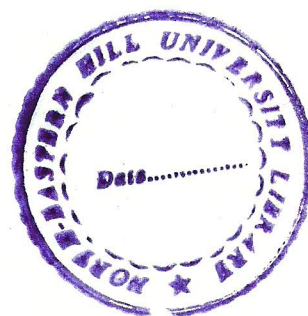


**PLANT REGENERATION FROM PROTOPLASTS
AND FUSION-MEDIATED PRODUCTION
OF CYBRIDS IN RICE**

By
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The North-Eastern Hill University

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DECLARATION

I, Miss Bijoya Bhattacharjee, hereby declare that the subject matter of the thesis entitled "Plant regeneration from protoplasts and fusion-mediated production of cybrids in rice" is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/ Institute.

This is being submitted to the North-Eastern Hill University for the degree of Doctor of Philosophy in Botany.


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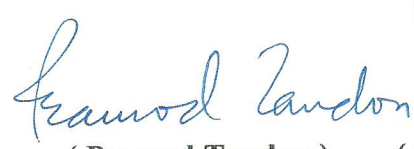
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Bijoya Bhattacharjee
(BIJOYA BHATTACHARJEE)

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ABBREVIATIONS

<i>atp6</i>	gene for subunit 6 of F ₀ -ATPase complex
bp	base pair(s)
°C	degrees Celsius
cm	centimetre
<i>coxII</i>	cytochrome oxidase subunit II gene
Co.	company
Cont.	continued
Corp.	corporation
CPW	cell and protoplast washing solution
CTAB	Cetyl-trimethylammonium bromide
cv(s)	cultivar(s)
DC	Direct current
2,4-D	2,4-dichlorophenoxyacetic acid
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra acetic acid
e.g.	for example
<i>et al.</i>	<i>et alia</i> (Latin; and others)
EtBr	Ethidium Bromide
Fig.	figure
F1	first generation
F2	second generation
γ-ray	Gamma ray
gm	gramme
g.f.wt.	gramme fresh weight
EDTA	ethylene diamine tetraacetic acid
HCl	hydrochloric acid
IOA	iodoacetamide
Kao	Kao (1977) basal medium
kb	kilobase (s)
KD	kiloDalton
krad	kilorad
λ	lambda
l	litre (s)
Ltd.	limited
m	metre
M	molar
mg	milligramme (s)
mg l ⁻¹	milligramme (s) per litre
ml	millilitre (s)
mm	millimetre (s)

mRNA	messenger ribonucleic acid
mM	millimolar
μm	micron
μl	microlitre (s)
mW	milliwatt
°N	degrees North
No.	number
O.D.	optical density (absorbance units)
PCV	packed cell volume
pH	hydrogen potential; logarithm of the reciprocal of hydrogen ion concentration i.e. $\log_{10}(1/[H^+])$ or $-\log_{10}[H^+]$
pp	page
RNAase	ribonuclease
RNA	ribonucleic acid
rpm	revolutions per minute
°S	degrees South
SD	standard deviation
S.E.	standard error
Tris	Tris (hydroxymethyl) aminoethane
UK	United Kingdom
USA	United States of America
USSR	Union of Soviet Socialist Republics
UV	ultraviolet light
v/v	volume to volume
V	volt
w/v	weight to volume
x g	x gravity (relative centrifugal force)
%	percentage
x	magnification
<	less than
>	more than

CHAPTER 1 : INTRODUCTION

Cereals are the most important group of food plants, which constitute the centrepiece of world agriculture by providing 52% of the total food calories. Of the more than 250,000 species of angiosperms, a mere 29 provides most of our calories. The most important of these are the eight cereal grains, viz. wheat, rice, maize, barley, oats, rye, sorghum and pearl millet of the family Gramineae (Poaceae). Therefore, cereals are the major targets for the application of cellular and molecular genetic manipulations with particular focus on recovering fertile transgenic plants. Among the major cereal crops the only one that is grown almost exclusively as human food is rice which accounts 27% of the world's food production and contributes substantially to global food security (Paroda, 1998).

1. Introduction to rice crop

Rice is the world's single most important food crop and a source of calories for more than one third of the world's population. More than 90% of the total rice is produced and consumed in Asia where 60% of the world's population live. By 2025 AD, the global population is likely to touch 8.5 billion from the current level of 5.7 billion, of which more than 3 billion will be primarily rice consumers (Khush, 1997; Paroda, 1998). With increasing population, global rice production needs to be increased from the 1995 level of 460 million tonnes to 560 million tonnes by 2000 AD and to 980 million tonnes by 2020 AD (Swaminathan, 1998). The task of producing the additional rice to meet the demand of the early 21st century poses major challenges. The world's rice area, however, has changed little since the early 1980s. A significant increase in

rice area is unlikely in the future. In fact, rice area is going down in several countries due to the pressure of urbanisation, industrialisation and other economic factors. Therefore, in order to meet this required increase in rice production, varieties must be developed which have higher yield potential, superior grain quality, multiple resistance to diseases and pests and tolerance to abiotic stresses. Recent breakthrough in molecular and cell biology have provided tools which will increasingly be used as aids to conventional plant breeding for developing rice varieties for the 21st century (Khush, 1997).

The advances in biotechnology offer enormous potential in enhancing the efficiency of breeding methods for rice improvement. Integration of the technology with conventional breeding has enabled the breeder to achieve several targets more quickly and efficiently, than was conceivable in the past. Transformation, somatic hybridization and cytoplasmic hybridization have already been employed in rice improvement programs. Transformation enables the integration of agronomically important genes into the plant genome, whilst somatic and cytoplasmic hybridization allows the transfer of genetic material between plants parasexually, thereby overcoming sexual incompatibility which may occur between desirable species combinations. However, for all these genetic manipulations, tissue culture is a prerequisite that allows recovery of plants from the manipulated cells.

1.1. Economic importance of rice

1.1.1. Cultivation of rice

The cultivation of rice dates to the earliest age of man and long before the era of which mankind have historical evidence. Rice was probably the staple food and the first cultivated crop in Asia. According to the Archaeological survey of India, four terraces of rice cultivation on the banks of the river Ravi in south-west Kashmir dates back to the Pleistocene or ice age (Grist 1986). Rice grows in diverse environments almost unparalleled in the plant kingdom. Natural dispersal and human selection have extended the cultivation of rice from the 55° north latitude (on the border between the USSR and China) to 43° south latitude (Central Argentina). It is also cultivated from sea level at the deltas of the great rivers to an altitude of 3,000 m (10,000 ft) elevation in the Himalayas (Swaminathan, 1984; Grist, 1986; Khush, 1997). Rice is cultivated in the cool climate of Nepal and India and in the hot desert of Pakistan, Iran and Egypt. Although typically grown on irrigated or rainfed, puddled, lowland soil, rice is the only crop that grows in the river deltas where water may rise as high as 4 m. Rice also grows without standing water, as an upland crop, particularly on rolling land.

1.1.2. Utilisation of rice

Rice is of vital importance to the economy of developing countries and nutritionally, produces more carbohydrate per hectare than any other cereal crop (De Datta, 1981). Rice accounts for 35 to 60% of the calories consumed by 3 billion Asians. The importance of rice in the diet accounts for over 70% of the daily calorie intake in

countries such as Bangladesh, Cambodia, Laos and Myanmar but drops to about 40% in countries such as China and India where wheat is also included in the diet. Rice is also an important staple food in Latin America, Africa and Middle East. Rice accounts for 8-9% of the protein for human consumption whereas wheat constitute 11-12% (Khush, 1997). Rice is planted on about 148 million hectares annually, or on 11% of the world's cultivated land. It is the only major cereal crop, which is consumed exclusively by human. In 1996, the world rice production was 553 million tons. The largest producer was China (187 million tons) followed by India (122 million tons), Indonesia (50 million tons), Bangladesh (27 million tons), Vietnam (24 million tons), Thailand (21 million tons) and Myanmar (20 million tons) (Khush, 1997). Thailand is world's leading rice exporter, selling about 4-6 million tons annually followed by USA which ranks 11th in production (produces 6 million tons) and exports about 40% of it.

1.2. The genus *Oryza* : origin, classification, distribution, morphology and brief genetics

1.2.1. The genus *Oryza*

The genus *Oryza* is a member of the grass family *Gramineae*, order *Glumifloreae*, class *Monocotyledoneae* and division *Angiospermeae*. *Oryza* consists of two cultivated and twenty one wild species. The two cultivated *Oryza* species are *Oryza sativa*, the Asian cultivated rice grown all over the world and *Oryza glaberrima* that is grown on a small scale in West Africa. Twenty one wild species of *Oryza* are scattered in Asia, Africa, Australia and Central and South America. It is estimated that 4,205,000 rice accessions

are conserved in various gene banks worldwide and of this, 10% are wild accessions. Over 80,640 cultivated accessions are preserved in Genetic Resources Center of International Rice Research Institute and of this, 15,000 are from India. The size of the national collection ranges from about 40,000 in China to 25,000 in India, 7,000 in U.S. and other countries have smaller collections (Swaminathan, 1984; Khush, 1997; Paroda, 1998). These germplasm are not only utilized as a source of genetic variability in plant breeding programs but also possess important genetic material for resistance to some insect pests (Swaminathan, 1984).

1.2.2. Origin of rice

The archaeological evidences suggest that the genus *Oryza* to which the cultivated rice belongs, probably originated at least 130 million years ago. The ancient super continent of Gondwana is believed to be the original habitat of this genus. When Gondwana broke up and became Africa, Antarctica, Australia, Malagasy, South America and Southeast Asia, *Oryza* species drifted into different geographic habitats. Today's species of this genus are distributed in all of these continents except Antarctica (Chang, 1976; Swaminathan, 1984; Khush, 1997). W G. Solheim II in 1966 discovered the earliest and most convincing archaeological evidence for domestication of rice in Southeast Asia (Solheim, 1966). Ancient India is undoubtedly one of the oldest regions where cultivation of *O. sativa* began (Khush, 1997). The excavation from Hastinapura revealed that rice had already existed by 1000 BC in Northern India. The oldest carbonized grains found in India date back to about 6750 BC. The second oldest

carbonized rough rice grain excavated in 1973 in Hemudu, a village near Ningpo, Central China, is estimated to be 6,000 to 7,000 years old (Matsuo *et al.*, 1997; Khush, 1997). The primary center of diversity for *O. glaberrima*, is probably the swampy basin of the upper Niger river, formed around 1500 BC and two secondary centers to the South-West near Guinean Coast which were formed 500 years later (Khush, 1997). The evolutionary pathways of two cultivated rice species are presented in Fig. 1.

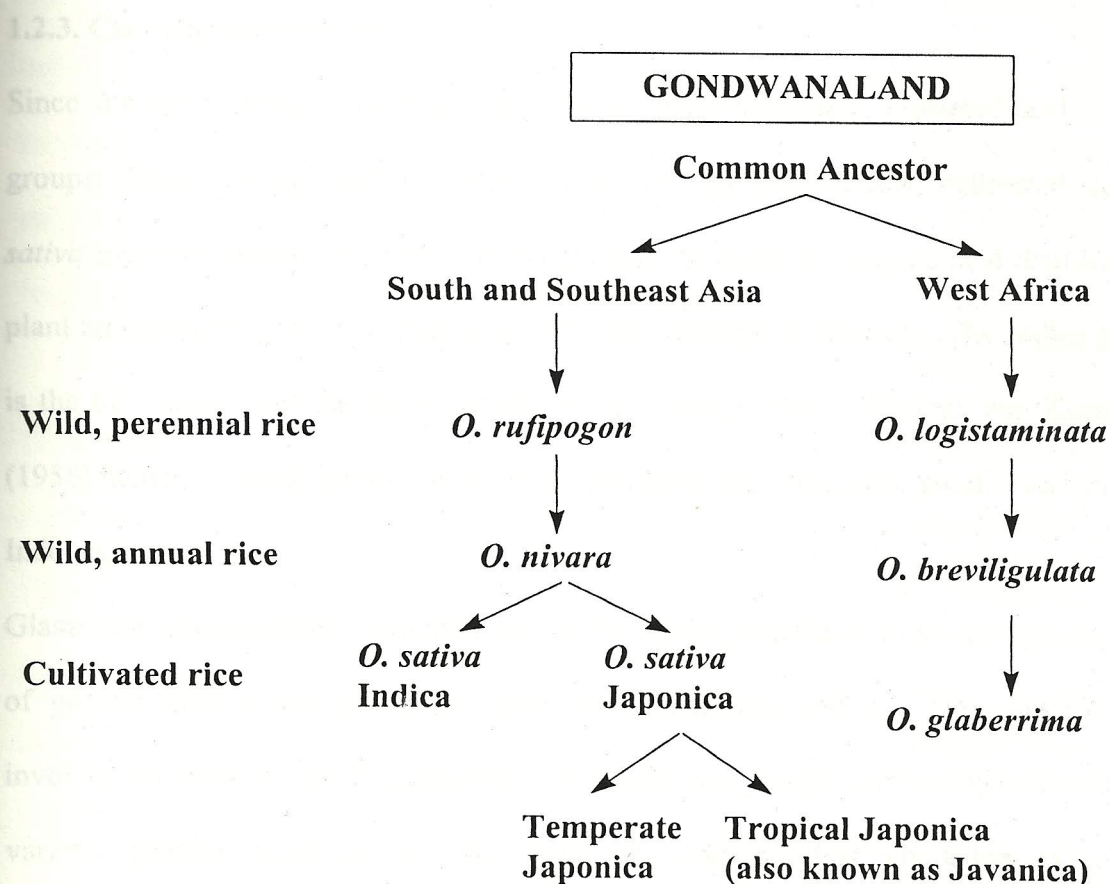


Fig. 1. Evolutionary pathways of two cultivated species of rice¹

¹Adapted from Khush (1997)

The cultivated rice, *O. sativa* and the African rice *O. glaberrima* are thought to be an example of parallel evolution in crop plants (Fig 1.). *Oryza sativa* probably derived from *O. nivara*, the wild annual rice, via the perennial rice, known as *O. rufipogon*. In another parallel evolutionary path, *O. glaberrima* was domesticated for annual *O. breviligulata*, which in turn evolved from perennial *O. longistaminata* (Chang, 1976; Khush, 1997).

