Somatic cell cryopreservation and protoplast regeneration of important disease-resistant wild rice *Oryza meyeriana* Baill*

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Abstract *Oryza meyeriana* Baill is one of the three wild rice species found in China. *O. meyeriana* possesses valuable characteristics but is reluctant in cell culture *in vitro*. In a series of experiments, callus with no regeneration ability was induced from young panicle of *O. meyeriana*. The callus was subcultured and propagated. Embryogenic cell clones were obtained after cryopreservation. Suspension cultures were established and protoplasts were isolated and regenerated into plants. Results of artificial inoculation of *Xanthomonas campestris* pv. *Oryzae* showed that the strong resistance did not change in the regenerated plants. The development of protoplast-to-plant system is an important progress towards utilization of *O. meyeriana* via cellular engineering. The experiments demonstrated that cryopreservation of plant calli was a new way to obtain embryogenic cell line.

Keywords: ***Oryza meyeriana*, cryopreservation, embryogenic cell line, protoplast culture.**

Wild germplasm consists of an important basis for genetic improvement of cultivated rice. *Oryza meyeriana* is one of the three wild *Oryza* species found in China[1]. Accessions originated in Yunnan Province exhibit strong resistance to bacterial leaf blight[2]. *O. meyeriana* grows on the hillside under trees and also possesses drought tolerance and shade tolerance. Rice breeders in China have attempted to transfer and utilize the useful genes in *O. meyeriana*. However, this wild rice has different genome compared with the cultivated rice, and gene transfer via sex hybridization is difficult because of the sexual incompatibility. In order to facilitate gene transfer via somatic hybridization, we have made a series of studies on the cell culture *in vitro* of *O. meyeriana*. In our experiments, the cell regeneration ability was improved through cryopreservation and the embryogenic cell clone was obtained. Protoplasts were isolated from cell suspension and cultured into plants. This is the first report on plant regeneration from protoplasts of the valuable disease-resistant germplasm *O. meyeriana*, which can serve as the starting point for protoplast fusion to transfer the resistance gene in *O. meyeriana*. The experiments demonstrated the enhancement effect of cryopreservation on the *in vitro* culture capacity of somatic cell of *O. meyeriana*.

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1 Materials and methods

1.1 Material

Accession of *O. meyeriana* originated in Yunnan Province, China was used in the present experiment. The plants were grown in the wild rice field of our institute under the normal management.

1.2 Methods

1.2.1 Callus initiation, subculture and regeneration. Young panicles of 0.2—1.0 cm long and embryos 20 d after flowering were collected. The explants were surface-sterilized and plated on the solid N6 medium supplemented with 2,4-D 2 mg/L to induce callus. Subculture of the calli was conducted on the same N6 medium at 4 weeks interval. Plants were regenerated from calli on the solid MS medium with 2 mg kenetin/L.

1.2.2 Cryopreservation of calli. Calli in size of 2—3 mm were selected and immersed in equal volume of cryoprotectant composed of 10% DMSO and 8% glucose in half strength of MS medium. After pretreatment in cryoprotectant for 60 min, the calli were frozen in the programmable freezer (KYRO10) and then stored in liquid nitrogen. When cryopreservation finished, the cryopreservation vials were taken out of liquid nitrogen and put into 40℃ water bath for rapid thawing. The calli were recovered by culture on the subculture media in the dark.

1.2.3 Establishment of cell suspension. The embryogenic calli were selected and transferred into liquid media to establish suspension culture following the method reported[3]. The suspensions were subcultured every week.

1.2.4 Protoplasts isolation and culture. Protoplasts were isolated from the suspensions on the third day after subculture. The enzyme solution contained 0.25% Cellulase RS and 0.012 5% Pectolyase Y-23 in CPW 13M. 1 g of suspension cells was added to 10 mL of enzyme solution and incubated at 26℃ overnight. After passing screens and being purified, protoplasts were cultured in KPR media using membrane nursing culture and mixture nursing culture methods[4]. When the protoplast colonies reached 1 mm in size, they were moved onto the subculture media or regeneration media and finally regenerated into plants. Plantlets of 10 cm high and with well-developed roots were transplanted into pots.

1.2.5 Determination of bacterial leaf blight resistance. Three strains of *Xanthomonas campestris* pv. *Oryzae*, Jangling 691 (isolated in Hubei Province, China), PXO61 (Philippines) and T7174 (Japan) were used. The density of bacteria was adjusted to 10^9/mL. Inoculations were carried out by chopping method. Plant reaction was scored according to lesion length 14 d after inoculation[5].

