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Article

Comparative Rice Bran Metabolomics across Diverse Cultivars and Functional Rice Gene-Bran Metabolite Relationships

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Abstract: Rice (*Oryza sativa L.*) processing yields ~60 million metric tons of bran annually. Rice genes producing bran metabolites of nutritional and human health importance were assessed across 17 diverse cultivars from seven countries using non-targeted metabolomics and resulted in 378-430 metabolites. Gambiaka cultivar had the highest number and Njavara had the lowest number of metabolites. The 71 rice bran compounds of significant variation by cultivar included 21 amino acids, seven carbohydrates, two metabolites from cofactors and vitamins, 33 lipids, six nucleotides, and two secondary metabolites. Tryptophan, ꩜-ketoglutarate, γ-tocopherol/β-tocopherol and γ-tocotrienol are example bran metabolites with extensive cultivar variation and genetic information. 34 rice bran components that varied between cultivars linked to 535 putative biosynthetic genes using to the OryzaCyc 4.0, Plant Metabolic Network database. Rice genes responsible for bran composition with animal and human health importance is available for rice breeding programs to utilize in crop improvement.

Keywords: rice bran; rice genes; rice cultivars; metabolic pathway; metabolomics

1. Introduction

Rice, a major global cereal crop, originates from *Oryza sativa* L. (Asian rice) or *Oryza glaberrima* Steud (African rice) [1]. *Oryza. sativa* is the primary source of calories for half of the world's population [2] and provides phytochemicals, vitamins, minerals, carbohydrates, and fats when consumed as whole grain rice [3]. Rice grows in over 100 countries [4]. As a result of its long history of cultivation and selection under diverse environments, *O. sativa* comprises over 400,000 varieties and has acquired a broad range of adaptability and tolerance to different water and soil regimens, from flooded lowlands to arid hillside slopes [5,6]. The 2017 global paddy rice production reported by Food and Agriculture Organization (FAO) of the United Nations was 756.7 metric million tons with over 502 million metric tons milled and more than 60 million metric tons of rice bran produced [7]. Polished white rice is the major product with rice milling yields of 65-70% and the by-products comprised of 20% rice husk and 8-12% rice bran [8].

Rice bran has a broad spectrum of health benefits [9-12], partially due to a diverse array of bioactive metabolites [13,14]. Rice bran bioactive components include, but are not limited to γ-oryzanol [15], tocopherols, tocotrienols [16], carotenoids [17], γ-aminobutyric acid [18], octacosanol [19], squalene [20], unsaturated fatty acids [21], phytosterols and phenolic compounds [22]. Dietary consumption of rice bran was shown to be feasible and tolerable to increase key nutrients and fiber intakes in children and adults [11,23-25], and provide health promoting properties in the prevention and control of major chronic diseases, such as diabetes [26], chronic inflammation [27], cardiovascular disease [25,28], as well as cancers of the colon, liver, prostate, and breast [11,24,29,30]. Until recently, rice bran has been largely under-valued and under-utilized for nutritional and medicinal applications [31], and this promising food has not received attention from rice breeders when compared to other traits of agronomic importance (e.g. yield, disease resistance) [32]. Given the bran component of whole grain rice is the fraction with highest nutritive value [33,34], and that extensive genetic variation exists in *Oryza sativa* germplasm, a continued exploration with regards to the nutritional and health properties of rice bran is merited.

Metabolomics has shown utility to study rice plant biology and compounds that are linked to tolerance to different stressors including abiotic stress [35], mineral toxicity [36], nutrient limitation [37,38], drought stress [39], and pesticide stress [40], suggesting extensive metabolome adaptability in rice. Metabolomics has also characterized natural and genetic variations in rice via phenotyping of brown rice seeds [41], cooked brown rice [42], mature seeds [43], embryo (a fraction of rice bran) and endosperm (white rice) [44], and leaves [39]. These findings support metabolomic approaches to improve plant function, enhance grain nutritional quality, and increase grain yield [6,45,46]. Metabolite profiling of rice bran from three USA rice cultivars showed appreciable variation of bioactive rice bran components and provided the rationale for larger, global scale investigations [13,47]. Variation in bran composition could substantially contribute to greater interest in whole grain rice for wider consumer acceptance.

There is a gap in knowledge between rice bran bioactive metabolites and their genetic variation among globally diverse rice cultivars. Given that metabolomics is a powerful tool that allows for a broad range of rice bran metabolite detection, the objective of this study was to investigate variation in the rice bran metabolome of 17 cultivars, and to identify gene-metabolite relationships relevant to the nutritional and medicinal quality of rice bran. Rice bran metabolites were hypothesized to exhibit biochemical variation across cultivars and reveal key rice gene-metabolite relationships of utility for rice bran traits with nutrition and health importance in future breeding programs.

2. Results

2.1. Classification of rice bran metabolite profiles and metabolic pathways by non-targeted metabolomics

The rice bran metabolome ranged in the total number of identified metabolites, with 378 metabolites identified in Njavara (India) rice bran, to 430 metabolites identified in Gambiaka (Mali) rice bran. **Table 1** shows rice bran metabolite number based on chemical classes and showed the cultivar(s) that had the highest and lowest total number of metabolites. RBT 300 had 122 amino acids, and Khao Gaew and Njavara cultivars had 99 amino acids. Gambiaka, Shwetasoke, and LTH had 53 carbohydrates, while Calrose had the smallest total number of 48 carbohydrates. On average, 39% of the rice bran metabolome were lipids across the 17 cultivars, and this represented the largest composition by chemical class. Basmati 217 and Shwetasoke had the largest number of lipids (166 total), whereas Jasmine 85 had the lowest number of lipids (146 total). For the nucleotides, Basmati 370 and Sawa Mahsuli had 38 metabolites, and Khao Gaew and Njavara had 29. Overall, the “red color” bran genotypes, i.e., Njavara and LTH had a broader range of secondary metabolites (22 metabolites), while Dorado, having brown

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bran, had 16 metabolites. Cultivars ranged from 0 to11 metabolites in rice bran peptides. It is noteworthy that the number of cofactors & vitamins did not vary greatly across rice bran cultivars (23-27 metabolites) (**Table 1** and **Supplementary Table S1**). Each of the chemical classes were subdivided into 53 metabolic pathways (**Supplementary Table S1**).

Table 1. Number of rice bran metabolites with confirmed annotation in each cultivar by cultivar and chemical class

Rice Cultivar **Chemical Class**	**Kenya- Basmati 217**	**Kenya - Basmati 370**	**Mali - Gambiaka**	**Mali - Shwetasoke**	**Mali - DM-16**	**Mali - Khao Gaew**	**Nicaragua - Dorado**	**Nepal – Sawa Mahsuli**	**India - Chennula**	**India - Njavara**	**USA - Calrose**	**USA – RBT 300**	**USA - Jasmine 85**	**USA - IAC 600**	**USA – LTH**	**USA – SHZ-2**	**Cambodia - Rang Jey**
Amino acid	119	117	120	117	115	99	117	117	105	99	119	122	110	113	119	117	108
Carbohydrate	51	52	53	53	52	50	51	50	51	50	48	49	50	53	53	50	51
Cofactors & vitamins	27	27	27	27	26	24	27	27	27	26	27	23	27	27	27	26	27
Lipids	166	165	165	166	159	164	161	155	155	151	165	161	146	163	165	150	156
Nucleotide	37	38	35	35	34	29	37	38	30	29	35	33	37	36	35	36	36
Peptide	0	2	11	11	11	7	3	11	1	1	11	1	11	10	8	11	2
Secondary metabolites	19	21	19	19	20	19	16	20	19	22	16	17	17	20	22	17	21
Total	**419**	**422**	**430**	**428**	**417**	**392**	**412**	**418**	**388**	**378**	**421**	**406**	**398**	**422**	**429**	**407**	**401**

Shading notes the cultivar with the largest and smallest number of identified metabolites

2.2. *Global rice bran metabolome variation for 17 cultivars*

Across all cultivars, the principal component 1 (PC1) accounted for 20.3% of the total variation in the dataset, and PC2 for 16% of the metabolite variation (**Figure 1**). These results indicate that there is a "core" rice bran metabolome and that the difference in the range of individual rice bran metabolites (ca. 60-90) can be seen across the different chemical classes of the metabolome.

