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Allelic variation in the microsatellite marker locus RM6100 linked to fertility restoration of WA based male sterility in rice

Kapil K. Tiwari[1], Sasmita Pattnaik, Ashutosh Singh[2], Maninder Sandhu, S. V. Amitha Mithra, M. Z. Abdin[1], Ashok K. Singh[2], Trilochan Mohapatra[3]*

National Research Centre on Plant Biotechnology, New Delhi 110 012, [2]Division of Genetics, Indian Agricultural Research Institute, New Delhi 110 012, [3]Central Rice Research Institute, Cuttack, Odisha

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Abstract

Determination of maintainer and restorer status of germplasm collections is a prerequisite for their use in development of hybrid employing CMS system. In the present study, restorer and maintainer status of 372 Indian genotypes was determined using fertility restoration (*Rf*) linked microsatellite marker RM6100. A total of seven alleles for the marker locus, including four novel and three known, were identified in the study material. Maximum frequency was observed for the 144bp allele (44.89%) while minimum frequency was for 138bp allele (0.27%). Though most of the novel alleles were minor, occurring in less than 5% frequency, cumulatively they accounted for 9.2% frequency in the study material. Of the known alleles, which were found in 90.8% (338 genotypes) of the tested rice genotypes, 36% had maintainer type allele (132bp allele similar to Pusa 6B) while the rest had restorer type (144 or 150 bp) alleles. The spikelet fertility data generated on a subset of the study material agreed well with the established associations for the already reported alleles. Of the novel alleles, two (141 and 147 bp) were represented in the spikelet fertility dataset, both of which restored fertility, though their representation was low. This allelic information would be useful in selection of parental lines for development of hybrids in rice.

Key word: Cytoplasmic male sterility, hybrid, microsatellite marker alleles, rice, RM6100

Introduction

Rice is a major source of food for more than half of the world population. To meet the future food demand for the growing population, enhancement of productivity in rice is essential. Exploitation of hybrid vigour through development and commercialization of hybrids is a practical approach for increasing productivity [1, 2]. In India, 69 hybrid rice varieties have so far been released, which are cultivated in an area of about 2.5 million hectares. All these hybrids are based on three line system that utilizes cytoplasmic male sterile (CMS) and fertility restorer lines ('R' lines) for facilitating large-scale production of hybrid seeds. CMS lines ('A' lines) have male sterile cytoplasm and are maintained by crossing them every time with iso-nuclear male fertile lines ('B' lines) carrying a normal cytoplasm. Hybrid vigour is brought about by use of appropriate combination of 'A' and 'R' lines, the latter harbouring fertility restoration (*Rf*) gene(s) in their nucleus.

Though several CMS systems are described in rice, the one based on wild abortive (WA) cytoplasm is most widely used in commercial hybrid seed production since 1970. WA cytoplasm is preferred because it is more stable and produces complete pollen sterility [3, 4]. For generating hybrids with higher level of heterosis, it is required to identify better combiners among the available germplasm/breeding lines, which are to be further classified as maintainer of male sterility or restorer of male fertility prior to their utilization in hybrid breeding. The molecular mechanism behind WA based male sterility was decoded recently by Luo *et al*. [4]. Screening rice genotypes for their ability to restore fertility is the first step in hybrid development so as to select male parents. Since the restorer genes are yet to be cloned and characterized, and screening for fertility restoration

*Corresponding author's e-mail: tmnrcpb@gmail.com
[1]Present address: Department of Biotechnology, Jamia Hamdard University, New Delhi 110 012
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by crossing is laborious, tightly linked co-segregating molecular markers of *Rf* gene(s) are being used for screening the parents in rice hybrid development [5, 6]. Many such markers namely RM258 [7], RM171, RM228 [8], RM224 [9] and RM6100 [10] have been used by different workers. Among them, RM6100, a co-dominant microsatellite marker has been identified to be tightly linked to the fertility restoration gene with the selection accuracy of 94.8% [5]. This marker has also been used for purity evaluation of hybrids [6].

