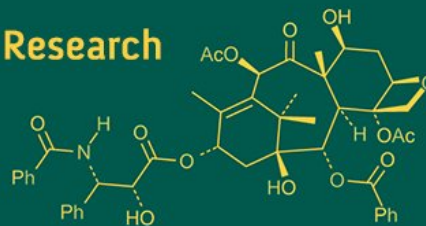


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
IJABR 2026; SP-10(5): 502-508
www.biochemjournal.com
Received: 24-02-2026
Accepted: 28-03-2026

Saikat Pal
Professor, Jayashankar
Telangana Agricultural
University, Rajendranagar,
Hyderabad, Telangana, India

J Suresh
Professor, Jayashankar
Telangana Agricultural
University, Rajendranagar,
Hyderabad, Telangana, India

B Srinivas
RARS, PJTSAU, Polasa,
Jagtial, Telangana, India

Yadla Hari
RARS, PJTSAU, Warangal,
Telangana, India

C Narendra Reddy
Professor, Jayashankar
Telangana Agricultural
University, Rajendranagar,
Hyderabad, Telangana, India

Corresponding Author:
Saikat Pal
Professor, Jayashankar
Telangana Agricultural
University, Rajendranagar,
Hyderabad, Telangana, India

Identification of cold-tolerant rice lines through field screening and molecular marker analysis (*Oryza sativa* L.)

Saikat Pal, J Suresh, B Srinivas, Yadla Hari and C Narendra Reddy

DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i5Sf.8415>

Abstract

Cold stress is one of the major abiotic constraints limiting rice productivity, particularly during the seedling stage in temperate, subtropical, and high-altitude regions. The present study was undertaken to identify cold-tolerant rice genotypes through field screening and molecular marker analysis. A total of 70 rice genotypes comprising 52 restorer lines, 16 CMS lines, and two check varieties (Resistant check Tellahamsa, susceptible check JGL1798) were evaluated across three environments during the Rabi 2021 season at Jagtial, Rudrur, and Rajendranagar. Seedling cold tolerance was assessed based on yellowing score using the Standard Evaluation System (SES) of IRRI. Considerable variation in cold tolerance was observed among the genotypes, with yellowing scores ranging from 1 to 9. Genotypes exhibiting lower yellowing scores (1-5) were classified as tolerant, while those with higher scores were considered susceptible. Among the evaluated lines, R1, R4, R32, R117, R131, R132, R136, R139, R150, R156, R161, R165, R190, R220, R308, SN127, SN464, SN760, CMS14B, CMS23B, CMS64B, CMS69B, JMS18B, and JMS23B showed superior tolerance under cold stress conditions. Twenty-four selected genotypes were further validated using four SSR markers associated with seedling cold tolerance, namely RM85, RM239, RM341, and RM561. Marker analysis revealed that RM85 and RM341 were the most informative markers linked to major cold tolerance QTLs qCTS3 and qCTS2, respectively. Genotypes R4, R32, R117, R156, R165, R190, SN127, and SN760 exhibited allelic similarity with the tolerant check Tellahamsa, indicating the presence of putative cold tolerance alleles. The integration of field screening and molecular marker analysis proved effective in identifying promising cold-tolerant rice lines. These identified genotypes can serve as valuable donor parents in breeding programmes aimed at developing rice hybrids and varieties with enhanced seedling cold tolerance and stable yield under low-temperature stress conditions.

Keywords: Cold tolerance, resistance, susceptible, SES, yellowing score, SSR marker

Introduction

Rice (*Oryza sativa* L.) serves as the primary food source for approximately 3.5 billion people worldwide, contributing up to 50% of the daily caloric intake for populations across Asia (Li *et al.*, 2023) ^[1]. As the second most extensively produced cereal crop globally, rice sustains more than half of the world's population (Kishor *et al.*, 2025) ^[2]. India has produced 152 million metric tons of rice from a total harvested area of 52 million ha with an average productivity of 4.39 MT/ha contributing to approximately 28% of global production during 2025-2026 (USDA). In Telangana, rice is the most important food crop, cultivated on nearly 1.21 crore acres (48.97 lakh hectares), producing 1.75 crore tonnes with an average productivity of 1,446 kg per acre (3,572 kg per hectare) (Directorate of Economics & Statistics, Telangana Socio-Economic Outlook, 2024-25). However, increasing rice productivity and ensuring yield stability under diverse environmental conditions remain major challenges for rice breeding programmes. Among the various abiotic stresses affecting rice production, low temperature stress during early seedling stages is a critical constraint that adversely affects seedling establishment, growth, and productivity (Andaya and Mackill, 2003) ^[3]. Exposure of rice seedlings to low temperatures leads to poor germination, reduced seedling vigour, and ultimately lower grain yield. Therefore, the development of cold tolerant rice varieties is essential to sustain rice production, particularly in regions prone to early-season low temperature stress.

