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Research article

Metabolomics characterizes metabolic changes of Apocyni Veneti Folium in response to salt stress

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ABSTRACT

Apocyni Veneti Folium (AVF) has been raised great interest in the antioxidant properties recently for the preservation of human health. However, little research was found on the integrate metabolites except our previous investigation on the variations of the bioactive constituents. To understand the salt-tolerant mechanisms of the halophyte, metabolomic platform based on ultra-fast liquid chromatography tandem triple time-of-flight mass/mass spectrometer was applied in this study. The results showed that metabolic profiles were separated and differentiated among groups based on multivariate statistical analysis; different metabolites belonged to various chemical classes. Besides, phenylpropanoid pathway and terpenoid biosynthesis were disturbed in all salt-stressed AVF and low salt-treated group appeared to be better than other samples in terms of relative contents (peak areas) of the wide variety of bioactive components and physiological variations of photosynthetic pigments, osmotic homeostasis, lipid peroxidation product and antioxidative enzymes. This study may provide additional insight into the salt-tolerant mechanisms and the quality assessment of AVF in a holistic level based on the plant metabolomics.

1. Introduction

Salinity has detrimental effects on almost all stages of plants including seed germination, plant development and growth. These effects are related to the activations of salinity-induced molecular networks involved in the stress perception, ion and osmotic homeostasis, signal transduction, regulation of stress-related genes, protein expressions and subsequently metabolisms (Patel et al., 2016; Mittler et al., 2004).

Plants possess diverse and complex metabolic pathways that generate metabolites (Dersch et al., 2016). Both primary and secondary metabolites are intermediates or end-products of cellular regulatory pathways and contribute to plant adaptation. The primary metabolites, including sugars, amino acids and lipids, are indispensable for the growth and development of plants by providing the necessary energy and molecular building blocks. A large number of the specialized compounds, named secondary metabolites, exhibit antioxidant activities including flavonoids, phenolics, terpenoids, saponins, cardiacglycosides, quinones, alkaloids and proanthocyanidins (Saeed et al., 2012). Enhanced synthesis of secondary metabolites under stressful conditions protect the cellular structures from oxidative effects, and are crucial for interactions with the environment by providing resistance against biotic and abiotic stresses (Sumner et al., 2003). Thus, it has been suggested that plants stressed by medium salinity may have the potential to be sources of polyphenols and alkaloids (Neffati et al., 2011; Wang and Liu, 2010).

There has been increasing interest in studying the omics tools to identify and understand salt tolerance components and mechanisms at molecular level (Qin et al., 2016), such as genomics, transcriptomics, proteomics and metabolomics (Jorge et al., 2016). Since a number of previous studies on the quality assessment of traditional Chinese medicines (TCMs) indicated that metabolic responses were critically important for plant adaptation and salt-tolerance (Dai et al., 2010; Zhou et al., 2018), plant metabolomics has become an invaluable tool to investigate abiotic stresses (Fiehn et al., 2000; Alvarez et al., 2008; Renberg et al., 2010; Ruan et al., 2010). Using a metabolomics platform, global patterns of plant metabolic changes in response to stress can be monitored (Kim et al., 2012). For the efficient ion production and enhanced selectivity, the wide ranges of measurements and the straightforward peak identification, LC-MS has been regarded as one of the most applicable and versatile methods in metabolomics (Li et al.,

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Abbreviations

AVF	Apocyni Veneti Folium
AVL	*Apocynum venetum* L
LC-MS	liquid chromatography mass spectrometry
GC-MS	gas chromatography-mass spectrometry
NMR	nuclear magnetic resonance
UFLC-Triple TOF-MS/MS	ultra-fast liquid chromatography tandem triple time-of-flight mass/mass spectrometer
PCA	principal component analysis
OPLS-DA	orthogonal partial least squares-discriminant analysis
VIP	variable importance in the projection
BPC	base peak chromatogram
ESI	electrospray ionization
HCA	hierarchical clustering analysis
SOD	superoxide dismutase
CAT	catalase
POD	peroxidase
t_R	retention time
m/z	mass-to-charge ratio
Chl	chlorophyll
TCA cycle	tricarboxylic acid cycle
Fructose-6-P	fructose-6-phosphate
Glucose-6-P	Glucose-6-phosphate
CoA	acetyl-CoA
MVA	mevalonate
IPP	isopentenyl pyrophosphate
DMAPP	dimethylallyl pyrophosphate
PEP	phosphoenolpyruvate
3-PG	3-phosphoglycerate

2007).

Halophyte *Apocynum venetum* L. (AVL) is one of the highly studied medicinal plants. It grows in a wide variety of saline habitats, and is characterized by a high physiological plasticity for salt tolerance limits. Apocyni Veneti Folium (AVF) is rich in bioactive compounds, including the most abundant constituents of polyphenols and flavonoids, which possess prominent antioxidant activities (Chen et al., 2018a). Accumulating evidences have illustrated that AVF is responsible for diverse pharmaceutical applications, such as antihypertension, antidepression, hepatoprotection and antioxidation (Xie et al., 2012). To the best of our knowledge, little information on its physiological response in terms of metabolomics to the salt stress is available. Therefore, it would be useful to investigate possible changes in metabolic levels and the possible pathways in the context of salinity.

In this study, AVL was exposed to varying saline concentrations (0, 100, 200 and 300 mM) for 40 days. A metabolic profiling method based on ultra-fast liquid chromatography tandem triple time-of-flight mass/mass spectrometer (UFLC-Triple TOF-MS/MS) coupled with multivariate statistical analysis, including principal component analysis (PCA), orthogonal partial least squares-discriminant analysis (OPLS-DA) and hierarchical clustering analysis (HCA), was employed to identify the significant metabolites and discriminate the groups. Additionally, the molecular responses were linked to the previous report on physiology (Chen et al., 2018b), and this report involving both primary and secondary metabolites in AVF on evaluating the effect of salinity stress on metabolic pathways. This strategy may help provide a deeper insight into the comprehensive understanding of the quality assessment of AVF and salt-tolerant mechanisms of halophytes in a holistic level.

