



Metabolomic profiling of five hulless barley (*Hordeum vulgare* L.) with different

Study on the metabolite difference of five hulless barley with different colors based on metabonomics approach

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Abstract Colored hulless barley (*Hordeum vulgare* L.) is a high-quality germplasm resource rich in nutrients, such as protein, β -glucan, flavonoids, amino acids, vitamins, microelements, and dietary fiber. However, a systematic evaluation of the metabolites present in colored cultivars is lacking. This study employed an untargeted metabolomics approach using ultra-high-performance liquid chromatography-tandem mass spectrometry (LC–MS) to analyze the metabolic profiles of five hulless barley cultivars with different grain colors. Six-hundred and eight metabolites in various chemical categories were detected; ABC transporters, flavonoid biosynthesis, and anthocyanin biosynthesis were found to be the most significant metabolic pathways. Principal component analysis and orthogonal partial least squares-discriminant analysis revealed significant metabolic differences among the samples, and could separate the colored barley cultivars from white barley. Black, blue, and purple grains rich in natural antioxidants were identified as promising ingredients for the development of cereal-based functional foods. These

findings showed that the nutritional function and quality of hulless barley were strongly correlated with its natural color, improving our understanding of the metabolic mechanisms and functional health value associated with different seed coat colors in different hulless barley cultivars. Our results thus provide a theoretical basis for the future study of hulless barley-related products.

Keywords Colored hulless barley · Metabolites · PCA · OPLS-DA · UPLC-ESI-MS/MS

Introduction

Barley (*Hordeum vulgare* L.) is a globally cultivated grain that is currently ranked fifth in the world in dry matter production (Roljević et al. 2020). Hulless barley (*H. vulgare* var. nudum Hook. f.) is a cultivar of barley with a lemma in the bare grain that is separated from the caryopsis, which is why it is referred to as hulless. Hulless barley is the only crop that can be cultivated in the Tibetan plateau and is thus the main staple food crop for Tibetans as well as an important feed for livestock (Ramakrishna et al. 2017; Zeng et al. 2015). Hulless barley is valued globally for its significant nutritional value, including protein, crop for Tibetans, amino acids, various vitamins, trace

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elements (zinc and selenium), and dietary fiber (Idehen et al. 2017; Yang et al. 2018).

Seed coat color is an important agronomic crop trait because of its unique biological activity and healthcare functions (Yao et al. 2018). The breeding of colored barley varieties has become increasingly prevalent in agriculture in recent years. Purple, blue, and black grains are considered as promising ingredients for developing functional grain-based foods, as they are a natural source of antioxidants, are rich in phenolic compounds called anthocyanins, and contain essential amino acids (Abdel et al. 2003, 2006; Shen et al. 2018; Zeven, 1991). The barley grain may have different pigmentation formed by the pigment deposited in the seed coat and aleurone layer at maturity. Studies have shown that the natural pigments in colored barley, which include anthocyanins, essential amino acids, and trace elements, are generally higher than those of ordinary white barley and have significant physiological functions. Therefore, there has been increasing focus on utilizing colored barley cultivars (Kim et al. 2007; Lin et al. 2018). The most studied seed coat colors are yellow, purple, red, and blue, which are thought to be associated with different subgroups of flavonoids (Shoeva et al. 2016).

The seed coat of barley is yellow due to the synthesis of proanthocyanidins in the seed coat (testa layer) (Aastrup et al. 1984). The anthocyanins synthesized in the pericarp and glume are related to red and purple seed coats (Zhang et al. 2019), while the anthocyanins synthesized in the aleurone layer of the grain result in a blue seed coat (Harlan 1914). These pigments are not present in the white barley grains (Shoeva et al. 2016). Colored hulless barley is a high-quality germplasm resource, and its nutritional function and quality are strongly correlated with its natural color (Ge et al. 2021; Yan et al. 2009; Yao et al. 2018). Qualitative and quantitative variations in the phytochemical profiles and nutrients, and especially the bioactive compounds of hulless barley, are believed to contribute to the differences in its health-promoting properties (Zhang et al. 2019). Colored barley varieties have been associated with greater beneficial health properties than the standard (white) cultivar because they produce pearling fractions rich in bioactive compounds (Bellido et al. 2009; Chen et al. 2013; Dawson et al. 2015; Fernie & Schauer 2009; Khakimov et al. 2014).

Metabolomics is a powerful tool that is widely used for the large-scale detection of small-molecule metabolites and is convenient for detecting hundreds of metabolites in cells at a specific time point, thus offering insight into the phenotype (Chen et al. 2013; Dawson et al. 2015; Fernie & Schauer 2009; Khakimov et al. 2014). There has been research interest in the metabolites present in hulless barley due to their unique nutritional characteristics and promising health benefits (Jung et al. 2000; Khlestkina 2013). However, specific compounds have been studied mainly through targeted metabolomics approaches, rather than thoroughly investigating all the phytochemical constituents in hulless barley. For instance, the flavonoid biosynthesis metabolic pathway has been well-studied (Badbro 1993; Lin et al. 2018; Suriano et al. 2020). To the best of our knowledge, a systematic evaluation and comparison of the metabolites present in different colored hulless barley cultivars are lacking. The accumulation of metabolites differs in different tissues, species, as well as temporally (Dong et al. 2014; Wang et al. 2016). In this study, five different colored hulless barley varieties were collected from China's main barley cultivation areas. An untargeted metabolomics approach using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) was used to analyze the metabolic profiles and variations in phytochemicals among these five different colored hulless barley varieties. We aimed to elucidate the metabolic differences among different colored hulless barley cultivars and provide a reference for further nutritional applications of colored hulless barley.

