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Identification of fertility restorers among Assam rice cultivars by phenotyping and molecular screening approaches

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Abstract

In three line hybrid rice seed production the combination of a CMS line, maintainer line and restorer line are involved in the development of hybrids. The wild abortive CMS is extensively used in commercial hybrid seed production. The fertility restoration of wild abortive CMS is governed by two independent dominant *Rf* genes *viz*., *Rf4* and *Rf3*. The present study was conducted with the objective of molecular characterization rice genotypes from Assam, India and to identify restorers for hybrid rice breeding programme. A total one hundred and eleven hybrids were generated and evaluated for their pollen and spikelet fertility. Based on molecular screening twenty five genotypes were identified to carry only *Rf4* gene and three genotypes identified to carry both *Rf3* and *Rf4* genes. These identified restorers in the present study would be a usable restores for hybrid rice breeding in north east India. Molecular diversity for fertility restoration using NTSYS package formed two clusters among the tested genotypes, cluster A represented a landrace Terabali from Assam and cluster B with all other genotypes.

Key words: Fertility restoration, *Rf3*, *Rf4*, WA-CMS, rice, hybrids

Introduction

Rice is staple food for half of the world's population. Worldwide, rice occupies an area of 166 million hectares with the production of 745 million tons of rough rice at an average productivity of 4.48 tons per hectare (FAOSTAT 2013). In India rice is cultivated in 44 million hectares, the largest area in the world with the production of 106 million tons, whereas China produces 144 million tons from 30.6 million hectares itself (RICESTAT 2014). This is due to the large-scale adoption of hybrid rice by China; while in India the progress in adopting hybrid rice has been slow with a total cultivated area under hybrid rice is about 2.5 million hectares.

In hybrid rice seed production using three line or CGMS (Cytoplasmic Genic Male sterility) system, the combination of a CMS line, maintainer line and restorer line carrying the restorer gene (*Rf*) to restore the fertility are indispensable for the development of hybrids. Identification of maintainers and restorers from elite breeding lines and landraces through test crossing (Ikehashi and Araki 1984; Virmani 1996) and their use in further breeding programme are the initial steps in three-line hybrid breeding (Siddiq 1996). In rice the wild abortive (WA) CMS derived from *O. sativa f. sp spontanea* is extensively used in commercial seed production (Lin and Yuan 1980; Li and Yuan 1986).

The most investigated fertility restoration trait of WA-CMS consistently explained that fertility restoration is governed by two independent dominant major genes (Li et al. 1982; Zhou 1983; Young and Virmani 1984; Li and Yuan 1986; Virmani et al. 1986; Govinda Raj and Virmani 1988; Bharaj et al. 1991; 1995; Teng and Shen 1994). The chromosomal location of Rf genes namely *Rf4* and *Rf3* was determined in chromosome 10 and 1 respectively through molecular analysis (Zhang et al. 1997; Yao et al. 1997). The process of screening for the trait of fertility restoration is laborious and time consuming as it involves test crossing with a set of CMS lines and evaluation of F_1 for pollen and spikelet fertility. Molecular markers are becoming increasingly useful in enhancing the efficiency in crop improvement.

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Revathi et al. (2013) reported that the molecular markers are useful tool for evaluating large number of germplasm/breeding lines for the fertility restoration trait.

The north eastern India is characterized by high rainfall, humidity, varied topography, heavy natural selection pressures and environmental stresses. Being a biodiversity hotspot (Myers 2000), the region is also rich in rice diversity. North eastern India has been highlighted as the repository of diverse rice varieties and valuable genes (Sharma et al. 1971; Vairavan et al. 1973). Rice is the principal crop of this region possessing around 72% of the total cultivated area. By evaluating genotypes from north eastern states morphologically and also by molecular analysis for the fertility restoration trait, a desirable restorer lines could be identified and utilized in hybrid rice breeding. In the present investigation sixty four rice genotypes from Assam agricultural university, Jorhat has been subjected to molecular analysis for the traits like fertility restoration, wide compatibility and diversity for fertility restoration trait to utilize them in hybrid rice breeding for developing new hybrids for north eastern India.

