RESEARCH ARTICLE

Association of molecular markers with physio-biochemical traits related to seed vigour in rice

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Abstract Eighteen physio-biochemical traits influencing seed vigour were studied for their association with molecular markers using a mini core set constituted from 120 germplasm lines. High genetic variation was detected in the parameters namely chlorophyll a, Chlorophyll b, total chlorophyll, carotenoids, total anthocyanin content, gamma-oryzanol, total phenolics content, superoxide dismutase, catalase, guaiacol peroxidase, total soluble sugar, total protein, seed vigour index -I and seed vigour index -II. Strong positive correlation of seed vigour index II was observed with amylose content, total anthocyanin content, catalase, total phenolic content and total flavonoid content while a negative association was observed for gamma-oryzanol content. High gene diversity (0.7169) and informative markers value (0.6789) were estimated from the investigation. Three genetic structure groups were observed in the panel population and genotypes were grouped in the subpopulations based on the seed vigour

trait. Differences in the fixation indices of the three sub populations indicated existence of linkage disequilibrium in the studied panel population. Association of the traits namely total flavonoids, superoxide dismutase, catalase, chlorophyll a, Chlorophyll b, total chlorophyll, carotenoids, starch, amylose, total anthocyanin, gamma-oryzanol, total phenolics with the molecular markers were detected by Generalized Linear Model and Mixed Linear Model showing $> 0.10 R^2$ value. Association of the trait, total flavonoids with marker RM7364 located on chromosome 8 reported in earlier study was validated in this investigation. The validated markers and the novel markers detected showing higher R^2 value will be useful for improvement of seed vigour in rice.

Keywords Seed vigour · Molecular markers · Marker-trait association · Biochemical traits · Physiological traits · Rice

Swastideepa Sahoo and Priyadarsini Sanghamitra have contributed equally to this work.

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Introduction

More than half of world population consume rice as staple food not only for calorie needs but also for their nutritional requirement. The future food demand of the world population is expected to increase by 33% in the coming thirty years (Manners and Etten 2018). As the basic biological input of agricultural production, a superior quality seed alone can contribute to about 30% increase in productivity (Hasanuzzaman 2015). The most potential trait of quality seed is the seed vigour that directly influences the crop productivity by delivering the genetic and yield potentials of the seed while ensuring uniformity in seed germination, seedling growth, establishment of seedling in the field and withstanding unfavourable environmental condition. Improvement of seed vigour of rice remains a primary

breeding objective of the agriculture and seed industry (Finch-Savage and Bassel 2016) as it is not only essential to enhance the yield but also can improve crop resilience against climate change effects and biotic impediments to crop yields (Daniel 2017). Genetic analysis of this trait is also essential as high seed vigour is important for direct seeding as it not only enhance crop establishment (Yamauchi and Winn 1996) but also increase the plant's ability to compete against weeds (Dingkuhn et al. 1999; Rao et al. 2007).

Seed vigour is a complex trait as it involves several physical, physiological and biochemical parameters responsible for metabolic events that are associated with seed germination and seedling growth. Further, quantitative inheritance nature of this trait is governed by many quantitative trait loci (QTLs) makes the genetic analysis difficult to identify the genetic factor contributing to this trait through conventional breeding. QTL analysis has been demonstrated an effective way to study such complex trait (Cui et al. 2002; Miura et al. 2002; Zhang et al. 2005; Fujino et al. 2008; Wang et al. 2010; Dang et al. 2014; Liu et al. 2014; Zhang et al. 2017). Most of these detected QTLs for rice seed vigour were found associated with various physical and physiological traits related to seed such as thousand seed weight, seed germination, germination rate, germination index, shoot length, root length, seedling length, seedling dry weight, radicle length, coleoptile length and mesocotyl length.

Only report of Cui et al. (2002) detected QTL related to biochemical traits such as amylase activity and reducing sugar associated with seed vigour in rice. Three QTLs for total amylase activity associated with seed vigour located in chromosome 3, 5 and 6 with phenotypic variance (PV) ranging from 4 to 11% and two QTLs were detected for α -amylase activity located in chromosome 6 explaining PV from 5.7 to 8.7%. Six QTLs were identified for reducing-sugar content associated with seed vigour located on chromosome 3, 5, 6, 10 and 11 with PV ranging from 2.9 to 18.4%. Apart from amylase activity and reducing sugar content, many other biochemical traits such as seed antioxidants: superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), nitrate reductase activity, glutamic acid decarboxylase activity, salicylic acid and seed protein content reported to be associated with seed vigour (Mahender et al. 2015; Huang et al. 2017) has not been elucidated for QTL analysis.

The previous QTLs analysis for seed vigour in rice were based on bi-parental segregating population by using simple sequence repeats (SSR) markers except the QTLs reported by Dang et al. 2014 which were analysed through association mapping. Identifying QTL in segregating population with limited genetic variation and recombination with a low resolution typically in the range of

10–30 cM (Flint-Garcia et al. 2005) has made association mapping, the popularly adapted QTL analysis approach to mine elite alleles in plants. Association mapping based on the linkage disequilibrium, enables researchers to identify QTLs with a higher resolution by targeting multiple alleles at individual loci, by exploiting recombination available in natural variation and by identifying association between marker and phenotype of larger number of traits (Anandan et al. 2016; Pradhan et al. 2016; Pandit et al. 2017) and has been successfully utilized dissecting several complex trait in rice (Pradhan et al. 2019, 2020). Genome mapping using SSR markers are widely employed to assess genetic diversity and genetic structure in rice and several other crops as they are hyper variable, co-dominant, robust, multi-allelic in nature, chromosome specific and greatly facilitated linkage map construction (Anandan et al. 2016; Barik et al. 2019, 2020). For association mapping, the genetic diversity and structure of the population need to be studied for detecting marker-trait association which could be useful for trait enhancement in molecular breeding programs. However, limited information is available on genetic analysis of this trait based on association of physio-biochemical parameters with SSR markers in a variable population.