2. Materials and methods

2.1. Chemicals and materials

Acetonitrile of HPLC grade were purchased from Merck (Damstadt, Germany). Standard compounds of glutamine, glutamic acid, proline, guanine, guanosine, aspartic acid, phenylalanine, epicatechin, rutin, hyperoside and quercitrin were purchased from Shanghai Yuanye Biotechnology (Shanghai, China); gallic acid and apigenin were obtained from Chinese National Institute of Control of Pharmaceutical and Biological Products (Beijing, China); fumaric acid, gallocatechin, epigallocatechin, cryptochlorogenic acid, kaempferol 3-*O*-rutinoside and amentoflavone were acquired from Chengdu Chroma Biotechnology (Chengdu, China); neochlorogenic acid, chlorogenic acid, catechin, isoquercitrin, avicularin, trifolin, astragalin, kaempferol, quercetin 3-*O*-sophoroside and quercetin were bought from Baoji Chenguang Biotechnology Co., LTD. (Baoji, China).

2.2. Salinity treatments

The experiment was carried out in the shelter where the conditions were similar to the open air. The main roots of AVL were two years old and originated from the same plant, which was identified by Professor Xunhong Liu (Department for Authentication of Chinese Medicines, Nanjing University of Chinese Medicine). They were planted in pots filled with 25 kg of soil (texture, loam; organic carbon, 36.6 g/kg; cation exchange capacity, 17.0 cmol(+)/kg^{-1}; pH, 5.0). Salt stress tests had been conducted since AVL was about 30 cm height. 4 levels of salt treatment concentrations: 0 (control, watering), 100 (low stress), 200 (medium stress) and 300 (high stress) mM NaCl treatments were designed with 3 replicates and 3 pots per replicate by pouring 2 L of solution. NaCl concentrations increased gradually by 50 mM NaCl every four days to reduce osmotic shock until the designated concentration was reached and lasted for 6 times (20 days). The harvest of AVF samples was performed after 12 h after the last salt treatment experiment. The collected samples of four groups were dried at room temperature under natural conditions.

2.3. Sample preparation

Naturally dried, salt-treated samples were powdered and passed through a 60 mesh sieve. Four groups of dried samples (20 g) were extracted twice with 200 mL of 70% (v/v) ethanol for 2 h under reflux followed by centrifugation at 3500 rpm for 10 min, and the supernatant was collected. After rotary evaporation and freeze-drying to a powder, a small portion was re-dissolved in 70% ethanol, forming a 1 mL solution containing 0.05 g of AVF, and the solution was centrifuged at 12,000 rpm for 15 min. Then, the supernatant was stored at 4 °C and filtered through a 0.22 μm membrane before being subjected to UFLC-Triple TOF-MS/MS analysis. The rest of the freeze-dried powder was stored for other analytical tests.

2.4. UFLC-triple TOF-MS/MS analysis of metabolomics

A UFLC system (SHIMADZU Corp., Kyoto, Japan) interfaced with Triple-TOF MS/MS and equipped with electrospray ionization (ESI) source was used to carry out the analysis. An XBridge BEH C_{18} column (100 mm × 4.6 mm, 3.5 μm) column was used for all the analysis. The mobile phase was consisted of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The samples were eluted using a linear gradient program as follows: 0–3 min, 5% B; 3–8 min, 5–18% B; 8–12 min, 18–20% B; 12–15 min, 20% B; 15–17 min, 20–60% B; 17–18 min, 60–80% B; 18–18.5 min, 80–5% B; 18.5–22.1 min, 5% B. The flow rate was 0.80 mL min^{-1}; the column was maintained at 30 °C and the injection volume was 5 μL. The Triple-TOF mass spectrometer was operated both in positive and negative ion modes. The operating parameters of the total ion chromatogram were set as follows: nebulizer pressure, 55 psi; drying gas pressure, 55 psi; a curtain gas pressure, 40 psi; source temperature, 550 °C; capillary voltage, 5500 V and −4500 V for positive and negative ion modes, respectively. Data were acquired for each sample from 50 to 1500 Da and dynamic range enhancement was applied throughout the MS experiment to ensure the accurate mass measurement.

2.5. Data processing and multivariate statistical analysis

The raw data of UFLC-Triple TOF-MS/MS was processed by Markerview 1.2.1 software. Through the analysis of the multistage tandem mass spectrometry, the characteristic peaks were extracted with mass spectrometry data peak matching, peak alignment and noise filtering. For the collection parameters, the intensity threshold was set at 10 counts and retention time (t_R) range 0–22 min. The parameter of peak to peak baseline noise was automatically calculated. Isotopic

