***Original Research Article***

**Molecular diversity analysis by reported genes/*QTL*s of advanced breeding lines of Rice (*Oryza sativa* L)**

**Abstract**

An investigation was conducted at Indian Institute of Rice Research (IIRR) farm located at Rajendranagar, Hyderabad. The experimental field was laid in RBD design using 30 advanced Breeding Lines ABL (SP-02, SP-03, SP-08, SP-13, SP-25, SP-34, SP-37, SP-55, SP-57, SP-61, SP-63, SP-69, SP-70, SP-72, SP-75, SP-80, SP-351, SP-352, SP-353, SP-354, SP-355, SP-356, SP-357, SP-358, SP-359, SP-360, NDR-359, IR-64 and JAYA), including one check variety BPT-5204. BPT-5204 used as quality check. For molecular characterization of advanced breeding lines seven genes (*Gn1& Gn2* for *grain number; SCM2* and *SCM3* for *strong culm; Gs3* for *grain size; Gw5* for *grain weight; Spl14* for *spikelet length*) and three *QTL*s (*Yld12.1, Yld2.1 and Yld4.1* for yield) were used. Upon genotyping, 30 advanced breeding lines, only one advanced breeding line SP-08 showed the presence of seven yield contributing genes/*QTL*s (*Gn1, Gn2, SCM2, SCM3, Gw5, Yld12.1, Yld2.1*) indicating presence of high potential for yield. Two advanced breeding lines showed the presence of six yield contributing genes/*QTL*s, they are SP-69 (*Gn1, Gn2, SCM3, Gw5, Spl14, Yld2.1*) and SP-70 (*Gn1, SCM3, Gw5, Gs3, Yld2.1, Yld12.1*). Whereas four advanced breeding lines showed the presence of five yield contributing genes. They are SP-37 (*Gn2, SCM3, Gw5, Yld12.1, Yld2.1*), SP-55 (*Gn2, SCM2, SCM3, Gw5, Yld2.1*), SP-75 (*Gn1, Gn2, SCM3, Gw5, Yld12.1*) and SP-61 (*Gn1, Gn2, SCM2, Gw5, Yld2.1*). The morphological and physiological parameters of advanced breeding lines were correlated with the molecular analysis. SP-08 showed better morphological and physiological parameters like number of tillers, LAD, LAI, CGR, NAR, and also showed the presence of seven (*Gn1, Gn2, SCM2, SCM3, Gw5, Yld12.1 and Yld2.1*) yield contributing genes/*QTL*s. Genotype, SP-70 showed better in several physiological parameters photosynthetic rate, SPAD meter readings, dry matter accumulation, and also contain six (*Gn1, SCM3, Gw5, Gs3, Yld2.1 and Yld12.1*) genes/*QTL*s. Genotype, SP-69 higher panicle length and also contain six corresponding genes governing (*Gn1, Gn2, SCM3, Gw5, Spl14 and Yld2.1*) genes/*QTL*s. Two advanced breeding lines namely SP-08, SP-70 can be further probed thoroughly for further increasing yield and yield attributes.

**1. Introduction**

 Rice (*Oryza sativa* L.) belongs to the family graminae and sub family Oryzoideae. As a cereal grain, it is the most important staple food crops in the world. In Asia, more than two billion people are getting 60-70 per cent of their energy requirement from rice and its derived products. In the twenty-first century, the world faces a serious challenge in that agricultural land area has sharply decreased in contrast to a population explosion. To solve the crisis of food shortage, there is necessity to increase crop productivity of rice as rice is the primary staple food for one third of the world population after wheat and maize.

Therefore identification of stable advanced lines having specific traits at whole plant level and their confirmation at molecular level would be highly appropriate. It is considered a model cereal crop in the world due to its relatively small genome size, vast germplasm collection, enormous repertoire of molecular genetic resources, and efficient transformation system (Paterson *et al*., 2005).

