Metabolite accumulation and inhibition of hypocotyl elongation by blue light induction in *Amaranthus tricolor* L.

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ABSTRACT: Red amaranth is an important fresh vegetable with valuable nutritional properties. Blue light is favorable for the accumulation of many metabolites and inhibits hypocotyl elongation in plants. However, the relation between metabolite induction and the inhibition of hypocotyl elongation by blue light in red amaranth is unclear. Here, we used LC-MS/MS-based widely targeted metabolomics to identify key compounds and to compare the metabolite composition of red amaranth under dark and blue light conditions. A total of 444 metabolites were identified, 37 of which differed between the two conditions. A pathway enrichment analysis also identified significant differences between the two conditions. Fifteen lipids, all up-regulated under the blue light, were significantly different. The amino acid content was lower under the blue light than under the dark condition. We found cyanidin chloride and anthocyanin 3-O-beta-D-glucoside which are the two components involved in the anthocyanin pathway. Our results verify the high nutritional quality of the red amaranth and suggest that blue light influences metabolite synthesis and hypocotyl elongation in the species.

KEYWORDS: *Amaranthus tricolor* L., blue light, metabolite profiling, hypocotyl elongation, lipids, anthocyanin

INTRODUCTION

Red amaranth (*Amaranthus tricolor* L.), a member of the family Amaranthaceae, originated in South or Southeast Asia and is widely distributed throughout warm and tropical regions worldwide [1,2]. Among leafy vegetables, amaranth is the most commonly cultivated species. Both leaves and stems are edible. *A. tricolor* L. has nutritional value owing to its rich profile of essential metabolites, such as protein, vitamins, phenolics, flavonoids, and alkaloids [3,4]. Fresh amaranth often provides 2 to 3 times the amount of nutrients found in other vegetables. Furthermore, medical compounds found in amaranth have broad applications as a component of complex therapies in traditional medicine in many countries [5,6]. The nutritional value of amaranth leaves is clearly established [7]. In particular, the amino acid profile of amaranth leaves is specifically characterized by its levels of methionine and lysine [8], which are the limiting amino acids in most plants. The amaranth lipids are natural organic compounds that reduce low-density lipoprotein blood cholesterol.

Light is one of the most important and easily controllable environmental factors, and it probably influences plant metabolite profiles [9,10]. For example, light can trigger the biosynthesis and accumulation of secondary metabolites [11]. Amongst various light spectra, blue light and red light have substantial effects on primary and secondary metabolism in plants [12]. However, blue light can be absorbed by cryptochrome in plants, which is the most operative light spectrum for the synthesis of anthocyanin and flavonoid compounds. Single-spectral blue light-emitting diodes increase anthocyanin accumulation by up-regulating the expression of anthocyanin biosynthesis genes in Chinese bayberry fruit [13]. Flavonoids are major blue light-absorbing pigments [14]. A previous report has revealed that blue light is the most effective light for promoting synthesis and accumulation of chlorogenic acid [15]. Compared with dark condition, blue light is favorable for the synthesis of betalains and carotenoids in the amaranth callus. Both metabolite synthesis and hypocotyl elongation in amaranth species were affected under blue light. However, little is known about metabolite synthesis and hypocotyl elongation inhibition by blue light in *A. tricolor* red amaranth.

Recently developed metabolomics technology provides a promising approach to reveal the physiological complexity of plants. Plant metabolomes could demonstrate comprehensive and quantitative arrays of metabolites in biological samples [16–18]. The use of mass spectrometry (MS)-based analytical platforms to identify stress-responsive metabolites associated with adaptation to adverse environmental conditions is fundamental in current plant biotechnology research programs for the development of stress-tolerant plants [19]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based widely targeted metabolomics is a more rapid and reliable approach compared with total scan ESI-based non-targeted metabolomics for the detection of a wide range of plant metabolites [20]. Thus, it may contribute to studies of plant systems at the molecular level, providing unbiased characteristics of total metabolite pools in plant tissues in response to environmental factors [21,22]. In this study, we used a widely targeted metabolomics approach to identify
relevant compounds in red amaranth hypocotyls and compare the metabolite compositions under blue light and dark conditions. Our results provide basic data for the future development and utilization of amaranth.

MATERIALS AND METHODS

Plant materials

*A. tricolor* ‘whole red’ amaranth seeds were placed on plastic Petri dishes covered with three layers of wet filter paper. Seed germination was carried out under a dark condition at 25 ± 2 °C. After 5 days, half of the sample was transferred to blue light, and the other half was maintained under the dark condition for 2 days. Then, the amaranth hypocotyls were cut off for metabolite analyses.