Materials and methods

Plant materials

The white cultivar '1127' and blue cultivar 'xiaolan-qingke' (XLQK) were collected from Tibet; the blue cultivar 'ganzilan' (GZL) and black-purple cultivar 'ganzi_160505' (GZ-160505) were collected from Ganzi Prefecture in Sichuan Province of China; and the purple cultivar 'beiqing_2' (BQ_2) was collected from Haibei Prefecture in Qinghai Province (Fig. 1a). When the plants were fully mature, they were harvested and dried. Once fully dried, the plants were threshed indoors, and the mature seeds were harvested

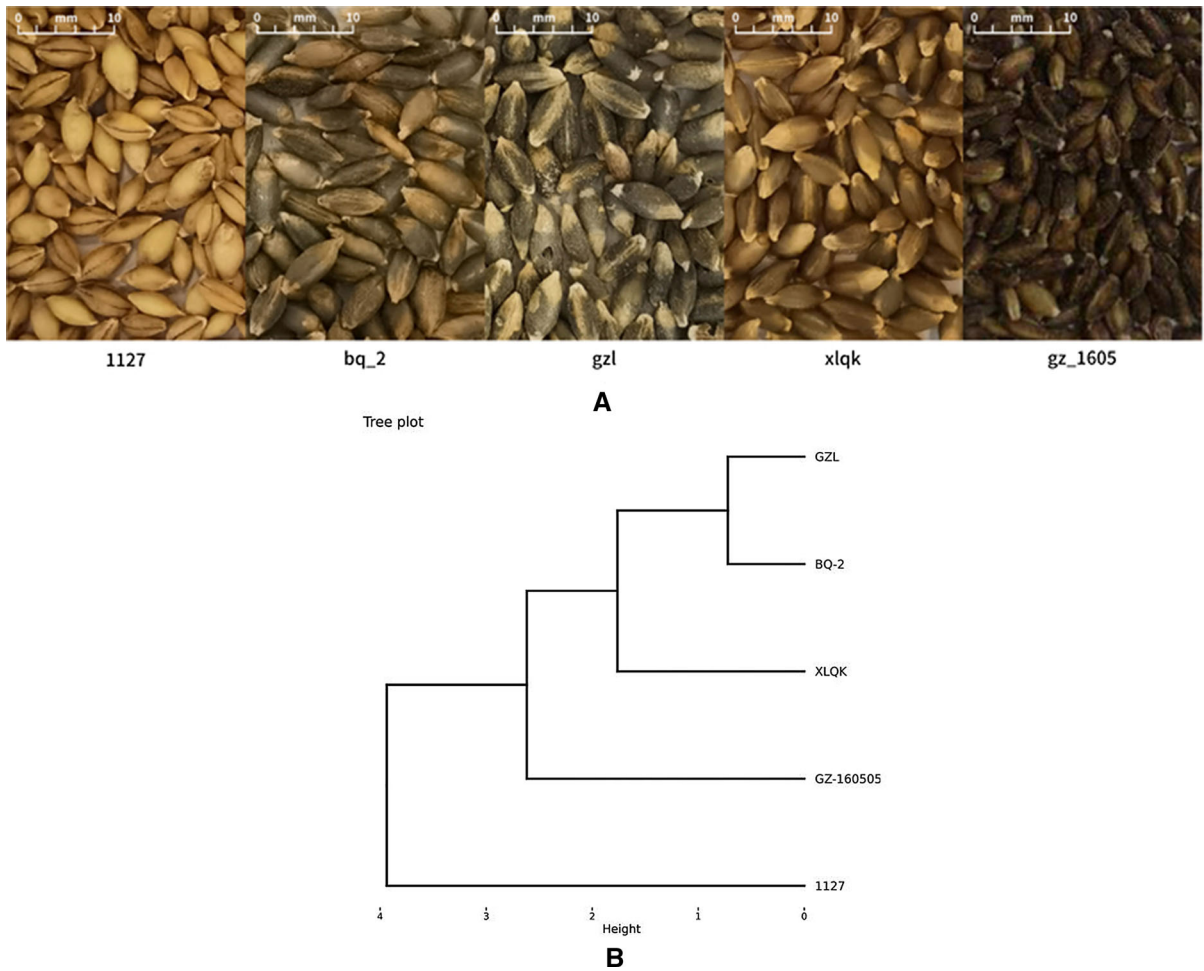


Fig. 1 **a** Grain coat color of the five hulless barley varieties. **b** Clustering dendrogram of the color difference values of the hulless barley cultivars

and stored at $-80\text{ }^{\circ}\text{C}$ until further analysis. Grain color was determined by scanning with a color difference meter (Konica Minolta CM-5).

