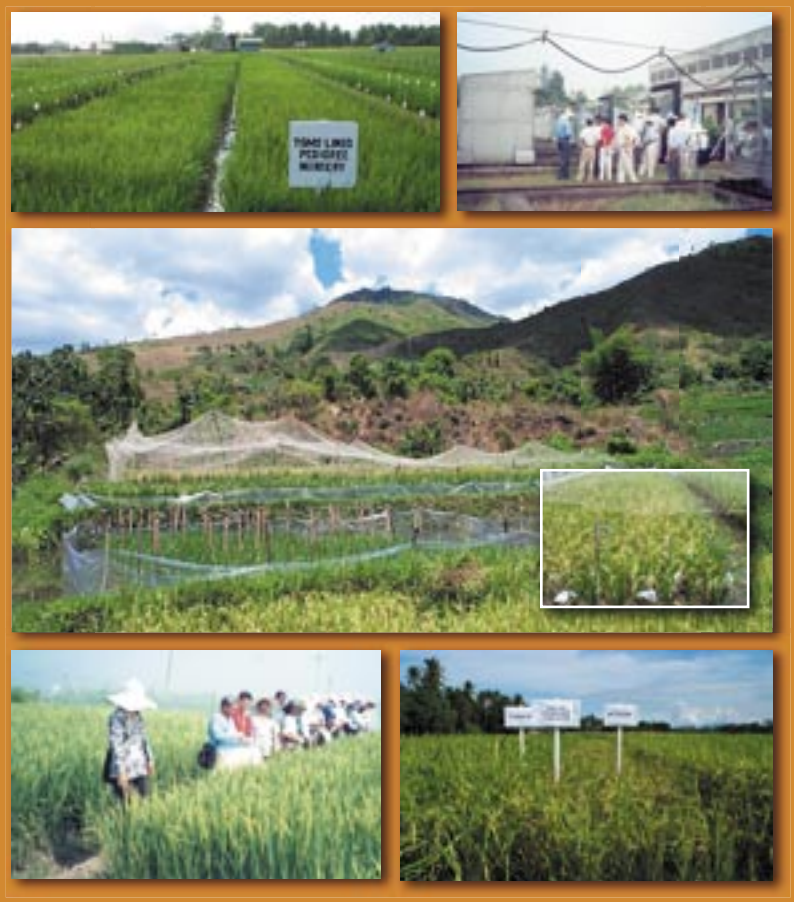
Two-Line Hybrid Rice Breeding Manual



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Contents

Foreword

Hybrid rice technology has contributed significantly toward food security, environmental protection, and employment opportunities in China for the past 25 years. Since the mid-1990s, this technology has also been developed and introduced to farmers in India, Vietnam, the Philippines, Bangladesh, and the United States, either independently or in close collaboration with IRRI. Several other countries, such as Egypt, Indonesia, Myanmar, Sri Lanka, Thailand, and the Republic of Korea, are now developing this technology in collaboration with IRRI.

The availability of adequately trained human resources is an essential prerequisite for developing and using hybrid rice technology. Hybrid rice breeding uses several concepts, skills, and procedures that are strikingly different from those used for inbred rice breeding. Two male sterility systems (the cytoplasmic genic male sterility and environment-sensitive genic male sterility system) have been used extensively to develop commercial rice hybrids in China and elsewhere. During the past 25 years, IRRI and China have offered several short-term training courses jointly and independently to develop the human resources in countries interested in developing this technology. In 1997, IRRI also published a "Hybrid Rice Breeding Manual" to serve the needs of those training courses. This manual described concepts and procedures to breed rice hybrids primarily using cytoplasmic genic male sterility and the fertility restoration system. Since then, considerable progress has been made in breeding rice hybrids using environment-sensitive genic male sterility.

In August 2000, China and IRRI held a collaborative international training course on hybrid rice breeding at the China National Rice Research Institute in Hangzhou, which was funded by the IRRI-ADB project on "Development and Use of Hybrid Rice in Asia." Several Chinese and IRRI scientists participated in this course as resource persons and provided the training materials focusing on two-line hybrid breeding using the environment-sensitive genic male sterility system. Based on these materials, and the experience of hybrid rice scientists from China and IRRI, this training manual on the two-line hybrid breeding method has been prepared, which expands upon the hybrid rice breeding manual published earlier by IRRI. The authors have described the concepts and procedures stepwise and in a systematic manner so that trainees can learn them easily. This should make an excellent manual for future hybrid rice breeding training courses organized at IRRI, in China, and in other countries.

I compliment the authors for preparing this training manual and thank Bill Hardy for editing it. The assistance of the Asian Development Bank, which provided financial support under RETA 6005 on Sustaining Food Security in Asia Through the Development of Hybrid Rice Technology, is gratefully acknowledged.

RONALD P. CANTRELL
Director General

Hybrid rice and heterosis breeding

Heterosis is a phenomenon in which F_1 hybrids derived from diverse parents show superiority over their parents in vigor, yield, panicle size, number of spikelets per panicle, number of productive tillers, etc.

- Heterosis is expressed in the first generation only.
- Heterosis varies according to the level of parental diversity and or presence of heterotic gene blocks in parental lines; indica × japonica crosses show maximum heterosis vis-à-vis any other combination between other subspecies. The crosses showing heterosis in descending order are indica × japonica > indica × javanica > japonica × javanica > indica × indica > japonica × japonica > javanica × javanica.
- Heterosis can be positive or negative. Both positive and negative heterosis can be useful depending on the trait, for example, positive heterosis for yield and negative heterosis for growth duration.
- Farmers tend to use a lower seed rate for hybrids than for conventional varieties because of their better seed quality and higher seed cost. However, it is necessary to purchase fresh seeds every season to raise a commercial crop.

Types of heterosis

Heterosis is expressed in three ways, depending on the reference used to compare the performance of a hybrid:

- Mid-parent heterosis is the increase or decrease in the performance of a hybrid in comparison with the mid-parental value.
- Heterobeltiosis is the increase or decrease in the performance of a hybrid in comparison with the better parent of the cross combination.
- Standard heterosis is the increase or decrease in the performance of a hybrid in comparison with the standard check variety of the region.

From the practical viewpoint, standard heterosis is the most important because we aim to develop hybrids that are better than the existing high-yielding varieties grown commercially by farmers.

How is heterosis measured?

Measurement of heterosis is quite simple. It is generally expressed as the percent increase or decrease in the performance of a hybrid in comparison with the reference variety or a parameter.

$$\text{Mid-parent heterosis (\%)} = \frac{F_1 - \text{mid-parent}}{\text{Mid-parent}} \times 100$$

$$\text{Heterobeltiosis (\%)} = \frac{F_1 - \text{better parent}}{\text{Better parent}} \times 100$$

$$\text{Standard heterosis (\%)} = \frac{F_1 - \text{check variety}}{\text{Check variety}} \times 100$$

Genetic basis of heterosis

Two major hypotheses have been proposed to explain the genetic basis of heterosis: the dominance hypothesis (Davenport 1908) and overdominance hypothesis (East 1908, 1936).

- **Dominance hypothesis**
  - This states that heterosis is due to the accumulation of favorable dominant genes in a hybrid derived from the two parents (Fig. 1).

    This was demonstrated in a pea hybrid whose parents had different dominant genes for node number and internodal length. The hybrid was much taller than either parent. The increased height was due to the accumulation of four dominant genes in the hybrid.

- **Overdominance hypothesis**
  - This states that the heterozygote (Aa) is more vigorous and productive than either homozygote (AA or aa). This has been proven in traits controlled by a single or a few genes. The heterozygote performs a given function, over a range of environments, more efficiently than either homozygote (East 1936).

Studies on the genetic basis of heterosis for polygenic traits in various crops have shown that heterosis is the result of partial to complete dominance, overdominance, and epistasis, and it may be a combination of all these (Comstock and Robinson 1952). Evidence of real overdominance for quantitative traits is hard to find. However, apparent overdominance caused by nonallelic interaction and linkage disequilibrium is a common contributor to heterosis (Jinks 1983).

Heterosis may also be due to the specific positive effects of the cytoplasm of the maternal parent on the nuclear component of the paternal parent. Differential heterosis observed between the same pollen parent and cytoplasmic male sterile (CMS) lines from different cytosterility sources is an example of this kind of heterosis.

Molecular basis of heterosis

Several molecular studies support the overdominance hypothesis (Stuber et al 1992, Yu et al 1997, Li et al 2000) except for a few that support the dominance hypothesis (Xiao et al 1995). Yu et al (1997) reported overdominance at several main-effect quantitative trait loci (QTLs) and a stronger additive epistasis affecting grain yield and its components in F_3 progenies from the most widely grown hybrid in China, Shan You 63. Zhang et al (2001) demonstrated the involvement of large numbers of two-loci interactions or epistasis as the genetic basis of quantitative traits and heterosis. Furthermore, Li et al (2000) concluded that most QTLs associated with inbreeding depression and heterosis in rice appeared to be involved in epistasis. And almost 90% of the QTLs contributing to heterosis appeared to be overdominant. Zhang et al (2001) assessed the relationship between gene expression and heterosis by assaying the patterns of differential gene expression in hybrids relative to their parents in a diallel cross. The analysis revealed that differentially expressed fragments occurring in only one parent of the cross were positively correlated with heterosis and fragments detected in F_1s but not in the respective parents were negatively correlated with heterosis. A total of 384 fragments recovered from gels were hybridized with mRNAs from seedling and flag-leaf tissues and thereby Zhang et al (2000) detected an overall elevated level of gene expression in the hybrid compared with the parents. Several fragments showed a higher expression in the highly heterotic hybrid than in the other hybrids.

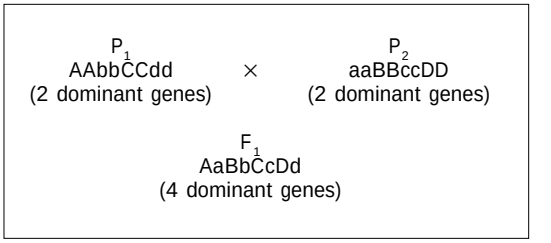


Fig. 1. Illustration of dominance hypothesis to explain genetic basis for heterosis.

Nonetheless, a lack of a clear understanding of the genetic or molecular basis of heterosis has not prevented plant breeders from exploiting this phenomenon to raise crop yields.

Approaches for using heterosis

Currently, hybrid rice technology mainly uses intrasubspecific heterosis, that is, indica × indica and japonica × japonica. The high-yielding

intrasubspecific hybrids yield nearly 15% to 20% more than the best inbred varieties grown under similar conditions. It has been quite difficult to create or widen the genetic difference among parents belonging to the intrasubspecific hybrids and they have almost reached their yield ceiling.

Indica and japonica (both tropical and temperate) are the two main subspecies of *Oryza sativa* in Asia. Among them, the indica and temperate japonica subspecies are the most apparently different in their morphological and agronomic traits apart from the genetic distance between them. Therefore, it is now well understood why the indica × japonica hybrids show maximum heterosis. But intersubspecific heterosis is limited because of high spikelet sterility and long growth duration. With the discovery of wide compatibility (WC) genes, it has been possible to exploit intersubspecific hybrids with normal seed setting and suitable growth duration. Efforts are under way in IRRI, China, and India to develop intersubspecific hybrids. Recently, Chinese scientists have developed super high-yielding rice hybrids from crosses involving indica/indica-japonica derivative parents.

Most of the interspecific crosses in cultivated species pertain to only *O. sativa* and *O. glaberrima*, which are heterotic but not so useful in terms of yield and plant stature. Most interspecific hybrids, resulting from wide hybridization, result in genetic variability and bring in desirable genes for resistance to several biotic and abiotic stresses, for example, *O. sativa* × *O. longistaminata*, *O. sativa* × *O. rufipogon*, and *O. sativa* × *O. perennis*. In rice, the interspecific F_1 hybrids cannot be used commercially.

Methods of using heterosis

The three-line method is based on cytoplasmic genic male sterility and the fertility restoration system and involves three lines—the CMS line (A), maintainer line (B), and restorer line (R)—for the commercial production of rice hybrids. The seed of the male sterile line is multiplied by crossing A and B lines in an isolation plot. Hybrid seed is produced by crossing the A line with an R line in isolation in another plot. Seed production techniques are now developed to produce up to 3 t ha^{-1} (mean 1.2 t ha^{-1}) of hybrid seed in the tropics and up to 6 t ha^{-1} (mean 2.7 t ha^{-1}) in subtropical and temperate regions of China.

In the two-line method, the two lines are involved in a cross for hybrid rice seed production. One is a male sterile line in which male sterility is genetically controlled by recessive genes, the expression of which is influenced by environment (temperature, photoperiod, or both) and the other is any inbred variety with a dominant gene for that locus. The male sterile lines in which sterility expression is controlled by temperature are known as thermosensitive genic male sterile (TGMS) lines and those in which expression is controlled by daylength are called photoperiod-sensitive genic male sterile (PGMS) lines.

Another two-line approach for hybrid rice seed production is by spraying chemical hybridizing agents (CHAs)—ethrel, ethyl 4′ fluoro oxanilate, or sodium methyl arsenate—that selectively sterilize the male reproductive organs of any one parent and planting the other line (not sprayed) close to the pollinator rows. China is the only country that used CHAs such as sodium methyl arsenate and zinc methyl arsenate on a commercial scale. Because of the inefficient seed production related to nonsynchronous tillering and flowering as well as health hazards associated with the use of arsenic compounds, CHA use in China was discontinued.

To use three-line and two-line rice hybrids, farmers have to buy fresh seed every season. This seed is produced by a proficient seed production agency in the public or private sector.

The one-line method involves the use of apomixis to develop F_1 hybrids. This represents true breeding so that farmers can use the harvest from the hybrids as seed for the next crop as with any inbred rice variety. Attempts to discover apomixis have not succeeded so far; however, research is still under way at IRRI, in China, and in some other countries using genetic engineering techniques.

Hybrid rice

What is hybrid rice?

Hybrid rice is the commercial rice crop grown from F_1 seeds of a cross between two genetically dissimilar parents.

- Good rice hybrids have the potential of yielding 15–20% more than the best inbred variety grown under similar conditions.
- To exploit the benefits of hybrid rice, farmers have to buy fresh seeds every cropping season.

Why hybrid rice?

The need for hybrid rice has been felt because

- Yield levels of semidwarf varieties of the Green Revolution era have plateaued.
- The demand for rice is increasing rapidly with the increase in population, especially in less developed countries.
- More and more rice has to be produced on less land and with less inputs.
- Hybrid rice varieties have already shown a 15–20% higher yield potential than inbred rice varieties under farmers' field conditions in several countries.
- Hybrids have also shown an ability to perform better under adverse conditions of drought and salinity.

How is hybrid rice developed?

Hybrid rice is developed by exploiting the phenomenon of heterosis. Rice, being a strictly self-pollinated crop, requires the use of a male sterility system to develop commercial rice hybrids. Male sterility (genetic or nongenetic) makes the pollen unviable so that rice spikelets are incapable of setting seeds through selfing. A male sterile line is used as a female parent and grown side by side with a pollen parent in an isolated plot to produce a bulk quantity of hybrid seed because of cross pollination with the adjoining fertile pollen parent. The seed set on male sterile plants is the hybrid seed that is used to grow the commercial hybrid crop.

Male sterility systems in rice

Male sterility can be defined as a condition in which the pollen grain is unviable or cannot germinate and fertilize normally to set seeds.

The following genetic and nongenetic male sterility systems are known for developing rice hybrids (Fig. 2):

- Cytoplasmic genetic male sterility
- Environment-sensitive genic male sterility
- Chemically induced male sterility

Cytoplasmic genetic male sterility

Male sterility is controlled by the interaction of a genetic factor S present in the cytoplasm and nuclear gene(s). It is now known that the male sterility factor S is located in the mitochondrial DNA. A line is male sterile when the male sterility-controlling factor S in the cytoplasm and recessive alleles (*rf*) of fertility-restoring genes are present in the nucleus. The maintainer line (B line) is iso-cytoplasmic to the CMS line since it is similar to it for nuclear genes but differs in cytoplasmic factor (N), which makes it self-fertile, but it has the capacity to maintain the sterility of the A line when crossed with it. A restorer or R line possesses dominant fertility-restoring genes (*Rf*) and it is dissimilar to or diverse from the A line. Crossing a restorer line as a pollen parent with a CMS (A) line as a female parent restores the fertility in the derived F_1 hybrid.

- The restorer gene in the dominant homozygous (*RfRf*) or heterozygous (*Rfrf*) state can restore the fertility in the F_1 hybrid despite the presence of sterility factors in the cytoplasm derived from the A line. The CMS system is illustrated in Figure 3.

Hybrid seed production using the CMS system

Hybrid seed production involves two steps: multiplication of the CMS line and production of hybrid seeds.

Multiplication of the CMS line

This requires crossing of the CMS line with its maintainer line by outcrossing by hand (for a small quantity of seed) or in the field under isolation by space or time (to produce a bulk quantity of seed). For successful production of the CMS line, it is grown in six or eight rows interspersed by two rows of a maintainer line in an alternating manner.

Because there is a small difference between the growth duration of A and B lines, their sowing dates are adjusted to achieve good synchronization of their flowering. Several other techniques (such as flag-leaf clipping, GA_3 application, and supplementary pollination by rope pulling or the bamboo pole method) are used to improve the outcrossing rate and seed yield of the CMS line.

Production of hybrid seeds

This involves the use of CMS lines with a selected restorer line (R line) by growing them in a specific female:male ratio in the field under isolation by space or time. The CMS line is usually grown in eight or ten rows interspersed with two rows of restorer lines in an alternating manner. The sowing dates of A and R lines are staggered to achieve synchronization of their flowering. To increase the outcrossing rate and hybrid seed yield, the techniques described in the previous section are used.

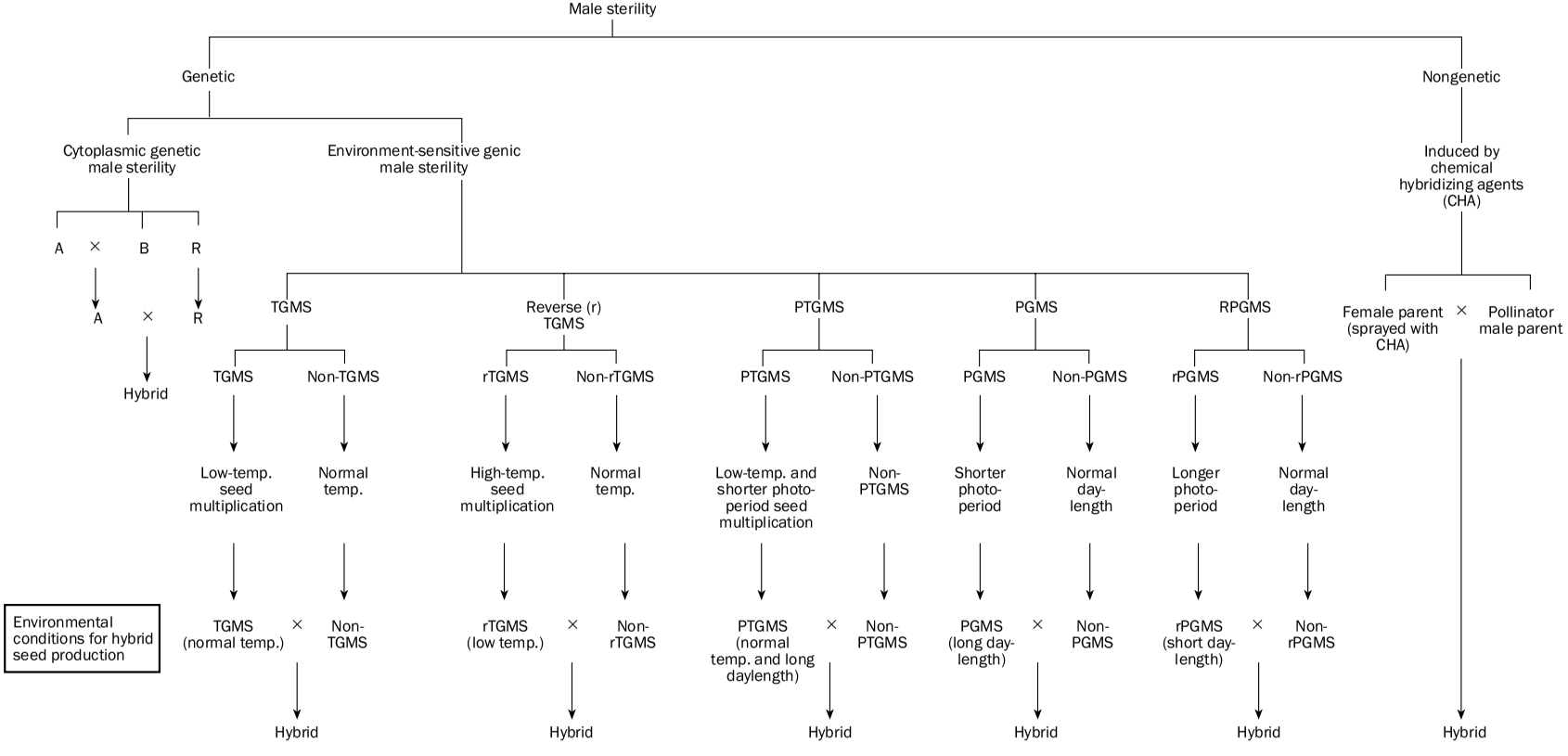


Fig. 2. Classification of male sterility systems in rice.

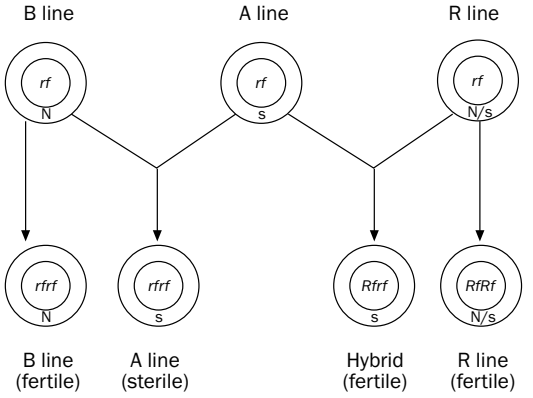


Fig. 3. Schematic description of the cytoplasmic genIc male sterility system. N = cytoplasmic factor, S = male sterility factor.

Environment-sensitive genic male sterility

This male sterility system is controlled by nuclear gene expression, which is influenced by environmental factors such as temperature, daylength, or both. This male sterility system was first observed in pepper by Martin and Crawford in 1951 and subsequently in different crops (Table 1). However, this system has been exploited commercially only in rice because of the pioneering work of Chinese scientists (Tables 2 and 3).

Advantages of the EGMS system

- There is no need for a maintainer line for seed multiplication, thus making seed production simpler and more cost-effective.

Table 1. Some reports on environment-sensitive genic male sterility systems in crop plants.

Crop	Environmental factor	Reference
Pepper	Temperature	Martin and Crawford (1951)
	Temperature	Peterson (1958)
	Temperature	Daskaloff (1972)
Cabbage	Temperature	Rundfeldt (1960)
Maize	Temperature	Duvick (1966)
	Temperature	He et al (1992, c.f. Yuan 1997)
Tomato	Temperature	Rick and Boynton (1967)
	Temperature	Abdallah and Verkerk (1968)
	Temperature	Steven and Rudich (1978)
	Temperature	Sawhney (1983)
Wheat	Daylength	Fisher (1972)
	Temperature	Jan (1974)
	Daylength and temperature	He et al (1992, c.f. Yuan 1997)
	Daylength and temperature	Tan et al (1992, c.f. Yuan 1997)
Barley	Daylength	Batch and Morgan (1974)
	Temperature	Sharma and Reinbergs (1976)
	Daylength	Ahokas and Hocket (1977)
Vicia faba	Temperature	Berthelem and Le Guen (1975)
	Light intensity	Duc (1980)
Cucurbits	Temperature	Rudich and Peles (1976)
Rice	Daylength and temperature	Shi (1981, 1985)
	Temperature	Zhou et al (1988), Sun et al (1989)
	Temperature	Maruyama et al (1991)
	Temperature	Virmani and Voc (1991)
Sesame	Temperature	Brar (1982)
Sorghum	Daylength and temperature	Murty (1995)
Soybean	Daylength	Wei et al (1994)
Brassica napus	Temperature	Xi et al (1997)
Wheat	Copper, boron, and molybdenum deficiency in soil	Agarwala et al (1979, 1980)
	Boron deficiency in soil	Rerkasem and Jamjod (1997)
Maize, barley, oats, and sunflower	Copper deficiency in soil	Dell (1981)

Table 2. Origin and fertility-sterility transformation behavior of photoperiod- and temperture-sensitive male sterile sources in rice.

Source	Varietal group	Origin	CDL[a] (h)/CSP/ CFP (°C)	Reference
Photoperiod-sensitive male sterile (h)				
Nongken 58S	Japonica	Spontaneous mutation, China	14.00–13.45	Shi and Deng (1986)
MSr 54A (B)	Japonica	Spontaneous mutation, China	14.00–13.00	Lu and Wang (1988)
CIS 28-10S	Indica	Spontaneous mutation, China	14.00–12.00	Huang and Zhang (1991)
26 Zhai Zao	Indica	Induced (R), China	14.00–12.00	Shen et al (1994)
EGMS	Japonica	Induced (C), USA	14.00–13.00	Rutger and Schaeffer (1989)
M 201	Japonica	Inducec (R), USA	14.00–12.00	Oard and Hu (1995)
Temperature-sensitive male sterile (°C)				
5460 S	Indica	Induced (R), China	28.0–26.0	Yang et al (1990)
IR32364	Indica	Induced (R), IRRI	32.0–24.0	Virmani and Voc (1991)
IR68945	Indica	Introgression from Norin PL 12, Japan	30.0–24.0	Virmani (1992)
IR68949	Indica	Introgression from Norin PL 12, Japan	30.0–24.0	Virmani (1992)
H 89-1	Japonica	Induced (R), Japan	31.0–28.0	Maruyama et al (1991)
Annong 1S	Indica	Spontaneous mutation, China	30.2–27.0	Tan et al (1990)
R 59TS	Indica	Induced (R), China		Yang and Wang (1990)
Xianquang	Indica	Breeding population, China	30.0–24.0	Cheng et al (1995)
26 Zhi Zao S	Indica	Induced (R), China	23.0–25.0	Shen et al (1993)
N5088 S	Indica	Introgression from Nongken 58 S, China	30.0–22.0	Zhang et al (1994b,c)
SM 5	Indica	Spontaneous, India	32.3–22.0	Ali et al (1995)
SM 3	Indica	Spontaneous, India	32.0–22.0	Ali et al (1995)
JP 2	Indica	Spontaneous, India	33.9–23.0	Ali et al (1995)
SA 2	Indica	Induced mutation (C) India	31.7–20.0	Ali et al (1995)
F 61	Indica	Induced mutation (C) India	30.9–22.0	Ali et al (1995)
JP 8-1A-12	Indica	Breeding population, India	30.9–20.0	Ali et al (1995)
JP 24A	Indica	CMS, India	33.8–23.0	Ali (1993)
JP38	Indica	Spontaneous mutation, India	24.0–30.5	Ali (1993)
Dianxin /A	Japonica	CMS, China	23.0–20.0	Lu et al (1994)
Hennong S	Indica	Cross breeding, China	30.0–29.0	Lu et al (1994)
IV A	Indica	Cross breeding, China	24.0–28.0	Zhang et al (1991)
J207S	Indica	Spontaneous mutation, China	31.0–>31.0	Jai et al (2001)

[a]CDL = critical daylength, CSP = critical sterility point, CFP = critical fertility point, R = irradiation, C = chemical mutagen. Several introgressed forms from Nongken 58S and Annong 1S developed by Yang (1997) and Mou et al (1998) not included here.

- Any fertile line can be used as a pollen parent (PP); therefore, the frequency of heterotic hybrids is higher among two-line hybrids than among three-line hybrids, thereby increasing hybrid breeding efficiency.
- Negative effects of sterility-inducing cytoplasm are not encountered.
- The EGMS trait is governed by major genes, thus enabling their easy transfer to any genetic background and thus increasing diversity among the female (EGMS) parents, which helps in reducing potential genetic vulnerability among the hybrids.
- Since there is no need for restorer genes in the male parents of two-line hybrids, this system is ideal for developing indica/ japonica hybrids because most japonica lines do not possess restorer genes.

