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ORIGINAL RESEARCH

Genome-wide association study for lignocellulosic compounds and fermentable sugar in rice straw

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Abstract

Cellulose and lignin are the two main components of secondary plant cell walls with substantial impact on stalk in the field and on straw during industrial processing. The amount of fermentable sugar that can be accessed is another important parameter affecting various industrial applications. In the present study, genetic variability of rice (*Oryza sativa* L.) genotypes for cellulose, lignin, and fermentable sugars contents was analyzed in rice straw. A genome-wide association study of 33,484 single nucleotide polymorphisms (SNPs) with a minor allele frequency (MAF) >0.05 was performed. The genome-wide association study identified seven, three, and three genomic regions to be significantly associated with cellulose, lignin, and fermentable sugar contents, respectively. Candidate genes in the associated genomic regions were enzymes mainly involved in cell wall metabolism. Novel SNP markers associated with cellulose were tagged to GH16, peroxidase, GT6, GT8, and CSLD2. For lignin content, Villin protein, OsWAK1/50/52/53, and GH16 were identified. For fermentable sugar content, UTP-glucose-1-phosphate uridylyltransferase, BRASSINOSTEROID INSENSITIVE 1, and receptor-like protein kinase 5 were found. The results of this study should improve our understanding of the genetic basis of the factors that might be involved in biosynthesis, turnover, and modification of major cell wall components and saccharides in rice straw.

1 | INTRODUCTION

Rice (*Oryza sativa* L.) is among the most consumed cereals in the world. Close to 1 billion tons of rice are consumed annually, which leaves more than 1.1 billion tons of straw behind (Santos et al., 2017). Dry rice straw is composed of about 35–47% crystalline cellulose with a high degree of polymerization, 18% branched low-molecular weight hemicellulose, and 19–24% lignin (Santos et al., 2017). This cell wall composition makes the plant mechanically strong to become resilient against lodging and to provide a first barrier against pests and diseases (Saeed, 2018). In addition to the use of rice straw in bioenergy production, that is, biofuels, bioethanol, and biomethane gas as fossil fuel replacement (Mahlia et al., 2020), it can be used in formulations of animal feeds, mushroom bed preparation

Abbreviations: CesA, cellulose synthase; CSC, cellulose synthase complex; GH, glycoside hydrolase; GO, gene ontology; GT, glycosyltransferase; GWAS, genome-wide association study; H^2_b, broad-sense heritability; LD, linkage disequilibrium; PC, principal component; PM, plasma membrane; QTL, quantitative trait loci; SNP, single nucleotide polymorphism; WAK, wall-associated kinase.

(Demont et al., 2020), heavy metal biosorption toward health and environmental preservation (Amer et al., 2017; Kardam et al., 2014), pulp and paper raw material production (Kaur et al., 2017), and medicine in forms of drug carriers by means of cellulose fibers (Yusefi et al., 2020). Rice straw can also be used to produce monomeric sugar required for production of biosurfactants (Makkar et al., 2011). The silica present in dried rice straw (~15%) can contribute to the ever-increasing demand of numerous industries (Oladosu et al., 2016).

Although agricultural residues are valuable resources for bioethanol production, the complex nature of plant cell walls limits the bioavailability of fermentable sugar; the recalcitrance of cell walls to depolymerization and fermentation is dependent on lignin content and the interrelationship of cellulose microfibrils with so-called matrix polysaccharides (hemicellulose) (Robak & Balcerek, 2018). Cellulose is considered to be the main producer of glycosyl residues as the most valued fermentable sugar via enzymatic hydrolysis. Cellulose is synthesized and assembled (Figure 1) in the plasma membrane (PM) by cellulose synthase (CesA) complexes (CSCs) that are initially being assembled in Golgi apparatus and delivered to PM (Polko & Kieber, 2019). Cellulose is an indispensable part of both primary and secondary cell walls. In the rice straw secondary cell wall, cellulose together with noncellulosic polysaccharides are immersed in a matrix of lignin, forming an abundant but indigestible composite (lignocellulose) (Donev et al., 2018). Lignocellulosic compounds are widely used as a raw material in the production of second-generation biofuels (Q. Liu et al., 2018; Tan et al., 2016). The degrees of lignification, cross-linking of polysaccharides to each other by ferulic acid, and crystallinity of cellulose cause recalcitrance in lignocellulosic materials (Gupta et al., 2011). Lignin inhibits saccharification processes aimed at producing simple sugar for fermentation to ethanol (Wegrzyn et al., 2010) and therefore acts as a hindrance in the process of biomass to biofuel. Physical and chemical pretreatments are, therefore, necessary to facilitate biomass digestion by removing some xylans and lignin to enable enzymes to gain access to the hydrophobic cellulose face (Baruah et al., 2018) and release simple sugar (saccharification). In a balancing act between food and biofuel production, sometimes the winners are dedicated energy crops including herbaceous crops such as switchgrass (*Panicum virgatum* L.), reed canary grass (*Arundo donax* L.) and bamboo (*Fargesia nitida* L.) (Glithero et al., 2015; Shortall, 2013). However, a win-win situation can be envisaged when cereals are being considered, as these crops produce both food grains and raw fermentable materials (straw + husk) that can be used to produce bioethanol (Townsend et al., 2017).

Many initiatives have been taken in recent years to improve the pretreatment or other aspects of industrial biomass processing to improve the yield of released fermentable sugar (Guragain & Vadlani, 2021; Østby et al., 2020). In addition to the process optimization of saccharification and fermentation (Sukma et al., 2019; Takano & Hoshino, 2018), attempts to change the plant cell wall structure to reduce its recalcitrance have been undertaken, and most have been dependent on silencing the genes that directly/indirectly produce the bulk of plant biomass to provide raw materials for bioprocessing next to other processing means (Kalluri et al., 2014). Therefore, knowing which genes and proteins define the amount and structure of the main constituents of cell walls in rice straw would be beneficial in designing future crops toward targeted applications.

Core Ideas

- GWAS was conducted for cellulose, lignin, and fermentable sugar contents in rice straw.
- GWAS was conducted for the first time for cellulose in rice straw.
- Many QTLs were found for rice straw composition, some of which are reported for the first time.
- New candidate genes were found in vicinity of genomic regions associated with studied traits.