Sample preparation and extraction for metabolomic analysis

The freeze-dried hulless barley seeds were crushed to a powder with a mixer mill (MM 400, Retsch) (30 Hz, 1.5 min). One hundred milligrams of powder were weighed and dissolved in 0.6 mL 70% methanol extract and then dissolved at $4\text{ }^{\circ}\text{C}$ overnight. The samples were stored in a bottle for UPLC-MS/MS analysis following centrifugation at $10,000\text{ g}$ for 10 min and then filtered through a $0.22\text{ }\mu\text{m}$ pore membrane.

High-performance liquid chromatography (HPLC) conditions

The data acquisition instrument system included an UPLC-ESI-MS/MS system (UPLC, Shim-pack UFLC SHIMADZU CBM30A system, www.shimadzu.com.cn/; MS, Applied Biosystems, 4500 Q TRAP, www.appliedbiosystems.com.cn/). A Waters ACQUITY UPLC HSS T3 C18 column ($1.8\text{ }\mu\text{m}$, $2.1\text{ mm}\times 100\text{ mm}$) was used. The mobile phase consisted of solvent A (pure water with 0.04% acetic acid) and solvent B (acetonitrile with 0.04% acetic acid). The gradient program was 95:5 V/V at 0 min, with a linear gradient to 5:95 V/V over 10 min, where it was held for 1 min at 5:95 V/V at 11.0 min, following which it was adjusted to 95% A 5.0% within 0.10 min,

where it was held for 2.9 min. The temperature was 40 °C and the injection volume was 4 µL. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (QTRAP)-MS.

ESI-QTRAP-MS/MS

Linear ion trap (LIT) and triple quadrupole (QQQ) scans were acquired on a 4500 QTRAP equipped with an electrospray ionization (ESI) Turbo Ion-Spray interface operating in positive and negative ionization mode and controlled by Analyst 1.6.3 software (AB Sciex).

The ESI conditions mainly included: electrospray ionization temperature, 550 °C; spray voltage (IS, 5500 V); and ion source curtain gas (CUR), 30 psi. The collision-activated dissociation (CAD) parameter was set to high. In QQQ, each ion pair was scanned based on the optimized declustering potential (DP) and collision energy (CE).

Qualitative and quantitative analysis of metabolites

Qualitative analyses of metabolites were carried out using the self-built database MWDB (Metware Biotechnology Co., Ltd. Wuhan, China) and public database of metabolite information. The isotopic signals, the repeated signals of K⁺ ions, Na⁺ ions, NH₄⁺ ions, and the repeated signals of fragments of other larger molecular weight substances were removed. Metabolites were quantified using the MRM model. The mass spectrum peak area of all substances was integrated, and the mass spectrum peak of the same metabolite in different samples was integrated and corrected (Markou and Kassomenos 2010).

Statistical analysis

The seed coat color differences were evaluated by cluster analysis. Cluster analysis is an analytical method that divides samples into several categories by measuring the similarity degree of the samples, ultimately aggregating them into one class step-by-step (Fraga et al. 2010). The color difference value of the hulless barley cultivars was used as the input into the cluster analysis.

All metabolites were analyzed using R software (www.r-project.org). Principal Component Analysis (PCA), hierarchical clustering analysis (HCA), and orthogonal partial least squares-discriminant analysis (OPLS-DA) were used to analyze the metabolic data according to previously described methods (Wang et al. 2016). The data were unit variance-scaled prior to the unsupervised PCA. The Pearson's correlation coefficient (PCC) between the samples was calculated using the 'cor' function in R, and the HCA results of the metabolites were presented in the form of a heatmap with dendrogram.

Variable importance in the projection (VIP) values extracted from the OPLS-DA results ≥ 1 and absolute Log₂FC (fold change) ≥ 1 were used to determine the differentially abundant metabolites between groups. The data were log-transformed (log₂) and mean-centered prior to OPLS-DA. A permutation test (200 permutations) was performed to confirm that the OPLS-DA model had not been overfitted.

The biological significance associated with hulless barley color was further evaluated through the functional analysis of metabolic pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG) compound database (<http://www.kegg.jp/kegg/compound/>) to annotate identified metabolites, and the annotated metabolites were then mapped to the KEGG pathway database (<http://www.kegg.jp/kegg/pathway.html>). Pathways with significantly regulated metabolites were then fed into MSEA (metabolite sets enrichment analysis), and their significance was determined based on the *P*-values of the hypergeometric test.

Results and discussion

Analysis of color differences in hulless barley

The color differences in the seed coat of hulless barley are shown as *L*a*b (L* represents the brightness, a* represents the red-green axis, and b* represents the blue-yellow axis) in Table 1, reflects the significant differences in the color characteristics of the five hulless barley cultivars (León et al. 2006). Analysis of the color value showed that the brightness factor L*, red factor a*, and blue factor b* decreased from white hulless barley to colored hulless barley. In the cluster analysis (Fig. 1b), the white hulless barley could be

Table 1 Color values of the seed coats of five hulless barley cultivars

Number	Cultivar	Seed coat color	L(brightness)	a (redness)	B (yellowness)
1	1127	White	56.27 ± 0.13	8.52 ± 0.26	24.83 ± 0.88
2	BQ-2	Purple	47.49 ± 0.62	3.61 ± 0.13	15.32 ± 0.12
3	GZL	Blue	49.14 ± 0.72	2.27 ± 0.15	12.94 ± 0.27
4	XLQK	Blue	46.17 ± 0.33	5.81 ± 0.28	19.34 ± 0.12
5	GZ-160505	Black-purple	36.61 ± 0.45	5.03 ± 0.25	8.37 ± 0.45

clearly distinguished from the colored hulless barley varieties.

