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Research Article

Identification of genomic regions linked to seed dormancy related traits using bulk segregant analysis in rice (*Oryza sativa* L.)

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Abstract
A total of 119 $F_{6:7}$ RILs of a cross between BPT 2231 (non- seed dormant parent) and MTU 1001 (seed dormant parent) were analyzed to identify the markers associated with seed dormancy. Parental polymorphism survey with 188 SSR markers revealed 10 polymorphic markers between the parents. The bulk segregant analysis results with 10 polymorphic markers revealed that four markers showed polymorphism between the bulks. The association of putative markers *viz.,* RM346, RM22565, RM7051 and RM10793 identified based on DNA pooling from selected segregants was analyzed by Single Marker Anaysis (SMA). The results of SMA revealed that RM22565 on chromosome 8 showed significant association with germination per cent at five days after harvesting indicating that the chromosomal region linked to the marker RM 22565 on chromosome 8 may be associated with seed dormancy. Out of the four polymorphic markers used in the present study, RM346 was notified as a seed dormancy linked marker from previous studies. The other three markers *viz.,* RM22565, RM7051 and RM10793 identified as seed dormancy linked markers in the present study, needs further validation on alternative set of population or a set of germplasm lines for their further utilization in the marker assisted breeding programme. Based on germination percentage, physiological parameters and genotyping studies, the RILs *viz.*, SD 3, SD 12, SD 15 and SD 68 were identified as donors for the future breeding programme for the development of seed dormant varieties.

Keywords: Rice, seed dormancy, SSR markers, BSA, Single Marker Analysis, RILs

INTRODUCTION

Rice is the predominantly consumed staple food crop in the world and a grain of life for more than 70 per cent of the Asians, holding great importance in Indian culture. Grain yield and quality enhancement are the major breeding objectives in rice improvement programmes. Non dormant seeds can germinate whenever the conditions are favorable including on the mother plant when the rain occurs before harvest; a phenomenon known as pre-harvest sprouting (PHS). Varieties cultivated under humid climate should possess pre-harvest sprouting (PHS) resistance in order to produce high quality grain. Hence, development of varieties with strong seed dormancy is an important objective in rice breeding programmes (Zhang *et al*., 2020). Seed dormancy is the temporary failure of a viable seed to germinate under favorable conditions. As the prevailing climatic conditions influence the expression of this character, screening of cultivars and selection of dormant genotypes based on phenotyping is problematic. Hence, utilization of molecular markers could be a useful tool to isolate the seed dormant genotypes possessing PHS resistance. Identification of genomic regions/QTLs involves comparative study of phenotyping and genotyping data of large mapping populations. Under genotyping, whole population genotyping (WPG),

selective genotyping (SG) and bulked segregant analysis (BSA) approaches are there, in which BSA approach successfully detected large effect of genomic regions associated with seed dormancy within a short time span with low cost when compared to other screening approaches.

MATERIALS AND METHODS

The experimental material comprised of 119 $F_{6:7}$ generation Recombinant Inbred Lines (RILs) (SD1 to SD 119) developed from a cross between BPT 2231 (non-seed dormant parent) and MTU 1001 (seed dormant parent). The parents and 119 RILs were grown in simple lattice design with two replications during *kharif* 2018-19. Each experimental unit in the field consisted of 2.4 m^2 and the spacing adopted was 20 x 15 cm between rows and between the plants, respectively. Each replication consisted of eighty plants, among these five plants were selected randomly for recording the data. The data was collected on different characters *viz.,* days to 50 % flowering, plant height, productive tillers, length of the panicle, test weight, grain yield, percentage of germination at 5 days & 10 days after harvesting, free amino acids and total soluble sugars. However, data on days to 50% flowering, test weight, germination per cent at 5 days and 10 days after harvesting, free amino acids and total soluble sugars were recorded on plot basis. For dormancy assessment, panicles from randomly selected plants in each replication was collected on 30th day after heading, when the grains were fully filled physiologically matured. The germination per cent at 5 days and 10 days after harvesting was assessed by following the procedure suggested by Wan *et al.* (1997). For evaluation of seed dormancy, 100 seeds were counted and placed in moist filter papers which were placed in Petri plates of 15 cm diameter and kept in incubator at 30^{o}C with 100 % humidity and then germination counts were taken on 7th day and 10th day after soaking. The seeds with a coleoptile longer than 2 mm were counted as germinated. Germination counts were taken for each genotype at different intervals (one at 5 days after harvesting and second at 10 days after harvesting) in two replications. After calculating the germination per cent of RIL population, they were categorized into different dormancy groups as weakly dormant (>80%), moderately dormant (50-79%) and strongly dormant (<50%). The time taken for attaining 80% germination after maturity was considered as the duration of dormancy (Voleti *et al*., 2013). The physiological parameters *viz.,* free amino acids and total soluble sugars were estimated by following the prescribed protocol of Moore and Stein (1948) and Hedge and Hofreiter (1962), respectively. The mean data was used for the analysis of heritability and standard deviation, as per Rao (2007).