2 Results

2.1 Embryogenic callus produced after cryopreservation

O. meyeriana is evidently different from cultivated rice in morphology and shows some characteristics of bamboo. We once intended to induce callus of *O. meyeriana* but did not succeed. In 1994, young panicles were used as explants and one piece of callus was obtained. The callus looks

adhesive, watery and gluey and no differentiation was observed. After propagation on the subculture media, the callus clones were frozen and stored in liquid nitrogen together with calli of other wild rice species[6]. Growth could be recovered after the cryopreserved calli were transferred and cultured on N6 media. It was found that a new type of granular calli began to grow out of the old gluey one after two cycles of subcultures. The newly formed calli were different in texture and had the compact structure. Green plantlets were easily regenerated from the calli on the regeneration media. The average rate was 90%. The regeneration ability was improved totally. It was found that several plantlets were commonly produced in one piece of callus (fig. 1). Obviously, a larger callus consisted of several smaller granular ones. The small granular calli had the potential of regeneration.

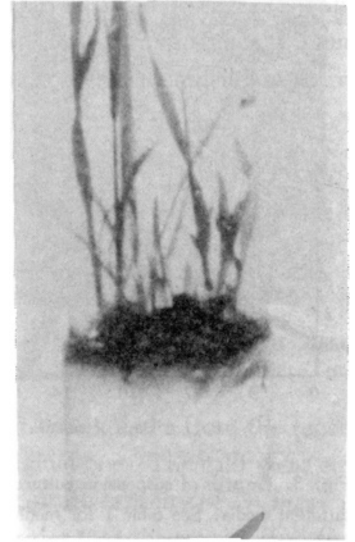

Fig. 1. Plantlet regeneration of cryopreservated callus of *O. meyeriana*.

Plant callus develops into plantlet via two pathways, that is organogenesis through differentiation and embryogenesis by embryoid development. Plant regeneration after cryopreservation showed obvious characteristics of embryoid development in *O. meyeriana*. Observations were made on the calli (fig. 2). It was found that there were a lot of embryoids in different development stages. Histological examination on the section of granular calli showed that the leaf primordium and root primordium had already developed. When the calli were transferred onto the regeneration media the embryoids germinated into plantlets. It was obvious that an embryogenic callus clone was produced after cryopreservation. The property of high regeneration rate has been maintained for three years in long-term culture of the embryogenic calli of *O. meyeriana*.

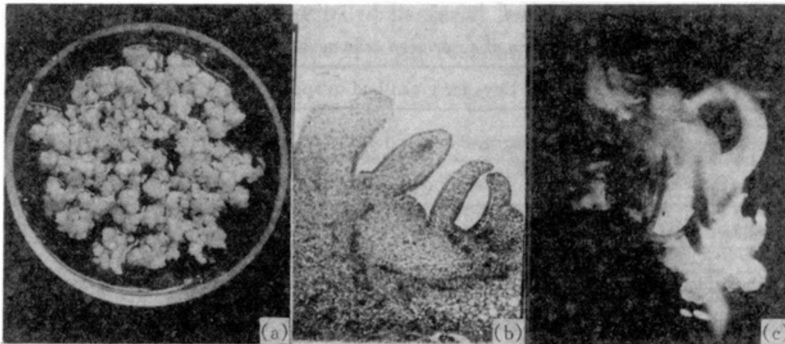

Fig. 2. Embryoid development in the cryopreserved calli of *O. meyeriana*. (a) Embryogenic calli; (b) development of leaf in the embryoid (×7); (c) germination of the embryoid (×7).

2.2 Establishment of suspension cell culture

The embryogenic calli with friable texture were obtained after several subcultures. The calli were transferred into liquid media. It seemed that the calli adapted to the liquid environment rapidly. The calli became dispersed and a suspension growing fast with thin cell wall and dense

cytoplasm was obtained in a month. There was no such browning, rooting occurring as usually found in some Gramineae. The results proved that the starting material is the most important factor in establishment of suspension culture.