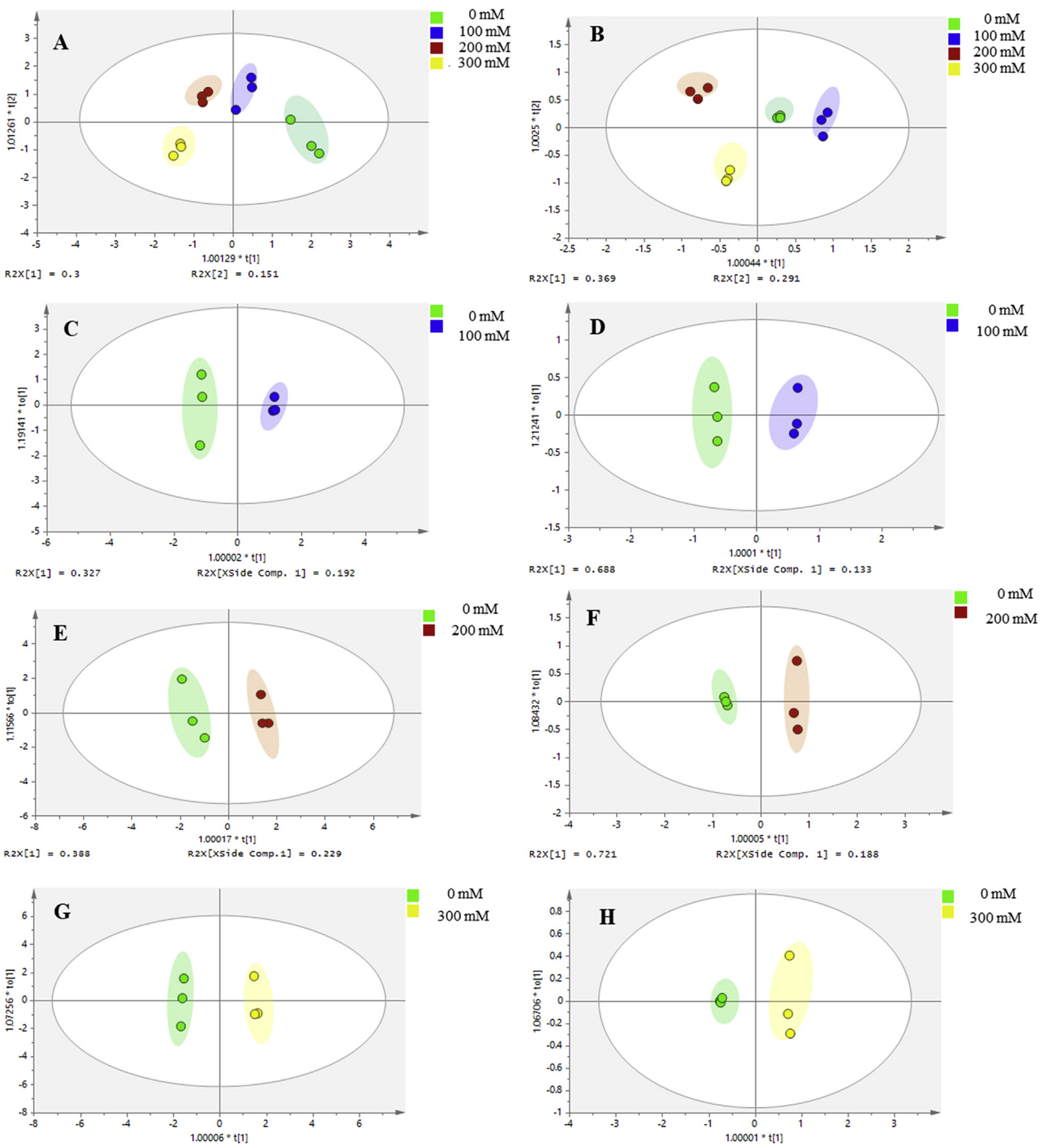

Fig. 1. OPLS-DA scores plots of AVF exposed to different levels of salt stress compared with control under positive (A, C, E and G) and negative (B, D, F and H) ion modes, respectively. A and B, case vs control; C and D, 100 mM salt vs control; E and F 200 mM salt vs control; G and H 100 mM salt vs control. 95% confidence regions are displayed for each treatment.

peaks were excluded for analysis. For the data analysis, a list of the intensities of the peaks detected was generated using t_R and mass data (m/z) pairs as the identifier of each peak.

Then the resulting three dimensional datum containing peak name (t_R–m/z pair), sample name and peak intensities were further exported to SIMCA-P software (Version 13.0, Umetrics AB, Umea, Sweden) for multivariate statistical analysis. Unsupervised PCA was run to obtain a general overview of the variance of metabolites, and supervised OPLS-DA was performed to obtain information on differences in the metabolic compositions between salt-treated samples and control. The ellipse that was shown in the PCA score plots of the models defines the 95% confidence interval of the modeled variations. VIP is the weighted sum of the squares of the OPLS-DA analysis (Jia et al., 2019). A metabolite with a VIP above 1 is regarded as significant. In addition, univariate analysis (one-way ANOVA followed by Duncan range test) was performed to compare the means with the significance level set as 0.05 by SPSS 19.0. Some of the differently expressed metabolites were identified by comparison with reference compounds (t_R and m/z) and a plant-oriented metabolic database (PMN, https://www.plantcyc.org/); while for compounds without standard references, the characteristic peaks were inferred though comparing MS/MS fragment ions using PeakView 1.2 software (AB SCIEX, USA), references on AVL and other *Apocynaceae* plants (An et al., 2013; Xie et al., 2012; Liang et al., 2010; Hao et al., 2013) and online resources, such as HMDB (http://www.hmdb.ca/), SciFinder (https://www.cas.org/products/scifinder) and PubChem (https://pubchem.ncbi.nlm.nih.gov/).

Hierarchical cluster analysis was introduced to cluster samples based on significantly changed metabolites from Markerview by Java Treeview 3.0 software. Clustering was based on the method of euclidean distance coefficient and average linkage. The mean values of all parameters were taken from the measurements of three replicates with the standard deviation calculated. Columns and rows represent samples exposed to different salinity concentrations and individual metabolite, respectively.

2.6. Biological pathway analysis

Biological pathway analysis was constructed based on significantly changed metabolites of salinity treated groups compared with control according to KEGG website (http://www.genome.jp/kegg/) and MetaboAnalyst 4.0 online (http://www.metaboanalyst.ca/). *Arabidopsis thaliana* (thale cress) was selected as a pathway library.

3. Results and discussion

Halophytes develop a robust defense system against excessive ROS-induced oxidative stress, since they are equipped with a powerful and sophisticate protective network (Chen et al., 2018b; Benjamin et al., 2019; Zhang et al., 2017). The phenotypic analysis reported in our previous study, including chlorophyll (Chl) and carotenoids content, osmolytes and antioxidant enzymes and was summarized in the supplementary materials (Table S1). Generally, results showed that Chl a, total Chl, Chl a/b and carotenoids were significantly increased in the presence of low and medium levels of salt; osmolyte contents were elevated significantly; while antioxidant enzyme activities varied differently. In the following parts, the metabolic responses will be linked to the phenotype changes.

3.1. Identification of significantly different metabolites between salt-treated samples and control

The representative base peak chromatogram (BPC) obtained from UFLC-Triple TOF-MS/MS both in positive and negative ion modes for the reference mixture and the representative AVF samples were shown in Fig. S1. Over 200 peaks of metabolites with unique m/z values were detected from the AVF extracts, including 71 identified compounds (Table S2).