Microsatellites are the most sought after codominant genetic markers in many plant breeding programs facilitating marker assisted selection. In general, they have high mutation rate [11] and hence have high allelic variability. Size variation of the microsatellites can be better captured by electrographs using automated fragment analysis system as compared to agarose/PAGE gel system. Three distinct alleles have so far been reported for RM 6100, one specific to maintainer genotypes and the other two tagging restorer types, using high resolution agarose/ PAGE gel system [5], which might not have captured all the allelic variants due to comparatively poor resolution. In the present study, we genotyped a diverse set of rice germplasm lines using automated fragment analysis system that gives single base resolution and thus provides an opportunity to identify all the alleles prevalent in the available germplasm set. This exercise is expected to result in accurate classification of the parental lines as maintainers or restorers.

Materials and methods

DNA isolation and genotyping

A total of 372 rice genotypes were collected from rice breeding centres across India (Supplementary Table 1). These lines were grown in field and leaf samples of 10 day old seedling of 20-25 individuals from each accession were collected and stored at -80^{0}C. DNA was isolated from seedlings following CTAB procedure [12]. For genotyping using automated fragment analysis system (ABI 3730xl), the forward primer for the RM 6100 marker was labelled with fluorescent dye, FAM. PCR amplification was performed according to protocol described earlier [13] with slight modification. PCR reaction mixture was prepared in a total volume of 10 µl containing 20 ng genomic DNA, 2.0 pmol of each primer, 1 µl of 10X buffer (0.1 M Tris pH 9, 0.5 M KCl, 15 mM $MgCl_2$, 0.1% gelatine), 200µM each of dNTPs and 0.33 U *Taq* DNA polymerase. The PCR conditions followed were: initial denaturation for 5 min at 94^{0}C; 35 cycles each of 30 sec denaturation at 94^{0}C, 30 sec annealing at 55^{0}C and 1 min extension at 72^{0}C; 20 min at 72^{0}C for final extension. To 1.0 µl of amplicon sample, 8.9 µl Hi-Di formamide and 0.2 µl of an internal size standard, ROX500 (Applied Biosystems) were added and mixed well. The samples were denatured at 95^{0}C for 5 min and loaded in the fragment analyzer for resolution. Raw results from fragment analyzer were examined using GENE MAPPER 4.1 software (Applied Biosystems) and amplicon size determined.

Sequencing of RM6100 alleles

Amplified products that corresponded to different alleles of fertility restoration gene-linked microsatellite marker RM6100 were purified by MinElute 96 UF PCR plate (Qiagen) and the purified products were used as template for sequencing. Sequencing was performed using forward and reverse primers in separate reactions following standard sequencing protocol (ABI) and the sequencing products were resolved in capillary based

Table 1. Marker locus allele type of the male parent and its correspondence with spikelet fertility in the resultant hybrids

Male parent allele type	No. of genotypes	Frequency of genotypes with specific allele type	Number of hybrids studied for spikelet fertility[@]	Spikelet fertility in hybrids (mean)	Spikelet fertility in hybrids (SD)
132bp	123	0.330	4	17.87	5.467
135bp	10	0.027	0	-	-
138bp	1	0.003	0	-	-
141bp	17	0.046	3	84.45	6.07
144bp	167	0.450	48	73.76	15.92
147bp	6	0.016	1	85.62	0
150bp	48	0.129	11	78.98	6.18

[@]Hybrids having the same marker locus allele as that of the male parent were included under the same allelic type.

automated sequencer ABI 3730xl. Trace files were base called and checked for quality using sequencing analysis v5.4 software. High quality sequences were used further for mining and characterization of the microsatellites. Contig formation using forward and reverse sequences, and sequence alignment were performed using BioEdit software [14].

