



Development of dual stress-tolerant rice variety Jyothi with *Saltol* and *Sub1* genes through marker-assisted backcross breeding

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Abstract

Abiotic stresses such as salinity, submergence, drought and extreme temperatures greatly affect rice production worldwide. Marker-assisted selection was effectively used to introgress QTLs for salinity tolerance (*Saltol*) and submergence tolerance (*Sub1*) into the background of elite rice variety Jyothi. *Saltol* introgressed BC₁F₂ lines of Jyothi with 79.3% of recipient parent genome was crossed with *Sub1* introgressed BC₂F₂ lines of Jyothi showing 95.9% of parent genome recovery. F₁ progenies were screened with *Saltol* and *Sub1* linked foreground markers and recombinant markers. F₁ heterozygous plants were selfed to produce F₂ generation. F₂ progenies homozygous for both the loci were selected. Phenotypic screening for salinity and submergence tolerance was performed to validate the introgressed genes. The plants able to tolerate both salinity and submergence stresses were selected for selfing to raise F₃ progenies. The pyramided lines were also similar to recurrent parent in agro-morphological and grain quality traits.

Keywords Rice · Abiotic stress · Saltol QTL · Sub1 gene · Marker-assisted selection

Introduction

Rice is the major food crop in Asian countries. The total production of rice in India has increased to 103.75 million tons in 2020–21 (Directorate of Economics and Statistics, 2nd advance estimates 2020–21). Salinity and submergence are the two major abiotic stresses greatly affecting rice production worldwide. Global warming and the resulting climate change lead to intrusion of seawater to coastal ecosystem and make wet lands salt affected and unsuitable for rice production (Wassmann et al. 2009; Thuy and Anh 2015). A total of 6.74 million hectare of land in India are affected by salinity resulting in huge economic loss (Biswas and Biswas 2014). According to a talk by V. Selvam, executive director of M.S. Swaminathan Research Foundation organized by Earth Journalism Network and The Third Pole, sea level is expected to rise by 16 and 32 cm by 2050 and 2100, respectively. Kerala state with a coastline of 590 km experiences the problem

of saline soils and also serious problems of water logging as they are situated below the mean sea level (Nambiar and Raveendran 2009). Therefore, there is an urgent need to develop rice varieties tolerant to several abiotic stresses.

Progress in the field of molecular biology, genetics, biochemistry and bioinformatics has led to the detailed study of major QTLs linked to salinity and submergence tolerance. These QTLs can be successfully introgressed into high yielding rice varieties for cultivation in problem soils (Ismail et al. 2007). Marker-assisted backcrossing is the most effective and economical way to introgress the desired trait while retaining the essential characters of elite rice varieties. The use of molecular markers in MAB greatly enhances the selection process by reducing the time period as compared to conventional breeding (Tanksley et al. 1989; Hospital 2003). The markers used in MAB include foreground, recombinant and background markers. Foreground markers are those that are within the locus and recombinant markers are the markers flanking the desired locus which will minimize the linkage drag. Background markers are used to accelerate the recovery of recurrent parent genome (Collard and Mackill 2008; Hasan et al. 2015).

The successful development of elite breeding rice varieties involves transfer of desirable genes from multiple parents. This process is called gene pyramiding.

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MAS-based gene pyramiding offers great opportunity to transfer multiple stress-tolerance genes into one single variety (Joshi and Nayak 2010). The developed variety expresses the introgressed genes from multiple parents simultaneously.

A major QTL for salt tolerance was mapped on chromosome 1 by using an F8 recombinant inbred line (RIL) of a Pokkali/IR29 cross (Bonilla et al. 2002). The mapping of *Saltol* QTL provided the opportunity to develop many salt tolerant rice varieties through introgression of this QTL using MAB (Vu et al. 2012; Linh et al. 2012; Babu et al. 2017; Quan et al. 2018). Fine mapping, positional cloning, transformation, expression and validation of the QTL conferring submergence tolerance *Sub1* located on chromosome 9, facilitated the introgression of this QTL into several high yielding rice varieties (Bailey et al. 2010; Collard et al. 2013; Mackill et al. 2012; Rahman et al. 2018).

