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REVIEW PAPER

Indigenization of hybrid rice development in Pakistan: Breeding prospects and approaches

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Abstract: Rice is an exportable commodity in Pakistan, earning a foreign exchange of almost two billion dollars every year. The demand for rice has recently been increased due to surging wheat prices in the country and food security issues. To cope with these issues there is an opportunity to exploit heterosis in the form of hybrid development in rice. Hybrid rice is one of the emerging avenues in Pakistan. Pakistan is importing almost 500 tons of hybrid seed every year from China and other countries at a cost of huge foreign exchange. Punjab Agriculture Department claimed hybrid system development in Basmati rice and its testing is in process. There is a possibility of hybrid system development in coarse rice by using a three-line and two-line hybrid system. The two-line hybrid system is an emerging hybrid system nowadays in China, Philippines, India, and Bangladesh. There is a need to import novel genetic material from China, IRRI and other sources for the strengthening of indigenous hybrid rice breeding. In a two-line hybrid system Japonica type rice genotypes were mostly utilized as a source of male sterility and restorer lines are used that can produce seed under hot climatic conditions. Pakistan has favorable climatic zones for the two-line hybrid system of rice like Swat is suitable for TGMS line maintenance and seed production. Jacobabad area of Sindh province and Jafarabad area of Baluchistan province are the best options for hybrid seed production under two-line hybrid rice production. In this article plenty of novel resources of *cms*, *Rf* and *tms* genes marker systems have been highlighted that can be utilized for the identification and characterization of indigenous rice germplasm. The objective of this article is to focus on the prospects and possibilities of hybrid rice system development in Pakistan. © 2020 Department of Agricultural Sciences, AIOU

Keywords: Hybrid rice, A, B and R-line hybrid system, Two-line hybrid, CMS and TGMS

Abbreviations: A-line = Male sterile line; B-line = Maintainer line; R-line = Fertility restorer line; TGMS = Thermo-sensitive genetic male sterility; GDP = Gross domestic product; *cms* = Cytoplasmic male sterility; *Rf* = Fertility restore; IRRI = International Rice Research Institute; Rf = Fertility restorer; *tms* = Thermosensitive male sterility.

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Introduction

Rice (*Oryza sativa* L.) belongs to the grass family, alongside corn (*Zea mays* L.) and wheat (*Triticum aestivum* L.) and it is one of the three crops on which humans subsist for their nutrition. Rice is presently the most important crop around the world, as it is consumed as a staple food in many countries of the world (Zhang, 2016). Rice grows in more than 100 countries and the global area under rice cultivation is around 158 million hectares. Worldwide consumption of rice has been slightly increased during the last few years as global consumption of rice was about 486.62 million metric tons during the year 2018-19, whereas it was 437.18 million metric tons during the cropping year 2008-2009 (Food and Agriculture Organization [FAO], 2019; Shahbandeh, 2019). Asia produces 90% of total rice in the world. Rice is the staple food of about 90 percent of the total population that comprise of Asia (Bandumula, 2018). As rice is the third-largest crop after wheat (*Triticum aestivum* L.) and cotton (*Gossypium hirsutum* L.) in terms of area under cultivation in Pakistan. During the *Kharif* season (beginning of autumn season in Pakistan) around 10 percent of the total cultivated area in Pakistan is under rice. Rice is a second main staple food, a high value cash crop of Pakistan and is a major source of earning foreign exchange (Akhter & Haider, 2020). The share of rice to the total value added in agriculture is three percent whereas it contributes six percent to the total GDP of Pakistan. During 2018-2019, area under rice crop was 2.810 million hectares (Mha), and the production was 7.202 million tonnes. Whereas, during 2017-2018 the area under rice crop was 2.901 Mha and production was 7.450 million tonnes for both coarse and Basmati rice.

Hence, rice is an important contributor to food security; alleviates poverty and malnutrition in Asia as well as around the world. Rice production is expected to increase every year as the world's population, and the demand for rice continues to grow to ensure food security (Muthayya et al., 2014). To increase rice production there is a dire need of improving the yield of rice crop as there exists scope for further improvement in productivity of rice through hybrid development rather than escalating the area under cultivation.

Pakistan consumes almost half of its rice production and the second half is exported. Pakistan is the main exporter of Basmati rice after India. Now the paradigm of rice export is shifting from Basmati rice to coarse rice due to novel export avenues and consumers of coarse rice. Basmati rice is preferably consumed in middle eastern countries, whereas coarse rice has a big exportable market in Europe, South Asia, and Africa. In coarse rice, hybrid variety is a better option than conventional rice varieties that have yield potential up to 15% or even 30%, higher resistance to abiotic factors and biotic factors due to heterosis (Virmani et al., 2003).

