. Thus about 5% of calli developed from protoplasts after fusion treatments was determined to be hybrids.

**Chromosome number**

The result of chromosome number in suspension cultured cells of two parental species, *N. glauca* and *N. langsdorffii* and somatic hybrid clones are given in Table 1. Both the parental species were found to have polyploid chromosome number, average being 47 and 35 per cell in *N. glauca* and *N. langsdorffii*, respectively. The average chromosome number in 8 clones ranged from 57 to 120, being 57-59 chromosomes in clones 1, 2; 66-69 in clones 3, 6, 7; 72-76 in clones 4, 8; whereas clone 5 had 120 chromosomes.

**Ribosomal DNA analysis**

DNA extracted from cultured cells were subjected to XbaI restriction endonuclease digestion, followed by agarose gel electrophoresis and blot-hybridization using $^{32}$p-labeled rRNA as probe. Eight hybrid clones used for chromosome observation were subjected to rDNA analysis. Results indicated every clone to contain rDNA from both the parents (Fig. 1).

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Chromosome number (number of cells observed)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. glauca</em></td>
<td>44 (2) 46 (7) 48 (6)</td>
<td>47 ± 1</td>
</tr>
<tr>
<td><em>N. langsdorffii</em></td>
<td>34 (7) 35 (4) 36 (5)</td>
<td>35 ± 1</td>
</tr>
<tr>
<td>Hybrid 1</td>
<td>57 (2) 58 (5) 59 (2) 60 (9)</td>
<td>59 ± 1</td>
</tr>
<tr>
<td>Hybrid 2</td>
<td>56 (7) 57 (4) 58 (2) 60 (3)</td>
<td>57 ± 2</td>
</tr>
<tr>
<td>Hybrid 3</td>
<td>66 (3) 68 (3) 69 (2) 70 (3) 71 (1) 72 (4)</td>
<td>69 ± 2</td>
</tr>
<tr>
<td>Hybrid 4</td>
<td>66 (3) 68 (1) 72 (6) 74 (3) 75 (1) 76 (4)</td>
<td>72 ± 4</td>
</tr>
<tr>
<td>Hybrid 5</td>
<td>109 (1) 110 (1) 112 (1) 115 (2) 118 (3)</td>
<td>120 ± 6</td>
</tr>
<tr>
<td></td>
<td>119 (2) 120 (3) 122 (2) 128 (5)</td>
<td></td>
</tr>
<tr>
<td>Hybrid 6</td>
<td>64 (1) 65 (2) 66 (9) 67 (5)</td>
<td>66 ± 1</td>
</tr>
<tr>
<td>Hybrid 7</td>
<td>66 (5) 67 (6) 68 (6)</td>
<td>67 ± 1</td>
</tr>
<tr>
<td>Hybrid 8</td>
<td>72 (3) 73 (1) 74 (2) 75 (2) 76 (5) 80 (4) 82 (2)</td>
<td>76 ± 3</td>
</tr>
</tbody>
</table>

Table 1. Summary of chromosome number in callus tissues of somatic hybrids and their parents (3)
Experiment 2. Molecular analysis of the somatic hybrid and pogenies

Leaf morphology and male sterility:
A cytoplasmic male sterile line, *N. tabacum* containing *N. debneyi* cytoplasm (2n=48), and *N. glutinosa* (2n=24) were used for somatic cell fusion. Although resulting hybrid plant was sterile due to the lack of anthers, upon pollination with an appropriate pollen donor fertile seeds can be produced. In this manner, we were able to carry out progeny analysis of nuclear and cytoplasmic characters contained in a somatic hybrid and its backcrossed generations. Stigmas of a hybrid plant obtained by protoplast fusion of male sterile *N. tabacum* containing *N. debneyi* cytoplasm and *N. glutinosa* were pollinated with either *N. tabacum* or *N. glutinosa* pollen. Plants thus obtained were repeatedly crossed with the same pollen donors to yield extensively back-crossed progeny.

In the progeny obtained by back-crossing with *N. tabacum*, all plants showed uniform leaf morphology, while in the population after the second back-crossing with *N. glutinosa*, some variation was observed (Fig. 2). Stable inheritance of male sterility
was demonstrated in the every progenies tested (Fig. 3).

**Chromosome number**

Young leaves were used for chromosome analysis. The somatic hybrid plant had the amphiploid chromosome number (2n=72), which is the summation of the two parental species, *N. tabacum* (2n=48) and *N. glutinosa* (2n=24). The chromosome number of 48 in plants resulting from pollination of the somatic hybrid with *N. glutinosa* indicates that the somatic hybrid is stable and produces normal gametes with 36 chromosomes (24 from *N. tabacum* and 12 from *N. glutinosa*). The meiotic behavior of the hybrid was not studied since it lacked anthers and formed the stigmatic-like organ. Extensive chromosome analysis was made in the population originating from the 2nd back-crossing with *N. glutinosa*, where variation in leaf morphology was observed. The individual contained chromosome numbers ranging from 32 to 38. It is evident that between 8 and 14 chromosomes of the *N. tabacum* genome were transmitted by the
female parent to give rise to plants with the observed chromosome constitution after pollination with *N. glutinosa*.

**Ribosomal DNA:**

In order to find the appropriate enzyme which discriminates between the rDNA of the two tobacco species, restriction fragment patterns generated with several restriction endonucleases were compared. Digesting of *N. tabacum* DNA with Hind III produced DNA fragments of 7.4 and 6.4 (minor band) $\times 10^6$ daltons hybridizing to labeled ribosomal RNA, whereas digestion of *N. glutinosa* DNA produced a $16 \times 10^6$ dalton rDNA. The DNA of the somatic hybrid contained all of these fragments (Fig. 4). The analysis of rDNA in successive back-cross generations with *N. tabacum* pollen showed that the *N. glutinosa* type rDNA was eliminated after the 2nd back-crossing. In the case of back-crossing with *N. glutinosa*, some individuals of the population from the 2nd back-cross contained *N. tabacum* type rDNA.
Fig. 4. Autoradiography of a blot-hybridization of $^{32}$P-labeled $-25S +17S$ rRNA to Hind III digests of nuclear DNA from leaves of (a) male sterile N. tabacum, (b) N. glutinosa and (c) their somatic hybrid$^{(15)}$. Numerals indicate molecular weight; $\times 10^6$ daltons.

Fraction I protein

Analysis of polypeptide compositions of Fraction I protein in these plants indicated the presence of small subunits of both parents, and N. glutinosa type large subunit (Table 2). Since somatic hybrid contained large subunit of N. glutinosa type (Fig. 5), chloroplast DNA was not altered by crossing a hybrid with N. tabacum ($\phi$). The second backcross was carried out using plants of the first backcross generation and N. tabacum cv. 'Samsun' as pollen donor. Type of Fraction I protein in six individuals of the second backcross generation indicated that one plant possessed small subunits of both parents, and five other plants contained N. tabacum type small subunit (Table 2). In the progeny of the third backcross generation, every individual expressed same large subunit (N. glutinosa type) and N. tabacum small subunit. Thus it must be concluded that nuclear informations in a somatic hybrid can be fixed by three repeated backcrosses with the same pollen donor.

Chloroplast DNA

Figure 6 shows profiles of gel electrophoresis of EcoRI-digested chloroplast DNAs of male sterile N. tabacum, N. glutinosa and a somatic hybrid. Nicotiana glutinosa chloroplast DNA lacks the 3.2, 2.2 and $1.8 \times 10^6$ dalton fragments which were characteristic of male sterile N. tabacum (i.e. N. debneyi) chloroplast DNA. Furthermore,
the chloroplast DNA of the somatic hybrid had the same fragment pattern as that of *N. glutinosa*. A similar analysis was made on back-cross progeny of the somatic hybrid with either *N. tabacum* or *N. glutinosa*. In the case of the progeny of the hybrid crossed with *N. tabacum*, the same EcoRI digestion pattern was observed. Similar results were observed with progeny which had been obtained by back-crossing with *N. glutinosa* twice.

Table 2. Polypeptide type of Fraction I protein in backcrossed progenies of a somatic hybrid with *N. tabacum*<sup>(2)</sup>

<table>
<thead>
<tr>
<th>Progeny No.</th>
<th>Polypeptide type of Fraction I protein&lt;sup&gt;(1)&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Large</td>
</tr>
<tr>
<td>First backcross</td>
<td></td>
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<tr>
<td>1</td>
<td>G</td>
</tr>
<tr>
<td>2</td>
<td>G</td>
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<tr>
<td>3</td>
<td>G</td>
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<td>4</td>
<td>G</td>
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<td>5</td>
<td>G</td>
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<tr>
<td>6</td>
<td>G</td>
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<tr>
<td>7</td>
<td>G</td>
</tr>
<tr>
<td>8</td>
<td>G</td>
</tr>
<tr>
<td>Second backcross</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>G</td>
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<tr>
<td>2</td>
<td>G</td>
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<td>3</td>
<td>G</td>
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<td>4</td>
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<td>5</td>
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<td>6</td>
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<tr>
<td>Third backcross</td>
<td></td>
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<td>1</td>
<td>G</td>
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<td>6</td>
<td>G</td>
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<tr>
<td>7</td>
<td>G</td>
</tr>
</tbody>
</table>

<sup>(1)</sup> G : *N. glutinosa* type large subunit.
<sup>(2)</sup> g : *N. glutinosa* type small subunit.
<sup>(2)</sup> t : *N. tabacum* type small subunit.
**Fig. 5.** Large and small subunit polypeptides of Fraction I protein resolved by iso-electrofocusing gel electrophoresis. (a) progeny of a somatic hybrid (?), (b) N. tabacum cv. 'Samsun,' (c) N. glutinosa male sterile line, and (d) a somatic hybrid. Arrows indicate positions of each small subunit polypeptide.

**Fig. 6.** A profile of agarose gel electrophoresis of EcoRI-digested chloroplast DNA from (a) male sterile N. tabacum, (b) N. glutinosa and (c) a somatic hybrid. Numerals indicate molecular weight; × 10^6 daltons.

**Discussion**

*Frequency of somatic hybrids*

It is an intriguing question that what portion of colonies after protoplast fusion contains real hybrid calli. To answer this question we carried out the experiment to estimate the frequency of hybrid formation in N. glauca - N. langsdorffii somatic hy-
bridization. Results suggest that about 5% of cells after fusion treatment was found to be hybrids regardless of selective or non-selective culture conditions.

**Chromosomal variation and rDNA**

Carlson *et al.* (4) created a first somatic hybrid of *N. glauca* (2n=24) and *N. langsdorffii* (2n=18) possessing amphidiploid chromosome number (2n=42). Later, Smith *et al.* (10) reported triple fusion in *N. glauca* and *N. langsdorffii* followed by loss of certain chromosomes to give differentiated plants with 56-64 chromosomes. Chupeau *et al.* (5) obtained somatic hybrid plants between *N. glauca* and *N. langsdorffii* with 28-143 chromosomes. Variation in chromosome number *in vitro* may appear as early as first subculture but is often associated with long-term culture. In *Nicotiana* with tissues containing endopolyploid or endoreduplicated cells and cultures derived from such explants are usually polyploid. Endomitosis and endoreduplication during growth of the cells in culture seem to be the most probable reason for polyploidy of parental cell clones used for somatic hybridization. The polyploid cells thus produced might have got advantage over the diploid cells thereby resulting into the high frequency of polyploid cells in the suspension culture. Among the 8 clones, 7 seem to be the result of 1:1 protoplast fusion of two parental genomes. The reduced number than the actual summation of the chromosome number of two parental species might have resulted from the loss of certain chromosomes of *N. glauca* or *N. langsdorffii* or of both during subsequent growth and proliferation of the hybrid cells. The loss of particular *N. glauca* or *N. langsdorffii* chromosomes could not be ascertained on the basis of chromosome morphology. Regardless of the instability of chromosome number of somatic hybrids and similar karyotype of two parents, rDNA can serve as an effective marker to detect hybrids. Ribosomal DNA could be applicable even to other plant hybridization where specific restriction endonuclease discriminates rDNA of hybrid parents. Since modification of rDNA by specific methylation was also noted (13), it would be needed to provide the stability of such analysis in plant development.

**Molecular genetics of the somatic hybrid and the progeny**

For the application of somatic hybridization into plant breeding, it is important to assess the inheritance of various characters in the progeny. In our case, the hybrid was male-sterile and self-pollination, therefore, was not possible. Progeny were obtained by cross-hybridization of a hybrid with other pollen donors. Elimination of the nuclear genome of *N. glutinosa* resulted in plants with normal morphology and such elimination was indicated by the type of the small subunit of Fraction I protein. However, when the hybrid was crossed with *N. glutinosa*, abnormal plant morphology was observed in the population after the 2nd back-crossing. Variations in plant morphology particularly in
growth habit and leaf shape may be attributed to the presence of a variable number of
*N. tabacum* chromosomes. Segregation of rDNA was also found in the individuals of the
same back-cross, but was not related to the chromosome number.

The outcome of large subunit of Fraction I protein and chloroplast DNA analysis
reveals maternal inheritance of the chloroplast DNA. There have been several reports
indicating the presence of a single type of chloroplast DNA prevailing in somatic hybrids
(1, 2, 6, 8, 9).

The transfer of cytoplasmic characters such as chloroplast DNA or male sterility
would be one of the most advantageous application of somatic hybridization in higher
plants. In this respect, the demonstrated stable inheritance of chloroplast DNA as well
as cytoplasmic male sterility is encouraging.

**Summary**

Restriction endonucleases discriminated between the ribosomal DNA genes (rDNAs)
contained in callus tissues derived from *Nicotiana* glauca, *N. langsdorffii*, and their
somatic hybrids produced by protoplasting fusion. Observation of chromosomes of select-
ed hybrids showed variation in number ranging from 57-128, whereas parents contained
34-48. Analysis of rDNA confirmed every hybrid clone to contain rDNA from both
parents.

Several nuclear and cytoplasmic characters of a somatic hybrid between male sterile
*N. tabacum* (*N. debneyi* cytoplasm) and *N. glutinosa*, and the progeny have been
analysed.

Nuclear ribosomal RNA genes were found to be a reliable marker to determine the
constitution of nuclear genomes in the progeny. The progeny obtained by back-crossing
with *N. tabacum* pollen maintained uniformity in leaf morphology. On the other hand,
variation in leaf morphology was observed in the second back-cross population obtained
with *N. glutinosa* pollen. This may be due to a variable contribution of *N. tabacum*
chromosomes.

The stable inheritance of large subunit of Fraction I protein, and chloroplast DNA
in the back-crossed generation was confirmed. Male sterility was consistently maintained
throughout several generations.

**Acknowledgements**

I am grateful to collaborative assistance from T. Ohgawara, T. Akiyama, M. Ono,
S. Kobayashi and D.S. Brar. Valuable advices and support from Drs. H. Harada and M. Sugiura are deeply appreciated.

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References

体細胞雑種における変異と分子解析

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茨城県新治郡桜村

特定の制限酵素の使用により、Nicotiana glauca, N.langsdorffii および、その体細胞雑種におけるリポソームRNA遺伝子（rDNA）が識別可能となりました。雑種の染色体数は、57–128であり、両親のそれは、34–48であった。しかも、全ての雑種に両親のrDNAが確認されました。

雌性不稔 N.tabacum（N.debneyi 細胞質）とN.glutinosaの体細胞雑種、および後代におけるいくつかの核と細胞質形質が検定された。リポソームRNA遺伝子は、後代における核ゲノムの構成を知る上で有効なマーカーであることが示された。

N.tabacum（花粉親）との交配によって得られた雑種後代では、葉の形態は安定であった。一方、N.glutinosaの場合、2代目植物間で変異が認められた。このことは N. tabacum 染色体の不均一な構成によるものと推定された。

フラクション1蛋白質（大サブユニット）、および葉緑体DNAは、安定に遺伝することが確かめられた。雌性不稔形質も安定に後代に伝達された。

質疑応答

菊池（生物研）：カルスのDNAを制限酵素で切ってサザン法でトランスファーして、リポソームのクローンと分子雑種を作る。その時にサザン法では主バンドしかつかまらないかもしれませんやが、その後はベクターとつなげて、例えばラムダのバンクにして、ブラーク雑種法でそのリポソームのクローンと雑種を形成するのを拾った場合に、本来の主バンド以外のものでどんなものが釣れてくるかということの解釈はなさっておられませんか？

内宮：今お見せした範囲のことしかっておりませんのでなんとも申し上げられませんが、こういったマーカーをもっと普遍的に使うということにもつながると思っています。私なりに解釈しますと、リポソームの遺伝子をマーカーにしていまが、実際にはそのスペーサー部分の変異によるものが多いため、たまたまりポソームの遺伝子が入ってきたものをバンドとして出てくるわけです。今のように差のはっきりしたものを扱う場合はいいのですけれども、目的とするものにこの方法が使えない時にはもっともした断片をクローンすることも必要になるかと思います。

菊池：もうひとつ、タバコの場合リポソームDNAの遺伝子のコピー数としてはどの位を考えておられますか？

内宮：タバコだけでなく、普通の場合はおそらく数百から多いもので千位あると考えら
れています。
平井（名大・農）：スベーサー領域の変異を見ておられるということでしたが、それならば変異が相当あるのではないかと思われるので、例えば種内での、N. tabacumの品種間での変異はみられませんか？
内宮：今まで見ましたのは種間での変異であって、種内での変異は見ておりません。聞こころでは、品種が違っても変異があるとのことですので、品種間の応用も考えられると思います。
足立（宮崎大・農）：このような細胞融合雑種の後代における遺伝子のエリミネーションあるいは脱落というものは、一般的にはランダムに起こっていると考えられるでしょうか、何らかの形で遺伝子なりに何かの因子によって制御されているような見逃しはないものでしょうか？この場合核の遺伝子についてですが。
内宮：大きなレベルでとえばタバコとダイズの融合では、タバコの染色体がどんなエリミネートされていくことは知られていますが、その場合ある程度の断片は残る。そのような断片を除去するためにもう一層タバコとバックヒューロンしますと、そのような特定の染色体が安定に維持されるという報告は知られています。ただそういったエリミネーションの機構がどのようにしておるか、それにかかわる特定の蛋白があるかどうかということはちょっと判らないと思います。
SEXUAL TRANSFER OF A PORTION OF PATERNAL GENOME BY MEANS OF IRRADIATED POLLEN

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Introduction

In order to introduce some desirable genes into crop plants, interspecific or intergeneric hybridization is frequently required. So far sexual hybridization has been used to incorporate some agriculturally important traits, e.g. disease resistance, into crop plants. However, sexual hybridization is limited to a few wild species closely related to a cultivated plant. The technique of ovule culture has been suggested as one of the methods to overcome the species barrier to sexual hybridization. In recent years, this technique has been applied successfully to several plant species (Kuwada and Mabuchi 1976, Reed and Collins 1978, Arisumi 1980, Takeshita et al. 1980, Shizukuda and Nakajima 1982, Douglas et al. 1983).

However, even if the hybrids are obtained, the elimination of the accompanying undesirable genes introduced into the hybrid plants by crossing will require a cumbersome procedures. In general, several generations of backcrossing and selection are required to eliminate the genes. The cumbersomeness can be canceled to some extent, if a small portion of paternal genome is introduced into an egg cell by fertilization and the zygote can be grown into a mature plant. We designate this plant as a partial hybrid.

The technique of ovule culture can not be successful in obtaining interspecific hybrids between any plant species. In some crosses, the obtained hybrid plants die in the young stages of development (Reed and Collins 1978). This phenomenon is considered to be a manifestation of hybrid inviability. If a portion of the paternal genome which brings about hybrid inviability can be eliminated, partial hybrids into which desirable genes are transferred will be obtained. And partial hybrids may offer a great promise for transferring desirable genes into crop plants from distantly related species.
It is well known that treatments with ionizing radiations lead to break the chromosomes of plant cells. Recently, Pandey (1975, 1978 and 1980) and Virk et al. (1977) showed that in genus *Nicotiana* the pollination with irradiated compatible pollen could cause particular genes from the pollen to be transferred to the egg cells and that the egg cells grew into maternal diploids in vivo. The combination of two techniques, i.e. the pollination after the destruction of chromosomes in pollen grains with ionizing radiations and the in vitro culture of the ovules, could offer an effective means of getting the partial hybrids to which particular genes or chromosomes are transferred from uncrossable pollen parents.