After normalizing the data based on the abundance of the metabolite, PCA was conducted. Fig. S2 showed the score plots of principal component 1 and principal component 2 conducted by the LC-MS analysis of AVF. The PCA scores plot could be readily categorized into four groups according to the saline concentration levels. In the loading

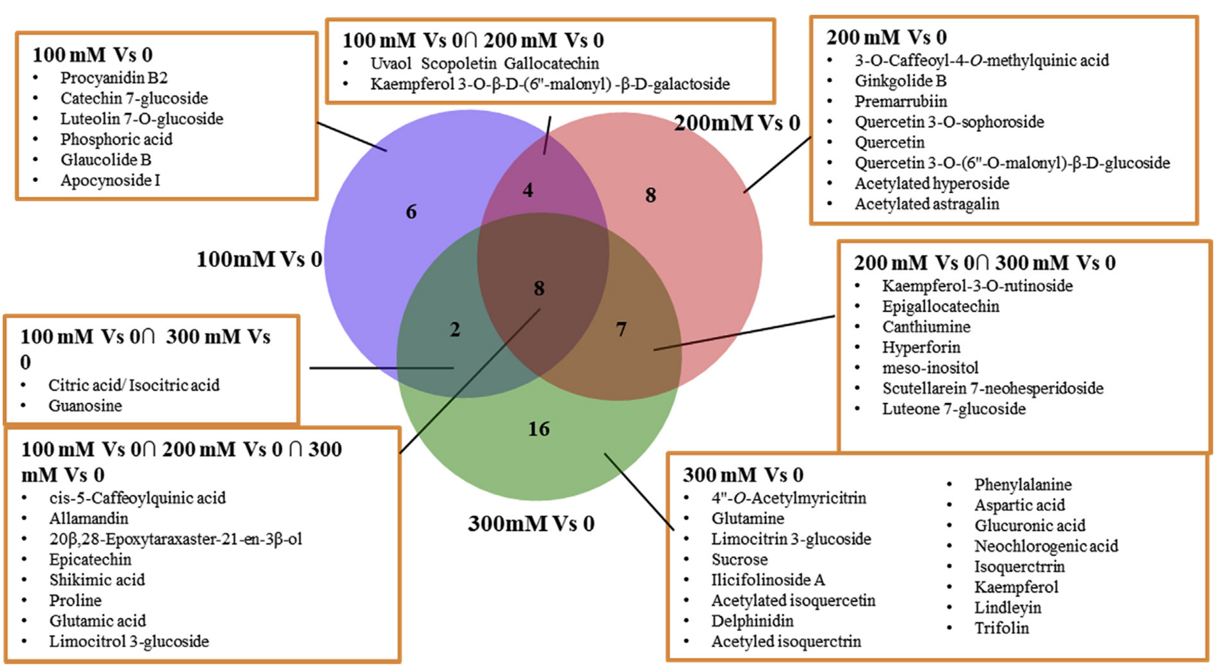

Fig. 2. Venn diagram analysis of differentially metabolites under salt treatment compared to the control group at a 5% level of significance. The overlapping regions of the Venn indicate the metabolites share between/among corresponding groups in the experimental comparisons, and the remaining regions shows the specifically metabolites.

plot, the further a metabolite is from the origin, the greater its contribution to the discrimination.

Supervised OPLS-DA was employed to the metabolomics data to determine the significant differences in metabolic profiles between salt-treated samples and control. R^2Y (cum) was used to estimate the “goodness of fit” of the model, and Q^2(cum) to estimate the ability of prediction (Jia et al., 2019). Besides, validation (Fig. S3) was performed in the discriminant analysis model to correct potential overfit of 2 OPLS-DA comparisons of salt treated group and control with the *p* value of 0.007 and 0.01 under positive and negative ion modes, respectively. The results of model validation indicated that the model was stable with strong ability of predict. Fig. 1A and B showed that plants treated with NaCl were clearly separated from the control, generally in a concentration-dependent manner. The significantly discriminating metabolites identified by the constituents of *Apocynaceae* family and online resource libraries were screened by variable importance in the projection (VIP) score. These metabolites were classified into different chemical classes, including flavonoids, phenylpropanoids, terpenes, alkaloids, amino acids, sugars, organic acids and inorganic acids (Table S3). However, this analysis was based on the combined data from the three treatments versus control, without considering the difference between concentrations. Thus, to elucidate the metabolic response at different concentrations of NaCl, OPLS-DA analysis for low vs control (100 and 0 mM NaCl, Fig. 1C and D), medium vs control (200 and 0 mM NaCl, Fig. 1E and F), and high vs control (300 and 0 mM NaCl, Fig. 1G and H) were separately conducted by referring to previous investigations (Zhao et al., 2018).

3.2. Metabolite profile changes responded to low concentration of NaCl

The low salt dose decreased the level of a number of flavonoids, such as limocitrol 3-glucoside, luteolin 7-*O*-glucoside and procyanidin B2, and increased the level of gallocatechin, epicatechin and catechin 7-glucoside (Fig. 2 and Fig. S3). Procyanidin B2 and luteolin 7-*O*-glucoside have the functions of radical scavenging and antioxidant by interacting with H_2O_2 (Sakano et al., 2006; Orhan et al., 2016), but their functions in the salty environment are still unclear. Flavanols, such as catechin, gallocatechin and epicatechin, have been demonstrated to have high antioxidant potential in grape seeds (Yilmaz et al., 2004) and in the medicinal halophyte *limoniastrum guyonianum* (Trabelsi et al., 2012). According to Griesser et al. (2015), prolonged drought stress leads to an increase of the formation of epicatechin, epicatechin gallate, kaempferol-3-*O*-glucoside and quercetin-3-*O*-glucoside; whereas the changes were not observed after the short term treatment. In agreement

