Euphytica (2007) 156:297–303
DOI 10.1007/s10681-006-9314-1

Use of herbicide-resistant genic male sterility in hybrid rice seed production

Sung Soo Kim · Jae Youn Jung · Seung Kwon Jeong · Dong Sun Lee · Li Juan Chen · Hak Soo Suh

Received: 14 July 2006 / Accepted: 17 November 2006 / Published online: 9 May 2007

Abstract One of the difficult problems in hybrid rice seed production is the low outcrossing frequency and requirement for much labor to produce hybrid seeds. In order to simplify the process of hybrid rice seed production, herbicide-resistant photoperiod sensitive genic male sterile (HRPGMS) rice was utilized in this study. The herbicide resistance gene *bar* was transferred into the photoperiod sensitive genic male sterile (PGMS) rice 920S by *Agrobacterium*-mediated transformation and the HRPGMS line YA3530ms with good agronomic characteristics was bred by applying conventional pedigree breeding technique. The seeds of HRPGMS and pollen parent were mixed with the ratio of 4:1 in weight, and were sowed in seedling box. The mixed seedlings of HRPGMS and pollen parent grown for 30 days were transplanted by the small transplanting machine in the field. The herbicide glufosinate ammonium was sprayed at 7 days after flowering to kill all the plants of pollen parent, whereas hybrid seeds were harvested from the survived HRPGMS parent at maturity. The outcrossing frequency of HRPGMS line from two combinations in 2002 and from five combinations in 2004 were compared with a control cultivated by the conventional 2-line system. As the result, the mean outcrossing frequency in HRPGMS of the treatments were 10.6–24.5% compared with 5.5% in PGMS of the control in 2002, and that were 24.7–32.0% compared with 7.5% in the control in 2004. Consequently, using HRPGMS in two-line system was proved to be a new method that would simplify the process of hybrid rice seed production and to increase outcrossing frequency without any artificial supplementary pollination processes.

Keywords Genic male sterility · Herbicide resistance · Hybrid seed production · Two-line system · Rice · Hybrid rice · Seed production · Breeding technique

S. S. Kim · J. Y. Jung · S. K. Jeong · H. S. Suh (✉)
School of Biological Resources, Yeungnam University, Gyeongsan 712-749, Republic of Korea
e-mail: hsuh@ynumail.ac.kr

D. S. Lee · L. J. Chen
Rice Research Institute, Yunnan Agricultural University, Kunming, Yunnan 650201, People's Republic of China

Introduction

Hybrid rice was reported to outyield inbred varieties by 15–20% (Virmani 2003; Yuan 2003). Hybrid rice has been utilized in China and other Asian countries for more than 20 years. However, the process of hybrid rice seed production is complicated and the rate of natural outcrossing in hybrid seed production fields is relatively low and artificial processes are essential to increase the outcrossing frequency.

Hybrid rice seed production involves the use of the 3- or 2-line system (Mao and Virmani 2003; Mou et al. 2003; Rongbai and Pandey 2002; Rutger 2001;

Virmani et al. 1982; Yuan and Virmani 1988). In the 3-line system, the cytoplasmic male sterile (CMS), maintainer and restorer lines are used. In 2-line system, the photoperiod sensitive genic male sterile (PGMS) or thermo-sensitive genic male sterile (TGMS) and pollen parent, should be sown, transplanted and harvested separately from alternating blocks. The additional artificial processes such as spraying gibberellic acid (GA_3), driving rod, rope pulling and/or utilizing motorized pollinator are essential to increase the outcrossing frequency (Jagadeeswari et al. 2004; Molina et al. 2003; Pandey et al. 2003; Suralta and Robles 2004; Zeng et al. 1999). The outcrossing frequency of the normal rice is lower than 0.5% in natural condition and that of the male sterile rice planted together with the pollen parent is 5.0–7.5% under natural conditions with no artificial treatments (Kim 2003). This level of outcrossing is insufficient for commercial hybrid seed production. Therefore, some of the artificial processes mentioned above are utilized. Outcrossing frequency in hybrid seed production can be increased up to 30% by using the artificial processes (Mao and Virmani 2003). However, these processes require much labor to produce sufficient hybrid seed in rice. So, hybrid rice is commercialized only in the countries where the labor cost is low.