Materials and methods

At research farm of Assam Agricultural University, Jorhat, Assam, India the selected rice genotypes were crossed with five CMS lines *viz*., IR 58025A, IR 68888A, IR68897A, IR79156A and IR80555A carrying WA cytoplasm during 2012 *kharif* season. One hundred and eleven test crosses were produced and evaluated to identify commercially usable restorers and maintainers during next 2013 *kharif* season with the ultimate objective of developing rice hybrids suitable for the state of Assam. The test hybrids along with their respective parental lines were transplanted one seedling per hill with spacing of 20 x 20cm.

Phenotyping

Pollen fertility

Pollen fertility study was done using anthers collected from spikelets at 1 to 2 days before anthesis. The anthers from each spikelets were smeared in a drop of 1% Iodine-potassium iodide (I-KI) solution (Virmani et al. 1997) on a glass slide and fertile and sterile pollens were counted in the three randomly selected microscopic fields. Stained, well filled and round pollen grains were counted as fertile, while unstained, shriveled and empty pollen grains were considered as sterile. Pollen fertility was calculated and expressed in percentage as given below:

$$\text{Pollen fertility } \% = \frac{\text{No. of stained pollen grains}}{\text{Total no. of pollen grains examined}} \times 100$$

Spikelet fertility

The panicles that emerged from the primary tiller were bagged before anthesis to avoid out crossing and the number of filled grains and chaffs in the panicle were counted at the time of maturity. The ratio of filled grains to the total number of spikelets was expressed as spikelet fertility percentage as given below:

$$\text{Spikelet fertility (SPF) } \% = \frac{\text{No. of filled spikelets in the panicle}}{\text{Total no. of spikelets in the panicle}} \times 100$$

Genotyping

DNA isolation and PCR analysis

Total genomic DNA was isolated from sixty four genotype (Table 1) of 20 days old young leaves by Mini-preparation method by Dellaporta et al. (1983). The polymerase chain reactions and cycling conditions were followed according to Balaji et al. (2012) for the both candidate gene based/ linked primers of *Rf* genes. The amplified PCR products along with 100 bp and 1kb ladders (Bangalore Genie, India) were separated on a 3.0% Seakem® LE agarose gel (Lonza, USA), and stained with ethidium bromide and documented using Gel documentation system (Alpha Innotech, USA). For the diversity analysis with respect to fertility restoration nineteen SSR markers which are linked to *Rf4* and *Rf3* genes located on chromosome 10 and 1 were utilized and also for identifying wide compatible genotypes S5 MMS marker system reported by Sundaram et al. (2010) and Revathi et al. (2015) were utilized. The presence or absence of a specific allele was scored as 1 or 0, respectively, and the matrix of 1 and 0 data was subjected to polymorphic information content (PIC) estimation. The PIC was calculated as per the given formula, Polymorphic Information Content (PIC) = $1-\Sigma P_{ij}^2$. Where Pij is the frequency of j^{th} allele of i^{th} locus, sum across all the alleles for the locus over all genotypes (Botstein et al. 1980). The data were entered in to binary matrix and subsequently analyzed using software package NTSYSpc ver 2.2 (Rohlf et al. 1994). Coefficient of similarity was

