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Pyramiding of $qDTY_{1.1}$ and $qDTY_{3.1}$ into rice mega-variety Samba Mahsuri-Sub1: physiological performance under water deficit conditions

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Abstract

Water deficit is a crucial factor causing huge loss to rice productivity. The present study aimed to develop a multiple stress tolerant genotype by pyramiding drought tolerant yield QTLs $qDTY_{1.1}$ and $qDTY_{3.1}$ into high yielding rice variety Samba Mahsuri Sub-1(SMS) through marker assisted pyramiding. To achieve this six introgression lines of SMS carrying $qDTY_{1.1}$ (SAB) were crossed with DRR-50, an Essentially Derived Variety of SMS carrying $qDTY_{3.1}$. The SAB lines are taller than SMS due to tight linkage between $qDTY_{1.1}$ and wild type *SD-1*. Therefore, F_2 generation of crosses were screened for recombinants between *SD-1* and $qDTY_{1.1}$. Phenotyping of 1530 F_2 plants representing three F_2 populations from 35 F_1 hybrids, identified 305 dwarf plants. Three dwarf F_2 plants along with three others carrying $qDTY_{1.1}$ and $qDTY_{3.1}$ were forwarded to F_3 generation. From the six F_3 (SABD) lines fourteen pyramided progenies were selected and forward to F_4 generation. The six SABD F_3 lines SABD-7, SABD-8, SABD-9, SABD-76, SABD-79 and SABD-80 along with parents were evaluated under moisture stress (MS) for various physiological parameters. Chlorophyll and relative water content were more, while canopy temperature and malonaldehyde (MDA) content were lesser in SABD lines compared to parents indicating tolerance under MS. Variance due to genotypes was highly significant for all the yield related traits except test weight. Based on seed morphology, agronomic characters and physiological parameters six superior lines SABD-9-3, SABD-9-2, SABD-9-6, SABD-9-7, SABD-76-2 and SABD-76-6 performing better under MS were identified, which could be released after multi-location evaluation.

Keywords Moisture stress · Marker assisted pyramiding · $qDTY_{1.1}$ · $qDTY_{3.1}$ · Rice

Introduction

Rice is cultivated in about 165.25 Mha area worldwide. However, average productivity of rice suffers because of many biotic and abiotic factors. Among the various abiotic factors, drought is an important factor causing huge loss in rice productivity. Nearly 34 Mha of rainfed lowland and 8 Mha of upland rice in Asia suffer from low moisture stress of varying intensities with 13.6 Mha area yearly affected in India alone (Singh et al. 2016). Rice is a staple food for half of the world's population and therefore, it's production also needs to keep pace with increase in population. Conventional breeding for low moisture tolerance in rice has been difficult and less efficient mainly because of complex genetic nature of inheritance and interaction of many secondary traits contributing towards tolerance (Valliyodan and Nyugen 2006; Heredia et al.

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2022; Melandri et al. 2022). The backcrossing approach has been used to incorporate one or few traits in an adapted or elite variety (Hasan et al. 2015). Conventional approach to transfer an allele of a desired trait from a donor variety to a popular cultivar through backcrossing takes a lot of time and moreover, the augmented variety may retain undesired chromosomal regions due to linkage drag. With the knowledge and availability of markers linked or lying within QTLs (Quantitative Trait Loci) MAS and Marker Assisted Backcrossing (MABC) has been successfully employed in improving resistance to various biotic and abiotic stresses in rice and in other crops as well (Miklas et al. 2006; Patto et al. 2006). QTLs with large effects on grain yield under drought stress, namely $qDTY_{1,1}$, $qDTY_{2,1}$, $qDTY_{2,2}$, $qDTY_{3,1}$, $qDTY_{4,1}$, $qDTY_{9,1}$ and $qDTY_{12,1}$ have been identified (Bernier et al. 2007; Dixit et al. 2012).

The availability of molecular markers linked to complex traits like drought (Ashraf 2010), submergence (Septiningsih et al. 2009), low phosphorous tolerance (Chin et al. 2010), etc. has made it possible to introgress desirable alleles into a single genotype, thereby providing resistance/tolerance. Rice breeding has observed targeted approach of integrating multiple stress tolerance genes/QTLs through MABC or MAS (Guo and Ye 2014; Singh et al. 2016) or genomic selection (Spindel et al. 2015; Melandri et al. 2020). These approaches are the necessary building blocks for generating climate resilient rice crop (Bhardwaj et al. 2022; Heredia et al. 2022). It is of vital importance that the QTLs and genes identified for imparting abiotic stress tolerance be incorporated into high yielding varieties in order to enhance their yield potential under stress conditions.

In hill regions of North Eastern India, rice is grown as a rainfed crop and is therefore, susceptible to moisture stress at both extremes of submergence and moisture deficit during a cropping season, or within the same geographical area due to varied topography. The current study aimed at pyramiding two drought yield QTLs, $qDTY_{1,1}$ and $qDTY_{3,1}$ into a mega-variety Samba Mahsuri-Sub1 (SMS) carrying submergence QTL *Sub1*, and evaluation of homozygous lines under water deficit conditions.

Materials and methods

This study was carried out at the College of Post-Graduate studies, CAU (Imphal), Umiam, Meghalaya, India during 2015–2020. The drought stress experiments for selected lines were conducted in 2017 and 2019/2020.

The Samba Mahsuri-Sub1 (SMS) introgression lines designated as SA and SB for QTLs $qDTY_{1,1}$ and $qDTY_{2,2}$, respectively, were previously obtained from simultaneous crosses of donor parents IR 86918-B-305 (D11.1) and IR 87728-367-B-B (D22.2) with recurrent parent SMS

