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ARTICLE

GC-MS Analysis of Metabolites in Filling Grains of Rice-Tartary Buckwheat (*Fagopyrum tataricum*) in Comparison to Conventional Tartary Buckwheat

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ABSTRACT

Rice-Tartary buckwheat (RTB) is a special germplasm of Tartary buckwheat. In this study, the appraisal of taste quality between RTB and conventional Tartary buckwheat (CTB) was presented, and the metabolites in kernels at three typical grain filling stage (GFS) were investigated. Unlike CTB, RTB showed thin shell seeds without longitudinal furrows at maturity, which was easily artificially dehulled. Sense organ test indicated that RTB exhibited better taste quality because of the higher values of appearance, viscosity, taste and summary were appraised. In total, 92 metabolites were identified in kernels using GM-MS metabolomics platform. The levels of most metabolites changed greatly during grain filling and a large numbers of metabolite-metabolite correlations were found by Pearson correlation coefficient analysis. ANOVA analysis identified 61 differentially expressed metabolites between RTB and CTB, while Venn diagram analysis screened 35 common differential metabolites. Compared with CTB, RTB showed similar levels of lysine and methionine, indicated that RTB own excellent nutritional value. Additionally, RTB exhibited significantly up-regulated levels of most sweeteners (sugars and polyols), which might contribute to better taste. This work provides the first comprehensive metabolomics analysis of kernels between RTB and CTB, which may potentially provide theoretical basis for further research.

KEYWORDS

Rice-Tartary buckwheat; metabolome; taste; amino acids; sugars; polyols

1 Introduction

Conventional Tartary buckwheat (CTB, *Fagopyrum tataricum* (L.) Gaertn) is one of cultispecies belong to *Fagopyrum* Mill [1,2]. As an edible pseudocereal, the high nutrition and health value of CTB have been wildly recognized recently [3–5]. Thus, more and more people enjoy to consume CTB as compensational food to achieve health benefits [6]. However, the kernel of CTB will be broken when directly processed due to the thick shell adhering to kernel [7], thus, the kernel of CTB is barely sold due to the difficulty of seed dehulling.

Rice-Tartary buckwheat (RTB) is a special germplasm with thin shell non-adhering to kernel [7,8]. It is feasible to directly process whole kernel of Tartary buckwheat without damaging of active ingredients with RTB [9,10]. Metabolomics analysis provides a powerful insight into understanding the basis of physiological metabolism during seed development and seed quality at maturity [11]. Till now, the dynamic changes of metabolites in CTB and RTB seeds during grain filling are rarely known. Unlike CTB (also well known as 'Kuqiao'), RTB is call 'Miqiao' that is acceptable as a daily functional cereal for the palatable taste cooking like rice, but the mechanism is poorly understood. Therefore, in this study, the metabolite profiling in kernels was analyzed by GM-MS to appraise the differential metabolites between CTB and RTB, and make a comprehensive understanding of physiological metabolism during grain filling and cereal quality at maturation.

2 Materials and Methods

2.1 Plant Growth and Sampling

RTB 'cv. Xiaomiqiao' and CTB 'cv. Jinqiao 2' were planted at the Experiment Station of the Research Center of Buckwheat in Guizhou Normal University, China (1146 m, 26°50′ N, 106°58′ E) during the autumn growing season in 2018.

The grains of Tartary buckwheat were sampled at early grain filing stage (10 d after heading), middle grain filing stage (20 d after heading) and maturity (30 d after heading) with 6 replications for further investigation (Fig. 1A).

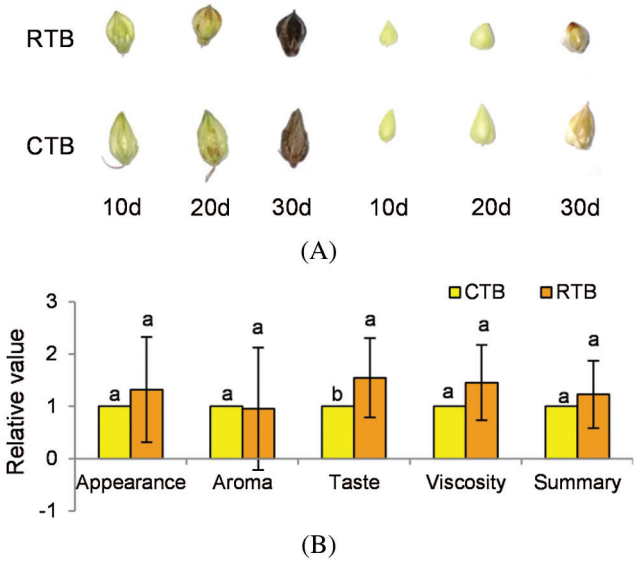

Figure 1: Phenotypic observation of grain and kernel during grain filling (A) and taste quality analysis of kernel (B) in RTB and CTB. Note: Different letters represent a significant difference ($p < 0.05$) between CTB and RTB by *t*-test, $n = 11$

2.2 Sense Organ Test

RTB and CTB seeds were artificially dehulled and cooked by electric rice cooker (MB-40EASY202, Midea, China). Twenty-two trained panelists were divided into 11 groups to participate in appreciating the sense organ test. The values of appearance, aroma, taste, viscosity and summary were appraised according to the method of Zhao et al. [12], compared with JQ2.

2.3 GC-MS Analysis

A 100 mg of samples was transferred to 5 mL centrifuge tubes and grinded by high flux organization grinding apparatus under liquid nitrogen condition. The preparation of samples for GC-MS detection was processed according to the methods of Lisec et al. [13] and Sangster et al. [14]. Then, all samples were injected into GC (Agilent GC 7890A-5975C, USA), respectively. GC was performed on a HP-5MS capillary column (5% phenyl/95% methylpolysiloxane 30 m × 250 μm i.d., 0.25 μm film thickness, Agilent J & W Scientific, Folsom, CA, USA). The injection temperature was 280°C and the split ratio was 1:20, while the interface was set to 150°C and the ion source was adjusted to 230°C. The temperature was controlled following by 60°C for 2 min, 10 °C/min rate up to 300°C and holding there for 5 min. Mass spectrometry was recorded from 35 to 750 (m/z).

The data of GC-MS was processed by G1701 MSD ChemStation and R (v3.3.2) and analyzed by the automatic mass spectral deconvolution and identification system (AMDIS, version 2.71) using National Institute of Standards and Technology (NIST), Wiley Registry and the Golm Metabolome Database (GMD). The qualitative standard was similarity greater than 70%.

Multivariate data analysis was performed by SIMCA-P (v13.0, Umetrics, Sweden) and R package *ropls*. The PCA and OPLS-DA were validated according to the method of Thévenot et al. [15]. The potential biomarkers and variable contribution were evaluated by the analysis of Variable Importance in the Projection (VIP) value (VIP > 1), ANOVA (p-value < 0.05) using SPSS (v19.0, IBM Corporation, Armonk, NY, USA).

