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Quality constituents of high amino acid content tea cultivars with various leaf colors

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Abstract: Green tea made from high amino acid content (HAAC) tea cultivars with different leaf colors exhibits many similar characteristics, such as slight bitterness, a rich aroma, and a refreshing and velvety taste. To determine differences in the key constituents of five HAAC tea cultivars with various leaf colors, the cultivars quality constituents were systematically analyzed and compared with that of a normal green tea cultivar. High theanine (Thea) content and the synthesis of Thea precursors are extremely important determinants of the character of these HAAC tea cultivars. The levels of most catechins, carotenoids, and chlorophylls in the HAAC tea cultivars were significantly lower than those in the normal tea cultivar, as was that of caffeine. The present study suggests that the inhibition of catechins (particularly epicatechins; ECs), chlorophylls, carotenoids, and caffeine biosyntheses in HAAC tea cultivars directs the metabolic network toward amino acid biosynthesis, particularly Thea biosynthesis.

Key words: HPLC, green tea, high amino acid content, bioactive profile, biosynthesis

1. Introduction

Tea is one of the most widely consumed nonalcoholic beverages worldwide because its water-soluble components have important health benefits (Khan and Mukhtar, 2013; Fang et al., 2014). The quality of processed tea is mainly determined by its metabolic constituents such as free amino acids, catechins, purine alkaloids (caffeine), and pigment (chlorophylls and carotenoids) (Cabrera et al., 2006; Li et al., 2016). Among these constituents, free amino acids have been reported to be the primary contributors to the taste of green tea and are the main constituent of the thearubigin fraction, which is responsible for much of the color of the black tea brew (Yamaguchi and Ninomiya, 2000; Chen and Zhou, 2005). Furthermore, they markedly intensify the taste of tea infusions by effectively counteracting astringency and bitterness (Chen et al., 2011). Theanine (g-glutamyl-L-ethylamide; Thea), a critical secondary metabolite, is one of the most abundant free amino acids present in tea leaves (Chu et al., 1997; Crozier et al., 2006). Thea has been extensively investigated in relation to human health because of its numerous physiological and pharmacological functions, such as nerve protection, blood pressure control, hematic fat reduction, antitumor properties, liver protection, and immunity enhancement (Hindmarch et al., 2000; Kakuda, 2002; Shimbo et al., 2005; Kimura et al., 2007). Therefore, the breeding of tea cultivars with HAAC (particularly Thea content) is crucial to enhancing the quality and health functions of tea.

Some tea germplasm resources with HAAC have been discovered, are crucial materials for tea breeding, and can produce tea with enormous economic and social benefits. For example, albino tea plants, a famous tea variation with young white shoots and higher amino acid levels than their green counterparts, have attracted much attention in recent years (Li et al., 1996; Du et al., 2006). Wang et al. (2014) indicated that the total free amino acid content is significantly higher in the leaves of a yellow leaf tea cultivar (Zhonghuang 2; ZH2) than in those of Longjin 43 (LJ43). In addition, Zhang et al. (2012) discovered a normal leaf color tea cultivar, Huangjincha 1 (Hu1), with HAAC. Some of the aforementioned studies focused only on albino *Camellia sinensis* cultivars with HAAC, whereas others focused on changes in tea growth. However, normal green tea cultivars with HAAC are relatively unexplored. Moreover, whether different leaf color tea cultivars with HAAC are metabolized through similar mechanisms remains unclear, and the mode of amino acid accumulation remains unknown.

Over several years, we have observed five notable HAAC cultivars: a white leaf tea cultivar Baiye 1 (BY1), two yellow leaf tea cultivars Zhonghuang 1 (ZH1) and ZH2, and two green leaf tea cultivars Hu1 and E1, all of which were planted in the tea garden of the Hubei Academy of Agricultural Sciences. To further identify and evaluate the metabolic mechanism of the tea cultivars with three colors and investigate their modes of amino acid accumulation,

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we systematically analyzed the metabolite profiles of the five HAAC tea cultivars and compared them with those of the normal green leaf tea cultivar Jinming 1 (JM1), which is the dominant cultivar in Hubei Province. The major chemical constituents in the shoots (one leaf and one bud) of these tea cultivars were determined through high-performance liquid chromatography (HPLC). Furthermore, the levels of 19 amino acids—including the critical differentiating amino acids Thea, glutamic acid (Glu), glutamine (Gln), asparagine (Asp), serine (Ser), alanine (Ala), leucine (Leu), and isoleucine (Ile)—were evaluated (Alcázar et al., 2007), and ethylamine, a precursor in Thea synthesis, was also detected in these tea cultivars (Deng et al., 2013). In addition, the chlorophyll, carotenoid, catechin, and caffeine levels in the shoots were measured. The metabolites that distinguished the five tea cultivars were identified through principal component analysis (PCA), followed by orthogonal partial least-squares discriminant analysis (OPLS-DA). The metabolic pathways involving the identified differential metabolites, such as pigment, catechin, and caffeine, were investigated. These results revealed the quality constituents' differences in the HAAC tea cultivars with different leaf colors. The aim of our investigation was not only to identify the advantages of the variations but also to provide a new viewpoint for their further application.