Results and discussion

Selection of maintainer and restorer lines is a critical step that determines the success of CMS based hybrid breeding. A rice microsatellite marker RM6100, reported to be tightly linked to fertility restoration gene located on the long arm of chromosome 10, which can distinguish maintainers from fertility restorers, was used for screening genotypes by capillary based high resolution approach using an automated fragment analysis system in the current study. We identified a total 7 alleles (132, 135, 138, 141, 144, 147 and 150bp) across 372 genotypes (Supplementary Table 1 available online at http//www.isgpb.co.in), as against three alleles known so far in literature [5]. The four novel alleles identified were of size 135, 138, 141 and 147bp carrying $(TCG)_6$, $(TCG)_7$, $(TCG)_8$ and $(TCG)_{10}$ microsatellites, respectively. As expected, alleles differed from each other by three or multiples of three nucleotides. This allelic variation was also confirmed by sequencing the amplicons representing all the seven alleles (Fig. 2). The identified alleles occurred in widely varying frequency ranging from 0.0027 to 0.449 (Fig. 1). Variation in microsatellite motif number occurs at higher rate as compared to the rest of the genome mainly due to replication slippage resulting in decrease or increase in the number of repeats [11]. Mutations in microsatellites are documented to be more common and the repeat length has higher probability to gain in size over losses in plants [15, 16]. In rice, it has been observed that the cultivated rice (*O. sativa.* L.) has higher molecular weight or higher number of repeats than the wild type (*O. rufipogon*) [17]. In our study, the alleles with higher number of repeats [144 and 150bp alleles having $(TCG)_9$ and $(TCG)_{11}$] were found as restorer type while alleles with lesser number of repeats [132bp with $(TCG)_5$] were found as maintainer type, which is in line with the proposition that restorer lines evolve from the maintainer lines [5].

Four of the seven alleles, occurring at or more than 5% frequency, were classified as major alleles while the rest occurring in lesser frequency were minor alleles. Of the novel alleles, only 141bp allele with 4.57% frequency was considered a major one while the rest were minor. Maximum frequency was observed for 144bp allele (44.89%), whereas the minimum frequency was for 138bp allele (0.27%) (Fig. 1). Genotypes homozygous for longer alleles, 144bp (same as the restorer line IR24) and 150bp (PRR78 type restorer allele), are classified as putative restorer lines in literature, whereas genotypes with 132bp allele, similar to that in Pusa 6B, a known maintainer line, are putative maintainer type. Based on this classification, in the material investigated, majority (58%) of the genotypes possessed restorer type alleles while 33% genotypes had maintainer type alleles. We analyzed spikelet fertility data on 67 hybrids generated by crossing as many lines with the CMS line Pusa 6A based on WA cytoplasm (Supplementary Tables 1 and 2 available online at http//www.isgpb.co.in). The spikelet fertility data recorded on the hybrids matched fairly well with the expectations; lower spikelet fertility was observed in the hybrids derived by crossing lines carrying maintainer type allele (132bp) with the CMS line. In contrast, when the male parents that possessed restorer type alleles (144 and 150bp) were used, the resultant hybrids were found to have higher spikelet fertility. Some level of spikelet fertility observed in the hybrids with the maintainer specific allele, contrary to the expectations of zero fertility, could be due to the presence of occasional out-crossing or because of breaking-down of linkage between the marker RM6100 and Rf gene in some of the lines used as male parent. Of the four additional alleles identified, we had spikelet fertility data for the major allele group (141bp) and one of the three minor allele groups (NDR-359 homozygous for 147bp allele). Though the number of male parents carrying 141 and 147 alleles, which were used in

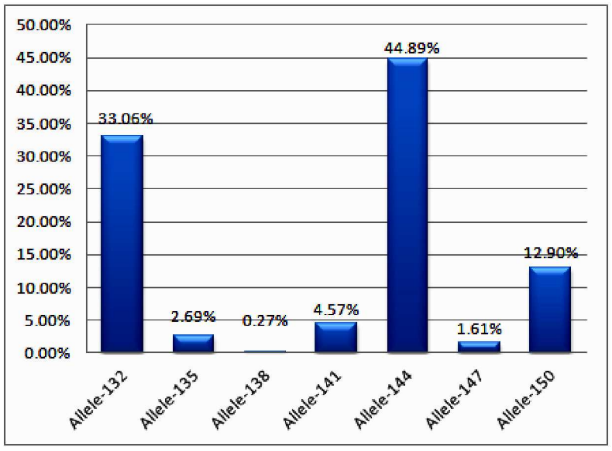

Fig. 1. Frequency (%) of rice genotypes with different RM6100 marker alleles in homozygous condition