1.2.3. Classification of rice

Since the third century BC, rice varieties in China have been clustered under three groups: 'hsien', 'keng' and glutinous. Kato *et al.* in 1928 divided cultivated rice *O. sativa* into two subspecies, Indica and Japonica, on basis of geographical distribution, plant and grain morphology, hybrid sterility and serological reaction. The Indica group is the old 'hsien' and the Japonica, the 'keng' (Grist, 1986). Moriga and Kuriyama (1958) added a third group Javanica to designate the bulu and gundil varieties of Indonesia.

Glaszmann classified the cultivated rice of the Asian countries into six groups on basis of genetic affinity using isozyme analysis (Glaszmann, 1987). This classification involves no morphological characters. When the six groups were compared with the varietal groups classified on the basis of morphological characters, Group I corresponded to the Indica and Group VI to the Japonicas. Javanica rices also belong to Group VI and designated as tropical Japonicas and the so-called Japonicas are referred to as temperate Japonicas (Khush, 1997).

The three ecospecies, Indica, Japonica and Javanica differ in their agronomic characters such as low temperature sensitivity, drought resistance, awn length, first internode length, grain shape and size and photoperiodic response (Oka, 1988). Indica rices are a large, diverse group of short day cultivars grown commercially in the tropics and sub-tropics. They generally are tall, heavy tillering, with pendant leaves and characterized by long, slender and non-glutinous grains. The Japonicas have darker green, erect leaves with moderate tillering, shorter, round and glutinous grains and are cultivated mainly in temperate zones and sub-tropics. The Javanica rices are found mostly in Indonesia, Java and to some extent, elsewhere. They are tall, insensitive to photoperiod and have a long vegetative phase, low tillering, long, broad and thick grains borne on long panicles (Grist, 1986; Oka, 1988; Khush, 1997).

1.2.4. Distribution of rice

According to most investigators, the cultivated species of *O. sativa*, spread from the Himalayan foot hills to Western and Northern India, to Afganistan and Iran and South India to Sri Lanka (Swaminathan, 1984; Khush, 1997). As early as 1000 BC, rice was a major crop in Northern India and Sri Lanka (Khush, 1997). Rice might have traveled from India to Madagascar and East Africa and then to countries of West Africa. The rice crop is believed to have been introduced to Greece and neighboring countries of Mediterranean. Rice grown in the Mediterranean regions, are Japonica while the rice grown in the Indian sub-continent is Indica. The Indica rice spread eastward to Southeast Asia and north to China. The Japonica rice was most likely domesticated

somewhere in northern parts of Southeast Asia or South China. It moved north to become a temperate Japonica. Around the beginning of 1st century, the temperate Japonicas were introduced in Korea and from Korea to Japan and to China. The Indica and Japonica rice was introduced from the mainland of Southeast Asia into Malaysia, Philippines, and Indonesia and from Philippines to Taiwan. The Portuguese introduced the tropical Japonica rice from Indonesia into Guinea-Bissau from where they spread to other West African countries. Lowland Indica rice was brought to Brazil and then to other Latin American countries by Spanish people. However, in all probability rice did not become an established crop in Europe much later perhaps in 15th or 16th century. The first record of rice in U.S.A dates from 1685, and it is believed to be introduced from Madagascar, South Asia and East Asia (Swaminathan, 1984; Khush, 1997).

The primary center of diversity for *O. glaberrima* is, from the swampy basin of the Niger southwest into two areas, near the Guinean coast. In West Africa *O. glaberrima* is a dominant crop grown in flooded areas of the Niger and Sokoto basins (Swaminathan, 1984).

1.2.5. Morphology of cultivated rice

Rice (*O. sativa* L) is an annual grass species with a more or less erect, cylindrical, smooth and hollow jointed stem or culm, flat sessile leaf blades and a terminal panicle (Grist 1986). A hull tightly encloses rice grain (caryopsis). Hull is composed of empty glumes, lemma and palea. The grain mainly consists of endosperm and embryo. Embryo is composed of an embryogenic axis, plumule and radicle. The plumule is

bound at the inner side by the scutellum or cotyledon, which lies next to the endosperm and is attached to the hypocotyl. The epiblast is seen as a protruding structure, which extends towards the upper end of the tip of the plumule and overlaps with the upper end of the scutellum. The radicle is enclosed in the coleorrhiza. When the grain germinates, the coleoptile emerges before the coleorrhiza. If grown in soil, the radicle protrudes first, but if the seed is submerged in water, the coleoptile emerges before the radicle. The radicle develops into root system and the young leaves emerge from the coleoptile.

The main stem or culm is differentiated from the growing point of the embryo, enclosed at first by the coleoptile. Stem has many nodes and covered by the leaf sheath. The leaves are alternate and borne in two ranks along the stem. The first leaf of the plant is the coleoptile. The second leaf, emerging through the lateral slit of the coleoptile, is reduced in size and has practically no blade. The remaining leaves are normal, except the uppermost or 'flag' leaf just below the panicle is slightly modified and plays an important role in assimilation of plant nutrients and therefore influences grain yield. The normal leaf has sheath, ligule, auricle and blade.

The inflorescence is a panicle, more or less lax, much branched and bearing spikelets. The spikelets are laterally compressed, oval, oblong or lanceolate, with or without awn. They are borne on a short pedicel, the rachilla dis-articulating below the lower floret and not produced beyond the uppermost floret. There are three florets in each spikelet but the two lower florets are reduced to sterile, scale-like lemmas while the terminal floret is fertile and forms the easily recognised 'grain'. The awn, when present, may be a mere

tip of about 1-2 mm or may be as much as 10 cm long. Inside the boat-shaped lemma and palea of the fertile floret the two lodicules, six stamens with slender filaments bearing versatile anthers and the pistil which consists of a one-celled ovary with a single ovule are the main organs. The style bears two plumose stigmas. At the time of flowering, the lemma and palea separates and the stigma protrudes followed rapidly by the anther bursting. Soon after pollination, the ovary gradually develops into the caryopsis and leads into mature seeds. (Grist, 1986).

Height of the plant is an important trait, related to the harvest index, growth duration, nitrogen response and lodging resistance. Apart from deep-water or so called floating varieties, the height of the rice plant is from 1 to 2 m but some deep water cultivars grow up to a height of more than 7 m. The dwarf varieties and some mutants grow to a height of little more than 0.5 m (Grist, 1986; Khush, 1997). Maturation period of rice plant depends on whether the variety is season-fixed or date-fixed. Some rice plants mature in less than 80 days from seed to seed while others like photo-period sensitive rice Rayada have a growth cycle of about 280 days (Grist, 1986; Khush, 1997).

1.2.6. Brief genetics of rice

The haploid chromosome numbers of *Oryza sativa* L. was first reported and presented by Kuwada in 1910, who demonstrated $n = 12$ ($2n = 24$). Studies by Audulov in 1931 provide strong evidence that the basic number of chromosome of the genus *Oryza* is 12. (Matsuo *et al.*, 1997). The two cultivated species of the genus *Oryza*, *O. sativa* and *O. glaberrima* are diploid $2n = 24$ and possess 12 pairs of chromosomes. They are

numbered according to the decreasing order of length at pachytene stage of sexual cell division. Thus, the longest chromosome is number 1, second longest number 2 and the shortest is number 12. Chromosomes of both the cultivated species and closely related wild species are similar and their genomes are designated as AA genomes.

The chromosomes of other wild species, however, differ from those of cultivated rice and they belong to genomes designated as BB, CC, DD, EE, FF and GG. A few of the tetraploid species have BBCC, CCDD and HHJJ genomes (Brar and Khush, 1997; Khush, 1997; Khush *et al.*, 1998). Somatic chromosome number, genomic composition and potentially useful traits of *Oryza* species are given in the Table 1.

Table 1. Somatic chromosome number, genomic composition and potentially useful traits of *Oryza* species (adapted from Khush, 1997)

Species	2n	Genome type	Distribution	Useful or potentially useful traits ¹
<i>O. sativa</i> complex*				
<i>O. sativa</i> L.	24	AA	World wide	Cultivated rice
<i>O. nivara</i> Sharma et Shastry	24	AA	Tropical and sub-tropical Asia	Resistance to grassy stunt virus, blast, drought tolerance
Table 1.Cont.				
<i>O. rufipogon</i> Griff.	24	AA	Tropical and sub-tropical Asia	Elongation ability, resistance to BB, source of CMS
<i>O. breviligulata</i> A. Chev. Et Roehr.	24	A ^a A ^b	Africa	Resistance to GLH, BB, drought tolerance
<i>O. glaberrima</i> Steud.	24	A ^a A ^b	West Africa	Cultivated rice
<i>O. longistaminata</i> A. Chev. Et Roehr.	24	A ^a A ^b	Africa	Resistance to BB, drought tolerance

Table 1 Cont.

<i>O. meridionalis</i> Ng.	24	A ^m A ^m	Tropical Australia	Elongation ability, drought tolerance
<i>O. glumaepatula</i> Steud.	24	A ^{gp} A ^{gp}	South and Central America	Elongation ability, source of CMS
<i>O. officinalis</i> complex				
<i>O. punctata</i> Kotschy ex Steud.	24	BB	Africa	Resistance to BPH, Zigzag leafhopper
<i>O. minuta</i> J.S. Presl. ex C.B. Presl.	48	BBCC	Philippines and Papua New Guinea	Resistance to sheath blight, BB, BPH, GLH
<i>O. officinalis</i> Walls ex Watt	24	CC	Tropical and sub-tropical Asia, tropical Australia	Resistance to thrips, BPH, GLH, WBPH
<i>O. rhizomatis</i> Vaughan	24	CC	Sri Lanka	Drought tolerance, rhizomatous
<i>O. eichingeri</i> A. Peter	24	CC	South Asia and East Africa	Resistance to yellow mottle virus, BPH, WBPH, GLH
<i>O. latifolia</i> Desv.	48	CCDD	South and Central America	Resistance to BPH, high biomass production
<i>O. alta</i> Swallen	48	CCDD	South and Central America	Resistance to striped stemborer, high biomass production
<i>O. grandiglumis</i> (Doell) Prod.	48	CCDD	South and Central America	High biomass production
<i>O. australiensis</i> Domin.	24	EE	Tropical Australia	Drought tolerance, resistance to BPH
<i>O. brachyantha</i> A. Chev. et Roehr.	24	FF	Africa	Resistance to yellow stemborer, leaf-folder, whorl maggot, tolerance to laterite soil
<i>O. meyeriana</i> complex				
<i>O. granulata</i> Nees et Arn.	24	GG	South and South East	Shade tolerance,

Table 1 Cont.

			Asia	adaptation to aerobic soil
Ex Watt				
<i>O. meyeriana</i> (Zoll. Et Mor. Ex Steud.) Baill.	24	GG	South East Asia	Shade tolerance, adaptation to aerobic soil
<i>O. ridleyi</i> complex				
<i>O. longiglumis</i> Jansen	48	HHJJ	Irian Jaya, Indonesia and Papua New Guinea	Resistance to blast, BB
<i>O. ridleyi</i> Hook. f.	48	HHJJ	South Asia	Resistance to stemborer, whorl maggot, blast, BB
Unknown genome				
<i>O. schlechteri</i> Pilger	48	unknown	Papua New Guinea	Increased tillering

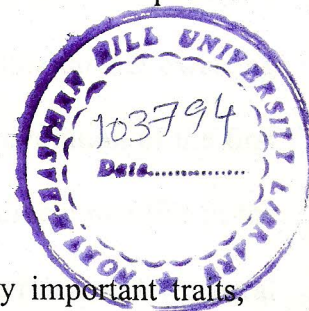
¹BPH = brown planthopper; GLH = green leafhopper; WBPH = white-backed planthopper; BB = bacterial blight; CMS = cytoplasmic male sterility.

