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Abstract

Background: Rice eating quality and nutritional quality is affected by both grain development and the post-harvesting process, but the dynamic metabolite changes among rice cultivars during these processes are unclear.

Results: We successfully identified 623 metabolites in four indica cultivars with different eating qualities using a widely-targeted metabolomics approach, and found that the metabolic variation became increasingly smaller and differed according to the stage and cultivar during grain development and post-harvesting. Our results suggested that the levels of sugars, amino acids, lipids, and flavones demonstrated cultivar-specific changes during grain development, and sufficient carbon supply during grain development may contribute to the formation of excellent eating quality. We further found that most of the metabolites, especially the nutritional metabolites, decreased significantly or even lost, whereas only few increased during post-harvesting, further indicating that the post-harvesting process reduced the metabolic molecular differences between rice cultivars with different eating qualities, and had an even greater influence on metabolites than genetic factors.

Conclusions: We found that a large number of metabolites changed significantly in the rice cultivars during grain development and the post-harvesting process, which facilitates further study of rice quality formation and has potential application in eating quality and nutritional quality improvement.

Keywords: rice, eating quality, nutritional quality, post-harvesting process, metabolomics

Background

Rice has been consumed for almost 5000 years and currently feeds almost half of the human population (Liu et al. 2013). With the improvement in living standards, rice quality is now a top consideration for customers and breeding programs. Rice quality is mainly related to grain appearance, milling quality, nutritional quality, and cooking and eating quality (Hori 2018). In addition, rice cultivars of different qualities are also used for medical, ceremonial, or special production purposes (Tian et al. 2009).

Rice quality, especially the eating quality and nutritional quality, is closely related to the chemical composition of the grains, including starch (mainly amylose and amylopectin), proteins, and lipids. These metabolites are synthesized and genetically regulated during rice grain development. The development of the rice grain can be divided into three basic stages: the embryonic development stage, the cell division stage, and the morphogenesis stage; the second stage of grain maturation, during which a large number of storage reserves accumulate; and the third stage of grain drying and dormancy (Deng and Wang 2013; Sreenivasulu et al. 2015). These three stages are associated with substantial spatiotemporal metabolic rearrangements regulated by global gene expression programs. Previous studies mainly focused on the mechanisms of rice quality formation at the level of gene regulation. For example, Zhao et al. found that *GS9* acts as a transcriptional activator to regulate rice grain shape and appearance (Zhao et al. 2018). It is reported that the natural variation in *OsGluA2* is involved in grain protein content regulation in rice (Yang et al. 2019). Wang et al. reported that a lipid transfer protein, OsLTPL36, is essential for grain development and grain quality in rice (Wang et al.2015). However, few studies have

63 investigated the mechanism behind the formation of rice quality at the global
64 metabolite level.

65 With the exception of genetic regulation during grain development, rice quality,
66 especially the eating quality and nutritional quality, is partially affected by the post-
67 harvesting processes, which can cause physical, chemical, and structural changes in
68 the grain (Corrêa et al. 2007). The post-harvesting processes, including harvesting,
69 threshing, drying, transportation, storage, rice processing, and milling, are primarily
70 influenced by human factors rather than genetic regulation. During these processes,
71 harvesting at the incorrect product maturity stage, excessive exposure to rain and
72 extreme temperatures, and microbial contamination can seriously affect rice quality
73 (Taiwo and Bart 2016). For example, rice milling is a crucial step whereby the husk
74 and the bran layers (including cuticular layer and aleurone layer embryo) are removed.
75 During this process, a large amount of nutritional metabolites, such as sterols, γ-
76 oryzanols, tocopherols, tocotrienols, and phenolic compounds, is lost in the rice bran,
77 which is a processing byproduct (Aguilar et al. 2007). However, few studies have
78 aimed to clarify how many beneficial metabolites are retained in the polished rice to
79 be consumed during the post-harvesting processes. Studying the metabolites
80 remaining in polished rice can improve the quality of rice, especially the nutritional
81 quality. For example, Zhang et al. (2019) reported that plant metabolic engineering
82 and synthetic biology strategies can be effectively and accurately applied in the
83 synthesis of specific micronutrients, phytonutrients, and/or bioactive components in
84 crops.

85 Finally, regardless of gene regulation during grain development or artificial
86 factors during the post-harvesting processes, the quality of rice is closely related to its

metabolites. It is thus of great significance that the changes in metabolites during grain development and the post-harvesting are explored to contribute to rice quality and breeding research. Metabolomics has provided an efficient large-scale solution that complements traditional and genomic approaches for investigating rice grain development and the post-harvesting processes (Chen et al. 2016; Sulpice 2019; Zhu et al. 2018). In this study, a widely targeted metabolomics was employed to elucidate the kinetic metabolic changes along the grain development and the post-harvesting processes in four indica cultivars with different eating qualities. Our results showed that the levels of a large number of metabolites changed significantly in the four rice cultivars during grain development and the post-harvesting process. Our findings provide a foundation for studies on rice quality formation and have potential application in rice quality improvement.

Results

Eating Quality Values of the Four Indica Cultivars

To investigate the changes in metabolites during grain development and the post-harvesting process, we selected four indica cultivars from a pedigree with different eating qualities: MXZ (MeiXiangZhan2), MSZ (MeiSiZhan), HHZ (HuangHuaZhan), and QXZ (QiXinZhan) (Figs. 1a and S1). The eating quality value results showed that MXZ, the largest conventional rice planted in Guangdong province of China at present, has the highest eating quality of 88. The eating qualities of HHZ, grown in over 4.5 million ha in southern China (Zhou et al. 2016), and MSZ were 86 and 84, respectively, while that of QXZ (negative control) was only 64.7 (Fig. 1a).

Overview of the Metabolic Profiles of All Rice Samples

In order to investigate the changes in metabolites during rice grain development and the post-harvesting process, samples of grains at 8, 15, 30, and 40 DAF were collected. Samples at 40 DAF were polished rice that had been dried and milled and lacked the endosperm and aleurone layers. These were collected in order to investigate the metabolite changes during the post-harvesting process and were subjected to metabolic profiling analysis using LC-ESI-MS/MS. A total of 623 metabolites were identified, including 95 amino acids and derivatives, 85 organic acids and derivatives, 67 lipids, 62 flavones, and 58 nucleotides and derivates (Fig. 1b and Table S1).

PCA was subsequently performed on the 623 metabolites to visualize the kinetic metabolome patterns of the developing rice grains. The developing rice grains of the four cultivars showed a similar dynamic pattern in the changes in their metabolomes (Fig. 1c). During the grain-filling stage at 8 DAF, there was clear and broad separation of the different cultivars. During the grain-desiccation stage from 15 to 30 DAF, the separation between the samples of 15 DAF and those of 30 DAF was almost indistinguishable. During the later stage at 40 DAF, the separation among the cultivars was smallest compared to those at 8, 15, and 30 DAF. These data indicated that as the grain develops, the metabolic variation among the different cultivars becomes smaller.

As PCA is not able to distinguish the exact contribution of each variable to the observed variation, two-way ANOVA (analysis of variance) and ASCA (ANOVA-simultaneous component analysis) were then conducted to deconstruct the metabolic variations derived from stage, cultivar, and their interaction. The abundances of 454, 588, and 498 metabolites were significantly affected by stage, cultivar, and their interaction, respectively (Figs. 1d, S2, and S3). Among these, the abundances of 623

134 metabolites were simultaneously affected by stage, cultivar, and their interaction. In
135 addition, ASCA revealed that 65.13%, 40.27%, and 49.78% of observed metabolic
136 variations could be explained by developmental stage, cultivar, and their interaction,
137 respectively (Figs. S2, and S3). Developmental stage score plots based on PC1 of the
138 corresponding submodels showed that the scores gradually decreased from 8 to 40
139 DAF, which is consistent with the PCA result shown in Fig. 1, indicating that the
140 metabolomes of the different cultivars had shifted to the same direction with time.
141 Cultivar score plots showed that HHZ and MSZ had the highest and the lowest score,
142 respectively (Fig. S3). The interaction score plot of HHZ decreased from 8 to 30 DAF
143 and increased at 30 DAF, whereas those of MSZ and QXZ demonstrated the opposite
144 trend. The interaction score plot of MXZ increased continuously from 8 to 40 DAF.
145 Furthermore, leverage/squared prediction error (SPE) plots were produced to correlate
146 the metabolic features with the experimental factors. Leverage/SPE analysis revealed
147 that the numbers of identified metabolomes responsible for the observed variations
148 derived from time, cultivar, and their interaction were 32, 16, and 36, respectively
149 (Tables. S2–4).

150 These results indicate that the metabolic variation among the different cultivars
151 is clear and wide at 8 DAF and becomes smaller from 15 to 40 DAF, and the
152 metabolites among the cultivars were affected by stage and cultivar.

153 **Carbohydrate Metabolism During Rice Grain Development**

154 Starch, an end product of photosynthesis in source tissues, is stored as energy reserves
155 in the rice grain and is composed of two major components, namely amylose and
156 amylopectin (Wang et al. 2013). Six of eight metabolites present in the starch
157 biosynthesis pathway were identified herein (Fig. 2a). We found that the levels of

158 sucrose (the main raw material for the synthesis of starch) and UDPG decreased
159 significantly from 8 to 15 DAF (grain-filling stage) in all four cultivars. It is worth
160 mentioning that the level of sucrose in MXZ was higher than that in the other
161 cultivars, and the degree of change in UDPG in MXZ was smaller than that in the
162 other cultivars from 8 to 30 DAF, indicating a sufficient carbon supply from the leaf
163 in MXZ during grain development. Interestingly, the levels of glucose in HHZ and
164 MXZ decreased dramatically, whereas those in MSZ and QXZ exhibited no
165 significant change from 8 to 30 DAF. The levels of fructose 6-phosphate (F6P),
166 glucose 6-phosphate (G6P), and glucose 1-phosphate (G1P) in QXZ and MSZ
167 demonstrated similar change patterns, that is, increased first and then decreased from
168 8 to 30 DAF. In striking contrast, the changes of F6P in HHZ and MXZ demonstrated
169 the opposite trend compared with that in MSZ and QXZ from 8 to 30 DAF. The levels
170 of G6P in MXZ decreased at 15 DAF and increased at 30 DAF, while those in HHZ
171 gradually decreased from 8 to15 DAF. The levels of G1P in MXZ and HHZ gradually
172 decreased from 8 to 15 DAF. These results indicated that the levels of metabolites of
173 starch biosynthesis exhibit cultivar-specific changes, such as F6P, G6P, and G1P in
174 HHZ and MXZ. Furthermore, sufficient carbon supply from the leaf during grain
175 development may contribute to the formation of excellent eating quality.

176 We compared the changes in the 19 carbohydrates identified among the four
177 cultivars during grain development (Fig. 2b). Most significantly, we found that the
178 levels of 57.9% (11 of 19) of carbohydrates demonstrated similar change patterns in
179 MSZ and QXZ during the grain development process. Three carbohydrates exhibited
180 cultivar-specific changes, including N-acetyl-D-glucosamine and threose in HHZ and

glucosamine in MXZ. We further found that the most significant difference in carbohydrates between the rice cultivars was at 8 DAF (Fig. 2c). We found that 10.5% of carbohydrates (two of 19) were significantly higher in HHZ than in MXZ, whereas 42.1% (eight of 19) were lower in MSZ and QXZ than in MXZ at 8 DAF (Table. S5). Additionally, 5.3% of carbohydrates (one of 19) were significantly higher in HHZ than in MXZ, whereas 21% (four of 19) were lower in MSZ than in MXZ at 30 DAF (Table. S6).

Amino Acid and Derivatives Metabolism During Grain Development

Amino acids are primarily utilized for the synthesis of grain-storage proteins, which are important factors affecting the eating quality and nutritional quality of rice and serve as precursors for the biosynthesis of secondary metabolites and as a source of energy (Amir et al. 2018). We identified 95 amino acids and derivatives, which were the most abundant component of the metabolic profiles (Figs. 1b and 3). We found that most amino acids showed cultivar-specific changes during grain development (from 8 to 30 DAF) (Fig. 3a). The levels of 36 amino acids in HHZ, 12 amino acids in MXZ, 11 amino acids in MSZ, and 6 amino acids in QXZ specificity increased significantly at 15 and 30 DAF as compared with those at 8 DAF. For example, the levels of L-tyramine in HHZ only increased significantly at 15 and 30 DAF as compared with that at 8 DAF.