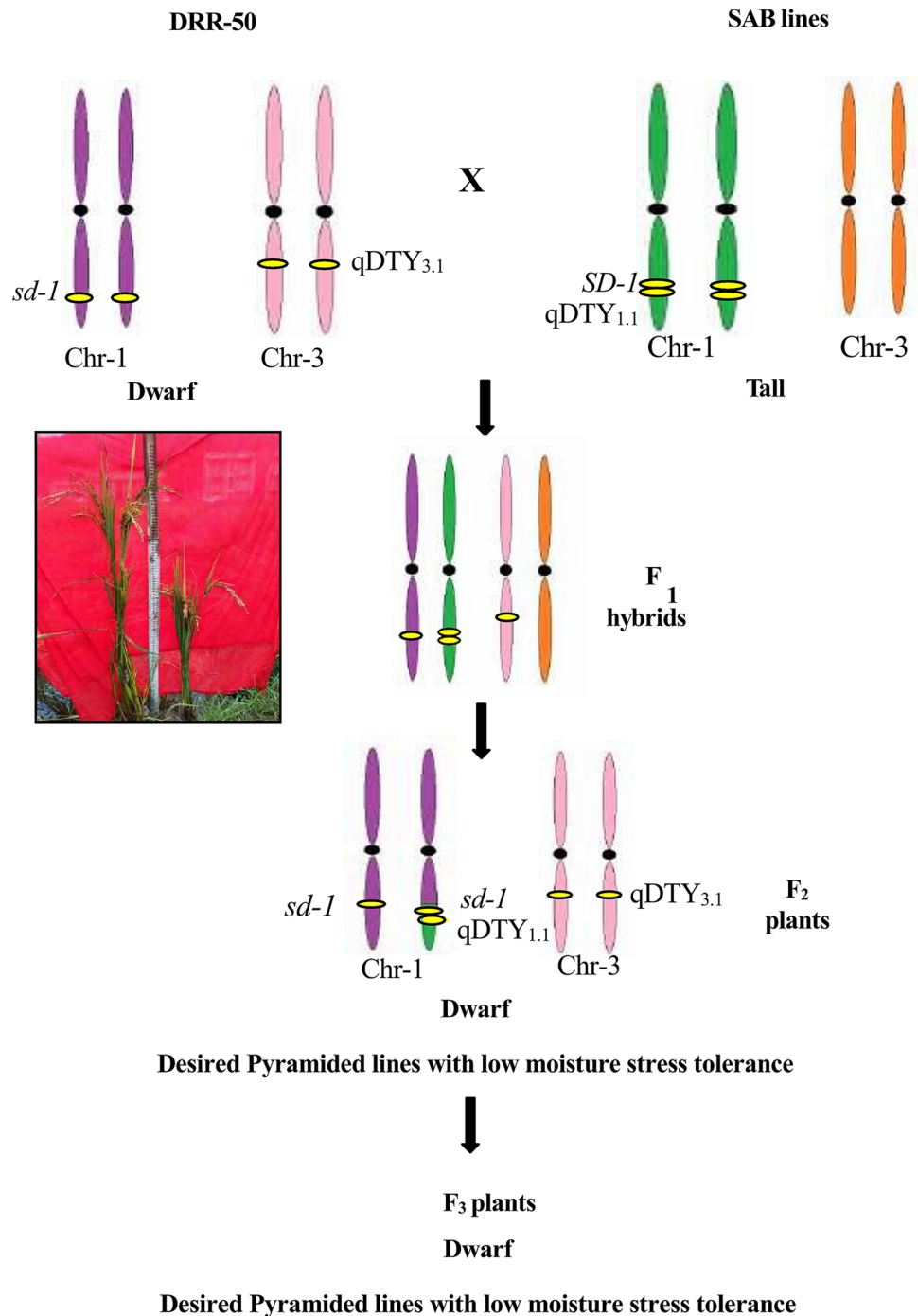
(Singh et al. 2016; Bhandari et al. 2019) (Fig. S1). The pyramided lines were generated by crossing SA and SB lines and designated as SAB. Better performing progeny of the lines SAB4-2, SAB4-7 and SAB7-13 (based on morpho-physiological traits superior than SMS) were identified (SAB 4-2-4; SAB 4-2-7; SAB 4-2-20; SAB 4-7-3; SAB 4-7-5 and SAB 7-13-5) (Fig. S1). DRR-50 carrying drought tolerant yield QTL $qDTY_{3,1}$ on chromosome number 3 and semi dwarfing allele *sd-1* on chromosome number 1, along with six SAB lines carrying $qDTY_{1,1}$ and *SD-1* wild type allele on chromosome number 1 were used in the current study. DRR-50 is an essentially derived variety obtained by cross between SMS and IR 81896-B-B-195 from ICAR-Indian Institute of Rice Research, Hyderabad (Fig. 1; Fig. S3).

The six SAB lines carrying drought-tolerant yield QTLs $qDTY_{1,1}$ and DRR-50 carrying $qDTY_{3,1}$ were grown under greenhouse conditions in pots. For pyramiding the drought QTLs into a single genotype, SAB lines were crossed with DRR-50. The emasculated panicles were hand pollinated and re-bagged. Mostly DRR-50 was used as a female parent and the SAB lines as a male parent because the DRR-50 flowering was late when compared to early flowering SAB lines. The F_1 hybrids were grown in greenhouse whereas, F_2 were grown under field conditions. Spacing maintained was 15 cm between plant to plant and the row to row distance was 20 cm.

Genotypic characterization of pyramided lines using QTL linked SSR markers

For validation of heterozygotes in F_1 plants, genomic DNA was extracted using CTAB method followed by PCR analysis using SSR markers. Briefly, total genomic DNA was extracted as described previously (Bhutia et al. 2021). Polymerase chain reaction (PCR) reaction volume was 10 μ L and contained 50–75 ng of template, 10X PCR buffer, 25 mM $MgCl_2$, 2 mM dNTPs, 10 pmol of forward and reverse primers, 0.5 U of Taq Polymerase (D6677, Sigma, India). PCR cycle comprised of one cycle of denaturation at 95 °C; 5 min, followed by 30 cycles at 95 °C; 45 s, 55 °C; 30 s, 72 °C; 30 s with final extension of 72 °C for 5 min. The amplicons were resolved on 2% agarose gels and visualized using ultraviolet transilluminator (Alpha Imager mini). The parents were surveyed with eleven SSR (Single Sequence Repeat) markers (RM3825; RM315; RM11943; RM431; RM12023 and RM12091 for $qDTY_{1,1}$ and RM15791; RM520; RM15925; RM416 and RM16030 for $qDTY_{3,1}$) previously reported (Vikram et al. 2011; Dixit et al. 2014). Among the markers surveyed namely RM431 and RM11943 for $qDTY_{1,1}$ and RM520 for $qDTY_{3,1}$ exhibited polymorphism between the parents DRR-50 and SAB lines.

Fig. 1 Crossing scheme for development of moisture stress tolerant pyramided rice lines. DRR-50 and SAB indicate lines carrying single QTLs $DTY_{3,1}$ and $DTY_{1,1}$, respectively. Inset shows pot photograph for height segregation



Two polymorphic markers RM431 and RM520 were used for foreground selection.

Phenotypic characterization under low moisture conditions

The F_1 plants were selfed to obtain F_2 . The F_2 progenies were grown in the field and segregation in height was observed. The tall plants along with dwarf plants were

further screened for the presence of $qDTY_{1,1}$ and $qDTY_{3,1}$ using peak foreground markers. Six confirmed pyramided lines (F_2 progeny) along with parents (SAB lines and DRR-50) were grown in pots under greenhouse condition for physiological studies. To test the effect of moisture stress, two sets of eight genotypes (six pyramided lines along with the parents) were grown and given moisture stress at vegetative and reproductive stages as described below. Each treatment consisted of eight plants for each genotype.