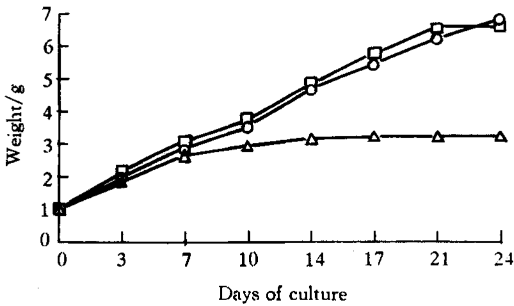

Fig. 3. Growth of suspension culture of *O. meyeriana* in different media. See table 1 for medium constituents. □, AA2m; ○, AA2; △, N6.

To identify suitable medium for the suspension of *O. meyeriana*, three kinds of liquid media, N6, AA2 and AA2m were tested (fig. 3). It was found that the two kinds of AA media were more suitable for establishing the cell suspension of *O. meyeriana* than N6. In the AA based media, calli grew faster at the beginning of suspension. Until the 21st day, the growth of cell clusters became slow in AA2m but kept fast in AA2. The growth speed of calli in N6 medium slowed down after 8 d and stopped after 14 d of culture.

Dispersing degree of the calli corresponded to the growth speeds in different media. In the AA based media, calli became dispersed rapidly and the cell clusters consisted of dozens of cells. Calli in N6 maintained larger granules in the course of suspension. The larger granules in liquid media might be poor in nutrition supply and aeration so that the growth was constrained.

The regeneration rates of the suspension cells were tested (table 1). Calli in suspension were transferred onto the MS regeneration medium. Although all of the calli from three kinds of media showed high percentages of regeneration, those from AA2 have the highest rate of 90%, followed by those from AA2m. However, calli from N6 regenerated faster than those from AA media. Green buds appeared in 20 d on calli from N6 but 30 d were needed for those from AA media. The results indicated that AA2 was the most suitable medium for the growth and embryogenic property maintenance of suspension of *O. meyeriana*.

Table 1 Regeneration of suspension cells maintained in different media

Media	Callus characteristics	Days for green bud to appear	Albino	Regeneration rate (%)
N6	compact, large granules	20	1	64
AA2	fine and friable granules	30—40	0	90
AA2m	fine and friable granules	30—50	0	82

N6, N6 salts and organic plus 2,4-D 2 mg/L, sucrose 30 g/L; AAm, sucrose in AA2 was replaced by maltose.

2.3 Protoplast isolation and culture

Suspensions maintained in three media were digested with enzyme solution. High yields of protoplasts up to 1.5×10^7—2.0×10^7 cell/gfw were isolated from suspensions in AA2 and AA2m. The yields from suspension in N6 were averaged 4.0×10^6 cell/gfw because of the large calli granules. The protoplasts contained sparse cytoplasm and were more fragile.

Protoplasts of *O. meyeriana* from suspension in AA2 were cultured using the membrane nursing method and mixture nursing method. The protoplasts were observed to divide after 2 d of culture (fig. 4(a)). After 15 d of culture, a patch of protoplast-derived calli formed on the membrane (fig. 4(b)). Based on the calli transferred onto the regeneration medium, the plating efficiency was 7.5×10^{-4}. One month after culture, the protoplast-derived calli were transferred to

regeneration medium and green plants were produced.

2.4 Culture capacity and disease resistance of the regenerated plants

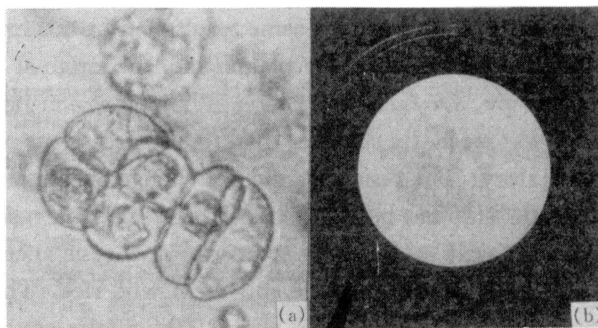

Fig. 4. Protoplast culture *O. meyeriana*. (a) Cell division after 2-d culture (×80); (b) micro-calli formed on the membrane.

The plants regenerated from protoplasts and suspension cells were grown in field to mature. These plants were identical to the original in morphology. Young panicles and embryos of the regenerated plants were cultured again to test the culture capacity. High rates of callus induction were obtained for the explants from the regenerated plants (table 2), in contrast to no callus formed from the original one. The calli were embryogenic and easy to regenerate plants. Results suggested that the culture capacity of *O. meyeriana* after cryopreservation could be inherited. It is reasonable to consider that the somatic embryogenic property was not just the result of physiological situation changed by cryopreservation process but that of genetic variation[7,8].