Metabolic profiling

In total, 608 metabolites were detected (Supplementary Table S1), including 147 flavonoids, 87 amino acids and derivatives, 78 phenolic acids, 74 lipids, 47 organic acids, 46 nucleotides and derivatives, 42 alkaloids, 10 lignans and coumarins, eight tannins, four terpenoids, and 71 others. The clustering heatmap of the metabolites (Fig. 2) showed significant differences in the metabolite levels of the five cultivars, with the colored hulless barley varieties being clearly distinguished from the white cultivar. This finding was generally consistent with the dendrogram based on the color difference values (Fig. 1b). GZL and BQ_2, although cultivated in different regions, were grouped into one category according to seed coat color and possessed similar metabolite contents. The white cultivar 1127 could be clearly distinguished from the other cultivars. This finding was demonstrated by clustering analysis of the samples and showed that the metabolites of hulless barley were strongly related to seed coat color (Fig. 3).

Differential metabolite analysis using PCA

The first two principal components of the PCA score plot were responsible for 51.31% (28.68% for PC1 and 22.63% for PC2) of the variation in the metabolite profiles. There was a clear separation of these five cultivars, suggesting that each group had a relatively distinct metabolic profile. The distinct separation of 1127 (white hulless barley) on the left side of the plot from the other four varieties, which clustered on the right side of the plot, confirmed the distinction of white hulless barley from the colored cultivars. The HCA and PCA results suggested that the differences in

the metabolite contents might be responsible for the differences in the seed coat color of hulless barley.

Differential metabolite analysis using OPLS-DA

OPLS-DA is a multivariate statistical analysis method for supervised pattern recognition that can maximize the differentiation between groups and identify differential metabolites. In this study, OPLS-DA was carried out to further verify the significantly different metabolites between the five hulless barley cultivars.

High predictability (Q^2) and strong goodness-of-fit (R^2X , R^2Y) of the OPLS-DA models were observed for comparisons between 1127 and XLQK ($R^2X = 0.771$, $R^2Y = 1$, $Q^2 = 0.989$; Fig. 4a), 1127 and GZ_160505 ($R^2X = 0.776$, $R^2Y = 1$, $Q^2 = 0.989$; Fig. 4b), 1127 and GZL ($R^2X = 0.773$, $R^2Y = 1$, $Q^2 = 0.987$; Fig. 4c), 1127 and BQ_2 ($R^2X = 0.768$, $R^2Y = 1$, $Q^2 = 0.986$; Fig. 4d), XLQK and BQ_2 ($R^2X = 0.744$, $R^2Y = 1$, $Q^2 = 0.979$; Fig. 4e), XLQK and GZL ($R^2X = 0.763$, $R^2Y = 1$, $Q^2 = 0.987$; Fig. 4f), XLQK and GZ_160505 ($R^2X = 0.748$, $R^2Y = 1$, $Q^2 = 0.982$; Fig. 4g), GZL and BQ_2 ($R^2X = 0.715$, $R^2Y = 1$, $Q^2 = 0.976$; Fig. 4h), GZL and GZ_160505 ($R^2X = 0.748$, $R^2Y = 1$, $Q^2 = 0.982$; Figure; 4i), and BQ_2 and GZ_160505 ($R^2X = 0.748$, $R^2Y = 1$, $Q^2 = 0.982$; Figure; 4j). The validation of the OPLS-DA models presented a high interpretation rate (R^2Y) and prediction degree ($Q^2 > 0.9$), indicating that the models were appropriate.

Differential metabolite screening, functional annotation, and enrichment analysis

The differential metabolites among the five cultivars were screened using a fold-change (≥ 2 or ≤ 0) and variable importance in the projection (VIP) value (≥ 1) of the OPLS-DA model. The screening results

