Seed Science Research

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Genome-wide association mapping for rapid and uniform germination traits associated with direct-seeded adaptation in rice

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Research Paper

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Cite this article: Behera S *et al*. (2025) Genome-wide association mapping for rapid and uniform germination traits associated with direct-seeded adaptation in rice. *Seed Science Research* 1–11. https://doi.org/10.1017/S0960258525100032

Received: 4 February 2025
Revised: 19 June 2025
Accepted: 3 August 2025

Keywords:
cgSSR markers; direct-seeded rice; GWAS; rice; rice breeding; seed germination traits

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Abstract

The genetic basis of rapid and uniform seed germination and its associated traits is crucial for improving seed vigour and seedling establishment for higher productivity in direct-seeded rice (DSR) systems. This study investigates the phenotypic diversity and genetic architecture of germination traits in 163 rice genotypes, using a genome-wide association studies (GWAS). An association panel of 163 diverse rice genotypes, including varieties, germplasm and breeding lines, was evaluated for seed germination traits over 2 years (2022 and 2023). The panel was genotyped using 295 simple sequence repeat (SSR) markers, including 80 random SSRs and 215 candidate gene SSRs linked to seed traits and morphological attributes. The genotyping of 163 lines with 295 markers revealed a range of genetic diversity, with polymorphic information content values between 0.04 and 0.93. Population structure analysis indicated the presence of two groups and four sub-groups. GWAS identified 80 significant marker-trait associations (MTAs) across 12 chromosomes at $P \leq 0.05$, which narrow down to 18 MTAs at $P \leq 0.01$. Twelve candidate genes are identified which were related with multiple traits, linked to important functions, such as seed-size regulation, nutrient mobilization and plant growth. Candidate gene-based SSR (cgSSR) markers such as M169 (OsMIK), M57 (THIS1), M66 (GW2), and M18 (OsBAK1), displayed pleiotropy including rapid and uniform germination (germination index, germination rate index and mean germination time) traits. The newly identified candidate gene markers associated with seed rapid and uniform germination traits can be leveraged in marker-assisted breeding programs to introduce diverse alleles for enhanced seed vigour and crop establishment. Markers closely linked to multiple traits hold significant potential for the simultaneous improvement of several traits.

Introduction

Rice (*Oryza sativa L.*) holds a primary position in global agriculture, serving as a staple crop in terms of both nutrition and calorie intake. It contributes to over 20% of the total caloric consumption for people worldwide (Fukagawa et al., 2019). Rice cultivation practices have gradually evolved with the resource availability, demand of food grain and nutrition. In recent years, there has been a rising preference for direct-seeded rice (DSR) which is an alternative to puddled transplanted rice, offering potential solutions for issues related to labour scarcity, water shortage, and methane emission arising in rice cultivation (Kumar et al., 2016). However, the widespread adoption of DSR faces certain challenges also especially during initial crop establishment, which includes slow germination rate (GR) and low seed vigour of the cultivars which causes delayed in establishment of seedling. Early vigour in rice is a characteristic of seed quality, describing by rapid and uniform germination, which is required for early plant establishment (Mahender et al., 2015).

In DSR cultivation, understanding various germination traits is crucial as they significantly influence the establishment of seedling, growth of crop, and plot yield. There are several parameters to access the germination potential of the genotypes, namely mean germination time (MGT) which indicates the average time taken for seeds to germinate, reflecting the uniformity and speed of seedling emergence, which is essential for achieving a uniform crop stand (Demir et al., 2008). The first day of germination (FGT) and the last day of germination (LGT) provide insights into the germination window, aiding in predicting the overall emergence period.

A shorter FGT and LGT indicate a more synchronized germination process. Germination rate index (GRI) and germination index (GI) are metrics used to evaluate the rate and vigour of seed which is essential for early seedling vigour. Germination percentage (GP%) is the total percentage of seeds that germinate, which reflecting the viability and quality of the seed lot (Talská et al., 2020). Time spread of germination (TSG) indicates the spread or uniformity of germination over time, with a narrower TSG being preferable for uniform seedling development (Kader, 2005). These traits collectively ensure the success of DSR by ensuring rapid, uniform, and complete germination, which are the key factors for high crop establishment and crop production in DSR systems (Mahender et al., 2015). Consequently, it is imperative for rice breeding to understand molecular mechanisms of these traits and the key quantitative trait loci (QTLs)/genes that regulate rapid and uniform germination of rice.

Advancements in molecular marker technologies has empowered plant breeders to take up novel methods in identifying QTL associated with complex traits (Sah et al., 2022). Further, the genome-wide association studies (GWAS) have been used as a promising technique to investigate possible causal alleles associated with quantitative traits. Association mapping identifies QTLs by analysing marker-trait association (MTA), allowing researchers to take use of inherent diversity and identify the region of the genome governing the trait (Muhammed-Azharudheen et al., 2022; Nayak et al., 2022; Sah et al., 2022). The use of GWAS through candidate gene-based SSR (cgSSR) markers has been shown to increase the precision of QTL finding by Nayak et al. (2022) and Muhammed-Azharudheen et al. (2022) in various phenotypic traits, seed vigour-related traits (Mohanty et al., 2025) of rice and reports are not available for seed germination-related traits.

There have been multiple QTLs reported for rice seed germination in recent years (Cheng et al., 2015; Hsu and Tung, 2015; Kretzschmar et al., 2015; Jiang et al., 2017; Jin et al., 2018; Yang et al., 2019; He et al., 2019; Yang et al., 2021). Wang et al. (2018) reported a total of 53 QTLs for germination, along with a putative gene known as OsSAP16. Additionally, using the GWAS approach, Li et al. (2021) and Peng et al. (2022) separately identified two candidate genes in rice, OsOMT and OsCDP3.10, respectively. The GR is controlled by the first cloned related gene, qLTG-3-1, which also encodes a protein with an unidentified function (Fujino et al., 2008). However, there is less reports of QTLs/genes on *indica* type rice for seed germination-related traits.