2. Materials and methods

2.1. Plant materials

This study employed six tea cultivars [*C. sinensis* (L.) O.Kuntze]: JM1, BY1, ZH1, ZH2, Hu1, and E1 (a HAAC tea germplasm found in the Hubei Germplasm Tea Repository, with HAAC characteristics that have remained stable for several years). The six tea cultivars, all aged 5 years, were planted in the tea garden of the Fruit and Tea Research Institute of the Hubei Academy of Agricultural Sciences (N 30.29518°, E 114.14673°) under the same conditions. Young shoots (one leaf and one bud) were collected in spring (Figure 1). Three independent biological replicates were obtained, and each replicate was collected from more than 20 randomly selected tea plants. All collected samples were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.2. Chemicals

The standards for Asp, Ser, Glu, Ala, Leu, Ile, histidine (His), cysteine (Cys), glycine (Gly), arginine (Arg), lysine (Lys), phenylalanine (Phe), proline (Pro), valine (Val), tyrosine (Tyr), and threonine (Thr) were purchased from Waters (Milford, MA, USA). Those for Thea, Gln, ornithine (Orn), ethylamine, epigallocatechin gallate (EGCG), epigallocatechin (EGC), catechin (C), epicatechin (EC), epicatechin-3-gallate (ECG), gallic acid (GA), catechin gallate (CG), gallocatechin (GC), gallocatechin gallate (GCG), and caffeine were purchased from Sigma-Aldrich (St. Louis, MO, USA). β-Carotene, β-cryptoxanthin, lutein, and zeaxanthin were purchased from CaroteNature (Lupsingen, Switzerland). HPLC-grade methanol and acetonitrile were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All other substances were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

2.3. Measurement of chlorophyll, carotenoid, amino acid, catechin, and caffeine levels

The amino acid content was determined using the method of Tai et al. (2015), with minor modifications. In brief, 0.1 g of a freeze-dried sample was extracted at 100 °C for 45 min in 10 mL of water, with the mixture shaken once every 15 min. The filtrates were sieved through a 0.22-μm nylon filter and analyzed using HPLC. Amino acids were detected using a Waters 2695 HPLC system equipped with a 2998 PDA detector. The Waters AccQ•Tag method with a Waters AccQ•Tag column (Nova-Pak C18, 4 μm, 150 mm × 3.9 mm) was used to detect various amino acids according to the protocol of the AccQ•Fluor Reagent Kit.

Chlorophylls were extracted and analyzed according to the method of Wei et al. (2012). In brief, 100 mg of a fresh sample was extracted using 5 mL of extraction solution (acetone/ethanol = 1/1, by volume). The extract was measured spectrophotometrically at 645 and 663 nm.

Carotenoids were extracted and analyzed as described previously with some modifications (Yu et al., 2007). In brief, 100 mg of a fresh sample was extracted using 3 mL of n-hexane/acetone/ethanol (50/25/25, by volume) and mixed thoroughly using a table concentrator for 30 min at 220 rpm. After centrifugation at 4000 rpm for 10 min, the residue was re-extracted, as described. The supernatant was transferred to a clean tube and dried at room temperature under a N_2 stream. The residue was resuspended in 5 mL of methanol–KOH (10 g KOH per 100 mL in methanol/water, 80/20, v/v) and incubated at 80 °C for 60 min. In total, 2 mL of H_2O and 3 mL of petroleum ether were added to the extracted carotenoids, and the residue was re-extracted twice, as described. The supernatant was transferred to a clean tube and dried at room temperature under a N_2 stream. The residue was resuspended in 200 μL of acetonitrile/methylene chloride/methanol (50/40/10, by volume) containing butylated hydroxytoluene [0.5% (w/v)] and filtered through a 0.22-μm organic membrane before HPLC was performed.

Catechins and caffeine were extracted and analyzed according to the method described by Tai et al. (2015), with minor modifications. In brief, 0.1 g of a freeze-dried sample was extracted using 3 mL of 80% methanol in an ultrasonic sonicator for 10 min at 4 °C. After centrifugation at 6000 rpm for 5 min, the residue was re-extracted twice, as described. The supernatants were combined and diluted

