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***Short Communication***

# A multiplex PCR system for testing the genetic purity of hybrid rice (*Oryza sativa* L.)

**Madhuchhanda Parida, Umakanta Ngangkham*,#, Jawahar Lal Katara, Manoj Kumar Yadav, Sanghamitra Samantaray and Trilochan Mohapatra[1]**

ICAR-National Rice Research Institute, Cuttack, Odisha 795 006; [1]Indian Council of Agricultural Research, New Delhi 110 012

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**Abstract**

**Commercial exploitation of rice hybrid for sustainable production and productivity largely depends on genetic purity of hybrid seed used. To detect genetic impurity in the hybrid seeds developed through the three-line system using wild abortive (WA) cytoplasmic-based male sterility, a multiplex PCR assay was designed . A total of six primers, namely, DRRM-Rf3-10, Rf4-STS, RM6100, CMS-WA, osWA352 and RMS-3-WA352 were designed based on fertility restorer genes *Rf3*, *Rf4* and *Wa352*. The primer combinations having RF4-STS and CMS-WA markers of *Rf4* and *WA352* genes, respectively showed clear and distinct PCR banding patterns among the WA-cytoplasm possessing 31A, fertile restorer PK117 lines and their cognate hybrid rice, Ajay. This multiplex PCR will be useful for assessment of genetic purity of hybrid rice seeds.**

**Keywords**: Hybrid rice, WA cytoplasm, genetic purity, multiplex PCR, cytoplasmic male sterility

Hybrid rice technology is one of the sustainable options for increasing rice production thereby supporting world food security (Katara et al. 2017). Hybrid rice shows yield increase of 15-20% as compared to high yielding varieties (HYVs) in similar growth conditions (Vermani et al. 2003; Katara et al. 2017). India is considered as one of the leading counties in hybrid rice technology as it has developed more than 73 rice hybrids for commercial cultivation (Katara et al. 2017). The hybrid rice production technology in India is mainly based on the three-line system involving cytoplasmic male sterility line (A), maintainer line (B) and fertility restorer line (R). The most widely used cytoplasmic male sterility (CMS) in rice hybrid of India is based on wild abortive (WA) cytoplasm (Ngangkham et al. 2010).

Successful reaping of yield potential of rice hybrid technology is largely depending upon the genetic purity of hybrid rice seed supplied to the farmers. It has been reported that there has been a yield reduction of 100 kg per hectare for every contamination of 1% in the hybrid seeds with other genotypes (Mao et al. 1996). Conventionally, GOT is used for genetic purity of hybrid seeds in which the seed sample are allowed to grow in the field for observation of morphological traits. The main disadvantage of this method is time, labour and influenced of morphological traits by environmental factors. Considering the above facts, PCR using molecular markers have been widely used for assessment of genetic purity, DUS testing in many commercial crops which were found to be fast, powerful, reliable, accurate, less time consuming and independent of environmental effects (Yashitola et al. 2002; Sundaram et al. 2008). Besides, multiplex PCR method shows its superiority over other PCR techniques in terms of sensitive, fast identification in a single reaction and economical (Satturu et al. 2018). Therefore, an attempt was made for the first time to develop a robust multiplex/single-tube PCR assay using primers derived for both the genes of CMS and

*Corresponding author's e-mail: ukbiotech@gmail.com

#Present address: ICAR Research Complex for NEH Region, Umiam, Meghalaya 793 103

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fertility restorer (*Rf*) to differentiate rice hybrids and inbreds/cultivars which could be useful for genetic purity assessment of hybrid rice developed in India.

The two parental lines of rice hybrid Ajay (H), 31A as WA based 'A' line and PK117 as fertility restorer 'R' line were used. Another seven lines were different rice hybrid varieties derived from different WA based 'A' and fertility restorer 'R' lines combinations. A different eight rice inbreds/cultivars were also included to assess the differentiation between the hybrid and inbred rice lines through gene-based molecular markers (Table 1).