The major countries which produce hybrid rice in the world include China with 18.6-million-hectare (Mha) area of cultivation that is over 63% of the total global rice zone, Bangladesh 7.35 Mha, India 1.5 Mha, Vietnam 6.45 Mha, Philippine 3.46 Mha, Indonesia 6.2 Mha, 5000 ha in the USA and Myanmar 5000 ha (Ludwig, 2012). The expected world market in 2020 for hybrid rice valued at 4194.5 million USD and during 2026 it is expected to be 7921 million USD.

The superiority of a hybrid over its parents is heterosis, which is the phenomenon in which two genetically dissimilar individuals are crossed and produce an F_1 hybrid that shows increased vigor over the better parent (Sah & Joshi, 2020). Heterosis is the way for increasing the production and productivity of rice. To meet global food demand, heterotic hybrid has great potential (Bano & Singh, 2019). Heterosis is the phenomenon in which the offspring of distant inbred varieties is superior to both parents in terms of yield, size of panicle, number of spikelets per panicle, number of (productive) tillers, and tolerance to stress (Gramaje et al., 2020). Moreover, good hybrid rice cultivars have the potential of yielding 20% to 30% more than the non-hybrid rice cultivars under similar conditions (Shih-Cheng & Loung Ping, 1980). Rice being a self-pollinated crop requires a male sterility system for the development of a commercial hybrid between promising parental lines. Male sterility causes infertility in pollens, rice spikelets became incapable for setting of seeds through self-pollination (Fan & Zhang, 2018). Hybrid rice production has been started in China and has now become increasingly popular among major rice-growing countries in the world. Hybrid rice cultivation has slightly higher cost of production due to the premium of hybrid seed as compared to conventional rice cultivation but net gain in the form of yield is 20% to 30% more in hybrid rice cultivation in Sindh province of Pakistan (Wagan et al., 2015).

Background of hybrid rice

The hybrid rice research program started under the leadership of Yuan Long Ping in China during 1964 and in 1970 they discovered the male-sterile rice plant in the wild rice population (Wang & Wang, 2017). In 1973 Professor Yuan and other researchers developed the first cytoplasmic male sterile system (three-line system) of hybrid rice production (Cao & Zhan, 2014). In 1974 first hybrid rice cultivar was released in China, which produced 20% more yield as compared to conventional cultivars of rice at that time. Commercial production of the first three-line hybrid rice combination was started in 1976 in China (Virmani, 2003; Rout et al., 2020). In the 1970's China successfully commercialized the hybrid rice production technology and got the first patent in 1989 in America (Longping et al., 1989). The two-line hybrid rice was released commercially in 1994 in China. The two-line hybrid system includes male sterility systems viz., thermo-sensitive genetic male sterility (TGMS), cytoplasmic male sterility, (CMS) and photoperiod-sensitive male sterility (PGMS). In 1973 the first japonica type photo-sensitive genetic male sterile line NoNgken58 was bred and that had a loss of function mutant of *pms3* gene (Ding et al., 2012). Thermo-sensitive male sterility hybrid system (TGMS) was started in 1987 in China (Zhang et al., 2016), and in China almost 71% of cultivars are generated by using *TMS5* mutants in the two-line hybrid system (Song et al., 2016). Recently IRRI has released TGMS based two-line hybrids for cultivation in the Philippine and other countries. Pakistan also has the scope and need for hybrid rice development with indigenous resources and germplasm for the three-line and two-line hybrid system.

Need of hybrid rice research in Pakistan

Being the second most important staple food, rice is also the third largest cash crop in Pakistan. The average yield of rice in Pakistan was 30-35 mounds per acre and for improving the socio-economic condition of farmers the low productivity is a major problem as they make less premium. The introduction of hybrid rice seed of coarse varieties helped many farmers to increase their per acre yield which in turn increases the income of farmers significantly, as after hybrid rice introduction, the farmers are taking yield of 100-110 mounds per acre and some progressive farmers are taking 136 mounds per acre (Akhter & Haider, 2020). Hybrids will not only produce higher yield but will also increase the income of farmers and ultimately it will help in building the country's economy by increasing the export of rice. The idea of introduction and development of high yielding hybrids of rice in Pakistan is not only to improve the domestic food security but also to increase the rice export to meet the global demand of rice especially the coarse rice that has high demand in the Asia-Pacific region and Africa (Akhter & Haider, 2020).

Status and trends of hybrid rice in Pakistan

With higher production over inbred varieties, hybrid rice has become popular in Pakistan. In the late 1990s Rice Research Institute, Kala Shah Kaku in a combined effort with International Rice Research Institute (IRRI) commenced a time-based hybrid program. This program was inaugurated by the Government of Punjab in 2000 by initiating a project named "Development of Hybrid Rice in the Punjab". This program also includes Basmati hybrid rice production. The breeders under this project developed several rice hybrids and in station yield trials of these hybrids performed well. However, due to unawareness of production technology of hybrid seed and minimal interest of the private sector, the rice growers got no interest in these hybrids.