We further compared the changes in the amino acids and derivatives identified in HHZ, MSZ, and QXZ with those in MXZ during grain development (Fig. 3b). We found that the most significant difference in amino acids between the rice cultivars

was at 8 DAF. A total of 28.4% (27 of 95) of amino acids in HHZ were significantly lower than in MXZ, whereas 14.7% (14 of 95) were lower in QXZ than in MXZ at 8 DAF (Table. S7). Furthermore, 26.3% (25 of 95) of amino acids were significantly higher in HHZ than in MXZ, whereas 24.2% (23 of 95) were lower in QXZ than in MXZ at 15 DAF (Table. S8). A total of 8.4% of amino acids (8 of 95) were significantly lower in HHZ than in MXZ, whereas 29.5% (28 of 95) were lower in MSZ at 30 DAF (Table. S9).

In addition, we further investigated eight types of essential amino acids in the four cultivars (Fig. S4). Except for L-threonine, the amino acids in MXZ, QXZ, and HHZ exhibited the same trend, and the levels of the amino acids in MXZ were always higher than those in the other two cultivars from 8 to 30 DAF. In addition, with the exception of L-tryptophan and L-threonine, the levels of the amino acids in HHZ demonstrated completely opposite trends from those in the other three cultivars, possibly due to the genetic relationship of the rice cultivars.

Lipid Metabolism During Grain Development

Storage lipids are vital components for maintaining the structure of grain storage substances and are valuable for rice eating quality and food texture, despite that lipids are only a minor nutrient compared to starch and protein. A total of 66 lipids were identified herein, including 34.8% (23 of 66) lysophosphatidylcholine (LysoPCs), 12.1% (eight of 66) lysophosphatidylethanolamine (LysoPEs), 13.6% (nine of 66) MAGs (monoacylglycerols), and 39.4% (26 of 66) other lipids (Fig. 4). We found that

lipids showed apparent cultivar-specific changes from 8 to 30 DAF. Most notably, the levels of 36 lipids, specificity MAG and LysoPC, increased significantly in HHZ, while those in MXZ decreased. Interestingly, compared with the other three cultivars, we found that the lipids with significant changes in HHZ were most significant only at 8 DAF. These results indicate that the early stage of rice development is the key period for lipid differentiation.

Flavones and Other Metabolites During Grain Development

In addition to metabolic molecules related to starch, protein, and lipid synthesis, we also identified many types of metabolites that are closely related to human health, such as flavonoids and vitamins. Flavones play an important role in human health due to their pharmacological properties as nutraceuticals and radical scavengers (Tapas, Sakarkar, & Kakde, 2008). We identified 62 flavones herein (Fig. 5) and found that the level of 12 and 15 flavones in MXZ and HHZ, respectively, showed cultivar-specific increases during grain development. Interestingly, the levels of the specific flavonoids in QXZ (the negative control) were significantly higher than the other three cultivars at 30 DAF, such as tricin, a potential multifunctional nutraceutical (Zhou and Ibrahim 2010).

In addition, we identified six vitamins, all of which belong to the vitamin B family (Fig. S5). The levels of vitamin B1 and vitamin B5 exhibited similar trends during grain development in the four cultivars. The levels of the other vitamins in MXZ, MSZ, and QXZ were significantly reduced, while those in HHZ showed completely opposite trends, possibly due to the genetic relationship of the rice

246 cultivars.

247 **Changes in Metabolites in Rice Grains During the Post-harvesting Processes**

248 We further explored the changes in rice grain metabolites during the post-harvesting
249 process and found that 49.28%–57.14% (307-356) of metabolites were significantly
250 decreased, while only 1.93%–4.98% (12-31) of metabolites were significantly
251 increased in the four cultivars (Fig. 6a). The metabolites with significant changes in
252 HHZ were the most varied among the four cultivars, showing significant variety
253 specificity. There were 307–358 metabolites (a total of 22 types of metabolites) that
254 were significantly reduced in the rice cultivars during this process, of which the
255 largest proportion was amino acids and derivatives, followed by organic acids and
256 nucleic acids and derivatives in the four cultivars (Fig. S6a). This may be due to the
257 loss of embryos during milling, as embryos are rich in proteins and other substances
258 that facilitate seed germination. We found that most of the nutritional metabolites
259 beneficial to human health were reduced dramatically during processing. For example,
260 the content of eight essential amino acids was significantly reduced by 70%–98%,
261 while the content of six vitamins and derivatives was significantly reduced by 76%–
262 91% in the rice cultivars (Figs. S4 and S5). We also noted that many metabolites
263 beneficial to human health were lost completely in the four cultivars, such as catechin,
264 reported to have anti-cancer, anti-virus, and weight loss functions (Xu et al. 2017). It
265 is worthy to note that the levels of chlorpyrifos, a broad-spectrum and moderately
266 toxic chlorinated organophosphate insecticide used in agricultural production (Chishti
267 et al. 2013), were significantly reduced by 76%–91% (Fig. S7). In addition, there
268 were only 12–31 metabolites from a total of 12 types of metabolites that were
269 significantly increased in the cultivars during this process, of which the largest

proportion was lipids (Fig. S6b). For example, 9-HOTrE and punicic acid in MXZ increased by 3.96 and 4.19 times, respectively.

We further analyzed the changes in metabolites between the rice cultivars during this process. We found that at 30 DAF (pre-harvest), the difference between metabolites in HHZ and MXZ was the largest, and the content of 82 and 45 metabolites was significantly higher and lower, respectively, while at 40 DAF (polished rice), the difference between them was the smallest, with only 42 significantly different metabolites (11 higher levels and 32 lower levels) (Fig. 6b and c). This result suggests that it is possible to reduce the differences in the metabolites of distantly-related rice cultivars during processing. In addition, we analyzed the differences in metabolites between the 40 DAF samples (polished rice)) and found that only 11–27 metabolites in MSZ, HHZ, and QXZ were higher than those in MXZ, while 30–72 metabolites were lower than those in MXZ. Among these, the metabolites that were higher in abundance in MSZ, HHZ, and QXZ mainly included amino acids and nucleotide derivates, while metabolites that were lower in abundance mainly comprised organic acids and derivates in MSZ, flavones in HHZ, and lipids in QXZ (Fig. S8). Interestingly, during this process, although the metabolite differences between the rice cultivars decreased, the unique substances increased at 40 DAF. At 30 DAF, there were only four and one unique metabolites in HHZ and QXZ, respectively; after processing, the unique metabolites in MXZ, HHZ, QXZ, and MSZ increased to 11, 8, 17, and 7, respectively (Fig. 6d). Many of these metabolites have medicinal properties; for example, taxifolin (detected in MXZ) was found to exhibit anticancer and neuroprotective effects (Kara et al. 2019), and quinic acid (detected in

293 QXZ) has been shown to possess radioprotection, anti-neuroinflammatory, and anti-
294 oxidant activities (Jang et al. 2017; Yan et al. 2018).

295 **Discussion**

296 Metabolites are the final products of cell activities and thus directly reflect rice quality.
297 Profound changes in metabolites occur during grain development and the post-
298 harvesting processes, and thus understanding the changes in metabolites underlying
299 these processes is important for both a basic understanding of rice biology and for
300 applied rice breeding. Here, our results showed that the levels of a large number of
301 metabolites changed significantly during grain development and the post-harvesting
302 process.

303 Rice grain development involves the synthesis, interconversion, and
304 accumulation of numerous metabolites to form and accumulate various
305 macromolecules, which directly affects rice eating quality and nutritional quality. The
306 PCA results showed that the metabolic variation among the different cultivars
307 decreased from 8 to 30 DAF (Fig. 1c), which is consistent with previous reports (Hu
308 et al. 2016). The most clear and wide separation among the cultivars was observed
309 from 8 to 15 DAF, which is the key phase (grain-filling stage) for the accumulation of
310 storage compounds in the grain. Drastic changes in metabolites, including sugars,
311 amino acids, lipids, and flavones, occurred during processing. For example, the levels
312 of sucrose (the main raw material for the synthesis of starch) and UDPG decreased
313 significantly from 8 to 15 DAF in all four cultivars (Fig. 2a). These results indicated
314 that significant changes in these primary and secondary metabolites may be closely
315 related to rice eating quality formation. We further observed that in MXZ (with the
316 best eating quality), the levels of sucrose were higher than in the other three cultivars

317 from 8 to 15 DAF (Figs. 2a). These results may indicate that sufficient carbon supply
318 during grain development may contribute to the formation of excellent eating quality.

319 The most important factor affecting rice quality is the genetic characteristics of
320 the cultivars during grain development. The two-way ANOVA and ASCA results also
321 showed that the abundances of 623 metabolites were simultaneously affected by
322 cultivar, stage, and their interaction (Fig. 1b and d). We found that the levels of sugars,
323 amino acids, lipids, and flavones demonstrated cultivar-specific changes. For example,
324 lipids only increased significantly in HHZ during gain development (Fig. 4a). We also
325 found that the eating quality value of MSZ quality was significantly better than that of
326 QXZ, but the levels of metabolites in the starch biosynthesis pathway (such as glucose,
327 G6P, F6P, and G1P) showed similar change patterns (Fig. 2a). This indicated that the
328 quality of MSZ exhibits varietal specificity in starch synthesis compared with MXZ
329 and HHZ, and that in addition to starch synthesis, other factors significantly affect
330 rice eating quality. It should be noted that although the eating quality of HHZ is
331 significantly better than that of QXZ and similar to that of MXZ and MSZ (Fig. 1a),
332 the changes in some substances showed completely opposite trends. For example, the
333 levels of amino acids, lipids, and vitamins in HHZ demonstrated completely opposite
334 trends from those in the other three cultivars (Figs. 3, 4, and S5), indicating that many
335 metabolite changes show varietal specificity, which is related to the genetic distance
336 between the cultivars. These results indicate that although rice cultivars have similar
337 eating qualities, their metabolites, especially nutrient molecules, differ significantly,

which provides a theoretical basis for the cultivation of excellent cultivars that balance both taste quality and nutritional quality.

The post-harvesting processes, including harvesting, threshing, drying, transportation, storage, rice processing, and milling, can cause physical, chemical, and structural changes in the grains (Corrêa et al. 2007). The PCA showed that the metabolic variation among the cultivars decreased obviously from 30 (mature grain) to 40 DAF (polished rice) (Fig. 1c), and we found that 49%–57% of the metabolites decreased significantly, whereas only 1%–4.9% increased (Fig. 6a). This indicated that there were significant changes in the type and content of metabolites during the process and that the metabolite differences between rice cultivars with different eating qualities were reduced. During this process, the levels of amino acids and their derivatives decreased most significantly, and even many metabolites beneficial to human health were completely lost, such as catechin. These significant changes are likely to be the result of rice milling processing, in which the husk and bran layers (including cortex and embryo) are removed. This is why the rice bran, a by-product of rice processing, contains high amounts of amino acids, lipids, fats, vitamins, and other cofactors that are beneficial to human health (Zarei et al. 2017). Although the levels of most metabolites were reduced or even lost (Fig. 6a), the content of a few substances increases significantly, such as B1 (Fig. S5). We also found that unique metabolites between cultivars increased following processing, such as taxifolin in MXZ (Fig. 6d). Although the content of beneficial metabolites may be low in white rice, it is possible to specifically increase the content of such substances using molecular biology techniques in particular rice cultivars (Zhu et al. 2019). Thus these results suggested that the metabolic differences between the rice varieties were

reduced, and that most metabolites were significantly reduced, whereas some specific or even unique metabolites were increased during these processes.

In addition, the metabolites with significant changes in HHZ were the most varied among the four cultivars during the post-harvesting process (Fig. 6a), indicating that the metabolite changes are cultivar-specific during the post-harvesting process due to the genetic relationship of the rice cultivars. We found that compared with MSZ and QXZ, the levels of metabolites in HHZ differed most significantly from MXZ at 30 DAF, while those in HHZ showed the least difference with MXZ at 40 DAF (Fig. 6b and c). This suggested that the post-harvesting process reduced the metabolic differences between the rice cultivars and had an even greater influence on the metabolites than the genetic factors. Finally, by comparing the significantly different metabolites between the 40 DAF samples (white rice), we found that most of the significantly different metabolites were higher in MXZ than in HHZ, MSZ, and QXZ (Fig. 6c), indicating that MXZ is a suitable material for studying rice quality and breeding due to the balance between eating quality and nutritional quality.