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Figure 1.

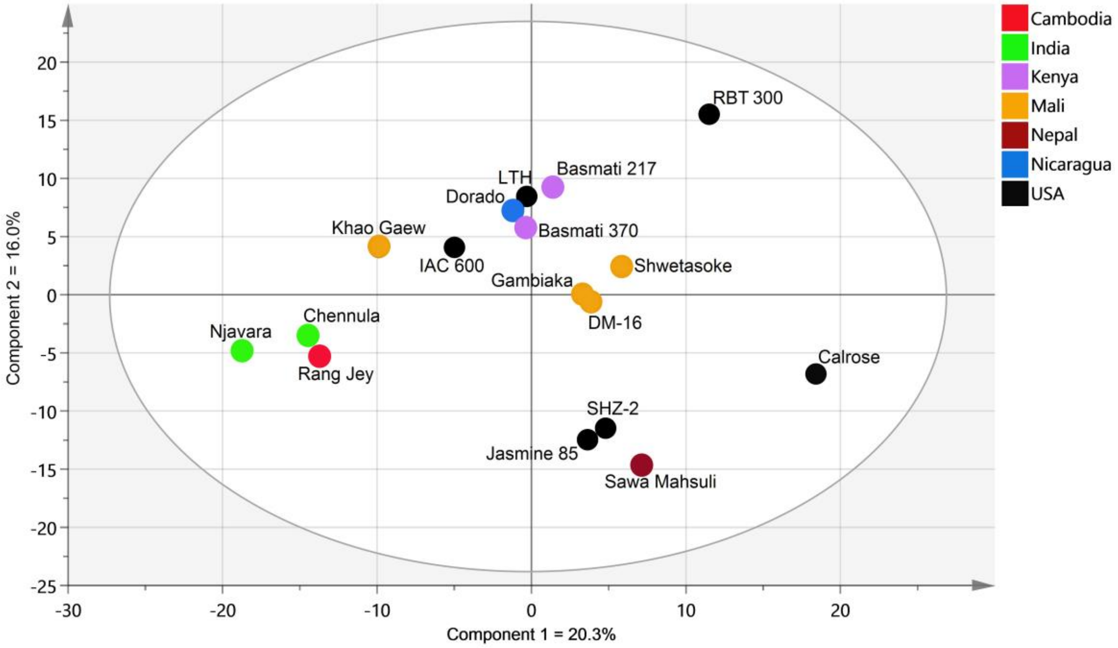

Figure 1. Principal Component Analysis (PCA) of rice bran metabolome for 17 rice cultivars. PCA was completed using median-scaled relative abundance of all bran in the 17 rice cultivars. The PC1 showed 20.3% variation, and PC2 showed 16% variation in the metabolite profiles. Colored dots indicate the country where the rice was produced.

2.3. *Comparison of rice bran metabolites across 17 cultivars*

A Z-score was used to represent the median-scaled relative abundance of each rice bran metabolite across all cultivars, and a threshold Z-score of ǀ2ǀ was applied to identify metabolites that differed appreciably between cultivars. This comparative Z-score analysis led to the identification of 71 rice bran metabolites that were significantly different across cultivars. The cultivar discriminating metabolites are shown in **Figure 2 (panel A-D)** and complete metabolite characterizations are included in **Supplementary Table S2**. Thus the 20% variation identified using comparative rice bran metabolomics (PC1) included 21 amino acids, seven carbohydrates, and two cofactors & vitamins, 33 lipids, six nucleotides, and two secondary metabolites.

2.3.1. Cultivar variation in rice bran amino acids

There were a total of 21 amino acids that differed among cultivars, and among these, many metabolites had previously-reported roles in human and animal health. **Figure 2A** shows that DM-16 rice bran had lower and higher expression of quinate and serotonin, respectively. Rang Jey showed the lower Z-score for tryptophan and tyrosine. Chennula rice bran had lower relative abundance of four amino acids namely lysine, N6,N6,N6-trimethyllysine, threonine, and arginine. Gambiaka rice bran showed higher relative abundance of N-methylproline, stachydrine, and trans-4-hydroxyproline when compared to other brans, while Njavara was lower in abundance of serotonin, asparagine, glutamate, glutamine, pyroglutamine, and taurine. Njavara was higher in relative abundance of N-acetylglutamate. Higher abundance of pipecolate and glycine was observed in Sawa Mahsuli among the cultivars. Khao Gaew and IAC 600 were the only cultivars with a lower relative abundance of methionine sulfoxide and aspartate, respectively. Basmati 217 and Basmati 370, Shwetasoke, Dorado, Calrose, RBT 300, Jasmine 85, LTH, and SHZ-2 were the cultivars with no significant Z-score changes across the class of amino acids.

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2.3.2. Cultivar variation in rice bran carbohydrates

Seven rice bran carbohydrates showed significant differences across cultivars (**Figure 2B**). Cultivars with lower abundances is some metabolites included arabonate/xylonate in Khao Gaew, glucosaminate, aconitate, and α-ketoglutarate in Njavara, and malate in IAC 600.Calrose was higher for abundance of malate, while Basmati 217 had highest abundance of erythritol. The remaining cultivars (i.e., Basmati 370, Gambiaka, Shwetasoke, and DM-16, Dorado, Sawa Mahsuli, Chennula, RBT 300, Jasmine 85, LTH, SHZ-2, and Rang Jey) showed no significant Z-score changes across the carbohydrate chemical class.

2.3.3. Cultivar variation in rice bran cofactors & vitamins

There was limited variation for cofactors & vitamins in bran amongst the cultivars, except for the vitamin E components γ-tocopherol/β-tocopherol and γ-tocotrienol, with the lowest relative abundances observed in Sawa Mahsuli rice bran (**Figure 2B**).

2.3.4. Cultivar variation in rice bran lipids

Lipids represented the largest percentage of the bran metabolome (39%) encompassing 146-166 metabolites across varieties (**Table 1**). **Figure 2C** shows the 32 lipid metabolites with significant Z-score below -2 and one lipid was significantly higher in abundance (Z-score above 2) when compared across cultivars. Chennula was the only cultivar that had an increased Z-score for laurate. RBT 300 (20 lipids), Rang Jey (5 lipids), Jasmine 85 (4 lipids), Njavara (2 lipids), and Chennula (2 lipids) were the cultivars with lower metabolite relative abundances. The significant metabolites in RBT 300 included linoleate, linolenate, myristate, myristoleate, palmitate, palmitoleate, diacylglycerol (14:0/18:1, 16:0/16:1), two isomers of linoleoyl-linolenoyl-glycerol (18:2/18:3), linoleoyl-linoleoyl-glycerol (18:2/18:2), oleoyl-linoleoyl-glycerol (18:1/18:2), oleoyl-oleoyl-glycerol (18:1/18:1), palmitoleoyl-linoleoyl-glycerol (16:1/18:2), palmitoyl-palmitoyl-glycerol (16:0/16:0), 1-linoleoylglycerol (18:2), 1-oleoylglycerol (18:1), 1-palmitoylglycerol (16:0), 12,13-dihydroxyoctadec-9-enoic acid (12,13-DiHOME), 9,10-DiHOME, and 9,10-epoxystearate. Rang Jey was lower in bran oleoyl-linoleoyl-glycerol (18:1/18:2), oleoyl-oleoyl-glycerol (18:1/18:1), 1-linoleoyl- glycerophosphoethanolamine (GPE) (18:2), 1-palmitoyl- glycerophosphocholine (GPC) (16:0), and 1-palmitoyl-GPE (16:0). Jasmine 85 showed a lower relative abundance of two isomers of palmitoyl-linoleoyl-glycerol (16:0/18:2), palmitoyl-oleoyl-glycerol (16:0/18:1), and palmitoyl-palmitoyl-glycerol (16:0/16:0). Lower relative abundance lipids in Njavara included glycerophosphoglycerol and GPC. Chennula showed low abundance in oleoyl-linoleoyl-glycerol (18:1/18:2) and 2-oleoylglycerol (18:1).