Genomic DNA was isolated from frozen fresh leaf tissue of 119 $F_{6:7}$ progenies along with both the parents (BPT-2231 and MTU-1001). Nano Drop (Thermo scientific) was used for estimation of the quality and quantity of DNA. The final DNA concentration was adjusted to 50 ng/µl. The PCR reaction was performed in volumes of 10 µl containing 1 µl of 10 pmol primer (both forward and reverse primer), 0.5 µl of 2.5 mM deoxyribonucleotides, 1 µl of GeNei 10X assay buffer (10 mM TrisHCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin) and 1 µl of 1 U/µl*Taq DNA polymerase* and 3 µl of sterile distilled water (GeNei^{TM}). PCR amplification with a PCR profile of 94°C for 5 min followed by 35 cycles of 1 min at 94°C, for 30 sec at 55°C and 1 min at 72°C followed by final extension at 72°C for 10 min with the Eppendorf Master cycler Germany. The PCR products were separated on 3% agarose gel and documented using a gel documentation system.

A total of 188 random SSR markers spanning all twelve chromosomes were used for parental polymorphism survey among the parents (BPT-2231 and MTU-1001) (Bharathi *et al.,* 2019 and Ye *et al.,* 2013). For the BSA, the DNA bulks of plants with high seed dormancy (Bulk A) and those with low seed dormancy (Bulk B) were prepared from $F_{6:7}$ phenotyped progenies. Pooling of DNA was done by taking equivalent amounts of total DNA, approximately 10 µl of 50 ng concentration from each of the ten high dormant and ten low dormant plants was done based on phenotyping results. The parents, two bulk DNA samples and DNA of individual $F_{6:7}$ plants were also used to screen with polymorphic SSR primer pairs analyzed with co-segregating markers to confirm their linkage with the seed dormancy. The SSR markers identified as polymorphic between the parents and the extreme bulks were used to screen the RIL population. DNA of 119 $F_{6:7}$ progenies and parents were analyzed to study the co-segregation of these markers. Scoring of the clearly resolved amplicons of SSR's was done as homozygote of the allele for high seed dormant parent (B), homozygote of the allele for low seed dormant parent (A) and heterozygote carrying the alleles from both parents (H) in the data sheet. The test of goodness of fit of the $F_{6:7}$population, χ^2 test was performed to the phenotyping and marker data by comparing an observed frequency distribution with an expected one. Marker-trait association was analyzed by simple marker analysis to know the association between the markers and the dormancy score using software map disto V.1.7.

RESULTS AND DISCUSSION

The performance of parents and RILs for grain yield and seed dormancy related traits is presented in **Table 1**. The mean grain yield of the RIL population ranged from 19.38 to 42.58 g, whereas the parents BPT 2231 and MTU 1001 recorded 32.6 g and 31.5 g respectively. The phenotyping data for germination per cent at five days and 10 days after harvesting revealed that dormant parent MTU 1001 recorded below 50% germination at five days and 10 days after harvesting while the non-dormant parent BPT 2231 recorded >80% germination even at five days after harvesting. Among the RIL population, 68

Table 1. Standard deviation of seed dormancy related traits

Character	Heritability (%)	Mean ± SE			Standard deviation of the progeny
		Parent 1 (BPT 2231)	Parent 2 (MTU 1001)	RIL population (Progeny)	
Grain yield per plant (g)	69	32.55±6.152	31.50±3.535	32.26±0.496	5.461
Germination (%) at 5 DAH	97	91.50±2.121	9±1.414	44.74±2.227	24.501
Germination (%) at 10 DAH	97	93.00±4.242	25.0±2.828	65.71±2.078	22.855
Free Amino Acids (mg g^{-1})	99	1.51±0.111	1.06±0.226	1.89±0.077	0.848
Total Soluble Sugars (mg g^{-1})	98	23.99±1.394	14.55±0.636	20.06±0.497	5.464

SE: Standard Error ; DAH: Days after harvesting

lines were categorized as strongly seed dormant lines (germination below 50%), 43 lines as moderately seed dormant (germination 50-79%) and 8 lines as weakly seed dormant at five days after harvesting (**Fig. 1**). Likewise, at 10 days after harvesting, 34 lines were categorized as strongly seed dormant, 42 lines as moderately seed dormant and 45 lines as weakly seed dormant (**Fig. 2**). Voleti *et al.* (2013) in a study involving 29 advanced breeding lines reported that MTU 1001 had nine weeks of seed dormancy and BPT 2231 was a non- seed dormant genotype. Bharathi *et al.* (2019) also reported similar findings with MTU 1001 for its strong seed dormancy in a study involving 32 rice genotypes.

Among the RIL population under study, seven lines *viz.,* SD 20, SD 30, SD 32, SD 45, SD 59, SD 65 and SD 80 recorded significantly superior grain yield than the parents coupled with low germination per cent than the dormant parent MTU 1001. The phenotyping results for seed dormancy related physiological traits revealed that SD 3, SD 68, SD 15, SD 20 and SD 27 manifested low values for both amino acids and total soluble sugars content than the dormant parent MTU 1001. Three lines *viz.,* SD 7, SD 12, and SD 83 recorded low soluble sugar content, while SD 33, SD 75 and SD 95 exhibited low amino acid content than the dormant parent. All these genotypes exhibited low germination per cent than the dormant parent MTU 1001 (<50 %) and were categorized as strongly dormant at five days after harvesting. Likewise, SD 5, SD 26, SD 39 and SD 56 manifested high values for total soluble sugars and free amino acids and recorded >80% germination at five days and 10 days after harvesting. Atwell *et al.* (1982) and Bewley and Black (1983) reported positive association of coleoptile elongation with continuous