Recent advances in plant cellular and molecular biology have greatly simplified the development of herbicide resistance in a wide range of crops. The ease with which theses genetic manipulations can be made opened up a range of new applications for herbicide resistance in crop protection, agronomic practice and seed production (Conner and Field 1995). It was demonstrated that using triazine resistance as a genetic markers offered new opportunity for hybrid seed production in *Brassica* (Conner and Christey 1997). Currently, the transgenic or naturally mutated herbicide-resistant rice were applied for hybrid rice seed production. The herbicide-resistant transgenic rice was used as the pollen parent in hybrid rice seed production process and the false hybrid seeds could be eliminated by testing herbicide resistance in F_1 seedlings (Xiao et al. 2005; Zhou et al. 2006). Zhang et al. (2002) found a herbicide (bentazon) sensitive mutant TGMS and the selfed seeds could be eliminated by spraying the herbicide bentazon at F_1 seedlings.

Here we report a new method of hybrid rice seed production using the transgenic herbicide-resistant photoperiod sensitive genic male sterile (HRPGMS) as female parent. The objective of this study is to simplify the process of hybrid rice seed production and to increase outcrossing frequency in producing hybrid rice seeds without artificial supplementary pollination processes.

Materials and methods

Transformation of herbicide resistance

The herbicide resistance gene *bar* was transferred to a PGMS line 920S by *Agrobacterium*-mediated transformation. The callus induced from scutellum of the mature seed of a PGMS line 920S was co-cultivated with the *Agrobacterium tumefacience* LBA4404 containing the herbicide resistance gene *bar*. The *E. coli* strain DH10B was used as recipient of pCAMBIA3300 vector containing the *bar* gene resistant to the herbicide glufosinate ammonium. Vector construction and rice transformation followed the procedures described by Lee (1999). Regenerated plantlets carrying pCAMBIA3300 vector were transferred onto hormone-free MS media to allow further root development at 25°C under continuous illumination. When transgenic plants had grown to about 10 cm height with a healthy root system, they were transferred to soil and grown in greenhouse.

Breeding HRPGMS with improved agronomic characteristics

The original transgenic HRPGMS line TR 18 obtained from transformation of the PGMS 920S was male sterile but its agronomic characteristics were poor for use as a breeding line. The original HRPGMS line TR 18 was crossed with the Korean rice variety Dongjinbyeo and the HRPGMS line YA3530ms with good agronomic characteristics was bred by applying conventional pedigree breeding technique. The field tests at Gyeongsan (35.9°N, 128.6°E, 58 masl.) in Korea showed that, HRPGMS YA3530ms heading in the summer (during June to July) was predominantly sterile with seed set of about 1%, while its panicles heading in autumn (from late of August to September) exhibited to be fertile with seed set from 30 to 50%. Normally, PGMS 920S heading in the summer was nearly completely sterile with seed set less than 3%.

Hybrid seed production using HRPGMS

We tried to produce hybrid rice seed following the process suggested in Fig. 1 and 2. The seeds of HRPGMS YA3530ms and two pollen parents Hwayeongbyeo and Ilpumbyeo, in 2002, and those of YA3530ms and five pollen parents Ilpumbyeo, Donganbyeo, Mangeumbyeo, Surabyeo, and Hwanambyeo, in 2004, were mixed with the ratio of four HRPGMS versus one pollen parent in weight, respectively. The mixed seeds were sowed in seedling box and were treated in following the ordinary seed growing process. The mixed seedlings grown 30 days were transplanted by using the transplanting machine (Daedong DP450). In control treatment, seeds of PGMS 920S and pollen parent Hwayeongbyeo was sowed separately in seedling box and was transplanted separately by hand with eight rows of PGMS 920S and two rows of the pollen parent. The experimental plot size was 10 m^2 per treatment and was arranged with the randomized complete block design with three replications. The commercial herbicide glufosinate ammonium solution (diluted to 540 ppm) was sprayed to the mixed cultivation plots at 7 days after flowering. At maturity stage, the seeds of different treatments in the mixed cultivation plots and the control plot (PGMS line) in 1 m^2 each were harvested. Seed set frequency of maternal parents in HRPGMS YA3530ms and PGMS 920S was evaluated from 100 panicles sampled randomly in each plot.