2.3.5. Cultivar variation in rice bran nucleotides

Six nucleotides varied among the cultivars. **Figure 2D** shows that Rang Jey rice bran had higher abundance of guanine and hypoxanthine, and lower abundance of adenosine when compared to the other rice cultivars. RBT 300 had higher relative abundance in 1-methyladenine and adenosine 5'-monophosphate (AMP). Khao Gaew showed a lower level of adenine amongst the cultivars.

2.3.6. Cultivar variation in rice bran secondary metabolites

Secondary metabolites that were expressed differently among the cultivars included two metabolites: 4-hydroxybenzoate and salicylate. **Figure 2D** shows that Rang Jey had the highest abundance of 4-hydroxybenzoate, and Khao Gaew had the lowest relative abundance for salicylate when compared to all other cultivars. There were no differences in the relative abundance of rice bran peptides, yet there was cultivar variation in the presence or absence of rice bran peptides (0-11 metabolites), whereby Basmati 217 was the only rice bran that had no peptide identified. Chennula, Njavara, and RBT 300 had 1 identified

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peptide (prolylglycine in Chennula and Njavara, and valylglutamine in RBT 300). There were seven cultivars with 11 peptides identified (Gambiaka, Shwetasoke, DM-16, Sawa Mahsuli, Calrose, Jasmine 85, and SHZ-2).

Figure 2.

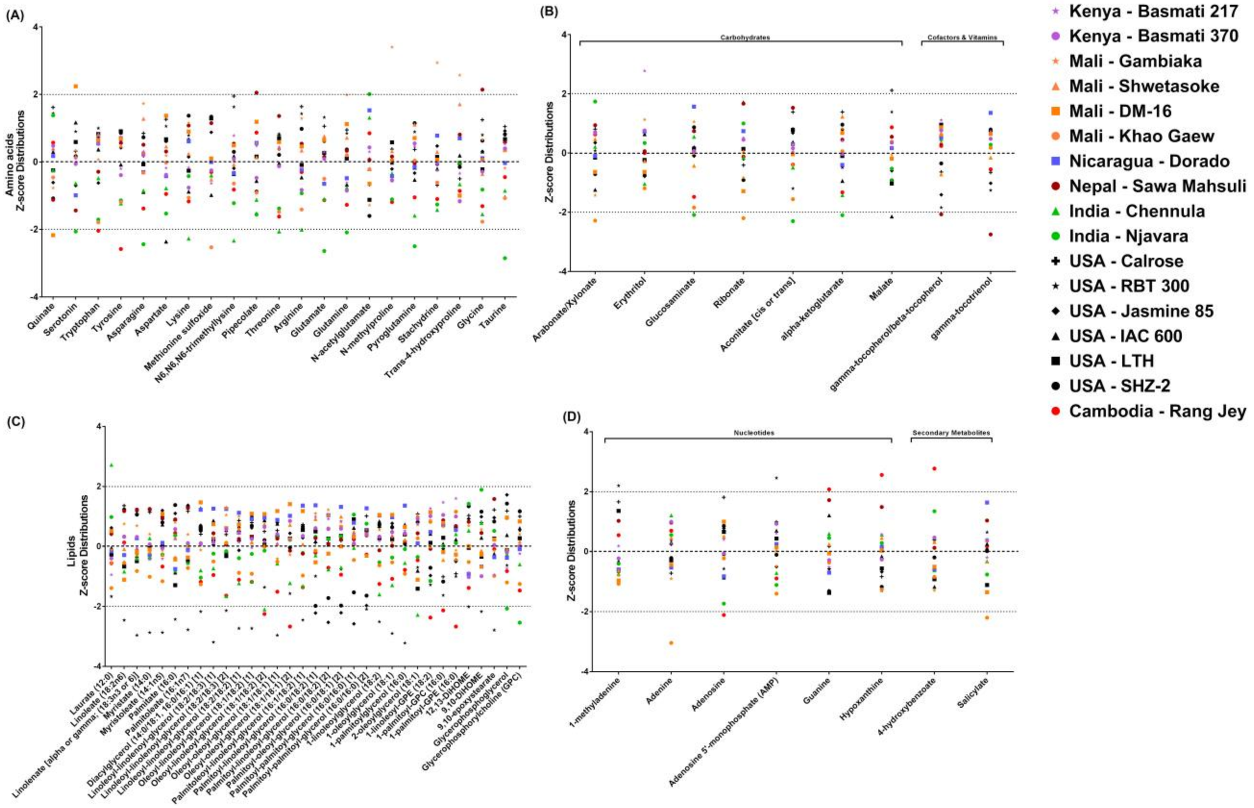

Figure 2. Comparison of discriminating rice bran metabolites across 17 cultivars. Based on the Z-scores obtained from the relative abundance of each metabolite, 71 rice bran components representing 15 metabolic pathways were significantly different among the cultivars and represented. The 21 amino acids (Panel A), 7 carbohydrates and 2 cofactors & vitamins (Panel B), 33 lipids (Panel C), 6 nucleotides and 2 secondary metabolites (Panel D) have Z-scores expressed as standard deviations from the mean and were calculated using the following formula: Z = (x- μ)/σ, "x" is relative abundance of the metabolite, "μ" is mean of relative abundance for the metabolite across 17 rice brans, and "σ" is the relative abundance standard deviation of same metabolite across 17 cultivars. Metabolites above 2 or below -2 in the panel A-D are highlighted for the largest variation and noted by cultivar and the country where the rice was grown. Colored symbols are associated with location where the rice was grown.

2.4. *Integrating rice biosynthetic genes with rice bran metabolites*

Using OryzaCyc database, 34 out of the 71 cultivar-discriminating metabolites had corresponding gene(s) identified in the database. Rice genes listed in **Table 2** were identified for 16 amino acids that differed across cultivars and were in the PMN databases for biosynthesis. Quinate and serotonin had five genes and two genes, respectively, that could regulate levels in the bran. Glycine is another amino acid with 8 genes directly-involved in its biosynthesis, yet there were 151 other possible rice genes in the database indirectly-involved in glycine biosynthetic pathways. Lipids were the majority of the methanol extracted rice bran metabolome and of the 33 differentially-abundant rice bran lipids, only eight had biosynthetic genes characterized (**Table 2**). Glycero-phosphocholine (GPC) was found to have seven biosynthetic genes, whereby linolenate, an omega-3 polyunsaturated fatty acid (PUFA) was linked to only one gene, but was indirectly associated with 332 genes that are common in biosynthesis of a standard fatty acid. **Table 3** lists three carbohydrates and two cofactors & vitamins metabolites with registered biosynthetic genes. There were two genes responsible for malate biosynthesis with an additional 150 genes that are common to biosynthesis of non-specified carbohydrates. Tocochromanols, also known as Vitamin E components (tocopherols and tocotrienols, collectively), have genes in rice that are clearly-defined [48] and γ-tocopherol/β-tocopherol had four genes while γ-tocotrienol had a single known gene. There were four nucleotides and one secondary metabolite with rice genes reported for biosynthesis (**Table 4**). Salicylate had three identified biosynthetic genes and 152 other possible genes may be involved in the carboxylate biosynthesis.