One set was treated with moisture stress at 55 days after transplanting. Plants were subjected to 7 days of low moisture stress at vegetative stage. Further, the same set was subjected to moisture stress at reproductive stage by withholding water for 15 days. The control set of plants were watered regularly.

Evaluation of pyramided lines for agronomic performance

Biometrical observations were recorded during appropriate stage of the crop. The height of the plant (cm) from the base to the tip of the panicle were measured. The total number of tillers per plant were recorded during harvest. The total number of panicles or productive tillers per plant were recorded during harvest. Length of panicles was measured in centimetres from the base to the tip of the panicle during maturity. Three panicles were randomly selected to count the number of spikelet and mean value of them were used for analysis. The matured grains of three panicles from each plant were counted and average was calculated from them. The number of fertile spikelets (filled grains) in the primary panicle of the selected plants were counted and recorded as percentage. Spikelet fertility (%) = [(number of filled grains/total number of grains) × 100]. The biological yield was content of dry matter (in gram) measured using weight of fully dried plant including panicles. The number of filled grains per plant were weighed in grams and recorded after harvest. The ratio of biological yield to grain yield were calculated as follows: HI = [(grain yield/biological yield) × 100]. A random sample of 100 grains were weighed on a precision balance to obtain the test weight.

Measurement of soil moisture content

Soil sample from five random pots each for treatment and control was collected and weighed. The soil weight before and after oven-drying of samples was measured. The moisture content (%) was calculated by subtracting the two soil moisture weight values.

Measurement of relative water content (RWC)

RWC was calculated by the method of Weatherley (1950). Briefly, random fresh leaves were harvested from each line grown under treatment and control conditions in three replicates. The fresh weight (FW) was measured immediately after harvesting; turgid weight (TW) was measured after floating the leaves in distilled water for 5–6 h and then dry weight (DW) was measured after 24 h of oven-drying (60 °C). RWC was calculated using the following equation: RWC (%) = [(FW–DW)/(TW–DW)] × 100.

Measurement of canopy temperature

Canopy temperature was measured by taking data on 48 samples per treatment both at vegetative and booting stage, with 5 days interval between each data point. Data was collected one hour before to two hours after solar noon (typically between 11:00 and 14:00 h). Care was taken to ensure that the plant surface was dry and not under shadow during data collection.

Measurement of chlorophyll content

Spectrophotometric method of Hiscox and Israelstam (1979) using dimethyl sulfoxide (DMSO) was used for estimation of chlorophyll content. Briefly, leaf sample (0.1 g) from both control and treatment conditions was harvested (the plant was at maximum tillering stage) weighed, chopped and put in opaque 2 mL tubes and stored at –80 °C for both control and treatment. A volume of 2 mL DMSO was added to each tube and kept in electric shaker for 1 h and absorbance measured at two different wavelengths viz., 663 nm and 645 nm. The chlorophyll content was calculated according to Arnon (1949) equation. For each sample, three replicates were taken.

Determination of lipid peroxidation

Lipid peroxidation status of plants was determined by measuring MDA (malondialdehyde) content (Heath and Packer 1968). A sample of 0.1 g frozen leaf was homogenized in freshly prepared 0.1% trichloroacetic acid (TCA) solution and centrifuged at 1500 rpm for 5 min. The supernatant of 0.5 mL was added to 1.5 mL of solution containing 20% TCA + 0.5% TBA, heated at 95 °C for 1 h and then cooled down to room temperature. Absorbance was measured at 532 nm followed by correlation for the non-specific absorbance at 600 nm. MDA content (μmol/g) was measured using extinction coefficient of 155 mM⁻¹ cm⁻¹ by the following formula:

$$\text{MDA} = \frac{\varepsilon(\text{Molar extinction coefficient})}{\text{Molecular weight of MDA}} \times (532 - 600) \times \frac{(\text{Volume of solution})}{\text{Weight of tissue}}$$

Statistical analysis

Phenotypic data including data for various traits was used for mean and variation analysis. Statistical analysis was carried out using *R* software. For each set of comparison, Duncan's multiplicative range test (DMRT) for differences in mean (n = 4) was carried out using alpha default value

as 0.05. Analysis of variance (ANOVA) was carried out using split plot design. In which, environment was the main factor or main plot factor and the genotypes were sub plots or subplot factor. Irrigated and irrigation withheld were the two environments.

Results

Generation of pyramided lines and identification of recombinants between *SD-1* and *qDTY_{1.1}*

Identification of double homozygotes (SAB) carrying the two drought tolerance QTLs on chromosome 1 and chromosome 2 was confirmed by using peak SSR markers RM431 and RM327, respectively. Three families based on highest background genome recovery of more than 90% (Bhandari et al. 2019) and seed morphology (similar to the recipient parent, SMS) were selected and thirty-five F_1 hybrids heterozygous for *qDTY_{1.1}* and *qDTY_{3.1}* were obtained. Two F_2 populations belonging to crosses DRR- 50 X SAB 4-2-20 and DRR- 50 X SAB 7-13-5 were grown in the field. About 1530 F_2 plants were screened both genotypically and phenotypically, and finally six SABD pyramided F_3 families were selected for phenotypic evaluation under moisture stress. Three F_2 plants were obtained in which SABD-8 was homozygous for both the QTLs, while SABD-7 and SABD-76 were homozygous for *qDTY_{3.1}* (RM520) and heterozygous for *qDTY_{1.1}*. Four F_2 recombinants were reported, in which SABD-9 and SABD-76 were homozygous and SABD-79 and SABD-80 were heterozygous for QTL *qDTY_{1.1}* (RM431), and were phenotypically semi-dwarf (*sd-1*).

Physiological evaluation of pyramided lines under low moisture conditions

SABD lines SABD-7, SABD-8, SABD-9, SABD-76, SABD-79, SABD-80 and parents (DRR-50, SAB lines) were assessed for drought response in greenhouse conditions during December 2019–May 2020 under well-watered and severe moisture stress conditions. Significant variation was observed for all investigated traits subjected to moisture stress and control under greenhouse conditions. At both vegetative and reproductive stages, plants were exposed to a dry spell (Fig. S2). After stress the soil moisture content gradually decreased in the vegetative stage whereas there was substantial reduction during the reproductive stage because of higher requirement for moisture during this stage. The moisture level during the reproductive stage declined significantly from 34 to 15 percent during 15-days stress and 21 percent during the vegetative growth stage (Fig. S2).

