Quality analysis and metabolomic profiling of the effects of exogenous abscisic acid on rabbiteye blueberry

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Blueberry, Anthocyanins, Metabolomics, ABA.
Abstract

**Background:** Blueberry is well known for its high content of bioactive metabolites, including anthocyanins, flavonols, and phenolic acids. This study aims to systematically study the effect of exogenous ABA application on ripening fruits in fruit growth, quality and anthocyanins content. The ripening process of blueberry fruit is divided into six stages for further analysis.

**Results:** In this article, nontarget metabolomics were performed to demonstrate the effect on metabolites level. Basic results showed that 1000 mg/ml ABA significantly promoted fruit ripening, accelerated fruit colouration and increased soluble solid and anthocyanins content. At the same time, the fruit firmness and the content of some bioactive metabolites were also reduced. In metabolites level, a total of 1145 metabolites and 575 metabolites were detected and annotated in the positive model and the negative model, respectively. Principal component analysis and endogenous ABA content shows the exogenous ABA application is mainly concentrated on the green fruit turning into red fruit, after which the effect begins to decrease. The important metabolites of flavonoid pathway were detected and the result shows that anthocyanins synthesis was increased and parts of other bioactive metabolites were decreased.

**Conclusion:** After comprehensive assessments, we believe that 1000 mg/ml exogenous ABA will have positive impacts on blueberry fruit quality and economic benefits. This finding provides a reference for different cultivation methods to improve the anthocyanin content of blueberry. Also, it provides a good research foundation for the next step of precise breeding for specific active compound of blueberry.

**Background**

Blueberry is the common name of plants with purple- or blue-coloured berries\(^1\). Cultivated blueberry belongs to the genus Vaccinium, mainly including northern highbush blueberry plants (Vaccinium corymbosum L.), southern highbush blueberry (primarily Vaccinium corymbosum L.), lowbush blueberry (Vaccinium angustifolium Aiton) and rabbiteye blueberry (Vaccinium ashei Reade)\(^2\).

During past years, increasingly clinical and animal model studies show blueberries and blueberries extracts have positive effects on the development of certain cancer and obesity complications\(^3,4\),
such as chronic inflammation\textsuperscript{5}, type 2 diabetes and cardiovascular disease\textsuperscript{6,7}.

Blueberry is rich in dietary fibres, vitamins, minerals and bioactive properties (including ascorbic acid, flavonols, hydroxycinnamic acids, pterostilbene, resveratrol, and anthocyanins) \textsuperscript{8,9}. Among them, anthocyanins play a major antioxidant role and are responsible for about 84\% of antioxidant capability\textsuperscript{10}. Blueberries have high content anthocyanins and contain up 27 different anthocyanins\textsuperscript{11}. The most common anthocyanidin aglycones are peonidins, pelargonidins, malvidins, delphinidins, cyanidins and petunidins\textsuperscript{12}. Purified blueberry anthocyanins alter development of obesity in mice fed an obesogenic high-fat diet\textsuperscript{13,14}.

In nonclimacteric fruits, abscisic acid (ABA), but not ethylene, which plays key role in fruits ripening in climacteric fruits such as apple, banana, and tomato, is recognized as an major regulator hormone in fruits ripening\textsuperscript{15}. Exogenous ABA were reported to effect ripening of non-climacteric fruits in field experiments, such as grape and strawberry\textsuperscript{16–19}. Influenced by exogenous ABA, the coloration, firmness and anthocyanins content of non-climacteric fruits were changed\textsuperscript{20,21}. The mechanism of exogenous ABA on grape berry ripening at 22 and 44 hours was systematic illustrated by RNA-seq and many transcription factors were verified\textsuperscript{15}. As a kind of non-climacteric fruit, blueberries start to accumulate anthocyains from ripening stage\textsuperscript{22}. Compared to the researches of exogenous ABA on grape and strawberry, there are few researches on blueberries. The fruits of southern highbush blueberry were treated with ABA at 200 ppm and 400 ppm at green mature period and the fruits and the firmness of berries were found to be increasing, suggesting a ripening delay effect\textsuperscript{23}. Northern highbush blueberry with 1000 mg/L ABA application were found that the coloration was accelerating and the anthocyanins content was increasing from green mature period to red period (12 days)\textsuperscript{24}.

