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HYBRID PURITY ANALYSIS OF RICE USING MICRO SATELLITE MARKERS

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Abstract– Rice is the staple food crop which can fight against hunger and poverty in most of the developing nations. Hybrids perform better than the traditional verities in both yield and quality aspects. But the main problem lies in the genetic purity of the hybrids. In this study, molecular test was conducted and compared with the grow out test to assess the genetic purity of the rice hybrid, JGLH1 at Seed Research and Technology Centre, Professor Jayashankar Telangana State Agricultural University (PJTSAU), Hyderabad. JGLH1 and its parents were used as materials, using SSR molecular marker technology, to select primers and identify genetic purity according to its polymorphism. The SSR marker, XA 21 was amplified on the DNA extracted from the rice hybrid, JGLH1, and its parents, CMS 64A and JBR7. The amplification of marker was conducted on the DNA of 20 hybrid leaves and 1 leaf from each parent, separately. The marker was amplified at 650 bp in the parent, CMS-64A, and 730bp in JBR7, and amplified at both the base pairs in their F1 progeny, JGLH1. This confirms the hybridity of the rice hybrid JGLH1. The marker was unveiled in all the 20 leaves of the hybrid rice sample ensuring the purity of the seed lot. The grow out test results also showed 100% purity without any deviations. It concludes that molecular marker, XA 21 is effective in hybrid purity evaluation of the rice hybrid, JGLH 1.

INTRODUCTION

Rice (*Oryza sativa* L.) is a major cereal crop that feeds over one third of the world's population (Sarangi *et al*., 2019). Moorthy *et al.* (2011) and Bora *et al.* (2016) estimated that 90% of the global rice production and consumption is in the Asian countries. India, China, Indonesia, and Vietnam are some of the top rice-producing countries in the world. India ranks first in terms of consumption and it is second in the production and first in the area of cultivation under rice, although it is the world's second-most-consumed cereal, after maize (Tiwari *et al*., 2018). Rice output in the country has not only doubled, but has also permitted self-sufficiency, with the Green Revolution (Singh *et al.,* 2020). However, due to a continual increase in population, rice consumption is expected to rise unquestionably in the near future. This means that more than half of the world's population is reliant on rice production and supply to secure food and nutritional security in coming future (Bora *et al*., 2016; Singh *et al*., 2020). The demand for rice and processed rice products have been gradually increasing across the globe. According to FAO, the world witnessed a growth of 100 million metric tons of rice production in the last 10 years. Rout *et al*. (2021) reported that the evolution of hybrid rice cultivars is one of the major factors for such a drastic increase over the last few years. Hybrids have a yield advantage of 20% over the high-performing inbred lines (Huang *et al.,* 2020) and have been widely cultivating in many regions. The total cultivation area of hybrid rice is anticipated to increase significantly in the coming years because of the increasing food demand.

Hybrid technology has been successfully exploited in rice for many years but face few challenges (Spielman *et al*., 2013). According to Li and Yuan (2010), maintaining the genetic purity of the seed lot is one of the major concerns in hybrid rice production as it is a self-pollinating crop. Mao *et al.* (1998) estimated that 1 % of genetic impurity in rice can cause a yield decline of 100 kg ha^{-1}. Therefore, maintenance of genetic purity in the parental and hybrid lines of rice seed is essential. Chetan Kumar and Prasad (2012) showed that

genetic impurity in rice is majorly due to the presence of pollen shredders, outcross with foreign pollen, and mechanical mixtures. Hence, the purity test of the hybrid lines is done to identify the seed mixtures present in the seed lot. Pourabed *et al.* (2015) reported that a genetic purity test is an inevitable procedure during the process of foundation and breeder seed certification. Highly qualified, and multiple purity tests have to be performed before the commercialization of any hybrid seed. Genetic purity test is usually conducted by two methods, Grow Out Test (GOT) and by using molecular markers. The former is time-consuming and laborious, whereas the latter one is relatively less time-consuming and more accurate (Nandakumar *et al*., 2004). Nagendra *et al.* (2020) reported that Currently, SSR (Simple Sequence Repeat) markers are the most popular molecular markers utilized for hybrid purity testing. The major objective of the current study is to confirm the hybridity and test the genetic purity of the rice hybrid, JGLH1 (CMS 64A × JBR7) using the SSR marker XA 21.

