The Plant Genome

ORIGINAL RESEARCH

Natural variation and underlying genetic loci of γ-oryzanol in Asian cultivated rice seeds

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Abstract

γ-oryzanol is the most studied component in rice (*Oryza sativa* L.) bran oil. It is not only associated with physiological processes of rice growth and development but also grain quality that is related to human health. Previous studies focused mainly on γ-oryzanol composition and content in various rice cultivars, while its biosynthetic and regulatory pathways remain unknown. Here we present the quantitative identification of γ-oryzanol in rice seeds across 179 Asian cultivated accessions using ultra-performance liquid chromatography–time-of-flight mass spectrometry (UPLC-TOF/MS), which revealed a significant natural variation in γ-oryzanol content among these tested rice accessions. In addition, we present, for the first time, the genome-wide association study (GWAS) on rice seed γ-oryzanol, which identified 187 GWAS signal hot spots and 13 candidate genes that are associated with variable γ-oryzanol content and provided the top 10 rice haplotypes with high γ-oryzanol content for breeding. Collectively, our study provides valuable germplasms for breeding rice cultivars rich in γ-oryzanol and genetic resources for elucidating genetic and biochemical bases of variable γ-oryzanol in rice.

1 | INTRODUCTION

Rice (*Oryza sativa* L.) is the prominent grain consumed by about 3.5 billion people worldwide. The rice bran oil derived

from the rice bran, a byproduct of the rice milling process, is one of the most health-benefiting oils, which can help to remove free radicals in human body and to prevent diseases (Saji et al., 2019). Rice bran oil is rich in bioactive chemicals including the γ-oryzanol, a mixture of phytosteryl ferulates, which is the most studied rice bran oil component so far. It is well known that γ-oryzanol is involved in various biological processes of growth and development in rice by maintaining the integrity and fluidity of membranes, regulating membrane lipid homeostasis and membrane permeability, and mediating

Abbreviations: admix, intermediate rice; cA, *Aus*; cB, Bengal fragrant rice; GWAS, genome-wide association study; HPLC, high-performance liquid chromatography; MLM, mixed linear model; MLMM, multilocus mixed model; PCA, principal component analysis; QTL, quantitative trait loci; SNP, single-nucleotide polymorphism; UPLC-TOF/MS, ultra-performance liquid chromatography–time-of-flight mass spectrometry.

signal transduction (Janson et al., 2009; Valitova et al., 2016). γ-oryzanol also plays a role in stress response such as drought (Liang et al., 2014). In rice seeds, γ-oryzanol exists mainly in the outer layer of the bran, such as the outer seed coat, seed coat, and the nacre layer (Kumar et al., 2015), and it is reported that 91% of γ-oryzanol is present in the oil body of rice bran (Nantiyakul et al., 2012).

Although more than 23 steryl ferulates have been found in γ-oryzanol (Aladedunye et al., 2013), four of them, including cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate, and β-sitosteryl ferulate, dominate in most rice cultivars (Xu & Godber, 1999; Cho et al., 2019). Currently, many qualitative and quantitative methods have been developed to measure γ-oryzanol, which reveals that both contents and compositions of γ-oryzanol vary significantly in different rice seeds depending on sources, genotypes, environmental factors, extraction methods, and determination methods (Bergman & Xu, 2003; Cuevas et al., 2017; Cho et al., 2019). The concentration of γ-oryzanol in conventional and organic brown rice bran is 60–65 mg 100 g^{-1} (Cho et al., 2012), while that in European, Thai and Korean rice bran is 26–63 (Andreas & Karl-Heinz, 2006), 56–108 (Chotimarkorn et al., 2008), and 0.69–61.6 mg 100 g^{-1} (Kim et al., 2015), respectively. In addition, content and composition of γ-oryzanol vary significantly in different rice products. For example, γ-oryzanol in rice bran oils, dehulled brown rice, polished rice, and embryos is 41.5–2,500 (Lu et al., 2014; Cuevas et al., 2017; Sawada et al., 2021), 6–170 (Finocchiaro et al., 2010; Huang & NG, 2011; Cho et al., 2019), 3–63 (Tsuzuki et al., 2019), and ~58 mg 100 g^{-1} (Fang et al., 2003), respectively. Furthermore, contents and compositions of γ-oryzanol differ significantly in rice cultivars with different colors. For example, the average γ-oryzanol content in nine black-purple, four red, four green, and three brown rice cultivars in Japan is 54.2, 47.3, 44.3, and 43.3 mg 100 g^{-1}, respectively (Tsuzuki et al., 2019). However, mechanisms underlying these observed natural variances remain unknown mainly because of the lack of large-scale measurement of γ-oryzanol in rice. The only quantitative trait locus (QTL) mapping for cycloartenyl ferulate and 24-methylenecycloartanyl ferulate was done using the reciprocal chromosomal segment substitution lines derived from a cross between 'Takanari' (*indica*, also known as Xian) and 'Koshihikari' (*japonica*, also known as Geng), which indicates that favorable alleles from *japonica* and *indica* can be used to improve 24-methylenecycloartanyl ferulate and cycloartenyl ferulate in *indica* and to further increase cycloartenyl ferulate in *japonica*, respectively (Nakano et al., 2018).

Besides its function in rice development and stress responses, γ-oryzanol has been increasingly demonstrated to have cholesterol-lowering and antioxidant effects in animals with significant human health promotion potentials (Faulds, 2010; Bunrungpert et al., 2019). Despite these

Core Ideas

- γ-oryzanol is a crucial metabolite that affects rice grain quality and human health.
- Understanding genetic control of γ-oryzanol will aid breeding for healthy rice.
- The natural variations in γ-oryzanol were determined in seeds of 179 Asian cultivated rice accessions.
- The genetic profile of γ-oryzanol was identified by GWAS.
- Our study provides resources for genetic improvement of γ-oryzanol in rice.

abovementioned various proven and expected biological activities, the biosynthetic pathway of γ-oryzanol in plants is still unclear. Considering its molecular structure, the biosynthesis of γ-oryzanol in plants may start with the synthesis of sterols and ferulic acid followed by the esterification of ferulic acid and sterols (Suzuki & Muranaka, 2007). However, the enzymatic system involved in the esterification reaction of ferulic acid and sterol is still vague. It is reported that microsomal phytosterol acyltransferase can convert phytosterols into sterol esters (Benveniste, 2002), while no esterases that esterify ferulic acid and sterols have been identified. Therefore, understanding the esterification of ferulic acid and sterols and its regulation is necessary to elucidate the biosynthesis of γ-oryzanol for the future improvement of rice grain quality. It is reported that sterol O-acyltransferase is involved in the biosynthesis of steryl ferulates in *Arabidopsis thaliana* (L.) Heynh. seeds (Kumar et al., 2015); however, the involvement of its rice orthologes in γ-oryzanol biosynthesis remains unknown.