In the present study, the combination of two techniques were applied to the following three interspecific crosses.

1. *Nicotiana rustica* × *Nicotiana tabacum*
2. *Nicotiana repanda* × *Nicotiana tabacum*
3. *Nicotiana rustica* × *Petunia hybrida*

In the first cross, complete mature hybrid plants have been obtained through the ovule culture technique (Shizukuda and Nakajima 1982). In the second cross, seedlings have been obtained through the ovule culture technique but they died in the young stage (Reed and Collins 1978). In the third cross, neither a hybrid plant nor a seedling has been obtained.

### Materials and methods

1. **Materials used**

   *Nicotiana rustica* L. cv Rustica, *Nicotiana tabacum* L. cv. Hicks 2, *Nicotiana repanda* Wild. and *Petunia hybrida* were used for the experiments. Seeds of *Nicotiana* were supplied from the Japan Tobacco and Salt Public Coorporation.

2. **Irradiation**

   Anthers were collected immediately before anthesis and stored in test tubes. They were exposed to gamma-rays with the Cesium 137 Gamma-ray Irradiation Facilities, Research Center for Nuclear Science and Technology, the University of Tokyo. Dosage ranging from 5 to 40 kR were achieved at a constant dose rate of 2.5 kR per minute. After the irradiation, the pollens were applied to the stigma of previously emasculated flowers. More than six flowers were pollinated at each dose.

3. **Ovule culture**

   Within a week after the pollination, the ovaries were cut off from the plants and
the surfaces were sterilized with 70% ethanol (30 sec) and with saturated solution of calcium hypochlorite (15 min). After the ovary wall was removed, the ovules which had already swollen were detached from the placentae and were inoculated on the solid medium in test tubes. And they were incubated at 28°C. Obtained plantlets were transplanted into pots and were cultivated till maturity in a greenhouse. The details of ovule culture were cited elsewhere (Shizukuda and Nakajima 1982, Shintaku et al. 1985).

4. Observation of chromosomes

Root tips obtained from the cuttings of lateral shoots were immersed in cold water (0°C) for 24 hours or pretreated with colchicine solution and were fixed in Farmer’s fluid. Number of chromosomes were examined under microscope after Feulgen staining.

Results

1) Production of partial hybrids

1.1) The variation in the characteristics of hybrid plants

Nicotiana rustica and N. tabacum have several morphological characteristics which make it possible to distinguish between them. Some of them are listed in Table 1. As reported in a previous paper (Shizukuda and Nakajima 1982), the hybrids between N. rustica and N. tabacum were efficiently produced through ovule culture, and they were homogeneous in their characteristics. The characteristics are also shown in Table 1.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>N. rustica (cv. Rustica)</th>
<th>N. tabacum (cv. Hicks 2)</th>
<th>Hybrid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flower</td>
<td>yellow</td>
<td>pink</td>
<td>pinky white</td>
</tr>
<tr>
<td>length</td>
<td>short (ca. 2cm)</td>
<td>long (ca. 5cm)</td>
<td>middle (ca. 3.5cm)</td>
</tr>
<tr>
<td>bud</td>
<td>spherical</td>
<td>ellipsoidal</td>
<td>ellipsoidal</td>
</tr>
<tr>
<td>Leaf</td>
<td>dark green</td>
<td>green</td>
<td>green</td>
</tr>
<tr>
<td>shape</td>
<td>heart</td>
<td>ellipse</td>
<td>ellipse</td>
</tr>
<tr>
<td>petiole</td>
<td>petiolar</td>
<td>sessile</td>
<td>intermediate</td>
</tr>
<tr>
<td>lamina</td>
<td>wavy</td>
<td>flat</td>
<td>flat</td>
</tr>
<tr>
<td>Plant height</td>
<td>low</td>
<td>high</td>
<td>high</td>
</tr>
</tbody>
</table>

After Shizukuda et al. 1983
In the present experiment, ten of the plantlets which were obtained through pollination with unirradiated pollen were grown till maturity in order to be used for the experimental control. It is verified that all of them had the same characteristics as the previously obtained hybrids had. Then we designated them as normal hybrids (Table 2).

The hybrid plants which were obtained by the cross-pollination with irradiated pollen grains varied widely in their characteristics. For example, flowers varied in color and length from those of normal hybrids to those of haploid mother plants (Plate 1).

Based on the characteristics listed in Table 1, the obtained hybrid plants were classified into three groups. In the first group, plants had characteristics which were not distinct from those of normal hybrids. In the second group, some characteristics of the plants were clearly different from those of normal hybrids. Then they were designated as anomalous hybrids. In them, the characteristics of *N. tabacum* expressed in the normal hybrids were lost to various extent and they showed resemblances to those of *N.*

Table 2. Number of hybrid plants obtained after irradiation of different dosesa

<table>
<thead>
<tr>
<th>Dose (kR)</th>
<th>Normal hybrid</th>
<th>Anomalous hybrid</th>
<th>Maternal hybrid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>11</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(43,48,48,48,48)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(36,36,40,43,48,48)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>17</td>
<td>24</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(24,25,26,36,36)</td>
<td>(48,48,48)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(36,42,42,43,48)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>48,48,48,48,48</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(46,47)</td>
<td>(48)</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(48,48)</td>
<td>(48,48)</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(48,96)</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>1</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(48)</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Totalb</td>
<td>44</td>
<td>63</td>
<td>7</td>
</tr>
</tbody>
</table>

a (Number of chromosomes)
b Total of plants obtained by pollination with irradiated pollen

After Shizukuda *et al.* 1983
Plate 1. The variation in flowers of hybrid plants obtained by pollinating with irradiated pollen. Upper most flowers are unirradiated controls (normal hybrids).

*rustica*. A few plants have the characteristics which were not observed in the parents, e.g. slender leaves. In the third group, the obtained plants have characteristics which were not distinct from those of *N. rustica* and they were fertile. Then we designate them as maternal hybrids.

The number of plants which belong to those three groups are shown in relation to exposure doses in Table 2. More than 50 percent of hybrid plants obtained by the cross-pollination with irradiated pollen belonged to the second group. About 7 percent of hybrid plants were maternal hybrids. Although the ratio of anomalous to normal hybrids increased as the exposure dose increased, the total productivity of the hybrid plants declined. It is considered that the higher doses of radiation affected the fertilization and/or the growth of zygotes through unknown mechanisms. Furthermore, it seemed that ovules which grew into calli increased with the ratio depending on the exposure dose. The hybrid plants which were ascertained to regenerate from callus were excluded from Table 2.
1.2) The variation in chromosome number of hybrid plants

It was reported previously that the normal hybrids obtained through the ovule culture technique had 48 chromosomes which were equal to sum of the haploid chromosomes of *N. rustica* (*n*=24) and those of *N. tabacum* (*n*=24) (Shizukuda and Nakajima 1982). The number of chromosomes in some anomalous hybrids varied in number from 24 to 48, except one plant which had 96 chromosomes. It is not curious that the anomalous hybrids had 48 chromosomes, since mutations and chromosome aberrations are induced with ionizing radiations. Though all of the chromosomes are very small and we can not discern difference among them, it is possible that the chromosomes which came from irradiated pollen of *N. tabacum* might lose their parts even if 48 chromosomes were observed. The important finding is that many anomalous hybrids have incomplete set of chromosomes less than 48. It is likely that in some of them only a few chromosomes were added to the haploid chromosomes of *N. rustica*. This result suggests that an irradiated pollen transfers an incomplete male genome into an egg cell and/or lesioned chromosomes are eliminated during embryogenesis. In any way, we can conclude that the irradiation of pollen before cross-pollination are effective for obtaining the partial hybrids into which an incomplete chromosome complement of male parent is introduced. And the results shown in Table 2 indicate that lower doses of gamma-rays (less than 15 kR) are enough to transfer the incomplete set of male chromosomes into female plants in this experimental system.

Examples of partial hybrids are shown in Plates 2 and 3. The hybrid plant in Plate 2 had flowers smaller than those of normal hybrids and relatively small and wavy leaves.

![Plate 2](image)

Plate 2. An example of anomalous hybrid (15kR). (a) a female parent (left), an anomalous hybrid (middle) and a normal hybrid (right). (b) a close-up of the anomalous hybrid.
Plate 3. An example of anomalous hybrid (15kR). (a) a normal hybrid (left), and an anomalous hybrid (right). (b) a leaf of normal hybrid. (c) a leaf of the anomalous hybrid. (d) a leaf of haploid plant of *N. rustica* obtained through anther culture. Flowers of the anomalous hybrid are shown in Plate 1 (bottom right).
like *N. rustica*. It had 36 chromosomes. The plant in Plate 3a had flowers which were similar to but smaller than those of *N. rustica*. It had 24 chromosomes which are equal to the chromosome number of the haploid female parent. But the characteristics of *N. tabacum* were slightly expressed in the morphology of leaves (Plates 3b, 3c, and 3d). Therefore we considered that a small portion of male genome was united with chromosomes of *N. rustica* and classified it into the anomalous hybrids.

All of the maternal hybrids had 48 chromosomes and were considered to be maternal diploids. Then the selfed progenies (M₁) of them were analyzed. The results showed that all of the maternal diploids were not different genetically from the maternal plants, i.e. *Nicotiana rustica*. It is concluded that pure lines can be obtained with the combination of two techniques i.e. the pollination with irradiated pollen and the ovule culture.

2) **Overcoming hybrid inviability**

2.1) Development of hybrid embryos *in vivo*

In the cross between *N. repanda* and *N. tabacum*, no viable seeds have been obtained *in vivo*. The histological observations revealed that the embryos of hybrid had developed into globular embryos and degenerated after that. When irradiated pollen were applied, seeds which were able to germinate were not obtained. However, the histological observations showed that some of them had embryos which had fully developed. Then the technique of ovule culture were used to make the embryos grow into seedlings.

2.2) Production of plantlet through ovule culture

Within 20 days after the inoculation of ovule on medium, many seedlings were obtained. At the 30th day, the number of seedlings after each exposure dose was counted (Table 3). The ratio of seedlings to cultured ovules decreased as the gamma-ray dose increased. No seedling was obtained after treatment with a gamma-ray dose of 40 kR. It is considered that the higher doses of radiation affected the fertilization and/or the growth of zygotes. It is concluded that the zygotes which were produced by the fertilization of non-irradiated or irradiated pollen grains can be grown up to seedlings through the technique of ovule culture.

The seedlings grew to plantlets having four to six leaves and a few slender roots. All of the plantlets obtained by pollination with non-irradiated pollen developed chlorosis and died. They were inviable as reported by Reed and Collins (1978). Although most of the plantlets obtained by pollination with irradiated pollen also died, several of them were able to survive.

The survived plantlets were subcultured once every month on the root-promoting medium. Some of them survived and continued to grow in culture vessels for more
Table 3. Production of seedlings through ovule culture

<table>
<thead>
<tr>
<th>Dose (kR)</th>
<th>No. of ovules inoculated</th>
<th>No. of seeds germinated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1503</td>
<td>69 (4.6 %)</td>
</tr>
<tr>
<td>5</td>
<td>910</td>
<td>27 (3.0 %)</td>
</tr>
<tr>
<td>10</td>
<td>1073</td>
<td>25 (2.3 %)</td>
</tr>
<tr>
<td>20</td>
<td>1000</td>
<td>13 (1.3 %)</td>
</tr>
<tr>
<td>30</td>
<td>879</td>
<td>8 (0.9 %)</td>
</tr>
<tr>
<td>40</td>
<td>867</td>
<td>0 (0.0 %)</td>
</tr>
</tbody>
</table>

Each figure is the sum of 3 individual experiments in each of which ovules were inoculated on the 4th, 5th and 6th day after cross-pollination. (After Shintaku et al. 1985)

than six months. As the exposure dose increased, the number of obtained seedlings decreased (Table 4). The ratio of survivor, however, increased with the increment of exposure doses. We can conclude that the irradiation of pollen before cross-pollination is effective in obtaining viable plantlets, and that there may be an optimal exposure doses to obtain plantlet efficiently.

2.3) Production of mature plants and their characteristics

The plantlets were potted in vermiculite and transferred into a greenhouse. Most of the transplanted plantlets showed poor growth and eventually died within a month. Only two of them were able to reach the flowering stage and maturity. Both of them were obtained after irradiation with a dose of 20 kR. It is considered that a compromise has to be made between using a high dose to cause maximum genome damage and

Table 4. Effect of gamma-irradiation on survival of hybrid plantlets

<table>
<thead>
<tr>
<th>Dose (kR)</th>
<th>No. of seedlings obtained</th>
<th>No. of plantlets survived</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>124</td>
<td>0 (0.0 %)</td>
</tr>
<tr>
<td>5</td>
<td>71</td>
<td>7 (9.9 %)</td>
</tr>
<tr>
<td>10</td>
<td>33</td>
<td>4 (12.1 %)</td>
</tr>
<tr>
<td>20</td>
<td>18</td>
<td>4 (22.2 %)</td>
</tr>
<tr>
<td>30</td>
<td>8</td>
<td>0 (0.0 %)</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>0 (--)</td>
</tr>
</tbody>
</table>

Each figure is the sum of 3 individual experiments in each of which ovules were inoculated on the 4th, 5th and 6th day after cross-pollination. (After Shintaku et al.)
Plate 4. Morphological characteristics of hybrid plants between *N. repanda* and *N. tabacum*.

(a) Morphology of leaves of *N. repanda*, hybrid (2n=47), hybrid (2n=46) and *N. tabacum* (left to right).
(b) Morphology of flowers *N. repanda*, hybrid (2n=47), hybrid (2n=46) and *N. tabacum* (left to right).

Note: Flower colors are white, light pink, light pink and pink, respectively (left to right).

producing viable mature plants, and that in the present work 20 kR appear to be this dose.

The phenotype of both mature plants was similar to that of the male parent, *N. tabacum*, with regard to the plant height and the branching habit. The leaves of the plants were intermediate in appearance between those of the parents, which the flower shape was similar to *N. tabacum*, the male parent (Plate 4). The flowers were light pink in color which is characteristics of the male parent. Thus, mature hybrid plants between *N. repanda* and *N. tabacum* could be obtained, which had never been reported.

The hybrid plants had 47 and 46 chromosomes respectively, which is less than the additive sum of the haploid chromosomes of *N. repanda* (n=24) and those of *N. tabacum* (n=24). We concluded that these plants were able to reach maturity because the chromosomes and/or chromosome segments bringing about hybrid inviability were eliminated. In conclusion, it is possible to overcome hybrid inviability to a certain extent by using the combination of two techniques, i.e. the pollination after the destruction of chromosomes in pollen grains with ionizing radiations and *in vitro* culture of crossed ovules.

3) Intergeneric cross

In order to investigate the utility of the combination of two techniques for obtain-
Table 5. Number of plants obtained through ovule culture

<table>
<thead>
<tr>
<th>Dose (kR)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>35</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of ovules inoculated</td>
<td>1879</td>
<td>3540</td>
<td>1758</td>
<td>3087</td>
<td>3196</td>
<td>2453</td>
<td>1454</td>
<td>895</td>
<td>845</td>
</tr>
<tr>
<td>No. of plants obtained</td>
<td>2</td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>12</td>
<td>5</td>
<td>46</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Diploids</td>
<td>1</td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>12</td>
<td>4</td>
<td>46</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Haploids</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Shintaku, unpublished data

ing intergeneric hybrids, *Nicotiana rustica* was pollinated with irradiated pollen of *Petunia hybrida*. We can obtain mature plants through the technique of ovule culture, whether the used pollen was irradiated or not. However, the ratio of mature plants to inoculated ovules tended to be higher in plants derived from the pollination with irradiated pollen than in plants derived from the pollination with non-irradiated pollen (Table 5). The mature plants were classified into two types. The first type of plants was sterile and had a half set of chromosomes, i.e. haploids. The second type was fertile and had complete set of chromosomes, i.e. diploids. It was expected that the diploid plants incorporated a few genes of *Petunia*, as reported by Pandey (1975) using interspecific cross in *Nicotiana*, then the selfed progenies of the second type of plants were analyzed. As it was difficult to discern the phenotype of progenies from that of *N. rustica*, we concluded that diploid plants produced through the ovule culture were inbred lines. We could not obtain the partial hybrid plants between *N. rustica* and *P. hybrida*, but it is interesting that the pure lines were effectively obtained through ovule culture after the pollination of irradiated pollen.

**Discussion**

Treatments of pollen with ionizing radiations have been carried out in many plant species with the following aims, (a) to overcome intra- and inter-specific pollen incompatibilities, and (b) to obtain haploid plants or maternal diploids (matromorphs).

In recent years, some unexpected observation was made during the experiments for overcoming self-incompatibilities in genus *Nicotiana* by using the mentor methods, in which the compatible pollen killed with lethal doses of ionizing radiations (mentor
pollen) was mixed with viable incompatible pollen and applied to the stigmata of unirradiated mother plants. Pandey (1975, 1978 and 1980a) reported that some characteristics of donor plants were introduced into the maternal diploids. And he stated that the pulverized chromatin of an irradiated compatible pollen was transferred into an egg cell by pseudo-fertilization and the egg cell developed into a diploid plant through the second fertilization with a viable self-incompatible pollen or through doubling of chromosomes at the onset of parthenogenesis (Pandey 1980b).

Effectiveness of irradiated pollen for raising the productivity of haploid plants was found by Katayama (1934) in *Triticum monococcum*. Ivanov (1938) also succeeded in getting several haploids and maternal diploids in *Nicotiana rustica*. After that, the possibility of obtaining instantly pure lines through parthenogenetic seed development has been investigated. However, Virk et al. (1977) threw a serious doubt on the possibility, because they found that the progenies of the diploid plants suspected of being maternal origin showed significant segregation for some quantitative characters and contained abnormal segregants. And they also reported that single gene was transferred from the pollen parent. Based on these unexpected observations, Pandey (1975) and Virk et al. (1977) proposed that this provides possible rapid means for transferring desirable genes without recurrent backcrossings.

Recently, Virk and Gupta (1984) reported that, in *Pisum sativum*, matromorphs were obtained by the pollination with irradiated pollen of *Lathyrus odoratus* and *Pisum sativum*, and stated that the induction of matromorphs appears to be a viable proposition for producing instant pure-breeding line in peas. In addition, they observed that a few genes were introduced into some of the matromorphs from the male parent.

Ever since the proposition of Pandey and Virk et al., experiments have been carried out aiming to study the possibility of facilitating the gene transfer and reducing the number of required backcross through pollen irradiation. And positive results were obtained by Jinks et al. (1981) and Caligari et al. (1981) in *Nicotiana*, by Powell et al. (1983) in *Hordeum*, by Snape et al. (1983) in *Triticum*, and by Daskalov (1984) in *Capsium*. These investigators reported that the second generation progenies (M₂) derived from crosses using irradiated pollen showed a closer resemblance to the maternal phenotype, and the degree of similarity increased with the increment of doses of radiation used. Powell et al. (1983) and Snape et al. (1983), however, claimed that Pandey's proposed mechanisms of gene transfer to explain this effects should be rejected. Instead, they proposed that their M₂ progenies were the products of conventional fertilization by pollen carrying some radiation damaged chromosomes. They considered that the radiation damage causes disturbance at M₁ meiosis which resulted either in the loss of parental chromosomes or in loss of function of the gamatophytes carrying them, and M₁ plants gave rise to M₂ families showing close resemblances to the maternal parents.
Furthermore, Caligari et al. (1981) and Wener et al. (1984) stated that Pandey's gene transfer mechanism could also be open in genus Nicotiana which was used as the material by Pandey. Based on cytological studies, these authors considered that the plants which showed great resemblances to the female are resulted from conventional radiation damage in the form of losses of whole chromosomes, part of chromosomes, or rearrangement.