A private company, Guard Agricultural, and Services (Pvt) initiated the introduction of Chinese exotic hybrids from China under the supervision of experts for assessment and promotion in Sindh and thus introduced GNY-50 and GNY-53 hybrids which were first-ever approved hybrids of rice in Pakistan. These hybrids got a low market rate as they belonged to the *japonica* type and had low quality as compared to inbred varieties (Akhter et al., 2007a). Moreover, the same organization, with the help of Chinese experts, put efforts into hybrid seed production in the lower divisions of Sindh and produce a low yield of two tonnes per hectare yield which is very uneconomical. Many private companies of seed initiated the import of hybrids from China. Due to the unavailability of an indigenous hybrid seed production system, hybrid seed is imported every year and overall, three thousand to four thousand metric tonnes of hybrid seed of rice is imported in Pakistan. Hybrids are not only useful due to its high yield but also increase the rural employment opportunities through the private sector's hybrid seed industries.

Nearly all hybrids that were imported from China to Pakistan have six to eight tonnes per hectare average yield and these hybrids are mostly prone to diseases, insect-pests, a high percentage of bursting, low recovery of head rice, low grain quality, and intolerant to high-temperature stress. Punjab is the main region of basmati rice production; however, hybrid rice got penetration in certain areas of southern Punjab like Sadiqabad, Multan, Rahim Yar Khan, Bahawalpur, and Dera Ghazi Khan. Sindh is the major region of hybrid production where coarse rice is produced. In Balochistan, it is cultivated in three districts i.e., Nasirabad, Jafferabad, and Usta Muhammad (Hina et al., 2019).

In Pakistan, three CMS lines were developed by Rice Research Institute Kala Shah Kaku (RRI KSK) Lahore, Pakistan. These CMS lines were developed in basmati rice. They have also maintained and evaluated nineteen exotic CMS lines of coarse rice (Akhter & Haider, 2020). All these lines of CMS belong to wild abortive (WA) source of cytoplasmic male sterility and the CMS line outcrossing rate is very low for achieving high-quality heterotic combinations that are ranging from 15-46 percent outcrossing. Some parental lines have been developed by RRI KSK that are resilient to BLB, submergence, and salinity tolerance through marker-assisted breeding by integrating resistant genes in parental lines (Akhter et al., 2007b; Akhter & Haider, 2020). Two rice hybrids have been developed by RRI, coarse type *KSK118H*, and basmati type *KSK111H*. These hybrids have been sent to NURYT during 2008 for the assessment of yield and adaptability at the different agro-climatic conditions. The KSK111H hybrid shows 26% increases in yield compared to check variety in NURYT trials. Regardless of the success in the development of the Basmati hybrid by RRI, there are still few restraints in the commercialization of these hybrids. First, the seed production of these hybrids is unachievable because of the availability of area for barrier isolation. Moreover, these hybrids also required favorable climatic conditions for seed production like high humidity and a specific temperature range which is difficult to attain (Akhter et al., 2017; Akhter and Haider, 2020). During November 2020 Variety Evaluation Committee (*VEC*) of Pakistan Agricultural Research Council, Pakistan recommended two hybrids of the private sector in the VEC meeting for general cultivation in Sindh Province.

During the period of 2012 in Pakistan, the cultivated area of hybrid rice was 202-347 hectares and the average yield per hectare recorded was 2.387 metric tonnes per hectare. Total rice-growing areas in Punjab is 1.76 million hectares out of which 3% area is under hybrid rice cultivation similarly in Sindh total rice-growing areas is 2 million acres out of which 55% area is under hybrid rice cultivation (Shaheen et al., 2017). Coarse rice hybrids have yield potential up to 15% or even 30% (Virmani et al., 2003), while in Basmati rice hybrid 26 % more yield potential have been recorded than conventional varieties (Akhter & Haider, 2020).

Possibilities of hybrid system development

Commercially there are two systems of hybrid rice development three-line hybrid system and a two-line hybrid system. The three-line system has stable male sterility, limited germplasm source, and requires an extra step for seed production. This process of development of hybrid is time consuming whereas, in a two-line system, it does not need a maintainer line, the chance of development of desirable heterotic hybrids increased in this system, multiple and diverse germplasm is available as parents (Rout et al., 2020). For commercial production purposes by changing the growing location of plants, the fertility of photo and thermo sensitive genic male sterility lines in a two-line system is switched on and off for self-propagation and seed production of hybrids. The two-line system is more efficient in the exploitation of germplasm and easier to handle. To produce a good grain quality hybrid with higher yield as compared to the three-line system (Wang, 2015).

