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PCR-based diagnostic protocol for *orfB* gene containing transgenic nuclear male sterile rice lines

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ABSTRACT

Three-line breeding strategy involving the use of WA-CMS lines is being extensively exploited for commercial cultivation of rice hybrids. Presence of an unedited 1.1-kb transcript of the mitochondrial orfB gene and its translated product leads to male sterility in rice. CMS based 3-line systems could enable breeders to use and recombine germplasm with fewer restrictions. Bringing the mitochondrial orfB gene into nuclear background may help to overcome such limitation. The male sterile lines possessing orfB gene in nuclear background were developed and characterized. Easy diagnostic tool is needed to identify the transgenic lines containing the orfB gene in order to study the inheritance pattern of the gene. In the present study, we developed PCR-based diagnostic tool for identifying nuclear male sterile lines with orfB gene.

Key words: *orfB gene, transgenic rice, PCR-based diagnosis*

Cytoplasmic male sterility (CMS) and nuclear-controlled restoration of fertility are widespread features of plant reproduction which provide useful tools to exploit heterosis in self-pollinated crops. Presently, about 90% of commercially cultivated rice hybrids have been derived through a three-line breeding strategy which involves the use of WA-CMS lines (Singh *et al.*, 2005; Zhang *et al.*, 2015). The intricacies of the molecular mechanism underlying the cytoplasmic-nuclear interaction in the WA-CMS system in rice have come to light to a great extent. Experimental evidence for the existence of a positive functional relationship between the presence of the unedited 1.1-kb transcript of the mitochondrial *orfB* gene as well as its translated product, and male sterility has been provided (Das *et al.*, 2010; Charaborty *et al.*, 2015). Expression of this 1.1-kb *orfB* transcript in WA sterile plants increased during the flowering stage. The male fertility could be restored by specific down-regulation of the unedited *orfB* transgene, with the concurrent increase in ATPase activity. The findings of Chakraborty *et al.* (2015) considered to be the keystones towards understanding the mechanisms that govern the WA-CMS operational system when the cytoplasm-nuclear interactive genetic elements play the crucial role.

It has been known that the unedited mitochondrial *orfB* gene when constitutively made to express transgenically in nucleus followed by transport of the gene product to mitochondria in a genotype independent manner gives rise to male sterility, in an otherwise male fertile genotype of *indica* rice. What has not been tested as yet in this respect is the stability of the acquired male sterility in terms of Mendelian pattern of inheritance in filial generations for their eventual use by the plant breeder. Maintenance of the dominant nuclear male sterile line can be achieved by back crossing with the non-transgenic same genotype of rice line, as pollen parent. The F_1 seed generated plants at their seedling stage of growth can be selected for male sterility on the basis of linked selectable marker.

The long term goal of the present study is to

promote the process of advancement of rice hybrid seed production technology endowed with higher out crossing rate as a result of which seed cost will be reduced. The cytoplasmic uniformity as like the WA cytoplasm will be reduced.Tansfer of nuclear based sterility is easy to any background as compared to CMS based male sterility (Wang *et al*., 2013).

The understanding that is recently acquired (Das *et al.*, 2010 and Charaborty 2015) on how the WA cytoplasm stands responsible for male sterility in rice and also how nuclear coded restorer of fertility (*Rf*) genes undo the influence of the WA cytoplasm to regain male fertility, it is expected that the same would provide important clues to genetically manipulate the WA-CMS system to our advantage in developing an alternative strategy for hybrid seed production in rice. This is expected to bring in further improvement in rice hybrid seed production technology with higher efficacy. The 3-line rice hybrid seed production protocol that is presently practised is based on some system of controlled pollination, and each system entails some compromise and sacrifice. For example, the CMS based hybrid seed production protocol requires development and maintenance of separate male and female (seed) gene pools. The progeny of the female gene pool is required to have a very reliable and stable male-sterile phenotype, when producing the hybrid seed. In fact only a subset of the female gene pool has the genetic constitution that confers the desired phenotype in a reliable manner. As a result of this, the female gene pools are often shallower or less diverse than the male gene pools. This is a major constraint placed on genetic gain. This can create additional risks associated with genetic, uniformity of the organellar genomes of the female gene pools. On the other hand, we see the possibility of evolution of a genetically transformed male sterility system, where the functional properties of the genetic factors responsible for the WA-CMS system, could be redesignated to function into a dominant nuclear male sterility system for hybrid seed production. This possibility could be classified in the same line of first generation of this sterility system (Mariani *et al.*, 1990; 1992), when the components of CMS, the sterile, maintainer and restorer lines will not be required. Potentially, such systems could enable breeders to use and recombine germplasm with fewer restrictions. It is believed that the possibility for development of such an approach would be facilitated, as our understanding on the role of the genetic elements that are operative in the 3-line hybrid seed production protocol is fairly rich.