In the present study, a panel of 163 genotypes used for genome-wide association analysis for identification of gens/QTLs governing rapid and uniform germination traits, which are important for DSR adaptation in rice. The present research was focussed on (i) relationship between the germination-related trait, (ii) identifying new gene/QTLs and markers associated with germination traits in rice and (iii) explore the candidate genes linked to these traits. The results of this study could support breeding rice varieties with rapid and uniform germination. The linked markers may be utilized in marker assisted breeding after validation.

Materials and methods

Experimental material

The association panel used in the experiment consisted of 163 different rice genotypes, including released varieties, germplasm and advanced breeding lines. Detailed information about these 163 lines is provided in the additional supplementary material as Table S1. The genotypes of the association panel were grown in the wet seasons of 2020 and 2021 through the panicle-progeny row as per the method suggested by Sahu et al. (2020) to obtained pure seed of the genetic materials. To raise a healthy crop, true-to-type panicles of each genotype were collected in both the years (2021 and 2022) to avoid any seed mixture. In the following season, true-to-type panicles were selected again to ensure the genetic purity of the genotypes. The bulk seeds from these progeny rows were evaluated for germination-related traits including rapid and uniform germination testing in 2022 and 2023.

Seed germination testing

The seeds from each genotype were sun dried for four days prior to use for testing to bring seed moisture content of 12 ± 1%. For the assessment of seed germination, each genotype's seeds were sown in four replications to minimize the potential impact of experimental variations. In each replication, 50 seeds were placed on two layers of filter paper within a 9 cm diameter petri dish. Subsequently, distilled water was added in equal quantity to each petri dish and kept at room temperature (approximately 25 ± 2°C) with ambient condition. Seed germination was determined based on the length of the roots equivalent to the seed's length and shoots reaching the size half of seed length, respectively, as described by Wang et al. (2010). Observations on germination were recorded daily basis up to tenth day. The MGT was calculated using the equation: $MGT = \sum(n \times d)/N$, where n referred to the number of seeds that germinated on each day, d represented the number of days from the beginning of the test and N denoted the total number of seeds that had germinated by the end of the experiment, following Ellis and Roberts (1981). The FGT is defined as the day on which the first germination event occurred. The LGT defined as the day on which the last germination event occurred. Additionally, the time span between the initial and final germination events in a batch of seeds was referred to as the TSG. Meanwhile, the GRI reflects the percentage of germination on each day of the germination period. GRI: $GRI = G1/1 + G2/2 + \cdots + Gx/x$, $G1 = GP\% \times 100$ at the first day after sowing, $G2 = GP\% \times 100$ at the second day after sowing. The computation of the GI involved the formula: $GI = GI = (10 \times n1) + (9 \times n2) + \cdots + (1 \times n10)$ where $n1$, $n2$ … $n10$ = number of germinated seeds on the first, second and subsequent days until the 10th day; 10, 9 … and 1 are weights given to the number of germinated seeds on the first, second and subsequent days, respectively (Kader, 2005). The GP% was determined by the percentage of seeds that germinated finally, (GP% = number of seeds germinated/total seeds × 100) (Zhang et al., 2018). The full phenotypic dataset of the 163 rice genotypes are provided in additional supplementary material as Table S2.

DNA extraction and genotyping of the association panel

Fresh leaves of all genotypes were collected from 20 days old plants, and genomic DNA was extracted using the CTAB method, as described by Doyle and Doyle (1987). After assessing both the quantity and purity of the DNA using a Nanodrop spectrophotometer, the concentration of the isolated DNA was adjusted to 20 ng/μL through dilution with nuclease-free water. To ensure proper amplification, the PCR reaction was carried out with a 10 μL reaction mixture including 1 μL of 20 ng template DNA, 2 μL each of 10 pmol forward and reverse primers, 3 μL of premix, and 4 μL of nuclease-free water, a cycling profile involving an initial step at 94°C for 4 minutes, followed by 38 cycles of denaturation at 94°C

https://doi.org/10.1017/S0960258525100032 Published online by Cambridge University Press

for 40 seconds, primer annealing at 55°C for 40 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 7 minutes was employed. The PCR products were separated on a 3.5 percent agarose gel using an electrophoresis unit. The size of the amplified fragments was determined using a 50 bp DNA ladder and a gel documentation system (Zenith Gel Pro CCD Gel doc, Biozen Laboratories, India). Gel images were analysed and scored using CLIQS Gel image analysis software, version 1.0 Totallab®.

A total of 295 simple sequence repeat (SSR) markers consisted of 80 random SSR markers and 215 cgSSR markers derived from well-characterized genes/QTLs related to seed traits, morphological traits and yield traits. The SSR genotype data comprising 295 SSR markers across 163 rice genotypes has been provided as Table S3 in additional supplementary material to support transparency and ensure the reproducibility of the study.

Primer designing

The genomic sequence of the cgSSR was retrieved from the Rice Genome Annotation Project. After downloading the sequence, polymorphic sites, including SSRs and insertions/deletions (InDels), were identified using the simple sequence repeat identification tool and NCBI BLAST (http://www.ncbi.nlm.nih.gov). Unique genomic sequences surrounding these polymorphic sites were used to design primer pairs with Primer3 software. For genic regions containing multiple adjacent SSR motifs, a single primer pair was designed to amplify all the SSRs within the target region. Primer design parameters were optimized for an annealing temperature of approximately 55°C. To ensure specific and reproducible amplification during PCR, the target amplicon size was set between 100 and 350 bp, with an ideal primer length of 20 bp.

Statistical analysis

Descriptive statistics, distribution of traits, first-degree statistics for mean and range, second-degree statistics for sample variance, third-degree statistics for skewness, and fourth-degree statistics for kurtosis were calculated using Microsoft excel. Correlation matrix plot (also called a pairs panel plot), generated using the pairs.panels function from the psych package in R. It visualizes the pairwise relationships between multiple variables. PowerMarker V3.25 software was used to calculate polymorphic information content (PIC), gene diversity (GD) and allele frequency (Liu and Muse, 2005). Two distinct methods were used to define the population structure of the panel. Principal coordinates analysis (PCoA) was executed using the Darwin software and familial relatedness among the individuals, was confirmed through VanRaden kinship analysis (VanRaden, 2008), which was shown as a heatmap using the GAPIT R package (Lipka et al., 2012).