Table 2. Metabolite-rice gene relationship identified from OryzaCyc (*Oryza sativa* japonica group), Plant Metabolic Network (PMN) database

Rice bran metabolites	Precursor	Biosynthesis pathway	No. of genes*	Gene ID	Gene(s) name
Amino Acids					
• Aromatic amino acid (PEP derived)					
L-quinate	Trans-5-O-caffeoyl-D-quinate	Caffeoylglucarate biosynthesis	5	GN7F-30156 GN7F-16973 GN7F-15850	LOC_Os02g39170.1 LOC_Os02g39590.1 LOC_Os06g47910.1
Serotonin	Tryptamine	Hydroxycinnamic acid Serotonin amides biosynthesis	2	GN7F-19639 GN7F-25663	LOC_Os08g04560.1 LOC_Os08g04540.1
Tryptophan	L-serine	Tryptophan biosynthesis	4+ (153)	GN7F-27027 GN7F-24368 GN7F-25293 GN7F-19428	LOC_Os03g58260.1 LOC_Os03g58290.1 LOC_Os06g42560.4 LOC_Os08g04180.1
Tyrosine	L-phenylalanine, L-Arogenate	Phenylalanine degradation V, Tyrosine biosynthesis II & III	3+ (153)	GN7F-27976 GN7F-19057 GN7F-18001	LOC_Os06g35050.1 LOC_Os06g49505.1 LOC_Os06g49520.1
• Aspartate family (OAA derived)					
Asparagine	L-aspartate, 3-cyano-L-alanine	Asparagine biosynthesis I & II, Cyanide detoxification I	5+ (153)	GN7F-32447 GN7F-23509 GN7F-23610 GN7F-15965 GN7F-23159	LOC_Os12g38630.1 LOC_Os06g15420.1 LOC_Os03g18130.1 LOC_Os02g42350.1 LOC_Os02g42330.1
Aspartate	L-asparagine, 3-cyano-L-alanine, Indole-3-acetyl-aspartate-N-β-D-glucose	Asparagine degradation I, Cyanide detoxification I, Indole-3-acetate conjugate biosynthesis II	3+ (153)	GN7F-15965 GN7F-23159 GN7F-27949	LOC_Os02g42350.1 LOC_Os02g42330.1 LOC_Os04g58600.2
Lysine	Meso-diaminopimelate	Lysine biosynthesis VI	1+ (153)	GN7F-25633	LOC_Os02g24354.1
Methionine sulfoxide	An acyl-CoA, An aldehyde, A carboxylic ester	Not in pathway	9+ (150)	GN7F-28729 GN7F-19329 GN7F-31811 GN7F-31843 GN7F-32115 GN7F-32205 GN7F-32376 GN7F-32723 GN7F-32765	LOC_Os09g34190.1 LOC_Os04g47120.1 LOC_Os01g12910.1 LOC_Os07g27870.1 LOC_Os04g35590.1 LOC_Os07g27960.1 LOC_Os02g32200.1 LOC_Os01g12920.1 LOC_Os01g65950.1
Threonine	O-phospho-L-homoserine, L-threonine 3-O-phosphate	Threonine biosynthesis from homoserine,	3+ (153)	GN7F-18835 GN7F-30196	LOC_Os01g49890.1 LOC_Os05g47640.1

		L-threonine 3-O-phosphate		GN7F-29436	LOC_Os08g17784.1
• **Glutamate family (α-ketoglutarate derived)**					
Arginine	L-arginino-succinate	Arginine biosynthesis I & II, Citrulline-nitric oxide cycle	3+ (153)	GN7F-20973 GN7F-32460 GN7F-32707	LOC_Os03g19280.1 LOC_Os03g60976.1 LOC_Os03g60992.1
Glutamate[1]	More than 35 precursors (top two: L-glutamine, 2-oxoglutarate)	More than 27 pathways (top two: 4-aminobenzoate biosynthesis, 4-aminobutyrate degradation)	91+ (157)	GN7F-17849 GN7F-25902 GN7F-28339 GN7F-27233 GN7F-19896	LOC_Os06g48620.1 LOC_Os04g52440.1 LOC_Os08g10510.1 LOC_Os02g02210.1 LOC_Os04g52450.1
Glutamine	More than 8 precursors (top 3: L-glutamate, A dipeptide with proline at the C-terminal, A γ L-glutamyl-L-amino acid)	More than 6 pathways (top 4: Ammonia assimilation cycle I & II, Glutamine biosynthesis I & II)	5+ (153)	GN7F-15709 GN7F-15901 GN7F-22516 GN7F-26393 GN7F-27460	LOC_Os03g50490.1 LOC_Os04g56400.1 LOC_Os03g12290.1 LOC_Os10g31820.1 LOC_Os02g50240.1
N-acetylglutamate	L-glutamate	Arginine biosynthesis II (acetyl cycle), Ornithine biosynthesis	6+ (150)	GN7F-20894 GN7F-19328 GN7F-17187 GN7F-31311 GN7F-32148 GN7F-32821	LOC_Os03g17120.1 LOC_Os07g39690.1 LOC_Os03g31690.1 LOC_Os03g46200.1 LOC_Os03g58010.1 LOC_Os03g58030.1
Pyroglutamine	An (γ-L-glutamyl)-L-amino acid	γ-glutamylcyclotransferase	2	GN7F-31386 GN7F-32110	LOC_Os03g63700.1 LOC_Os11g04420.4
• **Serine family (phosphoglycerate derived)**					
Glycine	L-cysteinyl-glycine,	γ-glutamyl cycle, Phytochelatins biosynthesis	8+ (151)	GN7F-30607 GN7F-16957 GN7F-25454 GN7F-26619 GN7F-28075 GN7F-16002 GN7F-26733 GN7F-19516	LOC_Os01g05810.1 LOC_Os04g38450.1 LOC_Os01g05820.1 LOC_Os05g34290.1 LOC_Os06g01260.1 LOC_Os12g35890.1 LOC_Os09g32290.2 LOC_Os01g21380.1
Taurine[2]	In Transport Reactions		4	GN7F-31312 GN7F-31505 GN7F-32616 GN7F-32729	LOC_Os09g29660.1 LOC_Os05g31080.1 LOC_Os03g20170.1 LOC_Os09g29670.1
Lipids					
• **Free fatty acid**					
α-linolenate	A phosphatidylcholine	No common pathways	1+ (334)	GN7F-18386	LOC_Os11g04940.1
Laurate	Lauroyl-CoA, A dodecanoyl- [acyl-carrier protein]	Palmitate biosynthesis II (bacteria and plants), Sporopollenin precursors biosynthesis	1+ (285)	GN7F-19329	LOC_Os04g47120.1
Linoleate	Linoleoyl-CoA	No common pathways	2+ (332)	GN7F-33079 GN7F-16232	LOC_Os02g44654.2 LOC_Os04g47250.1
Myristate	Myristoyl-CoA	No common pathways	0+ (325)	-	-
Palmitate	Palmitoyl-CoA, A palmitoyl-[acp], 1,2-dipalmitoyl-phosphatidylcholine, 1-palmitoyl-2-linoleoyl-phosphatidylcholine	Cutin biosynthesis, Sporopollenin precursors biosynthesis, Suberin monomers biosynthesis, Palmitate biosynthesis II	6+ (333)	GN7F-19329 GN7F-28729 GN7F-32532 GN7F-24830 GN7F-20654 GN7F-18158	LOC_Os04g47120.1 LOC_Os09g34190.1 LOC_Os01g66240.1 LOC_Os01g46250.1 LOC_Os01g73740.1 LOC_Os01g51360.1