*All species of *O. sativa* complex have the same genome, AA. However, genomic differences have been observed in samples from different continents. These differences are indicated by adding a superscript to the AA designation, e.g. A^BA^B for *O. glaberrima*.

1.3. Rice improvement through conventional breeding

Rice is a self-pollinated crop with less than 5% cross-pollination. Commercial varieties consist of homozygous, pure lines, which breed true-to-type. Natural crossing between commonly existing rice population produces new and mixed plant types in the field. The selection of special types from the mixed population was usually, achieved in the past by pure line selection and in a few instances by hybridization. This was done to not only suit various environmental conditions but also for the better yield. The rice breeding programme using hybridization was first initiated by Kano in 1904 in Japan (Grist, 1986). The International Rice Research institute in the Philippines that was set up in the mid-1960s undertook extensive rice breeding programs. Since then, rice

breeders of different countries of the world have developed new varieties by crossing carefully selected parents and eventually created many new cultivars with improved qualities.



1.3.1. Objectives of rice breeding

Initially, rice breeding programs were undertaken for agronomically important traits, such as tolerance to drought, flood, salinity and soil alkalinity mainly through selection from mixed natural population and through introduction. Now selection is used for the isolation of superior genotypes from segregating population following hybridisation (Poehlman and Sleper, 1995). One of the most important objectives of rice breeding is to develop varieties, which will assure the stable and maximum production. To attain this, objective of breeding programs are different in different countries and even within the country. However, the overall objectives include improvement in productivity coupled with disease and pest resistance, tolerance to abiotic stresses, grain quality and nutritional value.

1.3.2. Improvement in productivity

1.3.2.1. High yield technology

A major achievement of Green Revolution is the tremendous increase in the yields of cereal grain production including rice through conventional breeding and selection methods. This revolution in agriculture, started in 1960s, kept pace with population growth and assured food security to half of the world's population for over 3 decades

(Vasil, 1995). One single factor that brought about the yield breakthrough in tropical rice, as in case of wheat is the dwarfing gene Dee-Geo-Woo-Gen (DGWG), a spontaneous mutant isolated in China. Extensive exploitation of this unique dwarfing gene source in crosses with tall standard native varieties led to the evolution of the first high yielding dwarf variety Taichung (Native 1) followed by the miracle rice IR8 in the year 1966. Since then, several hundred high yielding varieties were released for general cultivation all over tropical Asia, Africa and Latin America (Paroda, 1998). This achievement increased the production at the rate of 3% per year, which was adequate to meet the demand of the rapidly growing world population. Since then, the world's rice production has been, doubled from 257 million tons in 1966 to 520 million tons in 1990. In the last 25 year nearly all of the rice growing countries in Asia including the most populous China and India attained self-sufficiency in rice production (Khush, 1997; Paroda, 1998). Recently, through the development of molecular genetic maps, through which, gene blocks governing component traits of quantitative characters known by quantitative trait loci (QTL) can be located and exploited in direct breeding, for improvement of complex characters like grain yield. Using molecular markers, probably it is for the first time that two valuable yield-related QTLs (*Yld 1-1* and *Yld 2-1*) could be found in the wild species, *O. rufipogon*. They were then, transferred to a hybrid background resulting in as much as 17% higher yield (Xiao *et al.*, 1996; Tanksley and MaCouch, 1997). The demand of rice is still increasing and yield is plateauing. In order to meet this ever-increasing demand, hybrid rice technology has been employed.

1.3.2.2. Hybrid rice technology

One of the major break-through of conventional breeding, is exploitation of heterosis using hybrid varieties. The successful development and utilization of maize hybrids beginning around 1930 was a landmark in crop breeding. It provided impetus to plant breeders to explore commercial exploitation of hybrid vigor or heterosis phenomenon in other crops. Rice is one of the most important crops in the world and is currently the focus of intensive efforts to increase yield through heterosis. Successful exploitation of hybrid vigor on commercial scale for last 20 years by Chinese has convinced many countries in and beyond Asia of hybrid technology as the potential strategy to step up the yield level. A farm-level study showed 15-16% yield advantage of hybrid rice over inbred rice in China. Comparison of average yield of hybrid rice and conventional rice varieties grown in China during 1981-1990 (Yuan et al., 1994) showed a 29% to 45% yield advantage of hybrids. During the year 1976 to 1995, hybrid rice technology had helped China to increase rice production by nearly 300 million tons (Yuan, 1998). Rj?

In 1991, about 17.6 million hectares of land was cultivated with hybrid rice in China, which is about 55% of the total area of rice (Yuan, 1994). The success of hybrid seed development and use in China led to the establishment of hybrid rice programs at IRRI in 1979 and at least 20 other countries; majority of which are developing ones including India during 1996. In addition to China, hybrid rice was planted in 102,000 hectare in Vietnam and about 65,000 hectare in India. Limited commercial cultivation of hybrid rice has also been reported in Bangladesh, Korea, Myanmar and Philippines (Pulver, 1998). Rice, being a self-pollinated crop, must involve use of an effective, male sterility

system to develop and produce hybrids. Cytoplasmic male sterile lines are normally hybridized with restorer lines for the production of hybrid seeds. Cytoplasmic male sterility (CMS) system or three line method of hybrid production involving CMS lines, maintainer lines and restore lines has been found to be the most effective and practical for developing rice hybrids. However, Two line method or PGMS (photoperiod sensitive genetic male sterility) and TGMS (thermo sensitive genetic male sterility) systems also hold promise to increase efficiency of breeding rice hybrids and reduce cost of seed production. Genetic male sterility (GMS) can also be used in hybrid seed production but due to its recessive nature, it entails rejection of 50% of the plants and thus CMS is preferred over GMS.

Among four different types of CMS systems, S1 (Chinsura Boro II or BT), S2 (Wild Abortive or WA), S3 (Gambiaca) and S4 (Not designated earlier) only two systems are currently used for hybrids seed production of Indica and Japonica rices. The level of heterosis in Japonica hybrids is not as good as in Indica hybrids. About 90% CMS line (A line) for the commercial production of the hybrids in China are based on a single source of wild abortive (WA) cytoplasmic male sterility. Records have shown that these varieties are significantly out-yielded over pure line cultivars (Yuan, 1994). During the last several years, this WA system of CMS has also been used extensively in hybrid rice programs of other countries to produce CMS lines in various genetic backgrounds (Virmani, 1994, Virmani, 1996).

1.3.3. Resistance to disease and pest

Rice productivity is effected by several biotic stresses. Rice is susceptible to many diseases and more than 200 insect-pests are known to herbivore on rice. Some of the major pests affecting rice production include bacterial blight, blast, and sheath blight, tungro, brown planthopper and stem-borers. Changes in insect's biotypes and disease races are becoming a continuing threat to increased rice production (Khush and Brar, 1991). Approximately 55% of the rice yield is lost by disease annually, whilst 10-30% is damaged by insects.

Although, pests and diseases can be controlled by using insecticides, pesticides and fungicides, the optimal and safe use of these chemicals is a growing concern. Exposure to these chemicals seriously affects farmer's health, productivity and environment. Development of new plant types with enhanced resistance to specific diseases and pests can overcome these problems. Whilst such a resistant variety can combat potential damage by pests and diseases for several years, new, emerging variants or biotypes of pests and diseases will still be able to break this resistance down (Diehl and Bush, 1984). Therefore, discovery of new sources of resistance and their introgression in the elite breeding lines is required for successful breeding programs in rice (Bennet *et al.*, 1993). The introgression of a gene for grassy stunt virus resistance from the wild species *O. nivara* to cultivated rice varieties, was the first example of useful gene transfer to rice. The resistant gene for brown planthopper (BPH) from *O. officinalis* into cultivated rice was also successfully transferred (Jena and Khush, 1990). In order to develop IR36, thirteen rice cultivars from six different nations and a wild species *O. nivara* were

utilized by the breeders (Innes, 1992). This rice variety was released as a result of international co-operation among the plant breeders, plant pathologists, entomologists, agronomists and plant physiologists in early 1980s. IR36 is resistant to many diseases and pests including green leaf hopper, bacterial blight, blast, stem borer, tungro, brown plant hopper and grassy stunt viruses. Furthermore, IR36 is tolerant to zinc and iron deficiency in soils and also to drought. Interestingly, wild rices are the important reservoir of useful genes for improvement. *Xa21* was originally identified in the wild species *Oryza longistaminata*, and subsequently transferred to the cultivated rice IR24, generating the near-isogenic line IRBB21 (Ikeda *et al.*, 1990; Khush *et al.*, 1990).

1.3.4. Tolerance to abiotic stress

The tremendous success of plant breeding programs in the 1960s enhanced rice production significantly. However, most of the high yielding rice varieties released in the 1960s were inadequately adapted to stress conditions. The enormous yield losses attributable to abiotic stresses, such as drought, cold, salinity, alkalinity and flooding, on the rice crop have been well documented (Anonymous, 1986). In such cases, new type of varieties that are tolerant to adverse soil condition, including salinity, drought, cold and alkalinity are required. For instance, *P. coarctata* has been identified as a source of salt tolerance. Production of intergeneric hybrids between IR28 or IR36 (salt-sensitive) rice cultivars and *P. coarctata* through sexual hybridization was reported (Jena, 1994). However, transfer of genes from sexually incompatible species to *O. sativa* and from wild species having genome other than AA to *O. sativa* through conventional breeding,

is difficult to achieve due to the low crossability and limited recombination between chromosomes of the different partners. Alternatively, somatic hybridization may provide a means of overcoming sexual incompatibility between the cultivars and wild species (Jelodar, 1996).

1.3.5. Enhancement in grain quality and nutritional value

Nutritive quality plays an important role where rice is principle source of dietary protein (Juliano and Villareel, 1993; Swaminathan, 1998). The nutritional aspect of rice becomes an important consideration in the context of improvement, because it supplies most of the energy and protein for the dependent population (Bajaj, 1991). Rice provides about 40% of the protein in the Asian diet. Among the cereal proteins, rice protein is considered the richest biologically by virtue of its high digestibility (80%), high lysine content and relatively better net protein utilisation. Yet, it is nutritionally handicapped due to its inherently low protein content (6-8%) and inevitable milling loss of as much as 15-20% (Swaminathan, 1998). More importantly, milling removes essential amino acids such as cysteine, methionine, lysine, also vitamin B1, and minerals (Fe, Ca) which are largely located on the outer layer of the endosperm. Consequently, there is an urgent need for improving quality of the grain protein so that the people consuming rice are supplied with adequate amount of protein. Breeding for high protein content undertaken at IRRI, India and other countries has so far met with limited success (Swaminathan, 1998). Complex mode of inheritance, non-linear relationship between yield and protein content and proneness to profound environmental conditions is

responsible for this slow progress. There may be two ways to minimise the losses in milled rice viz., (i) exploiting genetic variation in the distribution pattern of protein bodies, and (ii) differential resistance of outer layers to scrapping. Among several factors, thickness of aleurone layer seems to determine the level of milling loss (Pehu and Siddiq, 1986). Once their genetics and response to selection are established, breeding for 'Low protein loss' may become feasible (Swaminathan, 1998). Raj?

1.3.6. Limitations of conventional breeding methods

Achieving high yields had been the research priority in the sixties and seventies, whereas stability of yield performance became the thrust in seventies. Nearly all the high yielding varieties available then were vulnerable to a host of insect-pests and diseases and none was adapted to abiotic stresses of all kinds (Paroda, 1998). As we march into the next millennium, the world would face the most challenging task of sustaining even the current level of rice availability. Success in achieving the goals would depend on exploitable genetic variability for the desired traits that directly or indirectly contribute to the enhancement of yield and its stability. Primary and secondary gene pools have been the major sources of variability for breeders to make use off. Induced mutation is yet another potential but least exploited source of variability. However, there is a lack of genetic variability in cultivated rice and, in many cases, variability in the cultivated species for important economic traits is limited (Khush and Brar, 1991). However, at the same time, the wild rice germplasm has been found to constitute a rich reservoir of useful genes for varietal improvement. In this

case, related wild species can be used as a source of useful genes and offer great potential to incorporate such genes into commercial rice cultivars for resistance to major diseases, insects and tolerance to various abiotic stresses. However, crossability barriers limit the gene transfer from wild species to cultivated species. One of the most important barriers is the limited recombination among homologous chromosomes of cultivated rices and wild species and the introgression is poorly understood (Brar and Khush, 1997). The major problems in conventional rice breeding are summarized as follows:

- i) The rice breeders have to conduct breeding programs with the primary gene pool of the species.
- ii) The breeders are always confined to naturally occurring genetic variability
- iii) There is a very limited variability in the cultivated species.
- iv) Although wild species are an important reservoir of useful genes but transfer of these genes from wild species to cultivated species is very limited. It is due to several incompatibility barriers such as crossability and limited recombination between chromosomes of wild and cultivated species.
- v) The time taken to develop a new variety by conventional method is always long and time consuming. For instance, in a successful hybrid seed production programme, approximately 6-8 repeated back-crossings are necessary which requires 5-6 years for developing a new male sterile line.

vi) Development of improved rice cultivar, which can withstand diseases, insects and abiotic stresses for the adverse environmental conditions still, remains a major challenge to rice breeders.

vii) The extent of land required for conventional breeding methods is also very large.