It is premature to decide which of the proposed mechanism is correct. It might be said that the discrepancy between the proposed mechanisms may result from the difference of the experimental systems which they were used. Pandey has described his M₁ plants as being euploid, fertile, and largely maternal, that is, as matromorphs. However, the other investigators have not obtained matromorphs in the first generation (M₁). For example, Snape et al. (1983) stated that cytological studies of M₁ plants revealed hybrids with widespread aneuploidy and structural rearrangements in paternal genome. Moreover, the other investigators used self-compatible species whereas Pandey used self-incompatible species. And they used radiation doses considerable lower than those Pandey used. The mechanism proposed by Pandey should be tested with the experimental system in which the matromorphs were efficiently produced. But the major limitation of his experimental system is the very low number of seeds obtained and their poor germinability. We have shown that the ovule culture after the pollination with irradiated pollen made it possible to obtain matromorphs (Tables 2 and 5). As the induction of matromorphs was a very rare event in ovule culture after the pollination with non-irradiated pollen, it can be said that the irradiated pollen can cause the parthenogenetic growth of egg cells. Though we have already obtained about 90 maternal diploids (M₁) in Nicotiana rustica, there is no M₁ and M₂ plants of which phenotype was different from that of the maternal parent. In our experiments, the doses used were lower than those used by Pandey. Definite conclusions must await further investigations.

Regardless of the obscurity of the underlaying mechanisms, it is important to investigate how the technique of pollen irradiation facilitates the gene transfer and reduces the number of required backcross. Daskalov (1984) proposed a new procedure which were useful for facilitating gene transfer, especially in case of gene linkages. On the contrary, Zimar (1983) observed only minor effect on enzymic gene transfer after pollen irradiation in tomato (Lycopersicon), and Sanford et al. (1984) reported that the transfer of genes from the irradiated pollen is a very rare event and is not likely to be used in corn (Zea) improvement. It is important to study the combination of the techniques as we have tried in order to obtain M₁ plants derived from wide crosses using irradiated pollen.

Our intention, however, was not to use the phenomenon for facilitating the gene transfer in a backcross programme. We intend to investigate how this technique effects
the potential for interspecific and intergeneric transfer of genetic information to a certain species, which has not been successful by conventional technique of sexual hybridization. We succeeded in obtaining the partial hybrids of the cross *N. rustica* and *N. tabacum* using the combination of two techniques, i.e. the pollination with irradiated pollen and the ovule culture. The partial hybrids obtained in the present experiments had been added various number of male chromosomes to the female haploid chromosomes whereas Pandey and Virk and co-workers obtained directly maternal diploids to which a few genes transferred from the irradiated pollen. The fact is by no means a defect of our experimental system. On the contrary, it is an advantage that the amount of transferred male genome is changed depending on the applied radiation doses (Tabe 2). In some cases, it may be prefered to introduce some chromosomal segments rather than a single gene.

We have also shown that the production of partial hybrids is used as an effective means of overcoming hybrid inviability. Each plant species, which has evolved under different conditions on the earth since they were born, have built barriers to production of hybrids which they can not overcome by themselves. The phenomenon of hybrid inviability is one of these barriers. Then, if the barrier was eliminated with the technique of pollination with irradiated pollen, it might be possible to obtain novel plants which possess the impossible combinations of genes under the natural conditions. Our results suggest that the combination of two techniques is a candidate of techniques for realizing the possibility.

Application of the technique of ovule culture, however, is limited to the plant species of which gametic cells can fuse in vivo. Other techniques like the test tube fertilization and the protoplast fusion are needed to widen the range of possible crosses. The protoplast fusion have been suggested as a method to overcome the species barrier to sexual hybridization. It make it possible to cross between any remote species. However, somatic hybridization requires that complete plants are regenerated from the fused cell after two somatic cells are fused. While it has been expected to offer promise of obtaining new crop varieties, this technique has been applied successfully to only several plant species. It is considered that the fused cells are inviable because their metabolisms are disturbed by the fusion of two considerable different genetic systems. It could be said that the technique of irradiation is also used for overcoming the hybrid inviability of somatic hybrid cells. Further investigations for obtaing partial hybrids into which a portion of alien genome is transferred should be carried out by using various combinations of possible techniques.
SEXYAL TRANSFER OF A PORTION OF PATERNAL GENOME

Summary

The possibility of transferring sexually a portion of paternal genome between two uncrossable species was investigated in three crosses (i) Nicotiana rustica × N. tabacum, (ii) N. repanda × N. tabacum, and (iii) N. rustica × Petunia hybrida. Pollens were exposed to $^{137}$Cs gamma-rays (5–40kR) and were applied to stigmas of mother plants. On the 4–7th day after the pollination, the ovules were detached from the placentae and were inoculated on solid medium.

(i) N. rustica × N. tabacum

One hundred and fourteen mature plants were obtained through the ovule culture and they were classified into the following three groups on the basis of their morphological characteristics; (1) normal hybrids which were not distinguished from the hybrid plants obtained through crossing by unirradiated pollen, (2) anomalous hybrids which were clearly different from the normal hybrids, and (3) maternal hybrids which were not distinguished from the maternal parent, i.e. N. rustica and were fertile. More than 50 percent of the obtained hybrid plants were the anomalous hybrids. The morphology of anomalous hybrids varied from that of haploid mother plants to the normal hybrids and the chromosomes of these plants varied in number from 24 to 48. These results suggest that a portion of genome is transferred from N. tabacum (n=24) to N. rustica (n=24).

(ii) N. repanda × N. tabacum

Though many hybrid plants were produced, most of them died in the young stages. Two hybrid plants grew vigorously and reached the flowering stage. The observation of characteristics revealed that they were hybrids. They had 46 or 47 chromosomes. By using these techniques, it is possible to obtain aneuploid hybrids which have never been reported.

(iii) N. rustica × P. hybrida

Though many plants were obtained, most of them were maternal hybrids which were not distinguishable from maternal plants, i.e. N. rustica, and were fertile. The utility of the combination of two techniques, i.e. the pollination after the destruction of chromosomes in pollen grains with ionizing radiations and the in vitro culture of ovules for obtaining partial hybrids were discussed.
Acknowledgement

Much of the data included here is the result of the efforts by my collaborators. The experiments on interspecific hybrid of *N. rustica* × *N. tabacum* are mainly due to Mr. Naoki Shizukuda and were published. The experiments on interspecific hybrid of *N. repanda* × *N. tabacum* and on intergeneric hybrid of *N. rustica* × *P. hybrida* are mainly due to Miss Yurie Shintaku and will soon be published. Dr. Tetsuo Nakajima played the part of the leader. I thank all of these individuals. The present study was partially supported by a Grant-in-Aid for Special Project Research for the Ministry of Education, Science and Culture, Japan.

References


照射花粉を用いた花粉親の不完全なガノムの導入に関する研究

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東京都文京区皇学 1-1-1

遠縁交雑により有用遺伝子を栽培種へ導入する場合に、まず雑種を得ることの困難性が問題となる。その中には幼植物が得られたとしても成熟個体が得られない雑種致死という現象も存在している。また、たとえ雑種が得られたとしても、交雑によって同時に持ち込まれた好ましくない遺伝子の除去が必要である。これらの問題を同時に解決する一つの方法として、放射線を照射して染色体の断片化をはかった花粉を授粉し、花粉親のゲノムの一部だけを導入した「部分雑種」を、英語と呼ばれる利用して作出することが考えられる。本研究では、通常の交雑では初期胚が崩壊するために雑種が得られないNicotiana rustica × Nicotiana tabacum の組合せ、幼植物の段階で死滅する雑種致死であるNicotiana repanda × Nicotiana tabacum の組合せ、両方で雑種が得られていないNicotiana rustica × Petunia hybrid の組合せについて、上述の方法による「部分雑種」作出の可能性を検討した。それぞれの花粉を137Csガンマ線で照射し（5～40 kR, 2.5 kR/min）、あらかじめ除雑したそれぞれの花に授粉した。授粉後 4 ～ 7 日目に胚珠を摘出し、卵巣を抽出して胚珠培養を行ない雑種植物を育成した。

(i) N. rustica × N. tabacum

胚珠培養の結果、合計114個体の雑種が得られた。雑種植物は形態的形質（草姿、葉型、花型、花色など）に関する変異が著しく、正常な雑種（非照射花粉によって得られた雑種）型から母親であるN. rusticaの型に至る様々な段階の特性を持つ個体が見られた。これらの個体は、次の3群に分類できた(Table 2)。

正常型：非照射花粉によって得られた雑種と区別ができないもの
異常型：いずれかの形質において正常型と異なり、母親の特性が強く発現されているもの
母親型：N. rustica と区別できず、正常な仮性を持つもの

照射花粉の授粉によって得られた雑種植物のうち、50％以上が異常型に属し、約7％が母親型であった。照射線量の増加に伴ない異常型と母親型に属する個体の割合が増加したが、得られる個体数は逆に減少した。したがって、雑種個体が多く得られる線量は10～15 kRであった。

この組合せで、非照射花粉を授粉し胚珠培養によって得た正常な雑種植物は、すべての個体が形態的に正常で、両親（共にn=48）より受けついだ2n=48の染色体を持っている。これに反し、異常型雑種は、個体（96本）を除き48～48本の染色体をもっている。すなわちN. rusticaのゲノム（n=24）に種々の程度でN. tabacumの染色体、ある
いはその断片が加わっていると考えられる。

(II) N. repanda × N. tabacum

培養20日後に多くの発芽個体が得られた（Table 4）が、大半は4～6葉で枯死した。一部の幼植物は、発根培地上で長期にわたって生存した。しかし、それらの植木鉢に移植したところ、再び大部分が枯死した。そして20 kR区の2個体だけが旺盛に生長し開花するに至った。それらは、それぞれ46本、47本の染色体をもつ異数体であった。短姿、葉形、花形などについては両親のほぼ中間的特性を示し、花弁に淡い桃色が認められるなど2個体とも両親の雑種であることが確認できた。

(III) N. rustica × P. hybrid

培養の結果得られた個体の総てが旺盛に生育し、完全な稔性を持っていた。形質は母親であるN. rusticaと区別できず、後代（M₀）における形質の分離も観察されなかった。

以上の結果は照射花粉法と胚珠培養法を組合せることにより部分雑種の形成が可能となる、さらに雑種死亡の現象をも解消して自然条件下では存在しえない遺伝子の組合せをもつ植物体を作りうる可能性を示唆している。N. rustica × P. hybridにおいて部分雑種が得られなかったことから属間交雑までこの方法の適用範囲を拡大できるかどうかは不明である。しかし、この組合せで母系雑種が容易に出来るという事実は部分雑種の生成とは別の興味ある問題を提起している。

質疑応答

神山（三重大・農）：この材料では花粉は二核性ですから発芽過程で一回の核分裂をすることになり、染色体異常の影響がかなり出ると思われますが、その意味で成熟花粉で既に三核になっている植物で同じようなことを試みたばあい、かった結果になるのではとの期待もできますが、実験例かコメントを教えてください。

山元：コムギとオオムギではそうなるようですが、ただかけただけで培養は使っていないわけではないです。一応療母雑種ができるということのような報告はあります。そのへんにつきましては、出来るものは殆どニコチアナ属です。なにをやってもニコチアナ属で出てくるということから、進化という点からもなぜナス科といえるのかということをやってゆくのが、ほかのものに比べてゆっくりとおっかかりになるような気がいたします。
IN VITRO MUTATION BREEDING IN MEDICINAL PLANTS

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Introduction

Medicinal plants have not been subjected to genetic manipulation yet, although they have been utilized from time immemorial in human activities as well as ordinary crop plants. Unimproved genotypes gathered from wild habitats are still widely used. With the remarkable development of medicinal industry in recent years, the improved production efficacy of useful medicinal ingredient is requested. It may be realized by modern technology which have been applied to improvement of crop plants.

It is very important to elucidate the possibility of the application of mutation breeding which has successfully improved cultivated crops, to medicinal plants for the increment of yield in useful ingredient. Moreover, technologies pertaining to plant tissue culture has remarkably developed since 1960s and made it possible to regenerate plants from a single cell by its totipotency (Steward et al., 1958). This may lead to a new approach to screen a particular genotype from a plenty of cultured cells. And also it may lead to mass-production to identical genotypes by cloning of plant cells. By the combination of mutation breeding and in vitro culture technique, it would be possible to accelerate genetic improvement of such materials at primitive state as medicinal plants.

The present report deals with radiosensitivity referring to mutagenesis and phenotypic variation of regenerated plants in three species: *Carthamus tinctorius* L., *Datura alba* Nees and *Coptis japonica* Makino.

Materials and Method

Plant materials used in the present experiment are listed in Table 1. Each plant

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Table 1. Plant materials

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific name</th>
<th>Institution of procurement or place of collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Safflower</td>
<td><em>Carthamus tctorius L.</em></td>
<td>Tsukuba Medicinal Plant Research Station, National Institute of Hygienic Sciences (cultivated)</td>
</tr>
<tr>
<td>Chosen-Asagao</td>
<td><em>Datura alba</em> Need</td>
<td>Izu Experimental Station of Medicinal Plant, National Institute of Hygienic Sciences (cultivated)</td>
</tr>
<tr>
<td>Koseriba-Ouren</td>
<td><em>Coptis japonica</em> var. major Satake</td>
<td>Johoku-machi, Ibaraki-ken (native)</td>
</tr>
<tr>
<td>Seriba-Ouren</td>
<td><em>Coptis japonica</em> var. <em>dissecta</em> Nakai</td>
<td>Kuzuryu, Izumi-mura, Fukui-ken (cultivated)</td>
</tr>
<tr>
<td>Seribagata-Kikuba-Ouren</td>
<td><em>Coptis japonica</em> Makino</td>
<td>Toyama Prefectural Pharmaceutical Research Institute (cultivated)</td>
</tr>
</tbody>
</table>

material had uniform looking, though outcrossing seems to take place to some extent. Plants of *Datura* were used after selfing two times.

In *Datura* and *Coptis*, chronic gamma-ray irradiation was made in a gamma-field equipped with a 2,400 Ci ⁶⁰Co source and acute irradiation in a gamma-room equipped with a 1,200 Ci ⁶⁰Co source. For the determination of radiation injuries, canopy diameter, plant height, number of foliage and survival rate were measured after 2 year cultivation in a gamma field for chronic irradiation or after 2 year cultivation of acutely irradiated plants. Pollen fertility was determined for irradiated plants by staining with aceto-carmine.

Seeds and seedlings of *Carthamus* were irradiated in a gamma-room at a dose rate of 1 kR/hr. Height of 19 day seedling from irradiated seeds and increase of fresh weight of irradiated seedlings 18 days after irradiation were measured to determine radiosensitivity.

Calli of these three species were irradiated in a gamma-room at room temperatures. Calli were transferred to new culture medium immediately after irradiation. For determination of *in vitro* radiosensitivity, in *Coptis*, buds and/or shoot formation and coloring of calli by radiation injury were observed and in *Carthamus* fresh weight 3 to 4 week after irradiation were measured.
Table 2. Composition of culture media

<table>
<thead>
<tr>
<th>Composition</th>
<th>Coptis and Datura</th>
<th>Carthamus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic medium</td>
<td>MS</td>
<td>MS</td>
</tr>
<tr>
<td>Thyamine hydrochloride</td>
<td>0.4 (mg/l)</td>
<td>1.0 (mg/l)</td>
</tr>
<tr>
<td>Inositol</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>–</td>
<td>0.5</td>
</tr>
<tr>
<td>Pyridoxine hydrochloride</td>
<td>–</td>
<td>0.5</td>
</tr>
<tr>
<td>2,4-D</td>
<td>$10^{-5}$ (M)</td>
<td>– (M)</td>
</tr>
<tr>
<td>NAA</td>
<td>$10^{-4}$</td>
<td>$10^{-5}$</td>
</tr>
<tr>
<td>BAP</td>
<td>$10^{-6}$</td>
<td>$5 \times 10^{-6}$</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20-30 (g/l)</td>
<td>30 (g/l)</td>
</tr>
<tr>
<td>Agar</td>
<td>9 (g/l)</td>
<td>7 (g/l)</td>
</tr>
<tr>
<td>pH</td>
<td>5.4</td>
<td>5.7</td>
</tr>
</tbody>
</table>

MS medium was used for *in vitro* culture as the basic medium. Composition of this medium employed in the present experiments are shown in Table 2. As plant hormones, in *Datura* and *Coptis* $10^{-5}$ M 2, 4-D, $10^{-4}$ M NAA and $10^{-6}$ M BAP and in *Carthamus* $10^{-5}$ M NAA and $5 \times 10^{-6}$ M BAP was added to MS medium.

In *Coptis*, content of crude berberine, which is the most important medicinal ingredient contained in the species, in calli and embryoids were determined by thin layer chromatograph method. Berberine was extracted with methanol containing 1% hydrochloric acid, and the solution was concentrated by a vacuum evaporator. Thin layer chromatograms were run on G-type of silica gel plates of a Thin-Layer Chromatographic apparatus using solvent of butanol, acetic acid and water (7:1:2 (v/v)). Determination was made by a high speed chromato-scanner Shimadzu CS-920 with 365 nm wave length exciting light for the fluorescence of 550 nm wave length.

**Results**

1) *Carthamus tinctorius* L.

Callus was induced from leaves of young seedlings on MS medium with agar. Light yellow callus which showed continuous and stable growth was selected for the present experiment after several times of subcultures.
To investigate radiosensitivities of various tissues of *Carthamus tinctorius* L., seeds, 10 day seedlings and calli were irradiated with gamma-rays at a dose rate of 1 kR/hr. To determine radiation injury, height of plants grown from irradiated seed and increase of fresh weight in irradiated seedlings and calli were measured. RD$_{50}$ values of callus, seedling and seed were estimated to be 12 kR, 6 kR and 40 kR, respectively as shown in Fig. 1.

Effects of irradiation dose rate on radiation injury of callus are shown in Fig. 2. It was indicated that dose rate also has important influences on radiation injury of callus in the range of low total dose. Although irradiation of 6 and 12 kR at a dose rate of 0.5 kR/hr depressed growth of callus to 59.4% and 48.4% of control, respectively, irradiation of the same doses at a dose rate of 4 kR/hr caused 70 to 75% depression of
growth, which was comparable to the effect of 24 kR irradiation.

Although irradiated calli showed depression of growth, the growth pattern of irradiated calli after irradiation was similar to that of the control calli. The growth of callus of the first and the second culture after irradiation are shown in Fig. 3. All calli showed growth patterns of exponential curve. In the first culture after irradiation, the depression of growth rate in irradiated callus was obvious, and this depressed growth rate was not recovered until calli were transferred to fresh medium. In the second culture, however, irradiated callus grew at a similar to or even higher rate than unirradiated callus. It was suggested that injured cells in the irradiated callus might be selected out when these calli were transferred to fresh medium.

As the growth rate of callus in each culture is exponential, it was expected that radiosensitivity might change according to growing stage. Different radiosensitivity among three calli whose period of pre-culture was 4, 10 and 17 days respectively are shown in Fig. 4 (left). The callus of 4 day pre-culture showed higher sensitivity than calli of 10 day and 17 day pre-culture. The callus of 17 day pre-culture was the most radiation tolerant. But, as shown in Fig. 4 (right), in 7 to 8 weeks after irradiation, these three calli showed the same growth. It was also suggested that different mechanisms of radiation injuries might be working in 1 to 2 weeks and 3 or more weeks after the initiation of subculture.

2) *Datura alba* Nees

Radiosensitivity of air dried seed and callus of *Datura alba* Nees was investigated.
In the seed irradiation, both LD<sub>50</sub> and RD<sub>50</sub> of the stem height of plant were between 25 to 30 kR. RD<sub>50</sub> of the callus growth was around 15 kR at two dose rates of 200 R/hr and 400 R/hr.

In calli irradiated with LD<sub>50</sub> dose albino plants were observed at a frequency of 2 to 3% among regenerants.

3) Coptis japonica Makino

The response of growing Coptis plants to acute and chronic irradiation is determined and shown in Fig. 5. LD<sub>50</sub> and RD<sub>50</sub> of plant growth, flower induction and pollen fertility are shown in Table 3.

Regeneration of buds and/or shoots as well as plantlets were induced from explants of various organs. Induction rates of callus are shown in Table 4. The rate of callus induction from leaf blades was the highest. Tissues taken from base end of flower buds were exposed to gamma-rays and their rate of callus formation was also checked. Calli were differentiated even from explants which had been subjected to dose as high as 60 kR (at a dose rate of 250 R/hr) gamma-rays, although callus formation rates were very low as compared with the case of lower dosages.

Among the calli induced, there recognized three different types of callus, namely
brown, yellow, and colourless ones. They seemed to differ also in berberine content. Dark brown calli were more frequently induced from the basal end of flower buds than from leaf blades.