Plant metabolites play essential roles for plants during growth, development and environmental stress. To adjust to different conditions, plants can change metabolites in response to environmental signal through hormone pathways\textsuperscript{25}. With the deepening understanding of the function, biosynthesis and metabolic mechanism of plant metabolites, metabolomics has been shown to be a powerful tool
to understand how plants develop and respond to environment at metabolites level\textsuperscript{26}. Recently, the metabolic levels changes of chickpea (Cicer arietinum) under contrasting water regimes were demonstrated by nontargeted global ultrahigh-performance liquid chromatography/high-resolution mass spectrometry (UPLC-HRMS)\textsuperscript{27}; The effects of silver nanoparticles on cucumber (Cucumis sativus) were evaluated by gas chromatography-mass spectrometry (GC-MS) based nontarget metabolomics\textsuperscript{28}.

In this study, the fruits of rabbiteye blueberry ‘Brightwell’ were applied with 0, 500, 1000 mg/ml exogenous ABA in green mature period (Veraison). The main measured parameters included fruit skins color, fruit firmness, soluble solid content and total anthocyanins content. The 1000 mg/ml ABA treatment was more significant than 500 mg/ml ABA treatment in the four parameters above. Ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) was used to analyze the metabolites changes between 0 and 1000 mg/ml exogenous ABA treatment in six stages. By the comparison of metabolites changes, we thoroughly analyzed the effects of exogenous ABA on blueberry fruits from green mature period to purple mature period. This study provides a new insight to the application of exogenous ABA on ripening non-climacteric fruits in the metabolites level.

Results

Developmental stages and effects on maturity

The developmental stage in cv ‘Brightwell’ from ripening initiation to mature is divided into six stages (Fig 1a) by fruit color (Stage 1, the whole fruit is green; Stage 2, the top turn red; Stage 3, the side turn red; Stage 4, the whole fruit is red; Stage 5, the whole fruit turn purple; Stage 6, the whole fruit is purple) and size (Table 1). Fruit set and ripening initiation in ‘Brightwell’ is asynchronous and former researches found that exogenous ABA in ripening stage has no significant effects on the size of blueberry\textsuperscript{23,24}. Therefore, the fruits under different treatments were collected by developmental stages. The proportion of fruits in S6 (maturity) were counted in 20 days and 25 days after treatment (Fig 1b). Compared to 0 and 500mg/ml ABA treatment, fruits under 1000 mg/ml ABA treatment shows a higher percentage of maturity. In 25 days, there are about 49.7% fruits in S6 under 1000 mg/ml
ABA treatment and about 31% fruits in S6 under 0 mg/ml ABA treatment.

**Fruit colouration and total anthocyanins content**

Application of 1000 mg/ml ABA resulted a significant acceleration of fruit colouration (Fig 2a). From S3 to S4, a* value reflects the top, side and bottom of fruits of 1000 mg/ml ABA treatment turns red earlier than 500 and 0 mg/ml ABA treatment. In S5, the top and bottom turns green earlier. From S5 to S6, the b* value reflects the side and bottom of 1000 mg/ml ABA treatment turns blue earlier than 500 and 0 ABA group. From value of a* and b*, there are no notable difference in three groups in S6. Anthocyanins accumulation is consistent with the change of fruit colouration(Fig 3b). In S5 and S6, total anthocyanins content of 1000 mg/ml ABA treatment is obviously higher than the 0 and 500 mg/ml treatment. From S5 to S6, the anthocyanins content of 0 mg/ml treatment group is nearly doubled, while that of 1000 mg/ml treatment group increased by 13.89%.

**Fruit hardness, Brix and pulp cells**

Fruit hardness were accessed at 6 developmental satges. Compared to 0 and 500mg/ml ABA treatment, fruits hardness of 1000 mg/ml ABA treatment declined more sharply from S4 (Fig S1) and become softer in S6 (Fig 3a). Consist with fruit hardness, fruits of 1000 mg/ml ABA treatment contains more soluble solid in S6(Fig 3b). The pulp cells in S6 between 0 and 1000 mg/ml ABA treatment were chosen to do further observation(Fig 3c). The pulp tissues shows more fragments and the pulp cells become smaller in the 1000 mg/ml ABA treatment.