In summary, we found that a large number of metabolites changed significantly in the four rice cultivars during grain development and the post-harvesting process. This suggests that studies on rice quality improvement, particularly that of the eating quality and nutritional quality, should consider both the inheritance mechanisms of grain quality formation and factors relating to the post-harvesting process.

Conclusion

In the present study, 623 metabolites were identified in four indica cultivars from a pedigree with different eating qualities. We found that the metabolic variation became

increasingly smaller and differed according to the stage and cultivar during grain development and the post-harvesting process. The levels of sugars, amino acids, lipids, and flavones demonstrated cultivar-specific changes during grain development. Sufficient carbon supply during grain development may contribute to the formation of excellent eating quality. We further found that most of the metabolites decreased significantly, whereas only a few increased during the post-harvesting process. These findings provide new insight into rice quality formation and have potential application in quality improvement.

Methods

Plant Materials and Growth Conditions

Rice plants were planted in a paddy filed in Tianhe (23.2°N, 113.4°E), Guangzhou, during the late season in 2018. Three biological replicates of rice grains at 8, 15, 30, and 40 days after flowering (DAF) were collected, immediately frozen and shelled in liquid nitrogen, and stored at −80°C. In addition, the 40 DAF samples are polished rice that were dried, stored for 2 months, and milled with a small rice polishing machine; part of these polished rice samples was used for metabolite extraction and was stored at −80°C, while the other part was used for rice eating quality measurements.

Measurement of Rice Eating Quality

Seventeen grams of polished rice was washed three times with water. The washed polished rice was placed into a special small cup with 19.04 g of water and then placed into a rice cooker at 100°C and heated for 20 min. Finally, the rice eating quality was measured by the rice taste meter (SATA1B) produced by the Japan Satake

Corporation (Hiroshima, Japan). Each experiment was repeated three times, and data represent the mean ± standard deviation of three independent experiments.

Sample Preparation and Extraction

The freeze-dried grain was crushed using a mixer mill (MM 400, Retsch) with a zirconia bead for 1.5 min at 30 Hz. One-hundred milligrams of powder was weighed and extracted overnight at 4°C with 1.0 mL 70% aqueous methanol. Following centrifugation at 10,000 *g* for 10 min, the extracts were absorbed (CNWBOND Carbon-GCB SPE Cartridge, 250 mg, 3 mL; ANPEL, Shanghai, China, www.anpel.com.cn/cnw) and filtrated (SCAA-104, 0.22 μm pore size; ANPEL, Shanghai, China, http://www.anpel.com.cn/) before liquid chromatography-mass spectrometry (LC-MS) analysis.

High-performance liquid chromatography (HPLC) Conditions

The sample extracts were analyzed using an LC-electrospray ionization (ESI)-MS/MS system (HPLC, Shim-pack UFLC SHIMADZU CBM30A system, www.shimadzu.com.cn/; MS, Applied Biosystems 6500 Q TRAP, www.appliedbiosystems.com.cn/). The analytical conditions were as follows, HPLC: column, Waters ACQUITY UPLC HSS T3 C18 (1.8 μm, 2.1 mm*100 mm); solvent system, water (0.04% acetic acid): acetonitrile (0.04% acetic acid); gradient program, 95:5 V/V at 0 min, 5:95 V/V at 11.0 min, 5:95 V/V at 12.0 min, 95:5 V/V at 12.1 min, 95:5 V/V at 15.0 min; flow rate, 0.40 mL/min; temperature, 40°C; injection volume: 2 μL. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (Q TRAP)-MS.

ESI-Q TRAP-MS/MS

Linear ion trap (LIT) and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (Q TRAP) API 6500 Q TRAP LC/MS/MS System equipped with an ESI Turbo Ion-Spray interface, operating in positive ion mode and controlled by Analyst 1.6.3 software (AB Sciex). The ESI source operation parameters were as follows: ion source, turbo spray; source temperature, 500°C; ion spray voltage (IS), 5500 V; ion source gas I (GSI), gas II (GSII), and curtain gas (CUR) were set at 55, 60, and 25.0 psi, respectively; the collision gas (CAD) was high. Instrument tuning and mass calibration were performed with 10 and 100 μmol/L polypropylene glycol solutions in QQQ and LIT modes, respectively. QQQ scans were acquired as multiple reaction monitoring (MRM) experiments with collision gas (nitrogen) set to 5 psi. DP and CE for individual MRM transitions were done with further DP and CE optimization. A specific set of MRM transitions was monitored for each period according to the metabolites eluted within this period.

Qualitative and Quantitative Determination of Metabolites

Based on a self-established database (MWDB) and a public metabolite database, primary and secondary mass spectrometry data were subjected to qualitative analyses. For the qualitative analyses of some substances, interference from isotope signals; duplicate signals of K^+, Na^+, and NH_4^+ ions; and duplicate signals of fragment ions derived from other relatively large molecules were excluded (Fraga et al. 2010; Yang, et al. 2019).

Statistical Analysis

Principal component analysis (PCA), two-way analysis of variance (ANOVA),

454 ANOVA-simultaneous component analysis (ASCA), and heat maps were generated
455 in R (base package) version 3.5.0. The data were normalized prior to analysis. Two
456 screening criteria for significant differential metabolites were applied, including a fold
457 change of ≥ 2 or of ≤ 0.5 and a *P*-value < 0.05 (Student's *t*-test).

458 **Supplementary information**

459 **Additional file 1: Figure S1.** Rice pedigree analysis based on information from Ricedata
460 (http://www.ricedata.cn). **Figure S2.** Major patterns associated with time and cultivars
461 (developmental stage). **Figure S3.** ANOVA-simultaneous component analysis. **Figure S4.**
462 Relative changes in essential amino acids in the rice grains during the development. Blue, red,
463 green, and purple represent MXZ, MSZ, HHZ, and QXZ respectively. **Figure S5.** Relative
464 changes in vitamins in the rice grains during development. Blue, red, green, and purple
465 represent the MXZ, MSZ, HHZ, and QXZ samples, respectively. **Figure S6.** Classes of
466 metabolites significantly reduced (a) and increased (b) in the four cultivars during the post-
467 harvesting process (from 30 to 40 DAF). **Figure S7.** Percent of chlorpyrifos remaining in the
468 sample (40 DAF) after the post-harvesting process. **Figure S8.** Classes of metabolites
469 significantly lower (a) and higher (b) in the rice samples (40 DAF) in MSZ, HHZ, and QXZ
470 compared with MXZ. **Table S1.** A list of the 623 metabolites detected in this study. **Table S2.**
471 Time List of well-modeled metabolites. **Table S3.** Cultivar List of well-modeled metabolites.
472 **Table S4.** Interaction List of well-modeled metabolites. **Table S5.** Carbohydrate with
473 significant changes at 8 DAF. **Table S6.** Carbohydrate with significant changes at 30 DAF.
474 **Table S7.** Amino acid and derivatives with significant changes at 8 DAF. **Table S8.** Amino
475 acid and derivatives with significant changes at 15 DAF. **Table S9.** Amino acid and
476 derivatives with significant changes at 30 DAF.

477 **Abbreviations**

478 MXZ: MeiXiangZhan2; MSZ: MeiSiZhan; HHZ: HuangHuaZhan; QXZ: QiXinZhan;
479 DAF: days after flowering; PCA: principal component analysis; ANOVA: two-way
480 analysis of variance; ASCA: ANOVA-simultaneous component analysis; UPDG:
481 uridine diphosphate glucose; G-6-P: Glucose-6-phosphate; F-6-P: fructose-6-

phosphate; G-1-P: Glucose-1-phosphate; LysoPEs: lysophosphatidylethanolamine; MAGs: monoacylglycerols; LysoPCs: lysophosphatidylcholine.

Acknowledgement

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Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors declare no potential competing interests.

Author details

Yi-Bo Chen and Zhidong Wang contributed equally to this work.

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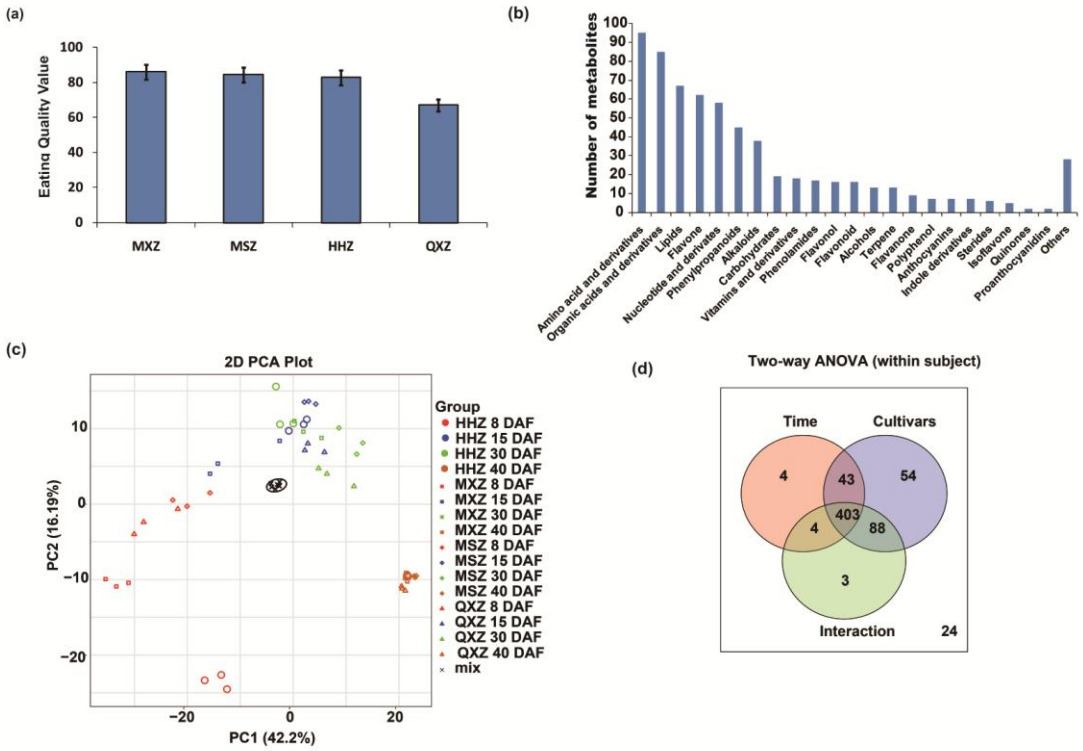
584 **Figs. 1-6 (Chen et al)**

Fig. 1. Chen et al.



