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ORIGINAL ARTICLE

Genetic variability and genome-wide marker association studies for starch traits contributing to low glycaemic index in pearl millet

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Abstract

Pearl millet grains are naturally rich in high quality starch, dietary fibre, polyphenols and important micronutrients. Grains from a random subset of the global diversity panel (PMiGAP) comprising 166 pearl millet accessions were assessed for total starch (TS), rapidly digestible Starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) content based on available glucose percentage after digestion at various time points. Highly significant genetic variations for SDS, RS and other starch traits were evident amongst the PMiGAP accessions leading to the identification of best accessions for use in the future pearl millet-breeding programmes. To identify potential candidate genes associated with these starch traits, genome-wide association studies (GWAS) were performed using 78K single-nucleotide polymorphisms (SNPs) well distributed across the seven chromosomes of pearl millet. A total of 902 SNPs showed a strong association with various starch traits at -log *p*-value range from 4.0–9.08. A total of 364 probable candidate genes were identified in the flanking regions of the significantly associated SNPs and high LD (linkage disequilibrium) region, which explains a correlation between nearby variants. Out of these, 19 probable candidate genes exhibited functional relationships with the starch biosynthesis pathway. Three starch synthase genes (Pgl_GLEAN_10026059 and Pgl_GLEAN_10027180) were found to be key probable candidate genes for SDS owing to their prior demonstrated involvement in amylose biosynthesis. Pgl_GLEAN_10018323, encoding β-amylase and Pgl_GLEAN_10009197, encoding α-amylase enzyme were identified as probable candidate genes for RDS content. The genetic variability being reported for SDS and RS in the germplasm panel, and the SNP markers associated with such variability, raises the possibility of developing pearl millet varieties with low glycaemic index using conventional and molecular breeding approaches.

KEYWORDS

candidate genes, genome-wide association studies, germplasm, marker-trait associations, Pearl millet, resistant starch, slowly digestible starch

1 | INTRODUCTION

The World Health Organization (WHO) reports that around 422 million people worldwide suffered from diabetes (http://www.who.int/diabetes/en/) with the majority of these living in low- and middle-income countries. Consumption of diets with high glycaemic index (GI) foods, low disease awareness amongst the public, limited health care facilities and the high cost of disease management are all factors contributing to an increasing prevalence of type 2 diabetes (T2D). Of the two types of diabetes (Type-1 and Type-2 diabetes), Type-2 diabetes (T2D) is a major form accounting for 90% of all diabetes cases globally. T2D arises as a result of poor insulin production or insulin resistance in the body system. This lack of insulin response means that T2D patients cannot utilize excess blood glucose leading to high blood glucose, a condition known as hyperglycaemia. Though advances in diabetic research have led to new therapeutic drug protocols being generated, dietary control is an incomparable and ultimate preventive-cum-treatment measure for this condition. Low GI foods, containing higher amount of slowly digestible starch and resistant starch, are recommended for managing T2D as they release glucose more gradually reducing the glycaemic and insulinaemic responses (Brouns et al., 2005; Haub et al., 2010; Robertson et al., 2003; Yamada et al., 2005; Zenel & Stewart, 2015). Starch is the most important constituent of carbohydrate and can be classified into three main fractions based on its in-vitro digestibility namely; rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS). Of these, a higher concentration of SDS and RS in food is particularly important in reducing the glycaemic and insulinaemic responses (Tuncel et al., 2019). RS in food completely resists digestion in the small intestine and passes straight on to the colon where it serves as a substrate for fermentation by the intestinal microbiome to produce short-chain fatty acids (Englyst & Cummings, 1985). Replacing available carbohydrate in the meal with SDS and RS, therefore, reduces postprandial glycaemia helping patients especially with T2D to normalize the glucose pressure.

Millets are collectively termed as nutraceutical cereals due to their nutritional value resulting from high micronutrient and antioxidant levels. Compared to other cereals, they are a better source of dietary carbohydrates consisting of a high proportion of SDS and RS. The popularity of millet when combined with these nutritional properties suggest that they could be an important target to be exploited in improving public health and preventing T2D (Bitzur et al., 2009; Sone et al., 2011). Amongst millets, pearl millet (*Pennisetum glaucum*), has the best credentials for treating T2D as it has relatively high SDS and RS content with a low GI, in addition to a high fibre content (β-glucans). Despite its potential, no systematic study has yet been conducted to understand the extent of genetic variations for SDS and RS content in pearl millet germplasm (Kam et al., 2016) and how such variations can be best utilized in pearl millet-breeding programmes.

The present study was planned to assess the extent of genetic variability for total starch (TS) and of its component traits—rapidly digestible (RDS), slowly digestible (SDS) and resistant starch (RS) using a subset of randomly selected 166 genotypes from within the world collection of 345 Pearl Millet inbred Germplasm Association Panel (PMiGAP; Sehgal et al., 2015). We also report the candidate genes associated with such variability as detected using genome-wide association studies (GWAS).

2 | MATERIALS AND METHODS

2.1 | Plant materials

The pearl millet inbred lines used in this study comprised of 166 accessions randomly picked from within the pearl millet inbred germplasm association panel (PMiGAP), which represents pearl millet genetic diversity from across the world (Sehgal et al. (2015). The 166 genotypes included in this study belonged to 22 different countries including 40 accessions from ICRISAT, 15 accessions from India, 22 from Niger, 10 from Nigeria, 9 each from Namibia, Zimbabwe, Togo and the remaining 52 from other locations (Table S1). Proportionally the largest number of genotypes included were from ICRISAT as a result of their great contribution to global pearl millet-breeding programmes. Other genotypes included were from African countries commonly used in pearl millet breeding across the globe. Seeds of each of the 166 accessions were multiplied by growing them in uniform field conditions at ICRISAT, Patancheru, India, following standard agronomic practices and seed multiplication protocols as described by Upadhyaya et al. (2008) and Ramya et al. (2018). Briefly, each accession was planted in three rows by maintaining 15 cm between plants

and 75 cm between rows. Fertilizer doses of 100 kg/ha of DAP (di-ammonium phosphate) were applied to the field. Thinning and weeding was done as per the plan described by Ramya et al. (2018). Each individual head was selfed before the emergence of the panicle and strict pollination was controlled to get pure seeds of each line. Seeds for each individual accession were supplied to IBERS, Aberystwyth University, by ICRISAT using standard Material Transfer agreements.