2.4 Statistical Analysis

Statistical analysis was performed by one-way ANOVA and t-test using SPSS (v19.0, IBM Corporation, Armonk, NY, USA).

3 Results and Discussion

3.1 Phenotypic Observation and Sense Organ Test

CTB showed three edges with obviously longitudinal furrows in both shell and kernel during grain filling, while RTB exhibited thin shell without longitudinal furrows at maturity (Fig. 1A). To identify the taste quality between RTB and CTB, the shell was artificially hulled, which confirmed that CTB was very difficult to dehull, whereas RTB was easily dehulled. The sense organ test of palatability characteristics was investigated by cooking like rice. Compared with CTB, RTB exhibited better taste quality because of higher values of appearance, viscosity, taste and summary were appraised (Fig. 1B, Tab. S1).

3.2 Metabolomics Analysis of Filling Grains between RTB and CTB Seeds

The metabolites in grains of cereals are not only related to physiological metabolism, but also determine nutritive value and taste quality [11]. In this study, The GC-MS metabolomics platform was performed and 92 metabolites (including 27 amino acids, 26 organic acids, 10 sugars, 6 phosphoric acids, 6 polyols, 3 fatty acids, and others) were positively identified in grains (Fig. 2A). The hot map was established using all metabolites of the samples, which indicated that the content of metabolites varied greatly during grain filling, especially amino acids and organic acids (Fig. 2C and Tab. S2).

PCA indicated that the metabolomes in grains sampled at different filling stage were different from each other by the value of PC1 and PC2 were 81.9% and 14.9%, respectively (Fig. S1). OPLS-DA was performed to maximize the distinction between groups, with the values of R2X, R2Y and Q2 were shown in Fig. S2.

The one-way ANOVA p-value ≤ 0.05 and the VIP value ≥ 1 were performed to screen DEMs using OPLS-DA models. In total, 61 and 41 DEMs in CTB and RTB were detected at different GFS, respectively. Compared with CTB, RTB showed 60, 50 and 54 of DEMs at 10 d, 20 d and 30 d after

heading, respectively (Fig. 2B, Tab. S2). Venn diagram showed 35 common differential metabolites amongst comparison groups (Fig. 2D).

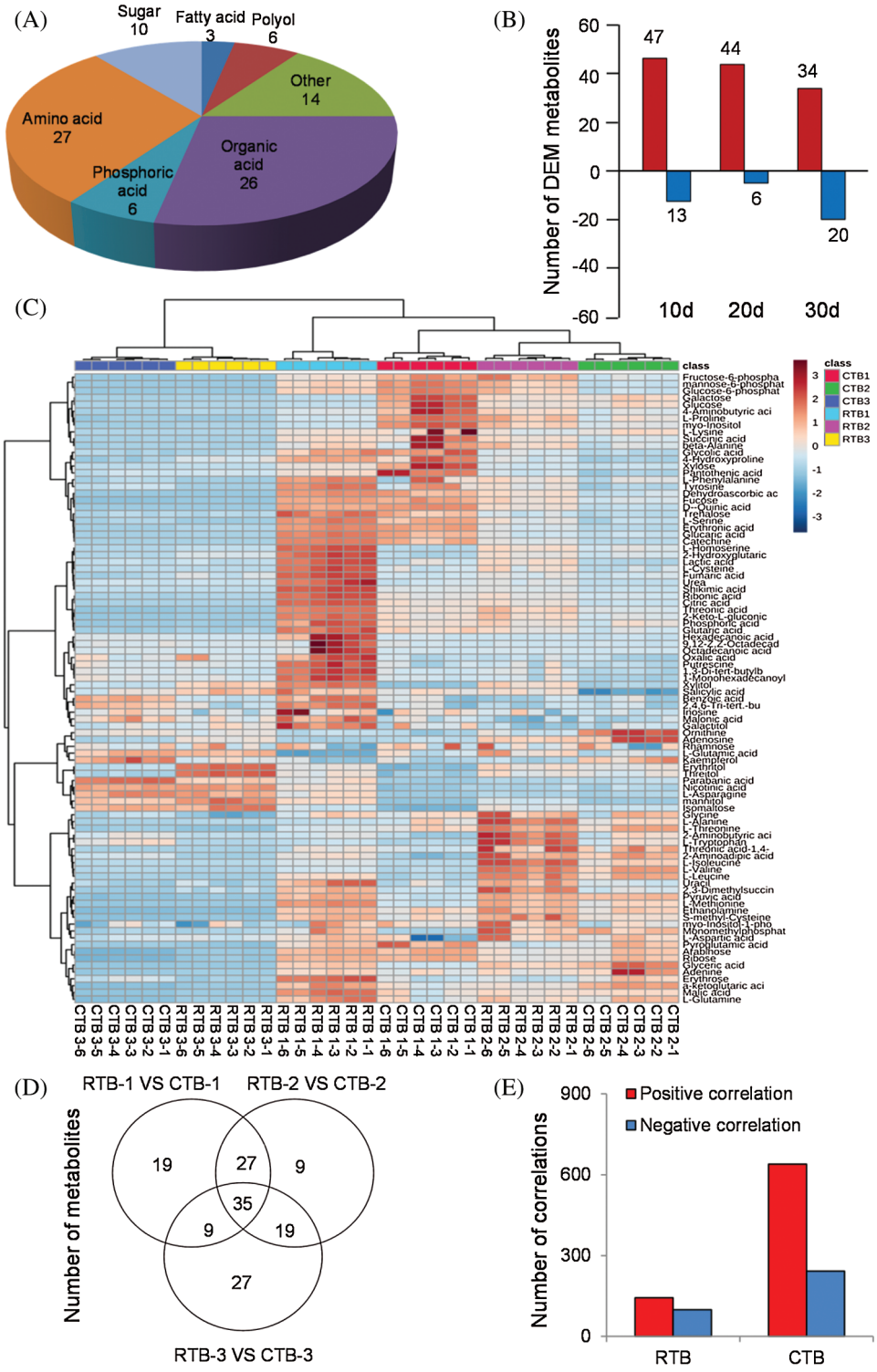

Figure 2: The groups (A), differential expressed metabolites (B), hotmap (C), common differential metabolites (D) and correlations (E) by GC-MS analysis

KEGG analysis indicated that the DEMs covered 41, 45 and 52 pathways or metabolisms at 10 d, 20 d and 30 d after heading, respectively (Fig. S3, Tab. S3). Among these, most DEMs involved in the pathways referring to amino acids metabolism (Tab. S3). The impact values indicated that the alanine, aspartate and glutamate metabolism at 10 d after flowering, beta-alanine metabolism at 20 d after flowering and glycine, serine and threonine metabolism at 30 d after flowering were the most diverse metabolic pathways during grain filling between RTB and CTB (Tab. S3). Protein-derived carbon is the substrate for the synthesis of most compounds. Therefore, it was conceivable that high levels of most amino acids were detected at early-GFS, but decreased to very low level at maturity (Tab. S2). Asparagine is a key nitrogen form of amino acids for long-distance transport to the seeds. It has been proposed that free asparagine levels are positive related to seed protein concentration, asparagine might act as a metabolite signal for protein accumulation in seed [16,17]. In this study, high levels of asparagine were detected at late-GFS rather than early-GFS in both CTB and RTB seeds. By contrast, the levels of glutamine were rapidly decreased at late-GFS. Meanwhile, the levels of glutamic acid were increased at late-GFS, while the levels of aspartic acid were decreased (Figs. 3A–3B). These results implied that, unlike other plants, the asparagine synthesis in Tartary buckwheat seeds was enhanced at late-GFS, which might influence seed protein accumulation.

