Variation of photosynthesis, secondary metabolites and antioxidant activities in third generation of spaceflight-induced Salvia miltiorrhiza

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1 Introduction

Salvia miltiorrhiza Bge. (Labiatae), a medicinal plant for treating dysmenorrhea, amenorrhea, and cardiovascular diseases, widely spread in the world. However, the yield of S. miltiorrhiza cannot meet the demand in the market. Accordingly, various treatments had been conducted to improve the quality and quantity of S. miltiorrhiza by space radiation (Shan et al., 2009) or genetic manipulation (Kai et al., 2011; Guo et al., 2013; Zhang et al., 2013; Peng, 2015; Pei et al., 2018).

Among these, space radiation has been widely applied in animal and plant mutation breeding programs. Since 1960s, many advanced countries have exposed plant materials to cosmic radiation during spaceflights as a common method to induce mutagenesis and that has been a part of their ambitious space programs (Halsted & Dutcher, 1987). In China, more than 29 batches of medicinal herb seeds have been sent into space by retrievable satellites and space crafts since 1987 (Li et al., 2020). Various factors, including strong radiation, microgravity, fine vacuum, low-intensity magnetic field, and high energy particles (Ohnishi et al., 2002), can produce a special surrounding, affecting metabolic activities, causing alterations in gene expression, gene regulatory response, alternative splicing, protein folding and endangering genomic stability of seeds, tissues, organs or individuals that entered into space (Maslinsky & Nechitailo, 2001; Beisel et al., 2019; Choi et al., 2019; Kruse et al., 2020; Angelos et al., 2021; Manian et al., 2021; Xu et al., 2021), thereby generating rare mutations in the mutagenized material and new germplasm resources.
Mutations often present substantial variations in their response to the same environmental challenge (Casanueva et al., 2012). Generally speaking, space radiation produces 5%–10% variability, but only 2%–3% are beneficial (Pan et al., 2005). It is, therefore, essential to select the profitable and steadily inheritable ones from multiple mutants for further investigation.

Space surrounding can affect the first generation of spaceflight-induced \textit{S. miltiorrhiza} (F1) in characteristics of morphology, physiology, and genetic to some extent. The rates of germination and emergence were increased; Earlier florescence, dark greener leaves, thicker leaves, heavier thousand seeds, and heavier fresh roots often appeared; conversely, seed setting rate per plant, leaf length and width were lower than control (Wang et al., 2007). The contents of chlorophyll, soluble protein, catalase (CAT) and superoxide dismutase (SOD) were largely increased, while the content of soluble sugar decreased compared with control (Yang et al., 2011). And mutation at genetic level happened because of gain and loss of some DNA bands (Shan et al., 2009; Beisel et al., 2019; Kruse et al., 2020).

The cultivars of \textit{S. miltiorrhiza} characterized with high yield and quality and closely associated with the primary and secondary metabolism, often attracted more attention. The primary metabolism produces metabolites and energy to maintain plant vital activity, and also provides precursors for synthesizing secondary metabolites that defend plant against outside threats (Erb & Kliebenstein, 2020). In our other work, we analyzed 224 spaceflight-induced accessions of the second generation (F2) in their active constituent contents and morphological traits, which suggested that a spaceflight environment could induce F2 accessions remarkably in the variation of root yield and active constituent content (Peng et al., 2014). We further analyzed 62 accessions of the third generation (F3) featured with high quality and quantity selected from the F2 accessions in the variations in morphological characters and active constituents and divided them into four grades (41–65 μg/g, 66–90 μg/g, 91–105 μg/g, 106–145 μg/g) according to the active constituent contents (total content of salvianolic acids and lipophilic tanshinones), Lines m16, m50, m51 and m57 from the four grades were further studied (Peng, 2015).

In this study, we evaluated the four lines in the primary productivity by photosynthetic characteristics and assess their quality by the secondary metabolites (salvianolic acids, tanshinones, total phenolics and flavonoids), and antioxidant activity of roots. Transcript levels of key enzyme genes, involved in phenolic acids metabolism, often attracted more attention. The primary metabolism produces metabolites and energy to maintain plant vital activity, and also provides precursors for synthesizing secondary metabolites that defend plant against outside threats (Erb & Kliebenstein, 2020). In our other work, we analyzed 224 spaceflight-induced accessions of the second generation (F2) in their active constituent contents and morphological traits, which suggested that a spaceflight environment could induce F2 accessions remarkably in the variation of root yield and active constituent content (Peng et al., 2014). We further analyzed 62 accessions of the third generation (F3) featured with high quality and quantity selected from the F2 accessions in the variations in morphological characters and active constituents and divided them into four grades (41–65 μg/g, 66–90 μg/g, 91–105 μg/g, 106–145 μg/g) according to the active constituent contents (total content of salvianolic acids and lipophilic tanshinones), Lines m16, m50, m51 and m57 from the four grades were further studied (Peng, 2015).

