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Research article

Physiological, transcriptional, and metabolic alterations in spaceflight-subjected *Senna obtusifolia*

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ABSTRACT

Senna obtusifolia is a widely used medicinal herb in Asian countries. To select elite cultivars, *S. obtusifolia* seeds were carried by "ShenZhou VIII" recoverable satellite to space. Three spaceflight-subjected lines (SP-lines), namely QC10, QC29, QC46, and their ground control line (GC-line) were cultivated on the ground. Previous studies demonstrated that biological traits and secondary metabolites are different between SP-lines and GC-line. Here, we combined physiological, transcriptional, and metabolic studies to compare the differences between SP-lines and GC-line. The results showed that activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), and monodehydroascorbate reductase (MDHAR) were dramatically increased in SP-lines as compared to that of GC-line. Transcript levels of *SOD*, *POD*, *CAT*, *APX*, and *MDHAR* were significantly up-regulated in SP-lines. Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents decreased in SP-lines. Seed yields of QC29 and QC46 were considerably higher than that of GC-line. Besides, QC29 had significantly higher aurantio-obtusin content. Pearson correlation coefficient analysis revealed positive relationships between POD and aurantio-obtusin, as well as APX and aurantio-obtusin. In conclusion, SP-lines have higher antioxidant gene expression level and antioxidant enzyme activity as compared to that of GC-line. With higher seed yield and aurantio-obtusin content, QC29 can be used to breed elite *S. obtusifolia* cultivars. This study provides a new insight in SP-lines and paves the way to breed elite *S. obtusifolia* cultivars in the future.

1. Introduction

Senna obtusifolia is an annual herb widely distributed in temperate and subtropical regions. Its ripe seeds have been used as a traditional medicine in many Asian countries to treat ophthalmic diseases, constipation and hyperlipidemia (Kim et al., 2009; Ju et al., 2010; Jung et al., 2016). Pharmaceutical studies have demonstrated that *S. obtusifolia* seeds have antifungal, inflammatory, and neuroprotective effects (Kim et al., 2009; Yang et al., 2012; Xue et al., 2018). Anthraquinones are believed to be the main bioactive compounds in *S. obtusifolia* seeds. Recent studies have proved that anthraquinones are major pharmaceutical components in *S. obtusifolia* seeds to inhibit angiotensin converting enzyme (Kim et al., 2009), β-secretase, and cholinesterases activities (Jung et al., 2016). Aurantio-obtusin is regarded as an exclusive anthraquinone component in *S. obtusifolia* seeds, because other anthraquinones such as emodin, aloe-emodin, and chrysophanol are also found in Polygonaceae plants. Furthermore, aurantio-obtusin and chrysophanol are selected as the quality marker for the quality control of *S. obtusifolia* seeds according to Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2015). Therefore, the contents of aurantio-obtusin and chrysophanol, especially aurantio-obtusin are crucial for *S. obtusifolia* seeds pharmaceutical application. In China, *S. obtusifolia* seeds (Juemingzi in Chinese) are a famous traditional Chinese medicine and have been regarded as the culinary and medicinal dual-purpose material by China Food and Drug Administration. In view of the significant medicinal value and promising culinary application, *S. obtusifolia* seeds are drawing more and more attention.

Space environment has unique characterization with multiple factors such as special magnetic fields, microgravity, and solar cosmic radiation, which may cause the changes at morphological, physiological, molecular, and metabolic levels (Badhwar, 1997; Kostov et al., 2002; Wei et al., 2006; Wu et al., 2011). Spaceflight-subjected is a method that carried materials into space, using space environment to induce mutations (Yan et al., 2010). Spaceflight-subjected method

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could generate many varieties with special traits, which makes this method a new approach to select new variations (Wen et al., 2004; Liu et al., 2007, 2009). To date, China has no long-term, manned space station, therefore spaceflight-subjected is a suitable method for space biology research. Major crops such as *Glycine max* (Zheng et al., 2014), *Capsicum annuum* (Xie et al., 2014), and *Triticum aestivum* (Xiong et al., 2017) have been carried by spaceships to space to generate varieties for breeding program. The new varieties generated by spaceflight not only facilitate identification of new functional genes, but also help in selecting new cultivars with high yield and quality (Ahloowalia and Maluszynski, 2001; Salmi and Roux, 2008; Zheng et al., 2008). However, research on spaceflight-subjected medicinal herbs is still scarce.

In our earlier studies, spaceflight-subjected method was employed to select *S. obtusifolia* lines with higher seed yield and secondary metabolite contents. In 2011, 10 g of seeds were averagely divided into two groups. One group (5 g) was carried by Chinese satellite “ShenZhou VIII” to space. Another group (5 g) was stored in the laboratory. After a 17-day flying in space, “ShenZhou VIII” satellite landed on schedule. Since 2012, the spaceflight-subjected seeds and ground control seeds were cultivated on the ground under same condition. After three years' research (2012–2014), we selected three spaceflight-subjected lines (SP-lines), namely QC10, QC29, and QC46 which had higher seed yield and aurantio-obtusin content than that of ground control line (GC-line).

We have identified some differences in biological trait and seed quality between SP-lines and GC-line (Mao et al., 2014, 2015). However, those research was mainly focused on the agronomic and morphological traits. Regardless a detailed understanding of the physiological and transcriptional traits of SP-lines is a prerequisite to select elite *S. obtusifolia* lines, little is known about the physiological and transcriptional characteristics of SP-lines. Herein, we investigated the differences of antioxidant enzyme activity and transcript level of antioxidant enzyme genes at different growing stages between SP-lines and GC-line. Besides, contents of five major secondary metabolites in *S. obtusifolia* seeds, namely aurantio-obtusin, chrysophanol, physcion, emodin, and aloe-emodin were measured by high performance liquid chromatography (HPLC). Changes in the biological trait and seed yield were compared. The objectives of this study were to i) evaluate the effects of spaceflight on physiological, transcriptional, and metabolic traits of *S. obtusifolia*, ii) explore the relationship among agronomic traits, antioxidant enzyme activities, and secondary metabolite contents, and iii) provide a theoretical basis for breeding elite *S. obtusifolia* varieties.

2. Materials and methods

2.1. Plant materials and sampling

QC10, QC29, QC46, and GC-line were planted in Institute of Soil and Water Conservation, Chinese Academy of Sciences (108°07′E, 34°29′N, altitude 518 m) during the growing season of 2015 and 2016. Seeds were sown on 11 April 2015 and 8 April 2016, respectively. Sowing time did not match in two years due to the weather condition. Each line was planted with three experimental plots respectively. Plant height and crown width of six plants of each line were measured at five different developmental stages: seedlings stage (35 days after planting, DAP), developing stage (55 DAP), buds stage (75 DAP), flowers stage (95 DAP), and pods stage (115 DAP). The intact young leaves were collected and immediately frozen in liquid nitrogen and stored at -80 °C until enzyme activity analysis and RNA extraction.

The chemical characteristics of the soil profile (0–20 cm) was evaluated. In 2015, the contents of available nitrogen, available phosphate, available potassium, and available ferrum were 18.91 mg kg^{-1}, 25.94 mg kg^{-1}, 180.56 mg kg^{-1}, and 7.78 mg kg^{-1}, respectively. The organic matter content was 1.58% and the soil pH was 8.54. In 2016, the surface soil (0–20 cm) contained 16.55 mg kg^{-1} available nitrogen, 21.52 mg kg^{-1} available phosphate, 188.21 mg kg^{-1} available potassium, and 7.56 mg kg^{-1} available ferrum. The organic matter content was 1.85% and the soil pH was 8.22.

