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Establishment of a Rice Protoplast Culture and Application of an Asymmetric Protoplast Fusion Technique to Hybrid Rice Breeding*

Tatsuhito FUJIMURA, Hiromori AKAGI, Masaaki OKA, Atsushi NAKAMURA and Rinnpei SAWADA

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Introduction

Rice is one of the most important crops in the world, and feeds about 40% of the world population. The yield of rice needs to be increased since a world food crisis has been predicted for early next century. Since rice shows heterosis to give a higher yield, hybrid rice may offer a solution to this predicted food crisis.

For practical large-scale production of hybrid rice (F_1) seeds, female strains must be genetically male sterile. Cytoplasmic male sterile (CMS) is a characteristic which can be used to produce stable male sterile lines. CMS occurs widely in higher plants and is due to incompatibility between nuclear and cytoplasmic gene products, which results in the failure to produce mature pollen grains[1,2]. Since CMS excludes the possibility of self-pollination, it is commercially useful for hybrid seed production[2]. In rice, CMS also occurs when the cytoplasm of a Japonica rice cultivar is replaced with that of an Indica rice cultivar or wild rice[3]. It takes about 6-9 years to convert fertile cultivars into CMS by the recurrent backcrossing method. Therefore, new methods which would enable the conversion of several fertile cultivars into CMS over a short period are desired. A donor-recipient protoplast fusion system has been developed in several dicotyledonous species. This new system should enable the CMS trait encoded in the mitochondrial genome to be transferred into the fertile line in a single step.

In this context, we established a protoplast culture method for rice[4] and then established a method for the practical production of CMS lines by an asymmetrical cell fusion (cybrid) technique[5], which enables the selective introduction of cytoplasm from CMS cells into cells of female candidates. Using 40 new CMS lines constructed using this method, we identified hybrid combinations which showed increased yields (>130%) compared to leading Japanese varieties. Here we describe the breeding of new hybrid rice using this technique.

Rice protoplast culture[4]

We established a reproducible system for the regeneration of rice plants from protoplasts in

藤村達人・赤木宏守・岡　正明・中村　淳・澤田倫平
イネプロトプラスト培養および非対称融合技術の開発，およびそれを利用したハイブリッドライスの育種
Plant Biotechnology Department, Life Science Institute, Mitsui Toatsu Chemicals Inc., Togo 1144, Mobara 297, Japan
三井東圧化学(株)ライフサイエンス研究所(〒297　千葉県茂原市東郷1144)

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1985[4]. Until then, protoplast culture of rice was possible only in special cell lines that readily released protoplasts. However, these had lost the capacity to regenerate rice plants.

We freshly isolated cell lines with high regeneration capacity from which protoplasts were readily isolated. The protoplasts isolated from these cell lines divided at a high frequency and formed many small cell clusters. Many plants regenerated from these calli. We optimized the conditions for protoplast isolation, protoplast culture and regeneration of rice plants using these cell lines.

Protoplasts were isolated from peripheral layers of cell clusters in suspension culture with gentle or no agitation in a solution of enzymes containing Macerozyme-R 10 (1%) and Cellulase-RS (4%). The protoplasts were very small (about 10 μm in diameter) and rich in cytoplasm, and had high regeneration capacity. They were purified and cultured in a shallow (less than 200 μm) layer of liquid medium in a plastic petri dish without any agitation.

The regenerated cells divided further and formed cell clusters (calli) from which plants regenerated at a very high frequency (>50%) in hormone-free medium. Since about 1% of the isolated protoplasts eventually regenerated to produce plants, this technique is readily applicable to transformation[6] and cell fusion[5], and has been used by other groups working on rice biotechnology.

Construction of rice cybrid plants[5]

An asymmetric protoplast fusion technique was developed to transfer cytoplasmic traits of CMS lines to fertile cultivars. The CMS strain MTC-9A was used as a cytoplasmic donor. Its cytoplasm had been derived from an indica rice cultivar, Chinsurah Boro II. On the other hand, the mutant strain used as a recipient originated from the cultivar Norin 8[7], and lacked the gene encoding aryl acylamidase I, which hydrolyzes the herbicide propanil. Aryl acylamidase I was used as a recessive genetic marker of recipient nuclei.

X-ray irradiation of protoplasts can cause the practical elimination of chromosomes or nuclei in somatic hybrid plants. We investigated the effects of various dosages of X-rays on CMS protoplasts. The number of colonies regenerated decreased with an increase in the X-ray dosage. At X-ray doses greater than 120 krad (2 krad/min.), no colony formation was observed, although most protoplasts were still alive based on a microscopic inspection, and limited cell division was apparent. Thus, protoplasts were routinely irradiated at 125 krad to ensure the complete inhibition of colony formation.

