RESEARCH COMMUNICATIONS


Received 28 July 1994; revised accepted 16 January 1995

Fertile plants regenerated from mesophyll protoplasts of cold-tolerant rice

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A protocol for regeneration of fertile plants from leaf-sheath-derived protoplast of Oryza sativa (L.), which is a pre-requisite for utilization of genetic manipulations at the cellular level without or with very little creation of genetic variation, is reported. Isolated protoplasts from inner leaf sheath of 7-day-old seedlings were cultured in modified N6 medium in the presence of feeder cells. The presence of feeder cells and reduction of osmoticum played an important role in obtaining the sustained divisions of mesophyll protoplasts. Among the three procedures, i.e. protoplast cultured in liquid medium, in 0.15% agarose and on top of a cellulose nitrate membrane in the presence of feeders, the latter procedure exhibited the highest number of protoplast-derived calli (protocalli). The protocalli obtained from 0.15% agarose-cultured protoplasts (without membrane) produced a larger number of plantlets.

REPRODUCIBLE plant regeneration from protoplasts is an essential pre-requisite for genetic manipulations such as somatic hybridization, cytoplasmic recombination and direct uptake of DNA. Plant regeneration from protoplasts has been achieved through callus suspensions in most of the grameneae, i.e. rice1,5, wheat6, sugarcane7, pearl millet8, maize9, barley10 and barley11. It is generally observed that the establishment of cell culture in cereals is not only difficult, but they also tend to become less regenerable and accumulate genetic changes12,13 on prolonged culture. Due to the above reasons, extensive efforts have been made14 since 1974 to induce sustained divisions and regeneration of plants from mesophyll protoplasts in cereals. Recently, Gupta and Pattanayak15 have reported the regeneration of plants from mesophyll protoplasts of rice. The reported protocol of Gupta and Pattanayak15 is not repeatable4,16. In this paper, we report a simple and reproducible procedure for induction of...
Figure 1. Fertile plants regenerated from mesophyll protoplasts of cold-tolerant rice: a, seedling of rice; b, purified protoplasts; c, dividing protoplasts (arrow indicating septa formation); d, four-cell stage; e and f, microcalli derived from mesophyll protoplasts; g, protocalli obtained on membrane; h, regenerated plantlets; i, plant in pot; j, mature panicle obtained from mesophyll protoplasts of RCPL-1C.
sustained divisions, callus formation and regeneration of fertile plants from mesophyll protoplasts of rice.