Genome-wide association studies (GWASs), also known as linkage disequilibrium (LD) mapping, provide the opportunity to find the correlation between phenotypes and associated markers in a high-resolution manner (Alqudah et al., 2020; Rosyara & Joshi, 2012). Choosing the right population in terms of diversity and number, availability of marker-enriched linkage groups for genotyping, and precision-phenotyping greatly enhances the resolution of GWAS findings toward the definition of candidate associated genes (Nayyeripasand et al., 2021; Nguyen et al., 2020). Rice straw GWAS have so far focused on biomass digestibility (Norton et al., 2018), lignin, and saccharification (Nguyen et al., 2020), whereas no such report has been presented for cellulose content. A small number of studies from other plants have shown that genes other than members of the *CesA* family and quantitative trait loci (QTLs) are involved in cellulose content (Houston et al., 2015; Kaur et al., 2017; K. Li et al., 2016; Miao et al., 2019; Niyitanga et al., 2019; Shiringani & Friedt, 2011; Thumma et al., 2010; Xu et al., 2017). For example, a barley (*Hordeum vulgare* L.) GWAS identified members of the glycosyltransferase (GTs) and glycosyl hydrolase (GHs) families as candidate genes associated with cellulose content (Houston et al., 2015). Wheat (*Triticum aestivum* L.) GWAS introduced β-tubulin and the auxin-induced protein 5NG4 as candidate genes (Kaur et al., 2017). Rice QTLs associated with lignin and fiber content were reported to be qADF-9, qADL-9, qADF-2, and qADF-3 (Bao et al., 2007). In another study, eight related lignin monomers and biomass

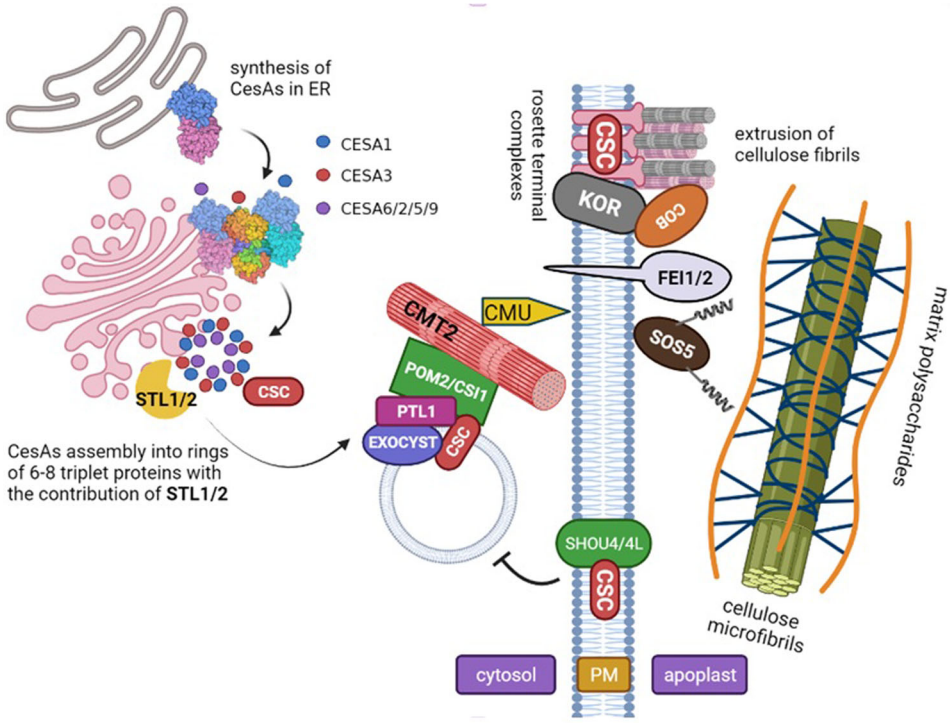

FIGURE 1 Trafficking of cellulose synthase complex (CSC) in plant cells. Cellulose synthase (CesA) proteins are being synthesized in endoplasmic reticulum and with the help of STELLO1 and 2 (STL1/2; GT75) are being assembled into CSC in Golgi apparatus (Zhang et al., 2016). Cortical microtubule-associated (CMT) vesicles carry CSC to plasma membrane (PM) (Crowell et al., 2009; Gutierrez et al., 2009) with the help of exocyst complex (Zhu et al., 2018). Exocyst is in interaction with CesA6 and POM2/CSI1, which the latter interacts with PATROL1 (PTL1) protein (Zhu et al., 2018). SHOU4/4L involves in regulating CesA exocytosis and therefore levels the cellulose synthesis (Polko et al., 2018). For proper CMT spacing during cellulose synthesis, CMT-interacting CELLULOSE SYNTHASE MICROTUBULE UNCOUPLING (CMU) is necessary (Liu et al., 2016). KOR (an endoglucanase) interacts with CesAs and defines their processivity (Vain et al., 2014). COBRA (COB) found in apoplast regulates the orientation of extruded cellulose microfibrils (L. Liu et al., 2013; Roudier et al., 2005). SOS5 (SALT-OVELY SENSITIVE5) and FEI1/2 (a leucine-rich repeat receptor like kinase) regulate cellulose biosynthesis (Basu et al., 2016)

digestibility QTL clusters were found for rice straw (Hu et al., 2018). A GWAS for maize (*Zea mays* L.) lignin presented xyloglucan endotransglucosylase/hydrolase among others (K. Li et al., 2016), while laccase and peroxidase genes were proposed for rice (Nguyen et al., 2020). For fermentable sugar content, a QTL for cellulose digestibility was reported in a recombinant inbred population of maize (Penning et al., 2014). In a rice biparental population, a broad region on chromosome 1 was identified to have an impact on straw digestibility (B. Liu, Gómez et al., 2016). Overexpression of the *OsAt10* gene, expressing a BAHD acyltransferase, altered the amount of saccharification in rice straw (Bartley et al., 2013). Later, overexpression of *OsAt10* in switchgrass enhanced saccharification of lignocellulosic biomass (G. Li et al., 2018). Rice GWAS for fermentable sugar revealed the probable involvement of *BdMYB48*, *OsIRX9*, and *CesA 11* in defining the content (Nguyen et al., 2020).

Here, we have used rice as a model plant with genomic data available to decipher the genes that might be involved in defining cellulose, lignin, and fermentable sugar contents. An understanding of the natural variability of cellulose and lignin contents, and the potential for cell wall saccharification in plants could, if associated with specific genomic regions, facilitate the enhancement of the industrial applications of rice.