Identification of cold tolerant genotypes is a crucial step in breeding programmes aimed at improving cold tolerance. Field screening under low temperature conditions, when combined with molecular marker analysis, provides an efficient approach for identifying tolerant genotypes and understanding the genetic basis of cold tolerance (Yang *et al.*, 2021) [4]. Among molecular markers, simple sequence repeats (SSRs) have been widely utilized for assessing genetic diversity, identifying tolerant genotypes, and facilitating marker-assisted selection due to their high polymorphism and reproducibility (Lee *et al.*, 2025) [5].

Material and Methods

The experimental material consisting of 52 restorer lines and 16 CMS lines were screened for seedling cold tolerance at three locations viz., Jagtial, Rudrur and Rajendranagar in 2021, *Rabi* season and based on yellowing score. Those identified cold tolerant lines were further confirmed genetically using four cold tolerant SSR markers viz., RM85, RM 239, RM341 and RM561. The list of CMS lines, restorers, and checks used in the study is presented in Table 1. Seedlings were scored for cold tolerance using the Standard Evaluation System for Rice (IRRI-SES, 2013) (Table 2) before uprooting them for transplantation at 20 and 30 Days after Sowing (DAS).

Table 1: List of 70 rice genotypes evaluated across three environments (E1, E2 and E3) for Seedling cold tolerance based on yellowing score

S. No	Genotype	S. No	Genotype	S. No	Genotype	S. No	Genotype
1	R1	19	R100	37	R204	55	CMS 23B
2	R4	20	R107	38	R211	56	CMS 46B
3	R6	21	R117	39	R217	57	CMS 52B
4	R9	22	R131	40	R220	58	CMS 59B
5	R16	23	R132	41	R234	59	CMS 64B
6	R22	24	R136	42	R237	60	CMS 67B
7	R32	25	R139	43	R247	61	CMS 69B
8	R42	26	R150	44	R255	62	CMS 75B
9	R45	27	R155	45	R272	63	JMS 11B
10	R46	28	R156	46	R276	64	JMS 13B
11	R55	29	R161	47	R300	65	JMS 14B
12	R58	30	R165	48	R308	66	JMS 18B
13	R59	31	R180	49	SN 127	67	JMS 23B
14	R67	32	R181	50	SN 464	68	JMS 24B
15	R69	33	R184	51	SN 760	69	JGL1798
16	R71	34	R190	52	SN 768	70	Tellahamsa
17	R93	35	R196	53	CMS 11B		
18	R95	36	R200	54	CMS 14B		

Table 2: Cold tolerance scoring using 0-9 Standard Evaluation System (SES - IRRI, 2013) scale (for seedlings)

Scale	Symptom	Tolerance Level
0-1	No damage to leaves; normal leaf color	Highly Tolerant
2-3	Tip of leaves slightly dried, folded, and light green	Strongly Tolerant
4-5	Some seedlings moderately folded and wilted; 30-50% seedlings dried; pale green to yellowish leaves	Tolerant / Moderately Tolerant
6-7	Seedlings severely rolled and dried; reddish-brown leaves	Sensitive
8-9	Most seedlings dead or dying	Highly Sensitive

Table 3: List of SSR Markers used in present study

S.No.	Primer Name	Forward Primer 5' → 3'	Reverse primer 3'→5'	Product size (bp)
1	RM85	CCAAAGATGAAACCTGGATTG	GCACAAGGTGAGCAGTCC	10
2	RM239	TACAAAATGCTGGGTACCCC	ACATATGGGACCCACCTGTC	144
3	RM341	CAAGAAACCTCAATCCGAGC	CTCCTCCCGATCCCAATC	172
4	RM561	GAGCTGTTTTGGACTACGG	GAGTAGCTTTCTCCCACCCC	190