Disadvantages of the EGMS system

- Since the sterility trait is conditioned by environmental factors, any sudden change such as temperature fluctuation because of a thunderstorm, typhoon, etc., will influence the sterility of EGMS lines.

Table 3. Two-line rice hybrids released up to 2001 in China.

Hybrid	Pedigree	Type	Year of release	Cultivation region/crop
Ejingza No.1	N5088S/R187	Japonica	1995	Yangtze Valley/second
Huajingza No. 1	7001S/1514	Japonica	1995	Yangtze Valley/second
Huajingza No. 2	N5088S/65396	Japonica	2001	Yangtze Valley/second
70 you 9	7001S/Wanhui 9	Japonica	1994	Yangtze Valley/second
70 you 99	7001S/99	Japonica	1997	Yangtze Valley/second
70 you 04	7001S/Xiushui 04	Japonica	1994	Yangtze Valley/second
Peiliangyou Teqing	Pei-ai 64S/Teqing	Indica	1994	Yangtze/single or second
Peiliangyou 288	Pei-ai 64S/R822	Indica	1996	Yangtze/single or second
Peiliangyou Yuhong	Pei-ai 64S/Yuhong No. 1	Indica	1997	Yangtze/single or second
Liangyou Peiju	Pei-ai 64S/9311	Indica	1999	Yangtze/single or second
Liangyou 923	W9593S/Shengyou No. 2	Indica	2001	Yangtze/single or second
Liangyou 681	Shuguang 612S/881	Indica	1999	Yangtze/single or second
Xiangliangyou 68	Xiang 125S/D 68	Indica	1998	Yangtze/first
8 Liangyou 100	Annong 810S/D100	Indica	1998	Yangtze/first
Tainliangyou 402	TianfengS/R 402	Indica	1998	Yangtze/first
An Liangyou 25	1356 S/Zao 25	Indica	1998	Yangtze/first
Peiza Shangqing	Pei-ai 64S/Shanqing	Indica	1997	South China/first and second
Jinliangyou 36	HS-3/946	Indica	2000	South China/first and second
Peiza Shuangqi	Pei-ai 64S/Shuangqizhan	Indica	1998	South China/first and second
Liangyou 2163	SE21S/Minghui63	Indica	2000	South China/first and second
Liangyou 2186	SE21S/Minghui86	Indica	2000	South China/first and second
Fu Liangyou 63	FJS-1/Minghui63	Indica	2000	South China/first and second
Pei Liangyou 275	Pei-ai 64S/275	Indica	1999	South China/first and second
Pei Liangyou 99	Pei-ai 64S/Gui99	Indica	1998	South China/first and second
Peiza Maosan	Pei-ai 64S/Maosan	Indica	2000	South China/first and second
Peiza Maoxuan	Pei-ai 64S/Maoxuan	Indica	2000	South China/first and second
South China/first and second Peiza 67	Pei-ai 64S/G67	Indica	2000	South China/first and second
Yunguang No. 8	N5088S/Yunhui 11	Japonica	2000	West China/single

- The multiplication of EGMS lines and hybrid seed production are restricted by space and season. This means that an EGMS line is used in a given region and season.

Characteristic features of ideal EGMS lines

- The proportion of male sterile plants in a population of more than 1,000 plants during the critical sterility period should be 100%.
- The pollen sterility of each male sterile plant should be more than 99.5%.
- EGMS lines should have clearly defined sterility-fertility alteration regimes.
- The male sterile phase should last for more than 4 consecutive weeks.
- Seed setting during the fertile phase should be more than 30%.
- The critical temperature or photoperiod for inducing sterility should be as low as possible for more stability of the EGMS lines, for example, <27 ºC (maximum) or <13 h photoperiod.

In addition, these lines should have a good plant type possessing high yield, acceptable grain quality, pest and disease resistance, and adaptability to the target environment.

Classification of the EGMS system

Depending on the environmental factor(s) influencing expression of the sterility-inducing gene(s), EGMS is classified in the following categories (Figs. 4–7):

1. TGMS: temperature-sensitive genic male sterility
2. rTGMS: reverse temperature-sensitive genic male sterility
3. PGMS: photoperiod-sensitive genic male sterility
4. rPGMS: reverse photoperiod-sensitive genic male sterility
5. PTGMS: photothermosensitive genic male sterility

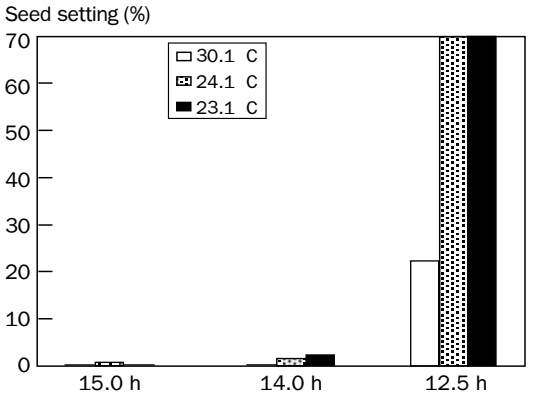


Fig. 4. Fertility of PGMS line N9044S in phytotron conditions.

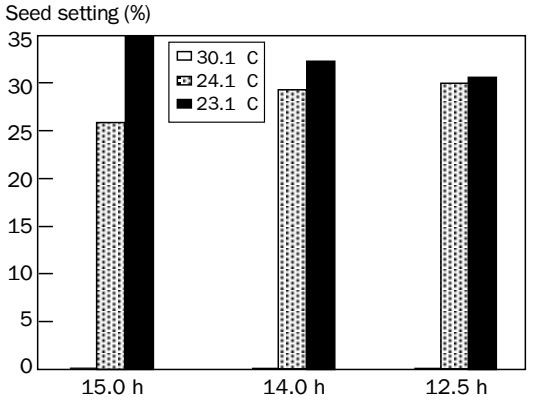


Fig. 5. Fertility of TGMS line W9046S in phytotron conditions.

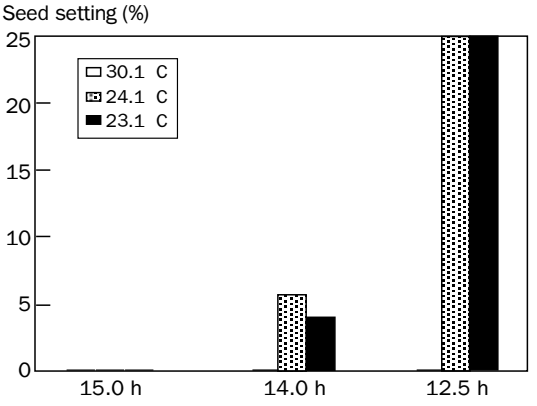


Fig. 6. High fertility of PTGMS line Xinguang S in phytotron conditions.

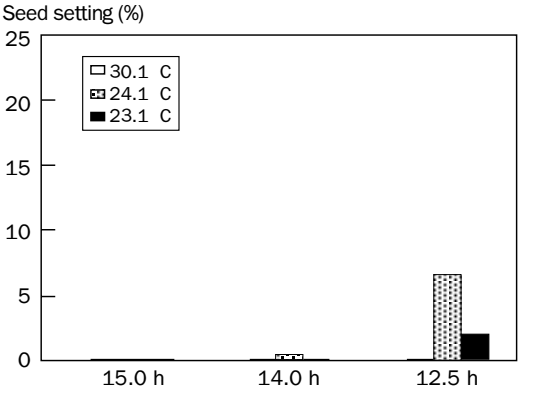


Fig. 7. Low fertility of PTGMS line Miai 64S in phytotron conditions.

TGMS lines are sensitive to the temperature for the expression of male sterility or fertility. For example, most TGMS lines remain male sterile at high temperature (day temperature >30 ºC/night >24 ºC) and they revert back to partial fertility at a lower temperature (day <24 ºC/>16 ºC night), for example, 5460S, IR68945, H89-1, and SA2.

Reverse TGMS (rTGMS) lines are sensitive to low temperature (<24 ºC day/>16 ºC night) for the expression of male sterility, whereas, at a higher temperature (>30 ºC day/24 ºC night), they become male fertile, which is just the reverse of the TGMS system, for example, JP 38, Dianxin 1A, and IVA.

PGMS lines are sensitive to the duration of daylength for the expression of sterility or fertility. For example, most PGMS lines remain male sterile under long-day (>13.75 h) conditions and revert back to fertility under short-day (<13 h) conditions, for example, N9044S and N5088S.

PGMS lines that express sterility under short daylength and fertility under long daylength are known as reverse PGMS (rPGMS). This category is yet to be found.

PTGMS lines are sensitive to both photoperiod and temperature. Temperature is the key factor since PTGMS lines become completely male sterile or fertile beyond a particular temperature range, that is, >30 ºC or <24 ºC, without any influence of photoperiod. But, within this temperature range (24–32 ºC), photoperiod influences the PTGMS lines, that is, longer photoperiod hours will enhance male sterility at lower temperatures

vis-à-vis a shorter photoperiod (i.e., 14 h at 30 ºC will make the PTGMS line sterile in comparison with 13 h at 30 ºC), for example, Nongken 58S, Xinguang S, and Miai 64S. Figure 7 characterizes the model for PTGMS lines.

Male sterility expression in EGMS lines is governed by a single nuclear recessive gene or pair of nuclear recessive genes that are sensitive to environmental conditions such as photoperiod, temperature, or a combination of both.

Under natural conditions, there is a constant interaction of photoperiod and temperature and it is therefore difficult to separate the effects of photoperiod and temperature on fertility. Using statistical methods, you can separate the effects of photoperiod (P), temperature (T), and P × T on the fertility of EGMS lines. EGMS lines can also be classified by evaluating them in a combination of photoperiod and temperature treatments in a two-way factorial analysis. Because of the limitation of available phytotrons, the best combinations of P and T should be selected.

While setting up P and T treatments, you need to consider (1) the characteristics of fertility responses to P and T in the EGMS line and (2) the ecological conditions in the target area in which the EGMS system will be deployed. In China, several EGMS lines were evaluated in a combination of three P and three T treatments (see Table 4).

Table 4. Photoperiod and temperature conditions in the phytotrons for evaluation of EGMs lines in China from 1993 onward.[a]

Time	Low temp. (ºC)	Medium temp. (ºC)	High temp. (ºC)
0500–0800	22.0	23.0	29.0
0800–1100	25.0	26.0	32.0
1100–1500	27.0	28.0	34.0
1500–1800	25.0	26.0	32.0
1800–0100	22.0	23.0	29.0
0100–0500	19.0	20.0	26.0
Daily average	23.1	24.1	30.1

[a]Illumination time: 12.5 h (0600–1830), 14.0 h (0530–1930), 15.0 h (0500–2000).

Data processing

Pollen and spikelet fertility of the test entries must be analyzed on time. Pollen samples must be collected at the time of heading by taking five apical spikelets from the panicle on the primary tiller from each plant and immersing them in a prepared fixative solution (alcohol:acetic acid, 3:1). Percent fertile pollen is observed under a microscope using the IKI staining procedure (Appendix 1). At the same time, panicles on primary and secondary tillers that head synchronously with the main stem (±4–5 days) are bagged. At maturity, spikelet fertility percent is calculated by the number of filled spikelets divided by total spikelets per panicle, multiplied by 100.

Both pollen and spikelet fertility data from the test EGMS lines need to be transformed in the form $\sin^{-1}\sqrt{x}$ before analyzing them further.

Two-factor variance analysis can be used to measure P, T, and P × T effects (Table 5).

EGMS lines can be classified on completion of the variance analysis as summarized in Table 6.

Different types of EGMS are characterized in the next section.

Characteristics of different EGMS types

PGMS. Fertility change in PGMS lines is characterized by significant P and P × T interaction effects ($P<0.05$) and nonsignificant T effects. PGMS lines are completely or highly sterile under the combinations of long daylength with high or low temperature and highly male fertile under the com-

Table 5. Results of two-factor variance analysis.

Source of variance	Degrees of freedom	Sum of squares	Mean of squares	Calculated F value	Tabular $F_{0.05}$ value
Replication	r – 1				
Treatment	pt – 1				
P	p – 1				
T	t – 1				
P × T	(p – 1)(t – 1)				
Error	(r – 1)(pt – 1)				
Total	rpt – 1				

Table 6. Classification of EGMS lines based on statistical significance of effects of various treatments.[a]

Photoperiod (P)	Temperature (T)	P × T	Classification category
*	ns	*	PGMS
*	ns	ns	
ns	*	*	TGMS
ns	*	ns	
ns	ns	*	PTGMS

[a]* = significant at the 5% level and ns = nonsignificant at the 5% level.

bination of short daylength with low temperature in the phytotron. Nongken 58S and most japonica EGMS lines derived from it belong to this group. An example of a typical PGMS line is N9044S. Figure 4 shows its behavior in response to P and T treatments.

PGMS lines are stably male sterile in the summer and high in fertility recovery in autumn under natural conditions in temperate regions. In Central China, most PGMS lines showed stable sterility, making them suitable for hybrid seed production in the summer and easy to be self-multiplied in autumn. But low temperature under long daylength or high temperature under short daylength induces partial fertility in such lines.

TGMS. Most indica EGMS lines studied belong to this group. These lines are characterized by nonsignificant P effects and significant T effects. TGMS lines are completely or highly sterile under high temperature and highly fertile under low temperature irrespective of photoperiod in the growth chamber. They are stable in male sterility in the summer and high in fertility recovery in autumn in the northern hemisphere under natural conditions in the tropical rice-growing regions but unstable in temperate regions. Typical examples of TGMS lines are W9046S (in China), Norin PL 12 (in Japan), and IR32364TGMS (at IRRI). Figure 5 shows the behavior of W9046S (an indica TGMS line derived from Nongken 58S) in response to P and T treatments.

TGMS lines have limited utility in temperate conditions because of their unstable sterility in the summer caused by the occurrence of sudden low temperatures. However, such lines can be easily multiplied with high yields in autumn because of the occurrence of low temperature. In tropical regions, where abnormally low temperature seldom occurs, TGMS lines can be used to produce hybrid seeds. These lines can be multiplied at high-altitude areas and/or in a season when the requisite temperatures occur under natural conditions for a prolonged period. Practically speaking, TGMS lines can be used in the plains during the summer for hybrid seed production and these can be multiplied in a mountainous area or in a season when the temperatures are low. Chinese scientists have also found that irrigating TGMS lines with deep cool water (with temperature >17–24 ºC) at the critical stage also helps to induce the fertility required for their seed multiplication.

P-TGMS. PTGMS lines show nonsignificant effects for both photoperiod and temperature but significant effects for P × T. Fertility changes in PTGMS lines are closely related to the combination of photoperiod and temperature. These lines are fertile only under the combination of short daylength and low temperature and are completely or highly sterile under all other combinations in the phytotron. About half of the EGMS lines studied belong to this group and most of them had originally been classified as PGMS. Xinguang S and Miai 64S are typical examples. Their behavior in response to P and T treatments is given in Figures 6 and 7, respectively.

PTGMS lines have stable sterility in the summer in large areas of China and can be used for hybrid seed production. However, PTGMS lines are difficult to multiply because of low fertility induction, which limits their wide use. Like TGMS lines, their multiplication is affected by abnormally high temperature in autumn.

Validation under natural conditions. To use different EGMS lines in the two-line hybrid rice breeding program safely, carry out a validation test under natural conditions to determine the best dates for hybrid seed production and EGMS seed multiplication.

Chemically induced male sterility

Since the early 1970s, attempts have been made to identify and use potential chemical hybridizing agents (CHAs) for hybrid rice seed production. Various chemicals tried so far include ethylene-releasing compounds, highly carcinogenic arsenic compounds, and growth hormones (Table 7). China is probably the only country where gametocides were used in commerical hybrid seed production, but their use has been reduced because they were found to be unsafe for human health. Rice hybrids developed by using CHAs

Table 7. List of chemical hybridizing agents and their efficacy on the rice plant.

Chemical	Male sterility induction	Reference
Ethrel	Partial to high	Perez et al (1973)
	Partial to high	Cheng and Huang (1978, 1980)
	Partial to high	Parmar et al (1979)
	Partial to high	Chan and Cheah (1981)
	High	Wang and Que (1981)
	Partial to high	Kaul (1988)
	Partial to high	Song et al (1990)
Ethrel + isourea	High	Kitaoka et al (1991)
Arsenates		
Sodium methyl arsenate (MG_2)	Complete	Chen et al (1986), Ali (1990, 1993)
Zinc methyl arsenate (MG_1)	Complete	Anonymous (1978)
Monosodium methane arsenate	Complete	Wang and Que (1981)
Oxanilates		
Ethyl 4′ flouro oxanilate	Complete	Ali (1990, 1993)
Ethyl 4′ metho oxyoxanilate	Complete	Ali (1990, 1993)
Ethyl 4′ bromo oxanilate	High	Ali (1990, 1993)
Ethyl 4′ chloro oxanilate	High	Ali (1990, 1993)
Other chemicals (with and without codes)		
RH 531	Complete	Perez et al (1973)
DPX 3778	Prevents anther dehiscence	Long et al (1973)
3-(-p-chlorophenyl) 6-methoxy-s-triazine –2-4 (1H,3H) dione tri-ethanolamine	High to complete	Zhangzing and Chunnong (1980)
Sodium sulfate	Complete	Wang et al (1981)
HRG 626	Complete	Takeoka et al (1990)
Ammonia sulfonic acid	High	Chen (1985)
HAC 123 + N 312	Complete	Luo et al (1988)
MHC	Complete	Song et al (1990)
CRMS	Complete	Wang et al (1991a,b,c)
Kasugamycin	Partial to high	Atsumi et al (1992)
AOA	High	Astumi et al (1992)

have been tested along with 3-line bred hybrids and were reported to give consistently comparable and often higher yields. Over the years, seed yields have increased from 0.4 t ha^{-1} with 40–60% seed purity to 1.5 t ha^{-1} with 80–90% seed purity. CHAs must be able to selectively induce total male sterility. The effectiveness of CHAs is highly stage-specific (i.e., these should be applied at the stamen and pistil primordia formation stage or stage IV) and genotype-specific (i.e., the gametocidal effect varies from variety to variety). In India, oxanilates, when sprayed at stage IV (meiotic stage) of rice development, were found to be effective and variety Pusa 150 was sterilized more effectively by the gametocidal spray than other varieties, thus indicating genotype specificity (Ali 1993). Figure 8 illustrates an example of two-line hybrid rice seed production using a CHA.

Properties of an ideal CHA

An ideal CHA should have the following properties:

1. Wide-spectrum action to induce sterility in successively emerging panicles.
2. Selective and total sterilization of stamens without affecting ovular fertility.
3. Be less phytotoxic, noncarcinogenic, and without residual toxicity that could harm human beings and animals.
4. Be easy to apply and economical.

Advantages of the two-line approach via CHA

1. A wide range of varieties can be used for making superior hybrid combinations.
2. The method of seed production is simpler than that of three-line breeding as it does

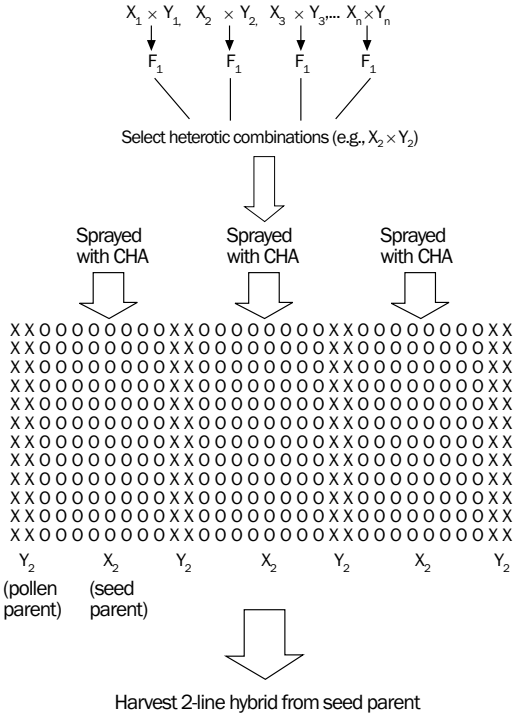


Fig. 8. Schematic description of the use of a CHA to develop two-line hybrids.

not require development of three lines (A, B, and R).

3. If the CHA is not effective because of nonsynchronization of flowering or continuous rain during the critical stage, heavy crop losses can still be averted because the yield of the sprayed unaffected female would still be good enough.
4. Partial CMS lines and EGMS lines can be made completely sterile with a spray of an ideal CHA.
5. The narrow genetic base for cytoplasmic genic male sterility, inherent in three-line rice hybrids, ceases to be a problem in CHA-derived hybrids.

Disadvantages of CHAs

1. Production of impure hybrid seeds if the CHA is not effective because of unfavorable weather conditions or nonsynchronized tillering and growth.
2. Health hazards of some CHAs (such as zinc methyl arsenate or sodium methyl arsenate).
3. High cost of the chemicals.

Comparative organization of two- and three-line hybrid breeding programs

Organization of a hybrid breeding program is strikingly different from the conventional inbred breeding program in rice. Three-line and two-line hybrid rice breeding have some similarities as well as differences in their organization. These are described in this chapter.

Similarities in three-line and two-line breeding nurseries

The breeding nurseries that are identical in both breeding programs are as follows.

Source nursery

This nursery contains elite lines that have the potential to become parents of commercial hybrids. Include the best available CMS, TGMS, and PGMS lines in this nursery. To raise the source nursery, grow 20 plants (with a single seedling per hill) per line in rows. Use these as single plants for testcrosses with the best available CMS or EGMS lines. To synchronize flowering and to make as many testcrosses between elite lines as possible and to make sterile lines, plant the sterile lines on three dates with a 10-day interval.

Testcross nursery

This nursery contains the testcrosses made in the source nursery along with the single-plant progenies of the male parents used to make the testcrosses. After growing every ten pairs of testcross and the corresponding male parent progenies, grow single rows of inbred and hybrid checks. In a three-line hybrid breeding program, screen the testcross progenies for pollen sterility/fertility, spikelet fertility, and other agronomic traits to identify potential maintainers and restorers and heterotic hybrids. In the two-line hybrid breeding program, screen the testcross progenies for pollen fertility, spikelet fertility, and other agronomic traits to identify the potential elite male parents and heterotic hybrids. Plant the F_1 and their male parents side by side in three rows each with a single plant per hill.

Combining ability nursery

This breeding nursery contains a set of crosses derived from promising CMS and restorer lines (in the three-line breeding program) and promising EGMS and elite pollen parent lines (in the two-line breeding program) made in a line × tester design to evaluate the parental lines for their combining ability, that is, their ability to produce superior progenies when crossed with several male parents.

Yield evaluation nurseries

The procedure adopted for heterosis evaluation for two- and three-line breeding is similar. It contains the following five different trials:

1. **Observational yield trial (OYT).** This contains hybrids derived from commercially usable CMS lines (showing stable pollen sterility, high general combining ability, and good outcrossing potential) and promising restorer lines identified in the testcross nursery. The trial also contains inbred check varieties of different growth duration. Groups of test hybrids are compared with a set of inbred checks and hybrids. Thus, the test hybrids are unreplicated, whereas the check varieties and hybrids are replicated across different groups. The OYT uses an augmented design in which test hybrids are

arranged in groups. Each plot is about 6 m^2, in which 125–175 single plants per hill are included. Data on agronomic traits, yield, and disease and insect pest resistance are recorded.

2. **Preliminary yield trial (PYT).** The promising hybrids that yield 15–20% higher than the check varieties in the testcross nursery, combining ability nursery, and OYT are forwarded for further evaluation in the PYT. Hybrids are grouped according to their maturity duration. This trial uses a randomized complete block design with 3–4 replications, with an individual plot size of 7 m^2. Data on agronomic traits, yield, disease and insect resistance, and grain quality (of heterotic hybrids only) are recorded. The plot design and data recorded are similar to those of the OYT.
3. **Advanced yield trial (AYT).** The promising hybrid entries from the PYT are included in the AYT. The plot design and data recorded are similar to those of the PYT, but the plot size is 10 m^2.
4. **Multilocation trials (MLT)/national hybrid trial (NHT).** The outstanding hybrid entries in the AYT are nominated into the MLT/NHT. The experimental design and data collected remain the same as in the AYT, except that the plot size is increased to 15–20 m^2.
5. **On-farm testing (OFT).** One to two hybrids performing consistently better than the regional check and local checks over 2–3 years are recommended to the OFT. The plot area is about 0.1 ha, with or without replication. The best local check variety (hybrid) is used as the control.

Hybrids that pass all the above trials and have resistance to major insect pests and diseases and acceptable grain quality are registered and/or released.

Differences in three-line and two-line breeding nurseries

1. Male sterile maintenance and evaluation nurseries

In three-line breeding, CMS lines are maintained and evaluated in a CMS line maintenance and evaluation nursery by growing A and B lines side by side. Their pollen sterility/fertility is monitored and lines are evaluated on a single-plant basis for phenotypic acceptability (on a 1–9 scale, where 1 = excellent and 9 = poor), days to 50% flowering, outcrossing ability, and other desired traits. The CMS lines are maintained by hand-crossing of A and B lines (on a single-plant basis) to maintain their purity. Hand-crossed seeds of the commercially usable CMS lines are produced in larger quantities (500–1,000 seeds) so that they can be used for nucleus seed production.

In the two-line breeding program, EGMS lines are maintained by selfing by growing them in appropriate daylength and temperature conditions that induce fertility. These EGMS lines are evaluated for sterility separately by growing them under suitable daylength and/or temperature conditions in the phytotron and/or field. Simultaneously, they are also evaluated for phenotypic acceptability, flowering, outcrossing rate, and other desired traits in comparison with suitable checks (such as popular commercial varieties, popular CMS lines, and the best available EGMS lines), which should be grown side by side.

2. EGMS breeding nurseries

These are described in the next chapter. A comparative flow chart of two-line and three-line hybrid rice breeding nurseries appears in Figure 9.

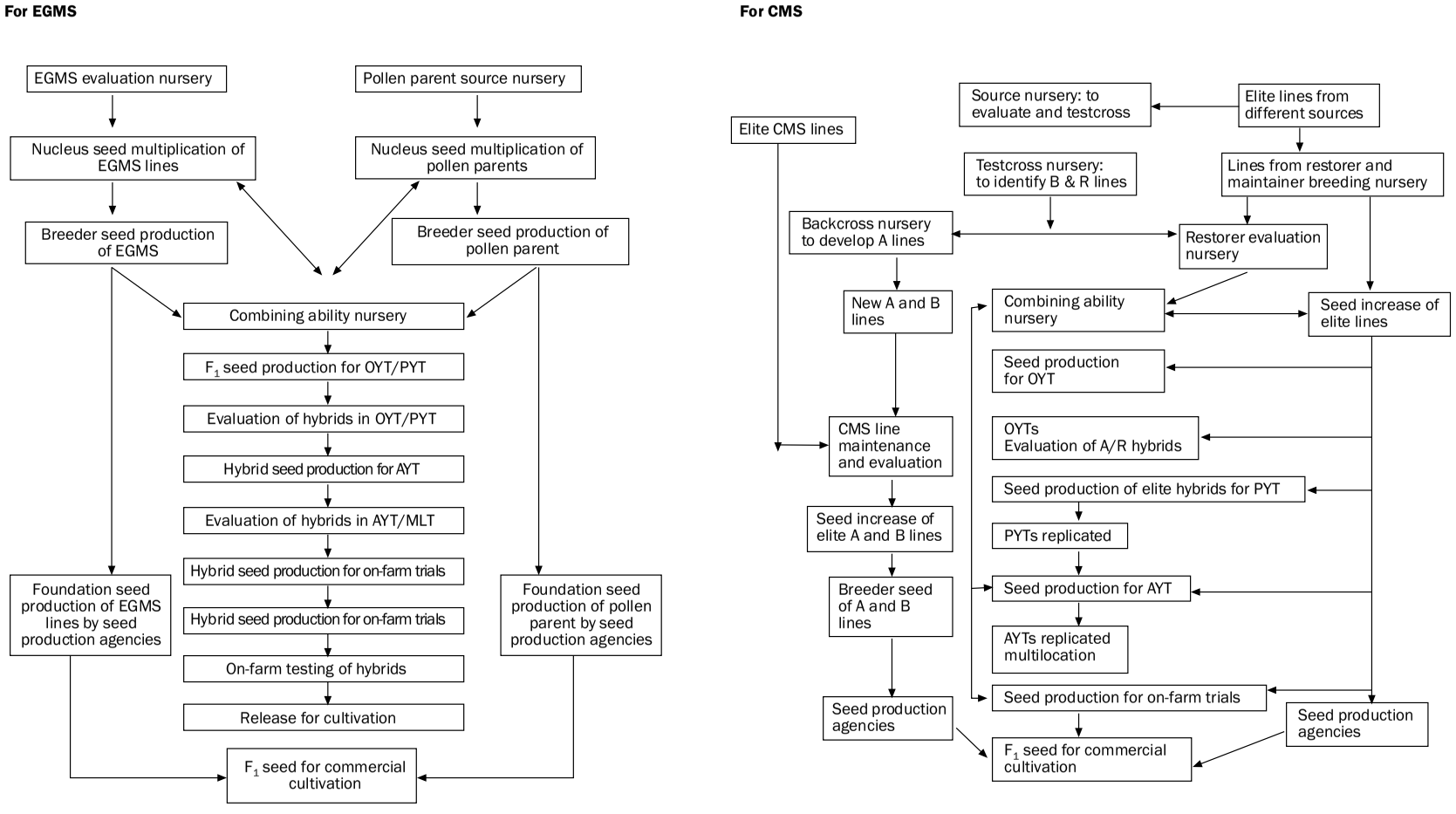


Fig. 9. Operational flow chart of hybrid rice breeding using EGMS and CMS lines. OYT = observation yield trial, PYT = preliminary yield trial, AYT = advanced yield trial, MLT = multilocational trial.