**Metabolite profiling**

The 0 and 1000 mg/ml ABA treatment were selected to do nonbiased global metabolomics(nontarget metabolomic) based on UHPLC-MS/MS. Quality control (QC) shows the quality of metabolomics is stable and adequate (Fig S2). A total of 1145 metabolites and 575 metabolites were detected and annotated in the positive model(Table S1) and the negative model (Table S2), respectively. Principal component analysis (PCA) (Fig 4) and Partial Least Squares Discrimination Analysis(PLS-DA) (Fig S3) were performed to systematically profile metabolic variances between two groups. Scores plot of samples are well separated in S1, S2 and S3, suggesting a clear distinction in the metabolite accumulation. On the contrary, the scores plot in S4, S5 and S6 are intersected in different degrees.
Metabolites were annotated by KEGG (Fig 5a) and Lipdmaps(Fig 5b). In Lipdmaps annotation, there are 33 metabolites in positive model and 10 metabolites in negative model involved in flavonoid metabolism. The differentially expressed metabolites in 6 stages were screened out (Fig 5c) according to the criteria of Fold change (FC) < 0.83 or FC > 1.2 and P < 0.05 and listed in tables (Pos, Table S3; Neg, Table S4). Top 10 metabolites of up-regulation and down-regulation in fold were separated shown in table 2 and table 3. Two compounds are noteworthy: gallic acid are strikingly up regulated from S1 to S3 and chlorogenic acid has declined significantly and continuously from S1 to S6.

**ABA concentration and stress-resistance**

The ABA content in the control group continued to increase from S1 to S4, but began to decrease from S4 to S5, and then increased from S5 to S6 (Fig 5a). The mean values of ABA content in ABA group are higher than control group at 1-3 stage, but the lower than control group at 4-6 stage (Fig 5b). Proline is a crucial osmotic adjustment in plants and will be accumulated to a higher lever when plants are in environmental stress. Trehalose-6-Phosphate serves as a signaling molecule which is negative correlation with the response of plants to conditions that result in starvation. At stage 1 to 3, the proline content of 1000 mg/ml group is significantly higher than control group and the trehalose-6-Phosphate of 1000 mg/ml group is significantly lower than control group.

**Flavonoid pathway**

For flavonoid pathway, 1000 mg/ml group differs from control group in the important metabolites level and the differences were found and annotated by KEGG (Fig 6; Fig S6). In addition, the pathway combines with the RNA-seq data of pre-véraison grapes with exogenous ABA at 44h to show the change of vital enzymes. The red anthocyanins cyaninidin are consist with the colouration change and its content increased more than control group from S3. Similarly, the blue anthocyanins petunidin-3-glucoside increased more than control group when the blueberry fruits turn purple. Quercetin, catechin and epicatechin are downstream metabolites of flavonoid 3'-hydroxylase (F3’H), showing a persistent lower content from S1 to S6. Myricetin and epigallocatechin are downstream metabolites of flavonoid flavonoid-3’,5’-hydroxylase (F3’5’H) and lower than control group in S5 and S3, respectively.
Bioactive metabolites and antioxidant capability

Due to changes in the flavonoid metabolic pathway, other bioactive metabolites already found in blueberries were further investigated. The representative metabolites among catechins, flavonols, tannins and phenolic acids in mature fruits were shown (Fig 7). Overall, most of their content value has been reduced more or less under the 1000 mg/ml ABA treatment. Two metabolites shown in table 2 and 3 were noteworthy: gallic acid which is higher at 1-3 stage back to normal level in the ripe fruit, but the chlorogenic acid are still at low level in the ripe fruit. Because of the changes in bioactive metabolites, we further studied the antioxidant capability of fruits. The reducing ability of 1000 mg/ml ABA treatment was higher than the 0 mg/ml treatment (Fig 8a). In addition the DPPH radical scavenging activity (Fig 8b) assay also shows the same result.

Discussion

As a nutritious fruit, blueberries are rich and famous in antioxidant anthocyanins and bioactive metabolites\textsuperscript{8,10}. The aim of this study is to find and illustrate a conventional method to increase the anthocyanins content of blueberries. Former researches on non-climacteric fruits found that exogenous ABA on veraison (The beginning of fruit maturity) could increased total anthocyanins content, such as grapes\textsuperscript{17,20}, strawberries\textsuperscript{21}. Based on that, physiological experiments and nontarget metabolomics were combined to systematically and comprehensively demonstrated the effects of high concentration ABA on mature green fruits of blueberry in metabolites level. We found that application of ABA on the beginning of maturity of blueberries has both advantages and disadvantages\[[]\]

