Identification of Ploidy Level of the Regenerated Plants by Anther Culture in Rice

Kazuhiro Nakamura, Hiroaki Suzuki, Kazumi Hattori and Yuzo Futsuhara

Faculty of Agriculture, Nagoya University, Chikusa, Nagoya, 464-01

Summary

In the regenerated plants by the anther culture of rice, panicle length, plant height and glume length were measured. Only the glume length was able to separate regenerated plants into three distinct classes. The cytological analysis of these plants revealed that the three classes based on the glume length consisted of haploids, diploids and triploids, respectively. Similar results were obtained in the regenerated plants derived from irradiated callus. With irradiation as a physiological disorder, the glume length was a stable index of the ploidy level of rice plants.

Key Words : rice, anther culture, ploidy, doubled haploid, nucleotype, glume length.

Introduction

It is a well-known phenomenon that chromosome numbers and ploidy level are highly variable in regenerated plants derived from callus. The resulting plants by anther or pollen culture are not only haploid but also diploid, and some plants become polyploids or aneuploids (Oono 1975, Che *et al.* 1985). This diploid is the doubled-haploid. The first doubled-haploid rice lines were obtained by Niizeki and Oono in 1968. The doubled-haploid is of major interest for the improvement of autogamous species. Since the time of breeding is saved by using doubled haploids for the fixation of pure lines. Therefore, many efforts have been made to increase the efficiency of doubling by colchicine treatments (Tanaka and Nakata 1969, Burk *et al.* 1972) and by reculture (Kasperbauer and Collins 1972, Heszky *et al.* 1989). The identification of ploidy level is essential for the study on the doubling of haploids.

For the identification of the ploidy level, the most accurate way is by counting the chromosome numbers directly by cytological observations. But this method needs skillful cytological microtechniques and entails laborious work. Therefore indirect and simple methods have been developed.

The amount of nuclear DNA was determined by flow microfluorometry (Galibraith *et al.* 1983) and by cytophotometry (McLeish and Sunderland 1961). These methods are rapid, accurate and sensitive but they need expensive apparatus. Hamaoka *et al.* (1991) presumed the ploidy level of plants via anther culture in Chinese

cabbage by the number of chloroplasts in a guard cell of stomata. Ho *et al.* (1990) also used the number of stomatal chloroplasts for the rapid determination of the ploidy level in maize. It is a relatively simple method. However, these methods are not useful in a field assessment where many plants are involved.

In the present study, we tried to establish the simple and easy method for the identification of the ploidy level of rice plants. Some morphological traits of the regenerated plants by the anther culture of rice were measured. Moreover, the same measurements were examined on the regenerated plants derived from irradiated callus.

Materials and Methods

Callus culture and plant regeneration

Rice plants (*Oryza sativa* L. cv 'Nipponbare') were grown in pots (three plants per pot). When the auricle of a flag leaf stood 3-4 cm above the auricle of the leaf beneath it, the stem including a panicle was sampled and stored at 10 °C for 10 days. After this cold treatment, the panicle was removed from the stem, and anthers excised from a spikelet were placed on a medium solidified with agar (0.8 %, w/v) containing N 6 (Chu 1978) nutrients, 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 30 g/l sucrose in 6 cm diameter petri dishes. The induced calli were transferred to regeneration medium in culture bottles to stimulate the formation of shoots and roots. The regeneration medium was the same as that used for callus induction except for the supplement of 0.2 mg/l 1-naphthaleneacetic acid (NAA) and 4 mg/l kinetin as growth hormones. Cultures were carried out under continuous light of about 2,500 lux at 28 °C. After hardening, the plantlets regenerated from callus were transferred to soil in plastic containers and were grown to maturity in a greenhouse.

Irradiation

Irradiations were carried out to calli one day after transfers to regeneration medium. The calli were exposed to 20, 40, 80 Gy of ⁶⁰Co γ-rays at a rate of 2 Gy per min.

Morphological analysis

Panicle length, culm+panicle length and glume length of the main culm of the regenerated plants were measured. Glume length is the average of ten glumes randomly selected. Awns were cut before the measurement.

In this paper, culm+panicle length of the main culm was defined as plant height.

Cytological analysis

Some tillers were excised from the regenerated plants and they were soaked in water at 30°C in the incubator. After 2-3 days, they were rooted. Young root-tips were collected and pretreated with 2 mM 8-hydroxyquinoline for 2 hr at 30 °C, followed by 75 mM KCl for 20 min at room temperature. They were treated with the enzyme solution (6 % Cellulase Onozuka R-10, 4 % Pectolyase Y-23, pH 4.0) for 60 min at 37 °C. After fixation in ethanol/acetic acid solution (3 : 1 v/v) for a few minutes, the slides were prepared as described by Kurata and Omura (1978). They were stained in 10 % Giemsa solution (33 mM phosphate buffer, pH 6.8) for 60 min.

