Cell Division of Rice and Soybean and Their Fused Protoplasts

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Protoplasts from callus tissues of rice, *Oryza sativa* L., and soybean, *Glycine max* (L.) Merrill., were cultured in liquid media. Cell division occurred in protoplasts of both plants and resulted in the formation of calluses or cell colonies after one or two months. Protoplasts of both plants were fused by using polyethyleneglycol (PEG)-high pH-high Ca^{++} technique. The percentages of hybrid protoplasts obtained by fusion of rice and soybean were approximately 1~3 percent. The hybrid protoplasts gave rise to cell clusters after two weeks of successive culture. The cell clusters of hybrid cell lines could be distinguished by features of nucleus and cytoplasm from those originated from non-hybrid cells of rice and soybean.

Introduction

The recently developed technique of protoplast culture has led to successful cell division and regeneration of whole plants in many dicotyledonous plants. Soybean is one of the instances of successful protoplast culture (KAO *et al.* 1970). *In vitro* culture technique of cereal plants is, however, still in a primitive state. Many attempts have been made to culture cereal protoplasts, but success has been reported so far only in rice (DEKA and SEN 1976), corn (POTRYKUS *et al.* 1977, 1979) and pearl millet (VASIL and VASIL 1979, 1980). We report here a successful protoplast cultures of soybean, rice and their fused protoplasts.

Materials and Methods

Seeds of 20 varieties and 4 strains of rice were germinated aseptically on the medium of MURASHIGE and SKOOG (1962) (MS medium) containing 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and subsequently obtained calluses from parts of hypocotyls after one month in the dark at 25°C. These calluses were divided and sub-cultured every 4 weeks. Soybean (var. Harosoy) seeds also were germinated on the medium of NITSCH and NITSCH (1969) with no growth regulator, and the hypocotyls from 7-day-old seedlings were transferred onto the same medium as used in rice callus induction. This callus was sub-cultured every three weeks. 1~2 g of fresh calluses were used for the isolation of protoplasts. 20 ml of enzyme solution containing 0.4% macerozyme R-10, 1% driselase, 2% cellulase "Onozuka" R-10, 0.7 M mannitol (pH 5.8) was used for rice calluses and the solution of 0.4% macerozyme R-10, 4% cellulase "Onozuka" R-10, 0.7 M mannitol (pH 5.8) was used for soybean calluses. The enzyme solutions containing calluses were incubated in the dark at 28°C for 14~17 hr. in a shaker bath. The protoplasts were separated from indigested cellular material by 4 sheets of gauze, followed by centrifugation at 80 xg for 4 min. at each time. The rice protoplasts were cultured in MS medium containing 29 mM sucrose, 0.7 M mannitol, 4 mg/l 2,4-D and 2 mg/l

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Received December 10, 1980

benzyladenine (AB). After one month, they were diluted by MS medium containing 0.4 M mannitol. The obtained cell colonies were then transferred to MS solid medium with 8 g/l agar and 2 mg/l 2,4-D. The protoplasts of soybean were cultured in modified 8p medium (KAO and MICHAYLUK 1975) containing BA instead of zeatin and eliminating coconut water. After two weeks of culture, they were diluted by fresh medium. The formed cell colonies were transferred to solid medium of the modified 8p.

The fusion of rice and soybean protoplasts was performed by a somewhat modified method of MELCHERS *et al.* (1978). A solution containing 5 g polyethyleneglycol 1540 (PEG)/10 ml distilled water, 10 mM CaCl_2 , 0.7 mM KH_2PO_4 and 0.1 M glucose was added to the mixed protoplasts of rice and soybean and incubated for 20 min. at 25°C. The incubated mixture was diluted with 0.08 M CaCl_2 (pH 10). After 20 min. the protoplasts were washed 4 times with a solution containing 0.6 M mannitol and 0.05 M CaCl_2 . They were then cultured in the liquid medium of modified 8p described on the above. Falcon plastic dishes (35×10 mm) were used for all protoplast cultures. The dishes were sealed with parafilm® and placed in a plastic moisture box at 25°C in the dark for 2~3 days. The protoplast population density ranged from 10^4 to 10^5 per ml. The cells regenerated from the protoplasts were gradually exposed to stronger light (up to 2000 Lux) after they divided a few times.

