SUPPLEMENTARY MATERIAL

Comparative Metabolomics of Two Saline-alkali Tolerant Plants *Suaeda glauca* and *Puccinellia tenuiflora* based on GC-MS platform

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Abstract

Suaeda glauca and *Puccinellia tenuiflora* are two important saline-alkali tolerant plants that can improve the soil properties. For exploring the different tolerance mechanisms between them, GC-MS-based metabolomics was used to comprehensively evaluate the primary metabolites response, a total of 51 different metabolites were present in different quantities through PLS-DA score plot analysis. The identified compounds were mainly 11 sugars, 7 amino acids, 5 alcohols and 18 organic acids; they play an important role in responding to the saline-alkali stress and distinguish between *S. glauca* and *P. tenuiflora*. All identified metabolites classes showed similar trend to largely accumulate in *P. tenuiflora* roots and *S. glauca* shoots, this reveals that the two plants used different physiological strategies to cope with saline-alkali stress. Identified metabolites were annotated to the biological pathways involved in the KEGG database showing that energy and defensive or protective metabolites were highlighted in saline-alkali condition.

Keywords Salt-alkali stress; agroforestry; physiological response; primary metabolites

EXPERMENTAL

Materials collected

S. glauca and *P. tenuiflora* materials collected from *Suaeda glauca* community and *Puccinellia tenuiflora* community of HulunBuir Grassland in China on July, 2017(115°31′00″～121°34′30″, 47°20′00″～50°50′30″). The plants were identified and authenticated by Prof. Mu Liqiang and voucher speciemens were given the numbers SG2365 for *S. glauca* samples and PT3342 for *P. tenuiflora* samples and both samples were pressed and saved in room No. 505 at Key Laboratory of Plant Ecology, Northeast Forestry University, Harbin 150040, China.

S. glauca community connected to *P. tenuiflora* community were selected as sample plot, and based on the distance greater than 500 km compared with the other sample plot. Total of three sample plot were used to experiment. Each community provided 2 plants, a total of 6 repetitions. And each plants were divided in to roots, stems and leaves 3 parts. The samples were collected and saved in drikold, respectively. Soil samples for the analyses were taken around the selected plants collected along the vertical length of 20 cm depth for the representative saline alkali mapping units. 4 repetitions.

The detection of soil salinity and alkalinity

Soil samples were dried at room temperature during 2 weeks, pulverized and then sieved through a 2 mm mesh sieve. The saturation paste extract was prepared as described by Elisha et al (Njuemugai, 2004). The soil PH was measured with a glass electrode PH meter (pHM-2000, Eyela, Rikakikai Co., Tokyo, Japan) in the saturation paste. All chemical determinations were conducted from the saturation paste extract. The contents of Na and K were determined by flame photometry (410, Corning, Halstead, England), those of carbonates and bicarbonates by titration with HCl in the presence of phenolphthalein and methyl orange indicators respectively, the contents of chlorides by titration with silver nitrate with potassium chromate as an indicator and those of sulphates by colorimetry (double beam spectrophotometer, UV-140-02, Shimadzu) after precipitation with barium chloride. The standard methods were those in Elisha et al (Njuemugai, 2004).

GC-MS analysis

The method referenced the article of Chen Qi (Chen Qi et al., 2017), Samples were weighted 60 mg and mixed with 360 μL of cold methanol and 40 μL internal standards (0.3 mg/mL 2-chlorophenylalanine in methanol), and then homogenized using a tissue lyser system

(Tissuelyser-192, Shanghai, China). After ultra sonication for 30 min, 200 μL chloroform and 400 μL water were added to the sample. The mixture was vortexed for 2 min and sonicated for 30 min, and then the sample was centrifuged at 10,000g for 10 min at 4℃. Finally, 400 μL of the supernatant was transferred to a glass sampling vial for vacuum-dry at room temperature. The residue was derivatized using a two-step procedure. First, 80 μL methoxyamine (15 mg Ml-1 in pyridine) was added to the vial, vortexed for 30 s and kept at 37℃ for 90 min followed by 80 μL BSTFA (1 % TMCS) and 20 μL n-hexane at 70℃ for 60 min. After derivatization of leaves tissue, each 1 μL solution was injected into the Agilent 7890A-5975C GC-MS system (Agilent Corporation, USA) with a split ratio of 30 to 1. 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. The temperature of injection and ion source was set to 260℃ and 230 °C, respectively. Electron impact ionization (-70 eV) at full scan mode (m/z 30−600) was used, with an acquisition rate of 20 spectrum/second in the MS setting. The QC sample was prepared by mixing aliquots of the tissues samples to be a pooled sample, and then analyzed using the same method with the analytic samples.