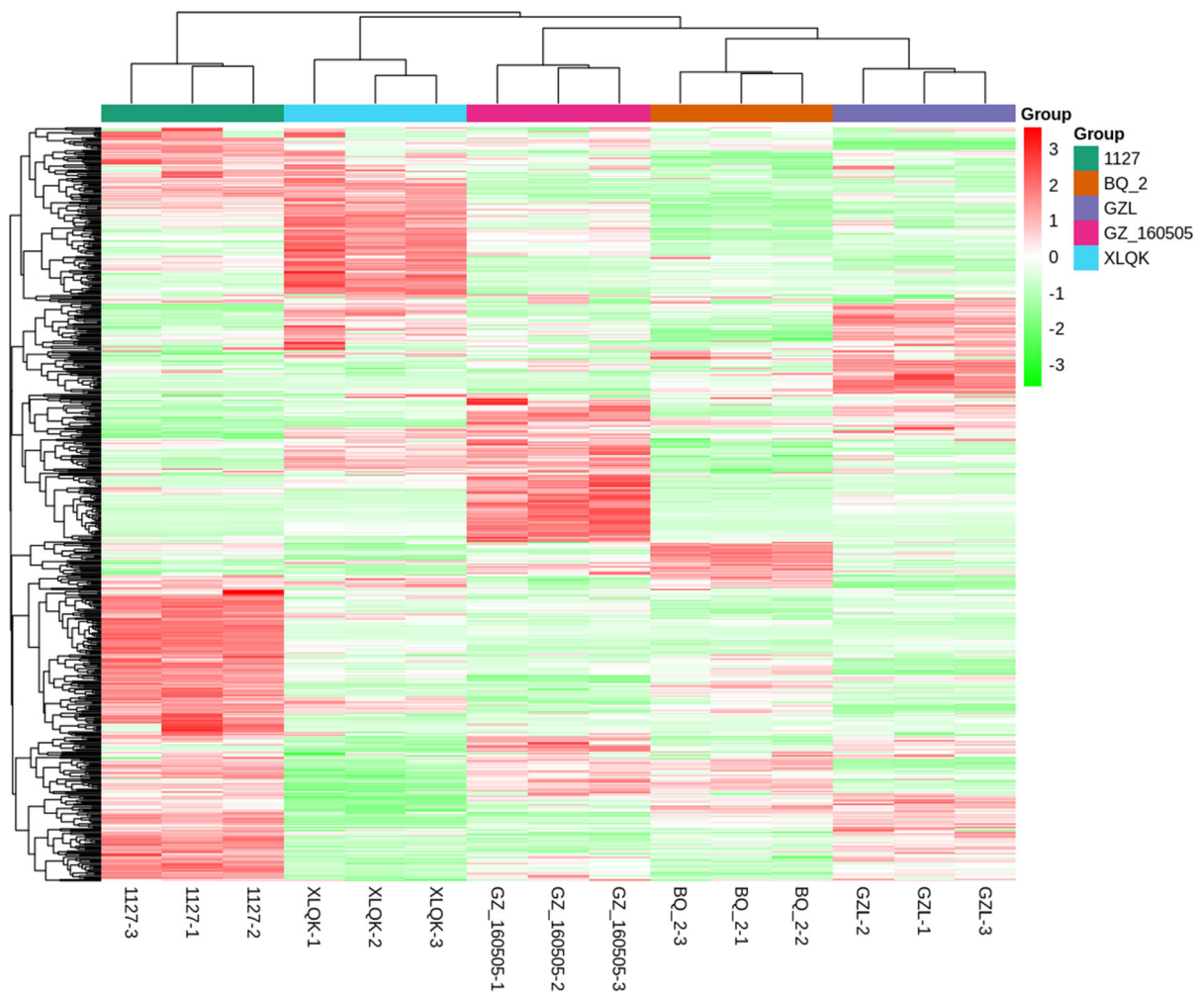


Fig. 2 Metabolic profiles reflected using a clustering heatmap. The content of each metabolite was normalized to complete linkage hierarchical clustering. Green and red colors indicate low and high abundances, respectively

are shown in Supplementary Table S2 and are illustrated using volcano plots (Fig. 5a–d) and Venn diagrams (Fig. 5e, f). There were 181 significantly different metabolites between 1127 and BQ_2 (134 down-regulated, 47 up-regulated), 219 between 1127 and GZ_160505 (115 down-regulated, 104 up-regulated), 207 between 1127 and GZL (131 down-regulated, 76 up-regulated), and 246 between 1127 and XLQK (146 down-regulated, 100 up-regulated). Compared with white hulless barley, there were more down-regulated metabolites in the colored hulless barley, and these metabolites could clearly distinguish the colored hulless barley from the white hulless barley. The screening results also showed that, in total, about one third of the differentially abundant

metabolites were flavonoids (62/181 in 1127 vs. BQ_2; 84/219 in 1127 vs. GZ_160505; 65/207 in 1127 vs. GZL; and 76/246 in 1127 vs. XLQK). These metabolites were mainly flavonoids and may constitute the representative differential metabolites for the different colored varieties. A difference in phenolic compound composition with grain color was previously observed in both barley and hulless barley (Abdel et al. 2012), which corroborates our results.

Using the intersection of each comparison group in the Venn diagram (Fig. 5e), a total of 70 common differential metabolites were observed, and each comparison group possessed unique differential metabolites. The Venn diagram in Fig. 5f shows the overlapping and unique metabolites among the