This study aimed to explore the natural variations of γ-oryzanol and to uncover its underlying genetic mechanisms in 179 Asian cultivated rice seeds using metabolite-based genome-wide association study (GWAS). The identified individual rice cultivars with higher γ-oryzanol and possible candidate causal genes provided valuable germplasm and genetic resources for future breeding of high nutritional quality rice cultivars, respectively.

2 | MATERIALS AND METHODS

2.1 | Plant materials

A total of 179 Asian cultivated rice cultivars (Supplemental Table S1) derived from the 3K Rice Genomes Project (Rellosa et al., 2014) that are rich in genotypic variations were used

in this study. This population included *indica* (XI-1A, XI-1B, XI-2, XI-adm, XI-3), *japonica* (GJ-tmp, GJ-sbtrp, GJ-trp, GJ-adm), *Aus* (cA), Bengal fragrant rice (cB), intermediate rice (admix), and other subgroup cultivars. Seeds of all accessions were planted in Hainan (18.48° N, 110.02° E), China, from December 2019 to April 2020. Harvested and natural dried uniform and full seeds were stored at −20 ˚C until analysis.

2.2 | Extraction of γ-oryzanol

γ-oryzanol in seeds was extracted as previously described (Cho et al., 2019) with minor modifications in sample grinding, extraction volume, and extract concentration. Rice seeds were freeze-dried with a freeze dryer, milled using a grinder (Ergo Chef), and filtered (mesh size: 0.33 mm). The 0.1-g fine powder sample was mixed immediately with 1 ml dichloromethane-methanol (2:1, v/v) in an amber glass tube, which was ultrasonically extracted at 40 ˚C for 20 min (KQ-500VDE), centrifuged at $2,000 \times g$, then the supernatant was transferred to a new glass tube. The extraction was repeated twice more with 0.6 ml and 0.4 ml of extraction solvent for the second and the third extraction, respectively. Three extracts were combined, then evaporated and concentrated overnight in a lyophilizer (Labconco). After being dissolved with 0.5 ml methanol and ultrasonically extracted for 10 min, extracts were filtered through 0.22-μm organic filter membrane (ANPEL SCAA-104) into the high-performance liquid chromatography (HPLC) sample bottle insert tubes. All samples were extracted and examined three times within one day.

2.3 | Measurement of γ-oryzanol

Ultraperformance liquid chromatography (UPLC)–time-of-flight mass spectrometry (TOF/MS) (UPLC1290/TOF/MS 6230, Agilent) was used to measure the four major components of γ-oryzanol qualitatively and quantitatively in rice seeds. Details of UPLC-TOF/MS conditions, chromatographic column, flow rate, mobile phase composition, and detection are shown in Supplemental Table S2. The γ-oryzanol standard (>98.0%) was bought from FUJIFILM Wako Pure Chemical Industries. All solvents used, including methanol, acetonitrile, dichloromethane, and isopropanol, were HPLC grade bought from Sigma-Aldrich. The molecular weight of the major components of γ-oryzanol were identified by TOF/MS by comparing with the γ-oryzanol standard. The external standard method was used to quantitatively calculate γ-oryzanol major component in rice samples. A serial diluted standard solution (0, 10, 20, 30, 40, 50, and 100 μg ml γ-oryzanol^{-1}) was used to build the standard curve. Under the same HPLC conditions, samples were analyzed and amounts of γ-oryzanol were calculated based on the established standard curve. The total γ-oryzanol was a simple sum of all detected four individual major components. The unit of each component and total content of γ-oryzanol was expressed as milligrams of γ-oryzanol equivalent per 100 g dry wt. (mg 100 g^{-1} dry wt.). All extracts and HPLC analysis were performed in triplicate experiments, and the data was presented as mean ± standard deviation (SD).

2.4 | Data multivariate analysis

The software IBM SPSS Statistics 25.0.1.0 and GraphPad Prism 8 (GraphPad, Inc, Chicago) were used to quantitatively analyze the major components and total content of γ-oryzanol in different rice cultivars and to draw graphs, respectively. Microsoft Excel was used to test the Pearson correlation between different γ-oryzanol components, and *P* value of .01 (two-tailed) was used as the significant correlation threshold. The software Plink was used for principal component analysis (PCA), and R software was used to read the test.eigenvec file, while the software GraphPad Prism 8 was used to draw the PCA graph of each metabolite based on the population structure.

2.5 | Genome-wide association study

Based on the high-density single-nucleotide polymorphisms (SNPs) in the 3K Rice Genomes Project (http://snp-seek. irri.org/_download.zul), GWAS of four γ-oryzanol categories content and total content was carried out. The PCA, phylogenetic tree construction, and population relationship cluster diagram were performed with 3,366,822 SNPs in 179 cultivars. Genome association and prediction integrated tool (GAPIT) (Lipka et al., 2012) was employed to perform GWAS and multilocus mixed model (MLMM) (X. Li et al., 2015) was chosen with top three principal components as a covariant after comparing it with mixed linear model (MLM) (Yu et al., 2006).

To determine the candidate interval of significantly associated SNP sites, the plink files were used to calculate the indicator *P* value using Genetic type 1 Error Calculator software (Duggal et al., 2008), which gave 2×10^{-6} as the suggestive *P* value. The significant hits were assigned when the peak SNP correlation exceeded the best threshold. Manhattan plots were drawn by the ggplot2 package in R (Villanueva & Chen, 2019). The quantile-quantile plot in R package was used to show the effect of correlation analysis under this model.