The acquired MS data from GC−MS were analyzed by Chroma TOF 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 and peak intensities. The internal standard was used for data quality control (reproducibility). 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. 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-P13 software package (Umetrics, Umeå, Sweden). Principle component analysis (PCA) 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. 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, the Student's t-test. Metabolites with both multivariate and univariate statistical significance (VIP >1.0 and $p < 0.05$) were extracted. Those variables with VIP > 1.0 are considered as be relevant for group discrimination.

Supplementary Tables and Figures

Table S1. The soil indicator around *S. glauca* and *P. tenuiflora*

	PH	Na^+/K^+	CO_3^{2-}(mg/l)	HCO_3^-(mg/l)	Cl^-(mg/l)	SO_4^{2-}(mg/l)
S1	9.74±0.14**	32.96±2.34**	0.018±0.003	0.037±0.009**	.090±.014*	0.20± .021
S2	8.53±0.25**	21.20±2.62**	0.015±0.001	0.005±0.001**	.037±0.009*	0.09±.011

S1: the soil around *S. glauca*, S2: the soil around *P. tenuiflora*; Significantly: *P<0.05, Extremely significantly: **P<0.01,

Table S2. The significantly different metabolites between *S. glauca* and *P. tenuiflora*

	Metabolite	Vip	Change	P-val
Sugar	Sucrose	1.82	P>S	**
	Tagatose	1.68	P>S	**
	Melezitose	1.60	P>S	*
	D-talose	1.37	P>S	*
	Fructose	1.13	P>S	*
	Trehalose	1.18	P>S	*
	Sorbose	1.32	S>P	*
	Fucose	1.25	S>P	*
	6-deoxy-D-glucose	1.16	S>P	*
	Galactonic acid	1.14	S>P	*
	D-arabitol	1.11	S>P	*
Amino acid	O-succinylhomoserine	1.08	P>S	*
	Isoleucine	1.30	P>S	*
	N-ethylglycine	1.30	S>P	*
	3-hydroxynorvaline	1.23	S>P	*
	Ornithine	1.09	S>P	*
	Proline	1.08	S>P	*
	Glycine	1.02	S>P	*
Alcohols	Cuminic alcohol	1.55	P>S	*
	Myo-inositol	1.24	S>P	*
	2-aminoethanethiol	1.24	S>P	*
	Dihydrocarveol	1.15	S>P	*
	Dodecanol	1.14	S>P	*
Acids	L-gulonic acid	1.75	P>S	**
	Cumic Acid	1.19	P>S	*
	Gallic acid	1.48	S>P	*
	Citric acid	1.34	S>P	*
	2-ketobutyric acid	1.31	S>P	*
	Glycolic acid	1.30	S>P	*
	Fumaric acid	1.23	S>P	*
	3,4-dihydroxybenzoic acid	1.22	S>P	*
	Phenylacetic acid	1.20	S>P	*
	Palmitic acid	1.19	S>P	*
	4-hydroxy-3-methoxybenzoic acid	1.19	S>P	*
	Oxalic acid	1.17	S>P	*
	Stearic acid	1.10	S>P	*

	Benzoic acid	1.09	S>P	*
	Hydrocinnamic acid	1.08	S>P	*
	Dioctyl phthalate	1.08	S>P	*
	2-methylfumarate	1.07	S>P	*
	P-anisic acid	1.06	S>P	*
Others	Methyl heptadecanoate	1.37	S>P	*
	Nonanoic acid methyl ester	1.19	S>P	*
	Methyl octanoate	1.11	S>P	*
	Vinylphenol dimer	1.33	S>P	*
	5-methoxytryptamine	1.25	S>P	*
	Glutamine	1.18	S>P	*
	Naringenin	1.06	S>P	*
	Phenylacetaldehyde	1.11	S>P	*
	Phosphate	1.82	S>P	*
	Adenine	1.09	S>P	*