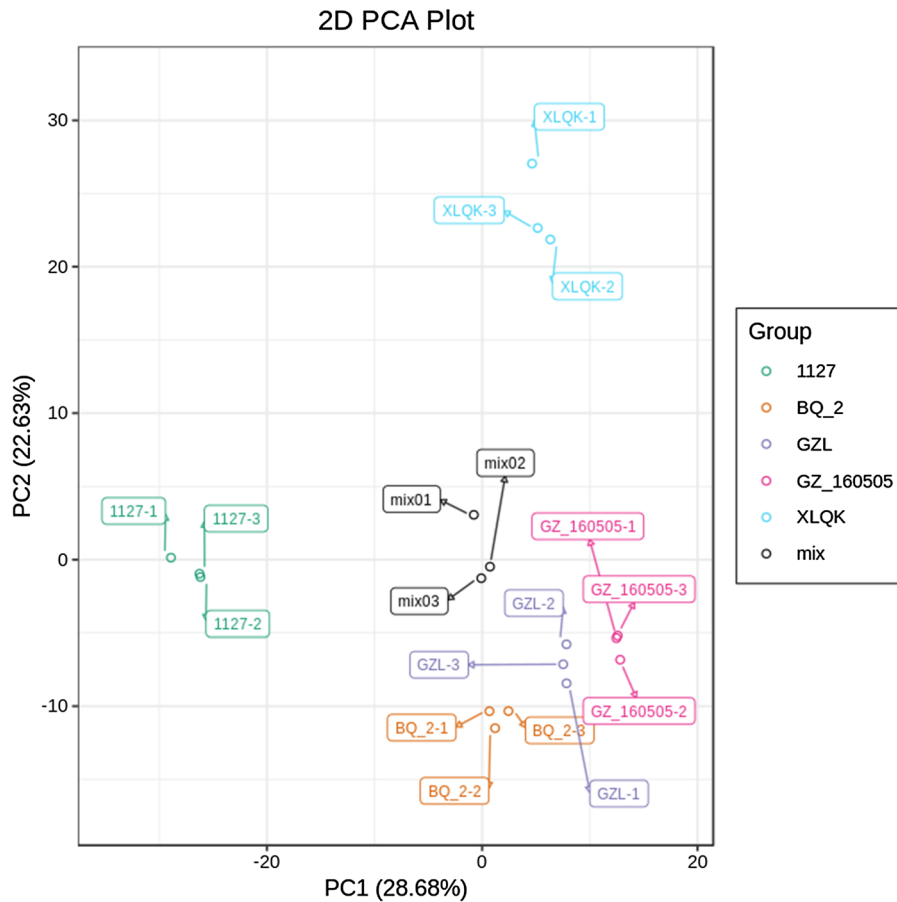


Fig. 3 Principal component analysis (PCA) score plots of the five hulless barley cultivars

comparison group. There were 28 metabolites that were only present in the colored hulless barley, 19 were flavonoids and three were phenolic acids. These metabolites were primarily flavonoids and may be considered as representative differential metabolites for colored hulless barley.

To gain further insight into the differences in flavonoids between colored and white hulless barley, differential flavonoids among the comparison groups were compared (Supplementary Table S3). The results showed that, except for BQ_2 (26/62 in 1127 vs. BQ_2), most of the flavonoids (69/84 in 1127 vs. GZ_160505; 47/65 in 1127 vs. GZL; and 62/76 in 1127 vs. XLQK) were up-regulated as the color of the seed coat changed from white to purple-black. Many flavonoids were up-regulated in the colored hulless barley, indicating that colored hulless barley is rich in flavonoids.

Based on the intersection of each comparison group (Supplementary Table S3), 14 common differential flavonoid metabolites were up-regulated among the comparison groups 1127 vs. BQ_2, 1127 vs. GZ_160505, 1127 vs. GZL, and 1127 vs. XLQK. Rutin was detected in all the samples, though the differences among the colored hulless barley and white cultivar were nonsignificant. Except for cyanidin 3,5-O-diglucoside and peonidin 3-O-glucoside in BQ_2, most of the anthocyanins were up-regulated in the colored varieties compared with the control. Isoflavones are exclusively present in legumes, such as soybeans, and play important roles in plant defense and nodulation (Jung et al. 2000; Kim et al. 2007; Shoeva et al. 2016). In the present study, three isoflavones were detected in all hulless barley varieties, of which afzelechin(3,5,7,4'-tetrahydroxyflavan) was down-regulated and 2'-hydroxyisoflavone was up-regulated, whereas arcarpene 2 was down-

