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RESEARCH

# Development of breeding lines with multiple stress tolerance to cold, drought, and high-temperature stress in rice (*Oryza sativa* L.)

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**Abstract** Farmers and breeders recognize that multiple abiotic stresses occurring simultaneously are more harmful to crops than individual stress conditions. In this study, pedigree-based breeding was employed to develop multiple abiotic stress-tolerant rice breeding lines (MTU 1010/IRINMST-291), using stringent phenotypic selection for germination and seedling stage cold stress tolerance, reproductive stage drought stress, and reproductive stage high-temperature stress. Among the developed lines, IRINMST-007, IRINMST-338, IRINMST-418, and IRINMST-129 emerged as high-performing families under non-stress, drought, and high-temperature conditions during the reproductive stage. Additionally, IRINMST-007, IRINMST-418, and IRINMST-129 showed strong cold tolerance at the seedling stage and germination stage. These breeding lines have the potential to address food security challenges posed by abiotic stresses. A total of 110 parental polymorphic markers were used for single-marker analysis in the 180 selected $F_5$ recombinant inbred lines. RM5344 were found associated with percent spikelet fertility at reproductive stage high-temperature stress with a phenotypic variance of 24%. RM5911 was found to be associated with grain yield at reproductive stage drought stress with a phenotypic variance of 11%. RM1019 was found associated with seedling growth under cold stress with a phenotypic variance of 18%. RM5344 was found associated with survival rate at seedling stage cold stress with a phenotypic variance of 19%. RM555 was found associated with vigor of germination at germination stage cold stress with a phenotypic variance of 18%. These markers can be used for molecular breeding programs to develop multi-stress tolerant rice varieties.

**Keywords** Cold stress · Drought stress · High-temperature stress · Single marker analysis · Rice breeding

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## Introduction

Multi-stress tolerance is indeed crucial for crops to withstand various environmental stresses, ensuring

stable yields and improved agricultural practices. Developing multi-stress tolerant rice varieties can provide numerous benefits to farmers, including increase crop yield, improve crop stability and enhance food security even in challenging multi-stress conditions. Rice serves as the primary source of dietary energy for nearly half of the global population, particularly for more than 3 billion people in Asia, where 90% of the world's rice is both produced and consumed. By 2050, the global population is expected to reach 10 billion (United Nations, 2017). To meet food demand, crop production must increase by 70–100% (Tomlinson 2013). Compounding this challenge is climate change, which threatens agricultural productivity through increased temperatures and heightened drought stress (Jagadish et al. 2012; Mottaleb et al. 2016). In rainfed areas, rice crops are especially vulnerable to drought at all developmental stage, including seedling, vegetative, and reproductive phases. Modern rice varieties are highly sensitive to drought, even under mild conditions, leading to significant yield losses (Torres and Henry 2018). The ongoing rise in global temperatures due to climate change poses a significant risk to rice production. Average temperatures are projected to increase by 2–3 °C over the next 30–50 years (Hatfield and Prueger 2015). Rice cultivation is typically concentrated in regions where day and night temperatures drift near the optimum thresholds of 28 °C and 22 °C, respectively (Das et al. 2014). While rice can maintain normal growth and grain yield within a temperature range of 27–32 °C, temperatures exceeding 32 °C have detrimental effects on all stages of growth and development (Aghamolki et al. 2014). For every 1 °C increase in global temperature, rice yields are estimated to decline by 10%. High-temperature stress is particularly damaging during the flowering stage, leading to low seed set and significant yield losses (Jagadish et al. 2010; Bahuguna et al. 2015; Wang et al. 2016).

Even low temperatures can also adversely affect rice plants during germination, vegetative growth, and reproductive stages. The optimal temperature for germination and seedling development in rice ranges from 25 to 30 °C. Cold stress occurs when temperatures fall below 17 °C, leading to poor germination, seedling injury, weak crop establishment, and reductions in yield stability and productivity (Koseki et al. 2010). Specially at seedling stage, cold-induced injuries manifest as necrosis, chlorosis, leaf rolling, and stunting (Lou et al. 2007). To address all these challenges, genetic improvement in rice is urgently needed to achieve high-temperature stress tolerant breeding lines with high productivity. Among all rice varieties, the recipient parent MTU 1010 (Cottondora Sannalu) is a short-duration, high-yielding, long-slender variety released by the Andhra Pradesh Rice Research Institute (APRRI), Maruteru and also widely cultivated in India during the *Rabi* season. While rice growing farmers require varieties with higher grain yields and higher stability of yield across diverse environments.

To address food security challenges posed by abiotic stresses such as drought and high temperatures during different growth stages of rice and alleviate poverty among farmers reliant on rainfed ecosystem (Venuprasad et al. 2008). In light of this, development of multi stress tolerant breeding lines is essential. These breeding lines are expected to perform better under multi-stress conditions on farmers' fields. For this purpose, we used the newly identified donor parent IRINMST-291, which exhibits tolerance to cold stress at the germination and seedling stages, as well as tolerance to drought and high temperatures during the reproductive stage. This donor parent was crossed with MTU 1010, a popular rice variety that is susceptible to cold stress during germination and seedling stages and also high-temperature stress during the reproductive stage but exhibits moderate tolerance to drought stress during the reproductive stage. While genotyping of the progeny was performed using polymorphic markers to conduct single-marker analysis for assessing tolerance to various abiotic stresses.

## Materials and methods

### Selection of parental material

The plant materials used in this research included the recipient parent MTU 1010 and the donor parent IRINMST-291, an improved breeding line derived from the cross Swarna$^3$/Moroberekan* (Dixit et al. 2014), which possesses tolerance to drought, high temperatures, and cold stress. MTU 1010 is a long-slender, high-yielding, short duration variety derived from a cross between IR-64 and Krishnaveni. The experiment was carried out at the International Rice

Research Institute-South Asia Hub (IRRI-SAH), ICRISAT, Patancheru (78°16′ E longitude, 17°32′ N latitude, and 540 m above sea level) from wet season of 2013 to dry season of 2017.