Person correlation coefficient analysis indicated that there were 881 and 252 significant correlation coefficients ($p < 0.01$, $r^2 \geq 0.49$, FDR ≤ 0.01) in CTB and RTB, respectively. Out of these significant correlations, 639 were positive and 242 were negative in CTB, whereas 143 were positive and 242 were negative in RTB (Fig. 2E).

3.2.1 Analysis of Nutrients between CTB and RTB

For human body, there are 8 essential amino acids that cannot be synthesized by itself. Tartary buckwheat seeds contain all 8 of the essential amino acids, of which levels varied greatly during grain filling (Figs. 3A–3C). These essential amino acids belong to 3 amino acids families: Aspartic acid family, Alanine family and Aromatic amino acid family. Typically, the similar change pattern of most amino acids during grain filling was observed in the same amino acids family pathway, such as leucine and valine belong to Alanine family; threonine and isoleucine belong to Aspartic acid family (Figs. 3A–3B). But, some amino acids involved in the same metabolic pathway changed differently during grain filling (Figs. 3A–3C). It was probably because of the complicated relationship of metabolites, such as competition, promotion and suppression.

Lysine and methionine are the first and second limiting amino acids, of which levels are generally used to evaluate the nutritional value of cereals [18]. CTB shows excellent lysine proportion of protein, which is better than the mode value of WHO/FAO and egg [19]. In this study, no significantly difference was detected in the levels of lysine and methionine by one-way ANOVA between RTB and CTB at 30 d after heading (Fig. 3B), which indicated that RTB also exhibited excellent nutritional value as CTB.

3.2.2 Analysis of Sweeteners between CTB and RTB

CTB is well-known as ‘Kuqiao’ in China because of the bitter taste. In history, CTB is a staple food for only Yi people in China [20]. It is conjectured that the bitter feature limited the consumption of Tartary buckwheat. Sugars not only act as signals for metabolism in seed during grain filling, but also are crucial constituents that influence the sweet taste [21]. In this study, 9 sugars were identified, while 6 sugars including arabinose, fucose, glucose, galactose, ribose and xylose in RTB were detected to be significantly higher than CTB (Fig. 3D). Meanwhile, polyols are well-known as sugar-free sweeteners. Xylitol, mannitol and maltitol are suggested as sucrose replacers in food [22]. Compared with CTB, the levels of most polyols were found to be higher in RTB regardless of GFS (Tab. S2). Especially, the levels of xylitol, mannitol, maltitol and threitol in RTB were detected to be significantly higher at maturity (Fig. 3D). The previous work found that the level of major storage components including starches and proteins in RTB were similar to CTB [10]. Therefore, the high level of sugars and polyols might be responsible for the better taste of RTB.