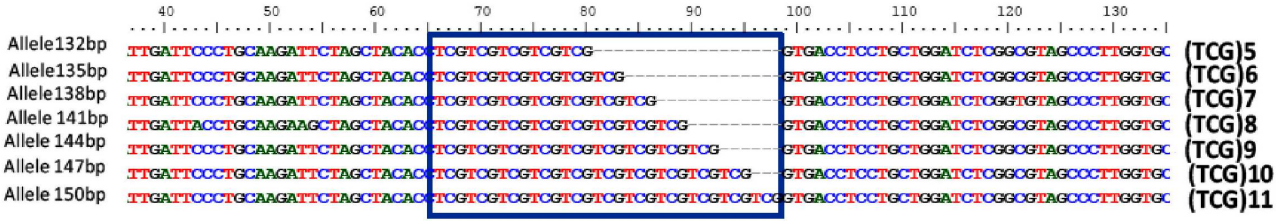

Fig. 2. Confirmation of allelic variation at RM6100 marker locus by sequencing

crossing, were 3 and 1, respectively, the resultant hybrids had higher spikelet fertility indicating that these two new RM6100 marker locus alleles are most likely linked to male fertility restoration.

The minor alleles were individually represented by a small fraction of the genotypes used in the study. However, cumulatively they were present in 9.2% of the study material. Hence, it is important that spikelet fertility data needs to be generated in larger-scale for the novel allelic types for confirmation of the relationship between these allelic forms and the nature of fertility restoration. Nevertheless, the present study has enabled identification of novel allelic forms, which has relevance in the light of their application in practical hybrid breeding in rice. The problem encountered with respect to misclassification of restorers and maintainers, while carrying out routine agarose or PAGE based resolution, can be avoided by resorting to automated capillary based electrophoresis as done in the present study. For instance, in a previous report [5], 5.2 percent of genotypes failed to represent true association between the type of alleles and their maintainer and restorer status, which could be due to misclassification. Further characterization of these novel alleles is essential for better utilization of the marker RM6100. Inclusion of more genotypes for identification of RM6100 alleles will throw light on stability of the minor alleles identified so that their utility in practical breeding could be assessed. This study is expected to guide selection of parents in development of rice hybrids and further help characterization of the linked marker RM6100 for fertility restoration.

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Supplementary Table 1. Allelic status of RM6100 across 372 genotypes