F_1 seed purity test

The seeds of different treatments harvested from the experimental plots in 2002 were directly seeded to test seed purity of the hybrids. Five hundred seed from each experiment plot were tested for F_1 seed purity. For the mixed cultivation plots, the herbicide glufosinate ammonium solution was applied to the young F_1 seedlings at five-leaf stage to identify the herbicide-sensitive plants from any surviving pollen parents. At heading and maturity stages, plant uniformity and the agronomic performances including plant height, heading date and seed set frequency of F_1 plants in different treatments from the mixed cultivation plots and the control plot were investigated.

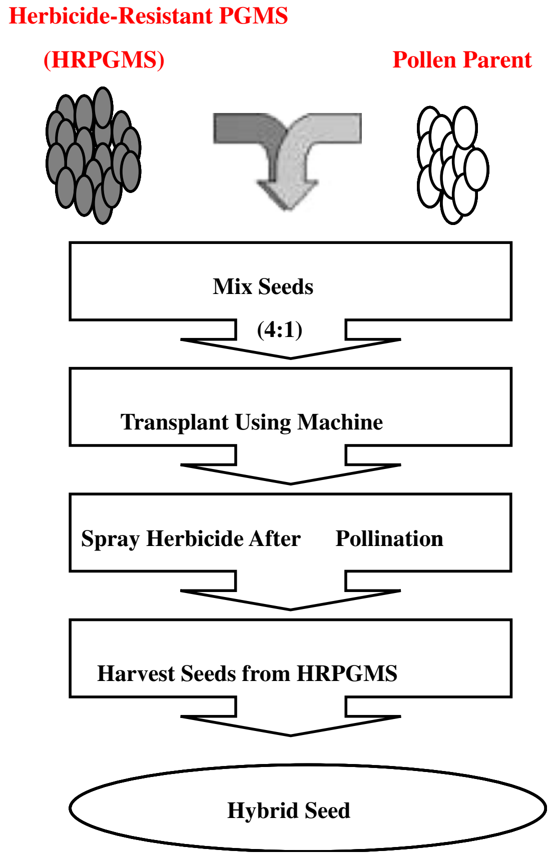


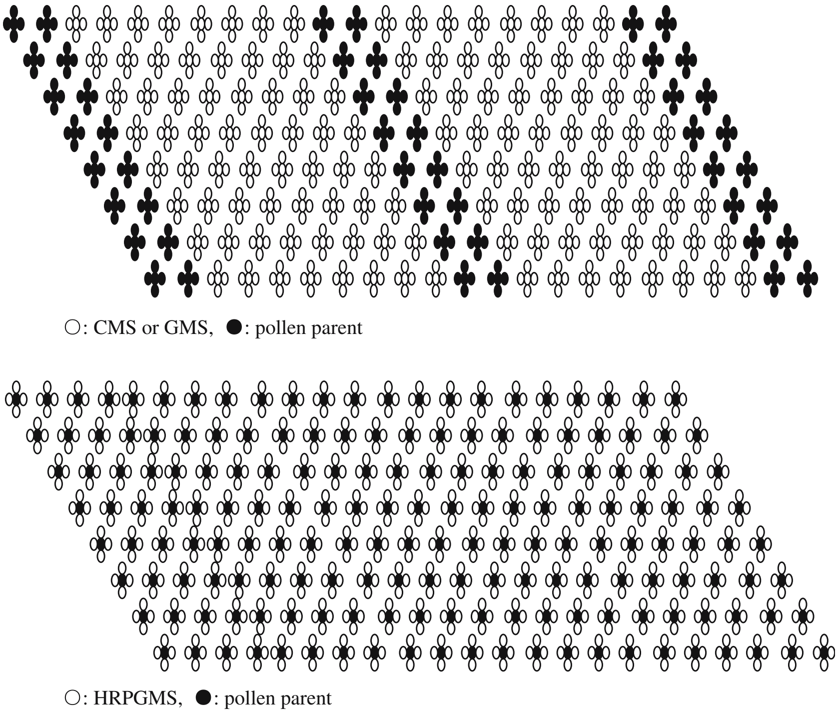
Fig. 1 A new two-line system of hybrid rice seed production using the HRPGMS line; the seeds of HRPGMS and pollen parent will be mixed with the ratio of 4:1 in weight, and sowed in seedling box. The seedlings will be transplanted in field by transplanting machine, and the herbicide will be applied to kill the pollen parent after pollination. The hybrid seeds from the survived HRPGMS line will be harvested and any remaining selfed-pollinated seed derived from the pollen parent eliminated by sensitivity to the herbicide

Results and discussion

Elimination of pollen parent by herbicide treatment

The pollen parent in the mixed cultivation plots died within 1 week after spraying the herbicide and the HRPGMS plants survived well (Fig. 3). Because the

Fig. 2 Diagram of planting methods of the conventional two- or three-line system in hybrid rice seed production (*upper*) and that of the new two-line system using the HRPGMS suggested in this study (*lower*)