585

586 Fig. 1. (a) Eating quality values of MXZ, MSZ, HHZ, and QXZ; (b) metabolite classes and
587 numbers detected in the samples; (c) principal component analysis (PCA) of the metabolomes
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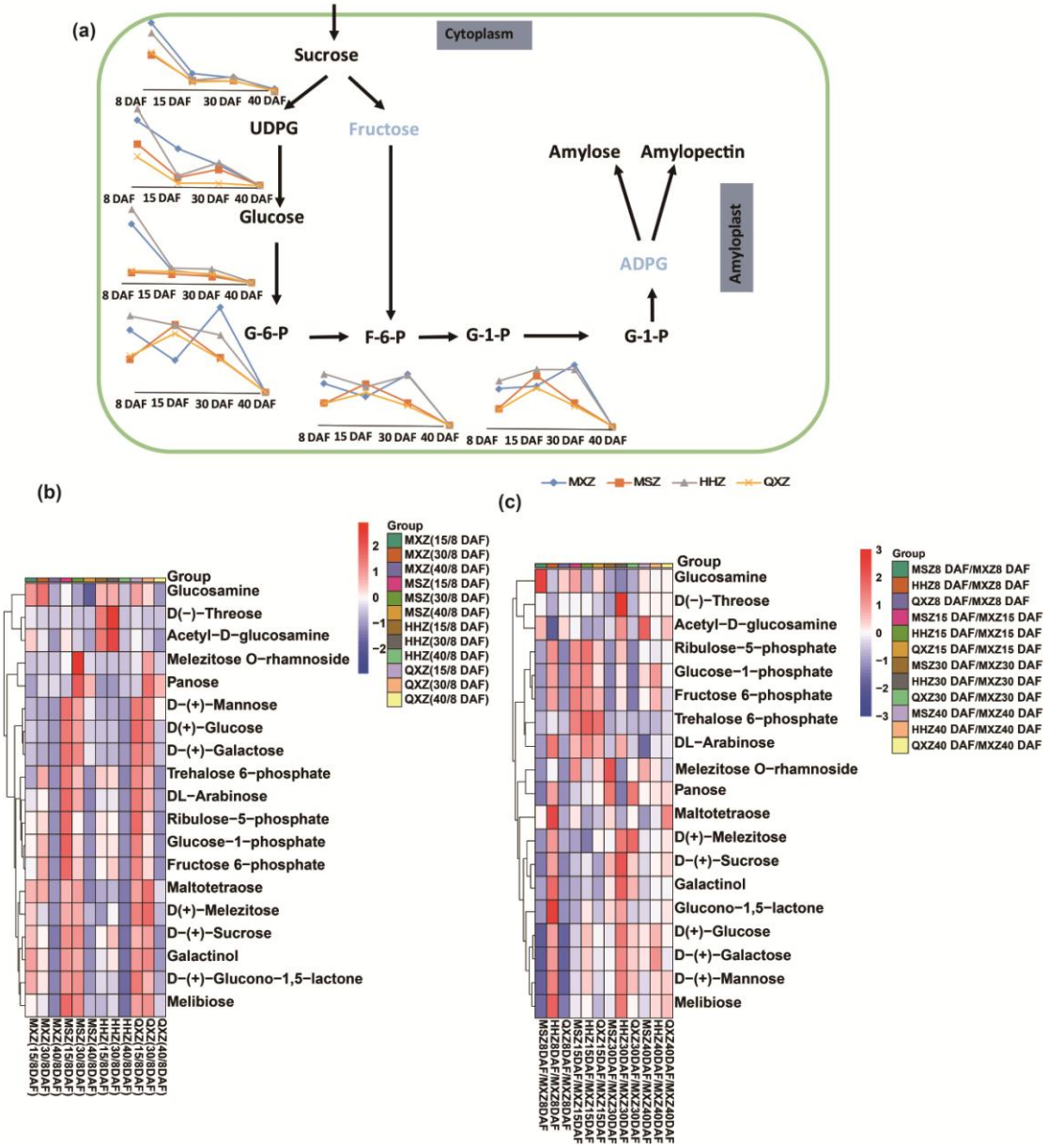
589 represent samples at 8, 15, 30, and 40 DAF, respectively. The circle, square, rhombus, and
590 triangle denote the grain metabolomes of HHZ, MXZ, MSZ, and QXZ, respectively; (d) Venn
591 diagram summary of the results from the two-way ANOVA.

Fig. 2. Chen et al.



592

593 Fig. 2. (a) Changes in metabolites mapped to the starch biosynthesis pathway in the four rice
594 cultivars at 8, 15, 30, and 40 DAF. Abbreviations: UPDG, uridine diphosphate glucose; G-6-P,
595 Glucose-6-phosphate; F-6-P, fructose-6-phosphate; G-1-P, Glucose-1-phosphate; (b) and (c)
596 Heatmap of carbohydrate metabolite changes in the rice grains at 8, 15, 30, and 40 DAF
597 among the four cultivars (b) or compared with MXZ (c). Ratios of fold changes are given by
598 shades of red or blue according to the scale bar. Data represent the mean values of three

599 biological replicates for each cultivar and time point. For full metabolite names, refer to Table

600 S1.

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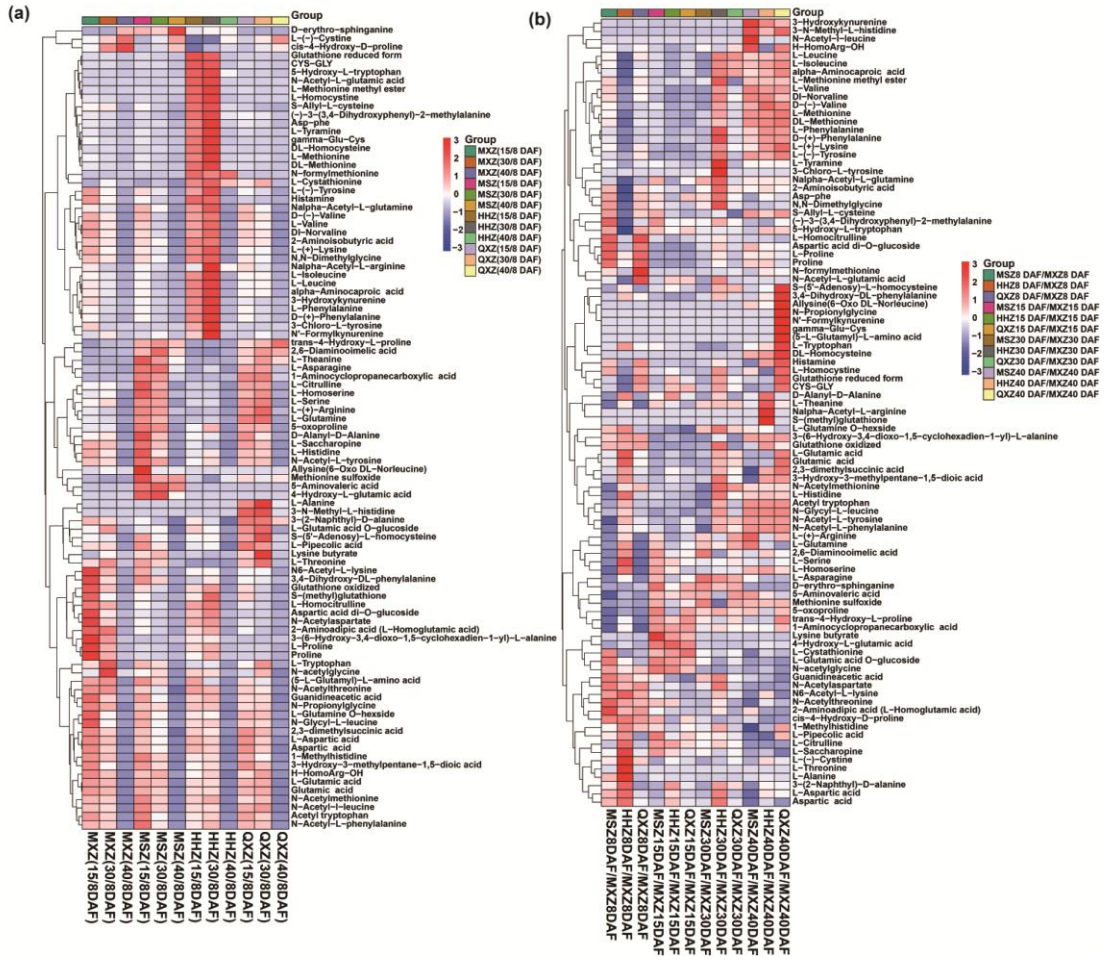
Fig. 3. Chen et al.



602

603 Fig. 3. Heatmap of the changes in amino acids and derivatives in the rice grains at 8, 15, 30,

604 and 40 DAF among the four cultivars (b) or compared with MXZ (c). Ratios of fold changes

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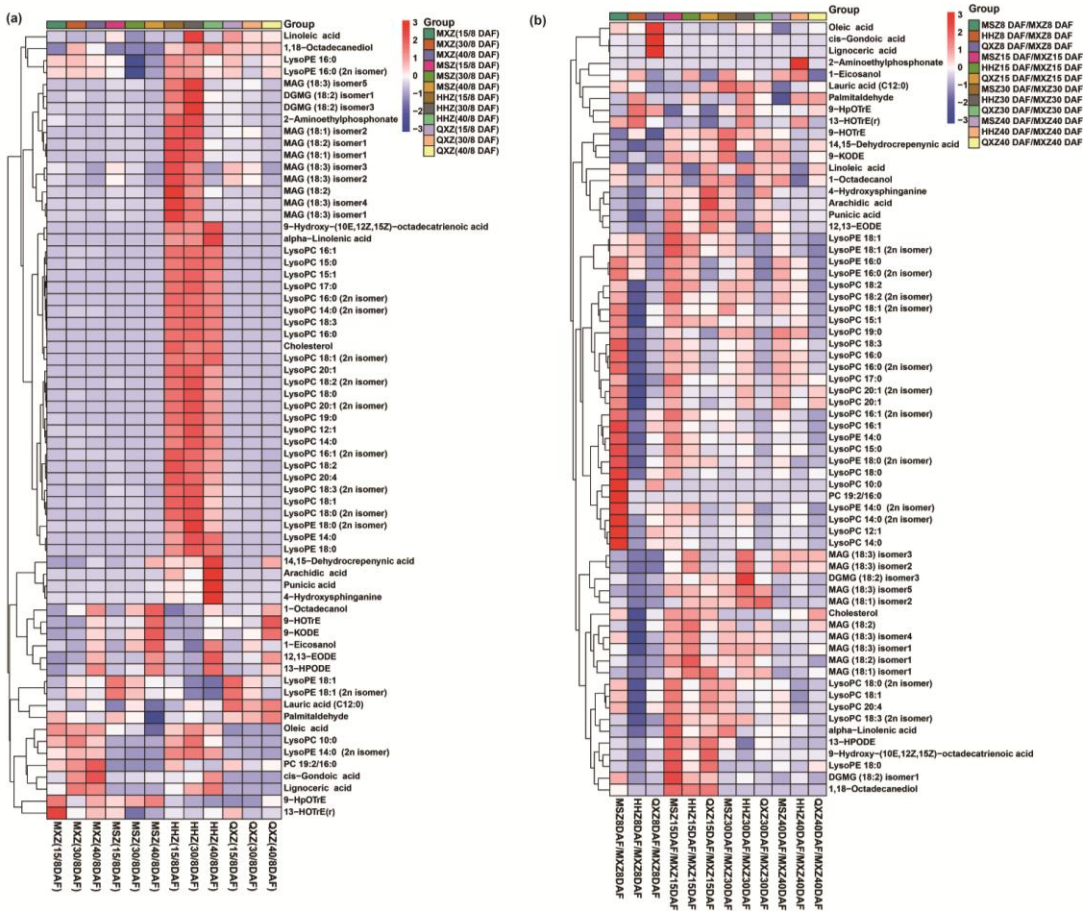
608

Fig. 4. Chen et al.



609

610 Fig. 4. Heatmap of lipid metabolite changes in the rice grains at 8, 15, 30, and 40 DAF among
611 the four cultivars (b) or compared with MXZ (c). Ratios of fold changes are given by shades
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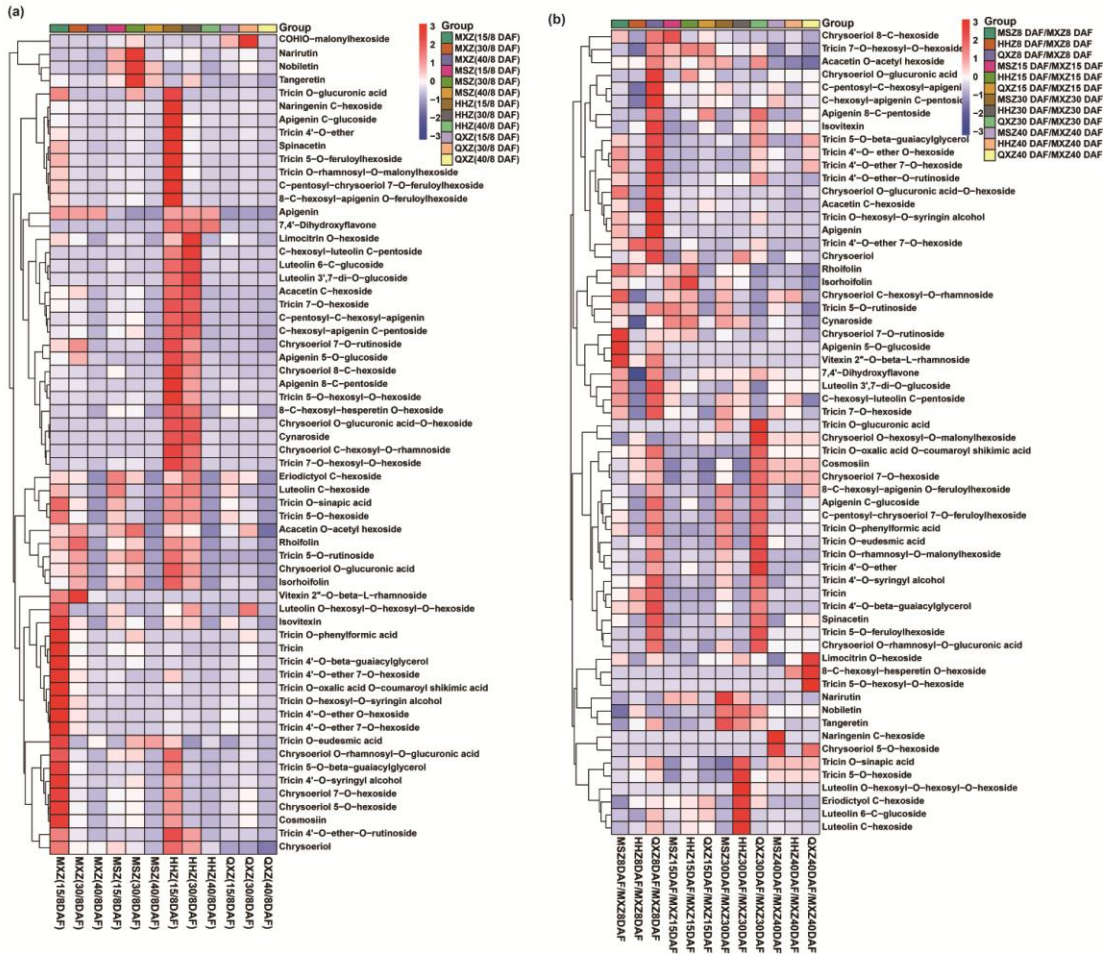
Fig. 5. Chen et al.