The best linear unbiased prediction (BLUP) estimates play a crucial role in reducing errors by minimizing the cumulative discrepancies across different sets and replications (Piepho et al., 2008). BLUP values of seed germination phenotypes and marker genotype data were evaluated for MTA using a mixed linear model developed in the GAPIT package of R software (Lipka et al., 2012). Combining the MLM model with an effective mixed-model association (EMMA) method allowed for the simultaneous rectification of population structure (VanRaden kinship analysis) and recognition of significant MTA (Kang et al., 2008). Boxplot was generated using R package ggplot2 (Wickham, 2016) to evaluate how effectively the significant candidate markers can distinguish phenotypic differences among the 163 rice lines.

Result

Phenotypic variation

The phenotypic variation of 163 genotypes was analysed by descriptive statistics using seven phenotypic observations. The MGT varied between 2 and 3 days with an overall mean of 2.37 days, while the FGT ranged from 1 to 2 days with an overall mean of 1.07 days. The range of LGT was 3.00–7.00 days with an average of 4.13 days, while some other trait like GRI ranged from 31.67 (EC-811544) to 83.33 (CR Dhan 407), with average value of 58.97; the GI ranged from 1448 (EC-811544) to 2192 (CR Dhan 500) with average value of 1946.55. The TSG ranged from 1.00 to 6.00 days with a mean of 3.06 days and the GP% ranged from 80% (Bindli, CR Dhan 507, EC-811544) to 100% (in 36 genotypes) with a mean of 93.89%. The third-degree statistic, skewness positively distributed for most traits except for the traits GI and GP%. The fourth-degree statistic kurtosis <3 in all traits except FGT showed platykurtic distribution, High kurtosis observed in FGT which showed leptokurtic distribution (Table 1).

Correlation analysis among the germination-related traits

Figure 1 depicts the correlation between germination-related traits. In this analysis, the values in parentheses represent Pearson correlation coefficients (r), and the asterisks indicate the level of statistical significance ($P \leq 0.05^{*}$; $P \leq 0.01^{**}$ and $P \leq 0.001^{***}$). Several significant correlations identified between traits. where MGT has a strong positive correlation with LGT ($r = 0.64^{***}$) and TSG ($r = 0.55^{***}$) and showed a moderate positive correlation with FGT ($r = 0.49^{***}$). Conversely, MGT showed strong negative correlations with GRI ($r = -0.88^{***}$) and GI ($r = -0.66^{***}$) and sowed a negative correlation with GP% ($r = -0.31^{***}$). FGT had a moderate negative correlation with GRI ($r = -0.41^{***}$) and showed a negative correlation with GI ($r = -0.29^{***}$). LGT also exhibits a strong positive correlation with TSG (0.98^{***}) and showed negatively correlated with GRI ($r = -0.35^{***}$) and GI ($r = -0.31^{***}$). The GRI and GI ($r = 0.80^{***}$) showed strong positive correlation with each other. Further, GP% showed a strong positive correlation with GRI ($r = 0.55^{***}$) and GI ($r = 0.92^{***}$).

In addition to Pearson correlation analysis, the coefficient of determination (R^2) was calculated as the square of the Pearson correlation coefficient (r), representing the proportion of variance in one variable that is predictable from the other. In this analysis, the R^2 value between LGT and TSG was the highest at 0.9604 (96.04%), followed by GP% and GI at 0.8464 (84.64%). The R^2 between MGT and GRI was 0.7744 (77.44%), while GRI and GI showed an R^2 of 0.6400 (64.00%), MGT and GI had an R^2 of 0.4356 (43.56%), and MGT and LGT had 0.4096 (40.96%). The association between MGT and TSG shared an R^2 of 0.3025 (30.25%), and GRI and GP% also had an R^2 of 0.3025 (30.25%).

Allelic diversity and population structure

The 295 markers were used to genotype 163 lines. The informativeness of cgSSR and SSR markers were estimated through GD, allele frequency and PIC. The GD ranged from 0.04 to 0.94, with the allele frequency ranged from 0.11 to 0.98 and the PIC ranged

https://doi.org/10.1017/S0960258525100032 Published online by Cambridge University Press

Table 1. Trait variation and distribution pattern of seven germination-related traits analysed on association panel

Trait	Mean	Sample Variance	Kurtosis	Skewness	Range	Minimum	Maximum	Count
MGT	2.37	0.07	1.84	0.68	1.73	2.00	3.00	163
FGT	1.07	0.06	10.24	3.48	1.00	1.00	2.00	163
LGT	4.13	1.45	−0.91	0.61	4.00	3.00	7.00	163
GRI	58.97	112.85	−0.46	0.23	51.67	31.67	83.33	163
GI	1946.55	19084.41	0.43	−0.73	744.00	1448.00	2192.00	163
TSG	3.06	1.39	−0.75	0.63	5.00	1.00	6.00	163
GP%	93.89	28.29	−0.11	−0.80	20.00	80.00	100.00	163

Mean germination time (MGT), first germination time (FGT), last germination time (LGT), germination rate index (GRI), germination index (GI), time spread of germination (TSG), and germination percentage (GP%).