Therefore, in the present investigation, association analysis was performed in a panel population containing 48 genotypes shortlisted through phenotyping of 18 physio-biochemical traits from 120 diverse genotypes covering five states of India by using 50 SSR markers, to study the population genetic structure, diversity and association of molecular markers with biochemical traits related to seed vigour in rice.

Materials and methods

Plant materials

The freshly harvested seeds of 120 diverse germplasm lines, collected from Gene bank of ICAR-NRRI, Cuttack were grown in the experimental plot of the Institute during 2018–2019 (Supplemental Table 1). These lines were collected from five states known for availability of high rice diversity viz., Assam, Madhya Pradesh, Kerala, Odisha and Manipur. The collections from secondary centre of origin of rice *i.e.*, Jayapore tract of Odisha, have been included in the germplasm list for the study.

The harvested seeds were stored for 3 months to break the seed dormancy and then used for estimation of physio-biochemical traits. A small set of germplasm lines highlighted in the Supplementary Table 1 were used for preparation of panel from 120 diverse germplasm lines for

genetic structure and association study of the physio-biochemical traits with molecular markers.

Phenotyping for physio-biochemical traits and statistical analyses

Seed vigour indices (seed vigour index I and II) were estimated by sowing 50 seeds in three replications following top of paper method by incubating at 30 °C. The final germination percentage and seedling length were recorded on 10th day. The seedlings (five) used for recording of the seedling length from each replication were subsequently oven dried at 70 °C for 48 h after removing the cotyledon and seedling dry weight was expressed in gram per seedling. Seed vigour indices were calculated using the formula suggested by Abdul-Baki and Anderson (1973). Leaf of same seedling (10 days old) were also used for estimating the total chlorophyll content (TCC), chlorophyll a (Chl. a) and chlorophyll b (Chl. b) following the procedure given by Arnon (1994) and carotenoids by following the protocol of Davis (1976). The estimations of moisture %, starch %, amylose % and protein % were estimated by near infrared spectroscopy (NIRS) using the dried dehusked grain. For seed antioxidants, total flavonoids content (TFC) as catechine equivalent (mg CEt 100 g⁻¹), total anthocyanin content (TAC: mg 100 g⁻¹), gamma-oryzanol (mg 100 g⁻¹), total phenolics content (TPC) as catechol equivalent (mg CE100 g⁻¹) and total soluble sugar % (TSS %) were estimated. While for seed antioxidant enzyme, super oxide dismutase (SOD: unit g⁻¹), catalase (CAT: unit min⁻¹g⁻¹) and guaicol peroxidase (PEROX: unit min⁻¹g⁻¹) were estimated. TAC in the samples was estimated by the procedure of Fuleki and Francis (1968). Estimation of gamma-oryzanol was done according to Bucci et al. (2003) with minor modifications. TPC was determined by modified protocol of Zilic et al. (2011). TFC was estimated as per the procedure of Eberhardt et al. (2000). TSS content of rice was estimated calorimetrically by the Anthrone method (Jayaraman 1981). Activity of SOD was determined as per the procedure of Madamanchi et al. (1994). The measurement of PEROX was performed following the method of Putter (1974). CAT activity was measured following the method of Aebi (1984). For the analysis of biochemical traits, the samples were dehulled by the Satake rice huller, Japan at moisture content of 10–12%. The kernels were grounded by a grinding machine (Glenmini grinder) and sheaved through 100 mesh size and then stored at 4 °C for the experiment. Three replicates of each physio-biochemical trait were analysed and data calculated based on dry matter basis except for chlorophyll and carotenoid content which were estimated based on fresh weight basis.

The estimation of critical difference (CD) and coefficient of variation (CV %) were estimated by using Cropstat software 7.0. The formula given by Singh and Chaudhary (1985) was used to calculate phenotypic, genotypic and environmental coefficient of variation. Broad sense heritability and genetic advance for various traits were computed as suggested by Allard (1960). Pearson's correlation coefficients were analysed to find out the relationship among the various biochemical traits, based on the mean values of the 48 genotypes and presented in correlation matrix heatmap.

Genomic DNA isolation, PCR analysis and selection of SSR markers

Genomic DNA of the germplasm lines was extracted from 15 days-old plant by adopting CTAB method (Murray and Thompson 1980). The 50 SSR (simple sequence repeat) markers were taken from the database (<http://gramene.org/>) available in the public domain (Supplementary Table 2). The isolated DNA was quantified by resolving the DNA fragments in gel electrophoresis. PCR analysis was done using the markers selected based on positions covering all the chromosomes to illustrate the diversity and to identify the polymorphic loci among the 48 rice germplasm lines (Table 1). Conditions of reaction was set to initial denaturation step (2 min, 95 °C), followed by 35 cycles of denaturation (30 s, 95 °C) and annealing/extension (30 min, 55 °C), extension (2 min, 72 °C), final extension (5 min, 72 °C) and store at 4 °C (infinity). The PCR products were electrophoresed using 3% agarose gel containing 0.80 g/ml ethidium bromide. To determine the size of amplicons, 50 bp DNA ladder was used. The gel was run for 4 h at 2.5 V/cm and photographed using a Gel Documentation System (SynGene).