Results

In Fig. 1a, individual histograms for panicle length, plant height, and glume length of 386 plants from unirradiated callus (control) were shown. On the basis of panicle length, two classes of a shorter class and a longer class whose modes were in 8.4-9.0 cm and in 13.2-13.8 cm, respectively were observed. But the separation of the regenerated plants into two separate classes was not clear because of some overlap between the classes. On the basis of plant height, two classes similar to panicle length were observed. Their modes were in 32-34 cm and in 48-50 cm for the shorter and longer classes, respectively. Their separation was not clear too.

On the basis of glume length, three classes (short, medium and long classes) were observed. Short class and medium class seemed to be similar to those of pani-

cle length and plant height, but long class was unique and was divided only by glume length. It was a much smaller class than the other classes. The mode of each class was in the range of 4.8-5.0 mm, 7.2-7.4 mm, and 8.2-8.4 mm. There was no overlap among classes in this character allowing the separation of regenerated plants into three distinct classes.

Twenty-three samples were randomly selected from these three classes (10 from a short class, 10 from a medium class and 3 from a long class) and cytological observations were carried out (Fig. 2). Three types based on glume length, a short class, a medium class and a long class consisted of haploids, diploids and triploids, respectively.

As for the 77 plants from irradiated callus (20 Gy-43 plants, 40 Gy-26 plants, 80 Gy-8 plants), results almost agree with those obtained in control plants from unirradiated callus (Fig. 1b). Compared with control, based on panicle length and plant height the distributions of putative diploids were not so different from control but the distribution of putative haploids was skewed towards the shorter direction. On the basis of glume length, distributions were the same as that of the control except that the class of triploids was not observed. It was only based on glume length that plants were classified into haploids and diploids.

Discussion

Generally, ploidy levels of rice plants are identified by total estimations of morphological features such as fertility, culm length, glume length, awns and so on (Nishi and Mitsuoka 1969). But in the regenerated plants from callus total estimations are affected by somaclonal variations (Larkin and Scowcroft 1981) and epigenetic

Fig. 1. Distribution of glume length, plant height and panicle length in regenerated plants.
(a) of 386 plants derived from unirradiated callus (control)
(b) of 77 plants derived from irradiated callus

Fig. 2. Mitotic chromosome in root tip cells of the regenerated plants from unirradiated callus. (a : haploid, $2n=x=12$; b : diploid, $2n=2x=24$; c : triploid, $2n=3x=36$)

variations (Binns 1981, Meins 1983).

In the regenerated plants by the anther culture of rice, the phenotypes on which somaclonal and epigenetic variations have no much influence and by which ploidy levels are able to be distinctly identified were searched. Fertility is a familiar trait for identification of ploidy level. Haploids, diploids and triploids are generally sterile, fertile and semisterile, respectively. In this study, fertility was not examined. Almost all the regenerated plants showed sterility because of epigenetic variations via culture or low temperature in winter even in a greenhouse (data not shown). Therefore the identification of ploidy level by fertility was impossible.

Panicle length, plant height and glume length were measured (Fig. 1a). It was possible to identify the ploidy level of plants (haploid, diploid and triploid) only by the glume length. The separating point of haploids from diploids was 5.6 mm and diploids from triploid was 7.8 mm. Of 386 control plants, haploids, diploids and triploids were 194 (50.3 %), 183 (47.4 %) and 9 (2.3 %), respectively. This result was supported by the result of Oono (1975). He obtained 39.3 % of haploid, 48.5 % of diploid, 7.6% of triploid and 1.5 % of tetraploid.

With irradiation treatment, distributions of panicle length and of plant height were distorted (Fig. 1b). In particular, the distributions of putative haploids were more skewed than diploids. This different response is in accordance with many reports that haploids were more radiosensitive than diploids (Galun and Raveh 1975, Krumbiegel 1979, Nielsen *et al.* 1985). Glume length distribution was not affected by exposing the callus to irradiation. It was apparent that glume length was not easily influenced by epigenetic variations as physiological disorder but by ploidy level or 'nucleotype'. The term 'nucleotype' was suggested (Bennett 1971) to describe those conditions of the nucleus that affect the phenotype independently of the informational content (the genotype) of the DNA. Recently, mutagen treatments to culture cells (Weber and Lark 1980, Ukai 1988, Wang *et al.* 1988) and selections of culture cells under stress (Vajrabhaya *et al.* 1989, Wenzel and Foroughi-wehr 1990) have been done. So the stability of glume length as an index of ploidy level is very important.

Triploids were a very small class in this study. If tri-

ploids have been a large class as diploids, distributions of triploids and diploids may overlap.