herbicide was sprayed at 7 days after flowering, most of the spikelets in the panicles of pollen parent died before the milky stage. However, it should be recognized that, if the timing of the herbicide application was delayed, some early flowering panicles of pollen parent would reach the milky stage and the seeds would have the ability for germination after harvest, even though they may have withered before harvest. In contrast, if the timing of the herbicide application was too early the yield of hybrid seeds would be decreased due to reduced pollen dehiscence.

Fig. 3 Plant performance following a field herbicide treatment after pollination. The brown colored plants are the pollen parent eliminated by herbicide and the green plants are surviving HRPGMS plants. The seeds harvested from the surviving HRPGMS plants are F_1 hybrid seeds

Xiao et al. (2005) and Zhang et al. (2002) used the herbicide-resistant pollen parent in hybrid rice seed production in two-line system, and the selfed seeds of male sterile line would be removed from hybrid by spraying herbicide in F_1 seedlings. They used the herbicide-resistant line as pollen parent to identify the selfed seeds after production of hybrid seeds. We used the herbicide-resistant male sterile line to simplify the seed production process. Similarly, hybrid seed purity would be improved by removing the selfed seeds of the pollen parent by herbicide application to F_1 seedlings in this two-line system with HRPGMS. In fact, various innovative methods for hybrid seed production using herbicide resistance as genetic markers have been designed (Conner and Field 1995). Conner and Christey (1997) also successfully developed new approach for hybrid seed production in *Brassica* by using triazine-resistant, CMS lines for 'female' parents of F_1 hybrid seed, showing that non-hybrids of triazine-sensitive broccoli (*Brassica oleraea* L. var. *italica*) progeny could easily be eliminated by a seed treatment with triazine application.