Total genomic DNA from the young leaves of rice was extracted using Modified CTAB DNA PCR was carried out with the reaction components in a 10µl reaction volume; 20 ng/µl of template DNA, 0.2 µM of each dNTP, 0.2µM each of forward and reverse primers, 1U of T*aq* DNA polymerase enzyme (DreamTaq, Thermo Scientific, USA) and 1X PCR buffer. The thermal cycling profile was set up as follow: 94°C for 5 min (initial denaturation), followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, extension at 72°C for 1 min and followed by a final extension of 10 min at 72°C in Thermal Cycler (Eppendorf AG, Hamburg, Germany). The PCR products along with 100-bp DNA ladder (BR Biochem Life Sciences, New Delhi, India) were separated on 3.5% MetaPhoragarose gel (Lonza, Rockland, ME, USA) electrophoresis. In the case of Multiplex PCR Assay, two pair of primers was used instead of single pair of primer in the same PCR reaction mixture and condition.

The CMS of WA-based cytoplasm in rice is restored independently by two different nuclear-encoded *Rf* genes, *Rf3* and *Rf4* which are located on chromosomes 1 and 10, respectively (Katara et al. 2017). Three markers, DRRM-RF3-10 linked to the *Rf3* gene and RM6100 andRf4-STS linked to the *Rf4* gene were selected. The DRRM-Rf3-10 marker yielded a PCR product of 140 and 160 bp fragment size in non-restorer A-line and restorer R line thereby differentiating between the A and R lines (Suresh et al. 2012; Ramalingam et al. 2017).

For *Rf4* gene, two linked/gene-based markers namely, RM6100 and RF4-STS have been widely used for screening of *Rf4* gene (Sheeba et al. 2009; Ngangkham et al. 2010; Ramalingam et al. 2017). Using RM6100, a PCR product of 160 bp and 175 bp were observed in 31A as A-line and PK117 as restorer line as shown in Fig**.** 1A. It confirms previous report (Katara et al. 2017). Hence, this RM6100 marker can differentiate the restorer and non-restorer lines by yielding 10 bp difference between the A and R lines. Another STS marker, RF4-STS provides a 1,488 bp amplicon specific to CMS and 1383 bp to restorer lines and showed significant co-segregation in large mapping population (Ngangkham et al. 2010). In the present study, this STS marker gave a similar size of amplification in A-line and R lines indicating the usefulness and robustness of the marker while differentiating the sterile WA cytoplasm and fertility restorer lines of rice (Fig. 1A).

Recently, the gene responsible for WA male sterility was cloned and characterized as chimeric mitochondrial gene known as *WA352* encoding a 352 amino acid protein which interacts and inhibits the normal function of a nuclear encoded-mitochondrial protein, COX11 preferentially in the anther tapetum of male-sterile WA line (Luo et al. 2013). Markers such as CMS-WA, WA-352, and RMS-3 specific to *WA352* gene were selected to differentiate normal and WA-

**Table 1.** List of the rice genotypes used in the present study

| S. No. | Genotypes | Remarks | S. No. | Genotypes | Remarks |
|---|---|---|---|---|---|
| 1 | 31A | A line (male sterile) | 10 | PA6444 | Hybrid |
| 2 | PK117 | R line (restorer) | 11 | Gayatri | Inbred |
| 3 | Ajay | Hybrid | 12 | Varshadhana | Inbred |
| 4 | Rajalaxmi | Hybrid | 13 | Savitri | Inbred |
| 5 | CRHR32 | Hybrid | 14 | Durga | Inbred |
| 6 | DRRH-2 | Hybrid | 15 | Samlei | Inbred |
| 7 | US382 | Hybrid | 16 | Utkalprabha | Inbred |
| 8 | US12 | Hybrid | 17 | Pooja | Inbred |
| 9 | BS6444G | Hybrid | 18 | Swarna-sub1 | Inbred |

