



Article

Influence of Citrus Rootstocks on Scion Growth, Hormone Levels, and Metabolites Profile of ‘Shatangju’ Mandarin (*Citrus reticulata* Blanco)

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Abstract: Dwarfing rootstocks are a valuable genetic resource for managing high-density plantations. The selection of the appropriate scion/rootstock combination is key to improving crop performance and sustainable production in a particular environment and specific training systems. ‘Shatangju’ mandarin scion cultivar grafted onto ‘Flying Dragon’ rootstock tends to be dwarfing and develops short stature plants. To obtain insight into potential mechanisms underlying rootstock-induced dwarfing effects, we conducted a rootstock trial to examine the influence of 11 different rootstocks based on their growth vigor, antioxidants, and hormonal levels of the scion cultivar. The phenotypic observations revealed that size reduction in the ‘Flying Dragon’ rootstock is due to lower node number, shorter internodal length, and a reduced trunk diameter of the scion compared with more vigorous rootstocks. Antioxidant analysis showed that ‘Shatangju’ mandarin grafted onto ‘Flying Dragon’ and ‘Trifoliate Orange’ rootstock had significantly lower peroxidase (POD) activity than other tested rootstocks. The hormonal analysis indicated that there were markedly lower amounts of abscisic acid (ABA) in ‘Shatangju’ mandarin grafted with ‘Flying Dragon’ rootstock. In addition, trees grafted with ‘Sour Pummelo’ and ‘Flying Dragon’ depicted minimum amounts of gibberellins (GA₂₄). Moreover, several metabolites associated with organic acids, flavonoids, amino acids, and alkaloids responded differently in plants grafted with ‘Flying Dragon’ (dwarfing) and ‘Shatang Mandarin’ (vigorous) rootstocks. This study concluded that ‘Flying Dragon’ rootstock with a strong dwarfing effect has been proposed to improve high-density cultivation methods. These findings will provide useful insights for future research associated with rootstock-mediated dwarfing mechanisms of citrus rootstocks.

Keywords: dwarfing; ‘Flying Dragon’; antioxidants; vigor; morphological; biochemical analysis

1. Introduction

Citrus fruits are widely grown worldwide in tropical and subtropical regions [1,2]. China has taken a prominent position in the citrus industry worldwide, with about 41 million tons of production spread over 2.7 million hectares of cultivation area [3]. These

are popular due to their better yield, improved quality, nutritional characteristics, and adaptability to a wide range of soil and climate conditions [4]. These are rich sources of vitamins, sugars, minerals, dietary fibers, and phytochemicals [5,6].

Commercial fruit plants typically consist of two separate genotypes: the scion part and rootstock, which makes it complicated to investigate shoot–root communications [7]. High-density farming is a significant advancement in the planting of modern orchards for maximum yield return and efficient mechanized management [8,9]. Dwarf rootstock material is a major factor in this development [10,11]. Various morphological, biochemical, and physiological parameters of the scion part are influenced by rootstocks [12,13]. Moreover, these significantly affect the shoot growth traits of grafted plants, including shoot length, internodal length, trunk cross-sectional area (TCSA), branch composition, and higher yield production [14,15]. To date, only a few dwarfing citrus varieties are available because of the long-term and expensive process of conventional breeding. In rootstock breeding, this process may take longer (25 years and more). The examination of rootstocks has been a central topic in citrus research for about half a century and has rapidly expanded globally.

Compared to vigorous rootstocks, citrus dwarfing rootstocks reduce canopy volume by at least 60% [16]. The term “dwarfing” can only be given to rootstocks that lower tree volume by at least 75%, restricting tree height to 2.5 m at the maturity stage [17]. Flying Dragon would be one such dwarfing rootstock. The ‘Flying Dragon’ (*Poncirus trifoliata* (L.) Raf) is most often used as the rootstock of citrus in different regions because of its resistance to phytophthora disease, nematodes, suitability for heavy soils, and induction of dwarfism of scion part [18]. Furthermore, producing superior dwarf citrus germplasm is the main objective of horticulture breeders in developing modern citrus orchards. Thus, identifying size-controlling rootstocks and screening for superior rootstocks from hybrid seedlings are required to improve cultivation methods [19]. Many researchers have argued the physiological mechanisms behind rootstock-induced size reduction in the past; however, the related phenomena have not been clarified. It has been reported that the dwarfing effect caused by rootstock may be linked with mineral transport [20], carbohydrates accumulation [21], phenolic contents [22], anatomical characteristics [23], and hormonal levels [24,25].