Hybrid lines development mechanisms and resources

For hybrid seed production two types of male sterility are used: three-line and two-line systems (Shakiba & Scott, 2019). According to Professor Yuan, three breeding methodologies are used for the development of hybrid rice i.e., three-line method (CMS), two-line method (PTGMS), and one-line method (Apomixis). Furthermore, there are three ways for exploiting the degree of heterosis, which include inter-varietal hybrids, inter-sub specific hybrids, and interspecific hybrids (Wang et al., 2015). In hybrid rice breeding male sterility and fertility restoration systems are used. Male sterility is a condition in which plants are unable to produce viable pollen and male reproductive systems fail to produce viable pollen in anthers, but the female reproductive portion remains viable to produce ovary and egg cells. This condition favors outcrossing in rice that is a key tool to exploit heterosis breeding. Male sterility in rice is controlled either by mitochondrial genes or nuclear genes or in a combination of both phenomena. Male sterility is either sporophytic or gametophytic. Sporophytic male sterility is governed by genes located at sporogenous tissues that cause abnormality in meiocytes and tapetal tissues as result pollens are aborted. The male sterility due to gametophyte affects microspore and pollen development and causes pollen abortion (Rout et al., 2020). Sporophytic male sterility is useful and frequently used for hybrid development in rice.

Three-line hybrid system

Commercial production of hybrid seed is possible via male sterile lines and these lines are used as female parents. In this system three lines viz, A, B, and R are used for hybrid seed production. The CMS A-line is used as a female parent, the R-line which is a restorer line used as male parent whereas B-line is used as a maintainer in the three-line system. B-line is isogenic to A-line with the differences in male sterility and fertility. The male sterility in this system is governed by cytoplasmic genes or the interaction of mitochondrial and nuclear genes (Amist & Singh, 2020). The cytoplasmic male sterility system (CMS) is the practical and most effective way of exploiting heterosis in rice (Li et al., 2007; Huang et al., 2014). Globally this method for the development and commercialization of hybrid is more popular in almost all crops (Singh & Kumar, 2004; Li et al., 2007; Fujii & Toriyama, 2009). Globally most of the hybrids in rice are developed by using the three-line system (Li and Xin, 2000; Li et al., 2009).

Several CMS sources in different genotypes of rice have been discovered and identified from East and Southeast Asia (Table 1). Almost 11 sources of cytoplasmic male sterility are reported that are exploited commercially for hybrid development, these sources include CMS-WA CMS-HL, CMS-DA, CMP-IP, CMS *O. perennis* CMS-GAM, CMS-KR, CMS-BT, CMS-ARC, and CMS-TN (Shakiba & Scott, 2019). The CMS-WA was identified in *Oryza rufipogon* and to produce CMS-WA lines having the wild rice cytoplasm backcrossed with the Indica rice (Luo et al., 2013). The nuclear donor in CMS-wild abortive was "Zhen Shan 97" and sterility source from weedy rice with non-viable abortive type pollen (Luo et al., 2013). Whereas, in CMS-DA the nuclear source was from the "Xue Qin Zhao" line and the sterility source was from the dwarf *Oryza sativa f. spontanea*. The cytoplasm was from Indonesian rice in CMS-IP whereas the nuclear donor was II-32 line of rice. "*Dissi type*" was the cytoplasm source and the "297" rice line was the nuclear gene source in CMS-DI. The cytoplasmic source was *Oryza sativa f. spontanea* and "Lian-Tang-Zao" was a nuclear source in CMS-HL. The cytoplasmic sterility source from "Chinsurah Boro II" and the nuclear gene source was "Taichung 65" in CMS-BT (Shakiba & Scott, 2019).

Table 1 Sources of cytoplasmic male sterility in rice

Cytoplasmic male sterile sources	Associate open reading frame	Cytoplasm source	Reference
BT-CMS (Gametophytic)	*B-atp6-orf79*	Chinsurah Boro II/Taichong 65	Wang et al. (2006)
LD-CMS (Gametophytic)	unknown	Lead Rice (variety; *Burmese indica*) x Fujisaka 5 (japonica type variety)	Rout et al. (2020)
Dian1-CMS (Gametophytic)	unknown	Landrace rice (indica) of Yunnan high altitude cytoplasm	Huang et al. (2014)
HonglianL-CMS (Gametophytic)	*atp6-orfH79*	Wld rice (red-awned) (*Oryza rufipogon*) cytoplasm	Wang et al. (2013)
Chinese Wild rice (CW-CMS)	*orf307*	*Oryza rufipogon* Griff.	Fujii et al. (2010)
Wild Abortive-CMS (Sporophytic)	*rpl5-WA352*	Wild abortive rice (*Oryza rufipogon*) cytoplasm	Tang et al. (2017)
Kalinga-I-CMS (Sporophytic)	unknown	Kalinga-I (*indica*) cytoplasm	Rout et al. (2020)
D-CMS (Sporophytic)	unknown	*Indica* rice Dissi D52/37	Rout et al. (2020)
CMS-RT102 (Sporophytic)	*rpl5-orf352*	*Oryza rufipogon*, W1125	Rout et al. (2020)
GA-CMS (Sporophytic)	unknown	Gambiaca (*indica*) cytoplasm	Rout et al. (2020)