In the present work, *Saltol* and *Sub1* QTLs from FL-478 and Swarna Sub1 donor parents, respectively, were transferred into the background of most popular rice variety of Kerala, Jyothi through marker-assisted gene pyramiding. Simultaneous and stepwise gene pyramiding scheme was followed. The newly developed rice variety Jyothi, which is tolerant to both the abiotic stresses, can be successfully cultivated in the low-lying coastal areas.

Materials and methods

Plant material

FL-478 and Swarna Sub1 were used as donors of saline tolerance and submergence tolerance, respectively. The recurrent parent Jyothi is the most popular rice variety of Kerala but sensitive to salinity and submergence. Simultaneous and stepwise gene pyramiding pattern was followed. The schematic representation of hybridization detail is given in Fig. 1.

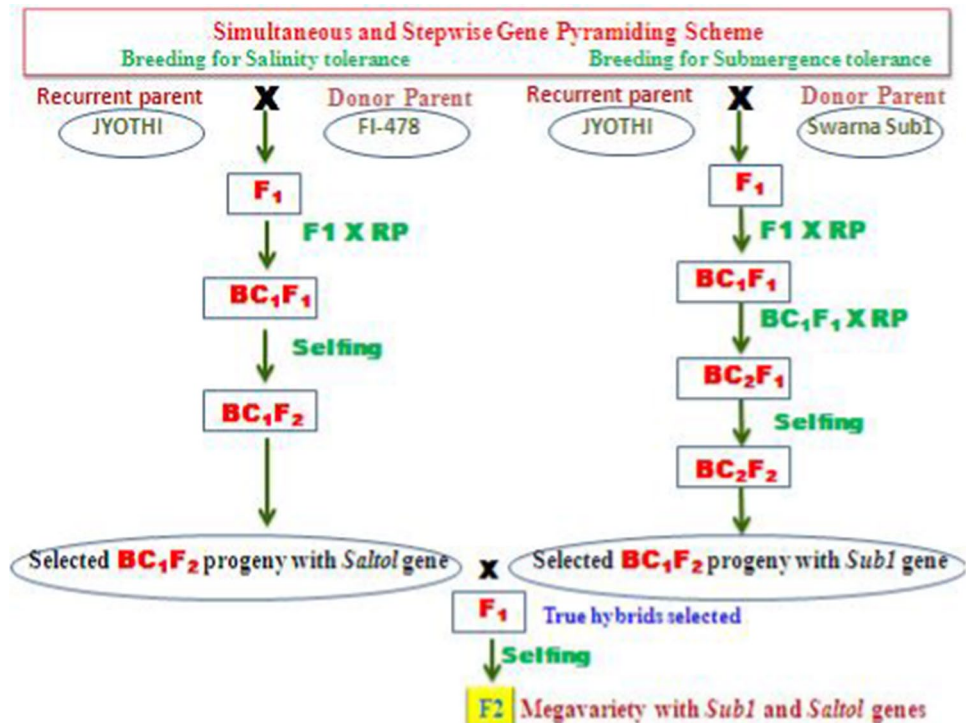
DNA isolation and quantification

DNA was extracted from young leaf tissues following modified CTAB method (Doyle and Doyle 1987) and dissolved in 1X TE buffer. DNA was quantified spectrophotometrically using NanoDrop 2000c (Thermo Scientific), and the concentration was adjusted to 25 ng/μl for use in polymerase chain reaction (PCR).

SSR marker analysis

PCR amplification was carried out in a total volume of 20 μl containing a final concentration of 25 ng of genomic DNA, 1X Taq Buffer with 2.5-mM MgCl₂, 400 μM of dNTPs, 1 unit of Taq polymerase enzyme and 0.4-μM each of forward and reverse primers. The PCR program involved an

Fig. 1 Breeding scheme for gene pyramiding



initial denaturation at 94 °C for 5 min followed by 31 cycles of denaturation (94 °C for 5 min), annealing at 55–65 °C depending on the GC content of the primers for 40 s and extension at 72 °C for 40 s. PCR reactions were carried out in Thermocycler (Applied Biosystems, Veriti 96-well thermocycler). SSR markers linked to *Saltol* and *Sub1* were used for foreground screening. The markers flanking on either side of the *Saltol* and *Sub1* locus were used for recombinant selection. The details of primers used are given in Table 1.