2.2. Antioxidant enzyme activity assays

The extraction of antioxidant enzyme was based on Yin (2013) method with some modifications, i.e., 0.2 g frozen leaves were finely ground in liquid nitrogen with 1% polyvinylpyrrolidone and 1.5 mL 50 mM potassium phosphate buffer (pH 7.8, 1 mM EDTA-Na$_2$ and 0.5% Triton X-100). The homogenate was centrifuged at 12,000 *g* for 20 min at 4 °C. The supernatant was collected and used for superoxide dismutase (SOD, EC1.15.1.1), peroxidase (POD, EC1.11.1.7), ascorbate peroxidase (APX, EC1.11.1.1), catalase (CAT, EC1.11.1.6), and monodehydroascorbate reductase (MDHAR, EC1.6.5.4) analysis. MDHAR activity was expressed as μmol·g^{-1} FW (fresh weight), and other four enzyme activities were expressed as U mg^{-1} protein minute FW. All enzyme activity was carried out with three replicates.

SOD activity was determined according to method described by Beauchamp and Fridovich (1971). One unit of SOD activity was defined as the quantity of SOD required to produce a 50% inhibition of NBT (Nitroblue tetrazolium) at 560 nm.

APX activity was determined by measuring the absorbance decrease at 290 nm per minute (Kong et al., 2015). 4 mL reaction system consisted of 200 μL supernatant, 3.4 mL phosphate buffer (50 mM, pH 7.2), 200 μL ascorbic acid and 200 μL H_2O_2. One unit of APX was defined as decreasing 0.1 in absorbance at 290 nm in 1 min.

CAT activity was determined according to the method described by Aebi et al. (1974) with some changes. 3 mL reaction mixture comprised of 0.5 mL sodium phosphate buffer (50 mM, pH 7.0), 0.5 mL 20 mM H_2O_2 and 2 mL supernatant. Decrease of the absorbance was observed at 240 nm. One unit of CAT was defined as decreasing 0.1 in absorbance at 240 nm in 1 min.

POD activity was based on Hammerschmidt et al. (1982) method. 2 mL reaction mixture contained 0.5 mL potassium phosphate buffer (50 mM, pH 7.5), 0.5 mL 3.4 mM guaiacol, 0.5 mL 0.9 mM H_2O_2, and 0.5 mL supernatant. The absorbance increase at 470 nm was recorded for 1 min. One unit of POD activity was defined as the changes in absorbance of 0.01 in 1 min.

MDHAR activity was determined according to Arrigoni et al. (1981) with some modifications. 1 mL reaction system contained phosphate buffer (50 mM, pH 7.2), 20 μL 2.5 mM AsA, 20 μL 0.1 mM NADH, 10 μL AsA enzyme (EC 1.10.3.3) and 20 μL supernatant. Decrease of absorbance in 340 nm due to NADH oxidation was measured to calculate the MDHAR activity. One unit of MDHAR activity was defined as the decrease of absorbance of 0.1 in 1 min.

2.3. Determination of proline, hydrogen peroxide and malondialdehyde contents

Proline content was estimated using the method described by Shi et al. (2012) with some modifications, i.e., 0.5 g leaves were ground in liquid nitrogen and extracted with 3% (w/v) sulfosalicylic acid, and then 2 mL of ninhydrin and 2 mL of glacial acetic acid were added. The homogenate was boiled for 40 min and the reaction was stopped in an ice bath. 5 mL toluene was added and incubation at room temperature for one day. Proline content was calculated at 520 nm absorbance by making the standard curve and expressed as U mg^{-1}·protein·min FW.

Malondialdehyde (MDA) was measured by thiobarbituric acid (TBA) method with some modifications (Gutteridge, 1982). 0.5 g leaves were homogenized with 5 mL of 10% trichloroacetic acid (TCA). The homogenate was centrifuged at 5000 *g* for 10 min. 2 mL of supernatant and 2 mL of 10% TCA containing 0.6% (w/v) TBA were added. The homogenate was boiled for 30 min, and cooled in an ice bath and then centrifuged at 10,000 *g* for 10 min. The absorbance of the supernatant was measured at 532 nm. The non-specific absorption value at 600 nm was subtracted. MDA content was expressed as μmol·g^{-1} FW.

Hydrogen peroxide (H_2O_2) content was determined following the method described by Yin et al. (2010). 1 g leaves were finely ground with 5 mL 0.1% trichloroacetic acid. The homogenate was centrifuged at 10,000 *g* for 15 min. 0.5 mL of the supernatant was added to 0.5 mL potassium phosphate buffer (10 mM pH 7.0) and 1 mL KI (1 M). H_2O_2 content was calculated at 390 nm by making the standard curve. H_2O_2 content was expressed as mmol·g^{-1} FW.

2.4. Cloning of antioxidant enzyme genes

Universal primers for gene clone were designed by CODEHOP (http://blocks.fhcrc.org/codehop.html). PCR reaction was conducted in 50 μL volume containing 25 μL 2 × Taq Mix (TransGen, China), 19 μL ddH_2O, 2 μL forward primer (1 mM), 2 μL reverse primer (1 mM), 2 μL cDNA template (50 ng/μL). The program was initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 52–56 °C for 40 s and 72 °C for 1.5 min, the final extension at 72 °C was held for 7 min in a Veriti 96 Thermal Cycler (Applied Biosystems, USA). PCR products were purified and ligated with PMD19-T Vector (Takara, Japan). DH5α competent cells (Tiangen, China) were used for transformation. After PCR identification the positive spots were sequenced by AuGCT Technology & Services Company (Beijing, China). The primer sequences have been listed in Table 1.

2.5. Antioxidant enzyme genes expression analysis

Total RNA was extracted from *S. obtusifolia* leaves using RNA Prep Plant Kit (Tiangen, China) in accordance with manufacturer's protocol. The quality and concentration of RNA were examined by agarose gel electrophoresis and NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). The first strand cDNA for qRT-PCR analysis was synthesized by using PrimeScript RT reagent Kit (Takara, Japan). Primers used for qRT-PCR identification have been listed in Table 1. 18S ribosomal RNA was used as internal control (Li et al., 2016). Real-time PCR was performed according to manufacturer's instruction of SYBR Premix Ex Taq II Kit (Takara, Japan). PCR reaction was conducted in 20 μL volume containing 10 μL 2 × SYBR Premix, 7 μL ddH_2O, 1 μL forward primer (1 mM), 1 μL reverse primer (1 mM) and 1 μL template cDNA (60 ng/μL). PCR reaction was performed on a CFX 96 Thermal Cycler (BioRad, USA) using the protocol that initial denaturation at 95 °C for 30 s, followed by 40 cycles each at 60 °C for 5 s. Quantification of the gene expression was performed with △△Ct method. qRT-PCR experiments were performed with three biological and two technical repeats. The results were represented as means ± SD (Standard deviation).

2.6. Determination of secondary metabolite contents by HPLC

The contents of five major secondary metabolites, i.e., aurantio-obtusin, chrysophanol, physcion, emodin, and aloe-emodin of *S. obtusifolia* seeds were determined according to Mao et al. (2017) with some modifications, i.e., Waters X-Bridge column (5 μm, 250 mm × 4.6 mm) was used in this study. Standard references of aurantio-obtusin (111900–201504), chrysophanol (110796–201520), physcion (110758–200611), emodin (110756–200110) and aloe-emodin (110795–201308) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The detection wavelength was 284 nm. Separation was achieved by elution using a linear gradient with acetonitrile (A) and 0.03% phosphoric acid (B). The gradient condition was 0–15 min 40% (A), 15–30 min 40–90% (A), 30–40 min 90% (A). The column temperature was maintained at 30 °C. The flow rate was at 1.0 mL min^{-1}. The secondary metabolite content was performed with three replications.