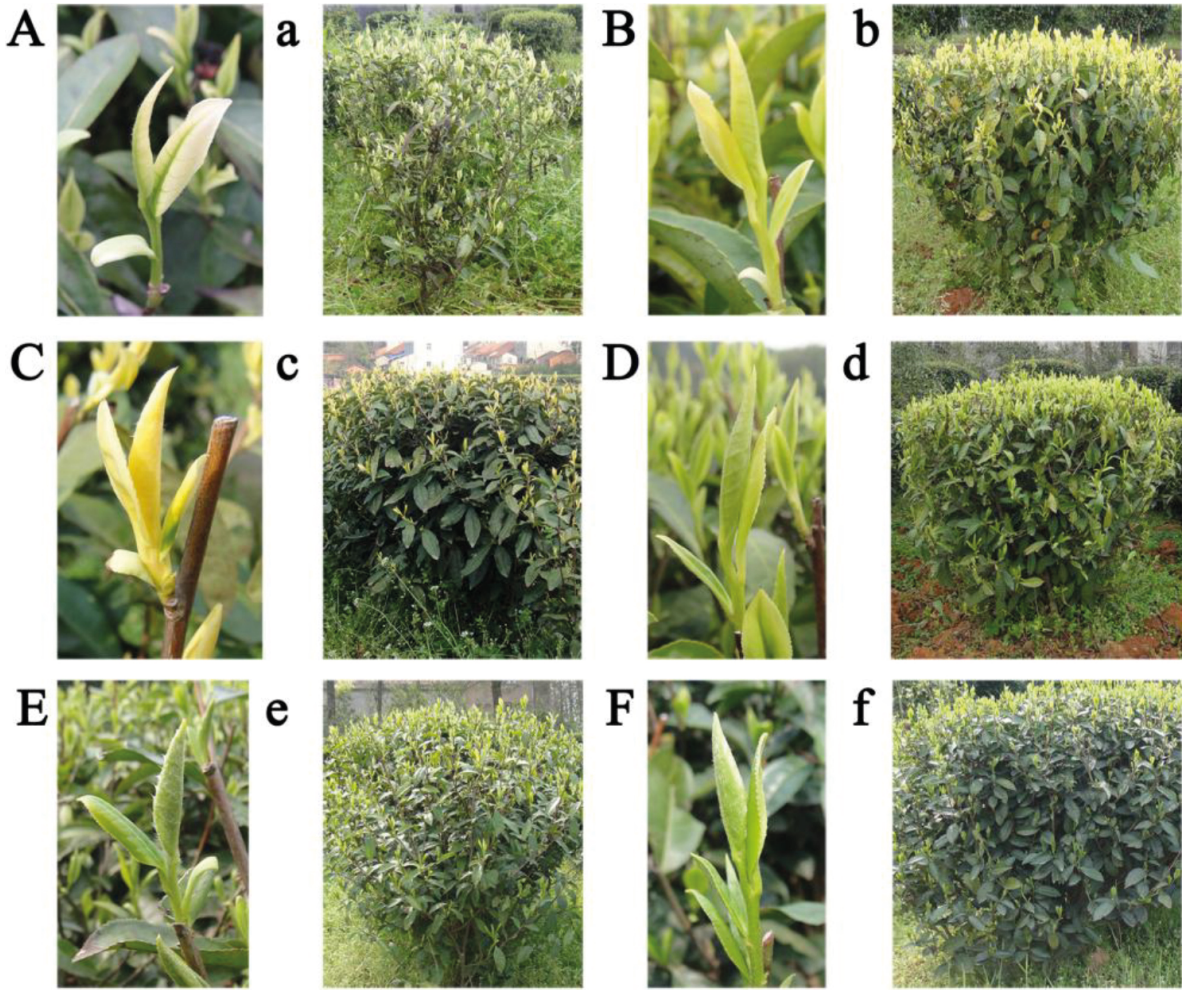

Figure 1. Morphological differences between the HAAC and normal tea cultivars. The plants and new shoots of the tea plants are shown. A and a, BY1; B and b, ZH1; C and c, ZH2; D and d, Hu1; E and e, E1; F and f, JM1.

with 80% methanol to a volume of 10 mL. Subsequently, they were filtered through a 0.22-μm organic membrane before HPLC was performed.

2.4. Data analysis

Data from three independent biological replicates are shown as means ± standard deviations. Statistical analysis was performed through one-way analysis of variance by using SPSS (ver. 17.0, SPSS Inc., Chicago, IL, USA). The normalized datasets were analyzed using SIMCA-P+11.5 (Umetrics, Umea, Sweden) for multivariate statistical analysis. PCA was conducted to detect the intrinsic variation among the different HAAC tea cultivar samples. OPLS-DA was performed to maximize sample separation. The data points on the OPLS-DA loading plot were assigned a variable of importance. In general, the data point of a metabolite with a smaller influence on cluster formation lies closer to the origin. Component P1 in the loading plot is the principal component and indicates the sample variation among cultivars, whereas component P2 is the Y-orthogonal component and models the variation within a specific cultivar. The distinguishing variables between the five HAAC tea cultivars and the normal tea cultivar JM1 were identified using the loading plots and variable influence on projection (VIP) value thresholds (VIP > 1) obtained from the OPLS-DA models.

3. Results and discussion

3.1. Differences in the free amino acid content in HAAC tea cultivars

In total, 19 amino acids were detected in the shoots of all HAAC tea cultivars, and these were compared with those detected in the normal green leaf tea cultivar JM1 (Table). The average total free amino acid content in BY1, ZH1, ZH2, Hu1, E1, and JM1 was 41.28 ± 0.58, 57.21 ± 1.58, 53.95 ± 1.33, 42.71 ± 0.48, 45.65 ± 0.96, 29.51 ± 0.21 mg g^{-1} dry weight in shoots, respectively. The concentration of

Table. Comparison of amino acid and ethylamine contents between the HAAC tea cultivars and normal tea cultivar JM1 (mean ± standard deviation).

Content (g kg^{-1}, DW)	HAAC tea cultivars					JM1
	BY1	ZH1	ZH2	Hu1	E1	
Asp	3.02 ± 0.08**	5.18 ± 0.31**	4.75 ± 0.57**	3.43 ± 0.08**	2.68 ± 0.07*	2.29 ± 0.07
Ser	1.67 ± 0.14**	1.81 ± 0.14**	1.24 ± 0.07**	0.67 ± 0.02	2.05 ± 0.19**	0.77 ± 0.04
Glu	5.45 ± 0.19**	7.88 ± 0.27**	7.27 ± 0.32**	6.22 ± 0.26**	6.54 ± 0.28**	4.47 ± 0.18
Gly	0.21 ± 0.04**	0.15 ± 0.02	0.13 ± 0.04	0.05 ± 0.01*	0.12 ± 0.02	0.13 ± 0.04
Gln	5.91 ± 0.12**	11.85 ± 0.28**	6.96 ± 0.55**	2.25 ± 0.14**	3.10 ± 0.20**	3.95 ± 0.11
His	0.52 ± 0.02	0.85 ± 0.05**	0.49 ± 0.06	0.20 ± 0.02**	0.57 ± 0.04**	0.46 ± 0.01
Arg	2.09 ± 0.10	4.45 ± 0.08**	5.61 ± 0.49**	1.34 ± 0.02**	4.91 ± 0.54**	2.35 ± 0.25
Thr	1.14 ± 0.02**	0.85 ± 0.06*	0.72 ± 0.05	0.40 ± 0.05*	0.97 ± 0.20**	0.60 ± 0.11
Ala	0.66 ± 0.05**	1.30 ± 0.08**	0.70 ± 0.08**	0.43 ± 0.03	0.82 ± 0.06**	0.46 ± 0.01
Pro	0.94 ± 0.13**	0.54 ± 0.02	0.76 ± 0.04	0.79 ± 0.08	0.57 ± 0.10	0.68 ± 0.11
Thea	18.47 ± 0.36**	21.52 ± 0.47**	24.62 ± 0.91**	26.20 ± 0.24**	22.36 ± 1.18**	12.76 ± 0.27
Cys	0.07 ± 0.01**	0.08 ± 0.01**	0.01 ± 0.00**	0.05 ± 0.00*	0.01 ± 0.00**	0.05 ± 0.00
Tyr	0.12 ± 0.02*	0.08 ± 0.01	0.09 ± 0.03	0.08 ± 0.01	0.09 ± 0.01	0.09 ± 0.00
Val	0.38 ± 0.06**	0.12 ± 0.03	0.13 ± 0.03	0.10 ± 0.02	0.22 ± 0.02**	0.10 ± 0.01
Orn	0.12 ± 0.01**	0.09 ± 0.01*	0.08 ± 0.04*	0.15 ± 0.01**	0.09 ± 0.01*	0.04 ± 0.00
Lys	0.18 ± 0.02**	0.20 ± 0.01**	0.18 ± 0.02**	0.12 ± 0.01	0.13 ± 0.01	0.13 ± 0.02
Ile	0.08 ± 0.00**	0.07 ± 0.01**	0.06 ± 0.00	0.06 ± 0.00*	0.06 ± 0.00**	0.05 ± 0.00
Leu	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.02	0.08 ± 0.01	0.08 ± 0.00	0.07 ± 0.01
Phe	0.15 ± 0.01**	0.11 ± 0.00**	0.09 ± 0.00**	0.09 ± 0.01**	0.12 ± 0.01**	0.07 ± 0.00
Ethylamine	0.68 ± 0.02**	0.73 ± 0.04**	0.71 ± 0.08**	0.77 ± 0.03**	0.85 ± 0.02**	0.45 ± 0.02
Total amino acid	41.28 ± 0.58**	57.21 ± 1.58**	53.95 ± 1.33**	42.71 ± 0.48**	45.65 ± 0.96**	29.51 ± 0.21