Genome-wide SSR markers were used for recurrent parent genome recovery analysis. The marker data were analyzed using the software Graphical Genotyper (GGT2.0). The homozygous recipient allele, homozygous donor allele and heterozygous allele were scored as ‘A,’ ‘B’ and ‘H,’ respectively. Recurrent parent genome recovery in the selected *Saltol* and *Sub1* introgressed F₂ lines of Jyothi was calculated using the statistics function of GGT 2.0 software.

Phenotypic screening

Screening for salinity tolerance

The rice varieties under study were subjected to phenotypic screening for salinity tolerance at seedling stage using hydroponics based on the standard protocol of IRRI, Manila, Philippines (Gregorio et al. 1997). The set up was prepared with monolayer plastic tubs and trays, plastic net sheets along with fabricated seedling floats (acrylic make) with holes. Two pregerminated seeds were sown per hole on the seedling float. The sheet was floated in distilled water for 3 days. After 3 days, the distilled water was replaced with

Yoshida nutrient solution (Yoshida et al. 1976) having a pH of 5.5 salinized with common salt to obtain the desired EC of 12 dSm⁻¹. The nutrient solution was renewed after every 8 days, and its pH maintained at 5.5 (adjusted by adding either 1N NaOH or 1N HCl). Visual salinity tolerant rating was done according to the modified standard evaluation score (SES) developed at IRRI, Manila, Philippines, as shown in Table 2. Progenies with a phenotypic score of 3 similar to the donor parent were selected for further screening.

Screening for submergence tolerance

Germinated seedlings of the selected F₂ plants along with donor and recurrent parents were sown in pots. IR64 and FR13A were used as submergence sensitive and submergence tolerant check varieties, respectively. Fourteen days old seedlings were completely submerged in tanks filled with turbid water of 1-m height for 14 days following standard protocols (Neeraja et al. 2007). The survival percentage of plants was scored 14 days after de-submergence according to the IRRI standard evaluation system (IRRI, 1988) as shown in Table 3. Progenies with a phenotypic score of 3 similar to the donor parent were selected for further screening.

Analysis of pyramided lines for agro-morphological and yield traits

Eight *Saltol/Sub1* introgressed F₃ lines of Jyothi were subjected to field trial during Kharif 2017 at paddy field of Rice Research Station, Vyttila. The 21-day-old seedlings of the selected F₃ progenies along with the donor and recurrent

Table 1 SSR markers used for foreground and recombinant selection

Marker	Type of marker	Abiotic stress resistance	Chromosome
AP3206	Foreground	Salinity	1
RM3412b	Foreground	Salinity	1
SUB1BC2	Foreground	Submergence	9
ART5	Foreground	Submergence	9
RM1287	Flanking	Salinity	1
RM140	Flanking	Salinity	1
RM8303	Flanking	Submergence	9
RM23958	Flanking	Submergence	9

Table 2 Modified standard evaluation system (SES) score of visual salt injury at seedling stage in rice

Score	Observation	Tolerance
1	Nearly normal growth, no leaf symptoms	Highly tolerant
3	Nearly normal growth, but leaf tips or few leaves whitish and rolled	Tolerant
5	Growth severely retarded; most leaves rolled; only a few are elongating	Moderately tolerant
7	Complete cessation of growth; most leaves dry; some plants dying	Susceptible
9	Almost all plants dead or dying	Highly susceptible

Table 3 Standard evaluation system (SES) score for submergence tolerance in rice (IRRI 1988)

Survival %	Score	Observation	Tolerance
100	1	Minor visible symptom of injury	Highly tolerant
95–99	3	Some visible symptom of injury	Tolerant
75–94	5	Moderate injury	Moderately tolerant
50–75	7	Severe injury	Susceptible
1–49	9	Partial to complete death	Highly susceptible

parental lines were transplanted in the field. Ten plants were randomly selected from each replication for each of the genotypes, and the following observations such as 50% flowering, days to maturity, plant height, panicle length, number of productive tillers per plant, number of grains per panicle, 1000 grain weight and grain yield recorded. The data from various agro-morphological and yield traits were statistically analyzed by Web Agri Stat Package 2.0 (ICAR Goa).

