



Identification of multiple abiotic stress tolerant donors for climate-resilient rice (*Oryza sativa*) development

KOUSHIK CHAKRABORTY¹, SOMNATH ROY², P JENA¹, G K DASH³, M BARIK⁴,
MOTILAL BEHERA¹, P SAR², J SENAPATY¹, M J BAIG¹ and PADMINI SWAIN^{1*}

ICAR-National Rice Research Institute, Cuttack, Odisha 753 006, India

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ABSTRACT

The increasing occurrence of adverse weather events is continuously challenging agricultural production globally. The wide genetic base of rice (*Oryza sativa* L.) and the availability of a diverse set of germplasm can be explored to identify multiple abiotic stress-tolerant donors for rice improvement. Therefore, an experiment was conducted during 2018–21 at ICAR-National Rice Research Institute, Cuttack, Odisha to evaluate diverse rice germplasm (68 accessions) for vegetative stage drought and submergence stress and for their anaerobic germination potential. Under drought stress, 49 accessions showed a drought score (DS) of 1 indicating a tolerance response. The early vegetative score indicated that 58 accessions were highly vigorous, while the drought recovery score indicates that >90% of the genotypes showed good recovery after the drought cycle. The same genotypes showed an average survival rate (SR) of 48% under two weeks of complete submergence. Among them, 11 were found highly tolerant with >70% SR and 26 genotypes scored positive *SUB1A* locus by SNP-based functional marker AEX1. Nearly 50% of the genotypes scored positive for *SUB1A*, based on both AEX1 and Sub1A203 markers, which nearly matched the phenotyping result. Out of 68, only 4 accessions showed more than 50% germination under anaerobic conditions. A multivariate analysis showed that 11 genotypes had both drought and submergence tolerance, while 6 genotypes were having tolerance to drought and anaerobic germination. We found only 1 accession (IC516149) tolerant to all these 3 stresses which can be identified as a potential donor for multiple abiotic stresses.

Keywords: Anaerobic germination, Drought, Early vegetative vigour, Submergence

Rice (*Oryza sativa* L.) is the world's most important staple crop and is a primary source of energy and nutrition for about half of the world's population. It is grown in a diverse range of ecosystems, and under changing climatic conditions it gets exposed to different environmental stresses reducing grain yield (Pathak *et al.* 2021). Rice has a unique ability to grow in wide hydrological environments ranging from aerobic to deep-water conditions. Rice landraces and traditional varieties possess diverse anatomical, morphological and physiological features that enable their cultivation in different agro-ecological zones and water regimes. Flood and drought tolerance may coexist in rice and the molecular mechanisms of drought and flood tolerance may be regulated by cross-talking among various

signaling pathways (Bin Rahman and Zhang 2022). Fukao *et al.* (2011) highlighted a cross-talk between drought and submergence tolerance in rice. Recently, Ikmal *et al.* (2021) suggested the presence of known drought-tolerant QTLs (*DTY*) improved *SUB1*-mediated submergence tolerance ability in rice.

Crops achieve drought tolerance through the modulation of complex interactions among morphological, physiological and biochemical traits (Chakraborty *et al.* 2022a). In the past decades, intensive research on drought tolerance resulted in the identification of several QTLs for grain yield and various drought-related traits (Kumar *et al.* 2014). On the contrary, submergence imposes an opposite kind of stress on the plants which ultimately resulted in restriction in growth and metabolism due to prolonged exposure to an anaerobic environment (Chakraborty *et al.* 2022b). Submergence tolerance is vital for yield security in areas where flash flooding is prevalent. High anaerobic germination potential is pre-requisite for direct-seeded rice (DSR), which is now in demand in rain-fed rice growing areas due to various climatic and economic reasons (Ismail *et al.* 2009). Genetic variability is the raw material for any crop improvement programme. Under changing climatic conditions, when

¹ICAR-National Rice Research Institute, Cuttack, Odisha;

²ICAR-National Rice Research Station, Hazaribag, Jharkhand;

³MS Swaminathan School of Agriculture Centurion University of Technology and Management, Paralakhemundi, Gajapati, Odisha;

⁴School of Applied Sciences, Centurion University of Technology and Management, Jatni, Odisha. *Corresponding author email: Padmini.Swain@icar.gov.in

crops face multiple stresses, it is demanding to develop climate-smart multi-stress tolerant varieties. Keeping the above facts in mind, the present investigation was designed to identify unique landraces/germplasm having tolerance to multiple abiotic stresses, viz. drought, submergence and high anaerobic germination ability as potential donors for climate-smart varietal development for rain-fed ecology.

