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Novel male sterile line development through marker-assisted backcross breeding suitable for aerobic planting in rice

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Abstract

Aim : Availability of suitable hybrids for drought or cultivation under aerobic situation is scarce in rice. Therefore, there is a dire need for developing of potential male sterile line for the production of promising hybrids. In this way, present study conducted was development of male sterile line suitable for aerobic rice hybrids in limited generations with the use of marker assisted backcrossing.

Methodology : The present study was conducted using strategy of marker assisted background selection for the development of new malesterile line in rice. A male sterile line KCMS31A was used as a donor parent for male sterility and a promising genotype MAS99 (early maturing and suitable for aerobic condition) as a recurrent parent. Backcrossing was carried out from 2013 to 2014. Phenotypic foreground selection was carried out in each generation by pollen fertility test. Based on parental polymorphism survey, 96 polymorphic SSR markers were identified and utilised for the marker assisted background selection in BC_3F_1 generation. Further, BC_4F_1 plants were evaluated for morphological characters.

Results : Analysis of data showed that all the plants were totally male sterile and the plants coded as BC_3F_1 -5-7 (96.88%), BC_3F_1 -5-10 (96.88%), BC_3F_1 -5-15 (96.88%) and BC_3F_1 -5-17 (95.83%) were found to be recovered maximum recurrent parent genome and they were advanced to BC_4F_1 . Evaluation of new BC_4F_1 male sterile line pertaining to morphological traits revealed that the trait means were on par with the original fertile counterpart MAS 99 B line.

Interpretation : The results confirmed the supremacy of marker assisted selection in backcross breeding aimed at the recovery of recurrent parent genome as quickly as possible and this is the first effort made by authors to develop male sterile line fit to utilise in hybrid rice programme under aerobic condition.

Introduction

Cytoplasmic genetic male sterility results due to interaction of nuclear genes and mitochondrial genes (Maureen and Stephane, 2004). The role of cytoplasm in causing male sterility in rice was first reported in 1954 (Sampath and Mohantu, 1954). Later, Katsuo and Mizushima (1958) also observed a similar phenomenon in the progeny of first backcross of *Oryza sativa* F. *spontanea* / *Oryzasativa* c. *eujisakos*. Since, rice is a self-pollinated crop, crossing work involves tedious hand emasculation and pollination, hence, the availability of suitable male sterile lines would render hybridization process easier (Hittalmani and Shivashanker, 1987). Among different male sterile sources, wild abortive male sterility is extensively used male sterile source in hybrid rice seed industry, i.e, approximately 90% of hybrids (Rajand Virmani, 1987; Yao *et al.*, 1997; Zhang *et al.*, 2002). But till now male sterility source has not been diversified for aerobic condition. Aerobic rice is considered as promising rice cultivation system as water scarcity is increasing in the world (Kumar *et al.*, 2015). Compared with lowland rice, water requirement in aerobic rice is reduced by more than 50%, and water productivity increases by 60% (Nareshbabu *et al.*, 2010; Gandhi *et al.*, 2011). Hittalmani and Shivashanker (1987) demonstrated that rice can be cultivated in dry sowing (aerobic) while attempting crosses using local rice of Karnataka. So there is a dire need to develop male sterile line suitable for aerobic condition. It has been reported that backcross breeding is a well-known technique of transferring a target trait from donor line in to the genetic background of a recipient line. However, for recovery of entire recurrent parent genome, it needs repeated backcrosses (up to 7 times). This process is time consuming and the rate of recovery of the recurrent parent genome based on phenotypic selection is not efficient (Young and Tanksley, 1989; Neeraja *et al.*, 2006; Liu *et al.*, 2006; Semgan *et al.*, 2006). Hence, an alternative for this is to go for marker assisted backcrossing to retrieve the recurrent parent genome in limited generations. The efficiency of selection significantly upturned by the usage of molecular markers in backcross programme. This strategy is rapid and very effective compared to phenotypic selection (Hospital, 2009). Markers can be used in combination with or to replace phenotypic screening for the target background which is referred as 'background selection' (Tanksley *et al.*, 1989; Hospital and Charcosset, 1997; Bouchez *et al.*, 2002). A minimum of two backcrosses are required in case of very short distances between molecular marker and the gene of interest (Hospital, 2001). Whereas, Bai *et al.* (2006) showed that only three backcrosses were enough to recover 98% of recipient parent genome. The success of this approach depends on the strategy of selection, marker density, size of population, recurrent parent genome and design of experiment (Frisch and Melchinger, 2005). More recently in Vietnam, QTL *SUB1* has been introgressed using MABB approach into an elite rice variety (Luu *et al.*, 2012). Limited availability and low variation of male sterile lines leads to genetic vulnerability. Therefore, breeders can transfer male sterility to the existing putative B lines through repeated backcrosses to diversify male sterile lines (Manivannan *et al.*, 2011).