VIP, variable importance in the projection; Significantly: *P<0.05, Extremely significantly: **P<0.01,

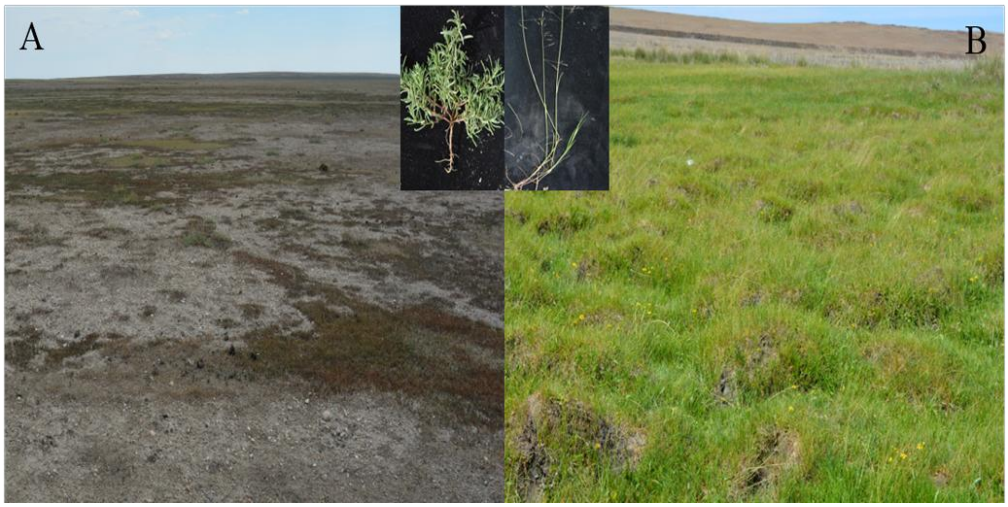


Figure S1. The appearance of *S. glauca* community and *P. tenuiflora* community. A: *S. glauca* community and *S. glauca*; B: *P. tenuiflora* community and *P. tenuiflora*.

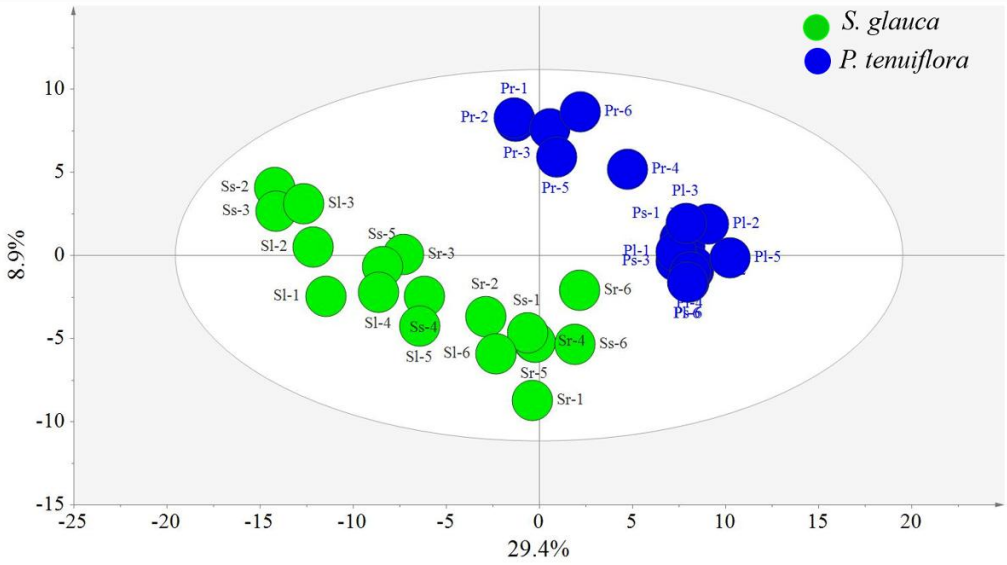


Figure S2. The PLS-DA score plot of primary metabolites in *S. glauca* and *P. tenuiflora*. Sr: *S. glauca* root; Ss: *S. glauca* stem; Sl: *S. glauca* leaf; Pr: *P. tenuiflora* root; Ps: *P. tenuiflora* stem; Pl: *P. tenuiflora* leaf.