Differentiation of embryoid took place when the calli were subcultured onto media which did not contain auxins, that is 2, 4-D or NAA. The embryoid produced shoots and/or roots and developed to plantlet after continued culture though it produced secondary embryoid as well.

Berberine content in embryoid was determined, since it may be presumed that the
berberine content of a regenerated plant may be positively correlated with that of cells in embryo which produced it. Mean and bias from the normal distribution of berberine content in embryos after different irradiation treatments are shown in Table 5. Determination was made on each culture flask. In the present experiment difference in berberine content among embryos developed in each culture flask was greater than among different parts of callus which produced embryo. Variations within culture flask was small. The highest mean berberine content was detected in the embryos derived from leaf bades. Gamma-ray irradiation caused the decrease of berberine content in every embryo derived from all parts of plant. But in embryos which derived from leaf blades after chronic irradiation of 30 kR, mean berberine content was remarkably high, since one sample with extraordinarily high berberine content was contained.
Table 5. Normality of distribution of crude berberine content in embryoids derived from different organs of *Coptis japonica* var. *dissecta* Nakai

<table>
<thead>
<tr>
<th>Source of explant</th>
<th>Exposure (kR)</th>
<th>Mean of crude berberine content (%)</th>
<th>Normality</th>
<th>Skewness</th>
<th>Kurtosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal end of flower bud</td>
<td>0</td>
<td>0.145</td>
<td></td>
<td>0.110</td>
<td>-1.772</td>
</tr>
<tr>
<td></td>
<td>8 (acute)</td>
<td>0.095</td>
<td></td>
<td>0.203</td>
<td>0.381</td>
</tr>
<tr>
<td>Leaf blade</td>
<td>0</td>
<td>0.225</td>
<td></td>
<td>0.717</td>
<td>-1.336</td>
</tr>
<tr>
<td></td>
<td>8 (acute)</td>
<td>0.248</td>
<td></td>
<td>1.840**</td>
<td>2.608**</td>
</tr>
<tr>
<td></td>
<td>chronic (pooled)</td>
<td>0.273</td>
<td></td>
<td>1.498**</td>
<td>0.952</td>
</tr>
<tr>
<td></td>
<td>30 (chronic)</td>
<td>0.260</td>
<td></td>
<td>0.961</td>
<td>-1.563</td>
</tr>
<tr>
<td></td>
<td>60 (chronic)</td>
<td>0.093</td>
<td></td>
<td>2.234*</td>
<td>4.992*</td>
</tr>
<tr>
<td>Leaf petiole</td>
<td>0</td>
<td>0.111</td>
<td></td>
<td>0.108</td>
<td>-1.726</td>
</tr>
<tr>
<td></td>
<td>60 (chronic)</td>
<td>0.089</td>
<td></td>
<td>1.241**</td>
<td>1.304</td>
</tr>
</tbody>
</table>

* Significant at 5% level  
** Significant at 1% level

Skewness and kurtosis of the distribution in the content were statistically significant in irradiated calli (Table 5). This means that distributions of berberine content were biased by gamma-ray irradiation. Significant positive values of shewness and kurtosis indicates that there were increased frequencies of embryoids of lowered berberine contents and that there were high frequencies of embryoids with a particular value of berberine content.

As shown in Table 6, three different varieties showed different rates of callus induction and embryoid formation calculated on the basis of culture flask. The number of plants in the table shows the total number of plantlets which developed from embryoids and were transferred to pots in a greenhouse. Two or more plants were taken from a culture flask. *Coptis japonica* var. *dissecta* Nakai had the highest potential to produce regenerated plant. On the other hand, in var. major Satake more than 80% calli developed to embryoid. In all these three varieties, although induction of callus was more efficient from leaf blades than from leaf petioles, and the number of plantlet developed from embryoid was higher in calli from leaf petioles than from leaf blades, rates of the embryoid formation from the calli induced from both organs did not differ, being 70 to 80%. These results suggested that there were different potentials to induce callus or embryoid and to regenerate plantlet.
Table 6. Induction of callus and embryoid differentiation in *Coptis japonica* varieties

<table>
<thead>
<tr>
<th>Clone</th>
<th>Explant</th>
<th>Number of explant</th>
<th>Rate of callus formation (%)</th>
<th>Rate of embryoid formation (%)</th>
<th>Number of plantlet obtained (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Coptis japonica</em> var. <em>major</em> Satake</td>
<td>Leaf blade</td>
<td>49</td>
<td>63</td>
<td>85</td>
<td>9 (18)*</td>
</tr>
<tr>
<td></td>
<td>Leaf petiole</td>
<td>46</td>
<td>45</td>
<td>83</td>
<td>12 (26)</td>
</tr>
<tr>
<td><em>C. japonica</em> var. <em>dessecta</em> Nakai</td>
<td>Leaf blade</td>
<td>105</td>
<td>82</td>
<td>86</td>
<td>10 (10)</td>
</tr>
<tr>
<td></td>
<td>Leaf petiole</td>
<td>65</td>
<td>61</td>
<td>66</td>
<td>23 (35)</td>
</tr>
<tr>
<td><em>C. japonica</em> Makino</td>
<td>Leaf blade</td>
<td>119</td>
<td>72</td>
<td>78</td>
<td>7 (6)</td>
</tr>
<tr>
<td></td>
<td>Leaf petiole</td>
<td>54</td>
<td>51</td>
<td>70</td>
<td>12 (22)</td>
</tr>
</tbody>
</table>

* Number of regenerated plant / Number of explant × 100.

Because tissues of *Coptis* released some alkaloid compounds to the media, the accumulation of a large quantity of berberine in the culture media may be expected after *in vitro* culture of either callus or embryoid. In the present experiment, 10 g medium was used to culture 1 to 3 g of calli, and the crude content of berberine was found to be around 0.01% in agar medium. If callus cells could have berberine at a high content, the agar medium which have the cells on it would be proportionally high in content of the ingredient. Correlation coefficients in content between tissue and medium were high (0.7 to 0.9) and statistically significant. It is concluded that estimation of berberine content in culture cells can be easily performed, with no damage on cells, by determining concentration of the ingredient in medium.

**Discussion**

Genetic manipulation might improve agronomic traits of a medicinal plant to a large extent, since they have never been subjected to the modern breeding trials.

Before applying mutation breeding techniques to medicinal plant species, radiosensitivity of the species should be determined as a first step. It was shown that *Coptis* has low radiosensitivity and can tolerate as high exposure as 60 kR of gamma-rays. Radiosensitivity of *Datura* was intermediate in radiosensitivity 25 to 30 kR of LD50. *Carthamus* showed rather high radiosensitivity.
Table 7. Radiosensitivities of plant callus

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>RD_{50} (kR)</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pisum sativum</td>
<td>2.5</td>
<td>2) Burholt, D.R. and Van't Hof, J. (1974)</td>
</tr>
<tr>
<td>Petunia inflata</td>
<td>5</td>
<td>Bapat, V.A. (1976)</td>
</tr>
<tr>
<td>Daucus carota</td>
<td>5 – 10</td>
<td>Miszke, W. et al. (1979)</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>5.2</td>
<td>3) Venketeswaran, S (1966)</td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td>10</td>
<td>3) Bajaj, Y.P.S. et al. (1970)</td>
</tr>
<tr>
<td>Pharbitis nil</td>
<td>20</td>
<td>Rao, P.S. et al. (1976)</td>
</tr>
<tr>
<td>Arachis hypogaea</td>
<td>100</td>
<td>Verma, D.P.S. and Van Huystee, R.B. (1971)</td>
</tr>
</tbody>
</table>

1) RD_{50} values on the callus growth.
2) RD_{50} value on the increase of cell number.
3) Suspension culture.

It is generally observed that in higher plants sensitivity to gamma-rays is higher in the order of growing plant, callus and seed (Venketeswaran et al. 1966, Bajaj et al. 1970, Bapat et al. 1976, Rao et al. 1976). However, the irradiation effect of callus may change with irradiation conditions and stages of cells, as shown here in *Carthamus*. Radiosensitivities of calli irradiated with gamm-rays in various species are shown in Table 7. *Nicotiana tabacum* L. (Venketeswaran 1966, Opatrny 1974) and *Phaseolus vulgaris* L. (Bajaj et al. 1970) show the comparable radiosensitivity to *Carthamus* in this experiment. As compared with these species, *Pisum sativum* (Burholt et al. 1974), *Petunia inflata* (Bapat 1976) and *Daucus carota* (Miszke 1979) show higher sensitivity, and *Citrus sinensis* (Spiegel-Roy et al. 1973) and *Pharbitis nil* (Rao et al. 1976) are radiation tolerant. As RD_{50} value of callus of *Arachis hypogaea* L. (Verma et al. 1971) is considerably higher than that of the other species, further examination may be needed of this species.

In *Coptis*, there were variations in berberine content among the embryoids derived from irradiated plant organs. And the distribution of berberine content was biased after gamma-ray irradiation. But, it is not clear at the moment whether the biases observed were caused by mutational change or by any physiological effects e.g. radiation damage. Moreover, there have been a few reports which showed that phenotypic manifestations of a particular character in cell level are not always similarly manifested at plant level (Handro, 1981). Regenerated plants of *Coptis* are now under cultivation in our field for examination of berberine content.
Berberine is released to the culture media from the callus cells and embryoids. And a close correlation was found in content between cultured materials and media. This phenomenon may enable us to select clones with high berberine content without destruction of cultured materials. Since determination of berberine content in culture medium could be performed in a very large scale by a simple colour metric system, \textit{in vitro} mutagenesis could be effectively practiced in improvement of \textit{Coptis} varieties.

There were different grades of totipotency in \textit{Coptis}. Some genotypes never regenerated to plantlet so far. In case of applying \textit{in vitro} selection technique to plant species, the choice of genotypes which regenerate plantlet at a high frequency is important to elevate breeding efficacy.

\section*{Summary}

To investigate radiosensitivity of \textit{in vitro} cell, callus of \textit{Carthamus tincrorius} L. was irradiated with gamma rays. Radiosensitivity of callus was intermediate between that of dormant seed and growing seedling. The effect of irradiation dose rate was observed. Irradiation effect varied due to preculture period in each culture. Depression of growth by irradiation was recovered in the second subculture after irradiation.

Berberine content was investigated in culture of \textit{Coptis japonica} Makino. Embryoids derived from leaf blade shows higher concentration than those derived from leaf petiole and basal end of flower bud. Irradiation to embryoid caused decrease of its content on the average, but some irradiated embryoids showed high content of berberine.

In \textit{Datura alba} Nees and \textit{C. japonica} Makino, regenerated plants were obtained. The number of regenerated plants depended on tissues from which callus was derived and on line of material plants.

It is suggested from this experiment that choice of genotype which shows a high frequency of plant regeneration and of condition of mutagenic treatment which induce mutants at highest efficiency as possible were important for breeding efficacy.

\section*{Acknowledgements}

This study was performed through special coordination fund of the Science and Technology Agency of Japanese government.
IN VITRO MUTATION BREEDING IN MEDICINAL PLANTS

References

薬用植物における組織培養の手法を用いた突然変異育種
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薬用植物は洋の東西を問わず古から利用されてきたが、それは単に野生植物の中から選抜した系統を栽培利用してきたにすぎず、近代的な育種手法が薬用植物に応用された例は少ない。今日薬用植物の利用が急速しているため、この栽培特性と薬用品質との画を改めても大幅に望まれているが、一般に薬用植物には多年生、生育の遅さおよび品種内の遺伝子型の不均一性などの理由で育種が困難であるものが多い。近年急速に進歩した組織培養法による単一群の遺伝子型の系統を大量に増殖することが可能であるため、この技術と突然変異誘発による変異拡大を組み合わせて薬用植物の育種の困難を解決できると考えられる。本報ではCarthamus tinctorius L., Datura alba NeesおよびCoptis japonica Makinoについて組織培養を試み、また変異源としてガンマ線を照射して、これに対する培養細胞の反応と変異の拡大について調査した。

カルスの誘導と培養はMS基本培地に2,4-D, NAAおよびBAP等の植物ホルモンを添加した培地で行ったが、ガンマ線の照射は、総照射については放射線育種場ガンマ・フィールドまたは急照射については放射線育種場ガンマ・ルームで行った。

1) 放射線感受性: Carthamus tinctorius L.の幼苗の葉から誘導したカルスについてガンマ線急照射をして、カルスの示す放射線反応を調査した。カルスの放射線感受性はほぼ種子の4倍まで生体(幼苗)の1/3だった。カルスの場合には照射線量率の効果が観られ、線量率の大きい場合に障害が大きかったが、この効果はとくに照射線量が少ない場合にみとめられた。また減代培養操作後のカルスと後のカルスでは感受性が異なっていた。照射したカルスの生育率は照射後の培養期間中回復しなかったが、1回の減代培養後には無照射のカルスと同等かそれ以上の生育率を示した。

2) 藥用成分の変異: Coptis japonica Makinoのカルスについて、ペルベリン含量の変異を調査した。カルスをその由来した器官で区分すると、葉身由来するカルスが葉柄または花芽由来のものよりも高い含有量を示した。含有の変異により不放射線の影響を調べるために、カルスに放射線を照射したところ、カルスのペルベリンの平均含有量は低下したが、30 kRの細照射をした葉身由来のカルスの中に高含有のものがみられた。またカルスは培地中にペルベリンを放出しているが、カルスのペルベリン含量と培地のペルベリン濃度とは0.7～0.9の相関がみられたため、この相関を利用した細胞系の選抜法が考えられる。

以上のように放射線照射によってCoptis japonica Makinoの薬用成分含量に変異がみられることが示されたが、この変異が遺伝変異か生理的変異かは明らかでないため、こ
の変異したカルス系統から植物体を再分化させた。しかし、植物体の再分化率はカルスの
由来した器官と供試系統に依って大きく異なっていた。育種素材としての系統の選択はそ
の後の育種の効率を大きく左右すると考えられる。また Carthamus tinctorius L. での
調査で示されたように、カルスの放射線に対する反応はカルスの状態と照射条件で異なる
ため、照射線量と照射条件の決定には注意を要する。
なお当研究は科学技術庁科学技術振興調整費によって行なわれたことを付記する。

質 疑 応 答

長田（基生研）：培養細胞をプレートするいろいろなコロニーが出ますが、そのコロ
ニーによって成分含有量が違うということでした。いまお話しでは放射線をあてたあとカ
ルスを作らせ、再分化させてその平均値をとっているようですが、その平均値に意味があ
るのか、その幅、つまり特異値がでてくることに意味があるのでしょうか、平均値には意
味がないとお考えのようですが?

矢頭：最終的には個体個々の話だと思います。平均値よりもどういう突然変異体がでて
くるかという個体レベルの話になるかと思います。ですからカルスの中のどこから不確定
を採るかについても、ひとつのカルスが必ずしもひとつの突然変異とは限らず、セクターに
分れていることもありますのでその辺の検討も必要ではないかと考えています。

河合（三和生業）：成株になるのが10％位と低いようですが、不定胚の定義はどう考え
ていますか。またベルベリン含有量の測定で、葉や茎といった由来組織によって含有量が
異なっていると理解しましたが、植えつき回数はどのくらいでしょうか?

矢頭：不定胚の定義ですが、今回のオウレンについてはそれほど厳密ではありません。結
果からいえば個体をもちだせる状態になってきたものを不定胚に分類したとして表現し
ています。植えつき回数ですが、大体30日位で植えつきしていますので、カルスになって
から1回から2回植えつきしたものです。

河合：いわゆるキャリーボーナ効果つまり前の組織から持ちこまれる効果があると思わ
れる例がありますが、そのあたりはどうでしょうか?

矢頭：分かるのではないかと思っています。しかし、いま維持しているカルスの中に
は1年から2年以上生きているカルスもありますので、それらではカルスの由来組織とい
うよりも、カルス間での差のほうがかなり大きくなっています。いまお話しのような由
来組織の影響というのはカルス培養の初期に見られる程度ではないかと思います。
HIGH FREQUENCY OF SOMACLONAL MUTATIONS IN CALLUS CULTURE OF RICE

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Introduction

The establishment of \textit{in vitro} cell culture systems in which physiologically and genetically stable plant cells multiply at a satisfactory rate is one of the most important premises for exploring the unique possibilities of tissue culture techniques in both basic studies and applications to plant production and genetic improvement. Cultured plant cells often habituate to cultural conditions, show alterations in their nutrient or auxin requirements and loose totipotency (Butcher 1977, Yeoman and Forche 1980). Furthermore, increasing numbers of experimental data indicate that the chromosome number and structure are highly variable (as reviewed by D'amato 1977, Sunderland 1977, Bayliss 1980). Phenotypic variations of regenerated plants from cultured cells (described as regenerated plants hereunder) have been known to occur since the late 1960's (as reviewed by Skirvin 1978, Larkin and Scowcroft 1981). In rice, the presence of abnormal or variant regenerated plants has been documented since the first success in plant regeneration, e.g., morphological variants from seed callus (Nishi \textit{et al.} 1968), and albinos (Niizeki and Oono 1968) and polyploids (Nishi \textit{et al.} 1969) from pollen callus. Albinos, morphological variants and polyploids were frequently observed in rice anther culture studies. Variations in plant height and panicle length were also observed in regenerated haploid plants obtained from a single pollen callus (Oono 1975).

Changes of chromosome number, aneuploidy and polyploidy, were often observed in regenerated plants in \textit{Nicotiana tabacum} (Sacristán and Melchers 1969, Ogura 1978), \textit{Saccharum} spp. (Heinz \textit{et al.} 1971), \textit{Pelargonium} (Bennici 1974), \textit{Lycopersicon peruvianum} (Sree Ranulu 1976), \textit{Allium} spp. (Roy 1980). Plants regenerated from mesophyll protoplasts of tobacco also exhibited variations in morphogenesis accompani-
ed by altered chromosome numbers (Takebe et al. 1971). Chromosome structural changes were observed in regenerated plants in Lollum spp. (Ahloowalia 1976), Allium sativa (Novak 1980), Hordeum vulgare X H. jubatum (Orton 1980), Haworthia setata (Ogihara 1981). Mutants resistant to streptomycin (Maliga et al. 1973), picloram (Chaleff and Keil 1981) in Nicotiana tabacum and to Helminthosporium toxin in Zea mays (Gengenbach et al. 1977) were isolated by in vitro selection procedures. Morphological variants were also observed in regenerated plants of Nicotiana tabacum (Burk and Matzinger 1976), Zea mays (Green 1977), Saccharum officinarum (Heinz et al. 1977, Liu and Chen 1978), Ananas comosus (Wakasa 1979), Solanum tuberosum (Wenzel et al. 1980). However, genetical analyses of such variants have so far been limited. Chaleff and Keil (1981) reported that the morphological changes observed in a plant regenerated from cultured cells of tobacco were merely physiological changes acquired during the culture of cells through many passages in subculture.

We therefore planned to clarify the nature and causes of variations in cultured cells as well as regenerated plants in rice, for developing breeding methods by using in vitro techniques both for broadening the genetic variability and establishing genetically stable cell lines.

A preliminary observation had already demonstrated the frequent occurrence of mutations in rice plants regenerated from cultured cells (calluses) (Oono 1978). The present study was undertaken to investigate the frequency of mutations carried by regenerated plants and the phenotypic characteristics of the mutants obtained in rice.

**Materials and Methods**

Dehulled rice seeds of a pure line (progeny of a doubled haploid of a spontaneous haploid plant of the variety Norin 8 of Oryza sativa L.) were inoculated on the callus induction media after sterilization for 20 minutes with a 10% (W/V) calcium hypochlorite aqueous solution followed by rinsing twice with sterile water. The media were Miller's basic medium (1963) with modifications in the concentration of yeast extract (0.2 g/l), of MgSO$_4$·7H$_2$O (35-380 mg/l), of ferric monosodium ethelendiaminetetraacetate (32-320 mg/l), and the addition of 30 g/l of sucrose, 10 g/l agar, and 10$^{-5}$ mol 2,4-dichlorophenoxyacetic acid (2,4-D).