2 | MATERIALS AND METHODS

2.1 | Plant material

A previously genotyped set of global rice accessions from 82 countries (Zhao et al., 2011) was received from the T. T. Chang Genetic Resources Center, International Rice Research Institute (IRRI) and grown in Sari Agricultural University (Northern Iran) on 2017–2019 in three replicates. One hundred seventy of the grown accessions were randomly selected for association mapping of cellulose, lignin, and fermentable sugar in rice straws (Supplemental Table S1). Accessions were TEJ (temperate *japonica*), IND (*indica*), AUS (aus), ARO (aromatic), TRJ (tropical *japonica*) ADMIX subpopulations. Single nucleotide polymorphisms (SNPs)

information for the rice 44.1 K SNPs array (Zhao et al., 2011) was downloaded from the Gramene portal (http://gramene.org). The rice accessions were sown in plots of 2 × 2 m^2 with 25 cm within rows spacing. Superphosphate triple (180 kg/ha at plowing): urea (100 kg/ha at seedling stage): potash (80 kg/ha at plowing stage) were given to plants. Plots were hand harvested at maturity and straw was stored at 25 ˚C.

2.2 | SNP genotyping data

The development and sequencing of a SNP array hybridization for the rice population have previously been described by Zhao et al. (2011). Briefly, previously published 44,100 SNP data from a 44K SNP array, resulting in genotype data from 33,484 high-quality SNP markers, were used for GWAS.

2.3 | Measurement of cellulose, lignin, and fermentable sugar contents

At the stage of complete maturity, first internodes (from the top) were randomly collected from each accessions in three replicates, ground with a mill, and filtered with a 0.1-mm mesh. Crystalline cellulose content was determined using the Updegraff acetic acid/nitric acid method (Updegraff, 1969) with modifications as described in Pettolino et al. (2012). Briefly, 1 ml of acetic acid:water:nitric acid (8:2:1) was added to 50 mg dried tissue, vortexed, and incubated at 100 ˚C for 4 h. The tubes were cooled to 22 ˚C and centrifuged in a swing-out rotor at 10,000 rpm for 10 min. The pellet was washed four times with dH_2O, vortexed in between, and the repelleted by centrifugation at 10,000 rpm for 10 min, followed by a 90% ethanol wash. The tubes were dried at 80 ˚C and the amount of cellulose was measured as dry weight.

Lignin was measured using the Klason method (Dence, 1992). Briefly, 1 ml 72% (v/v) sulfuric acid was added to 100 mg dried tissue, vortexed, and incubated at 22 ˚C for 2 h. Water (30 ml) was added, vortexed, and centrifuged at 13,000 rpm to pellet lignin. The tubes were dried at 80 ˚C to determine lignin content. If a visible pellet was not obtained with one step centrifugation, the second round was carried out.

Fermentable sugar were determined in two steps: chemical pretreatment and a hydrolytic process optimized by Lee et al. (2017). Cut pieces of rice straw (2–3 cm) were sieved through a 0.36–1.00 mm mesh and pretreated with 1% (v/v) of sulfuric acid at 95 ˚C for 60 min (Ong et al., 2012). The treated straw was washed with dH_2O, dried at 60 ˚C, and used for enzymatic hydrolysis. Straw (100 mg) was pretreated with sulfuric acid, incubated with 0.1 M of citrate buffer (pH = 6.0), and 0.1 ml of cellulase (Accellerase 1000; Sigma-Aldrich) at 50 ˚C for 48 h at 100 rpm on a rotary shaker (Hsu et al., 2010). The concentrations of reducing sugar were analyzed using the di-nitrosalicylic acid reagent and compared to a standard glucose curve (Lee et al., 2017). Each 10 ml of sample solution was mixed with 1 ml of di-nitrosalicylic acid reagent and heated in boiling water for 5 min. The solution was cooled down to 22 ˚C and the absorbance was measured at 540 nm.

2.4 | GWAS analysis

Analysis of population structure among rice accessions was performed by principal components analysis (PCA) in TASSEL (Bradbury et al., 2007). The PCA analysis and corresponding plot were generated using GAPIT, the genomic association and prediction integrated tool (Lipka et al., 2012). The kinship matrix was obtained using TASSEL v.5 and visualized in GAPIT (Zhang et al., 2010). To determine the size of LD blocks, pairwise LD between the markers was visualized using the LD heatmap package in R (https://CRAN.R-project.org/package = LDheatmap). Association analyses were performed using the genotypes of accessions with 33,484 SNPs and phenotyping data obtained from 170 accessions in GAPIT by Bayesian-information and Linkage-Disequilibrium Iteratively Nested Keyway (Blink) model (Huang et al, 2019). A QTL was considered significant when markers were associated with cell wall components content at $-\text{Log}_{10}(P) > 4$. Broad-sense heritability of lignin, fermentable sugar, and cellulose was estimated in rptR package using phenotypic data (Stoffel et al., 2017).

2.5 | Candidate gene finding and analyses

We extended marker intervals by 200 kb in both directions (400 Kb window) to take account of map order uncertainty and LD. This window of 400 Kb was chosen due to the very slow LD decay in rice genome (Mather et al., 2007). To identify genes underlying the QTLs of cell wall content, genes overlapping the physical regions of these QTLs (i.e., in the vicinity of their associated SNPs) and any gene deposited on the Rice Annotation Project database (http://rice.plantbiology.msu.edu/) were assessed. The co-expression gene analysis of candidate genes was carried out using Genevestigator (https://genevestigator.com/) and RiceFrend (https://ricefrend.dna.affrc.go.jp/). Expression pattern of the candidate genes was determined by RNA-Seq assay in eight different tissues (leaves, shoots, seed, endosperm, embryo, anther, pistil, and panicles) and was retrieved from RGAP database (http://rice.uga.edu/), in which the expression level was reported based on FPKM. The KEGG (https://www.kegg.jp/) and PANTHER (http://pantherdb.org/)

TABLE 1 Descriptive statistics of celloluse, lignin and fermentable sugar content in a collection of rice (*Oryza sativa* L.) genotypes

Descriptive statistic	Cellulose	Lignin	Fermentable sugars
	——µg/mg——		g/l
Average	485.62	273.02	9.74
Maximum	593.08	395.51	15.16
Minimum	240.55	98.88	2.49
SD	58.85	73.14	2.70
CV	0.121	0.268	0.277
H^2_b	0.69	0.70	0.63

Note. H^2_b, maximum, minimum, standard deviation, coefficient of variation, and average values in the collection are represented.

analysis tools were used for understanding the function of candidate genes.