*Oryza sativa* (L.) cvs. RCPL1-1C and Meghalaya-1, having red seed colour and commercially cultivated in high-altitude areas (1000 msl) of the northeastern hills of India, were used for protoplast culture. IR-65, a commercially cultivated variety released by the International Rice Research Institute, Manila, Philippines, was used for feeder cells. Dehusked, undamaged healthy seeds of RCPL1-1C and Meghalaya-1 (specific gravity 1.2) were surface-sterilized in 70% ethanol for 1 min and in 0.1% mercuric chloride for 5 min, followed by three washings in sterilized distilled water. Then they were cultured in MS\textsuperscript{17} half-strength semisolid medium under 16/8 h day/night photoperiod (3000 Lux intensity) at 26 ± 1°C for 7 days. On the 8th day, 100 seedlings were selected for protoplast isolation. The basal whist portion attached to the roots and the dark green portion (2–3 mm) at the base of the first leaf were discarded. The green inner sheath between these two portions (Figure 1 a) was cut into fine pieces and incubated for 1 h pre-plasmolysis in CPW–20M medium (CPW salts\textsuperscript{18} with 20% mannitol). The CPW–20M was replaced by 15 ml of an enzyme solution containing 0.25% cellulase Onozuka-RS (Yakult Honsha Co., Tokyo, Japan), 0.05% pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan) and 5 mM MES buffer, dissolved in CPW–15M medium. The mixture was incubated in a 90 mm Petri dish in the dark at 50 rpm on a gyratory shaker for 3 h, followed by 1 h stationary period at 26 ± 1°C. The released protoplasts were filtered through nylon sieves of 64, 45 and 30 μm and washed twice in CPW–15M by pelleting at 1000 rpm. Washed protoplasts were purified by floating over 5 ml of 0.8 M sucrose at 500 rpm for 5 min and again washed twice in CPW–15M medium. The viability of protoplasts was determined by Fluorescein diacetate (FDA) staining\textsuperscript{20} and cell contamination was checked by staining with Calcofluor white\textsuperscript{20}. One ml of protoplasts was cultured at a density of 1 × 10\textsuperscript{6} protoplasts/ml in N6Z (N6\textsuperscript{16} supplemented with 1.5 mg l\textsuperscript{-1} 2,4-D, 0.2 mg l\textsuperscript{-1} zeatin, 500 mg l\textsuperscript{-1} casein acid hydrolysate and 0.8 M glucose) (without sucrose at pH 5.8), in N6Z with 0.15% sea plaque agarose (LGT, FMC, Rockland, USA) in the well formed by feeder cells, and in N6Z with 0.15% sea plaque agarose on top of a 47 mm Whatman cellulose nitrate filter membrane (Whatman Paper Ltd., Kent, UK) of 0.8 μm pore size. The cultures were sealed by paraffilm and kept in the dark at 26 ± 1°C. The feeders were prepared from 3–4-month-old fast-growing fine cell suspension of immature embryo-derived calli of rice cv. IR-65. 1.5 ml of the settled volume of cell suspension was vigorously mixed-up with 10 ml of N6Z medium supplemented with 0.5 M glucose and 0.6% agarose (low melting, Sisco Research Pvt. Ltd., Bombay, India). From this mixture, 1.5 ml was poured in the inner periphery of a 35 mm Petri dish in the form of a ring for protoplast culture in the well, and 5.0 ml of this mixture (0.6% agarose was replaced by 0.4% agarose) was spread in a 55 mm Petri dish for protoplast culture on top of the cellulose nitrate membrane. The first reduction of osmoticum was made on day 8 by 0.5 ml of N6Z medium supplemented with 0.4 M glucose, followed by addition of N6Z medium supplemented with 0.2 M glucose every week for resuming the sustained divisions and vigorous growth of protoplasts. After the 20th day, the cultured protoplasts were transferred from the well of the 35 mm Petri dish to a new 60 mm Petri dish with the help of a wide-mouth pasture pipette containing 2 ml of N6Z medium supplemented with 0.2 M glucose and the membrane was transferred from feeder cells to a Whatman pre-filter pad moistened by 3.0 ml of N6Z medium supplemented with 0.2 M glucose. On the 30th day, the protoplast-derived calli were transferred onto the callus proliferation medium (N6Z supplemented with 2.0 mg l\textsuperscript{-1} 2,4-D, 30 g l\textsuperscript{-1} sucrose (instead of glucose) and 0.8% sea plaque agarose). After two weeks, the embryoid-like structures containing calli were transferred on to MS\textsuperscript{17} medium (MS salts with 3.0 mg l\textsuperscript{-1} kinetin, 0.5 mg l\textsuperscript{-1} NAA and 0.7% agarose). The cultures were kept initially in the dark for 7 days and then transferred to a 16/8 h light/dark regime using an assortment of fluorescent light and 60 W incandescent lamp (3000 Lux) at 26 ± 1°C. At the 2–3-leaf stage, the developing plantlets were transferred to 1/2 MS semisolid medium for root development. Later on, the regenerants were hardened into 1/2 MS liquid medium (without sucrose) and then transferred in pots containing 1:1 mixture of sterilized soil and

Table 1. Effect of culture procedures on the number of visible colonies and plant regeneration

<table>
<thead>
<tr>
<th>Protoplast culture</th>
<th>On membrane with 0.15% agarose</th>
<th>In 0.15% agarose</th>
<th>In liquid medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>No. of visible colonies</td>
<td>Frequency of plant regeneration</td>
<td>Average no. of plantlets</td>
</tr>
<tr>
<td>RCPL1-1C</td>
<td>903</td>
<td>30</td>
<td>2.10</td>
</tr>
<tr>
<td>Meghalaya-1</td>
<td>1103</td>
<td>38</td>
<td>2.00</td>
</tr>
</tbody>
</table>

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compost. The data in the table represent the average of five independent experiments.