Calluses were obtained from 28 seeds (Exp. 1) and 47 seeds (Exp. 2) respectively. Each of the seed calluses was divided into several pieces at 44 (Exp. 1) or 66 (Exp. 2) days after the inoculation and transferred to the differentiation media, Murashige and Skoog's basic medium (1962) containing 10$^{-5}$ mol naphthaleneacetic acid (NAA), 70 g/l sucrose, and either 3 g/l yeast extract and 2 g/l caseinhydrolysate or 10 mg/l
benzyladenine. Plants regenerated from the calluses at 20-40 day after the transfer to the differentiation media. Plants regenerated from one and the same seed callus and their descendants were called a callus clone. More than 800 regenerated plants (D₁ plants) were transplanted in pots and grown in a greenhouse, then transplanted in an experimental field, thereafter their agronomic characters were investigated. One to 26 D₁ mature plants from 75 seed calluses and 8 to 215 D₂ plants from each of the D₁ plants were analyzed. In the second generation (D₂), 8 to 10 plants from each of the 762 fertile D₁ plants, 6382 D₂ plants in total, were grown as the D₂ line and examined for changes of characters: morphological characters, chlorophyll deficiency, ploidy, heading date, plant height, panicle length, number of grains and seed fertility. A total of 1491 D₂ plants showing changes of any of the above characters were selected as putative mutants. Plant progenies from 335 D₂ normal and putative mutants were grown as D₃ lines, each consisting of 9-19 plants, (6315 D₃ plants in total), in order to confirm the heritable nature of the changes observed in the D₂. D₂ lines were planted in two replications. And 150 D₃ lines (2642 D₃ plants) with typical short or tall plant stature, early or late heading date, low seed fertility, etc., were examined to analyze their breeding behavior. Agronomic characters of the D₂ and D₃ plants were statistically analyzed.

Results

1. Variations observed in D₁ plants

D₁ plants numbering 1121 regenerated from 75 seed calluses. Eighty three D₁ plants (7.4%) were albinos. In 762 mature D₁ plants which were investigated the plant height, flowering date, morphological features and seed fertility at maturity were recorded. Variation of these characters in the D₁ plants are presented in Fig. 1. Plant height of the D₁ plants ranged between 77 and 124 cm (101.4 ± 9.1 cm on the average). Heading date of the D₁ plants ranged between Aug. 9 and Sep. 1, with two groups in the frequency distribution (Aug. 11.6 ± 1.3 and Aug. 24.4 ± 3.7), probably due to the two different transplanting dates of the regenerated plants to the field.

Seed fertility of the randomly investigated 489 D₁ plants varied widely. Seed fertility was less than 80% in 58.7% of the D₁ plants and less than 40% in 18.2% of the D₂ plants. Morphological changes of some characters, e.g., grain density and long awn, were observed.

Chimerism of D₁ plants in seed fertility (difference in seed fertility of more than 20% between panicles on the tallest two culms on one D₁ plant) and in chlorophyll deficiency was also observed, with a frequency of 23.7% and 29.3%, respectively. One
of the twelve tetraploids was a 2x – 4x chimera, bearing one 4x panicle among 10 panicles.

2. Variations observed in D2 plants

As shown in Fig. 2, heading date of the 6382 D2 plants fluctuated between Aug. 19 and Oct. 7 (Sep. 2.4 ± 2.9 on the average versus Sep. 1.7 ± 1.7 in the control). Early- and late-heading variants were observed. They included D2 plants 14-day earlier, and more than 20-day later in heading date than the control (the original variety) and 3 non-heading plants until Oct. 31. Plant height of the D2 plants ranged between 29 and 135 cm (107 ± 11.2 cm on the average versus 116.9 ± 6.8 cm in the control). D2 population showed a distinct shift to low plant height. Frequency of D2 plants with a plant height of less than 90 cm was 4.1%, and plants less than 50 cm tall were mostly
malformed and/or completely sterile. The difference in the mean of heading date and plant height between the D₂ and the control was significant at 1% level. Seed fertility of the D₂ varied from 99.2% to 0% (Fig. 3). Frequency of D₂ plants with seed fertility of less than 80% and 40% was respectively 19.5% and 5.0%. Completely or partially sterile plants showed various types of pollen abortion such as completely shrivelled pollen, incomplete accumulation of starch, and mixtures of both, normal pollen and pollen of normal and small size.

D₁ panicle progenies were examined for the presence of segregation of chlorophyll-
Fig. 3. Variations of panicle size, grain shape, grain numbers and seed fertility (a) and chlorophyll deficiencies (b) among $D_2$ plants.
deficient mutants in the D2 (Table 1, Fig. 3). Chlorophyll mutants segregated in 34 out of the 438 tested panicle progenies. The mutation frequency of 8.4% was comparable to the mutation frequencies in M2 following X-ray or γ-ray irradiation of rice seed. Segregation frequency of chlorophyll mutants in the D2 lines (D1 panicle progenies) was higher than 20% in 22 (64.7%) of the 34 mutated D2 lines.

3. **D2 Variation in relation to D1 variation.**

As seen from Fig. 1 and 2, the variation range of D2 plant height was larger than that of D1 plant height. D2 plant height (mean plant height of D2 lines) was not correlated with D1 plants height in parent-offspring relation (r=+0.168, statistically not significant).

Distribution of seed fertility of D2 plants was different from that of D1 plants, plants with a seed fertility of less than 80% being apparently more frequent in the D1 than in the D2. Relationships of seed fertility between D1 and D2 are summarized as follows: (1) Plants with normal seed fertility (90% or higher) generally transmit normal seed fertility: (2) However, in progenies of 23 D1 plants with a seed fertility of 80% or more, D2 plants (29 plants in total) with a seed fertility of less than 80% segregated in 23 D2 lines (176 D2 plants in total): (3) in progenies of 30 D1 plants with a seed fertility of less than 30%, D2 plants (21 plants in total) with a seed fertility of less than 30% and D2 plants (157 plants in total) with a seed fertility of more than 80%, respectively segregated in 30 D2 lines (235 D2 plants): (4) in progenies of 5 D1 plants (43 D2 plants) with a seed fertility of less than 30% showed a seed fertility which exceeded 80%. Furthermore, all the 56 D2 plants from 2 D2 plants in the successive generations displayed a seed fertility of more than 90% and (5) correlation coefficient between D1 plant seed fertility and D2 fertility (in parent-offspring relation) was statistically significant (r=+0.365**).

Several D1 plants showed changes in morphological traits, e.g., compact panicle and awned glume, but these changes were not transmitted to the D2, suggesting that some of the variants regenerated from callus culture are unstable.

As already mentioned, 83 albino D1 plants were observed among 1121 regenerated plants. These albino D1 plants regenerated together with 32 green D1 plants, from 14 seed calluses. This finding indicates that the 14 calluses originated from two or more cells. Chlorophyll-deficient plants segregated in 9 D2 lines (albinos in two, viridis in four, striata in one and virid-albinos and xantha respectively in one) out of the 32 D2 lines from the 14 callus clones. Frequency of the chlorophyll-deficient D2 lines, 28.1% (9/32), was higher than that of the D2 lines, 16.9% (123/730 D2 lines), out of 61 callus clones from which green normal D1 plants exclusively regenerated. Variants for heading date, plant height and seed fertility were also observed in 32 D2 lines from the 14 seed
Table 1. Chlorophyll mutations in D₂ lines grown as panicle progenies of D₁ plants

<table>
<thead>
<tr>
<th>No. of tested D₂ lines</th>
<th>No. of D₂ lines segregating mutants</th>
<th>No. of D₂ lines segregating mutants</th>
<th>Mutation 2) frequency %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Albina Xantha Viridis Striata Others</td>
<td></td>
</tr>
<tr>
<td>438</td>
<td>34</td>
<td>(29.7) (24.3) (18.9) (10.8) (16.2) (100)</td>
<td></td>
</tr>
<tr>
<td>(Relative frequency %)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: 1) In three D₂ lines, two different types of chlorophyll mutants were segregated.  
2) Mutation frequency (%) = \[\frac{\text{Sum of different types of mutants deficient in chlorophyll in individual D₂ lines.} \times 100}{\text{No. of D₂ lines examined}}\]  

Callus clones. The frequency of the D₂ lines which segregated these variants was not significantly different from that of the 730 lines from the 61 callus clones. Since both of the above frequencies of D₂ lines segregating chlorophyll-deficient mutants were far higher than those presented in Table 1, studies using larger numbers of D₂ lines are required to draw conclusions on the frequency of D₂ lines segregating chlorophyll-deficient mutants, particularly with regard to the difference in the frequencies between callus clones which produced albino D₁ plants and those producing normal green D₁ plants exclusively.

4. Estimation of frequencies of mutants in D₂.

Mutations induced in cells of callus or regenerated plants (at the early stage of development) which participate in the development of the generative tissues of D₁ plant could be detected by the segregation of mutants in the D₂ lines or D₃ populations. Mutants showing drastic changes, e.g., chlorophyll mutants and dwarf mutants can be readily identified in the D₂. Mutants which do not show drastic changes can be detected or identified by investigations in further generations. Since the analysis of the mutations was continued in the D₃ generation in the present study, variants segregating in the D₂ lines were identified and regarded as mutants based on any one of the following criteria: seed fertility of less than 80% (not observed in normal rice plants), plant height of less than 90 cm (average minus 4 times of standard deviation (SD) in the control), heading data earlier than Aug. 27 or later than Sep. 11 (respectively average heading date minus 3 SD, or plus 5 SD in the control).

Among 6328 D₂ plants at maturity, 1491 plants were recorded as mutants on the basis of the above criteria. Frequency of the mutants was 23.4% at the exclusion of the lethal chlorophyll mutants. Table 2 presents the numbers of D₂ lines segregating...
such mutants in addition to those of polyploids, mutants showing drastic morphological changes, and viable chlorophyll mutants. As seen from the table, many $D_2$ lines were segregating mutants, and only 28.1% of the $D_2$ lines were not segregating any mutants. The average number of mutated characters per $D_1$ panicle ($D_2$ line) was 1.08 (826/762)

Table 2. Numbers and frequencies of $D_2$ lines segregating mutants of particular characters

<table>
<thead>
<tr>
<th>Changed Characters</th>
<th>Exp. 1 (28 callus clones)</th>
<th>Exp. 2 (47 callus clones)</th>
<th>total (75 callus clones)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>126 (38%)</td>
<td>88 (20.5%)</td>
<td>214</td>
<td>28.1</td>
</tr>
<tr>
<td>Ploidy (4x)</td>
<td>8</td>
<td>4</td>
<td>12</td>
<td>1.6</td>
</tr>
<tr>
<td>Fer.</td>
<td>102</td>
<td>171</td>
<td>273</td>
<td>35.8</td>
</tr>
<tr>
<td>Ht.</td>
<td>8</td>
<td>11</td>
<td>19</td>
<td>2.5</td>
</tr>
<tr>
<td>Hd.</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>0.4</td>
</tr>
<tr>
<td>Mor.</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>Ch.</td>
<td>10</td>
<td>17</td>
<td>27</td>
<td>3.5</td>
</tr>
<tr>
<td>Fer. &amp; Ht.</td>
<td>31</td>
<td>43</td>
<td>74</td>
<td>9.7</td>
</tr>
<tr>
<td>Fer. &amp; Hd.</td>
<td>6</td>
<td>8</td>
<td>14</td>
<td>1.8</td>
</tr>
<tr>
<td>Fer. &amp; Mor.</td>
<td>6</td>
<td>1</td>
<td>7</td>
<td>0.9</td>
</tr>
<tr>
<td>Fer. &amp; Ch.</td>
<td>14</td>
<td>45</td>
<td>59</td>
<td>7.7</td>
</tr>
<tr>
<td>Ht. &amp; Ch.</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>0.4</td>
</tr>
<tr>
<td>Hd. &amp; Ch.</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>Mor. &amp; Ch.</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>Fer., Ht. &amp; Hd.</td>
<td>3</td>
<td>8</td>
<td>11</td>
<td>1.4</td>
</tr>
<tr>
<td>Fer., Ht. &amp; Mor.</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0.3</td>
</tr>
<tr>
<td>Fer., Ht. &amp; Ch.</td>
<td>9</td>
<td>18</td>
<td>27</td>
<td>3.5</td>
</tr>
<tr>
<td>Fer., Hd. &amp; Ch.</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0.3</td>
</tr>
<tr>
<td>Fer., Mor. &amp; Ch.</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>Fer., Ht., Hd. &amp; Mor.</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>Fer., Ht., Hd. &amp; Ch.</td>
<td>1</td>
<td>8</td>
<td>9</td>
<td>1.2</td>
</tr>
<tr>
<td>Fer., Ht., Mor. &amp; Ch.</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Total No. of $D_2$ lines 332 430 762 100

See text for callus clone and mutants
Abbreviations for the mutated characters are as follows:
Fer.: seed fertility, Ht.: plant height, Hd.: heading date,
Mor.: morphological traits, Ch.: chlorophyll deficiency.
when five characters were examined. The results suggest the frequent induction of mutations in the process of callus induction up to D₁ plant regeneration.

5. Differences in mutation occurrence among seed callus clones.

Mean seed fertility of D₂ lines of individual callus clones was less than 90% in 49 callus clones (49/75=65.3%) and less than 80% in 10 callus clones (13.3%), versus 94.1% in the control. Segregation frequencies of plants with a low seed fertility were different among callus clones, for instance, no D₂ plants with a seed fertility of less than 80% were observed in 3 callus clones, while 18 of 34 D₂ plants (52.9%) of a callus clone showed a seed fertility of less than 80%, ranging between 0 and 80%.

Similarly, mean plant heights of individual callus clones, ranging between 97.4 and 114.1 cm, all significantly decreased at 1% level, in comparison with 116.9 cm for the control. The variations in plant height in 23 callus clones (30.7%) also significantly increased at the 1% level.

Mean heading date significantly changed to early maturity in 7 callus clones (9.3%) and to late maturity in 35 callus clones (46.7%) with the range of Aug. 29.9 to Sep. 2.9 versus Sep. 1.7 in the control. Chlorophyll deficient plants, morphological variants and tetraploids segregated in 3 callus clones, respectively.

Relationships between mean plant height, and mean seed fertility and heading date of the D₂ lines are shown in Fig. 4. The correlation coefficients between plant height and seed fertility, plant height and heading date, and seed fertility and heading date were respectively 0.393**, −0.069 and −0.256*.

Based on these results it is possible to consider that every callus clone differed from one another in some agronomic characters in the D₂ population and that the correlations of mutated characters showed a definite trend. It is also worth noting that the mean values of quantitative characters such as plant height and seed fertility decreased in all seed progenies in the D₂ in comparison with the control (Fig. 4, cf. Figs. 1, 2). Comparison of the D₂ lines among 75 callus clones, failed to detect any tendencies in the order of occurrence of the mutations.

Pleiotropy was observed in some of the mutations, resulting in mutants with changes of specific characters in the same way and to the same extent in combination. Estimated numbers of mutations, taking into account pleiotropic effects, in individual callus clones are presented in Table 3. Average number of major mutations induced in 75 callus clones was 3.75. And the largest number of mutations per callus clone was 12 in a callus clone (No.58) from which, 22 D₁ plants and 176 D₂ plants out of 22 D₂ lines were investigated. Mutants resulting from the 12 mutations were characterized by changes in the following characters: Tetraploidy, low seed fertility, complete sterility, decrease in plant height, dwarfism, early heading date, presence of albinos, xantha,
viridis, striata forms, diseased leaf and drooping leaf.

Ten D₃ lines as progenies of segregants with reduced seed fertility in 10 D₂ lines segregated partially sterile plants. Segregation pattern and pollen fertility of these partially sterile plants in the D₂-D₄ suggested that the reduced seed fertility in these

Table 3. Estimated number of mutations per callus clone

<table>
<thead>
<tr>
<th>No. of callus</th>
<th>Estimated number of mutations per callus clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>1 10 14 9 18 11 6 4 1 1 1</td>
</tr>
</tbody>
</table>

\[
\frac{(1 \times 10 + 2 \times 14 \ldots) = 3.75}{75} = 3.75
\]
lines was caused by chromosomal translocations.

6. Pattern of appearance of mutants in individual callus clones.

Appearance of mutants (occurrence of mutations) differed among the $D_2$ lines in a callus clone. Fig. 5 illustrates the appearance (segregation) of mutants (as defined in Section 5) in a callus clone (No.3). Part of the $D_2$ lines of this callus clone showed segregation of mutants with common characters which were changed in a similar way and the a similar extent. And the smaller clusters were always included into the larger ones. Mutants of particular characters, such as tetraploidy, changes in morphological characteristics, chlorophyll deficiencies (*tigrina* and *maculata*), dwarfism and early maturity in Fig. 4 were not frequently observed in the 762 $D_2$ lines examined (Table 2). Fig. 5 shows that these mutants were not randomly distributed but distributed in a limited number of $D_2$ lines forming clusters in their genealogy. Although genetic analysis, particularly allelism test of the mutants commonly observed in $D_2$ lines derived

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Fig. 5. Mutations found in a rice clone. The following abbreviations are employed: Mutants for seed fertility = Fer, heading date = Hd, plant height = Ht, and chlorophyll deficiency = *albina* and *xantha* segregated in $D_2$ lines.
from one and the same callus was not carried out, studies of the progenies of these mutants in the D₃ plants indicated that these mutants were segregants resulting from common and single mutations carried by the D₁ plants from one callus clone. Fig. 5 illustrates the distribution of mutations in the callus clone, and shows that some mutations, in e.g., Hd, Xantha and Ht, formed small clusters which were incorporated into large clusters of Fer. and 4x.

Based on these results, it is highly probable that those mutations common to the D₂ lines in a callus clone were not induced independently, but were induced in a cell which participated in the formation of the generation of D₁ plants from one callus clone. It can also be assumed that mutations carried by the large sectors occurred earlier than those in the smaller sectors in the process of callus induction up to D₁ plant regeneration. For instance, tetraploidy was induced at an earlier stage than the mutation of heading date and the mutation of plant height was also induced earlier than that of heading date, as seen in Fig. 5. Mutations which are considered to be induced independently in the seed callus are indicated in the dotted boxes in Fig. 5.

7. Genetic analysis of isolated D₃ mutants.

Mutants selected for chlorophyll deficiency, plant height, heading date, etc., were true-breeding in the D₃ (Fig. 6). The breeding behavior of eight mutants showing drastic changes was investigated in crosses between the mutants and the original varieties. As shown in Table 4, F₂ segregation ratio patterns of six of the mutants fitted to the 3 : 1 ratio, indicating that the mutations were controlled by single recessive genes.

<table>
<thead>
<tr>
<th>Representative characteristics of mutants</th>
<th>No. of plants</th>
<th>Chi-square test for fitness to 3:1 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Mutant</td>
</tr>
<tr>
<td>Narrow leaf</td>
<td>272</td>
<td>81</td>
</tr>
<tr>
<td>Short culm</td>
<td>373</td>
<td>117</td>
</tr>
<tr>
<td>Dwarf</td>
<td>203</td>
<td>60</td>
</tr>
<tr>
<td>Dwarf</td>
<td>329</td>
<td>89</td>
</tr>
<tr>
<td>Rolled leaf</td>
<td>183</td>
<td>45</td>
</tr>
<tr>
<td>Fine stripes</td>
<td>312</td>
<td>97</td>
</tr>
<tr>
<td>Yellow leaf</td>
<td>277</td>
<td>71</td>
</tr>
<tr>
<td>Brown spot leaf</td>
<td>258</td>
<td>59</td>
</tr>
</tbody>
</table>
8. Variability in isolated D₃ lines.