 So far, several high yielding and management responsive varieties have been developed and released for improved crop production. Among which Samba Mahsuri, a hybrid derived from the cross (GEB 24 x TN1) rice is otherwise called Sona Mahsuri/ Samba Mahsuri/ BPT-5204 which is premium quality aromatic and light weight rice. Due to its excellent grain character, variety being regularly used in hybridization programmes to meet current breeding objectives. Therefore, use of advanced breeding lines generated from BPT-5204 would only be appropriate and evaluation of available germplasm or mutants for various physiological and yield attributes is essential (Babaei *et al.*, 2011).

 *Semi dwarf1* (*sd1*), the first cloned rice dwarfing gene, has contributed most significantly to rice breeding (Ashikari *et al.,*1999). The gene *sd1* was first identified in the Chinese semi dwarf rice variety Dee-geo-woo-gen, which was used to develop the semi dwarf cultivar IR8 that produced record yields throughout Asia and formed the basis for the development of several high yielding semi dwarf varieties (Kulkarni *et al.,*2014). The number of productive tillers per plant is an important yield component in rice and other cereals because tillers are the panicle-bearing branches. The tiller number is controlled by *quantitative trait loci* (*QTL)* (Wang *et al*., 2008).

Krishna *et al.* (2013) reported that on *chromosome-12* expression of *QTL, qDTY12.1* (reproductive-stage drought stress), significantly associated with grain yield. This *QTL* explained phenotypic the variance of 23.8% and contributed an additive effect of 45.3% for grain yield under drought. The positive *QTL* allele for *qDTY12.1* was contributed by tolerant parent IR74371-46-1-1.

**2. Material and Methods**

 The investigation was conducted during *Kharif* season 2014 and 2015 at the research farm of Indian Institute of Rice Research (IIRR), Rajendranagar, Hyderabad located at Latitude 170 19’ N, Longitude 780 28’ E and at an altitude of 542 m above the Mean Sea Level. The experiment was laid out in a Randomized Black Design (RBD) with 30 high yielding advanced rice genotypes (SP-02, SP-03, SP-08, SP-13, SP-25, SP-34, SP-37, SP-55, SP-57, SP-61, SP-63, SP-69, SP-70, SP-72, SP-75, SP-80, SP-351, SP-352, SP-353, SP-354, SP-355, SP-356, SP-357, SP-358, SP-359, SP-360, NDR-359, IR-64 and JAYA), including one check variety BPT-5204. Treatments replicated thrice. 30 genotypes selected from high yielding advanced breeding lines (ABL). All these genotypes are derived from BPT-5204 through EMS mutagenesis process. BPT-5204 is a parent (wild type) was used as check.

**2.1 Molecular Analysis**

Advanced breeding lines used for molecular characterization are the pre released lines which are homozygous mutant lines. These constitute an important source of genetic variation for utilization in breeding of high yielding rice varieties and hybrids. These lines were characterized for genes and *QTLs*, seven yield associated genes *viz., Gn1, Gn2, SCM2, SCM3, Gs3, Gw5, Spl14* and three *QTLs viz., Yld12.1, Yld2.1 and Yld4.*1 (Table 1).

**2.1.1. Genotyping for yield *QTL*s**

**2.1.1.1 Collection and preservation of the leaf material**

Leaves were collected from 45-50 days old field grown plants in plastic bags and immediately kept in the ice box and stored at -80°C till further use.