As a part of the long term goal, the male sterile lines possessing *orfB* gene in nuclear background were developed and characterised (Chakraborty *et al.* 2015). In order to study the inheritance pattern, there is a need for easy diagnostic tool to identify the transgenic lines containing the *orfB* gene. In the present study, we developed PCR-based diagnostic tool for identifying nuclear male sterile lines with *orfB* gene.

MATERIALS AND METHODS

Plant materials

The plant materials having the *orfB* gene in nuclear background were developed at IIT, Khargpur (Chakraborty et al.2015). The *orfB* gene construct present in the plant has been depicted in Figure 1. The materials were received in the form of cross seeds between the male sterile IR64 rice line having the orfB construct and the maintainer IR64 line along with the parental lines.

Growth and maintenance of materials

The cross seeds were germinated in petriplates and transferred to pots and grown under controlled condition of transgenic glass house. The normal IR64 plants were grown in staggered planting to match the flowering date

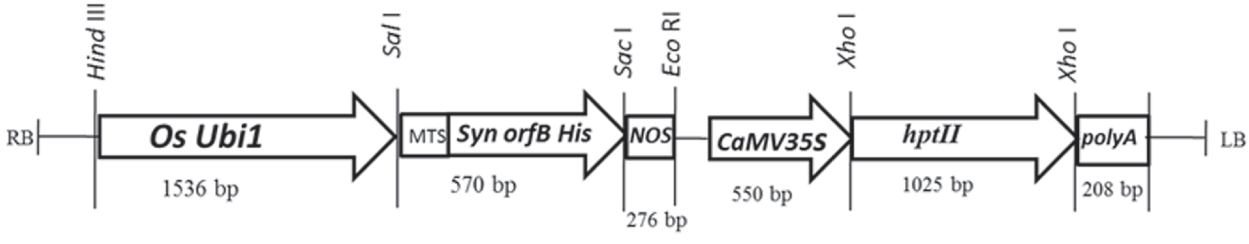

Fig. 1. Schematic diagram of *Syn orfB* with His-tag construct in pCAMBIA1300 vector (Chakraborty et al. 2015)

with the transgenic lines. The hybridization of the plants with normal IR64 was performed to maintain the lines.

Evaluation for spikelet sterility

The primary panicles of each plant of both male sterile and maintainer lines were bagged and allowed for selfing to check the sterility level of the plant. The number of grains and chaffs were counted and the mean percentage of sterility was calculated.

Pollen sterility test

Fertile pollens were differentiated from sterile pollens by staining in 1 % acetocarmine or 1 % KI followed by observation under a light microscope. Viable/fertile pollen showed a high level of staining, whereas non-functional/sterile pollen remained unstained. The percentage of viable pollens was calculated taking mean of five focus area.

Designing of primers

The *hptII* region of the construct was targeted for designing primers for selecting the plants containing the *orfB* gene. The primers were designed using primer3 Input (version 0.4.0) software available online at http://bioinfo.ut.ee/primer3-0.4.0/ and http://simgene.com/Primer3 considering GC content below 50% and Tm ~60^{0}C.

DNA Isolation

Leaves were collected from 21 days old seedling to extract genomic DNA for molecular screening for presence of *orfB* gene. Total genomic DNA was extracted after crushing in liquid nitrogen in microfuge tubes using CTAB extraction buffer (100 mM Tris-HCl pH 8, 20 mM EDTA pH 8, 1.3M NaCl, 2% CTAB) and chloroform-Isoamyl alcohol extraction followed by RNAase treatment and ethanol precipitation (Murray and Thompson, 1980). Agarose gel electrophoresis was used to estimate DNA concentration, and each sample was then diluted to approximately 30ng/µL.