Sample preparation and metabolite extraction

Fresh hypocotyls (1 g) were collected from amaranth seedlings and pooled to create a single biological sample. Three biological repeats were created for each treatment. All samples were stored at −80 °C until metabolite extraction. Frozen samples were freeze-dried under a vacuum and crushed using a mixer mill (MM 400; Retsch, Shanghai, China) with zirconia beads for 1.5 min at 30 Hz. Then, 100 mg of the powder sample was precisely weighed, and the extraction was done overnight at 4 °C with 1.0 ml of 70% aqueous methanol containing 0.1 mg/l lidocaine as an internal standard. Following centrifugation at 10,000×g for 10 min, the supernatant was absorbed and filtered (SCAA-104, 0.22-μm pore size; ANPEI, Shanghai, China) before LC-MS/MS.

Quality control measurements

Quality control (QC) samples were prepared by mixing equal volumes of the blue light and the dark condition samples to ensure the reproducibility of the mass spectrometric results. One in ten samples was included in the QC analysis.

ESI-Q TRAP-MS/MS analysis

Extracted compounds were analyzed using an LC-ESI-MS/MS system (UPLC, Shim-pack UFLC SHIMADZU CBM30A, http://www.shimadzu.com.cn/; MS/MS, Applied Biosystems 6500 QTRAP, http://www.appliedbiosystems.com.cn/). Briefly, 2 μl of sample extract was injected onto a Waters ACQUITY UPLC HSS T3 18 column (2.1 mm × 100 mm, 1.8 μm) operating at 40 °C and a flow rate of 0.4 ml/min. The mobile phases were acidified water (0.04% acetic acid) (Phase A) and acidified acetonitrile (0.04% acetic acid) (Phase B). Compounds were separated using the following gradient: 95:5 Phase A/Phase B at 0 min; 5:95 Phase A/Phase B at 11.0 min; 5:95 Phase A/Phase B at 12.0 min; 95:5 Phase A/Phase B at 12.1 min; and 95:5 Phase A/Phase B at 15.0 min.

The effluent was connected to an ESI-triple quadrupole-linear ion trap (Q TRAP)-MS. Linear ion trap (LIT) and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (Q TRAP), AB Sciex QTRAP6500 System, equipped with an ESI-Turbo Ion-Spray interface, operating in a positive ion mode and controlled by Analyst 1.6.1 (AB Sciex, Singapore). The operation parameters were as follows: ESI source temperature 500 °C; ion spray voltage (IS) 5500 V; curtain gas (CUR) 25 psi; and collision-activated dissociation (CAD) set to highest. QQQ scans were acquired as MRM experiments with optimized declustering potential (DP) and collision energy (CE) for individual MRM transitions. The m/z range was set to 50–1000.

Data evaluation and metabolite identification

MS data acquisition and processing were performed as described previously [20]. The original data were pre-processed (noise filtering, peak matching, and peak extraction) and then corrected.

Metabolites were identified by searching against the self-built database MWDB (metware database, Metware Biotechnology Co., Ltd., Wuhan, China) and the public databases MassBank (http://www.massbank.jp), KNAPSAck (http://kanaya.naist.jp/KNAPSAck), HMDB (http://www.hmdb.ca/), MoToDB (http://www.ab.wur.nl/moto/), and METLIN (http://metlin.scripps.edu/index.php). The primary and secondary spectral data were qualitatively analyzed using Analyst 1.6.1.

Multivariate statistical analysis

Unsupervised dimensionality reduction was performed by principal component analysis (PCA) for all samples using R v3.5.0 (http://www.r-project.org/). A supervised multiple regression orthogonal projection to latent structures-discriminant analysis, or orthogonal partial least-squares discriminant analysis (OPLS-DA), was performed using rplsp v1.19.8 in R (http://www.r-project.org/). The OPLS-DA model was further validated by cross-validation and a permutation test (n = 200).

Differential metabolite analysis

Metabolites with significant differences were selected according to the variable importance in projection (VIP ≥ 0.8) from the OPLS-DA model and fold change (FC ≥ 1.6 or ≤ 0.625) between the blue light and the dark condition samples. To assess the significance of differences in metabolite abundance, a Student’s t-test (two-tailed) was used (p = 0.05). Annotated metabolites were mapped to Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways for a pathway analysis and enrichment analysis. Pathways with Bonferroni corrected p-values of ≤ 0.05 were
To eliminate the effects of quantity on pattern recognition, we performed a hierarchical cluster analysis. This analysis showed two distinct groups associated with the blue light and the dark conditions (Fig. 2C). Thus, the combination of PCA and a clustering analysis revealed distinct metabolite profiles between the 'blue' and the 'dark' samples.