Researchers have shown that MAS99 B line has *rfrf* genotype, and hence is a putative maintainer line for wild abortive type male sterility. Thus, it can be potentially converted to a male sterile line using marker-assisted backcross breeding (MABB) method for accelerating the recovery rate of recurrent parent genome in fewer generations. Therefore, the present study aimed to develop male sterile line in MAS99 background from KCMS31A as donor male sterility source in limited backcrosses, which could be further exploited for hybrid rice seed production under aerobic condition. This is the first effort to develop a malesterile line suitable for aerobic condition using MABB.

Materials and Methods

Plant materials : In the present study an elite maintainer line MAS99 B line was used as male recipient parent, which was of short duration and suitable for aerobic condition. KCMS31 male sterile line was used as female donor parent. In kharif 2012, hybridization was affected between these two and 43F₁ plants were developed. First backcross was affected in summer 2013 and established 58 BC₁F₁ plants, selected plants which were synchronised in flowering with MAS99 were backcrossed subsequently to establish 32 and 45 plants in BC₂F₁ and BC₃F₁ respectively. Phenotypic foreground selection in each generations using pollen fertility test was carried out. In BC₃F₁ marker assisted background selection was carried out to obtain BC₄F₁. The resultant BC₄F₁ male sterile plants were evaluated along with parents for morphological traits according to standard evaluation system for rice (1996) under aerobic condition.

Aerobic rice cultivation : Under aerobic cultivation method, seeds were sown directly in unpuddled land unlike in wetland rice where rice was transplanted after growing in nursery. Standard spacing of 30 cm between rows and 25 cm between plants were maintained. The soil moisture was maintained at field capacity during entire crop growth period by irrigating at 5-6 days interval. The aeration of soil was maintained in contrast to anaerobic condition in case of puddled transplanted rice. Further, during reproductive stage irrigation was increased once in 3 days.

Sampling and DNA extraction : Young vigorously grown fresh leaf samples from KCMS31A×MAS99 cross derived BC₃F₁ progenies were collected from 25-day-old seedlings to extract genomic DNA of 45 individuals. The modified cetyltrimethyl ammonium bromide method was adapted to obtain DNA of individuals (Cao and Oard, 1997).

Marker assisted background selection : Parental polymorphism marker survey was initially conducted to identify polymorphic markers between the parents using 412 SSR markers that covered all 12 rice chromosomes. 96 polymorphic SSR markers on an average of 8 markers per chromosome were used for amplification of BC₃F₁ progenies along with parents. Scoring of BC₃F₁ genotypes was done based on recurrent and donor parent banding pattern, if the bands were like donor parent (KCMS31A)

then individuals were scored as 'A'. Similarly, if the bands were like recurrent parent (MAS99) then it was scored as 'B'. Since diploids have 2 alleles, we considered number of allele as twice the number of markers used, therefore for the present study total number of alleles became 192. Similarly, twice the number of A and B alleles were considered in calculation of recurrent parent genome recovery symbolically given below.

Recurrent parent genome recovery (%)

$$\frac{\text{Number of recurrent parent alleles (B)} + \frac{1}{2} \text{ of heterozygote alleles (H)}}{\text{Total number of marker alleles checked (A\&B)}} \times 100$$

Further, for pictorial visualisation of parental segments in BC_3F_1 progenies, resultant genotypic data was subjected to graphical genotypic analysis using GGT v.2.0 software package (Berloo, 2008).

Evaluation of new male sterile line : The performance of new male sterile line was evaluated along with parents KCMS31A and MAS 99 under field condition in two replications for morphological traits at BC_4F_1 generation. Observations were documented on sterility of pollen(%) using 2% acetocarmine dye, days to 50% flowering, plant height at the time of maturity (cm), days to maturity, length of panicle, number of spikelet's/panicle, spikelet sterility (%). An average of ten plants of new male sterile lines and parents observations were considered for comparison using 't'-test with the help of SPSS 16.0 software.

