METABOLOMICS ANALYSIS OF *ANABASIS APHYLLA* SEEDLINGS UNDER COLD STRESS

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Abstract. Cold severely affects plant growth and metabolism. The analysis of the characteristics of metabolite variations in seedlings under cold stress can help understand the physiological mechanism of *Anabasis aphylla* when responding to stress. In this study, gas chromatography-mass spectrometry technology was used to systematically analyze the changing characteristics of the metabolite profiles of *A. aphylla* under cold stress. A total of 116 types of differential metabolites were stably detected, of which 41 were up-regulated, while 75 were down-regulated. The metabolites of carbohydrates, amino acids, and organic acids formed the majority. The results showed that cold stress had a significant impact on the differential metabolites of *A. aphylla* seedlings. Via enrichment of metabolites such as γ- aminobutyric acid, α- ketoglutaric acid, and 1-kestose, *A. aphylla* could reduce the extent of damage caused by cold stress. The results of this study provide a basis for further analysis of the molecular and physiological mechanisms of the cold stress response of *A. aphylla*.
Keywords: *desert plant, differential metabolite, low temperature, carbohydrate, amino acids, organic acids*

Introduction

Cold is one of the most important abiotic stress factors to affect plant growth, plant development, geographical distribution, and crop yield. For cold-sensitive plants, cold stress from 1 to 10 °C can cause disorders in physiological processes such as water condition, mineral nutrition, photosynthesis, respiration, and metabolism, resulting in severe damage and even death of the plants (Wu et al., 2015). However, after a long evolutionary process, many plants that grow in a relatively low-temperature environment developed a well-established defense mechanism adapting to adverse environmental conditions. Studies have shown that the adaptation of plants to cold is related to the regulation of several metabolic pathways, particularly in the regulation of carbon metabolism, photosynthesis, and membrane lipids (Li, 2015). In Junggar basin of China, some desert plants have low temperature germination feature during snow-melting during the snow-melting period of early spring, these desert plants include *H. ammodendron*, *Haloxylon Persicum*, *Anabasis elatior*, *Lepidium apetalum* and *Anabasis aphylla* (Huang et al., 2003; Han et al., 2011; Peng et al., 2018). In the field, we found that germination speed of *Anabasis aphylla* was higher than other species, while its seedling regeneration probability is very low. Therefore, study on regeneration disorder mechanism of *A. aphylla* seedling is necessary for desert vegetation protection.

Anabasis aphylla is a semi shrub belonging to the Chenopodiaceae family. It is an important type of plant community for windbreak, sand fixation, and for the protection of the local ecological environment as well as surrounding land resources (Wang et al., 2017). The snow-melting period at Junggar basin happens within a 10-day period in March. During this time, the average daytime temperature is above 0 °C and the

maximum temperature is maintained at 7-14 °C, while the night-time minimum temperature drops below 0 °C (Zhou et al., 2009). Consequently, the ground surface is in a repeated freeze-thaw state during the snow melting period (Gornish et al., 2015). During field observations, we discovered that a large quantity of *A. aphylla* seedlings had already germinated under the snow at the snow-melting period, which indicates that *A. aphylla* has already adapted to the cold. In recent years, comparative transcriptomics, proteomics, and metabolomics studies combined with mutation analysis of cold resistance have significantly increased our understanding of the complex network of signaling pathways of the cold training process and the necessary molecular changes (Janda et al., 2014). Via proteomics studies, we found that the adaption of *A. aphylla* to cold was primarily achieved through changes in the energy metabolism. The proteins associated with the energy metabolism could be identified, such as the fructose-bisphosphate proteins, malate dehydrogenase proteins, citrate synthase proteins, oxygen-evolving enhancer protein, and photosynthetic proteins. However, the proteomic study mainly focused on the classification and identification of the associated proteins as well as on the analysis of the interaction between proteins and the function of the proteins, it cannot intuitively describe the physiological state of the plants under stress, while a plant metabolomics study is conducive to the overall study of the response of a biological system to changes in genes or the environment. The overall changes of the metabolites can directly reflect the physiological and pathological states of the organism and help to uncover complex plant stress response mechanisms (Teng et al., 2011). At present, metabolomics studies of plants under stress have been widely applied in species such as *Arabidopsis thaliana*, rice, potatoes, and *Festuca arundinacea* (Tarryn et al., 2016; Lin et al., 2017; Aliferis and Jabaji, 2012; Li et al., 2016). Studies have shown that under environmental conditions such as drought, salinity, or extreme temperature, the plant amino acid content increased significantly (Lanzinger et al., 2015; Annick et al., 2016), while the contents of several carbohydrates such as sucrose, lactose, and trehalose were also significantly increased induced by stress (Min et al., 2014; Wingler and Roitsch, 2008). Further studies have revealed that under adverse conditions, the contents of organic acids involved in the tricarboxylic acid (TCA) cycle showed increasing characteristics of metabolite changes at an early stage, while decreasing characteristics at a later stage (Widodo et al., 2009). However, a metabolomics study of *A. aphylla* seedlings under cold stress has not been reported yet. In this study, metabolomics technology was utilized for an analysis of the differential metabolites of *A. aphylla* seedlings under cold stress, thus providing a theoretical basis to explore the complex metabolic processes as well as their products and the secondary metabolic network structure.

Materials and methods

Plant materials and stress treatments

In the northwestern of China, it is mainly distributed around the piedmont alluvial fan, the Gobi Desert, and sand dune gravels areas (*Fig. 1*). *A. aphylla* seeds were obtained from the southern edge of the Gurbantünggüt Desert (E84°49′-85°23′, N45°21′-45°40′) in Xinjiang, China. All collected *A. aphylla* seeds were sterilized by soaking them in 10% H_2O_2 for 30 min, after which they were rinsed with distilled water. For the control treatment, the sterilized seeds were germinated on filter paper discs soaked with distilled water in Petri dishes at room temperature and a 16-h light/8-h dark