Advantages: The exogenous ABA could accelerate fruits ripening and colouration, which was consistent with the former results of other non-climacteric fruits\textsuperscript{17,20,21}. Besides this, we found the endogenesis ABA content and stress-resistance of blueberry fruits increased from S1 to S3 (about 15-20 days). Proline is positively correlated with plant stress-resistance and trehalose-6-phosphate is negative correlation with the response of plants to conditions that result in starvation\textsuperscript{34,35}. In these stages, the increase of proline content increased and decrease of trehalose-6-phosphate content
decrease indicates that the fruit have stronger resistance to adversity. Higher soluble solids and lower tannins means a better taste for consumers\textsuperscript{36}. Most of all, anthocyanins content is important for blueberry fruit, higher anthocyanins content means that blueberry fruits have more reducing ability. The reducing and DPPH assay also support the result that bluerry fruits under 1000 mg/ml ABA treatment have a stronger reducing ability.

**Disadvantages:** In this study, we found flavonals temporarily decreased during maturation and the final average value was lower than that of the treatment group under 1000 mg/ml aba treatment, such as myricetin, quercetin and kaempferol. This result is conflict with former research on grape\textsuperscript{17}, which show that the flavonols content is higher in mature fruits. However, the RNA-seq data of exogenous ABA on grapes at 22h and 44h shows the transcripts of chalcone synthase were down regulated at 22h and back to normal expression at 44h (Fig 7; Table S5). Considering the change of first enzyme in flavonaids pathway and our nontagert metabolomics’ results, flavonols content is unlikely to increase with the increase of anthocyanins content. To some bioactive compounds, such as catechin and epicatechin, are reported to have both antioxidant ability and pro-oxidant properties but their content is reduced under ABA treatment\textsuperscript{37}. Another possible negative impact is the reduction of fruit hardness. Fruit hardness may affect carry, store, transport and sell, but it has no effect on further processing of blueberries such as juice, preserved fruit and wine.

**Conclusions**

After comprehensive consideration, we believe that 1000mg/ml exogenous ABA will have a positive impacts for blueberry fruits on late green stage. Especially for blueberries, they are used for deep processing, such as juice, preserved fruit and etc. This finding provides a reference for different cultivation methods to improve the anthocyanin content of blueberry. Also, it provides a good research foundation for the next step of precise breeding for specific active compound of blueberry.

**Methods**

**Plant materials and ABA treatments**

Four years old rabbiteye blueberry ‘Brightwell’ were grown in experimental base of Baima district, Nanjing city, China. The rabbiteye blueberry ‘Brightwell’ were obtained from Institute of Botany,
Jiangsu Province and Chinese Academy of Sciences and identified by professor Wu Wenlong.

Blueberries of same size and growing condition were selected to do the treatment. Each treatment was arranged in a randomised complete block design with 6 replications and each block replication contains 6 shrubs.

The (+)-Abscisic Acid ( Purity 95%, Coolaber company, China ) was dissolved in double distilled H2O containing 5% (v/v) ethanol and 0.1% Tween 80. When most of blueberries fruits were in a growing stage of ‘green mature’ and the top of the fruit at the top of the branch has just begun to turn red, 0, 500 and 1000 mg L$^{-1}$ ABA solutions were sprayed on fruit clusters. Tiny sprayer were used to spray the ABA solutions on the peels untill the peels are wetted. The leaves and branches were carefully avoided.

In different development stage of fruits, 10 fruits of same size and weight were selected to determine fruit firmness, Brix and color and 30 fruits were immediately frozen in liquid nitrogen and stored at -80°C for later experiments.

**Physiological characterization**

**Fruit hardness**

The fruit hardness were determined by Fruit hardness tester ( Catno.9300, Takemura Electric Works Co., Japan ). The cone type tip was used and the tip was perpendicularly applied on the side surface of blueberry fruits. The value was measured at the moment of tip intrusion to the surface.

**Brix**

The Brix were determined by saccharometer ( PAL-1, Atago Co., Japan ). The juice were left on the prim for 20 seconds to do the measurement.

**The color of fruit peel**

Fruit peel color was measured by colorimeter ( Ci64, X-Rite, US ) and shown by the International Commission on Illumination a* and b* colour space co-ordinates\textsuperscript{29}. The a* value is negative for green and positive for red an the b* value is negative for blue and positive for yellow, both of the values range from -100 to 100. Due to the coloration of rabbiteye fruits starts from top to bottom, the top,
side and bottom of fruit peel were separately measured in the same stage.