MATERIALS AND METHODS

Plant materials: From a collection of >10,000 germplasm of ICAR-National Rice Research Institute, Cuttak, Odisha; Gene Bank, 68 genotypes (Table 1) were selected for the present study, which were found tolerant to drought stress from previous multi-season screening trials. All these 68 accessions were screened for tolerance to drought, submergence and anaerobic germination potential across seasons during 2018–21 at ICAR-National Rice Research Institute, Cuttak, Odisha.

Phenotyping for drought tolerance: Phenotyping for drought tolerance was conducted under managed drought screening facility at ICAR-NRRI, Cuttak in the dry seasons of 2018–2019. The rice genotypes were grown in a rain out shelter following the phenotyping protocol as described by Verulkar *et al.* (2010). In brief, the experiment was grown in a randomized complete block design with 3 replications and the genotypes were dry-seeded in a single row plot of 2 m in length, keeping a row-to-row distance of 20 cm. The crop was raised following the standard management practices under well-watered conditions up to 45 days after sowing (DAS). Then irrigation was stopped, and the experimental area was covered to protect it from rainfall, to impose moisture stress. Genotypes were scored around two weeks after withdrawal of irrigation when the peak stress developed (-60 kPa). Re-watering was done about

16 days after the withdrawal of irrigation. The data on the early vegetative vigor (EVV), drought score (DS), drought recovery score (REC) and relative water content (RWC) were recorded according to the IRRI-Standard evaluation system (SES) for rice (IRRI 2013).

Phenotyping for submergence tolerance: The genotypes were grown in field submergence tanks (1×b×h = 40 m ×8 m ×1.2 m) @4–5 seeds/hill with a spacing of 20 cm (row to row) and 15 cm (hill to hill), following a randomized block design with 3 replications in the wet seasons of 2019–2020. Twenty-five-day-old seedlings were completely inundated under 90±5 cm of water for 14 days. After 14 days of submergence, water was drained out and post-submergence data were recorded. The survival rate (recorded 7 days after de-submergence) and elongation ability of the plants due to submergence stress were calculated as described by Chakraborty *et al.* (2021).

Phenotyping for anaerobic germination: The anaerobic germination potential was assessed by creating hypoxic/anoxic stress during the germination process (Vijayan *et al.* 2018). Thirty seeds (per replication) of each genotype of 68 rice accessions along with checks were sown in a polypropylene tray containing well-pulverized sandy loam soil up to 4 cm thickness in 3 replications. Seeds were covered with 1 cm of soil and submerged immediately after sowing by pouring 10 cm of standing water above the soil surface inside the tray. The water was kept in such condition for 21 days. Control trays (without standing water) were also maintained along with the treatment trays where the soil surfaces were adequately moistened by the regular sprinkling of water. The hypocotyl length (cm) was measured in the treatment after 21 days of imposing stress from the germination count data, we calculated the germination percent (GP) which is the percentage of seeds that complete the germination process (Chakraborty *et al.* 2019). The anaerobic germination index (AGI) was calculated to find the actual germinability under anaerobic conditions

$$AGI = G_{pAG}/G_{pCONTROL}$$

Genotyping of SUB1 QTL: Total genomic DNA was extracted using Qiagen DNeasy Plant Mini Kit (Qiagen, India). The PCR reaction was done using 50 ng of template DNA, 0.5 µM of each forward and reverse primer, 0.2 mm of dNTPs, 1X PCR buffer with 20 mM MgCl₂ and 1 U of Taq DNA polymerase in a total volume of 25 µL. The PCR consisted of an initial denaturation for 5 min at 94°C, 35 cycles of 30 sec denaturation at 94°C, annealing at primer-specific temperature for 30 sec and extension at 72°C for 30 sec and final extension for 7 min at 72°C. The amplified products were separated by 3% agarose gel electrophoresis and visualized using a gel doc (gel imager, Invitrogen). The presence of SUB1A, SUB1B, and SUB1C were screened in 68 rice genotypes using AEX1, Sub1A203 and Sub1BC2 markers as reported by Septiningsih *et al.* (2009).