Molecular data analysis

The amplicons obtained were scored based on the presence or absence of the amplified products for each genotype-primer combination. The data entry was done into a binary data matrix as discrete variables. Number of alleles (N), observed heterozygosity (H), expected heterozygosity (He), major allele frequency (A) and polymorphic information content (PIC) for each SSR locus were estimated by using Power marker 3.25 (Liu and Muse 2005). The data analysis was performed and similarity matrix was generated from binary data using Jaccard's coefficients. Free Tree software was used to draw cladogram using unweighted pair group method arithmetic average (UPGMA) algorithm (Hampl et al. 2001; Pavalicek et al. 1999) and the cladograms were visualized by Treeview 32 software (Page, 1996). The detailed protocols followed for

Table 1 Genetic diversity parameters associated with the germplasm lines used for marker-trait association study

Marker	Major allele frequency	Genotype No.	Sample size	No. of obs.	Allele No.	Availability	Gene diversity	Heterozygosity	PIC	F
RM5310	0.3111	9	48.0000	45.0000	7.0000	0.9375	0.7681	0.0667	0.7313	0.9151
RM582	0.2500	6	48.0000	44.0000	6.0000	0.9167	0.7975	0.0000	0.7661	1.0000
RM13335	0.3043	5	48.0000	46.0000	5.0000	0.9583	0.7467	0.0000	0.7013	1.0000
RM328	0.6486	4	48.0000	37.0000	4.0000	0.7708	0.4894	0.0000	0.4135	1.0000
RM337	0.3125	10	48.0000	40.0000	7.0000	0.8333	0.7903	0.1750	0.7599	0.7835
RM340	0.4556	8	48.0000	45.0000	8.0000	0.9375	0.7084	0.1333	0.6695	0.8156
RM470	0.2222	13	48.0000	45.0000	9.0000	0.9375	0.8568	0.1333	0.8405	0.8476
RM472	0.3404	12	48.0000	47.0000	8.0000	0.9792	0.7922	0.1277	0.7655	0.8420
RM506	0.3723	8.0000	48.0000	47.0000	5.0000	0.9792	0.7374	0.1277	0.6929	0.8303
RM1812	0.3810	5.0000	48.0000	21.0000	5.0000	0.4375	0.7302	0.0000	0.6859	1.0000
RM6947	0.9583	2.0000	48.0000	48.0000	2.0000	1.0000	0.0799	0.0000	0.0767	1.0000
RM286	0.2805	10.0000	48.0000	41.0000	6.0000	0.8542	0.8070	0.0976	0.7794	0.8819
RM258	0.6000	2.0000	48.0000	25.0000	2.0000	0.5208	0.4800	0.0000	0.3648	1.0000
RM201	0.3750	5.0000	48.0000	40.0000	4.0000	0.8333	0.7009	0.0250	0.6445	0.9652
RM216	0.5217	11.0000	48.0000	46.0000	7.0000	0.9583	0.6680	0.1522	0.6333	0.7766
RM440	0.4063	8.0000	48.0000	48.0000	7.0000	1.0000	0.6838	0.1667	0.6260	0.7607
RM7571	0.5435	6.0000	48.0000	46.0000	5.0000	0.9583	0.6089	0.0217	0.5507	0.9651
RM1347	0.4583	4.0000	48.0000	24.0000	4.0000	0.5000	0.6076	0.0000	0.5280	1.0000
RM223	0.3750	14.0000	48.0000	36.0000	11.0000	0.7500	0.7905	0.2222	0.7680	0.7256
RM14978	0.3108	9.0000	48.0000	37.0000	8.0000	0.7708	0.8075	0.0270	0.7827	0.9674
RM18776	0.3191	9.0000	48.0000	47.0000	7.0000	0.9792	0.7924	0.1064	0.7639	0.8684
RM22034	0.3958	5.0000	48.0000	48.0000	4.0000	1.0000	0.6630	0.0208	0.5989	0.9692
RM24161	0.2609	7.0000	48.0000	46.0000	6.0000	0.9583	0.7961	0.0652	0.7651	0.9198
RM6275	0.8690	4.0000	48.0000	42.0000	4.0000	0.8750	0.2350	0.0238	0.2202	0.9010
RM50	0.4359	4.0000	48.0000	39.0000	4.0000	0.8125	0.6588	0.0000	0.5954	1.0000
RM85	0.4063	10.0000	48.0000	48.0000	6.0000	1.0000	0.6962	0.1250	0.6453	0.8239
RM222	0.2439	9.0000	48.0000	41.0000	8.0000	0.8542	0.8397	0.0244	0.8198	0.9717
RM247	0.4457	10.0000	48.0000	46.0000	8.0000	0.9583	0.7311	0.0870	0.6998	0.8835
RM3701	0.4375	13.0000	48.0000	48.0000	11.0000	1.0000	0.7235	0.1875	0.6891	0.7456
RM3735	0.2708	16.0000	48.0000	48.0000	8.0000	1.0000	0.8262	0.2292	0.8042	0.7276
RM103	0.2065	11.0000	48.0000	46.0000	10.0000	0.9583	0.8745	0.9565	0.8617	#####
RM14960	0.5111	3.0000	48.0000	45.0000	3.0000	0.9375	0.5946	0.0000	0.5147	1.0000
RM405	0.2667	6.0000	48.0000	45.0000	6.0000	0.9375	0.8040	0.0000	0.7756	1.0000
RM225	0.2396	10.0000	48.0000	48.0000	6.0000	1.0000	0.8095	0.2083	0.7814	0.7473
RM5793	0.4219	7.0000	48.0000	32.0000	6.0000	0.6667	0.7515	0.0313	0.7226	0.9597
RM20A	0.3143	10.0000	48.0000	35.0000	9.0000	0.7292	0.8347	0.0286	0.8178	0.9667
RM486	0.6136	7.0000	48.0000	44.0000	5.0000	0.9167	0.5586	0.0455	0.5089	0.9204
RM1113	0.2826	9.0000	48.0000	46.0000	8.0000	0.9583	0.8353	0.0217	0.8159	0.9745
RM6100	0.3387	7.0000	48.0000	31.0000	6.0000	0.6458	0.7622	0.0323	0.7255	0.9590
RM 7364	0.4524	9.0000	48.0000	42.0000	7.0000	0.8750	0.7163	0.0476	0.6804	0.9350
RM3423	0.5532	4.0000	48.0000	47.0000	4.0000	0.9792	0.6084	0.0000	0.5526	1.0000
RM547	0.1702	12.0000	48.0000	47.0000	11.0000	0.9792	0.8830	0.0213	0.8714	0.9764
RM205	0.3289	9.0000	48.0000	38.0000	8.0000	0.7917	0.7981	0.0263	0.7726	0.9679
RM229	0.3804	11.0000	48.0000	46.0000	7.0000	0.9583	0.7193	0.1522	0.6702	0.7926
RM235	0.2553	13.0000	48.0000	47.0000	8.0000	0.9792	0.8336	0.1064	0.8130	0.8749
RM167	0.2558	8.0000	48.0000	43.0000	8.0000	0.8958	0.8080	0.0000	0.7813	1.0000
RM7003	0.3824	7.0000	48.0000	34.0000	5.0000	0.7083	0.7279	0.0588	0.6818	0.9215