Results

Isolation and culture of rice protoplasts

20 varieties and 4 strains were used in an attempt to isolate protoplasts (Table 1). Protoplast release reflected clearly differences of genotypes (varieties or strains) of callus tissues used. Percentages of their isolated protoplasts in most of varieties and strains were less than twenty. Only 2 strains, A-58 cytoplasmic male sterile line and A-58 normal line showed satisfactory results of about 94 percent of protoplast production.

Table 1. Isolation of protoplasts from rice callus

Variety or strain	Protoplast isolation (%) [*]	Variety or strain	Protoplast isolation (%) [*]
1. Hōryu	5.30	13. Hokusetsu	14.29
2. Fukuyuki	6.41	14. Sasahonami	2.54
3. Yūkara	8.05	15. Sachiho	2.03
4. Sorachi	4.32	16. Yūnami	14.71
5. Tominishiki	33.09	17. Shiokari	13.85
6. Tomoemasari	6.31	18. Nōrin 20	3.70
7. Teine	56.41	19. Yukimochi	3.10
8. Iwakogane	14.45	20. Sōhōmochi	8.18
9. Shinsetsu	13.29	21. A-58 MS**	94.00
10. Eikō	7.94	22. A-58 Nor. ***	93.82
11. Himehonami	16.58	23. H-126 MS**	4.75
12. Toyohikari	6.82	24. H-126 Nor. ***	6.25

^{*}, $\frac{\text{Number of protoplasts}}{\text{Number of protoplasts} + \text{number of indigested callus cells}} \times 100$;

^{**}, Cytoplasmic male sterile line. ; ^{***}, Normal line.

Fig.1. Isolation and culture of protoplasts of rice and soybean.

a, Isolated protoplasts of rice callus cells. $\times$ ca. 480. b, Initiation of cell division of rice protoplast. $\times$ ca. 480. c, Formation of cell cluster of rice. $\times$ ca. 530. d, Formation of cell colony of rice. $\times$ ca. 480. e, Isolated protoplasts of soybean callus cells. $\times$ ca. 480. f, Initiation of cell division of soybean protoplasts. $\times$ ca. 500. g, Formation of cell clusters of soybean. $\times$ ca. 500. h, Formation of cell colonies of soybean. $\times$ ca. 110.

The isolated protoplasts of these two strains have opaque dense cytoplasm and the nuclei in the protoplasts could not be observed (Fig.1-a).

A newly formed cell wall sometimes could be observed within 24 hr. after isolation of protoplasts, by plasmolyzing the protoplasm. After a few days the cultured protoplasts in a liquid medium became vacuolated cells and displayed prominent nuclei. Cell division occurred in the vacuolated cells, followed by formation of 5 to 10 cell clusters within 2 weeks (Fig.1-b and c). At one month after dilution of MS medium containing 0.4 M

mannitol, the cell clusters grew up to 1 to 2 mm of visible cell colonies (Fig.1-d). They were transplanted onto solid medium with agar and subsequently formed calluses of 4 to 5 mm diameter after one month.

Isolation and culture of soybean protoplasts

The isolated protoplasts showed large vacuolated transparent features, but the nuclei in the protoplasts could not be recognized (Fig.1-e). Protoplasts changed their shape gradually from round to oval in a liquid medium and 31.68 percent of the protoplasts divided into 2 or 3 cells within 4 days (Fig.1-f). After a week some divided cells developed into small spherical cell clusters (Fig.1-g). The nuclei in the cell clusters were still not visible at this stage. These cell clusters developed into cell colonies in successive liquid cultures (Fig.1-h).

Fusion of rice and soybean protoplasts and culture of fusion products

The protoplasts isolated from rice callus tissues had dense cytoplasm, while those of soybean had vacuolated transparent cytoplasm. The differences in the appearance of the fused protoplasts depending on the feature of the parental cytoplasm were suitable for the recognition of fusion bodies (Fig.2-a and b). The percentages of hybrid protoplasts obtained by fusion of rice and soybean ranged from 1 to 3 percent. The frequency of fusion varied from one experiment to another. Success in protoplast fusion was largely determined by the viability of the protoplasts as provided by the isolation procedure