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2. Materials and methods

2.1. Plant materials

Detailed information about the first and second generation of space-induced \textit{S. miltiorrhiza} was described in Peng et al. (2014). The flown and controlled seeds were sowed in the green house on October 10, 2009 and then transplanted in eight rows of a research field on May 23, 2010 at Shaanxi Tasty Plants Pharmaceutical Co., Ltd. (Shangluo, China). The first generation, plants from flown seeds and their corresponding ground controls were cultivated and processed under the same condition and self-bred. Eventually, we achieved 136 accessions of the first generation after space flight (SP1) and 22 ground controls. For the second generation, we randomly chose 224 plants from 28 SP2 lines (eight individuals per line) and harvest on November 11, 2011, all of the selected lines had more than 10 plants. The third generation of space-induced \textit{S. miltiorrhiza} (F3) including Lines m16, m50, m51, and m57 and F1 was transplanted in a medicinal botanical garden of Northwest A&F University (latitude 34°29′, longitude 108°07′, elevation 520 m, Yangling, Shaanxi Province, China) in November 2013. The soil was highly viscous, the annual average temperature was 12.9 °C, the average annual sunshine duration was 2164 h, the annual total radiation was 4837 kJ/cm², the relative humidity was 65%, the frost-free period was about 221 d, and the average annual precipitation was about 637.6 mm. Yangling belonged to the warm temperate monsoon climate zone. Three individuals of each line with special traits (leaf shape, leaf color, active constituents) and the control (three individuals) were analyzed in photosynthesis-related properties and secondary metabolites.

2.2. Leaf gas exchange

Leaf gas exchange was performed in situ on the third fully expanded leaves using a portable photosynthesis system (LI-6400, LI-COR Inc., Lincoln, NE, USA) with red/blue LED light source with a 6-cm² clamp-on leaf chamber. Photosynthetic properties of the accessions were measured at an irradiance of 1500 μmol/m²/s, a CO₂ intensity of 400 μmol/m²/s, and a leaf temperature of 28 °C. For the determination of light response curves, light intensity was set up across the series of 2000, 1800, 1500, 1200, 1000, 800, 500, 200, 150, 100, 50, 20, 0 μmol/m²/s PPFD with the light auto-measured program. The relative humidity was 50%, and the air flow was 500 μmol/s. Each individual of the six lines and control were measured three times during 8:30 to 11:30 after 20-min was allowed to acclimate to chamber conditions for attached leaves, depending on the environmental conditions. Light-response curves were plotted using the mean values of Pn measured at each PPFD. Apparent quantum yield (AQY), light compensation point (LCP), light saturation point (LSP), and PPFD-saturated AN (Aₘₐₓ) were estimated with Photosynthesis Assistant (version 1.1.2 for Windows by Parsons and Ogston, Dundee Scientific, UK).

For the determination of CO₂ response curves, ambient CO₂ concentration in the cuvette was controlled with a CO₂ mixer across the series of 400, 300, 200, 100, 50, 400, 400, 600, 800, 1000, 1200, 1500, 1800 μmol CO₂ (mol air)⁻¹ with the CO₂ auto-measured program. Light intensity was dependent on respective light saturation point, and the rest of parameters were remained the same as measuring light response curves. The maximum carboxylation rate allowed by rubisco (Vₘₐₓ), rate of photosynthetic electron transport [based on NADPH requirement] (J), and triose phosphate use (TPU) were determined based on Farquhar et al. (1980). A biochemical model for C₅ leaf was based on Pn/Ci curve using methodology as suggested by Sharkey et al. (2007). The Rubisco Michaelis constants for CO₂ (Kc), O₂ (Ko), and the CO₂ compensation point in the absence of dark respiration (I₀) for analysis of the Pn/Ci curve were obtained from Bernacchi et al. (2001).

2.3. Chlorophyll fluorescence

Fv/Fm, the maximum photochemical yield of PSII in dark-incubated leaves, was performed in five fully night dark-acclimated leaves from each accession with a Portable Pulse Modulated Fluorometer (FMS-2, Hansatech, UK).

2.4. Stomatal density and size

Stomatal density (mm⁻²) and length of guard cells (μm) were determined by the method of nail polish imprinting on the third
or fourth youngest leaves. The abaxial sides of leaves were homogeneously colorless nail polish, and the stamps were tore and placed on slides, then were observed and photographed under 40 × magnification with a calibrated reticule in a fluorescent electronic microscope and software (Olympus BX51, Japan). Stomatal size (μm²) was calculated based on Franks (Franks & Beerling, 2009) multiplying guard cell length L by guard cell width, which was estimated as L/8 for grasses.

2.5. Determination of photosynthetic pigment content

After taking photosynthetic light and CO₂ response measurements, the leaves were cut and brought back to the laboratory with ice box. Pigments (chlorophyll and carotenoid, mg/g FW) were extracted using 80% (volume percentage) acetone and quantified with an ultraviolet visible spectrophotometer (UV-1700, SHIMADZU, Japan) at 645 nm, 663 nm, and 470 nm against the control using the following equations according to Arnon (1949).

Chla = 12.72A663—2.69A645
Chlb = 22.88A665—4.67A663
Car = (1000A470—3.27Chla—104Chlb)/229

2.6. HPLC analysis of main active components in roots of S. miltiorrhiza

The dried roots of S. miltiorrhiza were powdered and sieved through a 0.45-mm sieve. The powder (0.1 g) was extracted ultrasonically with 10 mL methanol–water solution (7:3, volume percentage) for 45 min and the extracts were centrifuged at 12000 rpm for 10 min and then filtered through a 0.45-μm Millipore filter. Then measurement of active components with HPLC referred to the method established in our lab (Liu et al., 2011).