Results

Parental polymorphism screening

A total of 600 SSR markers covering the 12 chromosomes were used to analyze the parental polymorphism. Among these, 109 markers showed polymorphism between Jyothi and respective donor parents FL-478 and Swarna Sub1. These polymorphic markers were used for further screening of *Saltol* and *Sub1* introgressed F_2 generations. Among the 13 foreground and recombinant markers linked to *Saltol* locus screened, six were found to be polymorphic between Jyothi and FL-478. Similarly, with respect to 10 foreground and 13

recombinant markers associated with submergence tolerance screened, three and four markers were found to be polymorphic between Jyothi and Swarna Sub1, respectively.

Foreground and recombinant selection

F_1 progenies derived from the hybridization between *Saltol* and *Sub1* introgressed lines of Jyothi were subjected to salinity screening. Withstanding plants were genotyped with foreground and recombinant markers linked to *Saltol* and *Sub1* locus. F_1 plants heterozygous at both the loci were selfed to produce F_2 progenies (Fig. 2). F_2 plants were then subjected to salinity and submergence screening according to the standard protocols, and plants showing a score of 3 for both salinity and submergence tolerances (Fig. 3) were genotyped with foreground and recombinant markers (Fig. 4 and Fig. 5.) The selected eight F_2 progenies homozygous for both the loci were selfed to produce F_3 progenies.

Lanes 1–15: F_1 progenies, lane 16: recurrent parent Jyothi, lane 17: donor parent and lane L: 100-bp ladder.

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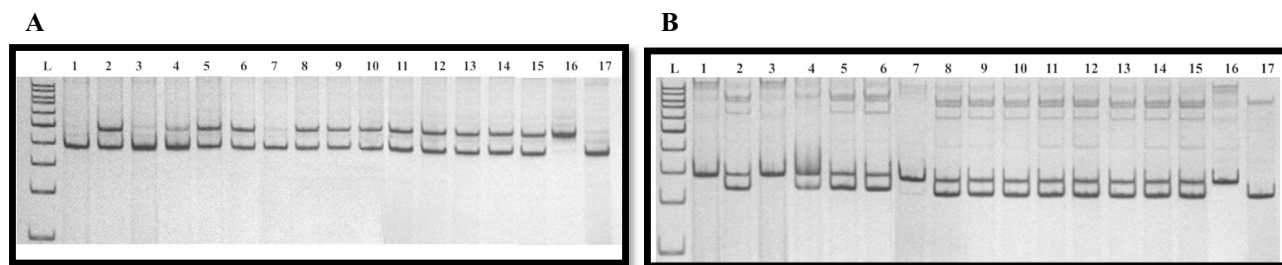


Fig. 2 Molecular screening of F_1 progenies with key *Saltol* marker AP3206 **A** and key *Sub1* linked marker Sub1BC2 **B**

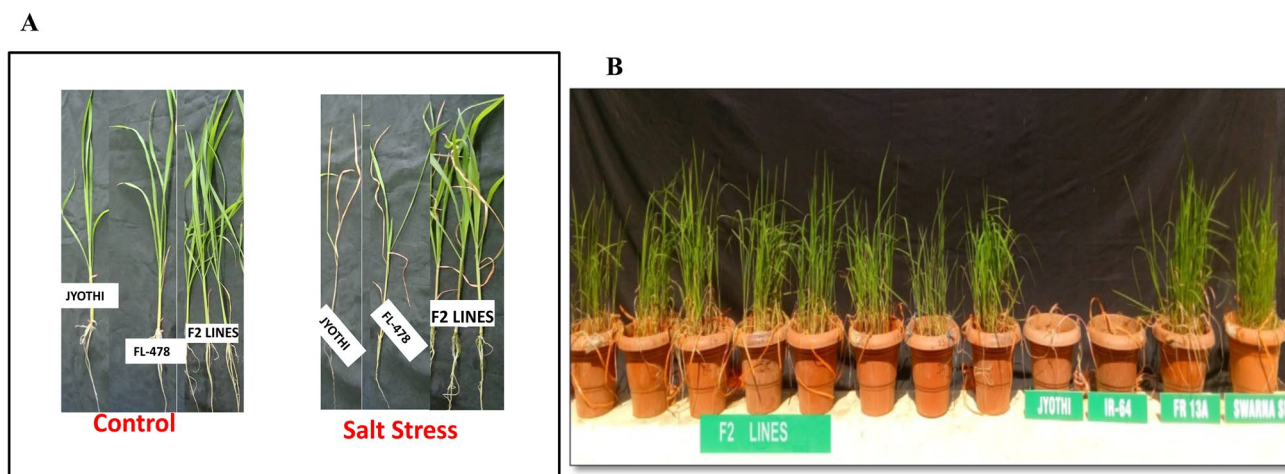


Fig. 3 Screening of F_2 progenies for salinity tolerance **A** and submergence tolerance **B**