Results and Discussion

Field screening for seedling cold tolerance

A total of 70 rice genotypes, comprising 68 inbred lines and two check varieties, were evaluated for seedling cold tolerance under field conditions. Among the 68 inbred lines evaluated, 16 were CMS (Cytoplasmic Male Sterile) lines and 52 were restorer lines. The inclusion of both CMS and restorer lines enabled the identification of cold tolerant parental lines that could be utilized in hybrid rice breeding programmes.

The genotypes were screened based on the yellowing score, which indicates the extent of leaf chlorosis and damage caused by low temperature stress at the seedling stage. Lower yellowing scores indicate better tolerance to cold

stress, whereas higher scores represent susceptibility. The yellowing scores recorded among the genotypes ranged from 1 to 9, indicating a wide range of variation in response to cold stress (Table 4.). This variation suggested the presence of considerable genetic diversity for seedling cold tolerance among the evaluated genotypes. Based on the yellowing score and visual seedling performance under cold stress conditions, the genotypes were classified into tolerant, and susceptible categories (Figure 1.). Genotypes R1, R4, R32, R117, R131, R132, R136, R139, R150, R156, R161, R165, R190, R220, R308, SN127, SN464, SN760, CMS 14B, CMS 23B, CMS 64B, CMS 69B, JMS 18B, JMS 23B showing lower yellowing scores (1-5) exhibited better seedling vigour and minimal leaf chlorosis and were

categorized as cold tolerant. In contrast, other genotypes with higher yellowing scores (6-9) showed severe yellowing, reduced vigour and poor seedling establishment and were considered susceptible to cold stress. This is in agreement with Andaya and Mackill (2003) [3], who used a visual scoring scale of 1 (tolerant) to 9 (susceptible) for Cold-Induced Yellowing Tolerance (CIYT) in rice seedlings and reported continuous variation among RIL populations, indicating the polygenic nature of the trait. The classification of genotypes into tolerant (score 1-5) and susceptible (score 6-9) categories based on yellowing scores is consistent with the methodology adopted by several researchers in evaluating seedling cold tolerance in rice germplasm (Saha *et al.*, 2022; Andaya and Mackill, 2003) [3, 6].

Among the evaluated genotypes, several lines exhibited good tolerance to cold stress, including R1, R4, R32, R117, R131, R132, R136, R139, R150, R156, R161, R165, R190, R220, R308, SN 127, SN 464 and SN 760 among the restorer lines. Similarly, among the CMS lines, CMS 14B, CMS 23B, CMS 64B, CMS 69B, JMS 18B and JMS 23B exhibited comparatively better tolerance with lower yellowing scores. The tolerant check Tellahamsa also showed a very low yellowing score, confirming its tolerance to cold stress.

Based on the field screening results, 26 genotypes consisting of 24 inbred lines (including both CMS and restorer lines) and two check varieties were selected for further studies for genotypic evaluation of cold tolerance.

Table 4: Seedling cold tolerance reaction of rice genotypes based on yellowing score evaluated across three environments (E1, E2 and E3)

S. No	Genotype	Yellow score_E1	Cold reaction_E1	Yellow score_E2	Cold reaction_E2	Yellow score_E3	Cold reaction_E3
1	R1	1	R	2	R	4	R
2	R4	2	R	3	R	1	R
3	R6	7		6		8	
4	R9	7		6		6	
5	R16	7		6		6	
6	R22	8		8		6	
7	R32	3	R	4	R	3	R
8	R42	6		8		7	
9	R45	8		6		9	
10	R46	7		6		9	
11	R55	6		7		6	
12	R58	6		8		7	
13	R59	7		6		7	
14	R67	7		6		6	
15	R69	7		8		6	
16	R71	7		8		6	
17	R93	7		8		7	
18	R95	7		6		9	
19	R100	7		7		7	
20	R107	6		9		7	
21	R117	4	R	3	R	2	R
22	R131	3	R	4	R	2	R
23	R132	3	R	2	R	3	R
24	R136	3	R	5	R	4	R
25	R139	3	R	2	R	3	R
26	R150	3	R	2	R	3	R
27	R155	8		6		6	
28	R156	5	R	2	R	4	R
29	R161	2	R	3	R	1	R
30	R165	3	R	5	R	3	R
31	R180	8		7		7	
32	R181	9		7		6	
33	R184	6		7		6	
34	R190	5	R	2	R	3	R
35	R196	7		6		6	
36	R200	6		6		8	
37	R204	7		7		6	
38	R211	8		6		8	
39	R217	8		9		6	
40	R220	3	R	4	R	2	R
41	R234	6		6		8	
42	R237	7		9		6	
43	R247	8		7		6	
44	R255	7		6		6	
45	R272	9		6		9	
46	R276	8		9		9	
47	R300	7		9		8	
48	R308	4	R	5	R	3	R