2.7. Seed yield assessment

At physiological maturity stage, ten *S. obtusifolia* plants from each experimental plot were harvested to assess the seed yield. The harvested seeds were dried at room temperature (25 ± 3 °C).

2.8. Data analysis

Data were analyzed by Dunnett's test using SPSS 22.0 (PASW STATISTIC, USA) software. *P* value indicates significant difference between SP-lines and GC-line at 0.05 level ($P \leq 0.05$). The correlation coefficients among agronomic trait, antioxidant enzyme activity, and secondary metabolite content were evaluated by Pearson correlation coefficient analysis.

3. Results

3.1. Agronomic traits measurement

The dynamic variations of plant height and crown width were shown in Fig. 1. In 2015, from 35 DAP to 75 DAP, SP-lines and GC-line had a similar growth regularity on plant height and crown width. At 95 DAP, the plant height and crown width of SP-lines were higher than that of GC-line (Fig. 1A and B), although these differences were not statistically significant ($P > 0.05$). In 2016, the values of plant height and crown width were lower than that of 2015. SP-lines also exhibited higher plant height and crown width than that of GC-line (Fig. 1C and D), which was similar to that of 2015. Significant difference of plant height was observed between QC10 and GC-line at 95 DAP. The crown width of QC29 and QC46 was significantly higher ($P \leq 0.05$) than that of GC-line at 115 DAP. The results indicated SP-lines displayed better growth as compared to that of GC-line.

Table 1
Primers used for gene clone and qRT-PCR identification.

Gene clone	Primer 5′-3′	qRT-PCR	Primer 5′-3′
SoSOD	F: GGGCTCCGGCTGGGYNTGGYT	qSoSOD	F: TCCTGGGCCTGGATGTATGG
	R: GGTAGTAGGCGTGCTCCCANAYRTC		R: TGCTTCAGCAGAGCGCAGAT
SoPOD	F: CGGATGCACTTYCAYGAYTGYT	qSoPOD	F: TGCTTCGTTAATGGGTGCGATGGA
	R: TGGCCAGGATGTCGGCRCANSWNAC		R: CCGTGAAGGTTGAGGTGTCGTCTA
SoMD	F: CCCCCGAGTGGTACAAGGARMANGGNAT	qSoMD	F: AGCACATTGCATGGCTCGTT
	R: CATGGGGAAGGCGGCNACRTCNCC		R: CCAATTCCCACCACAACCAT
SoAPX	F: GGCACGACGCCGGNACNTWYGA	qSoAPX	F: ACGAAGACGGGTGGACCTAA
	R: TGGCCGCCGGACARNGCNACDAT		R: TCAACTGCAACAACACCGGC
SoCAT	F: CCCATCCTGCTGGAGGAYTAYCAYBT	qSoCAT	F: CGGATCCCAGAACGTGTTGT
	R: CCTCGTCCCGGTGCADNAVRTTCAT		R: TCAGGGCTACCACGCTCATG
		18S	F: TTGAAGAACGGTGATGCAGGTA
			R: CACACTCTTGATGACTCCCACA

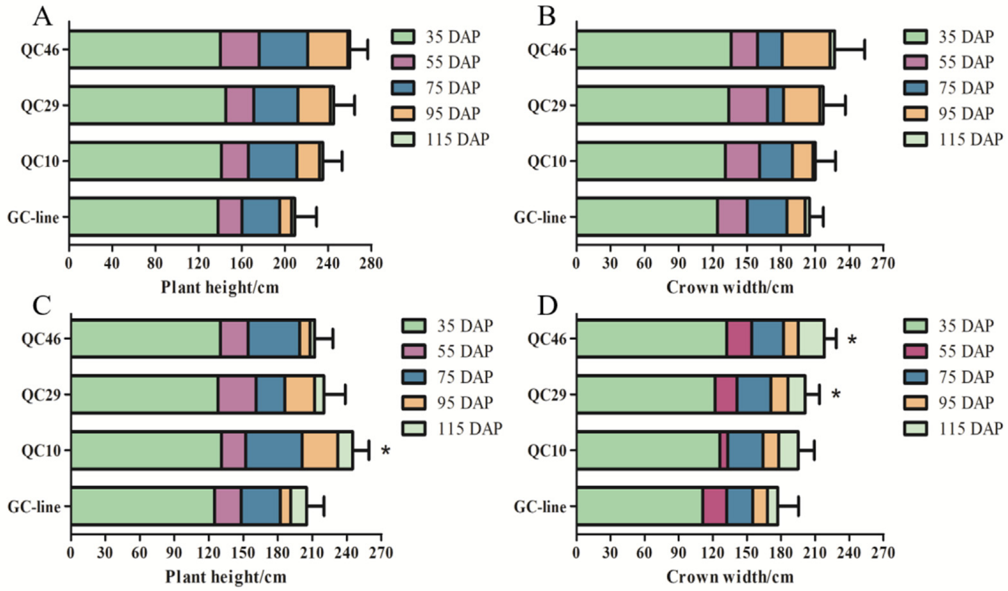


Fig. 1. Dynamic growth pattern of plant height and crown width of SP-lines and GC-line in 2015 (A and B) and 2016 (C and D). Dunnett's test set GC-line values as the standard and three SP-lines values are compared with GC-line values, respectively. Asterisks demonstrate significant difference were detected between SP-lines and GC-line ($P \leq 0.05$). Values are means ± SD (n = 6).

3.2. Cloning of antioxidant enzyme genes

Due to lacking of gene sequence information, we cloned the five antioxidant enzyme genes. PCR products were recovered and subcloned into PMD-19T vector and then sequenced. The 336 bp long band completely matched the superoxide dismutase enzyme domain identified using BLAST method (https://www.ncbi.nlm.nih.gov/cdd/). Thus, the fragment was named *SoSOD* and submitted to Genbank and gained the accession number (KY703223). We also cloned 204 bp *SoPOD* fragment (KY767019), 819 bp *SoCAT* fragment (KY767018), 364 bp *SoAPX* fragment (KY703224), and 677 bp *SoMDHAR* fragment (KY767020), respectively.

3.3. SP-lines had higher antioxidant enzyme activities

Five key antioxidant enzyme activities were determined at five different developmental stages with 20 days interval. In 2015, SP-lines showed higher SOD activity than that of GC-line. At 95 DAP, SOD activity of QC29 and QC46 was 1.96- and 1.85-fold to that of GC-line. SOD activity decreased at 115 DAP, and significant differences were detected between SP-lines and GC-line (Fig. 2A). As shown in Fig. 2B, QC29 and QC46 showed a remarkably higher POD activity compared to GC-line activity at all test points except at 35 DAP. Significantly higher CAT activity was detected in QC29 and QC46 at 75 DAP, 95 DAP, and 115 DAP (Fig. 2C). At 75 DAP and 95 DAP, APX activity largely enhanced in QC29 and QC46 (Fig. 2D). MDHAR activity of QC10, QC29, and QC46 was 1.86-, 2.43-, and 2.05-fold to that of GC-line at 95 DAP, respectively (Fig. 2E). SP-lines exhibited a significantly up-regulated proline content compared to GC-line content at 75 DAP, 95 DAP, and 115 DAP. The highest proline content of QC46 was found at 115 DAP which was 2.54-fold of GC-line content (Fig. 2F). Contrast to the higher antioxidant enzyme activities of SP-lines, SP-lines exhibited a lower MDA content. At 95 DAP, a dramatic decrease of MDA content was observed in QC29 and QC46 (Fig. 2G). H_2O_2 content presented an initial decrease and subsequent increase pattern. SP-lines presented a significantly lower H_2O_2 content as compared to GC-line at 95 DAP. At 115 DAP, all lines showed increment in H_2O_2 content and a higher increment was detected in GC-line (Fig. 2H).