Table 2 Induction rate of callus from explants of regenerated plants

Explants	No. of total culture	No. of calli induced	Induction rate (%)
Young panicle	42	20	47.6
Embryo	13	3	23.3

The most useful characteristic of *O. meyeriana* is the resistance to bacterial leaf blight. It is a prerequisite for utilizing the resistance through cell engineering that the plants regenerated from cultured cells *in vitro* must keep the genuine resistance. 65 plants regenerated from the cultured cells were inoculated with the typical strains of bacterial leaf blight from China, Philippines and Japan. All of the regenerated plants showed high resistance to the test strains as the original plants (table 3).

Table 3 Resistance to bacterial leaf blight (BB) of original plants and regenerated plants

Bacterium strains	Plants immune to BB (%)		Plants highly resistant to BB (%)		Plants resistant to BB (%)	
	original plants	regenerated plants	original plants	regenerated plants	original plants	regenerated plants
PXO 61	21	20	47	44	32	36
Jiangling 691	58	60	37	36	5	4
T7174	66	67	24	21	10	12

3 Discussion

Rice is one of the most important food crops in the world, especially in China and Asian developing countries. Due to the paddy field area decrease and population increase in the developing countries, it is of importance to utilize wild rice germplasm to improve yield potential and resistance level of cultivated rice. *O. meyeriana* possesses many valuable characteristics. Peng et al. first reported the immune resistance of *O. meyeriana* to bacterial leaf blight and many researchers

repeated the test and got the same results[2]. In this experiment, we determined the resistance of the somatic cell regenerated plants and the original plants to three strains of *Xanthomonas campestris* pv. *Oryzae* from China, Philippines and Japan. The results demonstrated that the resistance was evidently stronger than that of accessions of *O. rufipogan* and *O. officinalis* kept in our laboratory. The resistance did not change or lose after long term of culture *in vitro*. Gene for gene relationship has been recognized between plant resistance and pathogen avirulence. *O. meyeriana* is certainly a promising source for new resistance gene.

In China, there are three species of wild rice. They are *O. rufipogan*, *O. officinalis* and *O. meyeriana*. *O. rufipogan* and *O. officinalis* are sexually crossable to the cultivated rice and there have been reports on their gene transfer and utilization. *O. meyeriana* is distantly related to cultivated rice, and sexual incompatibility exists between the two species[9,10]. Gene transfer from *O. meyeriana* to cultivated rice has not been reported. We have made several thousands of spikelets of pollination between cultivated rice and *O. meyeriana*. No hybrid embryo was harvested. Transfer of the genes in *O. meyeriana* must depend on the protoplast fusion technique. Protoplast-to-plant system developed in this experiment is a key progress towards utilization of this important germplasm by somatic hybridization.

Up to now, genetic manipulation in Gramineae at cell level is not as easy as in dicotyls. The main obstacle is the reluctance of cell in culture. Some of the reluctant species are even impossible to initiate callus. Among the 20 wild species in *Oryza*, only several species such as *O. rufipogan* and *O. latifolia* were reported to be regenerated from protoplasts[11,12]. There is no report on tissue culture of *O. meyeriana* except our laboratory. In our research, we first induced a piece of callus from young panicle, then produced an embryogenic cell clone after cryopreservation of the calli. Cryopreservation in liquid nitrogen is considered an ideal way to conserve plant germplasm long term. Evidence on ability of cells to synthesize assimilates, resistance level, RFLP of the regenerated plants showed that the genetic properties did not change after cryopreservation. Cornejo isolated protoplasts from cryopreserved calli for gene transformation[13]. However, recent experiments showed that some properties of cell might change after liquid nitrogen cryopreservation. Repeat freezing-thawing process increased the colony number formed on callus. Cold tolerant lines were selected from the cryopreserved calli in wheat[14,15]. The property change in cryopreserved calli can be explained by physical situation change in the cell resulting from temperature shock or by the genetic variation. In the present experiment, the young panicle and embryo of the regenerated plants showed a high rate of callus induction and produced embryogenic calli, indicating that the embryogenic cell line might rise from genetic variation. At present, it is not known whether this embryogenic variation was directly caused by the freezing-thawing process or the cryopreservation just played a role of selection in the variation already existing in the cells of calli.

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