Final Technical Report

Cytoplasmic diversification for hybrid rice improvement: phase 2. R6632

Executive Summary

There is presently a need to develop alternatives to the widely used Wild Abortive male sterility system to facilitate the use of hybrid rice technology in India. In this project, putative alloplasmic lines have been produced by a robust protoplast fusion procedure employing the fragrant Indian rice cultivar Pusa Basmati 1 with *O. granulata.* This material will be sent to the Directorate of Rice Research, Hyderabad, India and the Central Rice Research Institute, Cuttack for evaluation and the development of new genotypes of hybrid rice with novel cytoplasms. This germplasm will contribute significantly towards the development and uptake of hybrid rice in India.

Background

Spectacular gains in rice productivity have been achieved in China through the adoption of hybrid rice technology. There is presently a requirement to utilise this technology in India. This necessitates the transfer of cytoplasmic male sterility genes into Indian rice cultivars. Male-sterile lines, which are unable to self-pollinate, are essential for a successful hybrid rice programme. The most commonly used system, cytoplasmic male sterility, utilises three lines, namely a cytoplasmic male sterile (cms) line with corresponding maintainer and fertility restorer lines. The wild abortive (WA) cytoplasm, by far the most commonly used source of cms, was developed by wide hybridisation between rice cultivars and a wild rice plant with aborted pollen. It is important to diversify the current narrow genetic background of hybrid rice which renders the crop vulnerable to disease and pest outbreaks and adverse environmental conditions. The experience of the devastation of the hybrid maize crop carrying the T-cytoplasm is an important reminder of the importance of this work. Considerable effort is now being directed, both in India and world-wide, towards establishing new sources of male sterility.

Wide hybridisation, involving sexual crosses and back-crossing, is currently being used at CRRI, Cuttack and IRRI, Philippines as well as other rice research centres around the world, to develop alloplasmic lines between cultivated rices and other

members of the genus *Oryza* in order to develop new sources of male sterility. Ideally, the new lines should be composed of the *O. sativa* nucleus, together with cytoplasm from the wild species. In practice, sexual crossing results in the inheritance of the mitochondrial and chloroplast genomes of the wild species together with the nuclear genomes of both the cultivated and wild rice species. Repeated backcrossing of the hybrids with rice breeding lines is employed to eliminate chromosomes of the wild species. To date, it has been possible to transfer only those cytoplasms from other closely related species ('A' genome) into *O. sativa*.

In phase 2 of the programme, somatic hybridisation has been exploited to circumvent the sexual barriers to cytoplasmic diversification. Populations of cybrid and somatic hybrid plants have been produced at Nottingham from protoplast fusions involving Pusa Basmati 1 and the distantly related rice *O. granulata* (G genome). These plants have been/are in the process of being sent to collaborating institutions in India for assessment of the extent of male sterility. Production of these plants represents completion of technology generation step TG5 of Activity 2.2 in the revised PSP Logical Framework (October 1998). The results obtained are an important progression towards realisation of the overall purpose: 'Novel sources of male sterility developed for use in hybrid rice breeding programmes in India'.

This project has been formulated after close collaboration between IRRI, the Directorate of Rice Research, Hyderabad, the Central Rice Research Institute, Cuttack and the University of Nottingham. During December 1994, N.W.Blackhall, at the request of the Plant Sciences Programme, travelled to India, visited the Directorate of Rice Research, Hyderabad and the Central Rice Research Institute, Cuttack and was able to converse directly with researchers involved in both wide hybridisation and hybrid rice research. The need to develop hybrid rice is a key element of the research portfolios of these Institutes and it is Indian Council for Agricultural Research (ICAR) policy to greatly increase the area of hybrid rice in India. This will help to increase rice production, an increase necessitated by increasing demand caused by population growth.

Project Purpose

The purpose of this project is to increase the production and stability of production of hybrid rice in India. The project will develop a range of plants containing diverse

cytoplasms derived from wild relatives of cultivated rice suitable for use in hybrid rice breeding programmes in India. A population of 55 plants has been produced and is in the process of being sent to collaborators in India in order to broaden the cytoplasmic (and genetic) background of hybrid rice.

This project is targeted towards the needs of rice farmers in high potential production systems in India. In the Eastern lowland region, it is difficult for breeders to produce male-sterile lines for distribution to farmers because all of the rice varieties adapted to this area restore fertility when crossed with conventional Wild Abortive hybrid rice. For the rest of India, there is an urgent requirement to diversify the currently narrow genetic background of hybrid rice, which renders the crop vulnerable to disease and pest outbreaks and adverse environmental conditions. The experience of the devastation of the hybrid maize crop carrying the T-cytoplasm demonstrates the need for cytoplasmic diversification in hybrid breeding programmes.