Isolated 150 D₃ lines (including 1 normal line) were observed for the breeding behavior of their agronomic characters. Agronomic characters of 14 D₃ mutants are shown in Table 5. The differences in agronomic characters between the D₃ mutant lines
Table 5. Agronomic characters of some isolated D₃ mutants lines

<table>
<thead>
<tr>
<th>No. of D₃ line</th>
<th>Days for heading</th>
<th>No. of panicles</th>
<th>Culm length</th>
<th>Panicle length</th>
<th>No. of grains</th>
<th>Seed fertility</th>
<th>1000-kernel weight</th>
<th>Characteristic features</th>
</tr>
</thead>
<tbody>
<tr>
<td>7VD-C</td>
<td>114.1</td>
<td>10.7</td>
<td>87.2</td>
<td>22.7</td>
<td>133.2</td>
<td>97.1</td>
<td>26.4</td>
<td>Control</td>
</tr>
<tr>
<td>25</td>
<td>113.6</td>
<td>9.8</td>
<td>84.5</td>
<td>23.2</td>
<td>136.1</td>
<td>96.3</td>
<td>26.3</td>
<td>Normal</td>
</tr>
<tr>
<td>94ᵃ</td>
<td>102.6**</td>
<td>12.2</td>
<td>71.7**</td>
<td>18.8**</td>
<td>88.5**#</td>
<td>79.1**#</td>
<td>24.9**#</td>
<td>Early maturation</td>
</tr>
<tr>
<td>75</td>
<td>118.0**</td>
<td>9.6</td>
<td>89.4</td>
<td>25.1**</td>
<td>145.7**</td>
<td>95.3**</td>
<td>25.6</td>
<td>Late maturation</td>
</tr>
<tr>
<td>30</td>
<td>111.3**</td>
<td>16.7**#</td>
<td>80.7</td>
<td>22.0</td>
<td>121.0</td>
<td>92.2**</td>
<td>27.4</td>
<td>Tiller</td>
</tr>
<tr>
<td>59</td>
<td>113.3**</td>
<td>9.4</td>
<td>97.8**</td>
<td>23.0</td>
<td>114.3**</td>
<td>95.6**</td>
<td>31.3**</td>
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<td>57.4**</td>
<td>17.5**</td>
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<td>7.2**</td>
<td>92.7**</td>
<td>26.2**</td>
<td>153.0**</td>
<td>90.3**</td>
<td>23.5**#</td>
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<td>9.4</td>
<td>87.3</td>
<td>19.7**</td>
<td>123.5</td>
<td>42.1**#</td>
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<td>9.5</td>
<td>80.1**</td>
<td>21.4**</td>
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<td>20.8**</td>
<td>105.8**#</td>
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<td>17</td>
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<td>74.1**</td>
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<td>100.7**</td>
<td>61.1**#</td>
<td>26.0**#</td>
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<td>92.5**</td>
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<td>19.5**#</td>
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<td>8.6</td>
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<td>21.1**</td>
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<td>48</td>
<td>118.6**</td>
<td>5.4**</td>
<td>74.6**</td>
<td>22.2  #</td>
<td>116.1**</td>
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<td>150</td>
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<td>4.5**</td>
<td>53.9**</td>
<td>19.1**#</td>
<td>62.3**</td>
<td>13.8**#</td>
<td>35.6**</td>
<td>Tetraploid</td>
</tr>
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</table>

** : Mean value was significantly different from that of the control at 1% level.
#  : Variance was significantly different from that of the control at 1% level.
a, b : Originating from one and the same seed callus respectively.
of a callus clone are seen in the table. For instance, D<sub>3</sub> lines, 7VD-94 and 7VD-28, are differed in their heading date, (though both headed earlier than the control), culm length, panicle length, and number of grains. Two D<sub>3</sub> lines, 7VD-15 and 7VD-76 were late in heading date but differed in their culm length and seed fertility.

Some of the lines showed inverted relations of the characters such as low plant height and large number of grains (7VD-45) and low plant height (43.7 cm) and long panicle (24.4 cm) (7VD-13). Frequency distribution of mean values of agronomic characters of 150 D<sub>3</sub> lines is shown in Fig. 7. Even D<sub>2</sub> populations, mutants with

Fig. 7. Frequency distribution of the mean values of 7 agronomic characters in 150 D<sub>3</sub> lines. The arrow shows the control.
vigorous agronomic characters could be isolated. Either mean values or variances in 149 mutant lines were significantly different from those in the control. Early- and late-heading mutants were isolated in 28 D₃ and 76 D₃ respectively. The other mutants isolated in D₃ were as follows; 19 and 10 respectively with smaller and larger numbers of panicles, 72 short and 23 tall, 77 with short and 15 with long panicles, 72 with a lower and 25 with a large number of grains (Fig. 6). In addition 135 D₃ lines showed a decrease in seed fertility.

Number and frequency of D₃ lines which were true breeding for individual characters are shown in Table 6. Observations were made on 6 characters of agronomic importance and additional 4 characters of little practical significance in 150 D₃ lines. Total number of characters investigated in 150 D₃ lines was 634 and among them 347 (58.4%) were found to be fixed (true breeding) on the basis of individual characters and individual D₃ lines. In 150 D₃ lines, 24 out of 10 characters examined were fixed and 125 D₃ lines were segregating for some of the characters. Only one character was altered in 14 D₃ lines, whereas several characters were altered in 135 D₃ lines. Therefore, the average number of altered characters carried in the D₃ lines was 3.7 (554/150) and 2.3 (347/150) characters were fixed in the D₃ lines. In eleven D₃ lines chlorophyll deficiencies such as *tigrina*, *maculata*, and *viridis* forms were fixed but 16 D₃ lines were

<table>
<thead>
<tr>
<th>Table 6. The mean value and variance in agronomic characters of isolated 150 D₃ lines</th>
<th>No. of fixed lines</th>
<th>No. of segregated lines</th>
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<tr>
<td></td>
<td>normal</td>
<td>with changes in mean value</td>
</tr>
<tr>
<td>Heading date</td>
<td>35</td>
<td>68</td>
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<tr>
<td>No. of panicles/plant</td>
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<td>Plant height</td>
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<td>Panicle length</td>
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<td>No. of grains/plant</td>
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<td>72</td>
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<tr>
<td>Seed fertility</td>
<td>12</td>
<td>22</td>
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<tr>
<td>Chlorophyll deficiency</td>
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<td>11</td>
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<tr>
<td>Morphology</td>
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<td>Lethality</td>
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<tr>
<td>Tetraploid</td>
<td>147</td>
<td>3</td>
</tr>
</tbody>
</table>

| total No. of mutations          | 347 (fixed)      | 287 (segregated)        |

**: Significant at the 1% level.
segregating for the albino or xantha traits. Those fixed traits were transmitted to the successive two generations of D₄ and D₅ in a stable manner.

Discussion and conclusions

It is evident that a high frequency of gene mutations is induced in cultured rice cells (callus). And true breeding of mutated characters could be observed in the D₅ with high frequencies. Larkin and Scowcroft (1981) in their review suggested that the origin of somaclonal variation involves karyotype changes, cryptic changes associated with chromosome rearrangement, transposable element, somatic gene rearrangements and gene amplification and depletion. However, in only a few of the studies mentioned above a genetic analysis of the variations obtained in the regenerated plants has been carried out (Evans and Sharp 1983). Orton (1983) discussed the detection and characterization of genetic variation in cultured somatic tissues and concluded that the use of morphological or physiological variation in the regenerated plants as a direct measure of somaclonal variation in cultured tissues is extremely tenuous. However, the use of totipotency of cultured cells is, at the present, the main advantage of somatic cell genetics in plants in comparison with animals. Furthermore, a new phenomenon such as the occurrence of putative homozygous mutation, which seems to be frequent could be observed (Oono 1985).

In this paper, we investigated the heritable nature of the changes in the agronomic characters observed in regenerated plants and their offsprings. Elimination of aneuploids and polyploids may be estimated based on the results showing that the frequency of aneuploid and polyploid cells in pollen callus in medium containing 2, 4-D in the course of anther culture was 23% (Oono 1975), while the frequencies of aneuploids and polyploids in regenerated plants were respectively 11.5% (Oono 1975) and 5% (Institute of Genetics 1976). The elimination of gene mutations may also take place in the process of callus induction up to plant regeneration. However, it is reasonable to assume that the elimination of gene mutations is far less frequent than that of aneuploid and/or polyploid cells.

It has often been debated whether somaclonal variation is present in the original tissues dissected for callus induction or is being induced in the process of culture. Variation in regenerated plants differed depending upon the origin of the explants in Pelargonium (Skirvin and Janick 1976) and in pineapple (Wakasa 1979) or by using old or new varieties in Solanum tuberosum (Shepard et al. 1980), suggesting that the variation may exist in these tissues. However, 15 vegetative haploid lines and 3 spontaneous diploid lines regenerated from a callus derived from a single rice pollen showed varia-
tions in plant height and panicle length which were transmitted to their progenies (Oono 1975). Regenerated plants obtained from haploid tissues and their offspring (Oono, 1984), and diploid tissues of other Japonica varieties, Koshihikari, Sasanishiki and Nipponbare and an Indica variety, Loctojan showed similar variations (unpublished data). Therefore, it is concluded that no mutation was present in the original tissues in the process of in vitro culture in rice.

The segregation patterns of the altered characters were different among 75 seed callus clones. The number of mutations carried by D₁ plants which were detected by the changes of five agronomic characters was 3.75/callus clone (281/75). Only 28.1% of the 762 D₂ lines were normal and the other D₂ lines segregated mutants for the investigated characters. Among the 6382 D₂ plants, 1491 D₂ (23.4%) were presumed to be mutants for one or more of the agronomic characters, and the changes of characters of about 300 D₂ examined showed that these changes were transmitted into the D₃. Isolated 150 D₃ lines showed 634 changed characters (average 4.2) among which in 347 cases the changes were fixed and in 287 cases the changes were segregated in the individual D₃ lines. In genetical studies F₂ segregation analysis of some of the characters demonstrated that single recessive genes were responsible for the changes. The frequent occurrence of mutation in regenerated plants is really comparable to that observed after the application of strong treatments of X-ray (Kawai 1963) and of alkylating agents (Sato 1966) to rice.

The culture conditions and/or the characteristics of cultured cells may be related to the high frequency of mutations in rice callus for the following reasons.

1) The plant tissue culture media were mostly developed to evaluate the growth responses of cultured cells or organs and not to evaluate the effect on the genetic stability of the cultured cells (Murashige and Skoog 1962, Miller 1963, Gamborg et al. 1968). Frequencies of chromosomal variation may change with the composition of the medium employed (Bayliss 1973, Niizeki 1974, Singh 1975). Different degrees of chromosomal variants of callus or regenerated plants were also observed in induction, growth and redifferentiation media. Modifications in the concentration of inorganic salts of callus induction media tend to increase the genetic variability of rice callus (manuscript in preparation).

2) The treatment with 2,4-D has been found to induce cytological abnormalities in Allium cepa (Croker 1953) and segregation of albinos in the second generation in barley (Monandas and Grant 1972). However, in our materials, the mutagenic effect of 2,4-D reveals by the occurrence of chlorophyll-deficient mutants was not significantly higher than in the control. Genetic effect was investigated in progeny of rice seedlings treated with 2,4-D at a concentration of 5 × 10⁻⁶ mol and NAA at a concentration of 10⁻⁵ mol for 30 days. The 2,4-D treated seedlings were grouped
into two categories, those with a callus formed around the scutellum and with abnormal growth and those with no visible callus formation and no abnormal seedling growth. Only the former group showed high genetic instability in the tests in the second generation, to the extent observed in the D₂ population derived from haploid tissues (unpublished data). A high frequency of chromosome variation was observed in rice pollen calluses induced after 55 days of anther inoculation in both the 5 X 10⁻⁶ mol 2,4-D medium and the 5 X 10⁻⁶ mol NAA medium (Oono 1975). There was no evidence to implicate 2,4-D as the direct causal agent of mitotic aberrations leading to changes in the chromosome number (Bayliss 1980). Therefore, the direct, mutagenic effect of 2,4-D could hardly account for the high frequency of mutations occurring in rice callus culture.

Useful characteristics in rice cultivation, such as early maturation, short culm, long panicle and large grain were identified in the somatic mutation of rice in the present experiments. And true breeding lines carrying single recessive gene mutations were obtained in the D₃ and are being maintained in subsequent generations. The high frequency and sequential somatic mutations in rice should facilitate the isolation of other agronomically important characters by the application of adequate selection pressures during in vitro culture (Gengenback et al., 1977, Chaleff and Parsons 1978, Nabor 1980).

The decrease in the mean values of plant height and seed fertility in callus derived progenies clearly suggests a deterioration in the population vigor, as in the case of treatments with powerful chemical mutagens or radiations of high doses. Similar depression effect on the qualitative characters was observed in doubled haploids obtained by anther culture (De Paepe et al. 1981) and in plants derived from protoplasts at younger and older stages of development (Prat 1983). It is reasonable to assume that changes which do not affect plant phenotype, or minor gene mutations, were also induced in the culture phase.

Mutations for reproduction mechanisms expressed as malformations and sterility are likely to decrease the effectiveness of the use of cell and callus in genetic manipulations including recombinant DNA, somatic hybrids, in vitro selection of mutants, etc. The recent development of molecular biology has revealed that the genes of higher plants show a high degree of polymorphism and considerable variability in structure (Piccini et al., 1982, Apples and Dvorak 1982). The central issue in the studies of genetic variation is to understand the processes involved and to control them in the in vitro system.
Summary

Genetic variation was studied in regenerated plants from seed callus (D₁) and their progenies (D₂, D₃). A total of 1,121 D₁ plants were regenerated from 75 seed calluses of a rice variety. In the second generation D₂, 762 lines (6382 D₂ plants in total) and in the third generation D₃ 150 lines (2642 D₃ plants in total) were analysed for the changes of 6 characters, i.e., heading date, plant height, panicle length, morphological characters, seed fertility and chlorophyll deficiency.

A proportion of 23.4% of the 6382 D₂ plants were mutants in the observed agronomic characters. D₂ lines showed segregation of mutants in a frequency of 71.9% (548/762). Only 28.1% of the 762 D₁ plants (D₂ lines) did not segregate mutants. The average number of characters altered per D₁ panicle (D₂ line) was 1.08 for the five characters examined. One hundred and fifty D₃ lines derived from typical mutants in D₂ were true breeding for the cultured characters detected in D₂ at a rate of 54.7% (347/634). Genetical analyses of 8 D₃ mutants (crossing with control) showed that the mutants examined resulted from single recessive gene mutations.

The D₂ lines derived from individual callus clones differed from one another in some agronomic characters. The mean values of characters such as plant height and seed fertility typically decreased in all seed progenies in the D₂ compared with the control. Agronomic characters of some of the true breeding mutant lines were investigated.

References

9. Chaleff, R.S. and Parsons, M.F. (1978) : Direct selection in vitro for herbicide resistant mutants of


高波段のスオマクロ駆動変異の育種について

藤井（遺伝研）：突然変異が多く出るということに興味があるのですが、放射線や化学変異剤ではゲノムの変化はまだないので、培養細胞ではよく出るようです。変異の数が増大という意味では育種的にも重要だと思いますが、他の二つの講演で示されたように、最近倉田になってきた遺伝子のクローニングやその遺伝資源としての保存・利用という点からは、変異を起さない方法を考えるべきだと思います。本来非常に変化することの少ない生物が培養によってどうしてそんなに変化が起こるのか、そのメカニズムの解明と、もうひとつ変化を起さないような変異の方法などについてどのように考えておられますか？

大野：なぜ遺伝的な変異が起るのかということは我々のほうで大きな問題になっているのですが、例えば有数の例は2、4-Dなどの植物ホルモンが変異原になるのではないかと考えられていたことと、もうひとつは培地の無機塩のバランスが悪いのではないかという二点についてはいろいろと検討した結果その可能性は無いかという結論になりました。変異が起こる原因は判りませんが、脱分化した状態が遺伝的な変異を起こす一番大きな原因になるのだろうと思います。なぜ脱分化した状態がそうなるのかはわかりませんが、現象的にはそのような状態がいろいろな不安定さの誘引条件になると現在考えており、脱分化した状態が何なのかが研究のターゲットになると考えています。

長谷川（大阪府立農業試験場）：従来の突然変異育種法と比較した場合、突然変異率の比較はどのようになるか、例えば細胞あたりの突然変異率の比較でよいのでしょうか？それともうひとつ、そのような方法で細胞質型質の突然変異は得られるものでしょうか？

大野：あとの方の細胞質突然変異体は得られると思います。解析は進んでいませんが実際例れば雄性不稔なものを見られましたし、ミトコンドリアで変異が起こっているのがわかりますから。失敗の方の突然変異率の比較は、厳密には特定遺伝子座について比較しなければなりませんが、一般には葉緑体突然変異などが突然変異の比較のよい例だと思います。そのような率では対比できるだけの効率で突然変異が起こっているといえると思います。

河合：アルビノがD1で出ますが、やく培養の場合と比較して出方は違いますか？

大野：2倍体の場合は最初のVDの実験のように8.7%です。分化当代のアルビノが何なのかがよく判りませんので検討しているところですが、ハブロイドのやく培養よりも明らかに少ないと思います。ただ、ひとつ細胞からの分割の回数がどのくらい早いかという観察の意味での比較ができませんので、実際に同じ率で起こっているかどうかはわかりません。ただ現象的には分化個体当たりのアルビノの出現率は低いですね。
GENETIC ENGINEERING OF PLANT CELLS

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Introduction

It must be admitted that until recently the genetic engineering of plants retarded in terms of the development of vectors and that of transformation systems compared to microbes and animal cells, but very recent progress on this subject is remarkable. Furthermore, its development is unique and provides interesting experimental systems. In this review such progress will be presented from the extension of the author's talks to the 23rd Gamma Field Symposium.

Before going into main topics one of important characteristics of plant cells will be noticed. It is so-called totipotency (Skoog and Miller 1958; Steward et al. 1963) that enables regeneration of whole plants from somatic cells in various species, which is a sharp contrast to animal cells. Thus whole plants can be obtained from genetically modified somatic cells. This implies that the genetic engineering of plant cells can be extended to that of whole plants. These genetically modified plants can be then utilized as starting materials of plant breeding. Then chances of recombination of plants increase unlimitedly, while so far recombination has been restricted to closely related species via sexual crosses. This possibility could give some resolutions to global food crisis due to the increase of world population, because the introduction of drought-, salt- and cold-resistant genes, which have not so far been available by conventional ways to crop plants, will make extreme climate areas such as deserts and polar regions utilisable for cultivation. Thus the genetic engineering of plants will be very important for the future of human beings.

As the two necessary requirements for genetic engineering are the development of vectors and that of transformation systems, these two requirements will be introduced successively in the following.
Plant Vectors

The utilization of Ti plasmids from *Agrobacterium tumefaciens* became realistic and people are now discussing their practical application. Thus this author will try to depict this subject possible in details. Transposable elements which have been discovered and studied by B. McClintock and DNA viruses will be also considered as a view of vectors.

1) **Ti plasmid**

Ti plasmid was found as a causative agent for crown gall tumorigenesis by a crown gall research group in Ghent University (Zaenen *et al.* 1974; Van Larebeke *et al.* 1974), but there have been tremendous amounts of works up to this time which were found to be in vain, since a hypothesis of Tumor-inducing Principle (TIP) was presented by Braun and Mandel in 1948, which was supposed to transfer from *A. tumefaciens* to plant cells upon infection. Thus it is necessary to draw the nature of crown gall in general briefly in the beginning.

Crown gall is a typical plant tumor caused by infection with a Gram-negative soil bacterium, *A. tumefaciens*. After induction of tumors crown gall grows persistently on a medium such as Murashige and Skoog medium lacking auxin and cytokinin after removal of causative bacteria by heat or antibiotics treatment. Thus one of characteristics of crown gall is autotrophy for plant hormones. Another is transplantability that bacteria-free crown gall can be grafted onto other healthy plants and the implanted tissues (Bomhoff *et al.* 1976) (Fig. 1). The host range of this bacterium is dicots and such as octopine, nopaline or agropine which have never been detected in normal tissues (Bomhoff *et al.* 1976) (Fig. 1). The host range of this bacterium is dicots and gymnosperm, but there have been disputes whether monocots can be infected by this bacterium. Traditionally this reply was negative, but recent study showed that some monocots could be infected by this bacterium, which will be cited again.

After the discovery of Ti plasmid, Ti plasmids were separated from various plant tissues and places and they were classified into octopine-, nopaline- and agropine-type Ti plasmids from the inductive utilization of respective opines (Guyon *et al.* 1980). The induced crown gall produced opines corresponding to each type of inciting plasmid. From these relations was presented the “opine concept”. Thus opine production can be utilized as a genetic marker of crown gall.

It has been shown by the crown research group in University of Washington in Seattle that a part of Ti plasmid was transferred to crown gall upon infection and a name of T-DNA was given to this fragment (Chilton *et al.* 1977). This conclusive evidence of the transformation of plant cells made the use of Ti plasmid as a means of
Fig. 1. Opines.