3 | RESULTS

3.1 | Variation of phenotypic traits

Phenotypic variation in 170 rice accessions was estimated for cellulose content using three biological replicates, and for lignin and fermentable sugar using two biological replicates. The average amount of cellulose in our study was equal to the amount of cellulose reported in other studies for rice (46.5%) (Siro & Placket, 2010). The typical lignin content observed in rice straw was at a similar level to that of grasses in general and higher than that found in dicots but lower than woody species (Abramson et al., 2013). Although we used a different method for lignin measurement than that used by Nguyen et al. (2020), similar results (26.3%) were obtained. Our mean values for cellulose, lignin, and fermentable sugar content were 485.6, 273, and 9.7 g/l in rice straw, respectively (Table 1). The broad-sense heritability (H^2b) was 0.69 for cellulose, 0.70 for lignin, and 0.63 for fermentable sugar (Table 1). These data indicate the greater contribution of accessions genotype in defining straw cell wall polysaccharide contents.

3.2 | PCA and population stratification results

The results showed relatively higher genetic relatedness among accessions within subpopulations. Population structure of rice collection justified by principal component $(PC)_1 = 8.5\%$ and $PC_2 = 7.4\%$ (Figure 2a). GAPIT was used to characterize population structure and PC_2 against PC_1 scree plot from GAPIT showed the selection of PCs for association study. Results were illustrative of three main groups (Figure 2b). The kinship matrix summarized the distribution of the pairwise relative relationship coefficients among the accessions in the association panel based on SNPs' information (Figure 3). As expected, genetic relatedness was greater within populations as opposed to between populations.

3.3 | GWAS results and candidate gene identification

To identify the genetic loci responsible for the variation in cellulose, lignin, and fermentable sugar contents in rice accessions, GWAS was conducted with SNP data using the BLINK model in GAPIT (Huang et al., 2019). BLINK model revealed eight, five, and five significant marker-trait associations with $-\log_{10}(P) > 4$ for cellulose, lignin and fermentable sugar, respectively (Figure 3). In the vicinity of significant SNP markers (400 kb window), several co-located genes were retrieved from RAP db (http://rice.plantbiology.msu.edu/) (Supplemental Table S2). Among the co-located genes with the associated SNP markers for each trait, we introduced nine, six, and four genes as candidate genes for cellulose, lignin, and fermentable sugar, respectively (Table 2). Candidates were selected based on whether the function of the genes had been characterized before in rice or if similar genes in other species had known roles in cell wall biosynthesis or modification. For cellulose, we identified significant associations for seven genomic regions (on chromosomes 2, 3, 6, 9, and 11) and a total of five QTLs (Figure 4a; Table 2). The strongest QTL was located on chromosome 3 at position 0.99 Mbp. For lignin, three genomic regions were tagged by a total of five SNPs on chromosomes 3 and 4. The strongest QTL was located on chromosome 4 at position 30.27 Mbp (Figure 4b; Table 2). For fermentable sugar, five SNPs were identified that together tagged 3 genomic regions located on chromosomes 1, 8, and 9. The most strongly associated QTL was located on chromosome 8 at position 17.38 Mbp (Figure 4c; Table 2).

3.4 | Analysis of candidate genes

Co-expression analysis of the identified candidate genes using Genevestigator are summarized in Table 2. Several genes including *CesA*s, *GH*s, and *GT*s were identified as co-expressors with most of the candidate genes. Network analysis using RiceFREND (https://ricefrend.dna.affrc.go.jp/) showed co-expression pattern of the candidate genes. For each candidate gene, up to six direct interactions were detected in the gene networks (Supplemental Table S3). For example, in the case of *LOC_Os11g34390* (*GT6*), it showed co-expression with *LOC_Os02g49140* (similar to α-galactosyltransferase), *LOC_Os06g41770* (DNA-binding domain containing

TABLE 2 Single nucleotide polymorphisms association with cellulose, lignin and fermentable sugar, and respective candidate genes

Trait	QTL	Chr	No. of AM	Peak marker	-log$_{10}$*P*-value	MAF	Position	Candidate gene name	Candidate gene ID	Co-expressed gene list obtained by Genevestigator
							bp			
Cellulose	qCLu2.1	2	1	id2016311	4.54	0.25	35,428,623	*GH16*	LOC_Os02g57770	*CESA5, CESA6, GT8, CESA1, GT43*
	qCLu2.2	2	1	id2010581	4	0.27	24,708,094	*GT8*	LOC_Os02g41520	*CESA7, GT43*
	qCLu3.1	3	2	id3000583	4.95	0.30	990,399	*GH16*	LOC_Os03g02610	*CESA1, CESA5, CESA6, GT8*
								peroxidase	LOC_Os03g02920	*CSLC7, GT43, COBRA, CESA8, CESA1, GT8*
								OsFBX76	LOC_Os03g02550	*GH17, CSLC2, CSLH1, GH3*
	qCLu3.2	3	1	id3001415	4.15	0.25	2,572,897	receptor-like protein kinase 2	LOC_Os03g05140	–
								xyloglucan galactosyltransferase KATAMARI1	LOC_Os03g05110	–
	qCLu6.1	6	1	id6000456	4	0.42	698,632	CSLD2	LOC_Os06g02180	*CESA6, CSLC7, GH16*
	qCLu9.1	9	1	id9004032	4.28	0.36	14,540,527	–	–	–
	qCLu11.1	11	1	id11007727	4.40	0.12	19,960,878	GT6	LOC_Os11g34390	–
Lignin	qLig3.1	3	1	id3010511	4.28	0.15	22,849,697	gibberellin-regulated GASA/GAST	LOC_Os03g41060	*GH16*, UDP-glucuronate

(Continues)

TABLE 2 (Continued)

Trait	QTL	Chr	No. of AM	Peak marker	-log$_{10}$*P*-value	MAF	Position	Candidate gene name	Candidate gene ID	Co-expressed gene list obtained by Genevestigator
	qLig4.1	4	3	id4010278	4.94	0.18	30,279,061	Villin protein	LOC_Os04g51100	*GH16, GH10, CSLH2, COBRA*, cell wall adhesion
								peroxidase precursor	LOC_Os04g51300	PS II 11 kD protein, PS I reaction center subunit III, PS I reaction center subunit IV A
								MADS-box family	LOC_Os04g49150	*GH35*, Sucrose-UDP glucosyltransferase 3
								OsWAK1/50/52/53	LOC_Os04g51030	COBRA7
	qLig4.2	4	1	id4010301	4.1	0.21	29,248,898	GH16	LOC_Os04g51450	–
FM	qFM1.1	1	2	id1006103	4.1	0.10	7,739,829	receptor-like protein kinase 5	LOC_Os01g13800	–
								BRASSINOSTEROID INSENSITIVE 1	LOC_Os01g14510	*GT14*
	qFM8.1	8	2	ud8001139	5.64	0.22	17,381,899	retinal pigment epithelial membrane protein	LOC_Os08g28410	–
	qFM9.1	9	1	id9007481	4.1	0.27	22,070,019	UTP–glucose-1-phosphate uridylyltransferase	LOC_Os09g38030	*GH10*

Note. QTL, quantitative trait loci; AM, [a]ssociated markers; MAF, minor allele frequency; FM, fermentable sugar.