Table 1 continued

Marker	Major allele frequency	Genotype No.	Sample size	No. of obs.	Allele No.	Availability	Gene diversity	Heterozygosity	PIC	F
RM25181	0.2024	13.0000	48.0000	42.0000	9.0000	0.8750	0.8608	0.1190	0.8452	0.8648
RM152	0.2083	6.0000	48.0000	24.0000	6.0000	0.5000	0.8229	0.0000	0.7977	1.0000
RM2634	0.5294	5.0000	48.0000	34.0000	4.0000	0.7083	0.6280	0.0588	0.5734	0.9090
Mean	0.3885	8.1000	48.0000	41.5400	6.4400	0.8654	0.7169	0.0852	0.6789	0.8837

population structure, AMOVA and cluster analysis using STRUCTURE 2.3.6, GenAIEx 6.5 and Darwin 5 softwares were described in earlier publications (Pradhan et al. 2016; Pandit et al. 2017, 2020; Pradhan et al. 2019, 2020). To derive the optimal number of groups (K), STRUCTURE was run with K varying from 1 to 10, with 10 runs for each K value. To determine the true value of K, adhoc statistic ΔK was followed (Evanno et al. 2005). Parameters were set to 150,000 burn-in periods and 150,000 Markov Chain Monte Carlo (MCMC) replications after burn-in with an admixture and allele frequencies correlated model.

Association analysis

The marker trait association analysis was carried out by using TASSEL5 software. To find the genetic association between phenotypic trait of the rice accessions and SSR makers, two statistical models, General linear model (GLM) and Mixed linear model (MLM) in TASSEL 5.0 were followed (Bradbury et al. 2007). Significantly associated markers with the traits were identified based on the markers' R^2 and p-values. Procedure followed in previous publication was followed to calculate the false discovery rate (FDR) and adjusted p values (q values) (Pandit et al. 2017).

Results

Genetic variations for the eighteen physio-biochemical traits

A total of 120 germplasm lines were phenotyped for 18 physio-biochemical traits (Supplemental Table 1). A mini core population containing 48 genotypes was constituted by including all the phenotypic groups of each studied 18 traits and marked (*) against the genotypes in the Supplementary Table 1. Mean values of eighteen physio-biochemical traits for each genotypes and analysis of variation

for the traits showed differences among the genotypes (Supplementary Table 1). Chl. a, Chl. b, total chlorophyll content, carotenoids, TAC, gamma-oryzanols, TPC, SOD, CAT, PEROX, TSS, total protein, SVI-I and SVI-II showed high PCV and GCV. However, traits like moisture content, starch, amylose and TFC had low PCV and GCV (Table 2). The present study revealed about high heritability (broad sense) for all the studied traits (Table 2). However, maximum traits showed low genetic advance except gamma-oryzanols, TPC and SVI II (Table 2). Adira 2 pallakkad was observed with highest SVI I, AC 9043 was observed with highest SVI II, Adira 3 pallakkad was observed with highest TAC. Highest TPC and TFC were observed in AC9028 and Adira 1 pallakkad, respectively. Gamma-oryzanol and protein content was observed highest in AC2067 and Bharati, respectively. The genotypes identified could be used as donor parent in the future breeding programme.

Nature of association among seed vigour related traits

The association among 18 physio-biochemical traits revealed a strong positive correlation ($r \geq 0.7$) of chlorophyll a with chlorophyll b; total chlorophyll with carotenoids, moisture, starch; carotenoids with moisture; amylose with TAC and CAT; TAC with CAT (Fig. 1). SVI-II showed significant moderate positive correlation (r : 0.5-0.7) with amylose, TAC, CAT whereas weak significant positive association with TPC and TFC ($r < 0.5$). Negative association of SVI-II was observed with gamma-oryzanol.