Hormonal regulations have been proposed as a mechanism through which rootstocks affect scion vigor by modulating root–shoot chemical signaling [26–28]. Changes in cell division and elongation mediated by various hormones, i.e., auxin, cytokinin, gibberellin, abscisic acid, and brassinosteroids, resulted in the dwarf type phenotype [21,29,30]. Previous studies have shown that size-controlling rootstocks contain lower levels of growth-promoting hormones (GA, IAA, and Ck) and higher levels of the growth-inhibiting hormone (ABA); however, a phenomenon regarding the hormonal regulation of scion vigor is still lacking [31]. These hormones also play a key role in controlling and improving citrus tree growth and production [32]. In addition, a positive correlation was found between plant growth and cytokinin levels in peach rootstock; in contrast, a negative correlation was found between IAA and plant vigor [33]. Furthermore, alterations in plant growth directly impact canopy spread, chlorophyll contents, stomatal conductance, and photosynthetic activity. These aspects affect scion yield when grafted on a range of size-controlling rootstocks. Khan et al. [1] investigated the nutrient uptake of Salustiana sweet orange grafted onto five rootstocks and revealed that the range of size-controlling rootstocks had a considerable effect on the capacity to absorb macro- and micro-nutrients. Many researchers have noted significant variations in the chlorophyll content and photosynthetic activity of mandarin and orange scion cultivars grafted on different rootstocks [34].

Citrus is commercially produced by grafting worldwide, and rootstocks play an important role in the growth and development of grafted plants. ‘Shatang Mandarin’ (*Citrus reticulata* cv. ‘Shatangju’) is one of the superior native citrus cultivars in Southern China. The high-density citrus cultivation favored by the utilization of dwarfing rootstocks could greatly promote the citrus yield. The current research is mainly focused on analyzing the changes in hormone levels, antioxidant enzymes, metabolites profile, and scion growth of

'Shatangju' citrus grafted onto 11 different rootstocks, aiming to provide detailed information about dwarfing features that could help them to choose rootstocks.

2. Materials and Methods

2.1. Plant Materials and Culture Conditions

The experiment was conducted in Guangzhou, China (Latitude 23°160 N; longitude 113°360 E) in 2015. The uniform healthy seedlings were grown in planting boxes comprising a combination of garden soil:peat:sand = 3:2:1, *v/v/v*. In March 2016, the scions of 'Shatangju' were collected and grafted onto 1-year-old rootstocks viz., 'Shatang Mandarin' (*Citrus reticulata* Blanco cv. Shatangju), 'Sour Orange' (*Citrus aurantium* L.), 'Goutou Orange' (*Citrus aurantium* L.), 'Sour Pummelo' (*Citrus maxima*), 'Rough Lemon' (*Citrus jambhiri* Lush.), 'Xiangcheng Orange' (*Citrus junos* Sieb), 'Citrange' (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L. Raf.), 'Red Tangerine' (*Citrus reticulata* Blanco), 'Red Limonia' (*Citrus limonia* Osbeck), 'Trifoliolate Orange' (*Poncirus trifoliata* L.), 'Flying Dragon' (*Poncirus trifoliata* Raf. var. *monstrosa*). In December 2018, the grafted plants were transplanted to the Xiaokeng base of the Conghua Agricultural Extension Center. The plants were drip irrigated and exposed to conventional practices. The experiment was carried out in Randomized Complete Block Design (RCBD), and three biological replicates were made (every replicate containing two plants). A total of 66 plants were used in this study.