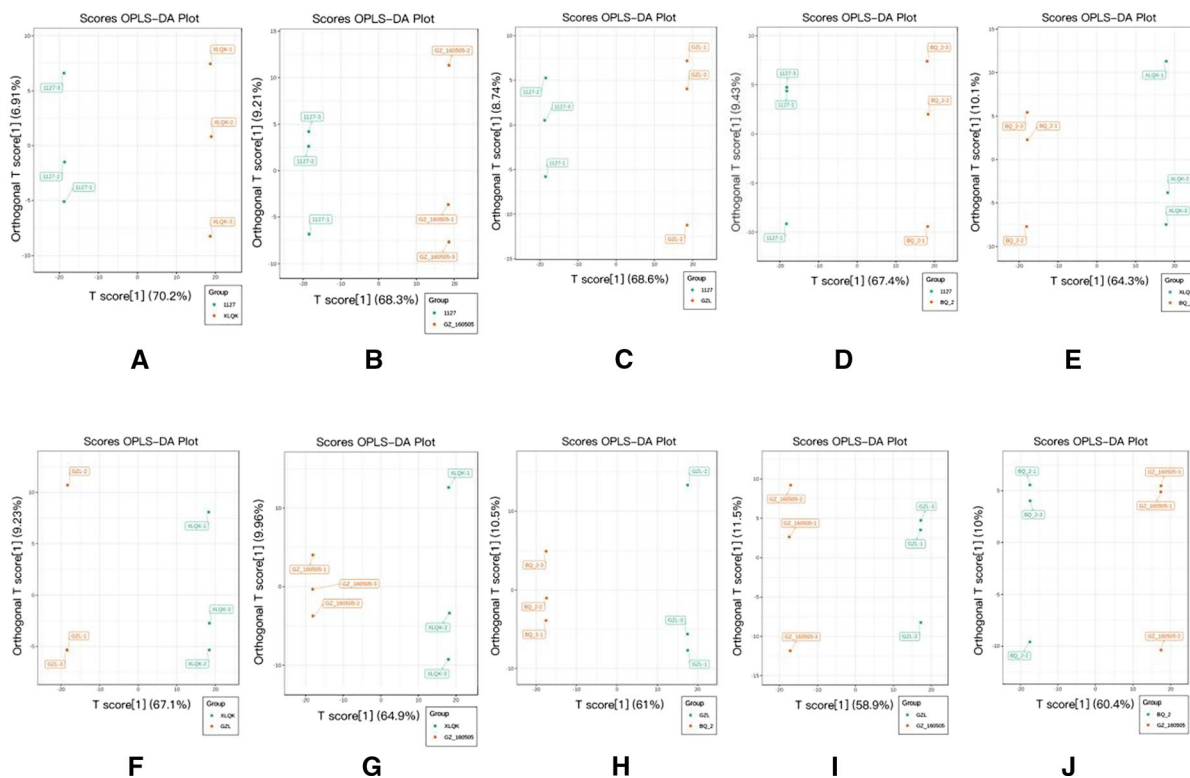


Fig. 4 Differential metabolite analysis using orthogonal least squares-discriminant analysis (OPLS-DA). (A–J) OPLS-DA model plots for the comparison group of 1127 vs. XLQK, 1127

vs. GZL, 1127 vs. BQ_2, XLQK vs. BQ_2, XLQK vs. GZL, XLQK vs. GZL, 160505, GZL vs. BQ_2, GZL vs. GL_160505, and BQ_2 vs. GL_160505

regulated in BQ_2 and up-regulated in GZL_160505 and XLQK.

Differential metabolic pathways among the samples

The differentially abundant metabolites among the five hullless barley cultivars were annotated using the KEGG database (<http://www.genome.jp/kegg/>) in order to obtain the detailed pathway information (Fig. 6a–d, Supplementary Table S4). The KEGG classification results and enrichment analyses indicated that the majority of differential metabolites were involved in metabolic pathways, including metabolic pathways (44/84 [52.38%], 1127 vs. BQ_2; 45/86 [52.33%] in 1127 vs. GZL_160505; 57/95 [60%] in 1127 vs. GZL; 65/113 [57.52%] in 1127 vs. XLQK), biosynthesis of secondary metabolites (22/84 [26.19%] in 1127 vs. BQ_2; 23/86 [26.74%] in 1127 vs. GZL_160505; 27/95 [28.42%] in 1127 vs. GZL; 34/113 [30.09%] in 1127 vs. XLQK); ABC

transporters (12/84 [14.29%] in 1127 vs. BQ_2; 8/86 [9.30%] in 1127 vs. GZL_160505; 14/95 [14.74%] in 1127 vs. GZL; 19/113 [16.81%] in 1127 vs. XLQK); flavonoid biosynthesis (9/84 [10.71%] in 1127 vs. BQ_2; 13/86 [15.12%] in 1127 vs. GZL_160505; 7/95 [7.37%] in 1127 vs. GZL; 13/113 [11.50%] in 1127 vs. XLQK); and anthocyanin biosynthesis (5/84 [5.95%] in 1127 vs. BQ_2; 6/86 [6.98%] in 1127 vs. GZL_160505; 6/95 [6.32%] in 1127 vs. GZL; 6/113 [5.31%] in 1127 vs. XLQK). Enrichment analysis (Fig. 6a–d) also indicated that the differential metabolites of the comparison groups were mainly involved in flavonoid biosynthesis, flavone and flavonol biosynthesis, and anthocyanin biosynthesis. The result is similar to a previous study on colored barley germplasm that found that the total average concentrations of anthocyanins in the blue and purple groups were significantly higher than those in the black group (Kim et al. 2007). Our findings also support earlier studies that found that seed coat color is related to anthocyanin synthesis (Kim et al. 2007; Shoeva et al.