49	SN 127	3	R	1	R	2	R
50	SN 464	4	R	3	R	2	R
51	SN 760	3	R	1	R	4	R
52	SN 768	6		7		8	
53	CMS 11B	9		6		6	
54	CMS 14B	3	R	4	R	2	R
55	CMS 23B	5	R	2	R	3	R
56	CMS 46B	8		9		8	
57	CMS 52B	6		8		7	
58	CMS 59B	8		6		6	
59	CMS 64B	4	R	2	R	2	R
60	CMS 67B	7		7		8	
61	CMS 69B	4	R	3	R	3	R
62	CMS 75B	8		7		6	
63	JMS 11B	7		6		6	
64	JMS 13B	7		6		8	
65	JMS 14B	6		7		8	
66	JMS 18B	3	R	4	R	2	R
67	JMS 23B	3	R	2	R	3	R
68	JMS 24B	8		6		6	
69	JGL1798	3	R	2		4	R
70	TELLAHAMSA	1	R	2		2	R

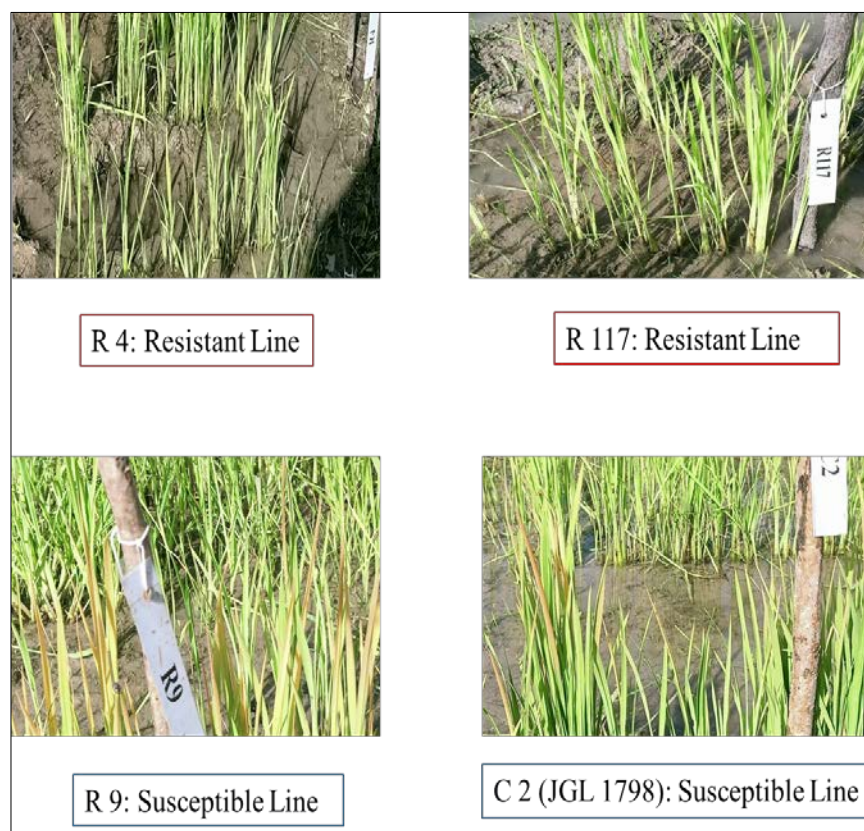


Fig 1: Visual comparison of seedling cold tolerance responses among rice genotypes under field conditions

The preliminary selection was primarily based on lower yellowing scores and better seedling performance under cold stress conditions, indicating their potential as sources of seedling cold tolerance. The selected genotypes exhibited lower yellowing scores ranging from 1 to 5, indicating better tolerance to cold stress compared to other evaluated lines. The identification of these tolerant lines provides valuable genetic resources for the development of rice hybrids with improved seedling cold tolerance and stable yield performance under low temperature conditions.