Inheritance of EGMS

Procedure for carrying out inheritance studies on the EGMS trait

The following stepwise procedure explains how inheritance studies on the EGMS trait are conducted:

- Purify the EGMS lines and fertile parents through bagging.
- Prepare all generations for genetic analysis and obtain sufficient seeds. The generations involve P_1, P_2, F_1, BC_1F_1, BC_2F_1, and F_2.
- Plant different generations with 10–20 P_1, P_2, and F_1 plants, 60–120 BC_1 plants, and more than 200 F_2 plants.
- Observe the pollen and spikelet sterility of each plant by putting the plants under appropriate sterile environmental conditions during the sensitive stage.
- Classify the sterile:fertile plants accordingly.
- Use the χ^2 test to analyze the segregation pattern and determine the genes that control EGMS.

Inheritance of TGMS

Genetic studies in Japan, at IRRI, and in India (Maruyama et al 1991, Borkakati and Virmani 1993, Ali 1996) indicated that the TGMS trait in Norin PL 12, IR32364S, and SA 2 was controlled by a single recessive gene. Allelic relationship studies indicated that the TGMS genes in Norin PL 12 and IR32364S mutants were different. TGMS line 5460s developed in China carries the *tms*$_1$ allele on chromosome 8, whereas IR32364S carries the *tms*$_3$ allele on chromosome 6. The TGMS allele in Norin PL 12 is designated as *tms*$_2$ (Fig. 10). On the basis of this information, other TGMS sources from India have also been studied for allelic relationships and Reddy et al (2000) found a new nonallelic TGMS trait designated as *tms*$_4$ in SA 2, a sodium azide-induced TGMS mutant. Other details are given in Table 8.

The transfer of the Norin PL 12 gene (*tms*$_2$) into IRRI cultivars such as IR68945S, IR68949S, and IR68294S revealed varied sterility-fertility–altering conditions, indicating a change in the expression of the TGMS trait in a different genetic background.

Inheritance of PGMS and PTGMS

Single-locus genetic model

In an initial study, many conventional japonica and indica varieties were reciprocally crossed to Nongken 58S (NK58S) and all the F_1s were fertile. In F_2 populations of reciprocal crosses between NK58S and three conventional japonica rice lines, a 3:1 ratio of fertile:sterile plants was observed in each population grown under long-day conditions. The sterility was therefore considered to be controlled by a single recessive nuclear gene. Yang et al (1992a) designated the PGMS gene as *Ps*. It was also noted that minor genes might also be responsible for the sterility, as few sterile F_2 plants were 100% sterile. Many other studies showed that fertility segregation in crosses between NK58S and NK58 was controlled by a single nuclear gene.

Two-loci genetic model

Many studies showed that PTGMS in NK58S-derived P(T)GMS lines was conditioned by recessive alleles at two loci.

The two-loci genetic control of PTGMS was

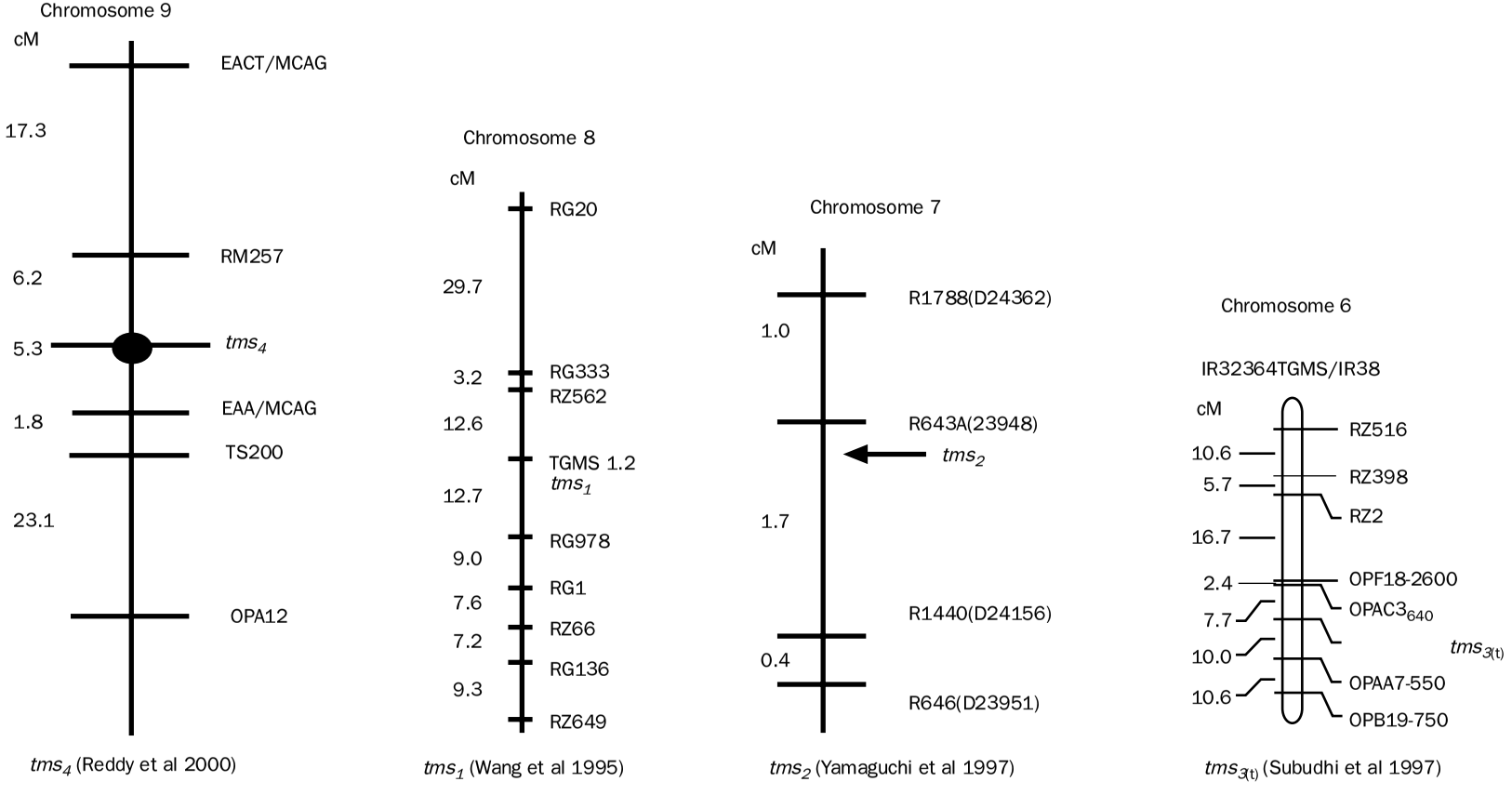


Fig. 10. Location of TGMS genes on the linkage map in rice.

Table 8. Different alleles for the TGMS trait among available TGMS sources.

Gene symbol	TGMS source
tms_1	JP 1 and 5460S
tms_2	IR68945 (Norin PL 12)
tms_3	JP 8-8-1-S, 1C 10, IR32364
tms_4	SA2
tms_5	Annong S-1
$rtms_1$ (reverse TGMS)	J207 S

well demonstrated in the work of Mei et al (1990). The F_1, F_2, and BC_1F_1 populations were derived by crossing NK58, NK58S(R), 1149, and NK58S with each other. NK58S(R) is a fertility revertant mutant of NK58S, whereas l149 is a fertile line developed from the cross of NK58S and japonica variety Nonghu 26. A χ^2 test indicated that sterility in NK58S was conditioned by recessive alleles at two loci. The fertility-restoring alleles in NK58S(R) and l149 are allelic to each other, but they are nonallelic to that in NK58.

Designating one gene as ms^{ph} and the other as rf^{ph}, the genotypes of NK58S, NK58, and NK58S (R8), a fertility revertant mutant of NK58S, were the following:

Nongken 58S: ms^{ph} ms^{ph} rf^{ph} rf^{ph}
Nongken 58: Ms^{ph} Ms^{ph} rf^{ph} rf^{ph}
Nongken 58S(R): ms^{ph} ms^{ph} Rf^{ph} Rf^{ph}

Complete dominance is displayed at the Ms^{ph} locus and plants possessing Ms^{ph} would be fertile. When partial dominance is displayed at the Rf^{ph} locus, plants possessing Rf^{ph} alone would be partially fertile.

Diverse genetic model

Results obtained from crosses between NK58S and japonica varieties other than NK58 were quite complex. Although the fertility segregation in the majority of crosses was attributed to a single dominant gene or two dominant genes, it was also evident that multiple genes were involved in the genetic control of PTGMS.

The two genes segregated independently and the interaction between them varied greatly depending on the genetic background. Different segregation patterns such as 9 fertile:6 partially fertile:1 sterile, 9 fertile:3 partially fertile:3 partially sterile:l sterile, and 9 fertile:3 partially fertile:4 sterile were observed (Sheng 1992). A bimodal distribution was generally observed in the fertility segregation but, on close scrutiny, the segregation was found to be continuously distributed in each population. Mei et al (1990) showed that the fertility segregation ratio varied greatly depending on the criteria for the classification of sterile and fertile plants. Although Mei et al (1990) suggested that PTGMS was quantitatively inherited, other reports suggested that the complexity of PTGMS was conditioned by a major gene and multiple minor genes, partial dominance of sterile alleles, and dosage effects. In addition, it was suggested that both major and minor genes might be responsible for the genotypic difference in the low-sterility-inducing temperature. The concept that the genetic control of PTGMS is now governed by a set of multiple genes having major and minor effects is well accepted.

Allelic relationship studies

The PTGMS genes in NK58S-derived japonica lines were allelic to the NK58S gene. In some of the NK58S-derived indica lines, genes that were nonallelic to NK58S were also found. Among PTGMS lines having independent origins, the sterility in both Hengnong S-l and 5460S was controlled by two recessive nuclear genes, and that in Annong S-l was controlled by a recessive nuclear gene. The allelism test indicated that the genes in NK58S, Annong S-l, 5460S, and Hengnong S-l were nonallelic to each other. It is now understood that five genes were involved in the genetic control of NK58S, NK58S-derived P(T)GMS lines W6154S and W7415S, Annong S-1, and 5460S (Table 9).

Genetic mapping of PTGMS

By employing marker-gene stocks, Zhang et al (1990) found that the major gene for PTGMS ms^{ph} in NK58S was linked to *d-1* on chromosome 5 with a recombination fraction of 28.4% and it segregated independently from the other 33 marker genes located on the remaining 11 chromosomes of rice. This result was confirmed by the detection

Table 9. Genotypes proposed for PTGMS in five lines.

Line	Genotype
Nongken 58S	S_1S_1 S_5S_5
W6154S	S_2S_2 S_5S_5
W7415S	S_1S_1 S_4S_4
Annong S-1	S_2S_2
5460S	S_3S_3 S_5S_5

of closer linkage (20.3 cM) between *ms*^{ph} and another marker gene, *gh-l* on chromosome 5. Isozyme markers *Cat-1* on chromosome 6 and *Adh-l* on chromosome 11 were also each linked to a gene conditioning PTGMS in NK58S.

The PTGMS genes were mapped precisely using RFLP markers by a new mapping approach using the bulked extremes and the recessive class for increasing mapping efficiency (Zhang et al 1994). In a cross involving NK58S-derived PTGMS indica line 32001S as the PTGMS parent, two PTGMS loci, *pms*$_1$ and *pms*$_2$, were located on chromosomes 7 and 3, respectively. The effect of *pms*$_1$ was 2–3 times larger than that of *pms*$_2$ and dominance was almost complete at both loci. In two crosses involving NK58S as the PTGMS parent, two PTGMS loci were located in both populations. One locus was *pms*$_1$ on chromosome 7 and the other was *pms*$_3$ on chromosome 12 (Fig. 11). Using the F_2 population of NK58S/NK58, the locus relevant to the fertility difference between NK58 and NK58S was confirmed to be *pms*$_3$ on chromosome 12.

Taking all the mapping studies into account, genes conditioning PTGMS have been assigned to six of the 12 rice chromosomes: chromosomes 3, 5, 6 , 7, 11, and 12 (Zhang et al 1990, Zhang Q et al 1994, Wang et al 1997, Mei et al 1999). Only a small number of crosses were used in the mapping studies and the PTGMS parents involved only NK58S and an NK58S-derived PTGMS line. Moreover, no reports to date have determined minor genes for PTGMS. We could expect that many genes are responsible for PTGMS. There might be two groups of genes: some genes condition photoperiod sensitivity, whereas others control male sterility. Studies to distinguish between genes for photoperiod sensitivity and for male sterility would be as important as studies to identify gene locations.

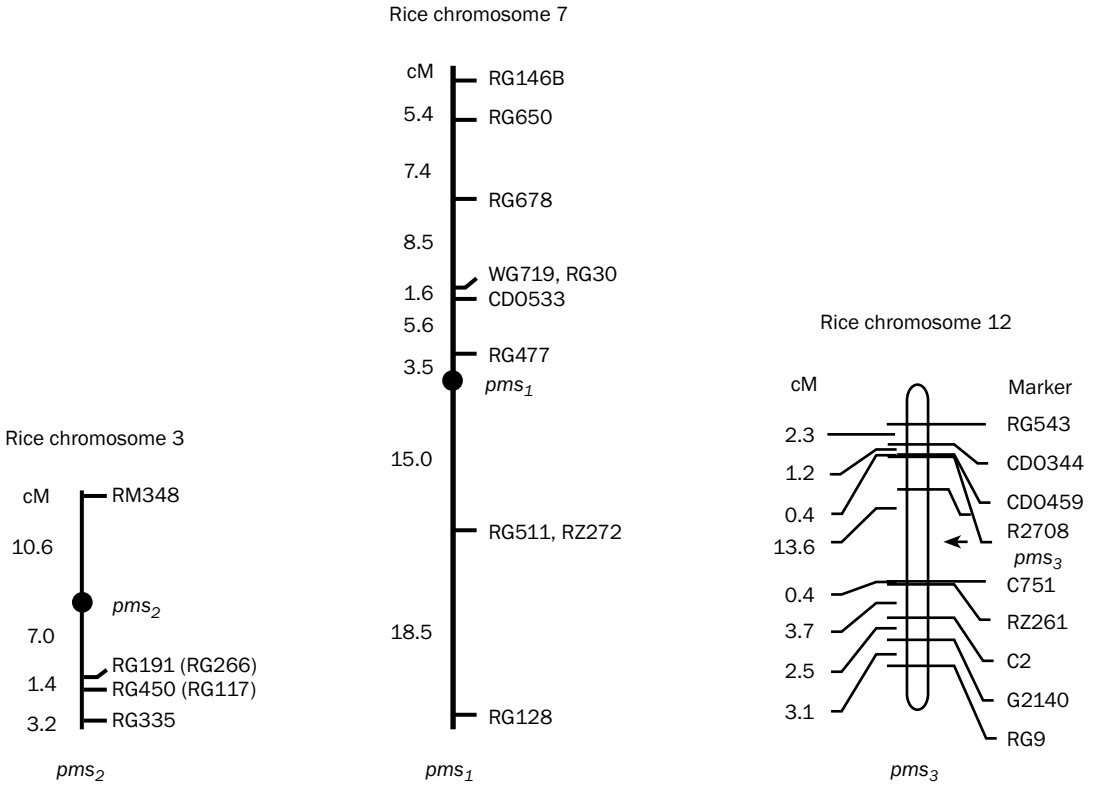


Fig. 11. Location of PGMS genes on the linkage map in rice (Zhang et al 1994, Mei et al 1999).

Breeding procedures for developing EGMS lines

EGMS lines can be developed by any of the following methods:

- Screening of existing varieties
- Induced mutagenesis
- Hybridization followed by pedigree selection, anther culture, backcrossing, and marker-aided selection (MAS)

Screening of existing varieties for EGMS

Existing varieties can be screened to detect spontaneous EGMS mutants. A large number of rice germplasm materials should be screened over seasons involving various high-temperature or daylength regimes to identify any spontaneous mutants or lines showing differential pollen and spikelet sterility during different temperature or daylength regimes occurring during the panicle initiation stage onward. Rice germplasm consisting of photoperiod-sensitive varieties or varieties adapted to high altitudes provides a higher probability of success when using this method.

During this screening,

1. Select plants in which earlier panicles are partly fertile and later ones are almost sterile or vice versa according to the environmental conditions. These plants are easily identified by the combination of partly filled bending panicles and sterile erect panicles in the same plant.
2. Study pollen sterility of younger panicles and determine whether sterility is higher than 99% (see Appendix 1 for the detailed procedure).
3. Multiply the suspected plants by separating the tillers and ratooning them.
4. Evaluate plants for their fertility behavior under different temperature (TGMS) and photoperiod (PGMS) using the growth chamber or phytotron or under field conditions (as mentioned in Chapter 2).

Induced mutagenesis

EGMS lines, particularly TGMS lines, can also be developed by the mutation breeding method described below.

1. Select the best available high-yielding rice cultivars that are photoperiod-sensitive, cold-tolerant, and adapted to high altitudes for inducing EGMS mutants. These lines stand a better chance for inducing such mutations.
2. Select any of the physical (gamma rays, fast neutrons) or chemical (sodium azide, ethyl methane sulfonate, EMS, methyl methane sulfonate, MMS, N-methyl-N-nitrosourea, MNU, etc.) mutagens.
3. Treat the seed material with an appropriate dose of mutagen and grow it as the M_1 generation, for example, gamma rays—25 Kr; sodium azide—0.002 M, pH 3 for 6 h; EMS—0.1%, pH 7.0 for 6 h; MNU—1.5 mM for 1 h.
4. Select 1 or 2 panicles from single plants in the M_1 generation.
5. Grow the M_2 progenies under appropriate temperature (such as >30/24 ºC) and daylength (such as >13.45 h) conditions and select plants showing complete sterility or differential fertility of panicles within the same plant. Multiply selected plants by separating their stubbles and evaluate them

under appropriate fertility induction in the growth chamber (as shown in Tables 10 and 11) or under natural field conditions. Those that revert to fertility are suspected EGMS plants. These may be TGMS, reverse TGMS, PTGMS, PGMS, or reverse PGMS depending on their behavior.

Hybridization method

This is an important and simple method to develop new EGMS lines in diverse genetic backgrounds by using already identified and characterized EGMS lines. Under this method, the known EGMS source is crossed as a female parent to any of the popular high-yielding rice varieties (HYV) possessing good combining ability, disease and pest resistance, and acceptable grain quality. This method is generally used in a situation in which the available EGMS line is unadapted or unacceptable for one reason or another. The crosses made above can be handled in any of the following procedures:

Pedigree selection

a) Pedigree selection procedure using method I

1. Grow F_1 plants from the crosses made to obtain F_2 seeds.

Table 10. Simultaneous screening of suspected EGMS lines for identification of type of EGMS.[a]

Category of growth chamber settings	Expected reaction of suspected EGMS on pollen sterility/fertility
High temperature (32 °C) and long photoperiod (14 h)	Completely sterile (PGMS/TGMS/PTGMS) Fertile (reject/reverse PGMS/reverse TGMS)
High temperature (32 °C) and short photoperiod (12 h)	Completely sterile (TGMS/PTGMS/reverse PGMS) Fertile (PGMS/reverse TGMS)
Low temperature (24 °C) and long photoperiod (14 h)	Completely sterile (PGMS/reverse TGMS) Fertile (TGMS/PTGMS/reverse PGMS)
Low temperature (24 °C) and short photoperiod (12 h)	Completely sterile (reverse PGMS/reverse TGMS accordingly) Fertile (TGMS/PGMS/PTGMS)

[a]The same set of lines must be placed in all four chambers simultaneously.

Table 11. Design of experiment to identify and differentiate PTGMS from TGMS lines.[a]

Temperature (°C)	Photoperiod (daylength duration)		
	12 h	13 h	14 h
>32	Sterile (TGMS and PTGMS) (1)	Sterile (TGMS and PTGMS) (2)	Sterile (TGMS and PTGMS) (3)
30	• Fertile (partial) TGMS (4)	• Fertile (partial) (TGMS) • Less fertile than the value in box 4 (PTGMS) (5)	• Fertile (partial) (TGMS) • Less fertile than the value in boxes 4 and 5 (PTGMS) (6)
28	• Fertile (partial) (TGMS) • More fertile than the value in box 4 (PTGMS) (7)	• Fertile (partial) (TGMS) • Less fertile than the value in box 7 (PTGMS) (8)	• Fertile (partial) (TGMS) • Less fertile than the value in boxes 7 and 8 (PTGMS) (9)
26	• Fertile (partial) (TGMS) • More fertile than the value in box 7 (PTGMS) (10)	• Fertile (partial) (TGMS) • Less fertile than the value in box 10 (PTGMS) (11)	• Fertile (partial) (TGMS) • Less fertile than the value in box 11 (PTGMS) (12)
24	Fertile (TGMS and PTGMS) (13)	Fertile (TGMS and PTGMS) (14)	Fertile (TGMS and PTGMS) (15)

[a]Lines that show influence of temperature and photoperiod are PTGMS, whereas the lines that show no influence of photoperiod are TGMS. Pollen fertility % is a better measure than spikelet fertility provided anthers from the top five spikelets at panicle emergence are used for making a squash preparation with 1% IKI stain and the pollen fertility/sterility of the emerged panicle is related to the given treatment in the phytotron.

2. Grow F_2 populations of the crosses made under appropriate temperature (for TGMS) or daylength (for PGMS) conditions to identify sterile single plants that combine most of the useful traits of both parents. Grow the popular HYV and the donor EGMS line beside the F_2 population to select comparable plants. Raise stubbles of the selected sterile single plants under fertility induction conditions to produce F_3 seeds. Seed setting on the ratooned plants identified earlier as male sterile will confirm the EGMS trait.
3. Grow F_3 progenies of the selected EGMS plants under appropriate fertility induction temperature or daylength conditions to select desirable plants and harvest F_4 seeds. Concurrently evaluate the F_3 progenies in sterility-inducing temperature or daylength conditions to select completely sterile F_3 progenies to advance the generation.
4. Handle F_4 and F_5 progenies in the same manner as F_3 progenies.
5. To confirm stability for sterility of an EGMS line in any given generation, trials can be conducted under varying temperature and daylength regimes in various places.
6. Characterize identified TGMS or PGMS lines for specific defined environmental conditions at the sensitive stage both in the growth chamber and under field conditions.
7. Test the specific TGMS and PGMS lines across various locations and seasons to identify suitable locations for hybrid seed production and EGMS self seed multiplication.

b) Pedigree selection procedure using method II
This method is deployed when we do not have a low temperature/shorter photoperiod conducive to fertility near where the research is conducted or the specific environmental condition is not available during the same season of selection.

1. Grow the F_2 population under a sterility-inducing temperature regime. Raise the standard controls as mentioned earlier besides the EGMS and the other elite parent used for hybridization to develop superior EGMS lines. Select desired fertile plants in the segregating population.
2. Grow the F_3 to F_5 generations under sterility-inducing temperature and select 8–10 desirable fertile plants from the progeny rows segregating for sterility. The reason for selecting so many fertile plants in the segregating population is to ensure the probability of selecting at least one heterozygous fertile plant that would segregate for sterility in the next generation.
3. Grow F_5 and F_6 populations under sterility-inducing temperature or longer photoperiod. Select the most desirable male sterile plants and ratoon them.
4. Transfer the ratooned male sterile plants to a phytotron or glasshouse with a day/night temperature of 25/19 ºC or shorter daylength (12 h) to induce fertility.
5. Select those plants that revert to fertility under low-temperature or shorter photoperiod conditions and collect their seeds. These are suspected TGMS/PGMS plants.
6. Grow progenies of the suspected TGMS/PGMS plants under sterility-inducing temperature/photoperiod conditions in the field and select those plants as TGMS/PGMS that give completely male sterile progenies.

Backcrossing after hybridization

Backcrossing is the most suitable method when we need to transfer oligogenes in a recessive condition to an already established variety as the recurrent parent.

The procedure involves the following steps: Select a stable and suitable TGMS/PGMS donor with well-defined critical sterility/fertility points and cross it with an established variety to which the TGMS/PGMS trait has to be transferred.

1. Grow the F_1 generation.
2. Grow the F_2 generation to select suspected EGMS plants; ratoon these plants to confirm their TGMS/PGMS nature.
3. Backcross the EGMS plants with the recurrent parent.
4. Repeat steps 2 and 3.
5. After every two backcrosses, one generation of selfing is required to verify that the recessive EGMS trait is being carried forward as depicted in Figure 12. After six generations of backcross, self the BC_6F_1 to select sterile plants under high temperature (>30 ºC day/24 ºC night)/long photoperiod (14 h daylength). Ratoon plants may be sent to the growth chamber or suitable natural conditions conducive to fertility reversion.
6. The TGMS development procedure is shown in Figure 13 but the same is applicable to

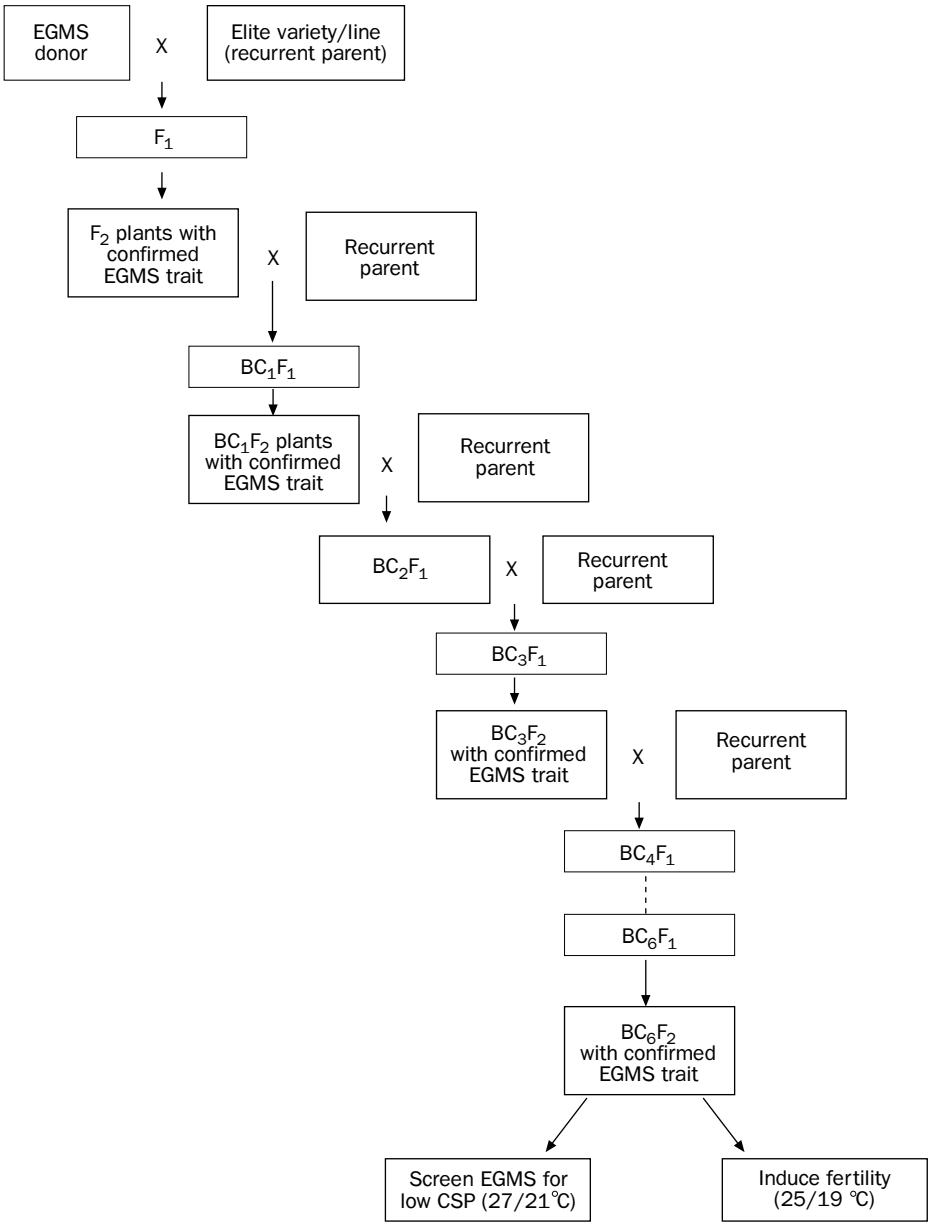


Fig. 12. Backcross procedure for developing TGMS/PGMS lines.