In addition, the correlations were detected to be positive significant for SOD, CAT with Chl a; total chl, carotenoids, moisture with chl b; total flavonoids, SOD, PEROX with total chlorophyll; starch, total flavonoids and TSS with carotenoids; starch, total flavonoids and PEROX with moisture; total flavonoids, SOD and PEROX with starch; TPC and SOD with amylase and TAC; CAT with TPC and

Table 2 Statistical parameters for various physio-biochemical traits estimated from the 48 mini core rice population related to seed vigour

Traits	PCV %	GCV %	h^2 %	GA	CD ₅ %	CV %
Chlorophyll a (mg/g)	82.94	82.8	99.64	1.65	0.1	4.96
Chlorophyll b (mg/g)	87.9	87.8	99.96	0.93	0.02	1.8
Total chlorophyll (mg/g)	82.26	82.24	99.94	2.5	0.06	2.02
Carotenoids (mg/g)	67.4	67.35	99.87	0.73	0.03	2.47
Moisture (%)	4.7131	4.6353	96.7244	1.1058	0.233	0.9
Starch (%)	5.7491	5.5316	92.5755	6.9206	6.27	4.2
Amylose (%)	9.88	9.44	91.4	4.43	1.39	2.9
Total anthocyanin content (mg/100 g)	105.1654	105.0952	99.8666	0.5652	0.311	2.8
Gamma-oryzanols (mg/100 g)	32.1317	32.1293	99.9851	31.4362	2.049	2.2
Total phenolics content (mg/100 g)	139.2568	139.1209	99.8048	32.9253	2.165	1.8
Total flavonoids (mg/100 g)	4.9477	4.8514	96.1442	1.2957	1.025	1.4
Superoxide dismutase (unit/g)	73.0405	73.0387	99.9951	0.1995	0.613	1.7
Catalase (unit/min/g)	99.32	91.26	99.89	0.4	0.1	3.22
Peroxidase (unit/min/g)	66.2241	58.5479	78.1609	0.0003	0.28	2.8
Tss (%)	38.0617	38.0575	99.9778	0.0069	0.45	4.4
Total protein (%)	21.6773	21.6424	99.6782	3.9816	0.179	0.8
Seed vigour index I	77.37	77.21	99.6	0.53	0.03	4.9
Seed vigour index II	56.28	56.15	99.52	600.18	40.81	3.9

CV: Coefficient of variation; ECV: Environmental coefficient of variation; GCV: Genotypic coefficient of variation; PCV: Phenotype coefficient of variation, h^2 : Heritability; GA: Genetic advance; CD: Critical difference

SOD. Significant negative correlation was observed for gamma-oryzanol with amylose, TAC, SOD and CAT; PEROX with amylose, TAC, TPC and CAT.

Molecular diversity estimation using SSR markers

Genetic diversity parameters estimated from the mini core germplasm lines using 50 SSR markers are presented in Table 1. A total number of 322 alleles were detected by using 50 markers. The total number of alleles per marker varied from 2 (RM6947, RM258) to 11 (RM223, RM3701, RM547) with a mean value of 6.44. A higher value of specific allele frequency was observed in Ac. 6221 detected by marker RM6947. The mean frequency for specific allele of the population in the study was 0.385. A high gene diversity value of 0.883 was obtained by marker RM547 while a low value of 0.0799 was observed in RM6947. The mean gene diversity value was 0.7169. The allele heterozygosity in the population varied from 0.00 (RM1812, RM6947, RM258, RM1347, RM 50, RM14960, RM405, RM3423, RM16, RM152) to a maximum value of 0.9565 showed by marker RM103. The mean heterozygosity in the population detected by 50 primers was 0.0852. The PIC in the genotypes estimated by primers varied from 0.0767 (RM6947) to 0.8617 (RM103) with a mean value of 0.6789 in the population.

Cluster analysis

The mini core population was phenotyped for 18 physio-biochemical traits related to seed vigour. All the 48 genotypes taken for study were broadly clustered into two groups based on euclidean distance from the mean values. Two genotypes were in cluster I whereas, rest 46 in cluster II. All these genotypes were clustered broadly on the basis of SVI- I. Adira-2, Pallakad and Kapanthi in Cluster I showed highest and second highest SVI- I, respectively. The genotypes in Cluster II had medium to low vigour index I. Cluster II was further grouped into two sub-clusters namely sub-cluster I and sub-cluster II. Sub-cluster I accommodated 30 genotypes while rest 16 in subcluster II. The genotypes in sub-cluster I mainly accommodated the low to low-medium vigour indices containing genotypes whereas the sub-cluster II had moderate to moderately high vigour indices. Both sub-cluster I and sub-cluster II were again divided into two sub-sub-clusters. The sub-sub cluster IA showed 11 genotypes with very low SVII and sub-sub cluster IB contained 20 genotypes which were with low to medium in their vigour indices. In sub-cluster II, sub-sub cluster IIA accommodated 10 genotypes showing medium seed vigour index I where as sub-sub cluster IIB had 6 genotypes which were of moderately high seed vigour indices containing genotypes. The genotypes showing relatively higher starch content and other biochemical properties were clustered into high to moderately

Fig. 1 Heat map showing Pearson's correlation coefficients for physio-biochemical traits. Chl a: chlorophyll a; Chl b: chlorophyll b; Total chl: total chlorophyll; TAC: total anthocyanin content; TPC: total phenolic content; TFC: total flavonoid content; SOD: superoxide dismutase; CAT: catalase; PEROX: peroxidase; TSS: total soluble

sugar; SVI-I: seed vigour index I; SVI- II: seed vigour index II; Significant correlations are colored either in red (positive: dark red at 0.01 and light red at 0.05 level) or blue (negative: dark blue at 0.01 and light blue at 0.05 level) hues, while correlations that were not significant are shown in gray

high seed vigour containing types, whereas the genotypes having relatively lower starch and higher moisture content clustered into low to low medium classes in the analysis (Fig. 2).

Genetic structure analysis

The Bayesian based population STRUCTURE analysis grouped the population into three sub populations on the basis of peak ΔK value at $K = 3$ using the genotyping results of 50 SSR primers (Fig. 3). The genotypes with $\geq 80\%$ probability were assigned to the corresponding subpopulation and the rest as admixtures (Supplementary Table 3). The sub-population 1 consists of 10 genotypes, sub-population 2 includes 16 genotypes, sub-population 3 with 12 genotypes and rest are admixtures. The overall proportion of genotypes in the inferred clusters was 0.245, 0.443 and 0.312 in sub-population 1, sub-population 2 and sub-population 3, respectively. The values of the three fixation indices (F_{st}) of the three populations were found to be 0.3556, 0.2025 and 0.2714 for sub-population 1, sub-population 2 and sub-population 3, respectively.