Table 1. List of genotypes and their pedigree

S.No	Designation	Pedigree	S.No	Designation	Pedigree
1	IET 17854	Phalguna /Surekha	33	TTB 286-2-731	Mugi Sali/IET 8002
2	IET 21840	Swarna/Geetanjali	34	Joria	Land race
3	IET 18645	R 672/R 371-1	35	Koimurali	Land race
4	IET 20800	OR 909-4-89/Pankaj	36	IET 19189	Savitri/ Padmini
5	IET 21480	NA	37	Chilarai	IR 24/ CR 44-118-1
6	IET 19916	CR563-1014/BG90-2//IR42	38	Mulagabhoru	Jaya/Mahsuri
7	IET 18648	Mahalaxmi/OR633-7	39	IET 19185	Pankaj/T 141
8	Krishna E	Pureline selection in Krishna	40	Jalashree	Pankaj/FR 13A
9	Luit	Heera/Annada	41	Kolong	Chilarai/Kalinga III
10	Prafulla	Akisali/Kushal	42	Dikhow	Heera/Annada
11	Mitra Sali	Pureline selection in Manohar Sali	43	Haccha	CRM 53/IR 64
12	Dhirendra	Pureline selection in Manohar Sali	44	Jalkunwari	Pankaj/FR13 A
13	Kmj-14S-1-2-17	Mahsuri/Malbhog	45	Bishnuprasad	K 343-29-1-1/ Suweon 334
14	Purnendu	Patnai 23/Jaladhi 2	46	IR09F434	PSBRc82 introgressed with *SUB1A* gene from FR13A
15	Kmj-13A-1-12-3	Mahsuri/Luit	47	Manohar Sali	Lati Sail/Guachari
16	Joymoti	Jaya/Mahsuri	48	Kmj 1-19-1	IR 8/Manohar Sali
17	Kanaklata	Jaya/Mahsuri	49	Tamdao	Introduction from Vietnam
18	Ranjit	Pankaj/Mahsuri	50	IET 20775	NA
19	Swarna-Sub 1	Swarna introgressed with *Sub1A* gene from FR 13A	51	Kapilee	Heera/Annada
20	Swarna	Vasistha/Mahsuri	52	Kmj-13S-3-1-3	Mahsuri/Luit
21	IR 64	IR 5657-33-2-1/IR 2061-465-1-5-5	53	Ketekijoha	Savitry/ Bhadshabhog
22	IR 64-Sub 1	IR 64 introgressed with *Sub1A* gene from FR 13A	54	IET 20771	BPT 1235/WGL1437-7
23	Kmj-13A-6-1-2	Mahsuri/Luit	55	Jyotiprasad	K 343-29-1-1/Suweon 334
24	Kmj-13B-1-13-3	Mahsuri/Luit	56	Disang	Heera/Annada
25	Bahadur	Pankaj/ Mahsuri	57	IET 21850	Jaya/Pusa Basmati 1
26	Kasalath	Pureline selection in land race	58	Terabali	Land race
27	IET 19208	Jagabandhu/IR 64	59	IET 21469	Swarna/Krishnabhog
28	Badal	Pureline selection in land race	60	IR 58025A	
29	Pankaj	Peta/Tongkai Ratan	61	IR 68888A	
30	Piyajihari	Land race	62	IR 68897A	
31	Longai	Pusa 2-21/China 63	63	IR 79156A	
32	Bali Ghugoor	Land race	64	IR 80555A	

calculated by using Jaccard's coefficient by SIMQUAL sub-function and cluster analysis was performed by agglomerative technique using the UPGMA (Un-weighted Pair Group Method with Arithmetic mean) method by SAHN clustering sub-function of NTSYSpc 2.02.

Results

Phenotyping

The selected rice genotypes were crossed with different CMS/A lines and one hundred eleven F_1 hybrids were produced. These F_1 hybrids were

evaluated during next season for their pollen and spikelet fertility according to Virmani et al. 1997 (Table 2). The pollen fertility percentage ranged from 0% (Terabali and Kasalath) to 100% (Mitra Sali) and the spikelet fertility percentage ranged from less than 1 % (Kasalath, Teraboli,) to 91% (Dhirendra). Among the selected genotypes for hybrids production twenty five genotypes *viz*., Purnendu, Ketekijoha, Mitrasali, Jalkunwari, Kmj-1-19-1, Joymati, Prafulla, Luit, Tamdao, Chilarai, Manohar Sali, Ranjit, IET 21840, IET 19189, IET 20771, IET 20800, IET 18648, IET 21480, IET 21850,Disang, IET-17854,IET-19916,IET-20775, Dhirendra and Bahadur were identified as restorers based on pollen and spikelet fertility percentage. The maximum numbers of restorers were identified with IR 58025A hybrid combination indicating that IR 58025A as good combiner with better out crossing ability in comparison with other A lines in hybrid rice breeding. The genotype Terabali was identified as maintainer for IR 68888A and Kasalath was identified as maintainer for IR79156A and IR80555A respectively (Table 3).