To obtain the loci with the lowest *P* value, the ±100-kb genome window near the most significant SNP in each peak obtained from GWAS was used as the QTL segment to screen
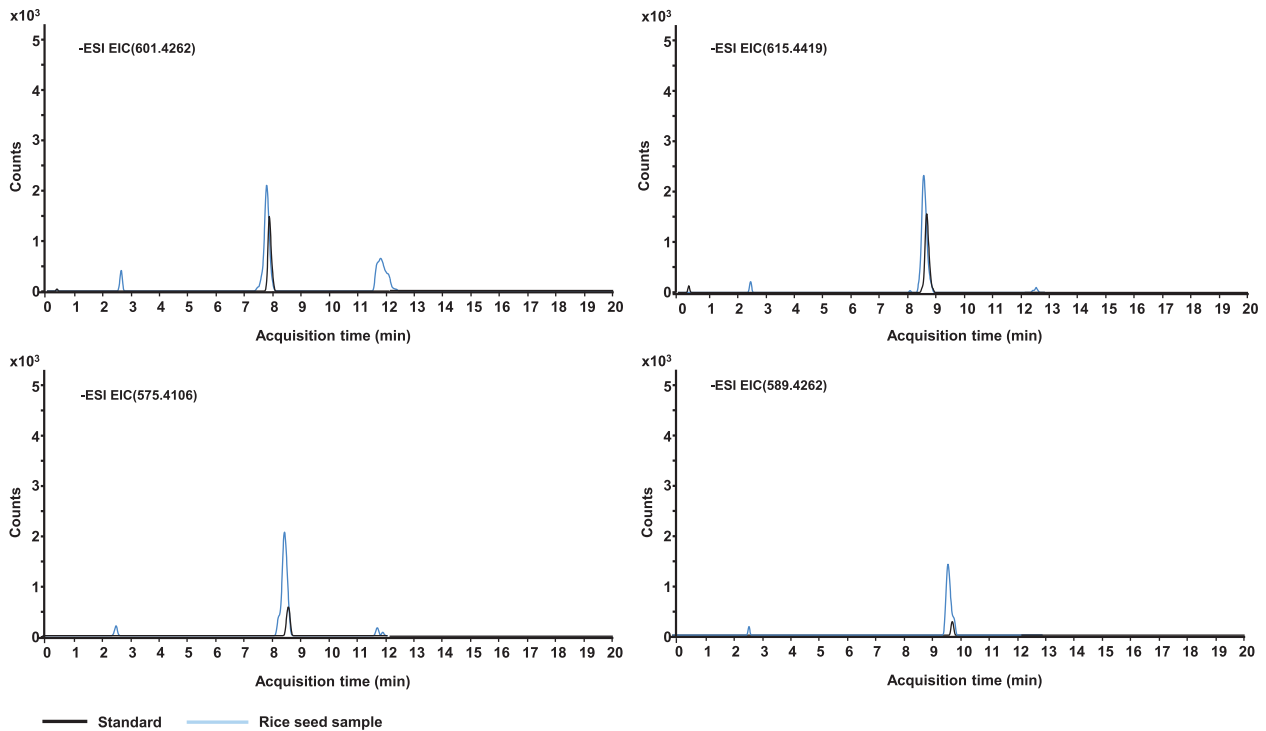
FIGURE 1 Quantitative determination of γ-oryzanol major components in rice seed samples (grey) and in γ-oryzanol standard samples (light blue): (a) cycloarteryl ferulate, (b) 24-methylenecycloartanyl ferulate, (c) campesteryl ferulate, and (d) β-sitosteryl ferulate

candidate genes. A manual search for candidate genes with a putative role in γ-oryzanol was carried out considering all the annotated genes included in the above indicated genomic regions through the screening of the *O. sativa* genomic reference sequence (RGAP: http://rice.uga.edu). To identify the candidate genes, homologous genes in *Arabidopsis* (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/#search) and expression pattern of candidate genes (https://ricexpro.dna.affrc.go.jp/RXP_0012/gene-search.php) were analyzed using data from public database. Expression patterns of candidate genes were visualized using Microsoft Excel.

2.6 | SNP effects analysis

The 3K_RG_SNP database SNP-Seek (http://snp-seek.irri.org) was used to perform analysis of major allele, minor allele, and the corresponding SNPs in promising lines with high γ-oryzanol identified in this research, while Excel was used for SNP effect analysis.

2.7 | Promoter analysis of candidate genes

The online PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to analyze the promoter *cis*-acting elements in candidate genes.

3 | RESULTS

3.1 | The measurement of γ-oryzanol by UPLC-TOF/MS

In this study, a modified UPLC-TOF/MS method was used to analyze the four major components of γ-oryzanol in both standard (commercial reagent) and rice seed samples. Although UPLC is principally similar to HPLC, UPLC has a faster sample processing speed and uses fewer solvents; TOF/MS also has better ion resolution (Xu & Howard, 2012). Our results confirmed the advantages of UPLC over HPLC in sample processing speed, solvent use, and ion resolution.

The chromatograms obtained from the γ-oryzanol in standard solutions and rice seed samples are shown in Figure 1. The molecular weight of cycloartenyl ferulate (Figure 1a, [M-H]⁻), 24-methylenecycloartanyl ferulate (Figure 1b, [M-H]⁻), campesteryl ferulate (Figure 1c, [M-H]⁻), and β-sitosteryl ferulate (Figure 1d, [M-H]⁻) was 601.4262, 615.4419, 575.4106, and 589.4262 m/z, respectively. The retention time of them was between 7 and 10 min. The electrospray ionization mass spectrometry (ESI-MS) data were shown in Supplemental Figure S1.

To quantitatively measure the four individual components of γ-oryzanol, the peak area of each component in seed samples was compared with that in the standard samples

**TABLE 1** Statistics on the composition and content of rice γ-oryzanol (Mean ± SD; mg 100 g^{-1} dry wt)

γ-oryzanol	Cycloarteryl ferulate	Methyl-enecycloartanyl ferulate	Campesteryl ferulate	β-sitosteryl ferulate	Total γ-oryzanol
Average	7.90 ± 0.47	21.78 ± 1.42	10.90 ± 0.85	11.38 ± 0.87	52.08 ± 3.35
Minimum					
3K ID	CX108	CX111	CX142	CX108	CX142
Content	1.04 ± 0.18	4.24 ± 0.30	0.55 ± 0.07	0.36 ± 0.13	9.84 ± 0.68
Maximum					
3K ID	CX101	IRIS_313-11202	IRIS_313-11245	IRIS_313-11202	IRIS_313-11245
Content	26.41 ± 0.3	113.09 ± 10.55	58.86 ± 4.98	57.78 ± 0.07	246.68 ± 9.79

(Figure 1) and the composition of each component was then calculated using the formula derived from the standard curve of each component (Supplemental Figure S2).

3.2 | Natural variations of γ-oryzanol

Based on the UPLC-TOF/MS results, the average total content of γ-oryzanol detected in these 179 rice accessions was 52.08 ± 3.35 mg 100 g^{-1} dry wt. (Table 1). The highest and the lowest level of total content of γ-oryzanol detected among these 179 rice accessions were 246.68 ± 9.79 and 9.84 ± 0.68 mg 100 g^{-1} dry wt., respectively, a difference of ~25×. The average contents of cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate, and β-sitosteryl ferulate were 7.90 ± 0.47, 21.78 ± 1.42, 10.90 ± 0.85, and 11.38 ± 0.87 mg 100 g^{-1} dry wt., respectively. Furthermore, the highest and the lowest level of individual steryl ferulates, namely cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate, and β-sitosteryl ferulate, detected among these 179 rice accessions were 26.41 ± 0.3 and 1.04 ± 0.18 mg 100 g^{-1} dry wt., 113.09 ± 10.55 and 4.24 ± 0.30 mg 100 g^{-1} dry wt., 58.86 ± 4.98 and 0.55 ± 0.07 mg 100 g^{-1} dry wt., and 57.78 ± 0.07 and 0.36 ± 0.13 mg 100 g^{-1} dry wt., respectively, a difference of 25.40×, 26.67×, 17.98×, and 160.50×, respectively.