Research Activities and Outputs

Phase 2 of the project built upon the foundation established during Phase 1 and utilised the robust cybridisation and plant regeneration protocols established at Nottingham. Wild rice species with genome symbols CCDD (*O. latifolia*), EE (*O. australiensis*) and FF (*O. brachyantha*), and GG (*O. granulata*) have been used in this study. As a result of work carried out in Phase 1, Pusa Basmati 1 cell suspensions with proven capability of producing protoplasts and plant regeneration were available for Phase 2.

Development of cell suspension cultures

The procedures employed for initiation of callus cultures and initiation of cell suspensions has been published as a book chapter (reproduced in appendix 1: Callus initiation, maintenance and shoot induction in rice. Published in Methods in Molecular Biology, Vol 111: Plant Cell Culture Protocols Ed R.D. Hall 1999 Humana Press, Totowa pp19-29). Suitable callus cultures were obtained of *O. australiensis*, *O. granulata* and *O. latifolia* but not of *O. brachyantha.* Consequently, *O. brachyantha* was not used in further studies. Rapidly growing suspension cultures were developed for *O. australiensis, O. granulata* and *O. latifolia* from which protoplasts of *O. australiensis* and *O. granulata* were successfully isolated.

Similarly for the cultivated rice varieties Gayatri, Savitri and Swarna (all from Eastern India) cell suspension cultures were developed as described for Pusa Basmati 1 (An improved procedure for plant-regeneration from indica and japonica rice protoplasts. Jain R.K *et al.*, Plant Cell Rep. 1995, 14:515-519). However, following discussions with colleagues at CRRI, it was revealed that Gayatri and Savitri are genetically very similar and so only Savitri was used in subsequent studies. It had been planned to employ varieties IR65597 and IR655898, which are representative of the improved rice ideotype developed by IRRI since a plant regeneration protocol had been developed as part of other studies at Nottingham. However, further studies revealed that more than 75% of the regenerated plants became albino upon transfer from *in vitro* conditions to growth in the glasshouse. This was considered an unacceptably high frequency and so regrettably these varieties were excluded from further studies. Variety Basmati 370, which has a higher grain quality than Pusa Basmati 1, was used for establishment of cell suspension cultures. It had also been planned to use variety IR64 for which plant regeneration protocols had been developed at DRR. However discussions with Dr N.P. Sarma of DRR revealed that these protocols had been found to not be reproducible following the departure of one of his scientists.

Protoplast fusion.

Equipment developed at Nottingham (Equipment for the large-scale electromanipulation of plant protoplasts. Jones *et al.,* 1994 Biotechniques 16: 312-321) was used to fuse protoplasts of the wild rices (*O. australiensis* and *O. granulata*) with the cultivated rices (Pusa Basmati 1, Basmati 370, Swarna and Savitri). In some experiments, a pre-treatment with irradiation from the ^{137}Cs source at the University of Nottingham was used to partially destroy the nucleus of the wild rices, converting them into cytoplasmic donors. In other experiments, no pre-treatment was used.

Plant regeneration

As in all studies with rice protoplasts, plant regeneration was the technically most demanding part of the project. Highly efficient protocols utilising layers of nurse cultures had previously been developed at Nottingham (An improved procedure for plant-regeneration from indica and japonica rice protoplasts. Jain R.K *et al.,* Plant Cell Rep. 1995, 14:515-519). Of the 8 combinations of wild rices fused with cultivated which were assessed, plants were successfully regenerated from the cross

Pusa Basmati 1 (+) *O. granulata*. Plants were successfully regenerated from unfused protoplasts of Basmati 370, Swarna and Savitri, indicating that genetic incompatibilities were preventing development of heterokaryons involving these varieties. Similarly, no plants were regenerated from fusion experiments involving *O. australiensis.* The use of irradiation pre-treatments was found to be extremely detrimental to the growth of callus derived from fusion mixtures.

Fifty five putative hybrid plants have been produced during phase 2. These plants are in the process of being transferred to CRRI and DRR.

Contribution of Outputs

During the first 6 months of phase 3, transfer of these plants to India will be completed and the plants will be acclimatised and grown in glasshouse conditions at the 2 institutions. During the year 2000 growing season, the plants will be transferred to field conditions and data will be collected on pollen fertility and spikelet emergence in order to identify the male sterile plants. Data for the cybrids will be compared to the values obtained for the corresponding seed-, callus- and protoplast-derived plants. Simultaneously, selected plants will be grown under glass-house conditions at Nottingham and data will be collected on pollen fertility.

Using clones of the plants maintained *in vitro* at Nottingham, the degree of cytoplasmic diversity amongst the male sterile plants will be ascertained and compared with the conventional sources of male sterility based on the WA cytoplasm and cytoplasms produced in India. These studies will build on experience gained at Nottingham in the characterisation of the WA cytoplasm using RFLP and PCR-based assays of the mitochondrial genome. Microsatellite markers will be used to characterise the chloroplast genome. An essential part of the project is diversification of the cytoplasm used in hybrid rice. It is therefore essential that the extent of diversity within the material generated at Nottingham is determined. The subsequent stages in the achievement of DFID's development goals are summarised in Table 1.