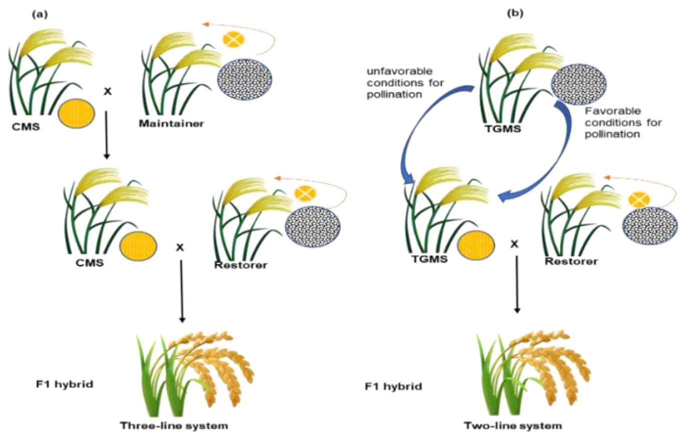

Fig. 1 Three-line hybrid system in rice includes cytoplasmic male sterile line named A-line, fertility restorer B-line and fertility maintainer R-line (a), two-line hybrid system that includes thermo-sensitive male sterile line fertility restorer line.

Two-line hybrid system

The second method of heterosis breeding is a two-line system. Two techniques used in this system include chemical emasculation and thermo and photosensitive genic male sterility system (Virmani, 1999; Virmani & Ahmed, 2001). During 1970 China started the use of the chemical emasculation methods to produce rice hybrids. Photoperiod sensitive genic male sterile (PGMS) lines are sterile and with daylight fluctuation regain fertility, whereas TGMS lines regain fertility when temperature fluctuates (Fan & Zhang, 2018). The major advantage of the two-line system is that this system does not require a maintainer line, any fertile line can be used as a pollen parent in this system and this trait makes it flexible to produce hybrids on large scale (Virmani et al., 2003; Singh et al., 2013; Nowinszky et al., 2018). The two-line system is genetically controlled by major nuclear recessive genes. Thermosensitive male sterile lines would be male sterile that carries *tms* genes when exposed today/night temperature ranging about 32℃/24 ºC and become male fertile at temperature 24 ºC/18 ºC. The photosensitive genetic male sterile line would be male sterile when the day length exceeds 13.45h. At International Rice Research Institute (IRRI), Ali et al. (1995); Sanchez and Virmani (2005) identified several TGMS lines. They developed the TGMS line by pyramiding the *tms* gene.

Another horizon according to the Chinese is one-line system, apomixis which is the asexual reproduction of plants reported in approximately 4000 plant species, in which seeds are formed without the fusion of male gamete with female gametes (Grossniklaus et al., 2001). Seeds cloned from a single-parent plant (Wang, 2020). Apomixes does not exist in rice but is reported in pearl millet. Scientists have been hopeful to transfer this gene from pearl millet to rice for apomixes (Fiaz et al., 2020). Genetic engineering is another way of inducing sterility in rice.

Male sterility induced in rice by genome editing

Another cutting-edge technology known as genome editing by targeted mutagenesis is also used in genome editing of rice and other major crops. In rice hybrid development *CRISPR/Cas9* genome editing technology has been used for development of the TGMS line by mutating *tms5* gene. For targeted mutagenesis 10 sites have been targeted in the exon region of *tms5* gene and the tms5ab construct has been established. This system shortened the sterile line development but also aids in hybrid vigor exploitation (Zhou et al., 2016). Barman et al. (2019) also reported an experiment for the generation of TGMS line for a two-line hybrid system in rice by targeting the *tms5* gene by using *CRISPR/Cas9* in Indica rice cultivar named YK17 that is widely cultivated in China. They generated two mutants *tms5-1* and *tms5-2* and reported that both mutants can produce seed at optimum temperature 22 ºC but when cultivated in areas having temperature 24℃ and 26 ºC their pollens were aborted, and no seed was set. A mitochondrial gene (*orf79*) associated with cytoplasmic male sterility has been knocked out by using TALENs technology and demonstrated that this gene is strongly responsible for male sterility in rice (Kazama et al., 2019).