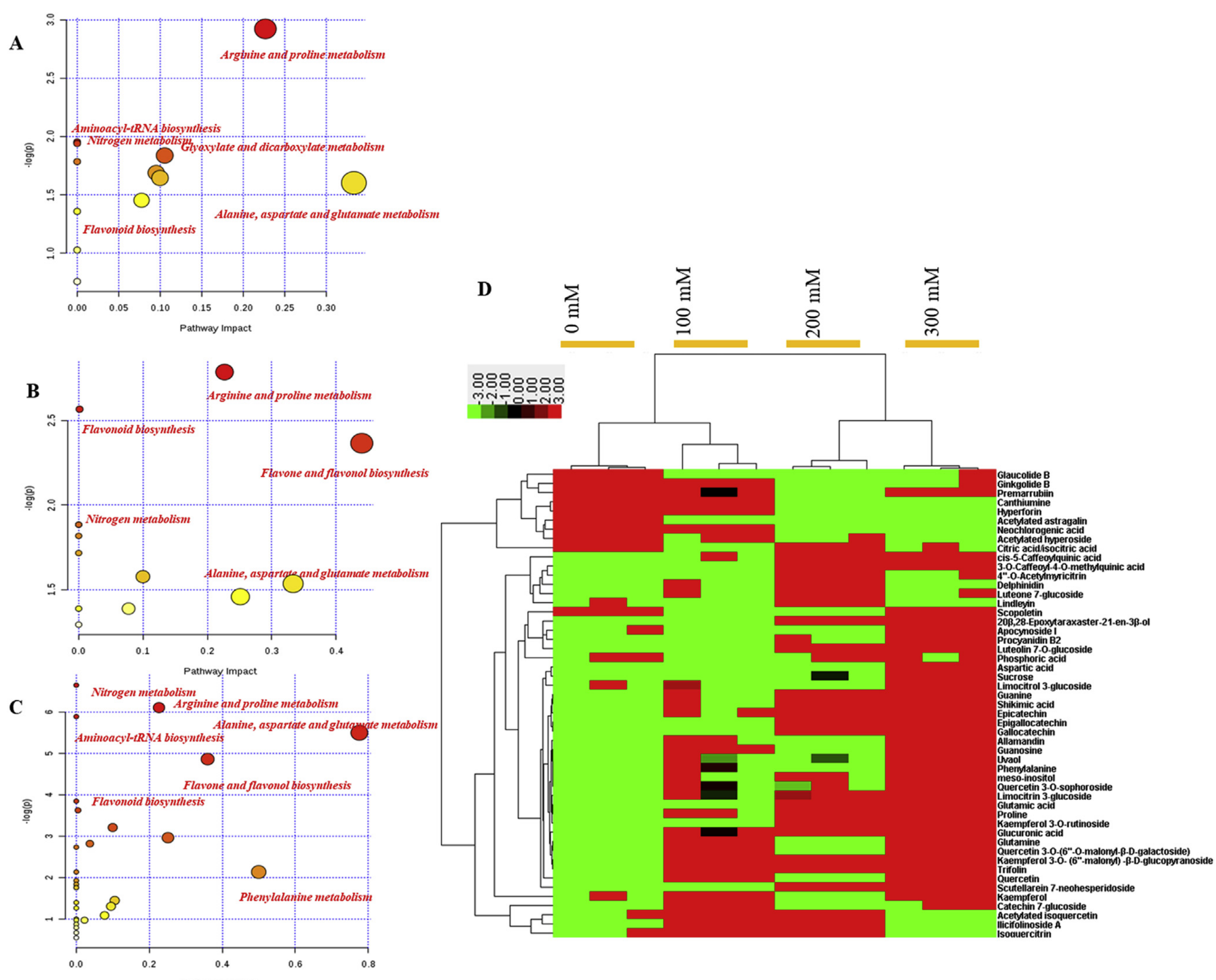

Fig. 3. Pathway enrichment analysis and hierarchical clustering analysis. The biological pathway analysis of A, B and C stands for the significantly changed biological pathway due to exposure to different concentrations of NaCl compared to the control. (100 mM vs 0, 200 mM vs 0 and 300 mM vs 0, respectively). D shows 46 differential metabolites of AVF samples subjected to salt stress.

with our findings, higher amounts of flavonols formed in response to abiotic stress were also observed in grape berry stems in an earlier study (Doshi et al., 2006). Indeed, polyphenolic compounds participate in plant protection against ROS under salt stress. A variety of flavonoids are tested for antioxidant activity and can effectively scavenge various free radicals though regulating oxidative stress-mediated enzyme activity or the chelation of transition metals to protect DNA indirectly (Melidou et al., 2005). Besides, metabolites acting as natural antioxidants and nutrient supplements, such as kaempferol, catechin, epicatechin and quercetin, contain potent antioxidant activities and provide functional value for the plant by modulating cell-signaling pathways (Williams et al., 2004; Hernández et al., 2009; Mishra et al., 2015).

This low level of NaCl also increased the levels of cis-5-caffeoylquinic acid (1.52-fold), allamandin (1.9-fold) and scopoletin (1.52-fold), and decreased 20β,28-epoxytaraxaster-21-en-3β-ol (0.69-fold) and apocynoside I (0.3-fold) compared to the control. Scopoletin, one of the coumarin derivatives which attracted much attention for plant growth regulatory, antioxidant, cytotoxic and antimicrobial properties (Al-Amiery et al., 2012; Saleh et al., 2014), was reported to accumulate in leaves of wheat subjected to chilling stress (Moheb et al., 2011) and under both salinity and non-salinity conditions in coumarin-pretreated seedlings (Saleh and Madany, 2015). Similar results were observed in our work. As an ionone glucoside, apocynoside I (terpenes) has been isolated from the roasted AVF (Murakami et al., 2001). However, its role related to salt stress has not been reported so far. In addition, the ubiquitously distributed terpenes contribute to both direct and indirect defenses (Mithöfer and Boland, 2012) such as the interaction with different transcription factors in response to oxidative stress (Arbona et al., 2013; Benjamin et al., 2019); and salinization can significantly produce a pronounced effect on terpenes composition of *rosemary* (Tounekti et al., 2011). In addition, the levels of organic acids, such as citric acid/isocitric acid, were decreased significantly. However, a significant increase was shown in shikimic acid (2.45-fold). Citric acid is a key metabolic component in the glyoxylate and dicarboxylate metabolism and tricarboxylic acid cycle (TCA cycle), which generate energy and provides adaptive flexibility to unfavorable environments. The results were consistent with the study that the short-term salinity induced decreases of TCA cycle intermediate probably due to salt-shock effects (Zhang et al., 2011). Such responses not only efficiently relieved the salt-induced hyperammonia but also generated compatible osmolytes to maintain osmotic balance. Shikimic acid is a precursor for the aromatic amino acids, many alkaloids, tannins and lignin. The up-regulated shikimic acid was present in ultraviolet-induced metabolic disruption of *Melissa officinalis* than in the control group, which may have a direct or indirect association with the biosynthesis of phenylpropanoids (Kim et al., 2012). The increased level of guanosine perhaps suggests that salinity caused alterations of DNA and RNA biosynthesis/degradation. Metabolic adjustments also play important roles in attaining a new balance of energy and metabolites (Obata and Fernie, 2012) and can be a way to counteract a lower water potential under salinity environment (Deinlein et al., 2014).