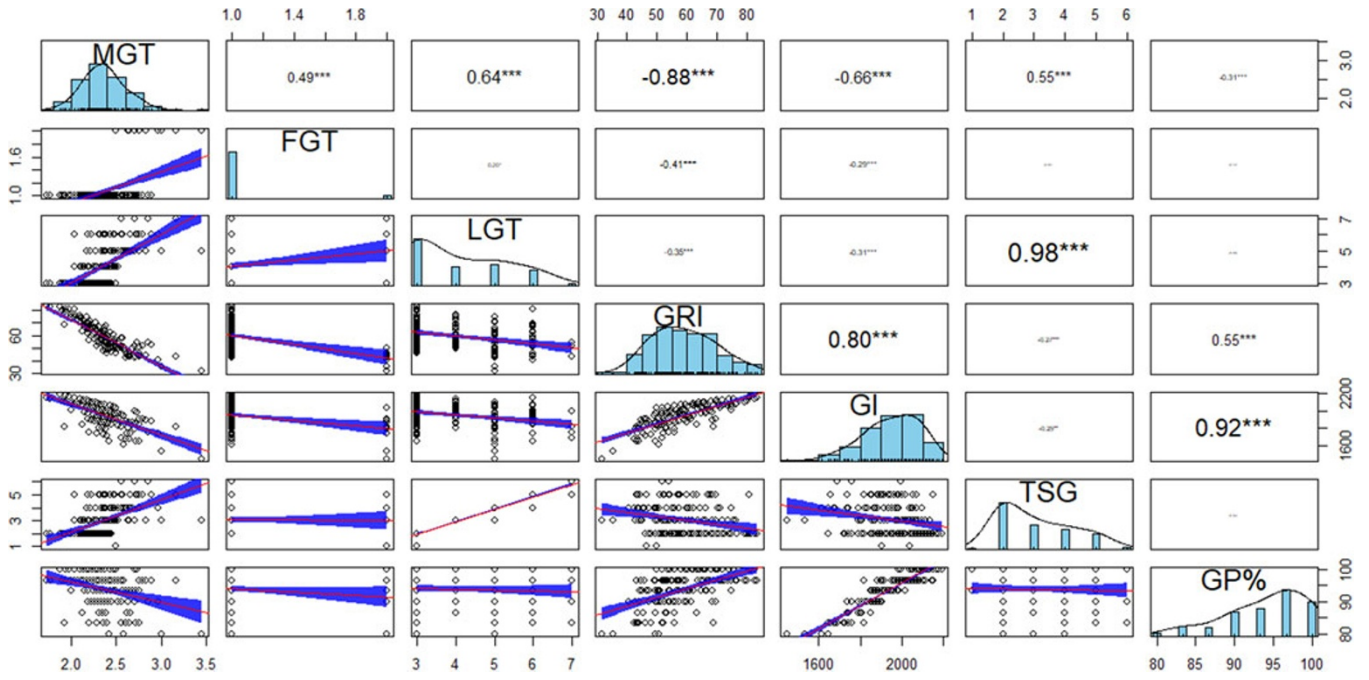

Figure 1. Pairwise correlation matrix and trend of distribution among germination-related traits. MGT (mean germination time), FGT (first germination time), LGT (last germination time), GRI (germination rate index), GI (germination index), TSG (time spread of germination), GP% (germination percentage).

from 0.04 (M88 and RM20633) to 0.93 (Marker: Gn1a-indel3) (supplementary Table S1).

Population structure was analysed using association panel through PCoA and kinship heatmap (Figures 2A and 2B). The first part (2A) was a 2D-PCoA plot, which illustrates the distribution of rice lines across three principal components (PC1, PC2 and PC3). The first three principal components, which together account for 26.96% of the total variation in the dataset (11.83% by PC1, 8.76% by PC2 and 6.37% by PC3). The PCoA reveals the presence of three distinct groups, as indicated by the clustering of data points in separate regions within the plot and the three distinct groups. These groups are colour-coded using three different colours (Figure 2A). The second part (2B) is a heat map of the kinship matrix, which showed the genetic relatedness among the individuals in the population. The heat map is colour-coded, with darker shades representing higher genetic relatedness and lighter shades indicating lower relatedness. The VanRaden kinship algorithm with GAPIT uncovered the existence of two major groups from kinship (groups 1: 80 genotypes; group2: 83 genotypes) which further partitioned to four sub-groups within the association panel (Figure 2B). The largest sub-population contained 82 genotypes in sub-group 4, followed by 64 in sub-group 2, 16 genotypes in sub-group 1, and 1 genotype in sub-group 3.

Genome wide association studies

Genotypic data from 295 markers of the association panel was used to conduct a genome-wide association analysis for 7 seed germination-related traits. A total of 80 significant MTAs at $P \leq 0.05$ were observed across 12 chromosomes using the MLM approach in conjunction with the EMMA algorithm (supplementary Table S2). Further, the level of significance was increased to $P \leq 0.01$ which showed a total of 18 MTAs (11 MTAs were associated with cgSSR markers 2 MTAs were gene based and 5 were associated with random SSR markers) across 7 traits (Table 2).

Three markers were associated with FGT, namely M202, M111 and RM593. The marker M202, linked to the gene OsAAP11B