PCR Amplification and visualization of products

DNA amplification reaction was performed in a volume of 20µl containing 1.5mM Tris HCL (pH 8.75), 50mM KCL, 2mM $MgCl_2$, 0.1% TritonX-100, 200µM each of dATP, dCTP, dTTP, dGTP, 4pmole of each forward and reverse primers (Table 1), 1 unit of Taq Polymerase and 30ng of genomic DNA. Amplification was performed in a Programmable Thermal Cycler (Verity, Applied BioSciences). The reaction mixture was first denatured for 4 mins at 94^{0}C and then subjected to 35 cycles of 1 min denaturation at 94^{0}C, 1 min annealing at 55^{0}C, and 1 min extension at 72^{0}C; and then a final

Table 1. Amplification details of the primers designed for identification of male sterile transgenic lines

Primer name	Sequence of primer (5'->3')	Tm (^{0}C)	GC content	Expected size of product (bp)	Observed size of product (bp) in the transgenic IR64	Observed size of product (bp) in the maintainer IR64
Orf_hptII_0_F	AGTTTAGCGAGAGCCTGACCTAT	60.00	47.8	195	200	Not detected
Orf_hptII_0_R	ATGTAGTGTATTGACCGATTCCTTG	60.50	40.0			
Orf_hptII_1_F	AGTTTAGCGAGAGCCTGACCTAT	60.00	47.8	196	200	Not detected
Orf_hptII_1_R	CATGTAGTGTATTGACCGATTCCTT	60.50	40.0			
Orf_hptII_2_F	AGTTTAGCGAGAGCCTGACCTATT	61.10	45.8	195	200	Not detected
Orf_hptII_2_R	ATGTAGTGTATTGACCGATTCCTTG	60.50	40.0			
Orf_hptII_3_F	AGTTTAGCGAGAGCCTGACCTATT	61.10	45.8	196	200	Not detected
Orf_hptII_3_R	CATGTAGTGTATTGACCGATTCCTT	60.50	40.0			
Orf_hptII_4_F	GTTTAGCGAGAGCCTGACCTATT	60.30	47.8	194	200	Not detected
Orf_hptII_4_R	ATGTAGTGTATTGACCGATTCCTTG	60.50	40.0			
Orf_hptII_5_F	GCGAAGAATCTCGTGCTTTC	60.10	50.0	165	170	Not detected
Orf_hptII_5_R	TCTCGCTAAACTCCCCAATG	60.21	50.0			
Orf_hptII_6_F	CTCGATGAGCTGATGCTTTG	59.70	50.0	165	170	Not detected
Orf_hptII_6_R	GATGTTGGCGACCTCGTATT	59.96	50.0			

extension for 10 mins at 72^{0}C. Aliquots of 10ml of DNA products from PCR amplification were loaded in 2.5% agarose gel containing 0.8mg/ml Ethidium Bromide for electrophoresis in 1X TBE (pH 8.0). 50bp DNA ladder was used for determination of size of amplicons. The gel was run at 60 volts (2.5V/cm) for 4 hrs and photographed using a Gel-Doc System (SynGene).

RESULTS AND DISCUSSION

Phenotypic expression of *orfB* gene in nuclear background

The *orfB* gene in cytoplasmic background leads to cytoplasmic male sterility. When the *orfB* gene is brought to nuclear background (Das et al. 2010), the phenotypic expression of the gene was studied. The percentage of filled grains and the chaffs from bagged panicles were considered to check the fertility and sterility level respectively of the plants. The normal IR64 variety showed 11.96% sterility whereas the transgenic line showed 100% sterility (Table 2). To confirm the expression of the gene, the pollen viability test was carried out by acetocarmine staining as well as KI staining. In the normal IR64 variety, 98.2% pollens were observed to be deeply stained indicating their viability, whereas in the transgenic male sterile line all the pollens observed were sterile. Although there was limited phenotypic study by Chakraborty *et al.* (2015), the present extensive study confirms their result.