Background selection

A total of eight F_2 progenies survived after salinity submergence screening were selected after genotypic screening with respective foreground and recombinant markers. These progenies were subjected to background screening with the

polymorphic markers. Marker data generated from the background screening of the selected F_2 progenies were analyzed with GGT 2.0 software, and the recurrent parent genome content was found to be in the range of 72.1–82.9% (Fig. 6). Graphical genotype of the best plant MC-F3-4 with maximum recurrent parent genome recovery is shown in Fig. 6.

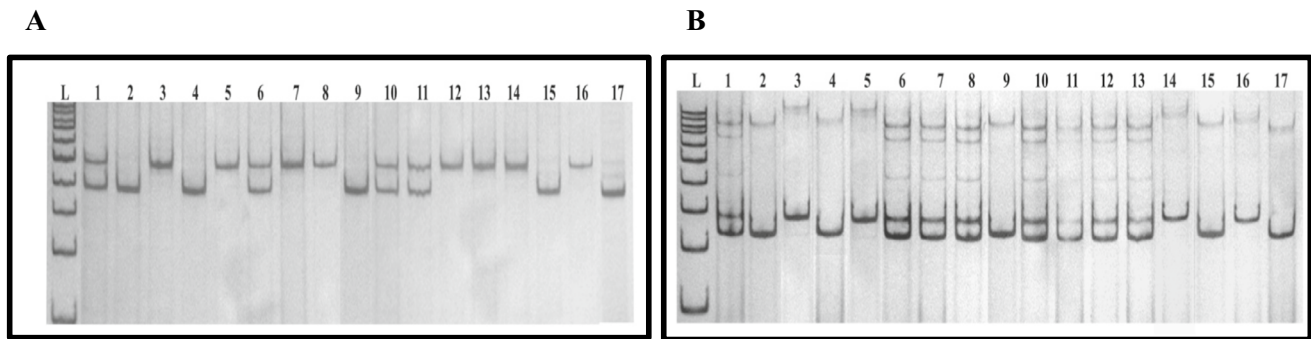


Fig. 4 Molecular screening of F_2 progenies with key *Saltol* marker AP3206 **A** and key Sub1 linked marker Sub1BC2 **B**

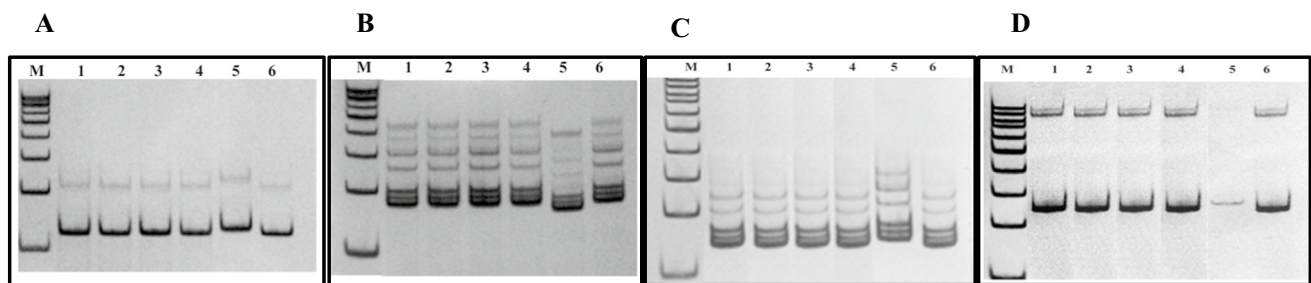


Fig. 5 Molecular screening of F_2 progenies with key *Saltol* linked recombinant marker RM8303 **A**, RM23958 **B** and key Sub1 linked recombinant marker RM1287 **C** and RM140 **D**. Lanes 1–4: F_2 progenies, lane 5: recurrent parent Jyothi, lane 6: donor parent and lane M: 100-bp ladder