		(bacteria and plants), Phospholipid remodeling (phosphatidylcholine, yeast)			
Palmitoleate	A palmitoleoyl- [acyl-carrier protein]	No common pathways	4+ (333)	GN7F-31362 GN7F-31587 GN7F-31765 GN7F-32681	LOC_Os04g46710.1 LOC_Os04g46730.1 LOC_Os02g44134.1 LOC_Os02g44200.1
• Oxylipins					
9,10-epoxystearate	oleate, A hydroperoxy-fatty-acyl-[lipid]	Cutin biosynthesis, Poly-hydroxy fatty acids biosynthesis	10+ (332)	GN7F-31064 GN7F-30659 GN7F-18468 GN7F-27809 GN7F-16745 GN7F-21459 GN7F-19209 GN7F-18601 GN7F-29035 GN7F-15971	LOC_Os10g37070.1 LOC_Os08g05620.1 LOC_Os01g24810.1 LOC_Os10g05020.1 LOC_Os04g03890.1 LOC_Os10g37100.1 LOC_Os04g33370.1 LOC_Os02g01890.1 LOC_Os08g05610.1 LOC_Os06g46680.1
• Phospholipid Metabolism					
Glycerophosphorylcholin (sn-glycero-3-phosphocholine)	A 1-acyl-sn-glycero-3-phosphocholine	No common pathways	7	GN7F-33055 GN7F-29598 GN7F-18763 GN7F-31398 GN7F-32181 GN7F-32498 GN7F-32638	LOC_Os01g07960.3 LOC_Os04g57370.1 LOC_Os04g09540.1 LOC_Os01g42690.1 LOC_Os04g57390.1 LOC_Os05g51050.1 LOC_Os08g42680.1

*Numbers in brackets indicate the number of common genes known to be responsible for synthesis of a standard alpha amino acid and a standard fatty acid.

[1] Only the top 5 genes (most studied) were included in the table.

[2] There are no identified genes for taurine biosynthesis in rice. However, genes that are responsible for its transport from environment into the rice plant are identified.

Table 3. Metabolite-rice gene relationships identified from OryzaCyc (*Oryza sativa* japonica group), Plant Metabolic Network (PMN) database

Rice bran metabolites	Precursor	Biosynthesis pathway	No. of genes*	Gene ID	Gene(s) name
Carbohydrate **• TCA cycle**					
α-ketoglutarate (2-oxoglutarate)[1]	L-glutamate, D-*threo*-isocitrate	9 pathways (top 4: Alanine degradation II, Glutamate degradation I, Arginine biosynthesis II, Ornithine biosynthesis)	11+ (160)	GN7F-23833 GN7F-16177 GN7F-28651 GN7F-23252 GN7F-26515	LOC_Os03g58040.1 LOC_Os02g43470.1 LOC_Os04g45970.1 LOC_Os05g03830.1 LOC_Os07g27780.1
cis-aconitate	Citrate	Glutamine biosynthesis III glyoxylate cycle, TCA cycle II (plants and fungi)	4+ (150)	GN7F-24702 GN7F-28499 GN7F-23691 GN7F-25422 GN7F-25644	LOC_Os10g03960.1 LOC_Os06g19960.1 LOC_Os03g04410.1 LOC_Os08g09200.1
Malate	Acetyl-CoA,	Glycolate and glyoxylate	2+ (150)	GN7F-24000	LOC_Os04g40990.1

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	Glyoxylate, Fumarate	degradation II, Glyoxylate cycle, Superpathway of glyoxylate cycle and fatty acid degradation, TCA cycle II (plants and fungi)		GN7F-21211	LOC_Os03g21950.1
Cofactors & vitamins					
• Tocopherol metabolism					
β-tocopherol	δ-tocopherol, S-adenosyl-L-methionine	Vitamin E biosynthesis (tocopherols)	4	GN7F-31982 GN7F-31239 GN7F-31334 GN7F-25500	LOC_Os10g41970.1 LOC_Os03g26200.1 LOC_Os08g02600.1 LOC_Os02g47310.1
γ-tocotrienol	2,3-dimethyl-6-geranylgeranyl-1,4-benzoquinol	Vitamin E biosynthesis (tocopherols)	1	GN7F-24601	LOC_Os02g17650.1

*Numbers in brackets indicate the number of common genes known to be responsible for synthesis of a standard carbohydrate.
[1]Only the top 5 genes were included in the table.

Table 4. Metabolite-rice gene relationship identified from OryzaCyc (*Oryza sativa* japonica group), Plant Metabolic Network (PMN) database

Rice bran metabolites	Precursor	Biosynthesis pathway	No. of genes*	Gene ID	Gene(s) name
Nucleotides					
• Purine metabolism					
Adenine[1]	S-methyl-5'-thioadenosine, adenosine, N6-dimethylallyladenine, Trans-zeatin, cis-zeatin, N1-ethyladenine, N1-methyladenine	S-methyl-5'-thioadenosine degradation I, Adenine and adenosine salvage II, Cytokinins degradation	18	GN7F-25353 GN7F-26929 GN7F-32781 GN7F-32797 GN7F-19530	LOC_Os08g44370.1 LOC_Os09g39440.1 LOC_Os05g33644.1 LOC_Os05g33630.1 LOC_Os06g37500.1
Adenosine	S-adenosyl-L-homocysteine, Trans-zeatin riboside, Isopentenyl adenosine	S-adenosyl-L-methionine cycle II, L-methionine degradation I (to L-homocysteine), Cytokinins degradation	3+ (8)	GN7F-20280 GN7F-19530 GN7F-20388	LOC_Os02g12780.1 LOC_Os06g37500.1 LOC_Os01g09260.1
Adenosine 5'-monophosphate[1]	Adenosine triphosphate (ATP)	More than 97 pathways (top 4: Trans-zeatin biosynthesis, Adenosine nucleotides degradation I, 4-hydroxybenzoate biosynthesis I (eukaryotes), L-arginine biosynthesis I (via L-ornithine)	299+ (24)	GN7F-23647 GN7F-23504 GN7F-28551 GN7F-25622 GN7F-25996	LOC_Os02g46970.1 LOC_Os06g44620.1 LOC_Os08g34790.1 LOC_Os08g14760.1 LOC_Os01g24030.1
Hypoxanthine	Inosine	Adenosine nucleotides degradation I	5	GN7F-25353 GN7F-21573 GN7F-26929 GN7F-32781 GN7F-32797	LOC_Os08g44370.1 LOC_Os03g31170.1 LOC_Os09g39440.1 LOC_Os05g33644.1 LOC_Os05g33630.1
Secondary metabolites					

• Benzenoids					
Salicylate	Methylsalicylate	Unknown	3 + (152)	GN7F-26541 GN7F-21475 GN7F-28107	LOC_Os05g30760.1 LOC_Os01g37650.1 LOC_Os01g25360.1

*Numbers in brackets indicate the number of common genes known to be responsible for biosynthesis of a nucleotide or a carboxylate.
[1] Only the top 5 genes were included in the table.

2.5. *Integration of rice bran metabolites, metabolic pathways and rice genes*

Figure 3 shows the Pathways Enrichment Score (PES) for 15 metabolic pathways in the cultivars that contained one or more metabolites with significant Z-scores among the 71 discriminating rice bran metabolites. A complete list of all PES is shown in **Supplementary Table S3**.

2.5.1. Amino acid metabolic pathway enrichment scores and gene associations

Amino acids contained four metabolic pathways with PES distinctions including aromatic amino acid [phosphoenolpyruvate (PEP) derived]p, aspartate family oxaloacetate (OAA) derived, glutamate family (α-ketoglutarate derived), and serine family (phosphoglycerate derived) that involve the 21 amino acids that differed among the cultivars including four aromatic amino acid (PEP derived), seven aspartate family (OAA derived), nine glutamate family (α-ketoglutarate derived), and two serine family (phosphoglycerate derived) and shown in **Supplementary Table S2**. DM-16 rice bran had low and high abundance of quinate and serotonin, respectively, which have the highest PES for the aromatic amino acid (PEP derived) pathway (PES= 24.8). Chennula rice bran had the highest PES for the aspartate family (OAA derived) pathway (PES= 3.9) and the low relative abundance of three amino acids namely lysine, N6,N6,N6-trimethyllysine, and threonine explained the difference. Gambiaka rice bran had the highest PES for the glutamate family (α-ketoglutarate derived) pathway (PES= 6.9) and high relative abundance of N-methylproline, stachydrine, and trans-4-hydroxyproline explain the difference. Sawa Mahsuli rice bran had the highest PES for the serine family (phosphoglycerate derived) pathway (PES= 6.0) when compared to other cultivars. Differentially-higher abundance of glycine in this cultivar was the contributor to this difference (**Figure 3**).