2.2. Measurement of Plant Growth Parameters

From March 2019, measurements were recorded concerning scion growth, which included plant height (cm), the diameter of rootstock (mm), the diameter of the scion (mm), apex shoot length (cm), average internodal length (cm), and node number and number of branches for each graft combination. (1) The plant height was measured with a measuring tape and expressed in cm. (2) Crown width was measured with a measuring tape in two directions (east–west and north–south), respectively. (3) The diameter of the trunk was measured with a Digital Vernier Caliper at three different points: the scion stem (3 cm above the graft union) and rootstock stem (3 cm below the graft union). (4) The number of nodes was calculated by counting the number of nodes on shoots above the graft union. (5) The internodal length was calculated by dividing the length of the scion by the total number of nodes. (6) The number of mature branches was calculated by the counting method on the trunk that can produce shoots.

2.3. Determination of Soluble Sugars Contents

The concentration of soluble sugar was analyzed using the anthrone method, as explained earlier by Shi et al. [35]. Then, 0.1 g of ground samples were combined with 2 mL of ethanol 80% (*v/v*) at 80 °C for 30 min. Subsequently, 100 µL of extracts were added to 2 mL of anthrone, and the mixture was heated for 10 min. At 630 nm, the absorbance was measured, and the concentration was determined by deploying a calibration curve with sucrose standard as a point of reference in the calculation.

2.4. Determination of Soluble Protein Contents

The content of soluble protein was estimated using the procedure of Coomassie brilliant blue G-250 as followed in the study of Yang et al. [36].

2.5. Determination of Enzymatic Antioxidant Activities

A total of 0.5 g of leaf tissue was mixed in 5 mL of extraction buffer (phosphate buffer, pH 7.5, containing 0.1 mM EDTA and 4% polyvinylpyrrolidone). The mixture was centrifuged at 12,000× *g* for 20 min, and to determine antioxidant enzymes, the supernatant was used [37]. The enzymatic antioxidant activity such as peroxidase (POD) and superoxide dismutase (SOD) was determined according to the methods described by Chen and Wang [38].

2.6. Determination of Endogenous Hormones Analysis

The endogenous hormones such as auxins, cytokinins, abscisic acid, 1-amino-cyclopropane-1-carboxylic acid (the ethylene precursor), salicylic acid, jasmonic acid, and gibberellins from the leaf samples were extracted using ultrahigh-performance liquid chromatography coupled to electrospray ionization tandem spectrometry (UPLC/ESI-MS/MS) according to the method explained by [39]. The hormone quantification was estimated using a standard curve method and expressed as ng g^{-1} fresh weight.

2.7. Method of Determining Metabolites

The biological samples were placed in a Lyophilizer (Scientz-100F) for vacuum freeze-drying and ground (30 Hz, 1.5 min) to powder with a grinder (400 revolutions per minute, Retsch, Germany). Then, 100 mg of the powder was weighed and dissolved in 1.2 mL of 70% methanol extract, vortex 30 min for 30 s, 6 times in total, and placed at 4 °C refrigerator overnight. After centrifugation (12,000 rpm, 10 min), the supernatant was removed using a microporous membrane (pore size 0.22 μm). The sample was filtered and stored in an injection bottle for UPLC-MS/MS analysis (SHIMADZU Nexera X2, <https://www.shimadzu.com.cn/>, accessed on 15 March 2022) and tandem mass spectrometry (Tandem mass spectrom, MS/MS) (Applied Biosystems 4500 QTRAP, <http://www.appliedbiosystems.com.cn/>, accessed on 15 March 2022).

2.8. Statistical Analysis

All data were subjected to analysis of variances by using SPSS 18.0 Statistics (SPSS Inc., Chicago, IL, USA) for correlation analysis and analysis of variance (ANOVA) to perform the statistical analysis. The differences among treatment means were evaluated by least significant difference (LSD) multiple comparison tests at $p \leq 0.05$, and different lowercase letters were used to represent significant differences among treatments. The experimental result correlation map was produced by Origin 2019, Tbtools, and Hplot web pages, and the picture-related layout was completed in Adobe illustrator 2020 and In Design 2020.