Significance level: *P < 0.05; **P < 0.01.

total free amino acids was significantly higher in the shoots of all five HAAC tea cultivars than in the shoots of JM1. The two yellow leaf tea cultivars had concentrations nearly two times that of the control cultivar, and other cultivars had concentrations almost 1.5 times higher. Evidence has demonstrated that amino acid types and contents impart distinct characteristics to various tea cultivars (Alcázar et al., 2007). We discovered that Asp, Ser, Glu, Gln, Arg, and Thea were the predominant amino acids in all the tea cultivars investigated. Among these amino acids, Thea was the most abundant free amino acid, accounting for approximately 50% of all free amino acids in all tea cultivars. The Thea content of the five HAAC tea cultivars was approximately twice that in JM1. We suggest that high Thea content is extremely important for the formation of the HAAC tea cultivars investigated. Glu and ethylamine are essential for the synthesis of Thea precursors (Deng et al., 2010), and Glu has been extensively evaluated in food science because of its unique taste characteristic, referred to as umami (Yamaguchi and Ninomiya, 2000). We observed that the Glu and ethylamine contents were significantly higher in the five HAAC tea cultivars than in JM1. Increased Glu level is caused by salt stress, which consequently leads to improved Thea biosynthesis in tea seedlings (Deng et al., 2013). Therefore, high Glu and ethylamine contents indicate the provision of rich substrates for Thea biosynthesis in the HAAC tea cultivars. Furthermore, Glu and Gln can be transformed into one another in the glutamine synthetase/glutamate synthase cycle (Tabuchi et al., 2007). Notably, the Gln content was markedly higher in the three albino tea leaves compared with JM1, whereas the Gln content in Hu1 and E1, the two normal green leaf HAAC tea cultivars, was lower than that of JM1. Li et al. (2015) stated that the inhibition of chlorophyll biosynthesis leads to a shift toward amino acid biosynthesis during the albescent stages of Anji Baicha. Therefore, we suggest that high Gln content is caused by the inhibition of chlorophyll biosynthesis in the three

albino mutant tea leaves, whereas low Gln content results from the activation of Thea biosynthesis in the two normal green leaf tea cultivars with HAAC. Content of Arg, a semiessential amino acid derived from Glu, was higher in the HAAC tea cultivars than in JM1, except in BY1 and Hu1. Asp content was higher in the HAAC tea cultivars than in JM1, and some amino acids such as Lys, Thr, and Ile (which are derived from Asp) had similar levels among all the HAAC tea cultivars. Content of Ser (derived from 3-phosphoglycerate) was higher in the HAAC tea cultivars than JM1, except in Hu1. Furthermore, we detected some lower-content amino acids, most of which were significantly more abundant in the HAAC tea cultivars, such as Phe, a precursor of catechin synthesis. Altogether, we suggest that amino acid (particularly Thea and Glu) biosynthesis is extremely prevalent in the five HAAC tea cultivars and is a main cause of the characteristics of these HAAC tea cultivars.

3.2. Differences in the chlorophyll and carotenoid contents of the HAAC tea cultivars

The chlorophyll contents of the five HAAC tea shoots were compared with those of JM1. The levels of chlorophyll a, chlorophyll b, and chlorophyll a+b in the shoots of all five HAAC tea cultivars (particularly the three albino mutant plants) were significantly lower than those in JM1 (Figure 2A). Tanaka et al. (2007) indicated that Glu acts as the direct precursor of 5-aminolevulinic acid in the C5 pathway, and 5-aminolevulinic acid is the critical precursor of chlorophyll synthesis. Feng et al. (2014) suggested that the suppression of chlorophyll biosynthesis leads to increased Glu level in albino mutant tea shoots that are forced into the Thea biosynthesis pathway. Some studies have demonstrated that changes in total chlorophyll content exhibit an opposite pattern to those in amino acid content (Li et al., 1996; Wang et al., 2014; Li et al., 2015). Therefore, we suggest that the inhibition of chlorophyll biosynthesis leads to the activation of amino acid (particularly Thea and Glu) biosynthesis. The three albino mutant tea cultivars having low chlorophyll levels may have been due to abnormal chlorophyll biosynthesis. Tanaka et al. (2007) also indicated that plants grown under strong light have higher chlorophyll a/b ratio, photosystem II/I ratio, and adenosine triphosphate synthase and Rubisco activities but lower chlorophyll and light-harvesting complex contents. This finding is consistent with our results, which demonstrated that the chlorophyll a/b ratio in the shoots of the HAAC tea cultivars except ZH2 was significantly higher than that in the JM1 leaves (Figure 2B). Furthermore, Feng et al. (2014) reported that an increased chlorophyll a/b ratio is usually associated with improved photosynthetic capacity in albino mutant tea cultivars. Therefore, we suggest that the higher chlorophyll a/b ratio of the HAAC tea cultivars modulates their photosynthetic apparatus to compensate for the changes in photosynthetic efficiency.