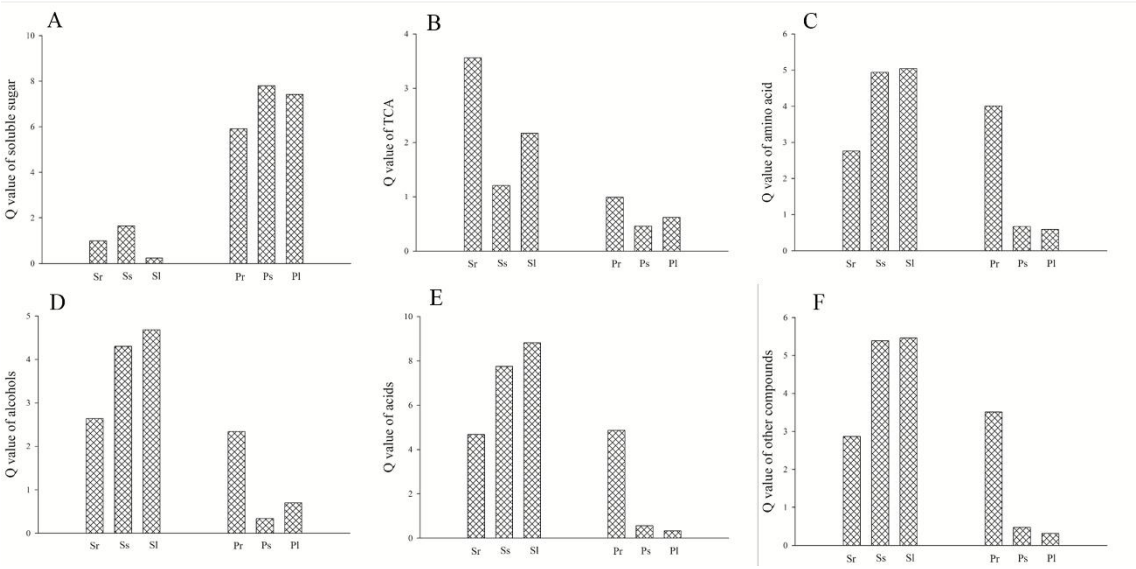
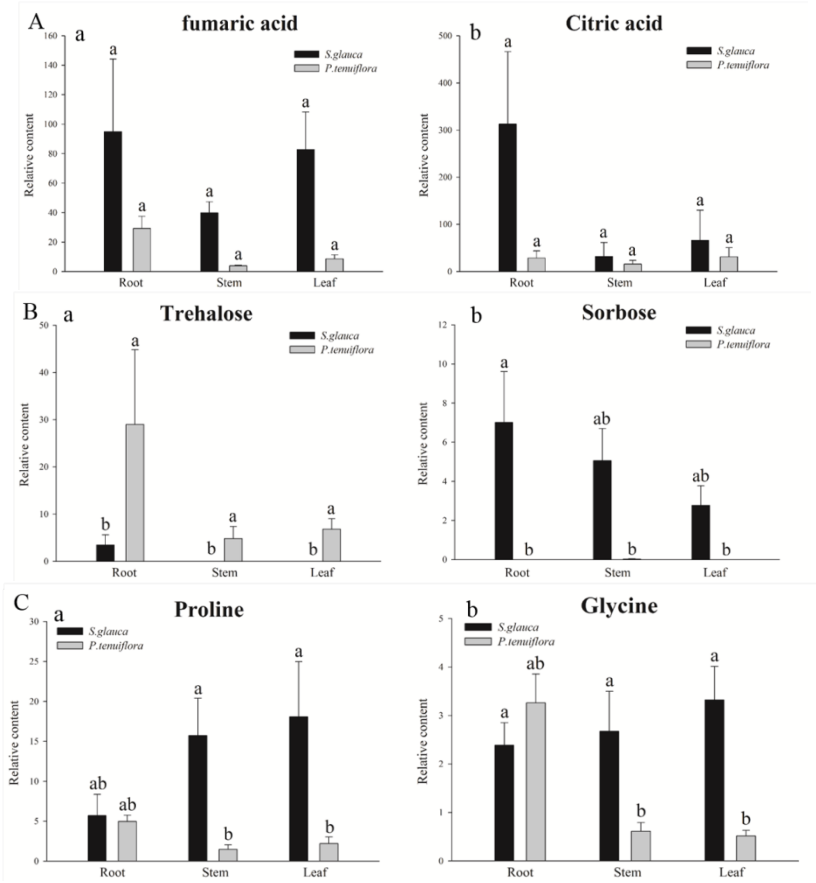