Statistical analysis: Phenotyping data from different experiments were subjected to analysis of variance and descriptive statistics using Graphpad Prism version 9.5.1. Principal component analysis (PCA) was performed

Table 1 List of 68 genotypes used for the present study

Parijat	Mahulata	AC24296	IC516149
PAU9	AC27675	ZHU11–26	IC516561
Mahsuri	Kamesh	Anjali	IC516278
Khandagiri	IET18208	AC35678	IC516313
Satyabhama	Indira	AC35679	IC516130
Bhanja	IET18816	Annapurna	Pathara
Udayagiri	IET18736	Surendra	Dagad Desi II
Rameswari	IET18720	Khitish	AC26685
Jogesh	IET18716	Moroberekon	EC267999
Badami	IET18727	IC516 334	EC268310
Dubraj	IET17509	AC35764	EC307066
Konark	N22	IC516390	EC305939
Lalkanda 41	Swarnaprava	AC34992	EC205302
Gurjari	CR–143–2–2	IC516366	EC268283
AC38209	Swarna	IC516283	EC306146
Brahmanakhi	IR20	IC516583	EC268177
AC3577	Salumpikit	IC516008	Sahabhangi dhan

using the standardized data and the number of principal components was selected based on parallel analysis.

RESULTS AND DISCUSSION

Genetic variation for drought tolerance: Overall, EVV, DS and REC varied widely under moisture stress (CV = 38.69%, 77.83% and 44.69%, respectively). Whereas RWC showed relatively low variation among accessions (CV=10.42%). The variability in different traits recorded in 2 year data are presented in Table 2. Under drought stress, 49 accessions showed a drought score (DS) of 1 indicating a tolerance response. Genotypes like Sahabhangi dhan, Anjali, AC35678, Khitish, Moroberekon, etc. comes under this group; 17 genotypes showed a moderate tolerance response (DS=3–5). Genotypes PAU-9, Udayagiri, Konark, Kamesh, IET18727, N22 are placed in this group. Only Mahsuri and IR-20 showed a sensitive response (DS=7). Based on EVV values, 58 accessions were scored as highly vigorous. The drought recovery score indicated that >90% of genotypes were recovered after the drought cycle. The RWC of leaves varied from 49.4% (Mahsuri)–80.7% (EC305939). It is well established that under water stress a reduction in RWC leads to various mechanical and oxidative stresses that lead to pollen sterility, and ultimately yield loss (Lonbani and Arzani 2011). Tolerant plants maintain a higher RWC to combat drought stress as the water content in leaf sheaths is maintained during drought stress (Dien *et al.* 2019).

Genetic variation for submergence tolerance: The genotypes showed variable responses to 2 weeks of complete submergence. The plant height before imposing stress varied from 26 cm in IR20 to 40.7 cm in AC24296. While plant height after completion of stress ranged between 47 cm (AC34992) to 92 cm (IC516278). The survival rate (SR) and elongation ability (EA) of the studied genotypes varied considerably with a CV of 47.53% and 31.33%, respectively

(Table 2). The average SR was 48.06% and varied from 0% (N22, Moroberekon, EC205302, and Sahabhangi dhan) to 100% (IC516366). Eleven genotypes showed SR of >70%. Maximum EA was obtained in IC516278 (203.2%) whereas EC267999 recorded the lowest (25.8%). Forty-three accessions showed considerable elongation (EA>100%). All these lines showed low to moderate internode elongation suggesting they might have employed a quiescence strategy for survival (Colmer and Voesenek 2009).

The marker AEX1 designed to amplify the functional SNP (C-to-T) in *SUB1A* conditioning submergence, revealed 231 bp amplicons in 26 test genotypes (Fig 1). The survival rate of these genotypes varied from 0–85.7%. Although EC205302, IR20, Brahmanakhi, Surendra genotypes scored positive for *SUB1A* but showed a susceptible response (SR=0–24.5%) to submergence during phenotyping. Whereas AC38209, IET18727, IC516366, and EC305939 scored negative for *SUB1A* but showed a survival rate of more than 70% during two weeks of complete submergence which might follow *sub1A-1* independent submergence tolerance (Singh *et al.* 2020). The marker Sub1A203, present in the exon of the *SUB1A* locus, showed desired amplicons in all those genotypes found positive for AEX1. Additionally, it showed amplification in few other genotypes such as Parijat, Mahsuri, Jogesh, Badami, Dubraj, Konark and IC516366 (Fig 1).