Our reports on physiological variations showed that the level of peroxidase (POD) was significantly increased induced by 100 mM NaCl, as well as ascorbic acid, the end product of ascorbate and aldarate metabolism (Urano et al., 2009). As a non-enzymatic free radical scavenger and a key substance in the network of antioxidants, ascorbic acid has been shown to play multiple roles in plant growth, such as regulating the normal reactive oxygen species in plant cells together with other small molecules (Wang et al., 2011; Moradi and Ismail, 2007). A significant increase was shown in ascorbic acid, proline and glutamic acid under salt stress to protect the body from endogenous damage of oxygen free radicals (Chen et al., 2018b). The up-regulation of osmotic adjustments and photosynthesis pigments may help maintain ion homeostasis and protect enzymes and proteins (Kim et al., 2007). Both enzymatic and nonenzymatic antioxidant defense systems play crucial roles in the normal growth of halophytes under salinity conditions, confirming that metabolomics can be used to early detect plant responded to environmental stress (Hall, 2006). The most significantly impacted biological pathways were shown in Fig. 3A; however, considering that the majority of metabolites are secondary metabolites, insufficient pathways can be referenced in the library.

3.3. Metabolite profile changes responded to medium concentration of NaCl

The changes in the metabolic levels of AVF stressed by 200 mM NaCl compared to the control were shown in Fig. 2 and Fig. S3. In addition to the changes observed at 100 mM NaCl (e.g., increases in key components of the phenylpropanoids and terpenoids, flavonoid biosynthesis, especially flavone and flavonol biosynthesis, such as gallocatechin (6.5-fold), epicatechin (2.4-fold), cis-5-caffeoylquinic acid (2.5-fold) and allamandin (1.9- fold); significant decreases in limocitrol 3-glucoside (0.44-fold), acetylated astragalin (0.77-fold), acetylated hyperoside (0.88-fold)), the medium concentration of salt altered additional metabolic levels. For example, the accumulate of a number of flavonoids(scutellarein 7-neohesperidoside (1.74-fold), epigallocatechin (3.49-fold), 3-*O*-caffeoyl-4-*O*-methylquinic acid (1.74-fold), quercetin 3-*O*-sophoroside (1.44-fold), quercetin (0.94-fold)), indicating that 200 mM NaCl significantly and extensively affected flavonoid metabolism. Published studies have showed that quercetin can increased several antioxidant enzyme activities and activated endogenous defense systems (Han et al., 2007); in particular, moderate salinity was able to induce the synthesis of quercetin, quercetin-3-*O*-glucoside and quercitrin (Sgherri et al., 2017). The decreased level of quercetin possibility indicates that this compound is used to synthesis other flavonoids to defense stress. Besides, medium salt stress led to a significant elevation of meso-inosito (1.69-fold) as well. Meso-inosito has critical functions in membrane biosynthesis and protection and plant cell signaling (Fiehn et al., 2000), and can be employed for the osmotic stress management purposes especially under salinity. Similar observation has been made for the salt-stressed *Actinidia* (kiwifruit) leaves (Klages et al., 1999) and tobacco under prolonged salinity with high-dose salt (500 mM NaCl) (Zhang et al., 2011).

Our previous study found the medium level of salt triggered the up-regulation of pigments, such as Chl and carotenoids, antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), as well as osmolytes, such as proline. Such enhancement of proline biosynthesis is a typical response of plant cells to osmotic stress and provides an extra compatible osmolyte, storage carbon and nitrogen, scavenge ROS and regulate intracellular pH (Miller et al., 2010; Jaleel et al., 2007). Thus, AVF exposed to salt stress triggered the enzymatic and nonenzymatic antioxidant defense system via various pathways, e.g., arginine and proline metabolism, flavonoid biosynthesis, flavone and flavonol biosynthesis (Fig. 3B).

3.4. Metabolite profile changes responded to high concentration of NaCl

The highest concentration of NaCl (300 mM) induced the most significant and widest alteration of metabolic level in AVF (Fig. S3 and Table S3). It is noteworthy that shikimate-phenylpropanoid pathway compounds (4"-*O*-acetylmyricitrin, delphinidin, kaempferol, limocitrin 3-glucoside, limocitrol 3-glucoside, luteone 7-glucoside, acetylated isoquercitrin, cis-5-caffeoylquinic acid and neochlorogenic acid) and other metabolites (lindleyin and ilicifolinoside A) were significantly decreased compared to the control. However, the upstream metabolites of phenylpropanoid pathway (epigallocatechin, epicatechin, scutellarein 7-neohesperidoside, kaempferol 3-*O*-rutinoside and gallocatechin) were significantly elevated. Quercetin *O*-sophoroside and delphinidin exhibite significant antioxidant activity (Wu, 2011; Afaq et al., 2007). Interestingly, metabolites of ilicifolinoside A, isoquercitrin, trifolin, 4"-*O*-acetylmyricitrin, limocitrin 3-glucoside and kampferol were significant reduced, while these compounds were observed no significant changes at low and medium doses of salt. The reason for their decrease under stress-related aspects probably indicated an insufficient effect of antioxidant defense system because of the excess consumption of secondary metabolites. In plants, the precise roles of most secondary metabolites mentioned above remains unclear; although investigations indicate that some of them plays protective roles during abiotic stresses.