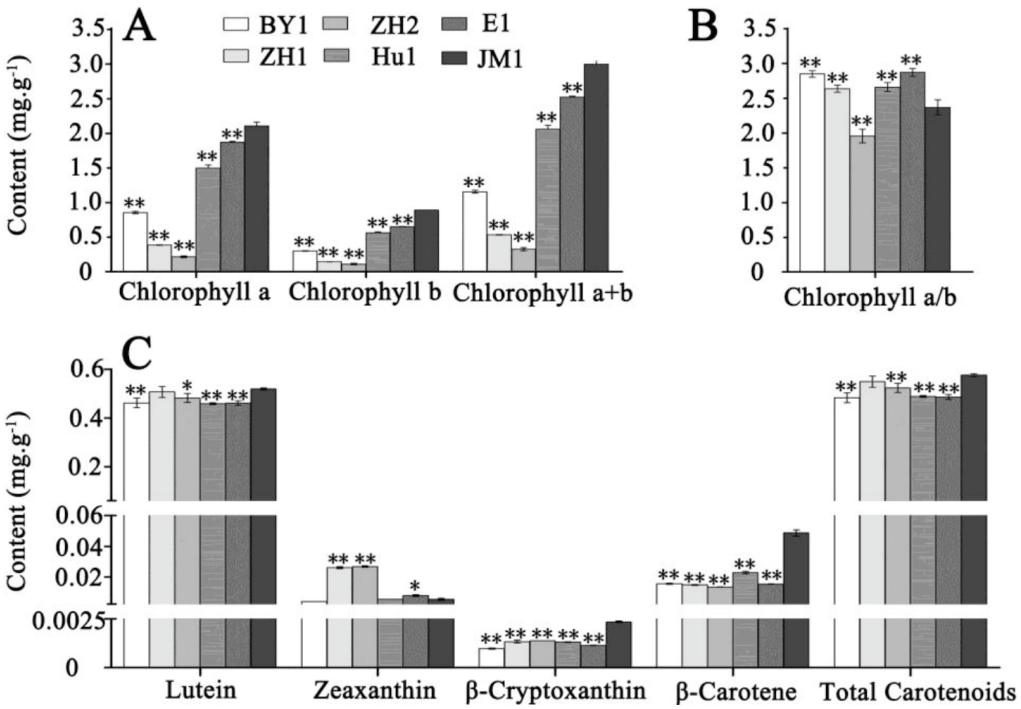

Figure 2. Pigment contents of the HAAC tea cultivars: A, chlorophyll contents; B, chlorophyll a/b ratio; and C, carotenoid contents in all tested tea cultivars. *P < 0.05; **P < 0.01.

Carotenoids are lipid-soluble pigments that are essential components of photosynthetic apparatus. The carotenoid contents of the five HAAC tea cultivars were compared with those of JM1. In total, four carotenoids were detected in the shoots of all tea plants. Lutein was the most abundant carotenoid, followed by β-carotene, zeaxanthin, and β-cryptoxanthin (Figure 2C). The five HAAC tea cultivars had significantly lower β-carotene, lutein, and β-cryptoxanthin levels as well as total carotenoid levels than JM1. Evidence has revealed that nitrogen assimilation into amino acids is controlled by light and metabolism at the molecular level (Lam et al., 1996). Feng et al. (2014) demonstrated that the inhibition of carotenoid biosynthesis in albino leaves promotes amino acid biosynthesis. This is consistent with our results that a lower carotenoid content promoted amino acid biosynthesis in the HAAC tea cultivars. Notably, the zeaxanthin content of the two yellow leaf tea cultivars ZH1 and ZH2 was significantly higher than that of JM1; however, no significant differences were observed in the other cultivars (Figure 2C). Zeaxanthin accumulation can be triggered by adverse environmental conditions, such as excess light, desiccation, and low temperature, leading to overexcitation of the photosystem through the violaxanthin cycle (Fernández-Marín et al., 2011). Therefore, we suggest that zeaxanthin biosynthesis is crucial for maintaining photosynthetic efficiency and capacity in yellow leaf HAAC tea cultivars.

3.3. Differences in the catechin and caffeine contents of the HAAC tea cultivars

Catechins are the primary astringent substances in tea and are an important determinant of tea quality and taste (Narukawa et al., 2010; Liu et al., 2015). Catechins have multiple effects on human health and play crucial antibacterial, antiviral, antiradiation, antiaging, and anticancer roles (Higdon et al., 2002). Eight catechins were identified and quantified using a typical HPLC system. The total catechin content varied from 97.82 to 140.76 mg g^{-1} dry weight in the shoots of the HAAC tea cultivars, which was significantly lower than that in JM1 (Figure 3A). The concentrations of individual catechins were compared (Figures 3A and 3B); EGCG was the most abundant catechin, accounting for approximately 50% of all catechins. The EGCG content was lower in the HAAC tea cultivars than in JM1, except for Hu1. Hu1 had the lowest C content among the examined tea cultivars, and that of ZH2 was approximately twice that of JM1. However, the other tea cultivars did not exhibit marked differences compared with JM1. The EC and ECG contents of the HAAC tea cultivars were significantly lower than those of JM1, whereas the contents of GA (a precursor of catechin synthesis), CG, GC, and GCG were nearly undetectable, without any significant differences between the shoots of the normal and HAAC tea cultivars. The EGC content was also significantly lower in ZH1, ZH2, and E1 but