## Breeding methodology

The pedigree-based breeding approach followed with the generation advancement (Fig. 1) till the homozygosity fixation of the breeding population**.** MTU 1010 was crossed with IRINMST-291, to produce $F_1$ seeds. Based on the hybridity test the true $F_1$s were identified and advanced further by selfing to generate the $F_2$ population. The superior single plant selection method was employed at every generation till the uniformity attains at the same time the population was screened for multiple abiotic stress such as cold stress tolerance at germination and seedling stages ($F_3$ families), drought stress tolerance at reproductive stage ($F_4$ families) and combaining high-temperature (at reproductive stage), drought and cold stress tolerance ($F_5$ families), while selecting superior abiotic stress tolerance lines from $F_5$ generation and advanced in to $F_6$ generation The suitable checks for each stress were utilized during the screening. At the end identified promising lines ($F_{6:7}$) evaluated for yield and

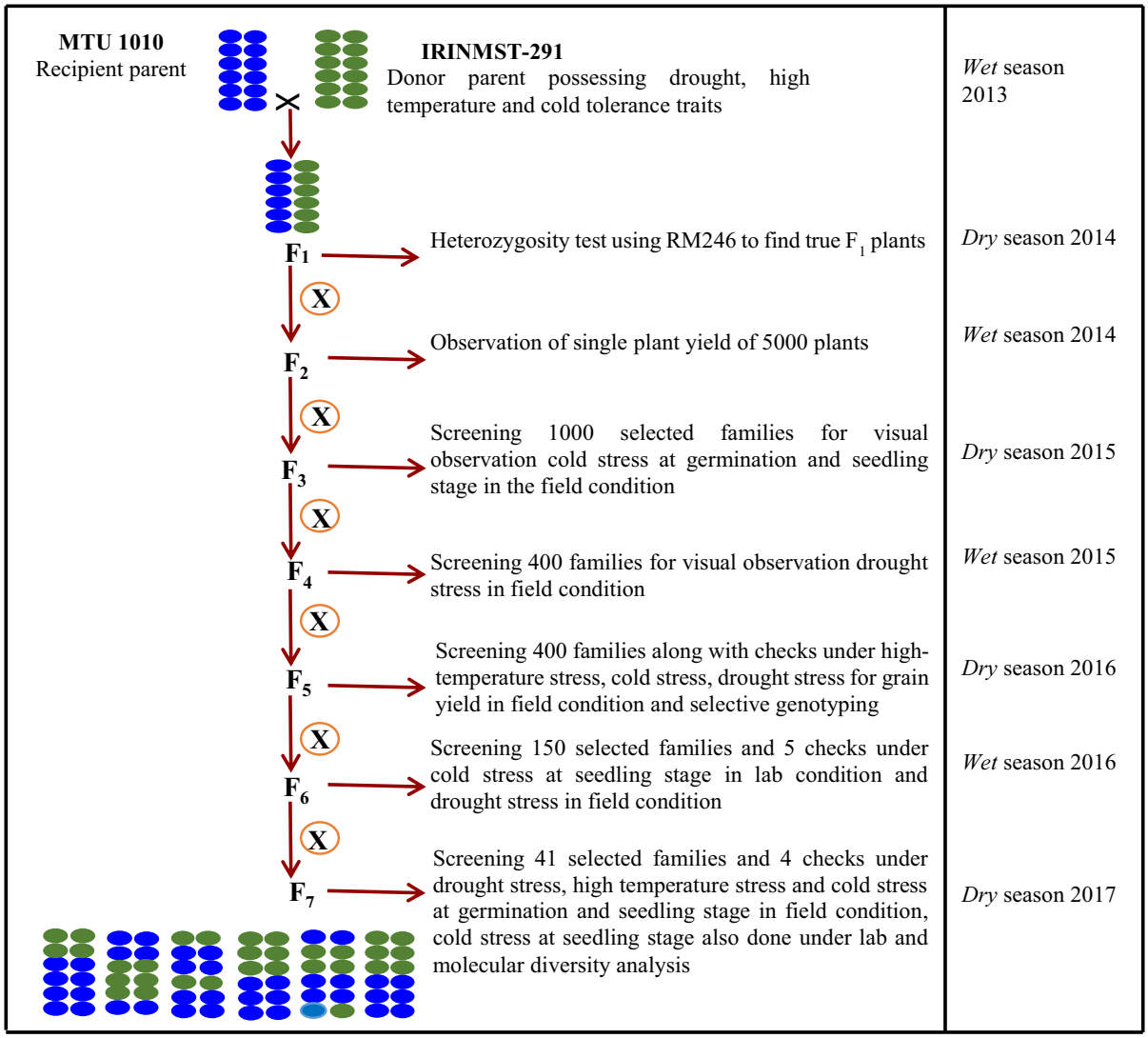


**Fig. 1** Development of a breeding population (MTU 1010/ IRINMST-291) for cold, high- temperature, and drought tolerance traits

component traits along with the relevant check varieties to identify the promising breeding lines possess with high yielding and multiple abiotic stress tolerance.

Experimental design and observations

The early generation of breeding lines were evaluated in the augmented design in the different conditions. The selected advanced generation breeding lines or recombinant inbred lines (RILs) were evaluated under three conditions: irrigated control (non-stress), drought stress at the reproductive stage, and high-temperature stress at the reproductive stage. The field experiment laid out with randomized complete block design (RCBD) in two replications. Twenty-five-day-old seedlings of the parental lines and selected RILs were transplanted in 4.8 square meter plot area. The phenotypic observations were made in both stress and non-stress conditions such as days to 50% flowering (No.), plant height (cm), productive tillers (number), panicle length (cm), flag leaf length (cm), number of filled grains/panicle (number), 1000-grain weight (g), spikelet fertility (%), grain type and grain yield (kg/ha).

Evaluation of breeding lines for drought stress at reproductive-stage

The basic requirement for open field drought stress evaluation the field topography should be alleviated and away from water sources or irrigated and flooded rice fields. A set of 41 advanced breeding lines and the relevent check varieties (4) were included in the experiments. In the non-stress trial we followed the general package of practices with irrigation up to maximum tillering stage. Rice is highly sensitive to drought stress at the reproductive stage (The critical period from 16 days before flowering to 10 days after flowering). In lowland fields, depending on the soil's moisture-holding capacity, drought typically becomes severe within 15–20 days. To simulate drought conditions, the field should be drained 23–25 days after transplanting. To monitor the ground water level, at least three groundwater tubes (1.10 m deep, with 1 m in the soil and 10 cm above ground) should be installed in each replicate 1 week before the draining of field. During severe drought stress, the field should be inspected at 1:00 PM to decide on potential irrigation. If all susceptible checks exhibit severe leaf rolling with a minimal likelihood of recovery upon watering and the trial as a whole shows signs of stress the field should be flood-irrigated. Six hours after irrigation, the field must be drained to initiate the next cycle of stress. This cycle of stress and irrigation should continue until harvest. The water table depth should be recorded after the initiation of stress. In severe stress treatments, the gravimetric soil moisture and soil water potential typically decline to very low levels (approximately −60 kPa).

Evaluation of breeding lines for high-temperature stress at reproductive-stage

The rice is highly sensitive to high-temperature stress during the anthesis. Temperatures exceeding 35 °C negatively impact the entire anthesis process, from anther dehiscence to fertilization, which takes between 45 min and 4 h, depending on the genotype.