photoperiod until seedlings were approximately 3 cm in length. For the cold treatment, 3-cm-long seedlings were kept in a -3 °C growth chamber for 12 h each day for one week. For each treatment, 3-g seedlings were selected as samples and quickly frozen in liquid nitrogen individually and stored at -80 °C for metabonomics analyses. Both treatments had eight biological replicates.
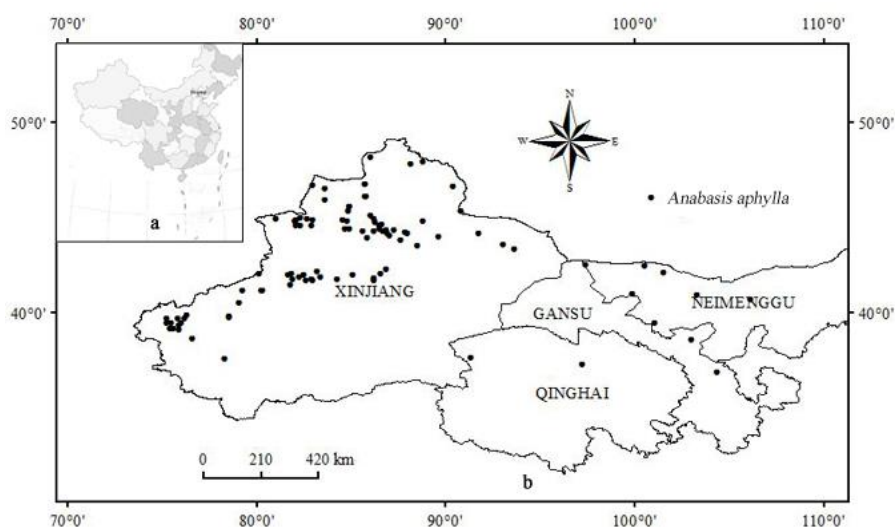
Figure 1. *Distribution of A. aphylla in northwestern China. (a) Map of China; (b) distribution of A. aphylla in northwestern China*

Sample pretreatment

Precision weighed 60 mg *A. aphylla* samples into 1.5 mL centrifugal tube, after adding 360 μL cold methanol and 40 μL internal standard (L-2-chlorine-phenylalanine, 0.3 mg mL^{-1}, methanol configuration) and homogenate (ground in Tissuelyser-48 (60 Hz, 2 min)). Then extracted by homogenate ultrasound at 30 min and sequentially added 200 μL chloroform and 400 μL water, again 30 min extracted by ultrasound and centrifuged for 10 min (14000 rpm, 4 ℃) at cold. Thereafter, collected 500 μL supernatant placed into glass derivative bottle, then dried with rapid centrifugal concentrator and added 80 μL methoxy pyridine hydrochloride solution (15 mg. mL^{-1}), after 2 min vorticity shock later, carried on oximation reaction 90 min in 37 ℃ shock incubator, then removed and 80 μL of N,O-bis trifluoroacetamide (BSTFA) containing 1% trimethylsilyl chloride (TMCS) derivative reagent and 20 μL n-hexane were added, after 2 min of vortex oscillation, reaction 60 min at 70 ℃. Later the samples were taken and placed at room temperature for 30 min for gas chromatography-mass spectrometry (GC/MS) metabolomics analysis (Lin et al., 2017).

GC/MS analysis

Each 1 μL aliquot of the derivatized solution was injected in split less mode into the Agilent 7890A-5975C GC-MS system (Agilent, USA). Separation was carried out on a non-polar DB-5 capillary column (30 m × 250 μm I.D., J&W Scientific, Folsom, CA), with high purity helium as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. The GC temperature programming began at 60 °C, followed by 8 °C min^{-1} oven temperature

ramps to 125 °C, 4 °C min^{-1} to 210 °C, 5 °C min^{-1} to 270 °C, and 10 °C min^{-1} to 305 °C, and a final 3 min maintenance at 305 °C. The electron impact (EI) ion source was held at 260 °C with a filament bias of -70 V. Full scan mode (m/z 50−600) was used, with an acquisition rate of 20 spectrum/second in the MS setting.

Quality control (QC) sample

QC sample was prepared by mixing aliquots of the all samples to be a pooled sample, and then analyzed using the same method with the analytic samples. The QCs were injected at regular intervals (every eight samples) throughout the analytical run to provide a set of data from which repeatability can be assessed.

Data analysis

The acquired MS data from GC-MS were analyzed by ChromaTOF software (v 4.34, LECO, St Joseph, MI). Briefly, after alignment with Statistic Compare component, the CSV file was obtained with three-dimension data sets including sample information, retention time-m/z and peak intensities, and the internal standard was used for data quality control (reproducibility). After internal standards and any known pseudo positive peaks, such as peaks caused by noise, column bleed and BSTFA derivatization procedure, were removed from the data set, and the peaks from the same metabolite were combined, the detectable metabolites of samples in GC-MS were 293 in total. The data set was normalized using the sum intensity of the peaks in each sample.

The data sets resulting from GC-MS were separately imported into SIMCA-P + 14.0 software package (Umetrics, Umeå, Sweden). Principle component analysis (PCA) and (orthogonal) partial least-squares-discriminant analysis (PLS-DA) were carried out to visualize the metabolic alterations among experimental groups, after mean centering and unit variance scaling. Variable importance in the projection (VIP) ranks the overall contribution of each variable to the (PLS-DA) model, and those variables with VIP > 1.0 are considered relevant for group discrimination.

In this study, the default 7-round cross-validation was applied with 1/seventh of the samples being excluded from the mathematical model in each round, in order to guard against overfitting.

Metabolite identification

All of the differentially expressed compounds in treated group were selected by comparing the compounds in the treated group with the control using the multivariate statistical method and Wilcoxon-Mann-Whitney test. Metabolites with both multivariate and univariate statistical significance (VIP > 1.0 and $p < 0.05$) were annotated with the aid of available reference standards in our lab and the NIST 11 standard mass spectral databases and the Feinh databases linked to ChromaTOF software. The similarity of more than 70% can be considered as reference standards.

Results

Chromatograms of cold stress and control treatment

Figure 2 shows the result of overlapping GC-MS total ion current (TIC) of the quality control samples (QC). This was a preliminary study of the reproducibility of the method.