The freshly isolated protoplasts from the leaf base (Figure 1 b) were small (8–15 μm in diameter) with dense cytoplasm, which displayed red fluorescence under UV light, a characteristic of mesophyll protoplasts. On an average 6–10 x 10⁶ protoplasts from 1 g fresh weight of leaf tissue were released. Undigested cells and cell debris were effectively removed by sucrose density gradient technique, which was monitored by Calcofluor white. More than 90% viable protoplasts were determined by fluorescein diacetate. Protoplasts regenerated the cell wall after the 3rd day, changing the shape from spherical to oval after the 4th day; the first division after the 6th day of culture (Figure 1 c) indicated the re-entry of protoplasts into the cell cycle. Further divisions (Figure 1 d, e) led to the formation of microcalli (Figure 1 f), i.e. after the 15th day. Reduction of osmoticum with fresh medium every week was found to be essential to maintain the sustained divisions and vigorous growth of protoplasts.

Support of feeder cells has been found essential for sustained divisions of protoplasts 2–4, 10, 11, 22–24. However, in the present investigation, the protoplasts of RCPL1-1C showed divisions and formation of microcolonies up to the 32-cell stage without feeder cells. Such divisions were not found in the other variety. The feeders prepared from fast-growing cell suspension were found most suitable for sustained divisions of protoplasts.

Among the three culture procedures, i.e. protoplasts cultured in liquid medium, in 0.15% agarose in the ring formed by feeder cells and in 0.15% agarose on top of a membrane, culture protoplasts on membrane (Figure 1 g) was more effective for obtaining a larger number of protocalli (Table 1). Perhaps, this could be attributed to better aeration and direct contact with feeder cells.

The use of callus proliferation medium for embryoid formation from protoplast-derived calli was an important step for obtaining a large number of plantlets. The embryoid-like structures were separated and transferred on MS-based plant regeneration medium with various combinations of growth regulators. The MS medium (MS salts with 3.0 mg l⁻¹ kinetin and 0.5 mg l⁻¹ NAA) was suitable for obtaining a larger number of plantlets (Figure 1 h). The differentiation of embryoids was observed as early as 14 days after the transfer on MS-based medium. These organized structures first developed into coleoptile and eventually into plants (Figure 1 I). A variation in the frequency of plant regeneration was also observed. The calli derived from protoplasts cultured in 0.15% agarose showed the highest frequency of plant regeneration, followed by protoplasts cultured in liquid medium and on membrane. The average number of plantlets was also higher in protoplasts cultured in 0.15% agarose (Table 1). Hardening of plantlets in 1/2 MS liquid medium (without sucrose) was an essential step for successful transplantation of plantlets in soil. Sixty-seven fertile plants from RCPL1-1C and about 48 plants from Meghalaya-1 were obtained. The panicle (Figure 1 J) derived from mesophyll protoplasts had red seeds, confirming that the plants had been regenerated from mesophyll protoplasts and not from the feeder cells derived from white-seeded genotype.

Thus, a reproducible procedure for regeneration of fertile plants from mesophyll protoplasts of *Oryza sativa* (L.) has been developed. This finding would be useful for in vitro genetic manipulations in rice plants at the protoplast level without/with little creation of variation.


ACKNOWLEDGEMENT. JNG is grateful to the Department of Biotechnology, India for a fellowship.

Received 12 September 1994, revised accepted 4 January 1995
In vitro generation of sheep-pox-virus-induced T cell clones

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In vitro studies pertaining to the development of sheep pox virus (SPV)-induced T cell clones have been attempted. Long-term cultures with PBMC of SPV-infected and/or immunized sheep were initiated in the presence of specific SPV antigen. The SPV-induced cultures could be maintained for 63 days by alternate antigen ‘starve and feed’ cycle without addition of antigen-presenting feeder cells. Significant alteration in CD3:CD4 ratio was observed in the cultured cells, and when CD3:CD4 ratio was stable, the subcloning was done by the limiting dilution technique. The long-term-cultured cells and cloned cells were characterized on the basis of microscopical examination, E rosette technique, monoclonal probe, interleukin-2 receptor expression and by determining the cytotoxic potential against histocompatible-virus-infected lamb testicular labelled target cells by 31Cr release assay.