Figure S3. The Q value of different kinds of metabolites between *S. glauca* and *P. tenuiflora*. A: Q value of soluble sugar; B: Q value of TCA; C: Q value of amino acid; D: Q value of alcohol; E: Q value of acids; F: Q value of other compounds; Sr: *S. glauca* root; Ss: *S. glauca* stem; Sl: *S. glauca* leaf; Pr: *P. tenuiflora* root; Ps: *P. tenuiflora* stem; Pl: *P. tenuiflora* leaf.



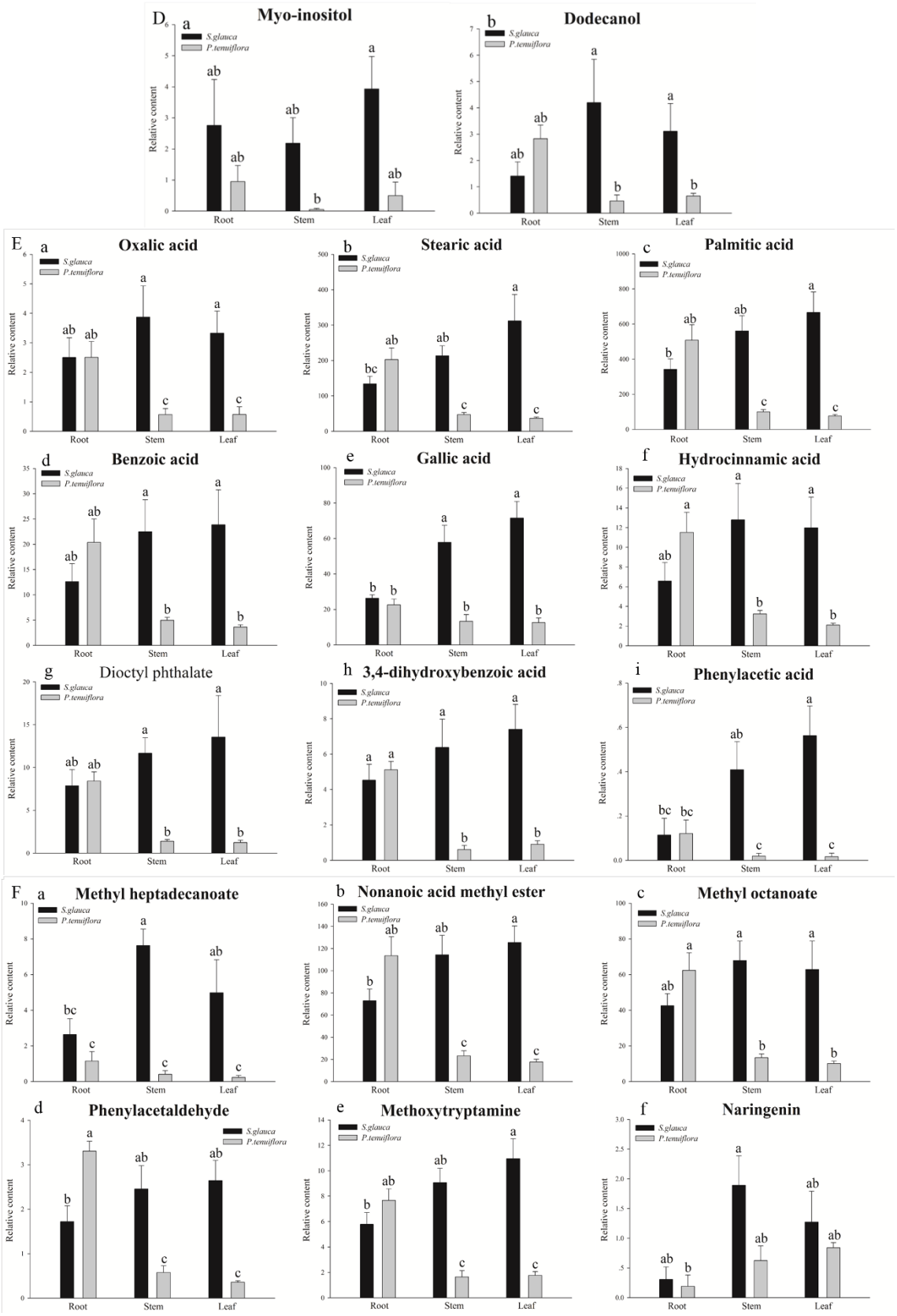


Figure S4. The relative content of significantly different metabolites in *S. glauca* and *P. tenuiflora*. **A:** Relative content of TCA; a. fumaric acid, b. citric acid; **B:** Relative content of soluble sugar; a. trehalose, b. sorbose; **C:** Relative content of amino acid; a. proline, b. glycine; **D:** Relative content of