To obtain only somatic hybrids, recipient protoplasts were treated with iodoacetamide (IOA) for inactivation. At 15 mM, IOA completely inhibited cell division. In further control experiments, donor and recipient protoplasts were treated with X-rays and IOA, respectively, and then fused separately and cultured, or mixed and cultured without fusion. In contrast to the experiment described above, many colonies were regenerated when IOA was used at concentrations lower than 25 mM. Recipient protoplasts, therefore, were treated with 30 mM IOA to reduce the possibility of colony formation by non-hybrid cells.

Colonies regenerated only after irradiated (125 krad) donor protoplasts were fused with IOA-treated (30 mM) recipient protoplasts, while no colonies were formed without cell fusion. Metabolic complementation between nuclear and cytoplasmic compartments restores the capacity for cell division in fused protoplasts. About 3 weeks after the transfer of hybrid calli to plant-regeneration medium, plantlets were regenerated from these calli.

Cybrid plants must be subjected to asymmetric protoplast fusion to develop hybrid rice in which only the cytoplasmic traits of the donor are transferred to the recipient. The origins of the mitochondrial genomes of these regenerated plants were determined from the restriction patterns

of their mitochondrial DNAs. All of the plants had fragments specific to the donor in addition to fragments specific to the recipient. Thus, these plants were cytoplasmic hybrids (cybrids) of MTC-5A and the mutant of Norin 8.

To determine the source of the nuclei of these cybrid plants, aryl acylamidase I was assayed. All of the cybrid plants analyzed lacked aryl acylamidase I activity, as did the recipient. Since these cybrid plants had 24 or 48 chromosomes, the nuclei or chromosomes of the donor may have been destroyed by X-rays. Thus, the plants we produced by asymmetric protoplast fusion were cybrids which derived their nuclei solely from the recipient and their cytoplasm from both the donor and recipient. Therefore, we can conclude that only cytoplasmic traits of CMS rice were transferred to fertile cultivars by donor-recipient protoplast fusion using X-rays and IOA.

Conversion of fertile cultivars to cytoplasmic male sterility by asymmetric protoplast fusion[8)]

Since the cytoplasmic traits of CMS rice had been introduced into the cybrid plants, we expected them to be CMS. To test this notion, we evaluated sterility and the restoration of fertility in rice cybrids and their progenies. We used two CMS lines as cytoplasmic donors and the fertile cultivar Sasanishiki as a recipient, since a CMS line of Sasanishiki which had been bred by the recurrent backcrossing method was available.

Cybrid plants were created by donor-recipient protoplast fusion of X-ray-irradiated CMS parents with IOA-treated Sasanishiki. Examination of the morphological features, the chromosome number and the mitochondrial genomes of the cybrid plants showed that they had inherited only the mitochondrial genome of the donor. Thus, this technique for transferring CMS traits to fertile rice is reproducible.

Among 142 regenerated cybrid plants, we selected 72 plants that had a normal morphology, and studied their seed fertility. More than 80% of these cybrid plants were not self-seeding. The remaining 20% fertile cybrid plants might be the result of the elimination of CMS traits by segregation after the inter-parental recombination of mitochondrial genomes[9,10)]. All of the cybrid plants set many seeds after they were pollinated with normal pollen of Sasanishiki, indicating that they were fertile female. The progenies of sterile cybrid plants backcrossed with Sasanishiki were not self-seeding. This demonstrated that the cytoplasmic traits caused male sterility of cybrid plants. Therefore, the cybrid plants were CMS.

The CMS introduced here is a gametophytic system, which can be restored by the single dominant gene Rf-1[11)]. To determine whether the CMS of cybrid plants was caused by the cytoplasmic traits from CMS lines, we examined the restoration of fertility in CMS cybrids by the Rf-1 gene. Fifteen BC 1 plants of 8 lines of sterile cybrids were crossed with MTC-10 R, which has the single dominant gene Rf-1 for fertility restoration. A high proportion of fully fertile plants was observed in all of the F_1 progenies. All except one progeny had panicles which set seeds at a frequency of 75-95%. The range of seed fertility of the F_1 progenies was similar to that of 'Sasanishiki A', which had been bred by the recurrent backcrossing method. Thus, cytoplasmic traits introduced from both MTC-5A and MTC-9A resulted in CMS.

The CMS traits of the cybrid plants were stable for at least 7 generations of backcrossing with Sasanishiki. Using the donor-recipient protoplast fusion method, it takes about 8 months (from callus induction to seed set by crossing with recipient cultivars) to produce new CMS lines, while the conventional recurrent backcrossing method takes about 6 years. We concluded that the cybrids produced by donor-recipient protoplast fusion can be useful for creating new CMS rice cultivars for

hybrid rice production.