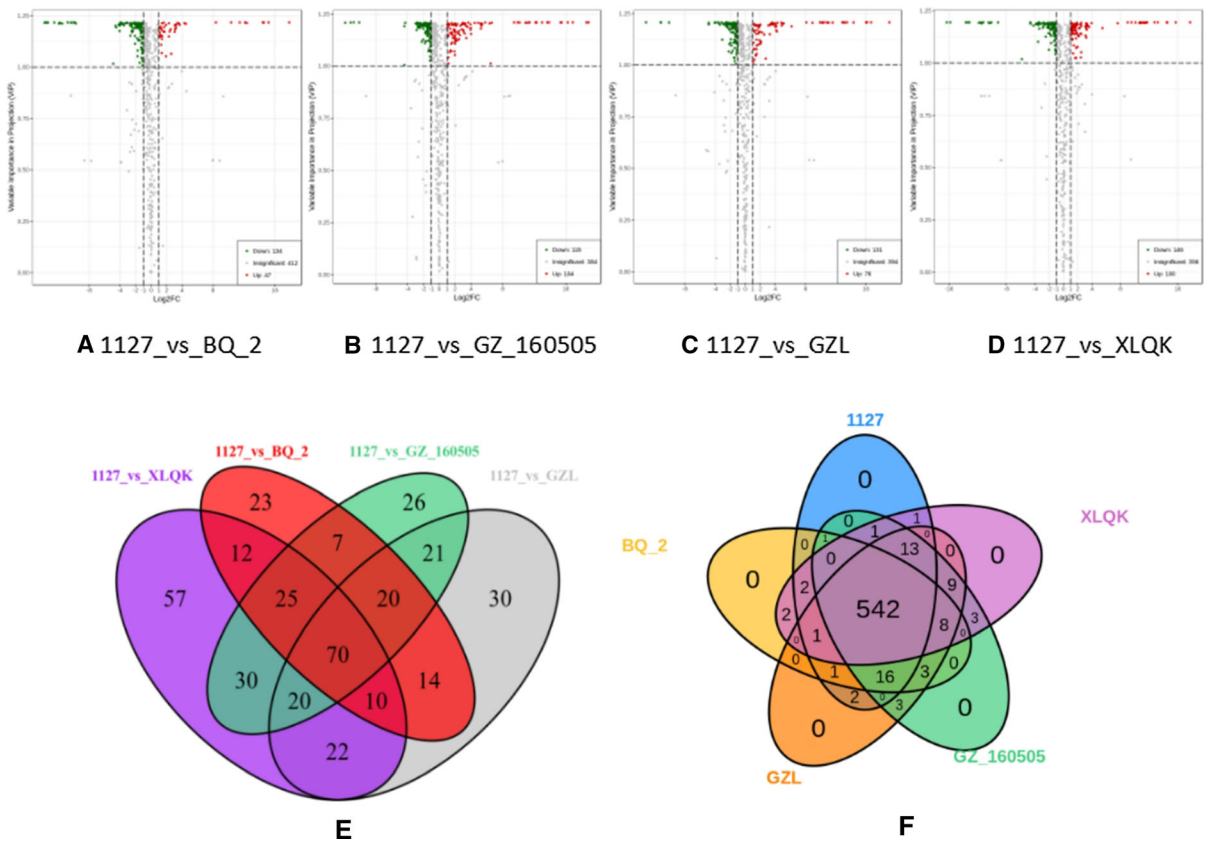


Fig. 5 Differential metabolite analysis of colored hulless barley compared with white hulless barley. A–D: The volcano plot shows the differential metabolite abundance levels between BQ_2, GZ_160505, GZL, and XLQK compared with 1127. Green dots represent down-regulated differentially abundant metabolites; red dots represent up-regulated differentially

abundant metabolites; and black dots represent metabolites detected but that are not significantly different. E: Venn diagram showing the overlapping and unique differential metabolites among the comparison groups; F: Venn diagram showing the overlapping and unique metabolites among the comparison groups

2016; Takahashi 1955), though we primarily detected flavonoids. Our future research will focus on the mechanisms of anthocyanin synthesis in the pericarp, glume, and aleurone layer of different colored hulless barley varieties.

Conclusions

The present study focused on the metabolic diversity, particularly flavonoids, of five hulless barley varieties and annotated 608 metabolites, including flavones, amino acids, and phenolic acids. The metabolic profiles of the colored hulless barley varieties could be effectively discriminated from that of the white cultivar, and the differences were mainly attributed to metabolites associated with ABC transporters,

flavonoid biosynthesis, and anthocyanin biosynthesis pathways. In addition, 18 flavonoids and three phenolic acids were detected and quantitated in the colored hulless barley, but not in the white cultivar. Except for BQ_2, more flavonoids were up-regulated in the colored hulless barley compared to the white cultivar. These changes may be responsible for the observed variability in hulless barley color, including increased amounts of certain phenolic acids or flavonoids. Colored hulless barley cultivars are rich in flavonoids and possess specific metabolite profiles that differ from white hulless barley, and these flavonoids also vary between the different colored cultivars. These findings improve our understanding of the metabolic mechanisms and functional health value associated with different seed coat colors in different hulless barley cultivars.

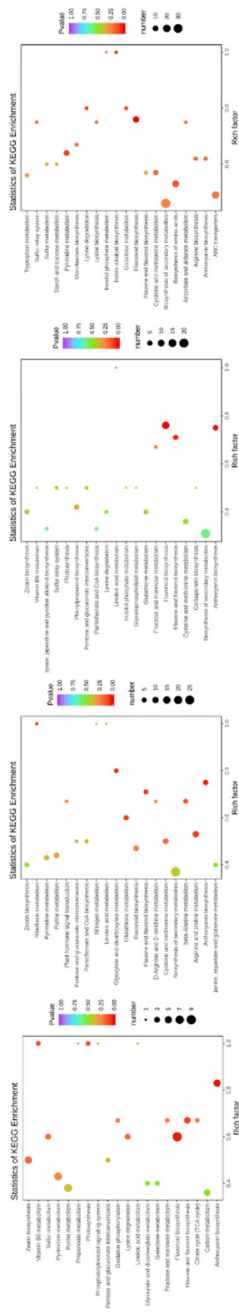


Fig. 6 Enrichment map of the differential metabolites to distinct KEGG pathways. Differential metabolites in BQ_2 (A), GZL (B), GZL_160505 (C), and XLQK (D) compared with those in 1127 were mapped to distinct metabolic pathways

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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