Genotype	RM6100 alleles (bp)	Genotype	RM6100 alleles (bp)	Genotype	RM6100 alleles (bp)
Sabarmati	132/132	Sagarsdamba	132/132	JGL-3828	132/132
Jyoti	132/132	Rajavaellu	132/132	Salampikit	132/132
CO-39	132/132	Kanchana	132/132	Dudhua Dhan	132/132
Surekha	132/132	PR-111	132/132	BL-245	132/132
Type-3	132/132	SKAU-23	132/132	Madhumati	132/132
Punshi	132/132	HUR-36	132/132	Panika	132/132
ADT-36	132/132	Narendra Usar Dhan II	132/132	Dobeeja-1	132/132
Punjab Basmati-2	132/132	Tapaswani	132/132	Dobeeja-2	132/132
Sonamahsuri	132/132	Dharaheera	132/132	Kharamunga	132/132
PLA-1100	132/132	Prachi	132/132	Nanu	132/132
Pant Dhan-4	132/132	Leimaphou	132/132	Sonasal	132/132
Sarathi	132/132	Mahanadi	132/132	Kudrat-3	132/132
VL-16	132/132	Palam Dhan 957	132/132	HMT-2	132/132
Pathara	132/132	MTU 1010	132/132	DV-85	132/132
Pusa-169	132/132	MTU 4870	132/132	Heibao	132/132
MTU-5293	132/132	Narendra Usar Dhan III	132/132	MR-219	132/132
ASD-16	132/132	RNRM-7	132/132	MR-220	132/132
Ananda	132/132	PNR-519	132/132	IR773841235367B	132/132
HKR-120	132/132	Pant Dhan-16	132/132	IR7738412353672B	132/132
MTU-2067	132/132	Anjali	132/132	IR78908	132/132
ADT-39	132/132	Phougak	132/132	IR-66	132/132
CSR-10	132/132	Barani Deep	132/132	IR-70	132/132
Divya	132/132	Pant Sugandh Dhan 15	132/132	Super Basmati	132/132
MTU-2077	132/132	Jagbhandu	132/132	Pusa-1173	132/132
Chandan	132/132	JGL3844	132/132	CR2496	132/132
Pusa Basmati-1121	132/132	Sukaradhan-1	132/132	CR2340-7	132/132
IR-64	132/132	Sumati	132/132	CR2364-25	132/132
VL dhan-221	132/132	HUBR-21	132/132	CR2494	132/132
Kavya	132/132	CSR-36	132/132	CR2499	132/132
Haryana Basmati-1	132/132	Jogesh	132/132	HPR-2104	132/132
PMK-1	132/132	Uphar	132/132	RAU-3061	132/132
ASD-18	132/132	Jaldubi	132/132	NDR-8015	132/132
Lunishree	132/132	Abhiskek	132/132	FL-478	132/132
Urbasi	132/132	Karma Mahsuri	132/132	MAS-25	132/132
Mahalaxmi	132/132	MTU-1075	132/132	MAS-26	132/132
HKR-126	132/132	Pusa-1401	132/132	MAS-9461	132/132
Birupa	132/132	NDR-9830135	132/132	JR-353	132/132
Manika	132/132	Sahbhagi Dhan	132/132	P-149003	132/132
Orugullu	132/132	MTU-1064	132/132	NDR-6237	132/132
NDR-6138	132/132	Intan	144/144	Kasturi	144/144

Genotype	RM6100 alleles (bp)	Genotype	RM6100 alleles (bp)	Genotype	RM6100 alleles (bp)
Piz-5	132/132	Annpurna	144/144	Jayathi	144/144
C101LACACC.NO18	132/132	MTU-3626	144/144	Erramallalu	144/144
C101A51Piz5	132/132	Suphala	144/144	Swarnamukhi	144/144
Pusa 6A	132/132	Parijat	144/144	PNR-162	144/144
Pusa 6B	132/132	Rasi	144/144	Himalaya-799	144/144
TKM 6	135/135	Shatabdi	144/144	Pant Dhan-10	144/144
Lalat	135/135	SKAU-5	144/144	Pant Dhan-11	144/144
Bhirgu Dhan 1	135/135	Himdhan	144/144	Ajaya	144/144
VL-9891	135/135	Prasad	144/144	Nilagiri	144/144
Browngora	135/135	TKM-9	144/144	Khandagiri	144/144
MI-48	135/135	Nagina-22	144/144	PNR381	144/144
Dular	135/135	MTU-7029	144/144	Samanta	144/144
Neelabatti	135/135	Sarjoo-52	144/144	Bhanja	144/144
Lajakulibadan	135/135	Shubhadra	144/144	Badami	144/144
Basmatisathi	135/135	IR-36	144/144	Meher	144/144
CG-20	138/138	Himalaya-1	144/144	Santephap-3	144/144
Sathi	141/141	Himalaya-2	144/144	UPLRI-7	144/144
Aditya	141/141	IR-24	144/144	SKAU-27	144/144
Naggardhan	141/141	Govind	144/144	Himalaya-2216	144/144
PMK-2	141/141	VLK-39	144/144	RP2421	144/144
Pusa-205	141/141	Pratap	144/144	Pantdhan-12	144/144
CSR-27	141/141	Kalinga-III	144/144	Bhadrakali	144/144
K-429	141/141	Gauri	144/144	Mahisugandha	144/144
WGL-23985	141/141	Daya	144/144	Poornima	144/144
BJ-1	141/141	Manhar	144/144	Shyamala	144/144
Dubraj	141/141	Pothana	144/144	Mahamaya	144/144
Tripura Medicinal Rice	141/141	Karjat-1	144/144	MTU-1001	144/144
Khaodaenkari	141/141	Rambha	144/144	Gautam	144/144
TaiPei-309	141/141	Himalaya-741	144/144	Joymati	144/144
Tetep	141/141	Sambha Mahsuri	144/144	Keshava	144/144
NDR8011	141/141	MTU-5249	144/144	ADT-42	144/144
DHMAS70G16429	141/141	Kanak	144/144	Kesava	144/144
Bhumansan	141/141	NDR-118	144/144	Vanprabha	144/144
Jaya	144/144	Heera	144/144	ADT-43	144/144
IR-8	144/144	Bhubana	144/144	Surendra	144/144
IR-20	144/144	Ananga	144/144	Sebati	144/144
Hema	144/144	Kalyani-II	144/144	Lalithagiri	144/144
Kalinga -I	144/144	Abhaya	144/144	Gajapati	144/144
Bhoi	144/144	Haldimuri	144/144	IRBB-4	144/144
Ramachandi	144/144	Jhumkhasa	144/144	IRBB-10	144/144
CSR-13	144/144	Khaopongkra	144/144	IRBB-13	144/144