Literature Cited

- Bennett, M. D. (1971) The duration of meiosis. *Proc. Roy. Soc. Lond. B.* 178 : 277-299.
- Binns, A. N. (1981) Developmental variation in plant tissue culture. *Env. Exp. Bot.* 25 : 325-332.
- Burk, L. B., G. R. Gwynn, and J.F. Chaplin (1972) Diploidized haploids from aseptically cultured anthers of *Nicotiana tabacum*. A colchicine method applicable to plant breeding. *J. Hered.* 63 : 355-360.
- Chu, C. C. (1978) The N6 medium and its applications to anther culture of cereal crops. In *Proc. Symp. Plant Tissue Culture*. Science Press, Beijing, 43-50.
- Chu, Q., Z. Zhang and Y. Gao (1985) Cytogenetical analysis on aneuploids from protoclonal lines of rice (*Oryza sativa* L.). *Theor. Appl. Genet.* 71 : 506-512.
- Galbraith, D.W., K. R. Harkins, J. M. Maddox, N. M. Ayres, D.P. Sharma and E. Firoozabady (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science* 220 : 1049-1051.
- Galun, E. and D. Raveh (1975) *In vitro* culture of tobacco protoplast : survival of haploid and diploid protoplast exposed to X-ray radiation after different times after isolation. *Rad. Bot.* 15 : 79-82.
- Hamaoka, Y., Y. Fujita and S. Iwai (1991) Number of chloroplasts in haploids and diploids produced via anther culture in *Brassica campestris*. *Plant Tissue Culture Letters* 8 : 67-72.
- Heszky, L.E., L. S. Nam, I. Simon-kiss and K. Lokos (1989) Organspecific and ploidy-dependent somaclonal variation ; a new tool in breeding. *Acta Biologica Hungarica.* 40 : 381-394.
- Ho, I., Y. Wan, J. M. Widholm and A. L. Rayburn (1990) The use of stomatal chloroplast for the rapid determination of ploidy level in maize. *Plant Breeding* 105 : 203-210.
- Kasperbauer, M. J. and G. B. Collins (1972) Reconstitution of diploids from leaf tissue of anther-derived haploids in tobacco. *Crop Sci.* 12 : 98-101.
- Krumbiegel, G. (1979) Response of haploid and diploid protoplasts from *Datura innoxia* Mill. and *Petunia hybrida* L. to treatment with X-rays and a chemical mutagen. *Env. Exp. Bot.* 19 : 99-103.

- Kurata, N. and Omura, T. (1978) Karyotype analysis in rice I, A new method for identifying all chromosome pairs. *Jpn. J. Genet.* 53 : 251-255.
- Larkin, P. J. and W. R. Scowcroft (1981) Somaclonal variation - a novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet.* 60 : 197-214.
- McLeish, J. and N. Sunderland (1961) Measurement of deoxyribonucleic acid (DNA) in higher plants by Feulgen photometry and chemical methods. *Expl. Cell Res.* 24 : 527-540.
- Meins, F. (1983) Heritable variation in plant cell culture. *Ann. Rev. Plant Physiol.* 34 : 327-346.
- Nielsen, E., E. Selva, C. Sghirinnzetti and M. Devereux (1985) The mutagenic effect of gamma rays on leaf protoplasts of haploid and diploid *Nicotiana plumbaginifolia*, estimated by valine resistance mutation frequencies. *Theor. Appl. Genet.* 70 : 259-264.
- Niizeki, H. and K. Oono (1968) Induction of haploid plant by anther culture in rice. *Nogyogijutsu* 23 : 327-328.
- Nishi, T. and S. Mitsuoka (1969) Occurrence of various ploidy plants from anther and ovary culture of rice plant. *Japan J. Genet.* 44 : 341-346.
- Oono, K. (1975) Production of haploid plants of rice by anther culture and their use for breeding. *Bull. Nntl. Inst. Agric. Sci.* D26 : 139-222.
- Tanaka, M. and K. Nakata (1969) Tobacco plants obtained by anther culture and the experiment to get diploid seeds from haploids. *Jpn. J. Genet.* 44 : 47-54.
- Ukai, Y. (1988) Effect of gamma-ray irradiation on growth of calli derived from mature embryos or seeds in barley. *Japan. J. Breed.* 38 : 292-300.
- Vajrabhaya, M., T. Thanapaisai and T. Vajrabhaya (1989) Development of salt tolerant lines of KDML and LPT rice cultivars through tissue culture. *Plant Cell Reports* 8 : 411-414.
- Wang, A. S., D. S. K. Cheng, J. B. Milcic and T. C. Yang (1988) Effect of X-ray irradiation on maize inbred line B73 tissue cultures and regenerated plants. *Crop Sci.* 28 : 358-362.
- Weber, G. and K. G. Lark (1980) Quantitative measurement of the ability of different mutagens to induce an inherited change in phenotype to allow maltose utilization in suspension culture of the soybean, *Glycine max* (L.) Merr. *Genetics* 96 : 213-222.
- Wenzel, G. and B. Foroughi-wehr (1990) Progeny tests of barley, wheat, and potato regenerated from cell cultures after *in vitro* selection for disease resistance. *Theor. Appl. Genet.* 80 : 359-365.