2.5.2. Carbohydrate metabolic pathway enrichment scores and gene associations

The carbohydrate chemical class contained two metabolic pathways with significant PES and included amino sugar and nucleotide sugar, and the citric acid cycle (TCA cycle). Seven discriminating metabolites explained these changes including four amino sugar and nucleotide sugar, and three TCA cycle. Rice genes involved in biosynthesis of metabolites under the TCA cycle metabolic pathways are shown in **Table 3**. There were no genes identified for metabolites under the amino sugar and nucleotide sugar metabolic pathway. Khao Gaew rice bran had the highest PES for the amino sugar and nucleotide sugar pathway (PES=20.3) and this was due to the low relative abundance of arabonate/xylonate and ribonate. Njavara rice bran had the highest PES for the TCA cycle across all cultivars (PES= 9.9) due to glucosamine, aconitate and α-ketoglutarate having the lowest abundance among the cultivars (**Figure 3**).

2.5.3. Cofactors & vitamins metabolic pathway enrichment scores and gene associations

Cofactors & vitamins were associated with the tocopherol metabolic pathway, whereby the Sawa Mahsuli rice bran had the highest score (PES= 9.6) due to lower relative abundance γ-tocopherol/β-tocopherol and γ-tocotrienol as compared to other cultivars (**Figure 3**). Rice genes involved in biosynthesis of metabolites under the tocopherol metabolic pathway are shown in **Table 3**.

2.5.4. Lipids metabolic pathway enrichment scores and gene associations

The 33 lipid metabolites that explained these changes in PESs included seven free fatty acids, 14 glycerolipids (diacyl), four glycerolipids (monoacyl), three lyso-phospholipids, three oxylipins, and two phospholipids. Rice genes involved in biosynthesis of metabolites under free fatty acid, oxylipins, and phospholipid pathways are shown in **Table 2**. There were no genes identified for metabolites under monoacyl and diacyl glycerolipids, and lyso-phospholipids metabolic pathway. RBT 300 had the lowest relative abundance for the majority of lipids (20 lipids) which explain the highest PES for the free fatty acid pathway (PES= 3.1), glycerolipids (diacyl) pathway (PES= 12.8), glycerolipids (monoacyl) pathway (PES=5.7), and oxylipins pathway (PES= 6.5). Rang Jey rice bran had the highest PES for the lyso-phospholipids pathway (PES= 3.6) and the low abundances of 1-linoleoyl- glycerophosphoethanolamine (GPE) (18:2), 1-palmitoyl-GPC (16:0), and 1-palmitoyl-GPE (16:0) explain this significance. Njavara rice bran was another cultivar with highest PES for phospholipid metabolism (PES= 4.6) and low abundance of glycerophosphoglycerol and GPC explain this significance (**Figure 3**).

2.5.5. Nucleotide metabolic pathway enrichment score s and gene associations

Within the nucleotides a PES of 2.7 was identified for purine metabolism, with Rang Jey rice bran having the highest score. This PES was a result of the lower relative abundance of adenosine in this cultivar compared to others (**Figure 3**). Rice genes involved in biosynthesis of metabolites under the purine metabolic pathway are shown in **Table 4**.

2.5.6. Secondary metabolite pathway enrichment scores and gene associations

Secondary metabolites that were expressed differently among the cultivars included two benzenoids pathway metabolites namely, 4-hydroxybenzoate and salicylate. Rang Jey had the highest relative abundance for 4-hydroxybenzoate, and Khao Gaew had the lowest relative abundance for salicylate, thus the highest pathway enrichment score for Rang Jey (PES= 4.3) followed by Khao Gaew (PES= 3.2) (**Figure 3**). Rice genes involved in biosynthesis of salicylate are shown in **Table 4**. No genes were identified for 4-hydroxybenzoate in the database.

Figure 3.

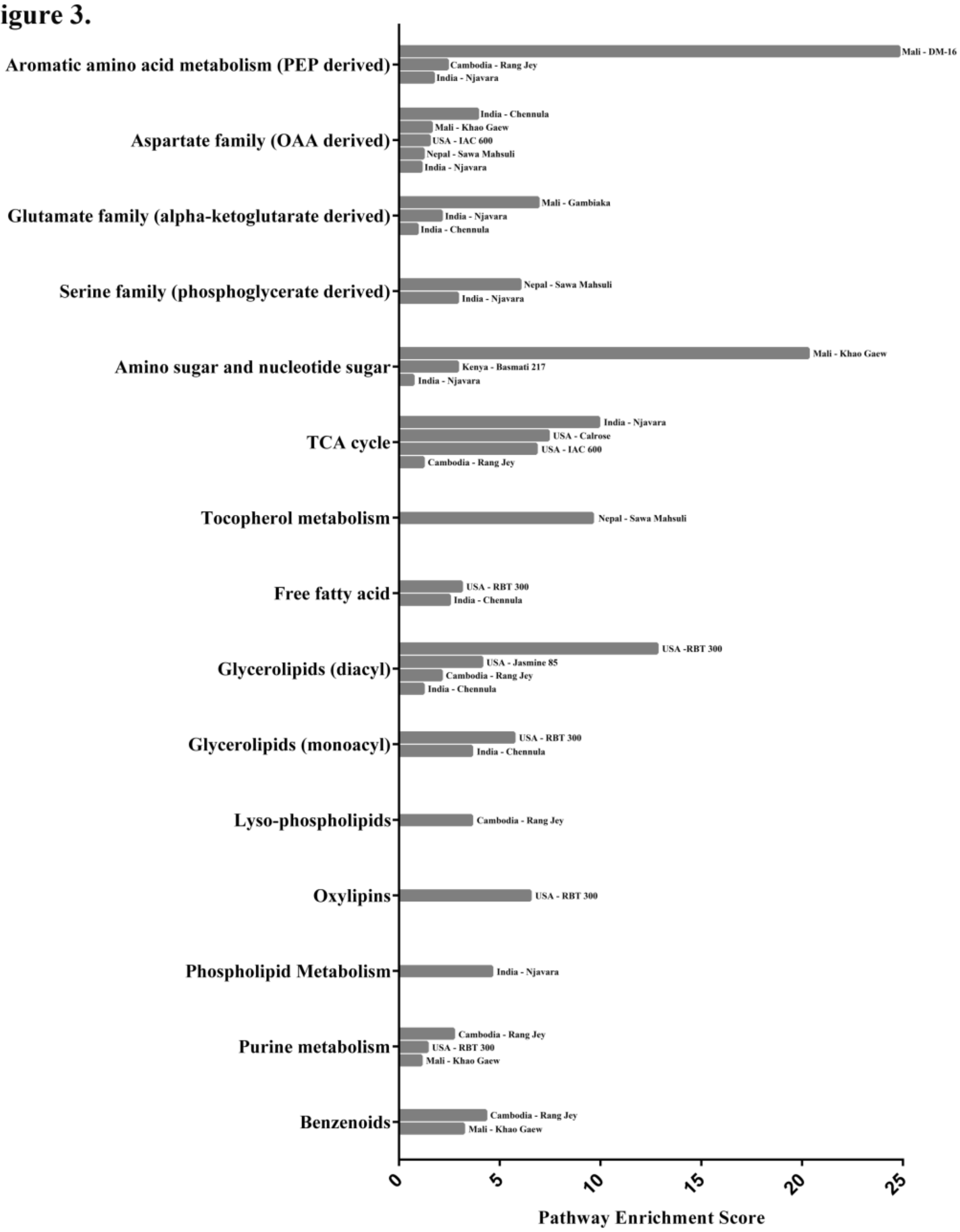

Figure 3. Cultivars with significant Pathway Enrichment Score (PES) of 15 metabolic pathways. Metabolic pathways with enrichment scores greater or less than 1 are shown in this figure and indicate that the pathway for the cultivar contained 1 or more metabolites with a statistically-significant Z-score.