The allele-frequency divergence of sub-population 1 and subpopulation 2 was 0.2090; sub-population 1 and sub-population 3 estimated to be 0.2053 while in between sub-population 2 and sub-population 3 observed to be 0.1787 computed using point estimates of P on the basis of net nucleotide distance. The expected heterozygosity (average distance) among the individuals in sub-population 1 was 0.5399; among the individuals in sub-population 2 was 0.6342 while the distance was 0.5977 for sub-population 3.

The structure analysis classified the population among the sub-populations based on the seed vigour of the genotypes present in the population at peak value of ΔK at $K = 3$ (Fig. 2). Majority of the good seed vigour containing genotypes were found in the subpopulation 3 (SP3) while moderate vigour possessing genotypes were in subpopulation 1 (SP1). The poor seed vigour germplasm lines were found in subpopulation 2. The alpha value of the population was low ($\alpha = 0.0461$) estimated by the structure analysis at $K = 3$. The distribution of the alpha-value of the panel population was leptokurtic distribution while F_{st} values were distributed almost symmetrically in all the 3 sub-populations at $K = 3$ (Supplementary Fig. 1).

Fig. 2 Cluster Analysis of 48 genotypes based 18 biochemical traits. The name of each genotype is listed in the Table 1

Marker-trait association with seed vigour influencing traits in rice

The marker-trait association analysis was performed to know the association of seed vigour traits using GLM and MLM deploying TASSEL 5 software. The marker-trait associations were trimmed at both less than 1% and less than 5% error of association. The traits, gamma-oryzanol, TPC, TFC, SOD and catalase were found to be above the threshold level and detected to be associated with SSR markers using the GLM approach (Table 3). RM222, RM225 and 547 were detected to be associated with total phenolics content showing > 0.1 R^2 value. Markers RM582, RM337, RM223, RM3701, RM3701, RM7364, RM547 and RM229 were found to be associated with total flavonoid content with high R^2 value. The association of markers with traits namely chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, starch, amylose, total anthocyanin, gamma-oryzanol and total phenolics were detected to be associated with SSR markers by using MLM approach. A stronger association was obtained for TPC, total flavonoids, SOD and catalase with the markers showing > 0.10 R^2 value by GLM model (Table 3). However, traits like chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, starch, amylose, total anthocyanin, gamma-oryzanol and total phenolics showed higher R^2 value and strongly associated with the markers using the markers by MLM analysis (Table 4). The false discovery rate (FDR) analysis showed the positive associations detected with GLM and MLM models to be significant.

The Q–Q plot also confirmed the association of the markers with various seed vigour influencing traits in rice (Fig. 4).

Discussion

In the investigation, the genotypes were found to be different from each other with respect to the different physio-biochemical parameters. High genotypic coefficient of variation was observed for the traits namely, total chlorophyll content, chlorophyll a, chlorophyll b, carotenoids, moisture content, starch content, amylose content, TAC, gamma-oryzanols, TPC, SOD, CAT, PEROX and total protein content. Also these compounds showed direct or indirect association with seed vigour index in this study. Physio-biochemical traits namely, superoxide dismutase, catalase, peroxidase, nitrate reductase activity, glutamic acid decarboxylase activity, salicylic acid and seed protein content are reported to be associated with seed vigour (Mahendra et al. 2015; Huang et al. 2017; Baek et al. 2018). Earlier reports of high variations and genetic advance for various traits in rice were reported by many workers (Anandan et al. 2016; Pradhan et al. 2016; Pandit et al. 2017; Pradhan et al. 2019, 2020). Positive correlations between phenols and antioxidant activity were reported earlier in some edible seed species (Bolivar and Luis 2010). Donor lines were identified in earlier germplasm screening study for TAC, TPC and TFC traits in rice. They showed that TAC varied from 1.29 to 93.67 mg/100, while TPC varied from 191.35–594.21 mg/100 g^{-1} in their

Fig. 3 **a** Graph of ΔK value for estimation peak value between successive K values for measuring the highest ΔK and **b** population structure obtained based on inferred ancestry value on the basis 50 molecular markers ($K = 3$)

studied germplasm lines. The results for TFC content in that population varied from 191.35 to 594.21 mg/100 g⁻¹ and gamma-oryzanol content was 31.00–93.44 mg/100 g⁻¹ (Sanghamitra et al. 2017, 2018). Wide variation in TAC, TPC and TFC was also observed in this investigation. The grain protein content in rice up to 16.41% was reported in earlier publications in the germplasm line, ARC10063 (Mahender et al. 2016; Pradhan et al. 2019). In this investigation, the population showed up to 22.67% grain-protein content that was detected in the germplasm line, Ganrohribuna.

The use of 50 markers for diversity parameter analysis indicated clear differentiations of the genotypes based on the studied parameters (Table 1). A total of 322 alleles amplified in the population indicated a good number of alleles present in the population. Also, the gene diversity detected in this study is high. A very high PIC value from

the population indicated that the markers used were good and informative. Many earlier results also report high genetic diversity parameters in various rice populations (Garris et al. 2005; Jin et al. 2010; Zhao et al. 2013; Salgotra et al. 2015). Many reports of existence of moderate and low genetic diversity were also available in rice (Agrama et al. 2007; Chen et al. 2011; Zhang et al. 2011; Singh et al. 2013; Pradhan et al. 2019, 2020). The sample population used in the present investigation represent from five states where rich genetic diversity of rice exist (Patra and Dhua 2003; Latha et al. 2013; Vanlalsanga and Singh 2019). Also, germplasm lines collected from secondary centre of origins were included in the study. Hence, selection of parental lines from the population is effective for a breeding programme. Therefore, the population will be useful for future study and breeding purpose.