Genetics and molecular markers of fertility restorer genes

The restorer line R carries the *Rf* gene that is responsible for fertility restoration (Virmani et al., 2003). The cross between the CMS nuclear gene and *Rf* gene can be categorized into two groups such as sporophytic which includes CMS-WA or gametophytic which includes CMS-BT (Shakiba & Scott, 2019). Restorer genes which are used mostly for commercial production of hybrid rice were identified as *Rf1*, *Rf3*, *Rf4* located on chromosome 1 and 10 (Chen et al., 2013; Katara et al., 2017). So far, almost 10 fertility restorer genes have been identified as mentioned in the Table 2. Seven out of 10 have been characterized and are found to be dominant in nature. *Rf17* restores fertility in the Chinese wild type male sterility system in heterozygous conditions (Rout et al., 2020).
Chen et al. (2013) mentioned a marker system named In Del *Rf1a* for the selection of restores genes in Japonica rice with BT-type CMS. They identified 574 bp In Del (Insertion/Deletion) of *Rf1a* locus and their results depicted no deletion of 574 bp in Indica rice and it could restore fertility in BT-type CMS with gene *Rf1a*. This gene is located on chromosome number 10 in the rice genome. Itabashi et al. (2011) cloned *Rf2* gene of fertility restoration in lead type rice and demonstrated that this gene encodes 152 amino acid-based protein that is rich in glycine domain. They detected mRNA expression of *Rf2* in anthers and concluded that the *Rf2* fertility restorer gene is not responsible to produce pentatricopeptide proteins unlike other fertility restorer (*Rf*) genes. They identified locus LOC-S02g173801 on chromosome number 2 on the rice genome and developed CAPs 42-1 marker for the detection of these genes.

Fertility restorer genes like *Rf3* and *Rf4* have been identified by Katara et al. (2017) in Indica rice by using markers system DRRM *Rf3-10* and Rm6100, respectively. These genes are located on chromosome number 1 and 10 and genotypes with double dominant genes have more fertility restoration ability than a single dominant gene. The genotypes identified by using this system can be utilized directly in hybrid breeding. The fertility restorer genes *Rf1* and *Rf4* respond to BT-CMS and wild abortive-CMS systems, respectively (Liu et al., 2004). Fertility locus (*Rf)* in Honglian type cytoplasmic male sterility (HL-CMS) that is different from *Rf1* and *Rf4* loci (Liu et al., 2004). They constructed fine mapping of BCF_1 population by using RAPD and microsatellite markers i.e., RM3150, RM1108, RM5373, and thus concluded that these markers amplified one locus *Rf5* that flanked at a distance of 0.9 cM and 1.3 cM, respectively with these markers. Another loci named *Rf6(t)* co-segregates with markers i.e., RM5373 that is flanked with SBD07 and RM6737 at 0.4cM genetic distance. These fertility gens (*Rf5* and *Rf6t*) are located on chromosome number 10. The *Rf5* gene also restore fertility in Boro II type of CMS lines in *japonica* type rice and partially restore fertility in Honglian-type CMS lines (Zhang et al., 2016)

Fertility restorer genes *Rf98* encodes pentatricopeptide repeats (PPR) in genotypes that have RT98-type of male sterility (Igarashi et al., 2016). In this line, male cytoplasm was recovered from Chinese wild rice *Oryza rufipogon* and nuclear genes from *Oryza sativa* L. By constructing a fine mapping and 170Kb region has been revealed that encoded seven PPR that is a candidate for *Rf98* gene. Out of seven genes, PPR762 is responsible for fertility restoration in RT98-type male sterility (Igarashi et al., 2016). The fertility restorer gene *Rf17* identified using a cleaved amplified polymorphic sequence marker and SSR marker located on chromosome number 4 at a 0.6 cM genetic distance from the near SSR region (Fujii & Toriyama, 2005). It has been confirmed restoration of fertility governed by single gene while characterizing CW-type of cytoplasmic male sterility in BC_1F_1 population and making a cross between W1-R and a maintainer line (Fujii & Toriyama, 2005). By using next generation pyrosequencing, whole genome sequence of the mitochondrial genome of CMS line RT102A and restorer line RT102C confirmed the candidate gene *orf* is responsible for fertility restoration in RT102-type of CMS system (Okazaki et al., 2013). The fertility restorer loci *orf*352co-transcribed *rpl4* ribosomal protein.

Several studies have been attempted for the identification and characterization of thermosensitive male sterile genes (*tms*). So far, 13 *tms* genes have been identified, some of them have been mentioned in Table 3. Wang et al. (1995) identified the *tms1* gene in line 5460S with the RZ562-RG978 marker system on chromosome number 8, Lopez et al. (2003) identified and characterized the *tms2* gene in Norin PL12 rice genotype by utilizing RM11-RM2 markers. The locus *tms2* resides on chromosome number 7. Reddy et al. (2000) reported the *tms4* gene on chromosome number 9 in genotypes SA2 by using marker RM257. Jia et al. (2000) reported *tms5* thermosensitive male sterility gene is located at chromosome number 2 in the genotype Annong S-1. They identified this gene by using the RM174 marker system. Lee et al. (2005) utilized the RM3351 marker system and reported the *tms6* gene in Indica rice line Sokcho-MS that resided on chromosome number 2. Hussain et al. (2012) identified *Tms8* by using the RM21, RM224 marker system in F6, and this gene is located on chromosome number 11. Sheng et al. (2013) reported *tms9* in Zhu1S by using Indel 37, Indel 57 marker system and this gene resides on chromosome number 2. Peng et al. (2010) identified and characterized the *tmsX* gene in the XianS rice genotype that is located on chromosome number 2 in the rice genome and they utilized RMAN81, RMX21 marker systems for the identification of this gene. Studies of these genes and marker systems provide useful information and can be utilized for screening of local rice germplasm for the setup of indigenous hybrid development. Up till now, no such genetic information about these is available for local rice germplasm.