Figure 2 shows that the reproducibility of the retention time and the corresponding intensity of *A. aphylla* QC sample mass peaks were very good, indicating the whole analysis method to be stable and reliable, making it useful for subsequent analysis. The typical TIC diagram of each set of sample is also presented (*Figs. 3* and *4*).
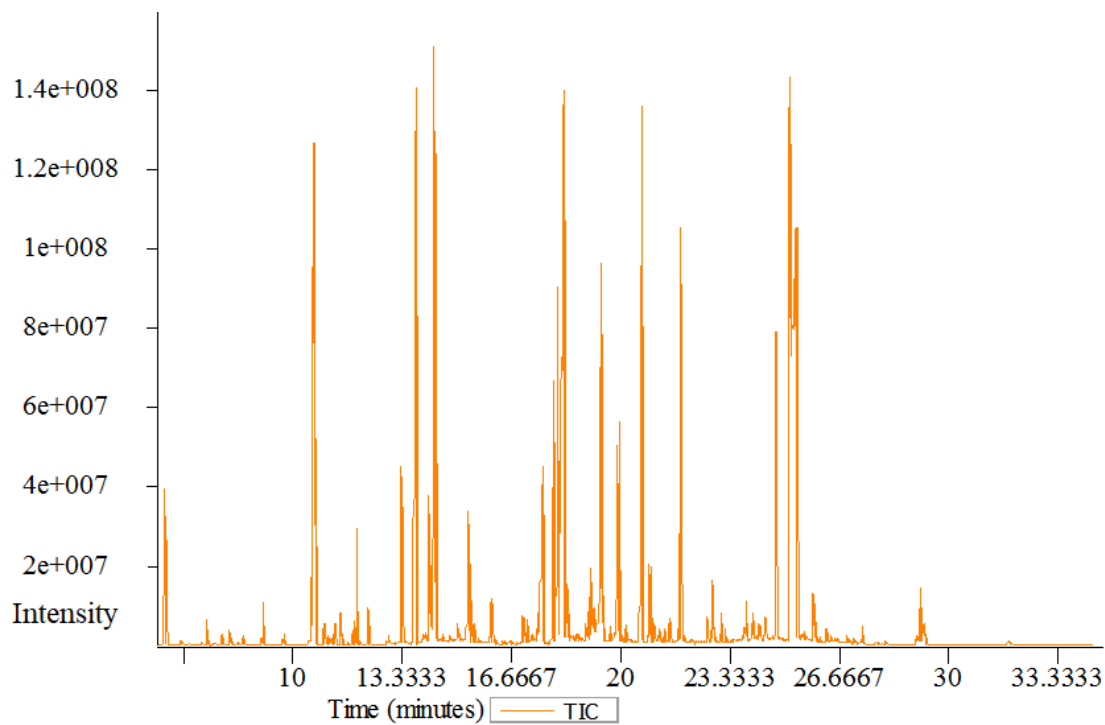

Figure 2. *Overlap total ion chromatography (TIC) of the quality control samples (QC)*
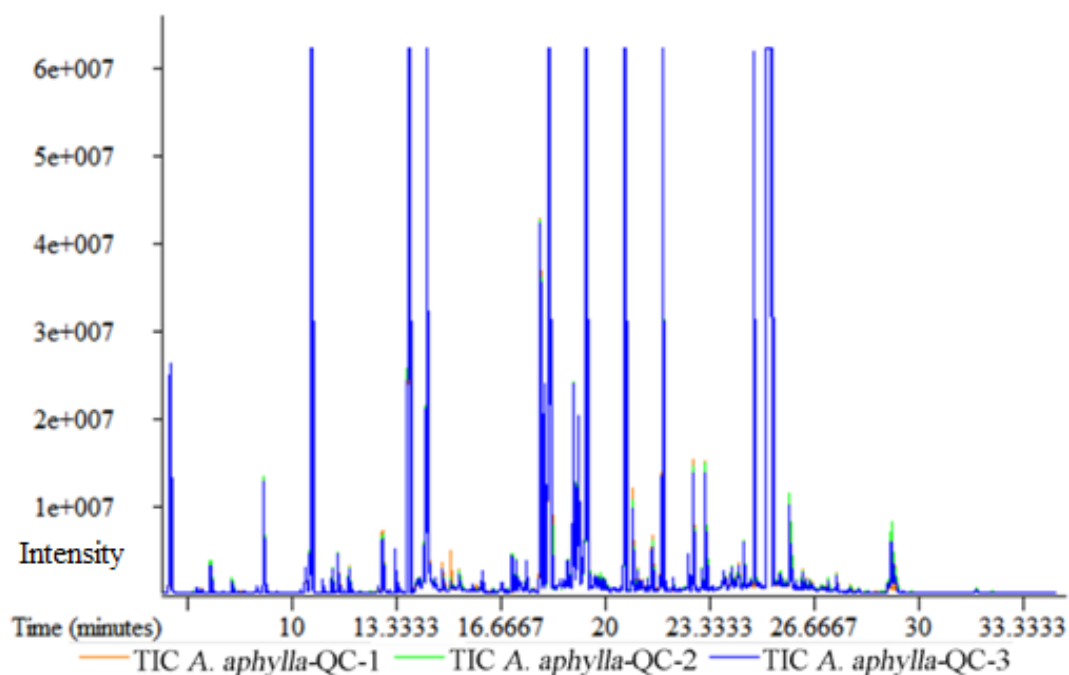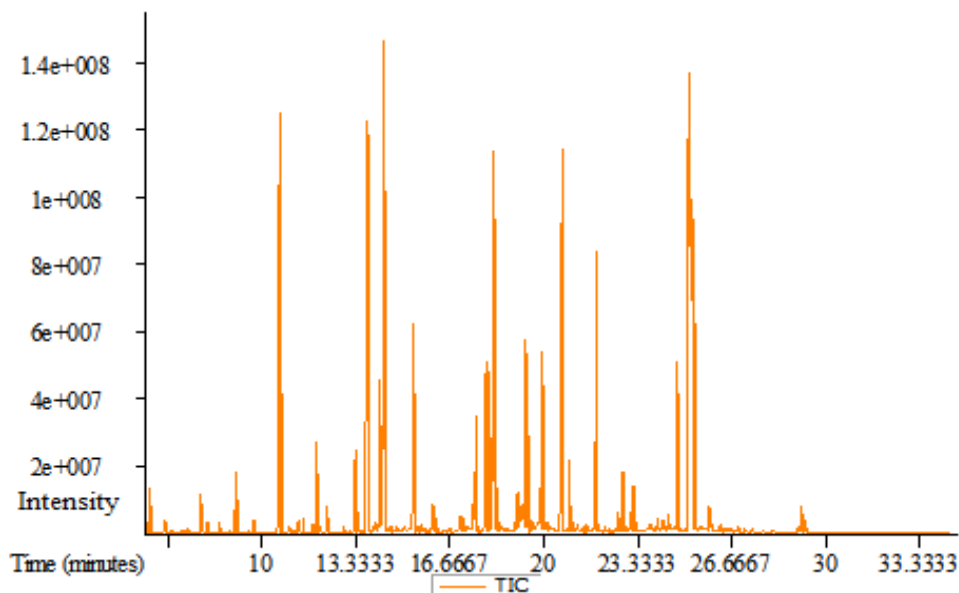