*Protocol followed for high-temperature stress screening*

To assess high-temperature stress tolerance, the genotypes were grouped according to their heading times. Seeds of each genotype were germinated in seedling trays filled with sterilized soil after breaking dormancy at 47 °C for 48 h. Eighteen-day-old seedlings were transplanted into plastic pots or buckets filled with clay-loam soil, supplemented with 2.5 g of muriate of potash (KCl) and 2.5 g of single super phosphate (SSP). Each pot contained a single plant with three replications and was maintained in a net house under natural temperature and light conditions.

At the panicle initiation stage, heading panicles were marked, and plants were transferred to an indoor growth chamber (Conviron®), where the temperature gradually increased from 29 to 39 °C between 7:30 and 2:30 PM. Panicles that anthesized before transfer to the growth chamber were removed. Watering was done only after 2:30 PM to maintain the target temperature stress period of 39 °C (7:30–2:30 PM). The temperature and humidity settings inside the growth chamber are detailed in Supplementary Table 1. A thermocouple placed above the canopy was used to validate air temperature and humidity inside the chamber.

*Evaluation of genotypes after stress imposition*

After 10 days of high-temperature exposure, the plants were returned to the net house under natural temperature and light conditions. At physiological maturity, the filled and empty spikelet from the marked panicles were counted to calculate spikelet fertility. Spikelet fertility (%) was calculated as the ratio of filled spikelets to total spikelets. The mean spikelet fertility across five marked panicles per pot was used as the basis for evaluating high-temperature tolerance (IRRI SES 2014). Recipient MTU 1010 and donor IRINMST-291 were screened alongside known cultivars: Nagina22 (N22), a high-temperature tolerant variety (Mohapatra et al. 2014; Prakash et al. 2016), and IR64, a heat-susceptible variety (Jagadish et al. 2010), as illustrated in Supplementary Fig. 1.

*Phenotypic observations*

Three individual plants from each selected breeding line and check variety were sampled and harvested separately. The main panicle from each plant was used to calculate spikelet fertility by counting filled and unfilled spikelets. Remaining panicles from the breeding lines were collected separately and used to calculate grain yield under high-temperature stress. To ensure synchronized flowering across breeding lines, sowing was scheduled based on flowering times. This strategy allowed uniform high-temperature treatment, minimizing phenotypic observation errors.

Cold stress or low temperature tolerance screening at germination and seedling stage

*Plant growth chamber conditions*

The 10–14 days healthy seedling to be keep in Conviron Plant growth chamber with temperature (10 °C/10 days), light (12 h light + 12 h dark) and humidity 70–80% to be maintained. Observation of seedling growth is 1 = dark green seedlings, 3 = light green seedlings, 5 = yellow seedlings, 7 = brown seedlings, 9 = seedlings dead (IRRI SES 2014) and survival rate (%) after cold treatment is calculated as the Number of surviving plants/Total number plants treated × 100 (Zhang et al. 2011). The screening of recipient, donor parent, and derived breeding lines along with reported check was mentioned in Supplementary Fig. 2.

*Field conditions and weather data*

Cold tolerance screening was conducted under two conditions: (1) laboratory (growth chamber and (2) field conditions at IRRI-South Asia Hub, ICRISAT, Patancheru, Telangana, India, and Bihar Agricultural University, Sabour, Bhagalpur, Bihar, India.

*IRRI-South Asia hub weather records* During the dry seasons of 2016 and 2017, the average minimum (night) temperature was 13.90 °C and 12.66 °C, respectively, while the average maximum (day) temperature was 30.86 °C and 29.08 °C in both seasons, recorded from December to January.

*Bihar agricultural university weather records* During the dry season of 2017, the average minimum (night) temperature was 8.7 °C, and the average maximum (day) temperature was 22.6 °C, recorded from December 2016 to January 2017.

*Cold tolerance check varieties*

The screening included recipient MTU 1010, donor IRINMST-291, and cold-tolerant checks IR83222-8-1-1-1-1-1-1 (Jena et al. 2010) and K-332 (Lone et al. 2018). Cold tolerance at the germination stage is primarily evaluated using germination vigor and seedling survival rate (Han et al. 2006). The cold tolerance screening at germination and seedling stages, alongside evaluations of drought and high-temperature stress, is shown in Fig. 2.

Molecular study of breeding lines

*DNA isolation, PCR amplification, and electrophoresis*

Young and healthy leaf samples from 41 breeding lines, along with the recipient and donor parents, were used to isolate genomic DNA following the IRRI protocol (TPS buffer). The isolated DNA was diluted to a final concentration of 100 µl with TE buffer. The quantification of DNA was done on 0.8% agarose gel electrophoresis. Polymerase Chain Reaction (PCR) was carried out using 15–20 ng of template DNA,