Breeding of new CMS lines[12,13]

To convert fertile elite cultivars to CMS, the CMS traits of Chinsurah Boro II were transferred to 40 Japanese cultivars by asymmetric protoplast fusion. Most of the diploid cybrid plants were sterile and were not self-seeding, except cybrids that had a nucleus from Hoshiyutaka. The restorer genes for the Chinsurah Boro II cytoplasm are widely distributed in the tropics, where indica varieties are grown[14]. Since Hoshiyutaka was bred by crossing japonica and indica rice, it may also contain this restoration gene. The remaining cultivars were believed to have no such restorer genes.

A PCR-based method of for specifically detecting a characteristic region downstream from atp 6 in the mitochondrial genome of Chinsurah Boro II[9,15], orf 79, which is closely related to CMS traits, was used to examine the cybrids. All of these cybrids were shown to have this gene, which suggests that the CMS trait was successfully transferred to these cybrids. We concluded that our method can be applied to most of the cultivars in Japan.

A novel CMS line (Bio-Mother I) was bred using this method from the cultivar 'Yukigesho' from 1991 to 1993[13]. We have already converted 40 Japanese cultivars as candidates for the female parent of hybrid rice. This large number of new CMS lines may be very useful germ stock for hybrid rice breeding.

Application of new CMS lines to hybrid rice breeding[16]

To be accepted in Japan, a novel rice cultivar must 1) give a high yield, 2) be visually appealing, and 3) taste good. We made 800 hybrid combinations, which were then cultured in Chiba and Ibaraki prefectures in Japan, and examined these three features. Tentatively, 10 combinations were selected. These were then cultured at several experimental stations in Japan, and eventually two combinations were selected. These showed excellent yields that were 30 to 40% higher than those of the leading representative varieties in each region. In addition, they were of good enough quality for acceptance by Japanese consumers.

Conclusion

We have succeeded in breeding new hybrid rice varieties that are acceptable in a competitive Japanese market. We have been able to develop such excellent varieties through the use of techniques we have developed, which provide a large number of candidate combinations of new hybrid rice. Our methods of protoplast culture and cybrid construction are reproducible and applicable to the rapid and large-scale production of new CMS lines.

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References

1) Levings III, C. S., G. G. Brown, 1989. Cell, **56**: 171-179.

2) Newton, K. J., 1988. Ann. Rev. Plant Physiol. Plant Mol. Biol., **39**: 503-532.
3) Virmani, S. S., C. Shinjyo, 1988. Rice Genetics Newsletter, **5**: 9-15.
4) Fujimura, T., M. Sakurai, H. Akagi, T. Negishi, A. Hirose, 1985. Plant Tissue Culture Lett., **2**: 74-75.
5) Akagi, H., M. Sakamoto, T. Negishi, T. Fujimura, 1989. Mol. Gen. Genet., **215**: 501-506.
6) Tada, Y., M. Sakamoto, T. Fujimura, 1990. Theor. Appl. Genet., **80**: 475-480.
7) Matsunaka, S., 1974. In: "Plant Growth Substance 1973", p. 1182-1186, Hirokawa Publishing Co, Tokyo.
8) Akagi, H., T. Taguchi, T. Fujimura, 1995. Theor. Appl. Genet., **91**: 563-567.
9) Akagi, H., M. Sakamoto, C. Shinjyo, H. Shimada, T. Fujimura, 1994. Curr. Genet., **25**: 52-58.
10) Akagi, H., H. Shimada, T. Fujimura, 1995. Curr. Genet., **29**: 58-65.
11) Shinjyo, C., 1975. Sci. Bull. Coll. Agric. Univ. Ryukyus, **22**: 1-51.
12) Akagi, H., A. Nakamura, R. Sawada, M. Oka, T. Fujimura, 1995. Theor. Appl. Genet., **80**: 948-951.
13) Nakamura, A., H. Akagi, M. Oka, N. Arai, R. Sawada, T. Sano, K. Matsumura, S. Samoto, T. Fujimura, T. Tsuchiya, 1995. Breeding Sci., **44**(suppl. 1): 212.
14) Shinjyo, C., 1972. Jpn. J. Breed., **22**: 329-333.
15) Iwabuchi, M., J. Kyouzuka, K. Shimamoto, 1993. EMBO J., **12**: 1437-1446.
16) Oka, M., A. Nakamura, T. Sano, K. Matsumura, R. Sawada, T. Kamiguchi, S. Samoto, T. Tsuchiya, T. Fujimura, 1996. Proceedings of the 2 nd Asian Crop Science Conference, p. 722.