614

615 Fig. 5. Heatmap of flavones metabolite changes in the rice grains at 8, 15, 30, and 40 DAF
616 among the four cultivars (b) or compared with MXZ (c). Ratios of fold changes are given by
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618 biological replicates for each cultivar and time point. For full metabolite names, refer to Table
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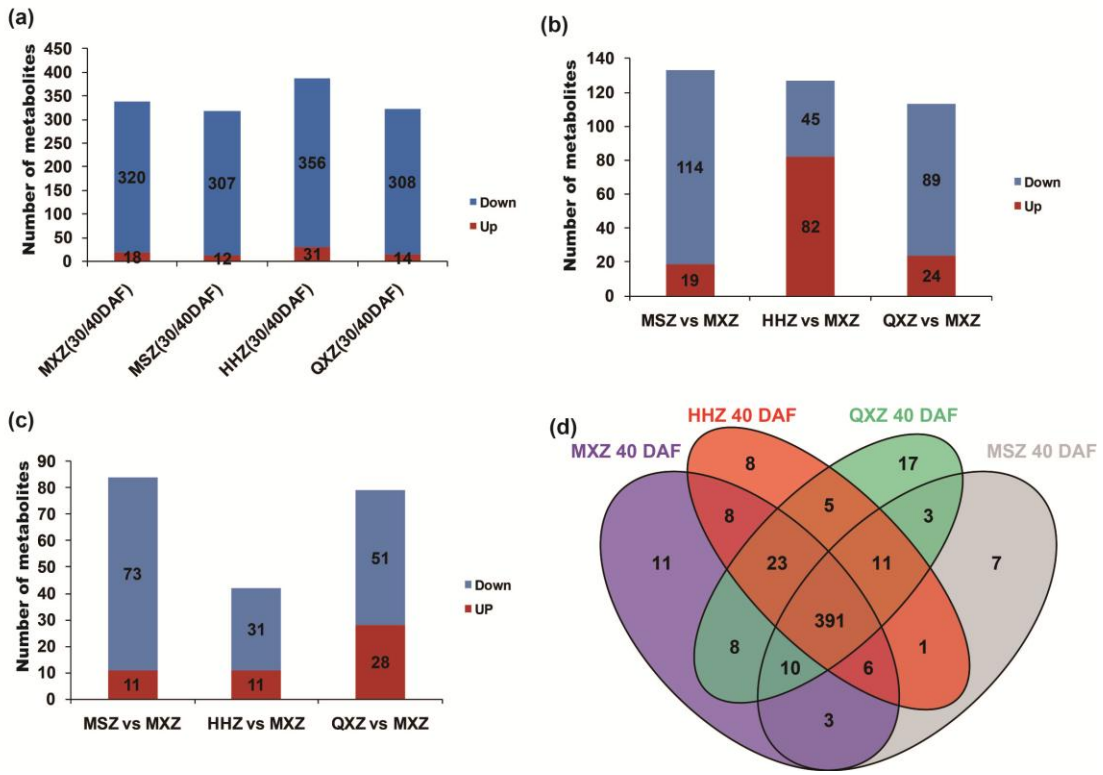
Fig. 6. Chen et al.



620

621 Fig. 6. (a) Number of metabolites with differential changes ≥2-fold and < 0.5-fold ($P < 0.05$)
622 in the four cultivars during the post-harvesting process (from 30 to 40 DAF). Number of
623 metabolites with differential changes in MSZ, HHZ, and QXZ compared with MXZ at 30
624 DAF (b) and 40 DAF (white rice) (c). (d) Venn diagram of significantly changed metabolites
625 in the four cultivars at 40 DAF (white rice).

626

627 **Supplementary Figs. S1-8 (Chen et al)**

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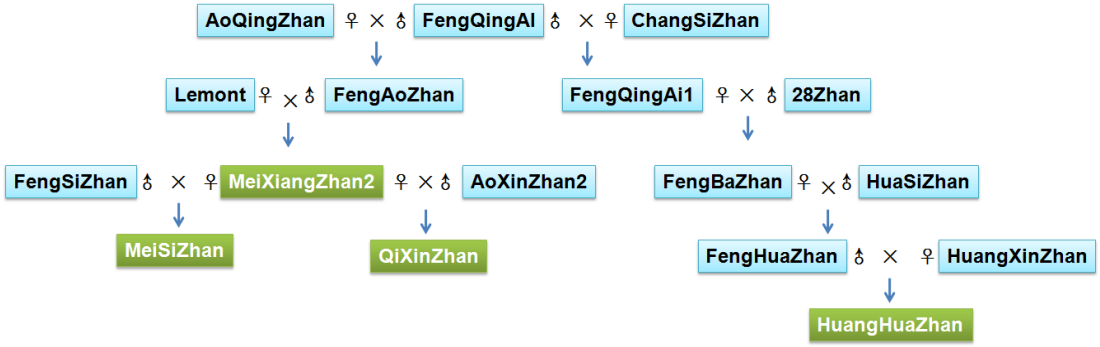
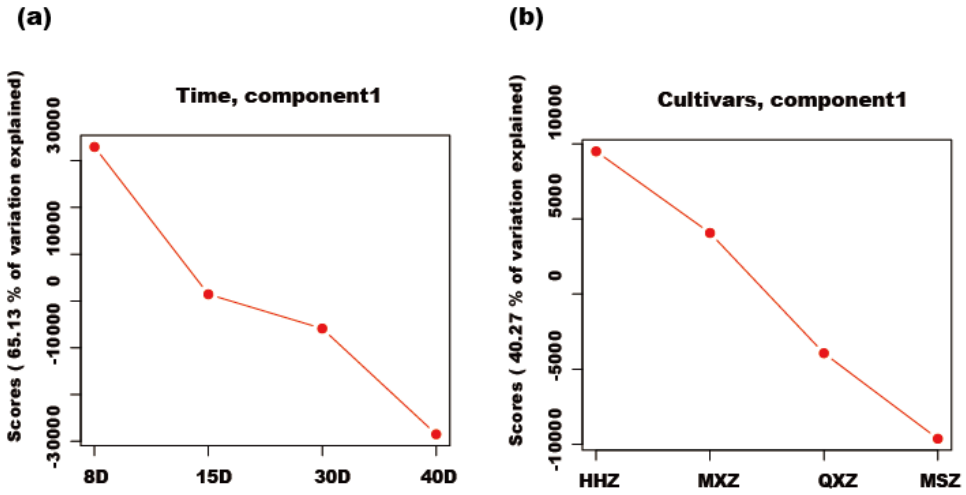


629

630 Fig. S1. Rice pedigree analysis based on information from Ricedata (http://www.ricedata.cn).



631

632 Fig. S2. Major patterns associated with time and cultivars (developmental stage).

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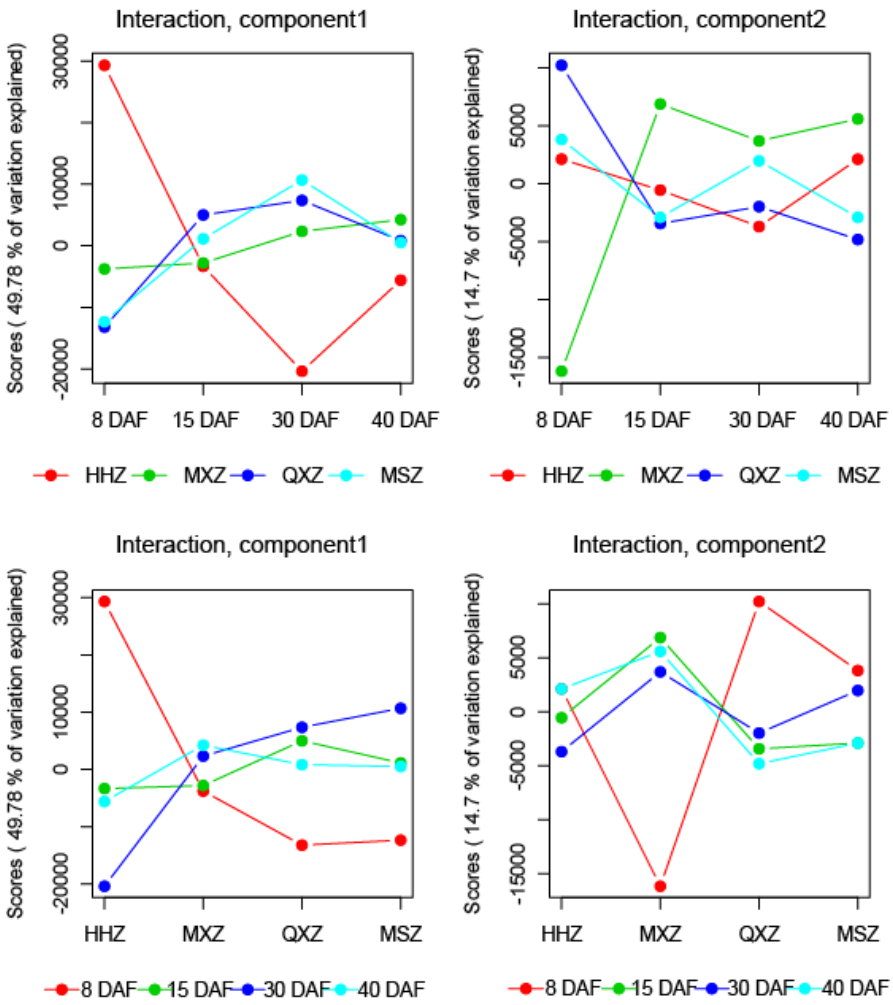

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635 Fig. S3. ANOVA-simultaneous component analysis.