As shown in Figure 2, levels of total γ-oryzanol in >68% rice accessions were in the range from 9.48 to 43.67 mg 100 g^{-1} dry wt. Cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate, and β-sitosteryl ferulate accounting for 16.8, 40.94, 20.46, and 21.46% of the total γ-oryzanol, respectively. Levels of cycloartenyl ferulate in ~60% rice accessions were in the range from 3.53 to 8.50 mg 100 g^{-1} dry wt., levels of 24-methylenecycloartanyl ferulate in >70% rice accessions were in the range from 4.24 to 19.79 mg 100 g^{-1} dry wt. Similarly, levels of campesteryl ferulate in >65% rice accessions were in the range from 0.55 to 8.88 mg 100 g^{-1} dry wt., levels of β-sitosteryl ferulate in ~80% rice accessions were in the range from 0.36 to 12.16 mg 100 g^{-1} dry wt. Less than
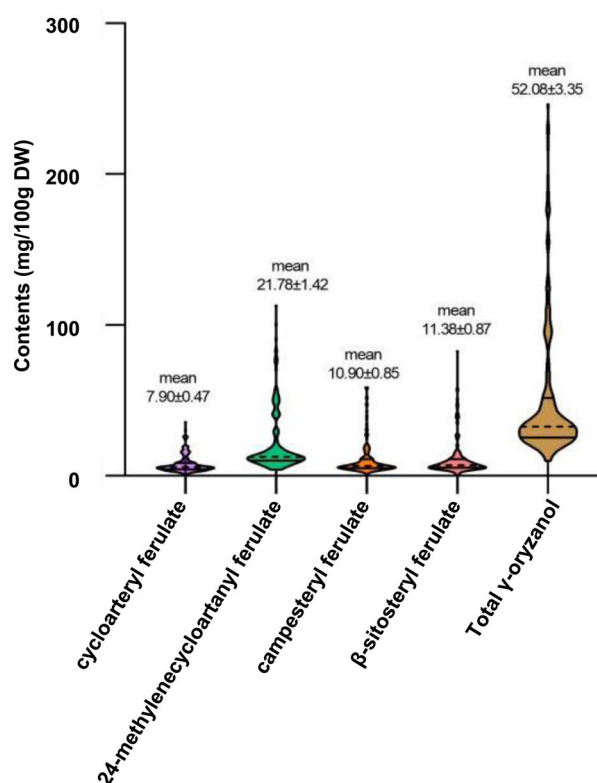
FIGURE 2 Violin diagram of the principal components and total content of rice γ-oryzanol

1% rice accessions contained either the highest individual or the highest total γ-oryzanol. The revealed significant natural variations among both total γ-oryzanol and individual components of γ-oryzanol among these detected 3K rice accessions highlighted the great potential to increase γ-oryzanol contents in Asian cultivated rice accessions.

Based on 3K rice subgroup classification, the 179 rice accessions used in this study could be easily classified into five specific categories, namely, *indica*, *japonica*, cA, cB, and admix (Supplemental Table S1). Therefore, PCA analysis was performed to conduct a comparative analysis between subgroups and to draw diagrams of the composition and total content of γ-oryzanol based on the genetic relationship between
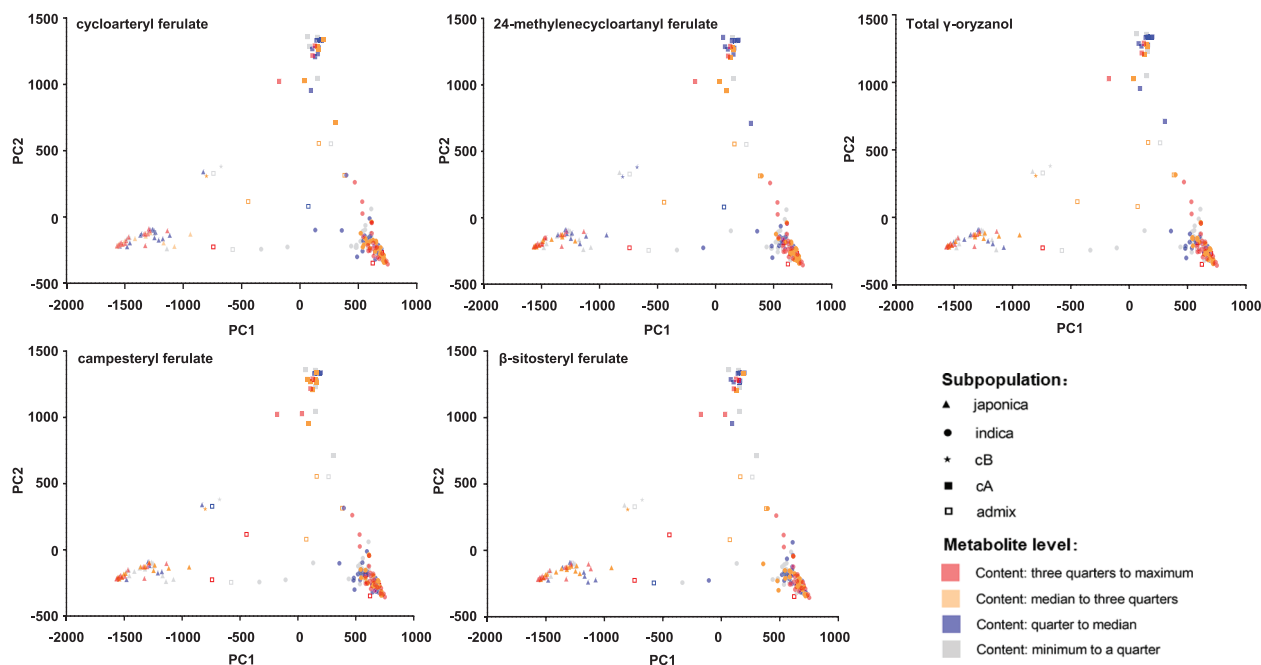
FIGURE 3 Principal component analysis diagram of composition and total content of γ-oryzanol based on population structure. cA, *Aus*; cB, Bengal fragrant rice; admix, intermediate rice

TABLE 2 Pairwise correlation coefficients of major component and total content of γ-oryzanol in rice

Trait	Cycloarteryl ferulate	24-methylenecycloartanyl ferulate	Campesteryl ferulate	β-sitosteryl ferulate	Total γ-oryzanol
Cycloarteryl ferulate	1	0.885[**]	0.764[**]	0.775[**]	0.888[**]
24-methylenecycloartanyl ferulate	0.885[**]	1	0.892[**]	0.844[**]	0.972[**]
Campesteryl ferulate	0.764[**]	0.892[**]	1	0.912[**]	0.954[**]
β-sitosteryl ferulate	0.775[**]	0.844[**]	0.912[**]	1	0.937[**]
Total γ-oryzanol	0.888[**]	0.972[**]	0.954[**]	0.937[**]	1

**Significant at the .01 probability level.

cultivars (Figure 3). It was evident that contents of γ-oryzanol in *japonica*, *indica*, and cA subgroups are more dispersed than those of the other two subgroups. Moreover, as compared with *indica*, *japonica* cultivars contained relatively higher contents of γ-oryzanol. However, difference in γ-oryzanol content between *indica* and *japonica* subgroups was not significant.