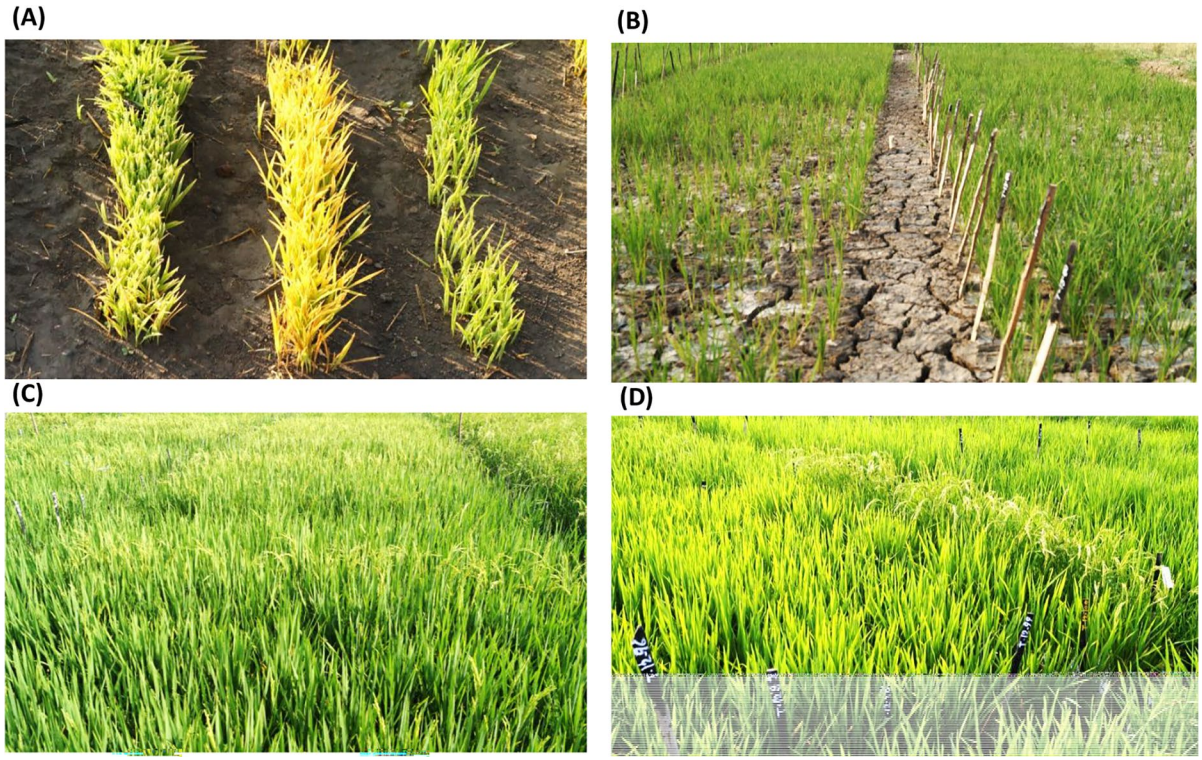


**Fig. 2** Screening of breeding population (MTU 1010/ IRINMST-291) for **A** cold stress **B** drought stress **C** high-temperature stress and **D** non-stress conditions

0.05 mM of dNTPs (Eppendorf, USA), 5 pM of each forward and reverse primer, 0.5 units of Taq DNA polymerase (Bangalore Genei, India) and 1X PCR reaction buffer (Bangalore Genie, India) in a total volume of 10 µl. PCR was carried out using Eppendorf Mastercycler (nexus) with initial denaturation at 94ºC for 5 min followed by 33 cycles of PCR amplification under the following parameters: 40 s at 94 °C, 45 s at 55 °C, and 45 s at 72 °C, followed by final extension at 72 °C for 10 min.

The PCR-amplified products were resolved on a 4% agarose gel in 1X TAE buffer at 110 V. After staining with ethidium bromide, the gels were visualized under UV light using a gel documentation system. Parental polymorphism was assessed using 1000 SSR markers, which were resolved on a 4% agarose gel. Among 1,000 SSR markers tested, 110 were identified as polymorphic between the recipient and donor parents. These markers were then utilized for molecular analysis of the breeding lines.

*Single marker analysis and cluster analysis based on molecular marker genotyping data*

The out of 400, 180 recombinant inbred lines (MTU 1010/ IRINMST-291) used for selective genotyping on the basis of phenotypic data under different abiotic stress condition. Single marker analysis was done using QTL IciMapping software. For diversity analysis of breeding lines along with recipient and donor parents was done using allele wise score of the amplified PCR product each primer. The allelic data format was subsequently analyzed using the computer package DARwin (V.6.0.5) (Perrier and Jacquemoud-Collet 2006). The construction of a dendrogram was done using the unweighted pair group method of arithmetic mean average (UPGMA) cluster analysis.

Statistical analysis

The mean values of agronomic parameters were calculated before performing statistical analyses. A factorial ANOVA was conducted, followed

by Tukey's post hoc test at significance levels of $p = 0.05$ and $p = 0.01$. The analysis was performed within each genotype under normal, drought, and high-temperature stress conditions using the Statistical Analysis System (SAS 9.4) software, North Carolina State University, USA. Single marker analysis was performed using the QTL IciMapping software.

## Results

### Identification of recipient and donor for drought, high-temperature, and cold tolerance

The cold stress screening at germination and seedling stages was conducted for MTU 1010 and IRINMST-291 alongside reported cold tolerance checks, IR83222-8-1-1-1-1-1-1-1 and K-332. Results from field conditions and the plant growth chamber confirmed that MTU 1010 is susceptible, while IRINMST-291 is cold-tolerant. High-temperature stress screening involving MTU 1010, IRINMST-291, the heat-tolerant check N22, and the susceptible check IR64, showed that MTU 1010 is susceptible, whereas IRINMST-291 is tolerant to high temperatures. Drought stress screening was performed with Sahabhagi Dhan as a drought-tolerant check. It was observed that MTU 1010 is moderately tolerant, while IRINMST-291 demonstrated full tolerance to drought.

### Performance of of breeding lines under non-stress condition

According to the Tukey grouping results, the best-performing breeding lines under non-stress conditions are identified as IRINMST-007, IRINMST-338, IRINMST-418, IRINMST-129, and IRINMST-057. The percentage yield superiority over best check IRINMST-291 was observed in IRINMST-007(20.30%), IRINMST-338(7.38%), IRINMST-418(1.20%), IRINMST-129(-0.09%) and IRINMST-057(-0.69%). Table 1 highlights the five top-performing breeding lines under normal conditions**.**

The result year wise showed that significant difference, in days of 50% flowering (DFF), plant height (PH in cm), number of productive tillers (NT), panicle length (PL in cm), flag leaf length (FLL in cm), filled grains per panicle (FGP), percentage spikelet fertility (% SF) and grain yield (GY in kg/ha) at 1% probability level but it was observed that no significant difference in test weight (TW). The replication wise result was observed that significant difference, in DFF, PH, NT, PL at 1% probability level but it was observed that no significant difference in FLL, FGP, % SF, TW, GY. In entry wise result was observed that significant difference, in DFF, PH, NT, PL, % SF, TW and GY at 1% probability level but it was observed that no significant difference in FLL and FGP. The interaction between year and entry was observed that significant difference, in DFF, PH and PL at 1% probability level but it was observed that no significant

**Table 1** Performance of yield potential trait of promising advanced breeding lines under non-stress condition

| S. No | Breeding lines | DFF | TW | FGP | % SF | GY (kg/ha) | % Sup | Tukey group |
|---|---|---|---|---|---|---|---|---|
| 1 | IRINMST-007 | 105.7 | 22.29 | 130.13 | 90.00 | 6617.0 | 20.30 | A |
| 2 | IRINMST-338 | 103.2 | 23.3 | 121.42 | 88.00 | 5906.0 | 7.38 | AB |
| 3 | IRINMST-418 | 105.5 | 21.71 | 129.17 | 86.46 | 5566.0 | 1.20 | BC |
| 4 | IRINMST-129 | 105.0 | 21.91 | 120.75 | 87.98 | 5495.0 | -0.09 | BC |
| 5 | IRINMST-057 | 107.7 | 20.05 | 125.38 | 80.12 | 5462.0 | -0.69 | BC |
| 6 | MTU1010 | 102.2 | 21.17 | 113.64 | 84.25 | 5062.3 | | |
| 7 | Sahbhagi Dhan | 102.7 | 19.86 | 107.17 | 79.53 | 3774.5 | | |
| 8 | IR 64 | 100.7 | 21.34 | 101.00 | 85.07 | 5417.6 | | |
| 9 | IRINMST-291 | 97.0 | 22.72 | 115.75 | 79.22 | 5500.3 | | |
| HSD @ 5% | | 7.92 | 4.32 | 50.80 | 17.23 | 995.65 | | |

DFF, days to 50% flowering; TW, 1,000 grain weight in gram; FGP, filled grains /panicle; % SF, percentage spikelet fertility; GY (kg/ha), grain yield (kilogram/hectare); % superior, percent superiority over best check; HSD, honest significant difference at 5% probability level

difference in NT, FLL, FGP, % SF, TW and GY (Supplementary Table 2).