Genotype	RM6100 alleles (bp)	Genotype	RM6100 alleles (bp)	Genotype	RM6100 alleles (bp)
Udayagiri	144/144	Khao khao	144/144	IRBB-21	144/144
Kharvela	144/144	Khao keo-4	144/144	HPR-2082	144/144
Indravati	144/144	Kharavda	144/144	HPR-2083	144/144
PR-116	144/144	IRAT-144	144/144	CR1009	147/147
PR-113	144/144	IRAT-240	144/144	Pant Dhan-6	147/147
PR-114	144/144	IR-72	144/144	NDR-359	147/147
Bameshwari	144/144	Pusa-1174	144/144	NDR-98303144	147/147
Pantdhan-19	144/144	CR143-2-2	144/144	HMT-1	147/147
Varalu	144/144	CR2363-26	144/144	NDR-6232	147/147
ADT-45	144/144	CR2407	144/144	CO-37	150/150
Pant Dhan-18	144/144	CR24616	144/144	T-23	150/150
PR-118	144/144	CR2461-9	144/144	Basmati-370	150/150
CSR-23	144/144	CR2361	144/144	Basmati-386	150/150
NDR-2026	144/144	CR2362	144/144	Shiva	150/150
HPR-2143	144/144	RR166645	144/144	Konark	150/150
WGL-14	144/144	SKAU-382	144/144	Hassanserai	150/150
Ghanteswari	144/144	Bada Bala	144/144	CSR-30	150/150
Samleshwari	144/144	MAS-109	144/144	PS-3	150/150
Chandrahasini	144/144	RR8585	144/144	Jaldi Dhan-13	150/150
Indirasona	144/144	P-1447	144/144	PS-5	150/150
WGL-32100	144/144	P-1306	144/144	Manaswini	150/150
MTU-1061	144/144	P-14470051	144/144	Chinoor	150/150
PAU-201	144/144	P-14630211	144/144	Saanwal basmati	150/150
HUR-105	144/144	P-1280	144/144	Basmati-564	150/150
HUR-43	144/144	NDR-9437	144/144	Dom Siah	150/150
Jaldi Dhan-6	144/144	Pusa-1342	144/144	Chimbalate Basmati	150/150
PR-120	144/144	P-1301	144/144	HBC-19	150/150
Punjab Mehak-1	144/144	IRBB-3	144/144	Kalajeera	150/150
Karjat-184	144/144	IRBB-5	144/144	Gangaba-ll	150/150
Pokali	144/144	IRBB-8	144/144	Badsahbhog	150/150
Gonrrabhog	144/144	IRBB-211	144/144	Thakurasuna	150/150
Lalankhanda	144/144	IRBB-59	144/144	Khosakani	150/150
BL-142	144/144	IRBB-54	144/144	Heerakani	150/150
Raskandam	144/144	IRBB-55	144/144	Pimpudibasa	150/150
Jhulhat	144/144	IRBB-60	144/144	Kalikati	150/150
Goalmalati	144/144	IRBB-1	144/144	Dhana prasad	150/150
Thakurbhog	150/150	Sirimula	150/150	Tilakchandan	150/150
Karpurakranti	150/150	Bindli	150/150	Pusa-1176	150/150
Tulsiphoola	150/150	ketekijoha	150/150	Shahpasand	150/150
Chinikamini	150/150	Thak-1	150/150	Seond basmati	150/150
Dhusara	150/150	Bhanta Phool	150/150	Bhanta Phool	150/150
Nagina-12	150/150	NDR-625	150/150	NDKN-3327	150/150
CRRI black aroma	150/150	SAF-122183	150/150	IRBB-14	150/150
PRR-78	150/150				