3.3 | Correlations between individual and total components of γ-oryzanol

The correlation between the individual component and total γ-oryzanol in rice seeds was further evaluated to correlate those parameters for future breeding applications. Results showed robust positive correlations between total γ-oryzanol and various individual components (Table 2). Among them, the strongest positive correlation was found between total γ-oryzanol and 24-methylenecycloartanyl ferulate. Among four individual components, the positive correlation between campesteryl ferulate and β-sitosteryl ferulate was the strongest followed by those between 24-methylenecycloartanyl ferulate and campesteryl ferulate, and between cycloartenyl ferulate and 24-methylenecycloartanyl ferulate. Considering that 24-methylenecycloartanyl ferulate correlated highly with total γ-oryzanol and each of the other three individuals (campesteryl ferulate, cycloartenyl ferulate, and β-sitosteryl ferulate), there must be a highly correlated metabolic network in rice seeds that are responsible for γ-oryzanol production.
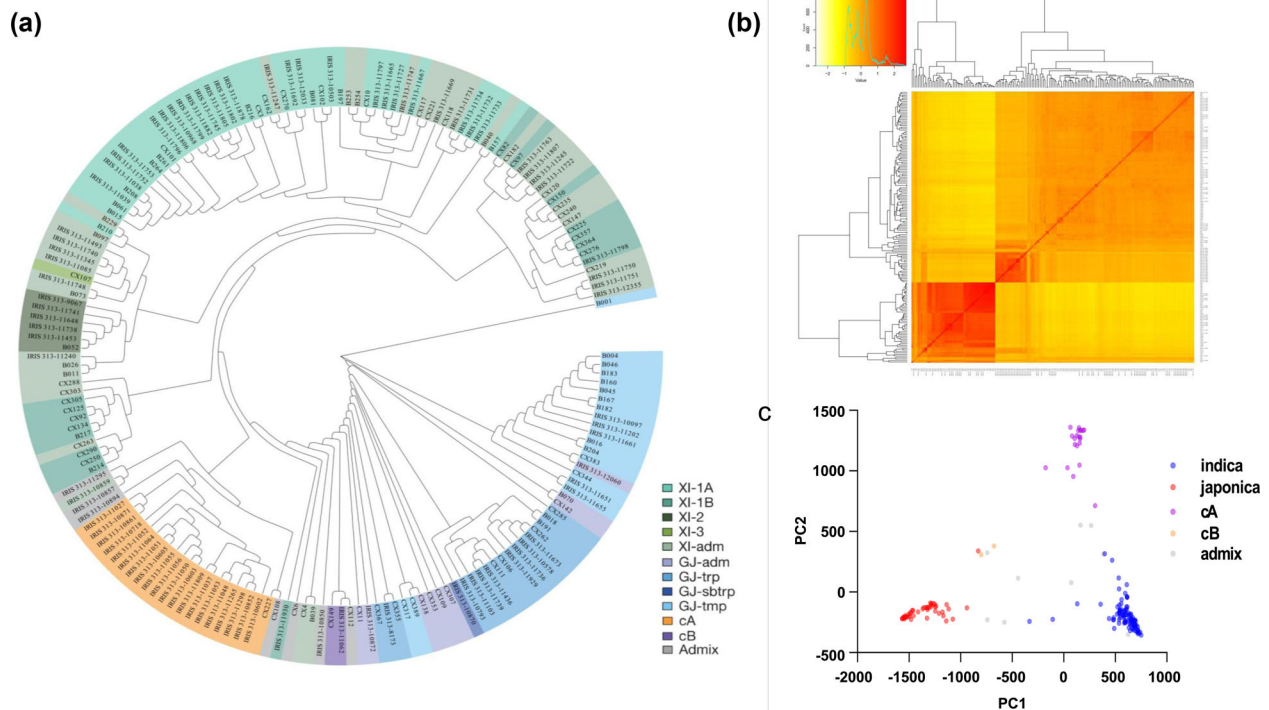
FIGURE 4 Population structure of 179 rice accessions used in this study: (a) population evolutionary tree; (b) kinship values; (c) variation of the top two principal components. cA, *Aus*; cB, Bengal fragrant rice; admix, intermediate rice

3.4 | GWAS analysis of γ-oryzanol

GAPIT was used to evaluate the population structure of these 179 rice accessions (Figure 4a), and the cluster diagram of kinship values (Figure 4b) showed a moderate genetic distance among these accessions. Next, the whole accessions were subdivided into 12 subgroups based on the species' source information, which was further classified into five distinct cultivar groups at the macro level: *indica*, *japonica*, *Aus*, basmati types, and a small group of admixed types. The PCA results showed that the population structure was clear and extensively abundant (Figure 4c), indicating that GWAS study on this collection could be properly carried out. We also performed power analysis based on the broad-sense heritability (H^2), which showed that the H^2 values of the cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate, β-sitosteryl ferulate and total γ-oryzanol were .993, .994, .989, .993, and .993, respectively. These analyses results indicated that γ-oryzanol is significantly affected by genetic factors.

Because the MLM (Yu et al., 2006) did not fit well (Supplemental Figure S3), the MLMM (X. Li et al., 2015) was selected for GWAS analysis to associate levels of four individual components and the total γ-oryzanol detected in 179 rice accessions with corresponding SNP markers. In total, 187 SNPs (*P* value $\leq 2 \times 10^{-6}$) for five metabolic traits were iden-

tified. Among them, 26, 28, 10, 43, and 80 SNPs were found for trait cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate, β-sitosteryl ferulate, and total γ-oryzanol, respectively. The QTL were found to be concentrated on chromosomes 6, 1, 7, and 12 (Figure 5). In particular, some QTL, such as *qMT06*, *qMCT07*, *qCS03*, *qCMT07*, *qMST12*, were found to be highly associated with multiple individual components of γ-oryzanol likely because of gene linkage or pleiotropy (Table 3, Figure 5).