### Performance of breeding lines in drought stress condition

As per the Turkey grouping results, the best-performing breeding lines under drought stress conditions are IRINMST-007, IRINMST-129, IRINMST-057, IRINMST-418, and IRINMST-338. The percentage yield superiority over best check IRINMST-291 was observed in IRINMST-007 (18.02%), IRINMST-338 (7.40%), IRINMST-418 (1.44%) IRINMST-129 (0.15%) and IRINMST-057 (-0.67). The top-performing breeding lines under drought stress conditions are presented in Table 2.

The water table declined progressively to below the depth of 60 cm for a major part of the stress period but in severe stress (100 cm) was observed. The result year wise showed that significant difference, in days of 50% flowering (DFF), plant height (PH in cm), panicle length (PL in cm), flag leaf length (FLL in cm) and test weight (TW) at 1% probability level and number of productive tillers (NT), percentage spikelet fertility (% SF) at 5% probability level but it was observed that no significant difference in filled grains per panicle (FGP) and grain yield (GY in Kg/ha). The replication wise result was observed that significant difference, in DFF, PH, FLL, FGP and TW at 5% probability level but it was observed that no significant difference in NT, PL, % SF, and GY. In entry wise result was observed that significant difference, in DFF, PH, FGP, % SF, TW and GY at 1% probability level but it was observed that no significant difference in NT, PL and FLL. The interaction between year and entry was observed that significant difference, in PH, NT at 1% probability level, and FLL at 5% probability level but it was observed that no significant difference in DFF, PL, FGP, % SF, TW and GY (Supplementary Table 3).

### Performance of of breeding lines in high-temperature stress condition

The result of Tukey grouping showed that the best-performing breeding lines under high-temperature stress conditions are IRINMST-007, IRINMST-113, IRINMST-338, and IRINMST-418. The percentage yield superiority over best check IRINMST-291 was observed in IRINMST-007 (14.79%), IRINMST-338 (9.04%), IRINMST-418 (2.39%), IRINMST-192 (1.55) and IRINMST-113 (1.39). The top-performing breeding lines under high-temperature stress conditions are presented in Table 3.

The result year wise showed that significant difference, in days of 50% flowering (DFF), plant height (PH in cm), flag leaf length (FLL in cm) at 1% probability level, and number of productive tillers (NT) and filled grains per panicle (FGP) at 5% probability level but it was observed that no significant difference in panicle length (PL in cm), percentage spikelet fertility (% SF), test weight (TW) and grain yield (GY in Kg/ha). The replication wise result was observed that significant difference, in NT at 1% probability level, and FGP

**Table 2** Performance evaluation of yield potential trait of promising advanced breeding lines under drought stress

| S. No | Breeding lines | DFF | TW | FGP | % SF | GY (kg/ha) | % Sup | Tukey group |
|---|---|---|---|---|---|---|---|---|
| 1 | IRINMST-007 | 122.03 | 19.51 | 67.41 | 44.94 | 2571 | 18.02 | A |
| 2 | IRINMST-338 | 121.68 | 20.42 | 67.58 | 43.78 | 2339 | 7.40 | AB |
| 3 | IRINMST-418 | 122.36 | 18.99 | 62.78 | 43.01 | 2209 | 1.44 | ABC |
| 4 | IRINMST-129 | 126.34 | 19.17 | 62.87 | 43.68 | 2181 | 0.15 | ABCD |
| 5 | IRINMST-057 | 126.09 | 19.00 | 57.06 | 39.86 | 2163 | −0.67 | ABCD |
| 6 | MTU1010 | 124.56 | 16.51 | 52.94 | 42.79 | 2005 | | |
| 7 | Sahbhagi Dhan | 127.06 | 17.79 | 47.30 | 41.12 | 2095 | | |
| 8 | IR 64 | 127.93 | 18.66 | 35.95 | 25.23 | 816 | | |
| 9 | IRINMST-291 | 117.69 | 19.90 | 58.95 | 43.40 | 2178 | | |
| HSD @ 5% | | 11.64 | 4.97 | 19.94 | 8.72 | 471 | | |

DFF, days to 50% flowering; TW, 1,000 grain weight in gram; FGP, filled grains /panicle; % SF, percentage spikelet fertility; GY (kg/ha), grain yield (kilogram/hectare); % Superior, percent superiority over best check; HSD, Honest significant difference at 5% probability level

**Table 3** Performance evaluation of yield potential trait of promising advanced breeding lines under high-temperature stress

| S.No | Breeding lines | DFF | TW | FGP | % SF | GY (kg/ha) | % Sup | Tukey group |
|---|---|---|---|---|---|---|---|---|
| 1 | IRINMST-007 | 95.98 | 19.87 | 108.19 | 78.06 | 4394 | 14.79 | A |
| 2 | IRINMST-338 | 97.73 | 20.88 | 102.51 | 78.39 | 4174 | 9.04 | AB |
| 3 | IRINMST-418 | 97.75 | 19.50 | 102.01 | 78.5 | 3920 | 2.39 | ABC |
| 4 | IRINMST-192 | 96.75 | 18.95 | 109.54 | 78.09 | 3888 | 1.55 | ABCD |
| 5 | IRINMST-113 | 99.23 | 18.00 | 98.65 | 71.02 | 3881 | 1.39 | ABCD |
| 6 | MTU1010 | 97.98 | 18.24 | 68.44 | 35.01 | 2184 | | |
| 7 | Sahbhagi Dhan | 96.23 | 18.60 | 76.57 | 58.03 | 2814 | | |
| 8 | IR 64 | 101.00 | 18.95 | 60.67 | 38.27 | 2044 | | |
| 9 | IRINMST-291 | 96.19 | 18.86 | 105.37 | 76.87 | 3828 | | |
| HSD @ 5% | | 6.90 | 4.18 | 40.22 | 16.97 | 595 | | |

DFF, days to 50% flowering; TW, 1,000 grain weight in gram; FGP, filled grains /panicle; % SF, percentage spikelet fertility; GY(kg/ha), Grain yield (kilogram/hectare); % Superior, percent superiority over best check; HSD, Honest significant difference at 5% probability level

and TW at 5% probability level but it was observed that no significant difference in DFF, PH, FLL, PL, % SF, and GY. In entry wise result was observed that significant difference, in PH, % SF, and GY at 1% probability level, and NT, FLL, and FGP at 5% probability level, but it was observed that no significant difference in DFF, PL and TW. The interaction between year and entry was observed that significant difference, in GY at 1% probability level but it was observed that no significant difference in DFF, PH, NT, PL, FGP, % SF, and TW (Supplementary Table 4).