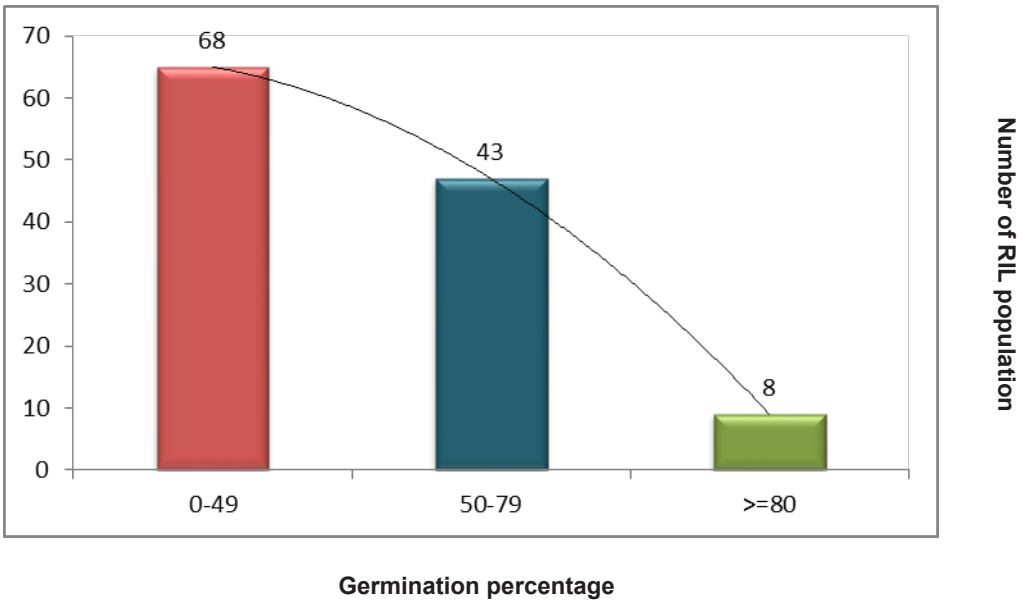


Fig. 1. Distribution of RIL population for germination (%) at 5 days after harvesting

0-49: High seed dormancy count; 50-79: Moderate seed dormancy count; >80: Low seed dormancy count

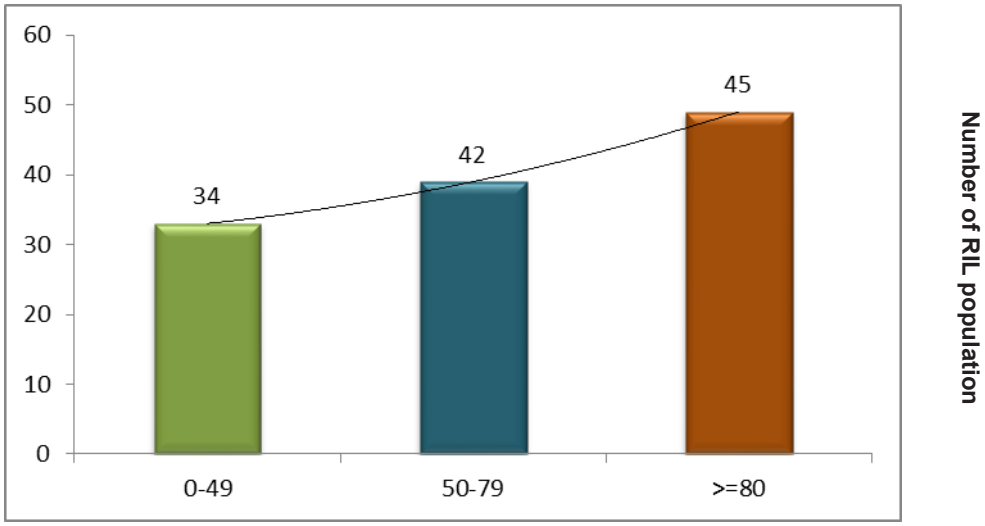


Fig. 2. Distribution of RIL population for germination (%) at 10 days after harvesting

0-49: High seed dormancy count; 50-79: Moderate seed dormancy count; >80: Low seed dormancy count

supply of soluble sugars to the developing embryo. In the present investigation, the genotypes which recorded low content of free amino acids also exhibited low germination per cent and showed high seed dormancy.

For genotyping studies, a total of 72 SSR markers which are in public domain along with 116 randomly selected SSR markers were used for parental polymorphism assessment between the parents BPT 2231 (non- seed dormant parent) and MTU1001 (seed dormant parent). Out of these 188 SSR markers used for polymorphic survey, ten markers (**Fig. 3**) exhibited distinct polymorphism between the parents with base pairs difference ranging from 5 bp (RM477) to 75 bp (RM22565) (**Fig. 3 to 5**). Bulk segregant analysis , though mostly applied to study qualitative traits, has also been applied to study traits like drought by Venuprasad *et al.* (2009); Kanagaraj *et al.* (2010); Salunkhe *et al.* (2011); Boopathi *et al.* (2013), Vikram *et al.* (2012) for identification of QTLs for drought tolerance in paddy, Tiwari *et al.* (2016) for mapping QTLs for salt tolerance in paddy and Waghmare *et al.* (2021) identified the QTLs for heat tolerance in paddy. Babu Motagi *et al.* (2018) studied the genetic and molecular components of rust resistance in Peanut (*Arachis hypogaea* L.) using BSA.