Because rice bran oil is mainly distributed in the embryo rather than in the endosperm (McCaskill & Zhang, 1991; Faria et al., 2012), likely candidate genes responsible for variable γ-oryzanol were searched based on (a) location ≤100 kb to the most significant peak SNPs (Bai et al., 2016), (b) annotation with or biologically associated with lipid traits, and (c) high expression in the embryo and in the endosperm. Thus, a shortlist of a total of 13 candidate genes was generated (Table 3). Among them, five candidate genes (*LOC_Os06g06350*, *LOC_Os06g06520*, *LOC_Os07g10130*, *LOC_Os07g10600*, and *LOC_Os12g42090*) were highly associated with total γ-oryzanol and 24-methylenecycloartanyl ferulate in tested rice seeds according to the high positive correlation between two traits (Table 2). Interestingly, they were associated with other different individual components. For example, *LOC_Os07g10130* was associated also with campesteryl

TABLE 3 List of quantitative trait loci (QTL) and candidate genes (multilocus mixed model, P value $\leq 2 \times 10^{-6}$)

Trait	QTL	PVE %	Pos Chr	Pos bp	P value	Candidate gene locus	Description	Orthologous genes in *Arabidopsis*
24-methylenecycloartanyl ferulate	qMT06	3.53	6	3,045,019	1.48×10^{-6}	LOC_Os06g06350	AMP-binding enzyme	AT1G64400/LACS3
Total γ-oryzanol		3.19				LOC_Os06g06520	GDSL-like lipase/acylhydrolase	AT4G01130/AtGELP76
24-methylenecycloartanyl ferulate	qMCT07	4.95	7	5,519,759	4.78×10^{-7}	LOC_Os07g10130	squalene synthetase	AT4G34640/SQS1
Campesteryl ferulate		3.62						
Total γ-oryzanol		4.36						
Cycloarteryl ferulate	qCS03	3.37	3	2,028,812	5.47×10^{-7}	LOC_Os03g04340	C-methyltransferase	AT1G20330/SMT2
β-sitosteryl ferulate		3.62						
Cycloarteryl ferulate	qCMT07	4.28	7	5,686,218	1.30×10^{-6}	LOC_Os07g10600	Cycloartenol-C-24-methyltransferase1	AT5G13710/SMT1
24-methylenecycloartanyl ferulate		4.49						
Total γ-oryzanol		4.02						
24-methylenecycloartanyl ferulate	qMST12	3.42	12	26,013,014	2.76×10^{-11}	LOC_Os12g42090	Methyltransferase domain containing protein	
β-sitosteryl ferulate		3.70						
Total γ-oryzanol		3.00						
Campesteryl ferulate	qC09	2.31	9	20,178,636	7.72×10^{-7}	LOC_Os09g34090	Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating	AT2G26260/3BETAHSD/D2
Campesteryl ferulate	qC061	2.67	6	2,721,447	8.86×10^{-7}	LOC_Os06g05900	Methyltransferase	AT2G30920/COQ3
						LOC_Os06g05910	Methyltransferase domain containing protein	
Campesteryl ferulate	qC062	2.48	6	2,811,696	1.33×10^{-6}	LOC_Os06g06230	SGNH hydrolase-type esterase	AT5G45920
						LOC_Os06g06250	GDSL-like lipase/acylhydrolase	AT5G45910/GELP10
β-sitosteryl ferulate	qS01	3.64	1	39,348,864	1.64×10^{-6}	LOC_Os01g67530	AMP-binding enzyme	
						LOC_Os01g67600	CXE carboxylesterase	AT5G14310/CXE16

Note. PVE, phenotypic variation explained; Chr, chromosome; Pos, position.
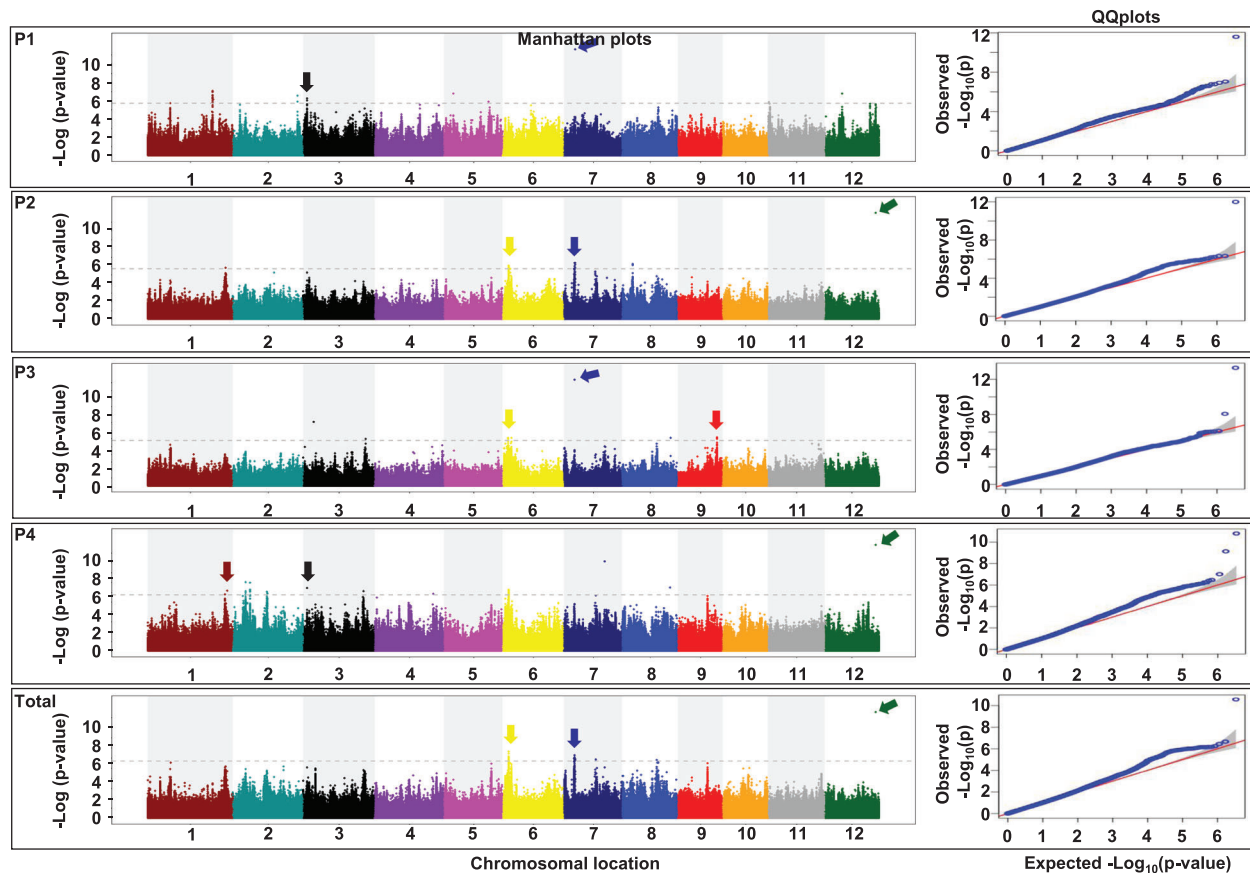
FIGURE 5 Manhattan plots and quantile–quantile plots of genome-wide association mapping for main components and total contents of γ-oryzanol in rice seed

ferulate, *LOC_Os07g10600* with cycloartenyl ferulate, and *LOC_Os12g42090* with β-sitosteryl ferulate (Table 3). In addition, five and two candidate genes were associated only with campesteryl ferulate and β-sitosteryl ferulate, respectively (Table 3). However, no single gene was found to be associated only with cycloartenyl ferulate (Table 3).