Figure 3. *Total ion chromatography (TIC) of A. aphylla from cold stress samples*

Figure 4. *Total ion chromatography (TIC) of A. aphylla from control treatment samples*

PCA, PLS-DA, and OPLS-DA analysis of A. aphylla seedlings under cold stress

PCA, PLS-DA, and OPLS-DA analyses were conducted on the *A. aphylla* samples in both the cold treated group and the normal temperature control group, and the scores of the two groups are shown in *Figures 5, 6,* and *7*. The PCA score diagram shows that the results of the eight duplicates from the cold treated group and the control group were well divided into two groups and were clustered together respectively, indicating that the error of the pretreatment method and the instrument analysis system were relatively small and that the reliability of the data was high, indicating that the method can be used for subsequent analysis. Using PLS-DA and OPLS-DA for further analysis of the GC-MS data from the control group and the treatment group, the results showed that the cold treated *A. aphylla* seedlings were clearly separated from the control group. Both the fitting degree and the predictive value of each model indicated good reliability of the models and the metabolites of the *A. aphylla* seedlings under cold stress showed significant changes compared to the control group.
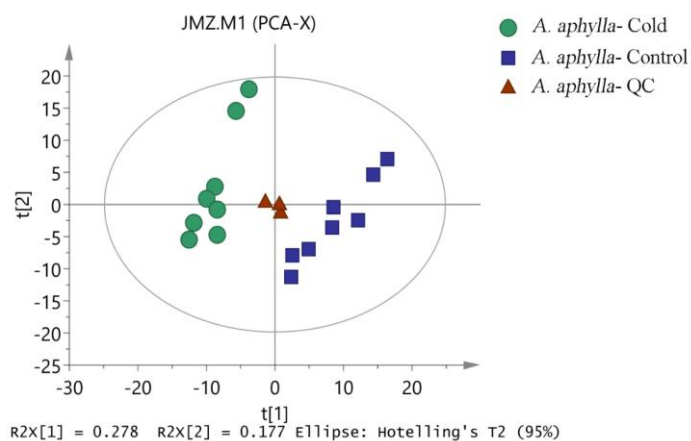
Figure 5. *PCA scatter plot of cold stress and control treatment A. aphylla samples*
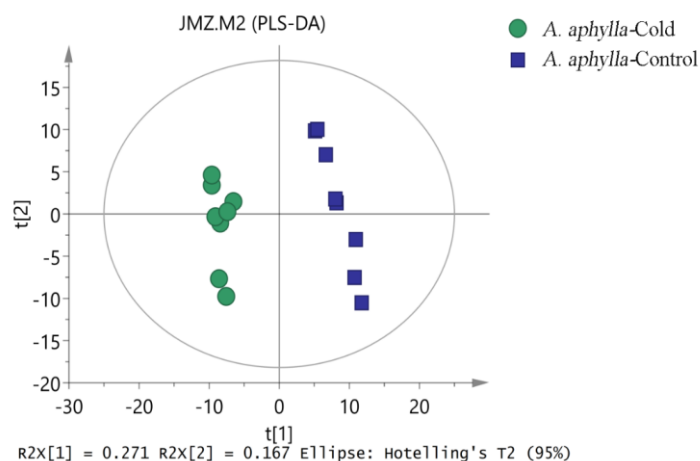
Figure 6. PLS-DA scatter plot of cold stress and control treatment A. aphylla samples
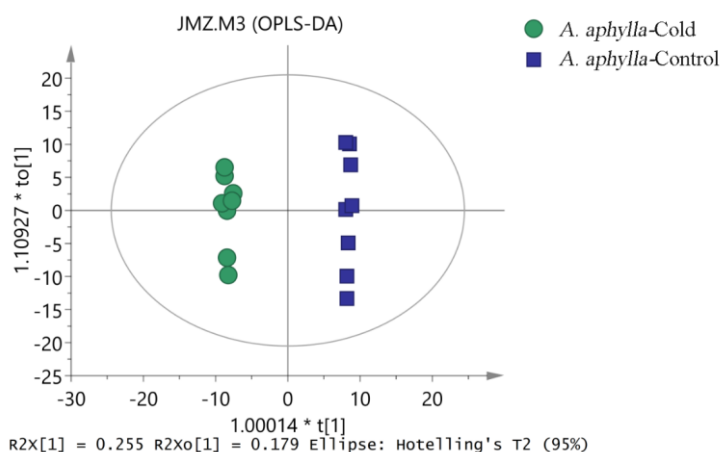
*Figure 7. OPLS-DA scatter plot of cold stress and control treatment A. aphylla samples*

Differential metabolite analysis under cold stress

293 metabolites were detected from the samples of the treated group and the control group, of which 116 metabolites with VIP > 1, $p < 0.05$ were screened out as differential metabolites. Among these, 41 were up-regulated, while 75 were down-regulated. The mass-to-charge ratio and the abundance of the characteristic fragmentation pattern of each chemical compound were compared to the standard fragmentation pattern in the NIST database and the Feihn metabolomics database to obtain the name of metabolites. The detected metabolites mainly included organic acids, amino acids, carbohydrates, alkaloids, phytohormones, organic bases, quinones, flavonoids, and other substances. Among these, the most abundant identified materials were amino acids, organic acids, and carbohydrates, including 21, 15, and 8 different types of substances in each category, respectively (*Table 1*); therefore, the materials in the three categories were the main focus for subsequent discussion and analysis. The metabolites in the focused discussion are listed in *Figure 8*. Some of these metabolites are associated with the ornithine cycle and the TCA cycle, and these identified metabolites are labeled in green in the diagram (*Figs. 9* and *10*).