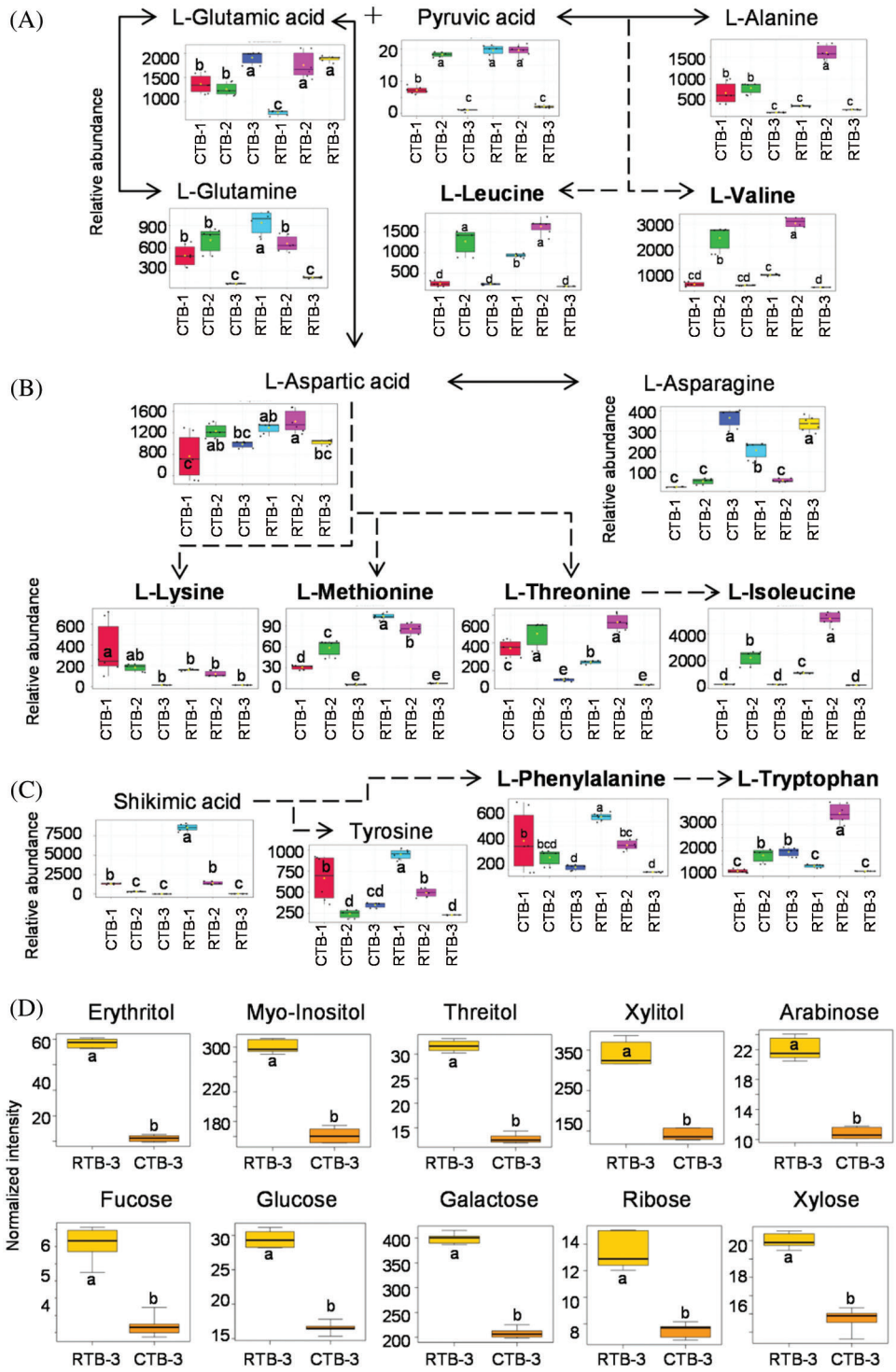

Figure 3: Boxplot-visualizations of Alanine family (A), Aspartic acid family (B) and Aromatic amino acid family (C) metabolites during grain filling and differential expressed sweeteners (D) at maturity between RTB and CTB. Note: Different letters represent a significant difference ($p < 0.05$) between CTB and RTB by one-way ANOVA in (A), (B) and (C) and *t*-test in (D), $n = 6$

4 Conclusions

RTB showed easily dehulled property because of the thin shell without longitudinal furrows adhering to seeds. Compared with CTB, RTB exhibited better taste quality by higher values of appearance, viscosity, taste and summary were appraised in sense organ test. In total, 92 metabolites were identified in kernels using GM-MS metabolomics platform. Among these, the significantly up-regulated levels of most sweeteners (sugars and polyols) in RTB seeds might contribute to the better taste. During grain filling, the levels of most metabolites changed greatly in both RTB and CTB seeds. ANOVA analysis identified 61 DEMs between RTB and CTB, while Venn diagram analysis screened 35 common differential metabolites. KEGG found that most DEMs involved in the pathways referring to amino acids metabolism. Unlike other plant, Tartary buckwheat exhibited unique protein-derived carbon change pattern, which might influence seed protein accumulation. This work provides the first comprehensive metabolomics analysis of kernels between RTB and CTB, which may potentially provide theoretical basis for further research.

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Author Contribution: YW and CL proposed the original idea. YW provided the experiment methods and supervised the experiment data collection. TS provided the materials. CW, WY, KL and QC participated in the fieldwork and the evaluation of the phenotype. DX performed sense organ test. CL and CW performed data analysis and drafted the manuscript. YW revised the manuscript. All authors have read and approved the final version of manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Appendix

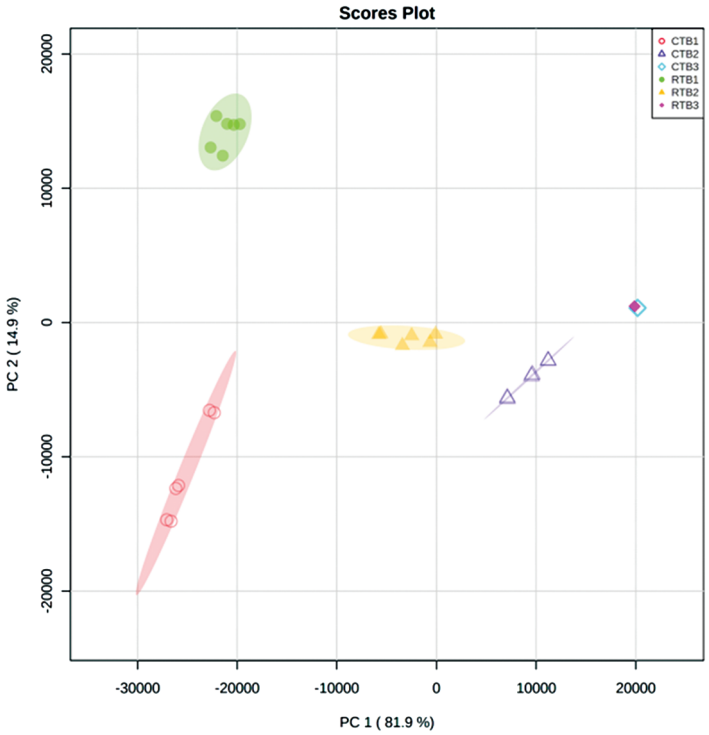

Figure S1: The PCA of metabolites in RTB and CTB during grain filling

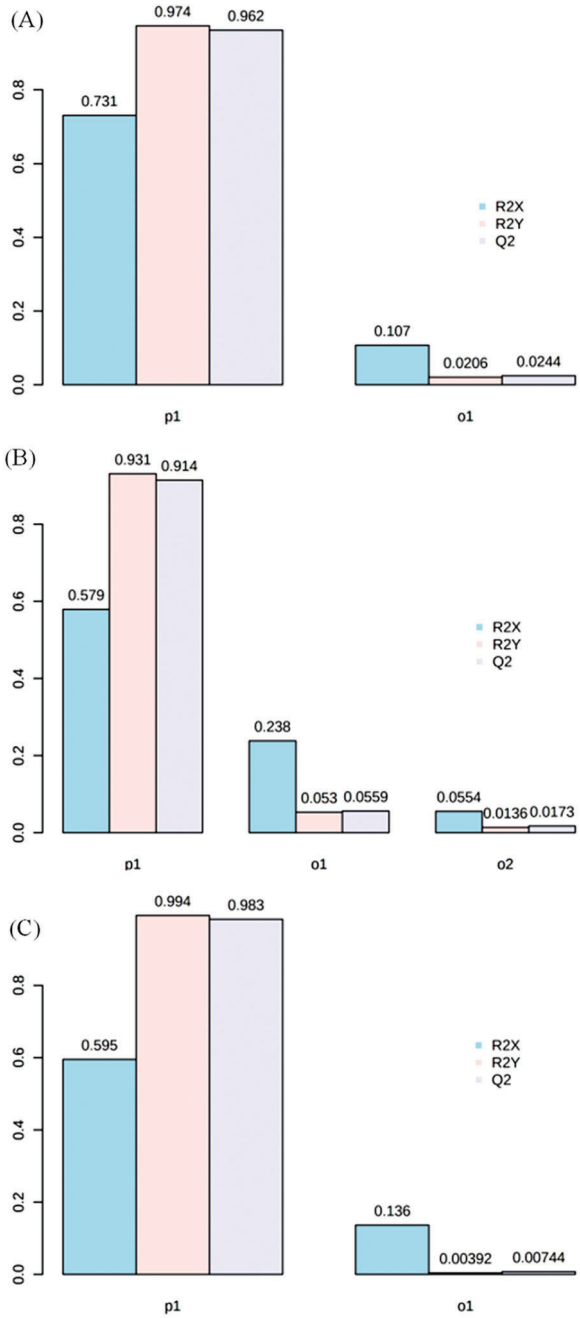

Figure S2: The value of R^2X, R^2Y and Q^2 in groups of RTB1 *vs.* CTB1 (A), RTB2 *vs.* CTB2 (B) and RTB2 *vs.* CTB2 (C) by OPLS-DA