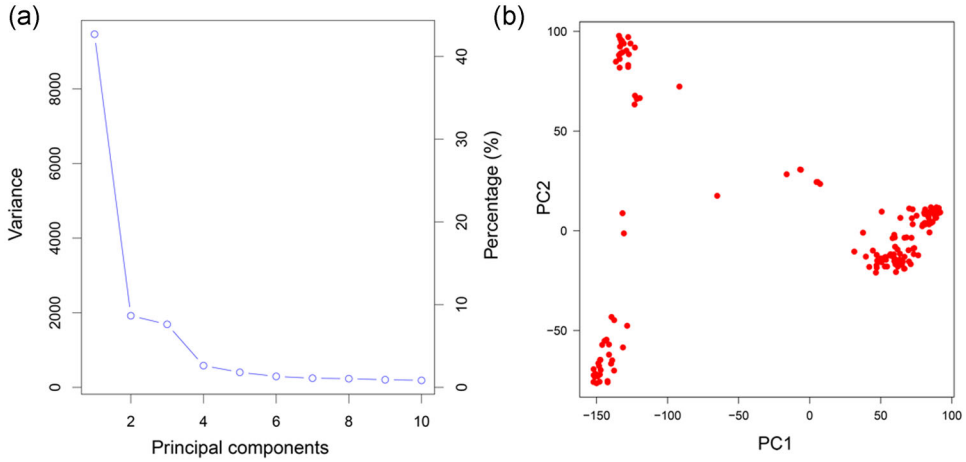

FIGURE 2 Principal component (PC) and population structure. (a) population structure of rice populations collection as reflected by PCs. First two PCs explain 8.5% and 7.4% of the variations, respectively. (b) PC_2 against PC_1 scree plot from GAPIT showing the selection of PCs for association study and results show three main groups

protein), *LOC_Os10g36760* (peptidase S28 family protein), and *LOC_Os06g44910* (similar to glutaredoxin). *SLD2* in first hierarchy was co-expressed with *LOC_Os10g35294* (DUF1218 family protein), *LOC_Os02g19510* (DUF707 family protein) and *LOC_Os03g21540* (resistance protein candidate).

Furthermore, gene expression analysis by RNA-Seq assay revealed differential expression of most of the candidate genes in different tissues. For example, in the case of cellulose content, peroxidase and GT6 genes showed highest expression in immature seed (5 DAP), *OsFBX76* and *GT8* in shoots, *CSLD2* and *LRR40* showed highest expression in pistil, and two *GH16* genes showed highest expression in panicles (Supplemental Table S4). In the case of lignin content, peroxidase and *GH16* were expressed in highest levels in leaves, villin protein in anthers, and MADS-box and gibberellin-regulated GASA in panicles. In the case of fermentable sugar, receptor-like protein kinase 5, UTP-glucose-1-phosphate uridylyltransferase, and BRASSINOSTEROID INSENSITIVE 1 showed highest expression in pistils, panicles, and shoots, respectively (Supplemental Table S4).

Using KEGG analysis, six genes associated with cellulose content were identified with a KEGG orthology identifier (Supplemental Table S5); these include *LOC_Os03g02610* (*K11752*), *LOC_Os03g02920* (*K00430*), *LOC_Os02g57770* (*K08235*), *LOC_Os03g05110* (*K20888*), *LOC_Os02g41520* (*K22809*), and *LOC_Os06g02180* (*K20924*). The KEGG pathway of four of the most putative genes, including CSLD2 (LOC_Os06g02180), is osa01000/or dosa01003 (glycosyltransferases, structural polysaccharides). The gene ontology (GO) analysis using PANTHER (http://pantherdb.org/) revealed cellular component, molecular function, and biological process of the candidate genes. For example, *CSLD2* (*LOC_Os06g02180*), is localized in membrane, PM, and Golgi apparatus, has molecular functions of transferase activity and cellulose synthase (UDP-forming) activity, and involves several biological processes including biosynthetic process, carbohydrate metabolic process, cellulose biosynthesis, multicellular organismal development, anatomical structure morphogenesis, cell differentiation, cell growth, and response to abiotic stimulus (Supplemental Table S5).

4 | DISCUSSION

Most studies on cell walls in plants to date have been performed on the model organism *Arabidopsis thaliana*. The results of these studies are not directly transferable to monocots and cereals due to differences in the cell wall structure of dicots and monocots. Rice straw has the potential to be refined and used in many industries as part of a circular bioeconomy. The initial step of refining (i.e., separation of macromolecular components) is the main burden due to the structural complexity of rice biomass and cell walls. To reduce the inherent recalcitrance, corresponding genes and proteins that define such complex structures need to be identified. A GWAS via establishing the correlation between phenotypes and genotypes has already promised the identification of such elements. Subsequent to such studies, candidate associated genes are required to be functionally characterized. The relevant genes can be used in developing fresh selection-cross breeding programs and new less-recalcitrant transgenic varieties. Here in a rice straw GWAS, we were able to find associated markers and their flanking genes to be the candidates for cellulose, lignin, and fermentable sugar contents. Interestingly, some of these genes are reported for the first time in this study, whereas