These limitations of the conventional breeding method necessitate the use of biotechnology and plant molecular biology in rice improvement.

1.3.7. Biotechnological approaches to rice improvement

In recent years, much attention has been directed to the newly emerging and invents of novel technologies in molecular and cell biology known as biotechnology. Advances in plant cell culture research, especially of major crop species, have played an increasingly critical role in the development of modern plant biotechnology (Vasil, 1990). In comparison to conventional methods, which aim to expand genetic variation through gene recombination with sexual reproduction, this technology enables to use much wider variation by overcoming the inter-specific barrier. More specifically, this method can transfer a character (or a gene) from an entirely different species or genus, thereby reveling out serious difficulties existing in sexual reproduction. The application of tissue culture and genetic engineering in rice crop improvement will be discussed in this section with special emphasis on mitochondrial gene transfer, for the production of cytoplasmic hybrids in rice.

1.4. Development of tissue culture systems for rice

1.4.1. General status of cereal tissue culture

Gottlieb Haberlandt in 1902 developed the idea of totipotency in plant cells (Haberlandt, 1902). According to his idea, plant cells are automatic in function, and in principle, are capable of regenerating to produce a complete plant. This scientific proposition has since become a basic principle in studies pertaining to plant tissue culture. The successful regeneration of plants from single cell and tissues cultured *in vitro* caused considerable upsurge of interest and activity in the possible combined uses of plant cell culture and modern molecular genetic techniques in the improvement of crop plant. The first successful tissue culture among cereals was reported from maize endosperm by La Rue in 1949 (La Rue, 1949). Fertile, diploid maize plants were first regenerated by Green and Phillips in 1975 from callus derived from the scutellum of immature embryos (Green and Phillips, 1975). Norstog succeeded with barley embryo culture in 1961 (Norstog, 1961). In 1973, the first barley plants were regenerated *in vitro* from anther cultures (Clapham, 1973). The importance of corrects nutritional and hormonal conditions were recognized by Skoog and Miller in 1957 and have influenced the techniques used since then (Skoog and Miller, 1957). Ry?

The modern work on tissue culture of cereals is based on, some important discoveries made in 1980-81 (Haydu and Vasil, 1981; Lu and Vasil, 1981; Vasil and Vasil, 1980). They were, the use of (i) meristemetic tissues (mature embryos, young inflorescence, immature leaf bases (at defined stages of development) as explants and (ii) nutrient media supplemented with a strong auxin, usually 2,4-dichlorophenoxy acetic acid. ? 4/2

These findings, led to the (i) establishment of long term callus cultures in which plant regeneration takes place via somatic embryo formation and (ii) establishment of cell suspension culture derived from embryogenic calli that yield totipotent protoplasts. These pioneering works show the ability to control organogenesis from various cereal callus, laid a foundation for subsequent advances in cereal cell and tissue culture and application of these techniques to plant breeding and genetic engineering of plants. Since then, an established plant regeneration systems has been reported for all major crops including wheat, rice, maize, barley, sorghum, sugarcane, oats, millet and few grass species under the family Gramineae (Srinivasan and Vasil, 1986; Chen *et al.*, 1988; Harris *et al.*, 1988; Prioli and Sondhall, 1989; Yamada, 1989; Roest and Gilssen, 1989; Hartke and Lörz, 1989; Lynch *et al.*, 1991; Yan *et al.*, 1990; Ghosh- Biswas *et al.*, 1994; Aftab *et al.*, 1996; Ahmed *et al.*, 1997; Southgate, 1996; Singh *et al.*, 1997; Elkonin *et al.* 1995; Bekele *et al.*, 1995; Khanna and Raina, 1997, Azhakanandam, 1999).

1.4.2. General status of rice tissue culture

The tissue culture of rice was initiated in the mid-1950s with culture of excised rice roots (Fujiwara and Ojima, 1955) and immature rice embryos (Amemiya *et al.*, 1956). Regeneration of rice plants was subsequently reported from root-derived callus (Kawata and Ishihara, 1968). In the same year, shoots were successfully obtained from callus initiated from rice embryos (Tamura, 1968). Since the early report of production of haploid rice plants from anthers (Niizeki and Oono, 1968), anther culture has been

widely employed in rice breeding. Since then, different useful technologies such as plant regeneration from suspension cultures (Lieb *et al.*, 1973; Abe and Futsuhara, 1986) and plant regeneration from protoplast culture has been developed for rice (Fujimura *et al.*, 1985; Yamada, 1989; Abdulla *et al.*, 1986; Kyoizuka *et al.*, 1987; Toriyama *et al.*, 1986; Masuda *et al.*, 1989; Jenes and Pauk, 1989; Li and Murai, 1990; Meijer *et al.*, 1991b; Jain *et al.*, 1995; Bhattacharjee and Gupta, 1995, Bhattacharjee *et al.*, 1998).

1.4.2.1. Callus induction and plant regeneration of rice

1.4.2.1.1. Embryo culture

Embryo culture is a technique in which scutella of immature and mature embryos excised from the ovules are aseptically raised in a liquid or semi-solid medium. Embryo culture was not applied to rice until the beginning of the 1950s. Amemiya *et al.* (1956) initiated studies pertaining to the immature embryo culture of rice plants. However, the use of the scutella of mature and immature embryos as explant sources was a breakthrough in cereal tissue culture, and is widely used even today to initiate callus cultures and plant regeneration from many rice varieties and other cereals. Cultured cells differ in properties among themselves. Heyser *et al.* (1983) classified the calli into two types: embryogenic and non-embryogenic. Embryogenic calli can regenerate a completely fertile plant, while non-embryogenic calli occasionally bear buds but can not bear any shoots. These two types of calli could be distinguished morphologically from each other. Embryogenic calli consist of small meristematic cells and produce many plants through somatic embryogenesis while the non-embryogenic calli consists of long

tubular and sometimes vacuolated cells which produce few or no plants (Siriwardana and Nabors, 1983). Embryogenic calli maintain their competence for long periods of time and give rise to genetically uniform and normal plant population. In rice, there are several reports on callus induction from immature embryos (Lai and Liu, 1976; Lai and Liu, 1982; Peng and Hodges, 1989; Wu and Zapata, 1992; Bhattacharjee and Gupta, 1995, Bhattacharjee *et al.*, 1998) and mature embryos (Maeda, 1967; Bajaj, 1991; Kavi Kishor and Reddy, 1986a; Yasuda *et al.*, 1990; Peterson and Smith, 1991; Datta *et al.*, 1992; [?]Torrizo and Zapata, 1992; Chowdhry *et al.*, 1993; Rance *et al.*, 1994; ²Bhattacharjee and Gupta, 1995; Bhattacharjee *et al.*, 1998; Azhakanandam, 1999. *aob?* Bhattacharjee *et al.*, 1999). Scutellum cells of immature embryos were found most responsive explants for tissue culture in rice. However, mature embryos are also suitable for tissue culture due to their availability throughout the year (Peterson and Smith, 1991; Datta *et al.*, 1992; [?]Torrizo and Zapata, 1992; Chowdhry *et al.*, 1993; Rance *et al.*, 1994; Bhattacharjee and Gupta, 1995) and provided that a high frequency of regeneration can be achieved (Rueb *et al.*, 1994). *aob?* The immature organs and meristematic tissues, which contain undifferentiated cells, are known to be more suitable for plant regeneration (Morrish *et al.*, 1987).

1.4.2.1.4. Plant regeneration through somatic embryogenesis and organogenesis

Somatic embryogenesis is the predominant mode of regeneration in cereals (Vasil, 1994). Both Japonica and Indica rice varieties can be regenerated from callus initiated from various parts of the plant through somatic embryogenesis (Rueb *et al.*, 1994; Rout

and Lucas, 1996) and organogenesis (Suzuki *et al.*, 1993). Plantlet regeneration through somatic embryogenesis has been reported in many rice varieties (Heyser *et al.*, 1983; Siriwardana and Nabors, 1983; Abe and Futsuhara, 1986; Wang *et al.*, 1987; Hartke and Lorz, 1989; Poonsapaya *et al.* 1989; Choudhry *et al.*, 1993; Rance *et al.*, 1994; Rueb *et al.*, 1994; Adkins *et al.*, 1995; Bajaj and Rajam, 1995; Khanna and Raina, 1998; Azhakanandam, 1999). Efficient plant regeneration, via somatic embryos, in rice depends on various factors including, genotype (Abe and Futsuhara, 1986; Hartke and Lorz, 1989; Choudhry *et al.*, 1993; Khanna and Raina, 1998; Azhakanandam, 1999), manipulation of medium (Siriwardana and Nabors, 1983; Bajaj and Rajam, 1995; Azhakanandam, 1999), carbohydrate source (Ghosh Biswas and Zapata, 1993), partial desiccation of callus (Rance *et al.*, 1994) and dehydration of callus using high concentration of gelling agent in the regeneration media (Azhakanandam, 1999). The addition of organic additives such as L-tryptophan, L-proline (Siriwardana and Nabors, 1983; Chowdhry *et al.*, 1993), abscisic acid (Peterson and Smith, 1991; Xu *et al.*, 1995), spermidine (Bajaj and Rajam, 1995), cytokinins such as 6-benzylaminopurine (BAP) and kinetin (Lee *et al.*, 1989; Datta *et al.*, 1990; 1992) to regeneration medium - all *as above?* potentially influence plant regeneration from rice callus. In rice, the interaction of plant phyto-hormones, such as 2,4-D and kinetin, is considered a key to induction of embryogenic callus and plant regeneration from such tissues (Peterson and Smith, 1991; Mitsuoka *et al.*, 1994). Somatic embryogenesis is useful, because the somatic embryos, like their zygotic counterparts, are directly or indirectly derived from single cells and therefore are non-chimeric in nature and possess a root-shoot meristem which is vital for

survival in soil. Plants derived from somatic embryos maintain uniformity and genetic fidelity (Vasil, 1995).

Improved plant regeneration through organogenesis has also been reported from rice callus tissues (Kawata and Ishihara, 1968; Nakano and Maeda, 1979). Osmotic stress, caused by a simple treatment like partial desiccation of calli, promotes organogenesis in calli and leads to a higher regeneration ability in calli (Tsukahara and Hirose, 1992; Rance *et al.*, 1994).

(1989)

1.4.2.1.5. Somaclonal variation in tissue culture-derived plants

Somaclonal variation is an important phenomenon observed in plants regenerated from tissue culture and can be utilized as a tool by plant breeders. Regeneration of plants through tissue culture should normally result in clones that are phenotypically and genetically identical to the progenitor plants. In many cases, however, regenerants exhibit deviation from the parental type (Brown *et al.*, 1991). This phenomenon is widespread in a range of plant species and defined as somaclonal variation (Larkin and Scowcroft, 1981). This topic has been extensively reviewed by several workers (Sun and Zheng, 1990; Peschke and Phillips, 1992; Ahmed and Sagi, 1993; Phillips *et al.*, 1994; Skirvin *et al.*, 1994; Karp, 1995). This variability (somaclonal variation) can however, be utilized for the development of new varieties Somaclonal variation has been observed in many cereal species such as wheat (Karp and Maddock, 1984; Cooper *et al.*, 1986), maize (Brettel *et al.*, 1986), sorgham (Bhaskaran *et al.*, 1987) and rice (Adkins *et al.*, 1990; 1995).

Nishi *et al.* in 1968 first reported the phenotypically variant plants of rice originating from seed-derived callus, including dwarf and twisted plants. Various factors such as cytoplasmic or nuclear mutation (Evans and Sharp, 1983), polyploidy and other chromosomal abnormalities (Ahloowalia, 1882; Orton, 1983), may be the reason for the origin of somaclonal variation. The variation in plants regenerated from haploid tissue is comparatively high (Lynch *et al.*, 1991). Male sterile somaclones have been identified from the Indica varieties IR24 and IR54 (Ling *et al.*, 1988). Zhang *et al.* (1989) analyzed plants regenerated from seed-derived callus of four Chinese varieties. Rice calli have also been screened for resistance to phytotoxin (Ling *et al.*, 1986). More recently, Azhakanandam (1999) has reported the characterization of somatic embryo- and seed-derived plants of the aromatic Indica rice, Pusa Basmati 1. Commonly observed variations in tissue culture-derived plants includes the number of tillers per plant, plant height, flag-leaf length, heading date, panicle length, fertility, and a number of seeds produced (Oono, 1978, 1981, 1985; Sun *et al.*, 1981, 1983; Schaeffer *et al.*, 1984; Murai and Kinoshita, 1986; Azhakanandam, 1999). Ry?