3.5 | In silico analysis of γ-oryzanol

In silico expression analysis results showed that these 13 candidate genes all have a specific expression in different developmental stages of the embryo and the endosperm, and 10 of them showed relatively higher expression in the embryo (Supplemental Figure S5). These expression data together with their association with various γ-oryzanol components indicated that these candidate genes could be causal genes for observed natural variation of γ-oryzanol in Asian cultivated rice.

In silico putative *cis*-elements analysis in the promoters of these 13 candidate genes showed that besides common *cis*-elements, such as TATA-box, all of them contained several elements related to seed development, stress response, hor-

mone response, and light response in their promoter regions (Supplemental Table S4), verifying their possible roles in seed development and suggesting their regulation by factors such as hormone and light. Notably, all of them contained at least one MYB binding or recognition site in their promoter regions, including the MYBHv1 binding site CCAAT-box, Myb-binding site, MYB recognition site, and MYB binding site involved in flavonoid biosynthetic genes regulation (MBSI), indicating a possible conservative regulatory mechanism for γ-oryzanol biosynthesis in Asian cultivated rice.

3.6 | Allele variation and SNP effect

To further analyze and excavate the elite alleles and haplotypes and to determine the most suitable parents for breeding, the major and minor alleles and SNP effects for each trait–SNP association loci were analyzed. The results (Table 4) showed that four major alleles, including SNPs at 217,851,666; 21,8018,125; 183,804,567, and 183,894,816 bp, had increasing positive effect among five metabolic traits, each with different percentage phenotypic variation explained; they were defined as elite allele that play impor-

TABLE 4 List of single-nucleotide polymorphisms (SNPs) and their SNP effects (multilocus mixed model, P value $\leq 2 \times 10^{-6}$)

| Trait | Peak SNP | Percentage variation explained | Allele[a] | SNP effect[b] | |
				Major allele	Minor allele
	bp	%			
24-methylenecycloartanyl ferulate	184,128,139	3.53	T/A	22.36	21.80
Total γ-oryzanol		3.19		51.38	55.12
24-methylenecycloartanyl ferulate	217,851,666	4.95	G/A	29.09	21.31
Campesteryl ferulate		3.62		16.63	10.49
Total γ-oryzanol		4.36		68.65	51.03
Cycloarteryl ferulate	81,236,985	3.37	T/C	7.47	9.07
B-sitosteryl ferulate		3.62		10.40	14.36
Cycloarteryl ferulate	218,018,125	4.28	C/T	8.11	7.93
24-methylenecycloartanyl ferulate		4.49		24.33	21.64
Total γ-oryzanol		4.02		58.22	51.78
24-methylenecycloartanyl ferulate	371,726,677	3.42	T/C	19.53	22.00
B-sitosteryl ferulate		3.70		9.76	11.60
Total γ-oryzanol		3.00		47.00	52.67
Campesteryl ferulate	290,651,186	2.31	A/G	10.10	11.09
Campesteryl ferulate	183,804,567	2.67	A/G	11.50	10.57
Campesteryl ferulate	183,894,816	2.48	T/C	11.60	9.95
β-sitosteryl ferulate	39,348,864	3.64	A/G	10.39	14.86

[a]Allele, major allele/minor allele; underlined base is the reference allele.

[b]SNP effect: The average trait value of this allele in the whole population.

tant roles in regulation of individual and total contents of γ-oryzanol.

In addition, we performed haplotype analysis on top 10 rice lines rich in total γ-oryzanol (Supplemental Table S5), all had at least one abovementioned positive peak SNP. Interestingly, three lines (IRIS_313-11202, IRIS_313-11739, and IRIS_313-12060) contained all the same alleles including two abovementioned positive peak SNPs, while an additional five lines (IRIS_313-11245, IRIS_313-11039, IRIS_313-11038, IRIS_313-11797, and IRIS_313-11240) contained all the same alleles including the two abovementioned positive peak SNPs. These results confirmed the roles of these alleles in the diversity of γ-oryzanol and provided germplasm resources for improving rice seeds quality and increasing the content of γ-oryzanol in rice.

4 | DISCUSSION

As an important biologically active substance in rice bran oil, γ-oryzanol plays an important functional role in both rice development, grain nutrition quality, and human health. Understanding the genetic and biochemical bases of γ-oryzanol in cultivated rice accessions, therefore, is of great significance, not only in rice breeding but also in rice fundamental studies. In this study, we explored the natural variation of γ-oryzanol in Asian cultivated rice and its underlying genetic mechanisms, which generated 13 candidate genes and laid a solid foundation for the further elucidation of the γ-oryzanol metabolism pathway and improvement of rice nutritional quality via marker-assisted breeding.

4.1 | Natural variation of γ-oryzanol in rice

To explore the γ-oryzanol diversity in rice, we first established a UPLC-TOF/MS-based method to qualitatively and quantitatively determine γ-oryzanol contents in tested 197 Asian cultivated rice lines, and our qualification and quantification results coincided with previous reports (Benveniste, 2002; Cho et al., 2012; Sookwong et al., 2016; Tsuzuki et al., 2017; Tsuzuki et al., 2019; Nakano et al., 2021), in which the

γ-oryzanol in rice contains four major individual steryl ferulates each had unique ESI-MS features. The total γ-oryzanol detected in this study ranged from 9.84 ± 0.68 to 246.68 ± 9.79 mg 100 g^{-1} dry wt., which was consistent with previous reports (Cho et al., 2012; Sookwong et al., 2016; Tsuzuki et al., 2019; Nakano et al., 2021). It is worthy to note that the γ-oryzanol content measured and presented in this study was from rice seeds (with hull), different from most previous studies, in which the γ-oryzanol content measured and presented were from dehulled brown grains. In this study, we found that although γ-oryzanol content can be detected in rice hull, its amount was very low, ~4.46% of the brown grain Supplemental Figure(S6). In addition, compositions of each individual contents detected in this study (Figure 2) were similar to those previously reported (Cuevas et al., 2017; Cho et al., 2019; Tsuzuki et al., 2019; Sawada et al., 2021). These results verified the accuracy of the established UPLC-TOF/MS method.