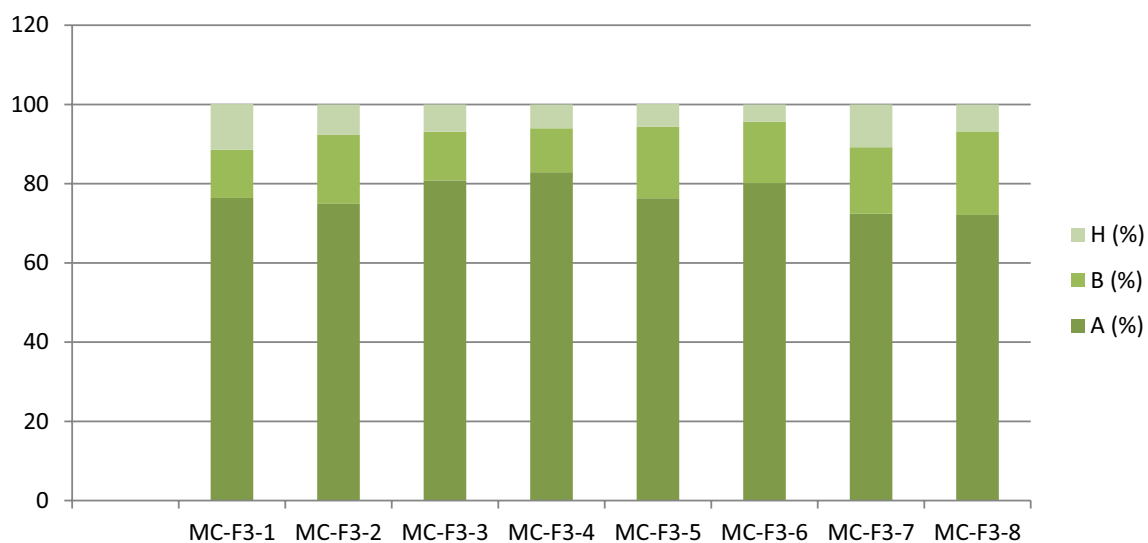


Fig. 6 Recovery of recurrent parent genome (percentage) in F_2 progenies

Agro-morphological traits and grain yield of pyramided lines

The selected eight pyramided lines along with the donor parents and recurrent parent were evaluated during Kharif 2017 at paddy field of Rice Research Station, Vyttila. Agro-morphological traits such as plant height, days to maturity, number of productive tillers, panicle length, number of filled grains per panicle, 1000 grain weight and seed length determine the yield of a rice variety (Moldenhauer and Nathan 2004; Sakamoto and Matsuoka 2008; Huang et al. 2013). Most of the pyramided lines performed better than the recurrent parent in the field. The agro-morphological characters of the pyramided lines were almost similar to the recurrent parent Jyothi. All the pyramided lines produced higher grain yield than the recurrent parent (Table 4).

The donor parent for submergence tolerance (Swarna-Sub1) showed longest days to maturity (144 days). However, all the pyramided lines had maturation days on par with the recurrent parent Jyothi which matured earlier (120). Varieties with duration of 110–135 days are more preferable as they produce better yield than those maturing earlier or later under most of the agronomic and climatic conditions (Jennings et al. 1979). A significant difference was observed for plant height among the pyramided lines, and it ranged from 91.51 to 111.43 cm with an average of 99.49 cm. The mean plant height of recurrent parent was found to be 95.97 cm for Jyothi, whereas in case of donor parent, it was found to be 114.47 cm for Swarna-Sub1 and 88.43 cm for FL-478. All the pyramided lines were found to be medium tall. With respect to productive tiller number, which is another important trait contributing to yield of a variety, seven out of the eight pyramided lines were found to bear on par or more number of productive tillers with respect to recurrent and donor parents.

The yield of a crop is an important parameter to be considered while crop improvement programs to select superior. Therefore, total yield of all the pyramided lines and recurrent and donor parents was calculated. Four of the pyramided lines had total yield on par with the recurrent parent (MC-F3-4, MC-F3-5, MC-F3-6 and MC-F3-7); whereas, three pyramided lines yielded on par with the donor parent (MC-F3-1, MC-F3-2 and MC-F3-8). One of the pyramided lines (MC-F3-3) showed better yield than both the recurrent and donor parents. All the pyramided lines were found to have long medium grains similar to the recurrent parent. Therefore, the pyramided lines were almost similar to the recurrent parent in terms of agro-morphological traits. Similar results were obtained in other gene pyramiding program (Pradhan et al. 2015; Hsu et al. 2020).