Fig. 2. Fusion of rice and soybean protoplasts and culture of fusion products. a, and b, Protoplast fusion of rice and soybean. Upside and underside are soybean and rice, respectively. $\times$ ca. 480. c, and d, Cytoplasmic mixing in hybrid protoplasts of rice and soybean. $\times$ ca. 610. e, and f, Cell clusters originated from hybrid protoplasts of rice and soybean. $\times$ ca. 470.

employed. The heterocyttoplasmic protoplasts shifted to cytoplasmic mixing within 3 hr. after fusion treatment (Fig.2-c and d). Various shapes from round to oval or oblong were observed in fused protoplasts between rice and soybean. Three types of cell clusters were observed after two weeks of liquid culture. The protoplasts of rice or rice+rice formed flat shaped cell clusters with transparent cytoplasm and prominent spherical nuclei

as indicated Fig.1-c. The protoplasts of soybean or soybean+soybean formed global transparent bodies in which the nuclei were not visible as shown in Fig.1-g. The hybrid protoplasts of rice and soybean formed global cell clusters which consisted of larger cells than those of non-hybrid protoplast origins. They had a conspicuous cytoplasm containing many starch grains and sometimes large irregular shape of nuclei (Fig.2-e and f). Experiments are now underway to study the precise behaviour of nuclei in the fused cells and also to isolate the cell clusters originating from the hybrid protoplasts of rice and soybean.

Discussion

During the last several years, many successful induction of protoplast fusion and occurrences of cell division in a number of species in dicotyledonous plants have been reported by KAO *et al.* 1974, KARTHA *et al.* 1974, MELCHERS and LABIB 1974, CONSTABEL *et al.* 1976, KAO 1977, SCHIEDER 1977, 1978, BINDING and NEHLS 1978, GLEBA and HOFFMANN 1978, MELCHERS *et al.* 1978, MENCZEL *et al.* 1978, NAGAO 1978, NEHLS 1978 and, KRUMBIEGEL and SCHIEDER 1979. On the other hand, successful fusion and cell division in combination with di- and monocotyledonous plants have been restricted in several cases (KAO *et al.* 1974). Protoplasts from soybean cultured cells which were used by KAO *et al.* (1974) appeared to provide particularly viable fusion partners for cereal plants such as barley and corn. The hybrid protoplasts of a combination of rice and soybean reported here extend the list of fusion products of di- and monocotyledonous plants with a capacity of cell division.

The problem in this study is how to select fusion products from parent protoplasts. The protoplasts from rice and soybean callus tissues were quite different in general appearance of cytoplasm so that fusion hybrids were distinctive. Therefore, it may be possible to use an inverted microscope and remove them for further culture, but since hybrid protoplasts have no cell walls there is a risk of injuring the protoplasts. Fortunately, at the early stage of cell clusters in liquid medium the hybrid products were distinguished from the non-hybrid products. It may be, therefore, possible to select only hybrid cell clusters by using a micropipette, as has been shown by GLEBA and HOFFMANN (1978), which give no damage to the cells. 8p medium exploited by KAO and MICHAYLUK (1975) was reported to make possible the culture of low density of protoplasts such as 5~25 cells per mL. Using a similar complex medium, the separated hybrid cell clusters may be successfully cultured into further growing cell colonies.

Acknowledgments

The authors wish to express their sincere appreciation to Dr. M. TAKAHASHI and Dr. T. KINOSHITA, Plant Breeding Institute, Faculty of Agriculture, Hokkaido University, for their valuable guidance and kindly providing the seed of some rice strains.

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イネとダイズおよびその融合プロトプラストの細胞分裂

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イネとダイズのカルス細胞からのプロトプラストの分離また液体培地での培養を試みた。イネ系統A-58の細胞質雄性不稔型および正常型、およびダイズ品種ハロソイがプロトプラストの分離および培養に適していることが判明した。両植物のプロトプラストは液体培地中で細胞分裂を開始し、1~2ヶ月後にコロニーを形成した。イネのコロニーは寒天培地へ移植することによりカルスを形成した。

両植物のプロトプラストの融合はポリエチレングリコールと high pH-high Ca^{++} 法を組合せることによって行った。雑種プロトプラストは両植物の細胞質の性状の違いにより判別でき、その出現頻度は 1~3% であった。融合処理後 3 時間で細胞質は混和し、2 週間後には細胞塊を形成した。雑種細胞は多くの澱粉粒や不整形の核を有し、両親単独由来の細胞塊とは明らかに区別することが出来た。