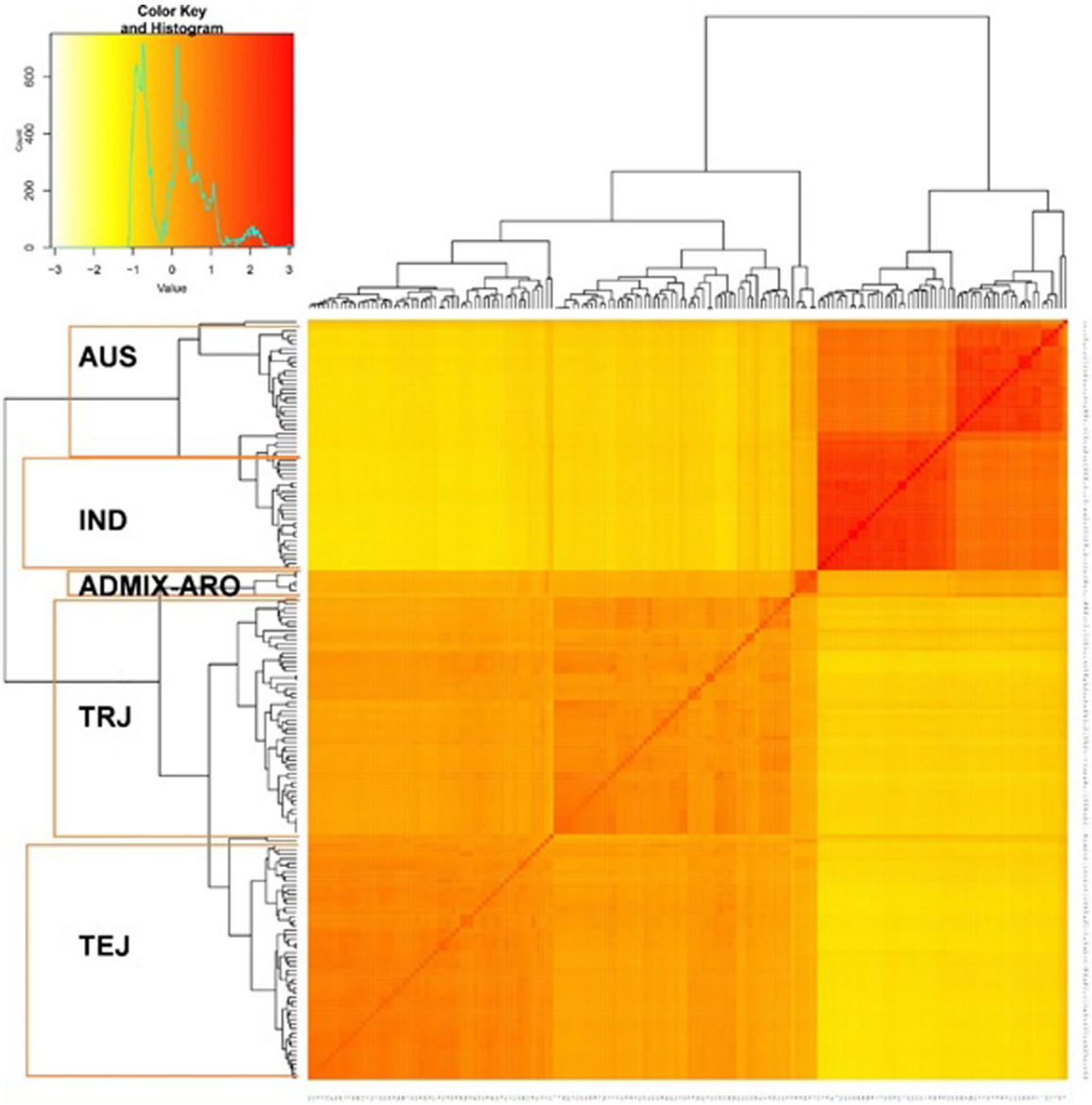

FIGURE 3 Phylogenetic tree in the form of a kinship plot that efficiently separates the 170 accessions in five main geographical clusters of subpopulations: TEJ (Temperate *japonica*), IND (*indica*), AUS (aus), ADMIX-ARO (aromatic + ADMIX), TRJ (Tropical *japonica*). Red indicates the highest correlation between pairs of individuals and yellow indicates the lowest correlation. A hierarchical clustering tree based on the pairwise kinship values for all accessions is displayed along the top and left axes

others were observed in earlier reported data. The levels of lignin and cellulose in our population were similar to those previously reported for rice (Santos et al., 2017).

4.1 | Cellulose content candidate genes and their co-expressors

Based on the marker-trait associations, eight rice genes are reported to be associated with cellulose content (Table 2). Galacturonosyltransferase 9 (GAUT; GT8, Table 2) (Pharr et al., 1981) was the one of the candidate genes with role in pectin and/or xylan biosynthesis (M. Li et al., 2019). Xylans and cellulose intertwine by hydrogen bonds to form a strong and flexible structure (Scheller et al., 2010). Brown et al. (2005) showed that some GT8 family members co-express with *CesA7 (IRX3)* in Arabidopsis, which is involved in secondary cell wall synthesis (Hernández-Blanco et al., 2007). Xylan glucuronosyltransferase is the other reported function for GT8 family members. In this role, it is involved in secondary cell wall thickening at interfascicular fibers and xylem cells (Lee et al., 2012). The deposition and arrangements of glucuronic acids on xylans may play a great role to shape and strengthen the wall (Lyczakowski et al., 2017). Interestingly, xylan backbone biosynthetic family (GT43) was the other co-expressor (Table 2). GT43 family members have shown to be