3. Results

3.1. Effect of Rootstocks on Scion Growth

Rootstocks influenced the growth vigor of grafted citrus trees (Table 1). The morphological traits, including plant height (cm), crown width (cm), number of nodes, average internodal length (cm), the diameter of rootstock (mm), and the diameter of the scion (mm), were found to be significantly unlike. 'Shatangju' scion cultivar grafted onto 'Flying Dragon' rootstock had the lowest plant height (75.67 cm) and weakest growth vigor (Table 2). Stronger growth vigor and maximum plant height were obtained for 'Shatangju' scion cultivar grafted onto 'Shatang Mandarin' (181.86 cm) and 'Sour Orange' (142.77 cm) rootstocks. Trees grown onto 'Flying Dragon' rootstocks produced a shorter internodal length (4.62 cm), while the 'Shatang Mandarin' rootstock resulted in the most extended internodal length (24.23 cm). Moreover, lower values of crown width were recorded with 'Red Limonia', 'Trifoliolate Orange', and 'Flying Dragon' rootstocks, especially compared to other selected rootstocks. Trees grafted onto 'Shatang Mandarin' rootstocks produced longer values of trunk diameter of the scion, trunk diameter of rootstock, and apex shoot length, whereas the 'Trifoliolate Orange' and 'Flying Dragon' rootstocks resulted in the smallest values of all these morphological traits (2.13 cm).

3.2. Effect of Rootstocks on Soluble Sugar and Soluble Protein Contents

The data regarding the soluble sugar and protein contents of 'Shatangju' mandarin scion grafted onto different rootstocks showed dissimilarly (Figure 1). The soluble sugar contents of 'Shatangju' mandarin scion grafted onto 'Red Tangerine' rootstock was significantly lower compared with other tested rootstocks. In contrast, higher soluble sugar contents were found in plants grafted with 'Trifoliolate Orange' rootstock (Figure 1A). Further, we found that there were no significant differences between 'Shatang Mandarin',

‘Goutou Orange’, ‘Red Limonia’, and ‘Flying Dragon’ rootstocks. In terms of soluble protein contents, the ‘Citrange’ rootstock was markedly higher, followed by the ‘Flying Dragon’ rootstock, whereas ‘Xiangcheng Orange’ rootstocks displayed low values of soluble protein contents (Figure 1B).

Table 1. The vigor, description, and origin of different rootstocks used in this study.

Rootstock	Origin	Parentage	Characteristics
‘Shatang Mandarin’	China	<i>Citrus reticulata</i> Blanco cv. Shatangju	Vigorous
‘Sour Orange’	Asia	<i>Citrus maxima</i>	Vigorous
‘Goutou Orange’	China	<i>Citrus aurantium</i> L.	Vigorous
‘Sour Pummelo’	Southeast Asia	<i>Citrus maxima</i>	Semi-dwarf
‘Rough Lemon’	India	<i>Citrus jambhiri</i> Lush.	Semi-dwarf
‘Xiangcheng Orange’	China	<i>Citrus junos</i> Sieb	Semi-dwarf
‘Citrange’	USA	<i>Citrus sinensis</i> L. Osbeck × <i>Poncirus trifoliata</i> L. Raf.	Semi-dwarf
‘Red Tangerine’	Southeast Asia	<i>Citrus reticulata</i> Blanco	Semi-dwarf
‘Red Limonia’	India	<i>Citrus limonia</i> Osbeck	Dwarf
‘Trifoliolate Orange’	China	<i>Poncirus trifoliata</i> L.	Dwarf
‘Flying Dragon’	Japan	<i>Poncirus trifoliata</i> Raf. var. <i>monstrosa</i>	Dwarf

Table 2. Scion morphological characteristics of 1-year-old ‘Shatangju’ scion cultivar grafted onto different rootstocks.

