

## Somatic Cell Hybridization in Rice and Birdsfoot Trefoil

Niizeki, M., S. Nakajo, and T. Harada

Laboratory of Plant Breeding, Faculty of Agriculture, University of Hirosaki, Hirosaki, Aomori-ken 036, Japan.

Asymmetric somatic hybrid calli, which have only the nuclei of birdsfoot trefoil (Lotus corniculatus L.), were produced by protoplast fusion between rice (Oryza sativa L.) and birdsfoot trefoil, and analyzed for their mitochondrial DNAs (mtDNAs) and chloroplast DNAs (cpDNAs). In the hybrid calli, novel mtDNA fragments were detected in Southern blots. This result shows that some kind of alteration such as intergenomic and/or intragenomic recombinations of mtDNA occurred in the hybrid calli. On the other hand, the cpDNA fragment patterns of all hybrid callus lines observed by Southern analysis were found to be identical with those of birdsfoot trefoil. Therefore, it is suggested that the cpDNAs of these hybrid calli sorted out unidirectionally. Interestingly, some regenerated plants from the hybrid calli were tolerant of low temperatures and low sunlight intensity.

### Introduction

The hybridization of distantly related species by protoplast fusion has been a practical tool for removing the barriers of incompatibility in sexual crossing of agriculturally important plant species. The cell division of somatic hybrids originating from fused protoplasts has been observed in several combinations of plant species belonging to different families (Gleba and Sytnic 1984). Callus formation has also been reported in somatic hybrids between the species of different families (Kao 1977, Niizeki *et al.* 1985, Sala *et al.* 1985). By irradiation of the protoplasts of one parent, or by spontaneous chromosome elimination of one parent, Smith *et al.* (1989) and Niizeki *et al.* (1989) obtained asymmetric somatic hybrid plants between the species of different families. In such hybrid calli and plants, new and sexually impossible genetic combinations can be constructed in the nucleus, chloroplast and mitochondrial genomes. The hybrid calli and plants may also give new data concerning the mechanisms of various cellular activities and morphologies which are not observed in the sexual hybrid cells.

Recently, several researchers have reported successful chloroplast transformation in higher plants (De Block *et al.* 1985, Cornelissen *et al.* 1987, Haring and De Block 1990, Svab *et al.* 1990, Staub and Maliga 1992). However, there are two difficulties that need to be overcome in order to bring about a stable chloroplast transformation. The difficulties arise from a number of plastid genomes in higher plant cells, and the double-layered envelope of organelle. Therefore, somatic hybridization will play an important role in the improvement of cytoplasmic genomes.

### Materials and Methods

The plants used in this study are rice (Oryza sativa L.), strain A-58 and birdsfoot trefoil (Lotus corniculatus L.), cv. Viking. The calli of rice were induced from the scutellums of seeds on the MS medium (Murashige and Skoog 1962) with 2 mg/l 2,4-dichlorophenoxyacetic acid. The calli of birdsfoot trefoil were induced from the hypocotyls of young seedlings on the MS medium with 4 mg/l 1-naphthaleneacetic acid and 2.5 mg/l kinetin. About 1-2 g of

fresh calli were used for the isolation of protoplasts. An enzyme solution containing 4% Cellulase Onozuka RS and 1% Macerozyme R10 was used for the rice calli and a solution of 4% Cellulase Onozuka RS, 1% Macerozyme R10 and 0.2% Pectolyase Y23 was used for the birdsfoot trefoil calli. The mixed protoplasts of the two species were treated with polyethylene glycol (PEG) by applying a somewhat modified method of Melchers *et al.* (1978). The modified method has been reported by Niizeki *et al.* (1985). Selection systems of hybrid calli will be described in the results and discussion.

MtDNA and a mixture of nuclear DNA and cpDNA were isolated from 2-3 g of calli of somatic hybrids and their parents by a somewhat modified method of Milligan (1989). After the first centrifugation of ground calli with a cold isolation buffer (1.25 M NaCl, 50 mM Tris pH 8.0, 5 mM EDTA, 0.1% BSA, 0.1% 2-mercaptoethanol) mtDNA could be obtained from the pellet, and the mixture of nuclear DNA and cpDNA could be isolated from the aqueous phase. For Southern hybridization, mitochondrial genes and the *Pst*I and *Bam*HI fragments of cpDNA were used as probes. Probe labelling, Southern hybridization and signal detection were performed by using the ECL (enhanced chemiluminescence) gene detection system (Amersham, UK).