2.2 | Estimation of various components of Starch

Starch digestibility analysis was performed for Rapidly Digestible Starch (RDS), Slowly Digestible Starch (SDS) and Resistant Starch (RS) fractions (Englyst et al., 1992, 1999). All analytical measurements of the milled samples were performed in duplicate (two replicates) of each accession to minimize any measurement errors on the starch assay of individual accession. Samples were milled with a centrifugal mill (Fritsch Pulverisette 14, 0.5-mm screen) and sample weights of 0.5 g were analysed. After a pretreatment simulating gastric conditions, samples were incubated with an excess of amylolytic enzymes under conditions controlled for temperature, pH, viscosity and mixing. Sub-samples were taken at 20 min and 120 min as measures of the rate and extent of starch digestion, with released glucose determined by High Pressure Ion Chromatography (HPIC) with pulsed amperometry detection using a CarboPac PA20 column (Thermo Scientific) and a 20-mM Potassium hydroxide eluent. The RDS fraction is the glucose in the 20 min sub-sample and the SDS fraction is the release between 20 and 120 min. The RS fraction is the starch remaining unhydrolysed after 120 min, which is determined following alkali dispersion and enzymatic hydrolysis. Available starch content (AvST) was derived by adding the RDS and SDS content and used as an estimate of the amount of starch content available in the small intestine for absorption from food. The RS/TS ratio was calculated to find-out the RS percentage corresponding to total starch (TS). These fractions were also used in identifying best performing PMiGAP genotypes for use as donors in pearl millet-breeding programme for developing varieties that are high in SDS and RS and low in GI.

2.3 | Statistical Analyses for phenotypic variability in PMiGAP genotypes

The mean values were calculated for each of the 166 PMiGAP genotypes for all the starch components measured viz. rapidly digestible (RDS), slowly digestible (SDS), resistant (RS), total starch (TS), available starch (AvST) and RS/TS ratio expressed as a percentage. Broad sense heritability (H^2) was estimated from the variance components obtained by fitting both replications and genotypes as random terms as $H^2 = \sigma 2g/(\sigma 2g + \sigma 2e)$, where $\sigma 2g$ is the genotypic variance component and $\sigma 2e$ is the residual variance component. The best linear unbiased prediction (BLUP) values were estimated in R package from the replicates value of each PMiGAP inbred lines. A linear mixed model in R package (lme4) was used to predict the random effects where genotypes have been considered as a random effect (Bates et al., 2015). Population-wide outlier removal has been performed by assessing deviation from a normal distribution. The BLUP values were used as the input data for the association mapping analysis.

The statistical analysis was performed using the JMPv.8 software (SAS Institute, 2008) to compare the range of mean values of the starch traits. The critical difference and coefficient of variation values were calculated using mean data by the JMPv.8 software. One factor analysis of variance (ANOVA) was performed using the replicated mean data of the 166 PMiGAP lines to identify significant differences amongst these PMiGAP lines.

2.4 | SNP identification and genotyping

A total of 166 accessions of the PMiGAP association panel were used for genome-wide association analysis. SNP data were derived from the publicly available database (Varshney et al., 2017) where 345 Pearl Millet inbred Germplasm Association Panel (PMiGAP) lines were resequenced by the International Pearl Millet Genome Sequencing Consortium (ftp://cegresources.icrisat.org/). The initial SNP dataset containing 28 million SNPs were filtered for site coverage (90%) and minimum minor allele frequency (MAF) of 0.01 for each of the 166 accessions as described in Yadav et al. (2021) using the Tassel ver. 5.2.64 software tool. Other SNP filtering criteria used in this study were no SSR motifs, no InDel markers, only bi-allelic SNPs and SNP quality score ≥30. The filtering criteria used included flanking sequence length (100bp), no repeats in flanking sequences (mono- and di-nucleotide), GC content on the flanking sequences (minimum 30.0%) and with minimal flanking markers (maximum 1 SNP from both sides). Genetic variant annotation for SNPs and its effect on genes and protein were predicted using SnpEff (http://snpeff.sourceforge.net/).

2.5 | Hierarchical and Bayesian model-based cluster analysis

Hierarchical cluster-based analysis of 166 accessions was performed using DARwin6.0.021 (http://darwin.cirad.fr/darwin; Perrier et al., 2003). A pair-wise dissimilarity matrix was calculated using Jaccard's dissimilarity coefficient to assess the genetic variability amongst the individuals. A phylogenetic tree was generated using the unweighted neighbour-joining (NJ) method keeping 1000 bootstrap replicates. Principal coordinate analysis (PCoA) was conducted for 166 individuals for clustering.

Population structure for 166 accessions of the PMiGAP association panel was then further assessed using the STRUCTURE software (Falush, 2003; Pritchard et al., 2000; Rosenberg et al., 2002). Bayesian model-based cluster analysis was performed using basic parameters described by Sehgal et al. (2015). A total of 10,000 burnin iterations followed by 20,000 Monte Carlo Markov Chain (MCMC) replications were implemented to assign the subpopulation grouping for each K ranging from 1 to 16. A total of 6 repetitions were carried out for each value of K. The optimum K was analysed using Structure Harvester by following the Evanno method to determine delta K (Earl & vonHoldt, 2012; Evanno et al., 2005). Optimum K was then considered as input in Genome-wide association analysis (GWAS) analysis.

2.6 | Genome-wide association analysis

GWAS was carried out using Fixed and Random Model Circulating Probability Unification (FarmCPU) implemented in the Genome Association and Prediction Integrated Tool (GAPIT) on an r platform for each of the traits such as RDS, SDS, RS, TS, AvST and RS/TS. A FarmCPU was used to identify associations for each trait utilizing the kinship matrix (K) and Q-matrix as random effects. SNP markers with a p-value < 0.001 were considered to be significant but a threshold to declare a marker as highly associated was set to $-\log10 \leq 3.0$. Q–Q plots and Manhattan plots were generated using the r package qqman (https://cran.r-project.org/web/packages/qqman/index.html).