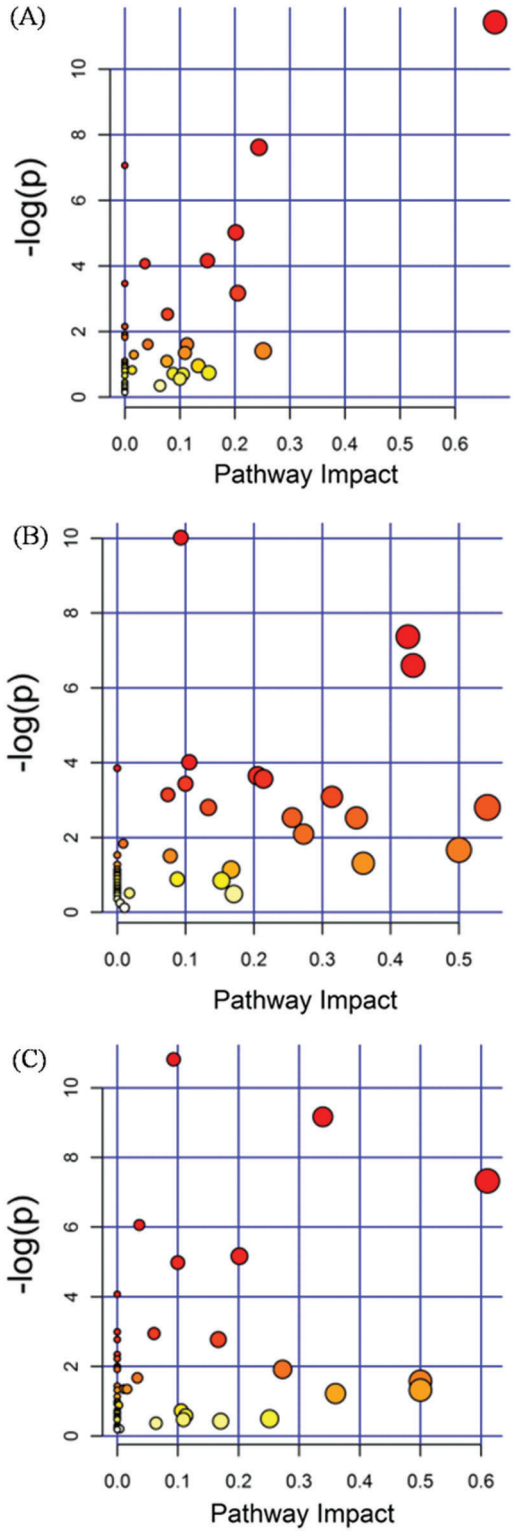

Figure S3: The analysis of KEGG in groups of RTB1 *vs.* CTB1 (A), RTB2 *vs.* CTB2 (B) and RTB2 *vs.* CTB2 (C) by OPLS-DA

Table S1: The taste quality analysis of kernel

RTB	Appearance	Aroma	Taste	Viscosity	Summary
Group 1	2.00	1.50	2.50	2.50	1.50
Group 2	2.50	2.50	1.00	2.00	0.50
Group 3	0.50	0.50	1.50	1.50	0.50
Group 4	1.00	−0.50	1.50	2.00	2.00
Group 5	1.00	−1.50	1.50	0.50	0.50
Group 6	0.50	1.00	2.00	2.00	2.00
Group 7	0.50	0.50	0.50	1.50	2.00
Group 8	3.00	2.00	2.50	0.00	1.50
Group 9	2.50	2.00	2.50	1.00	1.50
Group 10	1.00	1.50	0.50	1.50	0.50
Group 11	0.00	1.00	1.00	1.50	1.00
Mean	1.32	0.95	1.55	1.45	1.23
SD	1.01	1.17	0.76	0.72	0.65

Note: The value of each indicator in CTB was set as 1.00

Table S2: The differential expressed metabolites (DEMs)

Metabolites	RTB1	CTB1	VIP	*p* value	*q* value	log2fc_CTB1/RTB1
Pyruvic acid	19.55	7.22	1.13	0.005	0.007	−1.44
Lactic acid	359.09	107.70	1.13	0.005	0.007	−1.74
Oxalic acid	180.23	91.71	1.09	0.005	0.007	−0.97
2-Aminobutyric acid	11.32	8.12	1.05	0.005	0.007	−0.48
Monomethylphosphate	50.01	17.03	1.01	0.005	0.007	−1.55
Malonic acid	2.18	1.34	1.06	0.005	0.007	−0.70
L-Valine	753.39	339.09	1.10	0.005	0.007	−1.15
Urea	89.24	18.27	1.12	0.005	0.007	−2.29
Benzoic acid	22.16	15.62	1.04	0.005	0.007	−0.50
1,3-Di-tert-butylbenzene	51.65	31.31	1.13	0.005	0.007	−0.72
L-Leucine	933.58	248.94	1.14	0.005	0.007	−1.91
Phosphoric acid	14938.26	7832.00	1.08	0.005	0.007	−0.93
Nicotinic acid	11.27	4.65	1.12	0.005	0.007	−1.28
L-Isoleucine	1092.24	253.23	1.14	0.005	0.007	−2.11
L-Proline	280.79	595.69	1.12	0.005	0.007	1.09
Glyceric acid	81.89	35.39	1.14	0.005	0.007	−1.21

(Continued)

Table S2 (continued).

Uracil	11.80	7.12	1.06	0.005	0.007	−0.73
Fumaric acid	140.31	61.30	1.14	0.005	0.007	−1.19
Glutaric acid	7.98	4.51	1.11	0.005	0.007	−0.82
S-methyl-Cysteine	15.53	10.08	1.00	0.005	0.007	−0.62
Putrescine	41.56	24.52	1.12	0.005	0.007	−0.76
L-Homoserine	77.20	3.57	1.14	0.005	0.007	−4.44
Malic acid	2780.80	1301.26	1.09	0.005	0.007	−1.10
Parabanic acid	19.19	3.77	1.10	0.005	0.007	−2.35
Erythritol	30.06	10.49	1.14	0.005	0.007	−1.52
Threitol	18.61	11.46	1.11	0.005	0.007	−0.70
L-Methionine	103.89	30.67	1.14	0.005	0.007	−1.76
4-Aminobutyric acid	325.35	1166.47	1.01	0.005	0.007	1.84
2,4,6-Tri-tert.-butylbenzenethiol	32.23	21.46	1.08	0.005	0.007	−0.59
Threonic acid	42.86	20.86	1.14	0.005	0.007	−1.04
L-Cysteine	32.65	6.89	1.13	0.005	0.007	−2.25
2-Hydroxyglutaric acid	36.33	5.94	1.14	0.005	0.007	−2.61
a-ketoglutaric acid	12.04	6.47	1.07	0.005	0.007	−0.90
2,3-Dimethylsuccinic acid	6.87	2.47	1.14	0.005	0.007	−1.48
L-Glutamic acid	774.71	1367.84	1.08	0.005	0.007	0.82
L-Asparagine	206.58	25.92	1.11	0.005	0.007	−2.99
Xylose	68.48	207.94	1.03	0.005	0.007	1.60
2-Aminoadipic acid	32.10	12.40	1.11	0.005	0.007	−1.37
Xylitol	550.28	14.63	1.14	0.005	0.007	−5.23
Ribonic acid	184.20	59.48	1.14	0.005	0.007	−1.63
L-Glutamine	936.13	503.31	1.05	0.005	0.007	−0.90
2-Keto-L-gluconic acid	157.87	80.73	1.13	0.005	0.007	−0.97
Shikimic acid	8561.28	1316.42	1.14	0.005	0.007	−2.70
Citric acid	19992.02	7587.98	1.14	0.005	0.007	−1.40
Erythrose	33.45	16.86	1.13	0.005	0.007	−0.99
D(-)-Quinic acid	34786.22	40427.38	1.06	0.005	0.007	0.22
Galactose	2363.94	21326.59	1.11	0.005	0.007	3.17
Glucose	165.26	1578.22	1.08	0.005	0.007	3.26