Seed set frequency

Without artificial supplementary pollination, the mean seed set frequency on the HRPGMS in the experimental plot of mixed cultivation of the HRPGMS YA3530ms and Hwayeongbyeo, and of YA3530ms and Ilpumbyeo were 11 and 25%, respectively, while that from the PGMS of the control plot was 5% in 2002 (Table 1). In 2004, the mean seed setting percent in the plot YA3530ms and Ilpumbyeo was 32%, YA3530ms and Donganbyeo 27%, YA3530ms and Mangeumbyeo 25%, YA3530ms and Surabyeo 27%, and YA3530ms and Hwanambyeo 26%, while that of control plot was 8% (Table 2). The seed set frequency of the HRPGMS line pollinated from mix-planted pollen parent was substantially higher than that from PGMS planted separately in control plot in 2002 and 2004. The combination HRPGMS YA3530ms and Ilpumbyeo and the control PGMS 920S and Hwayeongbyeo were tested two times in 2002 and 2004. The outcrossing frequency of the two combinations in 2004 were higher than those in 2002, respectively. This variation might be caused by differences in heading date between the two parents resulting from changes in the environment between 2002 and 2004.

Hybrid rice seed production was usually carried out by separate planting and separate harvesting of the CMS and restorer pollen parent in the three-line system, and by those of PGMS or TGMS and pollen parent in two-line system (Virmani 2003; Yuan 2003). These materials were mostly planted by hand and require considerable work and labor for hybrid rice seed production. The new method suggested in our study could simplify the process of hybrid rice seed production. The outcrossing frequency obtained in the new method without artificial supplementary pollination processes was almost same as that obtained in the conventional two-line or three-line system with application of some artificial processes. This can be attributed to the close proximity of the male sterile and pollen parents resulting from the mixed field planting. The former methods relied on planting the male sterile and pollen parents in alternating field blocks requiring pollen dispersal over greater distances. We have observed that synchronization of heading in the HRPGMS and pollen parents, and determining the optimal time for herbicide application were the key points in implementing the new method.

A common problem during F_1 hybrid seed production is inadequate pollen transfer, resulting in poor yield of hybrid seed by the 'female' parent. In other crops, it has been noted that the transfer of pollen from 'male' parent to the 'female' parent can be substantially improved if the distance between the two parents can be reduced. If the 'female' parent has a genetic marker for herbicide resistance, the 'male' and 'female' parents could be grown as a random mixture, rather than as separate alternating blocks in the field, resulting more efficient pollen transfer, and therefore higher yield and more economical production of hybrid seed. As mentioned in the previous study (Conner and Christey 1997), the atriazine resistant, CMS *Brassica* plants could be grown as a random mixture with a male parent, thereby allowing

Table 1 Seed set frequency in the mix-planted plots with the HRPGMS YA3530ms and two pollen parents Ilpumbyeo and Hwayeongbyeo, and that of the control plot planted separately with the PGMS 920S and the pollen parent Hwayeongbyeo, in year 2002

Combinations		Seed set frequency (%)	
Male sterility	Pollen parent	Mean*	Range
YA3530ms	Ilpumbyeo	24.5[a]	11.0–35.8
YA3530ms	Hwayeongbyeo	10.6[b]	6.8–15.9
920S (control)	Hwayeongbyeo	5.0[c]	1.0–13.8

* Means followed by the different letters within column are significantly different at 1% level by Duncan's multiple range test

Table 2 Seed set frequency of the mix-planted plots with the HRPGMS YA3530ms and five pollen parents Ilpumbyeo, Donganbyeo, Mangeumbyeo, Surabyeo, and Hwayeongbyeo and that of the control plot planted separately with the PGMS 920S and the pollen parent Hwayeongbyeo, in year 2004

Combinations		Seed set frequency (%)	
Male sterility	Pollen parent	Mean	Range
YA3530ms	Ilpumbyeo	32.0[a]	11.2–52.8
YA3530ms	Donganbyeo	26.8[b]	10.3–42.3
YA3530ms	Mangeumbyeo	24.7[c]	6.1–43.3
YA3530ms	Surabyeo	26.7[b]	5.6–43.6
YA3530ms	Hwanambyeo	26.3[b]	7.2–45.4
920S (control)	Hwayeongbyeo	7.5[d]	4.7–15.7

* Means followed by the different letters within column are significantly different at 1% level by Duncan's multiple range test

more effective pollen transfer for greater efficiency of hybrid seed production.