1.4.2.1.6. Cell suspension culture

Lieb *et al.* (1973) were the first to report suspension cultures of rice from root callus using modified Heller's medium (Heller, 1953). Since then, several basal media, including R2 (Ohira *et al.*, 1973), MS (Murashige and Skoog, 1962), LS (Linsmaier and Skoog, 1965), B5 (Gamborg *et al.*, 1968), CC (Potrykus *et al.*, 1977), N6 (Chu *et al.*, 1975) and AA (Muller and Grafe, 1978) have been used to initiate suspension cultures

from various calli derived from different explants of many varieties of rice. R2 medium, has more often been used for Indica rices (Torrizo and Zapata, 1992; Ghosh Biswas and Zapata, 1993; Jain *et al.*, 1995) while AA medium, which contains four different amino acids as source of carbon, has been used for the initiation of Japonica rice suspension cultures (Abdulla *et al.*, 1986; Guiderdoni and Chair, 1992; Su *et al.*, 1992). The nitrogen source of the medium also plays an important role in the initiation and establishment of suspension culture in rice (Toriyama and Hinata, 1985; Thompson, 1986). However, the establishment of embryogenic cell suspension cultures seems to be under the control of the plant genotype.

To establish suspension culture of rice, different explants such as root, anther, leaf base, immature panicle, stem base, immature and mature embryos are used (Abe and Futsuhara, 1991). Friable embryogenic calli are commonly used as source for the initiation of suspension cultures in rice. Suspension cultures are initiated with a small amount of embryogenic calli in a suitable liquid medium. They are maintained on a shaker in the dark and subcultured with regular interval to increase highly dispersed fine and rapidly proliferating cells rich in cytoplasmic substances. A fine embryogenic cell suspension consists of small clumps composed of 10-20 cells, each actively proliferating, densely cytoplasmic with minimum number of highly vacuolated cells (Abe and Futsuhara, 1991). To maintain the embryogenicity of the cells they are subcultured at 3 to 7 days, with regular interval. Once established, these cultures exhibit a rapid growth with cell doubling time of 27 to 32 hours (Karlson and Vasil, 1986). A

significant feature of an established rice suspension culture is that it appears to possess a higher percentage of totipotent cells than other explants or callus (Hodges *et al.*, 1991). Plantlets regeneration, directly from suspension cells, have been reported in Japonica as well as Indica rice (Abe and Futsuhara, 1991; Binh *et al.*, 1992; Ghosh Biswas and Zapata, 1992; Tsukahara *et al.* 1992). Plant regeneration has been achieved from long term suspension cultures initiated from root-derived (Zimny and Lörz, 1986; Abe and Futsuhara, 1986; and seed-derived (Ye, 1984; Honda *et al.*, 1992; Kobayashi *et al.*, 1992) embryogenic calli of Japonica rice. Abe and Futsuhara (1991) reported that the regeneration capacity of embryogenic cell suspensions of Japonica varieties, in culture, was higher than for Indica, Indica-Japonica hybrids and Javanica types. Ozawa and Komamine (1989) established a system of high-frequency embryogenesis from long-term cell suspension cultures initiated from excised immature embryos of rice. In rice, embryogenic cell suspension cultures have been used as the main source material for the enzymatic isolation of protoplasts (cells without cell wall). The suspension cells are also amenable to *Agrobacterium*-mediated transformation (Hiei *et al.*, 1994). However, the establishment and maintenance of embryogenic suspension cultures particularly of Indica rice varieties, presents difficulties. It has been observed that the morphogenic competence of suspensions usually declines with successive subculture over prolonged period of time (Abe and Futsuhara, 1991). Suspension cultures of cereals lose their morphogenic potential in about 6 months (Jahne *et al.*, 1991) to 12 months (Shillito *et al.*, 1989) and tend to accumulate genetic changes (Chang *et al.*, 1991; Wang *et al.*, 1992; Karp, 1993) during prolonged culture. To overcome this

problem, competent cultures can be preserved over liquid nitrogen (cryopreservation) for ensuring a constant supply of suspension cells over the most responsive state later (Krautwig and Lörz, 1995).

1.4.2.1.7. Protoplast culture

A fundamental requirement for nearly all applications in biotechnology is the regeneration of whole plants from cells/tissues or protoplasts cultured *in vitro*. Due to the fact that protoplast represents a true single cell system, they offer a valuable baseline system for plant genetic manipulation. They are also used for various fundamental studies, such as membrane transport, metabolic regulation and studies on gene expression after gene transfer by direct DNA delivery into protoplasts (Krautwig and Lörz, 1995). Protoplasts are the versatile tool for genetic manipulation of plant cells. Reproducible plant regeneration from protoplasts is therefore, an essential prerequisite for practical application of the techniques like direct DNA uptake, somatic hybridization and cytoplasmic hybridization.

The first report of enzymatic isolation of protoplasts (Cocking, 1960) offered a novel and promising technique to the plant cell and tissue culture. It open a new area of research and protoplasts can now be obtained from nearly all plants tissues and cultured cells. Many studies were carried out on rice protoplasts (Deka and Sen, 1976; Lai and Liu, 1982, Wakasa *et al.*, 1984). Although formation of callus from protoplasts of rice has reported in some of these studies, but none of them succeed in regenerating plants from the calli derived from rice protoplasts. The first successful plant regeneration from

protoplasts was reported from Japonica rice (Fujimura *et al.*, 1985) and since then, a reproducible system has been developed from protoplasts of Japonica, Indica and Javanica rices.

In contrast to most dicotyledonous species, where the protoplasts isolated even from mature tissues have the ability to dedifferentiate and to re-enter the cell cycle, the majority of monocotyledonous plants, which include the most important crop species seem to be recalcitrant to tissue culture, especially protoplast culture. Later, it was reported that cultured cells provides the best source of protoplasts with division capacity in the family Gramineae (Thompson, 1986; Rueb *et al.*, 1994). However, due to the difficulties involved in the culture of protoplasts isolated directly from the plant tissues, embryogenic calli derived from mature and immature scutella and leaf base-derived calli were used as a source material for initiation of cell suspension.

Since 1985, substantial progress has been made in plant regeneration from protoplasts of Japonica, Indica and Javanica rices where mostly embryogenic cell suspensions have been utilized as a source material for the isolation of large number of totipotent protoplasts. Plant regeneration from the protoplasts of Japonica rice has been successfully accomplished for a range of varieties (Fujimura *et al.*, 1985; Yamada *et al.*, 1986; Abdulla *et al.*, 1986; Kyozyuka *et al.*, 1987; Toriyama *et al.*, 1986; Masuda *et al.*, 1989; Jenes and Pauk, 1989; Li and Murai, 1990; Meijer *et al.*, 1991b; Jain *et al.*, 1995; Bhattacharjee and Gupta, 1995). Indica rice varieties are the most widely cultivated in the tropical regions of the world, and are the most important food source for half of the world's population. Hence, attention has also been focused on the improvement of these

varieties using biotechnology including protoplast to plant regeneration systems. In general, it is difficult to culture and regenerate plants from the protoplasts of Indica rices. Since Kyoizuka *et al* (1989) established a protocol for regeneration of Indica rices using nurse culture methods, a number of researchers have reported plant regeneration from Indica rices. Most of them have utilized embryogenic cell suspensions derived from embryogenic callus obtained from scutellum of immature embryos (Lee *et al.*, 1989; Lee and Kim, 1991; Su *et al.*, 1992; Yin *et al.*, 1993), mature seeds (Kyoizuka *et al.*, 1988; Wang *et al.*, 1989; Torrizo and Zapata, 1992; Datta *et al.*, 1992b; Ghosh Biswas and Zapata, 1993; Timothy and Rangaswamy, 1993; Jain *et al.*, 1995; Ramaswamy *et al.*, 1995) and anthers (Datta *et al.*, 1990a; Ghosh Biswas and Zapata, 1991). There have been few reports on culture and plant regeneration of Javanica rice protoplasts. Plantlets have been regenerated from protoplasts of Javanica rices using embryogenic cell suspensions, initiated from calli obtained from scutellum of mature seed (Wang *et al.*, 1989; Suh *et al.*, 1992) and immature embryos (Suh *et al.*, 1992) and also from immature panicles (Li *et al.*, 1992; Utomo *et al.*, 1995).

In order to avoid difficulties, time and labour involved in the establishment of cell suspensions and variations due to prolonged *in vitro* culture in the regenerated plants; protoplasts derived from materials other than cell suspensions is clearly of considerable interest. Successful plant regeneration has been achieved from protoplasts isolated directly from rice immature embryo-derived callus (Wu and Zapata, 1992), scutellar tissues of immature zygotic embryos of rice (Ghosh Biswas *et al.*, 1994) and mesophyll tissues of leaf base and sheath explants (Gupta and Pattanayak, 1993). However, the

fact that protoplast yield from intact tissues and plating efficiency were comparatively low or that protoplasts could not be reproducibly induced to regenerate plants (Ghosh Biswas *et al.*, 1994a), has prevented these options from being adopted as routine source material for protoplasts isolation and culture. ? Ref

Success in rice protoplast culture depends on many variables. This includes genotype, explant source, age of cell suspension, medium composition, culture conditions, use of nurse culture and selection of carbon source in plant regeneration media (Abdulla *et al.*, 1986; Kyoizuka *et al.*, 1987; Li and Murai, 1990; Datta *et al.*, 1990; Hodges *et al.*, 1990; 1991; Su *et al.*, 1992; Torrizo and Zapata, 1992; Ghosh Biswas and Zapata, 1993). a co p? Ref

The effect of pre-culture treatment on rice protoplasts, in the term of heat shock (at 45°C, 5 min followed by 20 sec on ice or 45°C for 8 min followed by 10 sec in ice water), to improve plating efficiency has been reported by Thompson *et al.*, (1987) and Lin M-Yu *et al.*, (1991) respectively. ?

The use of feeder cells has greatly influenced protoplast culture in cereals (Rhodes *et al.*, 1988; Shillito *et al.*, 1989; Prioli and Sondahl, 1989; Funatsuki *et al.*, 1992; Krautwig and Lörz, 1995). Feeder cells from several species have been used to support cell division from cultured protoplasts of Indica and Japonica rices. This includes, cultivated rice species (Lee and Kim, 1991; Wen *et al.*, 1991; Su *et al.*, 1992; Guiderdoni and Chair, 1992; Timothy and Sree Rangasamy, 1993, Bhattacharjee and Gupta, 1995), wild rice species, *O. ridleyi* (Torrizo and Zapata, 1992; Jain *et al.*, 1995) and allied graminaceous plants e.g *Panicum maximum*, *Lolium multiflorum*, *Panicum serobiculatum* (Timothy and Sree Rangasamy, 1993; Jain *et al.*, 1995). Two different 9

types of nurse culture methods have been established using feeder cell system and adopted by several researchers. In one method, protoplasts are suspended in agarose-solidified protoplast culture medium inside 'donut' holes surrounded by nurse as established by Kyozuka *et al.*, (1987; 1988). In the second method, protoplasts are suspended in agarose-solidified protoplast culture medium and plated on the surface of a filter membrane positioned on the top of agarose-solidified feeder cells embedded as nurse as reported by Lee *et al.*, (1989). In the second method, membrane filters (0.8 μ m) were used as a partition between the nurse cells and protoplasts (Wen *et al.*, 1991; Su *et al.*, 1992; Guiderdoni and Chair, 1992; Torrizo and Zapata, 1992; Ghosh Biswas and Zapata, 1993; Timothy and Rangasamy, 1993; Bhattacharjee and Gupta, 1995, Jain *et al.*, 1995).

The presence of feeder cells in the protoplast culture of Japonica rice varieties improved protoplast to plant regeneration system by increasing plating efficiency and plant regeneration frequency. It was demonstrated that in some Indica varieties feeder cells were necessary to induce protoplast-derived colony formation and plant regeneration (Bhattacharjee and Gupta, 1995; Jain *et al.*, 1995).

Several workers (Kyozuka *et al.*, 1988) have done extensive work on the effect of different sources of carbohydrate on plant regeneration of rice. The use of maltose instead of sucrose, as a carbon source, is an important modification in plant regeneration medium that enhances frequency of plant regeneration (Torrizo and Zapata, 1992; Ghosh Biswas and Zapata, 1994; Jain *et al.*, 1995).

It has been reported that the water content of rice callus tissue is an important factor in relation to plant regeneration. A poorly regenerating callus can be changed to a vigorously regenerating one by exposure to water stress (Liu and Lai, 1991; Binh *et al.*, 1992; Tsukahara and Hirosawa, 1992).

1.4.2.1.8. Protoclonal variation

The development of a protoplast-to-plant regeneration system in rice has led to the generation of protoclonal variation in protoplast-derived plants (protoclones) and their seed progeny (Lynch *et al.*, 1991). ^{over} Variations in agronomic characteristics were ^{as ob?} observed among protoclones of rice. In rice protoclones, changes in agronomic characters, such as heading time, plant height, number of tillers per plant, number of fertile tillers, days to maturity, panicle length, grain size (Abdullah *et al.*, 1989; Sukekiyo and Kimura, 1991; Mezencev *et al.*, 1995), chlorophyll content of plants (Guiderdoni and Chair, 1992) and ploidy level have been reported (Guiderdoni and Chair, 1992; Kawata *et al.*, 1992). ^{a)}

Among protoplast derived rice plants, variation was also found for nuclear DNA content (Brown *et al.*, 1990). The degree of variation was lower in many lines of protoplasts regenerants than in callus-derived plants (Müller *et al.*, 1990). ⁱⁱ⁾ Somaclonal, protoclonal and molecular variations have been reported to exist in the progeny of plants regenerated from both callus and protoplast-derived tissues of rice (Abdullah *et al.*, 1989; Zheng *et al.*, 1989; Saleh *et al.*, 1990; Müller *et al.*, 1990; Davey *et al.*, 1991).