Publications

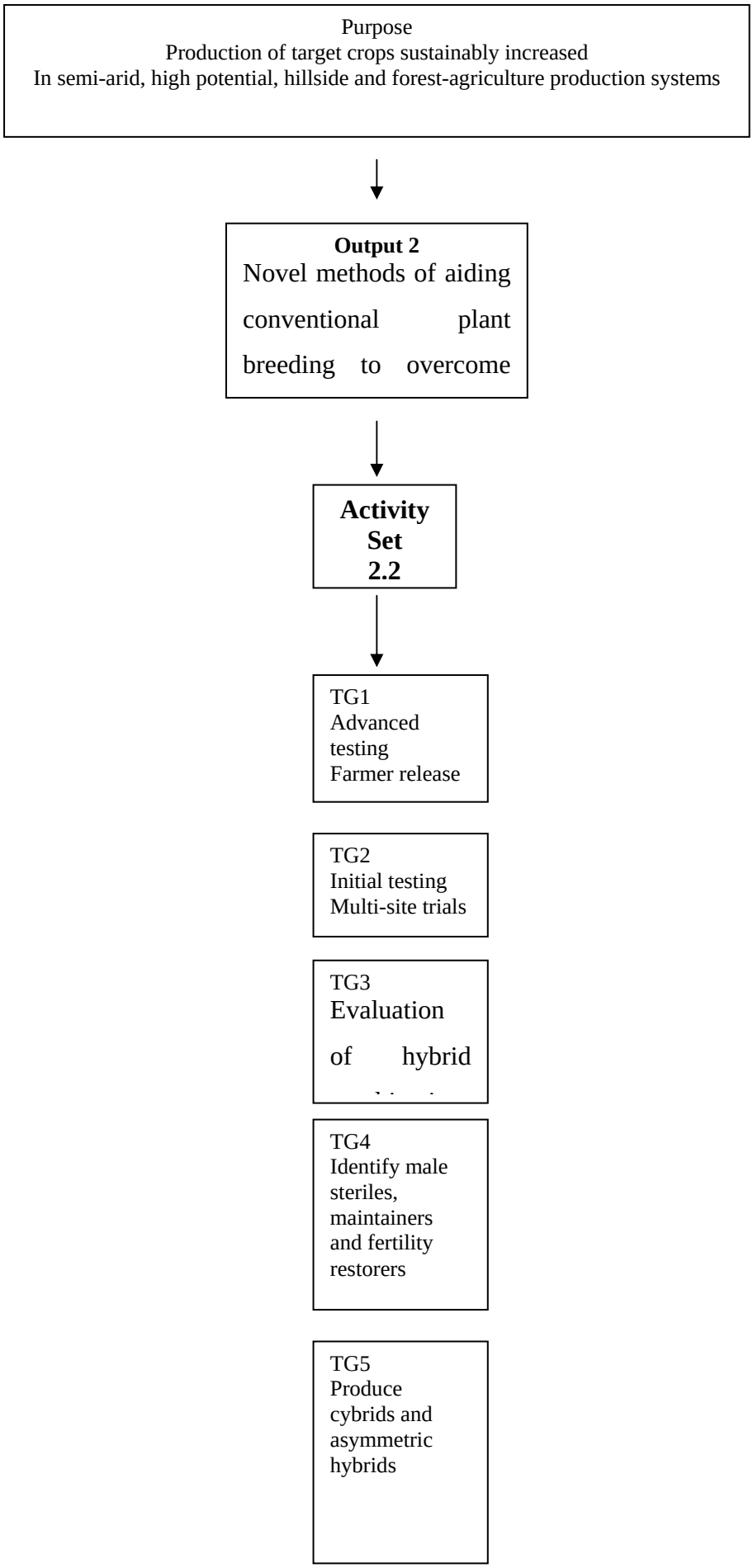
Blackhall NW, Jotham JP, Azhakanandam K, Power JB, Lowe KC, Cocking EC, Davey MR (1999) Callus initiation, maintenance and shoot induction in rice.

Methods in Molecular Biology, Vol 111: Plant Cell Culture protocols (Ed RD Hall). Humana Press, Totowa, pp19-29

Jelodar NB, Blackhall NW, Hartman TPV, Brar DS, Khush G, Davey MR, Cocking EC, Power JB (1999) Intergeneric somatic hybrids of rice [*Oryza sativa* L. (+) *Porteresia coarctata* (Roxb.) Tateoka]. Theor. Appl. Genet. 99:570-577

Blackhall NW, Akagi H, Fujimura T, Jotham JP, Davey MR, Power JB, Cocking EC (1998) Diversification of cytoplasmic male sterility systems through somatic cell hybridization. Advances in hybrid rice technology. Proceedings of the 3rd Intl. Symp. on Hybrid Rice, Nov. 1996, Hyderabad, India. (Ed. SS Virmanii, EA Siddiq, K Muralidharan), International Rice Research Institute, Manila, pp 257-264.