The differential metabolites were screened by combining the VIP values and the fold change. We selected and compared metabolites under the dark and the blue light conditions with a fold change of $\geq 1.6$ (upregulated) or $\leq 0.625$ (downregulated). These metabolites were screened using VIP $\geq 0.8$ from the OPLS-DA model. In total, 37 differential metabolites were identified between the two samples (Fig. 3A and Table S1). Of these, 12 metabolites were downregulated, and 25 were upregulated in the 'blue' samples compared with the 'dark' samples (Fig. 3A). The 37 metabolites can be categorized as lipids, amino acids and derivatives, phenolic acids, organic acids, alkaloids, and nucleotides and derivatives (Fig. 3B and Table S1).

To eliminate the effects of quantity on pattern recognition, we applied a log10 transformation of peak areas for each relative differential metabolite and performed a hierarchical cluster analysis (Fig. 4).

**KEGG classification and enrichment analysis of differential metabolites**

We mapped the 187 metabolites to the KEGG database. Most of the metabolites were mapped to 'Metabolism', as expected. A few metabolites were involved in 'Environmental Information Processing', 'human diseases', or 'Genetic Information Processing', suggesting that they might have health effects and confer resistance (Fig. S1). In particular, we found cyanidin chloride and anthocyanin 3-O-beta-D-glucoside which are the two components involved in the anthocyanin pathway (Table S1).

Subsequently, we conducted a KEGG pathway enrichment analysis to identify differences in metabolic pathways between the two samples. The enrichment analysis showed that metabolites were mainly involved in pathways related to lipids, amino acids, and the biosynthesis of other secondary metabolites (Fig. S2). The top 20 KEGG enrichment pathways were involved in the metabolism of amino acids, lipids, and so on (Fig. S3).

**DISCUSSION**

**Amaranth is an important medicinal plant and vegetable**

MS-based analytical platforms for the profiling of stress-responsive metabolites allowing plants to adapt to adverse environmental conditions are fundamental in current plant biotechnology research programs for the characterization and development of stress-resistant crop varieties. A combination of metabolomics and MS analysis provides a powerful tool for understanding the molecular mechanisms underlying stress responses.

Fig. 1 Circular diagram of all metabolic components in *A. tricolor*. Considering significantly enriched. The calculated p-value was subjected to FDR correction, taking FDR $\leq 0.05$ as a threshold. Pathways meeting this condition were defined as significantly enriched pathways for differential metabolites.

**RESULTS**

**Widely targeted metabolome analysis**

To better understand differences of components of the 'blue' and the 'dark' samples, we performed widely targeted LC-MS/MS-based metabolite profiling. In total, 444 metabolites were identified, including 84 flavonoids, 74 lipids, 73 amino acids and derivatives, 54 phenolic acids, 37 alkaloids, 36 nucleotides and derivatives, 33 organic acids, 3 lignans and coumarins, 2 tannins, 1 terpene, and 47 other metabolites (Fig. 1 and Table S1).

**Multivariate analysis of identified metabolites**

The 444 metabolites were evaluated by PCA. Two principal components, PC1 and PC2, were extracted, and 37.8% and 18% of the variance were explained, respectively. The metabolites could clearly separate the two samples and the QC samples (Fig. 2A), suggesting that there were significant differences in metabolic phenotypes between the 'blue' and the 'dark' samples.

The OPLS-DA model based on the entire metabolite contents of the core samples in pairs to evaluate the differences between the blue light and the dark conditions ($R^2 X = 0.791$, $R^2 Y = 0.996$, $Q^2 Y = 0.97$; Fig. 2B).

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tolerant plants [19]. Plant metabolomes could generate comprehensive and quantitative arrays of metabolites in biological samples [16–18].

Previous studies of metabolites in amaranth have focused on specific classes of metabolites, such as betalains (betacyanins and betaxanthin), phenolic acids, flavonoids, and alkaloids. These compounds have been isolated in various fresh plant tissues obtained at different developmental stages and/or under diverse environmental conditions [23–25]. However, the metabolic profiles of A. tricolor have not been thoroughly investigated. We performed LC-MS/MS-based widely targeted metabolomics to understand hypocotyls under the blue light and the dark conditions. We identified 444 metabolites, 37 of which showed differential accumulation in the ‘blue’ and the ‘dark’ samples. Thus, our results provide novel evidence for the broad application of amaranth in traditional medicine.