Our quantification data clearly showed natural variations in composition and contents of γ-oryzanol across these 179 rice accessions (Figures 2 and 3), which was in line with previous studies (Cho et al., 2019), providing useful rice germplasms for breeding rice cultivars with high γ-oryzanol contents for human consumption or industrial use through conventional hybridization. Remarkably, the result comparing different subgroups indicated that although *japonica* subspecies contain relatively higher contents of γ-oryzanol than *indica* subspecies, the difference between these two subspecies is not significant (Figure 4). This result was somewhat not consistent with a previous study, in which the total γ-oryzanol content of some *japonica* rice cultivars is significantly higher than that of *indica* rice (Kato et al., 2017). This inconsistency could be derived from the different cultivars and measurement methods used.

Correlation results showed that there is a significant correlation between different components of γ-oryzanol, which was consistent with previous studies (Cho et al., 2019), suggesting the existence of a genetically regulated network of γ-oryzanol in rice and providing insights into biosynthetic pathways of γ-oryzanol in plants. The high correlation between total γ-oryzanol and 24-methylenecycloartanyl ferulate revealed in this study could be used for quick screening for rice cultivars rich in total γ-oryzanol. Interestingly, correlations among individual γ-oryzanol components (Table 2) were confirmed by GWAS analysis (Table 3), in which several candidate genes were associated more than one individual component of γ-oryzanol.

4.2 | GWAS analysis on γ-oryzanol

Integrating metabolomics and genetics can provide new insights into the genetic and biochemical bases underlying the natural variations in metabolites. Previously, QTL mapping for γ-oryzanol in rice has been reported using recombinant inbred lines, backcross inbred lines, and chromosomal segment substitution lines (Kato et al., 2017; Nakano et al., 2018). However, no GWAS analysis has been carried out for rice γ-oryzanol. Taking the advantage of abundant SNP data of the 3K Rice Genomes Project, GWAS analysis for rice γ-oryzanol was performed, using MLMM (Figure 5) but not MLM (Supplemental Figure S3), for the first time in this study, which revealed in total 187 GWAS signal hot spots and obtained 13 candidate genes that might be responsible for observed natural variations in γ-oryzanol. Our results confirmed previous findings that GWAS is a reliable choice for mining and identifying important genes because of the relatively short linkage disequilibrium fragments (Y. Li et al., 2015) and that GAPIT is reliable in population structure and relative kinship estimates (Lipka et al., 2012; Rellosa et al., 2014).

4.3 | Candidate genes associated with variable γ-oryzanol

All these identified candidate genes or their *Arabidopsis* orthologues, according to the annotation in Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) and the Arabidopsis Information Resource (TAIR) (https://www.arabidopsis.org/), are related to lipid metabolism, likely involving in the biosynthesis of γ-oryzanol. It is worth noting that orthologues of *LOC_Os03g04340* and *LOC_Os07g10600* have been identified in *Arabidopsis*, both code for sterol methyltransferases contributing to the synthesis or metabolism of the phytosterol 24-methylene cycloartenol, the precursor of 24-methylenecycloartanyl ferulate (Diener et al., 2000). Based on GWAS analysis, *LOC_Os07g10600* likely participates in the biosynthesis of 24-methylenecycloartanyl ferulate. The *Arabidopsis* orthologue of *LOC_Os07g10130* is the only functional squalene synthase to produce squalene, the first committed precursor for sterol, brassinosteroid, and triterpene biosynthesis (Busquets et al., 2008). Overexpressing *SQS1* in mesophyll protoplasts significantly increased the biosynthesis of triterpenoid (Johnson et al., 2014). Overexpressing of the maize orthologue *GmSQS1* in *Arabidopsis* increased the production of phytosterols in seeds (Nguyen et al., 2013). Combined with GWAS analysis, *LOC_Os07g10130* might play an important role in the synthesis of 24-methylenecycloartanyl ferulate, campesteryl ferulate, and thus, total γ-oryzanol. The *Arabidopsis* orthologue of *LOC_Os09g34090* encodes a 3β-hydroxysteroid dehydrogenase/C-4 decarboxylases involved in the biosynthesis of phytosterols, and overexpressing of it altered sterol composition and auxin transport (Kim et al., 2012). In GWAS analysis, this gene was highly associated with campesteryl ferulate, therefore, *LOC_Os09g34090* pos-

sibly regulates the synthesis of campesteryl ferulate, a pathway is closely related to phytosterols.

Functions of other left candidate genes have not been reported in any plant species; however, in silico expression and *cis–trans* element analyses of those 13 putative candidate genes not only verified their involvement in seed development but also suggested a possible conservative regulatory mechanism of γ-oryzanol in rice (Supplemental Figure S5; Supplemental Table S4). Although the real causative ones are not identified in this study, the overlapping of the correlation between the identified candidate genes and variable components or total γ-oryzanol indicated that the composition and content of γ-oryzanol in rice is clearly under genetic control. This work, therefore, facilitates future elucidation of the genetic network for γ-oryzanol and for breeding high-content γ-oryzanol rice cultivars using molecular marker-assisted approaches.

5 | CONCLUSION

This study revealed the significant natural variations of γ-oryzanol across 179 Asian cultivated rice accessions, providing valuable germplasms for breeding high γ-oryzanol rice cultivars through conventional hybridization. In addition, this study, for the first time, identified 13 candidate genes that could be responsible for observed variable γ-oryzanol in rice. Further functional analyses of these genes are ongoing. In summary, our research lay a solid foundation and a good start point for uncovering the pathway of γ-oryzanol in rice.

DATA AVAILABILITY STATEMENT
High-density SNP data for the accessions used in genome-wide association analyses are publicly available and can be retrieved at OryzaSNP database (http://snp-seek.irri.org/_download.zul). All other datasets generated for this study are included in the Supplemental Materials.

AUTHOR CONTRIBUTIONS
Wenli Sun: Data curation, Formal analysis, Investigation, Validation, Visualization, Writing-original draft. Jin Shi: Conceptualization, Resources, Software. Jun Hong: Conceptualization, Methodology, Supervision. Guochao Zhao: Resources, Visualization. Wensheng Wang: Resources. Dabing Zhang: Conceptualization. Wei Zhang: Methodol-

ogy, Supervision. Jianxin Shi: Conceptualization, Funding acquisition, Supervision, Writing-review & editing.

CONFLICTS OF INTEREST
The authors declare no conflict of interest.

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