**The total anthocyanins content**

The total anthocyanins content were determined by the double pH differential method and some adjustments are made\(^30\). Absorbance of the extract was measured at 510 and 700 nm in buffers at pH 1.0 (hydrochloric acid-potassium chloride, 0.2 M) and 4.5 (acetate acid-sodium acetate, 0.2 M). Total anthocyanins content was calculated using a molar extinction coefficient of 29,600 (cyanidin-3-glucoside) and absorbance of \( A = [(A_{510} - A_{700})_{\text{pH} \ 1.0}-(A_{510} - A_{700})_{\text{pH} \ 4.5}] \).

**Light microscopy**

Semithin section analysis was performed to observe the fruit pulp cells according the following method\(^31\). In brief, the 1mm x 3mm specimens were fixed with 0.1-M phosphate buffer (pH 7.2) containing 2.5% (v/v) glutaraldehyde and washed thrice with 0.1-M phosphate buffer and then soaked in 1% (v/v) osmic acid for 2 hours. Specimens were washed again using 0.1-M phosphate buffer as above and dehydrated through a gradient ethanol series. Ultimately, the specimens were embedded and polymerized in Spurr's resin. Semithin sections (2 μm) were stained with 1% methylene blue and then photographed under a Zeiss Axio vert A1 microscope. Images are representative of at six observed samples.

**Reducing power assay**

Reducing power assay were adapted from the following method and the ascorbic acid (0.1 mg/ml) was used as positive control\(^32\). Briefly, 0.15g samples homogenized with 1.35 mL of phosphate buffer (0.2 M, pH 6.6) and centrifuged at 5000 \( \times \) g for 10 mins. The supernatant (1mL) was mixed with 1 mL of potassium ferricyanide solution (1%, w/v). The mixtures were then reacted at 50 °C for 20 min. Then, 1 mL of trichloroacetic acid (10%, w/v) solution was added to stop the reaction immediately. After centrifugation, 1 mL of supernatant was mixed with 1 mL of distilled water and 0.2 mL of ferric chloride solution (0.1%, w/v). At 10 min later, the 96 plates were shaken sufficiently and measured spectrophotometrically at 700 nm.

**DPPH radical scavenging activity assay**
DPPH radical scavenging activity were measured by the following method and the ascorbic acid (0.5 mg/ml) was used as positive control. DDPH(0.1 mM) was dissolved in ethanol. Sample slurry was added to 3 DPPH reaction systems. Absorbance was measured at 515 nm after 30 min to reach a steady state.

The scavenging activity of the DPPH radical was calculated as follows:

**See formula 1 in the supplementary files.**

The reaction system: Ai, 1mL ethanol + 1mL DDPH solution; Aj, 1mL sample slurry + 1 mL DDPH; Ao, 1mL sample slurry + 1mL ethanol.

**Metabolite analysis**

**Metabolite extraction**

About 5g blueberry fruits of each treatment were thoroughly grounded with liquid nitrogen. Then, 100mg of homogenate was taken and resuspended with prechilled 80% methanol and 0.1% formic acid by well vortexing. The samples were incubated on ice for 5 min and then were centrifuged at 15000 rpm, 4°C for 5 min. Equal supernatant was diluted to final concentration containing 60% methanol by LC-MS grade water. The samples were subsequently transferred to a fresh Eppendorf tube with 0.22 μm filter and then were centrifuged at 15000 g, 4°C for 10 min. Finally, the filtrate was injected into the LC-MS/MS system analysis.

**UHPLC-MS/MS Analysis**

LC-MS/MS analyses were performed using a Vanquish UHPLC system (Thermo Fisher) coupled with an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Fisher). Samples were injected onto an Hyperil Gold column (100×2.1 mm, 1.9μm) using a 16-min linear gradient at a flow rate of 0.2 mL/min. The eluents for the positive polarity mode were eluent A (0.1% FA in Water) and eluent B (Methanol). The eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (Methanol). The solvent gradient was set as follows: 2% B, 1.5 min; 2-100% B, 12.0 min; 100% B, 14.0 min; 100-2% B, 14.1 min; 2% B, 16 min. Q Exactive HF-X mass spectrometer was operated in positive/negative polarity mode with spray voltage of 3.2 kV, capillary temperature of 320°C, sheath gas flow rate of 35 arb and aux gas flow rate of 10 arb.
Metabolite obtainment