Table 2 Molecular markers associated with fertility restorer genes in rice

Fertility restorer genes	Location on chromosome no.	Marker	Gene	Reference
Rf1a, Rf1b	10	*InDel Rf1a*	PPR791, PPR8-1 Rf1B	Akagi et al. (2004); Chen et al. (2013)
Rf2	2	CAPs42-1	LOC-S02g173801	Itabashi et al. (2011)
Rf3	1	Rf3–10-DRRM	---	Katara et al. (2017)
Rf4	10	RM6100	PPR782a	Katara et al. (2017)
Rf5(t)	10	RM3150	PPR791	Liu et al. (2004)
Rf6	10 & 8	RM5373	----	Liu et al. (2004)
Rf17	4	SNP 7–1, AT10.5–1	PPR2	Fujii & Toriyama (2005)
Rf98	10	UK	PPR	Igarashi (2016)
Rf102	12	UK	UK	Okazaki et al. (2013)

Table 3 Genetics and molecular markers associated with thermosensitive genetic male sterility genes in rice

Gene	Source	Chromosome no.	Markers	Reference
tms-1	5460S	8	RZ562-RG978	Wang et al. (1995)
tms-2	PL12-Norin	7	RM11-RM2	Lopez et al. (2003)
tms-3	IR32364S	6	OPAC3640-OPAA7550	Subudhi et al. (1997)
tms-4	SA2	9	RM257	Reddy et al. (2000)
tms-5	Annong S-1	2	RM174	Jia et al. (2000)
tms-6	Sokcho-MS	2	RM3351	Lee et al. (2005)
tms-8	F61	11	RM21, RM224	Hussain et al. (2012)
tms-9	Zhu1S	2	Indel 37, Indel 57	Sheng et al. (2013)

Breeding methodology in hybrid rice production

A breeding method which is normally used for hybrid parental lines is the pedigree selection. The conventional breeding method which is utilized for the development of parental lines is the single seed descent (SSD) method and for identification of desired progenies/ desirable plants, marker-assisted selection (MAS) method used. Other methods like top-crosses, back cross, single cross, test cross, three-way cross and combining ability test can be utilized for the improvement purpose of maintainer, which is B line, restorer which is A-line, CMS and EGMS (Shakiba & Scott, 2019). For male-sterile plant identification pollen staining is the process and during this process pollens collected from the floral parts of the plant are observed under the microscope. Pollen that appears translucent is sterile whereas the pollen which appears dark in color is fertile. Based on the percentage of fertility and sterility the florals are divided into five groups: a full sterile group with 100% sterility, a sterile group with 90 to 99% sterility, a partial sterile with 70 to 89 % sterility whereas the sterile group contains 31 to 70% sterility; a fertile with 21 to 30% and a fully fertile 0 to 20% (Shakiba & Scott, 2019).

Seed production in CMS lines in two-line and three-line systems

In a two-line system, CMS seed production is possible in two ways, first one via ratooning the desired male-sterile plants under optimum environmental conditions and the second method is for large scale mass production of male-sterile seeds in this method sterile plant seeds planted in such a way that the plants at reproductive stage meet the required photoperiod or temperature (Virmani, 2003).

Whereas in a three-line system, the production of CMS seed is possible by crossing the male sterile line (female parent) with its maintainer line (B) which is used as the male parent. The abortion of pollen in CMS line is because of interaction among the mitochondrial DNA which carries sterility factor (S) and CMS nucleus which carries *rfrf* homozygous recessive allele. Despite recessive alleles of restorers in the nucleus of the B-line and carrying the homozygous *rf* gene, mitochondrial is normal and consequently maintains fertility and via self-pollination of the B-line (Shakiba & Scott, 2019). Excellent crop management is vital for growing a hybrid seed of rice whereas the agronomic management practices for hybrid development is not similar to those for inbred varieties. The production of hybrid seed of rice involves three lines in CMS system, which are A-line, B-line and R-line and it is used for the hybrid rice seed production, and it involves three steps i.e., multiplication of A-line and B into R-line and hybrid seed production through the cross of A-line into R-line (Sah & Joshi, 2020).