2.7 | Identification of candidate genes affecting starch-related trait

For each trait, pair-wise LD (linkage disequilibrium) was calculated between the significant SNP and every neighbouring SNP in a 10 kb surrounding region and high LD (95% confidence bounds on D prime) using the HAPPI-GWAS programme (Slaten et al., 2020). Haploblocks were identified using the Haploview programme (Barrett et al., 2004) implemented in the HAPPI-GWAS program. A search for candidate genes was performed using the gene annotated GFF file from the database (ftp://cegresources.icrisat.org/) according to the positions of the closest flanking significantly associated SNPs. SNPs were filtered at a 5% minor allele frequency (MAF) and LD calculated based on D prime. The genes located within the high LD region of each associated SNP were considered as the probable candidate genes for starch-related traits. SNPs that were significantly associated with the trait but did not fall within the regions of high LD were not considered. Homology based starch metabolism, starch biosynthesis and amylose content related genes were identified. The functions of corresponding genes were predicted using the Blast2Go programme (Conesa et al., 2005).

3 | RESULTS

3.1 | Phenotypic variations for starch traits in the subset of PMiGAP germplasm panel

Phenotypic diversity was determined by measuring Rapidly Digestible Starch (RDS), Slowly Digestible Starch (SDS), Resistant Starch (RS), Total Starch (TS), Available Starch (AvST) and RS/TS in 166 diverse pearl millet (*Pennisetum glaucum*) inbred lines from within the PMiGAP panel (Table S1). The RDS, SDS, RS and TS content was expressed in g/100 g sample and RS/TS ratio is as % TS. The TS content in 166 pearl millet accessions ranged from 54.85 ± 0.2 to 73.85 ± 0.2 with an average of 67.11 ± 0.2. The RDS content, obtained after 20 min enzymatic digestion, ranged from 13.95 ± 0.1 to 34.08 ± 0.3 in 166 pearl millet accessions with an average of 22.01 ± 0.2. The SDS obtained after 120 min enzymatic digestion was used as an estimation of SDS. This ranged from 33.8 ± 0.1 to 51.4 ± 0.0 in 166 accessions with an average of 43.05 ± 0.2. The genotype PMiGAP121 showed the lowest RDS and SDS content (13.95 and 33.8, respectively), but the highest RS content (18.15). The highest RDS content (34.08) and SDS content (51.4) was recorded in PMiGAP293 and PMiGAP120, respectively (Table S2). Similarly, the genotype PMiGAP201 showed the lowest RS content (0.47). A maximum TS content of 73.85 was observed in PMiGAP120 with the lowest TS content 54.85 being observed in the PMiGAP001 genotype. The average AvST content was 65.09 ± 0.2 (with a range 47.85 ± 0.07 and 71.3 ± 0.0), which approximates the amount of the starch that would be passed to the large intestine for further digestion. The highest AvST content (71.3) was observed in genotype PMiGAP107 whereas PMiGAP221 showed the

lowest (47.85). Data were also reported as RS/TS ratio in percentages, which allowed the quantification of RS content based on the corresponding TS value. The RS/TS ratio ranged from 0.73 ± 0.03% (PMiGAP201) to 27.44 ± 0.02% (PMiGAP221) with a mean value of 2.98 ± 0.12% (Table S2).

3.2 | Phenotypic statistical analysis

The phenotypic distribution pattern for RDS, SDS, RS, TS, AvST and RS/TS starch-related values was assessed by plotting against frequency to assure the dataset particularly relevant for studying the genetic basis of these starch components. Normal frequency distribution curves were observed for the PMiGAP population for all the thirteen starch-related traits (Figure 1). The analysis of variance demonstrated significant genotypic variability for starch-related traits such as RDS, SDS, RS, TS, AvST (available starch) and RS/TS ratio and confirmed the existence of highly significant genetic differences amongst the 166 accessions for starch traits at the p-value < 0.0001 (Table 1). Such highly significant genotypic variance observed for starch traits confirmed the suitability of the subset of the PMiGAP used (of 166 individuals) for the association analysis. Additionally, all the starch-related traits showed high broad sense heritability >90.00% confirming least effect of replications on the phenotypic starch values observed for each accession (Figure S1).

Pearson's correlation analysis revealed that out of 15 possible pairs, 11 trait pairs were significantly correlated at the p-value < 0.01 (Figure 2). In addition, out of 15 possible pairs, 7 trait pairs showed positive correlation and 8 trait pairs showed negative correlation at the p-value < 0.01. Correlation analysis further highlighted the positive relationship for RDS with TS ($r = 0.38$) and AvST ($r = 0.61$) (Figure 2). A negative correlation was observed between RDS and RS ($r = -0.53$) but no significant correlation was found between RDS and SDS ($r = -0.09$). We noted that RS was negatively correlated with RDS with an $r = -0.53$, and with SDS having an $r = -0.14$), but no significant correlation with TS with $r = 0.04$ (Figure 2).

3.3 | Identification and distribution of SNPs

An initial set of 28 million SNP variants generated by resequencing the Pearl Millet inbred Germplasm Association Panel (PMiGAP) was filtered by minor allele frequency (<0.01) and site depth (>90%). The SNPs were distributed over all the seven chromosomes of pearl millet with the resulting 78080 retained for analysis. SNP variant rate ranged from 41.46 to 63.47 SNP per MB with an average of 49.77 SNPs per 1MB of the genome. The maximum SNP change rate was observed on chromosome 5, which was 63.47 SNP per 1Mb region of the genome, and the minimum on chromosome 6 (37.95). On average, one SNP change was observed at every 20,037 bases in the genome. The highest number of SNPs were detected on chromosome 1 (14,502 SNPs; 18.57%), followed