Seed purity of F_1

The seeds harvested from the experimental plots of different treatments and the control in 2002 were grown to test hybrid seed purity. Judging from the frequency of herbicide resistance and the morphological traits of the F_1 plants, on an average, 96 and 95% of the seeds from HRPGMS mixture cultivation plots (combinations of YA3530ms/Ilpumbyeo and YA3530ms/Hwayeongbyeo) and 95% from PGMS plants in the control plot (920S/Hwayeongbyeo) were hybrid seeds. Other seeds were rare selfed seed set from the male sterile parents (YA3530ms, 920S) or rare selfed seed harvested from the herbicide-sensitive pollen parents (Ilpumbyeo and Hwayeongbyeo) that were not completely eliminated by the herbicide application in the field (Table 3). This result implied that the seed harvested from these mixed population would consist of hybrid seed from the 'female' parents, which is herbicide-resistant, and non-hybrid seed resulting from self pollination of 'male' parent, which is herbicide-sensitive (Conner and Christey 1997).

A common requirement for the commercial production of hybrid crops is seed purity of hybrids. One major problem arising during hybrid seed production is pollen contamination. The development of herbicide-resistant plants provided ideal lines for 'female' parents of F_1 of hybrid seed. When randomly planted with a small proportion of herbicide-sensitive pollen donor, it has been supposed that the non-hybrid seed resulting from the self pollination of pollen parent could be prevented from developing by spraying out the pollen parent after flowering, but before seed set. However, the complete elimination of such seeds cannot be guaranteed due to difficulties in attaining uniform herbicide application over all individuals in a population. Alternatively, we can overcome these issues by treating the F_1 seed with the herbicide to eliminate one source of contaminating seed. Conner and Christey (1997) demonstrated a simple seed treatment for the effective elimination of triazine-sensitive brassica progeny.

For utilization of this new method in commercial hybrid rice seed production the following problems should be considered: improving hybrid seed purity by using the stable HRPGMS as female parent, deciding the best time of herbicide spray by testing amount of outcross and survived seeds from the pollen parent, and synchronizing of heading stage of the HRPGMS and pollen parent by checking the data of heading in 2 or 3 years before using.

Table 3 Seed purity of the F_1 hybrid seed produced from the plot mixed planted with the HRPGMS YA3530ms and two pollen parents Ilpumbyeo and Hwayeongbyeo, and that from the control plot planted separately with the PGMS 920S and the pollen parent Hwayeongbyeo in 2002

Combinations		Seed purity of F_1 hybrid (%)	
Male sterility	Pollen parent	Mean*	Range
YA3530ms	Ilpumbyeo	95.2 ns	84.6–97.5
YA3530ms	Hwayeongbyeo	95.7	85.3–99.6
920S(control)	Hwayeongbyeo	95.3	85.5–98.7

* Means followed by ns are not significantly different at 5% level by Duncan's multiple range test

Acknowledgments This study is supported partly by Biogreen 21 project, Rural Development Administration, Korea, and by Molecular Genetics and Breeding Research Center, Korean Science and Engineering Foundation (R11-1999-062-03003-0).

References

Conner AJ, Christey MC (1997) A seed treatment for eliminating non-hybrid plants when using atrazine resistance as a genetic marker for hybrid seed production. Ann Bot 80:561–564

Conner AJ, Field RJ (1995) Herbicide-resistant crops: a new approach to an old problem or a radical new tool? In: McLean GD, Evans G (eds) Herbicide-resistant crops and pastures in Australian farming systems. Bureau of Resource Sciences, Canberra, pp. 53–71

Jagadeeswari P, Sharma SP, Dadlani M (2004) Effect of different chemicals on traits favouring outcrossing and optimization of GA_3 for seed production of cytoplasmic male sterile line in hybrid rice. Seed Sci Technol 32(2):473–483

Kim SS (2003) Hybrid rice seed production using herbicide-resistant photoperiod sensitive genetic male sterility in rice. MS thesis, Yeungnam University, Korea, pp. 59