For BSA, two bulks possessing distinct and contrasting phenotypes (*i.e.,* low seed dormancy bulk and high seed dormancy bulk) were prepared based on germination per cent of RIL population phenotyped at five days and 10 days after harvesting and also on physiological parameters *i.e.,* total soluble sugars and free amino acids. Low seed dormancy bulk (**Table 2**) consisted of 10 lines *viz.,* SD 5, SD 26, SD 39, SD 56, SD 73, SD 98, SD 102, SD 103, SD 114 and SD 119 recorded >80% germination at five days after harvesting. Likewise, the high seed dormancy bulk (**Table 3**) was constituted by pooling the genotypes which recorded low germination per cent at 10 days after harvesting. The 10 lines which were constituted as bulk B (high seed dormancy) *viz.,* SD 3, SD 12, SD 15, SD 32, SD 59, SD 68, SD 72, SD 81, SD 87 and SD 113 recorded <30% germination.

Ten polymorphic markers (**Table 4**) were used for screening of parents MTU 1001, BPT 2231, bulk A, and bulk B along with individuals of $F_{6:7}$ populations used in respective bulks. Out of these 10 markers, four markers RM346 (chromosome 7), RM 2256 (chromosome 8), RM 7051 (chromosome 4) and RM 10793 (chromosome 1) clearly distinguished low seed dormant bulks from high seed dormant bulks as that of the parents (**Fig. 4 & 5**). The $F_{6:7}$ RILs were genotyped with these four primers (RM346, RM22565, RM7051 and RM10793) to study their possible association with seed dormancy. Segregation pattern with marker RM 346 recorded a high seed dormancy allele of donor in 43 RILs and low seed dormancy allele of recipient was amplified in 47 RILs (**Fig. 6**). Similarly, with RM 22565 marker, 67 RILs showed donor allele and 52 RILs manifested recipient allele (**Fig. 7**). The marker RM 7051 recorded in 57 RILs having similar alleles as that of seed dormant parent MTU 1001 and 58 RILs showed similar amplicon size of non-dormant parent BPT 2231. Likewise, the amplification pattern of RM 10793 marker revealed that 67 RILs manifested similar alleles to that of the dormant parent, while 42 RILs reported similar alleles as that of non-seed dormant parent BPT 2231.

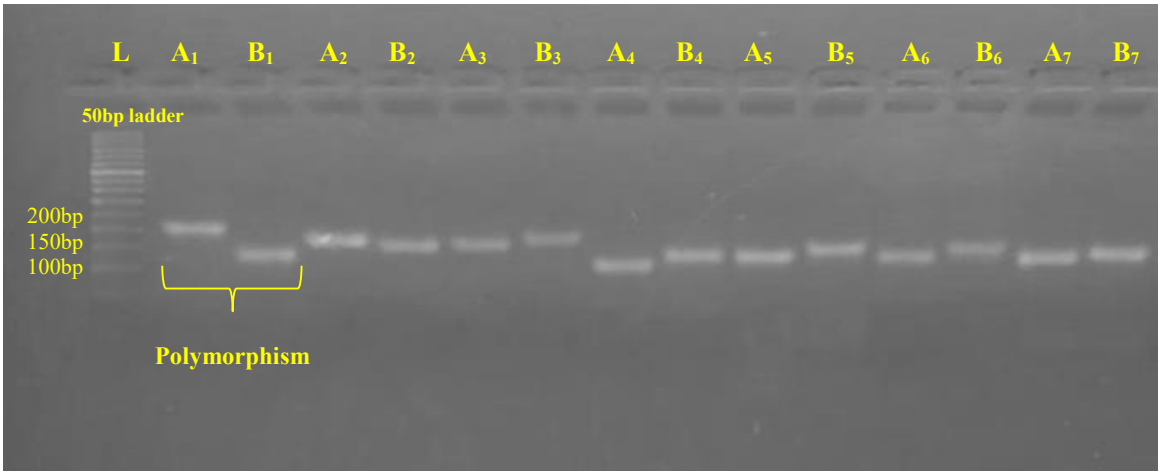


Fig. 3. Parental polymorphism between the parents BPT 2231 and MTU 1001

A: BPT 2231; B: MTU 1001, 1: RM346; 2: RM3295; 3: RM493; 4: RM22565; 5: RM8094; 6: RM260; 7: RM562

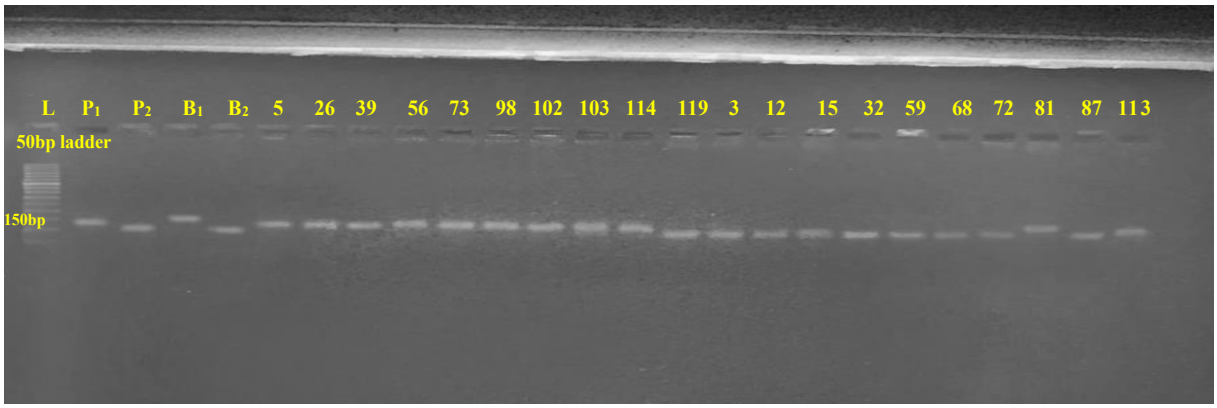


Fig. 4. Bulk segregant analysis using marker RM346

P_1: BPT 2231; P_2: MTU1001; B_1: Low seed dormancy bulk; B_2: High seed dormancy bulk; 5 to 114: Low seed dormancy individual line from bulk B_1; 119 to 113: High seed dormancy individual lines from B_2