PGMS since the screening procedure varies only under the BC_nF_2 (n = 1, 3, 5) generation. To develop PGMS through the backcross procedure, long-day conditions (>14 h) will be required for screening in the BC_1F_2, BC_3F_2, and BC_5F_2 generations to identify the recessive sterile genotypes.

Anther culture or pollen culture following hybridization

Anther culture or pollen culture accelerates the breeding process and increases selection efficiency in the breeding of EGMS lines over conventional techniques. To use this method, rice breeders should work closely with a tissue culture expert.

1. Make specific crosses to combine stable TGMS or PGMS lines with promising local cultivars. Grow F_1 plants of these crosses.
2. Produce dihaploid lines through anther culture using anthers or pollen grains from the F_1 plants, adopting standard available protocols for rice (Fig. 13). (See also Appendix II.)

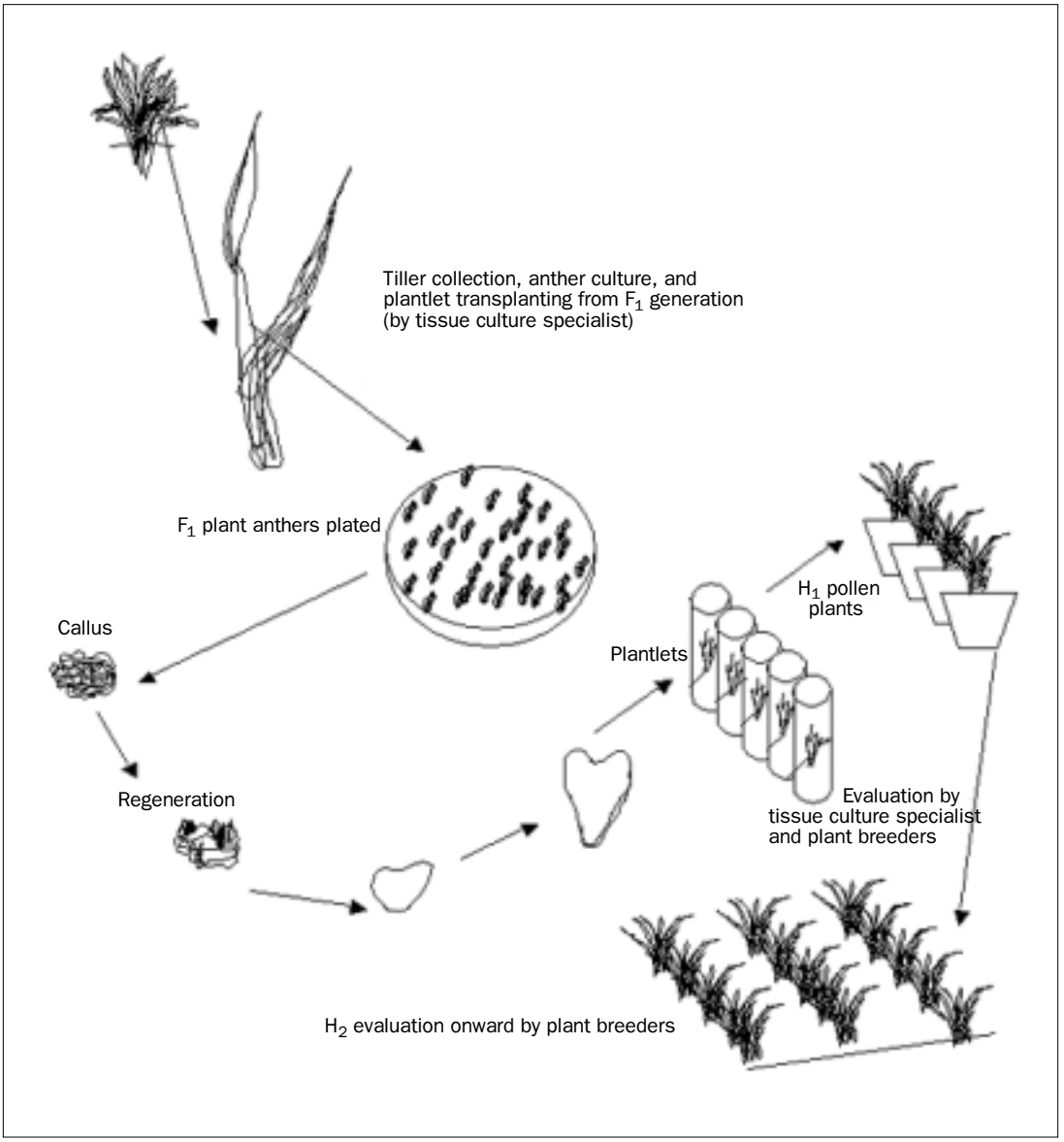


Fig. 13. Procedure for producing and evaluating dihaploid EGMS lines.

3. Seeds obtained from the dihaploid plants should be sown and raised under sterility-conducive conditions for the selection of male sterile lines. Monitor the pollen sterility of anther/pollen culture–derived plants.
4. Ratoon sterile dihaploid plants and place them under low temperature and shorter photoperiod to induce fertility and get selfed seeds.
5. Screen for TGMS and PGMS lines based on the criteria for ideal EGMS lines.
6. TGMS lines such as 6442S, 1286S, HS-1, and HS-5 and one PTGMS line, Lu Guang 2S, have been developed through anther culture and extensively tested in Jiangxi, Sichuan, and Fujian provinces of China (Fig. 14).

EGMS gene transfer and pyramiding through marker-aided selection (MAS) after hybridization

Several closely linked markers have been identified with TGMS and PGMS genes to use the MAS approach. These markers are listed in Table 12 and can be used for selection of the TGMS/PGMS trait in segregating F_2 and backcross breeding populations without actually screening them under specific field or phytotron conditions. The MAS approach can enhance the speed and efficiency for selecting EGMS plants without exposing them to conditions for expression of the EGMS gene.

MAS also allows pyramiding of different sources of TGMS or PGMS/PTGMS alleles into a common genetic background. It would be inter-

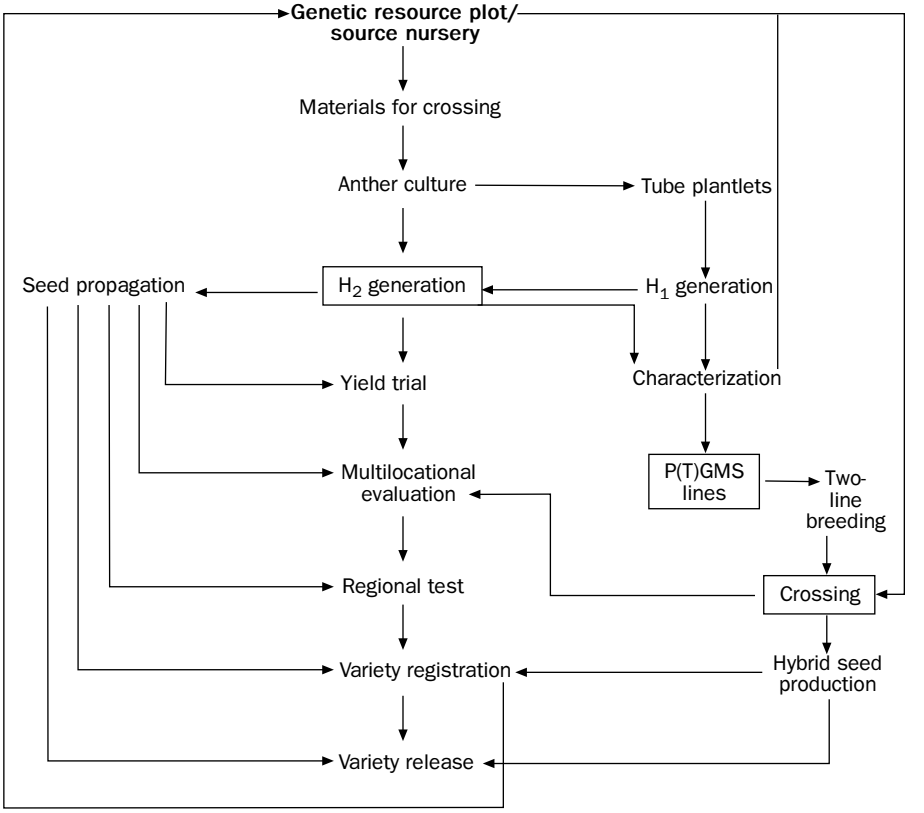


Fig. 14. Procedure for indica two-line hybrid rice breeding through anther culture (modified from Zhu et al 1999).

Table 12. Closely linked flanking molecular markers for EGMS genes.

Trait	Gene	Chromosome	Closest flanking markers
TGMS	tms_1	8	RZ562–RG978
	tms_2	7	R643A–R1440 (D24156)
	tms_3	6	$OPAC3_{640}$–$OPAA7_{550}$
	tms_4	9	RM257–TS200
	$rtms_1$	10	RM239–RG257
PGMS/PTGMS	pms_1	7	RG477–RG511, RZ272
	pms_2	3	RG348–RG191 (RG266)
	pms_3	12	R2708–RZ261/C751

esting to study the manifestation of pyramided EGMS genes under different environmental conditions. Pyramiding different sources of alleles may improve the EGMS lines for their critical sterility points (CSP) and their critical fertility points (CFP). A low CSP–low CFP line will be useful in tropical conditions. Four different TGMS genes—tms_1, tms_2, tms_3, and tms_4—exist, while there are three reported PGMS genes—pms_1, pms_2, and pms_3. Efforts are under way to pyramid different TGMS genes in China, at IRRI, and in India (Fig. 15).

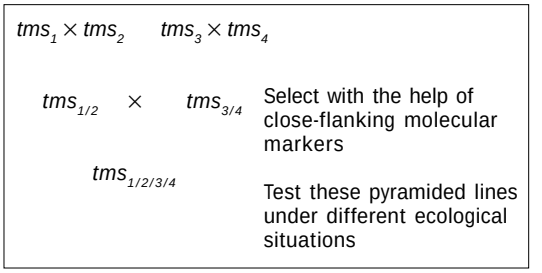


Fig. 15. Procedure for TGMS gene pyramiding.

Characterizing EGMS lines under field and controlled conditions

Photoperiod varies in a gradual and periodical manner year-round based on the latitude and solar terms. For a given place, the photoperiod on one day over different years is the same and, on a given date, the photoperiod in different locations at the same latitude is also the same. Likewise, the daylength at different latitudes in the northern hemisphere is the same on the spring (21 March) and autumn equinox (23 September) because the sun radiates vertically on the equator on those days (Table 13). The longest photoperiod is observed on the summer solstice, when the sun radiates vertically on the Tropic of Cancer, and the shortest photoperiod is on the winter solstice, when the sun radiates on the Tropic of Capricorn. Data in Table 13 also indicate that the higher the latitude, the longer the photoperiod on 22 June (summer solstice) and the shorter the photoperiod on 22 December (winter solstice). Likewise, in the southern hemisphere, similar conditions exist except that the winter and summer solstice changes. The winter solstice occurs in the southern hemisphere on 22 June when the daylength is shortest, whereas the summer solstice occurs on 22 December when the daylength is longest. The higher the latitude, the longer the observed photoperiod. Under the winter solstice on 22 June in the southern hemisphere, the higher the latitude, the lower the photoperiod or daylength (Table 13).

The temperature at any given location is influenced by factors such as solar radiation, latitude, altitude, local topography, and atmospheric and marine phenomena such as El Niño and La Niña. Many locations in the tropics still have less influence from the abovementioned environmental factors on temperature and therefore those locations are quite stable in temperature and can be easily identified based on 25 years of meteorological data.

Characterization of EGMS lines under field conditions

Detailed meteorological data (such as minimum and maximum temperature, daylength, sunshine hours, humidity, etc.) are essential to characterize EGMS lines at a given location. It is better if data of the past 10–15 years are available. To characterize given EGMS lines, the following procedure can be used:

1. Identify 3–4 distinct periods of high and low temperatures during the year. Likewise, determine the longer and shorter daylength durations during the year and over locations.
2. Seed/plant EGMS lines at 15–25-day intervals in such a way that their heading coincides with the high temperature or longer photoperiod.
3. Study pollen fertility of the EGMS lines from the top five spikelets of primary panicles under the microscope.
4. Relate the pollen sterility data to temperatures/daylengths prevailing during the period of 15–25 days before heading ("tracking technique"). The temperature or daylength that is just sufficient to make the plant completely pollen sterile must be noted among the several temperature or daylength regimes to which plants were exposed during the period. Such a temperature point or daylength at which complete pollen sterility is obtained is termed the critical sterility point and the number of days

Table 13. The natural photoperiod change with dates and latitudes in the northern hemisphere.

Latitude (°N)	Spring equinox 21 March	Summer solstice 22 June	Autumnal equinox 23 Sept.	Winter solstice 22 Dec.
		(h.min)		
40	12.09	15.01	12.12	9.20
30	12.08	14.04	12.12	10.13
29	12.06	13.58	12.06	10.14
28	12.06	13.52	12.06	10.20
27	12.06	13.48	12.06	10.24
26	12.06	13.44	12.60	10.28
25	12.06	13.38	12.06	10.32
24	12.06	13.34	12.06	10.36
23	12.06	13.30	12.06	10.40
22	12.04	13.26	12.06	10.44
21	12.04	13.22	12.06	10.48
20	12.04	13.18	12.06	10.52
19	12.04	13.14	12.06	10.56
18	12.04	13.10	12.06	11.00
17	12.04	13.06	12.06	11.04
16	12.04	13.02	12.06	11.08
15	12.04	12.58	12.06	11.12
14	12.04	12.54	12.04	11.16
13	12.04	12.52	12.04	11.20
12	12.04	12.48	12.04	11.22
11	12.04	12.44	12.04	11.26
10	12.04	12.40	12.04	11.30
9	12.04	12.36	12.04	11.34
8	12.04	12.32	12.04	11.38
7	12.04	12.30	12.04	11.40
6	12.04	12.26	12.04	11.44
5	12.04	12.22	12.04	11.48
4	12.04	12.18	12.04	11.52
3	12.04	12.16	12.04	11.54

before heading during which this behavior is expressed is designated as the sensitive stage.

5. Likewise, determine the critical fertility point (i.e., the lowest temperature at which maximum pollen fertility is achieved) by using the tracking technique. Verify the CSP and CFP information for each of the EGMS lines under growth chamber or phytotron conditions after the field characterization studies have been done.

Characterization of EGMS lines under controlled conditions

1. Controlled conditions can be created in the growth chamber. A minimum of 4 to a maximum of 15 growth chambers are needed to characterize EGMS lines.
2. The total number of growth chambers should be equal to the photoperiod × temperature treatments while keeping relative humidity constant at 75%. The number of seedlings per EGMS entry should be equal to the number of growth chambers times the number of plants in each treatment. For example, three temperature and three photoperiod treatments for a total of nine growth chambers are required. At the rate of 10 seedlings per entry per growth chamber, a total of $3 \times 3 \times 10 = 90$ seedlings per entry will be needed for evaluation. The number of seeds to be sown for the experiment should be three times more than the number of seedlings required per entry for the experiment. This will make an allowance for poor germination, discarding of seedlings because

of nonuniform growth, and varied growth duration of EGMS lines.

3. Sowing and nursery management. Use the following procedures for sowing and nursery management.
   - Sow the seeds of EGMS lines with similar duration and a standard set of controls in the nursery at the same time.
   - If there is a significant difference in growth duration among the EGMS lines, sow these in two stages (the first stage for late-maturing material and the second stage for early maturing material).
   - Manage the nursery using routine management to eliminate obvious mixed-rice seedlings and weeds.
   - Select uniform and healthy rice seedlings at the 5-leaf stage for transplanting two plants per plastic pot with a single plant per hill.
   - Use any convenient method to label the pots. As an example, 5-digit labeling can be used for each plant, that is, 30817 means number 3 photoperiod/temperature treatment, 08 is the number of the EGMS line, and 17 is the number of the individual plant.
   - Treat high-photosensitive japonica and indica EGMS lines with a short-day pretreatment (10 h/28 ºC of photoduration and 14 h/25 ºC of dark duration for 10 days) to speed up their development.
   - Adjust growth chambers prior to their use for the experiment. These should be allowed to function for one or two days using the specified temperature and relative humidity level. A placement plan for each of the pots within a growth chamber should be prepared and the chambers should be sprayed with appropriate chemicals a few days before moving the plants to control pests and diseases.
   - Keep the progenies of suspected EGMS plants in separate growth chambers with varying day and night temperature regimes and separate daylength durations. For easy adaptation of the EGMS lines, temperature or daylength should be kept as available in the natural situations exemplified as follows:

Situation 1 (maximum-minimum difference of 6 ºC)

Day temperature (ºC)	24	25	26	27	28	30	32
Night temperature (ºC)	18	19	20	21	22	24	26

Situation 2 (maximum-minimum difference of 8 ºC)

Day temperature (ºC)	24	25	26	27	28	29	30	31	32
Night temperature (ºC)	16	17	18	19	20	21	22	23	24

The lines identified to adapt to specific situations can be deployed accordingly.

- Place the plants in growth chambers at the critical stage. The critical stage for photoperiod or temperature sensitivity is during 5–15 days after panicle initiation (PI). The suspected EGMS plants grown in pots are observed for PI. The plants that are used for determining PI by physical opening must not be used for the experiment.
- Plants must be placed inside the growth chamber at the PI stage.
- Plants must be placed for 2 weeks or up to heading in the growth chamber for treatment.
- After the plants are treated in the growth chamber, they need to be examined for pollen sterility from the primary tiller (see Appendix1).
- Observations on pollen sterility must be recorded in relation to temperature and photoperiod.
- Collect a pollen sample from the primary tiller and bag the same panicle for spikelet fertility percent (bagged). Likewise, the pollen and spikelet sterility of the first three panicles from each plant need to be recorded separately as panicles 1, 2, and 3.
- The CSP and CFP vary from genotype to genotype. It is therefore essential to know the CSP and CFP of each EGMS line before it is used for seed production (Table 2).
- Sterile plants that remain completely sterile in different temperature or photoperiod regimes are not considered as EGMS types and are discarded.
- The EGMS lines that have been strictly evaluated in phytotrons for their fertility alteration are advanced for evaluation under different ecological conditions to further ascertain their suitability for hybrid seed production and self seed multiplication.

Evaluation of EGMS lines

EGMS lines are also evaluated for their phenotypic acceptability, outcrossing rate, and combining ability as per the methods described for evaluating CMS lines. The operational flow chart describing the procedure for using the EGMS system is presented in Figure 9.

After a thorough examination of the EGMS lines in the growth chamber for their fertility alteration behavior, they need to be evaluated across several selected environments. The purpose of this evaluation is primarily to identify suitable regions or locations for EGMS self seed multiplication and hybrid seed production. This evaluation also provides information on their range of adaptability and their resistance to diseases and insect pests. The key to successful multilocation evaluation lies in the timely layout of the trials and consistent observations. It is therefore highly recommended that one researcher be in charge of the multilocation evaluation.

Selection of locations based on environment

The location must represent the rice production region and must be in the vicinity of the research institute/station, with specialists equipped with minimum scientific instruments (e.g., microscope, refrigerator, hot dry oven, daily weather-recording gadgets, seed germinators, etc.) required for evaluating EGMS lines.

Criteria used for identifying locations

a. Locations that have a different latitude within the same longitude.
b. Locations with distinct differences in topography and temperature within the same latitude.
c. Locations with different elevations within the same region.

There should be proper representation of ecological conditions for better understanding of the behavior of EGMS lines under different agroclimatic conditions.

For the unified evaluation of EGMS lines, the sowing and transplanting dates must be in accordance with the respective locations identified because of the different ecological conditions (Fig. 16). For all other purposes, a standard evaluation procedure must be adopted for drawing meaningful conclusions, that is, planting design, pollen and spikelet fertility, and their method of data collection should be consistent.

The identification of appropriate areas and seasons for large-scale commercial production of TGMS and hybrid seed needs special attention, unlike for the PGMS lines, for which the daylength is quite stable across a given region. On the basis of analysis of about 10–15 years of meteorological data, areas and ideal periods for seed production in different parts of the world can be identified.

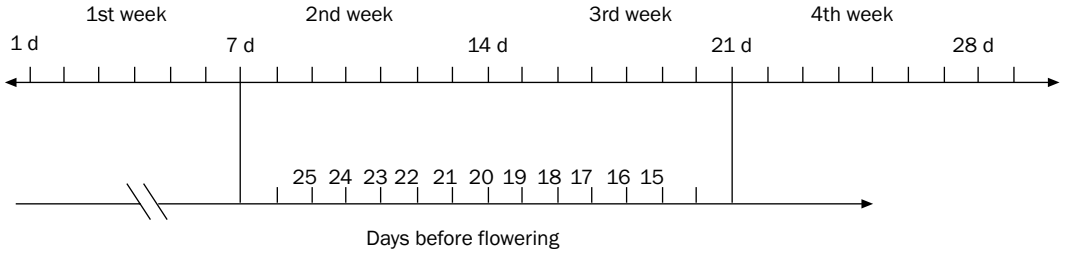
- Places located 500 and 700 m above sea level are highly suitable during May to September for hybrid seed production and during November to March for TGMS self seed production.
- In the choice of place, in hills or coastal plains or interior plains and plateau regions, extra care needs to be taken to ensure that the temperature range is <40 to > 16 ºC, beyond which physiological sterility occurs.
- Four weeks of stable high temperature (>30/ >24 ºC day/night) are required for hybrid rice seed production interspersing the sensitive stage of stage II (secondary branch primordial) to stage IV (stamen and pistil primordial stage) of the panicle development stage of rice (Fig. 16).
- Similarly, four weeks of low temperature (<26/>16 ºC night) are required for TGMS self seed multiplication, interspersing the sensitive stage of stage II (secondary branch primordial stage) to stage IV (stamen and pistil primordial stage) of the panicle development stage of rice (Fig. 16).
- The identified place must be suitable for rice cultivation with space or time isolation.
- Places with naturally chilled irrigation water of about >17–24 ºC can be useful for TGMS self seed multiplication under high-temperature fluctuations. The chilled irrigation water is effective from stage IV (stamen and pistil primordial stage) to stage V (meiotic division).

All the data collected should be stored in a computer and sent to the researcher in charge for analysis and interpretation.

Ideal locations with four consecutive weeks with stable temperature or photoperiod

A. Hybrid seed production

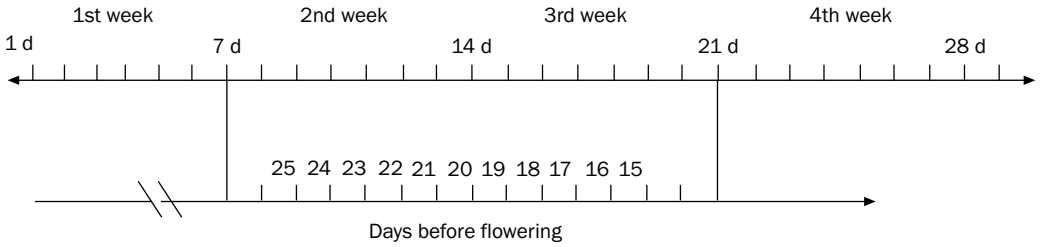
Minimum of four continuous weeks with stable high temperature (>30/24 °C day/night) or long photoperiod (>14 h)



- Adjust sowing date accordingly so that 15–25 days before flowering falls between the second and third week of high temperature.

B. EGMS self seed multiplication

Minimum of four continuous weeks with stable low temperature (<24/>16 °C day/night) or short photoperiod (<12 h).



- Adjust sowing date accordingly so that 15–25 days before flowering falls between second and third week of low temperature.

Fig. 16. Graphic illustration for adjustment of sowing time for EGMS for hybrid seed and self seed production in ideal locations.

Developing pollen parents for two-line hybrids

A pollen parent line is defined as a male parental line that has the ability to restore the fertility in the F_1 of the EGMS line under a sterile phase regime that ensures complete male sterility. Most conventional inbred lines are the source of pollen parent lines or pollen parents. For hybrid rice seed production using EGMS, any pollen parent line can restore fertility in the F_1, unlike CMS lines, for which a set of restorer lines alone can restore fertility in the F_1. It was estimated that about 97% of the japonica and indica inbred lines restored the fertility of japonica PGMS (such as Nongken 58S) and indica TGMS (such as W6154S) lines.

Characteristic features of an elite pollen parent

The following are the characteristic features of an ideal elite pollen parent:

- **Strong fertility-restoring ability.** When a cross is made with the EGMS line, the hybrids have a normal seed setting percentage (>80%) and are less affected by changes in environmental conditions.
- **Good general combining ability.** When crossed with different EGMS lines, the F_1 hybrids from many crosses perform well.
- **Good agronomic characters.** The pollen parent should be a high-yielding inbred line with favorable traits for outcrossing, for example, good anther dehiscence, good anther protrusion, large anther size, high pollen load, etc.
- **Genetic distance.** Considerable genetic distance from the EGMS lines will be the key to enhanced heterosis.

Breeding methods for identifying pollen parents for two-line hybrids

The following breeding methods can be used to identify pollen parents for two-line hybrids: screening available elite inbred lines by testcrossing with available EGMS lines, hybridization, induced mutagenesis, and anther culture. Among these methods, the testcross and hybridization methods are the most common.

Screening available elite inbred lines by testcrossing with available EGMS lines

This is the most effective method to screen for suitable pollen parents from existing rice germplasm. In China, most pollen parents for two-line hybrid rice breeding were screened from existing inbred lines. For example, the pollen parent in hybrid Liangyou Peijiu (Peiai64S/9311) was Yangdao b (9311), a medium-season indica variety developed by the Lixiahe Agricultural Institute, Jiangsu Province. The high-yielding indica variety Teqing developed by the Rice Institute, Guangdong Academy of Agricultural Sciences, was used as the pollen parent for the TGMS line Peiai64S and developed a hybrid, Liangyou Peite, in Hunan Province.

Three fundamental steps are required in making testcrosses, as discussed below.