PCR based diagnosis for transgenic lines

The transgenic IR64 male sterile lines are to be maintained through crossing with the normal counterpart. It is expected that there will be mendelian segregation of the nuclear *orfB* gene and the plants have to be identified before progressing to next generation for maintenance. To identify the lines by allowing for selfing through bagging is time and labour consuming where the major limitation is that the sterile plants can be identified after maturity only. So, molecular diagnosis of the sterile plants before flowering can be useful for crossing of the selected plants only. Using a PCR based marker for the synthetic *orfB* gene did not work due to presence of endogenous *orfB* gene in cytoplasmic background leaving the only option to confirm through southern hybridisation which was again time consuming and costly. Hence, we went for PCR based diagnosis targeting the *hptII* selectable marker region to identify the lines. For the purpose, seven primers were designed and used to test their usability to screen the transgenic lines at the segregating stage. The primers designed were tested in the parental lines, i.e. normal IR64 and transgenic IR64 possessing the *orfB* gene in nuclear background. All the primers designed were able to amplify the target region in the transgenic line containing the *orfB* gene in the nuclear background. The maintainer IR64, the normal counter part of the transgenic line did not show any amplification (Figure 2) with all the primers except the Orf_hptII_6 where it showed a larger amplicon of 260bp being distinct from the target band of 165bp. The primer Orf_hptII_0 showed multiple bands along with the target band of 195bp, hence tried with higher annealing temperature of 58^{0}C where the only target amplicon was observed. The markers used, expected product size and the actual products visualised in both the parents are presented in Table 1. The markers Orf_hptII_0, Orf_hptII_1, Orf_hptII_2, Orf_hptII_3, Orf_hptII_4 and Orf_hptII_5 were observed to be effective. This confirmed that the primers designed are able to distinguish the transgenic lines with *orfB* gene from the normal maintainer line. Hence, this tool can be used in identification of the transgenic rice lines with the construct having *hptII* gene as the selectable marker. Also, these markers can be useful for screening the progenies and studying the segregation pattern of the transgene. Shimizu et al. (2002) and Christou et al.

Table 2. Impact of *orfB* gene on spikelet fertility and pollen viability

Parental line	Presence of *orfB* gene in nuclear background	Percentage of sterile pollen	No. of grains	No. of chaff	Spikelet sterility (%)	Remark
Transgenic male sterile IR64 line	Present	100	0	278	100.00	Complete sterility
Maintainer IR64 line	Absent	1.8	243	33	11.96	Fertile line

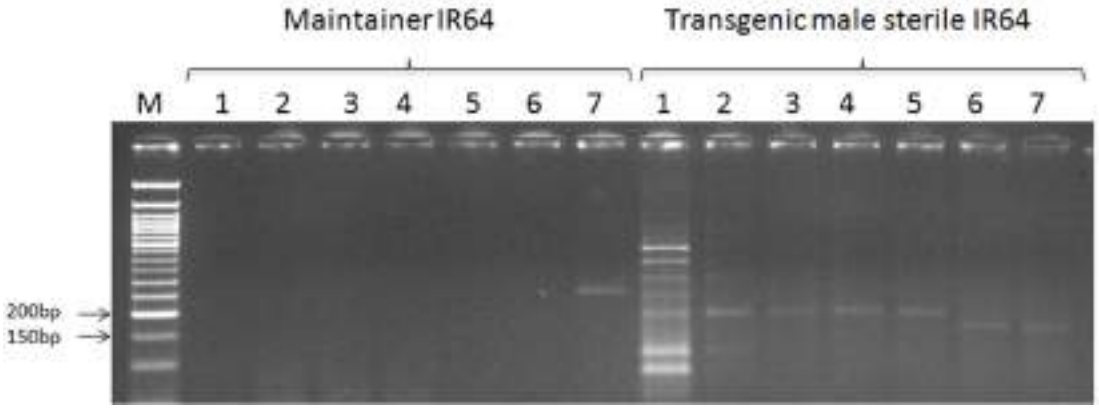

Fig. 2. Electrophoregram showing amplification pattern of maintainer IR64 and transgenic male sterile IR64 with the designed primers. The numbers denote the serial number of the primers depicted in table 1.

(1991) also used PCR based diagnosis of transgenic rice. PCR based diagnostic tool was also useful to identify mycobacterium strains. Babekova et al. (2008) also proved PCR-based diagnosis for identifying transgenic Bt-rice lines by targeting the maize ubiquitin promoter and *Cre* gene.

Genetic male sterility based hybrid is an innovative and futuristic approach. The cost of hybrid seed production will be reduced considerably if this innovative approach will be effective in future. The transgenic lines carrying *orf* gene can be verified by the primers Orf_hptII_0, Orf_hptII_1, Orf_hptII_2, Orf_hptII_3, Orf_hptII_4 and Orf_hptII_5. These primers can also be used for purity analysis in case of adulteration of maintainer line and hybrid based on this *orf* gene. Hence, this research aspect has a bright prospect in future.

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