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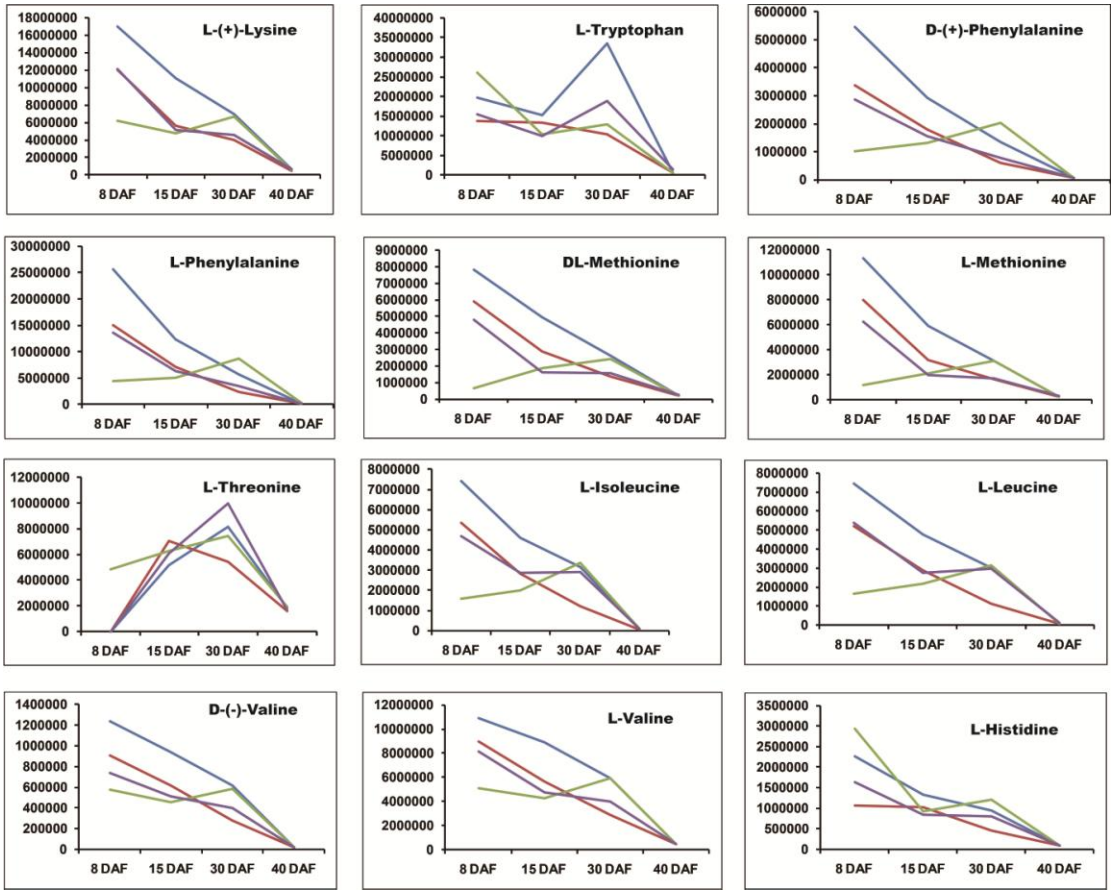
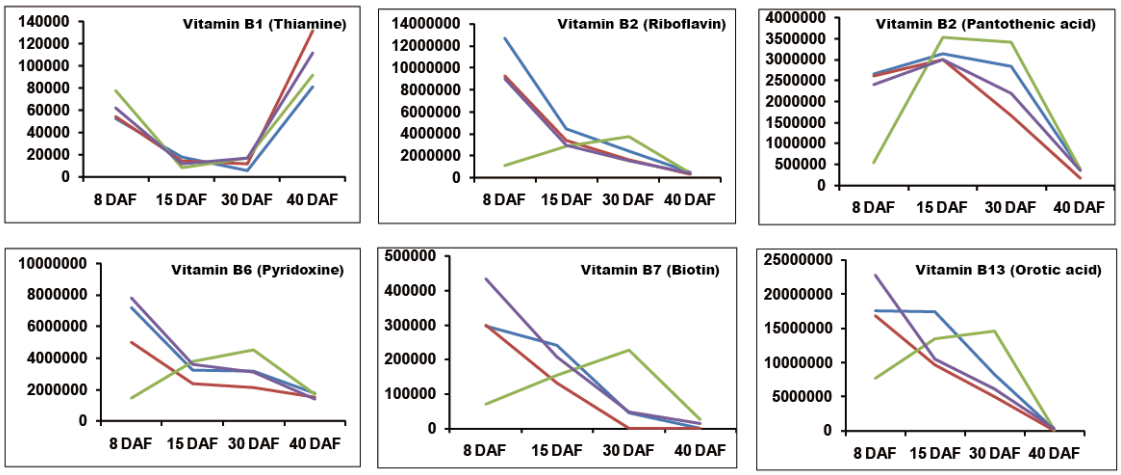


637

638 Fig. S4. Relative changes in essential amino acids in the rice grains during the development.
639 Blue, red, green, and purple represent MXZ, MSZ, HHZ, and QXZ respectively.



640

641 Fig. S5. Relative changes in vitamins in the rice grains during development. Blue, red, green,
642 and purple represent the MXZ, MSZ, HHZ, and QXZ samples, respectively.

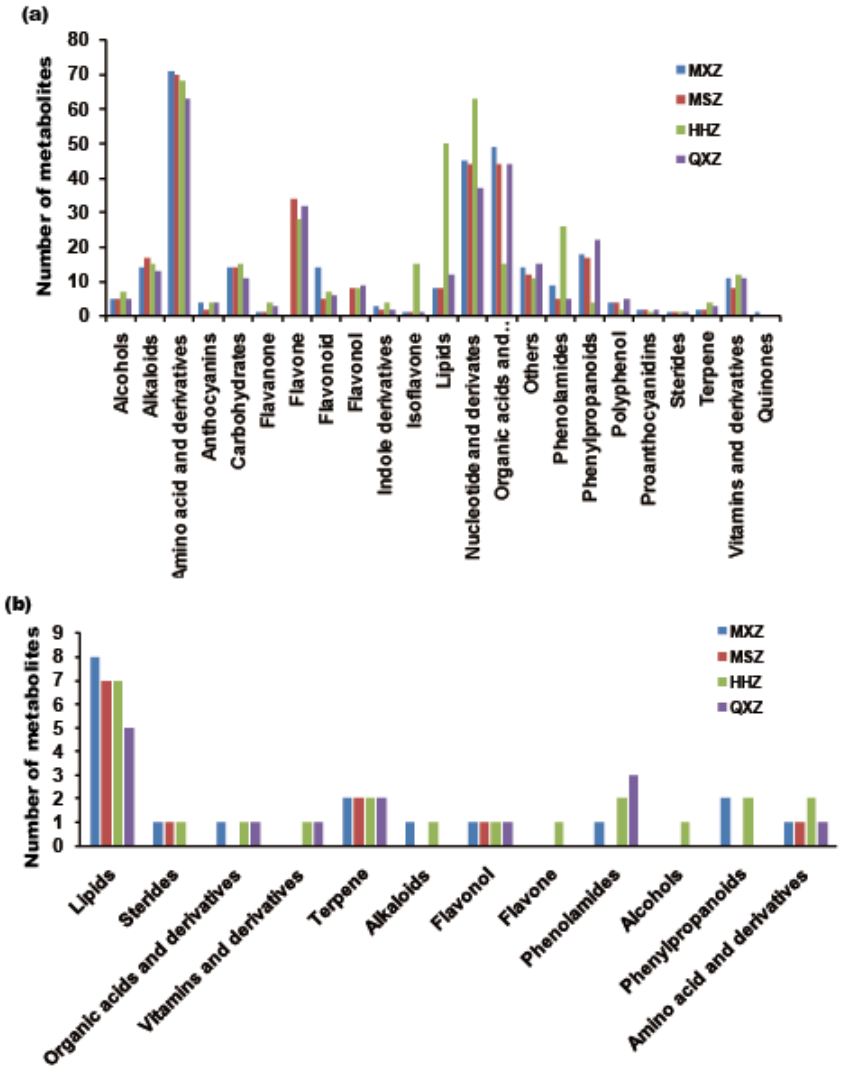


Fig. S6. Classes of metabolites significantly reduced (a) and increased (b) in the four cultivars during the post-harvesting process (from 30 to 40 DAF).

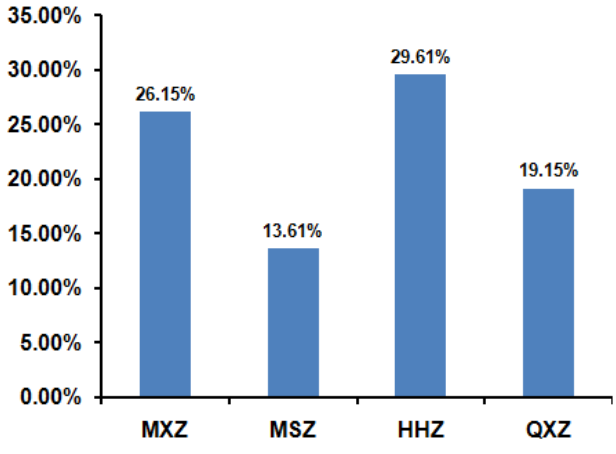
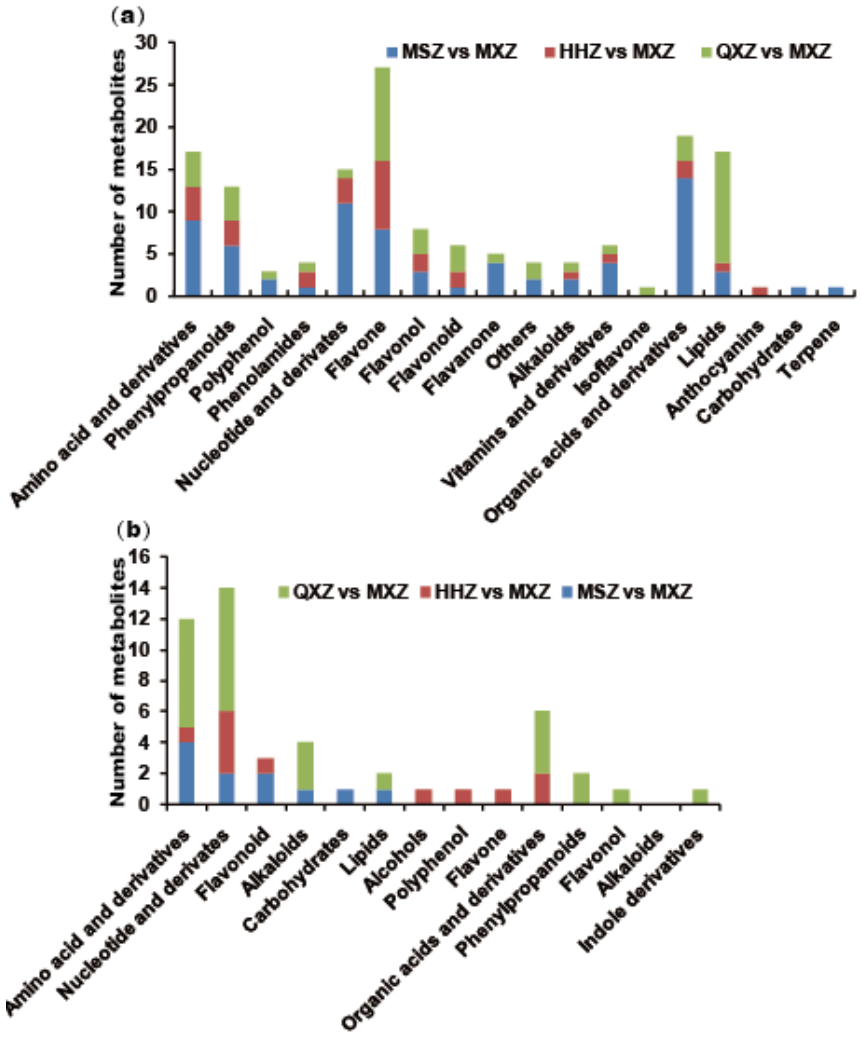


Fig. S7. Percent of chlorpyrifos remaining in the sample (40 DAF) after the post-harvesting process.



649

650 Fig. S8. Classes of metabolites significantly lower (a) and higher (b) in the rice samples
651 (40 DAF) in MSZ, HHZ, and QXZ compared with MXZ.