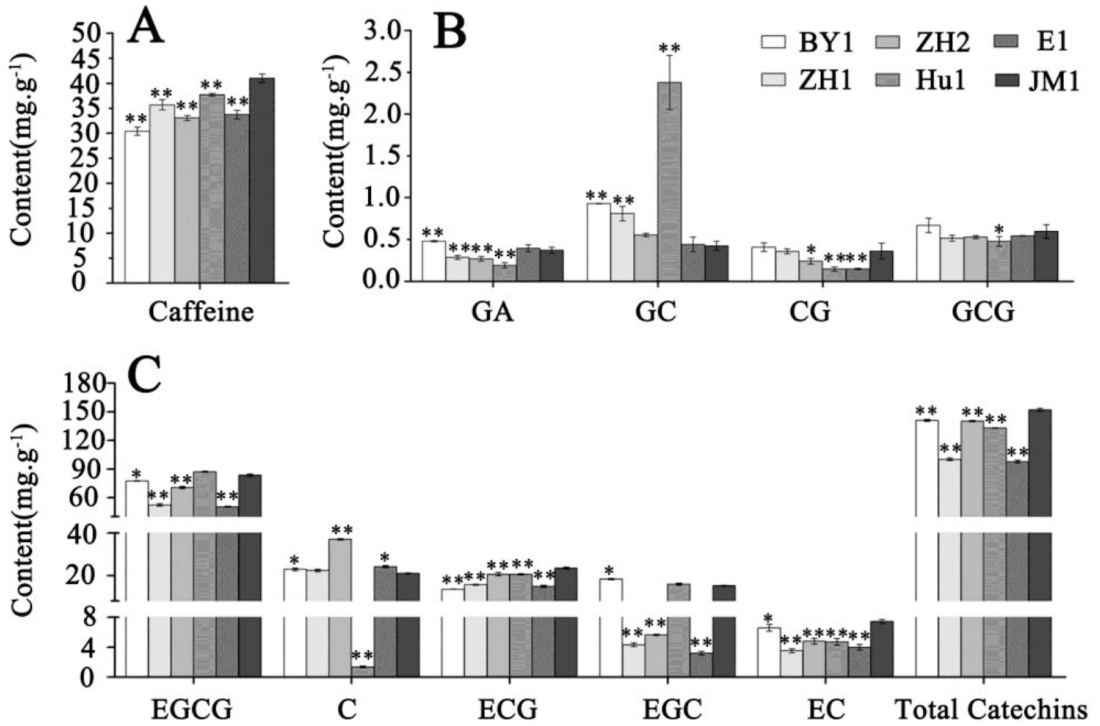

Figure 3. Contents of catechins (A, B) and caffeine (C) in different tea cultivars. *P < 0.05; **P < 0.01.

was not substantially different in the other tea cultivars compared with that in JM1. Caffeine is the main factor associated with the bitterness of tea infusions (Narukawa et al., 2010). The HAAC tea cultivars had a significantly lower caffeine concentration than JM1 (Figure 3C). Xiong et al. (2013) indicated that catechin and caffeine contents change in an opposite pattern to amino acid content. Feng et al. (2014) suggested that the inhibition of catechin and caffeine biosyntheses in albino leaves promotes amino acid biosynthesis. The present results thus suggest that lower catechin (particularly epicatechins) and caffeine contents promote amino acid biosynthesis in the shoots of HAAC tea cultivars and that HAAC tea cultivars are more suitable for producing quality tea with less astringency and bitterness.

3.4. Differential metabolites of the five HAAC tea cultivars and JM1 based on PCA and OPLS-DA

PCA and OPLS-DA are the basis for metabolomic analysis, which provides a summary or overview of all samples. In addition, subtle changes, groupings, trends, and outliers can be identified using these supervised analysis tools (Trygg et al., 2007). To determine the differential metabolites in the five HAAC tea cultivars and JM1, PCA and OPLS-DA score plots were constructed on the integrated HPLC datasets of different tea cultivars. The PCA analysis results (Figure 4A) reveal that the samples from the five HAAC tea cultivars are clearly separated from that of the normal tea cultivar JM1, indicating a marked difference between the metabolic profiles of each of the five HAAC tea cultivars and JM1. An overlap was identified among the three albino tea cultivars, suggesting that parts of their metabolic profiles are similar. In addition, the two normal green leaf HAAC tea cultivars had different metabolic profiles. In the OPLS-DA models, the results of clustering of different tea cultivars were similar to the PCA analysis (Supplementary Figure). A broad range of metabolites with varying influences on class separation were assigned (Figure 4B). ECs, carotenoids, caffeine, and chlorophylls were the most essential contributors to the variance between the cultivars. A metabolite with VIP > 1 can be considered a significant metabolite for the separation of sample groups within the OPLS-DA models (Szeto et al., 2010). A list of the metabolites with VIP > 1 was generated through OPLS-DA (Supplementary Table), and the order of these metabolites (from the most abundant) was as follows: EGCG, Ser, ECG, ethylamine, EC, Ala, EGC, Pro, His, β-cryptoxanthin, Phe, β-carotene, Thr, and Glu. These compounds represent the data points with the highest contribution to cluster formation within the metabolite profiles. The metabolic pathways of catechins (particularly