2.7. Determination of total phenolics (TP) and flavonoids (TF)

Total phenolics of the samples were evaluated by the method described by Yesil-Celiktas et al. (2007), with some modifications: 0.2 mL of sample extract was mixed with 1 mL of 1 mol/L Folin Ciocalteu’s phenol reagent (Sigma) and 0.8 mL of Na₂CO₃ (7.5%, mass to volume ratio). The mixture was incubated at 37 °C for 30 min and then immediately cooled. The absorbance of the final solution was recorded at 765 nm. A calibration curve was constructed with different concentrations of gallic acid as standard. Total phenolics values were expressed as gallic acid equivalent (mg of GAE g⁻¹ DW).

Total flavonoids of the samples were measured according to Falleh et al. (2011), with some modifications: 1.6 mL of 60% ethanol and 40 μL of 5% NaNO₂ solution were added into 200 μL of sample extract, and then samples were vortexed and kept at ambient temperature for 6 min. After that, 40 μL of 10% AlCl₃ was added into the solution and allowed to react for 6 min. A total of 120 μL of 4% NaOH was added and kept at ambient temperature for 10 min. Sample absorbance was measured at 510 nm using a spectrophotometer against a prepared blank solution. A calibration curve was constructed with different concentrations of (+)-catechin equivalent (Sigma) as standard. Total flavonoid values were calculated as (+)-catechin equivalent (mg of CE g⁻¹ DW).

2.8. Determination of antioxidant activities

2.8.1. DPPH

The effect of the extracts on DPPH radical was estimated using the method of Yesil-Celiktas et al. (2007), with minor modifications. 1 mL of 80 μmol/L DPPH in methanol was mixed with 1 mL of extract solution with different concentrations. The mixture was vortexed and kept in dark for 45 min at room temperature. The absorbance of the samples was determined at 517 nm. BHT was used as the reference positive control. DPPH radical scavenging activity was calculated as follows: % DPPH radical scavenging activity = [(A₀—A₁)/A₀] × 100, where A₀ was the absorbance of DPPH radical in methanol and A₁ was the absorbance of the sample extract. The extract concentration providing 50% inhibition (IC₅₀) was obtained by plotting inhibition percentage versus extract concentration.

2.8.2. ABTS radical scavenging activity

The method of Tawaha et al. (2007) was improved for the ABTS assay. The stock solution which was kept in dark for 16 h at room temperature contained equal volumes of 7 mmol/L 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2.45 mmol/L potassium persulfate. The resultant ABTS + solution was diluted with methanol until an absorbance of (0.7 ± 0.02) at 734 nm was obtained. Varying concentrations (0.25, 0.5, 1, and 2 mg/mL) of the extract were allowed to react with 2 mL of the ABTS + solution and the absorbance was recorded at 734 nm. The percentage inhibition was calculated as: ABTS radical scavenging activity %=([Acontrol—Asample]/Acontrol] × 100, where Acontrol is the absorbance of ABTS radical methanol and Asample is the absorbance of ABTS radical + sample/standard. All tests were carried out on three separate occasions. Trolox was used as reference compound. The extract concentration providing 50% inhibition (IC₅₀) was obtained by plotting inhibition percentage versus extract concentration.

2.8.3. CUPRAC

The subsequent CUPRAC assay referenced as Apak et al. (2004) was performed as follows: 1 mL CuCl₂, 1 mL Nc solution, and 1 mL NH₄Ac solution were added to × mL of the M-β-CD-containing final analyte mixture, followed by adding (1.1 – x) mL water. The absorbance of the final solution (4.1 mL in total) at 450 nm was read against a reagent blank after 30 min incubation at room temperature. The calibration curves were constructed under the described conditions, and their trolox-equivalent antioxidant capacities, ratio of the molar absorbivity of each compound to that of trolox in the modified CUPRAC assay, were calculated.

2.8.4. FRAP

The ferric reducing antioxidant power (FRAP) assay described as Ou et al. (2002) was used to measure the antioxidant potential of “antioxidants” to reduce the Fe³⁺/tripyridyl-s-triazine complex present in stoichiometric excess to the blue ferrous form. FRAP reagent was freshly prepared by mixing together 10 mmol/L 2,4,6-tripyridyl-s-triazine (TPTZ) and 20 mmol/L ferric chloride in 0.25 mol/L acetate buffer, pH 3.6. Plant sample (200 mL) was added to 3 mL of FRAP reagent at intervals of 30 s. The absorbance was read at 593 nm after 4 min incubation at ambient temperature against distilled water.