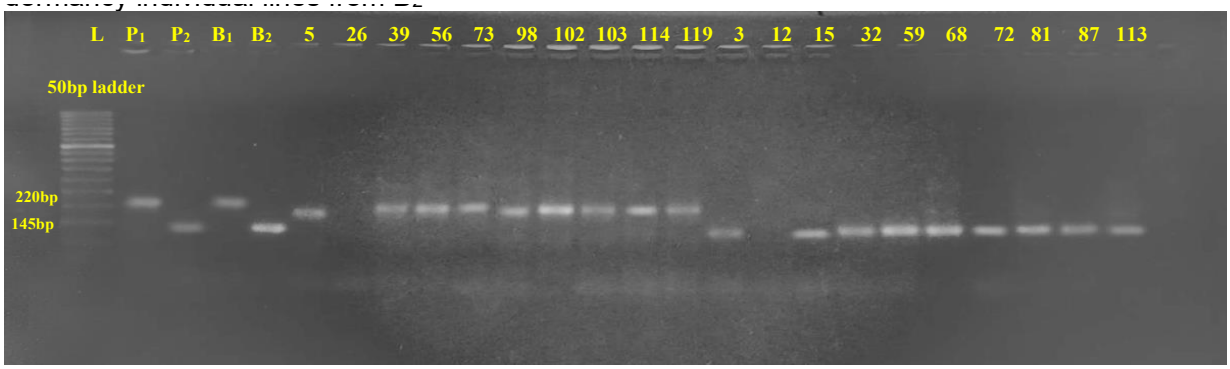


Fig. 5. Bulk segregant analysis using marker RM22565

P_1: BPT 2231; P_2: MTU1001; B_1: Low seed dormancy bulk; B_2: High seed dormancy bulk; 5 to 114: Low seed dormancy individual line from bulk B_1; 119 to 113: High seed dormancy individual lines from B_2

Table 2. Germination (%) and physiological parameters of genotypes used as bulk A for bulk segregant analysis

S. No.	Designation	Germination (%) at 5 days after harvesting	Germination (%) at 10 days after harvesting	Free amino acid content (mg/100g)	Total soluble sugar content (mg/100g)
1	SD 5	93	94	2.90	27.53
2	SD 26	85	89	3.18	13.14
3	SD 39	81	84	1.03	26.89
4	SD 56	71	82	1.41	30.17
5	SD 73	78	86	3.36	20.05
6	SD 98	86	92	1.01	26.52
7	SD 102	81	95	2.80	18.06
8	SD 103	94	96	2.85	20.59
9	SD 114	88	89	1.81	11.63
10	SD 119	95	98	0.95	25.40
11	BPT 2231 (non-seed dormant parent)	90	96	1.51	25.00

Table 3. Germination (%) and physiological parameters of genotypes used as bulk B for bulk segregant analysis

S. No.	Designation	Germination (%) at 5 days after harvesting	Germination (%) at 10 days after harvesting	Free amino acid content (mg/100g)	Total soluble sugar content (mg/100g)
1	SD 3	7	29	1.00	14.10
2	SD 12	5	10	3.09	12.78
3	SD 15	27	27	1.00	9.22
4	SD 32	19	27	2.12	17.43
5	SD 59	2	31	2.26	30.90
6	SD 68	0	40	0.85	13.13
7	SD 72	4	42	2.48	21.26
8	SD 81	25	28	1.30	29.49
9	SD 87	13	27	3.32	15.65
10	SD 113	7	22	1.30	15.80
11	MTU 1001 (seed dormant parent)	9	25	1.06	14.38

Table 4. List of the polymorphic markers between parents BPT 2231 and MTU 1001

S. No.	Name of the Marker	Sequence		Chromosome
		Forward Sequence	Reverse Sequence	
1	RM346	CGAGAGAGCCCATAACTACG	ACAAGACGACGAGGAGGGAC	7
2	RM3295	TCGTGTCATGCGATCGAC	GCTTCGACTCGACCAAGATC	5
3	RM493	TAGCTCCAACAGGATCGACC	GTACGTAAACGCGGAAGGTG	1
4	RM22565	TCCACGCGTTGTCGTAGAAATTTAGC	AGCCCGAGCACCATGAAACACC	8
5	RM8094	AAGTTTGTACACATCGTATACA	CGCGACCAGTACTACTACTA	1
6	RM260	ACTCCACTATGACCCAGAG	GAACAATCCCTTCTACGATCG	12
7	RM562	CACAACCCACAAACAGCAAG	CTTCCCCCAAAGTTTTAGCC	1
8	RM7051	CTCGATGAGCTTGGCGTC	TTCAGTGTTCATCGCCTCTG	4
9	RM10793	GACTTGCCAACTCCTTCAATTCG	TCGTCGAGTAGCTTCCCTCTCTACC	1
10	RM447	CCCTTGTGCTGTCTCCTCTC	ACGGGCTTCTTCTCCTTCTC	8