Table 1. Proposed PSP Logical Framework



Appendix 1

Callus initiation, maintenance and shoot induction in rice

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1. Introduction

Embryogenic suspension cultures provide the most widely employed source of totipotent cells for protoplast isolation in rice (*Oryza sativa* L.), since mesophyll-derived protoplasts of this important cereal rarely undergo sustained mitotic division leading to the production of tissues capable of plant regeneration. Cells from embryogenic suspensions provide an alternative to immature zygotic embryos for biolistic-mediated production of fertile transgenic rice plants *(1)* and are also amenable to transformation procedures employing agrobacteria *(2).* Currently, protocols are available for regenerating fertile plants from cell suspension-derived protoplasts of the three major sub-groups of rice varieties, namely japonica *(3),* javanica *(4)* and indica *(5)* rices.

Previous reports have stated that genotype and explant source are important parameters in determining the success of plant regeneration from cultured tissues of rice *(6-10).* In japonica rices, callus cultures can be produced relatively easily from almost any part of the plant, including roots, shoots, leaves, leaf-base meristems, mature and immature embryos, young inflorescences, pollen grains, ovaries, scutella and endosperm. Such tissues can be induced to regenerate plants *(11).* Conversely, indica rices are more recalcitrant in culture *(12-13).* In the procedures described in this chapter, scutella from mature seeds are used as the source of callus for both indica and japonica rices.

The establishment and maintenance of embryogenic cell suspensions is generally difficult, with morphogenic competence of suspensions usually declining with successive subculture over prolonged periods *(14).* However, following the development of reproducible protocols for cryopreservation of rice cell suspensions *(15, 16),* it has been possible to devise strategies to overcome difficulties associated with the loss of totipotency and the requirement to re-initiate periodically new cultures capable of plant regeneration. Samples of the cell suspensions should be cryopreserved as soon as possible after initiation. The cultures should be resurrected from frozen stocks immediately there are indications of loss of embryogenic potential of the suspensions.

Frequently, it has been noted that the initiation and growth of embryogenic callus requires media containing auxin (specifically 2,4-dichlorophenoxyacetic acid 2,4-D), whereas for the development of embryos into plants, auxins should be omitted from the culture medium. The regeneration of rice plants has been found to be enhanced by media lacking auxin but supplemented with reduced concentrations of cytokinins, such as 6-benzylaminopurine (BAP) or kinetin *(17,18).*

Micropropagation provides a means of rapidly multiplying material of both cultivated and wild rices as well as genetically modified plants (e.g. transgenics, somatic hybrids and cybrids). The ability to multiply plants *in vitro* is especially important for wild rices (*Oryza* species other than *O. sativa*), for which only limited supplies of seed may be available. Indeed, wild rices are an important genetic resource, since they possess resistances to biotic and abiotic stresses. These *Oryzae* can also be used to generate alloplasmic lines for the development of novel cytoplasmic male sterility systems.

2. Materials

2.1. Initiation of Embryogenic Callus from Mature Seed Scutella

1. Seeds of *Oryza sativa* cvs. Taipei 309 and Pusa Basmati 1 (obtained from the International Rice Research Institute [IRRI], Los Baños, Philippines).
2. "Domestos" bleach (Lever Industrial Ltd., Runcorn, UK) or any commercially available bleach solution containing approx. 5% available chlorine.
3. Sterile purified water: water purified by distillation, reverse osmosis or ion exchange chromatography, which has been autoclaved (121°C, 20 min., saturated steam pressure).
4. MS basal medium: based on the formulation of Murashige and Skoog *(19).* This medium can be purchased in powdered form lacking growth regulators (Sigma, Poole, UK), to which 30 g/L sucrose is added. The medium is semi-solidified by the addition of 8 g/L SeaKem LE agarose (FMC BioProducts, Vallensbaek Strand, Denmark), pH 5.8 (see Table 1). Autoclave.
5. LS2.5 medium: based on the formulation of Linsmaier and Skoog *(20)* supplemented with 2.5 mg/L 2,4-D and semi-solidified by the addition of 8 g/L SeaKem LE agarose, pH 5.8 (see Table 1). Autoclave.
6. Nescofilm: Bando Chemical Ind. Ltd., Kobe, Japan.

2.2. Micropropagation of Cultivated and Wild Rices

1. Seeds of *Oryza sativa* cvs. Taipei 309 and Pusa Basmati 1, *O. australiensis* and *O. granulata* (obtained from IRRI).
2. "Domestos" bleach: as in Section 2.1.2.
3. Sterile purified water: as in Section 2.1.3.
4. MS basal medium: as in Section 2.1.4.
5. Autoclavable culture vessels e.g. 175 mL capacity screw-capped "Powder-Round" glass jars (Beatson Clark and Co. Ltd., Rotherham, UK).
6. Micropropagation medium: MS medium (as in Section 2.1.4) with 2.0 mg/L BAP and 50 g/L sucrose, semi-solidified by the addition of 8 g/L SeaKem LE agarose, pH 5.8. Autoclave.
7. Glazed white ceramic tiles (15 cm x 15 cm), wrapped in aluminium foil and autoclaved.