Scion/Rootstock	Plant Height (cm)	Crown Width (cm)	Rootstock Diameter (mm)	Scion Diameter (mm)	Internodal Length (cm)	Number of Nodes	Number of Branches
‘Shatang Mandarin’	181.8 a	149 a	26.5 a	31.2 ab	24.2 a	6.80 bcd	10.2 cd
‘Sour Orange’	142.7 b	93.7 c	19.9 bc	26.9 bc	14.02 d	7.80 abc	11 bcd
‘Goutou Orange’	133.3 bc	113.5 b	20.2 bc	34.01 a	18.3 bc	6.60 cd	9.40 de
‘Sour Pummelo’	126.6 bcd	92 cd	21.9 b	34.7 a	9.63 e	8.40 a	13.8 a
‘Rough Lemon’	122.8 bcde	83.2 cde	17.5 cd	24.7 cd	19.2 b	6.80 bcd	13.4 ab
‘Xiangcheng Orange’	116.8 cde	68.6 ef	16.8 cd	24.5 cd	13.01 de	8.20 ab	14.4 a
‘Citrange’	108.4 def	79.2 cde	17.7 bcd	24.4 cd	14.8 cd	6.80 bcd	12.2 abc
‘Red Tangerine’	102.3 ef	76.3 de	16.0 cde	23 cd	13.2 de	6.40 cd	9.60 cde
‘Red Limonia’	92.7 fg	72.5 ef	17.4 cd	23.7 cd	12.7 de	6.60 cd	10.6 cd
‘Trifoliolate Orange’	89.1 fg	66.2 ef	13.4 de	25.5 cd	12.8 de	4.80 e	7.40 ef
‘Flying Dragon’	75.6 g	56.8 f	12.4 e	20.1 d	4.62 f	5.80 de	6.60 f

The data are means of three biological replicates. Different letters indicate significant differences by LSD ($p \leq 0.05$).

3.3. Effect of Rootstocks on Enzymatic Antioxidant Activities

In this study, rootstock behaved differentially regarding enzymatic antioxidant activities in the leaves of the ‘Shatangju’ scion cultivar (Figure 2). The leaf SOD activity of the ‘Shatangju’ scion cultivar grafted with the ‘Trifoliolate Orange’ rootstock was significantly lower than that of the other rootstocks. In contrast, maximum SOD values were recorded with the ‘Sour Orange’ rootstock (Figure 2A). Regarding POD activity, the result relative to ‘Rough Lemon’, ‘Sour Orange’, and ‘Citrange’ rootstocks showed noticeably higher values than other tested rootstocks (Figure 2B). In contrast, minimum values of POD activity were obtained with ‘Trifoliolate Orange’ and ‘Flying Dragon’ rootstocks.

3.4. Endogenous Hormone Levels

‘Shatangju’ citrus scion cultivar grafted with a range of size-controlling rootstocks varied in leaf auxin contents (Figure 3). The contents of TRA were higher for ‘Shatangju’ scion grafted onto ‘Flying Dragon’ and ‘Trifoliolate Orange’ rootstocks, whereas minimum TRA levels were recorded for plants grafted onto ‘Shatang Mandarin’ and ‘Goutou Orange’ rootstocks (Figure 3A). Regarding TRP and MEIAA, the result relative to the ‘Trifoliolate

Orange' rootstock was significantly high, whereas other rootstocks displayed low values (Figure 3B,F). Similarly, the IAA-Trp contents showed the highest with 'Flying Dragon' and 'Rough Lemon' rootstocks, whereas 'Goutou Orange' rootstock produced lower IAA-Trp contents (Figure 3C). The contents of IAN were higher in plants grafted with 'Sour Orange' and 'Sour Pummelo' rootstocks than other tested rootstocks (Figure 3D).