Table 1. *Identified sugars, amino acids and organic acids species in A. aphylla seedlings under cold stress*

| Metabolites	R.T. (min)	Quant mass	VIP value	FC (cold/control)	*p* value (*t* test)	Up/down
Saccharide						
Ribose	16.05798	307	1.01775	2.05672	0.02891	↑
1-Kestose	17.92873	438	1.35691	3.31756	0.00054	↑
D-Talose	19.40036	160	1.44748	7.50492	0.0001	↑
Tagatose	18.96745	307	1.45601	8.46888	0.00007	↑
Sedoheptulose	24.60103	333	1.10726	0.65934	0.02607	↓
Trehalose	26.08805	332	1.19952	0.80285	0.00735	↓
Cellobiose	22.49745	308	1.32735	0.14212	0.00067	↓
Melezitose	24.79442	199	1.48625	0.4118	0.00005	↓
Amino acids						
Isoleucine	10.94246	158	1.49586	1.69923	0.00006	↑
γ-aminobutyric acid	14.30521	304	1.40075	1.23911	0.00073	↑
Beta-Glutamic acid	14.03546	217	1.36979	1.50227	0.00081	↑
Phenylalanine	15.50448	91	1.30639	1.38467	0.00572	↑
Cycloleucine	15.18698	156	1.18226	2.17144	0.00577	↑
Proline	11.03825	142	1.12251	1.22459	0.03674	↑
Asparagine	13.3133	128	1.45597	0.5259	0.00018	↓
Sarcosine	8.40767	116	1.4032	0.22582	0.00083	↓
Citrulline	18.34069	256	1.31285	0.76186	0.00111	↓
Alanine	7.82152	116	1.26208	0.40557	0.00501	↓
Histidine	19.9032	317	1.18169	0.81064	0.00984	↓
Ornithine	15.34038	316	1.14275	0.75554	0.00987	↓
L-homoserine	13.17861	218	1.17977	0.75721	0.02349	↓
Aspartic acid	14.14461	232	1.06868	0.73052	0.02481	↓
O-acetylserine	12.40576	174	1.06425	0.81941	0.03432	↓
Threonine	12.27561	293	1.08858	0.88137	0.03566	↓
Methionine	13.51492	256	1.54807	0.65116	0.00001	↓
Trans-4-hydroxy-L-proline	11.21533	140	1.55369	0.25781	0	↓
L-Cysteic acid	14.40056	241	1.39356	0.65681	0.00074	↓
L-kynurenine	23.18388	322	1.22941	0.49746	0.00801	↓
Xanthurenic acid	22.55864	304	1.07547	0.47683	0.01136	↓
Organic acid						
Malonic acid	12.46042	307	1.44983	2.35443	0.00009	↑
Saccharic acid	21.47683	333	1.44611	2.00206	0.00018	↑
Tartaric acid	19.97148	275	1.34254	1.63333	0.00047	↑
Palmitic acid	21.43681	313	1.30646	1.20218	0.00616	↑
Alpha-ketoglutaric acid	14.79833	304	1.24162	1.74297	0.00657	↑
4-hydroxybutyrate	10.04681	233	1.23565	1.46819	0.00927	↑
3-Hydroxypropionic acid	8.52303	177	1.19535	1.32059	0.01215	↑
3,4-dihydroxybenzoic acid	7.5046	313	1.03061	2.13249	0.02131	↑
Benzoic acid	10.32072	179	1.02761	2.22846	0.02507	↑
Mucic acid	17.14024	333	1.56701	1.53627	0	↑
Gluconic acid	20.87028	333	1.37666	0.47108	0.00025	↓
Citramalic acid	13.46879	247	1.38831	0.7327	0.00092	↓
Glutaric acid	12.58547	261	1.31443	0.52929	0.00155	↓
Lactic acid	7.1533	190	1.16019	0.49001	0.01089	↓
Gallic acid	9.51771	370	1.19889	0.75783	0.01563	↓
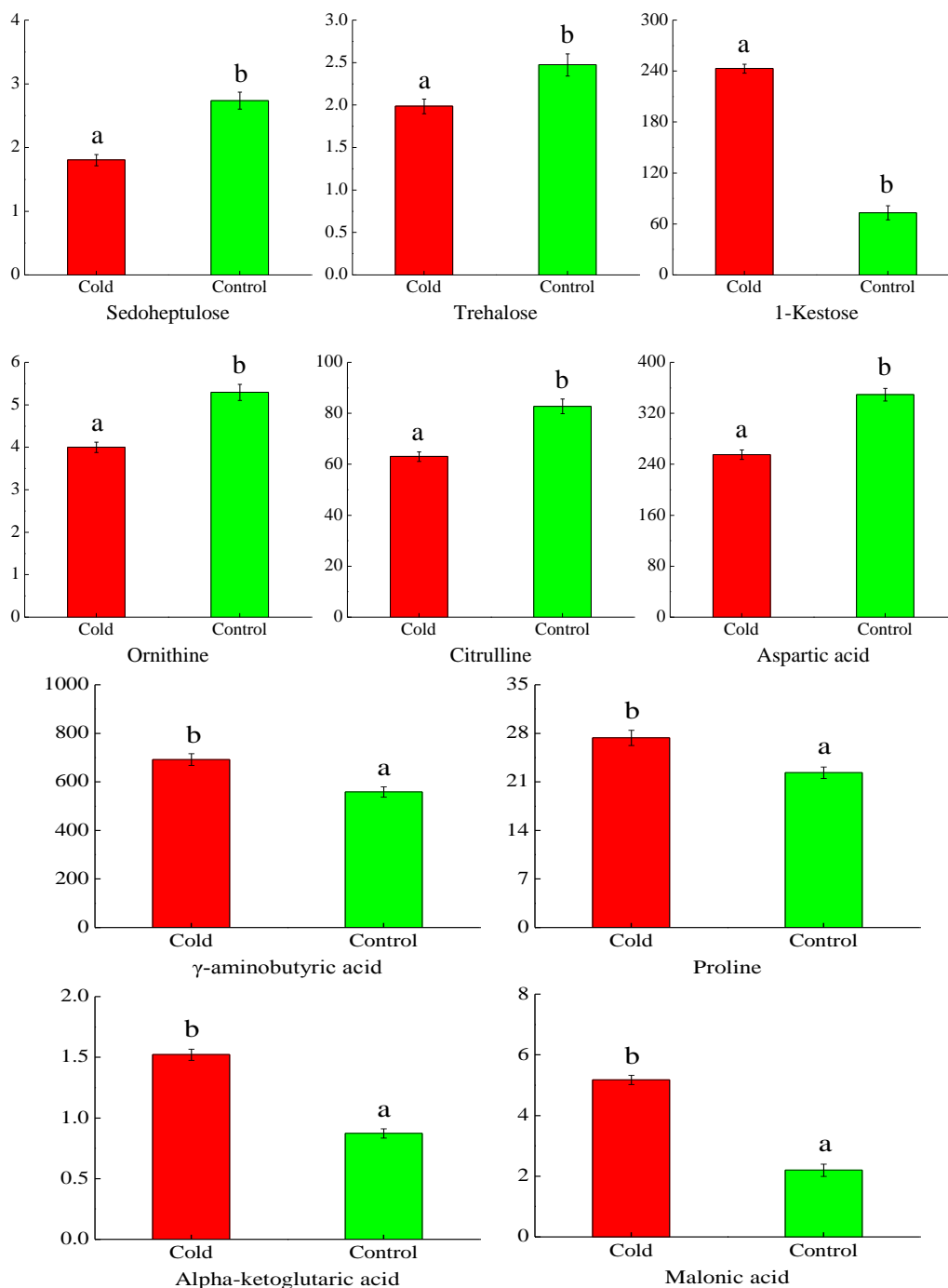
Figure 8. *Sedoheptulose, trehalose, 1-Kestose, ornithine, citrulline, aspartic acid, 4-aminobutyric acid, proline, alpha-ketoglutaric acid and malonic acid content in A. aphylla under cold stress and control treatment*