Molecular screening for seedling cold tolerance

In order to validate the cold tolerant genotypes identified through field screening, molecular screening was carried out using four SSR markers, namely RM85, RM341, RM561 and RM239, which were reported to be associated with seedling cold tolerance in rice (Lou *et al.*, 2007) ^[7]. The forward & reverse primer sequence and expected product length have been depicted in Table 3. Among these markers, RM85 and RM341 were observed to be closely linked to major cold tolerance QTLs and therefore considered more reliable for identifying tolerant genotypes. SSR markers have been widely validated as reliable tools for tagging cold tolerance QTLs and facilitating marker-assisted selection

(MAS) in rice breeding programmes (Andaya and Mackill, 2003; Pradhan *et al.*, 2016) ^[3,9].

The marker RM85 is linked to the qCTS3 locus and located on chromosome 3, which explains approximately 13.8% of the Phenotypic Variation (PVE) for seedling cold tolerance. Similarly, the markers RM341 and RM561 is associated with the qCTS2 locus located on chromosome 2, explaining approximately 27.42% of the phenotypic variation for seedling cold tolerance, with a high LOD score of 15.09 (Lou *et al.*, 2007) ^[7]. These QTLs are known to play an important role in regulating cold tolerance during the early seedling stage in rice.

For molecular analysis, the selected genotypes were screened along with two reference checks, namely Tellahamsa, which served as the resistant check, and JGL1798 (JGL1798), which served as the susceptible check. The allelic pattern observed in the resistant check was used as a reference to identify genotypes possessing the putative cold tolerance allele.

Marker RM85

The SSR marker RM85 exhibited clear polymorphism between the resistant and susceptible checks (Figure 2.). The resistant check Tellahamsa produced a PCR-amplified product of 107 bp, whereas the susceptible check JGL1798 showed an amplified product greater than 107 bp.

Genotypes exhibiting the same 107 bp amplified product as the resistant check were considered to possess the putative cold tolerance allele.

The polymorphism detected by RM85 in the present study is in agreement with earlier reports validating this marker for cold tolerance screening. Pradhan *et al.* (2016) ^[9] reported that individual SSR markers such as RM85 and RM341 could be effectively utilized in Marker-Assisted Selection (MAS) breeding programs. Furthermore, the combined use of non-paired markers RM284, RM239, and RM85 was found to be more effective than the combination of RM284 and RM85 alone. The qCTS3 locus located on chromosome 3 and detected by RM85 in the present study has also been identified as a stable QTL region in several independent studies conducted by Andaya and Mackill, 2003 ^[3] and Han *et al.*, 2007 thereby confirming its usefulness as a functional marker for seedling cold tolerance.

Based on PCR-amplified product sizes, the genotypes R4, R32, R190, and SN 760 exhibited the same allele size as the resistant check Tellahamsa, indicating the presence of the qCTS3 cold tolerance allele. These genotypes may therefore serve as potential sources of seedling cold tolerance associated with the qCTS3 locus. In contrast, the remaining genotypes displayed different allele patterns compared to the resistant check, suggesting the absence of the specific cold tolerance allele linked to RM85.