1.4.3. Utilization of protoplast technology

1.4.3.1. Transformation: Direct gene transfer to rice protoplasts

Genetic transformation is one of the most versatile tools in modern biology. In higher plant, genetic transformation was first attained by the use of a natural gene transfer system possessed by *Agrobacterium tumefaciens* (Schell, 1987). Unfortunately, Graminaceous species including rice, wheat, maize and barley were thought to be outside the host range for *Agrobacterium* infection and therefore, alternative methods like direct DNA transfer had to be devised for transformation of these plant species. Because of lack of rigid cell wall, protoplasts are excellent recipients of a variety of macromolecules including foreign DNA. It was established that DNA taken up by protoplasts could be stably integrated into the host chromosome. Gene transfer by direct delivery of DNA into protoplasts has been successful either through chemical treatment with polyethelene glycol (PEG) or through electroporation.

1.4.3.1.1. Polyethelene glycol induced gene transfer

Incubation of protoplasts with long chain cations, such as poly-L-arginine, poly-D-lysine and polyethelene glycol (PEG) can induce protoplasts to take up foreign DNA from the medium. Since the first reported use of PEG to introduce plastids into tobacco protoplasts (Krens *et al.*, 1982), this agent has been extensively used to mediate plasmid uptake into a number of protoplast systems, including rice (Davey *et al.*, 1989). Since then many rice varieties have been transformed using this method (Datta *et al.*, 1990b;

Zhang *et al.*, 1991; Peng *et al.*, 1992; Datta *et al.*, 1992a; Xu *et al.*, 1993; Ayres and Park, 1994; Ghosh Biswas *et al.*, 1994a; Cornejo *et al.*, 1995; Lee, 1996). Ref?
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1.4.3.1.2. Direct gene transfer via electroporation

Electroporation-mediated gene transfer has been widely utilized for transformation of plant protoplasts although it was developed for animal cell transformation (Neumann *et al.*, 1982). Electroporation-mediated gene transfer into protoplasts has been reported in rice by several researchers throughout world (Shimamoto *et al.*, 1989; Yang *et al.*, 1988; Zhang *et al.*, 1988; Battraw and Hall, 1990; Tada *et al.*, 1990; Schuh *et al.*, 1993; Ayres and Park, 1994; Chaudhury *et al.*, 1994; Lee, 1996; Bhattacharjee *et al.*, 1997). Ref?
Ref.?

1.4.3.1.3. Limitations of protoplast-mediated transformation

Establishment and maintenance of embryogenic suspensions is technically difficult with many Indica rices as the morphogenic competence decline during prolonged sub-culture (Abe and Futsuhara, 1991). Moreover, direct DNA uptake methods often result in multiple copies of genes incorporating into the genome, rearrangement and fragmentation of genes, high frequency of sterile plants (Flavell, 1994) and non-Mendelian inheritance of transgenes (Peng *et al.*, 1995; Aldemita and Hodges, 1996). With the recent development of *Agrobacterium tumefaciens* - mediated transformation in rice these problems in direct DNA transfer can be avoided (Chan *et al.* 1992; Chan *et al.* 1993; Hiei *et al.*, 1994; Rashid *et al.*, 1996; Aldemita and Hodges, 1996; Dong *et al.*, 1996; Cheng *et al.*, 1998; Azhakanandam, 1999, Khanna and Raina, 1999).

14.3.2. Protoplasts fusion

Sexual hybridization between closely related species has been used for years to improve cultivated crops. Introduction of new traits has been based mainly on sexual crosses between different genotypes within or between closely related species. However, due to the presence of various reproductive barriers, gene transfer has been restricted to sexually compatible species or at best to a few wild species closely related to cultivated crop and thus limiting the possibilities of modifying and improving crop plants (Waara and Glimelius, 1995). In most of the cases, many desirable and agronomically interesting traits were found only in distantly related species or even in unrelated species. Since they constitute a genetic resource potential, considerable efforts have been made to identify and isolate these genes and transfer them into crops. Through the rapid development of somatic cell genetics, methods now exist for transferring genes across sexual barriers and over taxonomic distances. Wherever interesting genes have been identified and isolated, they can be transferred through transformation, but for most traits the genes have not yet been identified. Somatic cell fusion might be a useful tool in overcoming the limitation of sexual hybridization because a cluster of genes can be transformed through this method.

Fusion of somatic cells in an original form is impossible in plant species because plant cells have a cell wall consisting of cellulose and are joined to each other with pectin. However, when cell wall is removed from the cell (mechanically or enzymatically), fusion of these somatic cells (known as protoplasts) permits hybridization of both sexually compatible and incompatible species (somatic hybridisation). This is

accompanied with fusion of cytoplasmic organelles like mitochondria and chloroplasts between species (cytoplasmic hybridization). Kuster (1909) was the first to point out the possibility of producing somatic hybrids through protoplast fusion and made the early attempts in this direction. However, at that time no methods were available to provide large number of protoplasts for the fusion experiments. Therefore, the experiments to establish a technique for the production of hybrids from somatic cells remained unsuccessful. With development of the method of enzymatic isolation of protoplasts (Cocking, 1960; Takabe^{et al.}, 1968) and their subsequent regeneration into plants (Vasil and Vasil, 1980), made protoplast system available for somatic hybridization. Since the first report on protoplast fusion-derived somatic hybrid between *Nicotiana tabacum* x *N. langsdorfi* (Carlson *et al.*, 1972), several successful experiments were made on (i) somatic hybridization (to produce different combination of somatic hybrids) and (ii) cytoplasmic hybridization (to transfer cytoplasmic traits like cytoplasmic male sterility) in several plant families through protoplast fusion. Ry2

1.4.3.2.1. Somatic hybridization

One of the most important practical uses of protoplast culture is the somatic hybridization, which has opened up new possibilities for the parasexual hybridization of plants. Somatic hybridization permits nuclear and organelle genomes to be inherited biparentally whereas sexual hybridization allows only the nuclear genomes to be inherited biparentally and organelle genomes are inherited uniparentally. When the trait of interest is present in a species that is sexually incompatible with the other species,

transfer of this trait is achieved by somatic hybridization through fusion of protoplasts. Technically, there are no limitations in the fusion of protoplasts of different species. The absence of cell wall enables the isolated protoplasts to be fused chemically, electrically or by combination of these two processes. Therefore, somatic hybridization is a unique tool for the transfer of useful genes and traits between plants to create a novel source of germplasm.

In rice, sexual crossing between Indica and Japonica varieties frequently results in sterility in the F1 (Ikehashi and Araki, 1986), and weak plants in the F2 (Oka, 1988). Due to non-availability of reproducible protoplast-to-plant regeneration system and the low frequency of plant recovery from protoplasts, successes in the production of somatic hybrids in rice has been limited for quite sometime. The first somatic hybrid plants of rice and barnyard grass (*Echinichloa oryzicola* Vasing) were reported by Terada *et al.* (1981). Earlier somatic hybrid callus formation of *Oryza sativa* x *Glycine max* and *Oryza sativa* x *Daucus carota* was reported by Niizeki *et al.* (1985) and Sala *et al.* (1985) respectively. Following the establishment of successful plant regeneration protocol from rice protoplasts interspecific somatic hybrids between cultivated rice and wild species was reported by Hayashi *et al.* (1988). Later, the use of electrofusion in obtaining somatic hybrids in rice was demonstrated by several workers, e.g. diploid somatic hybrid plants between two *O. sativa* cvs. Murasakidaikoku and Yamahoushi (Toriyama and Hinata, 1988); putative hybrid plants between two *O. sativa* varieties (Lee and Kameya, 1989); heterokaryons between *O. sativa* and *Porteresia coarcta* (Finch *et al.*, 1990); hybrid plants between *O. sativa* and *Lotus corniculatus* L. (Nakajo

see
7 Ref.

et al., 1994); assymetric hybrid plant between *O. sativa* and *Daucus carota* (Kisaka et al., 1994) and somatic hybrid plant between *O. sativa* and *Porteresia coarctata* (Jelodar, 1996).

1.4.3.2.2. Cytoplasmic hybridization

Another important application of protoplast fusion technology is cytoplasmic hybridization that is used when the DNA of the cytoplasmic organelles encodes the trait of interest. Higher plant cell contains apart from others, two cytoplasmic organelles, viz. plastids and mitochondria. These organelle possess relatively small genomes compared to the nucleus. This DNA codes for approximately 10% of the polypeptides necessary to ensure organelle functioning and the remaining 90% are encoded by nuclear genes and imported into cytoplasm through the membrane of the organelle. Cytoplasmically encoded traits are usually inherited maternally through female parent in higher plants. A few traits encoded by cytoplasmic organelles have been reported, such as herbicides resistance, non-chromosomal stripe mutants of maize, T-toxin sensitivity of T (Texas) male sterile cytoplasm in maize and cytoplasmic male sterility (Vasil I.K., 1982; Leaver and Gray, 1982; Levings, 1983; EcKenrode and Levings, 1986; Newton and Coe, 1986; Londsdale, 1987).

CMS is a characteristics which can be used to produce stable male sterile lines for a successful hybrid rice breeding programme. Mitochondrial DNA determination of CMS is well-documented (Srivastava and Gupta, 1990; Leaver and Gray, 1982). In conventional breeding method, an intensive and laborious backcrossing is needed to

introduce CMS in the desired species to produce a new CMS line. To bypass the backcrossing, direct transfer of isolated mitochondria or mitochondrial genomes encoding CMS trait in the fertile plant would be of great significance. Cytoplasmic hybridization or hybridization, involving a donor recipient protoplast fusion system has been developed in several dicotyledonous species viz *Nicotiana* (Zelcer *et al.*, 1978), *Brassica* (Menczel *et al.*, 1987), *Daucus* (Tanno-Suenaga *et al.*, 1988) and *Petunia* (Zahar *et al.*, 1983) to accomplish the transfer of CMS in one step.

Donor-recipient protoplast fusion has been proved a successful tool in achieving transfer of cytoplasmic genomes from one fusion partner to the other. In this method, protoplast of irradiated donor parent is usually fused with iodoacetamide-inactivated recipient parent. Irradiation of CMS donor protoplasts with a lethal dose of X-ray or Gamma-ray can successfully eliminate the nucleus of donor protoplasts (Dudits *et al.*, 1980; Ichikawa *et al.*, 1987; Sidrov *et al.*, 1987) whereas, iodoacetamide treatment of the protoplast prevents cell division (Nehls, 1978) of the recipient protoplast. As a result, fused product contains cytoplasm from the donor parent and nucleus from the recipient parent. In effect, in the cytoplasmic hybrid, CMS traits encoded in the mitochondrial genome of a sterile line is transferred into a desired fertile line in a single step.

1.4.3.2.2.1. General status of cytoplasmic hybridization in plants

Extensive work has been done on cybridization, which has far-reaching implications in plant breeding. Transfer of CMS via protoplast fusion has been successfully demonstrated in several dicotyledons, viz. *Daucus carota* (Tanno-Suenaga *et al.*, 1988),

Brassica napus (Yarrow *et al.*, 1986; Barsby *et al.*, 1987a; 1987b; Menczel *et al.*, 1987; Cardí and Earle, 1997), *Nicotiana* (Aviv and Galun, 1980; Menczel *et al.*, 1983) and *Petunia* (Izhar *et al.*, 1983). In monocots, there are relatively few reports on transfer of CMS through protoplast fusion mainly due to the lack of reproducible protoplast-to-plant regeneration system. Among the important cereal crops, rice is the most important one on which a lot of work has been done to transfer the CMS trait through protoplast fusion.

1.4.3.2.2.2. Current status of cytoplasmic hybridization in rice

Yang *et al.* (1988) produced cybrid calli between CMS line A-58, which carries CMS cytoplasm of Chinsura Boro II and fertile Japanese cultivar Fujiminori. After electrofusion between irradiated protoplasts of A-58 and iodoacetamide-treated protoplasts of Fujiminori; 14 calli were regenerated, some of which were cybrid as shown by isozyme patterns of peroxidase as markers of nuclear DNA and plasmid-like DNA as marker of mitochondria. Regenerated plantlets were also obtained from one of the five cybrid calli (Yang *et al.*, 1989). Morphological features, isozyme patterns chromosome numbers and restriction patterns of the mitochondrial DNA indicated that these plants were cybrids. However, the nature of the sterility of these cybrid plants was unclear. CMS cytoplasm was successfully transferred to a fertile Japanese cultivar directly from Chinsurah Boro II through asymmetric protoplast fusion (Kyoizuka *et al.*, 1989). Sixteen plants with diploid chromosomes were grown to maturity. Only one of these plants was male sterile and the progeny inherited male sterility. The restoration of

gametophytic fertility by the *Rf-1* gene suggested that the CMS trait of this cybrid plant were identical to those of Chinsurah Boro II. Production of cybrid plants also have been reported by Akagi *et al.* (1989, 1995a, 1995b, 1995c) using donor-recipient protoplast fusion method. More than 80% of the cybrid plants did not set seeds upon selfing. (Akagi *et al.*, 1989, 1995a, 1995b). It was further shown that male sterile cybrid plants were female fertile. In rice cybrids, occasional modification of mtDNA has been observed (Akagi *et al.*, 1989, 1995b) and about 20% of the cybrid plants between Indica and Japonica subspecies have been reported to be fertile (Akagi *et al.*, 1989; 1995a; 1995b).