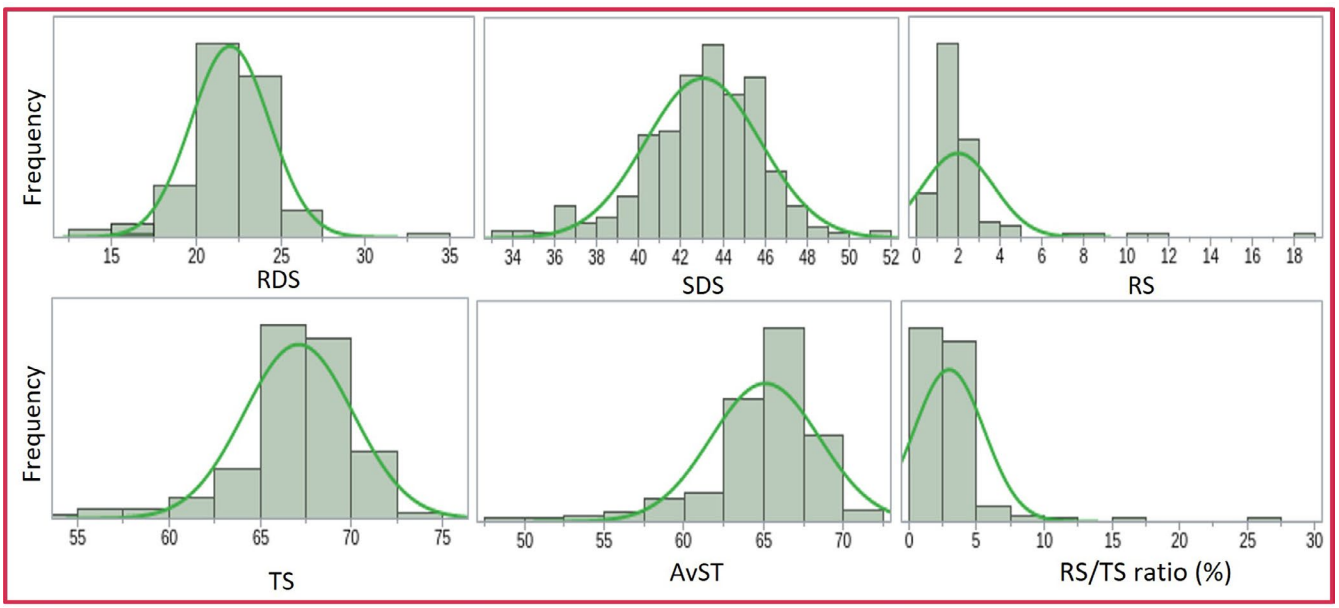

FIGURE 1 Histogram distribution pattern for starch-related estimated values recorded for RDS (Rapidly digested starch), SDS (Slowly digested starch), RS (Resistant starch), TS (Total starch), AvST (available starch) and RS/TS ratio (%) in pearl millet germplasm association panel (PMiGAP) of 166 genotypes

Traits	Source	df	Sum of squares	Mean square	*F* ratio	Prob > *F*
RDS	Genotypes	165	1760.6087	10.6704	64.663	<0.0001*
	Error	166	27.3924	0.165		
SDS	Genotypes	165	2423.3847	14.6872	50.8341	<0.0001*
	Error	166	47.9613	0.2889		
RS	Genotypes	165	958.69766	5.81029	206.184	<0.0001*
	Error	166	4.6779	0.02818		
TS	Genotypes	165	2916.501	17.6758	75.2367	<0.0001*
	Error	166	38.9993	0.2349		
AvST	Genotypes	165	3796.9993	23.0121	107.8488	<0.0001*
	Error	166	35.4201	0.2134		
RS/TS ratio	Genotypes	165	2191.31	13.2807	225.7657	<0.0001*
	Error	166	9.7649	0.0588		

TABLE 1 ANOVA results for RDS (Readily digested starch), SDS (Slowly digested starch) RS (resistant starch), TS (Total starch), AvST (available starch) and RS/TS ratio (%) starch-related traits in 166 pearl millet lines (one factor)

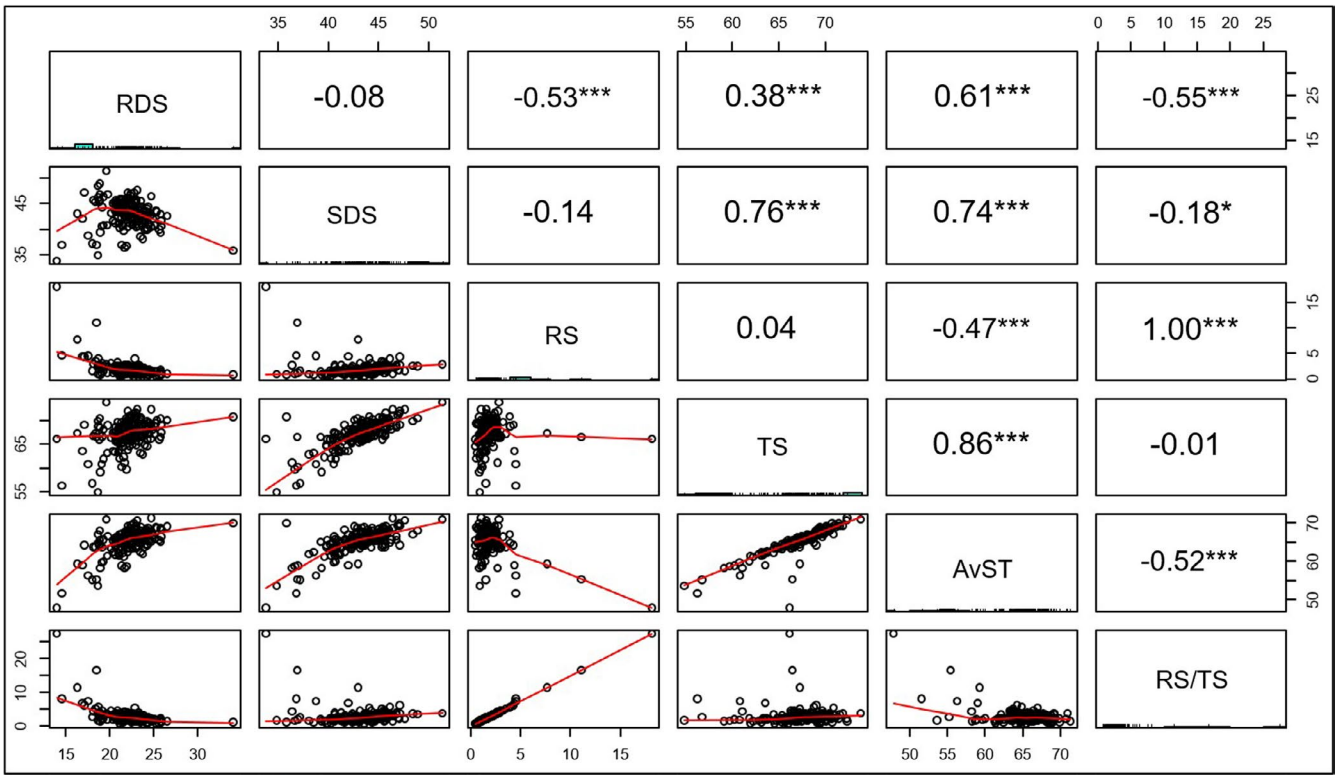

FIGURE 2 Prediction of correlation between starch-related estimated values for RDS (Rapidly digested starch), SDS (Slowly digested starch), RS (Resistant starch), TS (Total starch), AvST (available starch) and RS/TS ratio (%). Scatterplot matrix and Pearson's correlation coefficients (*R*) for each pair of traits and * represented the correlation significance at the >alpha (0.5)