Flavonoids are among the most effective pharma-

Fig. 2 Differential fruit chemotype between the ‘Dark’ (dark condition) and the ‘Blue’ (blue light) samples. (A) PCA analysis of metabolites identified from ‘Dark’ and ‘Blue’. Equal volumes of the ‘Dark’ and the ‘Blue’ samples were mixed for use as a quality control (QC). (B) OPLS-DA score plot; and (C) Cluster analysis of metabolites from samples of ‘Dark’ and ‘Blue’. The color indicates the level of accumulation of each metabolite, from low (green) to high (red).
The compositions and abundance of amino acids are key indicators of nutritional quality. A protein content of 12–38% DM has been reported in the leaves of amaranths [27, 28]. The composition of amino acids in amaranths was similar to that of animals, with an extraordinarily high lysine content, which was 2 or 3 times higher than those in wheat or maize, respectively [29]. Previous studies have shown that *A. spinosus* contains 18 kinds of amino acids, 8 of which are essential amino acids [30]. Our widely targeted metabolomic analysis identified 73 amino acids and their derivatives, including the 18 kinds of amino acids (Table S1). This result verifies the high nutritional quality of amaranths as a very promising vegetable crop.

Lipids are natural organic compounds. The chemical and lipid compositions of amaranth seeds and oils have been studied. The oil content of amaranth seeds is predominantly unsaturated fatty acids, mainly oleic and linoleic acid [31, 32]. Furthermore, the lipid fraction contains many biologically active substances, such as tocopherols, sterols, and phospholipids, which make the amaranth oil a valuable source of useful compounds for human health [31]. Previous studies have focused on seeds; however, we performed a widely targeted metabolomic analysis of the amaranth hypocotyls. We identified 74 lipids, and 15 of which were significantly upregulated under the blue light condition.

**Loss of late-acting enzymes might result in a loss of anthocyanins in amaranth**

Anthocyanins are the most important flavonoids. However, they cannot coexist with betalains in the same plant [33, 34]. The transcriptional downregulation of late-acting enzymes might result in a loss of anthocyanins based on a comparative genetic study [33]. Compared to the other plants with anthocyanins, *Mirabilis jalapa* L. is lack of anthocyanins because...
part of the anthocyanidin synthase (ANS) gene was deleted [35]. Although we found cyanidin chloride and anthocyanin 3-O-beta-D-glucoside which are the two components involved in the anthocyanin pathway. Hence, it is possible that the loss of late-acting enzymes results in a loss of anthocyanins in amaranth.

Blue light simultaneously affected metabolite accumulation and hypocotyl elongation

Light is one of the most important and easily controllable environment factors and is a key determinant of various metabolites and hypocotyl elongation in plants [9, 10, 36]. Among various light spectra, blue light can be absorbed by plant cryptochromes, which are photolyase-like blue-light receptors. Blue light treatment can significantly increase the expression of structural genes in plant metabolic pathways, resulting in the synthesis of secondary metabolites. In longan embryogenic calli, blue light regulates the expression of HY5, PIF4, and MYC2, thereby promoting the accumulation of flavonoids and anthocyanins in blue light and the dark conditions. A pathway enrichment analysis also identified significant differences between the two conditions. Lipid metabolism plays an important role in hypocotyl elongation and metabolite synthesis in amaranth. Our results provide a novel reference for studies of the metabolism and hypocotyl elongation of amaranth under blue light and dark conditions.

CONCLUSION

A total of 444 metabolites were identified in amaranth, and 37 of which (including lipids, amino acids, and flavonoids) differed between the two samples under the blue light and the dark conditions. A pathway enrichment analysis also identified significant differences between the two conditions. Lipid metabolism plays an important role in hypocotyl elongation and metabolite synthesis in amaranth. The amino acid content was lowered. We found cyanidin chloride and anthocyanin 3-O-beta-D-glucoside which are the two components involved in the anthocyanin pathway. Our results verify the high nutritional quality of amaranths, as a very promising vegetable crop, and suggest that blue light influences both metabolite synthesis and hypocotyl elongation in the red amaranth.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2022.044. Table S1 is available from the authors upon request.

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REFERENCES

Appendix A. Supplementary data

Fig. S1 KEGG classification of all metabolites.

Fig. S2 KEGG classification of differential metabolites.
Fig. S3  Top 20 of KEGG enrichment.