1.5. Male sterility and plant breeding

Male sterility in flowering plants is of tremendous importance not only in molecular and developmental studies of stamen / pollen grains and evolutionary studies on the origin of dioecy but also in its commercial application in hybrid seed production programme in plant breeding. The existence of male sterility in a particular species eliminates the laborious and often difficult task of hand emasculation, which is otherwise required for the production of hybrid seed. Male sterility enables the combination of traits from two parental lines, with the ultimate goal to suppress negative traits from either parent with the genes from the other parent and to superimpose the positive traits carried by both parents. This results in a vigor that equals or exceeds either parental line. Several natural sources of genetic and cytoplasmic male sterility have been used for many years. The occurrence of male sterility has been exploited in many crop species such as corn,

sorghum, sugar beet and rice in order to exploit heterosis (Yuan, 1977; Virmani and Shinjyo, 1988). However, in crops where natural sources of male sterility are not available or not suitable, e.g. in tomato, hand emasculation and manual hybridization is still being carried out.

1.5.1. Male sterility in higher plant

In higher plants, structural and functional disturbances in pollen development can cause gametophytic abortion or production of non-viable pollens. These aberrations in pollen development constitute male sterile phenotype, which either can be induced or can occur naturally due to nuclear-cytoplasmic incompatibility or other intrinsic biological factors. Male sterility, although generally defined as the condition when viable pollen is not produced, is variable in expression and can range from the complete absence of stamens to the failure of anther dehiscence and release of normal viable pollen (Gabelman, 1956; Frankel and Galun, 1977). The conversion of stamens to different types of floral organs also represents as male sterile condition. Thus, one or more aberrations in stamen development and microsporogenesis can result in male sterility (Sawhney and Shukla, 1994).

1.5.2. Types of male sterility

Several systems for classifying male sterility have been proposed by various researchers. Summarizing these proposals, Kaul (1988), classified male sterility into three types on a genotypic basis. These are, (i) genetic male sterility (GMS) - controlled by nuclear

genes, (ii) cytoplasmic male sterility (CMS) - controlled by male sterility inducing cytoplasm and (iii) genic- cytoplasmic male sterility (GCMS) - resulting from a nuclear cytoplasmic interaction. In practice, however, differentiation of later two groups is difficult, since a certain degree of interaction between the cytoplasm and the nucleus does occur in most cases of CMS plants. Therefore, the later two types are considered under the same heading 'cytoplasmic male sterility'.

1.5.2.1. Genetic male sterility

Genetic male sterility also known as nuclear or genetic or Mendelian male sterility, originating through spontaneous mutation, is a common phenomenon in nature. Generally, a single recessive gene can control this trait. Mutant nuclear genes affecting male gamete and organ development are designated as male sterile genes. These male sterile genes affect male fertility and thus, create complete or partial male sterility. They usually are recessive but there are a few that are dominant (Ms), and both are typically expressed in specific sporophytic tissues at different stages. (Horner and Palmer, 1995).

Dominant male sterility can be used for hybrid seed formation, if propagation of the female line is possible (e.g. via *in vitro* clonal propagation). On the other hand, recessive sterility can be used if sterile and fertile plants are easily discriminated. Commercial utility of genetic male sterility system is limited however by the high expenses involved in clonal propagation and rouging out the female rows of self-fertile plants. Mutations resulting in genetic male sterility have also been induced in several important crop plants. Both ionizing radiation and chemical mutagens such as ethyl

methyl sulphate (EMS) and ethylene imine (EI) have been used (Kaul, 1988) for this purpose.

1.5.2.2. Cytoplasmic male sterility

Cytoplasmic male sterility is a well-characterized valuable exception in nature that prevents production of functional pollen grains but does not affect female fertility. CMS occurs widely in higher plants and is due to incompatibility between nuclear and cytoplasmic gene products which results in a failure to produce mature pollen grains (Newton, 1988). This male sterile system relies on cytoplasmic factors/organelle genes that affect adversely the development of one or more types of cells in the anther during microsporogenesis. This type of sterility is maternally inherited, since all cytoplasmic organelles are inherited from the egg cell only and generally leads to complete sterility under normal environmental conditions.

In general, CMS results either from inter-specific crosses or from intra-specific crosses. Histological studies have demonstrated that tapetal abnormality occurs in about 35% of recorded cases of CMS in different species (Kaul, 1988, Laser and Lersten, 1972) where tapetal abnormalities were observed at different stages of microsporogenesis. Although CMS plants are unable to self-fertilize, their ovules are fertile and normal seed set can be obtained when they are fertilized with pollen from normal plants. Since CMS eliminates the possibilities of self-pollination, it has commercial application in the production of hybrid seed for economically important plants (Newton, 1988).

Specific nuclear genes (restorer genes) allow pollen formation in plants with CMS cytoplasm. In a CMS system, when the restorer gene is dominant, pollen fertility is restored, but when it is recessive, the plant remains sterile. Hybrid rice breeding has so far adopted this mechanism, which is called the 'three line method'. The seed parent (sterile line) has cytoplasm, which is effective in preventing pollen formation in the plant. The pollen parent (fertile) must have a specific nuclear gene that is adaptive to the cytoplasm of the seed parent, so the F1 variety can develop pollens and become fertile. This is known as restorer line. Since the seed parent is unable to produce self-fertilized seeds, it is necessary to have a cultivar that has the same nucleus as of the CMS lines, with the exception that the cytoplasm is normal and produces fertile pollens, to maintain CMS lines. Such a cultivar is called maintainer line. The maintainer and its corresponding CMS are normally isogenic but differ in their cytoplasmic background.

1.5.3. Cytoplasmic male sterility in rice

Sampath and Mohanty (1954) and Weeraratne (1954) first reported the role of cytoplasm in causing male sterility in rice. Subsequently, Katsuo and Mizushima (1958) also observed a similar phenomenon in a progeny of the first backcross *O. sativa* f. spontanea/*O. sativa* cv. Fujisaka 5. The first CMS line in cultivated rice was developed from the cytoplasmic source of Chinsurah Boro II variety designated as BT. Following this, many CMS lines were developed in rice (Virmani and Edwards, 1983). However, at that time, researchers did not deploy the CMS lines for hybrid seed production. In order to develop commercial F1 rice hybrids of rice, the first cytoplasmic male sterile

Line was developed in China in 1973 from a male sterile plant occurring naturally in a population of wild rice (*Oryza sativa* f. *spontanea*) growing on the Hainan island in 1970 (Yuan, 1977). This plant was designated as wild rice with aborted pollen (WA). Since then, a number of CMS lines have been developed from various wild and cultivated rice accessions (Lin and Yuan, 1980). CMS in rice has been classified into four groups, (i) S1 (CMS-boro or CMS-bo or BT), (ii) S2 (Wild abortive), (iii) S3 (Gambica) and (iv) S4 (not designated earlier) (Young *et al.*, 1983, Virmani *et al.*, 1986). However, only one source (viz., WA type) has been extensively used in hybrid seed production. The practical use of CMS in developing hybrid varieties in grain crop is possible only when the effective restorer lines are identified and/or developed. Therefore, the understanding and knowledge of CMS and fertility restoration system is crucial in using these genetic tools more effectively in F1 hybrid seed production.

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1.5.4. Fertility restoration in F1 hybrid in rice

In order to deploy CMS system to develop fertile F1 hybrid seeds on commercial basis, availability of an effective fertility restorer system is very essential. The fertility restorer, generally designated as 'restorer line', carries dominant nuclear gene(s) for fertility restoration (*Rf*) which is (are) able to override the sterility factor present in the cytoplasm of the CMS plant. Two types of interactions are observed between fertility restorer gene and cytoplasm. One occurs at diploid level, known as sporophytic and other one at haploid level, known as gametophytic. Fertility restoration in the CMS-BT (S1) male sterile lines of rice is controlled by a single dominant gene (*Rf*) which has

gametophytic effect and cause pollen fertility, in F1 hybrids (Shinjyo, 1969). This *Rf* gene of CMS-BT (S1) cytoplasm is located on chromosome C (Shinjyo, 1975), now designated as chromosome 10 using trisomic analysis (Bharaj *et al.*, 1995; Kinoshita, 1995; Virmani, 1996).

On the other hand, the effect of two restorer genes for CMS-WA (S2) cytoplasm is sporophytic and gives normal pollen and spikelet fertility in F1 hybrids. Govinda Raj and Virmani (1988) found that mode of action of the two restorer genes for CMS-WA cytoplasm varied with the cross; certain crosses show dominant epistasis, while others showed recessive epistasis or epistasis with incomplete dominance. Further studies showed that, one of the two fertility restorer genes for CMS-WA cytoplasm was stronger in action than other or one is dominant and the other is incompletely dominant (Virmani, 1996). Bharaj *et al.*, (1995) located the two restorer gene of CMS-WA cytoplasm using trisomic analysis. The stronger restorer gene (*Rf*-WA-1) was located on chromosome 7 and the weaker restorer gene (*Rf*-WA-2) was located on chromosome 10. Due to the fact that, the success of hybrid rice technology is dependent on the heterotic potential of the CMS and fertility restoration system, it is very important to develop restorer lines in Indica rices and more specially for Japonica rices which are mostly non-restorers and therefore requires restorer genes to be incorporated into them for hybrid seed production programme.

1.5.5. Molecular basis of cytoplasmic male sterility

Mitochondrial genomes of plants have been implicated in specifying the trait cytoplasmic male sterility, a defect, which causes pollen abortion, but give no reduction

in female fertility. CMS is due to incompatibility between nuclear and cytoplasmic gene products, which results in a failure to develop mature pollen grains. Numerous studies have shown that mutations in the mitochondrial genome are associated with CMS and that the CMS phenotype can be alleviated by the presence of appropriate (usually dominant) restorer genes in the nucleus. A wide range of mitochondrial mutations can cause CMS and thus there must be a correspondingly diverse spectrum of functions among fertility restorer genes. A significant advance in the understanding of fertility restoration has come with the realisation that mitochondrial gene expression may be controlled in a tissue specific manner.

1.5.5.1. Molecular biology of cytoplasmic male sterility in higher plant

In most cases of CMS, it has been shown that the mitochondrial genome is involved in the male sterile trait (Lonsdale, 1987; Newton, 1988; Levings and Brown, 1989; ^{Ry?} Hanson, 1991, Srivastava and ^uGupta, 1991). Earlier research on CMS in crop plants generally focused on the molecular organization of the heterogenous population of mtDNA molecules.

Plasmid like molecules of the main mitochondrial genome have been described in several fertile and CMS lines of *Zea mays* (Kemble and Bedbrook, 1980; Dale *et al.*, 1981; Ludwig *et al.*, 1985); sorghum (Pring *et al.*, 1982; Chase and Pring, 1985;), wheat [?] (Henda *et al.*, 1984), *Brassica* (Palmer *et al.*, 1983), sugar beet (Powling, 1981), *Vicia faba* (Boutry and Briquet, 1982, Negruk *et al.*, 1982) and rice (Yamaguchi and Kakiuchi, 1983; Kadowaki *et al.*, 1986; Mignouna *et al.*, 1987; Shikanai *et al.*, 1987,

Saleh *et al.*, 1989). In maize, CMS has been associated with alteration in the sequence organization of the mitochondrial genome (Levings *et al.*, 1979). Molecular analysis of mtDNA has been used to distinguish three CMS lines of maize from one another and from a normal male sterile line. In maize, CMS-C, rearrangement involving portions of the mitochondrial gene *atp9*, *atp6* and *coxII*, and a region of mtDNA homologous to cpDNA, may be associated with the CMS trait. On the other hand, a novel mitochondrial gene T-*urf13* is responsible for CMS and susceptibility traits born by CMS-T maize (Levings and Dewey, 1988). The 13 KDa polypeptide encoded by the T-*urf13* gene is a component of inner mitochondrial membrane. However, its effect is reduced in the presence of the fertility restorer genes, *Rf1* and *Rf2* (Forde and Leaver, 1980). Evidences from transgenic experiments indicate that *urf13* is toxic to cell viability in bacteria as well as insect cell culture. CMS in petunia and the mt-locus that encodes it (*pcf*) is believed to be associated with CMS. *pcf*, like T-*urf13* is a fusion gene. *pcf* contains 5' flanking and coding region of *atp9*, part of each exon of *coxII* and carboxyl terminous and 3' flanking sequence of an unidentified open reading frames, *urf5* (Young and Hanson, 1987). Two mitochondrial genes, *nad3* and *rps12*, are located closely downstream of the petunia *pcf* gene. In petunia, however, the *pcf* gene encodes a 25 KDa polypeptide (Nivision and Hanson, 1989) which is completely absent in fertile line and much reduced in presence of fertility restorer gene. Thus, in CMS-T maize and petunia, restored genes reduced the abundance of unique polypeptides that associated with the CMS. In sorghum, mutation of the *coxI* gene may play an important role in causing CMS.