Table 2. Pollen and spikelet fertility percentage for restorer and maintainer identification (Virmani et al. 1997)

Pollen fertility (%)	Category	Spikelet fertility (%)
0 – 1	Maintainers (M)	0
1.1 – 50	Partial Maintainers (PM)	0.1-50
50.1 – 80	Partial Restorer (PR)	50.1-75
>80	Restorer (R)	> 75

Genotyping

Molecular screening for the presence of *Rf4* gene, the SSR markers namely RM 6100 (Singh et al. 2005), PPR3 (Ngangkham et al. 2010) and for *Rf3* DRRRF3-10 (Balaji et al. 2012) primers were utilized. Based on molecular screening twenty five genotypes were identified to carry *Rf4* gene and three genotypes showed the presence of both *Rf3* and *Rf4* genes (Table 4).

Among twenty eight genotypes with *Rf4* gene, seventeen genotypes were confirmed as complete restorers based on pollen and spikelet fertility. Among

Table 3. List of hybrids and their pollen and spikelet fertility in per cent

S.No.	Cross	Pollen fertility	Spikelet fertility	S.No.	Cross	Pollen fertility	Spikelet fertility
1	IR 58025A/Pankaj	82.00	81.20	20	IR80555A/Manohar Sali	80.00	83.00
2	IR 58025A/IET 21840	80.00	80.70	21	IR68897A/Manohar Sali	80.00	82.00
3	IR 58025A/Purnendu	80.00	81.47	22	IR79156A/Manohar Sali	67.00	81.34
4	IR 58025A/Ketekijoha	81.00	88.00	23	IR80555A/Tamdao	82.00	88.26
5	IR 58025A/Mitrasali	100.00	88.40	24	IR80555A/IET 21480	90.00	94.14
6	IR 58025A/Jalkunwari	82.00	90.32	25	IR80555A/IET 19189	85.00	84.05
7	IR80555A/Kmj 1-19-1	79.00	81.00	26	IR68897A/IET 20771	82.00	83.38
8	IR68888A/Kmj 1-19-1	77.30	87.00	27	IR68897A/Chilarai	80.00	80.65
9	IR80555A/Kmj 1-19-1	83.00	80.08	28	IR80555A/Chilarai	80.30	81.90
10	IR58025A/Joymoti	75.00	90.00	29	IR68897A/Dikhow	93.40	84.00
11	IR79156A/Joymoti	70.00	80.24	30	IR 58025A/Ranjit	80.00	87.86
12	IR58025A/Prafulla	87.00	82.41	31	IR58025A/IET 18648	84.00	85.00
13	IR58025A/Luit	86.00	88.04	32	IR68897A/IET 18648	90.70	82.00
14	IR 58025A/Jalashree	81.00	80.00	33	IR80555A/IET 18648	83.00	83.00
15	IR68897A/Jalashree	84.00	83.00	34	IR58025A/IET 20800	83.00	85.00
16	IR 58025A/Dhirendra	78.00	82.00	35	IR79156A/Kasalath	0.00	0.00
17	IR68897A/Dhirendra	87.00	81.00	36	IR80555A/Kasalath	0.00	0.00
18	IR79156A/Dhirendra	91.00	91.67	37	IR68888A/Terabali	0.00	0.85
19	IR 58025A/Bahadur	80.00	87.00				

Table 4. Molecular screening for *Rf* genes presence

Genotypes	*Rf* genes
Kolong, Dikhow, and Ketekijoha	*Rf3 & Rf4*
IET-21840, IET-20800, IET-21480, IET-19916, IET-18648, Luit, Purnendra, Joymoti, IR-64, KMJ-13A-6-1-2, Bahadur, Pankaj, Joria, Koimurali, IET-19189, Chilarai, Jalashree, Kolong, Dikhow, Jalkunwari, KMJ-1-19-1, IET-20775, Kopilee, KMJ-13S-3-1-3, Ketekijoha, IET-20771, Disang, and IET-21850	*Rf4*
Kolong, Dikhow, and Ketekijoha	*Rf3*
IET-17854, Krishna E, Prafulla, Mitrasali, Dhirendra, KMJ-13AB-1-12-3, Kanaklata, Ranjit, Badal, Mulagabhoru, Manohar Sali, Tamdao, Jyoti Prasad, Kasalath, Basanta Bahar, Piolee, and Terabali	Without *Rf4* & *Rf3*

the three genotypes with both fertility restorer genes (*Rf3* and *Rf4)* only one genotype found to be a complete restorer based on phenotyping. To identify the genotype which carries *S5* neutral allele the SSR primer multiplex marker system located on chromosome 6 (Sundaram et al. 2010 and Revathi et al. 2010) was utilized and three genotypes namely Kmj 135-3-1-3, Swarna and Swarna Sub 1 were identified to carry *S5* neutral allele and these genotypes would be potential genotypes for inter sub-specific hybridization program to achieve higher heterosis.