1. *Preliminary testcross.* Use existing elite lines or varieties to cross to EGMS lines, then conduct a preliminary evaluation of the F_1 hybrids based on their seed setting, yield components, grain quality, and resistance to diseases and insect pests, etc. At

least 10 single plants are needed for a preliminary testcross.

2. *Re-testcross.* From the preliminary testcross data, the varieties or lines that performed well in terms of strong restoration and that showed no segregation for agronomic characters can be advanced to the re-testcross to confirm the results of the preliminary testcross. About 50–100 single plants are needed for the re-testcross. In the re-testcross, yield will be considered as the key evaluating factor.
3. *Identification.* Only excellent pollen parents (varieties or lines) are selected for further use. After identification of suitable pollen parents, hybrid seeds will be produced for field evaluation and multilocation trials.

Hybridization

This is an important and very popular method for breeding pollen parents for two-line hybrids when there is a lack of such parents among existing varieties or lines.

1. *Single cross.* Under this method, two varieties or lines with desired traits are crossed and, in the F_2, selection is exercised to identify single plants that possess the desired traits. The standard selection pedigree procedure is followed to fix the lines. At the F_5–F_6 generation, a testcross with EGMS lines is made. The best-performing pollen parent showing high fertility restoration and heterosis in testcross F_1s is selected for the production of hybrid seeds and subsequent evaluation in the field.
2. *Multiple crosses.* This procedure uses two or more varieties or lines to develop ideal pollen parent lines. The procedure is almost the same as the single-cross method except that in the F_2 generation the selections must be made to combine the favorable traits from all the parents used in the multiple cross.
3. *Backcross.* For a pollen parent line with several good traits except for one or two undesirable traits, the backcross method of breeding is the most suitable for removing and replacing the undesirable traits with favorable traits from the donor parent. Select a stable and suitable variety or line as the trait donor and cross it with the pollen parent line (recurrent parent) that needs to be improved. Raise the F_1 generation and backcross it with the recurrent parent and BC_1F_1 seeds obtained. The selection strategy may vary according to the dominant or recessive trait. For a dominant trait, it is simple to select the plants with the target trait in the backcross generations. In the BC_5F_1 generation, self them once. After selfing, the selected plants are used to test-cross to screen the strong F_1 hybrids for evaluation. For the recessive trait, care must be taken to ensure that the trait is selected and confirmed by selfing after every two generations of backcrossing.

New strategies for developing pollen parent lines

The main difference in pollen parent and restorer lines is that the former may not have special restorer genes, that is, *Rf*, like the latter, thereby making them more convenient for using the heterosis between indica and japonica lines in two-line hybrid breeding.

To overcome hybrid sterility, longer growth duration, taller plant types, and incomplete grain filling, wide-compatibility genes can be used in the development of pollen parent lines.

Exploiting intersubspecific indica × japonica crosses through the wide-compatibility system

What is wide compatibility? It is well known that indica and japonica crosses result in hybrid sterility despite their high heterosis for various agronomic traits. But, it was found that certain indica and japonica hybrids showed normal spikelet fertility. One or both parents of these crosses must possess a dominant wide-compatability gene ($S5^n$) and such lines are designated as wide-compatibility varieties (WCVs). When such WCVs are crossed with indica or japonica, the hybrids show normal spikelet fertility.

The concept of wide compatibility in rice was first introduced by Ikehashi and Araki (1986) to explain hybrid sterility at the subspecies level. To study the interactions between the sporophyte and gametophyte, Ikehashi and Araki (1986) conducted a survey for WCVs via a triple testcross. They found that varieties such as Ketan Nangka, Calotoc, and CPSLO-17 showed wide compatibility. The fertility of their hybrids was linked to the

cytochrome (*C*) and waxy (*wx*) gene. The WC gene was located on chromosome 6 and three alleles of the locus were identified: *S-5*^{i} from indica rice, *S-5*^{j} from japonica rice, and *S-5*^{n} from WC rice. The sterility resulting from crosses between indica and japonica rice is due to the interaction between the alleles *S-5*^{i} and *S-5*^{j}. The *S-5*^{i}/*S-5*^{j} genotype produces semisterile panicles because of the partial abortion of female gametes carrying the allele *S-5*^{j}. Such abortion does not occur in *S-5*^{n}/*S-5*^{i} and *S-5*^{n}/*S-5*^{j} genotypes. The donor of *S-5*^{n} is referred to as a WCV (Table 14).

Developing pollen parents with wide compatibility. Pollen parents with wide compatibility were developed by introducing the WC gene into conventional indica and japonica rice.

Pollen parents are generally crossed with WC lines and then selection is made in the segregating generation using marker genes linked with the WC trait. Sometimes, the backcross method is used when the traits of WCVs are not ideal.

In China, at the National Rice Research Institute (CNRRI), pollen parent R2070 with WC was successfully developed by using the cross Minghui 63 (Lunhui422 and javanica line WL1312). R2070 can be used in both three-line and two-line hybrid rice breeding. The three-line hybrid II You 2070 (II-32A/R2070) and two-line hybrid Guangya 2 (M2S/R2070) performed well in the yield trial and in farmers' fields and both were registered in Zhejiang Province.

To evaluate indica/tropical japonica hybrids,

- Make several crosses between TGMS lines (WC) and tropical japonica varieties. If some tropical japonicas have the WC gene, they can be crossed to any indica TGMS line.
- Evaluate the hybrids in the OYT and a series of trials to identify the most promising hybrids with enhanced heterosis. The best hybrids that are grown in the locality should be used as standard checks. Emphasis should be given to monitoring spikelet fertility during the evaluation.

Developing tropical japonica pollen parents, the same method described for transferring the desirable tropical japonica pollen parent can be followed. However, the recipient tropical japonica should possess a WC gene.

When developing desirable indica pollen parents possessing wide compatibility, the frequency of elite indica lines is quite high. Hence, these can be good male parents developing indica/tropical japonica hybrids, if only WC genes are transferred into them. The procedure for this is as follows:

- Select indica lines with very good restoring ability and WC donors with a marker gene (a purple apiculus or *Amp3*^{2}).
- Make crosses between the indica line and WC donors.
- Grow F_1 and evaluate all crosses for spikelet fertility. Choose the highly fertile cross for further selection.
- Grow F_2 and select good recombinants looking like the restorer but with the apiculus pigmentation or *Amp3*^{2} allele.
- Grow F_3 and F_4 and select the best plants in the best families. Keep track of the original plant type of the pollen parent and the *Amp3*^{2} allele.
- In the F_5, test-cross the selected lines on a single-plant basis with a tropical japonica EGMS line having no WC gene.
- Evaluate the testcross progenies for fertility. Select lines that show high fertility. They are the pollen parents possessing WC genes.

Once the requisite pollen parents are developed, they can be test-crossed with the best available female parents and the experimental hybrids can be evaluated as discussed in Chapter 9 on "Evaluating two-line hybrids."

Table 14. Wide-compatibility varieties in different varietal groups.

Indica	Japonica	Tropical japonica
BPI 76	NK 4	Banten
Dular (aus)	Norin PL9	Calotoc
N22 (aus)	02428	CP-SLO
		Ketan Nangka
		Moroberekan
		Palawan
		Padi Bujang
		Pendec
		IR64446-7-3-2-2
		IR65598-112-2

Combining ability nursery

Assessing the combining ability of parental lines is extremely useful in a hybrid breeding program, especially when many prospective parental lines are available and the most promising ones are to be identified on the basis of their ability to give superior hybrids. The line × tester method (Kempthorne 1957) is commonly used for this purpose.

Definitions

- *Combining ability* refers to the ability of a genotype to transfer its desirable traits to its progenies.
- *General combining ability (GCA)* is the average performance of a parent in a series of crosses.
- *Specific combining ability (SCA)* is the deviation in the performance of a hybrid from the performance predicted on the basis of the general combining ability of its parents.

Type of lines to be evaluated

- The most stable CMS and EGMS lines possessing high phenotypic acceptability and a fair to excellent outcrossing rate.
- Effective restorers/pollen parents adapted to the target area.

Procedure using the line × tester design

- Let us suppose we have "l" lines (elite pollen parents) and "t" testers (elite CMS and/or EGMS lines).
- All the l lines should be crossed to each of the t testers to produce l × t experimental hybrids.

Composition of the combining ability nursery

- All the l × t hybrids along with the parents (lines + testers).
- Suitable check varieties may also be included for working out standard heterosis.

Field layout

- Choose a fairly homogeneous plot for growing the combining ability nursery in a replicated trial using a randomized complete block design (RCBD).
- Use several replications to ensure a minimum of 12 degrees of freedom for error to have statistically valid comparisons.
- Plant a single seedling per hill with a spacing of 20 ×15 or 20 × 20 cm.
- Plot size may depend on the amount of F_1 seed available. However, a minimum of 50 plants per plot is essential. The larger the plot size, the better it is for evaluation.
- Avoid collecting data from border plants. Each three-row plot of hybrids can be flanked by a border row of a check variety.

Statistical analysis

- If we have five lines (pollen parent lines) and four testers (EGMS lines), the total number of crosses will be 1 × t = 5 × 4 = 20.

Rep 1					Rep 2					Rep 3				
5	25	15	18	13	24	C	4	21	6	25	17	27	14	7
16	9	11	2	28	19	14	23	13	26	19	10	1	20	12
12	21	1	17	8	5	28	16	9	17	21	29	3	16	2
3	7	23	4	27	29	7	3	11	10	26	6	23	8	C
10	20	C	26	29	12	1	18	22	20	29	13	5	24	22
14	22	6	24	19	27	25	15	8	2	9	18	15	11	4

1–20 hybrids, 21–25 lines, and 26–29 testers. C = check variety.

Fig. 17. Layout of combining ability trial.

- Let these 20 crosses along with five lines and four testers (29 entries) be tested in an RCBD with three replications with one check variety (Fig. 17).

Analysis of variance

$$\text{Correction factor (CF)} = \frac{(\text{Grand total})^2}{\text{Total no. of observations}}$$

$$\text{Total sum of squares (TSS)} = \Sigma Yij^2 - CF$$

$$\text{Replication SS (RSS)} = \frac{\Sigma Y.J^2}{t} - CF$$

$$\text{Replication SS (RSS)} = \frac{\Sigma Yi^2}{r} - CF$$

$$\text{Error SS (ErSS)} = TSS - TrSS - RSS$$

Analysis of variance table[a]

Source	df	SS	MSS	F
Replications	(r – l) [2]		RSS/2	
Treatments	(t – l) [28]		TrMSS/28	
Error	(r – l)(t – l) [56]		ErMSS/56	
Total	(rt – l) [86]			

[a]df = degrees of freedom, SS = sum of squares, MSS = mean sum of squares, F = F value.

To test the significance of the genotypic difference, compare the calculated F (TrMSS/ErMSS) with the table value of F for 28 and 56 degrees of freedom at the 5% or 1% level of significance.

Treatment SS can be further partitioned into SS from parents, SS from crosses, and SS from the interaction of parents vs crosses.

$$\text{Treatment SS} = \frac{\Sigma C^2ij + \Sigma P^2ii}{r} - CF$$

Cij = observation for ijth cross
Pii = observation for ith parent
r = number of replications

$$\text{SS from crosses} = \frac{\Sigma C^2ij}{r} - CF \text{ (crosses with 19 df)}$$

$$\text{SS from parents} = \frac{\Sigma P^2ii}{r} - CF \text{ (parents with 8 df)}$$

$$\text{SS from interaction of parents vs crosses} = TrSS - SS \text{ (crosses)} - SS \text{ (parents) (with 1 df)}$$

ANOVA with parents and crosses

Source	df	SS	MSS	F
Replications	2			
Treatments	28			
Crosses	19			
Parents	8			
Parents vs crosses	1			
Error	56			

Test all sources of variation against error variance.

Line × tester analysis

Construct a two-way table.

Lines	Testers				
	1	2	3	4	Total
1	Yij				Yi..
2					
3					
4					
5					
Total	Y.J.				Y..

$$\text{SS from lines} = \frac{\Sigma Yi^2..}{r \times t} - \text{CF (crosses)}$$

where r = replications and t = testers.

$$\text{SS from testers} = \frac{\Sigma Y.J.^2}{1 \times r} - \text{CF (crosses)}$$

SS from lines × testers = SS (crosses) – SS (lines) – SS (testers)

ANOVA for line × tester analysis

Source	df	SS	MSS	F
Lines	4			
Testers	3			
Lines × testers	12			
Error	56			

ANOVA for line × tester analysis including parents

Source	df	SS	MSS	F
Replications	2			
Treatments	28			
Parents	8			
Parents vs crosses	1			
Crosses	19			
Lines	4			
Testers	3			
Lines × testers	12			
Error	56			
Total	86			

Note: MSS from lines and MSS from testers are to be tested against MSS from lines × testers. MSS from lines × testers is to be tested against MSS from error.

Sometimes line × tester analysis is done by using cross means (means of crosses over replications). In that case, MS from error that is used for testing the significance of MS (lines × tester) should be divided by the number of replications before testing.

Estimation of GCA effects:

i) GCA effects of lines

$$gi = \frac{Yi..}{tr} - \frac{Y..}{ltr}$$

where Yi.. = total of ith line over testers, Y.. = grand total, and ltr = number of lines, testers, and replications.

Work out GCA effects for g_1 to g_5. See whether $\Sigma gi = 0$.

ii) GCA effects of testers

$$gt = \frac{\Sigma Y.j.}{lr} - \frac{Y..}{ltr}$$

where Y.j. = total of jth tester over lines, Y.. = grand total, and ltr = number of lines, testers, and replications.

Work out GCA effects for g_5 to g_9. See whether $\Sigma gt = 0$.

iii) Estimation of SCA effects

$$Sij = \frac{Yij.}{r} - \frac{Y..}{rt} - \frac{Y.j.}{rl} + \frac{Y..}{ltr}$$

where Yij. = value of jth line with ith tester, Yi.. = total of ith line over all testers, Y.j. = total of jth tester over all lines, Y.. = grand total, and ltr = number of lines, testers, and replications.

Work out SCA effects for all hybrids. See whether $\Sigma_i \Sigma_j S_{ij} = 0$.

Testing the significance of combining ability effects

SE (standard error) (GCA for lines) = $\sqrt{\frac{Me}{rt}}$

SE (standard error) (GCA for testers) = $\sqrt{\frac{Me}{rl}}$

SE (standard error) (SCA effects) = $\sqrt{\frac{Me}{r}}$

SE (standard error) (gi – gj) line = $\sqrt{\frac{2Me}{rt}}$

SE (standard error) (gi – gj) testers = $\sqrt{\frac{2Me}{rl}}$

SE (standard error) (Sij – Skl) = $\sqrt{\frac{2Me}{r}}$

Me is the error mean sum of squares.

Interpretation of results

- The statistical significance of treatments indicates that the entries have genotypic differences between them. If the treatment differences are significant, we can use further partitioning.
- Partitioning of treatment SS into SS from crosses and parents helps to test the significance of these two components individually.
- The parents with higher positive significant GCA effects are considered to be good general combiners, whereas those with negative GCA effects are considered to be poor general combiners.
- The hybrids with significant SCA effects in a positive direction are considered to be the most promising ones.

Using the results

- The EGMS lines with good GCA are chosen for developing experimental hybrids for testing in observation yield trials.
- The pollen parents with good GCA will be used for crossing with EGMS lines to produce experimental hybrids for testing in observation yield trials.
- Hybrids with higher positive significant SCA effects are chosen for evaluation in preliminary yield trials.

Evaluating two-line hybrids

Many hybrids developed through the two-line approach using EGMS lines are heterotic. Therefore, an elaborate evaluation of the hybrids is required to identify the most promising ones in terms of yield heterosis per se and their suitability for high seed yields and self seed yields of the female parent during its multiplication. Experimental hybrids should be evaluated in a series of trials, using an appropriate statistical design to ensure unbiased comparisons. The choice of design depends on the number of entries and the quantity of hybrid seed available. During the initial stages, the number of entries to be tested is large and the quantity of hybrid seed available is limited; therefore, the hybrids are tested in unreplicated trials. However, in subsequent evaluation, hybrids need to be tested in replicated trials with a larger plot size. The performance of hybrids may be location-specific. Therefore, it is necessary to conduct multilocation trials to identify hybrids having wide adaptability and those that are specifically adapted to certain locations. Testing the performance of hybrids in farmers' fields along with local check varieties of the region is necessary before these hybrids are released for commercial cultivation.

Observation yield trial (OYT)

Composition

Re-testcrosses are made between the commercially usable EGMS lines and effective restorers identified in the testcross nursery. Use three to four check varieties representing different growth durations (such as very early, early, medium, and late).

Experimental design and field layout

- Since the number of experimental rice hybrids is large (100–500) and the amount of hybrid seed is limited, it is convenient to conduct the OYT by using the augmented design.
- In this design, the whole experimental area is divided into several blocks.
- The check varieties are replicated in each block, whereas the test entries are not replicated but are assigned to the remaining plots randomly.
- The yields of test entries are adjusted for block differences based on the yield of check varieties in each block.
- The block size is determined as follows:

If c = number of check varieties, v = number of test hybrids, and b = number of blocks, the number of test entries in a block (n) = v/b, the number of plots/block (P) = c + n, and the total number of plots (N) = b (c + n).

- The total number of blocks should ensure at least 12 df for error in ANOVA.

$$b > \frac{12}{c-1} + 1$$

- Let us take 40 hybrids and four check varieties.

$$\text{The number of blocks} = \frac{12}{4-1} + 1$$

$$= \frac{12}{3} + 1 = 5$$

Number of check varieties = 4
Number of test hybrids = 40
Number of blocks = 5
Number of hybrids per block = 40/5 = 8
Number of plots per block = 8 + 4 = 12

Total number of plots = 60

Layout for augmented design

- The plot size should be at least 5 m^2 for each entry.
- Plant a single seedling per hill with a spacing of 20 × 15 cm.
- First assign the check varieties randomly in each block.
- Assign the test hybrids randomly to the remaining plots.
- The field should be properly leveled.
- Take up gap filling 7–10 days after transplanting to obtain a uniform plant population.
- Care should be taken for a uniform distribution of fertilizers and plant protection chemicals.
- Uniform water control is a must for valid comparisons.

A worked-out example of the OYT is described below.

Layout and yield figures (t ha^{-1}) for OYT (augmented design).

Blocks				
1	2	3	4	5
17 (4.6)	23 (7.1)	B (4.0)	12 (7.9)	2 (5.0)
C (3.8)	A (4.0)	28 (5.0)	37 (5.3)	25 (3.2)
9 (5.6)	3 (5.6)	14 (3.6)	A (3.9)	B (3.2)
13 (5.3)	36 (7.0)	D (4.3)	8 (3.4)	27 (5.4)
D (5.7)	B (5.1)	24 (7.5)	33 (5.2)	16 (6.0)
29 (5.2)	7 (6.3)	30 (6.2)	C (4.2)	35 (2.6)
6 (4.9)	38 (4.6)	A (4.5)	5 (2.9)	D (4.6)
A (4.5)	15 (3.9)	C (3.9)	40 (6.8)	22 (3.9)
31 (3.2)	C (4.1)	39 (4.3)	26 (7.8)	A (3.8)
18 (2.6)	20 (5.3)	1 (3.6)	D (3.9)	4 (4.7)
B (4.6)	11 (5.0)	32 (5.9)	34 (3.8)	C (3.3)
21 (6.1)	D (4.9)	19 (5.4)	B (4.9)	10 (5.2)

Agronomic management for yield trials

The trial should be managed so that all entries receive appropriate water, fertilizer, and measures to control weeds, insects, and diseases. Yield trials should be harvested carefully.

Data recording

Collect data on the following parameters:

- Vegetative vigor (on a 1–9 scale).
- Days to 50% flowering.
- Visual score for spikelet fertility (on a 1–9 scale).
- Yield $plot^{-1}$ for conversion to yield ha^{-1}.
- Phenotypic acceptability score (on a 1–9 scale).

Statistical analysis

Construct a two-way table of check yields (in t ha^{-1}) and means.

Check variety	Blocks					Total	Mean
	1	2	3	4	5		
A	4.5	4.0	4.5	3.9	3.8	20.7	4.14
B	4.6	5.1	4.0	4.9	3.2	21.8	4.36
C	3.8	4.1	3.9	4.2	3.3	19.3	3.86
D	5.7	4.9	4.3	3.9	4.6	23.4	4.68
Total	18.6	18.1	16.7	16.9	14.9	85.2	–
Mean	4.65	4.52	4.17	4.22	3.72	–	4.26

- Compute the block effect: rj = Bj – M

where rj = block effect of jth block, Bj = mean of all checks in jth block, and M = grand mean of the checks.

Block effects of different blocks are

Block	rj
1	0.39
2	0.26
3	–0.09
4	–0.04
5	–0.52

See whether Σ rj = 0

- Construct a table of unadjusted and adjusted yields. The adjusted yield for each test entry is obtained by deducting the block effect from the unadjusted yield.

Adjusted (AD) and observed (O) yields (t ha^{-1}) of test hybrids in the OYT.

Hybrid	Block	Yield		Hybrid	Block	Yield	
		O	AD			O	AD
1	3	3.6	3.69	21	1	6.1	5.71
2	5	5.0	5.52	22	5	3.9	4.42
3	2	5.6	4.74	23	2	7.1	6.84
4	5	4.7	5.22	24	3	7.5	7.59
5	4	2.9	2.94	25	5	3.2	3.72
6	1	4.9	4.51	26	4	7.8	7.84
7	2	6.3	6.04	27	5	5.4	5.92
8	4	3.4	3.44	28	3	5.0	5.09
9	1	5.6	5.21	29	1	5.2	4.81
10	5	5.2	5.72	30	3	6.2	6.29
11	2	5.0	4.74	31	1	3.2	2.81
12	4	7.9	7.94	32	3	5.9	5.99
13	1	5.3	4.91	33	4	5.2	5.24
14	3	3.6	3.69	34	4	3.8	3.84
15	2	3.9	3.64	35	5	2.6	3.12
16	5	6.0	6.52	36	2	7.0	6.74
17	1	4.6	4.21	37	4	5.3	5.34
18	1	2.6	2.21	38	2	4.6	4.34
19	3	5.4	5.49	39	3	4.3	4.39
20	2	5.3	5.04	40	4	6.8	6.84

- To work out the standard errors for comparing the means, an ANOVA table is prepared by using the replicated data of check varieties.

ANOVA for check varieties. ns = nonsignificant.

Source	df	SS	MSS	F
Block	4	2.068	0.517	
Checks	3	1.804	0.601	2.32 ns
Error	12	3.096	0.258	
Total	19	6.968		

- The standard errors are worked out as follows for different comparisons:

- Difference between two check means

$$\sqrt{2MSE/b} = \sqrt{2 \times 0.258/5} = 0.32$$

- Difference between adjusted yields of two hybrids in the same block

$$\sqrt{2MSE} = \sqrt{2 \times 0.258} = 0.72$$

- Difference between adjusted yields of two hybrids in different blocks

$$\sqrt{2MSE\ (1 + 1/c)} = 0.80$$

- Difference between an adjusted yield of a hybrid and a check mean

$$\sqrt{MSE\ (b + 1)\ (C + 1)/bc} = 0.62$$

Use of results

- The test entries are classified based on different maturity groups and their performance is compared with that of the check variety of the corresponding duration by using the standard errors calculated for the purpose.
- The hybrids that yield significantly higher than the check varieties are identified and promoted for the preliminary yield trial.

Preliminary yield trials (PYT)

Composition

Identify promising hybrids in observation yield trials. Use hybrids showing apparent heterosis in the testcross nursery and significant heterosis in the combining ability nursery. Use check varieties of different growth duration (very early, early, medium, and late).

Experimental design and field layout

- The RCBD is ideal for conducting the preliminary yield trials. The steps involved are as follows:
- The number of blocks or replications should be such that the error degree of freedom should be at least 12.
- The ideal plot size is about 10 m^2.
- If the fertility gradient is unidirectional, the blocks should be perpendicular to the fertility gradient.

- Hybrids should be grouped according to their growth duration. Each group or subgroup should have 15–20 hybrids and suitable checks.

An example

- Let us use 16 hybrids and four check varieties to be tested in four replications.
- Divide the field into four equal blocks.
- Subdivide each block into 20 experimental plots.
- Assign the treatments to each plot randomly. Each treatment should appear in every block.

Layout of the PYT (with RCBD). Numbers in parentheses represent yield (in t ha^{-1}).

Rep 1	Rep 2	Rep 3	Rep 4
3 (5.3)	4 (8.3)	9 (4.7)	C (6.0)
D (4.3)	11 (5.6)	5 (7.3)	2 (7.1)
8 (6.5)	A (4.2)	7 (5.0)	13 (6.0)
10 (7.5)	2 (5.8)	C (6.9)	6 (5.9)
2 (6.0)	D (3.9)	10 (6.8)	B (6.0)
A (3.8)	6 (5.6)	1 (4.7)	11 (6.1)
7 (5.6)	3 (6.2)	15 (6.9)	4 (7.6)
4 (7.9)	13 (5.9)	D (5.2)	3 (6.0)
12 (6.0)	B (6.5)	8 (5.9)	12 (5.6)
6 (7.1)	9 (5.9)	14 (8.2)	7 (4.9)
14 (8.2)	15 (8.2)	A (4.6)	15 (7.8)
1 (3.8)	16 (4.9)	4 (8.0)	D (4.6)
B (5.9)	5 (7.6)	11 (5.9)	9 (5.0)
15 (7.9)	14 (7.6)	3 (5.8)	8 (6.7)
16 (5.8)	7 (4.6)	6 (5.3)	16 (6.1)
C (6.5)	10 (7.6)	2 (7.3)	A (5.0)
13 (7.2)	12 (5.9)	16 (5.9)	1 (5.1)
5 (6.9)	1 (4.6)	13 (6.3)	14 (7.2)
11 (4.9)	C (7.8)	12 (4.9)	10 (7.3)
9 (5.3)	8 (7.2)	B (5.2)	5 (8.1)

Data recording

Observations are recorded on the following parameters:

- Days to 50% flowering
- Plant height
- Spikelet fertility (%)
- Grain yield (kg ha^{-1})
- 1,000-grain weight
- Reactions to major diseases/insects

Statistical analysis

- Group the data by treatments (entries) and replications and calculate the treatment total (T), replication total (R), and grand total (GT).

Table of means. Yield in t ha^{-1}.

Treatments	Rep1	Rep2	Rep3	Rep4	Total	Mean
1	3.8	4.6	4.7	5.1	18.2	4.55
2	6.0	5.8	7.3	7.1	26.2	6.55
3	5.3	6.2	5.8	6.0	23.3	5.82
4	7.9	8.3	8.0	7.6	31.8	7.95
5	6.9	7.6	7.3	8.1	29.9	7.47
6	7.1	5.6	5.3	5.9	23.9	5.97
7	5.6	4.6	5.0	4.9	20.1	5.02
8	6.5	7.2	5.9	6.7	26.3	6.57
9	5.3	5.9	4.7	5.0	20.9	5.22
10	7.5	7.6	6.8	7.3	29.2	7.30
11	4.9	5.6	5.9	6.1	22.5	5.62
12	6.0	5.9	4.9	5.6	22.4	5.60
13	7.2	5.9	6.3	6.0	25.4	6.35
14	8.2	7.6	8.2	7.2	31.2	7.80
15	7.9	8.2	6.9	7.8	30.8	7.70
16	5.8	4.9	5.9	6.1	22.7	5.67
A	3.8	4.2	4.6	5.0	17.6	4.40
B	5.9	6.5	5.2	6.0	23.6	5.90
C	6.5	7.8	6.9	6.0	27.2	6.80
D	4.3	3.9	5.2	4.6	18.0	4.50
Total	122.4	123.9	120.8	124.1	491.2	6.14

- Compute the correction factor and various sums of squares as follows:

$$CF = \frac{(GT)^2}{N} = \frac{241{,}277.4}{80} = 3{,}015.96$$

$$\text{Total SS} = (3.8)^2 + (6.0)^2 \ldots + (4.6)^2 - CF$$

$$= 3{,}128.76 - 3{,}015.96$$

$$= 112.80$$

$$\text{Replication SS} = \frac{(122.4)^2 + \ldots + (124.1)^2}{t} - CF$$

$$= 3{,}016.32 - 3{,}015.96$$

$$= 0.36$$

$$\text{Treatment SS} = \frac{(18.2)^2 + (26.2)^2 + \ldots (18.0)^2}{r} - CF$$

$$= 3{,}110.82 - CF$$

$$= 94.86$$

$$\text{Error SS} = \text{Total SS} - \text{RSS} - \text{TrSS}$$

$$= 112.80 - 0.38 - 94.86$$

$$= 17.58$$

- Compute the mean sum of squares by dividing each sum of squares by its corresponding degree of freedom.

$$\text{Replication MSS} = \frac{RSS}{r-1} = \frac{0.36}{3} = 0.12$$

$$\text{Treatment MS} = \frac{Tr.SS}{t-1} = \frac{94.86}{19} = 4.99$$

$$\text{Error MS} = \frac{Er.SS}{(r-1)(t-1)} = \frac{17.58}{57} = 0.30$$

- Compute the F value for testing the treatment differences.

$$\text{F value} = \frac{\text{Treatment MS}}{\text{Error MS}} = \frac{4.99}{0.30}$$

$$= 16.6$$

- Compare the calculated F value with the table F value.
- Prepare the analysis of variance table by including all the computed values.