alcohol; a. Myo-inositol, b. dodecanol; **E:** Relative content of acids; a. oxalic acid, b. stearic acid, c. palmitic acid, d. benzoic acid, e. gallic acid, f. hydrocinnamic acid, g. dioctyl acid, h. 3,4-dihydroxybenzoic acid, i. phenylacetic acid; **F:** Relative content of other compounds; a. methyl heptadecanoate, b. nonanoic acid methyl ester, c. methyl octanoate, d. phenylacetaldehyde, e.methoxytryptamine, f. naringenin.

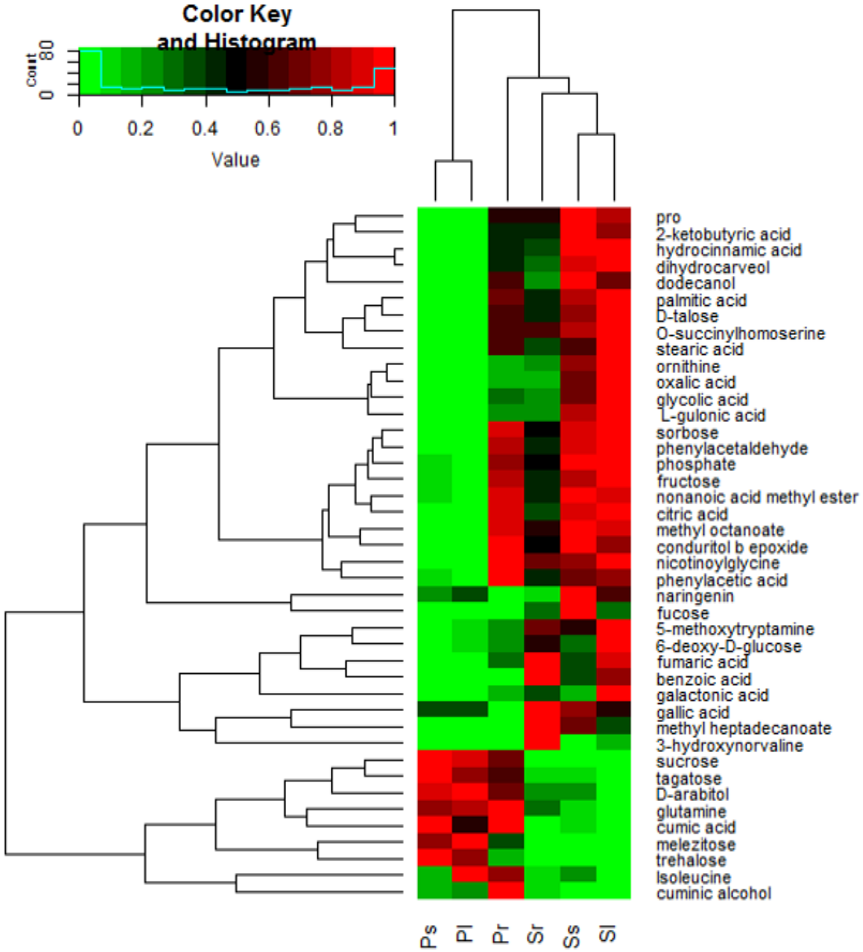


Figure S5. The heat map visualization of significant metabolites in *S. glauca* and *P. tenuiflora*. The content value of each metabolite was normalized to complete linkage hierarchical clustering; Sr: *S. glauca* root; Ss: *S. glauca* stem; Sl: *S. glauca* leaf; Pr: *P. tenuiflora* root; Ps: *P. tenuiflora* stem; Pl: *P. tenuiflora* leaf.