In case of Sub1BC2, an InDel-based marker that lies between *SUB1B* and *SUB1C* (38 bp insertion region), revealed an amplicon of 231 bp (FR13A type) in 7 genotypes. While in the rest of the genotypes, a 239 bp amplicon was detected. Among the genotypes, viz. PAU-9, Lalkanda 41 and CR-143-2-2 showed heterozygous bands. All the genotypes identified by this marker also scored positive for both AEX1 and Sub1A203, except Lalkanda 41, which showed susceptibility (38.1% SR) to complete submergence.

Table 2 Descriptive statistics of traits recorded under different abiotic stress conditions

Trait	Year	Mean	SD	Minimum	Maximum	CV%
EVV	1 st year (2018)	1.18	0.46	1.00	3.00	38.69%
	2 nd year (2019)	1.27	0.68	1.00	3.00	53.99%
DS	1 st year (2018)	1.71	1.33	1.00	7.00	77.83%
	2 nd year (2019)	2.41	1.61	0.00	7.00	66.91%
REC	1 st year (2018)	1.10	0.49	1.00	4.00	44.69%
	2 nd year (2019)	1.09	0.41	1.00	3.00	38.02%
RWC	1 st year (2018)	67.43	7.02	49.40	80.70	10.42%
	2 nd year (2019)	69.58	7.07	45.90	78.90	10.16%
SR	1 st year (2019)	48.07	22.85	0.00	100.00	47.54%
	2 nd year (2020)	49.05	23.16	0.00	100.00	47.22%
EA	1 st year (2019)	115.00	36.03	25.80	203.20	31.33%
	2 nd year (2020)	114.40	35.44	24.30	203.10	30.98%
AGI	1 st year (2020)	0.13	0.17	0.00	1.00	145.8%
	2 nd year (2021)	0.14	0.22	10.00	1.00	149.60%

EVV, early vegetative vigor; DS, drought score; REC, drought recovery; RWC, relative water content; SR, submergence survival rate; EA, per cent elongation under submergence; AGI, anaerobic germination index; SD, standard deviation; CV, coefficient of variation.

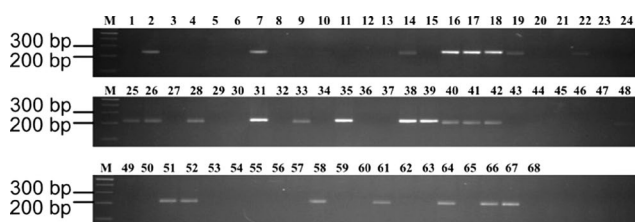


Fig 1 Detection of 231 bp amplicon specific to *SUB1A-1* allele in rice germplasm using AEX1 marker.

M, 100 bp DNA ladder; Genotypes are arranged (1–68) as mentioned in Table 1.

The genotypic data mostly matched with the phenotype evaluation of these genotypes for submergence tolerance, with some exceptions. Interestingly, we found some genotypes (AC38209, IET18727, EC305939, IET18208, IC516130 and Swarnaprava) were null for the *Sub1* gene however showed more than 60% survival.

Genetic variation for anaerobic germination tolerance:

Under anaerobic conditions, germination percentage (GP) ranged from 0–77% with IET18716 showing the highest GP under stress. Thirty-seven genotypes did not germinate at all under anaerobic conditions, while only 4 accessions showed more than 50% germination. Variability in the anaerobic germination index (AGI or S/C value) ranged from 0–1, with an average of 0.1412 (CV=149.5%). Hypocotyl length in these genotypes varies from 0–33 mm after 21 days of germination under anaerobic conditions. The growth of the coleoptile is first in tolerant genotypes but slow in case of sensitive genotypes. The two main important characteristics, i.e. elongation of coleoptiles and germination percentage are to be related to AG tolerance (Kretzschmar *et al.* 2015). Interestingly, only 12 genotypes showed a hypocotyl length of >20 mm. Coleoptile elongation is mainly targeted for the selection of tolerant genotypes which are germinating under the submerged condition as it is easy to phenotype

(Ismail *et al.* 2009).