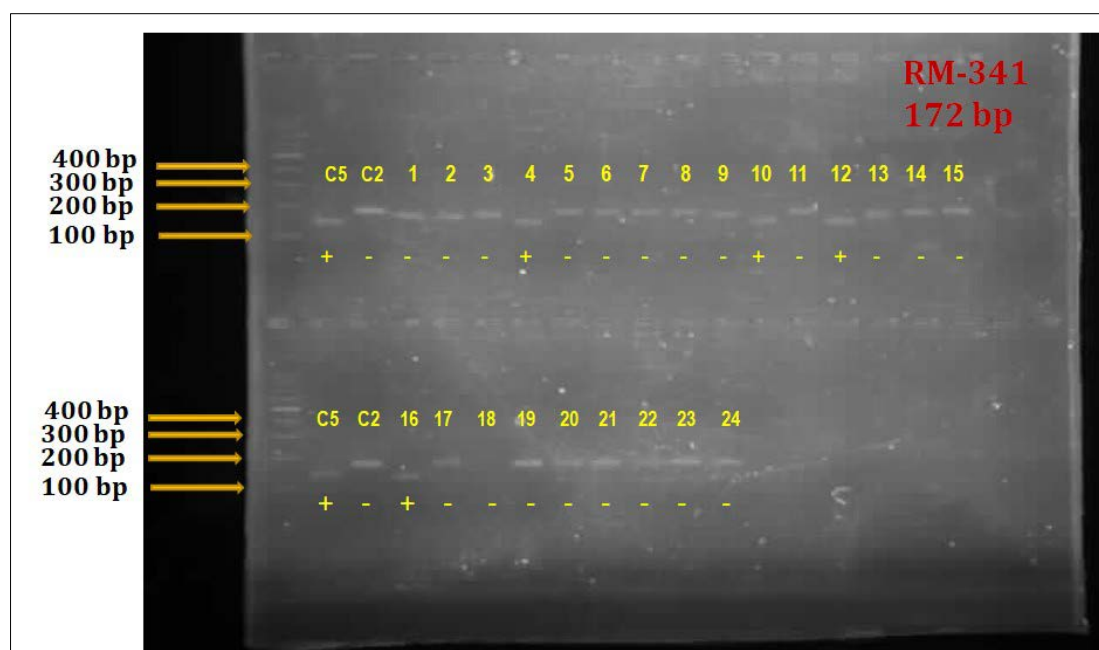
C5-Tellahamsa (Resistant), C2-JGL1798 (susceptible), 1-R1, 2-R4, 3-R32, 4-R117, 5-R131, 6-R132, 7-R136, 8-R139, 9-R150, 10-R156, 11-R161, 12-R165, 13-R190, 14-R220, 15-R308, 16-SN 127, 17-SN464, 18-SN760, 19-CMS 14B, 20-CMS 23B, 21-CMS 64B, 22-CMS 69B, 23-JMS 18B, 24-JMS 23B

Fig 2: Molecular screening of rice genotypes for cold tolerance using RM85 marker

Marker RM341

The SSR marker RM341, linked to the qCTS2 locus on chromosome 2, also showed clear polymorphism between the resistant and susceptible checks (Figure 3.). Three different alleles were amplified based on the product size and the resistant check Tellahamsa displayed a distinct allele pattern (band size 172 bp) which was used as a reference for identifying tolerant genotypes. Based on the allele scoring pattern, the genotypes R117, R156, R165 and SN127 showed the same product as the resistant check Tellahamsa, indicating the presence of the qCTS2 cold tolerance allele. The results obtained with RM341 are in agreement with earlier studies on the qCTS2 locus for seedling cold

tolerance in rice. A major QTL, qCTS-2, was identified on chromosome 2 flanked by RM561 and RM341, and was reported to have the highest LOD score among detected QTLs (Lou *et al.*, 2007; Zhao *et al.*, 2017) ^[7, 11]. Further studies confirmed that this locus explains a relatively high proportion of phenotypic variation (~27.42%) and is consistently associated with seedling cold tolerance across different genetic backgrounds. In addition, RM341 has been reported to be significantly associated with cold tolerance under different temperature conditions, indicating its reliability for identifying tolerant genotypes (Pandit *et al.*, 2017) ^[10].



C5-Tellahamsa (Resistant), C2-JGL1798 (susceptible), 1-R1, 2-R4, 3-R32, 4-R117, 5-R131, 6-R132, 7-R136, 8-R139, 9-R150, 10-R156, 11-R161, 12-R165, 13-R190, 14-R220, 15-R308, 16-SN 127, 17-SN464, 18-SN760, 19-CMS 14B, 20-CMS 23B, 21-CMS 64B, 22-CMS 69B, 23-JMS 18B, 24-7JMS 23B

Fig 3: Molecular screening of rice genotypes for cold tolerance using RM341 marker

Marker RM561

The marker RM561 was also used to screen the selected genotypes for polymorphism associated with cold tolerance. Most of the genotypes amplified same product size similar to the resistant check Tellahamsa (band size 190bp), indicating limited polymorphism at this locus and reduced ability to discriminate between tolerant and susceptible genotypes. This may be attributed to the narrow genetic variation present in the selected germplasm for this genomic region.