In 2016, the antioxidant enzyme activities exhibited similar fluctuation patterns to those of 2015. At 95 DAP, SOD activity of QC29 and QC46 was 1.47- and 1.56-fold compared to GC-line activity (Fig. 3A). POD activity of SP-lines was clearly higher than that of GC-line at 75 DAP (Fig. 3B). QC29 and QC46 showed remarkable increment of CAT activity at 95 DAP, which was 2.33- and 1.88-fold to that of GC-line (Fig. 3C). As illustrated in Fig. 3D, higher APX activity was detected in QC29 and QC46 at 95 DAP. MDHAR activity of QC29 and QC46 was strongly increased as compared to GC-line activity at 75 DAP. The highest MDHAR activity was found in QC46 at 95 DAP which was 1.72-fold to that of GC-line (Fig. 3E). Proline content tended to increase during the developmental stage, and SP-lines showed a comparatively higher content (Fig. 3F). MDA content of QC10, QC29, and QC46 were 75.8%, 78.4%, and 76.2% of GC-line content at 75 DAP, and reaching 80.1%, 82.5%, and 83.6% at 95 DAP, respectively (Fig. 3G). H_2O_2 content of QC29 and QC46 reduced significantly at 95DAP. H_2O_2 content of SP-lines decreased considerably at 115 DAP (Fig. 3H).

3.4. SP-lines exhibited higher antioxidant genes expression levels

SP-lines exhibited a higher gene expression level than that of GC-line. At 75 DAP, *SOD* expression level of QC29 and QC46 was 3.25- and 2.66-fold to that of GC-line, reaching 4.11- and 2.81-fold at 95 DAP (Fig. 4A). *POD* expression level of QC10, QC29, and QC46 was 1.73-, 2.95-, and 1.89-fold of GC-line expression level at 75 DAP, and changed to 2.41-, 3.52-, and 2.44-fold at 95 DAP, respectively (Fig. 4B). *CAT* expression of QC29 and QC46 was higher than that of GC-line at 35 DAP and 55 DAP (Fig. 4C). *APX* expression was up-regulated in SP-lines at 55 DAP and 75 DAP. Moreover, the expression level of *APX* was largely enhanced in QC29 and QC46 at 95 DAP and 115 DAP (Fig. 4D). *MDHAR* expression level of QC46 increased strongly at each test point compared to that of GC-line (Fig. 4E).

Similar to the results of 2015, higher expression level was detected in SP-lines, especially *SOD*, *POD*, and *MDHAR*. At 95 DAP, *SOD* expression of QC10, QC29, and QC46 was 1.99-, 3.21-, and 2.75-fold to that of GC-line, respectively. *SOD* expression decreased dramatically at 115 DAP, but SP-lines still exhibited a higher expression level (Fig. 5A). QC29 and QC46 demonstrated a clearly raised *POD* expression level compared to GC-line except at 35 DAP. At 95 DAP, *POD* expression level of QC10, QC29, and QC46 was 2.41-, 4.66-, and 3.74-fold to that of GC-line, respectively (Fig. 5B). *APX* expression of SP-lines showed remarkable differences with GC-line at 75 DAP and 95 DAP. A clearly increased expression of *APX* was also observed in QC46 at 75 DAP and 115 DAP (Fig. 5D). SP-lines exhibited a higher *MDHAR* expression level than that of GC-line at 55 DAP, 75 DAP, and 95 DAP (Fig. 5E).

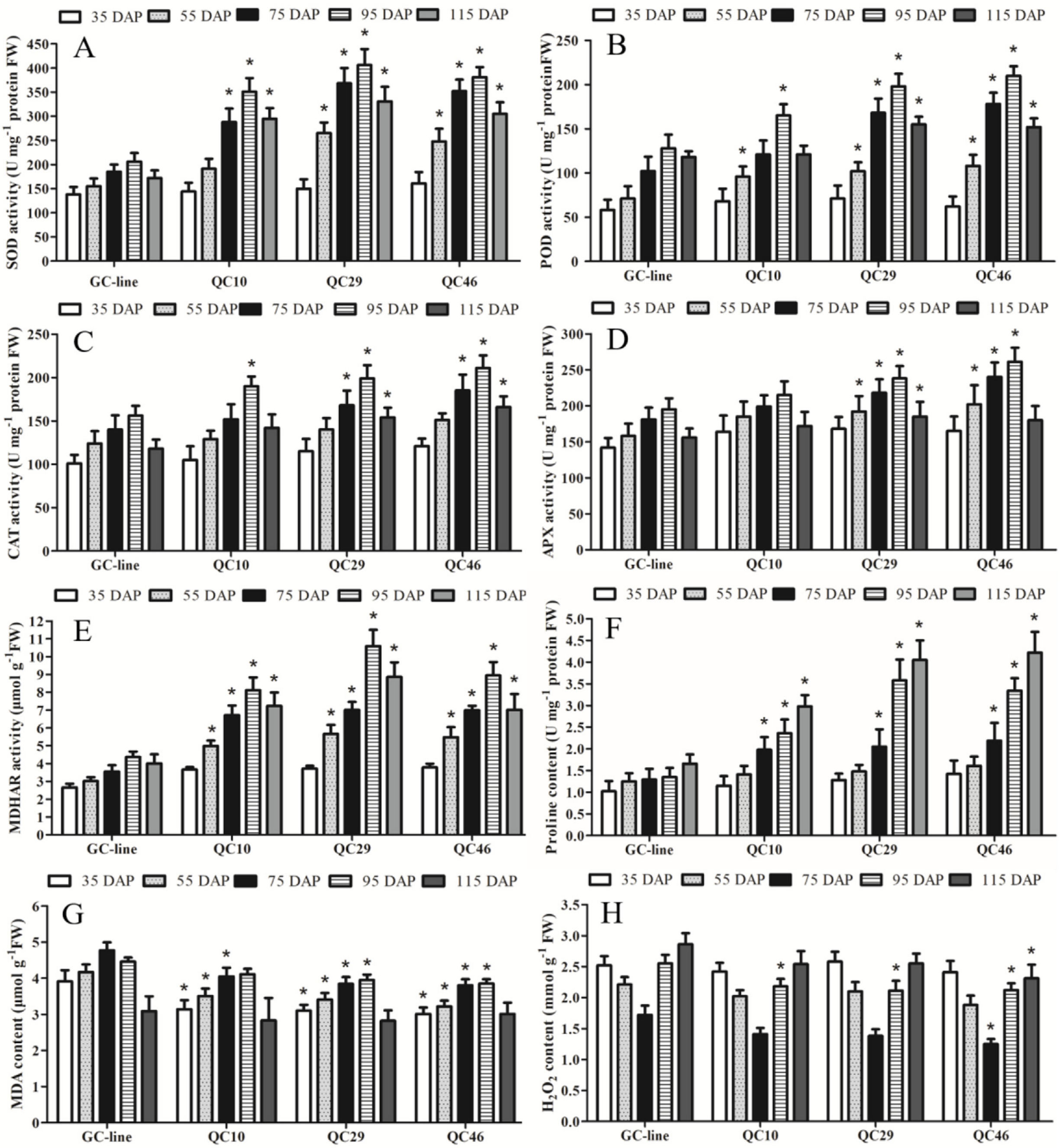


Fig. 2. Variations of antioxidant enzyme activities of SOD, POD, CAT, APX, MDHAR (A to E), and the contents of proline (F), MDA (G), and H_2O_2 (H) at five different developmental stages in 2015. Asterisks demonstrate significant difference were detected between SP-lines and GC-line ($P \leq 0.05$). Values are means ± SD with three replications (Dunnett's test).