Morphological symptoms were observed after 7-days stress in the form of leaf rolling and leaf drying (Fig. S2) suggesting water deprivation; while no symptoms were observed in control.

Pyramided lines maintained chlorophyll content under moisture stress

The highest chlorophyll content reported was 24 SPAD units and the lowest was 21 SPAD units after 7-day stress; while it was 22 SPAD units and 17 SPAD units after 15-day stress among pyramid lines, respectively. Interestingly, all pyramided SABD lines had higher chlorophyll content, both under irrigated and moisture deficit conditions as compared to their respective SAB parental lines, and the chlorophyll contents were comparable with DRR-50. During the 7-day stress, chlorophyll content of plants under stress was higher than control and slowly lowered for 15-day stress. Nevertheless, not much variation was found between control and treatment for the 15-day moisture stress. In response to 7-day moisture stress there was a significant increase in chlorophyll content in SABD-7, SABD-8, SABD-76 and SABD-79 under stress except SABD-80 and SABD-9 which maintained chlorophyll content (Fig. 2a). For 15-day stress high level of chlorophyll content was maintained in SABD-76, SABD-80 and SABD-9 both under control and treatment indicating greater tolerance for moisture stress relative to other pyramided lines (Fig. 2b).

Superior SABD lines identified based on canopy temperature under stress

The pyramided lines displayed lower canopy temperature as compared to the parents, suggesting a greater tolerance under stress conditions. In response to 7-day moisture stress, 34.2 °C was the highest and 30.6 °C is the lowest canopy temperature recorded (Fig. 2c). The highest canopy temperature reported after 15-days moisture stress was 34.8 °C in parents and the lowest 30.7 °C in pyramided lines (Fig. 2d). High canopy temperature was observed after 15-day moisture stress as compared with 7-day stress due to an increase in the intensity of soil moisture reduction. Canopy temperature during the 7-day moisture stress was maintained in all pyramided lines both in control and treatment except SABD-9. After 15-day moisture stress, canopy temperature was significantly higher in all pyramided lines except SABD-8 in which canopy temperature was maintained both in control and treatment (Fig. 2d). An increase in canopy temperature with intensity of moisture stress was observed. The highest increase in canopy temperature at 15-day moisture stress as compared to irrigated conditions was observed in SAB 4-7-3 and SABD-7. In SABD-8, SABD-80 and SABD-76 the canopy

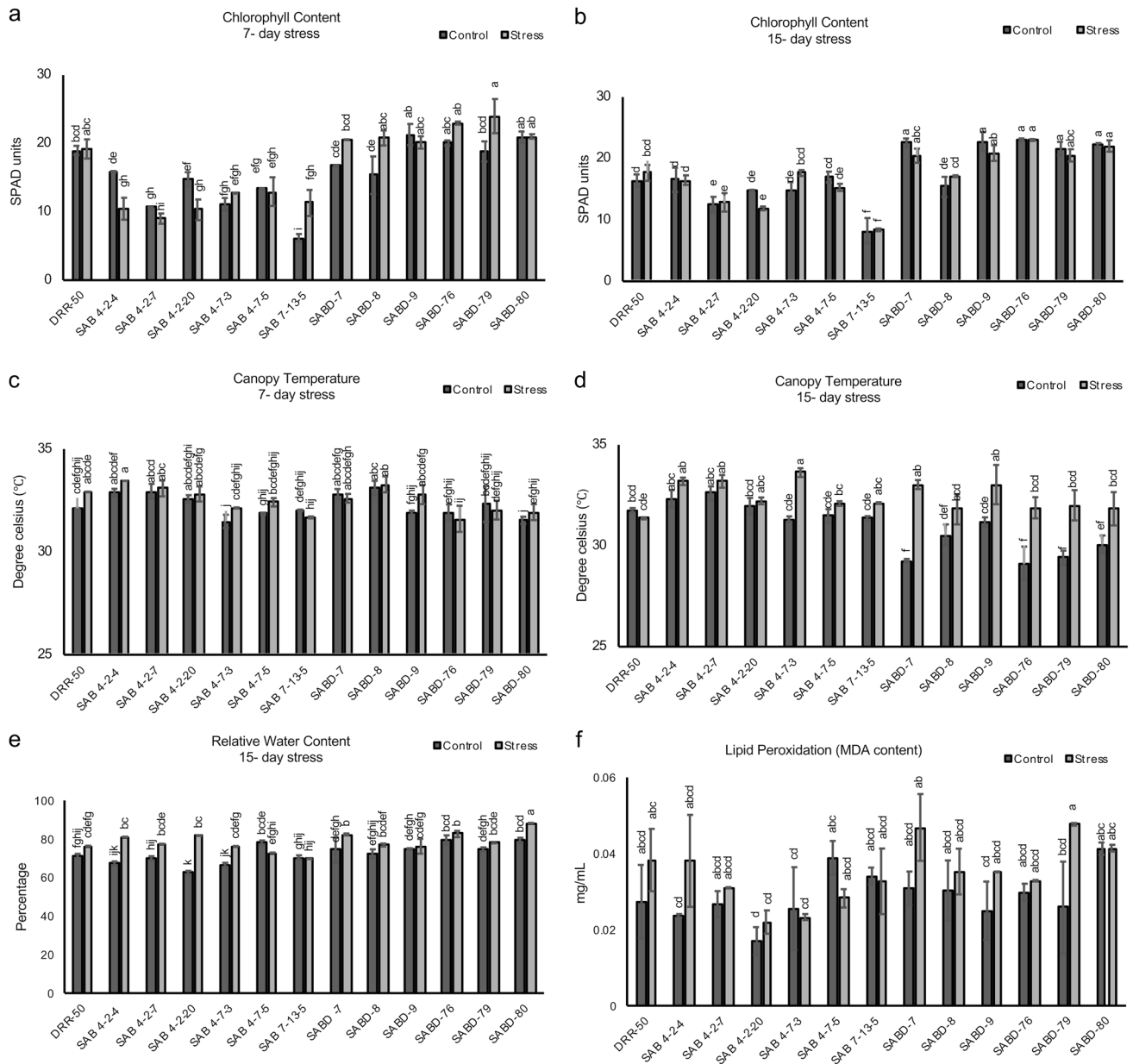


Fig. 2 Physiological parameters of pyramided lines after moisture stress. Variation in chlorophyll (**a**, **b**), canopy temperature (**c**, **d**), relative water content (RWC) (**e**) and **f**) MDA (malondialdehyde) content in pyramided lines after moisture stress. The difference in chlorophyll content (SPAD values) and canopy temperature ($^{\circ}\text{C}$) were measured at 7- and 15- days after stress induction. The RWC in stressed plants is expressed as percentage of RWC in control and

MDA content as a measure of lipid peroxidation after 15 days of moisture stress. DRR-50- donor line for $\text{DTY}_{3,1}$; SAB indicate lines carrying $\text{qDTY}_{1,1}$, whereas SABD indicate pyramided lines. Error bars represent ± 1 standard error (SE) of the technical variation, $n=4$. Different lowercase letters above the bars in (**a**–**f**) indicate significant differences ($P < 0.05$) between the groups using Duncan's multiplicative range test (DMRT)

temperature was significantly lower under stress compared to other pyramided lines.