Table 3 Marker-trait association for seed vigour traits in rice using GLM analysis

Traits	Marker name	Chr#	F	p	q	R ²
Gamma oryzanols	RM225	6	7.97361	0.00729	0.009208421	0.09196
Gamma oryzanols	RM167	11	7.60282	0.00866	0.009443478	0.08835
Gamma oryzanols	RM152	8	8.18886	0.00661	0.009208421	0.09403
Total Phenolics	RM286	11	7.98642	0.00725	0.009208421	0.10469
Total Phenolics	RM222	10	7.50856	0.00905	0.009443478	0.0994
Total Phenolics	RM225	6	19.77749	6.50E−05	0.00078	0.20896
Total Phenolics	RM547	8	7.7814	0.00797	0.009245714	0.10243
Total flavonoids	RM582	1	9.21813	0.00415	0.009208421	0.10738
Total flavonoids	RM337	8	10.2867	0.0026	0.009208421	0.11733
Total flavonoids	RM286	11	8.33154	0.00619	0.009208421	0.0988
Total flavonoids	RM223	8	10.2867	0.0026	0.009208421	0.11733
Total flavonoids	RM3701	11	9.02251	0.00453	0.009208421	0.10551
Total flavonoids	RM7364	9	10.2867	0.0026	0.009208421	0.11733
Total flavonoids	RM547	8	9.95274	0.00301	0.009208421	0.11427
Total flavonoids	RM229	11	11.67737	0.00144	0.009208421	0.12968
Superoxide dismutase	RM340	4	8.23683	0.00646	0.009208421	0.10126
Superoxide dismutase	RM472	1	8.93366	0.00471	0.009208421	0.1083
Superoxide dismutase	RM223	8	9.76938	0.00326	0.009208421	0.11648
Superoxide dismutase	RM50	6	7.74899	0.00809	0.009245714	0.09622
Superoxide dismutase	RM247	1	8.12212	0.00681	0.009208421	0.10009
Superoxide dismutase	RM103	6	7.34674	0.00977	0.00977	0.09198
Superoxide dismutase	RM14960	3	8.23683	0.00646	0.009208421	0.10126
Superoxide dismutase	RM3423	4	8.59727	0.00549	0.009208421	0.10493
Catalase	RM5310	1	26.7697	6.40E−06	0.0001536	0.38594

R²: indicate the % of total variation explained; *p* value: probability values; *F*- value: Value estimated from likelihood-ratio (LR) test or *F*-test and false discovery rate (FDR) or *q* values were obtained from QVALUE R package

Genetic structure analysis categorized the studied population into three sub-populations. The genetic makeup of individuals present within a population is determined by using the population genetic structure. The crop improvement genetic gain of the targeted trait will be quicker by using parental lines from a population with known genetic makeup. Therefore, estimation of population structure and further utilization of the structure result in crop improvement program is important to the rice breeders. Phenotype results also showed three category of population as per seed vigour index I. A fair degree of correspondence was observed for genetic structure and seed vigour index I. Majority of the good vigour index I genotypes were present in sub-population 3. Also, majority of the poor seed vigour index I genotypes were seen in sub-population 2. Moderately good genotypes are present in the sub-population I. For seed vigour and genetic structure analysis of population in rice, very few publications are available. A fair degree of correspondence of genetic structure and seedling vigour was reported by Anandan et al. (2016). But, for other traits, many publications on genetic structure for various populations are available. Genetic structure and

population was on high temperature stress tolerance in rice was reported by Pradhan et al. (2016). For low temperature stress tolerance and population structure in *indica* rice was reported by Pandit et al. (2017). The population structure correlation and grain protein content in rice is reported by Pradhan et al. (2019). Structure correlation with phenotype has been reported by many researchers (Anandan et al. 2016; Pradhan et al. 2016; Pandit et al. 2017; Agrama et al. 2007; Zhao et al. 2011; Zhang et al. 2012; Huang et al. 2011; Zhao et al. 2013; Huang et al. 2015; Muthukumar et al. 2015; Kumar et al. 2015; Pan et al. 2015; Swamy et al. 2017; Pradhan et al. 2019, 2020).

The seed vigour influencing traits namely gamma-oryzanol, TPC, total flavonoids, SOD and catalase were found to be above the threshold level and detected to be associated with SSR markers using the GLM approach (Table 3). The association of markers with chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, starch, amylose, total anthocyanin, gamma-oryzanol and total phenolics were observed to be associated with SSR markers by MLM model. However, a stronger association of specific SSR markers presented in the Table 4 with vigour were detected

Table 4 Marker-trait association for seed vigour traits in rice using MLM analysis