Besides, the levels of allamandin and 20β, 28-epoxytaraxaster-21-en-3β-ol were significantly elevated by 3.13 and 2.05 times, respectively. Phenylalanine metabolism was altered significantly compared to the control. The relative abundance of phenylalanine was elevated 90.9% compared to the control. This aromatic amino acid, a starting compound in the biosynthesis of phenylpropanoids and flavonoids, is a key metabolite for plant development, growth and biological responses to environmental stresses. The abundance of phenylalanine significantly was also observed increased in *Melissa officinalis* under abiotic stress, and this could be due to the enhanced phenylpropanoid pathway (Kim et al., 2012). The elevation of sucrose and transamination-related metabolites induced by such high level of NaCl treatment indicated that both sugars and glutamate-mediated proline biosynthesis were important for controlling salinity-induced osmotic pressure (Kim et al., 2007; Golldack et al., 2014). Under the abiotic stress, sugar utilization may be activated to generate more energy and synthesize more amino acids as defense measures against the external stress (Teo et al., 2011). According to the report, short-term salt stress to *Arabidopsis thaliana* cell cultures induced changes in the phenylpropanoid pathway for lignin production whereas the long-term stress induced changes in sucrose metabolism (Kim et al., 2007). Moreover, the change of aspartic acid can alter carbon fixation in photosynthetic organisms (Liu et al., 2019). The up-regulation of osmolytes and enzymes, and down-regulation of most flavonoids explained that AVF synthesized more energy to resist stress under severe conditions (Chen et al., 2018b). Under such severe salt stress, AVF cells seemed to adopt consistent metabolic responses to prevent hyperammonia through extensive and multiple metabolic pathways.

Severe stress of salt resulted in the perturbation of defense related metabolic pathways and some amino acid related pathways specifically (Fig. 3C). These changes indicated that the highest concentration of salt had a wide range of influence in AVF. Although primary metabolites identified in this study were not as many as secondary metabolites in this study, key intermediates of major metabolic pathways perturbed in carbon and nitrogen metabolism were observed.

3.5. Hierarchical clustering analysis

Clear differentiation was observed in the heat map based on a total of 51 different expressed metabolites, e.g., higher levels of metabolites, such as epigallocatechin, epicatechin, and allamandin in NaCl-treated samples (Fig. 3D) together with lower levels of citric acid/isocitric acid and limocitrol 3-glucoside compared to control. The similarity assessment for clustering was consistent with OPLS-DA analysis. A clear stress-induced trajectory for metabolomic changes was evident indicating the dosage dependence of AVF exposed to salt stress. Colors varying from green to red graphically indicate that the relative contents of metabolites are from low to high. It suggested that the quality of AVF exposed to low level of salt stress were better than other three groups of samples in terms of HCA combined with physiological changes of photosynthetic pigments, osmotic homeostasis, lipid peroxidation product and antioxidative enzymes.

3.6. Global visualization of pathway changes and proposed flavonoid biosynthesis of AVF exposed to salinity

To obtain a global perspective of the metabolic changes (Vogt, 2010), glycolytic pathway, ascorbate and aldarate metabolism, TCA cycle, terpenes biosynthesis, phenylpropanoid biosynthesis, alkaloid biosynthesis and nitrogen metabolism pathways were mapped into a network (Fig. 4). As mentioned above, the chemical interaction between plants and their environment is mediated mainly by the biosynthesis of secondary metabolites. Studies on medicinal plants indicate that flavonoids are ubiquitous secondary metabolites that are generally involved in signaling and antioxidant defensive systems (Blokhina et al., 2003). Furthermore, flavonoids play important roles in the color modification, antioxidative, uvioresistant, antihypertensive and

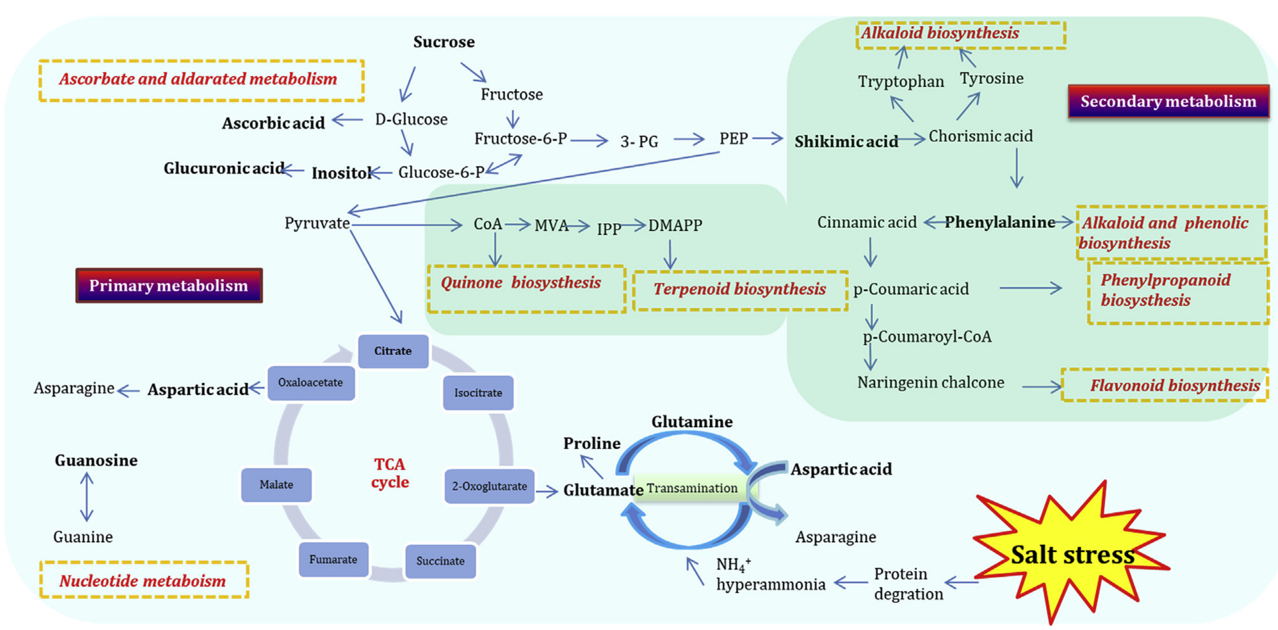

Fig. 4. Schematic diagram of proposed metabolic pathways of AVF exposed to salt stress. Central metabolic pathways (the glycolysis, TCA cycle, shikimate-phenylpropanoid biosynthesis, the amino acid metabolism, nucleotide metabolism, alkaloid biosynthesis, quinone and terpenoid biosynthesis, ascorbate and aldarate metabolism) are shown. The bold font indicates the compounds changed significantly in the experimental comparison. Fructose-6-P: fructose-6-phosphate; Glucose-6-P: Glucose-6- phosphate; CoA: acetyl-CoA; MVA: mevalonate; IPP: isopentenyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; PEP: phosphoenolpyruvate, 3-PG: 3-phosphoglycerate.