Pyramided line retained higher relative water content (RWC) under stress

Relative to their parents, the SABD lines recorded higher RWC. The pyramided lines were able to retain their cellular moisture content up to 65 percent under stress showing resistance compared to their parents after 15 days

of moisture stress (Fig. 2e). The highest RWC recorded among pyramided lines under stress was 65 percent and the lowest 59 percent. SABD-80, SABD-7 and SABD-8 maintained higher RWC compared to parents and other pyramided lines.

Pyramided lines selected on the basis of lower lipid peroxidation

Lipid peroxidation status as measured by MDA content is a measure of cell membrane stability. The findings of this experiment suggested that under conditions of moisture stress, there was an increase in MDA content. MDA content is negatively correlated with stress tolerance. The lowest recorded MDA content was 0.032 mg/mL, and the highest among the pyramided lines was 0.047 mg/mL after 15 days of water stress in vegetative stage (Fig. 2f). MDA content was significantly higher in SABD-7, SABD-79 and SABD-9 under water stress as compared to irrigated conditions. However, under stress condition, MDA content was significantly lower in SABD-9 as compared to SABD-7 and SABD-79, showing higher membrane stability under water stress. In SABD-80 and SABD-8 MDA content was maintained both under control and stress. The least MDA content, and thereby, the highest membrane stability was observed in line SAB 4-2-20.

Evaluation of pyramided lines and their parents for agronomic traits under moisture stress

After attaining maturity, plants of each line were harvested individually from both sets of pots, the irrigated set (control) and pots that were subjected to two periods of moisture stress. Considering each pot containing four plants as a replication, and irrigated and moisture stress conditions as two environments, Analysis of Variance (ANOVA) was performed to estimate the proportion of variance explained due to genotypes, environment and genotypes X environment (GXE) interactions (Table 1). Variance due

to genotypes for traits such as plant height, tiller number, panicle number, panicle length, spikelets per panicle, grains per panicle, spikelet fertility, biological yield, grain yield, harvest index were highly significant at 1% level of significance. Variance due to genotypes for test weight was not significant. Interestingly, variance due to genotypes is not expected to be significant as the experimental material is composed of NILs. This indicates the influence of *SD-1*, which was present in some of the NILs, on the agronomic traits. The variance among environments was identified to be significant for panicle number, panicle length and test weight. GXE interaction for traits tiller number, grains per panicle and harvest index were significant at 5% level of significance. The variance among replications was found to be significant for panicle number and test weight.

Characterization of pyramided lines with respect to agronomic characters

All the SAB lines were tall (129.95–159.4 cm) while DRR-50 was short (109.5 cm) in height. Segregation in plant height was observed in pyramided lines SABD-7, SABD-76, SABD-79 and SABD-80 as they were in heterozygous condition for QTL *qDTY_{1.1}*. All genotypes along with parents which were under moisture stress exhibited a slight decrease in height, relative to plants under control (Fig. 3a). Dwarf plants with drought resistance QTLs were of special significance in this study because such plants will be tolerant to both drought and lodging. The plants which had the characteristics of DRR-50 were dwarf and had short culm with more tillers compared to the SAB lines which were tall and had 2 or 3 tillers (Fig. 3b). The pyramided lines that were dwarf and had up to 7–8 tillers. This indicates the significant influence *SD-1* gene has on the agronomic traits. The plants under moisture stress, mainly pyramided lines SABD-7, SABD-76 and SABD-80 had more secondary tillers relative to plants under irrigated condition. As compared to SAB lines the pyramided lines and DRR-50 have more productive tillers and hence a greater number of panicles (Fig. 3c).

Table 1 Mean squares, component of variance as a % of total variance (CV) and heritabilities

Sources of variation	PH	TN	PN	PL	SP	GP	SF	BY	GY	HI	TW
Replication	4.05	0.24	1.09*	0.02	58.24	2.11	29.73	9.39	2.15	0.14	0.01*
Genotypes	3513.57**	8.33**	8.58**	10.93**	1774.92**	924.92**	356.25**	20.89**	5.75**	312.75**	0.01
Environment	52.97	2.33	0.02*	0.51*	610.27	1543.53	443.85	11.58**	3.95	291.55	0.07*
Genotypes × Environment	79.6	1.27*	0.93	1.36	433.31	494.69*	164.22*	4.46	1.08	74.97*	0.002
Error a	287.26	0.09	0.003	0.23	41.12	201.21	251.86	9.26E	0.78	127.34	1.71E-05
Error b	50.98	0.3	0.36	1.28	373.57	168.08	60.81	2.68	0.5	26.51	0.01

PH plant height, TN tiller number, PN panicle number, PL panicle length, SP spikelets/panicle, GP grains/panicle, SF spikelet fertility, BY biological yield, GY grain yield, HI harvest index, TW test weight