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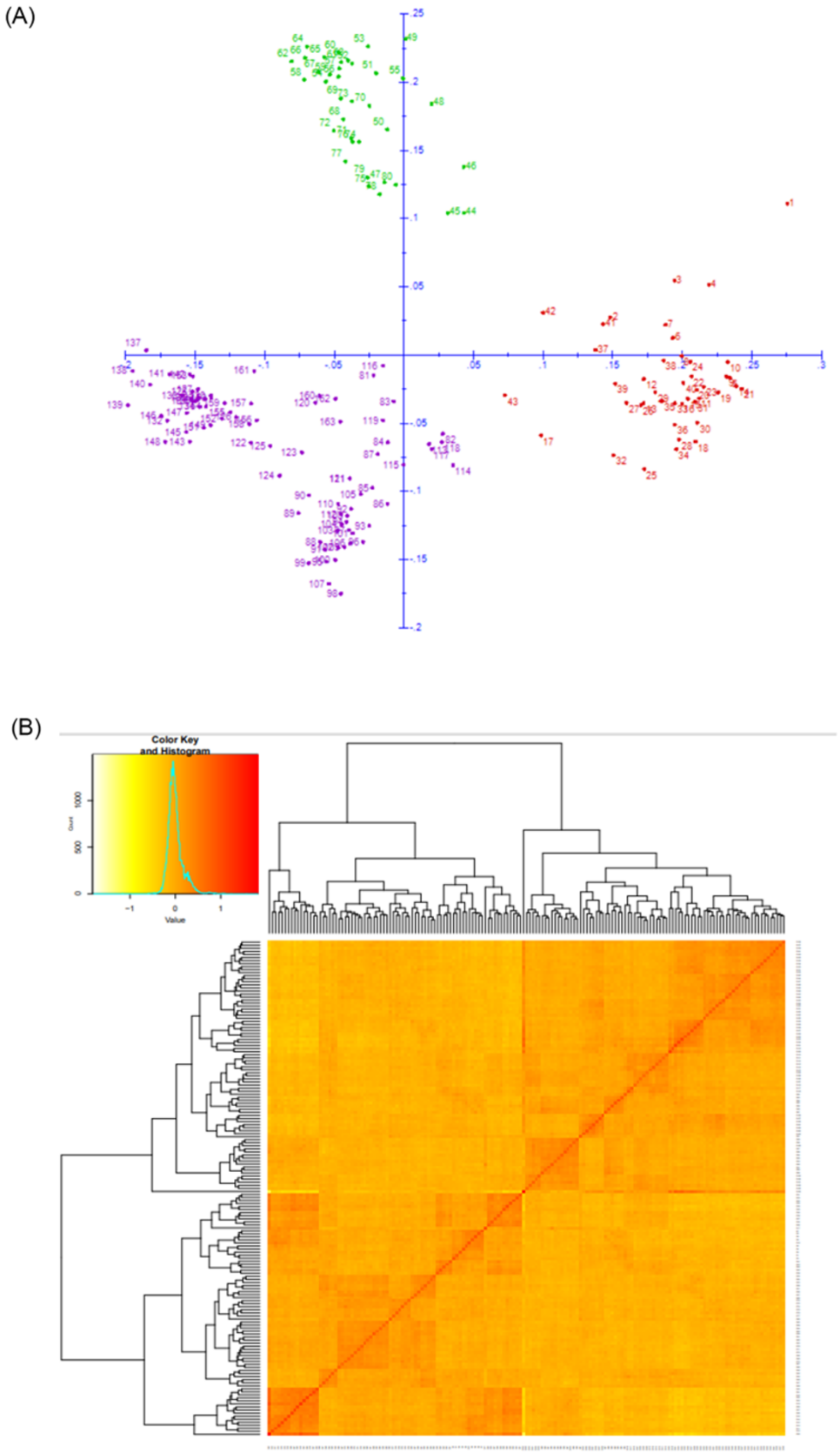

Figure 2. (A) A 2D representation of Principal Coordinate Analysis (PCoA) revealed three groups, each labelled with a different colour and (B) heat map of kinship matrix, the heat map shows the level of relatedness among the population. The darker areas show the level of relatedness between varieties and dark coloured line boxes at the top depicts clustering of sub-populations.

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Table 2. Significant marker-trait associations identified for seven germination-related traits based on MLM model at $P \leq 0.01$

Trait	Marker Name	Gene name	Chromosome	Position	*P* value	R^2%	Allelic effect
FGT	M202	OsAAP11B	12	4146619	0.004	7	−0.1
	M111	OsbHLH144	4	21276704	0.009	6	0.06
	RM593	–	5	2818823	0.009	6	−0.13
GI	M57	THIS1	1	31527177	0.008	10	−122.03
GP	Ghd7−05SNP-T	Ghd7−05SNP-T	7	9152402	0.003	13	3.89
	SPIKE-indel3	SPIKE-indel3	4	31204979	0.007	12	−2.7
	M200	OsAAP10C	1	38120760	0.007	12	1.33
GRI	M57	THIS1	1	31527177	0.007	11	−3.4
	M169	OsMIK	3	30247380	0.01	10	−2.45
LGT	M75	OsbHLH107	2	34356787	0.002	9	0.66
	RM431	–	1	38893890	0.008	7	0.42
MGT	M169	OsMIK	3	30247380	0.001	12	−0.14
	RM3643	–	4	19948112	0.003	11	−0.15
	RM490	–	1	6677230	0.007	10	−0.1
	M57	THIS1	1	31527177	0.009	10	−0.15
	M18	OsBAK1	8	4344171	0.01	10	−0.1
TSG	M75	OsbHLH107	2	34356787	0.002	9	0.67
	RM431	–	1	38893890	0.009	7	0.41

Mean germination time (MGT), first germination time (FGT), last germination time (LGT), germination rate index (GRI), germination index (GI), time spread of germination (TSG), and germination percentage (GP%).

on chromosome 12, had a negative allelic effect of −0.1 with an explained phenotypic variation of 7%; M111, associated with OsbHLH144 on chromosome 4, had a positive effect of 0.06 with an explained phenotypic variation of 6%, and RM593 on chromosome 5 had a negative effect of −0.13 with an explained phenotypic variation of 6%. The marker M57, associated with GI belongs to the gene THIS1 on chromosome 1, had negative allelic effect of −122.03 with an explained phenotypic variation of 10%. Three markers were associated with GP, namely Ghd7-05SNP-T on chromosome 7 had a positive allelic effect of 3.89 with the highest explained phenotypic variation of 13% among all traits; SPIKE-indel3 on chromosome 4 had a negative effect of −2.7 with an explained phenotypic variation of 12%; and M200 on chromosome 1 had a positive effect of 1.33 with an explained phenotypic variation of 12%.

Further, two markers were significantly associated with GRI viz; the M57, linked to the gene THIS1 on chromosome 1, exhibited a negative allelic effect of −3.4 with an explained phenotypic variation of 11%, and M169, associated with OsMIK on chromosome 3, had a negative effect of −2.45 with an explained phenotypic variation of 10%. For the LGT traits two markers were associated, i.e. M75, which is associated with OsbHLH107 on chromosome 2, showed a positive allelic effect of 0.66 with an explained phenotypic variation of 9%, and RM431 on chromosome 1 had a positive effect of 0.42 with an explained phenotypic variation of 7%. Five markers were associated with MGT. M169 on chromosome 3 had a negative allelic effect of −0.14 with an explained phenotypic variation of 12%. RM3643 on chromosome 4 and RM490 on chromosome 1 had negative effects of −0.15 and −0.1, respectively, with explained phenotypic variation values of 11% and 10%. M57, linked to THIS1 on chromosome 1, showed a negative effect of −0.15 with an explained phenotypic variation of 10%, and M18, associated with OsBAK1 on chromosome 8, had a negative effect of −0.1 with an explained phenotypic variation of 10%. Two markers were associated with TSG, namely M75, linked to the gene OsbHLH107 on chromosome 2, which had a positive allelic effect of 0.67 with an explained phenotypic variation of 9%, while RM431 on chromosome 1 had a positive effect of 0.41 with an explained phenotypic variation of 7%. A boxplot analysis was conducted using 11 MTAs ($P \leq 0.01$) which were associated with cgSSR markers. The analysis revealed that all markers in these MTAs were able to differentiate phenotypes based on the alleles they produced. Among them, four markers (5 MTAs) showed a significant mean comparison with the corresponding alleles: M75 with TSG, M18 with MGT, M169 with both GRI and MGT, and M202 with FGT (supplementary Figure S1).