For the last one and a half decades many efforts have been made to obtain antigen-specific T lymphocyte clones. The lymphocytes could be propagated in vitro and made specific to repeated exposures to antigens. T cell clones have been raised against most of the antigens, including peptides, proteins, PPD, hormones, MHC tumour, erythrocytes, inert metals like nickel, bacterial viral antigens, and mitogens. Details about cloning of T cells from human and murine source are available in the literature but the information about cloning of T cells against sheep pox virus (SPV)-specific clone is not available.

In the present communication we report studies on the development and characterization of SPV-specific and Concanaalvin A (ConA)-induced ovine T cell clones.

Healthy Muzzafarnagri sheep of either sex and lambs of known pedigree with no prior history of sheep pox disease or vaccination were used for this study.

The sheep were immunized with 0.1 ml dose (1000 TCID50) of live attenuated SPV/RF strain of sheep pox vaccine (B. P. Division, IVRI). The virulent Jaipur strain of sheep pox virus (B. P. Division, IVRI) was used. The lambs were given 2000 ID50 (skin-infective dose 50%) at four spots by intradermal route (0.1 ml at each spot) on the shaved abdominal region.

In an attempt to develop SPV-specific T cell lines and mitogen-induced clones, an experimental model was designed based on the method of raising murine T helper cell line and goat mononuclear cell specific against rabbit RBC antigen.

The PBMC from peripheral blood collected on 8th day post-immunization and 7th day post-infection were separated on Ficol Hapaque. The lymphocytes (4 x 107/ml) were suspended in GM and dispensed in the Mongo cells flasks. Predetermined inactivated sheep pox virus suitable for blastogenesis at optimum concentration (20 μg protein/ml) was incorporated in the culture flasks and the cultures were incubated at 37°C. On 6th day post-incubation, the live cells were harvested, counted and suspended in RPMI-1640 GM (2 x 105/ml) without antigen, in fresh flasks for 6 days. The period from 6th to 12th day post-incubation was designated as antigen ‘starve’ period. At the end of antigen ‘starve’ cycle, viable cells were harvested and subcultured in the presence of SPV antigen (20 μg protein/ml) in conditioned medium and incubated for further 6 days. This period of antigenic stimulation was designated as antigen ‘feed period’. On 6th day of antigen feed period, the viable cells were subcultured and maintained by antigen starve cycle. The cultures were thus maintained by alternate antigen ‘starve’ cycles for 42 days. Thereafter, the cultures were fed antigen at weekly intervals and were maintained up to 63 days. Similarly, the cultures were maintained for 47 days using ConA.

The spent media of the cultures were changed every 72 h with CM. At each stage parallel cultures were run in Leighton tubes (1.5 ml/tube) for microscopic examination (by Giemsa staining or indirect fluorescein antibody technique using monoclonal antibodies) and phenotype characterization.

Dead cells were removed by layering the cultured cell suspension over Ficol–Hapague (2:1) followed by centrifugation at 400 g for 15 min. The viable cells were recovered from the interface, washed twice with GM and dispensed as per the requirement.

The 40th day mitogen-induced cultures and 58th day SPV-induced PBMC were subcloned by the limiting dilution method in 96-well round-bottom microculture plates in the presence of IL-2 growth factor. Expansion of cells was done in 24-well costar plates (Cluster 24–205 Broadway, Cambridge) and the phenotype was characterized by monoclonal probe.

Phenotype characterization of long-term cultured and cloned cells was done on the basis of microscopical examination of culture flasks and stained smears, E rosette test, monoclonal probe using indirect immunofluorescence, IL-2 receptor expression and by determination of cytotoxic potential by 31Cr release assay using histocompatible-virus-infected lamb testicular labelled target cells. A periodical microscopical examination of cultures was carried out.