The raw data files generated by UHPLC-MS/MS were processed using the Compound Discoverer 3.0 (CD 3.0, Thermo Fisher) to perform peak alignment, peak picking, and quantitation for each metabolite. The main parameters were set as follows: retention time tolerance, 0.2 minutes; actual mass tolerance, 5ppm; signal intensity tolerance, 30%; signal/noiseratio, 3; and minimum intensity, 100000. After that, peak intensities were normalized to the total spectral intensity. The normalized data was used to predict the molecular formula based on additive ions, molecular ion peaks and fragment ions. And then peaks were matched with the mzCloud (https://www.mzcloud.org/) and ChemSpider (http://www.chemspider.com/) database to obtained the accurate qualitative and relative quantitative results.

Differential Analysis

Significant differences were calculated by one-way ANOVA tests or T-test in SPSS 19 (IBM, USA). Different letters and asterisk (*) in figure indicate statistical significance (P< 0.05). Unsupervised principal component analyses (PCA) and partial leastsquares discriminant analysis (PLS-DA) clustering method were performed by MetaboAnalyst (http://www.metaboanalyst.ca/). Variable importance in projection (VIP) is the weighted sum of squares of the PLS-DA analysis, which represent the contribution rate of metabolites difference in different groups; Fold Change (FC) is the ratio of the mean of all replicate quantitative values for each metabolite in the comparison group. Combined with T-test, differential metabolites are screened by the following criteria: VIP > 1.0, FC > 1.2 or FC < 0.833 and P value < 0.05.

Declarations

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Not Applicable

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Competing interests
The authors declare that they have no competing interests.

**Author contributions**

WLL, WLW and TYH designed experiments. WLL and WLW got the funds and experimental base. ZHX did the treatment and looked after the plants. CS, XMW and HYY did the experiments and measurements. TYH and SC performed LC-MS/MS analyses and analyzed data. TYH visualized the results and writes the manuscript. All authors approved the final manuscript.

**Availability of data and materials**

The datasets of nontarget metabolomics analysed during the current study are available in the Table S1 and Table S2. The raw data (26.8Gb) generated by nontarget metabolomics does not have a suitable database to upload, but can be obtained from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

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Tables

Table 1 Mean fresh fruit length, diameter and weight throughout ripening. Data are means ± SE (n = 10).

| Stage | Length(cm)   | Diameter(cm) | Weight(g)   |
|-------|--------------|--------------|-------------|
| S1    | 10.93±0.51   | 9.41±0.49    | 0.67±0.09   |
| S2    | 11.37±0.43   | 9.45±0.56    | 0.68±0.08   |
| S3    | 11.4±0.44    | 9.54±0.34    | 0.73±0.07   |
| S4    | 13.16±0.46   | 10.99±0.38   | 1.13±0.10   |
| S5    | 13.55±0.28   | 11.46±0.39   | 1.33±0.06   |
| S6    | 15.19±0.68   | 12.53±0.47   | 1.87±0.19   |