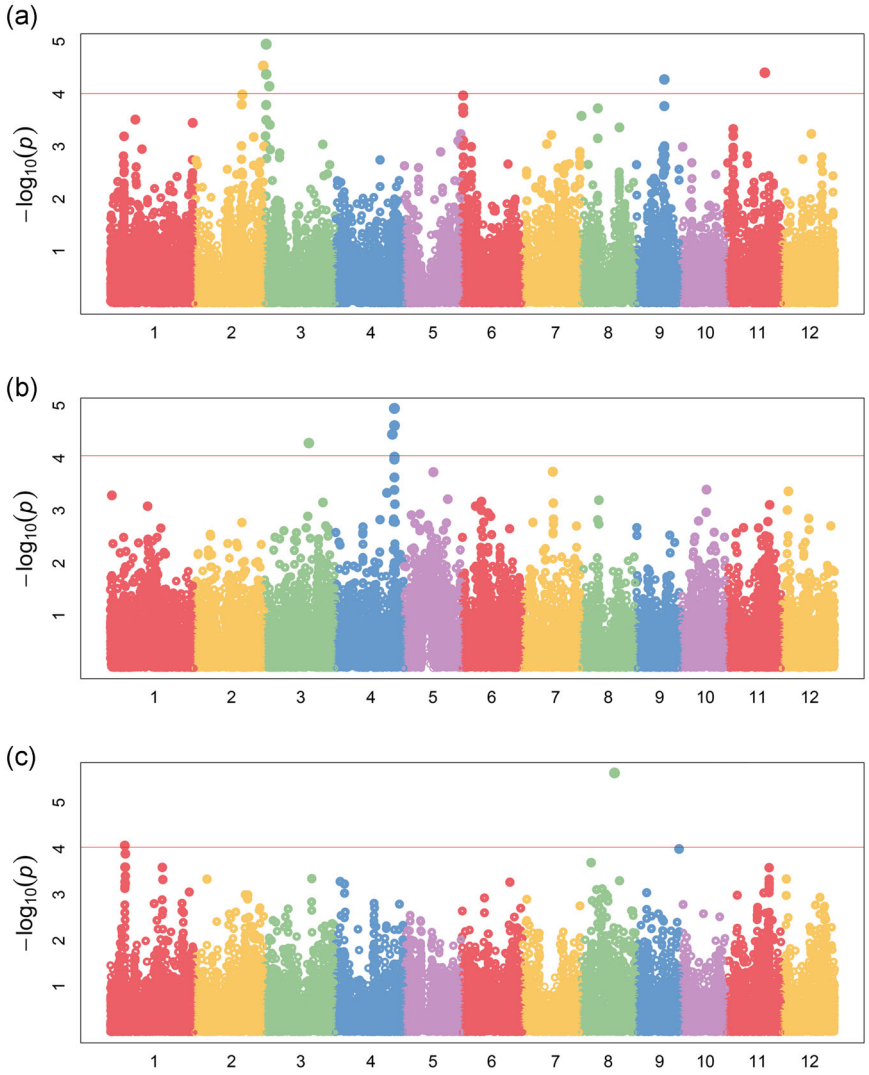

FIGURE 4 Manhattan plot showing quantitative trait loci significant single nucleotide polymorphisms ($-\text{Log}_{10}[P] > 4$; minor allele frequency > 5%) from genome-wide association studies (GWAS) using the BLINK model. The-$\log_{10}$ (p-values) from the GWAS are plotted according to genetic position on each of the 12 rice chromosomes in left side of each Manhattan plot. (a) cellulose, (b) lignin, (c) fermentable sugar

involved in secondary cell wall formation and the definition of cellulose orientation (Ratke et al., 2018; Wang et al., 2016).

Next candidate gene which was associated to cellulose content was xyloglucan galactosyltransferase KATAMARI 1 (LOC_Os03g05110). This protein belongs to GT47 family (A. Wu et al., 2019) and regulates actin microfilament organization. It is involved in cell wall biosynthesis (Tamura et al., 2007). Xyloglucans interact with cellulose in plant cells (Lopes et al., 2010) to make a network that provides flexibility; with proven function in cell elongation (Somerville et al., 2004). Considering the involvement of this gene in xyloglucan turnover and due to the association of cellulose with xyloglucan, it can be concluded that this gene can indirectly affects the amounts of cellulose, but its putative role must be investigated in future.

CslD2, a cellulose synthase-like protein belonging to GT2 and the closest to CesAs (Richmond & Somerville, 2001) was the other candidate gene found in our study. This gene has been shown to be involved in the synthesis of cellulose (Bernal et al., 2008; M. Li et al., 2009). *CSL* mutant analysis in Arabidopsis showed that *CSLD2*, *CSLD3* and *CSLD5* are required for early flower development in addition to stem interfascicular fibers and xylem vessels via their role on cell wall mannan content (Yin et al., 2011). *GH16, CesA6, and CesA7* genes showed to be co-expressed with *CSLD2* (Table 2). *CesA6* and *CesA5* roles were demonstrated in stunted *O. glaberrima* with compromised height by tungro spherical virus (Budot et al., 2014). It was demonstrated that a naturally occurring barley *CesA6* siRNA, not only reduces the abundance of primary wall *Ces*As, several *Csl* genes, and GT8, it is also correlated with the reduction of cellulose biosynthesis (Held et al., 2008). *CesA1* and *CesA8* found to be co-expressed with more than one co-located genes (Table 2). These two *Ces*As that are functionally belong to primary and secondary wall synthesis respectively, were demonstrated to complement each other (S. Li et al., 2013). GO enrichment analysis for CSLD2 revealed that it involves in cellulose biosynthesis (GO: 0016760) and has a cellulose synthase (UDP forming) activity (GO: 0030244) (Figure 5).

FIGURE 5 Gene ontology (GO) enrichment for CSLD2 (LOC_Os06g02180). The enrichment was done with GO terms: (a) biological process and (b) molecular function

Interestingly, a peroxidase gene (LOC_Os03g02920) showed association with cellulose content. It is co-expressed with many cellulose related genes including *CSLC7, GT43, COBRA, CesA8, CesA1*, and *GT8*. Furthermore, here we report the co-expression of COBRA genes with the associated genes of both cellulose and lignin contents (Table 2). COBRA modulate the orientation of cellulose microfibrils and have shown to have defining roles in both cellulose and lignin content (Gritsch et al., 2015; Sato et al., 2010).

4.2 | Lignin content candidate genes and their co-expressors

Based on the marker-trait associations, five rice candidate genes are shown to be associated with lignin content (Table 2). One of the candidate genes was villin protein (*LOC_Os04g51440*). Villin family proteins appear to participate in secondary cell wall formation and thickening (Obudulu et al., 2016). *GH10*, a co-expressor gene with villin, is involved in breaking down lignocellulosic materials and removing residual xylans from pretreated lignocellulosic feedstock (Velasco et al., 2019). Earlier, and in hybrid aspen, the possible roles of *GH10* and *GH16* were proposed via functional genomics and use of microarrays with the application of probes obtained from developing xylem (Aspeborg et al., 2005). Gibberellic acid-stimulated (GASA/GAST) protein, one of our candidate genes (*LOC_Os03g41060*) associated with lignin content, has been shown to be wall associated and involved in regulation of hydroxyl radical levels at specific sites to help in cell division and wall elongation (Furukawa et al., 2006; Trapalis et al., 2017). However, its clear link to lignin content has not been demonstrated.

Another candidate gene was a peroxidase (*LOC_Os04g51300*). It was reported that peroxidases are among lignin degrading and synthesizing enzymes (Falade et al., 2017). This gene was introduced as a candidate gene linked to saccharification potential in an earlier QTL mapping study (Liu et al., 2016). It is co-expressed with photosynthesis-related genes, including PS II 11 kDa protein and PS I reaction center subunit III and IV A (Table 2).