Diversity analysis with fertility restoration trait based SSR markers

A total nineteen *Rf* linked/candidate gene based markers were utilized for diversity analysis of sixty four genotypes. Out of nineteen markers, nine were linked to *Rf* genes on chromosome 10 and nine were located on chromosome 1 and another mitochondrial SSR marker DRRCMS which can able to distinguishing WA-CMS and their cognate maintainer lines (Rajendrakumar et al. 2007) were utilized for diversity analysis.

The data generated from nineteen SSR markers were utilized for calculating genetic similarity coefficient and construction of dendrogram. The molecular diversity pattern based on allelic variation ranged from one to six alleles and the average alleles were 2.58. The PIC value ranged from 0.17 (RM 6100) to 0.72 (RM 3148) and the diversity analysis was done using NTSYSpc2.2 software using UPGMA clustering method. The cluster analysis was used to group sixty four genotypes and also to construct a dendrogram. The dendrogram generated based on these marker data revealed the presence of two major clusters, *viz*., A and B. Cluster A represented a landrace Terabali from Assam land race which clustered separately indicating more molecular diversity with respect to fertility restoration from other genotypes and another cluster B having all other genotypes. The cluster B had two sub clusters viz B-I and B-II in which B-Ist cluster consisted of four CMS lines along with twenty five other genotypes and the B-IInd cluster with thirty four genotypes. The maximum similarity index of 1.0 was obtained between genotypes IR 68897A, IR 79156A and Swarna Sub 1 with Swarna and Jalashree with Jalkunwari indicating these genotypes are closely related based on their available pedigree information.

Discussion

For hybrid rice breeding, the identification of effective restorers and maintainers are the initial steps in three line system (Siddiq 1996). The pollen and spikelet fertility traits are important criteria at test cross nursery stage for identifying restorer and maintainer lines (Ikehashi and Araki 1984; Virmani 1996). The genotypic identity of maintainer and restorer lines cannot be determined without generation of test crosses with CMS lines. Therefore, breeding for hybrid rice is laborious and time consuming. Molecular markers are useful in facilitating plant breeding by increasing selection efficiency with the help of closely linked markers (Zhang et al. 1997). Nas et al. (2003) demonstrated the use of molecular markers for identification of restorer line. The usefulness of RM6100 in marker aided selection of restorer with selection accuracy of 97% and 94.87% was reported by Singh et al. (2005) and Sheeba et al. (2009) respectively. Revathi et al. (2013) indicated that molecular screening for fertility restoration can be a useful tool for identifying restorers from breeding lines of unknown restoration status with 80 to 85% efficiency without making and evaluating large number of tedious test crosses. In the present study, based on molecular screening, 28 lines were identified to carry *Rf* genes and among these 17 were identified to

be complete restorers based on pollen and spikelet fertility percentage indicating importance of test crosses in identifying restorers and maintainers.

The inheritance of fertility restoration for WA - CMS system was studied by Li and Yuan (1986) in China in the representative rice variety IR24, which restored complete fertility and used widely. It was reported that IR24 contains two pairs of major fertility restoring genes that are independently inherited. One pair of genes inherited from Cina, a late indica variety of Chinese origin while another pair was from SLo 17.