2.3. Initiation of Embryogenic Callus from Leaf Bases of Micropropagated Plants

1. LS2.5 medium: as in Section 2.1.5.

2. Nescofilm: as in Section 2.1.6.

2.4. Shoot Regeneration from Callus

1. Differentiation medium: MS medium (as in Section 2.1.4.) supplemented with 2.0 mg/L BAP, with 30 g/L sucrose, pH 5.8. Autoclave.
2. Nescofilm: as in Section 2.1.6.
3. Rooting medium: MS medium (as in Section 2.1.4.), supplemented with 1.5 mg/L α-naphthaleneacetic acid (NAA), pH 5.8. Autoclave.
4. Maxicrop liquid fertiliser solution: Maxicrop Plus Sequestered Iron, Maxicrop Garden Products, Gr. Shelford, Cambridge, UK.
5. Initiation compost: a 12:1 (v:v) mixture of M3 soil-less compost (Fisons plc., Ipswich, UK) and Perlite (Silvaperl Ltd., Gainsborough, UK).
6. Growth compost: a 6:1:1 (v:v) mixture of M3 soil-less compost, John Innes No. 3 compost (J. Bentley Ltd., Barton-on-Humber, UK) and Perlite.

2.5. Initiation of Embryogenic Suspensions

1. AA2 medium: modified AA medium *(21)* supplemented with 2 mg/L 2,4-D, pH 5.8 (see Table 1). Filter sterilise.
2. R2 medium: modified R2 medium *(22)* supplemented with 1 mg/L 2,4-D and 30 g/L maltose, pH 5.8 (see Table 1). Autoclave, (*see* Note 1).
3. Disposable sterile plastic 10 mL pipettes with a wide orifice (e.g. Sterilin 47110; Bibby Sterilin, Stone, UK), or 10 mL glass serological pipettes (e.g. Sterilin 7079-10N) with the ends removed to produce a wider orifice.

3. Methods

3.1. Initiation of Embryogenic Callus from Mature Seed Scutella of Cultivated Rices

1. Dehusk 100 seeds each of *O. sativa* cvs. Taipei 309 and Pusa Basmati 1.
2. Surface sterilise the seeds by immersion in a 30% (v/v) solution of “Domestos” bleach for 1 hour at room temperature.
3. Remove the “Domestos” solution using 5 rinses with sterile purified water.
4. Germinate the seeds by laying on the surface of 20 mL aliquots of MS basal medium in 9 cm diameter Petri dishes (9 seeds/dish). Seal the dishes with Nescofilm and incubate in the dark at 28 ± 1°C.

5. After 14 d, remove the coleoptiles and radicles and transfer the explants to 20 mL aliquots of LS2.5 medium in 9 cm diameter Petri dishes (9 explants/dish). Seal the dishes with Nescofilm and incubate as in 3.1.4.
6. Subculture every 14-28 d (*see* Note 2) by selecting the most embryogenic callus, i.e. tissue with a dry, friable appearance, and transferring 1-5 calli (each approx. 5 mm in diameter) to 20 mL volumes of LS2.5 medium in 9 cm diameter Petri dishes.

3.2. Micropropagation of Wild Rices

1. Dehusk 10 seeds each of *O. australiensis* and *O. granulata* (the number of seeds can be varied depending upon supply*).*
2. Surface sterilise the seeds and germinate as in Sections 3.1.2.-3.1.4.
3. Sub-culture the seedlings after 14 d. Aseptically remove the seedlings and place onto the surface of a sterile white tile. Trim the roots to their base using a scalpel and trim the leaves of the shoots to a length of 2 cm.
4. Transfer the shoot bases to screw-capped glass jars each containing 50 mL aliquots of micropropagation medium (see Section 2.2.6.). Immerse the bases of the shoots 5 mm below the surface of the medium.
5. Sub-culture the shoots every 28 d. At each sub-culture, use forceps and a scalpel to separate the multiple shoots (tillers) which develop from each explant. Select healthy micropropagules (tillers) and trim the roots and stems as in Section 3.2.3. Transfer to micropropagation medium as in Section 3.2.4.

3.3. Initiation of Embryogenic Callus from Leaf Bases of Micropropagated Shoots

1. Use separate micropropagules of *O. australiensis* and *O. granulata* plants obtained as in Sections 3.2.3.-3.2.5. Each micropropagule has hard white tissue at its base. This tissue is embryogenic and is used for callus initiation. Excise the tissue and cut into 4 mm^2 sections; culture the latter on 20 mL aliquots of LS2.5 medium in 9 cm diameter Petri dishes (8 tissue sections/dish).
2. Seal the Petri dishes with Nescofilm and incubate in the dark at 28 ± 1°C.
3. After 28 d, inspect the dishes for callus production by the explants. Select yellow coloured, rapidly dividing calli composed of small cell clusters, excise the tissue from the parent explants and transfer the tissues to LS2.5 medium every 28 d (8 tissues/dish; *see* Note 3, Fig. 1A-D).