*Significant at $P < 0.05$ level; **Significant at $P < 0.001$ level

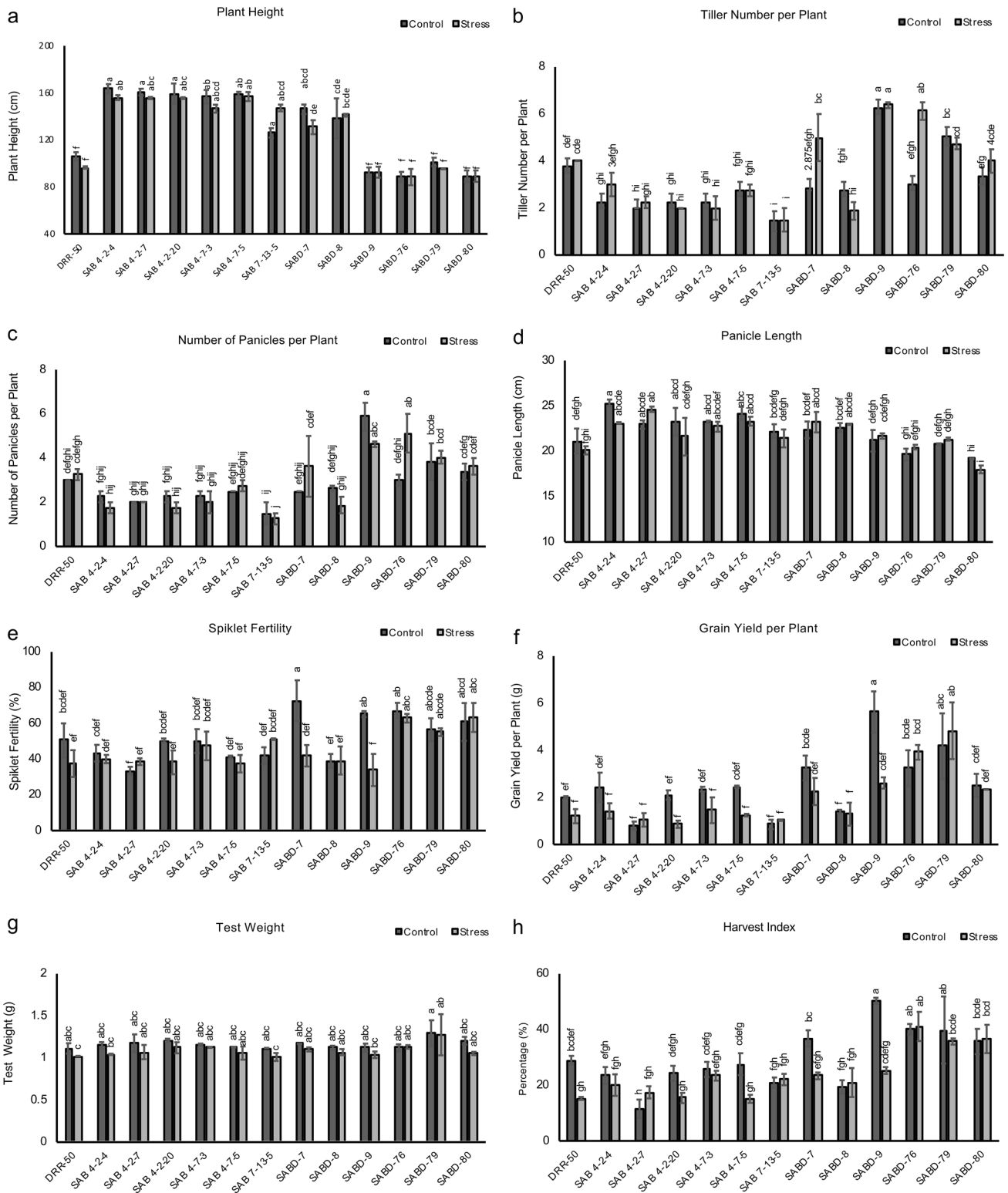


Fig. 3 Comparison of agronomic traits. **a** plant height, **b** tiller number per plant, **c** panicles per plant, **d** panicle length, **e** spikelet fertility, **f** harvest index, **g** grain yield per plant and **h** test weight of SABD lines with parents. Error bars represent ± 1 standard error (SE) of the

technical variation, $n = 4$. Different lowercase letters above the bars in (a–h) indicate significant differences ($P < 0.05$) between the groups using Duncan’s multiplicative range test (DMRT)

While SABD-9 had reduced number of panicles, other SABD lines maintained their number of panicles per plant in soil moisture deficit conditions as compared to irrigated conditions. The highest number of panicles per plant under moisture deficit condition was observed in SABD-76 followed by SABD-9. The SAB lines had longer panicles on an average compared to pyramided lines which had medium sized panicles (Fig. 3d). The panicles of secondary tillers were smaller. Highest panicle length was 26 cm recorded in SAB 4-2-4. No significant reduction in panicle length was observed in the lines due to moisture stress, except in the case of SAB 4-2-4 and SABD-80. Spikelet fertility was higher in pyramided lines, except in the case of SABD-8 compared to parents. There was non-significant difference between control and treatment in all the SABD lines except SABD-9 and SABD-7, where the spikelet fertility was reduced under stress condition (Fig. 3e). SABD-80, SABD-76 and SABD-79 retained the higher spikelet fertility in both control and moisture stress. The highest spikelet fertility under moisture stress was observed in the case of SABD-79 followed by SABD-76. Plants under stress were having lower harvest index compared to well irrigated plants (Fig. 3f). Harvest index was maintained in SABD-76, SABD-80, SABD-79 and SABD-8 both under control

and treatment. The highest harvest index under stress was observed in SABD-76 followed by SABD-79 and SABD-80. SAB 4-2-4, SAB 4-7-3 and SAB 7-13-5 also maintained their harvest index under moisture stress.

Grain yield in the case of all SABD lines, except SABD-8, was higher than SAB lines and DRR-50 under irrigated conditions. In general, plants under stress had lesser grain yield compared to plants which are irrigated continuously (Fig. 3g). While the majority of the SAB lines and DRR-50 showed significant decrease in grain yield when subjected to soil moisture stress, SABD-76, SABD-79 and SABD-80 maintained their grain yield under stress. The highest yield under moisture stress was observed in SABD-79 followed by SABD-76 and SABD-9. As expected, due to the higher grain yield, harvest index was more in pyramided lines relative to their parents.

Test weight was statistically similar in all the lines under study under irrigated condition (Figs. 3h and 4a, b), with the exception of SABD-79 and SABD-80 where some of the plants had bolder seed as compared to SMS (Fig. 4d), which may be due the presence of some donor alleles with minor phenotypic effect, which are still segregating in these lines. Such plants can be phenotypically selected against (rejected) to ensure that grain characteristics of Sambha Mahsuri Sub1