The Table 3 presents a set of significant markers associated with multiple traits at two levels of significance, $P \leq 0.05$ and $P \leq 0.01$. These markers had effects on multiple seed germination parameters. At $P \leq 0.05$, several markers show associations with traits such as GI, GRI, FGT, TSG and others. The marker M66 derived from the GW2 gene located on chromosome 2, was associated with GI, GRI, and TSG, similarly the marker M18, derived from the OsBAK1 gene on chromosome 8, was associated with GI, GRI, and MGT, while M57, derived from the THIS1 gene on chromosome 1, was linked to the same traits. Similarly, RM3643 on chromosome 4 and RM490 on chromosome 1 were also associated to GI, GRI, and MGT. Three markers

https://doi.org/10.1017/S0960258525100032 Published online by Cambridge University Press

Table 3. Significant Markers which are associated with multiple traits at $P \leq 0.05$ and $P \leq 0.01$

Marker Name	Gene name	Ch. No	Position	Associated traits
***P* ≤ 0.05**				
DEP1_S9	–	9	16411151	FGT, GP
M42	OsFBK12	3	3833957	GI, GRI
M45	OsDWARF	3	22538341	LGT, TSG
M113	OsHAP2I	10	34152417	LGT, TSG
M66	GW2	2	8114961	GI, GRI, TSG
M149	DSG1	6	2813004	GI, GRI
M165	OsLpa1	2	35175254	LGT, TSG
M167	OsMIK	3	30247380	FGT, GRI, MGT
M168	OsMIK	3	30247380	GI, GRI, MGT
***P* ≤ 0.01**				
RM431	–	1	38893890	LGT, TSG
RM3643	–	4	19948112	GI, GRI, MGT
RM459	–	5	20176610	GI, GRI
RM490	–	1	6677230	GI, GRI, MGT
M18	OsBAK1	8	4344171	GI, GRI, MGT
M57	THIS1	1	31527177	GI, GRI, MGT
M75	OsbHLH107	2	34356787	LGT, TSG
M169	OsMIK	3	30247380	FGT, GI, GRI, MGT
M200	OsAAP10C	1	38120760	GP, MGT

Mean germination time (MGT), first germination time (FGT), last germination time (LGT), germination rate index (GRI), germination index (GI), time spread of germination (TSG), and germination percentage (GP%).

M167, M168, and M169, derived from the OsMIK gene on chromosome 3, were associated with different combinations of FGT, GI, GRI, and MGT. Additionally, markers such as DEP1_S9, M42, M45, M113, M149, M165, RM431, RM459, M75, M200 were significantly associated with at least two germination-related traits. These 18 significantly associated markers consisting of 14 gene-based markers and 4 random SSR. These markers were observed to be associated with at least two germination-related traits and majority of them were cgSSR markers (Table 3). The detailed information about selected markers that showed pleiotropic effect for germination-related traits is given in supplementary Table S3. Also, the detailed information about 215 cgSSR markers are provided in additional supplementary Table S4.

The genes in the 200 kb vicinity (100 kb from both sides) around genomic regions with significant MTAs are taken for candidate gene/locus analysis, which were retrieved from the Rice Annotation Project database (https://rapdb.dna.affrc.go.jp/viewer/gbrowse/irgsp1/ accessed on 22 August, 2023). Based on their putative function for seed germination-related traits, and upon meticulous screening of the loci in the proposed region, a total of 12 major candidate genes were identified. These candidate genes included two on chromosome 1, three on chromosome 2, three on chromosome 3, one on chromosome 6, one on chromosome 8, one on chromosome 9 and one on chromosome 10. The putative functions of these genes are associated with seed development, grain characteristics and overall plant growth (Table 4).

Discussion

Germination is one of the most critical stages in plants and is associated significantly with seedling establishment and crop yield. Improvement in germination-related traits and seed vigour are important for DSR rice (Mohanty et al., 2025). Understanding the genetic basis for these traits could allow the development of rice varieties with improved germination capacity, uniform germination, speed of seedling emergence, uniform emergence period and faster seedling establishment to ensure optimal plant density in field, which is a key factor for high productivity in DSR systems. Before utilizing these traits in breeding for enhancement, it is important to identify the genomic regions associated with quantitative traits for accurate and rapid trait improvement. Association mapping serves as a potent method for uncovering QTL/genes in plants associated with quantitative traits. It is capable for identifying alleles governing various phenotypes. Using candidate gene-derived markers for association mapping has proven successful for this approach; however, linking these markers to genomic regions that impact multiple germination-related traits has significant implications (Molla et al., 2019; Sah et al., 2022).

In this study, the germination traits of 163 rice genotypes showed considerable phenotypic variation, indicating diversity in germination speed and uniformity. Lower MGT (2–3 days) and FGT (1–2 days) indicate faster germination and more robust early seedling growth (Matthews and Khajeh 2006) and Kader (1998). The variability in LGT (3–7 days) may be due to the genetic constitution of seed architecture. GRI (31.67–83.33) and GI (1448–2192) indicated differences in germination speed and uniformity, with higher values suggesting better crop establishment (Esechie, 1994; Sghayar et al., 2023) and higher rate of germination (Bench Arnold et al, 1991). TSG (1–6 days) indicates the degree of uniformity in germination. A lower TSG suggests that the genotypes are germinating in a more synchronized manner, whereas a higher TSG reflects more variation in the timing of germination (Kader 1998). GP% (80–100%) confirmed high overall GRs. These indices are important for selecting genotypes with superior germination traits, as both rapid and uniform germination are critical for improving rice crop establishment, yield and resilience in varying sowing condition (Sahu et al., 2020). FGT had strong positive skewness, indicating most seeds germinated early with a few delayed. GI and GP% had slight negative skewness, suggesting high germination with few low outliers. Kurtosis was <3 for most traits, indicating platykurtic distributions that suggest multiple genes may be involved in controlling these germination traits (Muhammed-Azharudheen et al., 2022). FGT showed high kurtosis, suggesting a leptokurtic distribution where most genotypes are clustered around a central value with fewer extreme deviations. Similar patterns of phenotypic variation in grain, panicle and yield traits have been reported by Nayak et al. (2022), Sah et al. (2022) and Singh et al. (2023).