Discussion

During growth and development, plants are often subjected to stresses such as environmental stress (temperature and moisture), biological stress (diseases, pests, and weeds), and nutritional deficiency stress (nitrogen, phosphorus, and potassium) (Hans et al., 2006). Plants can defend themselves against biotic and abiotic stress factors by

regulating their metabolic network to induce the production of a series of specific metabolites as response (Teng et al., 2011). Metabolomics can qualitatively and quantitatively analyze all metabolites within a specified biological sample under given conditions (Oliver et al., 2000). Therefore, using metabolomics to analyze changes of metabolites to study the metabolic regulation of plants under stress has gained increasing attention (Vicent et al., 2013).
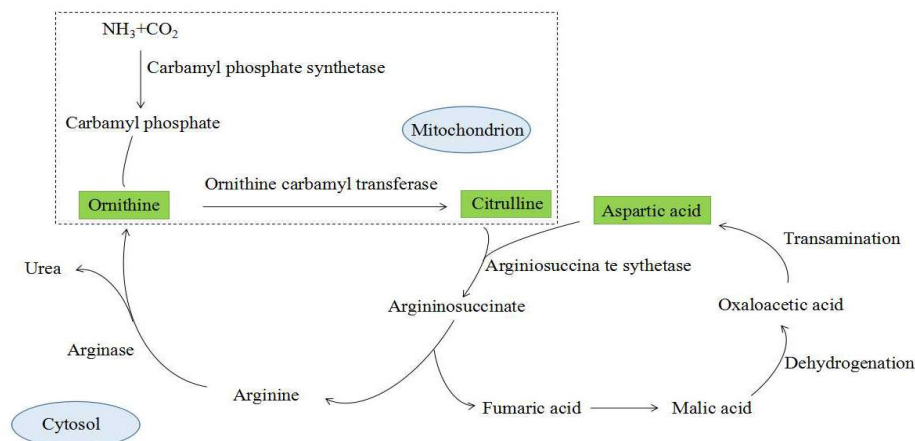
Figure 9. *Identified three amino acid metabolites are involved in the ornithine cycle*
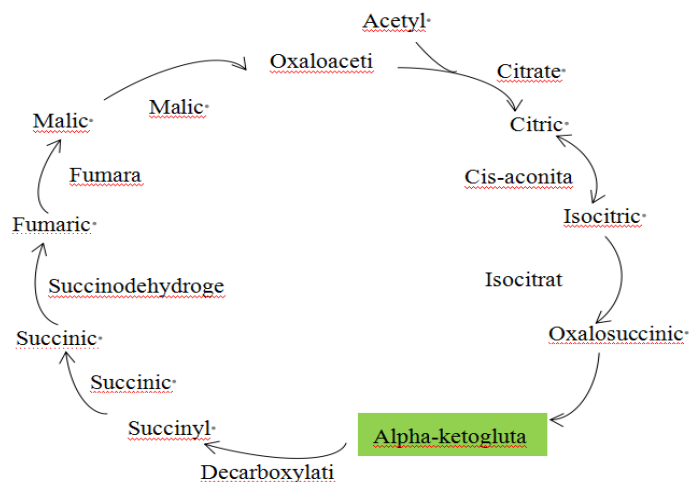
Figure 10. *Identified organic acid metabolites are involved in the TCA cycle*