Table 4 Agromorphological traits of pyramided lines and parental varieties

Progeny	Pl. Ht	50% flower-ing	Days to maturity	Total tillers	Productive tillers	Panicle length	Flag leaf length	Flag leaf width	Index leaf length	Index leaf width	Total no. of grains/panicle	No. of filled grains/panicle	Length (mm)	Breadth (mm)	L/B	1000 grain wt (g)	Total yield (kg)
MC-F3-1	111.43 ^a	89.00 ^b	119.00 ^b	11.61 ^{abc}	10.03 ^c	26.83 ^a	35.30 ^a	1.50 ^b	36.00 ^b	1.30 ^a	171.38 ^b	139.40 ^b	6.59 ^c	2.32 ^{bc}	2.88 ^b	28.70 ^{abcd}	7.04 ^b
MC-F3-2	105.01 ^b	89.80 ^b	119.80 ^b	10.16 ^{de}	9.64 ^{cde}	25.33 ^b	29.03 ^{cd}	1.50 ^b	32.33 ^d	1.00 ^b	168.88 ^b	136.53 ^b	6.71 ^{bc}	2.33 ^b	2.89 ^b	30.29 ^{ab}	6.81 ^b
MC-F3-3	101.44 ^{bc}	90.00 ^b	120.00 ^b	12.65 ^a	12.07 ^a	25.44 ^b	22.00 ^f	1.00 ^{de}	32.00 ^d	1.00 ^b	160.07 ^c	140.67 ^b	6.77 ^{bc}	2.34 ^b	2.90 ^b	27.70 ^{cde}	7.85 ^a
MC-F3-4	96.35 ^d	89.70 ^b	119.70 ^b	9.13 ^e	8.96 ^{def}	24.82 ^{bc}	22.03 ^f	1.00 ^{de}	32.00 ^d	1.00 ^b	136.87 ^d	105.39 ^{de}	6.84 ^b	2.36 ^b	2.86 ^b	26.67 ^{de}	5.43 ^{cd}
MC-F3-5	99.42 ^{cd}	89.77 ^b	119.77 ^b	7.56 ^f	7.68 ^f	24.61 ^{bc}	18.07 ^g	1.00 ^{de}	22.00 ^e	0.60 ^{cd}	133.87 ^d	117.07 ^c	6.84 ^b	2.28 ^{bc}	3.00 ^b	30.38 ^{ab}	5.84 ^e
MC-F3-6	99.52 ^{cd}	89.67 ^b	119.67 ^b	10.81 ^{cd}	8.93 ^{def}	23.73 ^c	30.04 ^{bc}	1.50 ^b	32.00 ^d	1.00 ^b	120.57 ^e	100.60 ^{ef}	6.64 ^{bc}	2.24 ^c	2.96 ^{ab}	29.62 ^{abc}	5.36 ^{cd}
MC-F3-7	91.237 ^e	89.77 ^b	119.77 ^b	9.6 ^e	8.09 ^{fg}	24.12 ^{bc}	17.02 ^g	1.3 ^c	30.00 ^d	1.00 ^b	132.93 ^d	112.10 ^{cd}	6.85 ^b	2.32 ^{bc}	2.91 ^b	28.19 ^{bcd}	5.95 ^c
MC-F3-8	91.51 ^e	89.63 ^b	119.63 ^b	12.00 ^{ab}	9.79 ^{cd}	23.75 ^c	18.97 ^g	1.00 ^{de}	32.00 ^d	0.80 ^{bc}	131.49 ^d	116.06 ^c	6.72 ^{bc}	2.30 ^{bc}	2.92 ^b	30.70 ^a	6.67 ^b
Jyothi	95.97 ^d	89.67 ^b	119.67 ^b	9.3 ^e	8.80 ^{ef}	21.63 ^d	26.03 ^e	1.12 ^d	29.37 ^d	0.45 ^d	109.77 ^f	91.37 ^{fg}	6.84 ^b	2.30 ^{bc}	2.82 ^{bc}	26.94 ^{de}	5.53 ^{cd}
Swarna Sub1	114.47 ^a	114.33 ^a	144.33 ^a	11.17 ^{bcd}	11.09 ^b	25.33 ^b	32.33 ^b	1.63 ^a	42.67 ^a	1.367 ^a	191.50 ^a	162.84 ^a	5.52 ^d	2.13 ^d	2.59 ^c	19.42 ^f	7.13 ^b
FL-478	88.33 ^e	83.00 ^c	113.00 ^c	9.24 ^e	8.04 ^{fg}	23.52 ^c	27.05 ^{de}	0.98 ^e	35.10 ^{bc}	0.78 ^c	99.30 ^g	84.38 ^g	9.73 ^a	2.97 ^a	3.27 ^a	26.31 ^e	4.95 ^d