1.5.5.2. Molecular biology of cytoplasmic male sterility in rice

Existence of a heterogeneous population of linear (1 - 150 kb) and circular (1 - 96 kb) molecules in rice mtDNA was established by electron microscopic study of the mtDNA (Wang *et al.*, 1989a). Size of the rice mtDNA was thought to be greater than 150 kb (Yamaguchi *et al.*, 1986), and on the basis of DNA restriction profile, perhaps up to 300 kb in size (Wang *et al.*, 1989a). However, in 1990, Hirai *et al.* reported the construction of a 350 kb rice mtDNA physical and genetic map. Later a genetic map of rice mitochondrial DNA was created by "walking" along the genome in a lambda phase library constructed from the mtDNA from green leaves. It was reported that rice mtDNA was organized as five basic circular DNAs, each of which shares homologous sequences with others. A master circle was created from the results of recombination across repeated sequences and its size was estimated to be 492 kb (Iwahashi *et al.*, 1992). In 1993, it was reported that the rice mt genome has a genetic complexity of about 300 kb (Narayanan *et al.*, 1993) and this is smaller than that of several other cereals like wheat (more than 400 kb) or maize (more than 500 kb). Further studies showed that the rice mitochondrial genome is organized as several circular, subgenomic molecules or mitochondrial chromosomes (Narayanan *et al.*, 1995).

Plasmid like DNA molecules also have been observed in both fertile and CMS lines of many rice varieties (Yamaguchi and Kakiuchi, 1983; Kadowaki *et al.*, 1986, 1988; Mignouna *et al.*, 1987; Shikanai *et al.*, 1987; Seleh *et al.*, 1989). These plasmids exist in linear or circular forms and can apparently replicate independently of the main mitochondrial genome.

The presence of double stranded RNA (dsRNA) has also been documented in rice, particularly in those varieties carrying BT cytoplasm and a nuclear male sterile line, Nongken 58S. However, dsRNA was not detected either in rice carrying WA cytoplasm or in the fertile maintainer of BT CMS (Wang *et al.*, 1990).

Among the four different types of CMS in rice (S1, S2, S3 and S4), intensive work has been done on the molecular mechanism of BT (S1) and WA (S2) type CMS. In case of BT type of CMS, the *atp6* gene has been implicated in sterility on the basis of (i) expression of a chimeric *atp6* gene in the CMS line, (ii) incomplete processing or editing of *atp6* transcripts in the male sterile line and (iii) expression of antisense transcripts from an open reading frame downstream from *atp6* gene. However, in WA CMS (S2), when *atp6* gene was studied, results were not the same as in case of BT CMS.

A simple amplified mitochondrial DNA fragment R₂₋₆₃₀ WA was obtained from wild WA male sterile cytoplasm by AP-PCR technique. Polymorphism between mtDNAs of male sterile and normal cytoplasm was detected by Southern hybridization with the probe of R₂₋₆₃₀ WA fragment. This fragment was sequenced by the didoxy chain-termination method. It is 609 bp in length. Its base components of A+T are 54.1%. A comparison of R₂₋₆₃₀ WA fragment and 1236 plant genes (including sixteen mitochondrial genes of rice) from Gene Bank reveals low homology (<50%). The nucleotide sequence of this DNA fragment contains a small inverted repeated sequence 5'- ACCATATGGT-3', which is located at the position from 262-272. In addition, it has a coding region of 20 amino acids residues, which is located at the position from 279-439. The results in this study imply that this specific DNA may associate with WA type

cytoplasmic male sterility in rice. The small inverted repeated sequence probably plays a role in the generation of male sterility (Xu *et al.*, 1995).

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Differences in transcription of *orf155* mitochondrial gene between WA CMS and fertile lines have been reported. All the fertile maintainer and restorer lines exhibit a single transcript of 0.7 kb in size. Equivalent steady state levels of this transcript were also present in the sterile line. However, an additional 1.1 kb transcript (of lower intensity) was found in the WA CMS cytoplasm (Seth *et al.* 1995). Later it was confirmed that, this gene encodes a membrane bound, 155 amino acids long polypeptide. In contrast, in wheat the same protein is 156 amino acids long (Srivastava *et al.*, 1997).

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1.6. Biotechnological manipulation and CMS transfer

Traditional method of transfer of CMS between different varieties through sexual crossing is accomplished by 5-6 recurrent backcrossings, which require 5-6 years if one crop is grown every year. This process, however, can be hastened through biotechnological manipulations using (i) protoplast fusion between CMS line and the variety which is to be sterilized and (ii) through transformation of the variety with foreign gene having capability to inhibit or hamper pollen development. Both the methods will be briefly discussed here.

1.6.1. Transfer of CMS through Protoplast fusion

Because of far-reaching implications of cell fusion for somatic and cytoplasmic hybridization, much effort in the past has centered on the development of efficient and

reproducible techniques for cell fusion. In an asymmetric protoplast fusion system, where the DNA of cytoplasmic organelles, such as chloroplasts or mitochondria encodes the trait of interest, only the organelles have to be transferred from one parent to the other. To attain such a transfer, a donor-recipient protoplast fusion technique was developed by Zelcer *et al.*, (1978); and further modified by Sidorov *et al.*, (1987). This technique provides an opportunity to study nucleo-organellar and organellar-organellar genomic interaction in the heterokaryotic cell and in any cybrid plant (Galun and Aviv, 1988). The scheme for donor-recipient cytoplasmic gene transfer involves inactivation of the protoplasts of one parent, which is used as donor by a physical mutagen to get rid of the nucleus. Cytoplasm of the other parent, the recipient, is inactivated by chemical treatment viz. iodoacetamide.

X or Gamma irradiation of protoplasts causes practical elimination of chromosomes or nuclei (Dudits *et al.*, 1980; Sidorov *et al.*, 1987; Ichikawa *et al.*, 1987) in somatic hybrid plants. This treatment has however, no deleterious or mutagenic effects on organelle genomes recovered in the progeny. Effect of various dosages of X-rays on protoplasts of CMS lines has been investigated. It has been reported that the gamma ray or X-ray dose was the most important factor in cybrid production. 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 (Yang *et al.*, 1988, Fujimura *et al.*, 1996). A dose of 50 to 60 krad gamma rays (source Cobalt-60) was required for virtually complete division-arrest of citrus protoplasts (Vardi *et al.*, 1987).

Metabolic inhibition method using anti-metabolite was first described in mammalian cell genetics (Wright, 1978) as a method to recover somatic hybrid. Chemicals such as iodoacetate or iodoacetamide are used for this purpose. Iodoacetamide is known to inhibit the division of protoplast (Nehls, 1978). Different doses of iodoacetamide work for different species without any deleterious effect on the progeny. Concentration of iodoacetamide is also an important factor in the production of cybrid plants.

With the development of an active cell fusion system for the production of somatic hybrid and cytoplasmic hybrids in a vast range of plants, a number of different fusion techniques have been reported. Protoplast fusion can be mediated by either chemical or electrical method. In case of chemical fusion, Carlson *et al.* (1972) used sodium nitrate as the fusogen. Since then, several other fusogens have been used for this purpose. Later the development of electrofusion gave a boost to the technology of somatic hybridization for *en masse* fusion of protoplasts. Electrofusion of protoplasts was first reported by Senda *et al.* (1979). Various modifications in the electrofusion system have been employed in order to use di-electrophoresis for protoplast alignment (Zimmermann and Schleicher, 1981; Zimmermann and Vienken, 1982; Watts and King, 1984; Jones *et al.*, 1996).

The electrofusion method has many advantages over the chemical fusion. This technique eliminates the use of potentially toxic chemicals. It is extremely rapid and, under appropriate conditions, can produce fusion rates in excess of 50% (Zimmermann and Schleicher, 1981; Waara and Glimelius, 1995). The fusion frequency in electrofusion is however, affected by several factors. Different populations of

protoplasts with different sizes exhibit different degrees of fusibility. Electrical parameters and the fusion medium also affect the fusion frequency (Pillai, 1990).

Considerable attention has been attracted by demonstrations of cell and protoplast fusion using electric field. Electrically induced protoplast fusion is a two step process. First, intimate membrane contact is established then localized bilayer disruption that initiate fusion. In this method, cell-to-cell contact is established by application of a high frequency AC (Alternate Current) electrical field. This creates charge on the surface of the protoplast, which results in the protoplasts being arranged in chains on the electrode. With cell contact established, fusion is initiated by the application of one or more short DC (Direct Current) pulses of sufficient magnitude to cause reversible membrane breakdown. Following electrofusion, protoplasts are successfully cultured in appropriate protoplast culture medium. In proper culture conditions these protoplasts divide and give rise to protocolonies which, in turn, can be regenerated into putative cybrid or hybrid plant.

1.6.2. Induction of male sterility through genetic engineering

Use of molecular biology for induction of male sterility in tobacco and oilseed rape was reported for the first time in 1990. Plants were engineered through transformation using chimaeric ribonuclease gene fused to a fragment of 1.5 kb TA-29 promoter having tapetum-specific expression. With the expression of these chimaeric genes, tapetum degenerated and pollen production was prevented. The plants ultimately showed male sterility (Mariani *et al.*, 1990). This male sterility system was a dominant genetic male

sterility and the ribonuclease gene was further linked to bialophos-resistant gene for use in hybrid seed production of oil seed rape. Later, Worrall [?]*et al.* (1992) reported induction of male sterility through premature dissolution of the microsporocyte callose wall in transgenic tobacco. Another report was published on antisense inhibition of flavonoid biosynthesis that resulted in male sterility in *Petunia* anthers (Van der Meer *et al.*, 1992).

1.6.3. Usefulness of the cybridisation in rice breeding

1.6.3.1. Development of new CMS lines

The cytoplasmic traits of Chinsura Boro II were transferred to 40 Japanese cultivars by asymmetric protoplast fusion in order to convert fertile cultivars to CMS. More than 80% of the diploid cybrid plants were sterile and did not set seeds on selfing except for the cybrids that had the nucleus from Hoshiyutaka (Akagi *et al.*, 1995a;). The restorer gene for Chinsura Boro II are widely distributed in the tropics where Indica varieties are grown (Shinjyo, 1972). Since, Hoshiyutaka was bred by crossing Japonica and Indica rice, it might have had the restorer gene. Cybrid plants were analyzed using PCR-based method for specifically detecting the mitochondrial gene region *orf79* which is a chimera of another mitochondrial gene (Akagi *et al.*, 1994) and all the sterile plants showed to have this region. Inter-parental recombination between two mitochondrial genomes occurs in rice cybrids and the recombinant genomes as well as the parental genomes segregate during protoplast culture (Akagi *et al.*, 1995c).

A novel CMS line, called 'Bio-Mother 1', was bred using the cybridization method from the cultivar 'Yukigesyo' during 1991 to 1993 (Nakamura *et al.*, 1995). To date, 40

Japanese cultivars have been converted to CMS lines as candidates for used in hybrid rice seed production. CMS cybrid plants were also produced using WA cytoplasm derived from 'wild abortive' type which expressed CMS in a sporophytic manner and is widely used for Indica subspecies.

1.6.3. 2. Application of new CMS lines in hybrid rice breeding

One of the most important features of hybrid rice technology lies in its yield advantage. In order to evaluate the yield performance, many F1 lines are necessary. For this purpose, candidates for female parents were converted to CMS through hybridization method and using these new CMS lines, F1 seeds of several candidate varieties were produced in Japan. These F1 seeds, showed yields that were 30 to 40% higher than those of the leading representative varieties. In addition, their quality was good enough for Japanese consumers (Oka *et al.*, 1995). In Japan, many hybrid rice varieties that are acceptable in the competitive Japanese market were developed using cybrid-derived CMS lines.

It takes about 8 months to produce new CMS lines by the hybridization method and two additional backcrossing is necessary for elimination of somaclonal mutation. Therefore, it takes about 2 years for establish the new CMS lines, while conventional recurrent backcrossing method requires 5-6 years. Thus, the hybridization method will be useful, especially, for conversion a large number of fertile cultivars into CMS, which is the most important, step in hybrid seed production.

1.7. Specific objectives of the thesis

The present investigation was undertaken with the following specific objectives:

- i) Callus induction, maintenance of embryogenicity and plant regeneration from different explants of CMS, maintainer and restorer lines of rice.
- ii) Initiation, establishment and maintenance of embryogenic cell suspensions from CMS, maintainer and restorer lines of rice.
- iii) Isolation, culture, induction of division and plant regeneration from suspension-derived protoplasts and
- iv) Transfer of cytoplasmic male sterility through protoplast fusion.