Trait	Marker name	Chr. No.	F	p	q	MarkerR ²
Chlorophyll a	RM235	12	9.91027	0.00306	0.009051176	0.2063
Chlorophyll b	RM7571	7	7.37544	0.00963	0.00983	0.1586
Chlorophyll b	RM3735	4	8.17324	0.00665	0.009051176	0.17576
Chlorophyll b	RM5793	7	8.46548	0.00582	0.009051176	0.18204
Chlorophyll b	RM6100	10	8.20189	0.00657	0.009051176	0.17637
Chlorophyll b	RM7003	12	8.20915	0.00655	0.009051176	0.17653
Total Chlorophyll	RM3735	4	7.33183	0.00983	0.00983	0.15497
Total Chlorophyll	RM5793	7	7.90928	0.00751	0.009296842	0.16718
Total Chlorophyll	RM235	12	8.66462	0.00532	0.009051176	0.18314
Carotenoids	RM582	1	9.5549	0.00358	0.009051176	0.19269
Carotenoids	RM223	8	7.54478	0.0089	0.009747619	0.15215
Carotenoids	RM547	8	9.3319	0.00395	0.009051176	0.18819
Starch	RM582	1	9.67922	0.00339	0.009051176	0.21372
Starch	RM223	8	8.64373	0.00537	0.009051176	0.19085
Amylose	RM440	5	14.19923	5.18E−04	0.005918667	0.3259
Total anthocyanin	RM6275	2	13.56365	6.67E−04	0.005918667	0.26187
Total anthocyanin	RM486	1	10.15084	0.00276	0.009051176	0.19598
Total anthocyanin	RM547	8	7.62178	0.00859	0.009747619	0.14715
Gamma oryzanols	RM286	11	8.86107	0.00487	0.009051176	0.17167
Gamma oryzanols	RM222	10	13.19993	7.72E−04	0.005918667	0.25573
gamma oryzanols	RM547	8	7.86276	0.00768	0.009296842	0.15233
Total phenolic compounds	RM286	11	8.4797	0.00579	0.009051176	0.1674
Total phenolic compounds	RM222	10	8.16149	0.00669	0.009051176	0.16112

R²: indicate the % of total variation explained; p-value: probability values; F-value: Value estimated from likelihood-ratio (LR) test or F-test and false discovery rate (FDR) or *q* values were obtained from QVALUE R package

for TPC, total flavonoids, SOD, chlorophyll a, Chlorophyll b, total chlorophyll, carotenoids, starch, amylose, total anthocyanin, gamma-oryzanol and total phenolics with the markers showing > 0.10 R² value. The FDR test confirmed the significant association of the markers with these traits. Therefore, these markers are useful in marker-assisted breeding for enhancement of seed vigour in rice. Very few reports are available for association marker-trait for seed and seedling vigour in rice. Genes/QTLs controlling seed colour traits have been reported by earlier researchers (Furukawa et al. 2007; Jin et al. 2009; Sweeney et al. 2006). Earlier reports on the control of total phenolics content was reported to be associated with SSR markers RM316, RM339 and RM316 present on chromosome 7, 8 and 9, respectively (Shao et al. 2011). In our study, the markers associated with total phenolic content were located on chromosome 6, 10 and 11 (Tables 3, 4). Therefore, the QTLs associated with phenolic content are different from others results. Flavonoid content in rice is reported to be associated with the markers RM339, RM316 and RM228 and located on chromosomes 8, 9 and 10, respectively (Shao et al. 2011). In this study, we found the association of the trait on chromosome 8 by RM337 (1.1 cM), RM223

(80.5 cM) and RM547 (58.1 cM) on different region from the report of Shao et al. 2011. The markers on chromosome 1 (RM582) and 11 (RM286, RM3701 and RM229) were not reported in earlier studies. However, the association of the flavonoid content controlled by chromosome 9 with RM7364 in our study is located at 1.7 cM. As per the report of Shao et al. 2011, the associated marker for the trait on the chromosome 9 was observed to be at 1.8 cM. Therefore, these QTLs may be same QTL controlling flavonoid content in rice. QTLs for Association of molecular markers-trait for seedling vigour were reported by Anandan et al. (2015, 2016). However, for marker-trait association on other traits have been reported by many other workers (Anandan et al. 2016; Pradhan et al. 2016; Pandit et al. 2017; Agrama et al. 2007; Zhao et al. 2011; Zhang et al. 2012; Huang et al. 2011; Zhao et al. 2013; Muthukumar et al. 2015; Kumar et al. 2015; Pan et al. 2015; Huang et al. 2015; Swamy et al. 2017; Pradhan et al. 2019, 2020).

In conclusion, a wide variation for starch content, phenolic content, chlorophyll content, carotenoids, moisture content, starch content, total anthocyanin content, gamma-oryzanols, total phenolic content, total flavonoids, superoxide dismutase enzyme, catalase, peroxidase, total soluble

Fig. 4 Quantile–Quantile (Q–Q) plot and distribution of marker–trait association from MLM analysis for biochemical traits related to seed vigour at $p < 0.05$

sugar, amylose content and total protein content were observed among the 120 germplasm lines used for association study. Promising donor lines containing higher intensities for each of these biochemical traits influencing seed vigour were identified. A mini core set of 48 lines were developed from 120 germplasm lines based on their 18 physio-biochemical features showed linkage disequilibrium in the panel population. Studies on eighteen physio-biochemical traits revealed their association with molecular markers. STRUCTURE software classified the

core population into three genetic structure groups. Higher values for gene diversity and polymorphic information content (PIC) were estimated from the population using 50 SSR markers. Various groups and sub-clusters obtained from the population showed similarity within the accommodated genotypes for their physio-biochemical traits. Stronger association of the traits namely total flavonoids, superoxide dismutase, catalase, chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, starch, amylose, total anthocyanin, gamma-oryzanol, total phenolics with the

molecular markers was detected showing $> 0.10 R^2$ value. The marker, RM7364 reported earlier for association of total flavonoids on chromosome 8 is validated in this panel population. The traits identified and markers detected in the association analysis will be useful for improvement of seed vigour in rice.

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Authors' contributions SKP conceived the idea, PS and SKP wrote, and PS, EP and SKP revised the paper. SS, NN, SP, EP and RB generated phenotypic and genotypic data. EP and KCM performed data analyses. SKP interpreted the data. All authors reviewed and approved the final manuscript.

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Data availability The data generated or analyzed in this study are included in this article.

Compliance with ethical standards

Competing interests We declare that there is no competing interest among the authors.

Consent for publication Not applicable.

Ethics approval and consent to participate Guidelines were followed in the ethics approval and consent to participate.

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