Results and Discussion

The inefficiency of selection based on phenotype has been noticed in previous studies emphasizing the limitations associated with traditional backcross breeding for complete recovery of recurrent parent genome (Yi *et al.*, 2009). With the advent of molecular markers gene mapping was achieved, which helped to identify molecular markers linked to gene of interest. Those markers could be used in marker assisted selection. Among different approaches, marker assisted backcross breeding (MABB) is one which could be exploited for transfer of specific genomic regions in to recipient parent background with high efficiency (Kumar and Hittalmani, 2000; Jena and Mackill, 2008).

The observations of pollen fertility test revealed complete male sterility of F_1 plants derived from KCMS31 x MAS 99 cross, indicated that MAS99 behaved as maintainer genotype. This paved the way for us to convert maintainer MAS 99 into new male sterile line (Fig.1). Subsequently, all the back cross derived progenies were also found to be completely male sterile (100%) even after three consecutive backcross with recurrent parent MAS99. In each generation, stringent phenotypic selection followed with a foremost attention on sterility and flowering synchronization with MAS99 genotype for the selection of plants. The observed stable male sterility even after several backcrosses confirmed that interaction of

Fig.1 : Marker assisted backcross breeding strategy followed to develop new male sterile line

Table 1 : Details of polymorphism survey of SSR markers on parents of BC_3F_1 progenies derived from KCMS31A x MAS99 cross in rice

Chromosome number	Total SSR markers screened	No. of polymorphic SSR markers	Percent polymorphism
1	75	15	20.00
2	35	8	22.86
3	38	6	15.79
4	30	12	40.00
5	43	10	23.26
6	35	7	20.00
7	33	8	24.24
8	42	4	9.52
9	46	8	17.39
10	28	4	14.29
11	36	3	8.33
12	42	11	26.19
Total	483	96	Average = 20.16

cytoplasm with new nucleus in such a manner would caused complete male sterility *i.e.*, without any adverse background effect, incomplete penetrance or variable expressivity etc. Because, in presence of these conditions, there will be a existence

Fig. 2 : A sample of electrophoresis banding pattern of RM471 and RM437 SSR markers showing amplification of 45 BC₃F₁s of cross KCMS31A (Donor) × MAS99 (Recurrent)

of partial fertile /sterile plants to some extent, similarly presence of complete male sterility was reported by Ahmadikha *et al.* (2015).

The parental DNA polymorphism is a pre requisite for any marker assisted selection. Hence, in this study a total of 412 SSR markers covering all 12 rice chromosomes were used to identify DNA polymorphic markers between two parents. The results showed that among 412 SSR markers, 96 were polymorphic (Table 1) indicating the presence of genetic variability at different locus on chromosome between parental materials. These polymorphic markers help us to determine the parent of origin of DNA segments. On an average, the percent polymorphic markers on parental survey was approximately 20.16 % with highest polymorphism in chromosome 4 (40%) and lowest polymorphism in chromosome 11 (8%). Further, these 96 polymorphic SSR markers were used for background selection to check the proportion of

recurrent parent genome recovery. Similarly, Hien *et al.* (2012) observed average polymorphism of 24% for salt tolerance between the parents and Ahmadikhah *et al.* (2015) reported 34% of polymorphism between donor and recurrent parent.

The marker assisted background selection has been advised for rapid recovery of recurrent parent genome in early generations of backcross population (Chen *et al.*, 2001; Joseph *et al.*, 2004). In the present study, an average of eight SSR markers per chromosome in background selection in the present study (Fig.2; Fig.3). In BC₃F₁ progenies derived from KCMS31A × MAS99 cross the observed proportion of recurrent parent genome using SSR marker data ranged from 87.50 to 96.88 %. Out of 45 plants analysed, 16 were found to be recovered more than or equal to expected recovery percentage of recurrent parent genome (MAS99). The highest recovery

Fig. 3 : A sample of electrophoresis banding pattern of RM11 and RM1370 SSR markers showing amplification of 45 BC₃F₁s of cross KCMS31A (Donor) × MAS99 (Recurrent)

was by plants BC₃F₁-10, BC₃F₁-15 and BC₃F₁-7 (96.88%), whereas the lowest recovery of 87.50% was recorded by the 40th individual (Table 2).