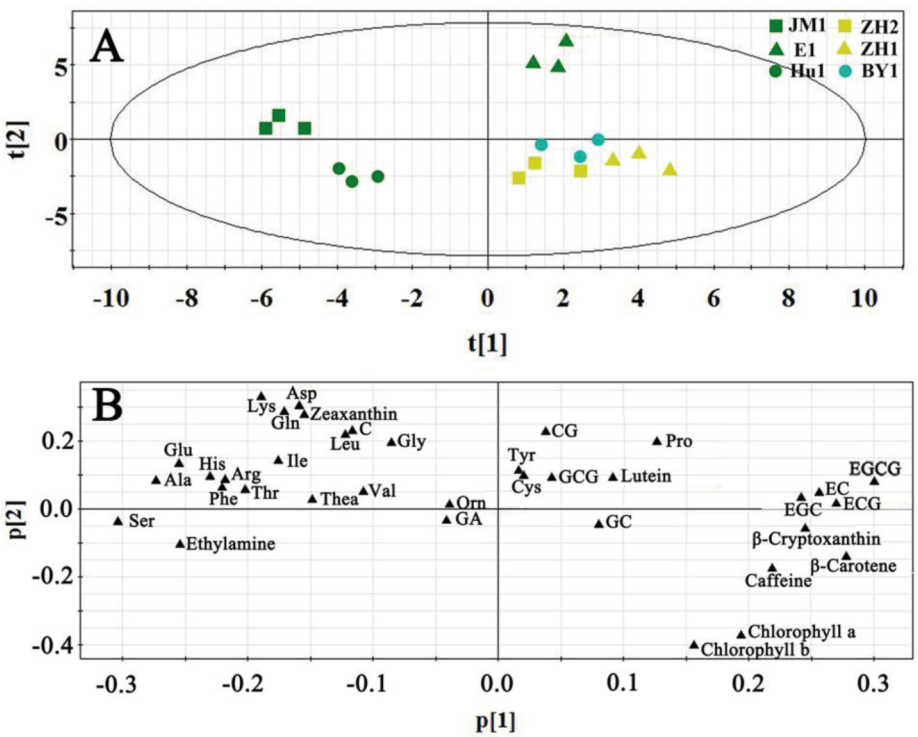

Figure 4. PCA score plot (A) and corresponding OPLS-DA loading plot (B) derived from the integrated HPLC datasets of the HAAC and normal tea cultivars.

ECs), carotenoids, caffeine, chlorophylls, and amino acids (particularly Thea) were disturbed in the five HAAC tea cultivars compared with JM1.

3.5. Differentiation of cultivars induces metabolic reprogramming that involves multiple metabolic pathways

Metabolic differences were observed in the five HAAC tea cultivars compared with JM1. Figure 5 displays the related metabolic pathways. The heat maps illustrate the levels of the differential metabolites in the five HAAC tea cultivars compared with JM1 (Figure 5). In our study, most amino acids were present in higher concentrations in the HAAC tea cultivars compared with JM1. Of these amino acids, Thea was the most abundant free amino acid, and Glu and ethylamine, as essential factors for the synthesis of Thea precursors, were also significantly more abundant in the HAAC tea cultivars than in JM1. Furthermore, the Gln content was markedly higher in the three albino mutant tea leaf cultivars but lower in the two normal green leaf HAAC tea cultivars. The present results indicate that the higher Gln content was due to inhibition of chlorophyll biosynthesis in the three albino mutant tea leaves, whereas lower Gln content was due to the activation of Thea biosynthesis in the two normal green leaf HAAC tea cultivars. The contents of caffeine and most catechins in the HAAC tea cultivars were significantly lower than those in the normal cultivar; however, Phe, a precursor of catechin synthesis, was significantly more abundant in the HAAC tea cultivars than in JM1. We concluded that the inhibition of catechin biosynthesis leads to a shift toward Phe biosynthesis in the HAAC tea cultivars. Moreover, Xiong et al. (2013) indicated that catechin and caffeine contents change in an opposite direction to amino acid contents. This finding is consistent with our data, which showed that leaves from normal cultivars contain more catechins and caffeine than those from HAAC tea cultivars. Using a cutoff VIP value of 1 in OPLS-DA, ECs play a crucial role in the clustering of cultivars. Feng et al. (2013) indicated that the inhibition of chlorophyll and carotenoid biosyntheses in albino leaves promotes amino acid biosynthesis. In our study, the chlorophyll contents of all five HAAC tea cultivars (particularly the three albino mutant plants) were significantly lower than those of the JM1 leaves. The contents of most carotenoids, including lutein, β-carotene,