3.5. Seed yield

In 2015, seed yields of QC10, QC29, and QC46 were 2.15, 2.29, and 2.34 kg, respectively, while the GC-line yield was 2.05 kg. Seed yield of SP-lines was higher than that of GC-line. Significant differences ($P \leq 0.05$) were observed between QC29 (2.29 kg) and GC-line (2.05 kg), QC46 (2.34 kg) and GC-line (2.05 kg), respectively. Seed yield of all lines reduced in 2016. The yields of QC29 and QC46 were 2.24 and 2.26 kg, both of which were significantly higher than that of GC-line (2.03 kg).

3.6. Analysis of secondary metabolite contents

Aurantio-obtusin and chrysophanol contents in seeds of SP-lines ranged from 0.806 to 0.815 and from 2.155 to 2.191 mg g^{-1} DW, while the contents of the two components in GC-line were 0.808 and 2.122 mg g^{-1} DW. The average contents of emodin, aloe-emodin, and physcion in SP-lines were 0.549, 0.271, and 0.135 mg g^{-1} DW, respectively. The contents of these components in GC-line were 0.545, 0.268, and 0.135 mg g^{-1} DW, respectively. In 2015, no significant difference of secondary metabolites were detected between SP-lines and GC-line. In 2016, secondary metabolites contents were higher than that of 2015 (Table 2). Aurantio-obtusin and chrysophanol contents of SP-lines ranged from 0.815 to 0.865 and from 2.218 to 2.315 mg g^{-1} DW,

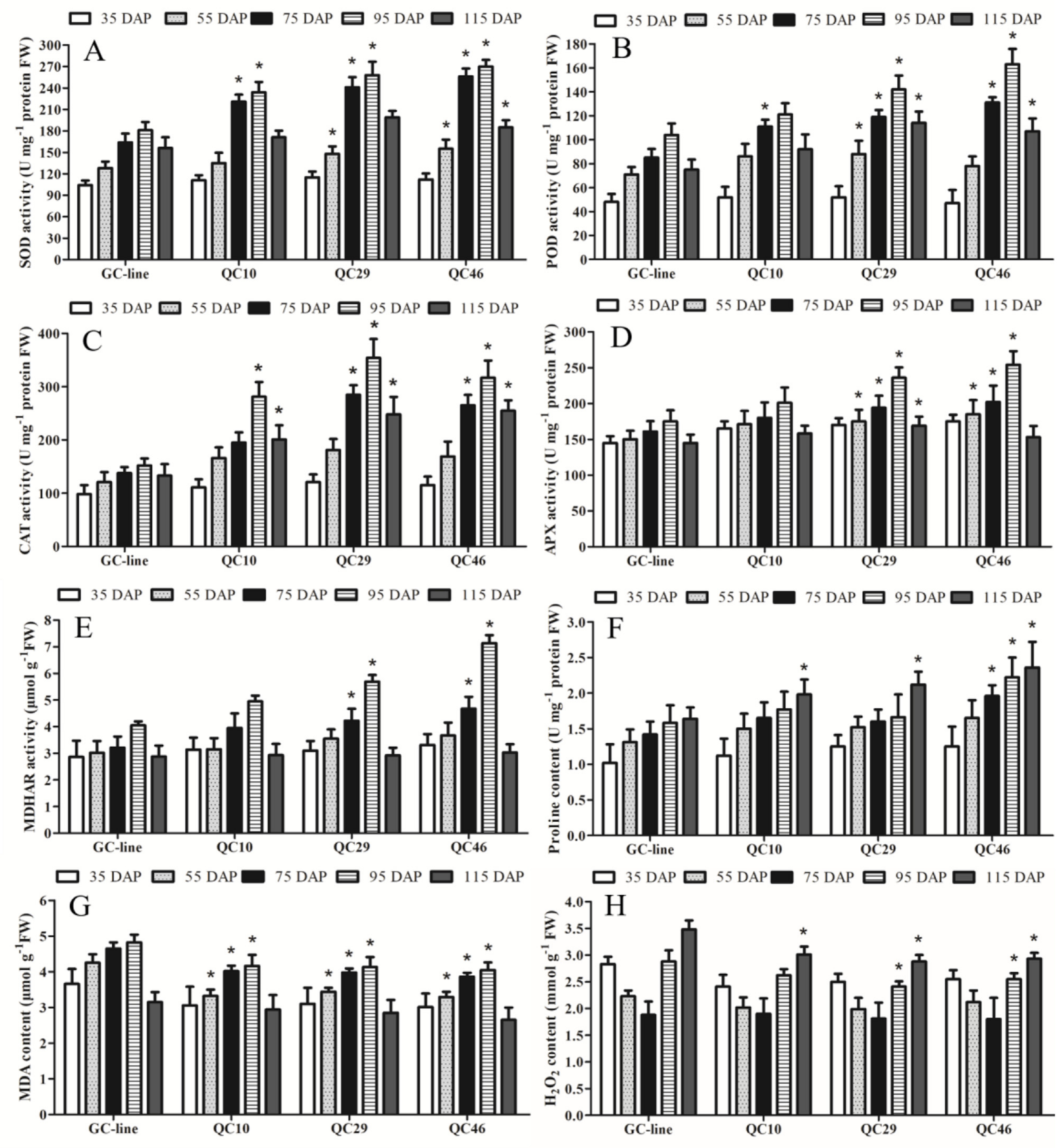


Fig. 3. Variations of antioxidant enzyme activities of SOD, POD, CAT, APX, MDHAR (A to E), and the contents of proline (F), MDA (G), and H_2O_2 (H) at five different developmental stages in 2016. Asterisks demonstrate significant difference were detected between SP-lines and GC-line ($P \leq 0.05$). Values are means ± SD with three replications (Dunnett's test).

while the contents of the two components in GC-line were 0.822 and 2.251 mg g^{-1} DW. Noticeably, aurantio-obtusin content of QC29 (0.865 mg g^{-1} DW) was significantly higher ($P \leq 0.05$) as compared to GC-line content (0.822 mg g^{-1} DW). The contents of aurantio-obtusin and chrysophanol meet the requirement of Chinese Pharmacopoeia (aurantio-obtusin ≥ 0.8 mg g^{-1} DW and chrysophanol ≥ 2.0 mg g^{-1} DW).

3.7. Pearson correlation coefficient analysis

To investigate the relationship among agronomic trait, physiological parameter and secondary metabolite content, we combined two years' data and performed the Pearson correlation coefficient analysis. Aurantio-obtusin and chrysophanol were the quality marker of *S. obtusifolia* seeds according to Chinese Pharmacopoeia, so they were chosen for Pearson correlation analysis. As shown in Fig. 6, five antioxidant enzyme activities exhibited a positive correlation with seed yield, and the most positive correlation was found between APX activity and seed yield (R = 0.878). Besides, aurantio-obtusin content showed significantly positive correlations with POD (R = 0.461) and APX activities (R = 0.447), respectively. Seed yield also exhibited a positive correlation with chrysophanol content (R = 0.495). Conversely, a