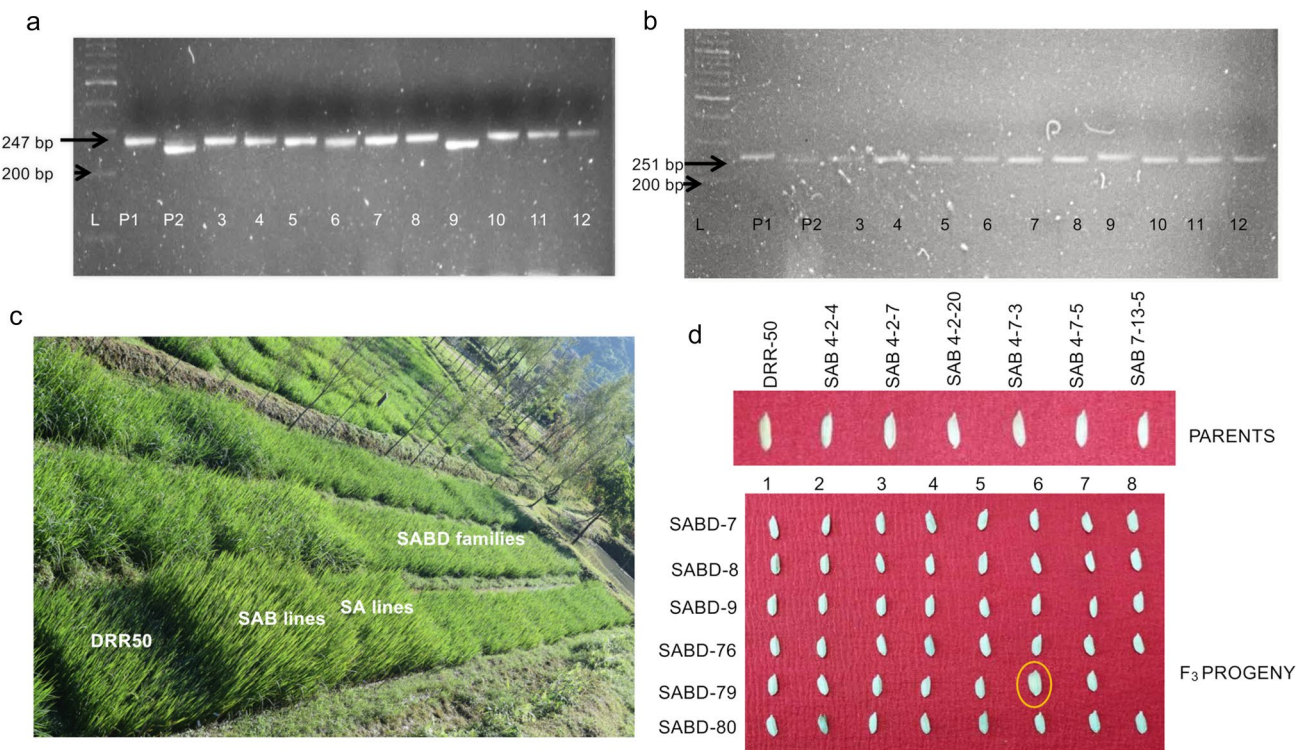


Fig. 4 Confirmation of pyramided lines with peak foreground markers **a** RM520 for qDTY_{3.1} and **b** RM431 for qDTY_{1.1}. L: Ladder (100 bp GeneRuler); P1:DRR-50; P2:SAB-4-2-20; lane 3 to 12: SABD 9-2, SABD 9-3, SABD 9-4, SABD 9-5, SABD 9-6, SABD

9-7, SABD 9-8, SABD 76-2, SABD 76-6, SABD 80-2. Variation in plant height (c) as observed at CPGSAS, Umiam and **d** seed morphology of donor, recipient, single and double pyramided rice lines

are fixed in the pyramided lines. Plants under stress were having lesser harvest index as compared to well irrigated plants due to impaired grain filling. SABD-79, SABD-76 and SABD-8 maintained test weight under moisture stress. Field evaluation under rainfed conditions revealed selected SABD lines to be similar in height as DRR50 (Fig. 4c).

Discussion

Our study reports the incorporation of two major QTLs for yield under drought stress conditions in a popular variety, Samba Mahsuri and the physiological performance of selected pyramided lines. Samba Mahsuri is a mega-variety for Eastern region which carries the submergence tolerance due to incorporation of Sub1 QTL (Singh et al. 2016). There are rice growing areas in Eastern India which encounter flooding as well as low moisture stress, albeit at different stages of rice growth. Therefore, a need was felt to introduce QTLs which would allow yields to be maintained even under water scarcity. Breeding for drought resistance and water-saving rice has been ongoing for many rice growing countries like China (Luo 2010), India (Mir et al. 2012; Singh et al. 2016), Philippines (Melandri et al. 2020, 2022), etc. to name a few. QTLs and genes identified are being pyramided into high yielding varieties in order to enhance their yield potential under drought stress conditions. However, the success of gene pyramiding depends upon several critical factors, including the number of genes to be transferred, the distance between the target genes and flanking markers, the number of genotypes selected in each breeding generation, the nature of germplasm, etc. (Raj and Sanghamitra 2010; Melandri et al. 2022). Drought grain yield QTLs showing large effects, viz. $qDTY_{1,1}$, $qDTY_{2,1}$, $qDTY_{2,2}$, $qDTY_{3,1}$, $qDTY_{3,2}$, $qDTY_{9,1}$ and $qDTY_{12,1}$ have been identified by IRRI; $qDTY_{1,1}$ and $qDTY_{2,2}$ have been transferred into the background of Samba Mahsuri-Sub1 background at CAU, Umiam (Singh et al. 2016). The BC₃F₄ introgression lines carrying individual QTLs from this study were used in the present study. The SAB lines were selected for double recombination on the carrier chromosomes using flanking markers, and for recipient (SMS) background using 50K SNP chip (Singh et al. 2015; Bhandari et al. 2019). Vikram et al (2015) reported that loss of the $qDTY_{1,1}$ allele during the green revolution was attributed to its linkage in repulsion with the *sd-1* semi dwarfing allele. During present study six F₂ plants SABD-9-3, SABD-9-2, SABD-9-6, SABD-9-7, SABD-76-2 and SABD-76-6 which were semi-dwarf (*sd-1*) and carrying $qDTY_{1,1}$ were obtained reflecting the breakdown of linkage between tall (*SD-1*) wild type and $qDTY_{1,1}$ on chromosome number 1. Our results suggest that targeting yield related QTLs effective under stress conditions can give lines which perform better for target trait as well

as maintain yield potential. Till date drought yield QTLs have been successfully introgressed in several genetic backgrounds like MR219 (Shamsudin et al. 2016), MRQ74 (Ikmal et al. 2018), Swarna-sub1 (Sandhu et al. 2019), etc. in different QTL combinations.

Low moisture stress tolerance being a quantitative trait, multiple physiological parameters might contribute to overall performance of a genotype (Heredia et al. 2022). During the current study, the level of soil moisture decreased to 21% in vegetative stress and 15% during reproductive stress, with plants exhibiting symptoms like leaf rolling and leaf drying. Similar results were observed in experiments where soil moisture decreased by 19.4% in the vegetative stage and 1.2% in the reproductive stage under water stress, confirming the crucial requirement for moisture during the reproductive stage as compared to the vegetative stage (Kitilu et al. 2019). RWC provides a measurement of the 'water deficit' of the leaf (Lugojan and Ciulca 2011), and may indicate a degree of stress expressed under drought and heat stress (Teulat et al. 2003; Wada et al. 2019). During the present experiment, higher RWC value was recorded for pyramided lines compared to parents at vegetative stage. A significant reduction in leaf water potential during vegetative stage under stress has been reported (Cha-Um et al. 2010). Based on RWC values pyramided lines SABD-80, SABD-76 and SABD-7 showed better tolerance to low moisture stress as these lines can retain higher moisture content even under limitation conditions.