by chromosomes 3 (12,480 SNPs; 15.98%), 2 (12,402; 15.88%), 4 (10,761; 13.78%), 5 (10,092; 12.92%) and 6 (9146; 11.71%) whereas minimal SNPs were observed on chromosome 7 (8697; 11.13%). Structural annotation of the 78,080 SNPs revealed the presence of 34,777 (~2.4%) SNPs in exonic regions, followed by 66,260 (46.75%) in intergenic regions. A total of 1527 (1.07%) suggested non-synonymous changes whereas 1922 (1.35%) were synonymous SNPs. A total of 12,319 (8.69%) SNPs were located in intragenic regions and 130 (0.009%) SNPs were present in 5′ UTR regions (Figure S2).

3.4 | Hierarchical and Bayesian model-based cluster analysis

Pair-wise Jaccard's genetic dissimilarity was calculated for the 166 accessions of pearl millet and the value ranged

from 0.11 to 0.80 with a mean value of 0.51. More than 70% of the pair-wise assessments displayed genetic dissimilarity higher than 0.5. DARwin-based Unrooted neighbour-joining cluster analysis revealed that the 166 accessions of pearl millet were divided into six groups (Figure S3). Most of the accessions originating from ICRISAT were grouped in two clusters; however, genetic diversity amongst these accessions was not as high as it was observed in the case of accessions. Principal coordinate analysis (PCoA), displayed no coherence for dispersion of the pearl millet germplasm lines in relation to the first two principal coordinates. Based on measured eigenvalues the first principal coordinate explained 23.78% of the total variation whilst the second coordinate could explain only 21.12%. The first five coordinates together could explain a total of 87.21% of the variation. Notably, the PCoA analysis inference did not show specific clustering of the accessions as observed on dendrogram analysis.

Bayesian cluster analysis using the STRUCTURE programme estimated the membership probability (Q-matrix) of each PMiGAP accession and combined them into a number of hypothetical subpopulations (1–16). The optimum K value was determined using the method of Evanno et al. (2005) and maximum ΔK peak height was observed at $K = 6$. Thus, Bayesian model-based clustering revealed that the 166 individuals were clustered into six groups ($K = 6$) (Figure S4). There is coherence in clustering of the accessions belonging to groups 1 and 2 by phylogenetic and STRUCTURE analyses, whereas accessions in phylogenetic groups 3 and 4 were clustered in one group by Bayesian analysis. Thus, for practical purposes, phylogenetic classification could form a basis for the selection of potential parents for hybridization studies.

3.5 | Genome-wide association analysis (GWAS) for markers trait association

A total of 78K high quality SNP variants were identified from the 166 PMiGAP lines for use in association analysis aimed at identifying loci associated with starch-related traits (such as RDS, SDS, RS, TS, AvST and RS/TS). The GWAS was conducted using the Fixed and random model Circulating Probability Unification (FarmCPU) tool implemented into the GAPIT software to avoid any confounding problem. A total of 6 starch-related traits were taken into considerations and the FarmCPU model-based association mapping showed 1132 SNP makers associated with these starch-related traits at p-value = <0.001 (Table S3). The markers associated with these traits (MTA) were distributed across 7 chromosomes. Chromosome 5 demonstrated the highest number of MTAs (382), followed by chromosome 4 (175 MTA), chromosome 2 (160 MTAs) and chromosome 1 (120 MTAs). Chromosome 7 showed the lowest number of MTAs. A total of 1.4% (1132) markers exhibited associations for all starch-related traits at the p-value = <0.001. The trait with the highest number of a MTAs was available starch (AvST) (331), followed by SDS (193), RDS (184 MTA), RS (161 MTA) and RS/TS (156 MTA). TS was the trait with the smallest number of MTAs detected (107 MTA).

A total of 85 markers were found to be associated with RDS (–log10 p-value ≥ 3.0). These makers were further visualized into Manhattan plots against their chromosomal positions and the observed p-values (on a –log10 scale) to demarcate the highly significant SNP markers. The Manhattan plot highlighted the most strongly associated SNPs for RDS where the –log10 p-value ranged from 3.0–4.94 (Figure 3). Chromosome 5 had the largest number of makers associated with this trait followed by chromosomes 1 and 2. Twelve SNP markers were found to be highly associated with the RDS trait at p-value 1.12×10^{-5}–9.8×10^{-5}. The Q–Q plots between observed and expected p-values of association for RDS revealed an appropriate distribution pattern explained by the model fitting involving population structure and kinship. Gnome-wide association analysis for slowly digested starch (SDS) showed 84 SNP markers to be significantly associated at –log10 p-value ≥ 3.0. The Manhattan and Q-Q plot visualization explained that significantly associated SNPs were recorded at the lowest p-value ranges from 9.7×10^{-4}–3.8×10^{-6} (Figure 3). Five SNP markers were found to be highly associated with the SDS trait at p-value 8.9×10^{-5}–3.8×10^{-6}. A further 78 SNP markers were visualized into a Manhattan plot using their chromosomal positions and the observed p-values (on a –log10 scale) and shown to be associated with the trait resistant starch (RS). The p-values of the detected associations ranged from 9.9×10^{-4}–1.7×10^{-5}. Q–Q plot visualization for RS demonstrated that –log10 p-value ranged from 3.0–4.7. Ten SNP markers were highly associated markers with the trait RS and p-value ranged from 1.7×10^{-5}–9.8×10^{-5}.