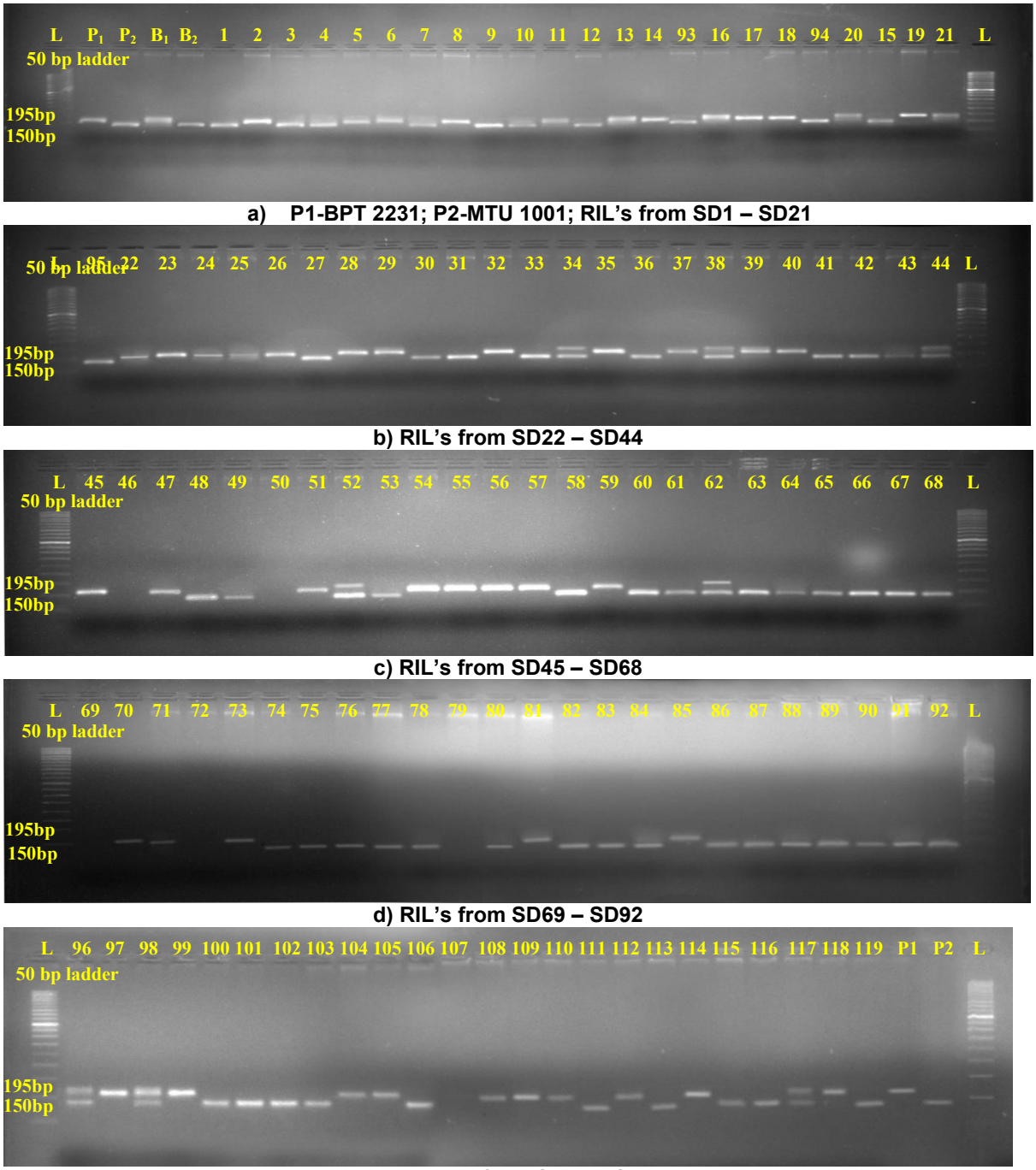
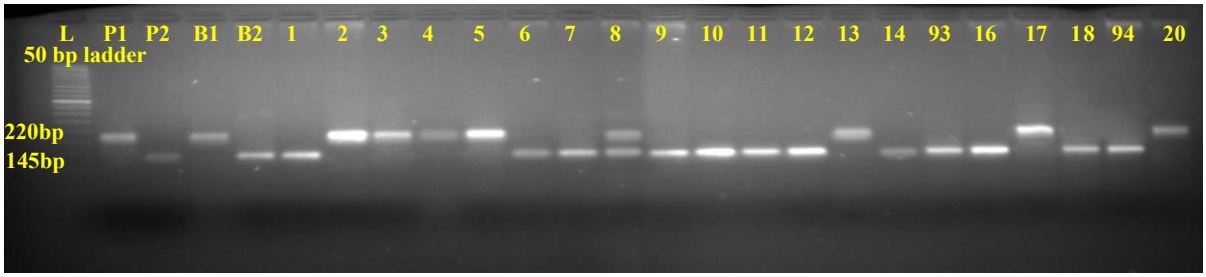
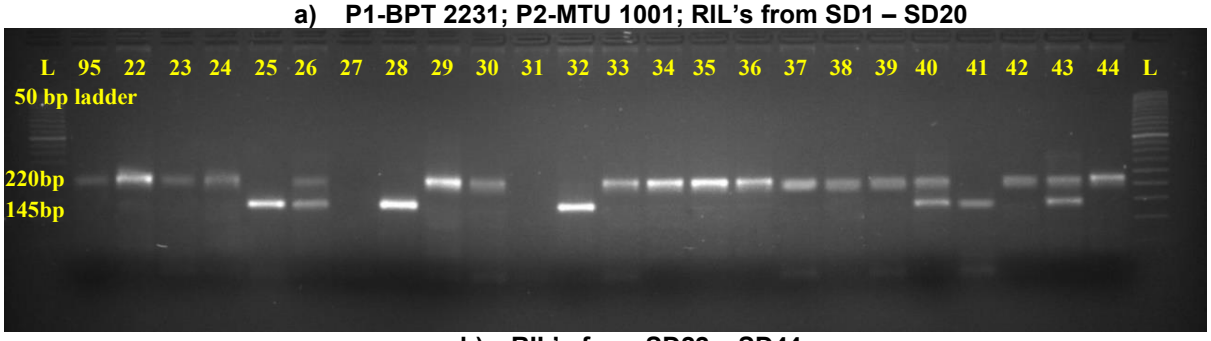