Figure 1 depicts the correlation between germination-related traits, highlighting several significant relationships among them. The MGT was positively correlated with FGT, LGT and TSG. This indicates that genotypes that start germination early (lower FGT) and complete it more quickly (lower LGT) tend to have a lower MGT, indicating faster and more synchronized germination (Orchard, 1977). A lower mean MGT in the genotypes and negative correlation means higher mean in GRI, GI and GP% indicates more vigorous seeds that can germinate uniformly and rapidly. Similarly, GRI and GI are positively correlated with each other and with GP%. Similar result also observed by Konyak et al. (2024). These results suggest that genotypes with rapid and uniform germination,

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Table 4. Putative functions of major candidate genes in the vicinity of significant MTA

Marker name	Gene name	LOC	Chr no.	Position (Mbp)	Description	Putative functions
DEP1_S9	DEP1	LOC_Os09g26999	9	16411151	Phosphatidylethanolamine-binding protein (PEBP) like domain protein, 426-amino-acid protein, homologous to the keratin-associated protein (KAP) 5–4 family.	Panicle architecture, panicle erectness.
M42	OsFBK12	LOC_Os03g07530	3	3.83415	F-box protein containing a Kelch repeat motif,	Regulation of leaf senescence, seed size and grain number.
M45	OsDWARF/brd1	LOC_Os03g40540	3	22538341	Cytochrome P450 85A1 (EC 1.14.-. -) (C6-oxidase).	Plant hormones involved in various growth and developmental processes
M113	OsHAP2I	LOC_Os10g25850	10	34152417	CCAAT-binding transcription factor, sub-unit B domain containing protein.	Associated with seed development, particularly in the endosperm, which is crucial for seed viability and nutrient storage.
M66	GW2	LOC_Os02g14720	2	8114961	RING-type E3 ubiquitin ligase.	Regulation of grain width and weight
M149	DSG1	LOC_Os06g06090	6	2813004	Mitogen-activated protein kinase, brassinosteroid (BR) signalling and homeostasis,	Regulation of grain size and plant height.
M165	Os LPA1	LOC_Os02g57400	2	35175254	Phytic acid metabolism.	Seed phytic acid content
M167	OsMIK	LOC_Os03g52760	3	30247380	Myo-inositol kinase, phytic acid (PA) biosynthesis,	Seed metabolism and energy storage.
M18	OsBAK1	LOC Os08g04270	8	4344171	FERTILIZATION-INDEPENDENT ENDOSPERM 2, FERTILIZATION-INDEPENDENT ENDOSPERM2, Fertilization-Independent Endosperm 2, Polycomb protein OsFIE2	Yield-related traits such as grain size and number
M57	THIS1	LOC_Os01g54810	1	31527177	Class III lipase,	Regulation of tillering, plant height, and spikelet fertility
M75	OsbHLH107	LOC_Os02g56140	2	34356787	Basic helix-loop-helix transcription factor,	Regulation of grain size.
M200	OsAAP10C	LOC_Os02g56140	1	38120760	Amino acid transporter, transmembrane domain containing protein	A positive regulatory factor for grain protein content and nutritional quality of rice seeds

Mean germination time (MGT).

reflected by lower MGT, FGT and LGT, exhibit higher efficiency in plant establishment.

A high level of allelic diversity reflects the genetic richness within the panel, which is essential for identifying markers associated with desirable traits. High GD and PIC values are indicative of markers that can effectively differentiate between genotypes, which are crucial for the success of association studies. To investigate the informativeness of markers, the PIC of each marker was calculated as a function of allele frequency in the population, and a PIC value of >0.5 was considered as high. Only 41 markers had a PIC value of <0.5. The PIC value of the remaining 254 markers was >0.5, with the highest PIC value of 0.93 (Gn1a-indel3). This suggests that the markers are highly informative for distinguishing between different genotypes and is useful for studying genetic diversity and mapping traits. Similar type of observation was also reported by Sah et al. (2022) for panicle characters and grain yield in rice. The population structure analysis using PCoA and a kinship heatmap revealed the presence of three distinct groups in the PCoA and four sub-groups in the kinship analysis. This suggests a substantial level of genetic diversity both within and among the identified groups. Similar type of result was also observed by Mohanty et al. (2025).

An association analysis using the MLM technique revealed significant MTAs across 12 rice chromosomes for seven seed germination-related traits. Eighty significant ($P \leq 0.05$) MTAs were identified. Further, the level of significance was increased to $P \leq 0.01$, which narrow down to 18 significant MTAs for seven germination-related traits, explaining 6–13% of phenotypic variance and elucidating key genetic factors influencing seed germination-related traits. Further boxplot analysis revealed that all significant markers (11 MTAs) were effective in differentiating phenotypes based on their respective allelic forms. Notably, the MTAs such as M75 with TSG ($P = 0.0015$), M18 with MGT ($P = 0.008$), M169 with both GRI ($P = 1.1e{-}06$) and MGT ($P = 0.0091$), and M202 with FGT ($P = 0.0077$) significantly distinguish phenotypes among the 163 rice lines. The analysis shows that these genetic markers are strongly linked to important rice

https://doi.org/10.1017/S0960258525100032 Published online by Cambridge University Press

traits and these MTAs are highly valuable for future breeding programs.