### Results and Discussion

When the rice protoplasts were fused with 15 mM IOA-treated protoplasts of birdsfoot trefoil, and were cultured using the agarose-bead method in the modified KM8P medium (Kao and Michayluk 1975) containing benzyladenin instead of zeatin and without coconut water, only hybrid protoplasts initiated cell division and formed colonies. In this case, rice protoplasts could not give rise to cell division in the modified KM8P medium. The hybrid colonies had the flavonoid pigment of rice, strain A-58, or were a brownish yellow in contrast to the green of birdsfoot trefoil colonies. Also, malformed shoots were derived from the early subcultured calli and near-normal shoots of birdsfoot trefoil from advanced subcultured calli. The microscopic observation of colony cells showed that most of the cells have the chromosomes of birdsfoot trefoil and a small number of rice chromosomes. Therefore, the regenerated malformed shoots might presumably be caused by the presence of a few rice chromosomes. After a considerable number of subcultures, reduction of the abnormality might be due to the disappearance of the rice chromosomes retained. In mtDNA of 6 hybrid callus lines digested by 6 restriction enzymes, some novel fragments were detected by the Southern analysis (Table 1). This result shows that some modification occurred in the mtDNA of hybrid callus lines. On the other hand, the Southern blots of cpDNA in the hybrid calli of 12 combinations of 4 restriction enzymes and 3 probes, the banding patterns of hybrid callus lines were the same as those of birdsfoot trefoil. This result shows unidirectionally nonrandom segregation of cpDNA, despite the fact that many researchers have reported that the cpDNA of somatic hybrids are sorted out randomly (Chen *et al.* 1977, Sidrov *et al.* 1981, Bonnett and Glimelius 1983, Ashahi *et al.* 1988). From the study of somatic hybrid between carrot and tobacco, Smith *et al.* (1989) suggested that the irradiation-induced chromosome instability in carrot nuclei resulted in a tobacco nuclear background which may provide a selective advantage to tobacco cpDNA. In the case of somatic hybridization in this study, the chromosomes of birdsfoot trefoil were predominant in the nuclei of hybrid callus cells. In addition, original fusion products were cultured in a modified KM8P medium, which did not allow the division of rice protoplasts. Therefore, such a medium may be a selective disadvantage for rice chloroplasts.

Some regenerated plants from the hybrid calli of rice and birdsfoot

trefoil had deep green leaves and vigorous growth under low sunlight intensity and low temperatures (20°C) in a green house in the winter season in Hirosaki, Japan (Fig. 1). It has been shown that the organization of mtDNA is correlative to the morphological and physiological functions of plants (Bonnett and Glimelius 1990, Newton *et al.* 1990, Honda *et al.* 1991). In the study of hybrid calli of rice and soybean, we (1985, 1986) suggested that nucleocytoplasmic interactions determine the colour of the callus and its susceptibility to streptomycin. Therefore, it is possible to assume that some kinds of alteration in mtDNA organization and/or nucleocytoplasmic interaction have an effect on the traits of callus cells such as the colours of the callus and its tolerance of low temperatures and low sunlight intensity.

Table 1. Southern blot of mtDNAs in 6 hybrid callus lines (A-F) of rice and birdsfoot trefoil

Restriction enzyme	<u>atpA</u>	Probe <u>rrn26</u>	<u>coxI</u>
<u>EcoRI</u>	A	ND	ND
<u>BamHI</u>	ND	ND	ND
<u>HindIII</u>	A	A	ND
<u>PstI</u>	ND	ND	ND
<u>SmaI</u>	A	ND	ND
<u>SalI</u>	A	ND	ND

A: Callus line A shows novel fragments which are different from those of parents. ND: No difference from the fragment pattern of birdsfoot trefoil in all callus lines.



Fig. 1. Shoots under the low temperatures (20°C) and low sunlight intensity in a green house in the winter season. A: Shoots of plants derived from the parental calli of birdsfoot trefoil, leaves of which are etiolated. B: Shoots of hybrid plants, leaves of which show deep green.