### Performance of breeding lines under cold stress at germination and seedling stage

Out of 41 selected breeding lines, 16 and 33 was observed highly cold tolerant at seedling stage and tolerant at germination stage respectively. Out of 16 breeding lines possessing highly cold tolerant at seedling stage, 14 breeding lines was also observed cold tolerant at germination stage. Highly cold tolerant at seedling stage of breeding lines were ranged seedling survival percentage from 90 to 100%. Cold tolerant at germination stage of breeding lines were ranged percentage vigor of germination from 55 to 94%.

### Performance of yield and related traits of breeding lines under multi-abiotic stress conditions

According to the Tukey grouping results, the best-performing breeding lines under non-stress, cold stress at germination and seedling stage, drought stress, and high-temperature stress at reproductive stage are identified as IRINMST-007, IRINMST-113, IRINMST-129, IRINMST-192, and IR IRINMST-418. The performance of the most promising breeding lines under non-stress, cold stress (at germination and seedling stages), drought stress, and high-temperature stress at the reproductive stage is summarized in Table 4.

The result year wise showed that significant difference, in days of 50% flowering (DFF), plant height (PH in cm), panicle length (PL in cm), filled grains per panicle (FGP), percentage spikelet fertility (% SF), test weight (TW) at 1% probability level and grain yield (GY in Kg/ha) at 5% probability level but it was observed that no significant difference in number of productive tillers (NT) and flag leaf length (FLL in cm). In conditions (normal, drought and high-temperature stress) was observed that significant difference, in DFF, PH, NT, PL, FLL, FGP, % SF, TW and GY at 1% probability level. In replication (year wise) was observed that DFF, PH, NT and FGP at 1% probability level but it was observed that no significant difference in PL, FLL, % SF and GY. In entry wise result was observed that significant difference, in DFF, PH, NT, PL, FLL, FGP, % SF, TW and GY at 1% probability level.

The interaction between year and entry was observed that significant difference, in DFF, PH, and PL at 1% probability level but it was observed that no significant difference in NT, FLL, FGP, % SF, TW and GY. The interaction between year and conditions (normal, drought and high-temperature stress was

**Table 4** The performance of promising multiple stress tolerant advanced breeding lines under seedling and germination stage cold stress tolerance, reproductive stage drought stress (RS_DS) tolerance, and reproductive stage high-temperature stress (RS_HT) tolerance and non-stress (NS) [a] Source: Standard Evaluation System for Rice, International Rice Research Institute (SES, IRRI), 2014. [b]Source: Han et al. (2006)

| S. No | Breeding line/s | Cold stress tolerance score | | Grain yield (kg ha$^{-1}$) | | | % Yield reduction | | RS_HT tolerance |
|---|---|---|---|---|---|---|---|---|---|
| | | Seedling stage[a] | Germination stage[b] | NS | RS_DS tolerance | RS_HT tolerance | RS_DS tolerance compared to NS | RS_HT tolerance compared to NS | Spikelet[a] fertility |
| 1 | IRINMST-007 | 1 | 1 | 6617 | 2571 | 4394 | 61.2 | 33.6 | 3 |
| 2 | IRINMST-338 | 4 | 1 | 5906 | 2339 | 4174 | 60.4 | 29.3 | 3 |
| 3 | IRINMST-418 | 1 | 1 | 5566 | 2209 | 3920 | 60.3 | 29.6 | 3 |
| 4 | IRINMST-129 | 1 | 1 | 5495 | 2181 | 3828 | 60.3 | 30.3 | 3 |
| 5 | IRINMST-057 | 9 | 2 | 5462 | 2163 | 3694 | 60.4 | 32.3 | 3 |
| 6 | IRINMST-192 | 3 | 1 | 5329 | 2111 | 3888 | 60.4 | 27.0 | 3 |
| 7 | IRINMST-113 | 1 | 1 | 5418 | 2146 | 3881 | 60.4 | 28.4 | 3 |
| 8 | MTU1010 | 7 | 2 | 5062 | 558 | 2184 | 89.0 | 56.9 | 7 |
| 9 | IRINMST-291 | 1 | 1 | 5500 | 2178 | 3828 | 60.4 | 30.4 | 3 |
| 10 | IR 64 | 7 | 2 | 5418 | 816 | 2044 | 84.9 | 62.3 | 7 |
| 11 | Sahabhagi Dhan | 9 | 2 | 3775 | 2095 | 2814 | 44.5 | 25.4 | 3 |
| Honestly significant difference (HSD) @ 5% | | | | 996 | 471 | 595 | – | – | – |

observed that significant difference, in DFF, PH, NT, PL, FLL, FGP, TW, and GY at 1% probability level but it was observed that no significant difference in % SF. The interaction among year, conditions (normal, drought, and high-temperature stress), and entry was observed that significant difference, in DFF, PH, PL, FLL, % SF, and GY at 1% probability level but it was observed that no significant difference in NT, FGP, TW, and GY (Supplementary Table 5).

## Genetic diversity analysis and single marker analysis

A dendrogram was constructed using UPGMA (Unweighted Pair Group Method with Arithmetic Averages) method comprising two main groups that are A and B (Fig. 3). Out of 41 families, 13 families belong to A group along with recipient parent MTU 1010 and 28 families belong to B group along with donor parent IRINMST-291. The best-selected breeding lines (IRINMST-007, IRINMST-338, IRINMST-418, IRINMST-129, and IRINMST-113) was performed better in non-stress, cold stress at germination and seedling stage, drought and high-temperature stress at reproductive stage is belong to A, B, B, B, and B group respectively.