2.9. Gene expression analysis of key enzymes involved in metabolic pathway by qRT-PCR

Total root RNA was isolated using the RNAprep pure Plant Kit (QIAGEN) and then reversely transcribed following the manufacturer’s instruction of PrimeScript™RT reagent Kit (Takara) to generate cDNA. qRT-PCR was performed in triplicate for each sample
on Bio-RAD CFX96 (USA). Primers presented in Table 1 were used to detect key enzymes expression levels of five accessions. The constitutively expressed actin gene was used as an internal control. The following protocol was performed for all qRT-PCR experiments: 30 s pre-denaturation at 95 °C, 1 cycle; 5 s denaturation at 95 °C, 30 s annealing using calculated T_{m,0} 40 cycles. Then a melting curve was performed: 65 °C to 95 °C, with 0.5 °C steps, hold for 5 s. Relative expression was calculated using comparative C_{t} method.

2.10. Data analysis

One-way analysis of variance (ANOVA) was performed to high-light differences in relevant variables between accessions and followed by ‘Tukey HSD’ post-hoc multiple comparison tests. Normality and homogeneity of variance of data were assessed prior to analysis. All tests for significance were considered statistically different with P < 0.05 or P < 0.01, unless otherwise indicated. All the data analyses were carried out using the “Statistical Package” for Social Sciences program (SPSS 16.0, SPSS Inc., USA). All the figures were plotted with OriginPro8.0 data analysis software (OriginLab Corporation, Northampton, MA). Calculation of IC_{50} values of was done using GraphPad Prism Version 4.00 for Windows (GraphPad Software Inc). Principal component analysis (PCA) was performed with SIMCA-P 11.0 to acquire a small number of linear combinations of parameters, which vary significantly among accessions and account for most of the variability in the original data.

3. Results

3.1. Leaf gas exchange

Photosynthesis is the motivity for plant growth and development and further drives its life processing circle. The stronger ability to synthesize primary metabolites is, the stronger competitiveness the plant in nature is; Specially, the utilization efficiency for light, CO_{2}, nutrition, and water. All of the parameters exhibited significant differences except C_{i} under the same condition (Table 2). The P_{n} of line m57 was the highest followed by line m50, control, and line m16, and there was no difference between m51 and m57. Cond and Fv/Fm were the lowest in m16 and showed significant difference only in this line. Tr was the highest in m50 and m51, but no significantly different in m50, m51 and CK. Line m57 showed the highest photosynthetic performance, with higher P_{n}, Cond, and lower Tr, which lays a foundation for its higher production. Lower P_{n}, Cond, Tr, and Fv/Fm were found in m16, suggesting that genes related to photosynthesis may be reversely induced.

To address their light and CO_{2} response performance, we determined light and CO_{2} response curves that enabled the analysis of light and CO_{2} utilization efficiency of the five accessions. As shown in Fig. 1A and Table 3, line m57 and m50 had a wide span of light utilization (1435 μmol/m^2/s and 1436 μmol/m^2/s), and specially, line m57 exhibited the highest capacity both for the utilization of weak light and strong light. On the contrary, line m16 had a narrow span of light utilization (542 μmol/m^2/s) with higher LCP and lower LSP. However, the higher AQY may be the necessity to survive in nature. For all the lines except for line m16, A_{max} did not statistically differ from each other, lines m57 and m16 also showed lower R_{d} than others. These data suggest that line m57 showed high light utilization and low consumption and could further be programmed for breeding and investigation for high production. Line m16, with a narrow span of light utilization, could be used for the analysis of photosystem alteration, that is, the space environment possibly mutated genes related to photosynthesis.

CO_{2} response curve is indispensable for exploring the biochemical progress of plants. Fig. 1B presented the CO_{2} response curves of S. miltiorrhiza lines. All parameters showed evident differences except for R_{d} (Table 4). Concretely, the apparent Rubisco activity (V_{c,max}) in vivo appeared to be higher in line CK and m57, and lower in line m50. The rate of electron transport used in the regeneration of RuBP (J) was higher in CK, line m57 and m16, and lower in line m50. CK and line m57 behaved to be higher in Triose-phosphate utilization (TPU), and lower in line m50 and m16, which also showed slower Gm. These data indicated that m57, with higher Rubisco activity, lower RuBP-regeneration limitation, and higher Triose-phosphate utilization, could efficiently make good use of CO_{2}, whereas m50 might be just on the contrary.

3.2. Stomatal dimensions

Stoma governs the flow of gases into and out from leaves, adjusts to climatic change, and shows especial importance in plant physiology, evolution and global ecology (Hetherington & Woodward, 2003). Stomatal size and density determine maximal stomatal conductance of CO_{2} to the cite of assimilation (Franks & Beering, 2009), which is positively correlated with plant photosynthesis (Hetherington & Woodward, 2003). As presented in Fig. 2, line m51 behaved the highest stomatal density but the smallest stomatal size, and line m16 exerted the lowest stomatal density but larger stomatal size. In general, leaves with higher stomatal density had smaller stomatal size.

3.3. Photosynthetic pigments in five S. miltiorrhiza accessions

Photosynthetic pigments are mainly composed of chlorophylls and carotenoids. Chl present an actively important role in the absorption, transportation, and conversion of light energy in plants. Carotenoids (Cars) serve as protective agents and play an important role in the light harvesting mechanism of photosynthesizing plants, protecting against reactive oxygen species (Domonkos et al., 2013). Line m57 had highest contents of Chla, Chlb, Chla + b, and Car, while m50 was on the contrary because of its yellowish green leaves (Fig. 3).