Association analysis was also performed for total starch (TS) content and fifty-seven SNP markers were found to be highly significant at –log10 p-value ≥ 3.0. Manhattan and Q–Q plot visualization revealed the high –log 10 p-value raged from 3.0 to 9.08 for these markers (Figure 3). Out of 57, 20 SNP markers exhibited a strong association with TS content based on the FarmCPU model with p-value ranging from 8.2×10^{-10} – 9.1×10^{-5}. These markers were found to be distributed on chromosome 2, 3, 5 and 7 for TS at lowest p-value ranged from 0.47–6.4×10^{-5}. Interestingly, 221 SNPs were significantly (–log10 p-value ≥ 3.0) associated with the trait available starch (AvST), which approximates primary digestion of millet grain starch inside the small intestine. Of these, 42 SNP markers exhibited

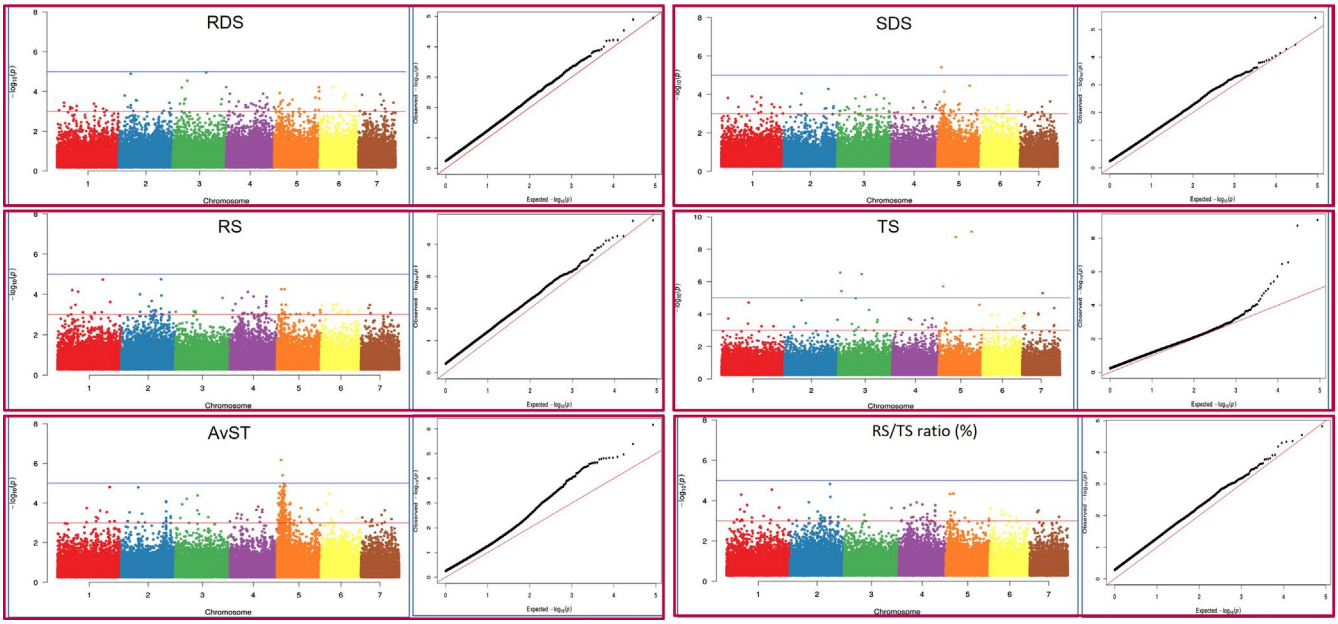

FIGURE 3 GWAS-based Manhattan plots built in the r package exhibiting significant P-values measured by FarmCPU model for six starch-related traits such as RDS (rapidly digested starch), SDS (Slowly digested starch), RS (resistant starch), TS (total starch), AvST (available starch) and RS/TS ratio (%)

significant association with AvST and *p*-value ranged from 6.7×10^{-7}–9.1×10^{-5} (Figure 3a). These SNPs for AvST trait were confirmed by the Manhattan plot with–log 10 *p*-values ranging from 3.0 – 6.1. Chromosome 5 had the greatest number of maker associations for this trait followed by chromosome 2 and 7. The *p*-value was ranged from 0.35–0.05. QQ (quantile-quantile) plots displayed linear distribution when plotted against the observed and the expected distribution of p-values for AvST trait (Figure 3). A total of 73 SNPs were significantly (–log10 *p*-value ≥ 3.0) associated with RS/TS ratio and 6 SNP markers had the strongest association with RS/TS ratio with *p*-value ranging from 1.4×10^{-5}–6.5×10^{-5}.

3.6 | Identification of candidate genes

The SNPs significantly associated with starch traits were mapped onto the *Pennisetum glaucum* reference genome assembly (http://ceg.icrisat.org/ipmgsc/genome.htmL) in LD blocks ($r^2 > 0.6$) in 20 kb windows. Genes within these regions, in addition to genes, which were the nearest neighbours to significant SNPs, which were mapped in LD blocks, were considered as candidate genes. Three hundred and sixty-four candidate genes were identified in the surrounding regions of the significantly associated SNPs. Out of these 364, 30 (0.08%) SNP flanking genes were predicted to have similar functions to starch biosynthetic pathway-related genes. Amongst them, starch synthase genes (PgSSIV; Pgl_GLEAN_10026059, Pgl_GLEAN_10018307) on chromosome 2 and (PgSSIV; Pgl_GLEAN_10027180) on chromosome 3 were identified (Table 2). Functional annotation revealed that these genes encode enzymes with molecular active sites involved in the initiation and formation of starch granules and the conversion of amylose and amylopectin. This suggests that these genes may be acting as co-factors in these pathways or that they may regulate genes through various chemical pathways to promote the accumulation of amylose and exhibit a key role in SDS accumulation. Pgl_GLEAN_10018323 encoding β-amylase was identified as a candidate gene for RDS content and is involved in the chemical reactions and pathways resulting in the breakdown of a polysaccharide, a polymer of many (typically more than 10) monosaccharide residues linked glycosidically. Similarly, Pgl_GLEAN_10010158, which encodes a lipase with an essential role in digestion, transport and processing of dietary lipids, showed association with RS trait.