1 and 2 show the schematic presentation of biosynthetic pathway of octopine and nopaline, respectively. Only representative opines in each group are shown.

Genetic manipulation decisive. On the other hand, from the identification of T-DNA the mechanism of crown gall tumorigenesis was elucidated in details and now this mechanism can be summarized as follows:

Firstly, some genes located in host bacteria are necessary for the attachment of bacteria to plant cells (Douglas et al. 1982). These genes reside at least two loci corresponding to 10-15 kb. Inactivation of these genes nullifies bacterial virulence.

Secondly, the VIR region of 40-45 kb locates in the left of T-NDNA as is shown in Fig. 2 is necessary for infection (Klee et al. 1983). This region composed of at least 6 loci corresponding to 10 genes are supposed to be related to excision and recombination.

Fig. 2. The structure of Ti plasmid. Only relative position of T-DNA and VIR region are shown.
of T-DNA with host DNA. Interestingly vir A and vir G were induced to activate upon attachment to plant cells. Vir C seems to be related to the determination of host range, because a narrow host range bacterium such as AG 152 which infects only grape vine lacks this region. The inactivation of VIR region negates the infectivity of Ti plasmid.

Thirdly, upon transformation T-DNA is transferred to plant cells and integrated into host DNA, but recent experiments showed that 25 bp in both borders of T-DNA is sufficient for transformation and the left border is dispensable (Wang et al. 1984). This means that genes on T-DNA do not play any role in transformation process per se. The genes on T-DNA function for the maintenance of tumorigenic state after transformation. This implies that tumor genes can be separated from transformation function (Leemans et al. 1982). Actually such plasmids have been developed in several laboratories as is described later.

From recent studies oncogenes were identified as auxin- and cytokinin-biosynthetic genes. These were firstly supposed by transposon mutagenesis in T-region. Garfinkel et al. (1981) found that the inactivation of tms locus (Fig. 3) turned virulent plasmids to less virulent and crown galls induced by such Ti plasmids produced shoots. The inactivation of tmr locus induced root upon infection. Then these two regions were supposed to be related to hormone production. Actually it was shown (Akiyoshi et al. 1983) that crown gall tissues induced by tms and tmr mutant plasmids induced less amount of auxin and cytokinin, respectively.

On the other hand, from the analysis of transcripts of octopine-type crown gall Willmitzer et al. (1982) identified 7 independent transcripts, to which was added one more transcript as is shown in Fig. 3. From the comparative studies of these transcripts with the results of mutagenesis in T-region, transcripts 1 and 2 were identified as tms locus and transcript 4 as tmr locus, respectively. More recent studies of the analysis of protein products in these regions together with gene cloning clearly showed that transcript 2 for tryptophane 2-monoxygenase (iaaM) and transcript 1 for indoleacetamide hydrolase (iaa H) (Schröder et al. 1984). These two genes catalyze the production of

![Fig. 3. The structure of T-DNA of octopine-type Ti plasmid.](image)
indole-3-acetic acid (IAA) from tryptophane. Transcript 4 was also identified as a gene for isopentenyl transferase (ipT), which catalyzed the production of cytokinins (Barry et al. 1984). Therefore, the cause of tumorous growth of crown gall is due to overproduction of auxins and cytokinins, which are produced by the introduced bacterial genes in plant cells. In this context it should be noted that plant cells produce these two hormones by themselves and in the case of auxin, IAA was synthesized from tryptophane via indolepyruvic acid or from indole via tryptamine as an intermediate. Thus in crown galls both eukaryotic and prokaryotic hormone genes function simultaneously. It is surprising that prokaryotic genes function in plant cells, as molecular biology teaches us functional discrepancy between prokaryotic and eukaryotic genes. So far clear function of other genes such as 5, 6a, 6b and 7 are not yet released. In the case of nopaline type crown galls much more transcripts were detected, but hormone producing genes were common in both cases (Bevan and Chilton 1982).

From the evidence that tumor genes could be separated from transformation function, vector using Ti plasmids became very promising. Actually a plasmid deleting oncogenes such as pGV 3850 (Fig. 4) has been constructed (Zambryski et al. 1983). This plasmid retained VIR region, border sequences of 25 bp and nopaline synthase gene as a genetic marker, while oncogenes were completely replaced by pBR 322. Thus when some gene cloned in pBR 322 is introduced into A. tumefaciens from E. coli by aid of helper plasmids of pGJ 28 and R64d1, the introduced gene can be inserted into T-region via homologous recombination. Then this gene can be introduced into plant cells via infection with plants or co-cultivation with mesophyll protoplasts which is shown later and whole plants retaining introduced genes can be regenerated from offsprings of the transformed cells.

Recently the introduction of foreign genes into plants was reported by several groups using Ti plasmid deleting oncogenes (so-called disarmed plasmid). One of such examples was the introduction of neomycin phosphotransferase II (NPT II) gene in the downstream of promoter region of small subunit of ribulose-bisphosphate carboxylase/oxygenase (Rubisco) to tobacco using pGV 3850 (Herrera-Estrella et al. 1984). Such examples were also extended to promoter region of chalcone synthase gene combined with NPT II. In both cases gene activation was shown to be controled by phytochrome system. Similar one was also presented by Brogle et al. (1984), where Rubisco gene of pea was introduced into petunia. This line of works is carried out toward the practical application and the introduction of resistant gene of herbicides such as Glyphosate into plants is planned, in which breeding of herbicide resistant plants is expected. This could be also applied to other herbicides including Alachlor and Atrazine (R. Fraley personal communication). In the near future this technique will be applied to other useful genes such as drought-, cold- and disease-resistant genes, but at the moment
isolation of such genes seems to be extremely difficult. Sooner or later improvement of vectors developed from Ti plasmids will be progressed further and then the genetic engineering of plants will become more familiar and productive and then contribute to the increase of food production in world-wide levels.

However, there has been a discussion that the application of Ti plasmid vectors will be confined to dicots, because the host range of *A. tumefaciens* harboring Ti plasmid were believed to be confined to dicots and gymnosperm. Recent reconfirmation concerning to this subject looks very promising. Hoooykaas-Van Slogteren *et al.* (1984)
observed the swellings of tissues of two monocots species belonging to Liliaceae and Amaryllidaceae after infection with *A. tumefaciens*. Then they could detect opine production in these swellings. On the other hand, tumor formation on asparagus and opine production in these tissues was reported by Hernalsteens *et al.* (1984) after infection with *A. tumefaciens*. Therefore, at least in some species of monocots *A. tumefaciens* can infect and transfer T-DNA into plant cells and this implies that the genetic engineering of monocots which have many important crop plants using Ti plasmids is possible.

Ri plasmid from *A. rhizogenes* found as a causative agent of a hairy-root disease showed essentially similar genetical characteristics to Ti plasmids. It is supposed to share a common ancestor with Ti plasmid and can be considered as a subgroup of Ti plasmid (White and Nester 1980; Willmitzer *et al.* 1982; Chilton *et al.* 1982). Thus Ri plasmid is also considered as a candidate for plant vectors, but detailed description will be avoided here, because most of descriptions tend to duplicate the sketch shown above. Only one point is added that transformants with Ri plasmids tend to regenerate easier than those with Ti plasmids.

2) Transposable Elements

Among several transposable elements found in plants, the *Activator (Ac)-Dissociation (Ds)* system discovered in maize and studied genetically by McClintock (1961) has been elucidated recently at the molecular level by cloning technique (Sutton *et al.* 1984; Pohliem *et al.* 1984; Doring *et al.* 1984). This view showed that both transposable elements retained inverted repeats of 11 bp at borders. Upon insertion into plant DNA 8 bp direct repeat is generated and *Ac*-mediated excision of *Ds* brought the 8 bp repeats into continuity, but with nucleotide alterations or deletions at the new junction. Although full details of transposable elements have not been released yet, general features have similarity to those of other eukaryotic and prokaryotic transposable elements. Thus plant transposable elements may be utilized for the vehicle for introducing foreign genes into plant cells, but at the moment they are still in the state of infancy and are discussing the possibility of plant vectors, although P elements from *Drosophila melanogaster* is now used as cloning vector in this organism.

On the other hand, the usefulness of transposable elements for the isolation of plant genes has been shown and looks very promising, as has been shown by Wienard *et al.* (1982). From mutnats of flower color variation of *Antirrhinum majus* whose chalcone synthase (CHS) was inactivated by the integration of a transposable element they obtained revertants after their transposition. By using cDNA to m RNA of CHS of parsley as a probe they could isolate a functional structural gene of CHS from genomic library, while simple isolation scheme might not encounter functional gene. Thus this
procedure is effective for the isolation of functional gene in plants.

3) DNA Viruses

DNA viruses which are quite rare in plant viruses are considered as candidates for plant vectors. Although in animal cells DNA viruses such as SV 40 have been successfully used as vectors, cauliflower mosaic virus (CaMV) which is thought to be one of the best candidate for this purpose is still not satisfactory, because in the replication cycle of this virus reverse transcription process is involved and so it is not a typical DNA virus, but a kind of retrovirus (Pfeiffer and Hohn 1983). Furthermore, there is no evidence of genetic transformation of host DNA and its host range is very narrow. Thus in general DNA viruses look very pessimistic for the application of vectors. On the contrary, recent report showed the introduction of bacterial dihydrofolate reductase gene (DHFR) into plant cells using CaMV as a vector. In this case this DHFR gene could be successfully inserted into the open-reading frame II of this virus, which did not decrease the infectivity of this virus and the expression of DHFR gene was detected enzymatically (Brisson et al. 1984).

There are some more DNA viruses such as gemini viruses (Ikegami et al. 1981), but basic study of these viruses is much more retarded than that of CaMV.

Transformation

Transformation system has been studied mostly by using protoplasts, because usual plant cells retaining thick cell walls are resistant to artificial manipulations. Furthermore, in the case of protoplasts regeneration of whole plants has been established in many cases. Thus whole plants can be regenerated from genetically manipulated protoplasts. This does not necessarily mean that genetic manipulation is confined only to protoplasts, but as the separation of single cell clones is always possible from protoplasts, protoplasts are preferable material for genetic manipulation.

Several methods have been developed so far for the introduction of genetic materials into plant cells; direct introduction of DNA into protoplasts, spheroplast-mediated transformation and co-cultivation of protoplasts with Agrobacterium etc. Therefore, some features of these examples will be described in the following. In this context Ti plasmids were chosen as experimental materials, as most of informations concerning to this subject are released from Ti plasmids.

1) PEG, PVA and PO method.

There are several reports for the introduction of DNA into protoplasts, where
naked DNA is introduced into protoplasts by aid of polymers such as polyethylene glycol (PEG), polyvinylalcohol (PVA), poly-L-ornithine (PO) and poly-L-lysine (PL).

Krens et al. (1982) showed the transformation of mesophyll protoplasts by isolated isolated Ti plasmids in the presence of PEG. In their case the analysis of T-DNA showed that it is rather variable and slightly different from bacteria-mediated transformants. Similar experiments have been reported also in the case of PL and PO (Davey et al. 1980; Nagata et al. 1979). However, reported transformation frequency was rather low and in the level of 10⁻⁶.

In this context it should be noted that recently Hain et al. (in press) showed the transformation of protoplasts by NPT II gene which was placed in the downstream of the nopaline synthase promoter and has poly A signal from octopine synthase. In this experiment DNA co-precipitated with calcium phosphate was taken up by protoplasts by aid of PVA 203. The resultant transformants were obtained by the selection on the medium containing kanamycin. Thus the selection was carried out solely by antibiotic resistance and so this method is versatile for any plant material and there is no more restriction to host range, as has been encountered in the case of other vectors including Ti plasmid.

It is interesting that polymers such as PEG and PVA were effective in introducing DNA into protoplasts, although both chemicals were originally found for the induction of protoplast fusion (Nagata 1984a). Polycation such as PL and PO was also effective in introducing DNA into protoplasts, while they were found originally for the uptake of plant viruses by protoplasts (Takebe and Otsuki 1969).

2) Liposome Method.

Liposome-mediated transformation of animal cells has been reported in several cases and Schaefer-Ridder et al. (1982) showed that TK⁻ cells were converted to TK⁺ by the introduction of TK⁺ gene encapsulated in liposomes at higher frequency than other methods. In the case of plant protoplasts liposome-mediated introduction of RNA into protoplasts has been shown very efficiently. To more than 90% of tobacco cultured cell protoplasts (3 × 10⁶) was introduced functional tobacco mosaic virus (TMV)-RNA encapsulated in liposomes, which could be detected by staining with a fluorescent antibody against TMV particle after one day of culture (Nagata et al. 1981; Nagata 1984b). For the successful introduction of liposomes into protoplasts PEG or PVA treatment in combination with high pH-high Ca washing was indispensable. The application of this technique to Ti plasmids seems to be difficult, because encapsulation of large plasmid such as Ti plasmid was so far troublesome, but introduction of smaller DNA is very efficient compared to other procedures.
3) Spheroplast Method.

Hasezawa et al. (1981) introduced *A. tumefaciens* spheroplasts into protoplasts from *Vinca rosea* by aid of PEG or PVA in combination with high pH-high Ca washing. The resultant transformants were obtained at rather high frequency and this method is expected to be useful for the transformation of materials which do not belong to host range of *A. tumefaciens*.

4) Microinjection.

Microinjection of DNA into protoplasts has been also tried in many laboratories, but so far handling of limited number of protoplasts and difficulty in injecting into nuclei may be obstacles to be overcome. Informations concerning to this procedure is still fragmentary (Steinbiss and Stabel 1983).

5) Co-cultivation Method.

So far the highest transformation frequency of plant cells was attained by co-cultivation of mesophyll protoplasts with *A. tumefaciens*, in which natural infection of this bacterium was utilized and so removal of bacteria using antibiotics is necessary (Márton et al. 1979; Wullems et al. 1981). According to this procedure up to 10% of protoplasts have been transformed at the optimal condition. By using this procedure foreign genetic materials including NPT II gene were introduced into protoplasts (Fraley et al. 1983). However, this method is confined to host range of *A. tumefaciens* because of the use of natural infection system of this bacterium.

Conclusion

As is shown above, the genetic engineering of plants has just started, but at the same time it is true that some examples of introducing foreign genes into plant cells have been demonstrated only in a model system. Therefore for practical application of this technique much more basic studies are necessary. In this context it should be noted that for these experiments disciplines of molecular biology, cell biology are absolutely necessary. At the same time background of cell and tissue culture is required. Then for the application of these results in practice plant breeding is also necessary. Such wide spectrum of disciplines have not been required for any previous subject. In several laboratories including Max-Planck-Institut für Züchtungsforschung in Cologne or Monsanto Co. in St. Louis such considerations have been carried out for the construction of experimental teams. So far such attention is very subtle in this country. In this respect if such attention is aroused from this paper, it would be far more than expected.
GENETIC ENGINEERING OF PLANT CELLS

Summary

In this paper the present status of genetic engineering of plants has been reviewed, which has just taken off. Both the development of vectors and that of transformation systems were considered. As for vectors special attention was payed for Ti plasmid, as the introduction of foreign genes into plant cells has been done by using this vector. As for transformation systems critical considerations of published methods have been done. Although much more improvements are necessary, the future of this discipline looks very promising.

References


植物細胞の遺伝子操作

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これまで植物細胞の遺伝子操作は、微生物や動物細胞に比べて遅れている点が多かったが、最近の進歩は著しくもあり独特の展開を遂げつつある。従って本書では先のシンポジウムでの講演を基として近況の概略を行なった。

遺伝子工学は、ベクターの開発とベクターの細胞内への導入とからなるので、それぞれの概略の紹介が行なわれた。

植物ベクター

植物ベクターの中では、Tiプラスマドを用いた遺伝子操作の進歩は著しく、Tiプラスマドベクターを用いた外来遺伝子の導入は、既にいくつかの例で示されている。

ところでTiプラスマドについてふれるとときにその発見の元となる植物腫瘍クラウンゴルの形成機構についてふれねばならない。Braunが、1948年に細菌より植物細胞に移行すると仮想した腫瘍誘導因子（TIP）は、1974年にSchellらによりTiプラスマドと同定され、更に形質転換の決定的証明が1977年に、Chiltonらによってなされてから、遺伝子工学のベクターとして注目されるようになったように、Tiプラスマドの研究は本来腫瘍機構の解明を発展点としている。

しかも最近の見解は、植物細胞の形質転換と、腫瘍状態の維持に関わる遺伝子とを完全に分離できることを明らかにしたので、この系を用いて、腫瘍形成を伴わない外来遺伝子の導入が可能となった。このようなベクターの例が、pGV3850であり、T－DNAから全て腫瘍遺伝子を省き、両端の25塩基対と、遺伝子マーカーとしてのノバリン合成酵素遺伝子を残すのみである。T－DNAの左側にあるVIR領域は、細菌よりT－DNAを植物細胞へ伝達し植物DNAとの組換えに関係あると想像されている。

一方、最近の遺伝子クローニングは、腫瘍遺伝子の正体をも明らかにしたが、それらはオーキシン合成に関わる、トリプトファンモノキシンガーチーとインドールアセトアミドヒドロラーゼおよびサイトカイニン合成のイソベンテニルトランスフェラーゼであることを明らかにしたが、この結果は原核細胞の遺伝子がそのまま真核細胞中でも働くという生物学的に極めて興味ある現象であることも明らかにした。

未だベクターとして実用化までは、時間がかかると予想されるが、将来の展望は極めて明るいと予想される。

ところで、これまでTiプラスマドは、Agrobacterium tumefaciensの宿主が、双子葉植物に限ると信じられてきたので単子葉植物には応用できないのではないかとわれてきだが、最近の知見では、ユリ科、アマリリス科植物では腫瘍形成のあることが明らかにされ、Tiプラスマドを用いる単子葉植物の遺伝子操作も有望となってきている。
一方、植物のトランスポーザー・エレメンツは、McClintockの研究にはじまるが、未だベクターとしての検討は近未来はまったばかりである。一方、これを用いた遺伝子の単離は有望と考えられている。

DNAウイルスは、カリフラワーモザイクウイルスでベクターとしての検討がなされたが、このウイルスはいわゆるレトロウイルスに属するため困難の点もあるが、最近これを用いて植物細胞へ抗生物質抵抗遺伝子の導入が報告された。

遺伝子導入法

植物プロトプラストを用いて、PEG、PVAなどの処理で遺伝子を導入する方法や、リボソーム、細菌スフェロプラストを取り込みさせる方法、更にはプロトプラストとAgrobacteriumの共存培養による形質転換法が開発され、特に共存培養によっては極めて効率良く細胞内への遺伝子導入が可能であることが示されている。

この分野の展望は明るいが、今後の発展のためには、分子生物学、細胞生物学、細胞培養、更には育種学の知見を自由に使いつつことが必要である。

質疑応答

小林（生物研）：ベクターについてですが、T-DNAを使ったものは双子葉植物に特異的でイネなどの単子葉植物には効果を示していないのが現状だろうと思います。先程理論的には完成したとのことでしたが、現実を拙者が見たイト、もう双子葉植物では現実のものになっているように思われます。しかし単子葉植物ではまだこれからのようにです。T-DNAの一部を変更したあるいはイネ科植物でも入る可能性があるのかどうか、それともイネ科では全く別のベクターを開発しなければならないのかどうか、そのあたりについておうかがいしたい。

長田：これは非常にむずかしい問題です。はじめの方の問題で、遺伝子が導入できてもこれが役立つかどうかはむずかしい問題だとおもいます。それから次におたずねの単子葉植物でどうかということにつきましては実はよく判りません。しかし未確認情報では、もうすでにTi-プラスミドを変修・改変でき、その選択は足であるというような話も聞きます。双子葉植物で調べはじめられているようにとりこみの初期の過程では細胞壁との接着が重要だとしますと、これをバイパスすれば可能であるという説でありましょう。未確認情報でどの程度のものか判りませんが、やってみるにこしたことはないと思っております。私自身のデータを持っておりませんが、案外できるのではないかと思われます。
総合討論

座長・志賀（生物研）：このガンマーフィールドシンポジウムはいままでに非常に活発に議論がすすめられてきたのですが、今回は出席者が多く講演会のようになりましました。しかしやはりシンポジウムですので一つ積極的なご討論をお願いいたします。