Supplementary Table 2. Spikelet fertility data of 67 hybrids

Genotype tested as male parent	Allelic form of of RM6100 in the male parent	Spikelet fertility of the hybrid	Genotype tested as male parent	Allelic form of of RM6100 in the male parent	Spikelet fertility of the hybrid
Ananda	132/132	17.99	Bhoi	144/144	75.17
Divya	132/132	24.59	Udayagiri	144/144	63.30
PNR-519	132/132	17.67	PR-116	144/144	70.43
Pant Dhan-16	132/132	11.21	PR-113	144/144	79.03
Naggardhan	141/141	77.88	Pantdhan-19	144/144	77.01
CSR-27	141/141	89.84	Varalu	144/144	80.00
BJ-1	141/141	85.62	Pant Dhan-18	144/144	83.57
Jaya	144/144	94.08	PR-118	144/144	80.54
Hema	144/144	74.54	HPR-2143	144/144	66.88
Kalinga -I	144/144	75.43	WGL-14	144/144	63.83
Rasi	144/144	30.37	Ghanteswari	144/144	90.04
Shatabdi	144/144	59.03	Samleshwari	144/144	88.27
Himdhan	144/144	77.41	Chandrahasini	144/144	51.41
Prasad	144/144	89.90	WGL-32100	144/144	83.95
Nagina-22	144/144	75.90	PAU-201	144/144	74.40
Sarjoo-52	144/144	81.04	HUR-105	144/144	76.02
IR-36	144/144	74.49	PR-120	144/144	92.00
IR-24	144/144	90.96	IR-72	144/144	83.51
Gauri	144/144	74.55	MAS-109	144/144	80.50
Himalaya-741	144/144	75.20	RR8585	144/144	81.64
Sambha Mahsuri	144/144	88.12	P-1447	144/144	65.52
Abhaya	144/144	18.78	NDR-359	147/147	85.62
Kasturi	144/144	59.03	Basmati-370	150/150	87.81
Himalaya-799	144/144	75.15	Shiva	150/150	73.96
Ajaya	144/144	78.03	Hassanserai	150/150	78.49
Pantdhan-12	144/144	75.90	CSR-30	150/150	77.73
Poornima	144/144	68.00	PS-5	150/150	78.88
Shyamala	144/144	64.88	Saanwal basmati	150/150	73.78
Gautam	144/144	79.96	Dom Siah	150/150	89.71
Keshava	144/144	87.72	Chimbalate Basmati	150/150	75.75
Vanprabha	144/144	24.49	Badsahbhog	150/150	83.80
Sebati	144/144	78.24	Seond basmati	150/150	68.96
Lalithagiri	144/144	91.60	PRR-78	150/150	79.89
Gajapati	144/144	70.43			