Furthermore, some candidate genes were found to be directly associated with starch-related biosynthetic pathways across all SNP data sets including Alpha-amylase (Pgl_GLEAN_10009197), which is a 1,4-α-glucan branching enzyme that hydrolyses 1,4-alpha-glucosidic linkages in starch-type polysaccharide. Similarly, UTP—glucose-1-phosphate uridylyltransferase (Pgl_GLEAN_10018054) is an important part of the sucrose biosynthesis pathway, providing Uridine diphosphate glucose to Sucrose-phosphate synthase, which converts UDP-glucose and D-fructose 6-phosphate into sucrose-6-phosphate,

TABLE 2 List of starch biosynthesis pathway-related candidate gene resided around significantly associated markers for starch-related traits such as RDS (rapidly digested starch), SDS (Slowly digested starch), RS (resistant starch), TS (total starch), AvST (available starch) and RS/TS ratio (%) in pearl millet

SNP	Chr	LD_start	LD_end	Gene_name	Gene_start	Gene_stop	Gene_description
2_78761117	2	78751117	78771117	Pgl_GLEAN_10026059	78754684	78760614	Starch synthase 4 (SSIV)
2_78764226	2	78754226	78774226	Pgl_GLEAN_10026059	78754684	78760614	Starch synthase 4 (SSIV)
2_209171246	2	209161246	209181246	Pgl_GLEAN_10018307	209174406	209177486	Starch metabolism
2_209175132	2	209165132	209185132	Pgl_GLEAN_10018307	209174406	209177486	Starch metabolism
2_209175137	2	209165137	209185137	Pgl_GLEAN_10018307	209174406	209177486	Starch metabolism
2_209175368	2	209165368	209185368	Pgl_GLEAN_10018307	209174406	209177486	Starch metabolism
2_209175370	2	209165370	209185370	Pgl_GLEAN_10018307	209174406	209177486	Starch metabolism
2_209175414	2	209165414	209185414	Pgl_GLEAN_10018307	209174406	209177486	Starch metabolism
2_209176605	2	209166605	209186605	Pgl_GLEAN_10018307	209174406	209177486	Starch metabolism
2_209177023	2	209167023	209187023	Pgl_GLEAN_10018307	209174406	209177486	Starch metabolism
2_240852290	2	240842290	240862290	Pgl_GLEAN_10018139	240861948	240865137	Glycosyl hydrolase
2_238687363	2	238677363	238697363	Pgl_GLEAN_10018323	238678164	238684627	Glycosyl hydrolase family 14 (Beta-amylase 2)
2_233332164	2	233322164	233342164	Pgl_GLEAN_10020483	233329809	233331973	Laccase-2
6_52460693	6	52450693	52470693	Pgl_GLEAN_10014787	52467150	52468373	GDSL-like Lipase/ Acylhydrolase
5_123963884	5	123953884	123973884	Pgl_GLEAN_10008133	123957354	123959329	Glutathione peroxidase
7_26545865	7	26535865	26555865	Pgl_GLEAN_10010158	26547619	26548916	Lipase
5_81743637	5	81733637	81753637	Pgl_GLEAN_10004165	81734759	81737196	Glycosyltransferases
6_228035770	6	228025770	228045770	Pgl_GLEAN_10028958	228030965	228032731	Triose-phosphate Transporter family
1_57711273	1	57701273	57721273	Pgl_GLEAN_10033951	57719920	57722359	Succinate dehydrogenase
1_177130077	1	177120077	177140077	Pgl_GLEAN_10033763	177133813	177139238	GST
3_271851504	3	271841504	271861504	Pgl_GLEAN_10004992	271851237	271852953	GDSL-like Lipase/ Acylhydrolase
3_278097000	3	278087000	278107000	Pgl_GLEAN_10018027	278095601	278101572	Fucosyltransferase family protein
6_189716192	6	189706192	189726192	Pgl_GLEAN_10020216	189701772	189706397	Glucose-6-phosphate isomerase
1_171668212	1	171658212	171678212	Pgl_GLEAN_10009197	171662291	171668844	Alpha-amylase (1,4-alpha-glucan branching enzyme)
1_171668441	1	171658441	171678441	Pgl_GLEAN_10009197	171662291	171668844	Alpha-amylase (1,4-alpha-glucan branching enzyme)
2_229744277	2	229734277	229754277	Pgl_GLEAN_10012155	229740512	229742334	Sugar (and other) transporter
2_241850181	2	241840181	241860181	Pgl_GLEAN_10018054	241852379	241856224	UTP-glucose-1-phosphate uridylyltransferase
3_295792947	3	295782947	295802947	Pgl_GLEAN_10027180	295793140	295800113	Starch synthase 4 (SSIV)
3_295792973	3	295782973	295802973	Pgl_GLEAN_10027180	295793140	295800113	Starch synthase 4 (SSIV)
3_295799961	3	295789961	295809961	Pgl_GLEAN_10027180	295793140	295800113	Starch synthase 4 (SSIV)
5_145069275	5	145059275	145079275	Pgl_GLEAN_10031509	145067338	145067670	Sugar (and other) transporter

UTP-glucose-1-phosphate uridylyltransferase (Table 2). Pearl millet predicted gene Pgl_GLEAN_10004992 having similar function to GDSL-like Lipase/Acylhydrolase, which is involved in many cell biological processes from maintaining lipid homeostasis to lipid signalling. A large number of candidate genes were predicted to be chloroplast-related, to encode mitochondrial proteins or proteins linked to stress defence mechanisms, growth and development. Other genes showed similarities with transcription factors including NAC domain transcript factors, Myb domain transcript and auxin-related transcriptome factors (Table S4).

4 | DISCUSSION

Pearl millet [*Penisetum glaucum* (L) R. Br.] is widely cultivated as a dietary staple in the arid and semi-arid regions of the world including areas of India and Africa. Consumption of proper carbohydrate plays a vital role in human health especially in regulating blood glucose level (Ludwig, 2002). Low glycaemic index (GI) foods and food products are, therefore, regularly recommended for diabetic patients as they avoid sudden spikes in blood glucose levels by releasing glucose more gradually (Ludwig et al., 2018). In general, RDS and SDS are the most important constituent of carbohydrate and are digested in the small intestine providing an important source of energy (Englyst et al., 1996).