Fig. 6. Genotyping of RIL population using RM346

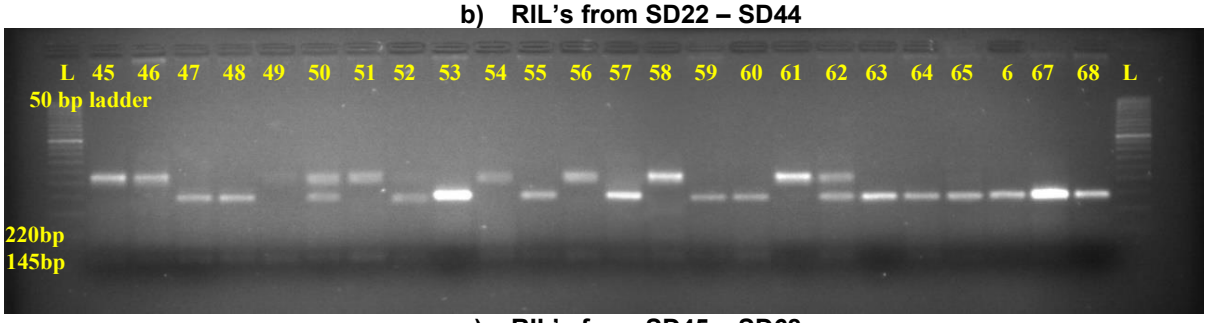
Among the RIL population under study, three RILs *viz.,* SD 3, SD 15 and SD 113 produced amplicon size , similar to MTU 1001, when amplified with two markers *viz.,* RM 346 and RM 7051. Likewise, four RILs *viz.,* SD 55, SD 68, SD 72 and SD 113 exhibited similar banding pattern with MTU1001 when amplified with RM 346 and RM 22565. SD113 also manifested similar banding pattern with that of MTU 1001 when it was amplified with three markers *viz.,* RM 346, RM 22565 and RM 7051, while SD 72 and SD 87 exhibited similar allele with RM 346,



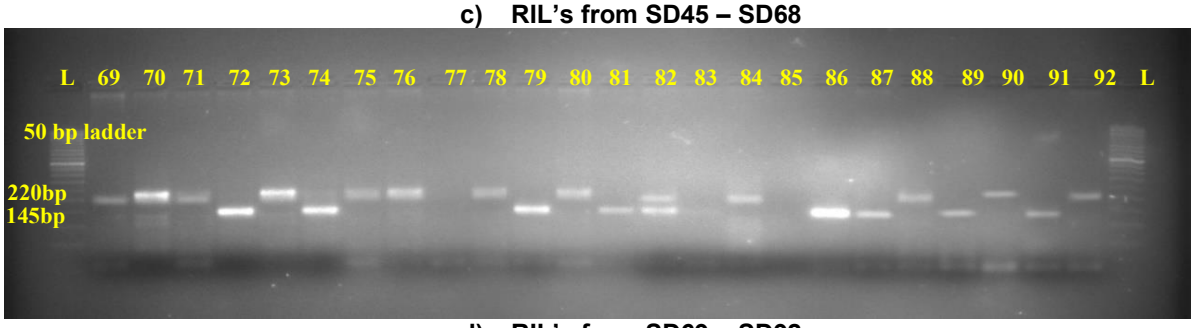
a) P1-BPT 2231; P2-MTU 1001; RIL's from SD1 – SD20