3.4. HPLC analysis for roots of five S. miltiorrhiza accessions

Secondary metabolites protect the plant itself against outside attack and provide beneficial substances with pharmacological effects for humans. Hydrophilic salvianolic acids and lipophilic tanshinones are the two major groups of biologically active components in S. miltiorrhiza. As shown in Fig. 4A, danshensu (DSS), cinnamic acid (CIA), ferulic acid (FEA), and caffeic acid (CAA), belonging to hydrophilic phenolic acid compounds, are intermediate products in phenylpropanoid metabolism pathway and showed no differences among the five accessions. rosmarinic acid (ROA), salvianolic acid B (SAB), and salvianolic acid A (SAA) as the end products in phenylpropanoid metabolism pathway were higher in m16 than others, which may indicate that while components in branch pathway were not induced notably, end-products were greatly affected by spaceflight. In addition, dihydrotanshinone (DDI), cryptotanshinone (CPS), tanshinones I (TSI), and tanshinones II A (TSIIA), main lipophilic tanshinones components in MEP (2-C-methyl-D-erythritol-4-phosphate) metabolism pathway were notably affected by spaceflight. Generally, DDI, CPS, and TSI were lower than TSIIA in S. miltiorrhiza. However, line m51 had higher contents of DDI and CPS than TSIIA (Fig. 4B). Taken together, line m16 behaves relatively weak photosynthetic ability but high contents of phenolic acid compounds. Line m57 overall presents...
strong photosynthetic ability and high contents of lipophilic tanshinones.

3.5. Total phenolics and flavonoids and antioxidant activities in leaves and roots

Total phenolics and flavonoids reflect the ability of plants to defense itself from outside attack. Four methods were used to evaluate the antioxidative ability, which could mutually confirm each other. As shown in Table 5, line m16 had high total phenolics and flavonoids contents in both leaf and root, exhibiting strong antioxidative abilities. Line m51 had a high content of total phenolics and total flavonoids in leaves, and exhibited a strong antioxidative ability, but the root did not show the respective performance. Neither leaf nor root of

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**Table 1**

| Genes | GenBank number | Forward (5'-3') | Reverse (5'-3') |
|-------|----------------|----------------|----------------|
| β-actin | DQ243702.1 | AGGAACCCACCGATCCAGACA | GGTGCCCCCTAGGTCTT |
| PAL1 | DQ408636.1 | ATAAGTTAAAGGGCCAGACGC | GAAAGCTTTCCAAACAG |
| PAL2 | GQ249111.1 | CATGTTTACCTAGGAGGTTG | TCTCCCTCCAAACACG |
| 4CL2 | AY237164.1 | GTGACAGTGATGCTGTTA | CGAAGACACCTGGCAC |
| TAT | DQ344606 | AGTATAGCTGCTGCTTCT | TGCTATCCCAACTCTCC |
| C4H | DQ355979 | TCCGGTTCCGCTTATCTT | CATGAGGAGGTGGCTT |
| HPPR | DK99741 | CCTCCACAGGAAAAAACCCAC | ACCGACAGCCTCTCAT |
| HPPD | EF157837.1 | TAGGGCCACGATCTCTTACG | TTTCCGATCCCTCACA |
| RAS | FJ308696 | GAGTTCGGTCCCTTCTATT | TGAAGCCAGAAACAG |
| CPR | HQ346179 | CCTCAATGCTGCTGCTTCA | AGTCCGACAAATCATAACT |
| HMGGR | FJ747636 | CCAACATGCTGCTGCTTACA | GATGGTGCGACGAACAG |
| DXR | FJ476255 | CATCCTGGTCCCTTCTTATG | ACTAAAGACTCCGGGTAGT |
| IPP1 | EF635967 | CCCACATGCTGCTGCTTCTT | TCTCCGCGTACATTATC |
| GGPPS | FJ643617 | ACAAGACACGTATCCAAAGC | TCTGGCTATGCAATTAG |
| R5 | EF639566.2 | CTCCCCAGACGATGCAAGAT | ATTCCTCCTACATATAG |

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**Table 2**

| Accessions | Pn (µmol/m²/s) | Cond (mol/m²/s) | Ci (µmol/mol) | Tr (mmol/m²/s) | Fv/Fm |
|-----------|----------------|----------------|---------------|----------------|-------|
| m16       | 3.14 ± 0.09b  | 0.061 ± 0.007b| 272.41 ± 7.22a| 4.09 ± 0.38c  | 0.801 ± 0.005ab |
| m50       | 10.67 ± 0.17b | 0.43 ± 0.05a  | 314.41 ± 8.24a| 11.94 ± 0.34ab| 0.440 ± 0.014 |
| m51       | 12.01 ± 0.55ab| 0.37 ± 0.09a  | 266.05 ± 17.60a| 15.75 ± 1.65ab| 0.840 ± 0.012b |
| m57       | 13.62 ± 0.50ab| 0.23 ± 0.04a  | 265.51 ± 6.49a| 6.41 ± 1.51bc | 0.832 ± 0.003ab |
| CK        | 10.12 ± 0.16ab| 0.27 ± 0.04a  | 262.02 ± 12.66a| 8.66 ± 0.68abc| 0.851 ± 0.007 a |
| P-value   | < 0.01        | < 0.01        | > 0.05        | < 0.01        | < 0.01 |

Note: Pn-photosynthetic rate; Cond-stomatal conductance; Ci-intercellular CO2 concentration; Tr-transpiration rate; Fv/Fm-maximum photochemistry quantum yield of PSII.