There were also 18 significantly associated markers showing pleiotropic effects, consisting of 14 gene-based markers and 4 random SSR markers. Using these 14 gene-based markers, 12 candidate genes associated with germination-related traits were identified with putative roles through annotation of the rice genome (Table 4). The marker DEP1_S9, associated with FGT and GP%, was derived from the DEP1 gene (LOC_Os09g26999) located on chromosome 9, which plays a crucial role in controlling panicle architecture and erectness (Huang et al., 2009). The marker M42, associated with germination-related traits such as GI and GRI, was derived from the OsFBK12 gene (LOC_Os03g07530) located on chromosome 3. The OsFBK12 gene linked to the F-box protein with a Kelch repeat motif, which is involved in regulating leaf senescence, seed size, and grain number (Chen et al., 2013). The involvement in seed size is particularly relevant for enhancing vigour and, larger grains tend to germinate more efficiently due to greater stored resources (Basu and Groot, 2023). The marker M45, associated with germination-related traits such as LGT and TSG, was derived from the OsDWARF/brd1 gene (LOC_Os03g40540) located on chromosome 3. The OsDWARF/brd1 gene encodes the cytochrome P450 85A1 protein, which plays a key role in BR biosynthesis and plant hormone regulation (Hong et al., 2002). The cytochrome P450 85A1 protein is also known for its strong expression during seed development in tomato (Montoya et al., 2005). The marker M113, associated with germination-related traits such as LGT and TSG, was derived from the OsHAP2I gene (LOC_Os10g25850) located on chromosome 10. This gene encodes a CCAAT-binding transcription factor and also associated with seed development, particularly in the endosperm. Since the endosperm plays a critical role in providing nutrients to develop embryos, understanding the function of OsHAP2I could help enhance the GR. The marker M66, associated with traits such as GI, GRI, and TSG, was derived from the GW2 gene (LOC_Os02g14720) located on chromosome 2. The GW2 gene is involved in regulating grain width, grain weight and also influence grain size, according to (Basu and Groot, 2023) larger grain size is directly correlated with increased seed vigour, which enhances germination potential and overall plant health. The marker M149, associated with traits such as GI and GRI, was derived from the DSG1 gene (LOC_Os06g06090) located on chromosome 6. This gene encodes a mitogen-activated protein kinase involved in brassinosteroid (BR) signalling and homeostasis, regulating grain size and plant height (Tian et al., 2021).

The marker M165, associated with LGT and TSG, is linked to the OsLPA1 gene (LOC_Os02g57400) located on chromosome 2. This gene is involved in phytic acid metabolism and impacts seed phytic acid content (Kim et al., 2008). High levels of phytic acid can inhibit germination by binding essential nutrients. Modifying this gene could lead to reduced phytic acid content, improving seed nutrient availability during germination (Follmer et al., 2021). The marker M167, associated with traits such as FGT, GRI and MGT, is linked to the OsMIK gene (LOC_Os03g52760) located on chromosome 3. This gene encodes myo-inositol kinase, a key enzyme in phytic acid biosynthesis, which impacts seed metabolism and energy storage, as reported in maize by Shi et al. (2005). The marker M18, associated with traits such as GI, GRI and MGT, is linked to the OsBAK1 gene (LOC_Os08g04270) located on chromosome 8. This gene plays a role in yield-related traits, including grain size and number (Deveshwar et al., 2020). Improved grain size and increased grain number are likely to enhance seed quality and germination capacity, making it an important candidate gene for rice breeding (Yuan et al., 2017). The marker M57, associated with GI, GRI, and MGT, is linked to the THIS1 gene (LOC_Os01g54810) located on chromosome 1. This gene encodes a class III lipase involved in regulating tillering, plant height and spikelet fertility (Liu et al., 2013). These factors contribute to overall seed production and quality, indirectly impacting the germination success of the produced seeds (Liu et al., 2013). The marker M75, associated with LGT and TSG, is linked to the OsbHLH107 gene (LOC_Os02g56140) located on chromosome 2. This gene encodes a basic helix-loop-helix (bHLH) transcription factor involved in regulating grain size.The marker M200, associated with traits such as GP and MGT, is linked to the OsAAP10C gene (LOC_Os01g56140) located on chromosome 1. This gene encodes an amino acid transporter that plays a positive role in regulating grain protein content and the nutritional quality of rice seeds. The involvement of amino acid transporter with grain protein content and the nutritional quality of rice seeds was also reported by Wang et al. (2023).

Conclusion

Germination traits are important for enhancing rice crop establishment, especially in DSR systems, where seedling emergence rapid and uniform germination ensures optimal plant density and productivity. In this study, significant variation was observed in 163 rice genotypes for traits like MGT, FGT, LGT, GRI, GI, TSG and GP%, indicating the potential for selecting genotypes with rapid and uniform germination suitable for DSR systems. A total of 18 significant MTAs ($P \leq 0.01$) were identified using MLM, explaining 6–13% of phenotypic variance across seven germination-related traits. The identification of eight candidate genes linked to germination traits supports the use of marker-assisted selection for improvement in rapid and uniform germination, seed vigour and germination efficiency. Markers like M18, M57, M66, M167, M168 and M169 exhibited pleiotropic effects, suggesting their potential for enhancing multiple traits simultaneously. These findings hold significant implications for the development of rice varieties with superior germination traits, which is essential for achieving high productivity in DSR systems. Further validation of these markers will enable their effective incorporation into rice breeding programs, paving the way for the creation of high-performing, germination-efficient DSR rice varieties.

- Significant variation in germination traits across 163 rice genotypes identified.
- 18 MTAs at $P \leq 0.01$ identified by GWAS analysis.
- 12 candidate genes are identified with putative roles in seed development, grain characteristics, and overall plant growth.
- Markers like M169 (OsMIK), M57 (THIS1), M66 (GW2) and M18 (OsBAK1) show pleiotropic effects, with germination traits like GI, GRI and MGT.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0960258525100032.

Acknowledgements. The authors gratefully acknowledge the financial support received from the ICAR-NRRI, Cuttack. The authors are also thankful to IRRI for sharing some of the genetic materials.

Competing interests. The authors have no competing interests to declare that are relevant to the content of this article.

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