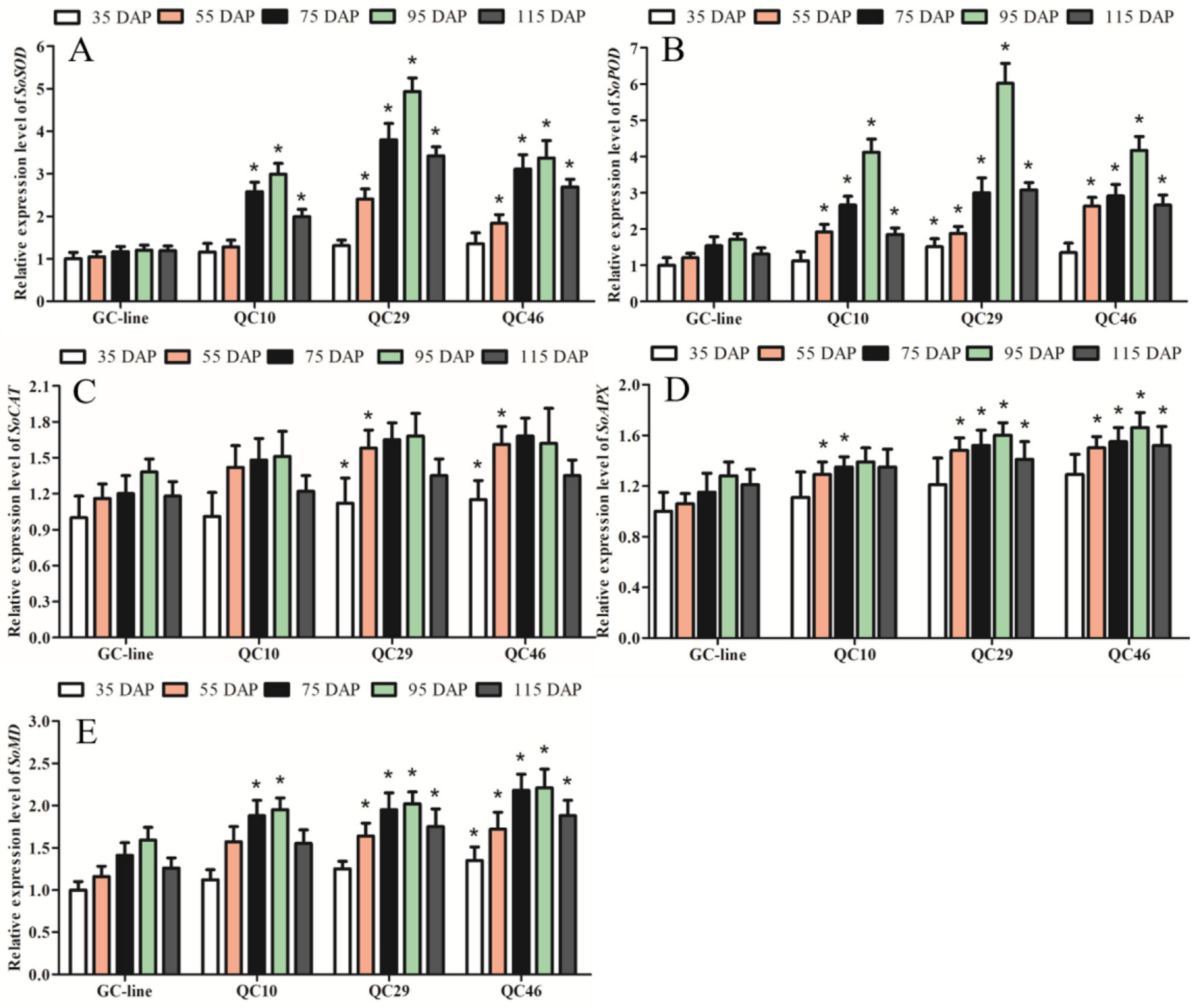


Fig. 4. Expression profiles of antioxidant enzyme genes at different developmental stages in 2015.18S RNA was used as the internal control. The transcript level of GC-line at 35 DAP was set to 1. Asterisks demonstrate significant difference ($P \leq 0.05$) of gene expression between SP-lines and GC-line. Values are shown as means ± SD with six replications (Dunnett's test).

significantly negative correlation between seed yield and H_2O_2 content was noticed ($R = -0.631$). Chrysophanol content showed negative correlations with H_2O_2 content ($R = -0.796$) and MDA activity ($R = -0.730$).

4. Discussion

In the present study, we performed a comprehensive study to analyze the differences on agronomic, physiological, transcriptional, and metabolic characteristics between SP-lines and GC-line. To our knowledge, this is the first attempt to understand the physiological and transcriptional traits of spaceflight-subjected *S. obtusifolia*.

4.1. SP-lines exhibited better physiological and transcriptional traits

SP-lines showed higher plant height and crown width compared to that of GC-line, although the significant difference ($P \leq 0.05$) was only observed in 2016 (Fig. 1). Our former studies also indicated that SP-lines exhibited the higher plant height, crown width, and bigger cross diameter (Mao et al., 2014, 2015). In addition, QC29 and QC46 exhibited significantly higher seed yield. These studies indicated that spaceflight could affect the agronomic traits of medicinal plants.

SOD, POD, CAT, APX, and MDHAR are the main protective enzymes and play pivotal roles in plant defense system. Higher antioxidant enzyme activity was observed in SP-lines, especially in QC29 and QC46 (Figs. 2 and 3). Proline content was increasing in both SP-lines and GC-line during the growing season, while the increase was more apparent in SP-lines (Figs. 2F and 3F). Higher MDA and H_2O_2 contents were detected in GC-line (Fig. 2G and H; Fig. 3G and H). These results were in accordance with previous studies on spaceflight-subjected *Acer mono* (Li et al., 2012) and *Brassica juncea* (Shen et al., 2012), which indicated that spaceflight-subjected materials had higher SOD, POD, and CAT activities. The results demonstrated that SP-lines may keep a better cell homeostasis by increasing the antioxidant enzyme activities and proline content to reduce the MDA and H_2O_2 contents.

Previous studies on spaceflight-subjected medicinal herbs not involved in transcriptional level due to lacking of antioxidant enzyme gene sequence. In this study, to elucidate the transcript variation of SP-lines, we firstly cloned the five antioxidant enzyme genes and deposited the gene sequence information to Genbank database. Parallel to results of antioxidant enzyme activity, the expression levels of *SOD*, *POD*, *APX*, *CAT*, and *MDHAR* were up-regulated significantly in SP-lines. This