The plants BC₃F₁-5-7(96.88%), BC₃F₁-5-10 (96.88%), BC₃F₁-5-15(96.88%), BC₃F₁-5-17 (95.83%), BC₃F₁-5-24 (95.83%) and BC₃F₁-5-42 (95.83%) were superior in genome recovery recurrent parent (Table 2), hence they were forwarded to BC₄F₁. Whereas, 64.4% of the plants *i.e.*, 29 plants recovered less than average recovery (93.75%). Thus, by using markers these plants were eliminated otherwise by conventional way we would have selected plants which recovered lesser 'MAS99' genome. Further, we may have to go for more number of backcrosses. This difference in recovery percentage was due to difference in

contribution of proportion of genomic regions of parents which was consequence of variation in occurrence of recombination and presence of linkage of genes present on the chromosome. Similarly, the MABB approach was utilized for development of variety resistance to rice stripe virus resistance using 47 markers, which accounts nearly four markers per chromosome (Pan *et al.*, 2009). In the same way, forty three SSR markers (<4 markers per chromosome) were used for background selection to develop new male sterile line in rice fit to wet land condition (Ahmadikhah *et al.*, 2015). Whereas Yi *et al.* (2009) used nearly three markers per chromosome to develop rice variety with improved cooking quality. Marker density used for background selection was more than the previous studies. Further, graphical genotyping

Fig. 4 : Genomic regions of recurrent parent and donor parent visualized by Graphical genotyping of 12 linkage groups of 45 BC_3F_1 s derived from KCMS31A $\times$ MAS99 cross in rice

Fig. 5 : Comparison of fertile pollens of MAS 99B plants (I) and sterile pollens of MAS 99A plants (II) visualized by light microscope (40x) after 2% acetocarmine staining

Table 2 : Proportion of recurrent parental genome observed in BC₃F₁ progenies of cross KCMS31A × MAS99 using rice SSR markers

Plant no.	Total no. of alleles	Recurrent parent allele	Donor parent allele	Percent recurrent genome recovered (1)	Expected recurrent parent genome recovery* (2)	Difference (2-1)
BC3F1-5-1	192	178	14	92.71	93.75	-1.04
BC3F1-5-2	192	178	14	92.71	93.75	-1.04
BC3F1-5-3	192	174	18	90.63	93.75	-3.12
BC3F1-5-4	192	180	12	93.75	93.75	0.00
BC3F1-5-5	192	178	14	92.71	93.75	-1.04
BC3F1-5-6	192	180	12	93.75	93.75	0.00
BC3F1-5-7	192	186	06	96.88	93.75	3.13
BC3F1-5-8	192	179	13	93.23	93.75	-0.52
BC3F1-5-9	192	176	16	91.67	93.75	-2.08
BC3F1-5-10	192	186	06	96.88	93.75	3.13
BC3F1-5-11	192	174	18	90.63	93.75	-3.12
BC3F1-5-12	192	174	18	90.63	93.75	-3.12
BC3F1-5-13	192	178	14	92.71	93.75	-1.04
BC3F1-5-14	192	184	08	95.83	93.75	2.08
BC3F1-5-15	192	186	06	96.88	93.75	3.13
BC3F1-5-16	192	178	14	92.71	93.75	-1.04
BC3F1-5-17	192	184	08	95.83	93.75	2.08
BC3F1-5-18	192	180	12	93.75	93.75	0.00
BC3F1-5-19	192	178	14	92.71	93.75	-1.04
BC3F1-5-20	192	174	18	90.63	93.75	-3.12
BC3F1-5-21	192	176	16	91.67	93.75	-2.08
BC3F1-5-22	192	182	10	94.79	93.75	1.04
BC3F1-5-23	192	176	16	91.67	93.75	-2.08
BC3F1-5-24	192	184	08	95.83	93.75	2.08
BC3F1-5-25	192	176	16	91.67	93.75	-2.08
BC3F1-5-26	192	176	16	91.67	93.75	-2.08
BC3F1-5-27	192	178	14	92.71	93.75	-1.04
BC3F1-5-28	192	176	16	91.67	93.75	-2.08
BC3F1-5-29	192	176	16	91.67	93.75	-2.08
BC3F1-5-30	192	182	10	94.79	93.75	1.04
BC3F1-5-31	192	178	14	92.71	93.75	-1.04
BC3F1-5-32	192	180	12	93.75	93.75	0.00
BC3F1-5-33	192	176	16	91.67	93.75	-2.08
BC3F1-5-34	192	178	14	92.71	93.75	-1.04
BC3F1-5-35	192	176	16	91.67	93.75	-2.08
BC3F1-5-36	192	178	14	92.71	93.75	-1.04
BC3F1-5-37	192	172	20	89.58	93.75	-4.17
BC3F1-5-38	192	180	12	93.75	93.75	0.00
BC3F1-5-39	192	176	16	91.67	93.75	-2.08
BC3F1-5-40	192	168	24	87.50	93.75	-6.25
BC3F1-5-41	192	178	14	92.71	93.75	-1.04
BC3F1-5-42	192	184	08	95.83	93.75	2.08
BC3F1-5-43	192	180	12	93.75	93.75	0.00
BC3F1-5-44	192	176	16	91.67	93.75	-2.08
BC3F1-5-45	192	176	16	91.67	93.75	-2.08
Average				92.86	93.75	
Min.				87.50		
Max.				96.88		