Analysis of variance (ANOVA) table.

Source	DF	SS	MSS	Computed F[a]	Table F 5%	Table F 1%
Replication	3	0.36	0.12			
Treatment	19	94.86	4.99	16.6**		
Error	57	17.58	0.30			
Total	79	112.80				

[a]**A highly significant F value indicates that the test entries differ significantly among themselves.

- Compute the coefficient of variation (CV)

$$CV = \frac{\sqrt{\text{Error MS}}}{GM[1]} \times 100$$

$$= \frac{0.30}{6.14} \times 100$$

$$= 8.92$$

- Compute the critical difference (CD)

$$CD = t\,0.05 \times \frac{\sqrt{2 \times EMS}}{r}$$

$$= 3.44 \times 0.387$$

$$= 1.33$$

- The hybrids with a difference of more than the CD value from the check variety are considered significantly superior to the check variety.

Use of results

- The performance of the hybrids is compared with that of the check variety of corresponding duration or the highest-yielding check variety.

[1]GM = grand mean.

- The hybrids that have a significantly higher yield than the check variety are identified and promoted to the advanced yield trial. A significant yield advantage of more than 1 t ha^{-1} would also be the ideal criterion for selecting the best hybrids after testing the significance.

Advanced yield trials (AYT)

Composition

Identifying promising hybrids in the preliminary yield trials. Use three or four check varieties of different duration (very early, early, medium, and late).

Experimental design and field layout

- The RCBD is ideal for conducting the AYT.
- The number of entries in the AYT is much lower than in the PYT. It is helpful to increase the plot size to 15 m^2.
- Entries should be divided into at least two maturity groups (1—very early and early; 2—medium and late).
- The field layout and agronomic management are similar to those for the PYT.
- Data recording is essential.

The following observations are recorded for the AYT:

- Plant height
- Days to 50% flowering
- Panicles m^{-2}
- Number of filled grains $panicle^{-1}$
- Spikelet fertility (%)
- Yield ha^{-1}
- 1,000-grain weight
- Reactions to major pests and diseases
- Remarks on special features
- Statistical analysis

The method of statistical analysis is the same as the one explained for the preliminary yield trials.

Use of results

- The performance of the hybrids is compared with that of the check variety of corresponding duration or the highest-yielding check variety.
- The hybrids that have significantly higher yield (>1 t ha^{-1}) than the check variety are promoted for multilocation trials.
- Mere statistical significance is not sufficient to consider a hybrid as promising. Therefore, an advantage of about 1 t ha^{-1} over the check variety is specified, which would result in a real benefit to farmers.

Multilocation yield trials (MLT)

The major objective of multilocation yield trials is to identify the hybrids that have a wider adaptability or those that are specifically adapted to a particular location. This exercise is essential as hybrids perform differently in different environments. This also provides an opportunity for breeders to see the performance of hybrids bred by them in other locations, even though these hybrids may fail to perform well in the location where they are developed. The concept of multilocation yield trials has really improved the efficiency of rice breeders and this is more so in hybrid rice breeding.

Composition

Identify the promising hybrids in the AYT from different centers, including those introduced from abroad. Use three or four check varieties of different duration (very early, early, medium, and late). If the trials are constituted based on duration, it would suffice to include a check variety of corresponding duration in the trial.

Experimental design and field layout

- The locations for MLT should be selected carefully so that each location serves as a distinct environment. The location selected should be in the proposed target area for the cultivation of hybrid rice.
- An RCBD is most commonly used for conducting MLT.
- It is necessary to have common guidelines for agronomic management and data collection from different centers.

General guidelines for conducting MLT

- Specify the design to be adapted—an RCBD with four replications.
- Specify the entries and the check varieties. Besides the common check, each center can choose a local check for comparison.
- The trial should be conducted during the same season at all locations.

- Specify the seedling age at transplanting—21–25 days old.
- The spacing adopted should be uniform—20×20 or 20×15 cm.
- Specify planting a single seedling per hill.
- The fertilizer dose may depend on the native soil fertility and recommendations in the local area.
- Plant protection should be need-based.
- The plot size should be uniform in all the locations to the extent possible (at least 15 m^2).

Agronomic management

Agronomic management should be uniform in all locations so as to have valid comparisons, except for some specific recommendations made for a particular location.

Data recording

Data sheets are circulated to all the cooperators for collecting data on important parameters, such as

- Plant height
- Days to 50% flowering
- Panicles m^{-2}
- Number of filled grains $panicle^{-1}$
- Spikelet fertility (%)
- Yield $plot^{-1}$
- Yield ha^{-1}
- 1,000-grain weight
- Reactions to pests and diseases
- Weather data of each location

The statistical analysis for $G \times E$ interactions and interpretation of results are covered in the chapter on $G \times E$ analysis.

Use of results

- The hybrids with higher yield potential and wider adaptability are identified based on stability analysis. These are promoted for on-farm testing in different areas, before their release for commercial cultivation.
- Those hybrids that are found to be suitable for a particular location are promoted for on-farm testing in that particular region only.

Two-line hybrid rice seed production

The production of two-line rice hybrids involves two major steps: (1) multiplication of EGMS lines and (2) hybrid rice seed production.

Each of these steps requires specific environmental conditions for seed production. Therefore, the locations/seasons for producing the two types of seed have to be different. In Chapter 5, we suggested that suitable specific locations must be identified for elite EGMS lines for their self seed multiplication. Locations must also be identified that are suitable for hybrid seed production using the same EGMS lines.

Multiplication of EGMS lines

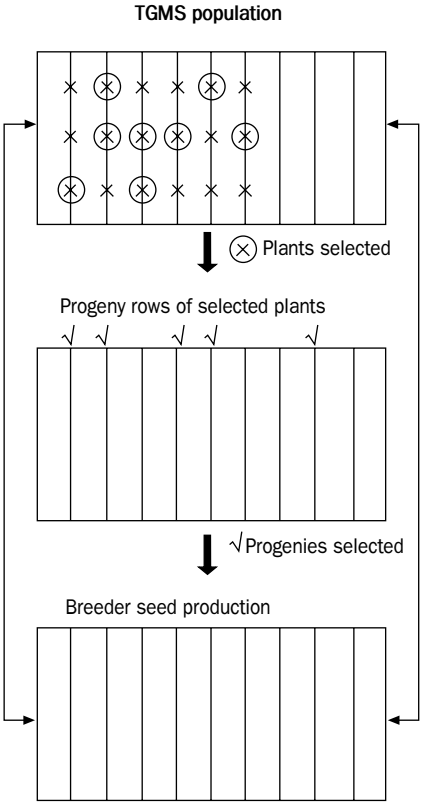
EGMS lines, if multiplied continuously for several generations without any selection, may segregate for CSP, thereby causing major problems in maintaining purity of the hybrid seeds. Therefore, nucleus and breeder seed production must be taken up on a continual basis.

Method I

- Nucleus seed production of an EGMS (TGMS or PGMS) line begins in the fertility-inducing environment. Seeding of TGMS or PGMS lines is arranged in such a way that the sensitive stage occurs when the temperature or photoperiod is favorable for a higher seed set.
- At the time of flowering, about 100 typical plants are selected from the population of an EGMS (TGMS or PGMS) line and their panicles are bagged. The selection process should be completed within 1 week.
- After the harvest, the selected plants are scored for spikelet fertility (based on the main panicle) and 50 plants with higher spikelet fertility (above 30%) are selected.
- Progenies of the selected plants are grown in the sterility-inducing environment. About 30 seeds are taken from each of the selected plants to grow single-row progenies and the remaining seeds are stored carefully. The balance of the seeds of the progenies that are uniform and completely male sterile must be marked and bulked to form the nucleus seed (Fig. 18).
- Nucleus seed of the EGMS line is used for producing breeder seed under strict isolation. Breeder seed for the EGMS line is produced in the fertility-inducing environment.
- The breeder seed produced under the direct supervision of the plant breeder has high genetic purity and is used for producing foundation seed of parental lines, which in turn will be used for producing hybrid seed.

Method II

- Select a completely male sterile plant with typical characteristics of the original EGMS line under a sterility-inducing environment.
- Ratoon the selected plant and clone it for as many plants as you need. Multiply the ratooned stubbles under a fertility-inducing environment. The nucleus seed will be harvested from the ratooned stubbles.
- The nucleus seed is used for producing breeder seed and the latter for producing foundation seed.
- Preserve the selected stubbles under favorable temperature conditions with good management as long as you need them. The new nucleus seed will be produced continuously.



Step 1 Select about 100 typical plants from a population of TGMS lines grown in a fertility-inducing environment and bag all the panicles. Select 50 highly fertile plants among them based on their higher spikelet fertility determined after harvest.

Step 2a Grow single-row progenies of 50 selected plants in a sterility-Inducing environment and mark those progenies that are completely male sterile.

Step 2b Bulk the balance of seed kept earlier as a reserve of the progenies selected in step 2a to form the nucleus seed.

Step 3 Multiply the nucleus seed from step 2b to produce breeder seed under strict isolation.

Step 4 Multiply the breeder seed to produce foundation seed under strict isolation.

Step 5 Repeat steps 1 to 3 in the breeder seed production plots to continue the process of producing nucleus and breeder seed.

Fig. 18. Procedure for nucleus and breeder seed production of TGMS lines (Virmani et al 1997a).

- With this system of multiplication, the EGMS line continues in the same generation and it will not segregate for its CSP. Its general combining ability will also not change.

In the tropics, since ratooning may not always be successful because of the high incidence of diseases and insects, method II can be risky.

- Foundation seed production. Fresh breeder seed should be used by seed production agencies to produce foundation seed of TGMS/PGMS lines in a strict isolation area with suitable temperature and/or daylength conditions.

Differences between EGMS and CMS line multiplication

- The fertility of the CMS line is controlled by the CMS gene and a pair of recessive nuclear fertility restorer genes. Expression of male sterility of CMS lines is stable over environments. The fertility of EGMS lines is controlled by a recessive nuclear gene alone, the expression of which is influenced by environmental conditions.
- The maintenance of the CMS line is through its cross pollination with the maintainer line. The yield of CMS seeds through this process largely depends on outcrossing and

other related traits of the CMS line. The maintenance of the EGMS lines, in contrast, is quite simple since it is through selfing under fertility-inducing environments, especially during the sensitive panicle development phase (stage II to stage VI). The seed yield of EGMS lines largely depends upon the CFP and the favorable environment during fertility alteration.

- The CMS line is purified through pair crossing (A × B) in which the A and B lines are selected based on their morphological characteristics. The purity of the EGMS line is maintained by evaluating the morphological characters and the CFP under specific locations.
- The yield of CMS line multiplication is about 2 t ha^{-1} under suitable conditions and it is unstable because of the changing weather conditions during the flowering period. Under favorable conditions, the yield of EGMS line multiplication could reach 4–6 t ha^{-1}. This yield is higher because it occurs due to self-pollination compared to seed yield in the CMS line, which occurs due to cross-pollination.

Similarity of CMS and EGMS line multiplication

- CMS line multiplication (A × B) requires strict isolation to prevent contamination by stray pollen. Under favorable environmental conditions, the EGMS line becomes fertile and seed setting can be as high as 75%; however, some male sterile spikelets can still receive pollen from another source. Therefore, strict isolation must also be provided to multiply pure EGMS lines.
- Strict roguing is necessary for CMS and EGMS line multiplication.

High-yielding techniques for PGMS line multiplication (Chinese experience)

Autumn-season multiplication

Short daylength can induce PGMS lines to become fertile under proper temperature conditions. In autumn, the daylength is getting shorter and a high percentage of seed setting can be achieved if the sensitive stage occurs under this condition. However, seed quality becomes inferior, especially when the temperature falls sharply in late autumn.

Winter-season multiplication in lower-latitude areas

For PGMS self seed multiplication, short daylength and low temperature in lower-latitude areas in the winter season are the most suitable environmental conditions. Seed quality is better than in late autumn because the temperature gradually rises at the time of seed maturity.

High-yielding techniques for TGMS line multiplication (Chinese experience)

Spring-season multiplication

The fertility of TGMS lines is mainly influenced by temperature. TGMS lines should be sown in early spring so that the sensitive stage matches the temperature for fertility induction based on local meteorological data. The yield may not be stable because of the occurrence of abnormally high temperature, and the long growing period may result in nonmatching of the sensitive stage with the proper temperature regime.

Autumn-season multiplication

The temperature is low enough for fertility induction in the autumn season. The TGMS lines should be sown at the proper time to make the sensitive stage coincide with the low-temperature period in order to obtain higher yields. Seed quality may be influenced by low temperature in the late maturity period.

High-altitude multiplication

In high-altitude areas (with altitudes from 800 to 1,000 m), moderate temperature and longer daylength conditions are suitable for the short growing period for PGMS and TGMS line self seed multiplication.

Chilled-water irrigation

Results showed that chilled-water (>17 ºC) irrigation can induce TGMS lines to produce fertile pollen similar to ambient low temperatures. The advantage of chilled-water irrigation is that it provides flexibility for adjusting the sensitive stage. The TGMS self seed production is high and more stable since the water temperature is relatively stable and controllable.

Seed plot selection. A sufficient water supply with suitable temperature is essential to the establishment of a seed plot for TGMS self seed multiplication. The water temperature should be higher than 17–18 ºC but lower than the CFP of the TGMS line to be multiplied.

Season selection. The joint effect of lower temperature and chilled-water irrigation is good for increasing EGMS self seed yield.

Irrigation time. Chilled-water irrigation starts from the young panicle differentiation of the stamen and pistil primordial (stage IV) and goes to the meiotic division of the pollen mother cell (stage VI).

Three-line hybrid rice seed production

The three-line hybrid rice seed production system involving CMS lines is a relatively stable method across normal rice-growing conditions, whereas the two-line hybrid rice seed production system involving EGMS lines has environmental limitations requiring strict adherence to the proper timing according to season and location. For successful hybrid rice seed production, the male sterility trait of the female parent and effective pollen load of the male parent with proper flowering synchronization are the key factors. For three-line seed production, only the flowering synchronization between the A and R lines is important for higher hybrid seed yields, whereas, for two-line seed production, both the male sterility expression under the sterile phase and the flowering synchrony between the EGMS and pollen parent influence seed purity and bring about higher hybrid seed yields.

Two safe-period determinations for hybrid seed production

- In three-line hybrid rice seed production, favorable climatic conditions for pollination are called the safe flowering period, which is important for increasing the outcrossing rate. In two-line seed production, the two safe-periods refers to the conducive environmental conditions that support first the induction of complete male sterility and second the facilitation of proper pollen movement from the pollen parent and fertilization of the EGMS parent.
- Therefore, the first safe-period determines seed purity, while the second safe-period determines hybrid seed yield.
- The first safe-period, the sensitive stage of fertility alteration, must be given priority if there is a need for an adjustment according to the prevailing environment at a given location.

The desirable climatic conditions for pollination are as follows: (1) temperature of 23–35 ºC (minimum-maximum) in a day, (2) relative humidity around 70–90%, and (3) no continuous rains (that last more than 3 days) during the pollination period.

Isolation of the hybrid seed production plot

- *Terrain isolation.* The selected seed plot is either isolated by mountains or hills or by other natural barriers.
- *Time isolation.* The hybrid seed plot should flower 20–30 days earlier or later than any other plot that could be a source of stray pollen for the seed production plot.
- *Distance isolation.* Keep a distance of 200 m between the seed plot and any other rice pollen source.

Determining the seeding interval for synchronization

- *Time method.* The seeding interval is determined by the difference in growth duration between the two parental lines. The one with longer duration must be sown early according to the number of days of difference between the two parents in terms of days to 50% flowering.
- *Leaf number method.* The total leaf number of a variety is relatively stable at the same site and in the same season in different years. The rate of growth in terms of leaf number is influenced by environmental temperature. By observing the leaf number of the early seeded parental line, you could determine the seeding date of the later one because the leaf number difference is rather stable between the two parents.
- *Heading date prediction*
  - *Remaining leaf number method.* The young panicle differentiation starts from the reciprocal third-leaf emergence (keeping the flag leaf as the first leaf and the leaf before the flag leaf as the 2nd leaf and the leaf before the 2nd leaf as the 3rd leaf) and the time taken for com-

plete panicle differentiation is about 30 days from the start to heading. This means that the parental line would head after 30 days, when the reciprocal third leaf emerges.

- *Stripping the young panicle.* The young panicle differentiation could be divided morphologically into eight stages and each stage takes about 3–4 days for developing (Table 15, Fig. 19).

Production of hybrid seeds for preliminary yield trials

For preliminary yield trials, the hybrid seed requirement is usually small, but numerous hybrids are produced simultaneously.

Planting techniques. To produce a small quantity of hybrid seeds for the OYT or PYT, the following are the planting designs: (1) chimney isolation, (2) modified chimney isolation, and (3) the isolation-free method.

i. Chimney isolation procedure

- The desired parental lines are sown on different dates to obtain synchronous flowering.
- Twenty-five-day-old seedlings of EGMS (female parent) and pollen parent lines are planted in alternate rows of five plants each at a spacing of 15 ×15 cm (Fig. 20).
- Frames of 1 × 1 × 1 m are prepared with either iron or aluminum angles.
- Cubicles of 1 × 1 × 1 m are stitched with muslin cloth, with a flap at the top.
- The metal frame is placed around a 1-m^2 area where the EGMS (female parent) and pollen parent lines are planted just before flowering.
- The frame is covered with a muslin cloth bag to prevent cross pollination.
- During the flowering period, the pollen plants are shaken to increase seed setting on the EGMS line. This can be facilitated by opening the flap.
- Pollen parent plants are harvested first and threshed separately. The EGMS line is harvested and threshed later to avoid possible seed admixture.

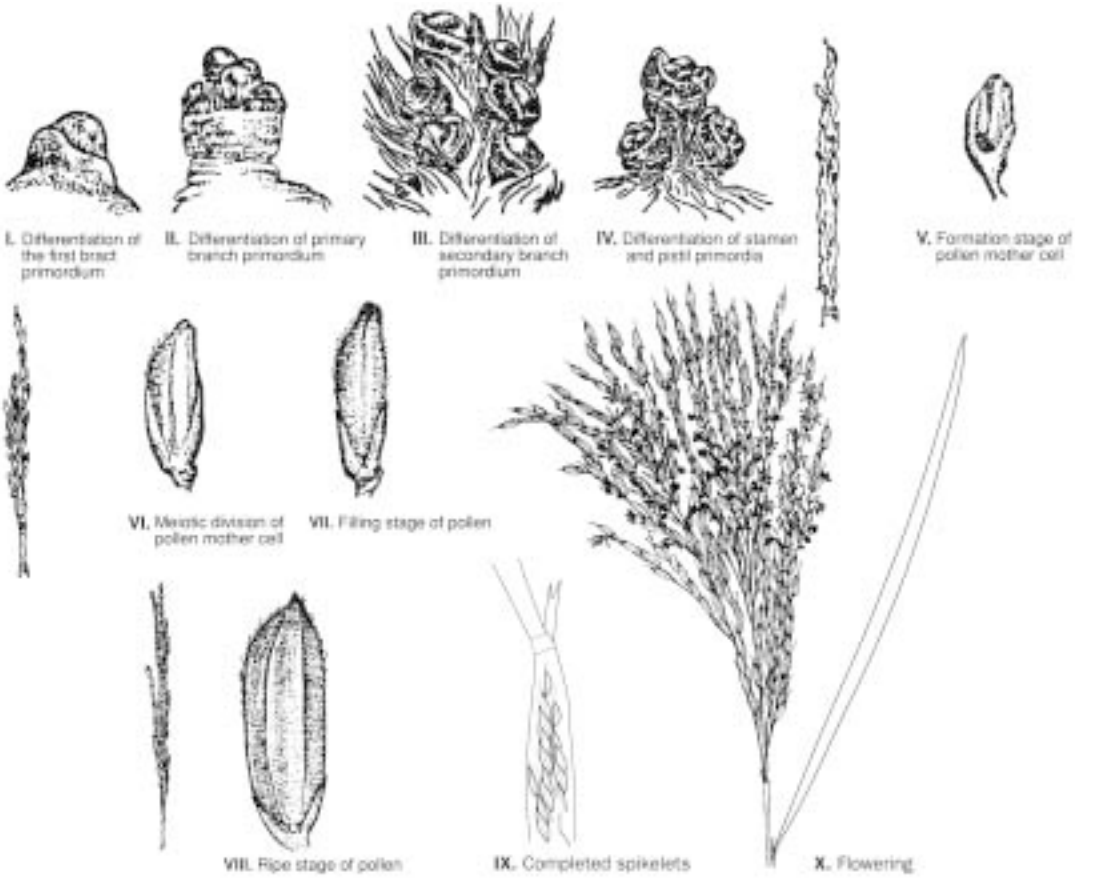
ii. Modified chimney isolation procedure

The chimney isolation method has been modified to overcome the problem of synchronization and to simplify the supplementary pollination. The basic layout is the same as that of the chimney method, except for the following differences:

- The EGMS and pollen parents are sown on different days to achieve maximum flowering synchronization.
- At the boot-leaf stage of the parental lines, 2-m-high barriers are erected to cover the three sides of a 1-m^2 plot, leaving a gap of 20 cm from the ground. The open side is covered by the barrier of the opposite plot. The space between the opposite plots is convenient for cultural operations, including supplementary pollination.
- Supplementary pollination is done by using sticks 3–4 times per day at peak anthesis during the flowering period of 7–10 days.

Table 15. Morphological stages in rice panicle development.

Morphological character	Morpho-physiological stages of rice panicle development (equivalent)
Invisible	First bract primordium differentiation (stage I) and primary branch primordium (stage II)
Little white hairs	Secondary branch primordium differentiation (stage III)
More large hairs	Stamen and pistil primordium differentiation (stage IV)
Can see the spikelet individually	Mid meiotic stage (stage V)
The lemma and palea are visible	Late meiotic stage (stage VI)
Spikelet has full size	Pollen tetrad stage (stage IV)
Panicle has green color	Pollen maturation (stage VII)
Panicle is enclosed in leaf sheath and is about to emerge	Heading stage (stage VIII, stage IX)



Stage no.	Development stage	Approx. days before heading	Approx. panicle length (mm)
I	Panicle primordia	30	0.2
II	Primary branch primordium	27	0.4
III	Secondary branch primordium	24	1.5
IV	Stamen and pistil primordia	20	2
V	Pollen mother cells	17	10–25
VI	Meiotic division	12	80
VII	Mature pollen	6	190–250
VIII	Ripe stage of pollen	4	260
XI	Completed spikelets	1–2	270
X	Flowering	–	

Fig. 19. Development stages of panicle formation to flowering.

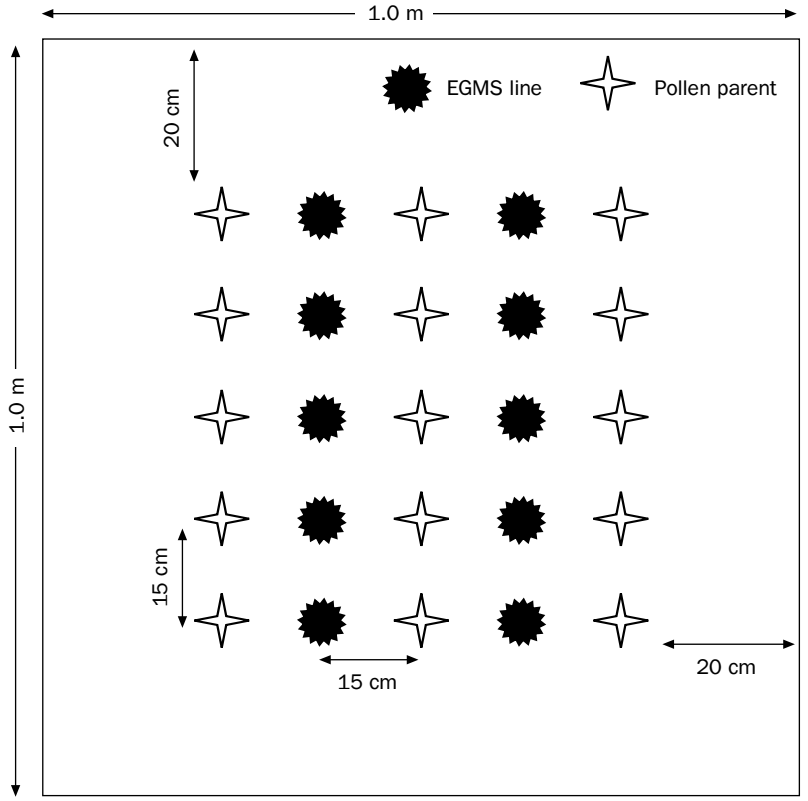


Fig. 20. Position of EGMS line and pollen parent line in the chimney isolation procedure.

iii. Isolation-free method

An isolation-free method developed at the International Rice Research Institute has been found to be more practical and popular in tropical countries. This method is ideal for producing small quantities of hybrid seed required for the OYT and PYT.