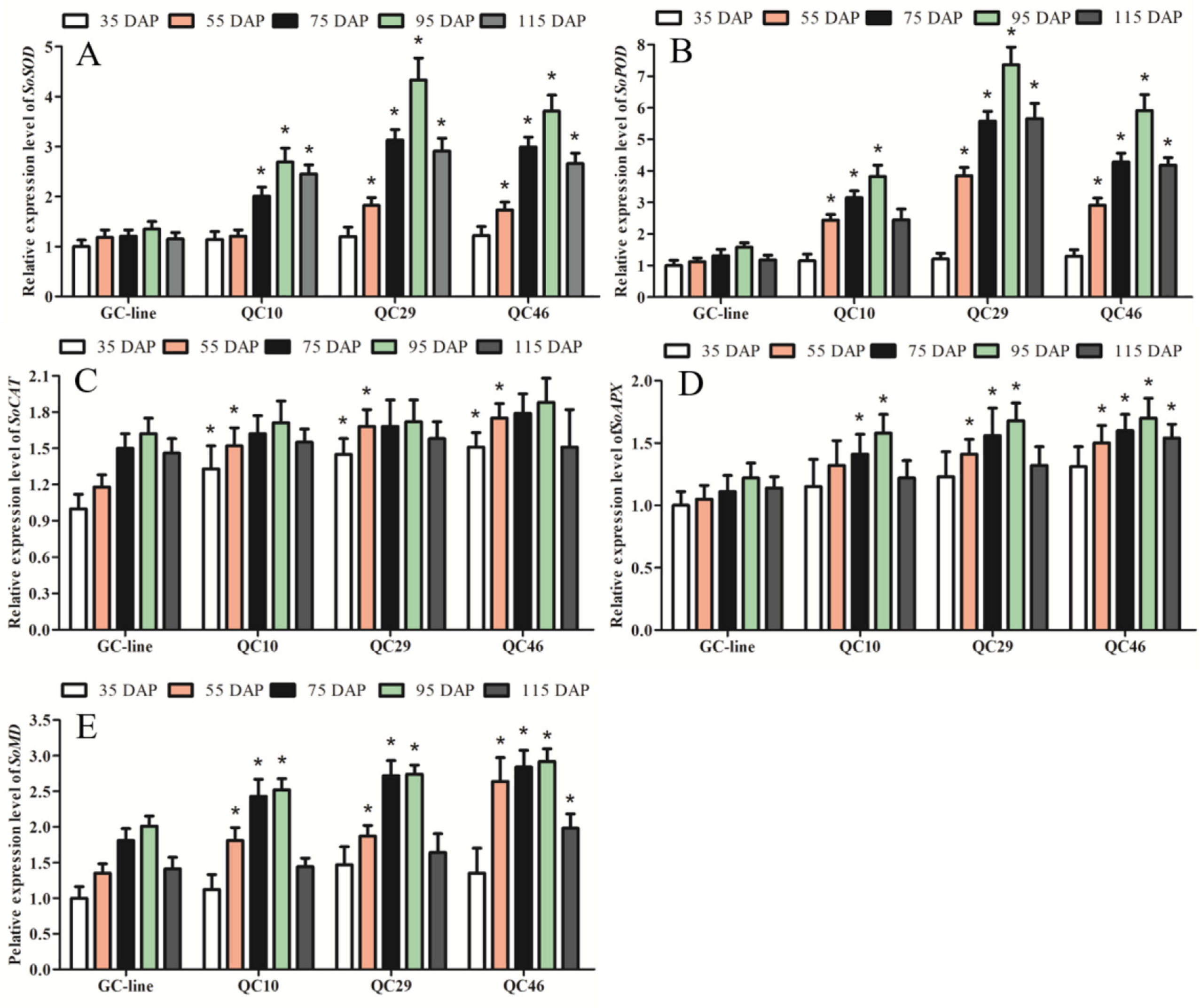


Fig. 5. Expression profiles of antioxidant enzyme genes at different developmental stages in 2016.18S RNA was used as the internal control. The transcript level of GC-line at 35 DAP was set to 1. Asterisks demonstrate significant difference ($P \le 0.05$) of gene expression between SP-lines and GC-line. Values are shown as means ± SD with six replications (Dunnett's test).

results not only indicated the up-regulated gene expression in SP-lines, but also validated the results of antioxidant enzyme analysis. Similar to our results, former studies on *Avena sativa* (Kong et al., 2015) and *Arabidopsis thaliana* (Kayıhan et al., 2016) also indicated that the increased antioxidant enzyme activities were attributed to the increments of antioxidant gene expressions. The results of physiological and transcriptional analysis showed that SP-lines had higher antioxidant enzyme activities and gene expression levels. Our results also implied that the alterations of antioxidant enzyme and gene transcript were the excellent traits of spaceflight-subjected materials.

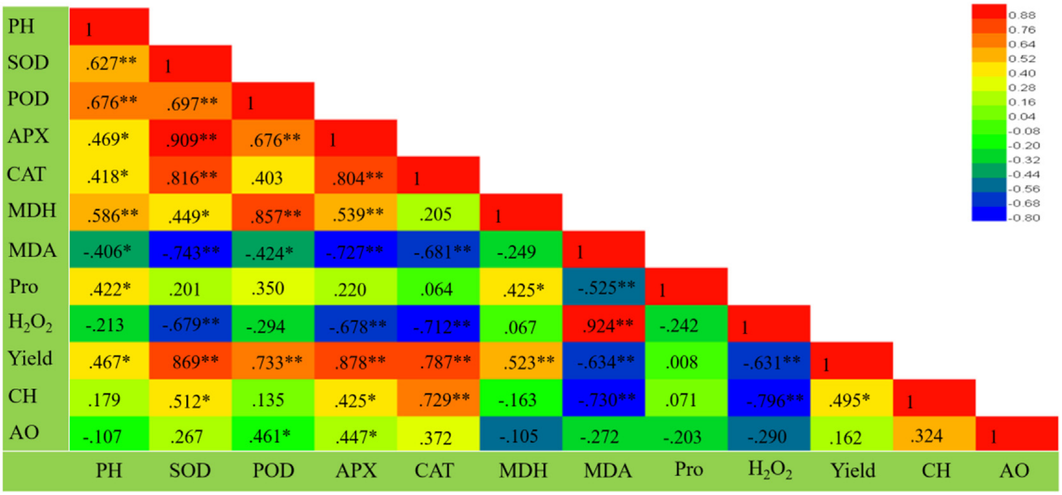
4.2. Spaceflight-subjected effects the secondary metabolite content

Former studies indicated that spaceflight-subjected could induce the

Table 2
Contents of five secondary metabolites in *S. obtusifolia* seeds (mg·g^{-1} DW).

		GC-line	QC10	QC29	QC46
2015	Aurantio-obtusin	0.808 ± 0.006	0.806 ± 0.005	0.815 ± 0.005	0.811 ± 0.008
	Chrysophanol	2.122 ± 0.012	2.155 ± 0.010	2.191 ± 0.015	2.156 ± 0.012
	Physcion	0.135 ± 0.015	0.133 ± 0.010	0.138 ± 0.018	0.135 ± 0.016
	Emodin	0.545 ± 0.012	0.550 ± 0.008	0.552 ± 0.005	0.544 ± 0.011
	Aloe-emodin	0.268 ± 0.011	0.271 ± 0.012	0.275 ± 0.010	0.272 ± 0.015
2016	Aurantio-obtusin	0.822 ± 0.005	0.815 ± 0.006	0.865 ± 0.008*	0.826 ± 0.008
	Chrysophanol	2.251 ± 0.010	2.245 ± 0.015	2.218 ± 0.010	2.254 ± 0.012
	Physcion	0.142 ± 0.008	0.146 ± 0.006	0.145 ± 0.005	0.141 ± 0.005
	Emodin	0.568 ± 0.015	0.558 ± 0.021	0.575 ± 0.012	0.572 ± 0.020
	Aloe-emodin	0.295 ± 0.013	0.288 ± 0.015	0.302 ± 0.016	0.298 ± 0.016

Values were expressed as the mean ± SD (n = 3). "*" demonstrates significant difference was detected between QC29 and GC-line at $P \le 0.05$ level.



	PH	SOD	POD	APX	CAT	MDH	MDA	Pro	H_2O_2	Yield	CH	AO
PH	1											
SOD	.627**	1										
POD	.676**	.697**	1									
APX	.469*	.909**	.676**	1								
CAT	.418*	.816**	.403	.804**	1							
MDH	.586**	.449*	.857**	.539**	.205	1						
MDA	-.406*	-.743**	-.424*	-.727**	-.681**	-.249	1					
Pro	.422*	.201	.350	.220	.064	.425*	-.525**	1				
H_2O_2	-.213	-.679**	-.294	-.678**	-.712**	.067	.924**	-.242	1			
Yield	.467*	869**	.733**	.878**	.787**	.523**	-.634**	.008	-.631**	1		
CH	.179	.512*	.135	.425*	.729**	-.163	-.730**	.071	-.796**	.495*	1	
AO	-.107	.267	.461*	.447*	.372	-.105	-.272	-.203	-.290	.162	.324	1