Discussion

Submergence and salinity are the two major abiotic stresses affecting rice cultivation in coastal areas. Climate changes increase the risk of sea level rise and flooding. Submergence and salinity can occur separately, or both the stresses can affect simultaneously. Therefore, modifying high yielding rice varieties to withstand submergence and salinity stresses has become crucial for meeting the ever-increasing demand. Advance techniques like marker-assisted breeding have enabled introgression of QTLs imparting tolerance to various abiotic stresses into the genetic background of high yielding mega rice varieties. In the current study, the two different QTLs for salinity tolerance and submergence tolerance were pyramided into one single genotype using marker-assisted simultaneous and stepwise gene transfer method. Similar methodology was followed by researchers to pyramid two or more stress-tolerance genes into a single genetic background (Singh et al. 2013; Jamaloddin et al. 2020).

The recombinant progenies were screened with SSR markers linked to *Saltol* locus (Chowdhury et al. 2016; Singh et al. 2018; Adak et al. 2020) and SSR markers linked to *Sub1* loci (Neeraja et al. 2007) to confirm the introgression of both the QTLs. The codominant nature of SSR markers makes it easier to detect the recombinant progenies even in the heterozygous state (Yang et al. 2016). Background markers were used to screen out the progenies possessing the maximum recovery of the recurrent parent genome. Background markers are used to accelerate the recovery of recurrent parent genome and thereby reduce the breeding cycle (Collard and Mackill 2008, Hospital and Charcosset 1997). Therefore, with the help of molecular markers, the recurrent parent genome could be restored in the pyramided lines and highlights the significance of marker-assisted breeding (Olalekan et al. 2019).

The backcross inbred lines pyramided with *Saltol* and *Sub1* loci were evaluated for their tolerance to salinity and submergence stresses. All the selected progenies could withstand the stress conditions similar to the respective donor parents; whereas, the recurrent parent exhibited complete susceptibility. This confirmed the effectiveness of the QTLs introgressed into the genetic background of Jyothi. Similar results were demonstrated in other introgressed progenies (Neeaja et al. 2007; Septiningsih et al. 2009; Rahman et al. 2018; Singh et al. 2018; Valarmathi et al. 2019).

The grain quality parameters and total duration of the pyramided lines were found to be very similar to the recurrent parent. The improved lines also exhibited higher grain yield than the recurrent and donor parents. Therefore, the

current study clearly demonstrates the success of marker-assisted breeding technique in pyramiding two QTLs imparting tolerance against both salinity and submergence stresses into the genetic background of high yielding mega rice variety of Kerala. This is the first ever work reported in Kerala.

Conclusion

Marker-assisted backcrossing was successfully used to pyramid the two major abiotic stress-tolerant traits into the most popular rice variety of Kerala within a short span of time. The size of each donor fragments was limited assisted with the use of foreground and recombinant markers. The parent genome recovery was also ensured with the use of rice genome background markers. The pyramided lines showed similar agro-morphological characters like the recurrent parent. The total grain yield was much better than the recurrent parent. Four best lines with higher grain yield were selected for further field trials necessary for future release procedures. The newly developed rice variety will be tolerant to both salinity and submergence with all the other favorable traits of the recipient parent retained. This variety can be successfully cultivated in low-lying coastal areas leading to increased rice production. The present study successfully demonstrates the application of marker-assisted gene pyramiding as an efficient tool to introgress multiple abiotic stress genes into a single variety. These developed pyramided lines can be used by breeders as donors suiting to their respective needs.

Declarations

Conflict of interests The authors have no relevant financial or non-financial interests to disclose.

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