The cells harvested at regular intervals from SPV-
stimulated and mitogen-induced sheep PBMC long-term cultures were characterized both morphologically and functionally. Morphological characterization was carried out by microscopical examination of antigen-stimulated cultures in culture flasks with or without Giemsa's technique. Blastogenesis appeared 72 h post-initiation of cultures. At 96 h, the blasts were marked, ranging from 8.34 to 9.25%. The blasts appeared as large, round or oval cells with a thin rim of cytoplasm and an almost central large nucleus. By 108–120 h, clusters of lymphoblasts appeared in the cultures. Subsequently, on day 6, cells larger than lymphocytes were seen floating throughout the culture flasks. These cells had a large eccentric nucleus and a wide rim of bluish cytoplasm in contrast to the lymphocytes, which had a thin rim of pink–bluish cytoplasm. By about 10 days, the size and number of large-cell colonies increased. About 10–20 colonies were seen per field under low power.

The 'larger' cells appeared to multiply constantly up to day 20 and 20–25 colonies, with 20–30 cells per colony seen under low power. On day 25, most of the cells in these colonies appeared to be degenerating, with the colony size decreasing gradually. At this stage only 20–25 cells per colony could be visualized. By day 28, most of the colonies disappeared, except for one or three which appeared to be scattered in the culture. On day 31 numerous 'small' cells were seen floating in the cultures, with one or two colonies seen per flask. On day 35–40 the colonies were hardly visible. Only 'small' and 'large' cells were observed in suspension cultures.

On day 45, small colonies reappeared in the culture and were seen up to day 55. On day 50, 'large' cells were predominant in number and small cells with two to three colonies per culture flask were observed. Thereafter, no appreciable difference in microscopical picture was observed.

'Small' cells appeared always floating as single duplets or quadruples. These cells had a very small nucleus and a thin rim of cytoplasm. Small cells were considered to be mostly young daughter cells or cells at the multiplying stage budding out from large cells.

The mitogen-stimulated PBMC long-term cultures showed a similar sequential microscopic picture with minor differences. The number of colonies per culture flask appeared more. The 'large' and 'small' cells were more distinct and colonies appeared throughout the culture period. Small cells appeared singly in duplets or in groups of 4–5 cells resembling a small colony.

The adherent cells, which form an integral part of the cultures, were always found in the culture flasks. These cells formed a thin sheet which embodied in it most of the dead cells. The sheet appeared in the background when examined under a microscope. In a few cultures the adherent cells appeared to inhibit further growth of 'large' and 'small' cells, resulting in increased dead cell count. The adherent cells were gradually eliminated on subsequent subcultures (made beyond day 15) by changing the culture flasks. The SPV-antigen-induced cultures were maintained up to 63 days and Con-A-induced cultures for 47 days.

E-rosette-forming cells from the SPV-specific sheep PBMC cultures were enumerated at weekly intervals. The results are given in Table 1.

The results of phenotypic characterization of SPV-antigen- and Con-A-stimulated long-term cultures of sheep PBMC were interesting. The cultured cells uniformly contained lymphocytes with CD4, CD8, CD3, and T19 markers. Stability in SPV-antigen-stimulated cultures with respect to CD4:CD8 ratio was observed between day 35 and 40 post-initiation with minimum T19+ cells. In Con-A-stimulated cultures stability with respect to CD4:CD8 ratio was observed between day 28 and 42 post-initiation. MAB T19+ cells continued to exist in the cultures till day 42.

SPV-specific PBMC were studied for expression of IL-2 R and cytotoxic potential on day 48 post-initiation.