**2.1.1.2 Genomic DNA isolation**

1. DNA was isolated by following the protocol of Zheng *et al.* (1995) with modifications as detailed below: Leaf pieces were cut into small bits in a sterile porcelin mortar and liquid nitrogen was added till the leaf bits were completely immersed. The leaf bits were then ground into fine powder using a sterile porcelin pestle. It was ensured by continuously adding liquid nitrogen that the leaf powder did not thaw while grinding.
2. Immediately after the grinding, 3-4 ml of DNA extraction buffer (50 mMTris-HCl pH 8; 25 mM EDTA, 300 mMNaCl and 1 % SDS) was added to the mortar and incubated at 650C for about 5min in a water bath. Once the solution was thawed, the contents from the mortar were transferred to 3-5 sterile, labeled 1.5 ml micro-centrifuge tubes using 1000l capacity micropipette using micropipette tips cut at their tip portion. The micro-centrifuge tubes were incubated for 15 min at 650 C.
3. After the incubation step, approximately 400 l of Phenol: Chloroform: Isoamylalcohol (25:24:1) mixture was added to each micro-centrifuge tube. It was ensured that the pH of phenol used was ~8.0. The contents were mixed well by inversion for about 10 min and centrifuged at 10,000 rpm (~8000 g) for about 10 min at room temperature.
4. After centrifugation, the supernatant was transferred from the micro-centrifuge tube into freshly labeled sterile 1.5 ml micro centrifuge tubes. Care was taken that the intermediate layer of insoluble proteins was not disturbed. If the supernatant was not clear, the phenol: chloroform: isoamyl alcohol purification step was repeated once again. To the supernatant, 5 µl of *RNase* (10 mg/ml) was added and incubated for 30 min at room temperature and again treatment with an equal volume of chloroform (400µl) was added and centrifuged at 10,000 rpm (~8000 g) for 10 min at room temperature.
5. To the clear aqueous supernatant, the 1/8th volume of 3M sodium acetate (pH 5.2) and an equal volume (500-600 µl) of chilled Isopropyl alcohol was added. The contents were mixed gently and centrifuged at 10,000 rpm (~8000 g) for 10 min at room temperature.
6. The supernatant was drained gently and about 200 µl of 70 % ethanol was added to the pellet collected at the bottom of the micro centrifuge tube. The tube was tapped gently so that the pellet was disturbed. Centrifugation is done at 10,000 rpm (~8000 g) for 10 min at room temperature. After this, the supernatant was drained and the pellet was washed with 70 % ethanol once again as described above.
7. Finally, the pellet was left for air-drying overnight at room temperature with the tube cap open. After complete drying of pellet, depending on the size of the pellet, about 50-100µl of sterile TE buffer (10 mMTris-HCl, pH 8.0 and 1 mM EDTA) was added to the tube for dissolving the pellet. The DNA solution was then stored at 4˚C until further analysis.

**2.1.1.3 Analysis of quality and quantity of isolated DNA**

The quality and quantity of isolated DNA were checked through Ethidium bromide stained Agarose gel electrophoresis. A 0.8 % Agarose gel was prepared for electrophoresis of DNA samples as follows

* 0.8 g of Agarose was weighed and added to 100 ml of 0.5 x TBE in a 250 ml conical flask.
* One liter of 10X TBE was prepared as follows: 108 g of Tris base (Sigma, USA) was taken in 500 ml double distilled water, to which 55 g of Boric acid (Qualigens, India) was added and mixed well by stirring. This was followed by the addition of 40 ml of 0.5M EDTA of Na4 EDTA and the volume was finally made up to 1000 ml using double distilled water. The solution after thorough mixing was filter sterilized using 0.45 m filter (Millipore, USA), autoclaved and stored at room temperature. This was used for the preparation of 0.5X TBE by diluting 10X TBE twenty times with sterile distilled water.
* 100 ml of 0.5X TBE was added to 0.8 g of agarose in a 250 ml beaker and boiled gently in a microwave oven.
* After the Agarose was properly melted (when the solution was crystal clear with no floating particulate matter), the solution was taken out of the microwave oven and allowed to cool down.
* Meanwhile, a gel-casting tray (CBS Scientific, USA) was washed thoroughly first with tap water and then with distilled water followed by rinsing with methanol.
* The gel-casting tray was then placed in a sealing mechanism given by the supplier (CBS Scientific, USA) and a comb (containing 8 lanes) was arranged in its slot on the gel-casting tray.
* When the boiled Agarose had cooled down substantially (to about 450C), 2 l of Ethidium bromide solution (10 mg/ml of distilled water) was added and mixed gently. Gloves were used while handling Ethidium bromide.
* The melted Agarose was poured in the gel casting tray by carefully avoiding air bubbles and allowed to polymerize (~20-30 min).
* After solidification, the gel was removed from its casting tray and put into a gel tank, which was then filled with 0.5X TBE buffer till the buffer reached 0.5 cm above the gel surface (~100 ml).
* Five microlitres of DNA was mixed with ~1-2 l of Bromophenol blue dye (0.0025 % Bromophenol blue in 40 % sucrose), loaded into the wells and the electrodes were connected to Power pack. (CBS Scientific, USA). ƛDNA solutions of known concentration (50 ng/l, 100 ng/l, 150 ng/l and 200 ng/l) were also loaded along with the samples.
* The samples were electrophoresed at 75 V for 20-30 min till the dye front reached 2/3rd of the running length of the gel. The gel was then visualized under UV light in an Alpha Innotech gel documentation system (Alpha Innotech, USA) for checking the quality and quantity of DNA. Based on comparison with the intensity of DNA solution of known concentration, the unknown samples were diluted to a final concentration of ~ 50 ng/l using 1X TE buffer.