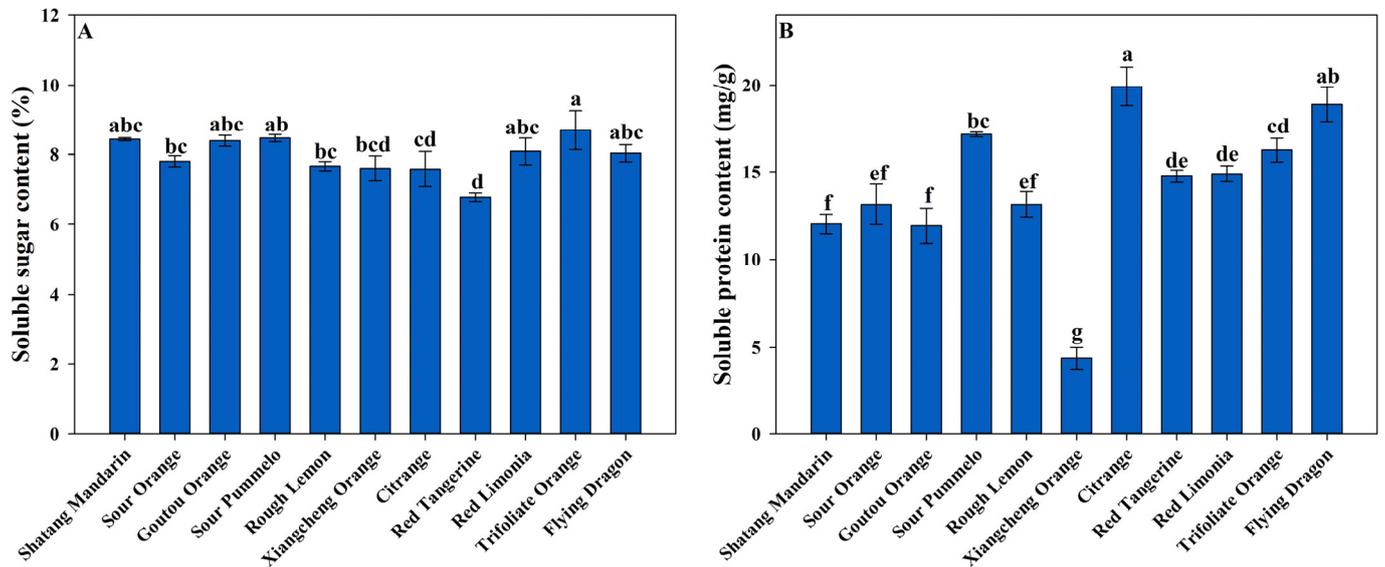


Figure 1. Soluble sugar and protein contents in leaf tissues of 'Shatangju' scion cultivar grafted onto different rootstocks. Soluble sugar content (A), soluble protein content (B). Error bars indicate the standard error of three biological replicates, and lowercase letters indicate significant differences between rootstocks by LSD ($p \leq 0.05$).