Selection of promising multiple stress-tolerant genotypes and implications in rice breeding: A total of 7 traits (drought: EVV, DS, REC and RWC; submergence: SR and EA; and anaerobic germination: AGI) were further considered in multivariate analysis to find the genotypes with tolerance to multiple stresses. The Spearman's rank correlation between the selected traits is given in Fig 2A. Drought recovery showed a significant positive correlation with EVV and DS. RWC had a negative correlation with DS and drought recovery. RWC also showed a significant negative correlation with the AG index. Principal coordinate analysis (PCA) extracted two components (PCs) explaining 58.75% of the total variance. On PC1 drought traits had higher positive loadings while on the PC2 greater loading were shown by AGI. The PC1 vs. PC2 biplot revealed that the genotypes placed in the top-bottom corner possessed a high value of EVV and REC (Fig 2B). Whereas genotypes in the bottom-left had higher RWC and EA. Genotypes in the top-left showed a higher value of SR. Hereof, from the position of the genotypes in the biplot and higher positive loading of AGI along with low loading of DS on PC2, we can conclude that the genotypes, viz. PAU-9, Sahabnagi dhan, Arnapura, IC516149 had high AGI and DS, indicating tolerance to both drought and anaerobic germination. While the genotypes, viz. Gurjari, Jogesh, Mahulata, AC38209, CR-143-2-2, AC35678, IC516366, Dagad Deshi-II allocated in the bottom-left showed both submergence and drought tolerance. Considering the results of trait variability and PCA, a few promising genotypes were identified from the present study, which possesses tolerance to at least 2 of the studied stresses (Table 3). Among them, we found IC516149 as the most promising genotype having tolerance to all 3 abiotic stresses, viz. drought, submergence and anaerobic germination.

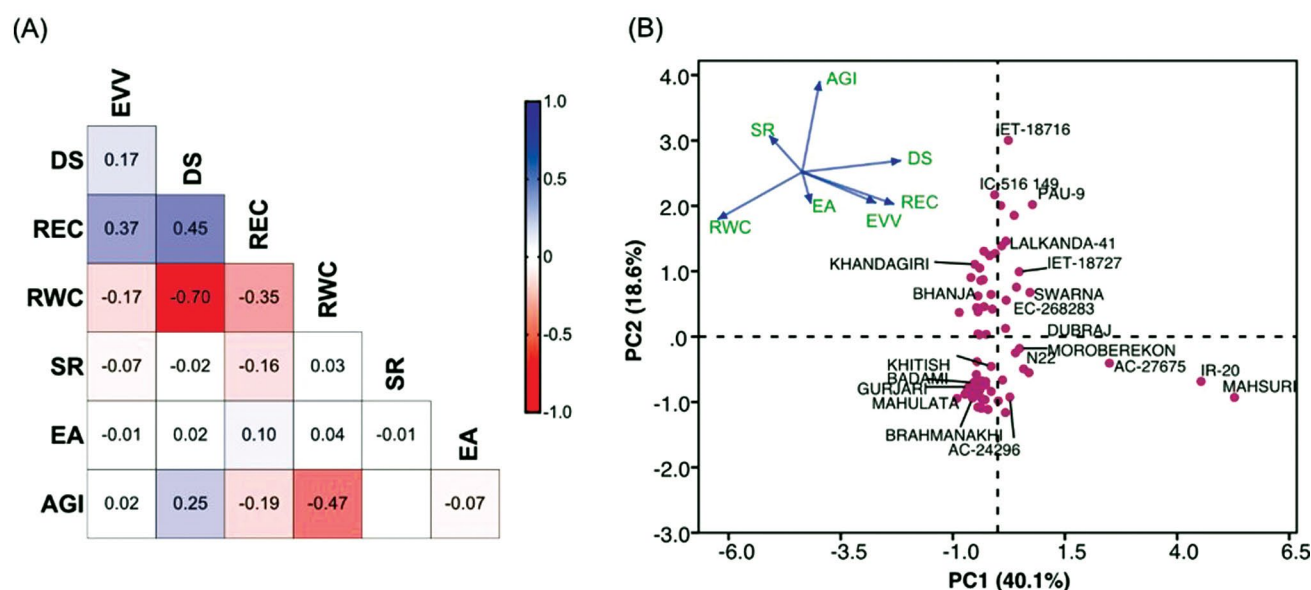


Fig 2 Correlation among the traits considered for multivariate analysis (A); Principal coordinate analysis (B).

Table 3 Promising rice genotypes identified as tolerant to multiple abiotic stresses

Combination of stress	Promising genotypes
Drought + Submergence	IC-516366, Gurjari, AC-38209, AC-35678, Khitish, Mahulata, IC-516008, IET-18727, EC-305939, IC-516149 and IET-18208
Drought + Anaerobic germination	IET-18716, PAU-9, Khandagiri, IC-516149, Arnnapurna and Sahabhagi dhan
Drought + Submergence + Anaerobic germination	IC-516 149

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