antibacterial properties of plants (Pitura and Arntfield, 2018; Wu et al., 2008). In this study, the existence of a potential flavonoid biosynthesis pathway was inferred under salt stress (Fig. 5). Specifically, started from the phenylpropanoid metabolism, flavonoid biosynthesis leads to the major subgroup pathways, namely flavone and flavonol, isoflavonoid and anthocyanin biosynthesis. Many flavonol derivatives are glycosylated to form stable flavonol glycosides through interactions with sugar molecules (Price et al., 1998; Dong et al., 2019). The proposed flavonoid biosynthesis pathway illustrates a paradigm to understand the transcriptional regulation and opens a new avenue to select regulatory gene(s) for metabolic engineering in the salt-tolerance and plant adaptation of AVL.

Since antioxidant metabolites, especially flavonoids, are predominant in AVF extracts, it is necessary to study how flavonoids and their oxidation products (re)distributed through plants and plant cells under oxidative stress, and how organelle-specific biosynthesis affect this (re)distribution. In addition, *Arabidopsis thaliana* is a valuable model species chosen for the pathway enrichment analysis; however, it is prominent in certain families of compounds and absent in others (Grotewold, 2005). Besides, a decrease in antioxidant capacity of phenolics is observed in order of procyanidin dimer, flavanol, flavonol and hydroxycinnamic acids; however only a fraction of plant metabolites have related antioxidant information (Wu, 2011; Manach et al., 2004). Therefore, more experiments remain to be conducted.

4. Conclusion

In this study, LC-MS based metabolomics coupled with multivariate statistical analysis could be a useful tool for determining the potential indicators and discriminating salt-induced AVF. First, PCA and OPLS-DA models showed that the metabolic levels of AVF were clearly dependent on salinity dose. The significantly altered metabolites, especially the secondary ones, could be closely related to the antioxidant property of AVF in response to salt stress. Meanwhile, flavonoid, phenylpropanoid and terpenoid biosynthesis were affected in all AVF samples exposed to salt stress. Such metabolomic changes induced by salt were reported for the first time on AVF and clearly involved a complex network. In summary, this study demonstrated a LC-MS based metabolomics was a powerful approach for understanding the salt-tolerant mechanisms and quality assessment of AVF.

Contributions

CHC and XHL provided resources and obtained grant funding to support the research. CHC, HML and CCW designed and conducted the experiments and performed detections and metabolite extractions. ZXL and LSZ conducted the metabolite analysis and data processing. CHC, HZ, YY, JJS and SYC analyzed the data and wrote the manuscript. All authors read and approved the final manuscript for its publication in Plant Physiology and Biochemistry Journal.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.043.

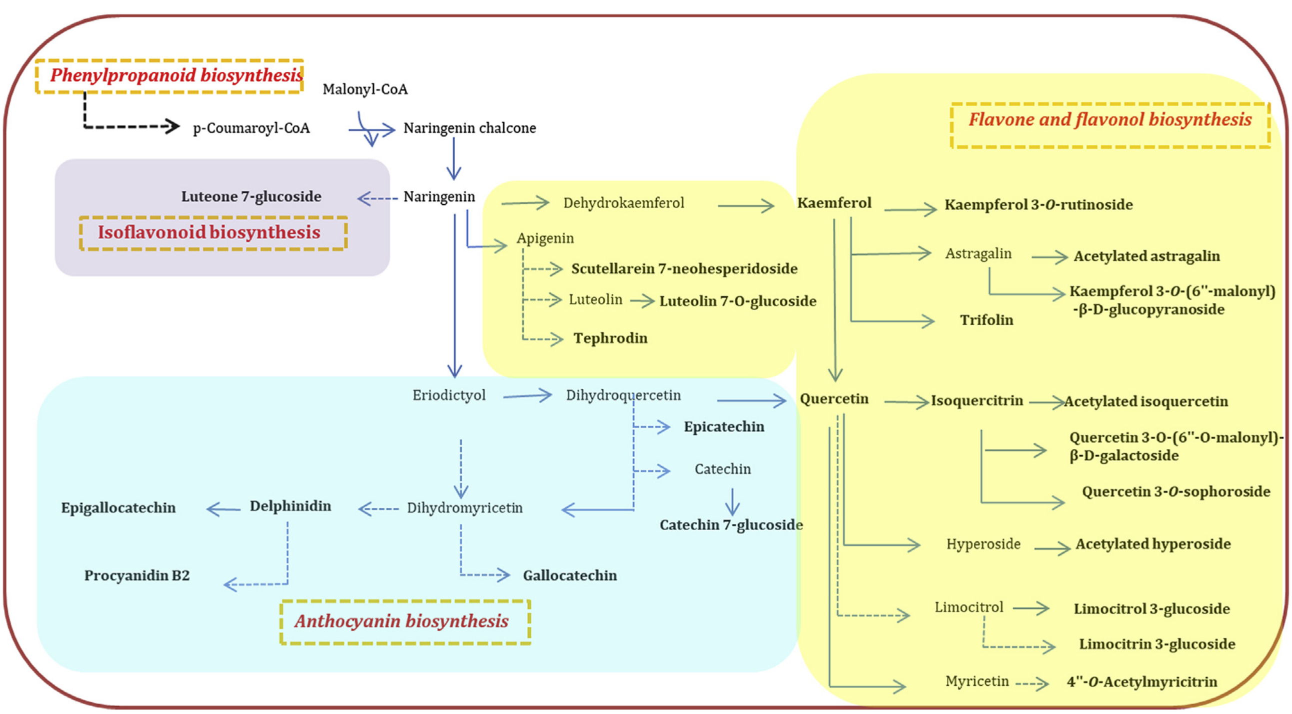

Fig. 5. Schematic representation of proposed flavonoid biosynthesis affected by salt. The bold font indicates the compounds changed significantly in the experimental comparison.

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