3.4. Shoot Regeneration from Callus

1. Transfer individual pieces of callus, obtained either from mature seed scutella or from leaf bases as in Section 3.3.1., to 9 cm diameter Petri dishes each containing 20 mL aliquots of differentiation medium (12 calli/dish, each approx. 3 mm in diameter, Fig. 2A). Seal the dishes with Nescofilm and incubate at 27 ± 2°C in the dark for 14 d, followed by transfer to the light (16 h photoperiod, 55 µmol/s/m^2, Daylight fluorescent tubes). Examine every 7 d for the regeneration of shoots and roots.
2. When shoots appear, transfer each shoot, together with a 3 mm diameter piece of the adjacent parental callus, to rooting medium (1-8 shoots/dish; Fig. 2B, C). Seal the dishes with Nescofilm and incubate at 27 ± 2°C in the light as in Section 3.4.1.
3. Transfer rooted shoots to initiation compost (see Section 2.4.5.) in 7.5 cm diameter plastic plant pots (Fig. 2D). Cover the regenerated plants with 20 cm x 20 cm clear polythene bags (*see* Note 4). Maintain the potted plants in a glasshouse under natural daylight with maximum day and night temperatures of 28 ± 2°C and 24 ± 2°C, respectively.
4. After 3 d, make 5 incisions with a pin into the top of the bags.
5. Four d later, remove, with scissors, one corner of each bag.
6. After a further 4 d, remove the other corner of each bag.
7. Every 2 d, cut off the top 1 cm of each bag, until the top-most leaves of the potted plants are exposed. Remove each bag.
8. Spray the plants daily with a 0.1% (v/v) aqueous solution of Maxicrop.
9. Transfer plants producing tillers and roots and which show healthy, vigorous growth to 15 cm diameter pots containing growth compost (see Section 2.4.6.).

3.5. Initiation of Embryogenic Suspensions

1. When sufficient dry, friable, callus has been obtained (approx. 5 weeks for cultivated rices and up to 24 weeks for wild rices, depending on the growth rate of the callus), initiate cell suspension cultures by transferring 1.5 g fresh weight of embryogenic callus (*see* Notes 5, 6) to a 75 mL capacity Erlenmeyer flask containing 18 mL of either AA2 medium [for *O. sativa* cv. Taipei 309 and *O. granulata* (*see* Note 7)] or R2 medium (for *O. sativa* cv. Pusa Basmati 1 and *O. australiensis*). Incubate at 28 ± 2°C in the dark on a horizontal rotary shaker (120 rpm, 4 cm throw).

2. Subculture the suspensions every 3-4 d. Allow the cells to settle, remove 50% of the supernatant and replace with an equal or slightly greater volume of medium. Gradually increase the volume of medium and the size of flasks in accordance with the rate of growth of the suspension cultures.
3. After 12-15 weeks, there should be sufficient quantity of small clusters of cells (*see* Note 8) to transfer 1 mL packed cell volume (PVC, *see* Note 9) of the small clusters, to produce a 'pipettable' suspension (*see* Note 8). This 'pipettable' culture is subcultured at 7 d intervals by transferring 1 mL PCV, together with 9 mL of spent medium, to a 250 mL capacity Erlenmeyer flask containing 42 mL of new medium (*see* Note 10). Maintain the 'stock' culture for a further 2 weeks until the new 'pipettable' suspension has become established. Repeat this procedure if the 'pipettable' culture fails to become established.
4. The growth characteristics of established cultures are determined by daily measuring the settled cell volume (SCV). Decant the cell suspension into a graduated centrifuge tube, allow to sediment under gravity (10 min) and note the SCV from the graduations (*see* Note 10).

4. Notes

1. R2 medium should be autoclaved at 116°C for 30 min, as sterilisation at 121°C for 20 min causes excessive caramelization.
2. Regular sub-culture of scutellum-derived callus will produce fast-growing, globular tissues. Fourteen d is the optimal time between transfers, but the tissues can be left for 28 d before sub-culture.
3. Transfer of leaf base-derived calli to fresh LS2.5 medium every 28 d is important, since compact embryogenic callus becomes more globular with time in culture and hence more suitable for the initiation of finely-divided cell suspensions.
4. Place polythene bags over the potted plants and secure the bags by placing the pots inside other 7.5 cm pots. The bags are required to maintain high humidity around the plants and to reduce transpiration. The humidity is reduced gradually by opening the bags as the plants become acclimatised.
5. In the case of *O. granulata*, approximately 2% of the stem bases produce fast-growing, fine yellow callus suitable for the initiation of cell suspension cultures. Transfer of this callus to new semi-solidified medium results in rapid tissue

proliferation, enabling initiation of cell suspensions after 8 to 12 weeks of culture of the callus on semi-solid medium.