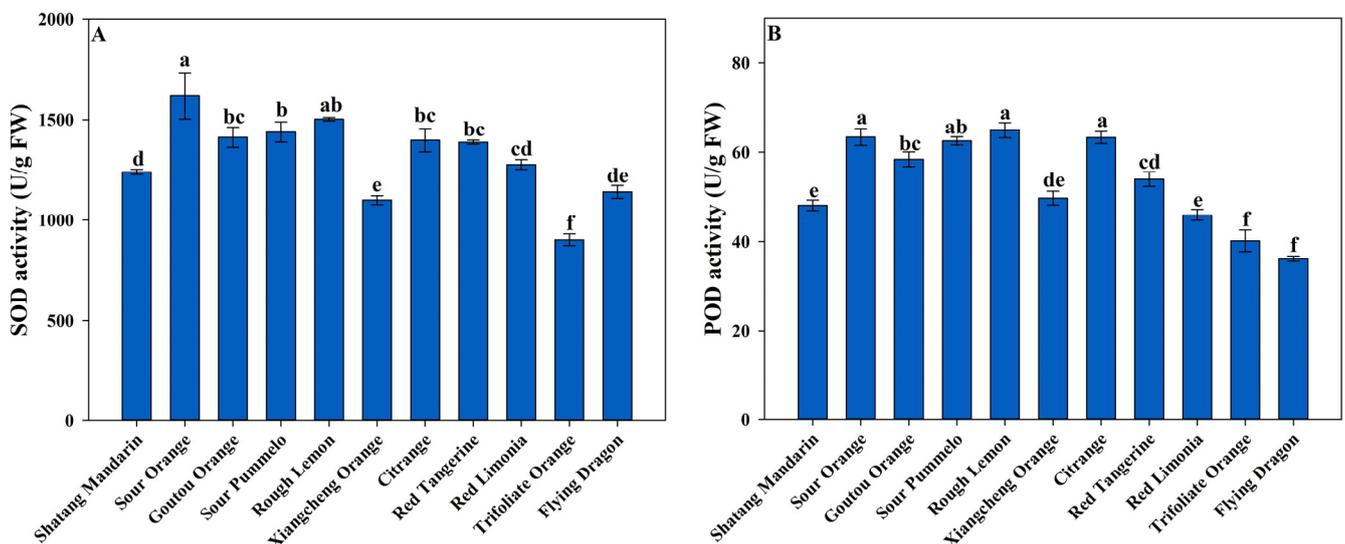


Figure 2. Enzymatic antioxidant activities in leaf tissues of 'Shatangju' scion cultivar grafted onto different rootstocks. SOD (A), POD (B). Error bars indicate the standard error of three biological replicates, and lowercase letters indicate significant differences between rootstocks by LSD ($p \leq 0.05$).

Different rootstocks significantly affected the endogenous cytokinin levels in grafted 'Shatangju' citrus plants (Figure 4). Dihydrozeatin (DZ) levels were relatively high in the 'Shatangju' scion cultivar grafted onto 'Xiangcheng Orange', 'Red Limonia', and 'Flying Dragon' rootstocks, whereas low DZ levels were obtained with 'Rough Lemon', 'Citrange', and 'Goutou Orange' rootstocks (Figure 4A). The contents of dihydrozeatin-O-glucoside riboside (DHZROG) were pointedly higher in plants grafted with 'Shatang Mandarin' root-

stocks, whereas minimum DHZROG levels were recorded for plants grafted onto ‘Trifoliolate Orange’ rootstock (Figure 4B). Similar Kinetin riboside (KR) levels were detected, being lowest in ‘Citrange’ and ‘Trifoliolate Orange’ rootstocks, whereas ‘Sour Orange’, ‘Goutou Orange’, and ‘Rough Lemon’ rootstocks displayed higher values of KR (Figure 4C). For the isopentenyl adenine riboside (IPR) levels, the result corresponding to ‘Goutou Orange’, ‘Sour Orange’, ‘Red Tangerine’, ‘Trifoliolate Orange’ and ‘Flying Dragon’ rootstock was markedly higher than in the other treatments (Figure 4D). In terms of cis-zeatin-O-glucoside riboside (cZROG) levels, the ‘Citrange’ rootstock was markedly lower than other rootstock treatments, whereas high cZROG levels were obtained with ‘Shatang Mandarin’, ‘Sour Pummelo’, ‘Red Tangerine’ and ‘Flying Dragon’ rootstocks (Figure 4F).