based sterile cytoplasm in rice. The primer CMS-WA was designed from the *WA352* gene as a dominant marker in such a way that it can amplify in the WA line but not in another cytoplasm (Yashitola et al. 2004). It produces a PCR fragment of 386bp in the WA cytoplasm, whereas there was no amplification in another cytoplasm thereby differentiating WA cytoplasm from another normal cytoplasm. This marker produced similar PCR fragment in 31A whereas no PCR product was observed in PK117 rice genotype (Fig. 1A). Another dominant marker, osWA352 produced a PCR product of 1432 bp only in WA cytoplasm but no amplification in normal cytoplasm (Luo et al. 2013). Using osWA352 marker,similar 1432 bp PCR fragment was observed in 31A line with no amplification in PK117 line. Both CMS-WA and osWA352 markers were found to be the dominant marker that differentiates WA cytoplasm from the other normal cytoplasm in rice. RMS-3-WA352 is a co-dominant functional marker specific to the *WA352* gene which was more robust and efficient as compared to the above two dominant markers (Pranathi et al 2016). This highly robust and efficient co-dominant marker was designed targeting a 20 bpInDel within the *WA352* gene of WA cytoplasm of rice (Pranathi et al 2016). As earlier reported, this marker amplifies 247 bp product from 31A, WA cytoplasm possessing A-line whereas a smaller 227 bp product was observed in PK117, fertile/normal cytoplasm possessing R line (Fig. 1A). Therefore, all the three markers based on WA352 gene were sufficient to distinguish WACMS line from other normal/fertile restorer line. The PCR was performed in the present investigation using all the above six primers with DNA template of 31A rice representing WA cytoplasmic male sterility A-line and PK117 representing fertility restorer line having *Rf3* and *Rf4* genes (Fig. 1A). All the primers successfully amplified with their expected PCR fragment size mentioned by the earlier groups (Fig. 1A).

Most of the assessment of genetic purity in rice earlier was carried out using singleplex PCR in which a single target is amplified in a single reaction tube.

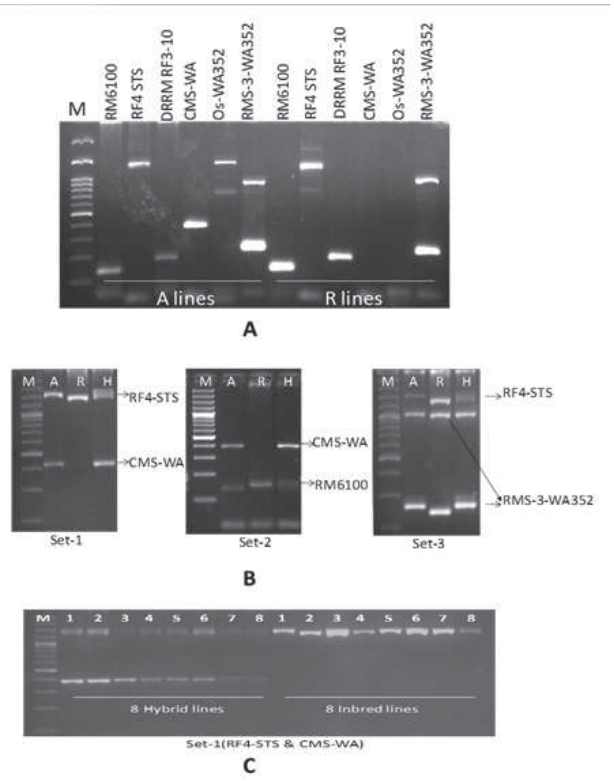


**Fig 1. A: Pictorial representation of all the six primers using the genomic DNA of A-line (31A) and R (PK117) line.. B: Differential patterns of PCR amplification of SET-1, -2, and -3 using DNA of A-line (31A), R (PK117), and H (Ajay) lines. C: PCR validation of SET-1 for differentiation of hybrids from inbreds lines. 1 to 8 (hybrid) denotes Ajay, Rajalaxmi, CRHR32, DRRH2, US382, US12, BS6444G & PA6444 and 1 to 8 (inbred) denotes Gayatri, Varshadhana, Savitri, Durga, Samlei, Utkalprabha, Pooja& Swarna-Sub1. M: 100 bp DNA ladder**