(Continued)

Table S2 (continued).

mannitol	1476.84	35.55	1.14	0.005	0.007	−5.38
Pantothenic acid	27.48	51.20	1.08	0.005	0.007	0.90
Hexadecanoic acid	178.87	43.76	1.05	0.005	0.007	−2.03
Glucaric acid	394.79	307.01	1.07	0.005	0.007	−0.36
myo-Inositol	639.33	2813.00	1.12	0.005	0.007	2.14
Fructose-6-phosphate	67.69	124.14	1.07	0.005	0.007	0.87
mannose-6-phosphate	173.34	293.92	1.08	0.005	0.007	0.76
Glucose-6-phosphate	42.47	64.76	1.08	0.005	0.007	0.61
1-Monohexadecanoylglycerol	65.50	40.48	1.13	0.005	0.007	−0.69
Adenosine	22.49	32.15	1.01	0.005	0.007	0.52
Isomaltose	87.55	16.16	1.12	0.005	0.007	−2.44
Catechine	8158.24	6115.57	1.01	0.005	0.007	−0.42
Metabolites	RTB2	CTB2	VIP	*p* value	*q* value	log2fc_CTB2/RTB2
Lactic acid	181.06	65.97	1.17	0.005	0.010	−1.46
Glycolic acid	1.99	1.42	1.15	0.005	0.010	−0.48
L-Alanine	1599.82	793.08	1.16	0.005	0.010	−1.01
2-Aminobutyric acid	22.41	12.81	1.13	0.005	0.010	−0.81
Urea	25.39	13.09	1.12	0.005	0.010	−0.96
L-Isoleucine	5122.19	2218.68	1.16	0.005	0.010	−1.21
L-Proline	484.06	332.38	1.11	0.005	0.010	−0.54
Succinic acid	72.46	43.21	1.14	0.005	0.010	−0.75
Glyceric acid	76.46	117.59	1.06	0.005	0.010	0.62
Uracil	10.81	7.51	1.15	0.005	0.010	−0.52
L-Serine	702.42	447.98	1.11	0.005	0.010	−0.65
Glutaric acid	3.71	2.67	1.03	0.005	0.010	−0.47
S-methyl-Cysteine	18.48	10.45	1.06	0.005	0.010	−0.82
beta-Alanine	51.48	28.24	1.09	0.005	0.010	−0.87
L-Homoserine	31.48	17.34	1.13	0.005	0.010	−0.86
L-Methionine	85.72	58.77	1.06	0.005	0.010	−0.54
4-Hydroxyproline	4.13	1.79	1.14	0.005	0.010	−1.21
4-Aminobutyric acid	571.64	305.50	1.14	0.005	0.010	−0.90
Threonic acid	27.89	15.34	1.14	0.005	0.010	−0.86

(Continued)

Table S2 (continued).

Erythronic acid	48.00	21.47	1.17	0.005	0.010	−1.16
2-Hydroxyglutaric acid	15.01	10.49	1.09	0.005	0.010	−0.52
a-ketoglutaric acid	6.86	12.13	1.18	0.005	0.010	0.82
2,3-Dimethylsuccinic acid	9.41	3.16	1.17	0.005	0.010	−1.58
L-Glutamic acid	1757.14	1257.63	1.03	0.005	0.010	−0.48
Xylose	37.48	25.56	1.08	0.005	0.010	−0.55
Xylitol	308.64	79.49	1.17	0.005	0.010	−1.96
Fucose	33.58	21.55	1.12	0.005	0.010	−0.64
Ribonic acid	64.78	40.10	1.11	0.005	0.010	−0.69
2-Keto-L-gluconic acid	96.10	61.15	1.10	0.005	0.010	−0.65
Shikimic acid	1392.14	303.54	1.17	0.005	0.010	−2.20
Ornithine	30.03	79.68	1.17	0.005	0.010	1.41
Citric acid	7505.07	3306.16	1.16	0.005	0.010	−1.18
Erythrose	16.48	23.12	1.07	0.005	0.010	0.49
Dehydroascorbic acid dimer	107.13	48.82	1.14	0.005	0.010	−1.13
D(-)-Quinic acid	19553.44	7430.09	1.18	0.005	0.010	−1.40
Tyrosine	494.19	244.00	1.16	0.005	0.010	−1.02
mannitol	778.50	140.46	1.18	0.005	0.010	−2.47
Pantothenic acid	19.15	11.76	1.14	0.005	0.010	−0.70
myo-Inositol	1699.23	933.55	1.15	0.005	0.010	−0.86
L-Tryptophan	2929.29	1333.11	1.15	0.005	0.010	−1.14
Fructose-6-phosphate	124.44	42.63	1.15	0.005	0.010	−1.55
mannose-6-phosphate	197.66	59.23	1.17	0.005	0.010	−1.74
Glucose-6-phosphate	44.72	15.87	1.16	0.005	0.010	−1.49
Salicylic acid	56.51	20.93	1.18	0.005	0.010	−1.43
Isomaltose	111.31	84.52	1.10	0.005	0.010	−0.40
Catechine	2536.31	794.15	1.18	0.005	0.010	−1.68
Kaempferol	9.70	18.19	1.10	0.005	0.010	0.91
L-Cysteine	13.31	4.90	1.03	0.013	0.023	−1.44
myo-Inositol-1-phosphate	53.11	36.88	1.01	0.013	0.023	−0.53
L-Lysine	120.48	182.52	1.00	0.031	0.045	0.60

(Continued)

Table S2 (continued).