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Table 1. E-rosette-forming cells in SPV-antigen-stimulated and Con-A-stimulated long-term in vitro sheep PBMC cultures

<table>
<thead>
<tr>
<th>Animal</th>
<th>Post-culture initiation intervals (days)</th>
<th>Percentage rosette formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td><strong>SPV-antigen-stimulated cultures</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamb L546</td>
<td>18</td>
<td>30</td>
</tr>
<tr>
<td>Sheep E67</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td><strong>Mean SE</strong></td>
<td>16.33</td>
<td>18.00</td>
</tr>
<tr>
<td>± 0.88</td>
<td>± 1.15</td>
<td>± 0.37</td>
</tr>
<tr>
<td><strong>Con-A-stimulated cultures</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E67</td>
<td>15</td>
<td>16</td>
</tr>
</tbody>
</table>

Values are averages of three readings taken from each culture. ND, Not done.
of the culture. The IL-2R expression was studied in IL-2 stimulated and unstimulated culture cells by thymidine uptake assay. The stimulation index was 3.43. The cytotoxic potential was assayed by \(^{51}\)Cr release assay using histocompatible-virus-infected lamb testicular cell targets. The counts per minute were taken after 1 h incubation with labelled target, at 100 : 1 E : T ratio.

After day 12 post-subcloning by limiting dilution, the cultured cells were assayed for surface markers. The cells in one row of the culture plate were positively reacting to CD\(_6^8\), which were expanded. In other rows CD\(_4^+\) and CD\(_8^+\) cells were in 3 : 2 ratio.

In the present study Con-A-induced cultures required 6-weekly mitogenic stimulation and regular change of conditioned medium on every alternate day without providing antigen-presenting cells (APC). The provision of CM was felt essential in the absence of APC. Long-term cultures require the presence of APC\(^{9-12}\). But, in contrast, several reports indicated long-term growth and maintenance of certain human T cell lines and clones in the absence of APC\(^{13,14}\). In this study an optimal growth with colony formation was observed in mitogen- and SPV-antigen-induced sheep PBMC long-term cultures derived from infected/vaccinated lambs. In long-term cultures, it was observed that E-rosette-forming lymphocytes increased from day 0 to 35 in antigen-stimulated and from day 0 to 21 in mitogen-induced cultures. The observations are consistent with earlier reports\(^{13,14}\). The overall increase in E rosette is believed to be due to the release of a soluble factor, the E rosette augmenting factor (E-RAF), which increases the total number of lymphocytes that form rosette with SRBC\(^{14}\).

Interesting information was collected on monoclonal probe from growing lymphocytes in long-term cultures. The CD\(_4^+\) : CD\(_8^+\) ratio was 2.25 on day 7, with a significant positive increase in cells bearing CD\(_4^+\) or CD\(_3^+\) molecule and a corresponding decrease in cells bearing CD\(_8^+\) molecule. The MHC class II molecule appeared on 48% lymphocytes, which implies that most of the T cells were activated. On day 14–21, the CD\(_4^+\) : CD\(_8^+\) ratio in the cultures decreased to 1.29, with a corresponding increase in lymphocytes bearing CD\(_8^+\) molecule; T\(_{16}^+\) reacting cells decreased below baseline values. It can be predicted that between day 7 and 14, CD\(_8^+\) (cytotoxic/suppressor) cells proliferated in response to in vitro antigen stimulation of in vivo primed cells, the resultant maturation of young precursor/daughter cells and appearance of CD\(_4^+\)-receptor-bearing cells with increased percentage reactivity. A gradual increase in CD\(_4^+\) cells was observed on subsequent days till day 35–49. The CD\(_4^+\) : CD\(_8^+\) ratio stabilized in cultures with higher decrease in percentage frequency of T\(_{16}^+\)-reacting cells and 75–82% cells expressed MHC class II. The functional characterization and monoclonal antibody approach revealed that T cells had also proliferated, which showed cytotoxicity potential against histocompatible-virus-infected lamb testicular labelled target cells. The subcloning and characterization of cloned cells were suggestive that both subsets of T cells bearing CD\(_4^+\) or CD\(_8^+\) molecule proliferated. Further studies regarding cloning and functional characterization of viral-antigen-specific clones are suggested.


ACKNOWLEDGEMENTS. We thank Dr M. Brandon, University of Melbourne, Australia, for the generous gift of antiswine leucocyte monoclonal antibodies and Laboratory Protocol. We also thank Dr Arunan, Scientis, Immunodynamics Laboratory, NII, New Delhi, for supply of some reagents and conjugates.

Received 29 June 1993; revised accepted 22 December 1994