6. For *O. australiensis,* approximately 90% of the stem bases produce embryogenic callus. Initially, the cell suspension cultures of *O. australiensis* consist of large clusters of callus (each in excess of 2 mm diameter), but after 8 to 12 weeks of subculture, the majority of the cell clusters are less than 1 mm in diameter.
7. Maintenance of cell suspensions of *O. granulata* is difficult because of the extreme viscosity of the cultures. It is necessary to shake the Erlenmeyer flasks vigorously immediately before sub-culture and to pay particular attention to the volume of cells (*see* Note 7) used for inoculation into new medium.
8. When a cell suspension is first initiated, the initial or 'stock' suspension consists of large cell clusters. As these clusters grow, they release smaller clumps of cells into the liquid medium. For experimental use, a 'pipettable' culture is initiated from the small cell clusters. The 'stock' culture is discarded once a 'pipettable' culture has been established.
9. PCV is measured by drawing a suspension of cells into a sterile 10 mL pipette (see Section 2.5.3.). The medium is expelled whilst holding the pipette tip against the bottom of the flask. The cells will be retained in the tip of the pipette, allowing the medium to escape. The PCV may be adjusted to 1 mL by drawing more suspension into the pipette, or by releasing packed cells from the pipette tip.
10. The maintenance of cell suspension cultures requires careful attention to the sub-culture procedure. Regular sub-culture, usually at 7 d intervals and use of the correct inoculation volume (1 ml PCV) are vital in maintaining totipotency. An excess of inoculum causes exhaustion of the nutrients early in the subculture cycle, resulting in necrosis within the centres of the cell clusters. Conversely, if too small an inoculum is transferred, the growth rate of the cells is reduced considerably, since the cells fail to reach the minimum inoculation density.
11. The doubling time of cultures varies, but is generally between 2 and 4 d.

References

1. Sivamani, E., Shen, P., Opalka, N., Beachy R.N., and Fauquet, C.M. (1996) Selection of large quantities of embryogenic calli from indica rice seeds for production of fertile transgenic plants using the biolistic method. *Plant Cell Rep.* **15**, 322-327.

2. Hiei, Y., Ohta, S., Komari, T., and Kumashiro,T. (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *The Plant J.* **6**, 271-282.

3. Thompson, J.A., Abdullah, R., and Cocking, E.C. (1986) Protoplast culture of rice (*Oryza sativa* L.) using media solidified with agarose. *Plant Sci.* **47**, 179-183.

4. Suh, S.C., Kim, H.I., and Park, W. (1992) Plant regeneration from rice protoplasts culture in javanica cv. Texmont. *Res. Rep. Rural Dev. Adm. (Suweon)* **34**, 1-9.

5. Jain, R.K., Khehra, G.S., Lee, S-H., Blackhall, N.W., Marchant, R., Davey, M.R., Power, J.B., Cocking, E.C., and Gosal, S.S. (1995) An improved procedure for plant regeneration from indica and japonica rice protoplasts. *Plant Cell Rep.* **14**, 515-519.

6. Lai, K.L., and Liu, L.F. (1982) Induction and plant regeneration of callus from immature embryos of rice plants (*Oryza sativa* L.). *Jpn. J. Crop Sci*. **51,** 70-74.

7. Lai, K.L., and Liu, L.F. (1986) Further studies on the variability of plant regeneration from young embryo callus cultures in rice plants (*Oryza sativa* L.*). Jpn. J. Crop Sci.* **55**, 41-46.

8. Lai, K.L., and Liu, L.F. (1988) Increased plant regeneration frequency in water-stressed rice tissue cultures. *Jpn. J. Crop Sci*. **57,** 553-557.

9. Abe, T., and Futsuhara, Y., (1984) Varietal difference of plant regeneration from root callus tissues in rice. *Jpn. J. Breed.* **34**, 147-155.

10. Abe, T., and Futsuhara, Y. (1986) Genotypic variability for callus formation and plant regeneration in rice (*Oryza sativa* L.). *Theor. Appl. Genet*. **72,** 3-10.

11. Bajaj, Y.P.S. (1991) Biotechnology in rice improvement, in *Biotechnology in Agriculture and Forestry, Vol. 14. Rice*. (Bajaj, Y.P.S., ed.), Springer-Verlag, Heidelberg, pp. 3-18.

12. Kyozuka, J., Otoo, E., and Shimamoto, K. (1988) Plant regeneration of protoplasts of Indica rice: genotypic differences in culture response. *Theor. Appl. Genet*. **76**, 887-890.

13. Lee, L., Schroll, R.E., Grimes, H.D., and Hodges, T.K. (1989) Plant regeneration from indica rice (*Oryza sativa* L.) protoplasts. *Planta* **178**, 325-333.