**2.1.1.4 Polymerase Chain Reaction (PCR):**

The genthe genomic DNA of 30 advanced breeding lines was subjected to PCR amplification as per the procedure described. PCR was carried out using a programmable thermo cycler (Applied Biosystem, USA). PCR reactions were carried out in 10µl reaction volume containing 30-50 ng of template DNA, 0.2 µM of each primer (both forward and reverse primers), 200 µM of deoxyribonucleotides, 1.5 mM MgCl2, and 1 unit of Taq DNA polymerase (Banglore Genei Private Limited, Banglore). The PCR profile adapted for amplifying SSRs was:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Stage | Steps | Temperature (oC) | Duration (min & sec) | No. of cycles |
| I. | Initial denaturation | 94 | 5 min | 1 |
| II. | Denaturation | 94 | 30 sec | 35 Cycles |
| III. | Annealing | 55/58 | 30 sec |
| IV. | Extension | 72 | 1 min |
| V. | Final extension | 72 | 10 min | 1 |

List 1: PCR profile adapted for amplifying SSRs

For SSR markers the above said protocol followed. Whereas for functional markers annelling temperature is 58 0C and remaining programme is same as above.

**Table 1: High yield contributing genes/*QTL*s and related markers with references**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **S.No** | **Gene/*QTL*s** | ***Chromosome*** | **Location** | **Markers** | **References** |
| 1 | *Gn1* | *Chromosome-1* | 5.5Mb-7.9Mb | Gn1A\* Gn1A17\* Gn1INDE\* RM10499 RM151 RM10382 | Miura *et al.*(2010)Yoshida *et al.*(2013)Liu *et al.*(2015) |
| 2 | *Gn2* | *Chromosome-2* | 32.7Mb-35.1Mb | RM250 RM208 | Zhao *et al.*(2015)Yoshida *et al.*(2013) |
| 3 | *SCM-2* | *Chromosome-6* | 25.9Mb-27.8Mb | SCM2-1\* SCM2-2\* SCM2-3\* SCM2-4\* RM20615 RM20458 | Ookawa*et al.*(2010) |
| 4 | *SCM-3* | *Chromosome-3* | 30.3Mb-28.07Mb | SCM3-1\* SCM3-2\* SCM3-3\* SCM3-4\* RM1350 | Ookawa*et al.*(2010) |
| 5 | *Gw-5* | *Chromosome-5* | 4.2Mb-6.8Mb | RM437 RM18161 RM18089 RM 18065 | Wan *et al.*(2008) |
| 6 | *Gs-3* | *Chromosome-3* | 16.60Mb-16.70Mb | DRR-GL | Ram kumar *et al.*(2010) |
| 7 | *SPL-14* | *Chromosome-8* | 22.3Mb-24.8Mb | Spl14-12\* Spl14-4\* RM23237 RM23386 | Miura *et al*.(2010) |
| 8 | *Yld-12.1* | *Chromosome-12* | 45.2Mb-67.3Mb | RM511 RM28166 RM28163 RM280130 RM28099 | Krishna *et al.* (2013)Venuprasad *et al.*(2009)Dixit *et al.*(2012) |
| 9 | *Yld-2.1* | *Chromosome-2* | 20.7Mb-25.8Mb | RM262 RM263 | Bernier *et al.*(2007)Dixit *et al.*(2012)Venuprasad*et al.*(2009)Swamy *et al.*(2013) |
| 10 | *Yld-4.2* | *Chromosome-4* | 25.8Mb-31.5Mb | RM261 RM16338 RM16373  | Sandhu *et al.* 2017 |