3. Discussion

This comparative, global rice bran metabolomics investigation led to the high throughput and sensitive identification of over 450 diverse rice bran metabolites/phytochemicals. Rice bran metabolites stem from multiple chemical classes and metabolic pathways involved in rice seed development. The rice bran metabolomics analysis of 17 global cultivars revealed substantial variation in both the presence and abundance of metabolites from distinct chemical classes (~36% of entire metabolite profile) as well as from a ‘core’ rice bran metabolite profile (~74% of metabolites). Rice bran has been shown to be feasible for dietary use [11,23,49] and is a valuable nutritional addition to polished rice, especially in the geographic regions where rice is one of the primary food sources. Moreover, rice is a staple crop in many

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low-middle-income countries where malnutrition remains a major problem [50,51]. Evaluating the nutritional and health benefits of rice bran is enhanced by using a global metabolomics approach. The 71 discriminating rice bran metabolites that varied among these 17 cultivars that were collected from diverse field environments should be considered in breeding due to the presence of nutritionally- and medicinally-valuable bran metabolites.

A few examples of amino acids with health benefits that were identified herein are quinate and serotonin from the aromatic amino acid metabolic pathway (PEP derived) that have shown to be anti-inflammatory [52] and antiemetic [53], respectively. This metabolome analysis showed that the abundance of quinate and serotonin were significantly lower and higher, respectively in DM-16 (produced in Mali, West Africa) when compared to other rice brans. The increased relative abundance of glycine and pipecolate was also observed in Sawa Mahsuli (produced in Nepal). Glycine was shown to have anti-diarrheal [54], anti-inflammatory [55], antioxidant [56], cancer chemoprevention [57], and anti-obesity properties [58]. Pipecolate was shown to have cancer chemoprevention properties as this phytochemical serves as a precursor to gut microbial secondary metabolites production such as such as rapamycin, swainsonine, virginiamycin, and marcfortine that exhibit anti-inflammatory, antitumor, and antibiotic properties [59].

Carbohydrates, vitamins and lipids of interest to improve animal and human health, differed in abundance across cultivars. Malate, a carbohydrate from the TCA cycle was detected and had higher relative abundance in Calrose (USA grown) when compared to other cultivars. In the human body, malate has importance to energy metabolism during both aerobic and anaerobic conditions [60,61], and it was shown that a deficiency of malate may be a major cause of physical exhaustion [61]. Malate has also been reported to have natural antimicrobial activity against *Salmonella typhimurium* and other microbial pathogens [62]. Given that rice bran, and in particular the Calrose cultivar, is a good source of malate, it could be used to replenish the endogenous malate in the body, and warrants further attention for nutritional and breeding programs.

The Sawa Mahsuli cultivar from Nepal showed lower relative abundance of γ-tocopherol and γ-tocotrienol when compared to other cultivars and should be considered as a genetic resource for crop improvement because total vitamin E contents found in rice bran are associated with health benefits [63]. γ-tocopherol, the primary form of vitamin E in food in the USA [64], and γ-tocotrienol, a safe and well-tolerated form of vitamin E [65], have demonstrated a broad range of disease fighting activities including but not limited to anti-inflammatory [66] and anti-hypertension actions [67].

In the context of lipid metabolites, two bran sources that demonstrate the value of nutritional breeding considerations are RBT 300 (USA derived) and Jasmine 85 cultivar (USA grown). RBT 300, a blend of rice bran from California where Calrose cultivars predominate, was shown to be lower in the relative abundance of lipids including linolenate (alpha or gamma), an essential fatty acid (omega-3 or omega-6, respectively), which is not synthesized by mammals [68]. Jasmine 85 was also shown to be low in relative abundance of four diacylglycerolipids, namely two isomers of palmitoyl-linoleoyl-glycerol (16:0/18:2), palmitoyl-oleoyl-glycerol (16:0/18:1), and palmitoyl-palmitoyl-glycerol (16:0/16:0). These diacylglycerols are important to the diet because they deliver two distinctly important fatty acids. For example, palmitoyl-linoleoyl-glycerol (16:0/18:2) consists of palmitic acid and linoleic acid. Palmitic acid exerts multiple fundamental biological functions [69]. On the other hand, linoleic acid has been shown to be anticarcinogenic [70] and reduces the risk of atherosclerosis in rabbits [71]. α-linoleic acid (ALA) acts as the precursor of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [72]. Thus, ALA from rice bran may have as many beneficial effects as EPA and DHA to promote human health. Studies on humans and rodents have shown that the synthesis of anti-inflammatory prostaglandin E1 was

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selectively elevated through γ-linoleic acid supplementation [73]. Future studies should consider using rice bran lipids from bran or whole grain rice as they may demonstrate beneficial effects when compared to consuming these lipids as isolated supplements.

Other metabolites from this study that varied by cultivar and should be considered for importance to human health were 4-hydroxybenzoate and salicylate. The 4-hydroxybenzoate had a higher relative abundance in Rang Jay (grown in Cambodia), and this compound has established antimicrobial properties [62,74] as well as antioxidant [75] actions. Salicylate, a phytochemical with a broad function in plant growth and development [76], is also an active component of aspirin and has well-documented anti-diabetic, anti-inflammatory, and cardio-protective properties [77,78]. It has potential to be improved through selective breeding programs in Khao Gaew (from Mali) as salicylate had the lowest abundance in this cultivar.

The rice bran phytochemical diversity observed among 17 cultivars produced in geographically diverse field environments included bran molecules that were present at very low levels and which have been typically overlooked in targeted studies. For example, taurine was only recently shown to exist in rice bran [13] and this study verifies its lower abundance across cultivars and regions. Taurine is a known antioxidant and anti-inflammatory agent, as well as a powerful scavenger of hypochlorous acid [79,80]. These effects of taurine may be also related to prevention of obesity by increasing energy metabolism in white adipose tissue [81]. In other studies, taurine was found to have antiepileptic actions [82] and to be neuroprotective against glutamate excitotoxicity [83].

Understanding the genes involved in biosynthesis of bran specific metabolites that are distinct from the rest of the grain is essential to improve nutritional and medicinal value of the bran and whole grain (brown) rice. Increasing our understanding of the rice genes involved in the biosynthetic pathways of bran composition, such as lipid biosynthetic pathways, are noteworthy as these genes were largely missing from the integrated database (**Table 3**). Enhancing rice bran amino acid and lipid contents, as well as many other phytochemicals, such as recently shown for tricin [84], may be beneficial to co-develop with high-yielding rice cultivars. Genome-wide association study (GWAS) using cultivar diversity panels [85,86] and Quantitative Trait Locus (QTL) mapping in structured bi-parental populations [6,87,88], when coupled with rice bran metabolomics, offer a novel means of gene discovery and crop improvement. This emerging field of phytochemical genomics, integrating genomic, proteomic and metabolomic approaches, has been used in crop improvement for barley [89], corn [90,91], potato [92], and tomato [93,94]. We put forth that the study and identification of rice genes underlying the nutritional and medicinal traits in rice bran deserve investigation to realize the tremendous potential impact of rice bran in global nutritional security.

Rice bran variation in composition of bioactive molecules has functional relevance to rice breeders that seek to improve bran for human and animal health. The results of this study support the feasibility that numerous phytochemicals that have reported medicinal mechanisms of action can be improved in rice bran through breeding [13,14,95]. Rice bran, regardless of varietal differences, has a valuable ‘core’ metabolome as well as a variable set of metabolites that differ among cultivars, which can be developed as an affordable food ingredient for a diverse global population that remains challenged to meet basic nutritional security needs. The identification of 71 metabolites and the ~1500 rice genes associated with the 15 metabolic pathways are significant results obtained herein that distinguish *Oryza sativa* cultivars and support the use of bran small molecules as bio-markers. The current understanding of the genetic basis for the type and quantity of metabolites and the metabolic pathways that exist in rice bran is sufficient to start breeding rice cultivars that contain optimal profiles for some rice bran metabolites that will benefit animal and human health.