Effects of cold on carbohydrate metabolites of A. aphylla seedlings

Carbohydrates play an important role in the life process, and it is the main source of energy required for all life to maintain alive. Our study on differential metabolites of *A. aphylla* seedlings under cold stress showed that carbohydrates played very important roles in the response of *A. aphylla* seedlings to cold stress. Among these, tagatose, D-talose, ribose, and 1- kestose accumulated in the seedlings under cold stress, while melezitose, cellobiose, trehalose, and sedoheptulose showed a trend of down-regulation. D- talose is an aldohexose, tagatose is a sweetening agent, often used in food and drug

industry, and cellobiose is the hydrolysis product of cellulose, which also forms the basic structural unit of cellulose. Many studies have focused on cellobiohydrolase; however, the significance of cellobiose in plants has been rarely reported (Fan et al., 2018). Melezitose has been identified from the nectar secretion of plants such as the North American pinon pine, the Douglas fir, leguminous plants, and the poplar (Aderkas et al., 2012; Tian et al., 2018). Some researchers have also indicated that melezitose was once isolated from the leave secretion of the Yunnan pine. It is mostly used in the food industry (Mu and Lu, 1984). Therefore, only sedoheptulose, trehalose, and 1-kestose, which play important roles in plants, were discussed here. Sedoheptulose belongs to the heptoses and is an important primary and secondary metabolite in the pentose phosphate cycle, photosynthesis, other carbohydrates synthesis, and the biosynthesis of shikimic acid and aromatic ammonia (Xie, 2009). Studies reported that as the primary product of plant photosynthesis, sedoheptulose may be converted from 1,7-diphosphate sedoheptulose produced from carbon source through the Calvin cycle and the aldolase reaction at the end. This is not only the main storage form of carbon in plants, it also plays a key role in the carbon balance of plant. Sedoheptulose in plant mainly exists in the forms of 7-phosphate sedoheptulose and 1,7-diphosphate sedoheptulose and is involved in plant photosynthesis (Keith, 2017). Lee et al. have described sedoheptulose in the leaves of *Cornus oficinalis* of Comaceae, and it was also found in *Sedum spectabile* of Crassulaceae (Lee et al., 1989; Tolbert and Zill, 1954). Trehalose is a non-reducing disaccharide, widely distributed in organisms. Genome sequencing results showed that trehalose biosynthesis genes are widespread in plants (Lunn et al., 2014). In most plants however, the content of trehalose is very low, indicating that trehalose may not directly participate in the plant metabolism, but may be involved in signal regulation, metabolic regulation, and gene expression regulation processes (Paul et al., 2008). The synthesis, decomposition, and regulation of trehalose are important components of the plant sugar signal transduction pathway (Chen et al., 2014a). Studies have found that trehalose has a significant effect on the protection of *Volvariella volvacea* under cold. Furthermore, a large number of studies have shown that in stress conditions such as freezing and refrigeration, trehalose can prevent protein denaturation and protect cells from damage (Wang et al., 2008). However, Veluthambi et al. suggested that too much trehalose can severely inhibit the growth of plants. This would be because the excessive amount of trehalose impairs sugar utilization and reduces phloem transport processes (Veluthambi et al., 1982). In this study, in the seedlings of *A. aphylla*, trehalose was found to be down-regulated under cold stress, which is similar to the results of previous studies (Maria et al., 2013). 1-kestose is a linear inulin-type fructan and fructan is the most widely distributed biopolymer in nature. The fructan metabolism is an important means for regulating environmental stress in plants and the accumulation of fructan in plants can enhance their stress resistance (Wu et al., 2011). Studies have shown that fructan is mainly distributed in plant cell vacuoles. Under cold or cold conditions, fructan in the vacuoles is de-polymerized and releases large amounts of free fructose, which can reduce the water freezing point in plant tissues, thus acting as a vacuole osmotic buffer and cold protective agent in plants (Wang, 2000). Scholars have reported that the winter wheat varieties with strong cold resistance have higher fructan contents; after the introduction of the levansucrase gene that synthesizes fructan into sugar beet and tomato, the obtained transgenic plants not only exhibited cold resistance, but also expressed functions of drought resistance, salt tolerance, and improved fruit quality (Tognetti et

al., 1989; Sevenier et al., 1998; Wang et al., 2004). The contents of the metabolites sedoheptulose and trehalose detected in this study under cold conditions were 0.66 times and 0.80 times that of the contents under normal temperature, respectively. However, under cold, the content of 1-kestose was 3.32 times that of the content under normal temperature, indicating that cold stress inhibits the photosynthesis and carbohydrate metabolism in *A. aphylla* seedlings but does not affect the normal growth of plants. *A. aphylla* may adapt to a cold environment by accumulating 1-kestose or other carbohydrate metabolites.

Effects of cold on amino acid metabolites of A. aphylla seedlings

During the metabolic regulation process of responding to abiotic stress, the synthesis and decomposition of metabolites in plants are in a delicate dynamic state of equilibrium in order to swiftly respond to the changes of the external environment and to maintain the normal metabolism of the plant (Zhou et al., 2017). In addition to carbohydrate substances, the contents of amino acids in *A. aphylla* under cold stress also differed. As the basic components of the biological functional macromolecule proteins and the important nitrogen metabolites in plants, amino acids play important roles in the nitrogen metabolism and in plant stress resistance (Song et al., 2012). By comparing *A. aphylla* seedlings under cold treatment to the control under normal temperature, a large number of amino acids and amino acid metabolic intermediates revealed to be produced under cold stress. Among these, only few amino acids were up-regulated such as isoleucine, γ-aminobutyric acid, proline, cycloleucine, phenylalanine, and β- glutamic acid, while asparagines, L-homoserine, O-acetyl serine, methionine, 4-hydroxybutyrate, cysteic acid, threonine, aspartic acid, ornithine, histidine, citrulline, alanine, sarcosine, and intermediate metabolites of tryptophan, kynurenine, and xanthurenic acid were down-regulated.