 \* Functional markers.

**3. Results And Discussion**

In view of characterization of advanced breeding lines for genes and *QTLs*, seven yield associated genes *viz., Gn1, Gn2* (*grain number*)*, SCM2, SCM3 (strong culm), Gs3, Gw5 (grain weight), Spl14 (filled grains)* and three *QTLs viz., Yld12.1, Yld2.1 and Yld4.1* (*grain yield*) were selected. The *Gn1*gene was characterized using three functional markers (Gn1A, Gn1A17 and Gn1INDEL) and three linked markers (RM10499, RM151 and RM10382). For *Gn2,* two linked markers (RM250 and RM208) were used. *SCM2*, major gene for *Strong Culm* was studied with four functional markers (SCM2-1, SCM2-2, SCM2-3 and SCM2-4) and two linked markers (RM20615 and RM20458). *SCM3* another important gene for strong culm present on *chromosome-3* was assessed with four functional markers (SCM3-1, SCM3-2, SCM3-3 and SCM3-4) and one linked marker (RM1350). Grain Weight is an important trait for yield improvement. This trait was assessed by taken *Gw5* gene. *Gw5* was assessed among the advanced breeding lines using four linked markers (RM437, RM18161, RM18089 and RM18065). Grain Size is important trait in crop improvement. This trait was characterized considering *Gs3* gene with functional marker, DRR-GL. The *Spl14*was assessed using two functional markers (Spl14-12 and Spl14-4) and two linked markers (RM23237 and RM23386). *Yld12.1* with five markers (RM511, RM28166, RM28163, RM28130 and RM28099), *Yld2.1* with two (RM262 and RM263) markers and*Yld4.1*with three (RM261, RM16338, RM16373) markers were assessed. The functional ladder size was used 50bp and linked markers ladder size was used 100bp. Three major traits (grain size, grain number per panicle, and panicle number per plant) are directly associated with yield, and these traits strongly depend on the genetic potential of rice. However, these traits are complex and quantitative in nature. Through *quantitative trait loci (QTL)* analysis with fine mapping or positional cloning using rice mutants, about 20 genes that are involved in yield-related traits have been isolated in rice (Liu *et al.* 2015).