4. Materials and methods

4.1. *Rice cultivars and heat stabilization of bran*

Rice bran was isolated from 17 rice cultivars that originated from 11 countries and were grown in seven countries including Cambodia, India, Kenya, Mali, Nepal, Nicaragua, and the United States, all having emerging interests in producing functional foods [9,10,23]. The phenotypes of all cultivars are described in **Table 5**. Immediately after the milling process, raw rice bran was heat-stabilized (110°C for 6 min) to prevent rancidity and then stored at -20°C until further processing for metabolomics.

Table 5. Classification and phenotypes of the seventeen rice cultivars used for bran metabolomics

Cultivar	Grain type	Bran color	Country of Origin	Growing Location
Basmati 217	Long	Brown	India	Kenya
Basmati 370	Long	Brown	India	Kenya
Calrose	Medium	Brown	USA	California
Chennula	Long	Brown	India	India
DM-16	Short	Brown	South America	Mali
Dorado	Long	Brown	Colombia	Nicaragua
Gambiaka	Long	Brown	Mali	Mali
IAC 600	Medium	Purple	Brazil	Arkansas
Jasmine 85	Long	Brown	Philippines	Arkansas
Khao Gaew	Long	Brown	Thailand	Mali
Li-Jiang-Xin-Tuan-Hei-Gu (LTH)	Medium	Red	China	Arkansas
Njavara	Long	Red	India	India
Rang Jey	Medium	Brown	Cambodia	Cambodia
RBT 300*	Medium	Brown	USA	California
Sawa Mahsuli	Long	Brown	Nepal	Nepal
Shan-Huang-Zhan-2 (SHZ-2)	Long	Brown	China	Arkansas
Shwetasoke	Long	Brown	Mali	Mali

* This rice bran is a commercial ingredient from a mixture of varieties grown in California.

4.2. *Rice bran metabolite extraction and sample preparation*

Metabolon Inc. (Durham, NC) performed the global, non-targeted metabolomics. Rice bran samples were extracted as previously described [13]. Briefly, samples were extracted with 80% methanol. Samples then underwent vigorous shake for 2 min (Glen Mills GenoGrinder 2000) and followed by centrifugation to precipitate protein and separate the small molecules from macromolecules. The attained supernatant extract (i.e., rice bran extract) was divided into several fractions for different modes of analysis by ultrahigh performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) including reverse phase chromatography with positive/negative ion mode electrospray ionization for non-polar compounds, and hydrophilic-interaction chromatography (HILIC) UPLC-MS/MS for the analysis of polar compounds. Prior to injection, samples were placed on TurboVap® (Zymark) evaporator to make sure that there is no organic solvent remained.

4.3. UPLC-MS/MS analysis

The non-targeted metabolomics analysis was based on previously-described methods [13]. Briefly, a Waters ACQUITY UPLC coupled with a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer was utilized. The dried rice bran extract was reconstituted in UPLC-compatible solvents (acidic or basic solvents) for each mode of analysis. For the acidic solution, the rice bran extract was once analyzed for hydrophilic compounds and once for hydrophobic compounds. For hydrophilic compounds, the extract was eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol, containing 0.05% perfluoropentanoic acid and 0.1% formic acid in gradient manner. For hydrophobic compounds, the extract was gradient eluted from the same mentioned C18 column and solvent, with added acetonitrile.

Similar to the acidic mode, in the basic solution, rice bran extract was once analyzed for hydrophilic compounds and once for hydrophobic compounds using a similar C18. For more hydrophobic and positive ion compound extraction, water, methanol and 6.5mM ammonium bicarbonate at pH 8.0 was used to elute the rice bran extracts from the C18 column. For more hydrophilic and more negative ion compounds, the extract was analyzed and eluted through interaction liquid chromatography (HILIC) column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile and 10 mM ammonium formate, at pH 10.8. Using dynamic exclusion, the mass spectrometry analysis was interchanged between MS and data-dependent MS^2 scans with the scan range of 70-1000 m/z.

4.4. Metabolite data extraction and compound identification

Biochemical identifications from UPLC-MS was completed using the Metabolon database and were based on three criteria: retention index, accurate mass match to the National Institute of Standards and Technology library within +/- 0.005 atomic mass units, and the tandem mass spectrometry (MS/MS) scores between the generated data from the experiment and standards [13]. Each rice bran metabolite was then cross checked for an associated number in the Kyoto Encyclopedia of Genes and Genomes (KEGG), Human Metabolome Database (HMDB), and PubChem databases.

4.5. Metabolic pathway analysis

Across all 17 rice bran varieties, the metabolome analysis comprised 53 metabolic pathways, and each metabolite was assigned to one pathway. Using the following equation, pathway enrichment score (PES) was calculated, where "k" correspond to the number of metabolites with a Z-score of +/- 2 or larger in a metabolic pathway, "m" correspond to the total number of metabolites identified in that pathway, "n" correspond to the total number of significant metabolites in the dataset, and "N" correspond to the total number of identified metabolites in the entire dataset:

$$\frac{k/m}{n/N}$$

Metabolic pathways that had PES less or greater than one indicated that the pathway contained one or more differentially-expressed metabolites compared to all other pathways.

4.6. Rice biosynthetic gene identification for selected rice bran metabolites

The *Oryza sativa* (japonica group-based) OryzaCyc 4.0, Plant Metabolic Network (PMN) database was used to identify rice biosynthetic genes that produce selected bran metabolites involved in human and animal health promotion. Bran metabolites selected for rice gene analysis had a Z-score of +/-2 or larger when comparing the 17 cultivars in the metabolomics dataset. The rice gene and bran metabolite linkages can be verified at (http://plantcyc.org/databases/oryzacyc/4.0). It is notable that a limitation arises from the fact that rice genome database is limited to japonica sub-population and may differ for indica sub-population [96].

4.7. Statistical analysis

Median-scaled relative abundance and Z-score for each of the metabolites and across all cultivars were calculated as previously described [13]. Z-score calculation was based on the median-scaled relative abundances and reported as standard deviations from the mean and was calculated using the following formula:

$$Z = \frac{x - \mu}{\sigma}$$

where relative abundance of the metabolite is expressed as "x", mean of relative abundance for the metabolite across 17 rice brans is expressed as "μ", and the relative abundance standard deviation of same metabolite across 17 cultivars is expressed as "σ". A notable Z-score for a metabolite shows that the relative abundance of that metabolite in a specific cultivar is lower or higher than the standard deviations from the mean of other cultivars. For each variety, metabolites with a Z-score greater than 2 or less than -2 (Z-score > |2.0|) were considered to be distinguishers of that variety. Furthermore, a principal component analysis (PCA) was completed using SIMCA (Sartorius Stedim Biotech) to assess the overall variability in the global metabolite profile of bran from 17 rice cultivars using median-scaled relative abundance values. However, the country in which the rice cultivar originated from or was grown in was not a variable in the PCA as a means to focus differences in rice varieties that are available to consumers in various countries.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Supplementary Table 1: List of all the identified metabolites in bran of 17 rice cultivars with their relative abundances, Supplementary Table 2: Z-scores and relative abundances of 71 significant metabolites, Supplementary Table 3: Metabolic pathway enrichment scores (PES) for discriminating metabolites between rice bran varieties.

Author Contributions: EPR and IZ designed and conducted the research; IZ conducted the metabolome analysis and wrote the manuscript. EL and IZ investigated the gene-metabolite relationship. JEL, AM, SV, and OK among others acknowledged below assisted in the rice bran collection from the field and classification from diverse countries. All authors read and approved the final manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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