Table 2 Top 10 metabolites of up-regulation in 6 stages. Log2FC indicates the degree of metabolites difference between 1000 mg/ml ABA treatment and 0 mg/ml ABA treatment.
| Stage 1                      | log2 FC | Stage 2                      | log2 FC | Stage 3                      | log2 FC |
|-----------------------------|---------|-----------------------------|---------|-----------------------------|---------|
| (+)-Abscisic acid           | 5.75    | MFCD19443524                | 4.05    | 3-Hydroxyvaline             | 4.38    |
| 1-(4-Pyridinyl)-4-piperidinecarboxamide | 5.24    | Gallic acid                 | 3.97    | 7-(Methylsulfanyl)[1,3]thiazolo[4,5-g] [1,3]benzothiazol-2-amine | 4.03    |
| Jasmonal                    | 4.98    | 1-(4,6-Dimethoxy-1-benzofuran-2-yl)ethanone | 3.61    | Gallic acid                 | 3.90    |
| Streptidine                 | 4.90    | 9-Oxo-9H-fluorene-2,7-disulfonyl dichloride | 3.36    | FMNH2                       | 3.79    |
| 3-Hydroxyvaline             | 3.97    | FMNH2                       | 3.52    | 1-(4,6-Dimethoxy-1-benzofuran-2-yl)ethenone | 3.78    |
| 7-(Methylsulfanyl)[1,3]thiazolo[4,5-g] [1,3]benzothiazol-2-amine Dehydrovomifoliol | 3.51    | Disisodecyl phthalate       | 3.29    | Quillia Acid                | 3.77    |
| Amide C18                   | 3.41    | 2,4-Dinitro-3'-nitrodiphenyl ether | 3.21    | (3beta)-3-((Nonyloxy)olean-12-ene | 3.52    |
| Gallic acid                 | 3.40    | taxifolin                   | 3.19    | Hexose                      | 3.47    |
| 2-(Difluoromethyl)-9,9-dimethyl-9H-fluorene | 3.29    | okenone                     | 3.05    | Disisodecyl phthalate       | 3.32    |
| Stage 4                      | log2 FC | Stage 5                      | log2 FC | Stage 6                      | log2 FC |
| Marrubin                    | 3.16    | Ethyl [(trifluoromethyl)sulfanyl]-4-pentenoate Mitobronitol | 3.17    | Cyclododecyne               | 2.90    |
| (+)-Staurosporine           | 3.13    | Mitobronitol                | 3.12    | Mitobronitol                | 2.54    |
| Bruceine A                  | 3.04    | 7-Isouquinolinyl trifluoromethanesulfonate | 3.13    | 7-Isouquinolinyl trifluoromethanesulfonate | 2.63    |
| Boldione                    | 3.02    | (+)-Staurosporine           | 3.12    | Mitobronitol                | 2.54    |
| 2-Chloro-6-(hydroxyamino)phenol | 2.89    | 1-(3,5-Dichloro-2,6-dihydroxy-4-methoxyphenyl)-1-hexanone nimidane | 3.08    | N-(3,4-Dimethylphenyl)-9,10-dioxo-9,10-dihydro-1-anthracenesulfonamide | 2.30    |
| 3,3-Bis(1,3-thiazol-2-ylamo)-1,2-propanediol 4-(Dodecyloxy)-2,2'-bis(ethoxymethoxy)-4'-ethynylbiphenyl | MFCD19443524 | 2.82    | 2.94 | DL-Carbidopa | 2.30    |
| 2.77 MFCD15146035           | 2.77    | 2.89 | Etaglucid                   | 2.22    |
| bengazole A                 | 2.54    | 2.60 | Diosmin                     | 2.20    |
| Quillia Acid                | 2.37    | 2.60 | (+)-Staurosporine           | 2.09    |
| DL-Carbidopa                | 2.57    | 2.57 | Tosufloxacin                | 1.91    |

Table 3 Top 10 metabolites of up-regulation in 6 stages.

Figures
| Stage 1 | log2F<sub>C</sub> | Stage 2 | log2F<sub>C</sub> | Stage 3 | log2F<sub>C</sub> |
|---------|----------------|---------|----------------|--------|----------------|
| 3-hydroxybenzylhydrazine | -6.032-Chloro-6-(hydroxyamino)phenol | -5.592-Chloro-6-(hydroxyamino)phenol | -5.12-Chloro-6-(hydroxyamino)phenol |
| 5,5'-Dehydrodivanillate | -4.697-Hydroxy-6-[(2-oxo-2H-chromen-7-yl)oxy]-2H-chromen-2-one | -4.797-Hydroxy-6-[(2-oxo-2H-chromen-7-yl)oxy]-2H-chromen-2-one |
| 2-Chloro-6-(hydroxyamino)phenol | -4.485,5'-Dehydrodivanillate | -4.42Cinnamttannin A3 | -4.38-Chloro-6-(hydroxyamino)phenol |
| Aldioxa | -4.29Cinnamttannin A3 | -3.66Aripiprazole | -3.83-Chloro-6-(hydroxyamino)phenol |
| Cinnamttannin B1 | -4.25DL-Carbidopa | -3.45Tetrakis(4-nitrosophenyl)silane | -3.58-Chloro-6-(hydroxyamino)phenol |
| Combretastatin A-1 | -4.17p-Chloroacetophenone | -3.42Cinnamttannin B1 | -3.48-Chloro-6-(hydroxyamino)phenol |
| hexanoyl-CoA | -3.96Chlorogenic acid | -3.23Chlorogenic acid | -3.22-Chloro-6-(hydroxyamino)phenol |
| all-trans-retinal | -4.161-(1H-Indol-3-ylcarbonyl)-9H-beta-carboline-3-carboxylic acid |
| (+)-Fluprostrenol | -3.96Chlorogenic acid | -3.23Chlorogenic acid | -3.22-Chloro-6-(hydroxyamino)phenol |
| p-Chloroacetophenone | -3.82aldioxa | -2.96Benzyl beta-D-xylopyranoside | -3.21-Chloro-6-(hydroxyamino)phenol |