- Selected pollen parent lines are grown side by side in 5×3-m plots. In each pollen parent line plot, four rows of pollen parent plants are planted as border rows at 20×20-cm spacing to provide isolation from adjoining plots. Four vacant spaces 40 cm in width are left in the middle of the plot, which are interspersed by single rows of pollen parent line plants. About 68 EGMS plants can be planted in these spaces at the time of flowering (Fig. 21).
- EGMS lines of experimental hybrids are staggered five times at 8–10-day intervals to ensure a continuous supply of EGMS plants at the flowering stage to synchronize the flowering of pollen parents in different seed production plots.
- When primary tillers of EGMS and pollen parent lines are in the boot-leaf stage, their flag leaves are clipped except for the two outermost border rows of pollen parent lines, which act as a barrier for pollen from adjoining plots.
- Three to five days after leaf clipping, the EGMS lines are uprooted (preferably in the morning, that is, 0600—0800) and are planted in the vacant spaces of the plots.
- To enhance outcrossing, supplementary pollination is advocated at the peak anthesis period. Care should be taken to shake only those pollen parent lines that are flanking the EGMS lines.
- The pollen parent line is harvested first and threshed separately, followed by the EGMS line bearing the hybrid seeds.
- By adopting this method, 3–5 g of hybrid seed can be obtained from each EGMS/CMS

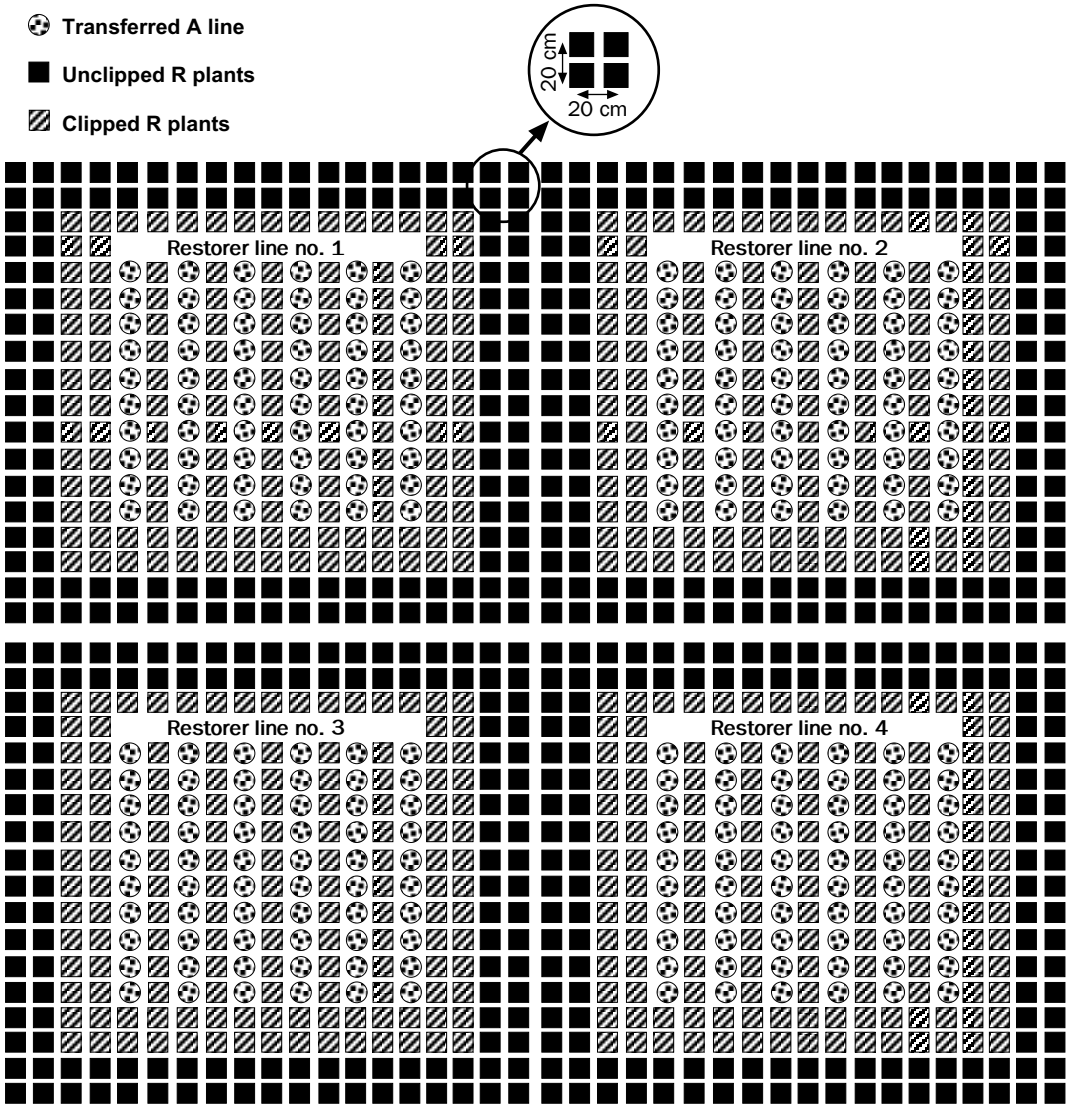


Fig. 21. Layout for isolation-free system for producing seeds of experimental two-line hybrids.

plant. A plot with 15–40 EGMS plants can yield 50–200 g of hybrid seed, which will be enough to conduct the OYT for two seasons (20 g per season) and replicated PYTs also for two seasons (100 g per season).

Seed production for AYT and MLT

- *Strict isolation method.* The hybrid seed required for conducting the AYT should be highly pure. About 1–2 kg of seed is required for this purpose. Therefore, the seed has to be produced in a larger area (100–200-m^2 plots) under strict isolation to ensure purity. The method is described below.
- *Isolation.* A space isolation of 50 m is ideal for hybrid seed production, which means that within this range no other rice varieties should be flowering except the pollen parent. If it is difficult to get space isolation, a time isolation of more than 21 days would serve the purpose. Distance isolation can be reduced to 30–40 m if the hybrid seed production plot is surrounded by an additional 15–20 rows of pollen parents.
- *Seeding sequence.* Parental lines of hybrid combinations differ in their growth duration.

Therefore, they have to be seeded on different dates so that their flowering will be synchronous. A late parent is sown first and an early parent is sown later, the difference being equal to the difference in their growth duration. The EGMS line is seeded only once so as to match the environmental conditions that favor complete male sterility. The pollen parent is seeded three times with 3-day intervals, such that the difference between the second sowing of the pollen parent and that of the EGMS line is equal to the seeding interval between the parental lines.

- *Row ratio and layout.* The optimum row ratio for hybrid seed production is 2–3 males:8–10 females. Pollen parent seedlings are evenly mixed and planted in three rows, at a spacing of 15 × 15 cm, leaving a space for an EGMS line in between. The EGMS seedlings are planted with a spacing of 30 × 15 cm. The spacing between the EGMS line and the adjacent pollen parent line should be 20 cm. Row direction should be perpendicular to the wind direction (Fig. 22).
- *Roguing.* Roguing is an important operation in a hybrid seed production plot to ensure purity of hybrid seeds. Rogues can be identified as those that are out of their row and early in booting, and based on other morphological characters. The off-types observed during different growth stages are to be removed. Before flowering, roguing is essential, especially in experimental hybrid seed production plots. Roguing at flowering is also extremely important as pollen from off-type plants can cause irreparable damage through cross pollination with male sterile plants.
- *GA_3 spray.* Spraying of GA_3 is recommended to obtain good panicle exsertion. A dose of 40–60 g ha^{-1} by a knapsack sprayer or 15–20 g ha^{-1} by a ULV sprayer is recommended for desired results. The spray liquid required is 500 L and 20 L for the knapsack and ULV sprayer, respectively. GA_3 should be sprayed two times, the first when 15–20% of the tillers have started heading and the second 2 days after the first spraying or when 35–40% of the panicles of the seed parent have emerged.
- *Supplementary pollination.* At the time of flowering, supplementary pollination is done by shaking the pollen parents with either a rope or bamboo sticks. This operation has to be done 3–4 times daily at peak anthesis for 6–10 days. The supplementary pollination technique using bamboo poles will substantially increase the experimental hybrid seed yield.
- *Harvesting and threshing.* Extreme care should be taken while harvesting and threshing the hybrid rice plots. Harvest and thresh the pollen parent first. Thoroughly check and remove any panicles of the pollen parent separately. The seed should be dried, processed, bagged, and properly labeled. The rogues must be removed before the flowering of the parental lines and before harvest. The male rows or the pollen parent lines must be harvested carefully and threshed separately. Before threshing, the threshing machine must be properly cleaned to avoid seed admixture. The labels must be carefully placed on each bag of hybrid seed along with the parentage, date of harvest, and field location.

Two-line hybrid rice seed production on a large scale

Isolation from a pollen source is equally important for large-scale seed production, especially in the initial stages, for a newly released hybrid. Total space isolation is the best way for large-plot seed production. But, a minimum of 200 m distance from any other rice pollen source is essential for ensuring seed purity.

Sowing and transplanting

For proper flowering synchronization, the male parental line should be raised on three different sowing dates to match the estimated heading date of the female line. Such a sowing plan will provide a continuous supply of pollen during the flowering of the female (EGMS) parent (Fig. 23).

At the time of transplanting, the top one-third part of the leaves may be chopped off from each uprooted nursery bundle of the pollen parent for its easy identification. The pollen parent can be transplanted first in two rows with a space of 45

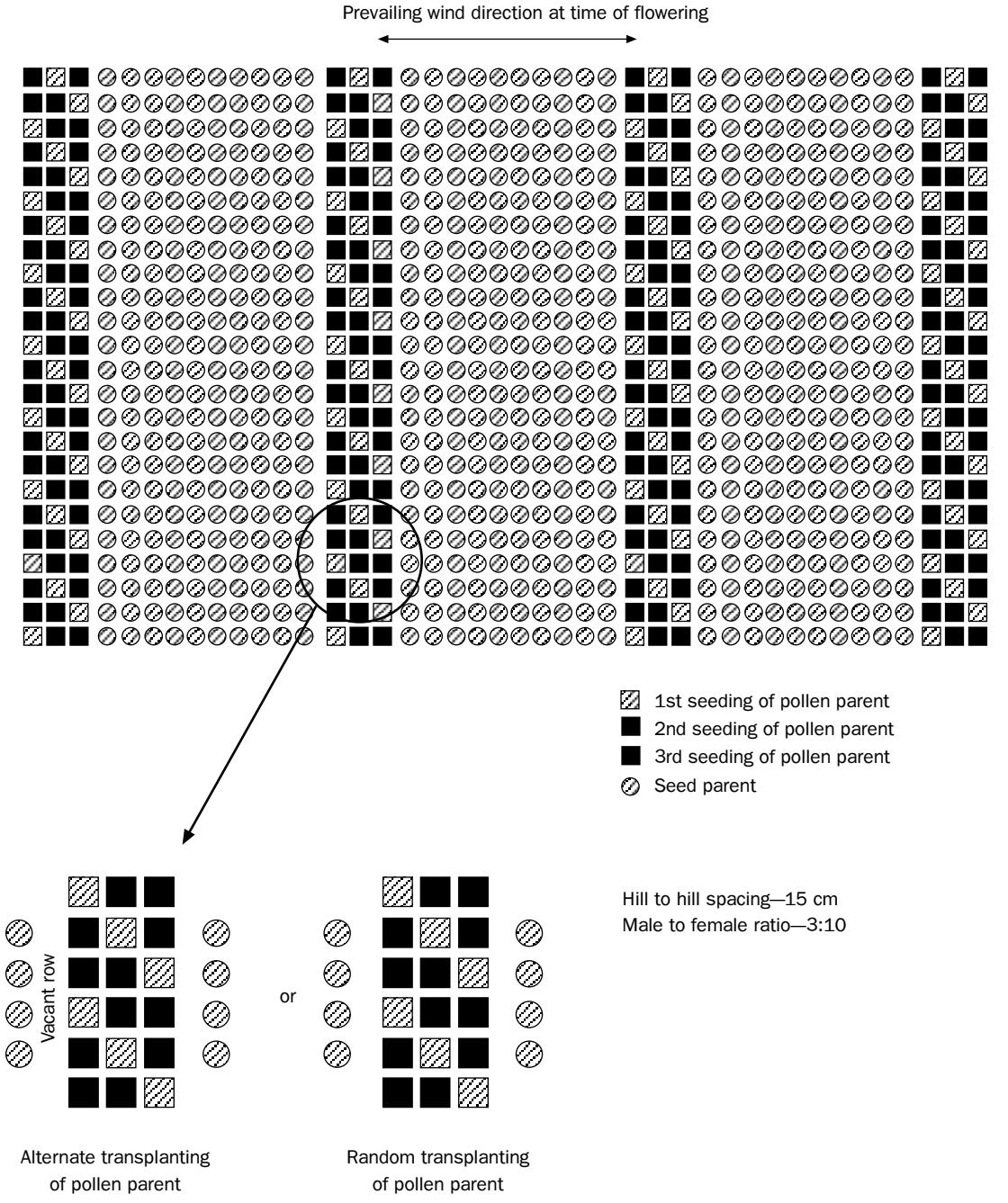


Fig. 22. Layout of breeder seed and hybrid seed production plots.

cm in between the two rows (Fig. 24). Later, the EGMS lines can be transplanted in eight or ten rows with 15-cm spacing in between them. A space of 30 cm between the pollen parent and EGMS line must also be maintained.

Before flowering, roguing is essential, especially in hybrid seed production plots. Rogues can be identified as those plants that are out of their row and early in booting, and based on other morphological characters. The rogues must be re-

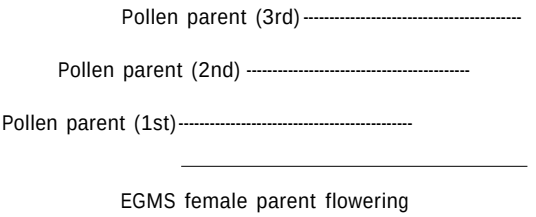


Fig. 23. Proper flowering synchronization between pollen and seed parent in a hybrid seed production plot.

moved before the flowering of the parental lines and before harvest.

Supplementary pollination techniques

Supplementary pollination techniques (i.e., pushing the plant over to facilitate pollen movement) using rope or bamboo poles will substantially increase seed yield.

Harvesting

The male rows or the pollen parent lines must be harvested carefully and threshed separately. Before threshing, the threshing machine must be properly cleaned to avoid seed admixture. The labels must be carefully placed on each bag of hybrid seed along with the parentage, date of harvest, and field location for further seed processing and packaging for distribution to farmers.

Problems and their solutions in hybrid seed production

Male sterility of EGMS lines, particularly TGMS and PTGMS lines, is highly influenced by temperature rather than by photoperiod. Among the problems, the most important is the adverse effect of temperature fluctuations caused by sudden/unforeseen local weather changes.

During hybrid seed production at high-temperature locations, a sudden drop in temperature (below the CSP) can be disastrous because of reversion to the fertile phase resulting in selfing in the female parent or the TGMS line. Hence, care must be taken to use TGMS lines of low CSP and low CFP.

Equally important is the identification of locations with stable temperature based on several years of meteorological data.

Further, the high-temperature regime can be prolonged for a minimum of 4 weeks during the sensitive phase.

Likewise, higher temperature (above the CSP) during TGMS seed multiplication at low-temperature locations can result in reduced percentage seed set, thus seriously affecting seed yield.

Aside from chemical remedies, the real solution to these problems lies in developing stable TGMS lines adapted to reasonable fluctuations in temperature during hybrid seed production and TGMS seed multiplication.

Rather than using a strictly temperature-sensitive sterility system, it is desirable to use TGMS lines slightly influenced by photoperiod because they are flexible regarding temperature fluctuations and are more stable for fertility-sterility expression.

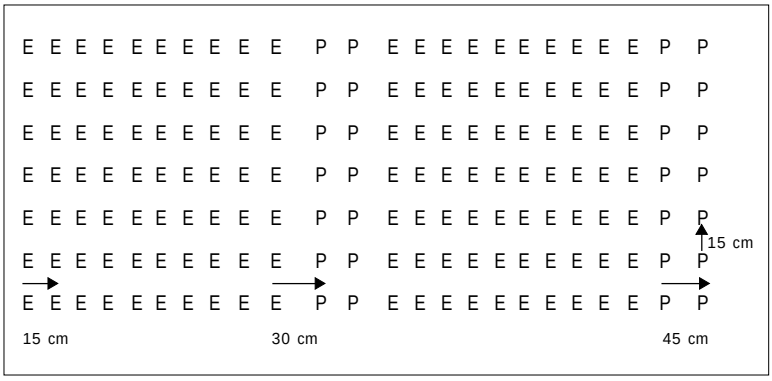


Fig. 24. Field layout for two-line hybrid seed production. E = EGMS line and P = pollen parent line.

Two-line rice hybrids: maintenance of genetic seed purity standards

Compared to three-line hybrids, the development of two-line hybrids was mainly limited by their purity. The purity of two-line rice is determined by the uniformity of the fertility transformation of EGMS lines, which is probably controlled by both major and minor genes. These minor genes are not always homozygous during the selection and evaluation of EGMS lines, which thus segregate during seed reproduction, which leads to the difference in the critical temperature of fertility alteration among individual plants within lines. When the environmental temperature is above the critical sterility point (CSP), the whole population of a TGMS line shows complete male sterility and looks uniform. However, once the temperature decreases, a difference among individual plants appears. Plants with a lower CSP are sterile, whereas plants with a higher CSP show partial fertility or even complete fertility. During hybrid seed production, plants with a low CSP are sterile and produce hybrid seeds, but plants with a high CSP become fertile and produce self seed at low temperature. This causes a mixture of hybrid seeds with self seeds, which results in a nonuniform hybrid rice production field. Therefore, purification of EGMS lines is critical to the production of two-line hybrid seed. The following methods are recommended for purification of EGMS lines:

1. Use nucleus seeds directly from the research center for seed reproduction of EGMS lines.
2. Apply anther culture for the purification of EGMS lines.
3. Introduce a recessive morphological marker into the EGMS line or a dominant morphological marker gene into the pollen parent line by gene transformation or conventional backcross breeding methods. The pseudo-hybrids can be identified and eliminated in the nursery, thereby increasing the purity of hybrid rice in the field.

1. Using nucleus seeds of EGMS for seed production

The nucleus seeds of EGMS lines produced must be used for only five generations since there can be a gradual change in CSP levels.

The procedure for purifying EGMS lines is as follows:

- Select about 100 plants with typical morphological characters of the original line from a population with less purity and plant them in pots.
- Transfer the pots into a glasshouse or phytotron with the controlled temperature of CSP until heading, when the selected plants develop into the secondary rachis–branch primordial differentiation stage (stage III).
- Select the plants with 100% sterility. When the plants begin heading, investigate pollen and spikelet fertility under the microscope.
- Ratoon the selected plants and adjust the controlled-temperature to CFP till heading. The self-pollinated seeds harvested are called nucleus seeds.
- Plant nucleus seeds from each selected plant again in rows. Compare their agronomic traits and fertility with those of the original line and harvest the seeds from the rows that are identical to the original line in bulk.

2. Using anther culture for purifying EGMS lines

Applying anther culture to purify EGMS lines by producing instant dihaploids that are completely homozygous for the EGMS trait can be highly useful for the proper maintenance of any given EGMS line. Such an approach will be highly useful for hybrid seed production.

EGMS lines that are genetically homozygous and uniform in morphology have a better photo- and thermoperiod response to fertility alteration. Those showing a significant difference in fertility among individual plants are difficult to purify effectively by conventional methods. Such lines should undergo anther culture for purification.

Choose a suitable sowing date to make EGMS plants head at the fertile phase. Alternatively, use the ratooned plants that passed the evaluation test in the growth chamber for purification. Make the ratooned EGMS plants tiller as much as possible to increase their panicle number for anther culture. Appendix II contains the detailed procedure for anther culture.

3. Transferring a recessive marker gene into EGMS lines

A recessive morphological marker is used to distinguish the pseudo-hybrid plants. The marker must have (1) single-gene control, (2) a clearly visible phenotype, (3) no obvious negative effect on other useful traits, and (4) no influence on EGMS trait expression.

The phenol reaction gene (*ph* gene) is one such marker that could be incorporated into TGMS lines. Paddy grains of varieties possessing this gene, when treated with solutions of phenolic compounds (such as phenol, catechol, hydroquinone, pyrogallol, and tyrosine), become uniformly black. A monogenic recessive gene controls the expression of this trait. If the pollen parent has this gene in the homozygous dominant form, the selfed seed of the TGMS line mixed with the hybrid seed can be identified easily by the appearance of black grains after staining treatment. The true hybrid F_1 seed material will appear brownish white after staining. These two classes of grains can then be separated easily by a color-sorting machine (Virmani and Maruyama 1995). This method can also be used by seed certifying agents to determine seed purity percentage.

The backcross method is also used to transfer the recessive marker gene into EGMS lines. The procedure is illustrated in Figure 25 using the pale green leaf marker gene *(pgl)* as an example.

4. Insertion of a dominant marker gene into the pollen parent

A dominant morphological marker is used to distinguish the pseudo-hybrid plants and it must have the following characteristics: (1) be controlled by a single gene, (2) have a visible phenotype, (3) have no negative effect on any other useful trait,

March 1991	IGM 19 (with *pgl*)	×	8902S
July 1991		F_1	
Oct. 1991	8902S	×	F_2 (plants with pale green leaves)
March 1992		B_1F_1	(out of 22 lines, 15 showed fertility transformation and M2S was the best among them)
Aug. 1992		B_1F_2	(36 individual sterile plants were selected from 661 plants with pale green leaves)
March 1993		B_1F_3	(22 lines with uniform characters and better fertility were selected from 36 lines)
Aug. 1993		B_1F_4	(22 lines were sown in intervals. After 1.5 months, the best one–M2S–was selected)
			M2S indica P(T)GMS line with pale green leaves

Fig. 25. Procedure for transfer of recessive marker gene *pgl* into EGMS lines.

and (4) have no influence on the restoration ability of the pollen parent line.

The protocol for transferring the herbicide (Basta) resistance gene (*Bar*) into a pollen parent using genetic transformation and/or backcrossing is described below.

a) Screen the pollen parents used widely in the commercial hybrids for their regeneration ability and those possessing good regeneration ability are selected.
b) For gene transformation, use mature seeds and immature embryos as explants. The *Bar* gene is inserted by a particle gun or *Agrobacterium*-mediated transformation.
c) Transplant about 50 transgenic plants (T_0) into the soil using appropriate agronomic management procedures.
d) Spray herbicide (Basta) on the regenerated plants to kill the sensitive plants. Conduct molecular analysis (such as Southern blotting) of the resistant plants.
e) Harvest separately seeds (T_1) from T_0 plants that were identified as single-copy inserted transgenic plants.
f) Sow T_1 seeds on a line basis. The seedling number of each line should be more than 50. Meanwhile, sow the original pollen parent line as a control. In addition, EGMS lines should be sown timely using routine agronomic management practices.
g) Spray herbicide Basta on the T_1 plants in the nursery and select the surviving seedlings from the lines that show a 3:1 segregation ratio for resistance to sensitivity. Then transplant them in the field as a single plant per hill. The ideal transgenic plants will be the ones that show no significant difference from their original pollen parent in phenotype.
h) At the heading stage, cross the transgenic plants (pollen parent + *Bar* gene) with the EGMS line and their control (pollen parent line) with the EGMS line at the same time and harvest their F_1 hybrid seeds.
i) Noncrossed panicles from the transgenic plants should also be bagged, then harvest self and crossed seeds (T_2) plant by plant.
j) Raise 24 F_1 plants per replication, replicated three times. Measure agronomic traits such as plant height, tiller number, resistance to diseases and pests, and yield. The perfect transgenic lines should have nonsignificant differences with their control in F_1 characters.
k) Sow T_2 seeds derived from T_1 plants (nearly 50 plants are required for each T_1-derived plot). Spray Basta in the nursery, select one plot in which all seedlings are resistant to Basta from each line, and transplant them.
l) Harvest the selfed seeds from the Basta-resistant plants; these will give a transgenic pollen parent possessing the *Bar* gene.
m) In the next year, use these transgenic *Bar* lines to test the production potential of the two-line transgenic hybrid. If the production potential of the hybrid is acceptable, produce the bulk F_1 seed. Carry out the normal hybrid trial to ascertain transgenic restorer lines with practical production potential.
n) Seed the bulk seeds of transgenic two-line hybrids in the seedbed and spray Basta on the seedbed to kill the selfed EGMS seedlings and other nonhybrid plants. Only true hybrid seedlings carrying the *Bar* gene will survive in the seedbed, which can be transplanted to the field.

Special note: Any field experiments with transgenic plants need proper approval and permission from the government and must be done in an isolated area for testing. Transgenic plants and their derived seeds are forbidden to serve as parents of a cross, animal feed, and human food without government approval. This approach, therefore, cannot be used freely by plant breeders. As a result, it has limited practical application.

The purity standards of two-line hybrids are based on grade, varietal purity, seed purity, germination percentage, and percent moisture content. The standards for China appear in Table 16.

The criteria of breeder seeds and foundation seeds of three-line hybrids appear in Tables 17 and 18.

Table 16. Quality standards for rice seed according to China national standards issued in 1996.

Type	Grade	Varietal purity (%)	Seed purity (cleanliness) (%)	Germination percentage	Moisture content (%)
Conventional rice	Basic seed Certified seed	99.9 98.0	98	85	13.0 (indica) 14.5 (japonica)
MS[a] ML RL	Basic seed Certified seed	99.9 99.0	98	80	13.0
Hybrids	Class 1 Class 2	98 96	98	80	13.0

[a]MS = male sterile, ML = maintainer line, RL = restorer line.

Table 17. Seed standards for the three parental lines.

Seed grade	Purity (%)	Cleanliness (%)	Germination (%)	Moisture (%)	Weed seeds (%)
A line					
Breeder	100	>99.9	>93.0	<13.0	0
Foundation	>99.9	>99.0	>90.0	<13.0	0
B line					
Breeder	100	>99.8	>98.0	<13.0	0
Foundation	>99.9	>99.0	>96.0	<13.0	0
R line					
Breeder	100	>99.8	>98.0	<13.0	0
Foundation	>99.9	>99.0	>96.0	<13.0	0

Table 18. Criteria for field identification of parental multiplication.

Seed grade	Sterility and sterile plants (%)	Restoring rate (%)	Off-type plants (%)
A Line			
Breeder	100.0	–	0
Foundation	100.0	–	<0.01
B line			
Breeder	–	–	0
Foundation	–	–	<0.01
R line			
Breeder	–	>85.0	0
Foundation	–	>85.0	<0.01

Future outlook for two-line rice hybrids

The exploitation of hybrid vigor in rice has shown the way to increasing rice production after yield stagnation with the use of semidwarf inbred rice varieties in irrigated ecosystems. The deployment of three-line rice hybrids in China and elsewhere in Asia has substantially increased the hopes of sustaining Asian food security. Hybrid rice technology has caught the attention of rice farmers outside China and, during the next ten years, several countries should have a large area covered with rice hybrids. There is a continuous need to reduce the cost and increase the efficiency of hybrid rice seed production. The discovery of the EGMS system in China and later in Japan, at IRRI, and in India has improved the chances of substantially reducing the cost of seed production by using two-line rice hybrids. These hybrids also help to increase heterosis beyond the level of three-line rice hybrids. The two-line hybrids have already created an impact in China, with their area reaching 2.6 million ha. Among the several two-line rice hybrids, Liangyou PeiJiu (Peiai64S/9311) gave the highest average yields of 11 t ha^{-1} on 10-ha farms in two successive years. The highest yield recorded was about 12.1 t ha^{-1}, a new record in these areas, clearly revealing the enhanced potential of two-line rice hybrids. Outside China, two-line rice hybrids are also being developed at IRRI and in Vietnam, India, Korea, the Philippines, Thailand, and Egypt.

Future research priorities include the following:

1. *Development of stable EGMS lines.* Stable elite EGMS lines with a precise fertility alteration mechanism hold the key to success in developing two-line commercial hybrid rice. The underlying genetic mechanism of fertility alteration needs to be understood clearly to properly enhance the efficacy of EGMS seed multiplication and hybrid rice seed production. Breeding of TGMS lines with a low CSP is important for developing two-line commercial rice hybrids in the tropics. The genetic characterization of the loci of the EGMS genes from different sources in relation to closely tagged molecular markers is useful for marker-assisted selection.
2. *Use of anther culture to develop and/or purify elite EGMS lines.* Anther culture techniques involving dihaploidization can be used to expedite the development and/or purification of EGMS lines possessing major genes and QTLs in influencing the PGMS/TGMS trait.
3. *Breeding for super high-yielding two-line hybrids.* Two-line hybrid rice technology involving EGMS lines allows the choice of a wider range of parental combinations and avoids the negative effects of male-sterility-inducing cytoplasm. Rice scientists at IRRI and in China can now use new plant type (NPT) lines developed in the tropical japonica and indica/tropical japonica background as male and/or female parents to develop hybrids with enhanced heterosis. Two-line breeding technology can overcome the major problems of wide incompatibility and the narrow range of restorers in exploiting indica-japonica heterosis.
4. *Incorporation of hybrids with resistance to biotic stress.* Two-line rice hybrids possessing multiple resistance to diseases and in-

sects can be developed more expeditiously than three-line hybrids since the desired resistance genes need to be incorporated in two rather than three parental lines.

5. *Abiotic stress tolerance.* Hybrid rice technology so far has been used under the irrigated rice ecosystem. Environmental degradation resulting from salinity and water shortages has posed a major threat to sustaining food production. Researchers at IRRI and in Egypt, India, and China have found that hybrid rice technology can be extended to saline-prone irrigated conditions because hybrids have performed exceedingly well under moderate to high saline soil conditions. Since the high cost of seed is a major constraint to the adoption of this technology by resource-poor farmers and two-line hybrid breeding and the seed production approach make it more cost-effective, this technology can be adopted by resource-poor farmers.
6. *Quality.* The negative influence of WA cytoplasm on certain quality parameters (such as grain chalkiness) allows the alternative use of EGMS-based two-line hybrid rice technology to overcome such drawbacks.