On the other hand, Multiplex PCR involves simultaneous amplification of multiple target sites in a single tube using a different pair of primer sets in combination. Six primers were integrated into three multiplex PCR primer set (SET-1, 2 & 3) by considering the PCR fragment size to avoid interfering with each other and requirement of one primer from each of the

**Table 2.** Different set/combinations of primers for *Rf*s and *WA352* genes and their expected PCR amplification patterns

| S.No. | Multiple x SET | Primers | PCR amplicon size (bp) | | |
|---|---|---|---|---|---|
| | | | A (male sterile) | R (male fertile) | H (male fertile) |
| 1 | SET-1 | Rf4-STS CMS-WA | 1488386 | 1383- | 1488, 1388386 |
| 2 | SET-2 | RM6100 CMS-WA | 160386 | 175- | 160, 175386 |
| 3 | SET-3 | Rf4-STS RMS-3-WA352 | 1488247 | 1383227 | 1488, 1383247 |

mitochondrial and nuclear genome in a SET as shown in Table 2.

In SET-1, Rf4-STS and CMS-WA markers of the *Rf4* gene and *WA352* gene were included, respectively. Similarly, two markers, namely RM6100 and CMS-WA of *Rf4* gene and WA253 gene, respectively was the component in SET-2. In the case of SET-3, Rf-STS and RMS-3-WA352 markers were combined for multiplexing. These three different primers combination as SET-1, -2 and -3 were used for multiplexing in DNA template of 31A, PK117 and Ajay (Fig. 1B). In SET-1, two distinct PCR fragments of 1488 and 386 bp were observed in 31A, one fragment of 1383 bp was observed in PK117 and three fragments of 1488, 1388 and 386 bp were observed in Ajay. The bands in the gel picture showed clear and distinct bands separating from each other (Fig. 1B Set-1) and differentiating among the three DNA of A, R and H lines. In the case of SET-2, two PCR fragments of 160 and 386 bp size in 31A, one fragment of 175 bp size in restorer PK117, and three fragments of 160, 175, and 386 bp size in hybrid Ajay were observed (Fig. 1B Set-2). However, in contrast to SET-1, the PCR bands of RM6100 in hybrid Ajay was smeared and not distinct which may be difficult to distinguish between the A and H lines for multiplexing PCR assay. In the case of SET-3, two bands of 1488 and 247 bp size in A-line, two bands of 1383 and 227 bp size in R line and three bands of 1488,1383 and 247 bp size in H line were observed (Fig. 1BSet-3). Similar to the SET-2, the PCR product of Rf4-STS in H line was smeared and not distinct which may also be difficult to distinguish between the A and H lines during multiplexing PCR assay. Comparison of all the three SET, SET-1 was found to be suitable for the assessment of genetic purity and identification of A, R and H lines in three-line rice hybrid system. Further, the multiplex PCR assay of the combination of the markers of SET-1, RF4-STS and CMS-WA were selected to assess the genetic purity in a random selection of eight hybrids and eight inbreds. Based on the PCR banding patterns, all eight hybrid lines were separated from the eight inbreds (Fig. 1C). So, these marker set of RF4-STS and CMS-WA could be useful to identify the contamination of inbred lines in rice hybrid varieties.

## Author Contribution

Conceptualization of research (UN, TM); Designing of the experiments (UN, TM, SS); Contribution of experimental materials (MP, JLK); Execution of field/ lab experiments and data collection (MP, UN, JLK, MKY, SS); Analysis of data and interpretation (MP and UN); Preparation of manuscript (MP, UN, SS).

## Declaration

The authors declare no conflict of interest.

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