The single marker analyses were done of selective genotyped 180 selected samples of $F_6$ under different abiotic stress condition with 110 markers. The result of single marker analysis was presented in Table 5. RM5344 and RM1019 were found associated with % spikelet fertility at reproductive stage high-temperature stress with phenotypic variance 24% and 14% respectively. RM5911 was found associated with yield at reproductive stage drought stress with phenotypic variance 11%. RM1019 and RM2441 were found associated seedling growth at seedling stage cold tolerance with phenotypic variance 18% and 12% respectively. RM5344 and RM210 was found associated with survival rate at seedling stage cold tolerance with phenotypic variance 19% and 18% respectively. RM555 and RM208 were found associated with vigor of germination at germination stage cold tolerance with phenotypic variance 18% and 16% respectively.

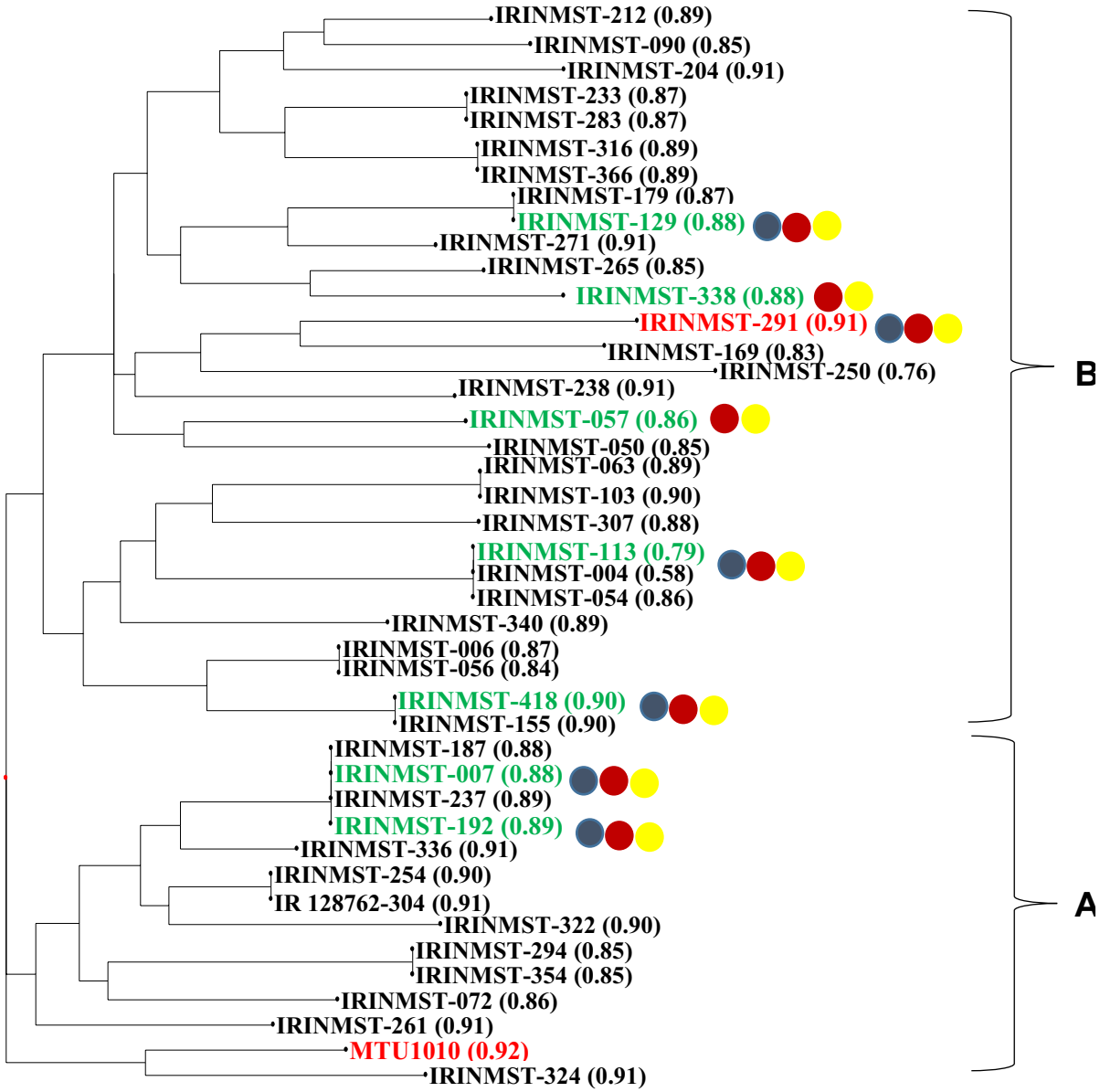


**Fig. 3** Unweighted Pair Group Method with Arithmetic Mean (UPGMA) Cluster Tree of 41 Recombinant Inbred Lines (MTU 1010/ IRINMST-291) along with recipient MTU 1010 and donor IRINMST-291 parent using 110 polymorphic microsatellites maker. Note: Seedling and germination stage cold stress tolerance; Reproductive stage drought stress tolerance, Reproductive stage high-temperature stress tolerance, and (): Similarity coefficient

## Discussion

Rice production across many countries is adversely affected by various abiotic stresses (Drought, heat, and cold stress) either individually or in combination. Developing rice varieties of high yielding coupled with enhanced tolerance to cold stress, drought stress, and high-temperature stress is critical to ensuring sustainable rice production in the future. The major objective of the present study is to develop breeding lines possessing tolerance at reproductive stage drought and high-temperature stress and cold tolerance at germination and seedling stage through conventional breeding.

**Table 5** Detection of QTLs for high-temperature tolerance, cold stress tolerance, and drought stress tolerance using single marker analysis (SMA)

| Trait | QTL | Chr. No | Position (Mb) | Marker | LOD | PVE (%) | Additive effect |
|---|---|---|---|---|---|---|---|
| % Spikelet fertility at reproductive stage high-temperature stress (SFRSHTS) | *qSFRSHTS7.2* | 7 | 2 | RM5344 | 5.72 | 24 | 4 |
| % Spikelet fertility at reproductive stage high-temperature stress (SFRSHTS) | *qSFRSHTS8.0* | 8 | 0 | RM1019 | 4.7 | 14 | 4 |
| Yield at reproductive stage drought stress (YRSDS) | *qYRSDS8.0* | 8 | 0 | RM5911 | 2.43 | 11 | −126 |
| Seedling growth at seedling stage cold tolerance (SGSSCT) | *qSGSSCT8.0* | 8 | 0 | RM1019 | 5.2 | 18 | −3 |
| Seedling growth at seedling stage cold tolerance (SGSSCT) | *qSGSSCT4.28* | 4 | 28 | RM2441 | 4.12 | 12 | −3 |
| Survival rate at seedling stage cold tolerance (SRSSCT) | *qSRSSCT7.2* | 7 | 2 | RM5344 | 5.38 | 19 | −32 |
| Survival rate at seedling stage cold tolerance (SRSSCT) | *qSRSSCT8.22* | 8 | 22 | RM210 | 4.32 | 18 | 31 |
| Vigor of germination at germination stage cold tolerance (VGGSCT) | *qVGGSCT2.4* | 2 | 4 | RM555 | 5.1 | 18 | 33 |
| Vigor of germination at germination stage cold tolerance (VGGSCT) | *qVGGSCT2.35* | 2 | 35 | RM208 | 4.17 | 16 | 33 |

QTL, quantitative trait loci; LOD, logarithm of odds; PVE, phenotypic variance explained

The drought screening was done at open field condition. The high-temperature stress screening of parental lines was done in the Plant growth chamber and screening of high-temperature stress of breeding lines was done in the open field condition. The screening of cold tolerance at germination stage was done in the open field condition and cold tolerance at seedling stage was done in Plant Growth Chamber and open field condition.