| Stage 4 | log2F<sub>C</sub> | Stage 5 | log2F<sub>C</sub> | Stage 6 | log2F<sub>C</sub> |
|---------|----------------|---------|----------------|--------|----------------|
| 3-Pheny1-4-(phenylsulfanyl)butanal | -4.12(1S)-1,5-Anhydro-1-benzyl-D-galactitol | -4.90Guanine | -7.02-Chloro-6-(hydroxyamino)phenol |
| T1940000 | -3.45tectorigenin | -3.51Benzyl beta-D-xylopyranoside | -3.91-Chloro-6-(hydroxyamino)phenol |
| Cinnamttannin B1 | -3.26Propicillin | -3.272-amino-2,3,7-trideoxy-D-lyxo-hept-6-ulosonic acid | -3.61-Chloro-6-(hydroxyamino)phenol |
| Melibiose | -3.071-(1H-Indol-3-ylcarbonyl)-9H-beta-carboline-3-carboxylic acid |
| hexanoyl-CoA | -3.07Benfotiamine | -3.00Chlorogenic acid | -3.43-Chloro-6-(hydroxyamino)phenol |
| Chlorogenic acid | -3.04Chlorogenic acid | -2.97Flufenamic Acid | -3.33-Chloro-6-(hydroxyamino)phenol |
| Epicatechin | -3.02Gallic acid | -2.82Silalfluofen | -2.95-Chloro-6-(hydroxyamino)phenol |
| 1-(1H-Indol-3-ylcarbonyl)-9H-beta-carboline-3-carboxylic acid | -2.82guajavarin | -2.35Fludiazepam | -2.92-Chloro-6-(hydroxyamino)phenol |
| Cinnamttannin A3 | -2.822-\{[3-Hydroxy-2-oxo-2,3-dihydro-1H-indol-3-yl]acetyl\}amino\}succinate | -2.20Cinnamttannin B1 | -2.89-Chloro-6-(hydroxyamino)phenol |
| 5-Methoxy-3-indoleacetic acid | -2.78Catechin | -2.20T1940000 | -2.88-Chloro-6-(hydroxyamino)phenol |
Figure 1

Ripening progress of blueberry (cv ‘Brightwell’) fruits used for further analyses. (a) The ripening progress is divided into six stages. (b) The fruit proportion of S6 stage was counted at 20 and 25 days.
Figure 2

Colouration and anthocyanins content of fruits under 0, 500 and 1000 mg/ml ABA treatment. (a) The color of top, side and bottom of fruits from S1 to S6. (b) Total anthocyanins content form S4 to S6.
Figure 3

Hardness, Brix and semithin section of fruits under ABA treatment. (a) The hardness of fruits under 0, 500 and 1000 mg/ml ABA treatment from S4 to S6. (b) The Brix of fruits under 0, 500 and 1000 mg/ml ABA treatment from S4 to S6. (c) Semithin section of pulp cells of fruits under 0 and 1000 mg/ml ABA treatment.
Figure 4

PCA analysis of metabolites under positive model and negative model in 6 stages. 1000 represents 1000 mg/ml ABA treatment and 0 represents 0 mg/ml ABA treatment.
Changes in relative ABA content and resistance to adversity. (a) ABA changes in fruits from S1 to S6 in control group. (b) relative ABA content in 0 and 1000 mg/ml ABA treatment in 6 stages, respectively. (c) L-proline and trehalose-6-phosphate content under 0 and 1000 mg/ml ABA treatment from S1 to S6.
Figure 6

Schematic diagram of the flavonoid pathway. The metabolites under 0 and 1000 mg/ml ABA treatment were shown from S1 to S6. The change of enzymes transcripts in grape under ABA treatment at 44h were downloaded from Pilati1 et al., (2017).
Figure 7

Relative abundance of bioactive constituents under 0 and 1000 mg/ml ABA treatment.
Reducing ability of fruits under 0 and 1000 mg/ml ABA treatment. Ascorbic acid was used as positive control. (a) The reducibility of fruits (b) Scavenging rate of DDPH.

Supplementary Files

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