High physical and genetic purity is a prerequisite to produce the best quality F_1 seed (Singh et al., 2013). The impurity in parental lines causes dissimilarity and variation time (period), plant height, plant type, and grain size which leads eventually to affect the quality of F_1 hybrids. The seed production should be carried out with the balance use of fertilizers, IPM, and in the area where irrigation is available easily. The production practice of seed which is used commercially worldwide is described below (Singh et al., 2013).

Prerequisites for hybrid seed production

The site required for hybrid development must have a daily mean temperature ranging from 24 ºC to 30 ^{o}C with relative humidity (RH) of 70 to 80%. Changes in day and night temperature should be eight to ten degrees Celsius with adequate sunlight and reasonable wind velocity. There should not be rain persistently for three days during of flowering stage. Heat (temperature) above 35 ºC and below 20 ºC will have adverse effects on seed yield (Singh et al., 2013). Almost all rice-growing areas of Punjab province are suitable for three-line hybrid seed production. For a two-line hybrid seed production system, there are certain limitations for sight selection and climatic conditions. Male sterile plants in the TGMS line can only be obtained in high-temperature conditions where the temperature exceeds 35 ºC to 40 ºC. Even self-seed production in TGMS can be obtained in cooler regions. Pakistan has favorable climatic zones for a two-line hybrid system of rice like Swat is suitable for TGMS line maintenance and seed production. Jacobabad area of Sindh Province and Jaffarabad area of Balochistan province are the best option for hybrid seed production under two-line hybrid rice production.

A field for hybrid seed development must be isolated as the pollen of rice with air can be transported to longer distances. Normally space isolation of seed plots to other varieties of rice is 100 m whereas for A-line multiplication it is better to ensure an isolation distance of 200 m however isolation distance of 3-5m is enough for R and B-line multiplication in varieties. If space separation (isolation) is not achievable then maintain a time separation of about 21- 30 days (between flowering stage). Seeding interval is the growth duration difference among the two parental lines. According to the difference between parental lines in relation to days to 50 % flowering, the one with a longer duration should be planted early.

Transplanting is commenced when seedlings attain the age of 21-35 days, which make sure timely flowering and heading of parental lines. To get synchronization at flowering, parental lines with long duration should be transplanted first. Transplant the parental lines in such a manner that A line has 10 days longer or 10 days' shorter growth duration than R-line (Singh et al., 2013). Transplanting of R-line seedlings must be in paired rows, transplant single plantlets per hill with a plant-to-plant distance of fifteen centimeter and row to row distance of thirty centimeters. Six rows of A-line seedlings transplant between the paired rows of R-line. 1-2 plantlets per hill or hole are transplanted with 15 into 15 cm leaving a space of 23 cm between the rows of A-line and R-line.

Parental lines (R and A-lines) planted in row ratio of 2: 8, 2: 10, and 3: 10 etc. The distance between the rows of the R line should be 30 cm whereas the distance between the rows of A line should be 15 cm. Space between the blocks of A-line and R-line should be 20 to 30cm whereas space between hills must be 15 cm. The direction of rows should be perpendicular to the wind for easy dispersal of pollen on the seeding parent (Singh et al., 2013). To attain the desired synchronization in flowering we can delay flowering in early developing parents and apply the quick releasing nitrogen fertilizers in early growth development stages of the panicle. Similarly, to enhance the flowering by 2 to 3 days, spray the (1%) phosphate solution on the later flowering parent.

Leaf-cutting of both parental lines A and R is useful to improve the out pollination and setting of seed. Erect and long Flag leaves that are erect and long may obstruct (block) the pollen dispersion of R into A and influence the rate of outcrossing. Female parents with wild abortive cytoplasm have incomplete or poor panicle initiation. GA_3 is used to obtain good panicle exertion. It not only exerts the panicle but also improves the stigma exertion, stigma receptivity, increases the opening duration of floret, and widens the angle of the flag leaf. The use of GA_3 not only increases the plant height by 10 to 15 cm but also adjusts the plant height of the R and A-line (Singh et al., 2013).

Conclusion

One of the strongest tools to break the yield barriers in rice is hybrid rice, it can escape drought due to its shorter duration and fits well in the cropping system. In Pakistan, hybrid rice is produced through three-line system A and R-lines exotic lines by a few foreign companies but an indigenous setup for hybrid seed rice production is not available. Demand of hybrid rice is very high and every year almost 500-ton to 700-ton hybrid rice is imported that drains huge foreign exchange. There is a need to import novel genetic material from China and IRRI to strengthen indigenous hybrid rice breeding. In this article, plenty of novel resources of CMS and *Rf* genes marker system have been highlighted that can be utilized for the identification and characterization of indigenous rice germplasm. By developing the hybrids which meet the specific quality requirements, the rice area, production, and productivity will be increased.

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