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Figures

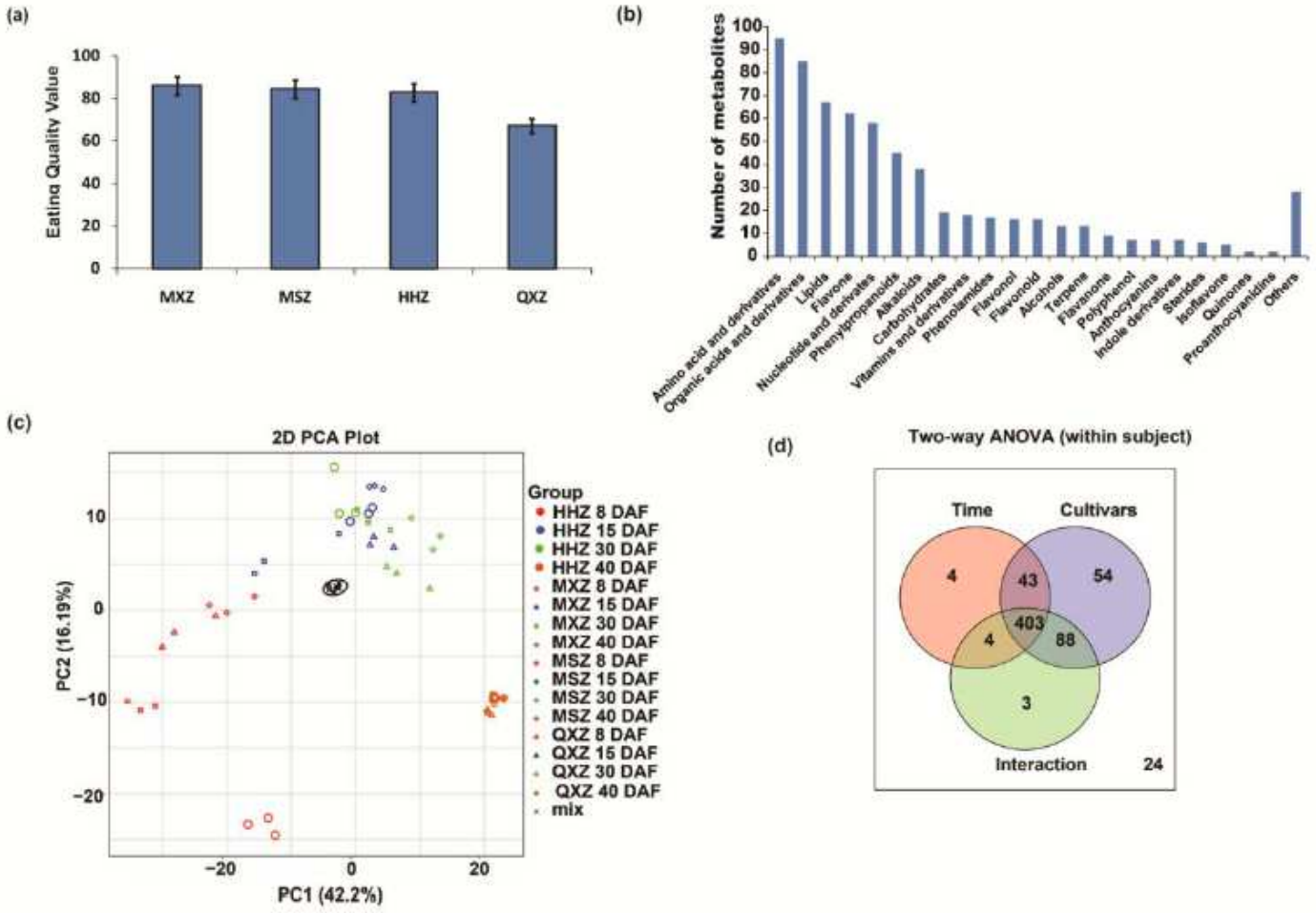

Figure 1

(a) Eating quality values of MXZ, MSZ, HHZ, and QXZ; (b) metabolite classes and numbers detected in the samples; (c) principal component analysis (PCA) of the metabolomes during grain development and the post-harvesting process. Red, blue, green, and orange represent samples at 8, 15, 30, and 40 DAF, respectively. The circle, square, rhombus, and triangle denote the grain metabolomes of HHZ, MXZ, MSZ, and QXZ, respectively; (d) Venn diagram summary of the results from the two-way ANOVA.

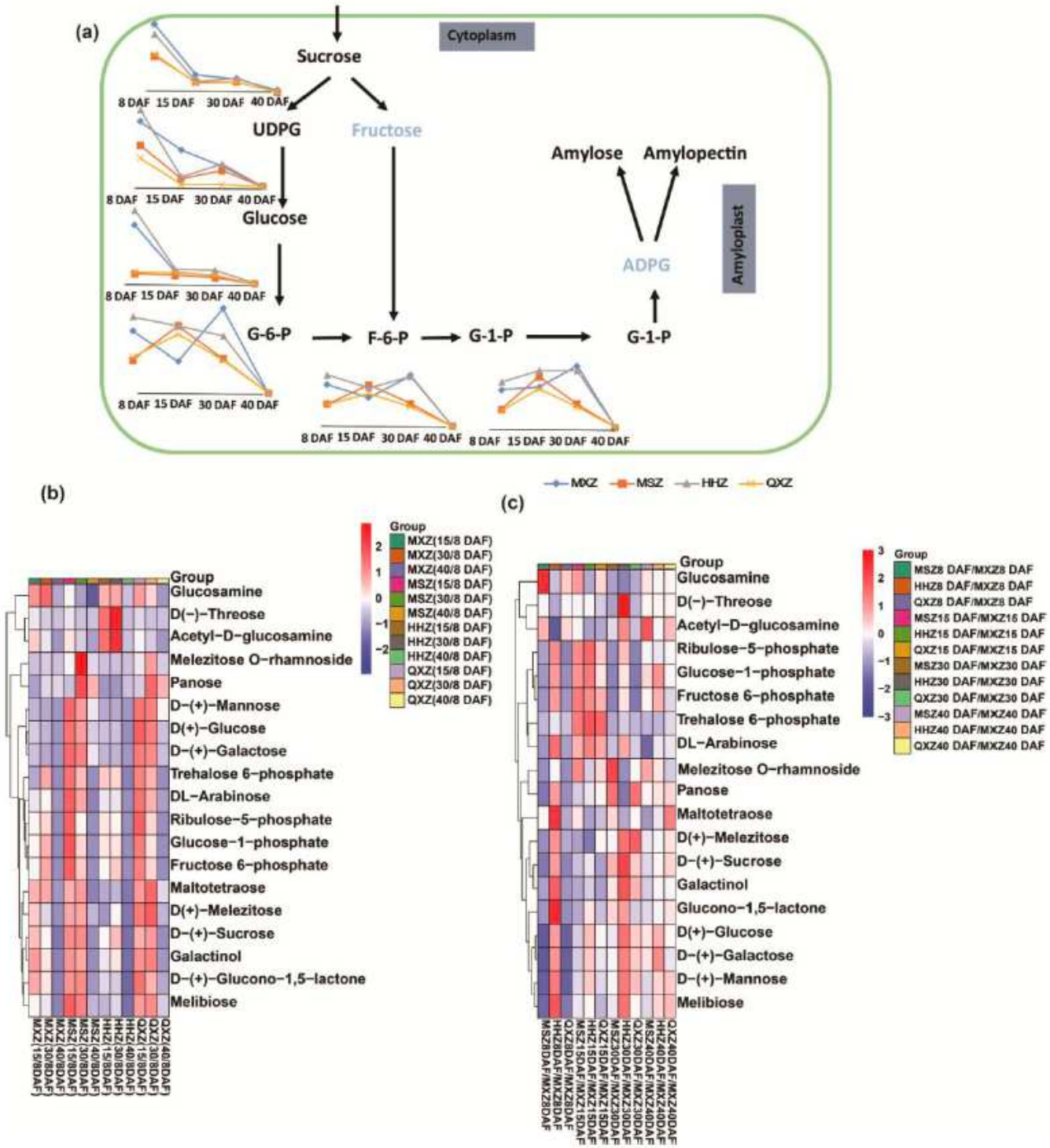

Figure 2

(a) Changes in metabolites mapped to the starch biosynthesis pathway in the four rice cultivars at 8, 15, 30, and 40 DAF. Abbreviations: UPDG, uridine diphosphate glucose; G-6-P, Glucose-6-phosphate; F-6-P, fructose-6-phosphate; G-1-P, Glucose-1-phosphate; (b) and (c) Heatmap of carbohydrate metabolite changes in the rice grains at 8, 15, 30, and 40 DAF among the four cultivars (b) or compared with MXZ (c). Ratios of fold changes are given by shades of red or blue according to the scale bar. Data represent

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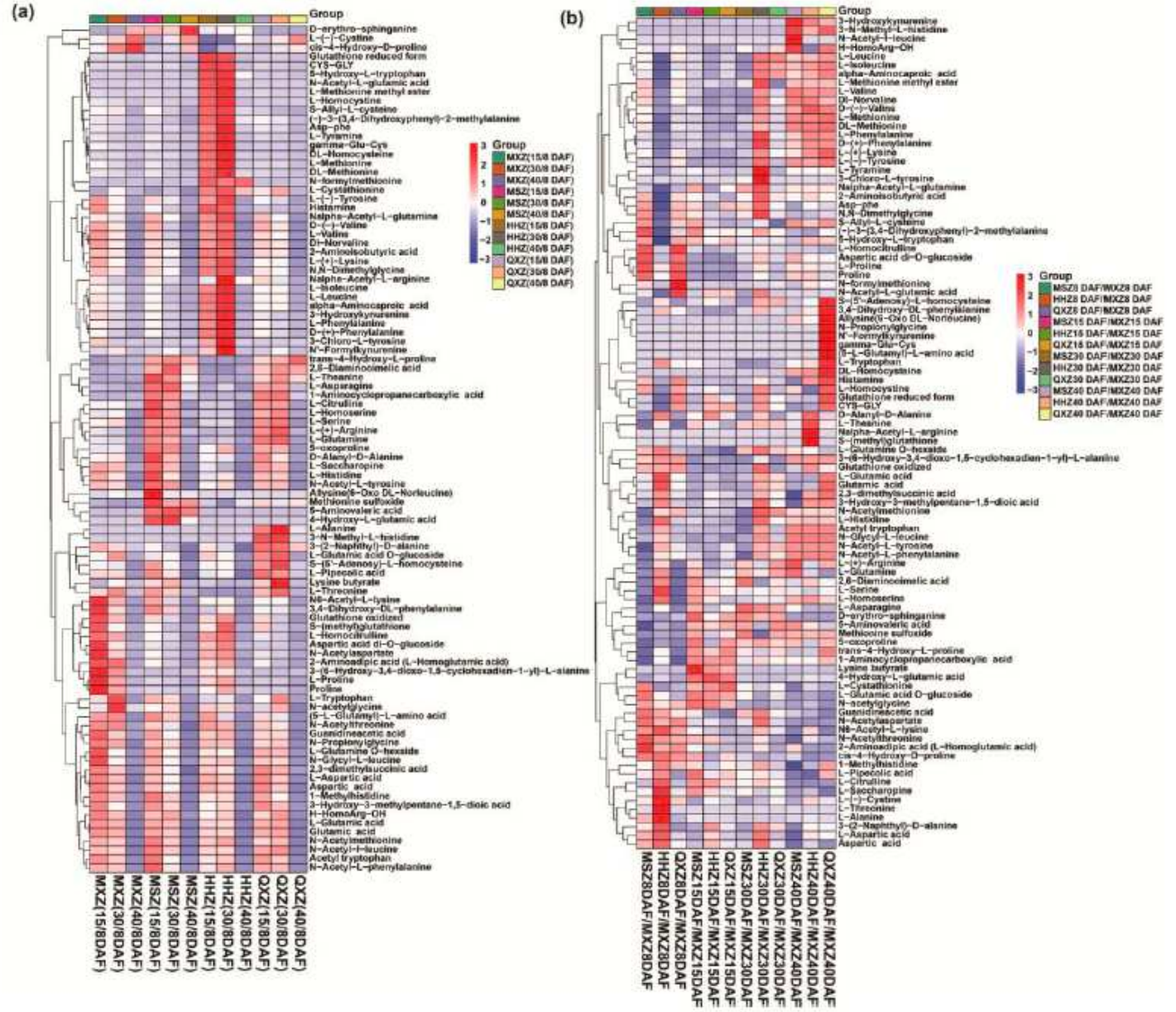

Figure 3

Heatmap of the changes in amino acids and derivatives in the rice grains at 8, 15, 30, and 40 DAF among the four cultivars (b) or compared with MXZ (c). Ratios of fold changes are given by shades of red or blue according to the scale bar. Data represent the mean values of three biological replicates for each cultivar and time point. For full metabolite names, refer to Table S1.