14. Abe, T., and Futsuhara Y. (1991) Regeneration of rice plants from suspension cultures in *Biotechnology in Forestry and Agriculture*, *Vol. 14. Rice* (Bajaj, Y.P.S., ed.), Springer-Verlag, Heidelberg, pp. 38-46.

15. Anthony, P., Jelodar, N.B., Lowe, K.C., Power J.B., and Davey, M.R. (1996) Pluronic F-68 increases the post-thaw growth of cryopreserved plant cells. *Cryobiology* **33**, 508-514.

16. Lynch, P.T., Benson, E.E., Jones, J., Cocking, E.C., Power, J.B., and Davey, M.R. (1994) Rice cell cryopreservation: The influence of culture methods and the embryogenic potential of cell suspensions on post-thaw recovery. *Plant Sci*. **98**, 185-192.

17. Raghava-Ram, N.V., and Nabors, M.W. (1984) Cytokinin mediated long-term, high-frequency plant regeneration in rice tissue culture. *Z. für Pflanzenphysiol*. **113**, 315-323.

18. Raghava-Ram, N.V., and Nabors, M.W. (1985) Plant regeneration from tissue cultures of Pokkali rice (*Oryza sativa*) is promoted by optimizing callus to medium volume ratio and by a medium-conditioning factor produced by embryogenic callus. *Plant Cell, Tiss. Org. Cult.* **4,** 241-248.

19. Murashige, T., and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473-497.

20. Linsmaier, E.M., and Skoog F. (1965) Organic growth factor requirements of tobacco tissue cultures. *Physiol. Plant.* **18**, 100-127.

21. Müller, A.J., and Grafe R. (1978) Isolation and characterisation of cell lines of *Nicotiana tabacum* lacking nitrate reductase. *Mol. Gen. Genet*. **161**, 67-76.

22. Ohira, K., Ojima K., and Fujiwara A. (1973) Studies on the nutrition of rice cell culture. I. A simple, defined medium for rapid growth in suspension culture. *Plant Cell Physiol*. **14**, 1113-1121.

Table 1

Formulation of Media - Macronutrients, Micronutrients, Vitamins and other Supplements

Component	Concentration, mg/L					
	MS	LS2.5	AA2	R2		
Macronutrients						
$CaCl_2$	332.2	332.2	332.2	147.0		
KH_2PO_4	170.0	170.0	170.0	170.0		
$MgSO_4$	180.7	180.7	180.7	120.4		
KCl		2940.0				
KNO_3	1900.0	1900.0		4040.0		
$NaH_2PO_4.2H_20$				312.0		
$(NH_4)_2SO_4$				330.0		
Micronutrients						
KI	0.83	0.83	0.83			
H_3BO_3	6.20	6.20	6.20	500.00		
$MnSO_4$	16.90	16.90	19.92	447.00		
$NaMoO_4.2H_2O$	0.25	0.25	0.2550.00			
$ZnSO_4.7H_2O$	8.60	8.60	8.60500.00			
$CuSO_4.5H_2O$	0.025	0.025	0.025	50.00		
$CoCl_2.6H_2O$	0.025	0.025	0.025			
$FeSO_4.7H_2O$	27.85	27.85	27.85			
Na_2EDTA	37.25	37.25	37.25			
NaFeEDTA				2.5		
Vitamins						
Myo-inositol		100.0	100.0	100.0		
Nicotinic acid		0.5		0.5		
Pyridoxine HCl		0.5		0.1		
Thiamine HCl		0.1	1.0	0.51.0		
Glycine	2.0	75.0				
L-Glutamine				877.0		
L-Aspartic acid				266.0		
L-Arginine			228.0			
L-Proline				560.0		
Other Supplements						
2,4-dichlorophenoxyacetic acid				2.5	2.0	2.0
Gibberellic acid				0.1		
Kinetin		0.2				
Sucrose	30000	30000		20000		
Maltose			30000			
pH	5.85.8	5.8	5.8			
Sterilisation	Autoclave		Autoclave	Filter	Autoclave	

Figure Legends

Fig. 1. Callus initiation from explants of *O. australiensis* (A) and *O. granulata* (B) after culture of the explants for 62 and 84 days, respectively (x 0.9). Embryogenic callus of *O. australiensis* (C) and yellow-coloured, friable callus of *O. granulata* (D) both suitable for the initiation of cell suspensions (x 3.8; x 2.7).

Fig. 2. Stages in plant regeneration from mature seed scutellum-derived callus of the indica rice cv. Pusa Basmati 1.

A. Embryogenic callus 28 d after sub-culture on LS2.5 medium (x 42).

B,C. Stages in shoot formation, 15 d after transfer of callus to differentiation medium in the dark, and 30 d after transfer to the light (x 33; x 25).

D. Rooted shoots ready for transfer to pots (x 1.0).