In our study, the glycaemic index (GI) was observed for the 166 pearl millet lines where the GI trend correlates with an increased proportion of SDS as compared to RDS. Thus, the majority of pearl millet accessions (123) had relatively high SDS content. PMiGAP211 and PMiGAP241 have low GI value. The combination of SDS and RS contents identified ten pearl millet lines, which have a low GI value but relatively high SDS content. The best entries comprising key starch measuring values are shown in Table S2. Based on SDS content and GI value ten accessions (PMiGAP051, PMiGAP120, PMiGAP148, PMiGAP167, PMiGAP173, PMiGAP211, PMiGAP212, PMiGAP221, PMiGAP235, PMiGAP241) containing high relative SDS content, low GI value and low GI with high relative SDS content were considered as best entries for further utilization in breeding programmes. Such accessions can either used directly (if found to possess superior agronomic traits as well) or be utilised as donors in generating elite pearl millet varieties combining high yield and low GI through molecular and genomics-assisted breeding methods. In pearl millet, relatively lower RDS levels were found (average of 22.0 ± 0.2 and ranged from 13.95–34.07 in 166 accessions studied) than observed in rice from 59.28 – 72.73, with a mean value of 65.42 (Zhang et al., 2020). This further confirms the superiority of pearl millet over rice in traits contributing to a low GI. Pearl millet has also been identified as a good source of RS with levels higher than major cereals like wheat and rice. In our study, we observed an average content 1.99 of RS (ranging from 0.08–1.99 in 166 accessions evaluated), which was slightly higher than observed in rice, which ranged from 0 – 3.47, with an average of 1.67 (Zhang et al., 2020). High levels of SDS within food is directly associated with a low glycaemic index (GI) and high SDS foods can, therefore, form an important part of the diet to help mitigate the risk of diverse chronic degenerative diseases such as type 2 diabetes and other obesity-related disorders (Jenkins et al., 2002; Parween et al., 2020).

Genetic improvement of traits contributing to low GI (more specifically SDS and RS) in pearl millet is almost non-existent. The scope for large scale phenotyping of starch digestibility by laboratory assay can be limited by the relatively low throughputs. Molecular markers, especially the SNP markers, hold great promise in simplifying the selection of such traits in plant breeding programmes. To this end, we have identified 902 SNPs linked to various starch-related traits, which on further validation have the potential for use in targeted plant breeding programmes for improving the starch profile in millet. For identifying the most appropriate SNPs, we studied the population structure identifying six clusters within the 166 accessions. As reported in earlier studies (e.g. Kanfany et al., 2020; Sehgal et al., 2015; Serba et al., 2019; Varshney et al., 2017), genotypes from similar geographies and agroecologies grouped together within clusters whilst those from diverse geographical and agroecological origins grouped into different clusters. Higher density markers used in the study also facilitated analysis of linkage disequilibrium (LD) for finding the most appropriate marker-trait associations. No correlation between the country of origin and genetic diversity was observed probably due to the relatively limited number of germplasm accessions used in this study. Similar observations were reported by Sehgal et al. (2015) where they also reported six subpopulations amongst the PMiGAP association panel of 345 entries. Moreover, population genomic analysis carried out by Kanfany et al. (2020) reported that the pearl millet inbred lines derived from diverse geographical and agroecological features demonstrated five subgroups mostly following pedigree differences with different levels of admixture. Serba et al. (2019) reported six subgroups utilizing model-based clustering and hierarchical clustering analysis of 82,112 SNPs in 398 pearl millet accessions in agreement with our findings.

Using 78K SNP markers and 13 starch-related phenotypic traits for 166 genotypes collected from different

parts of the world we report ninety-four high-confidence SNP markers associated with starch-related traits. Furthermore, a total of 364 genes were found to reside around the SNPs associated with starch traits such as RDS, SDS, RS, TS, AvST and RS/TS. Thus, 19 candidate genes including Starch synthase (SSIV), amylases, Beta-D-xylosidase, Glycosyl hydrolase, glucose phosphomutase, Glycosyltransferases involved in the synthesis of glucuronoxylan hemicellulose in secondary cell walls, Triose-phosphate Transporter family, Sugar (and other) transporter, Glucose-1-phosphate adenylyltransferase, beta-1,4-xylosyltransferase, Succinate dehydrogenase, GST fucosyltransferase family protein, Glucose-6-phosphate isomerase, Glucuronokinase 1 (Sugar-1-kinase) and UTP-glucose-1-phosphate uridylyltransferase were found to be directly associated with starch-related biosynthetic pathways across all selected SNP data sets that were found to be associated with starch phenotypes.

Of particular interest was the identification of starch synthase (PgSSIV) genes (Pgl_GLEAN_10026059, Pgl_GLEAN_10018307 and Pgl_GLEAN_10027180) are key genes for SDS and amylase for RS accumulation in pearl millet. In other studies, Parween et al. (2020) also investigated and found that alpha-amylase/branching enzyme is the key regulatory gene to enhance resistant starch in rice. Contrastingly, the findings by Zhang et al. (2020) who have previously reported that starch branching enzymes IIa (BEIIa) are closely associated with RS levels in Indica rice. In the same study, Zhang et al. (2020) also reported that a lipase gene (LOC_Os09g09360) was found to be associated with SDS through GWAS in rice.

5 | CONCLUSION

This is the first study in pearl millet to report the extent of genetic variation present in its germplasm for starch traits and also identifies SNP markers associated with them. It also reports the ten best germplasm accessions with potential for direct cultivations (if found high yielding) and/or for use as donors in breeding future pearl millet hybrids and cultivars possessing both high yield as well as low GI. Furthermore, this study also identifies candidate genes residing in regions adjacent to SNPs found to be associated suggesting their involvement in determining starch content in pearl millet. Validation of these genes is currently underway in our laboratory and will be reported in due course.

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CONFLICT OF INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

R.S.Y. conceived and supervised the completed study. C.B.Y. analysed the results and wrote the manuscript. Englyst as a supplier analysed starch traits in the entries. P.G., S.B. and R.K.S. contributed towards revising the manuscript. All authors have read, revised and approved the final manuscript.

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