個々のご講演のとおりまとめは省略させていただきますが、中島先生からは組織培養を用いていくのであるとおっしゃっておられる視点から、平井氏、内子氏からは細胞融合をした場合の細胞選択されるマーカーについてのお話をいただきたいわけですが、山元氏からはいわゆる不和合の花粉を放射線処置することによって、今まで進まないなかった違いの遺伝子をとりこむ新しい例のご紹介がありました。本日は久木村・矢頭両氏と大野氏から組織培養を使ってどのような変異が起きるかといった話をいただき、最後に長谷川氏から組織培養をやってる者の一番の夢である培養細胞に不確定の遺伝子をとりこませてそれを行わない新しい今まで不可能であった植物病原菌を作ることが理論的には可能になったとご講演がありました。大きく分けると三つの話になるかと思います。一つは組織培養をつかってどのように変異が拡大するかという話、いまひとつはプロトプラストを融合させる、いわゆる細胞融合によってどのように変異が拡大し、それを我々がどのように利用できるかという話、最後には遺伝子操作によって我々の夢であった変異の遺伝子を他の植物に入れるということがどのように可能なのか、ということです。

まず組織培養による変異の拡大についてはですが、今回突然変異体の選抜についてはふれられていませんが、やはり選抜の問題を抜きにして考えられませんので、その辺を含めた議論をしてゆきたいと思っています。はじめに何かご質問はございますか？

鵜飼（放育場）：培養法を使いますが色素体異常と突然変異の両方がかなり高い頻度で出ますが、私共放射線育種をやってきた歴史の中では色素体異常はなるべく少なくて、突然変異が多い処理を考慮してきたつもりです。培養のほうではそのような仕事はないものかどうか、それとそのような色素体異常は抑えず突然変異だけを高めるような培地条件を作ることが可能かどうかお聞きしたい。

大野：色素体変異を起こさないで遺伝子突然変異を起こせばよいというわけですが、今のところは色素体異常を起こす条件と、遺伝子突然変異を起こす条件が同じかどうかちょっと判らないと思います。現在では主要雑誌に発表される報告にも基本的には現象の話だけです。ですから遺伝子突然変異が起こってもその状態ではありませんし、ミトコンドリアのレベルでの遺伝子配列といった話です。したがって色素体変異を起こすということにはならないと思います。培養条件のあらゆる条件において細胞質突然変異を起こすという可能性もあるかもしれません。細胞質は細胞融合でよくとしもしてもスペクトラムの差が、培養条件によって差があるかどうかということについては、現象としては可能性はあります。ただ育種の目的で考えるなら、変異はなんべんなく起こって、あとは培養条件で選抜することで、突然
変異体の密度を高めればいいのではないかとも考えられます。

河合（三和生薬）：結局は細胞質のどちらかが消えてゆくことになりますが、分化するときにそこで選択がおきているのだということですと、細胞質と核の問で何かの作用があってそこで分化が起こるか起こらないかがきまるというふうに解釈してよろしいのでしょうか？

平井：そこはむずかしいところで、私もよくわからないのですけど、私の考えではひとつの細胞のなかに一種類の葉緑体をもつものと、二種類の葉緑体をもつ細胞を比べてみますと、一種類の葉緑体をもっている方がより分化しやすいのではないかと思いますが、これはあくまでも仮説ですのでこれからます確めなければならないと思います。ただ確めないうちにその仮説によって解釈するのはおかしなことですので、植物にとっては葉緑体は独自の遺伝情報をもっていますし、植物細胞をコントロールするためにはやはり葉緑体も核の制御のもとに置かなければならない。そのために葉緑体にコードされている遺伝子も完全なわけではなく、例えばルピノスコの場合は大サブユニットは葉緑体にあるけれども、小サブユニットは核にある。CP1でもα、β、εは葉緑体だがγ、δは核だといいます。そういう形で制御されているわけですから、その制御するところが複数になれば、多分細胞としてはやりにくく、分化能も悪くなるのではないかと思います。あくまでも仮説の延長上の推論ですが。

中島（東大・農）：葉緑体がランダムに分離するというお話ですが、細胞分裂の時にランダムになるためにはもっとも細胞質の中でも両方の葉緑体が全く同じように混在しているのではなくて、ある部分にはこちらが、他の部分にはもう一方がというようなことがなければならぬと思うのですがそのあたりはどうでしょうか？

平井：それは逆に、ひとつの細胞の中にも二種類の葉緑体がアトランダムに存在している。それがランダムに分離してゆくことで説明できるのがあの数式で、たとえばひとつの細胞にA、Bの2種類の葉緑体が5個ずつ10個あるとして、それが二つにわかれるとときに5個のA葉緑体と5個のB葉緑体にわかれられる確率は非常に低いものですが、ある一定の確率でそれは存在するわけです。そのつくりかさでAという葉緑体を持つ細胞が出来てしまうと、そのあとはAという葉緑体だけを持つわけですから、べつに細胞の中で分権がおいていなくても、その確率で説明できると思います。

西尾（野菜試）：ミトコンドリアも葉緑体と同じようになるのでしょうか？また、ミトコンドリアは一方の親、葉緑体は他方のものといった融合植物ができる可能性はあるでしょうか？

平井：私はミトコンドリアのことは全然やっておりませんので、私のデータとしては何もお話しできないのですが、ほかの研究者のデータによると、ミトコンドリアの場合葉緑体と全く違って2種類のミトコンドリアの組替えも起こるとわれています。種のミトコンドリアができているわけですから、むしろ片方の葉緑体を持ってミトコンドリアはもう一方のもののみというのはむつかしいのではないかと思います。

西村（放育場）：いま組換えの話がでましたが、融合細胞の中で二種の葉緑体間で遺伝子の組換えが起こる可能性はありますか？
平井：私達がみておりますのはひとつの細胞の中に2種類の葉緑体があるというところまでにして、1種類の葉緑体のなかに2種類の葉緑体DNAがあるかどうかまではみていません。すくなくとも組換えを起こすためには1種類の葉緑体の中に2種類の葉緑体DNAがなければ無理ではないかと思います。ただし培養細胞では葉緑体をなくすことができます。正確にはなくすというのはおそらくかもしれませんが、硬い形としての葉緑体ではなくてプロプラステチドみたいな形、色素体みたいな形にすることはでききますから、そういったことをしたあとでは、組替えの可能性はなきにしもあらずだと思います。ただし現在のところそのような報告はほとんどありません。

座長：組織培養を使って変異を拡大するという大野さんと久木村さんのお話がありましたが、組織培養を使って今後どのような選抜方法が考えられ、どのような変異が期待できるかといった点についてコメントをいただけませんか？

大野：突然変異の起こるかたは一般にはランダムで、培養条件で一番誘発の可能性が高い突然変異というのは現状ではあまり判りません。個体レベルでやるよりもin vitroでやるほうが実験的な目標としていいのではないかでしょう。基本的には薬剤耐性であるとか生化学的な変異体が考えられます。イントラく植物では、前駆物質を与えて実験することにはむつかしいので、前駆物質関連での選抜などで生化学的な突然変異体を得るのが可能あるのではないでしょうか。

座長：実際に選抜をされての経験をどうなたかコメントしていただけませんか？

島田（石川短大）：コムギのChinese Springという品種で再分化植物を多数見つけましたが、これらではほとんど変異体はありませんでした。染色体の変異はありませんでした。ですからイネのように染色体の変異や形態の異常はあるがイネの性質で、コムギではあまり無いのではないかと思っています。その後やく培養による半数体のカルスでは、Chinese Springは安定しているのですですが、それ以外の品種ではすごく変異しているものがありました。最近目についた論文に、コムギの再分化植物で染色体数がいちじるしく変異している、再分化植物の30％で染色体数が変異していたという報告がありました。もうひとつは、染色体数は安定していたが、形態的にたいへん変異していたという報告でした。やはりコムギも培養で変異しやすいものようです。しかし品種が違っていいますので、品種の差の意義は大きいと思います。私としてはむしろ安定するような方法をどうなたか教えていただければありがたいと思っています。

座長：野菜試で永い間色々な植物を再分化させた大沢さん、野菜での変異の起こたらとか選抜の可能性などについて教えてください。

大沢（野菜試）：私共の所でも沢山の野菜で脱分化組織から再分化するという仕事を中心にやっております。イチゴ、サトイモ、ブルッコリー、キャベツや、2％から5％位の率で肉眼的に変異だらと思われるものがみつかっています。あと大半のものはユリ科のものため十数種類の野菜ですが、脱分化組織からの再分化で特別目立った形の変異はありませんでした。島田さんのお話と同様に、野菜でも扱う品種等でかなり出てくる率が違います。特に培養していて再分化植物を作ったときの変異の出方とか、変異率をどういうレベルで
整理していけるのだろうかということも困っています。例えば、試験管一本から何本かの変異が出た場合に、十なら十、あるいは百なら百の試験管で何千本かの植物ができるわけですが、そのなかの一株だけのレベルで考えるかかなり高率になってくるけれども、それを全体に拡げると非常に低率になってしまうということもあります。プロトプラストでハクランから大量の植物を復元しました。当初はシェーマーからのジャガイモ等の成績も見ておりましたが、これはかなり今までになかった形のハクランが出るのではないかと楽しみにしていたのですが、じつは非常にきれいに摘ったハクランになっていってしまいまして、そういう点では期待はずれだったこともあります。変異を出そうと思って期待するとなかなか出なくて、抑制しようとすると出てしまうという、これがまあ実際に培養している人間の実感ではないかと思います。

阿部（名大・農）：私はイネで多株の系統を使いまして再分化の試験をしているのですが、アルビノ個体の出現率でみますと、品種間でかなり違うです。全然アルビノ個体が出ないものから、50％位るるものまであります。分化の形として不定胚形成というのと不定芽形成というのがありますが、イネの場合には不定芽よりも不定胚形成の場合、変異の出現率はひくく、アルビノ個体はほとんど出ません。いま Da で圃場に展開していまますが不定胚形成の場合は今のところ変異体はほとんどみられない状態です。私のやっているかぎりでは不定胚形成の場合は変異がでないようです。不定胚形成は由来組織が根の場合に起きるので、由来組織によっても変異の頻度は違ってくるように思います。

座長：組織培養を利用して変異をいかに拡大するかということでは大沢さんのいわれる、出そうすると出ないし、出ますまいすると出る、というところが実感ではないかと思いますが、今後皆さんのお研究を積み重ねることによって、さらに実際の育種にこれらの拡大した変異を選抜し利用する方法が確立されればと思います。

次に細胞融合に移りたいと思います。さっきはも質問がでておりましたが、細胞融合の問題では現在のところプロトプラストからの再分化系を作ることに集中していて、融合を始めているところはタバコを除いてはまだあまりないと思います。しかし細胞融合が今後どのように形質にどのように利用できるのかということは、すでにじめている人、これからはじめるとしている人の問題点であろうと思いますが、講師の平井氏と内宮氏にコメントをいただきたいと思います。

内宮：これはむっかりな問題で、お答えというよりはむしろ同じ悩みだと思いますが、目的とするものを細胞融合で作るのは目的とする材料を的確に選ぶということが大切なわけです。一般的に細胞融合をつかう場合には、少なくともこれまでの育種では破れないような障害を乗り越えられるといった育種目標を持つ必要があると思います。細胞質の遺伝子の導入、特に細胞質雄性不稔といった形質を導入するためには、細胞融合で良いチャンスが与えられるでしょう。実際にやってみますと、おそらくミトコンドリアに原因すると思いますし、細胞質雄性不稔の形質はたしかに雑種にうけつがれます。いままでの実験では種間あるいは属内であっただけなら似たものの間の細胞質の導入だったわけですが、これがもし非常に遠縁になった場合にはどうなるのかというのが今後重要かと思います。

つぎに核の情報ですが、こちらは細胞融合の場合はどうしても両方のゲノムがはいりこ
むとすることがありますので，両方のゲノムを持った場合とか一方の特定の遺伝子を保存させたいといった目的によって使いわける必要があると思います。そのへんはまた新しい技術が必要でしょう。例えば先端の放射線の利用にもなりますが，一方の核を放射線で照射しておいて違う植物と融合させる。その時に特定のマーカーを選抜することで，ある特定の遺伝子を奪った植物に入れるということをも少しずつ報告されています。

平井：内宮氏のお話に付け加えることはないのですが，融合をする場合に普通ではかけあわせのできない組合せで融合を狙うということかと思いますが，珍しい植物を作るという事ではなくて必要な遺伝子だけを融合で入れるということが今一番必要だと思います。そのためには放射線をかけて片一方の染色体をある程度壊しておいての融合というのが大事になると思います。

座長：ありがとうございました。細胞融合については農林水産省でも細胞融合の大型別冊という研究が進んでいるのですが，実際の融合にはなかなか成功していません。ただ芸ではかなりの成果があがっているのでそのへんを食糧研の柳さんにご紹介と今後の展望をお願いいたします。

柳（食糧研）：担子菌でヒトヨタケという茸について細胞融合を試みているのですが，ヒトヨタケの場合はプロトプラストが沢山出ますし，そのあとの再生も簡単にゆく条件がみつけられました。それでどこ最近私達がやりました実験では，栄養要求性のヒトヨタケを紫外線照射でとりまして，それを持って栄養要求性を持っているもの同士を融合させて，それを再生させるということができています。ヒトヨタケの場合は，先端の問題に関連するのですが，融合したプロトプラストから再生してゆくときに，高等植物の場合とは違いまして，カルスのような状態をまったくとられませんで，いきなりプロトプラストから菌糸ができます。プロトプラスト自体を脱分化というならば別ですけど，カルスといった状態を通らないで，プロトプラストという状態を経過しただけですでに変調というか元のものとは違った性質でもててくるのです。それには真の強い性質で，茸の方に会ったということでは子実体が発酵に作られるといった現象がみられます。今ところ私達はプロトプラスト化したあとに再生してくるコロニーを拾っていますから，再生率が非常に高くなってはやや活性の高いものを拾っているのではないかと考えているのですが，もっともほかの要因もあるかもしれません。

座長：ありがとうございました。そのほかこのことに関連して実際にこのようなことをやっているとか，ご提案とかございますか？

菊池（生物研）：細胞融合は動物などの系では染色体地図の作成などにもかなり使われておりますけれども，植物ではかなり遺伝のものの間で染色体の排除が起こると聞きましたが，実際にどのような植物の間でそのようなことが行なわれつつあるのか，あるいは考えられているのか，内宮氏におうかがいします。

内宮：遺伝のものの融合によって一方の染色体が排除されるという現象は，例えばタバコとダイズではタバコの染色体がほとんど排除されます。しかしある程度たとえはタバコの染色体の一本あるいはその断片は残っていくという現象があります。おそらくかなり詳しく調べてゆきますとほかの植物でもあると思います。ただそういった残っていた染色
体にどのような遺伝情報が乗っているかということは今のところ判らないわけです。やはり植物の体細胞組織を通じて実現的なものにするためには、今お話にありましたように、体細胞レベルでの色々なマーカー、あるいは染色体でのマーカーのマッピングが重要になってくると思います。栄養要求性といったことが実際に出ておりますが、それをいかにマップするかということだが、非常に難しいであるということもあるわけです。ただこの問題は色々な遺伝子が植物でもクローニングで実際に in situ のハイブリット形成といった方法で、遺伝子のマッピングができるようになってきています。今迄形質として葉の形や色として地図が作られていたものが、それ以外に例えば蛋白のサブユニットの遺伝子とかいったものの場所が判りつつある時期だと思います。ですからそういったものを組合せますと、体細胞の識別というような面においてこれからの利用されるのではないかと思っています。

菊池：今のことに関連して、動物細胞の方では遺伝子の脱乳を利用してヒトのマッピングができたということですが、その意義そのものはヒトの交配ができないからということで、植物の方の意義としてはちょっと違うのではないかと思います。ただ内宮さんが話されたように植物細胞には突然変異体がかぎられる住まいがないという現状は、細胞融合に限らずほかの色々な遺伝子を扱う上でも問題かと思います。ただ、いままでありましたダイズと N. glauca との場合はダイズの方が増殖の良いものを使って、glauca の方は葉の細胞を使っていますが、あの場合の脱乳の結果はもともと逆にしただろうかという点をいつも思っております。ヒトとネズミの場合は両方とも培養したもので、培養されたものと新鮮な葉から採った細胞との違いということを、植物の場合では考えなければならないのではないかと思います。

座長：最後に長田氏からお話のありました遺伝子操作について、ご質問なりコメントをうかがってこのシンポジウムを終わりたいと思います。

河合（三和生業）：たいへん御迷惑な質問かもしれませんが、全般的な総括になりますから、細胞融合とDNA組替えとではどちらが育種を考えた場合に推奨できるかという点について、御意見がありましたらお答えください。

長田：それはまた内宮さんとか平井さんとかのご意見もあるかと思うのですが、私は植物細胞に遺伝子を導入するのは興味としてやっているものですから、あまりそういうことは考えたことはありません。むしろ細胞融合にも少し手を染めてはいますが、プロトプラストも少しやっていますので、今この時点での実験からいったら融合のほうができると思います。ただ十年以上先はどうなるかは、いつも実現するかを別にすれば、例えば動原体みたいなものを人工的に作って人工染色体を作ったら、今我々が考えているものは随分違うものになるでしょうし、ですが今の時点でのいかはやはり私はまだ細胞融合に分があると思っています。

座長：大野氏はいかがですか、今と同じ質問に。

大野：結局細胞融合の場合には遺伝子が伝わるほど植物体の分化がいかなくなるし、染色体の消失もあるし、そういう意味でなかなか問題があります。永年特性とか栄養繁殖性であれば細胞融合でも可能性があります。雑種を作っていて F1 作成からあのの時間がかかるだろうということが細胞融合にあります。また細胞融合というのは基本的には雑
種を作るわけですが、遠縁交雑は胚培養などよりも程度の段階はゆくので、それから先の段階が細胞融合のターゲットになるでしょう。そもそももっと遠縁になれば遺伝子組替えの方のターゲットになるでしょう。基本的には利用の可能性の範囲がやや明らかになりそうと思います。遺伝子組替えの方は、一番の問題は構造遺伝子を実際にどうするかという一般的な戦術のないわけですから、たとえばトランススポゾンをいれてそれで拾いあげるというふふかたもありますが、特定の機能を持っていなければ育種になりませんから、そいつらが解決しなければなかなか使えないのではないかという気がします。

山元：私は実際にやっておりませんので、どちらがいいとはいえませんが、感じとしましては色々なレベルで仕事をやっていて、それでどれかが当たるだろうという形でやっていくよりしかたがないだろうという気がしているのです。私としては生殖過程を通して入れていくのが一番ととりとらえはあると思ってやっているのであります、どういう形質を狙うのかというのも勿論問題になってきます。もっと質的な形質を使うのであれば、大きな染色体を入れてゆかなければならないでしょう、遺伝子操作をやってたのでは全然気にあわないだろうと思います。これからますます色々なレベルで色々なことをやりながら進めてゆくよりしようがないのではないかでしょうか。

内宮：私も今のお話に同感です。自分でそう思ったことを忠実にやったらほうがいいのでしょうか。ですから一番いいのはあまり変わりに惑わされないでがんばっていくことだと思います。

平井：おそらくこの問題はどんな答えをしても間違いないということになるのだと思いますが、遺伝子操作の場合はある特徴の遺伝子が有るとか、遺伝子の数が少ないものは簡単かもしれませんが、そうではない例えば茎を大きくしたいとか特定の遺伝子だけで解決できないような問題はまだまだ細胞融合の方が有利なものではないかと思います。ただし細胞融合をやっているものとしては、どうも遺伝子操作の方がいいのではないかと考えているのですが。

長田：今のことの繰返しになりますが、要するに両方とも違った性質があるので両方とも必要だということではないでしょうか。

座長：司会がまとめなくてもスピーカーの方々のコメントが出まして、だいたい今回のシンポジウムの結論がでたのではないかなと思います。昨日の中島氏のお話ではありませんが、まだ実験段階のものでして、これを実際にいかに効率的にやっていくかということ、これから我々がしなければならない問題でしょう。その場合にやはり中島氏のご注意をいただいたことなどを守りながら、組織培養による変異をいかに育種に利用していくかということなどについて皆で努力していきたいと思います。