MATERIALS AND METHODS

Materials

The rice hybrid, JGLH1 along with their parents obtained from PJTSAU, Hyderabad, was used for the purity test. Four hundred seeds of the rice hybrid and 100 seeds of their parents were randomly drawn from the seed lot and sown in the field for grow out test. The sampling of leaves for the hybrid purity test was done by the two dimensional DNA sampling method given by Nas *et al*. (2000). A sample of 20 leaves from different plants of the rice hybrid JGLH1 has been collected from the field. Similarly, one leaf each was drawn from the parents and used for the molecular test. The experiment was conducted at the Seed Research and Technology Centre (SRTC), PJTSAU, Hyderabad, Telangana, India.

DNA Extraction

Total genomic DNA was extracted from Rice leaves by the CTAB method given by Murray and Thompson (1980) with some modifications. Approximately 3 seedlings of Rice was transferred to mortor and masticated to fine suspension with CTAB buffer (100 mM Tris, 20 mM EDTA, 1.4 M NaCl, 2% CTAB, pH 8.0). This fine suspension was taken into 2 ml Eppendorf tubes. These tubes were incubated in a water bath at 65 ^{o}C for 40 minutes with intermittent mixing for every 15 minutes.

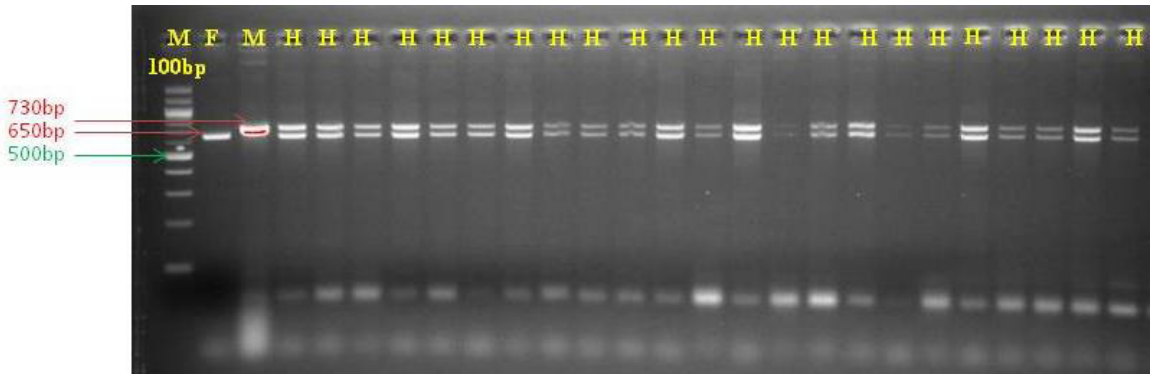
The incubated samples were allowed to attain room temperature and an equal volume of Chloroform: Isoamyl Alcohol (C:IAA= 24:1) was added, gently mixed for 15 minutes, and centrifuged at 10,000 rpm for 5 minutes. The aqueous layer obtained is transferred to 1.5 ml Eppendorf tubes. To these tubes containing supernatant, an equal volume of chilled Isopropanol was added and gently mixed by inverting the tubes. These tubes were incubated in a refrigerator for 15 minutes and centrifuge at 10,000 rpm for 5 minutes. The supernatant obtained is discarded and the pellet is washed with 100 µl of 70% Ethanol, followed by centrifugation at 10,000 rpm for 5 minutes. The pellet is air-dried and dissolved in 50-100 ìl sterile distilled water/ $T_{10}E_{0.1}$ buffer, where $T_{10}E_{0.1}$ buffer facilitates for longer storage whereas sterile distilled water for shorter storage. The samples were stored in a -20 ^{0}C refrigerator for further use.