Metabolites	RTB3	CTB3	VIP	*p* value	*q* value	log2fc_CTB3/RTB3
L-Tryptophan	734.25	1463.09	1.19	0.005	0.009	0.99
Parabanic acid	25.80	43.34	1.21	0.005	0.009	0.75
Kaempferol	13.18	21.36	1.15	0.005	0.009	0.70
2-Aminoadipic acid	15.79	24.96	1.20	0.005	0.009	0.66
Tyrosine	230.12	348.46	1.19	0.005	0.009	0.60
Shikimic acid	19.82	13.09	1.19	0.005	0.009	−0.60
mannitol	2535.99	1673.41	1.14	0.005	0.009	−0.60
L-Glutamine	211.29	130.70	1.21	0.005	0.009	−0.69
Fucose	6.08	3.69	1.18	0.005	0.009	−0.72
myo-Inositol	300.05	181.36	1.23	0.005	0.009	−0.73
Ethanolamine	46.27	27.75	1.19	0.005	0.009	−0.74
Ribose	13.38	7.53	1.18	0.005	0.009	−0.83
Glucose	29.44	16.55	1.22	0.005	0.009	−0.83
Galactose	399.59	208.28	1.23	0.005	0.009	−0.94
2-Hydroxyglutaric acid	11.49	5.92	1.23	0.005	0.009	−0.96
Malic acid	309.91	157.53	1.22	0.005	0.009	−0.98
a-ketoglutaric acid	1.73	0.83	1.17	0.005	0.009	−1.07
Pyruvic acid	2.13	0.99	1.05	0.005	0.009	−1.10
Arabinose	19.98	8.79	1.21	0.005	0.009	−1.18
Threonic acid	3.63	1.55	1.23	0.005	0.009	−1.23
D(-)-Quinic acid	266.03	110.99	1.13	0.005	0.009	−1.26
Xylitol	339.42	140.67	1.21	0.005	0.009	−1.27
Threitol	31.65	12.80	1.23	0.005	0.009	−1.31
Glyceric acid	4.78	1.86	1.23	0.005	0.009	−1.36
Adenosine	61.42	23.79	1.22	0.005	0.009	−1.37
Erythronic acid	17.77	6.76	1.23	0.005	0.009	−1.39
4-Hydroxyproline	4.27	1.58	1.17	0.005	0.009	−1.43
Erythritol	58.45	21.17	1.23	0.005	0.009	−1.46
L-Homoserine	8.38	2.90	1.23	0.005	0.009	−1.53
4-Aminobutyric acid	84.02	24.00	1.20	0.005	0.009	−1.81
L-Valine	206.65	295.25	1.20	0.005	0.009	0.51

(Continued)

Table S2 (continued).

L-Threonine	97.85	133.76	1.18	0.005	0.009	0.45
Glycine	97.55	130.42	1.08	0.005	0.009	0.42
Benzoic acid	15.64	20.79	1.21	0.005	0.009	0.41
Erythrose	13.38	17.38	1.13	0.005	0.009	0.38
2,4,6-Tri-tert.-butylbenzenethiol	21.83	28.01	1.12	0.005	0.009	0.36
L-Leucine	186.38	237.67	1.16	0.005	0.009	0.35
L-Isoleucine	184.99	234.46	1.18	0.005	0.009	0.34
Octadecanoic acid	36.80	45.94	1.14	0.005	0.009	0.32
2-Aminobutyric acid	9.66	11.85	1.14	0.005	0.009	0.29
Catechine	117.00	143.31	1.00	0.008	0.014	0.29
L-Phenylalanine	179.81	219.68	1.08	0.005	0.009	0.29
Hexadecanoic acid	43.17	52.71	1.18	0.005	0.009	0.29
L-Serine	326.78	391.51	1.12	0.005	0.009	0.26
Pyroglutamic acid	482.62	523.17	1.04	0.005	0.009	0.12
Glucaric acid	56.60	48.21	1.15	0.005	0.009	−0.23
L-Alanine	298.23	235.17	1.21	0.005	0.009	−0.34
Xylose	19.99	15.72	1.21	0.005	0.009	−0.35
2-Keto-L-gluconic acid	24.30	18.83	1.19	0.005	0.009	−0.37
Citric acid	1114.51	852.52	1.21	0.005	0.009	−0.39
Salicylic acid	67.34	50.82	1.14	0.005	0.009	−0.41
Fumaric acid	64.41	48.34	1.05	0.008	0.014	−0.41
Adenine	6.93	5.15	1.17	0.005	0.009	−0.43
Ribonic acid	14.96	11.03	1.02	0.005	0.009	−0.44

Table S3: The analysis of KEGG

Pathway	T	E	H	R p	L	HA	F	I	Compounds	Pathway	Links
Alanine, aspartate and glutamate metabolism	22	1.04	5	0.003	5.86	0.2	0.1	0.3	C00026, C00064, C00152, C00022, C00334	ath00250	http://www.kegg.jp/pathway/ath00250+C00026+C00064+C00152+C00022+C00334
Aminoacyl-tRNA biosynthesis	67	3.17	9	0.003	5.74	0.3	0.1	0.1	C00152, C00064, C00065, C00073, C00183, C00407, C00123, C00188, C00148	ath00970	http://www.kegg.jp/pathway/ath00970+C00152+C00064+C00065+C00073+C00183+C00407+C00123+C00188+C00148
Valine, leucine and isoleucine biosynthesis	26	1.23	5	0.006	5.09	0.5	0.2	0.0	C00188, C00123, C00183, C00407, C00022	ath00290	http://www.kegg.jp/pathway/ath00290+C00188+C00123+C00183+C00407+C00022
Pantothenate and CoA biosynthesis	14	0.66	3	0.025	3.68	1.0	0.5	0.2	C00022, C00183, C00864	ath00770	http://www.kegg.jp/pathway/ath00770+C00022+C00183+C00864

(Continued)

Table S3 (continued).

Pathway	T	E	H	R p	L	HA	F	I	Compounds	Pathway	Links
Arginine and proline metabolism	38	1.80	5	0.030	3.50	1.0	0.5	0.3	C00077, C00148, C00064, C01157, C00334	ath00330	http://www.kegg.jp/pathway/ath00330+C00077+C00148+C00064+C01157+C00334
Glycine, serine and threonine metabolism	30	1.42	4	0.050	3.00	1.0	0.7	0.4	C00065, C00188, C00263, C00022	ath00260	http://www.kegg.jp/pathway/ath00260+C00065+C00188+C00263+C00022
Citrate cycle (TCA cycle)	20	0.95	3	0.065	2.74	1.0	0.8	0.2	C00026, C00158, C00022	ath00020	http://www.kegg.jp/pathway/ath00020+C00026+C00158+C00022
Cysteine and methionine metabolism	34	1.61	4	0.073	2.62	1.0	0.8	0.2	C00073, C00065, C00263, C00022	ath00270	http://www.kegg.jp/pathway/ath00270+C00073+C00065+C00263+C00022
Ascorbate and aldarate metabolism	15	0.71	2	0.156	1.86	1.0	1.0	0.0	C00137, C00818	ath00053	http://www.kegg.jp/pathway/ath00053+C00137+C00818
C5-Branched dibasic acid metabolism	4	0.19	1	0.177	1.73	1.0	1.0	0.0	C00022	ath00660	http://www.kegg.jp/pathway/ath00660+C00022

(Continued)

Table S3 (continued).