Accessions were measured at an irradiance of 1500 µmol/m²/s, a CO2 intensity of 400 µmol/m²/s and a leaf temperature of 28°C. Each parameter was measured on three individuals of each line, three leaves per plant. Items indicated by different lower cases in each column are significantly different (determined by Tukey HSD test, P<0.05).

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**Fig. 1.** Light and CO2 response curves of third generation of *S. miltiorrhiza* accessions induced by spaceflight (means ± SD, n = 9). (A) For the determination of light response curves, light intensity was set up across the series of 2000, 1800, 1500, 1200, 1000, 800, 500, 200, 150, 100, 50, 20, 0 µmol/m²/s PPFD with the light auto-measured program. The relative humidity was 50%, and air flow was 500 l/mol/s. (B) For the determination of CO2 response curves, ambient CO2 concentration in the cuvette was controlled with a CO2 mixer across the series of 400, 300, 200, 100, 50, 400, 400, 600, 800, 1000, 1200, 1500, 1800 µmol CO2 (mol air)-1 with the CO2 auto-measured program. Light intensity was dependent on respective light saturation point, and the remaining parameters were remained the same as measuring light response curves. Each individual of each line was measured three times during 8:30 to 11:30 independently.
line m57 showed high total phenolics and flavonoids contents or strong antioxidative capacity.

3.6. Quantitative real-time PCR analysis of key enzyme genes transcript levels

The qRT-PCR analysis of eight key enzyme genes (PAL1, PAL2, 4CL2, C4H, TAT, HPPR, RAS, CRP) involved in phenolic acids pathway and five key enzyme genes (DXR, HMGR, IPPI, GGPPS, KS) involved in tanshinone metabolic pathway was carried out (Fig. 5). Gene transcripts (PAL1, PAL2, 4CL2, TAT) in the upstream of line m16 were lower than that in control, but expression levels of C4H, HPPR, RAS, and CRP were about 3-fold, 1.5-fold, 6.5-fold, and 1.5-fold higher than control, respectively. It was consistent with previous results that line m16 has highest contents of ROA, SAB, and SAA. It was also the highest line expressed five key enzyme genes in tanshinone metabolic pathway. Line m57 had high expression levels in PAL1, PAL2, and TAT.

3.7. Principal component analysis

Out of these significantly different parameters among S. miltiorrhiza accessions, PCA was used to extract three components with eigenvalues higher than one (Fig. 6). They altogether accounted for 84.2% of the total variability in the raw data. The parameters contributing most to PC1 (39.9%) were total phenolics and flavonoids.

Table 3
Estimated parameters of light response curves in five S. miltiorrhiza lines (means ± SD, n = 9).

| Accessions | LCP (μmol/m²/s) | LSP (μmol/m²/s) | AQY (μmol/mol CO₂/1) | Amax (μmol/m²/s) | Rd (μmol/m²/s) |
|------------|----------------|----------------|----------------------|----------------|---------------|
| m16        | 69 ± 16ᵇ       | 711 ± 30ᵇ     | 0.180 ± 0.020ᵇ      | 6.32 ± 0.80ᵇ   | 2.50 ± 0.22ᵇ  |
| m50        | 80 ± 4ᵇ        | 1516 ± 20ᵇ    | 0.035 ± 0.013ᵇ      | 14.28 ± 0.97ᵇ  | 2.98 ± 0.45ᵇ  |
| m51        | 71 ± 4ᵇ        | 1401 ± 19ᵇ    | 0.060 ± 0.018ᵇ      | 15.37 ± 1.08ᵇ  | 3.75 ± 0.70ᵇ  |
| m57        | 39 ± 5ᵇ        | 1474 ± 15ᵇ    | 0.049 ± 0.006ᵇ      | 15.45 ± 1.19ᵇ  | 1.80 ± 0.52ᵇ  |
| CK         | 73 ± 6ᵇ        | 1425 ± 20ᵇ    | 0.050 ± 0.006ᵇ      | 14.60 ± 0.33ᵇ  | 3.57 ± 0.68ᵇ  |
| P-value    | < 0.05         | < 0.01        | < 0.01               | < 0.01         | < 0.05        |

Note: AQY-apparent quantum yield; Amax-photon-saturated photosynthetic rate; LCP-light compensation point; LSP-light saturation point; Rd-dark assimilation rate. Each parameter was measured on three individuals of each line, three leaves per plant. Items indicated by different lowercases in each column are significantly different (determined by Tukey HSD test, P < 0.05) among accessions.