**Table 2: Profile of advanced breeding lines for yield contributing genes and *QTL*s**

|  |  |  |
| --- | --- | --- |
| **ABL** | **No of *QTL*s**  | **Name of the genes/*QTL*s** |
| SP-08 | 7 | *Gn1, Gn2, SCM2, SCM3, Gw5, Yld12.1, Yld2.1* |
| SP-70 | 6 | *Gn1, SCM3, Gw5, Gs3, Yld2.1, Yld12.1* |
| SP-69 | 6 | *Gn1, Gn2, SCM3, Gw5, Spl14, Yld2.1* |
| SP-75 | 5 | *Gn1, Gn2, SCM3, Gw5, Yld12.1* |
| SP-37 | 5 | *Gn2, SCM3, Gw5, Yld12.1, Yld2.1* |
| SP-55 | 5 | *Gn2, SCM2, SCM3, Gw5, Yld2.1* |
| SP-61  | 5 | *Gn1, Gn2, SCM2, Gw5,Yld2.1* |
| SP-351 | 4 | *Gn1, SCM2, Gw5, Yld 12.1* |
| SP-357 | 4 | *SCM3, Gw5, Yld2.1, Yld12 .1* |
| SP-360 | 4 | *SCM3, Gw5, Gs3, Yld12.1* |
| SP-57 | 4 | *Gn2, SCM2, SCM3, Yld 12.1* |
| SP- 25 | 4 | *Gn1, Gn2, Gw5, Yld12.1* |
| SP-63 | 3 | *Gw5, Gs3, Yld12.1* |
| SP-02 | 3 | *Gn2, Gw5, Yld2.1* |
| SP-353 | 2 | *Gw5*, *Yld12.1* |
| SP-359 | 2 | *Gw5*, *Yld12.1* |
| SP-72 | 2 | *Gw5*, *Yld2.1* |
| SP-80 | 2 | *Gn2, Gs3* |
| SP-03 | 2 | *Gn2*, *Yld2.1* |
| SP-34 | 2 | *Gn2*, *Yld2.1* |
| SP-355 | 2 | *SCM2, Gw5* |
| SP-352 | 1 | *Gw5* |
| SP-354 | 1 | *Gw5* |
| SP-356 | 1 | *Gw5* |
| SP-358 | 1 | *SCM3* |
| SP-13 | 1 | *Gn2* |

**3.1 Molecular Diversity**

**3.1.1 DNA Isolation**

DNA was isolated from the leaf tissue by following Murray’s (Murray and Thompson, 1980) protocol. The quantity of DNA was estimated in nanodrop (Thermo Fisher Scientific, United States) as well as in agarose gel.

**3.1.2 Gene Profiling**

PCR analysis was done for advanced breeding lines together with the positive controls and susceptible checks to identify the presence of 10 major known yield genes/*QTL*s namely *Gn1, Gn2, SCM2, SCM3, Gw5, Gs3, Spl14, Yld12.1, Yld2.1 and Yld4.1* using molecular markers as described in Umakanth*et al.* (2017). Gene-based (*SNP* and *STS*) markers as well as were used for genotyping linked SSR markers. The markers used for the gene profiling and positive controls for respective genes are listed table 1 figure 1.

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**Figure 1: Diversity analysis of breeding lines for genes through Darwin software**

Among 30 advanced breeding lines, seven lines clustered differently from rest of the lines. Two advanced breeding lines SP-08 and SP-70 (25, 27) formed one cluster, since these two lines have similar characters like more number of tillers, productive tillers, LAI, LAD, CGR, NAR, stomatal conductance, SCMR values, number of grains per panicle, Single plant yield hill, test weight, grain yield and harvest index.

Another three advanced breeding lines SP-03, SP-25 and SP-57 clustered with IR-64. Since all these lines share the similarly in various morpho-physiological traits (lowest number of productive tillers, photosynthetic rate and number of grains per plant) with IR-64.

**4. Conclusion**

Upon genotyping of 30 advanced breeding lines only one advanced breeding line SP-08 showed the presence of seven yield contributing genes (*Gn1+Gn2+SCM2+SCM3+Gw5+Yld12.1+Yld2.1*) indicating presence of high potential for yield (Table 2). Similarly, two advanced breeding lines, SP-69 and SP-70 contain six genes.

Four advanced breeding lines, SP-37, SP-55, SP-75 and SP-61 showed the presence of five yield contributing genes in different combinations. Five lines SP-351, SP-357, SP-360, SP-57and SP-25 contain four yield contributing genes in different combinations. Two lines, SP-02 and SP-63 contain three genes in different combinations (Table 2).

Seven advanced breeding lines showed the presence of two yield contributing genes and five advanced breeding lines showed only one gene (Table 2).

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