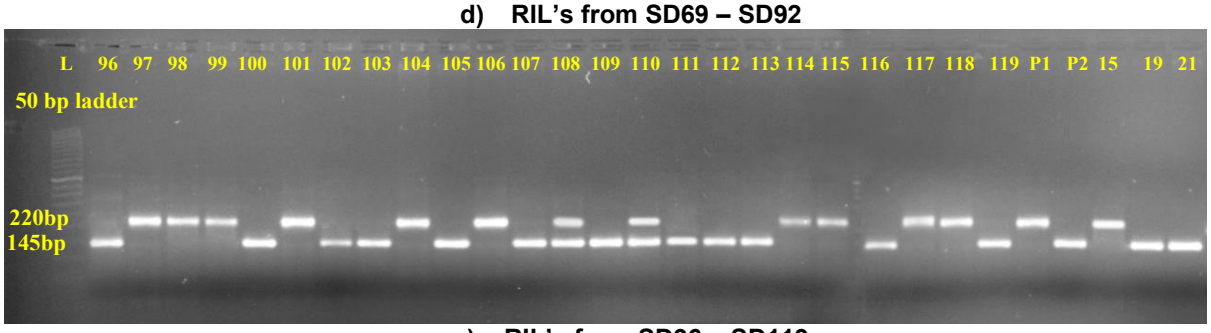
b) RIL's from SD22 – SD44



c) RIL's from SD45 – SD68



d) RIL's from SD69 – SD92



e) RIL's from SD96 – SD119

Fig. 7. Genotyping of RIL population using RM22565

RM 22565 and RM10793. Among the 119 RIL population studied, SD 12 manifested banding pattern as that of the dormant parent MTU 1001 with all the four polymorphic markers *viz.,* RM 346, RM 22565, RM 7051 and RM 10793. In phenotyping also, SD 12 recorded only 10 per cent germination at 10 days after harvesting, whereas the dormant parent exhibited 25 per cent germination at 10 days after harvesting. In addition, SD 12 also exhibited low content of total soluble sugars which might be one of the reasons for strong dormancy of this line. Furuthata *et al.* (2006) reported positive association of soluble sugars concentration with coleoptile elongation. Hence, SD 12 may be considered as strongly seed dormant line and can be utilized as donor for dormancy subjected to further testing and confirmation of results.

Out of all the lines which showed similar results in both phenotyping and genotyping (similar banding pattern with MTU 1001), four RILs *viz.,* SD 3, SD 6, SD 15 and SD 68 also recorded low content of free amino acids (1, 0.95, 1 & 0.85 mg/100 g, respectively) and total soluble sugars (14.10, 13.92, 9.22 &13.13 mg/100 g, respectively). Likewise, seven RILs *viz.,* SD 27, SD 33, SD 60, SD 75, SD 95, SD 107 and SD 118 which manifested similar amplicon size and banding pattern with dormant parent MTU 1001 with more than two markers also recorded low amino acid content ranging from 0.90 to 1.03 mg/100 g, whereas MTU 1001 recorded 1.06 mg/100 g free amino acids. Chaitanya *et al.* (2017), in a study of 48 rice genotypes reported that the available amino acid content will aid in high germination and plant survival under anoxia. Similarly, six RILs (SD 7, SD 12, SD 20, SD 28, SD 67 and SD 83) which were categorized as strongly dormant and also showed same banding pattern as that of MTU 1001 with more than two markers also recorded low content of total soluble sugars.

Considering phenotyping, genotyping and bulk segregant analysis studies, it is concluded that the identified recombinant inbred lines which showed strong seed dormancy also exhibited similar banding pattern as that of dormant parent MTU 1001 with four markers *viz.,*RM346, RM22565, RM7051 and RM10793 on chromosome 7, 8, 4 and 1, respectively. Among these four markers RM346 was identified and used as dormancy linked marker in previous studies, as QTL linked to RM346 *qSD7-2* was detected by Ye *et al.* (2013) in all the three populations of BC_1F_1, BC_1F_2 and BC_1F_3 of EM93-1/SS18-2//EM93-1 using RM346 marker. The other three markers *viz.,* RM22565, RM7051 and RM10793 were not reported in the earlier studies. Hence, these three markers identified in the present study needs further validation on alternative set of population or a set of germplasm lines for further utilization in the marker assisted breeding programme.

To determine the strength of association between the putative markers and the respective trait of interest, Single marker analysis was carried out using phenotypic data (germination % at five and 10 days after harvesting, free amino acids and total soluble sugar) and marker genotypic data. The results revealed that marker *i.e.,* RM22565 on chromosome 8 exhibited significant ($P<0.0001$) association with germination (%) at five days after harvesting. Similar results for significant association of seed dormancy and germination at five days after harvesting with RM106 on chromosome 2 were reported by Rathi *et al.* (2014). Several groups have attempted to identify the genomic regions/QTLs linked to dormancy and found some QTLs on different chromosomes of rice. Among those groups, some were identified QTLs related to dormancy which is a permanent solution for pre-harvest sprouting on chromosome 8. Dong *et al.* (2003) identified six QTLs on chromosome 1 (two QTLs), 4, 5, 7 & 8 using RIL population derived from the cross between the Asominori (*japonica*) and IR24 (*indica*) with 289 RFLPs for pre-harvest sprouting between the marker intervals R2976-C277 (map distance of 69 cM). Similarly, Gu *et al.* (2005) identified five QTLs on the 4, 7, 8 and 12 chromosomes for seed dormancy between the marker intervals RM531 and RM135B.

The recombinant inbred lines *viz.,* SD 3, SD 12, SD 15, SD 27, SD 68 and SD 59 exhibited low seed germination per cent even after the 10 days of harvesting and were categorized as strongly dormant. Hence, these RILs may be tested for their suitability in coastal areas during *kharif* season for further utilization in the breeding programme. Three markers *viz.,* RM22565, RM7051 and RM17093 were identified as dormancy linked and can be used as dormancy linked markers in the marker assisted breeding programme after validation studies on a set of germplasm lines. Based on genotyping studies, the marker RM 22565 on chromosome 8 was significantly associated with the region responsible for seed dormancy and indicating its usefulness in the marker assisted selection to screen the genotypes/cultivars to identify the strongly dormant genotypes.

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