Table 4
Estimated parameters of CO₂ response in S. miltiorrhiza based on biochemical model for a C₃ leaf using methodology (means ± SD, n = 9).

| Accessions | Vcmax (μmol CO₂/m²/s) | J (μmol (e⁻)/m²/s) | TPU (μmol CO₂/m²/s) | Rd (μmol/m²/s) | Gm (mol/m²/s) |
|------------|------------------------|-------------------|---------------------|----------------|--------------|
| m16        | 67.82 ± 15.84ᵇᶜ       | 75.58 ± 5.44ᵇᶜ    | 6.39 ± 0.83ᵇᶜ      | 1.56 ± 0.14    | 0.45 ± 0.07ᵇᶜ |
| m50        | 32.64 ± 2.32ᵇᶜ        | 31.03 ± 2.30ᵇᶜ    | 4.58 ± 1.06ᵇᶜ      | 1.58 ± 0.34    | 0.61 ± 0.33ᵇᶜ |
| m51        | 78.36 ± 16.13ᵇᶜ       | 63.09 ± 2.34ᵇᶜ    | 14.19 ± 2.26ᵇᶜ     | 1.42 ± 0.48    | 2.82 ± 0.89ᵇᶜ |
| m57        | 107.16 ± 15.79ᵇᶜ      | 70.20 ± 7.56ᵇᶜ    | 14.15 ± 2.03ᵇᶜ     | 1.25 ± 0.17    | 2.42 ± 0.40ᵇᶜ |
| CK         | 145.85 ± 12.05ᵇᶜ      | 90.18 ± 6.20ᵇᶜ    | 17.25 ± 3.38ᵇᶜ     | 1.52 ± 0.17    | 2.07 ± 0.62ᵇᶜ |
| P          | < 0.05                 | < 0.05            | < 0.01              | > 0.05         | < 0.05        |

Note: Vcmax-maximum carboxylation rate allowed by rubisco; J-rate of photosynthetic electron transport at the measured irradiance [based on NADPH requirement]; TPU-rate of triose phosphate use; Rd-day respiration; Gm-mesophyll conductance for CO₂ diffusion. CO₂ response parameters were standardized at 25 °C, under ambient conditions. Each parameter was measured on three individuals of each line, three leaves per plant. Different lower cases in the columns indicate significant differences (determined by Tukey HSD test, P < 0.05) among accessions.
noids in root, antioxidative indicators CUPRAC and FRAP of root, hydrophilic phenolic acid compounds in root, AQY (all positive loadings), carotenoid and tanshinones in root, DPPH (IC$_{50}$) and hydrophilic phenolic acid compounds in root, antioxidative indicators CUPRAC and FRAP of root, seven hydrophilic (a) and four lipophilic (b) components distributed in roots of the third generation of $S$. miltiorrhiza. Fig. 4. That may be conducive to its survival and competitive advantage in nature. Line m50 showed low content of pigments and had especially weak response ability to CO$_2$. Contents of secondary metabolites were low and antioxidative ability was weak. Line m51 had large stomatal density and small stomatal size, middle level of pigments and middle range of light utilization, strong photosynthetic ability. Besides, its secondary metabolites and antioxidative ability were in the middle level. Line m57 had high content of pigments and a good response to CO$_2$. Secondary metabolites and antioxidant ability were also high and strong. Together, compared with the control, spaceflight-induced lines presented various changes in photosynthetic ability and the contents of secondary metabolites. It is suggested that the uncertainty of spaceflight-induced variation often occurs. Therefore, spaceflight could create multiple mutations that may satisfy the needs of multiple kinds of researchers.

The results suggested that a correlation between the primary metabolism and photosynthetic pigments, stomatal size and density that favor photosynthesis. Total phenolics in leaf are significantly correlated with stomatal density, photosynthetic pigments, stomatal size and density. Total flavonoid in leaf is significantly related with photosynthetic pigments, and CO$_2$-responsive parameters. Total flavonoid in root are positively related with AQY, and negatively with $F_v/F_m$ and $A_{max}$. What’s more, lipophilic tanshinones is positively correlated with stomatal density, photosynthetic pigments, and CO$_2$-responsive parameters. Total flavonoid and total phenolics are major contributors to antioxidant activities, which are almost coincident with them. (Table 6). High AQY, low $F_v/F_m$ and $A_{max}$ may be the explanation for line m16 with low photosynthetic ability but high contents of secondary metabolites and strong antioxidative abilities in root.

### 4. Discussion

Spaceflight-induced breeding has long been perceived as an effective way to produce new species with high quantity and excellent quality, and this idea has been enforced by many successful cases of staple crops, such as maize (Zea mays) (Rhoades, 1950), rice (Oryza sativa) (Yu et al., 2013; Zeng et al., 2021), wheat (Triticum aestivum) (Tripathy et al., 1996), soybean (Glycine max) (Levine et al., 2001), rape (Brassica campestris), and cotton (Gossypium hirsutum). Research has also been launched in medicinal plants and great progresses have been made in Scutellaria baicalensis (Shan et al., 2008), Platycodon grandiflorum (Wang et al., 2004), Carthamus tinctorius (Gao et al., 1997), Glycyrrhiza uralensis (Wang et al., 2009), and Andrographis paniculata (Xia et al., 2014). The mutations induced by spaceflight often emerge in morphology, DNA and chromosomal integrity, and physiological and biochemical properties. Beneficial and undesirable mutants may both occur during mutagenesis. The probability of occurrence of mutants with good characteristics induced by spaceflight just happen only one in a million. Stably heritable mutants often appear on the third or fourth generation. Therefore, it is necessary to proceed from the primary and secondary metabolism aspects to investigate and select the third generation for the future application in cultivation and breeding program.