Lee S (1999) Binary vectors for efficient transformation of rice. J Plant Biol 42:310–316

Mao CX, Virmani SS (2003) Opportunities for and challenges to improving hybrid rice seed yield and seed purity. Hybrid rice for food security, poverty alleviation, and environmental protection. In: Proceeding of the 4th international symposium on hybrid rice. Hanoi, Vietnam, Los Banos, Laguna (Philippines), IRRI:85–95, 14–17 May 2002

Mou TM, Lu GX, Hoan NT,Virmani SS (2003) Two-line hybrid rice breeding in and outside China. Hybrid rice for food security, poverty alleviation, and environmental protection. In: Proceeding of the 4th international symposium on hybrid rice. Hanoi, Vietnam, Los Banos, Laguna (Philippines). IRRI:31–52, 14–17 May 2002

Molina OA, Tadeo BD, Agbayani RS, Cordero JC, Ramos RA, Lucas RJ, Ballesteros LM, Escobar RP, Guillermo RA, Sosa YP, Gaspar MG, Casimero VV, J. de-Lon C (2003) Mechanizing supplementary pollination in hybrid rice seed production. Philipp J Crop Sci (Philippines) 28(Suppl 1):21

Pandey S, Sharma SP, Dadlani M (2003) Effect of gibberellic acid application on floral and morphological traits, seed yield and storability of parental lines in hybrid rice (*Oryza sativa*). Indian J Agric Sci 73(7):376–380

Rongbai L, Pandey MP (2002) Genetic improvement of features for hybrid seed production and self seed multiplication of thermosensitive genic male sterile lines in rice. J Genet Breed 56(2):89–97

Rutger JN (2001) Induction of photoperiod sensitive genetic male steriles for use in hybrid rice seed production. Euphytica 120:399–400

Suralta RR, Robles RP (2004) Gibberllic acid (GA_3) effect on heading characteristics of ten cytoplasmic male sterile (CMS) lines and on hybrid rice seed production using IR58025a CMS line. Philipp Agric Sci 87(3):285–297

Virmani SS (2003) Advances in hybrid rice research and development in the tropics. Hybrid rice for food security, poverty alleviation, and environmental protection. Proceeding of the 4th international symposium on hybrid rice. Hanoi, Vietnam, Los Banos, Laguna (Philippines). IRRI:7–20, 14–17 May 2002

Virmani SS, Aquino RC,Khush GS (1982) Heterosis breeding in rice (*Oryza sativa* L.). Theor Appl Genet 63:373–380

Xiao GY, Yuan LP,Samuel SMS (2005) Strategy and practice of herbicide resistance gene utilized in two-line hybrid rice. Abstracts the 5th international rice genetics symposium, Edsa Shangri-la Hotel, Manila (Philippines). IRRI:p 245

Yuan LP (2003) Recent progress in breeding super hybrid rice in China. Hybrid rice for food security, poverty alleviation, and environmental protection. In: Proceeding of the 4th international symposium on hybrid rice. Hanoi, Vietnam, Los Banos, Laguna (Philippines). IRRI:3–6, 14–17 May 2002

Yuan LP, Virmani SS (1988) Organization of a hybrid rice breeding program. In: Hybrid rice. Manila (Philippines), IRRI:33–37

Zeng XC, Zhou X, Zhang W, Murofushi N, Kitahara T, Kamura Y (1999) Opening of rice floret in rapid response to methyl jasmonate. J Plant Growth Regul 18:153–158

Zhang J, Xu Y, Wu X, Zhu L (2002) A bentazon and sulfonylurea sensitive mutant: breeding, genetics and potential application in seed production of hybrid rice. Theor Appl Genet 105:16–22

Zhou XS, Shen SQ, Wu DX, Sun JW, Shu QY (2006) Introduction of a xantha mutation for testing and increasing varietal purity in hybrid rice. Field Crops Res 96:71–79