Most of the studies have demonstrated that two independent loci control fertility restoration in WA-CMS system (Zhou 1983; Young and Virmani 1984; Li and Yuan 1986; Govindaraj and Virmani 1988 and Bharaj et al. 1991), but Huang et al. (1986), reported that a single dominant gene controlled fertility restoration. Bharaj et al (1995) reported that the two fertility restorer genes were located on chromosomes 7 and 10. Based on the amounts of deviation from the expected segregation ratio, Bharaj et al. (1995) further inferred that one gene was stronger than the other. The stronger gene, designated as *Rf-WA-1*, was located on chromosome 7 and the weaker gene, designated as *Rf-WA-2*, on chromosome 10. In the present study, six genotypes were identified as restorers based on pollen and spikelet fertility percentage without the presence of *Rf4* and *Rf3* genes confirming the results of Bharaj et al. (1991) that modifiers are also playing role in fertility restoration.

Among PCR-based markers, SSR markers are the most favored because of their codominant nature, abundance, genome wide coverage and high reproducibility (McCouch et al. 2002). Yadav et al. (2013) demonstrated that trait linked SSR markers are more useful than random SSR markers in rice diversity analysis. Fertility restoration trait linked SSR markers were utilized for diversity analysis in the current study. The level of genetic diversity between two parents was the possible predictor of F_1 performance and higher level of heterosis were usually observed from parental lines of diverse in relatedness, ecotype, geographic origin, etc. (Lin and Yuan 1980; Yuan and Cheng 1986; Yuan 1985). The CMS lines, namely, IR 58025A ,IR 68888A, IR68897A and IR79156A were grouped together in B-I cluster, therefore, for developing high heterotic hybrids for Assam region the restorers may be chosen from the B-II cluster instead of B-I cluster to achieve higher heterosis.

It has been reported that some of the well-established restorer lines for WA-CMS such as IR24, IR36, IR54, IR9761-19-1, and IR2797-105-2-2 showed incomplete fertility restoration in CMS lines such as IR17492A possessing WA cytoplasm (IRRI 1986). The causes reported were the presence of either inhibitory genes for restoration in CMS lines or inter-varietal hybrid sterility (Govinda Raj and Virmani 1988). Virmani et al. (1997) also reported the differential reaction of rice genotypes with different CMS lines of the same cytoplasmic source. In the present investigation also the genotypes *viz*., Luit and Kanaklata showed varied level of fertility restoration from complete restoration to maintainer expression with different CMS lines having WA cytoplasm. Similar observations have also been reported earlier (Hemareddy et al. 2000; Bisne and Motiramani 2005; Krishnalatha et al. 2012). This recorded variation may be due to the pollen fertility restoring genes differing in their penetrance or expressivity in different genotypes (Umadevi et al. 2010) or due to existence of modifiers genes (Pande et al. 1990). It has been also reported that restoration reaction also influenced by environmental factors (Govind Raj et al. 1984). The CMS lines utilized in the present study though derived from the same WA source, more number of restorers were identified with IR58025A as compared to other CMS lines representing differential reaction in terms of fertility restoration. So far, 59 hybrids have been released for commercial cultivation in India (Viraktamath et al. 2012). Among these, 31 hybrids have been released from the public sector, surprisingly 15 out of thirty-one hybrids had IR 58025A (CMS) as female parent indicating better out crossing potential, combining ability for higher heterosis.

The NE India is also the home to many locally adapted aromatic and quality rice landraces. Despite their low-yield potential, these cultivars are grown for their high market and social values. Hence, it is essential to identify maintainers and restorers from local cultivars for developing rice hybrids that meets local consumer's preference. In this study the genotype Terabali was identified as maintainer by molecular and spikelet fertility studies. This genotype could be utilized in back cross breeding programme to develop new CMS lines with desirable traits of Terabali for producing new hybrids for north eastern region.

The present study identified more number restorers and partial restorers than maintainers. Lin and Yuan (1980) reported relatively a high frequency (20-30%) of restorers and a low frequency (< 5%) of the maintainers from among the tropical indica rice

from Southeast Asian countries. To conclude, the identified restorers and maintainer from local Assam genotypes may help in the development of new hybrids with desirable traits for north eastern region. The present study is an initial attempt for hybrid rice breeding in north eastern region and in same way large number of rice germplasm from north eastern region needs to be screened and tested for their pollen and spikelet fertility for identification of efficient parental lines. Development of highly heterotic hybrids specially for north eastern region may bring in Green Revolution as also targeted by the Government of India.

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