Canopy temperature (CT) has been used as non-destructive and indirect method of measuring the amount of stomatal conductance in plants including rice (Takai et al. 2010; Jiang et al. 2023). Also as a reliable indicator of drought stress in field (Melandri et al. 2020) with lower CT values indicating drought tolerance (Yan et al. 2012). In our study, as the intensity of moisture stress increased, the canopy temperature also increased from 7th day upto 15th day of stress. Growth rate surrounding heading date is critical for ensuring yield in rice (Takai et al. 2006). Zhang et al (2007) showed that soil moisture content significantly influences CT; lower the soil moisture content higher is the CT value. Correlation between high yield potential and stomatal conductance is well known for C3 plants like rice (Roche 2015; Melandri et al. 2020). It is now clear that sustained stomatal conductance allows for cooler canopies with continued photosynthesis even under dry conditions as CO₂ is able to diffuse in leaf through stomata. Delayed leaf rolling (Turner et al. 1986); better access to soil water and osmotic adjustment (Blum 1988); drop in transpiration, light interception and leaf dehydration (Kadioglu and Terzi 2007) conferred yield advantage under drought conditions (Singh and Mackill 1991). In our study SABD-9, SABD-79 and SABD-8 displayed delay in leaf rolling, drying

and performed better under moisture stress relative to the parents.

Lipids constitute a major portion of cell membrane and their stability indicates survival strategy at low moisture levels. Water deficit tends to increase reactive oxygen species (ROS) formation, eventually leading to lipid peroxidation (Hansen et al. 2006). Increased MDA content is an indication of oxidative damage under drought (Møller et al. 2007; Pandey et al. 2010; Melandri et al. 2022). Pyramided lines SABD-76, SABD-9, and SABD-8 recorded lower MDA content under moisture stress relative to their parents and other pyramided lines. Similarly, Sharma et al (2012) reported that tolerant genotypes were better equipped under drought stress having higher antioxidants activity and lower MDA content and electrolyte leakage which protect against oxidative stress. Therefore, the pyramided lines which recorded lower MDA content under stress were tolerant genotypes.

Chlorophyll loss is a negative consequence of water deficit. Chlorophyll content is a good indicator of photosynthetic efficiency of plants. The pyramided lines SABD-76, SABD-80 and SABD-9 recorded higher chlorophyll content under moisture stress conditions than the parents and other pyramided lines suggesting that these lines were more resistant to moisture stress. Sepehri and Golparvar (2011) reported that chlorophyll content of sensitive cultivars decreased but increased in resistant cultivars. Ormaetxe et al (1998) showed that severe water deficit inhibited photosynthesis and decreased chlorophyll quantity. It was observed that chlorophyll content during initial days of stress increased significantly, while in later stages as the stress increased the chlorophyll content decreased drastically. In a wheat experiment chlorophyll content decreased as drought was prolonged (Nikolaeva et al. 2010).

Variation for panicle number, panicle length and test weight was significant in soil moisture stress conditions. Results by Ruth et al (2017) revealed significant variation among four environments including drought stress whereas, GXE interaction was non-significant for all the traits except tiller number, grains per panicle and harvest index. Hence, there was no environmental influence on the stability of genotypes, indicating tolerance in general.

Height of the plant is one of the selection criteria used to identify drought tolerant genotypes under drought conditions. In our study parents exhibited a small decrease in height during stress. But the difference in plant height among pyramided lines were mainly dependent on the presence or absence of wild type *SD-1* allele.

A major yield contributing trait is panicle number with rice plants responding to drought by reducing the number of tillers. Pyramided lines in particular SABD-8 and SABD-9 registered a reduced number of panicles relative to the other

plants under moisture stress. Whereas, the other pyramided lines SABD-76, SABD-79 and SABD-80 recorded higher number of panicles compared with plants under control. SABD-76 and SABD-79 had the highest yield under moisture stress and maintained their yield as compared to irrigated conditions.

Stress tolerance is considered to be the product of many physiological and morphological characteristics (Bhardwaj et al. 2022). It is now suggested that breeding rice for drought resilience will benefit from integrating information from metabolic/biochemical traits representative of plant endophenome along with yield with stress indices (Singh and Choudhary 2003; Raman et al. 2012; Melandri et al. 2022). During present study there was significant reduction in the tiller number, 1000 grain weight; spikelet fertility, grain number per panicle; grain yield and spikelets per panicle under moisture stress conditions. Previously, rice cultivars with the least affected physiological and biometrical parameters displayed greater tolerance to drought (Sikuku et al. 2012; Melandri et al. 2020). Therefore, the pyramided lines SABD-9, SABD-76, SABD-79 and SABD-80 which performed better for the above mentioned traits under stress conditions and showed greater tolerance relative to the parents are eligible for further evaluation.

Conclusion

Breeding rice varieties with multiple stress tolerance is of great relevance in the scenario of climate change; Samba Mahsuri-Sub1 is not only a high yielding variety but is tolerant to submergence as well. The incorporation of two major drought tolerant QTLs in this variety would confer multiple stress tolerance and would be of great benefit especially for areas prone to both drought and flood. In this study, the seeds obtained from the dwarf pyramided lines morphologically resemble that of the recurrent parent but were more tolerant to low moisture stress as indicated by various parameters like RWC, lipid peroxidation, canopy temperature, chlorophyll content, etc. These physiological parameters have led to identification of six superior lines like SABD-9-3, SABD-9-2, SABD-9-6, SABD-9-7, SABD-76-2 and SABD-76-6 performing better under moisture stress. These lines after multi-location evaluation may be released as varieties possessing multiple stress tolerance for cultivation in rice growing areas experiencing multiple stresses.

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Author contributions MR, NS conceived and supervised the project. WT supervised the molecular work. PG contributed DRR-50 seeds. DC made crosses and performed physiological analysis. DC, DD, BL and MP performed molecular marker analysis. MR and DD selected the dwarf pyramided lines. DC, BL, MP and MR interpreted the data. DC wrote the original draft and WT and MR edited the manuscript. All authors read and approved the final manuscript.

Data availability The data that support the findings of this study are included as supplementary data.

Declarations

Conflict of interest Authors declare that there is no conflict of interest.

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