*Average recurrent parent genome recovery expected in traditional backcross method

Table 3 : Comparison of parents and novel male sterile line 'MAS99A' for morphological traits

Entry	Description	No. of plants	Days to 50% flowering	Plant height at maturity (cm)	Total number of tillers	Days to maturity	Panicle length (cm)	Spikelet per panicle
KCMS31A	Donor parent	10	106.60	66.17	25.80	138.80	17.45	126.87
MAS99B	Recipient parent	10	95.17	74.50	22.80	126.60	19.04	128.83
MAS99A	Developed in this research	10	96.07	72.73	21.73	130.67	18.41	127.12
T test between 2 & 3		10	n.s	n.s	n.s	*	n.s	n.s

n.s and *, indicate non-significance and significance at 5% level of probability respectively

was carried out for the selection of BC₃F₁ individuals with maximum of 'MAS99' genome (Fig.4).

Adaption of MABS allowed us to recognise plants which recovered maximum of recurrent parent genome up to 96.88% in BC₃F₁ itself which was 3.13% more than expected average recovery, thereby new male sterile lines could be developed in just four backcrosses. Similar results were reported by Chen *et al.* (2001); Joseph *et al.* (2004) and Gopalakrishnan *et al.* (2008).

Development of new male sterile lines is a pre-requisite to step up the hybridization programme under aerobic condition. As evidenced from 't-test' analysis, the trait means of 'MAS99A line' were statistically on par with original fertile counterpart MAS99 B line besides 100% pollen and spikelet sterility (Table 3; Fig.5). Indicated that the genome of recurrent parent was completely recovered, except some negligible proportion in BC₄ itself. As use of markers helped us to select and advance superior plants in BC₃ unlike in conventional backcross breeding it will take BC₆ or BC₇ generation to recover. The results of the present study again confirmed the power of MAS in back crossing for the recovery of recurrent parent genome in limited generations. Similarly using MABB, Ahmadikhah *et al.* (2015) reported that BC₄F₁ male sterile plants were comparable to the original maintainer fertile counterpart for agronomic traits. However, Luu *et al.* (2012) successfully introgressed *SUB1* into AS996 rice variety in just four backcrosses with 100% genome recovery. Whereas, *SUB1* was introgressed to swarna rice variety in just three back crosses with complete genome recovery (Neeraja *et al.*, 2006). Similarly, Hu *et al.* (2016) demonstrated that MABB is a very efficient approach to improve BPH resistance of elite rice cultivar of South China, 'Hemeizhan' in three backcrosses.

New rice male sterile line 'MAS99A' with short duration, suitable for aerobic condition was developed in just four back crosses in the present study with the application of MABB using SSR markers. It is expected that newly developed male sterile line will be useful in development of new heterotic and short duration

rice hybrids suitable for aerobic condition. This would help in expansion of hybrid rice cultivation to those areas of world where water availability is less so that an augmented production and productivity of rice could be achieved.

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