Several genes of WAK family (OsWAK1, 50, 52, 53b) were identified as candidate genes that co-located with associated SNPs with lignin content (Table 2). The OsWAK proteins have kinase activity and bind to pectin fragments in the cell wall (He et al., 1996). These genes have been shown to be required for cell wall expansion (Wagner & Kohorn, 2001). Mutations in some members of this gene family in the plants

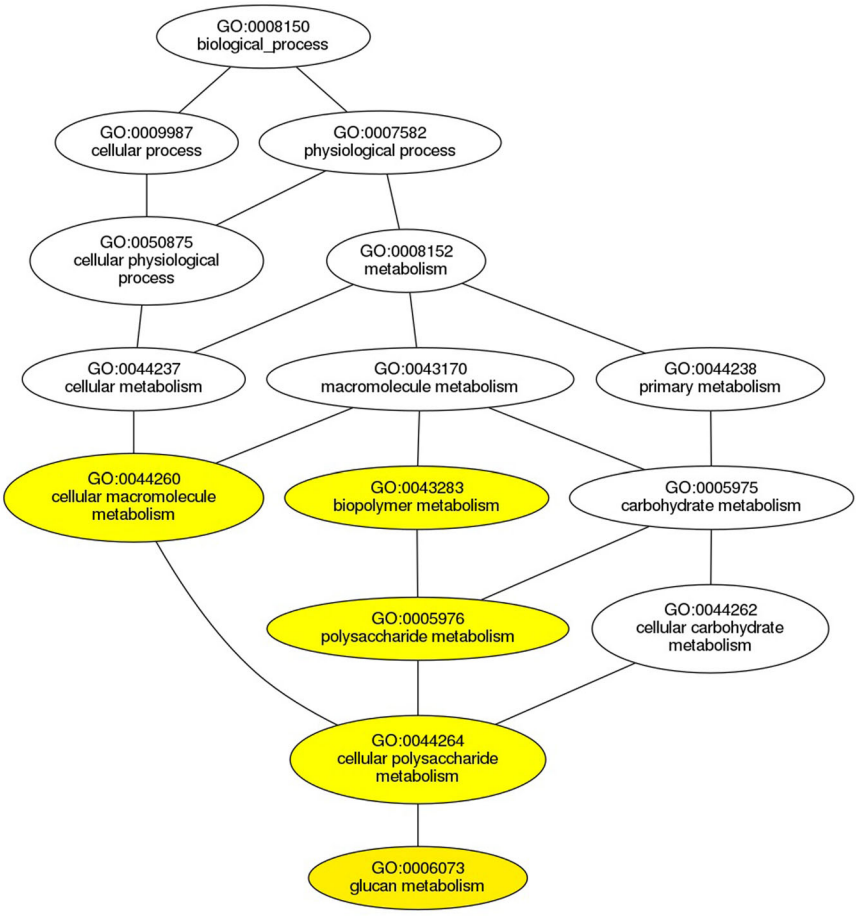

FIGURE 6 Gene ontology (GO) enrichment with GO term of biological process for GH16 (LOC_Os04g51450). The gene involves in polysaccharid metabolism and glucan metabolism

have been studied and it has been shown that they reduce plant growth. For example, mutation in WAK4 has stopped leaf growth (Lally et al., 2001). It has also been suggested that mutations in this gene family alter glucose metabolism (Kohorn et al., 2006). In rice, OsWAK gene family has 122 members (Zhang et al., 2005). OsWAKs found in this study, localize to cell/PM (Supplemental Table S4) as few other OsWAK proteins tested in rice (Cayrol et al., 2016). The relationship between each of these genes and the amount of lignin has not been studied yet, but due to their effect on cell wall expansion (Wagner & Kohorn, 2001), it is likely that they have an indirect effect on the amount of lignin.

Interestingly, GH16 (*LOC_Os04g51450*) was found as a candidate gene associated with lignin content. However, no reports considering the role of GHs on lignin content are available and we believe in the future that a more detailed analysis of the corresponding genes is required to be performed. Based on GO enrichment, the *GH16* gene localizes to cell wall, functions as a hydrolase/transferase, and involves in cellular polysaccharide metabolism (GO: 0044264) and glucan metabolism (GO: 0006073) (Figure 6).

4.3 | Fermentable sugar content candidate genes and their co-expressors

Sugar is not only important molecules for growth, development, and gene expression regulation in plants, they also serve various industrial applications. Thus, comprehensive understanding of the genes and proteins that establish sugar homeostasis within plant cells would have a strong influence in developing future crops. We have found four candidate genes in association to fermentable sugar content (Table 2). UTP-glucose-1-phosphate uridylyltransferase (also known as UGPase for UDP–glucose pyrophosphorylase) was among high-ranking candidate genes for fermentable sugar content, as its important regulatory role already demonstrated in carbohydrate metabolism (N. Li et al., 2014). Rice contains two UGPases, namely Ugp1 and Ugp2 (Chen et al., 2007). Tobacco plants overexpressing the corresponding gene showed an improved height growth (Coleman et al., 2006; Wang et al., 2011). In overexpression of a UDP-glucose pyrophosphorylase genes in Arabidopsis, the role of the corresponding protein in sucrose/polysaccharide metabolism, soluble sugar contents, starch, cellulose, and cell wall

biosynthesis was reported and suggested that the gene would be a fine candidate in improvement of fiber cell development (Coleman et al., 2006, 2007; N. Li et al., 2014; Wang et al., 2011). Another candidate gene was BRASSINOSTEROID INSENSITIVE 1, a ubiquitous leucine-rich repeat receptor of serine/threonine kinase (Friedrichsen et al., 2000). It has been shown that this gene negatively regulates cellulose synthesis in Arabidopsis by phosphorylating cellulose synthase 1 (Sanchez-Rodriguez et al., 2017). Because of the effect of this gene on the amount of cellulose, its effect on the released sugar must be investigated in the future.

5 | CONCLUSIONS

Genome-wide association study, as a forward genetic approach, is a powerful tool for detecting genes defining specific traits. In the present study, genetic diversity for cellulose, lignin, and fermentable sugar contents were analyzed in a panel of rice accessions via GWAS. Several genes were reported for each trait, and the probable roles of these genes in defining corresponding phenotype were discussed. Most of the candidate genes found for cellulose content were directly co-expressed with CesAs in rice straw. Candidate genes for lignin content were mostly kinases. A variety of kinases have been shown to be involved in lignin deposition in cells (Sulis & Wang, 2020). Among the associated genes with markers for fermentable sugar, a kinase with probable releasing capability of cyclic activated protein kinase into intercellular space was noted.

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AUTHOR CONTRIBUTIONS

Rahele Panahabadi: Formal analysis; Visualization; Writing-original draft. Asadollah Ahmadikhah: Conceptualization; Investigation; Project administration; Supervision; Validation; Visualization; Writing-review & editing. Lauren S. McKee: Investigation; Methodology; Project administration; Visualization; Writing-review & editing. Pär K. Ingvarsson: Investigation; Methodology; Software; Validation; Visualization. Naser Farrokhi: Investigation; Project administration; Supervision; Writing-review & editing.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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