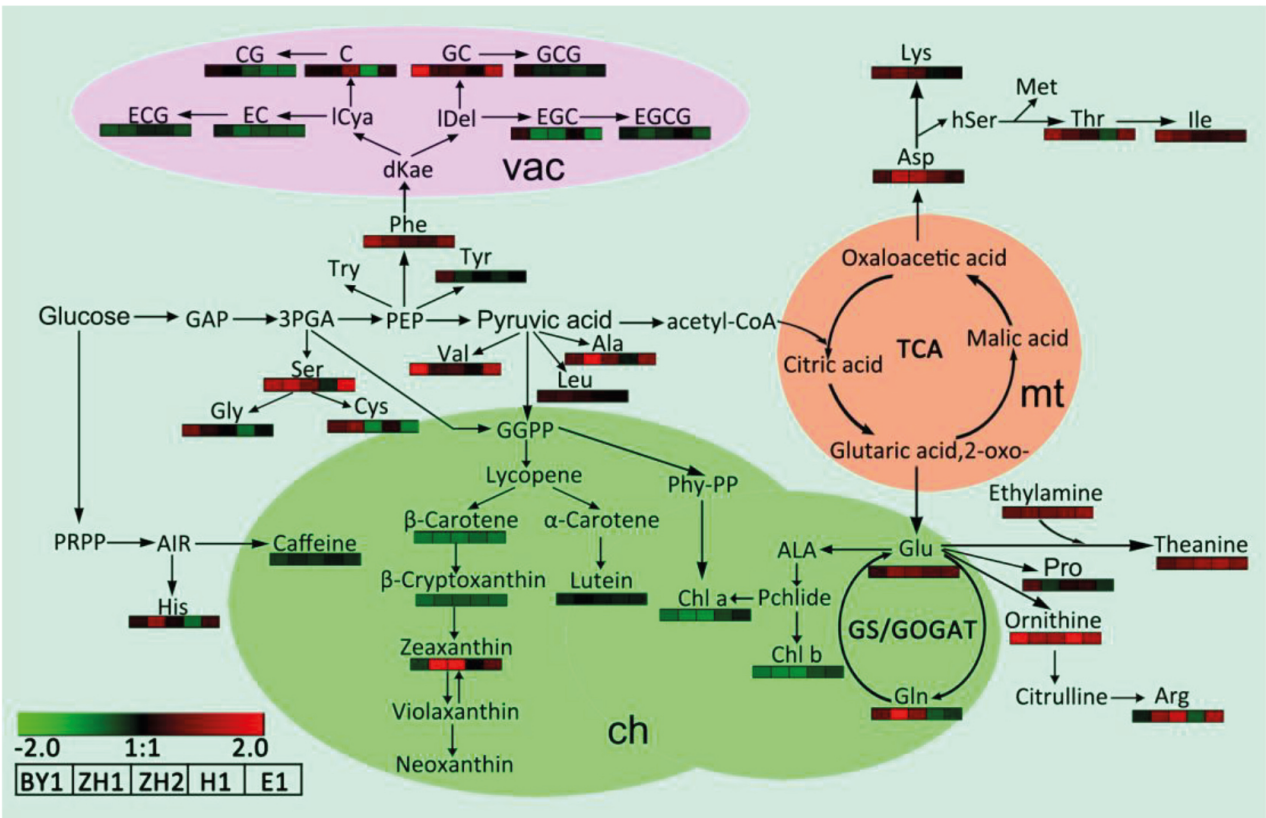

Figure 5. Differential metabolite contents in the shoots of the HAAC tea cultivars. Red and green colors denote the highest and lowest concentrations, respectively. The scale (ranging from −2 to 2) represents the fold difference in these differential metabolites in the five HAAC tea cultivars compared with JM1.

and β-cryptoxanthin, were lower in the five HAAC tea cultivars compared with the normal cultivar. Altogether, our results on the notable differences in metabolic pathways reveal that inhibition of the biosynthesis of catechins (particularly ECs), carotenoids, chlorophylls, and caffeine in the HAAC tea cultivar leaves leads to a shifting of the metabolic network toward amino acid (particularly Thea and Glu) biosynthesis, and the order of influence of the compounds on amino acid biosynthesis (from the strongest influence) was as follows: catechins (particularly ECs), carotenoids, chlorophylls, and caffeine.

4. Conclusion

Green tea products produced from HAAC tea cultivars exhibit the quality characteristics of high-grade green tea, with slight bitterness and astringency, a rich aroma, and a refreshing and velvety taste. The present study systematically analyzed the metabolic profiles of five HAAC tea cultivars and compared them with that of a normal green tea cultivar to investigate the mechanism of amino acid accumulation in HAAC tea cultivars. Our results reveal that quality constituents effectively distinguish between HAAC and normal green leaf tea cultivars. HAAC cultivars are characterized by high amino acid levels, along with the low levels of catechins (particularly ECs), chlorophylls, carotenoids, and caffeine. HAAC tea cultivars with different leaf colors share a common characteristic of strong Thea biosynthesis and enhanced synthesis of Thea precursors. Altogether, we suggest that inhibiting the biosynthesis of catechin, carotenoid, chlorophyll, and caffeine leads to a shift in the metabolic network toward amino acid biosynthesis (particularly Thea biosynthesis). The order of influence of the compounds on amino acid biosynthesis (from the strongest influence) was as follows: catechins (particularly ECs), carotenoids, chlorophylls, and caffeine. The present results improve the current understanding of the metabolic mechanisms involved in HAAC tea cultivars.

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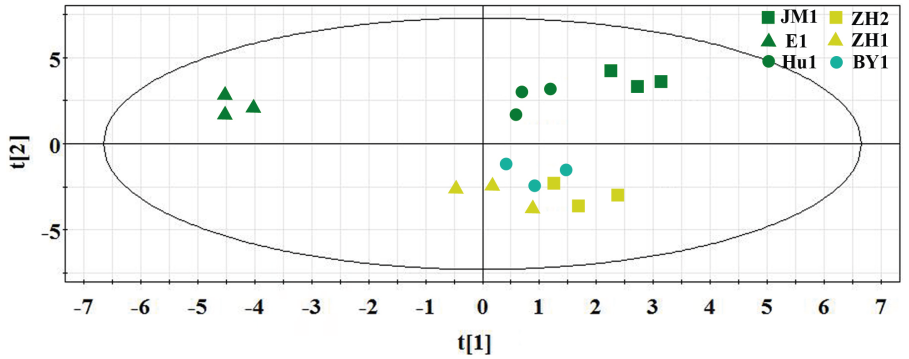

Supplementary Figure. OPLS-DA score plot (A) derived from the integrated HPLC datasets of the HAAC and normal tea cultivars.

Supplementary Table. Compounds with VIP values greater than 1, as determined using OPLS-DA.

No.	Name	VIP
1	EGCG	2.13
2	Ser	1.97
3	ECG	1.79
4	Ethylamine	1.67
5	EC	1.66
6	Ala	1.56
7	EGC	1.49
8	Pro	1.42
9	His	1.29
10	β-Cryptoxanthin	1.20
11	Phe	1.20
12	β-Carotene	1.12
13	Thr	1.07
14	Glu	1.04