γ-aminobutyric acid (GABA) is a non-protein amino acid found in many organisms and it is an important component of the free amino acid pool, which is widespread in every part of the plant (Yang et al., 2014). Under normal growth conditions, the GABA content is relatively low in plant tissue; however, under conditions of environment stress, physiological stress, or insect pest and other adverse conditions, the concentration of GABA will sharply increase. This reduces the damage of adverse conditions to plants, while enhancing plant resistance under stress through the stabilization of the cell membrane structure, reduction of activity oxygen damage, and regulation of biological macromolecule synthesis (Fait et al., 2008). Two major ways exist to synthesize and transform GABA in plants: glutamic acid decarboxylation and polyamine degradation. Glutamic acid metabolism plays an important role in plant growth, while GABA, as a glutamic acid enzymatic product, also plays an integral role in the life cycle of plants (Francisco et al., 2014). A study by Shelp et al. (2006) reported that plant endogenous GABA could stabilize and protect the thylakoid membrane against freeze damage *in vitro*, while exogenous GABA treatment could reduce the occurrence of cold damage and reduce the damage to plants. Proline is one of the components of plant proteins and it is widely distributed in plants in a free state. When the ambient temperature is lowered, the accumulation of proline plays an important role for protecting the stability of the cytoplasmic membrane of plants and for scavenging free radical damage (Matysik et al., 2002). Therefore, many researchers regard the content of proline in plants as one of the indexes to determine the cold hardiness of plants. It is generally assumed that the accumulation of proline under stress

may be a great help for plants to recover after stress relief since proline can be used as both carbon source and nitrogen source, and it can also provide a large amount of energy for cell recovery (Sun et al., 2017). Many studies have shown that under cold, proline contents in plants such as *Festuca arundinacea*, tea tree, *Cinnamomum camphora*, and *Hibiscus syriacusplants* showed a significant increase compared to control groups, indicating that plants can adjust their proline content in response to adverse environmental conditions of cold (Li et al., 2016; Yang et al., 2004; Wen, 2017; Li and Zou, 2016).

The ornithine cycle in plants plays an important role in their adaptation to cold stress. Except for a few free amino acids that are increased during the accumulation, the majority of the amino acids were down-regulated under cold stress. Aspartic acid is the precursor of the biosynthesis of amino acids such as threonine, isoleucine, and methionine, as well as purine and pyrimidine. Ornithine, citrulline, and aspartic acid participate in the *in vivo* biosynthesis process of ornithine in plants. During this process, arginine is hydrolyzed into ornithine and urea via arginase; the carbamoyl group on the carbamyl phosphate is transferred onto ornithine, thus forming citrulline by ornithine transcarbamylase. Citrulline is then transported from the mitochondria to the cytoplasm and is condensed with aspartic acid to form argininosuccinic acid. Argininosuccinic acid is decomposed into arginine via argininosuccinase, releasing fumaric acid. The fumaric acid that is generated from the ornithine cycle not only connects the ornithine cycle and the citric acid cycle, it can also be decomposed by urease to produce ammonia, which can be used in the synthesis of other nitrogen-containing compounds such as nucleic acids, hormones, chloroplast, heme, amine, and alkaloids (He et al., 2014). The results of this study showed that cold stress significantly affected the amino acid metabolism, and weakened the ornithine cycle process, while differential metabolites such as isoleucine, β-glutamic acid, phenylalanine, cycloleucine, γ-aminobutyric acid, and proline were significantly up-regulated, indicating that *A. aphylla* seedlings had adapted to the cold environment via accumulation of these amino acids. The reason for the decreases in the contents of the majority of amino acids might be due to an inhibition of the metabolism under cold, resulting in amino acid decomposition.

Effects of cold on organic acid metabolites of A. aphylla seedlings

Cold stress induced the accumulation of a large quantity of organic acids in *A. aphylla* seedlings. The content of organic acids directly reflects plant growth and metabolic activity (Zhao et al., 2013). Malonic acid, also known as maleic acid, is a succinic acid analogue that can be used as a competitive inhibitor of succinate dehydrogenase, thus inhibiting the corresponding respiratory process (Ye et al., 2012). In our study, the content of malonic acid in *A. aphylla* seedlings under cold stress was increased by 2.35 times compared to the control group. This indicates that the respiration of *A. aphylla* seedlings was decreased and the metabolic activity was reduced. Some studies have also reported that plant respiration was inhibited under the treatment of high concentration of malonic acid. At the same time, the consumption of soluble solids was decreased, enabling plants to slow their growth under stress to survive the hazards caused by an adverse environment (Guo et al., 2016).

Abiotic stress induces the production of large quantities of reactive oxygen species in plants, which may activate proteases and induce excessive production of NH_4^+ in cells. To avoid surplus of NH_4^+, plants usually convert excessive NH_4^+ into glutamic

acid/glutamine via transamination or convert it into α-ketoglutaric acid via glutamate dehydrogenase, entering the TCA cycle (Dai et al., 2010). Our results indicate that compared to the control group, the content of α-ketoglutaric acid was significantly increased under cold stress in the TCA cycle metabolism, indicating that the cold does not inhibit the TCA cycle, and that the energy metabolism was not compromised. Since the TCA cycle is the hub of communication among the metabolisms of carbohydrates, lipids, and amino acids, cold stress cannot only affect carbohydrate, lipid, and protein metabolisms, but can also directly affect the synthesis of several amino acids. Among the identified 15 organic acids, the relationships between many of these organic acids and plant stress resistance remain unknown. Tartaric acid is a dicarboxylic acid, and it exists in plants such as grapes, bananas, and tamarind, and is commonly used as antioxidant synergist, retarder, and chelating agent. Some studies suggest that tartaric acid is a good antidote, which is important for the germination and growth of plant seeds under heavy metal stress (Chen et al., 2014b). Some of the organic acids such as 4-hydroxybutyrate, mucic acid, glutaric acid, gallic acid, and 3-hydroxypropionic acid are also commonly used for chemical production, medicine and health care, food processing, and in research of separation and extraction technologies. The applications and significances of these organic acids in plants still require further exploration.

Conclusion

Gas chromatography-mass spectrometry technology was used in this study to analyze the metabonomic changes in *A. aphylla* seedlings under cold stress. A total of 116 differential metabolites were identified, including organic acids, amino acids, carbohydrates, alkaloids, auxins, organic bases, quinone compounds, flavonoid compounds, and other substances. In this study, most of the differential metabolites found in *A. aphylla* seedlings under cold stress showed a down-regulated trend. Through the analysis of carbohydrates such as sedoheptulose and trehalose, various amino acids, and the organic acid malonic acid, it was found that photosynthesis, respiration, carbohydrate metabolism and ornithine cycle of *A. aphylla* seedlings were all inhibited under cold stress; however, at the same time, *A. aphylla* seedlings were able to lower the extent of damage caused by adversity via accumulation of metabolites such as γ-aminobutyric acid, α-ketoglutaric acid, and 1-kestose. The results of this study provide a foundation for further analysis of the molecular and physiological mechanisms of the response of *A. aphylla* to cold stress.

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