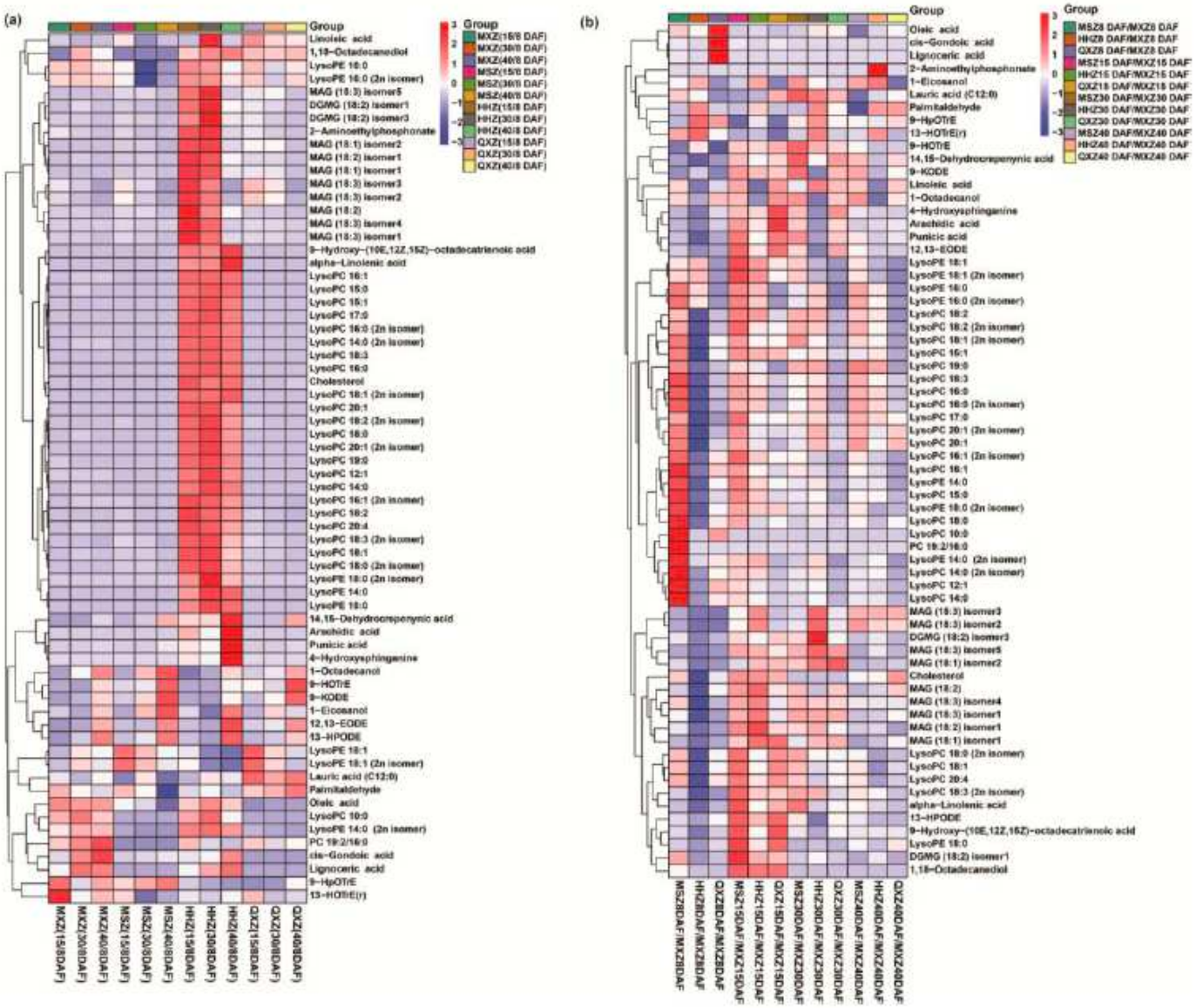

Figure 4

Heatmap of lipid metabolite changes in the rice grains at 8, 15, 30, and 40 DAF among the four cultivars (b) or compared with MXZ (c). Ratios of fold changes are given by shades of red or blue according to the scale bar. Data represent mean values of three biological replicates for each cultivar and time point. For full metabolite names, refer to Table S1.

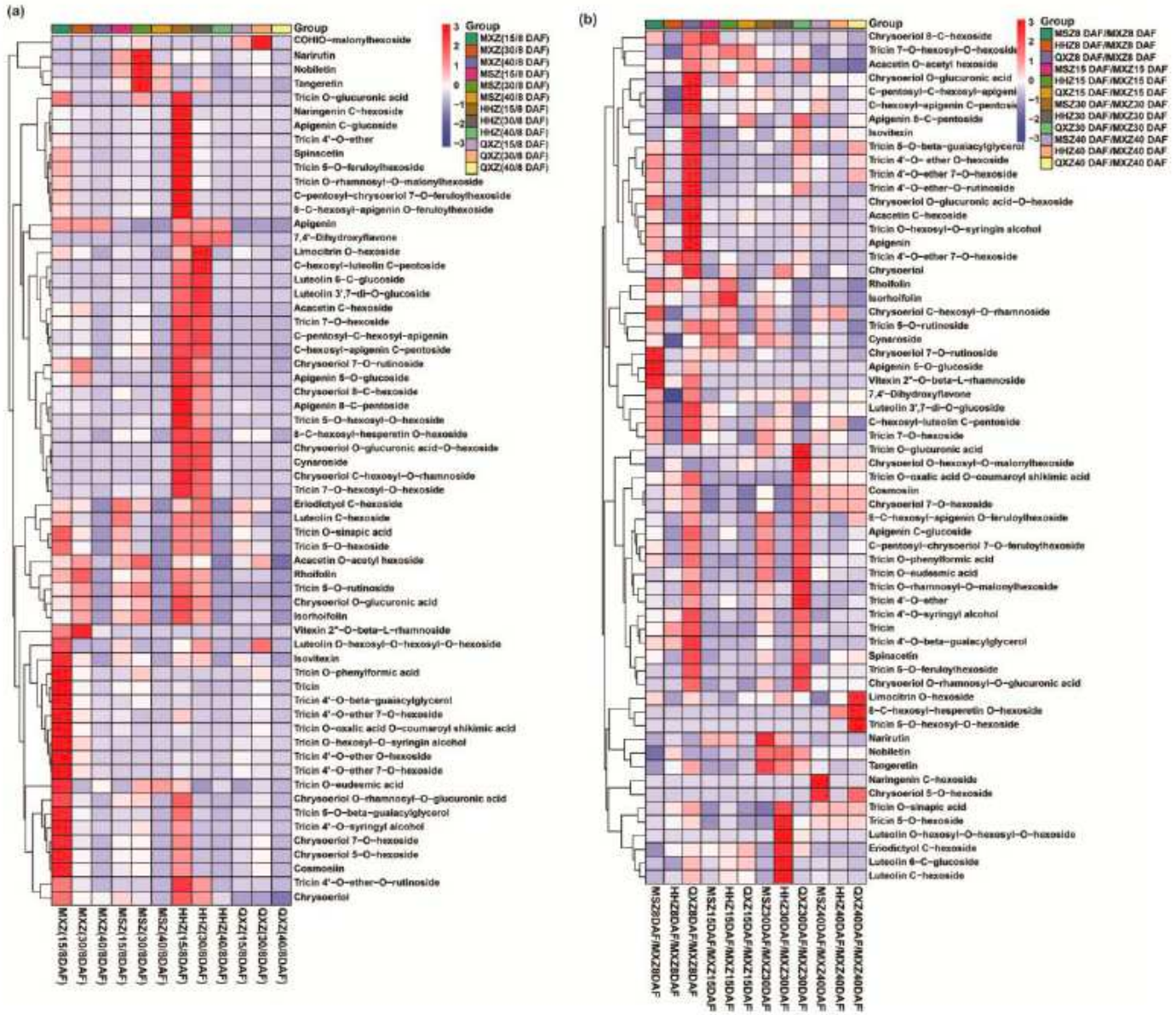

Figure 5

Heatmap of flavones metabolite changes in the rice grains at 8, 15, 30, and 40 DAF among the four cultivars (b) or compared with MXZ (c). Ratios of fold changes are given by shades of red or blue according to the scale bar. Data represent mean values of three biological replicates for each cultivar and time point. For full metabolite names, refer to Table S1.

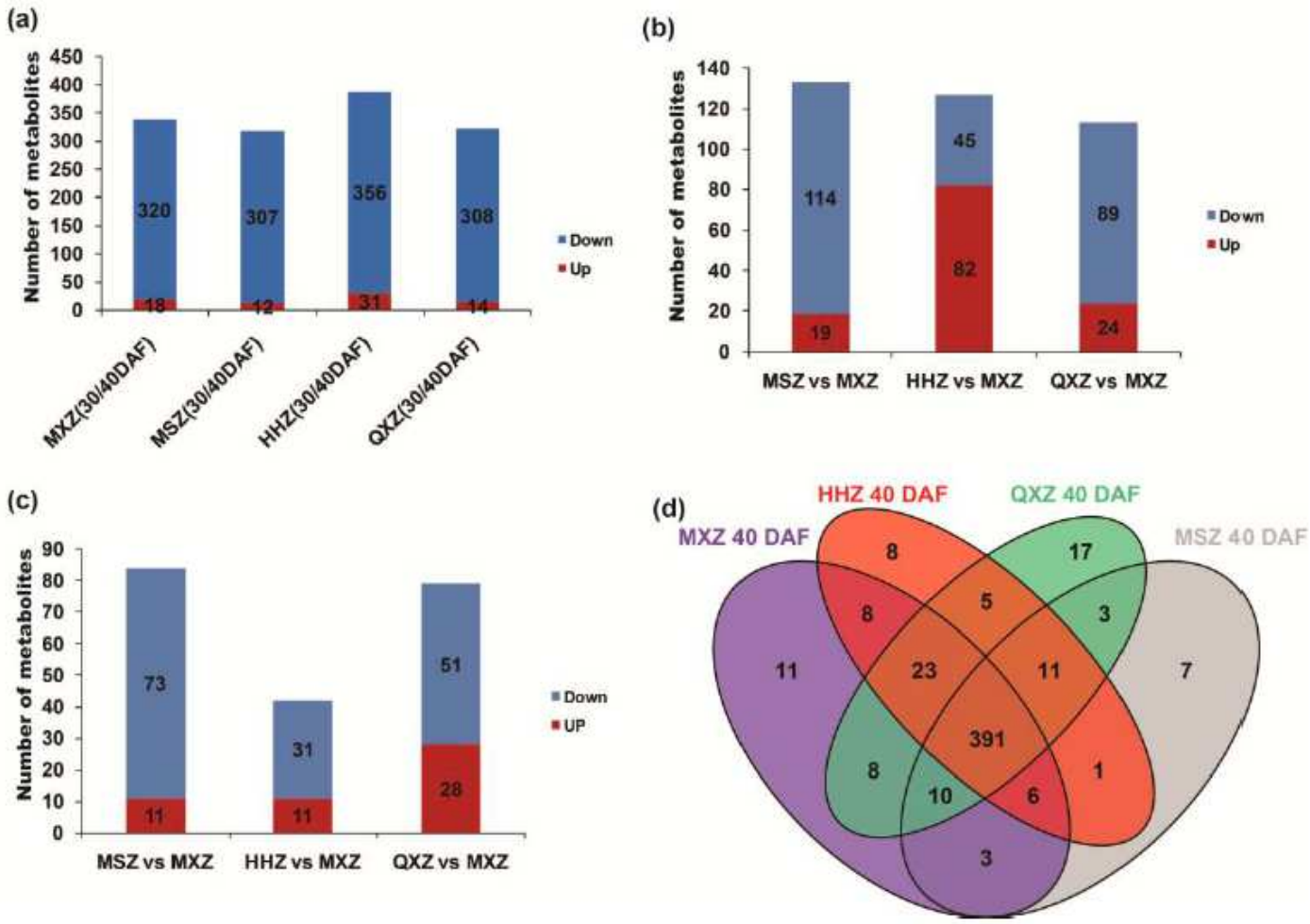

Figure 6

(a) Number of metabolites with differential changes ≥2-fold and < 0.5-fold ($P < 0.05$) in the four cultivars during the post-harvesting process (from 30 to 40 DAF). Number of metabolites with differential changes in MSZ, HHZ, and QXZ compared with MXZ at 30 DAF (b) and 40 DAF (white rice) (c). (d) Venn diagram of significantly changed metabolites in the four cultivars at 40 DAF (white rice).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterial.rar
- SupplementalFigures.pdf