In order to obtain distinctive materials, we have studied and determined the third generation of $S$. miltiorrhiza accessions induced by spaceflight. Based on our preliminary investigation on $F_1$ and $F_2$ $S$. miltiorrhiza induced by spaceflight, we have chosen four typical lines derived from four gradients that comprised 62 F3 accessions according to the total content of salvianolic acids and lipophilic tanshinones from low to high. Especially, leaf gas exchange characterization, secondary metabolites (salvianolic acids, lipophilic tanshinones, total phenolics and flavonoids), and antioxidant activity, were targeted and analyzed.

We found that line m16 presented weak photosynthetic ability may be due to its low stomatal density and large stomatal size, and low content of pigments, but high AQY and high contents of secondary metabolites and strong antioxidative ability (Tables 2–5, Figs. 2 and 3). That may be conducive to its survival and competitive advantage in nature. Line m50 showed low content of pigments and had especially weak response ability to CO$_2$. Contents of secondary metabolites were low and antioxidative ability was weak. Line m51 had large stomatal density and small stomatal size, middle level of pigments and middle range of light utilization, strong photosynthetic ability. Besides, its secondary metabolites and antioxidative ability were in the middle level. Line m57 had high content of pigments and a good response to CO$_2$. Secondary metabolites and antioxidant ability were also high and strong. Together, compared with the control, spaceflight-induced lines presented various changes in photosynthetic ability and the contents of secondary metabolites. It is suggested that the uncertainty of spaceflight-induced variation often occurs. Therefore, spaceflight could create multiple mutations that may satisfy the needs of multiple kinds of researchers.

The results suggested that a correlation between the primary metabolism and photosynthetic pigments, stomatal size and density that favor photosynthesis. Total phenolics in leaf are significantly correlated with stomatal density, photosynthetic pigments, and CO$_2$-responsive parameters. Total flavonoid in leaf is significantly related with photosynthetic pigments, and CO$_2$-responsive parameters. Meanwhile, total phenolics and total flavonoid in root are positively related with AQY, and negatively with $F_v/F_m$ and $A_{max}$. What’s more, lipophilic tanshinones is positively correlated with stomatal density, photosynthetic pigments, and CO$_2$-responsive parameters. Total flavonoid and total phenolics are major contributors to antioxidant activities, which are almost coincident with them. (Table 6). High AQY, low $F_v/F_m$ and $A_{max}$ may be the explanation for line m16 with low photosynthetic ability but high contents of secondary metabolites and strong antioxidative abilities in root.
PCA revealed three components (84.2%) among all these parameters, PC1 (39.9%) is almost a root-related index, PC2 (29.4%) is a leaf-related index, and PC3 (14.9%) is specifically about CO₂ response parameters (Fig. 6). These parameters can effectively distinguish the four classes of the F3 62 accessions, and representative index can be the indicator to select and analyze targeted lines regarding plant growth and plant secondary metabolism for further breeding programs. Investigations on the mechanism of spaceflight-induced changes in growth and metabolic pathway are worth more attention, for the purpose of making better manipulations on metabolic engineering.

5. Conclusion

Four representative varieties were chosen from the four main classes of F3 spaceflight-induced S. miltiorrhiza to investigate and analyze their primary and secondary metabolism and antioxidative abilities. Three principal components could be used to distinguish spaceflight-induced S. miltiorrhiza lines. We found two interesting lines. Line m57, with strong gas exchange ability, relatively higher secondary metabolites contents, and ascending antioxidative abilities, could be used as an appropriate material to investigate targeted breeding towards high production. Line m16, with weak photosynthetic ability, but with higher apparent quantum yield (AQY), more secondary metabolites, and strong antioxidative abilities, could be used to characterize important genes and reveal complex secondary metabolism pathways.

Authors’ contributions

Liang Peng, Zongsuo Liang, and Mei Ru designed the experiments. Liang Peng and Mei Ru performed the research. Liang Peng and Mei Ru analyzed the data and drafted the manuscript. Liang Peng, Zongsuo Liang, and Mei Ru revised the manuscript and con...
tributed reagents/materials/analysis tools. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 6

| Index | Leaves | Roots |
|-------|--------|-------|
| D     | 0.60** | 0.59* |
| S     | -0.48  | 0.43  |
| Clh   | 0.68** | -0.69* |
| Car   | 0.79** | -0.70* |
| Fe/Fm | -0.33  | -0.07 |
| A5O   | 0.09   | -0.24 |
| V_{OR} | 0.66  | 0.59* |
| J     | 0.69*  | -0.56* |
| TPU   | 0.70   | 0.63* |

Note: * and ** denote significant at 5% and 1% level, 1total phenolics, 2total flavonoids, 3lipophilic tanshinones.

Table 6: Correlations among secondary metabolites and primary metabolism related index.
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