Fig. 6. Pearson correlation analysis based on combined data of two years (2015 and 2016) of antioxidant enzyme activities, proline, H_2O_2, and MDA contents, seed yield and secondary metabolites. The correlation coefficients were presented by both color and number. Pearson correlation coefficient value is between 1 and -1, where 1 means that the variables are completely positive while −1 means negative. “*” and “**” indicate statistical significance at $P \leq 0.05$ and $P \leq 0.01$, respectively. PH: Plant height; MDH: MDHAR; Pro: Proline; CH: Chrysophanol; AO: Aurantio-obtusin. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

secondary metabolite variations in medicinal herbs. For example, Peng et al. (2014) demonstrated that the contents of caffeic acid, ferulic acid, rosmarinic acid, salvianolic acid A, and cinnamic acid in spaceflight-subjected *Salvia miltiorrhiza* were markedly increased as compared to that of ground control. Studies on *Glycyrrhiza uralensis* proved that four components in licorice root, isoliquiritigenin, isoliquiritin, liquiritigenin, and glabridin were different between spaceflight-subjected materials and ground control (Zhang et al., 2011, 2012; Dong et al., 2012). In this study, QC29 exhibited significantly higher aurantio-obtusin content in 2016. Although this value did not reach the significant level in 2015, it also exhibited a higher content than that of GC-line (Table 2). Concerning higher aurantio-obtusin content and seed yield, QC29 and QC46 were fantastic materials for breeding new *S. obtusifolia* varieties. Besides, QC29 could be used as the ideal material to elucidate the biosynthesis pathway of aurantio-obtusin.

4.3. POD and APX regulate aurantio-obtusin accumulation

QC29 exhibited significantly higher POD and APX activities at four different stages (except at 35 DAP) compared to that of GC-line (Fig. 2B and D; Fig. 3B and D). Moreover, either POD or APX activity showed significant positive correlations with aurantio-obtusin content in Pearson correlation analysis (Fig. 6). Based on these results, we proposed that POD and APX may participate in regulating aurantio-obtusin accumulation. Previous research have demonstrated that POD and APX were involved in regulating secondary metabolite content. For instance, *Uncaria tomentosa* increased flavonoid and phenolic contents when treated by exogenous salicylic acid, and the increment was positively related with POD enrichment (Sánchez-Rojo et al., 2015). Ma et al. (2013) addressed that POD was a key enzyme for synthesizing lignin, which was an important secondary metabolite in ginseng root. RNA-Seq analysis on the peanut mutant *pscb* revealed that its seed coat accumulated more phytomelanin through enhancing polyphenol oxidase and POD activity (Wan et al., 2016). Besides, except being an antioxidant enzyme, APX also showed multiple functions in plant cell. For instance, the thylakoidmembrane-attached isoform of ascorbate peroxidase (tAPX) involved in H_2O_2 metabolism and played a role in the regulation of γ-aminobutyric acid metabolism (Maruta et al., 2016; 2012). Furthermore, secondary metabolites played important roles in plant stress resistance (Holub et al., 2019). The increased aurantio-obtusin content in QC29 participate in resisting external stresses together with higher antioxidant enzyme activity. Taken together, we established a model to show the possible relationship between POD/APX and aurantio-obtusin (Fig. 7). We presumed that higher changes of POD and APX activities during the different developmental stages facilitated metabolite flux converts to aurantio-obtusin accumulation pathway, resulting in higher aurantio-obtusin content in QC29. To further elucidate the potential mechanism of excellent properties of QC29, transcriptome and metabolome methods have been employed to obtain a better understanding of SP-lines.

5. Conclusions

In conclusion, we conducted a comprehensive study to compare the differences between SP-lines and GC-line. SP-lines exhibited higher transcript level of antioxidant enzyme genes, antioxidant enzyme activities, seed yield and aurantio-obtusin content. We proposed that POD and APX may be involved in regulating aurantio-obtusin accumulation. These excellent traits of SP-lines can be used to breed elite *S. obtusifolia* varieties in the future.

Author contribution

MRJ and LZS conceived and designed the study. MRJ, LWR, HZG and BZQ performed the experiments. MRJ, LWR, XPG and LY carried out the data analysis. MRJ, LWR and XPG wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors claim no competing interests.

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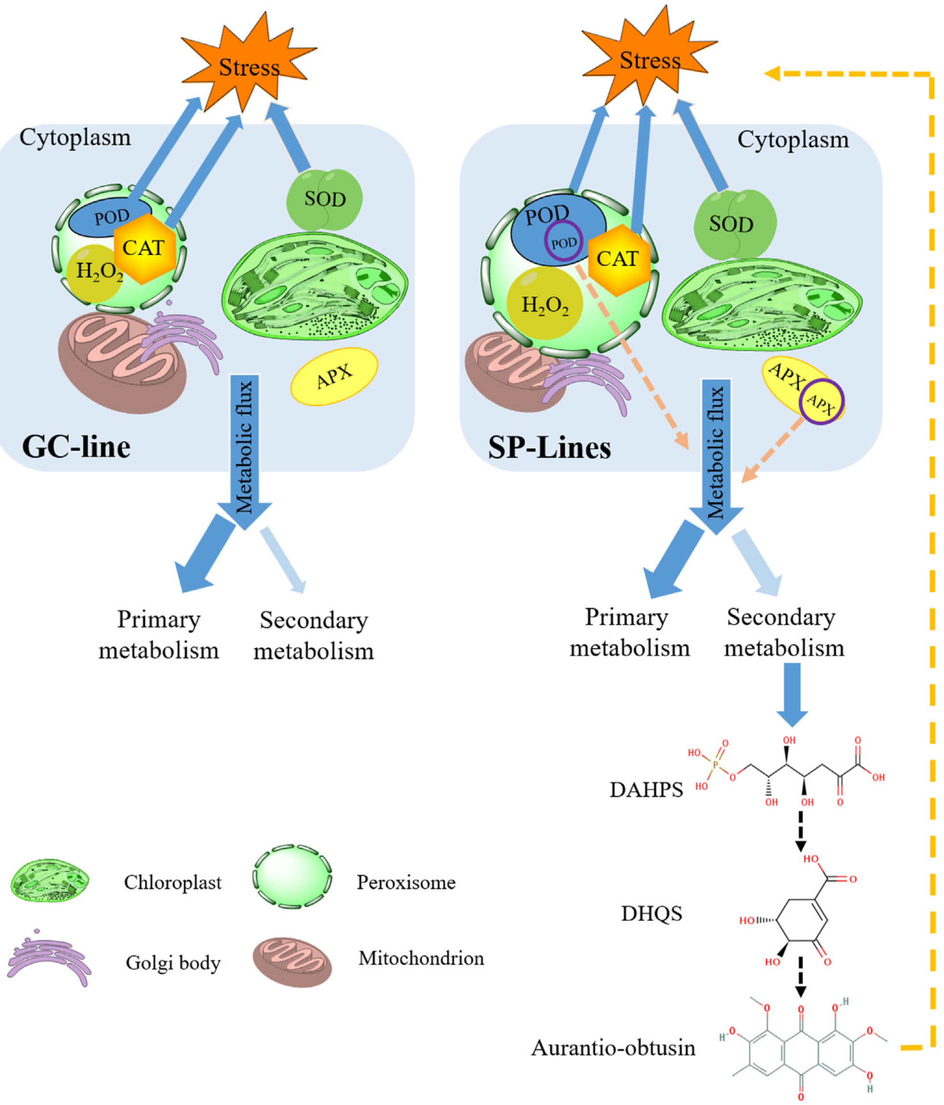

Fig. 7. A model for POD and APX regulation aurantio-obtusin accumulation. QC29 have higher antioxidant enzyme activity and offer a better protection. In addition, POD and APX may be involved in the shikimic acid and pentose phosphate processes in cytoplasm and enhance the accumulation of aurantio-obtusin. Consequently, the increased aurantio-obtusin would participate in resisting external stress which ensure a better seed yield of QC29.

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