RM561 is located near the *qCTS-2* QTL on chromosome 2 along with RM341 (Lou *et al.*, 2007) [7]. The comparatively lower effectiveness of RM561 in the present study suggests that it may be less closely associated with the functional allelic variation governing cold tolerance than RM341. Similar observations have been reported in earlier studies, where RM561 showed variable association with cold tolerance traits depending on the genetic background and environmental conditions (Yang *et al.*, 2018). Thus, the low polymorphism observed with RM561 indicates that not all flanking markers of a QTL are equally informative, and markers more tightly linked to functional genes tend to provide better discrimination (Pradhan *et al.*, 2016) [9].

Marker RM239

The SSR marker RM239 showed clear amplification across the evaluated genotypes. The resistant check Tellahamsa amplified product size is 144bp, which was used as a reference for identifying genotypes possessing the putative cold tolerance allele. Most of the evaluated genotypes exhibited the same band size as the resistant check, indicating low polymorphism at this locus and the presence of similar alleles among the selected lines. Although RM239 showed limited ability to discriminate between genotypes, it indicated the presence of conserved genomic regions associated with cold tolerance. The low polymorphism observed with RM239 is in agreement with earlier reports suggesting that this marker provides supplementary rather

than primary information for cold tolerance. Pradhan *et al.* (2016) [9] reported that RM239 is more effective when used in combination with other markers rather than independently. Similarly, Pandit *et al.* (2017) [10] observed that RM239 is associated with chilling tolerance under moderate temperature conditions, indicating its context-dependent utility. Thus, the results suggest that RM239 plays a supportive role in marker-assisted selection and is more informative when used alongside major functional markers.

The combined analysis of the four SSR markers revealed that several genotypes possessed alleles similar to the resistant check, indicating the presence of genomic regions associated with seedling cold tolerance. Among the markers, RM85 (*qCTS3*) and RM341 (*qCTS2*) were found to be the most informative for identifying tolerant genotypes. The genotypes carrying either RM85 or RM341 are R4, R32, R117, R156, R165, R190, SN127, SN760; which could serve as potential donors for improving seedling cold tolerance in rice breeding programmes. The integration of field screening with molecular marker analysis provided a reliable approach for identifying promising genotypes for further breeding.

This approach is well supported by earlier studies, which highlight that only a few cold tolerance QTLs are consistently expressed and useful in breeding despite the large number reported (Zhao *et al.*, 2017) [11]. Therefore, the use of validated markers such as RM85 and RM341 enhances the efficiency of marker-assisted selection (Andaya and Mackill, 2003; Lou *et al.*, 2007; Pradhan *et al.*, 2016; Pandit *et al.*, 2017) [3, 6, 9, 10]. The multi-marker approach further improves the accuracy of identifying genotypes with favorable alleles for stable cold tolerance.

Conclusion

The combined use of phenotypic screening and marker-assisted selection proved to be a reliable and efficient approach for identifying cold-tolerant rice lines. The

identified genotypes can be effectively utilized in hybrid rice breeding and varietal improvement programmes aimed at developing high-yielding rice cultivars with enhanced seedling cold tolerance and stable performance under low-temperature stress environments. Future studies involving fine mapping, gene characterization, and multi-location evaluation may further strengthen the utilization of these genotypes in climate-resilient rice breeding.

Acknowledgement

The first author is thankful to Council of Scientific and Industrial Research (CSIR) for providing Senior Research Fellowship (File number 08/1285(12045)/2021-EMR-I. The authors are thankful to PJTAU, Hyderabad; RARS, Warangal; RARS, Jagtial and RS&RRS, Rudrur for providing necessary facilities to carry out the experiment.

Competing Interests

Authors have declared that no competing interests exist.

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