Conventional breeding is one of the powerful approaches to develop new parental lines and high yield potential varieties under different abiotic stresses. The identified donor IRINMST-291 showed tolerance under cold stress, drought stress and high-temperature stress. The true $F_1$ plant derived from the recipient MTU 1010 and the donor IRINMST-291 to generate 5000 $F_2$ plants. On the basis of single plant yield (25–35 g), only 1000 $F_2$ plants was forwarded to $F_3$ for visual observation of cold stress at germination and seedling stage in the open field condition.

Finally, 400 breeding lines showed either cold stress at germination and seedling stage were forwarded for visual observation of drought stress in open field condition. 400 breeding lines were forwarded for $F_5$ generation for screening of cold stress, drought stress and high-temperature stress and plot yield potential in field condition. Out of 400 $F_5$ breeding lines, only 150 selected best breeding lines which performed better in combined stress or either of stress were advanced to $F_6$ along with 5 checks for screening of cold stress at seedling stage in lab condition and drought stress in open field condition. Further, 41 $F_7$ selected families along with 4 checks were screened under non-stress in open field condition, drought stress and high-temperature stress at reproductive stage in open field condition, and cold stress at germination in field condition and cold stress at seedling stage in open field and lab conditions.

Three breeding lines consistently outperformed others across multiple stress conditions, including non-stress, cold stress (germination and seedling stage), drought stress, and high-temperature stress (reproductive stage) are identified IRINMST-007, IRINMST-418, and IRINMST-129. The seven selected breeding lines (IRINMST-007, IRINMST-338, IRINMST-418, IRINMST-129, IRINMST-057, IRINMST-192 and IRINMST-113) showed the highest yield across different conditions with yield of 5418–6617 kg $ha^{-1}$ under non-stress, 2146–2571 kg $ha^{-1}$ under drought stress and 3881–4394 kg ha − 1 under high-temperature stress. The % yield reduction of seven promising lines under

reproductive stage drought stress compare to non-stress varies from 60.3 to 61.2. The % yield reduction of seven promising lines under reproductive stage high-temperature stress compare to non-stress varies from 28.4 to 33.6.

Among the 41 selected breeding families, 16 families exhibited strong tolerance at the seedling stage under both controlled (plant growth chamber) and field conditions. Of these, 14 families also demonstrated cold tolerance at the germination stage. Year-wise and condition-wise analyses (normal, drought, and high-temperature stress) revealed significant differences in agronomic traits, including plant height, number of tillers, and grain yield, across various conditions. Interactions between year and entry, as well as year and conditions, also showed significant differences for most agronomic parameters.

The drought at reproductive stage is most critical stage, which reduces number of grains per panicle, increases grain sterility, reduces grain weight and yield reduction by 13–35% (Pantuwan et al. 2002). The rise of temperature 40 °C in 2007 at flowering stage in Japan, resulting in 25% reduction in paddy rice yield (Hasegawa et al. 2011). Increasing the tolerance of rice during the most sensitive flowering stage to reproductive stage drought and high-temperature stresses is an ideal adaptation strategy for highly variable future climates (Horie et al. 1996). High-temperature and drought stress resulted in reduction of number of tillers, number of panicles and panicle length in both N22 and NH219 (Poli et al. 2013).

Low temperature is one of the major abiotic stresses affecting rice plant at different growth stages and it eventually reduces rice yield significantly (Sipaseuth et al. 2007). Water deficit at reproductive stage significantly reduces pollen viability (Liu 2003), spikelet fertility (Praba et al. 2009) and grain yield (Boonjung and Fukai 1996). It is recorded a significant reduction in spikelet fertility under high-temperature (HT), water stress (WS) and HT + WS (Rang et al. 2011). The evaluation of more than 25,000 introgression lines in elite rice genetic backgrounds has proven successful for improving tolerance to multiple abiotic stresses, such as drought, flooding, and extreme temperatures (Kumar et al. 2020). These findings reinforce the necessity of integrating strong genetic tolerance into rice breeding programs to mitigate losses caused by varying abiotic stresses and support food security under changing climate conditions.

RM5344 was found associated with % spikelet fertility at reproductive stage high-temperature stress with phenotypic variance 24%. RM5911 was found associated with yield at reproductive stage drought stress with phenotypic variance 11%. RM1019 was found associated seedling growth at seedling stage cold tolerance with phenotypic variance 18%. RM5344 was found associated with survival rate at seedling stage cold tolerance with phenotypic variance 19%. RM555 was found associated with vigor of germination at germination stage cold tolerance with phenotypic variance 18%. Dendrogram constructed using data of 110 SSR loci by DARwin (V.6.0.5) software package and based on UPGMA method. The two main groups, that is, A, and B of breeding lines have been obtained from the resulting dendrogram. Out of 41 breeding lines, 13 breeding lines belong to A group along with recipient parent MTU 1010 and 28 breeding lines belong to B group along with donor parent IRINMST-291. These identified markers in the study under multi-stress conditions can be effectively utilized in marker-assisted backcross breeding to improve existing rice varieties, enhancing tolerance to stresses like drought, high temperature, and cold. The efficiency of each marker can be tested through molecular profiling of a set of breeding lines or genotypes and their evaluation under targeted stress conditions.

## Conclusion

This study successfully developed stress-tolerant rice breeding lines with enhanced climate resilience to drought, high temperatures, and cold stress. Further field trials and validation studies are recommended in regions facing multiple abiotic stresses before varietal release to ensure stability across diverse environments. These multi-stress-tolerant rice lines offer promising solutions for climate-resilient agriculture and food security.

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**Data availability** No datasets were generated or analysed during the current study.

**Declarations**

**Conflict of interests** All the authors declare that they have no conflict of interests.

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