A multidisciplinary approach in developing superior EGMS lines and pollen parents can help to develop two-line rice hybrids suitable for the different ecological situations in which rice is grown. Despite the promise that two-line hybrid rice technology holds, it would be wise to have a harmonious balance in using two-line and three-line hybrids and conventional rice varieties in an appropriate manner in national rice production programs.

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Glossary

A

A line – the male sterile parent involving cytoplasmic or cytoplasmic genetic male sterility. It is also known as a CMS line.

adaptability – the ability of a genotype to adjust to a given environment and give a reasonably good yield.

allo-plasmic lines – CMS or restorer lines that have different cytoplasms.

anther – terminal part of the stamen that contains the pollen grains (male gametes).

anthesis – the action of opening of a flower or spikelet of rice.

apiculus – a small acute point or tip of a rice spikelet; extension of the lemma or palea.

apomixis – a kind of asexual reproduction through seed in which the embryo develops from a maternal cell without fertilization. The resulting seed has the same genetic constitution as that of the seed parent.

apparent heterosis – subjective superiority of a hybrid over its parents or a check variety based on visual observation.

augmented design – a statistical design used for evaluation of genotypes in which the check varieties are replicated and the test entries are not replicated but are allotted randomly to the blocks.

auricles – the small paired ear-like appendages on either side of the base of the rice leaf blade that may not be present in older leaves.

awn – a bristle-like extension of varying length originating from the lemma of the spikelet.

B

B line – the fertile counterpart parent of the male sterile A line of a cytoplasmatic or cytoplasmic genetic male sterility system that is used as a male parent to maintain the latter. It is also known as a maintainer line.

backcross method – a breeding method in which the F_1 hybrid is again crossed with either of its parents. The resulting progeny is called a backcross progeny.

backcross nursery – breeding nursery in which male sterile plants identified among the test-crosses (CMS ´ elite lines) are crossed with the respective male parents to transfer cytoplasmic male sterility into the nuclear genotype of the elite line.

boot – a rapidly growing panicle enveloped by the flag-leaf sheath. In tissue culture, this refers to the panicle collected when the distance between the collar of the flag leaf and subtending leaf is about 7 to 8 cm.

booting – bulging of the flag-leaf sheath because of the growing panicle inside.

border rows – the recommended number of rows of the male parental line grown on all sides of the hybrid seed production plot to minimize contamination by outcrossing with stray pollen.

bract – a leaf from the axis from which a flower arises.

breeder seed – breeder seed is the seed of the highest genetic purity and is produced by the agency sponsoring a variety; it is used to produce foundation seed.

C

caryopsis – a small one-seeded dry indehiscent fruit with a thin membranous pericarp adhering so closely to the seed that fruit and seed are incorporated in one body, forming a single grain, as in wheat and barley. In rice, brown rice is the caryopsis.

certified seed – seeds used for commercial crop production produced from foundation, registered, or certified seeds under the regulation of a legally constituted agency. In hybrid rice, it is F_1 seed produced directly from CMS × restorer lines grown as per certification standards.

CHA (chemical hybridizing agent) – any chemical that is used to induce male sterility in plants.

check variety – any popular or high-yielding variety widely grown in a region.

chemical mutagen – any chemical used to induce mutations artificially.

chemical hybridizing agent (CHA) – this is any chemical formulation, that is, auxins, antiauxins, growth regulators, arsenicals, oxanilates, ethylene-releasing compounds, halogenated aliphatic acids, etc., that has the ability to selectively sterilize the male gametes without affecting ovular fertility and is not phytotoxic.

CMS – the CMS line is governed by genetic factors present in the mitochondria of the cytoplasm responsible for inducing selective male sterility. But its pistil is normal and it can produce seeds when pollinated by any normal plant.

combining ability – the ability of a genotype (inbred, pure line, or synthetic) to transfer its desirable traits to its progeny: general—average performance of a strain in a series of crosses; specific—deviation from performance predicted on the basis of general combining ability of parental lines.

correlation coefficient – a measure of the degree of association between two variables that is computed as the ratio of the covariance of the two variables to the products of their standard errors. Its values vary between –1 and +1.

covariance – the mean of the product of the deviation of two varieties from their individual means.

critical difference – a statistical parameter computed to test whether the observed differences between the means of entries are significant or not.

critical fertility point (CFP) – the critical temperature or photoperiod experienced by the EGMS line during the sensitive stage results in maximum pollen and spikelet fertility.

critical sterility point (CSP) – the critical temperature or photoperiod experienced by the EGMS line during the sensitive stage results in complete pollen and spikelet sterility.

cross fertilization – the fertilization of an egg nuclei (ovule) of one parent by the pollen of another parent.

cytoplasm – all the protoplasm of the cell except the nucleus.

cytoplasmatic heredity/inheritance – the transmission of characters from parent to offspring through the cytoplasm of the germ cell.

D

daylength - number of light hours in a day.

diallel mating – a mating design in plant breeding in which a set of parents is crossed in all possible combinations.

dihybrid – a hybrid of two different genes; heterozygous for two pairs of alleles.

diploid (2n) – an organism having two chromosomes of each kind.

disomic – a plant having one or more chromosomes duplicated, but not the entire genome.

diverse – having or capable of having various forms or qualities.

dominance – intra-allelic/intragenic interaction with complete suppression of the effects of one allele by another.

E

effective accumulated temperature (EAT) – the total effective temperature in centigrade received by the plant from seeding to flowering. It is useful for predicting flowering.

EAT = Mean daily temperature (°C) – temperature higher than 30 °C – temperature of lower limit (18 °C)

emasculation – the process of removal of anthers from the florets so as to make the plant male sterile.

elite line – an improved breeding line or a variety.

endosperm – the nutritive tissue of the ripened ovary. It consists of the aleurone layer and the starchy tissue, and serves as the source of food for the germinating embryo.

environmental genic male sterility (EGMS) – male sterility–fertility transformation controlled by environmental factors such as temperature and photoperiod.

epistasis – the interaction of different genes in the expression of a trait.

F

F_1 – abbreviation for the first filial generation, usually the hybrid between two homozygous parents.

fertility restoration – an ability of a genotype to restore fertility to its progeny when crossed to a CMS line.

fertilization – fusion of the nuclei of male and female gametes.

flag leaf – the uppermost leaf (of rice plant) originating just below the panicle base.

flag-leaf clipping – a method of cutting 1/2 to 2/3 of the flag leaf from its tip in CMS and restorer lines to facilitate easy pollen dispersal.

floret – a unit of the spikelet, which includes the lemma, palea, and the flower.

flower, rice – the reproductive organ consisting of lemma, palea, two lodicules, six stamens, and the pistil.

foundation seed – seed stock produced from breeder seed by or under the direct control of a breeder or a research station. Foundation seed is the source of certified seed, either directly or through registered seed.

G

GA_3 – a form of gibberellic acid that is sprayed on CMS lines to obtain good panicle exsertion.

gamete – a mature reproductive male or female germ cell, sperm, or egg specialized for fertilization.

gametic (tissue or generation) – having "n" number of chromosomes (haploid), in contrast to zygotic tissue with 2n (diploid).

gametocide – organic or inorganic chemicals used for killing the functional sexual parts (pollen, ovule) of the plant. These may be selective for male or female parts.

gametophytic – in this system, the sterility/fertility reaction is imparted to the pollen by the genetic constitution of the pollen itself and is controlled by a single gene, which may have a large number of allelic forms.

genetic purity – trueness to type; seeds/plants confirming to the characteristics of the line/variety/hybrid as described by the breeder.

genetic shift – change in the genetic makeup of the line/variety/hybrid if grown over a long period, particularly in areas outside its adaptation.

genic male sterility – the type of male sterility governed entirely by the nuclear genes. It may be transmitted by either the male or female parent.

germination – the resumption of growth by the embryo and development of the young plant from the seed. Germination, precisely, is the emergence and development from the seed embryo of those essential structures that, for the kind of seed being tested, indicate the ability to develop into a normal plant under favorable conditions in the soil.

grain – the ripened ovary and its associated structures.

H

heading (flowering), rice – growth stage of the rice plant marked by the emergence of the panicle from the boot followed by anthesis.

heritability – broadly, the proportion of observed variance that is inherited, the remainder being due to environmental effects. Strictly, the proportion of variance caused by the additive effect of genes.

heterobeltiosis – refers to the phenomenon in which an F_1 hybrid obtained by the crossing of two genetically dissimilar parents shows superiority over the better parent in one or a combination of characters.

heterosis – refers to the phenomenon in which an F_1 hybrid obtained by the crossing of two genetically dissimilar parents shows superiority over mid-parental values in one or a combination of characters.

heterosis (standard) – refers to the phenomenon in which the F_1 hybrid obtained by the crossing of two genetically dissimilar parents shows superiority over the best standard check prevailing at that time in one or a combination of characters.

heterosis breeding – a method of breeding to develop an F_1 hybrid obtained by the crossing of two genetically dissimilar parents.

heterozygote – an individual having different alleles for any gene pair and producing two kinds of gametes.

heterozygous – hybrid for any gene pair, with different alleles for the gene being considered.

hill – a group of rice plants directly adjacent to each other because the seeds or seedlings were planted together. A hill may also consist of only one plant.

hybrid – the product of a cross between genetically dissimilar parents.

hybrid rice – the F_1 seed of rice bred for commercial use.

hybrid vigor – increased vigor of the hybrid over its parents in one or more characteristics.

hybridization – a breeding method in which two varieties are crossed to produce new variability and desired recombinants. The hybrids are allowed to self-pollinate and the segregating populations are handled by an appropriate method.

I

inbred – an individual resulting from the mating of closely related parents or by selfing.

i**nbred line** – a nearly homozygous line produced by continued self-fertilization.

inbreeding – the interbreeding of closely related individuals occurring naturally (as in a closed population) or as a deliberately chosen system of breeding and serving especially to preserve and fix desirable characters or to eliminate unfavorable characters from a suitably selected stock but tending to bring about an unwanted decline (as in size, vigor, or fertility) through the fixation of undesirable and often recessive characters when the initial stock is in any way defective.

indoor growth cabinets – small indoor chambers wherein temperature, humidity, and light are artificially controlled.

intersubspecific hybrid – a cross between different subspecies of a crop. For example, in rice, hybrids between indica and japonica lines are considered as intersubspecific hybrids.

isolation – the separation of one group from another so that mating between or among groups is prevented.

isolation (barrier) – the separation between two groups can be provided by topography surface features or artificial/natural obstacles to a height of at least 2.5 m for rice.

isolation-free method – a method of producing hybrid seed for experimental purposes without isolation but by providing crop barriers of 2–4 rows of the restorer lines.

isolation (space) – separation is provided by keeping a certain distance between two groups. A space isolation of 50–100 meters is ideal for hybrid rice seed production.

isolation (time) – separation is provided by growing two groups at different times of the crop season so that one group is already mature (stopped providing pollen) when the other group reaches flowering. Generally, a period of 21 days' difference in flowering is sufficient for rice.

isoplasmic – these are the CMS or restorer lines differing in nuclear genetic constitution but having common cytoplasm.

L

leaf number – total number of leaves developed on the main culm of a plant, which is a characteristic feature of each variety.

lodicules – the two scale-like structures adjoining the base of the palea that control the opening of the lemma and palea during anthesis.

M

maintainer line – a pollinator variety is used to pollinate a CMS line and produce progenies that remain male sterile. If there is no maintainer line, the male sterile line cannot be maintained and multiplied generation after generation.

male sterility – absence or nonfunction of pollen in plants.

mature grain stage (rice) – stage occurring during the ripening phase when the inside of the grain is at first watery but later turns milky in consistency.

milling yield – the estimate of the quantity of head rice and of total milled rice that can be produced from a unit of rough rice. It is generally expressed in percentage.

multilocation trial – yield trials conducted in different locations to study the adaptability of varieties/hybrids over environments.

N

nuclear genes – genes located on the chromosomes.

nucleus – a small quantity of genetically pure seed produced under the strict supervision of the plant breeder.

O

off-type – the plants/seeds of the same crop deviating significantly from the characteristics of the variety/hybrid as described by the breeder.

one-line breeding – this uses apomixis as a means to fix the heterosis of F_1 hybrids into true-breeding hybrids and is also known as the one-line breeding method.

outcrossing rate – the extent of cross pollination measured on the basis of seed set to the total number of spikelets.

outdoor growth cabinets – the small cabinets located outside where temperature and humidity are artificially controlled while light provided is natural.

ovary – the bulbous basal portion of the pistils containing one ovule.

overdominance – superiority of the heterozygote Aa over either homozygote AA or aa.

P

panicle – the terminal component of a rice plant that bears the rice spikelets.

panicle development – the growth stage of the rice plant in which the spikelets become distinguishable and the panicle extends upward inside the flag-leaf sheath.

panicle exsertion – growth stage of the rice plant marked by the emergence of the panicle from the boot.

panicle exsertion rate – the extent to which the panicle is exserted out of the flag leaf.

panicle initiation (rice) – growth stage that starts when the primordium of the panicle has differentiated and becomes visible.

partial restorer – a pollinator variety used to pollinate a male sterile line to produce F_1 male fertile progenies, which produce partial seed set upon selfing.

pedigree – the record of the ancestry of an individual or a cultivar.

p**edigree nursery** – a nursery consisting of segregating families in different generations derived from different crosses.

PGMS – photoperiod sensitive genic male sterile line. The genic male sterile plants that respond to the photoperiod or duration of daylength in terms of pollen fertility and sterility behavior.

phenotypic acceptability – breeders' shorthand to record their observations on overall acceptability of breeding lines or populations. This can be done using an acceptability score of 1–9. For example, 1 = excellent plant type and absence of diseases. Promote to the next level of testing and spread to other breeding programs. 3 = very good appearance. Promote to next level of testing. 5 = fair appearance, but has a few essential shortcomings (too early maturity, etc.). Use as parent in hybridization block. 7 = poor appearance, but has a few important traits that make it suitable as a donor. Make a few crosses. 9 = poor. Discard.

photoperiod – duration of daylength.

pistils – the female reproductive organ consisting of the ovary, style, and stigma.

plant growth substances – natural and synthetic compounds that elicit growth and developmental or metabolic responses. These substances are usually not metabolites in the sense that they are not intermediates or products of the pathways they control, and they are active at very low concentrations.

planting ratio – the ratio in which the male and female parental lines are planted to make a crossing block in hybrid seed production or maintenance of the CMS line.

plumule – the leaves of the young plant in any embryo It is enclosed by the coleoptile.

pollen – a mature reproductive male germ cell (microsporocyte) specialized for fertilization.

pollen fertility/sterility – the ratio of fertile/strerile pollen grains to the total pollen grains counted in 3–4 fields under a microscope and expressed in percentage. Fertility/sterility of pollen grains is determined by their stainability with 1% IKI stain.

Pollen fertility/sterility gradiation

% sterile pollen	Category	% fertile pollen
0–20	Fully fertile	81–100
21–40	Fertile	61–80
41–70	Partially fertile	31–60
71–90	Partially sterile	11–30
91–99	Sterile	1–20
100	Completely sterile	0

pollen load – the amount of air-borne pollen per liter per hour at peak anthesis on a specified day.

pollen parent – male parent of a cross combination.

pollination – transfer of pollen from the anther to the stigma of a flower.

progeny – offspring; individuals resulting from a mating.

pure line – a line that has been rendered almost homozygous by repeated self-pollination over generations.

purity – the composition by weight of the sample being tested and, by inference, the composition of the seed lot; the identity of various kinds of seeds and inert matter constituting the sample.

R

random mating – a system in which every individual plant in a population has an equal chance of becoming pollinated by any other individual.

randomization – allotting treatments to different plots without any bias.

recurrent selection – a method of breeding designed to concentrate favorable genes scattered among several individuals by selecting in each generation among the progenies produced by random mating of the selected individuals (or their selfed progenies) of the previous generation.

replication – repeating the experiment under identical conditions with the objective of reducing the experimental error.

restorer line – a pollinator variety is used to pollinate the male sterile line to produce F_1 progenies that are male fertile and thus produce seeds on selfing.

retestcross – a cross made beween a cytoplasmatic male sterile line and a test variety (identified to be a restorer in the testcross) to recheck the potentialities of the F_1 to give normal seed set upon selfing.

retestcross nursery – breeding nursery to evaluate the retestcross F_1s and corresponding male parents.

ripening phase (syn. maturity phase, grain-filling phase) – the period from pollination to harvest.

rogue – a variation from the standard of a variety or strain. Roguing is the removal of undesirable individuals to purify the stock.

row ratio – the proportion of seed parents and pollen parents planted to maintain cytoplasmic male sterile lines or to produce F_1 hybrid seed in a seed production plot.

S

secondary tillers – tillers arising from primary tillers.

second leaf – the first differentiated leaf with blade and sheath.

seed – the fertilized and ripened ovule of a seed plant comprising an embryonic plant accompanied by a store of food (as endosperm or perisperm), enclosed in a protective seed coat, and capable under suitable conditions of independent development into a plant.

seed dormancy – the ability of mature seeds to delay their germination after reaching physiological maturity.

seed parent – a female parent of a cross combination.

seed viability – in general, the state of being alive; ability of the seed to germinate and produce normal seedlings.

seedbed – the bed on which rice seeds are sown, consisting of soil (wetbed method) or banana leaves, plastic sheets, or concrete floor ("dapog method").

seeding sequence – the order of seeding the parental lines based on their growth duration so that they reach flowering at the same time.

seedling (rice) – from seed germination to early tillering; a juvenile plant.

self-fertilization – fusion of male and female gametes from the same individual.

source nursery – breeding nursery where all the genetic material, including sources imparting cytoplasmic male sterility, genotypes with specific traits useful for a hybrid breeding program, and elite rice lines showing high general and specific combining ability, is maintained for use in a hybrid breeding program.

spikelet – the basic unit of the rice inflorescence consisting of the two sterile lemmas, the rachilla, and the floret.

spikelet fertility – the number of filled spikelets to the total number of spikelets on a panicle.

sporophytic – in this system, sterility/fertility is imparted to the pollen by the mother plant upon which the pollen is borne and the genotype of the pollen has no bearing per se. It may be controlled by more than one gene with multiple alleles.

staggered planting – planting the restorer line on different dates to maintain a uniform and regular supply of the pollen to the spikelets of a cytoplasmic male sterile line that continues to bloom for a longer period.

stamen – the male reproductive organ consisting of the anther and the filament.

sterile – failing to produce or incapable of producing offspring.

stigma – the apex of the pistil of a flower, upon which pollen is deposited at pollination.

stigma exsertion rate – the proportion of spikelets with exserted stigma (either on one or on both sides) to the total number of spikelets in a panicle.

supplementary pollination – a method of shaking the male parent at the time of peak anthesis so as to disperse pollen grains to increase the seed set on a CMS line. This is particularly necessary when the wind velocity is less than optimum (2–3 m sec^{-1}).

synchronization (anthesis) – refers to the simultaneous opening of the spikelets of the seed and pollen parents.

synchronization (flowering) – refers to the simultaneous flowering of seed and pollen parents despite having different growth durations.

T

TGMS (thermosensitive genic male sterile) line – the genic male sterile plants that respond to the temperature in terms of their fertility/sterility behavior.

testcross – a cross made between a cytoplasmic male sterile line and a test variety to identify maintainers and restorers.

testcross nursery – breeding nursery where F_1 progenies of cytoplasmic male sterile lines and test varieties are screened for pollen sterility/fertility and spikelet fertility to identify maintainers and restorers.

thermosensitivity – sensitivity of a genotype to varying temperature regimes in terms of pollen or spikelet sterility/fertility.

three-line breeding – a breeding strategy to develop hybrids uses three important lines—CMS lines (A lines), maintainer lines (B lines), and restorer lines (R lines)—in a two-step seed production system as follows: (1) CMS line multiplication from an A × B cross in the field through natural outcrossing and (2) hybrid (A × R) seed production.

tiller – a vegetative branch of the rice plant composed of roots, culm, and leaves, which may or may not develop a panicle.

tillering – growth stage of the rice plant that extends from the appearance of the first tiller until the maximum number is reached.

topcross – a cross between a selection, line, clone, etc., and common pollen parent is called the topcross of a tester parent.

two-line breeding – breeding methodology in which only two lines, a male sterile line (photosensitive, thermosensitive, or chemically induced) and a pollen parent, are used to produce F_1 hybrids.

U

uniformity – the extent of similarity between the individuals of a population.

V

variance – the mean squared deviation of varieties from their mean.

vegetative phase – the period from germination to panicle initiation.

viability – the ability to grow and develop.

vigor – the capacity for natural growth and survival, as of seed, plants, or animals.

volunteer plants – unwanted plants growing from the seed (may or may not be the same crop) that remains in the field from a previous crop.

W

wide compatibility – the ability of a genotype to produce normally fertile progeny when crossed with both indica and japonica testers.

wide hybridization – a process of crossing between distantly related species.

Appendix I

Identifying male sterility

When rice plants start heading, male sterility is identified with the following methods:

1. **Visual inspection**
   At the complete flowering stage, observe the color and plumpness of the anthers in the male sterile plants directly with the naked eye. Shake the panicles slightly to examine the dehiscence of anthers. Pay attention to detecting any pore dehiscence that occurs at the basal part of the anthers. Male sterility in rice plants is expressed by anthers that are whitish/pale yellow, shriveled, and nondehiscent.
2. **Seed set on bagging**
   When the plants just start heading but their florets have yet to flower, cover the panicles with glassine paper bags to check whether self seed setting has occurred. In practice, two panicles are bagged for each plant. After 25 days, observe the seed setting in the bagged panicles. If no seed is set, the plant is considered to be completely male sterile. When a few seeds (i.e., 5–20%) are produced, the plant is considered to be partially male sterile. The spikelet fertility percent is calculated as the number of filled spikelets divided by the total number of spikelets (i.e., filled and unfilled spikelets) per panicle on a per plant basis multiplied by 100.
3. **Pollen viability study under the microscope**
   Collect five spikelets at the time of flowering and squash the anthers in 1–2 drops of 1% acetocarmine or 1% IKI (iodine potassium iodide) stain. When the plant just starts heading but its flowers have yet to flower, sample the five apical spikelets and immerse them in a tube with a screw cap containing fixative solution (alcohol:acetic acid, 3:1) and store them at room temperature. For microscopic observation under the light microscope, rinse the spikelets with distilled water, place the anthers from three or five spikelets on a slide, and crush them on the slide with a drop of 1% IKI stain. Observations for each microscopic field must have more than 60 pollen grains and be averaged for five different fields. The fertile pollen grains will be spherical and darkly stained, whereas the sterile pollen grains will be either unstained and spherical or unstained and irregular in shape. Pollen fertility percent is calculated as the number of stained spherical pollen grains with normal shape and size to the total number of pollen grains expressed in percentage. Many of the EGMS lines in the completely sterile phase, that is, under very high-temperature or long-photoperiod conditions, become pollenless and only the anther bag remains.

Classifying male sterility according to the morphology of sterile pollen

1. **Typical abortion type**
   The pollens are irregular in shape; some are triangular, some are shuttle shaped, etc. They are unstained with IKI solution. Pollen abortion occurs mainly at the one-nucleus stage. So, this type is also called the uninucleate abortion type. The CMS lines of the WA type correspond to this type.

2. **Spherical abortion type**
   Pollens are spherical and unstainable with IKI solution. Pollen abortion occurs approximately at the two-nuclei stage. So, this type is also called the binucleate abortion type. The Hong-Lian type CMS lines are representative of this type.
3. **Stained abortion type**
   Pollens are spherical, but partially or lightly stained with IKI solution. Pollen abortion occurs mainly at the three-nuclei stage, so the trinucleate abortion type is its other name. The boro-type CMS line are included in this type.
4. **No-pollen types**
   There are no pollen grains, indicating that abortion occurs even before the pollen mother cell formation stage. An example is SA2.
5. **Antherless type**
   Under extreme cases, this has been observed in certain EGMS lines with no anthers at all, leaving the female organs functional and intact.

For most EGMS lines, fertility to sterility alteration behavior has been well documented, especially for different pollen types with the gradual change in environmental conditions. When an EGMS line is placed during its sensitive phase under extremely high-temperature (e.g., >35 ºC) or long-photoperiod (15 h) conditions, no-pollen types and typical abortion types are observed. While under relatively less higher temperature near its CSP level (e.g., >32 ºC) or photoperiod >14 h, the unstained spherical and abortive types are observed. And with a further lowering of temperature or photoperiod, the fertile stained spherical pollen types and unstained spherical pollen types are observed. As the EGMS lines experience the environment that approaches the CFP level, that is, <24 ºC or <13.5 h, the fertile stained spherical pollen grains are observed in a higher percentage. This gradual change can be observed with changing environmental conditions within an EGMS line by collecting regularly the pollen grains upon flowering from subsequent flowering panicles.

Appendix II

Protocol for anther culture

This section is based on Xiang et al (1993), Li et al (1994a, 1995), and Pan et al (1993).

1. Young panicles at the meiosis stage as plant material for in vitro irradiation (0.5 to 3.0 KR). The panicles were pretreated for 7 d under 6–10 h of irradiation, followed by incubating anthers on M8 medium with 2 mg L^{-1} 2,4-D, 2 mg L^{-1} NAA, 1 mg L^{-1} KT, and 5% sucrose.
2. Select and plate the anthers in N6 media from the F_1 hybrids of TGMS × non-TGMS or PGMS × non-PGMS (Chu et al 1975) or in M8 media (Mei et al 1988).
3. To obtain a higher success rate of useful dihaploids, a large number of anthers must be plated in N6 or M8 media for more than 5,000 petriplates, with 100 anthers on each petriplate (6 cm). Callus initiation is <7% and the regeneration rate is <20%.
4. The callus obtained must be tried for the regeneration of plants.
5. Regenerated plants should be hardened in 1/2 MS liquid media followed by activated charcoal-sterilized soil medium, adopting standard procedures.
6. Regenerants should be taken to the field and raised under special fertility-conducive conditions.
7. Vigorous-looking plants that may be dihaploids should be selected and their seeds kept separate. Weak plants should be treated with colchicine for chromosome doubling since they may be haploids.
8. Seeds obtained should be sown and raised under sterility-conducive conditions for the selection of male sterile lines.
9. Sterile plants should be ratooned and brought to low temperature or shorter photoperiod for inducing fertility to get self seeds.
10. TGMS and PGMS lines should be screened based on the criteria for ideal EGMS lines.
11. Four TGMS lines, 6442S, 1286S, HS-1, and HS-5, and one PTGMS line, Lu Guang 2S, have been developed through anther culture and extensively tested in Jiangxi, Sichuan, and Fujian provinces of China.

Appendix III

Data to be recorded for hybrid rice experiments

Characters	Breeding nurseries				Evaluation trials[a]				
	Source nursery	EGMS nursery	Testcross nursery	Backcross nursery	OYT	PYT	AYT	MLT	On-farm
Days to flowering (d)	✓	✓	✓	✓	✓	✓	✓	✓	✓
Vegetative vigor (score)					✓	✓	✓	o	
Plant height (cm)					✓	✓	✓	✓	
Panicles m^{-2} (no.)					✓	✓	✓	✓	✓
Anther color and shape (ypl;ws)[b]		✓	✓	✓					
Pollen fertility (%)		✓	✓	✓					
Spikelet fertility (%)			✓		✓	✓	✓	✓	
Panicle exsertion rate (%)	✓	✓		✓					
Stigma exsertion rate (%)	✓	✓		✓					
Outcrossing rate (%)		✓		✓					
No. of filled grains $panicle^{-1}$					✓	✓	✓	✓	✓
Apparent heterosis (scale)			✓		✓	✓	✓	✓	✓
Grain yield (kg ha^{-1})					✓	✓	✓	✓	✓
Grain type (scale)	✓	✓	o	✓	✓	✓	✓	✓	✓
Phenotypic acceptability (scale)	✓	✓	✓	✓	✓	✓	✓	✓	✓
Reaction to pests/diseases					o	o	✓	✓	✓
Weather data							✓	✓	o

[a]OYT = observational yield trial, PYT = preliminary yield trial, AYT = advanced yield trial, MLT = multilocational trial.
[b]ypl;ws = yellow plumpy; white shriveled.
✓ = essential, o = optional.