## References

1. Asahi, T., T. Kumashiro and T. Kubo 1988. Constitution of mitochondrial and chloroplast genomes in male sterile tobacco obtained by protoplast fusion of Nicotiana tabacum and Nicotiana debneyi. *Plant Cell. Physiol.* 29:43-49.
2. Bonnett, H.T. and K. Glimelius 1983. Somatic hybridization in Nicotiana: Behavior of organelles after fusion of protoplast from male-fertile and male-sterile cultivars. *Theor. Appl. Genet.* 65:213-217.
3. Bonnett, H.T. and K. Glimelius 1990. Cybrids of Nicotiana tabacum and petunia hybrida have an intergeneric mixture of chloroplasts from P. hybrida and mitochondria identical or similar to N. tabacum. *Theor. Appl. Genet.* 79:550-555.
4. Chen, K., S.G. Wildman and H.H. Smith 1977. Chloroplast DNA distribution in parasexual hybrids as shown by polypeptide composition of fraction I protein. *Proc. Natl. Acad. Sci. USA* 74:5109-5112.
5. Cornelissen, M.J., M. De Block, M. Van Montagu, J. Leemanns, P.H. Schreier and J. Schell 1987. Plastid transformation: A progress report. In *Plant DNA Infections Agents*. Hohn T. and J. Schell Eds. Springer-Verlag Berlin Heidelberg New York Tokyo. pp. 311-320.
6. De Block, M., J. Schell and M. Van Montagu 1985. Chloroplast transformation by Agrobacterium tumefaciens. *EMBO J.* 4:1367-1372.
7. Gleba, Y.Y. and K.M. Sytnik 1984. Protoplast fusion and hybridization of distantly related plant species. In *Protoplast Fusion*. Springer-Verlag Berlin Heidelberg New York Tokyo. pp. 115-161.
8. Haring, M.A. and M. De Block 1990. New roads towards chloroplast transformation of higher plants. *Physiol. Plant.* 79:218-220.
9. Honda, H., K. Itoh and A. Hirai 1991. The heterogenous composition of mitochondrial DNA in somatic hybrid calli and the relatively simple composition of such DNA in regenerated leaves. *Jpn. J. Genet.* 66:279-289.
10. Kao, K.N. 1977. Chromosomal behaviour in somatic hybrids of soybean-Nicotiana glauca. *Mol. Gen. Genet.* 150:225-230.
11. Kao, K.N. and M.R. Michayluk 1975. Nutritional requirements for growth of Vicia hajastana cells and protoplasts at a very low population density in liquid media. *Planta* 126:105-110.
12. Melchers, G., M.D. Sacristán and A.A. Holder 1978. Somatic hybrid plants of potato and tomato regenerated from fused protoplasts. *Carlsberg Res. Commun.* 43:203-218.
13. Milligan, B.G. 1989. Purification of chloroplast DNA using hexadecyltrimethylammonium bromide. *Plant Mol. Biol. Report* 7:144-149.
14. Murashige, T. and F. Skoog 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Planta.* 15:473-497.
15. Newton, K.J., C. Knudsen, S. Gabay-Laughnan and J.R. Laughnan 1990. An abnormal growth mutant in maize has a defective mitochondrial cytochrome oxidase gene. *The Plant Cell* 2:107-113.
16. Niizeki, M., M. Tanaka and K. Saito 1986. Response of somatic hybrid callus between rice and soybean to streptomycin. *Jpn. J. Breed.* 36:75-79.
17. Niizeki, M., M. Kihara, K. Cai, R. Ishikawa and K. Saito 1989. Somatic cell hybridization among gramineous and leguminous species. *Proc. of the 6th Internatl. Congr. of SABRAO.* pp.501-504.
18. Niizeki, M., M. Tanaka, S. Akada, A. Hirai and K. Saito 1985. Callus formation of somatic hybridization of rice and soybean and characteristics of the hybrid callus. *Jpn. J. Genet.* 60:81-92.
19. Sala, C., M.G. Biasini, C. Morandi, B. Nielsen, B. Parisi and F. Sala 1985. Selection and nuclear DNA analysis of cell hybrids between Daucus carota and oryza sativa. *J. Plant Physiol.* 118:409-419.

20. Sidrov, V.A., L. Menczel, F. Nagy and P. Maliga 1981. Chloroplast transfer in Nicotiana based on metabolic complementation between irradiated and iodoacetate-treated protoplasts. *Planta* 152:341-345.
21. Smith, M.A., A. Pay and D. Dupid 1989. Analysis of chloroplast and mitochondrial DNA in asymmetric somatic hybrids between tobacco and carrot. *Theor. Appl. Genet.* 77:641-644.
22. Staub, J.M. and P. Maliga 1992. Long regions of homologous DNA are incorporated into the tobacco plastid genome by transformation. *Plant cell* 4:39-45.
23. Svab, Z., P. Hajdukiewicz and P. Maliga 1990. Stable transformation of plastids in higher plants. *Proc. Natl. Acad. Sci. USA* 87:8526-8530.