PCR and Gel electrophorosies

The SSR Markers XA 21 was amplified with the 20 samples of the rice hybrid JGLH1 and their parents. XA 21 primer is used for screening and its details are provided in Table 1. PCR and gel electrophorosis was conducted according to Lee *et al.* (2012). The annealing temperature of SSR primer pairs was standardized using temperature gradient PCR (Surecycler 8800). 4 different temperatures were analyzed in the range of 50-60 ^{o}C and standardized at 54 ^{o}C. PCR reactions were performed in 20 µl volume using a programmable thermal cycler (Surecycle 8800, Agilent). The reagent mixture included a 2ng template DNA, 10 pmol of both forward and reverse primers, (IDT, Germany), 2µl of 10X PCR buffer (10Mm Tris-HCl (pH 8.0); 50 mM KCl, 2mM $MgCl_2$, 1U of *Taq* DNA polymerase (NEB, UK) 0.4mM dNTPs (Bangalore genei). Ten templates

Table 1. Details of the rice hybrid and primer used in the study

Rice hybrid	Parents	Primer	Forward Sequence	Backward sequence
JGLH1	CMS 64A and JBR7	XA 21	AGA CGC GGA AGG GTG GTT CCC GGA	AGA CCG GTA ATC GAA AGA TGA AAG



Lane M: 100bp DNA ladder; Lane F: CMS-64A; Lane M: JBR-7; Lane H: JGLH1

Fig. 1. Amplification pattern obtained using SSR marker XA 21

were taken per each primer and they were amplified using the following PCR program.

The PCR temperature regime was as follows

	Step	Temperature	Duration
1.	Initial Denaturation	94 ^{0}C	5 minutes
2.	Denaturation	94 ^{0}C	45 seconds
3.	Annealing	54 ^{0}C	30 seconds
4.	Extension	72 ^{0}C	30 seconds
5.	Go to step 2-4 30 cycles		
6.	Final extension	72 ^{0}C	8 minutes
7.	14 ^{0}C forever		

The PCR amplified product was mixed with 2µl of 6X single dye and loaded on 3.0% Agarose gel. The gels were electrophoresed at a constant voltage of 100 volts, 400mA for 90 minutes. Later the Ethidium bromide-stained gels were documented in the gel documentation system.

RESULTS AND DISCUSSION

Marker analysis on Agarose gel revealed that the SSR marker Xa 21 confirms the hybridity of the rice variety, JGLH1, which is an F1 progeny of CMS-64A and JBR-7. The marker was amplified at 650 bp in the parent, CMS-64A (A-line), and 730bp in JBR7 (R-line), but the marker was amplified at both the base pairs in its offspring, JGLH1 (Hybrid). The presence of marker at both the base pairs ensures that the rice cultivar JGLH1 is a hybrid between the above-mentioned parents. All the 20 leaves of the rice hybrid sample showed the marker at both the base pairs validating the purity of the hybrid seed lot. The gel documentation showing the hybridity of JGLH1 was shown in Figure 1 along with the band lengths of its parents.

The Grow out test performed during Rabi 2019 was also in alignment with the result of the marker test. In the Grow-out trials, purity evaluation was conducted based on the morphological characters such as days to flowering, days to maturity, pollen sterility, presence of awns on spikelet, panicle length, flag leaf senescence, and nodal pigmentation. All the characters showed 100% purity without any major deviations.

Genetic purity test during various stages of breeding and before hybrid release is mandatory to avoid any impurities in the seed lots. (Anupama *et al.*, 2020) and Deshmukh *et al.* (2013) proved that molecular markers are efficient than the field test in the genetic purity studies. Many markers were identified to confirm the hybridity and check the genetic purity of hybrid rice lines. For example, Sundaram *et al*. (2008) identified 48 SSR markers and marker combinations, which recognized 10 CMS and restorer lines along with the hybrids. Kumar *et al.* (2021) also found 14 markers that are specific to 16 high yielding rice varieties. Singh *et al.* (2015) reported that apart from genetic purity, the presence of a validated marker on the hybrid DNA also proves the hybridity of a line between specific parents and also differentiates the hybrid from its parents. Thus hybrid purity tests can be useful in multiples ways such as confirming hybridity, testing genetic purity, and separating self and cross-pollinated lines (Pallavi *et al.*, 2011). The SSR Marker, XA 21 has been already identified as specific to the hybrid rice line, JGLH1, and its parents. The current study had reconfirmed that the marker XA 21 is the indicator of the hybrid rice line, JGLH1. The study clearly depicts that application of SSR markers for hybrid purity test is one among the best methods for the seed purity test.

CONCLUSION

The presence of XA 21 SSR marker at 650 bp and 730 bp in the parents, CMS-64A, and 730 bp, respectively and at both the base pairs in it hybrid, JGLH 1 confirms the hybridity of the rice hybrid, JGLH 1. The presence of marker at both the base pairs in all the samples tested ensures the hundred percent purity of the seed lot.

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