Pathway	T	E	H	R p	L	HA	F	I	Compounds	Pathway	Links
Glyoxylate and dicarboxylate metabolism	17	0.81	2	0.191	1.66	1.0	1.0	0.1	C00160, C00158	ath00630	http://www.kegg.jp/pathway/ath00630+C00160+C00158
Butanoate metabolism	18	0.85	2	0.208	1.57	1.0	1.0	0.0	C00022, C00334	ath00650	http://www.kegg.jp/pathway/ath00650+C00022+C00334
Valine, leucine and isoleucine degradation	34	1.61	3	0.215	1.54	1.0	1.0	0.0	C00183, C00407, C00123	ath00280	http://www.kegg.jp/pathway/ath00280+C00183+C00407+C00123
Glucosinolate biosynthesis	54	2.56	4	0.250	1.39	1.0	1.0	0.0	C00073, C00183, C00123, C00407	ath00966	http://www.kegg.jp/pathway/ath00966+C00073+C00183+C00123+C00407
Carbon fixation in photosynthetic organisms	21	0.99	2	0.262	1.34	1.0	1.0	0.0	C00022, C00085	ath00710	http://www.kegg.jp/pathway/ath00710+C00022+C00085
Amino sugar and nucleotide sugar metabolism	41	1.94	3	0.307	1.18	1.0	1.0	0.1	C00275, C00085, C00259	ath00520	http://www.kegg.jp/pathway/ath00520+C00275+C00085+C00259

(Continued)

Table S3 (continued).

Pathway	T	E	H	R p	L	HA	F	I	Compounds	Pathway	Links
Inositol phosphate metabolism	24	1.14	2	0.316	1.15	1.0	1.0	0.3	C00092, C00137	ath00562	http://www.kegg.jp/pathway/ath00562+C00092+C00137
Galactose metabolism	26	1.23	2	0.351	1.05	1.0	1.0	0.0	C00031, C00137	ath00052	http://www.kegg.jp/pathway/ath00052+C00031+C00137
Lysine biosynthesis	10	0.47	1	0.385	0.95	1.0	1.0	0.0	C00263	ath00300	http://www.kegg.jp/pathway/ath00300+C00263
Cyanoamino acid metabolism	11	0.52	1	0.415	0.88	1.0	1.0	0.0	C00065	ath00460	http://www.kegg.jp/pathway/ath00460+C00065
Methane metabolism	11	0.52	1	0.415	0.88	1.0	1.0	0.2	C00065	ath00680	http://www.kegg.jp/pathway/ath00680+C00065
Starch and sucrose metabolism	30	1.42	2	0.420	0.87	1.0	1.0	0.1	C01083, C00031	ath00500	http://www.kegg.jp/pathway/ath00500+C01083+C00031
Pentose and glucuronate interconversions	12	0.57	1	0.443	0.81	1.0	1.0	0.0	C00181	ath00040	http://www.kegg.jp/pathway/ath00040+C00181
beta-Alanine metabolism	12	0.57	1	0.443	0.81	1.0	1.0	0.0	C00864	ath00410	http://www.kegg.jp/pathway/ath00410+C00864

(Continued)

Table S3 (continued).

Pathway	T	E	H	R p	L	HA	F	I	Compounds	Pathway	Links
Nicotinate and nicotinamide metabolism	12	0.57	1	0.443	0.81	1.0	1.0	0.0	C00253	ath00760	http://www.kegg.jp/pathway/ath00760+C00253
Sulfur metabolism	12	0.57	1	0.443	0.81	1.0	1.0	0.0	C00065	ath00920	http://www.kegg.jp/pathway/ath00920+C00065
Glycerolipid metabolism	13	0.62	1	0.469	0.76	1.0	1.0	0.0	C00258	ath00561	http://www.kegg.jp/pathway/ath00561+C00258
Sphingolipid metabolism	13	0.62	1	0.469	0.76	1.0	1.0	0.0	C00065	ath00600	http://www.kegg.jp/pathway/ath00600+C00065
Nitrogen metabolism	15	0.71	1	0.519	0.66	1.0	1.0	0.0	C00064	ath00910	http://www.kegg.jp/pathway/ath00910+C00064
Fructose and mannose metabolism	16	0.76	1	0.542	0.61	1.0	1.0	0.2	C00275	ath00051	http://www.kegg.jp/pathway/ath00051+C00275
Purine metabolism	61	2.89	3	0.561	0.58	1.0	1.0	0.0	C00064, C00212, C00147	ath00230	http://www.kegg.jp/pathway/ath00230+C00064+C00212+C00147
Pentose phosphate pathway	18	0.85	1	0.585	0.54	1.0	1.0	0.0	C00121	ath00030	http://www.kegg.jp/pathway/ath00030+C00121

(Continued)

Table S3 (continued).

Pathway	T	E	H	R p	L	HA	F	I	Compounds	Pathway	Links
Zeatin biosynthesis	19	0.90	1	0.605	0.50	1.0	1.0	0.0	C00147	ath00908	http://www.kegg.jp/pathway/ath00908+C00147
Phenylalanine, tyrosine and tryptophan biosynthesis	21	0.99	1	0.642	0.44	1.0	1.0	0.1	C00493	ath00400	http://www.kegg.jp/pathway/ath00400+C00493
Pyruvate metabolism	21	0.99	1	0.642	0.44	1.0	1.0	0.1	C00022	ath00620	http://www.kegg.jp/pathway/ath00620+C00022
Glycerophospholipid metabolism	25	1.18	1	0.706	0.35	1.0	1.0	0.0	C00189	ath00564	http://www.kegg.jp/pathway/ath00564+C00189
Terpenoid backbone biosynthesis	25	1.18	1	0.706	0.35	1.0	1.0	0.0	C00022	ath00900	http://www.kegg.jp/pathway/ath00900+C00022
Glycolysis or Gluconeogenesis	25	1.18	1	0.706	0.35	1.0	1.0	0.1	C00022	ath00010	http://www.kegg.jp/pathway/ath00010+C00022
Pyrimidine metabolism	38	1.80	1	0.846	0.17	1.0	1.0	0.0	C00064	ath00240	http://www.kegg.jp/pathway/ath00240+C00064

Note: T represents Total, E represents Expected, H represents Hit, R p represents Raw p, L represents–LOG (p), HA represents Holm adjust, F represents FDR, I represents Impact