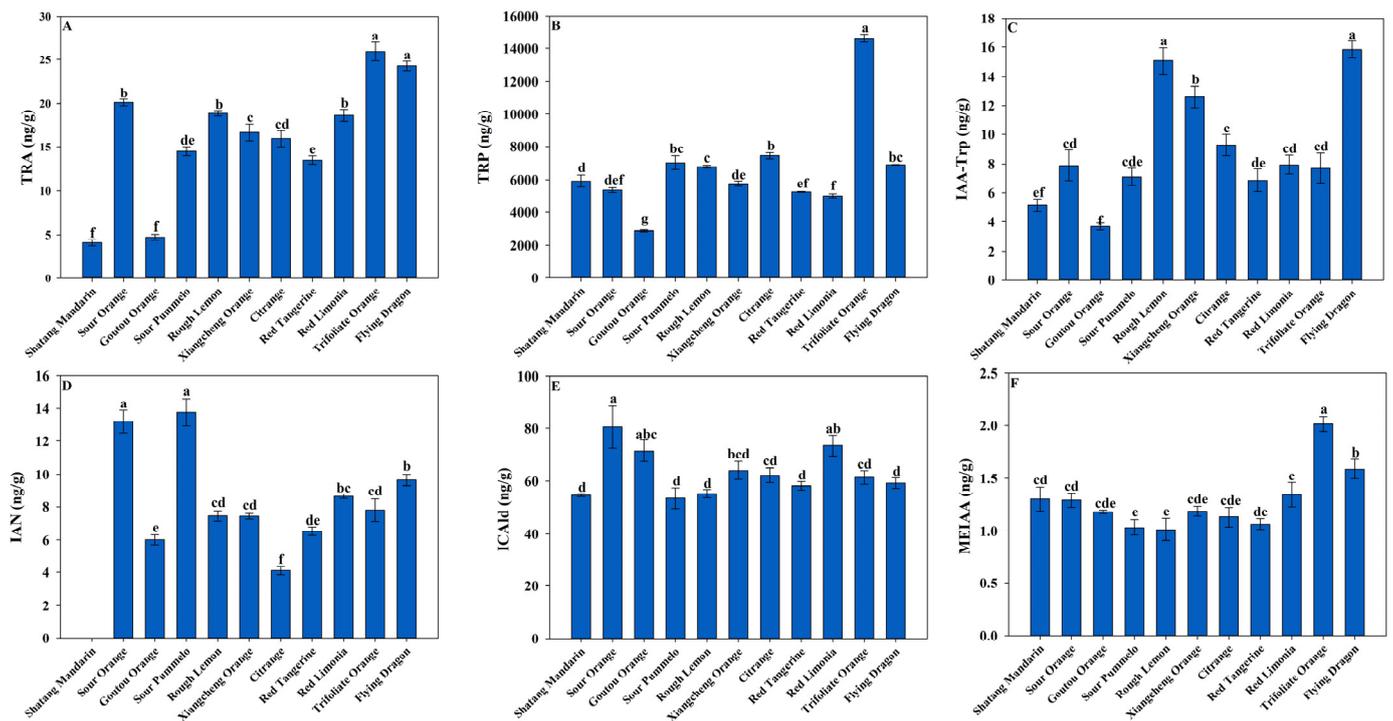


Figure 3. Endogenous auxin concentrations in leaf tissues of ‘Shatangju’ scion cultivar grafted onto different rootstocks. TRA = tryptamine (A), TRP = tryptophan (B), IAA-Trp = tryptophan (C), IAN = Indole-3-acetonitrile (D), ICAld = indole-3-carboxaldehyde (E) and MEIAA = methyl indole-3-acetate (F). Error bars indicate the standard error of three biological replicates, and lowercase letters indicate significant differences between rootstocks by LSD ($p \leq 0.05$).

Abscisic acid contents (ABA) were significantly higher with plants grafted with ‘Shatang Mandarin’ rootstock, whereas minimum values of ABA were recorded with ‘Flying Dragon’ rootstock (Figure 5A). Regarding ABA glucose ester (ABA-GE) contents, the result corresponding to the ‘Trifoliolate Orange’ rootstock was higher than other rootstocks. In addition, the contents of ABA-GE were not detected in the plants grafted with ‘Shatang Mandarin’, ‘Rough Lemon’, and ‘Flying Dragon’ rootstocks (Figure 5B). The contents of gibberellin (GA_{24}) were significantly higher for the ‘Shatangju’ scion cultivar grafted onto the ‘Goutou Orange’ rootstock, followed by ‘Sour Orange’, ‘Red Tangerine’, and ‘Shatang Mandarin’ rootstocks. In contrast, ‘Flying Dragon’ and ‘Sour Pummelo’ rootstocks displayed lower values of GA_{24} (Figure 5C). Moreover, the opposite trends were observed for GA_9 contents, whereas the ‘Flying Dragon’ rootstock showed a significantly higher level of GA_9 , and lower levels were recorded with ‘Shatang Mandarin’ rootstocks (Figure 5D). Furthermore, the contents of 1-aminocyclopropanecarboxylic acid (ACC) were significantly higher with the ‘Shatang Mandarin’ rootstock followed by the ‘Sour Pummelo’ rootstock; ACC contents were the lowest with ‘Trifoliolate Orange’ and ‘Flying Dragon’ rootstocks (Figure 5E). In addition, there were no significant differences in the content of remaining

rootstocks. 5-deoxystrigol (5DS) concentrations were lowest with ‘Shatang Mandarin’ and ‘Sour Pummelo’ rootstocks compared with the other tested rootstocks (Figure 5F).

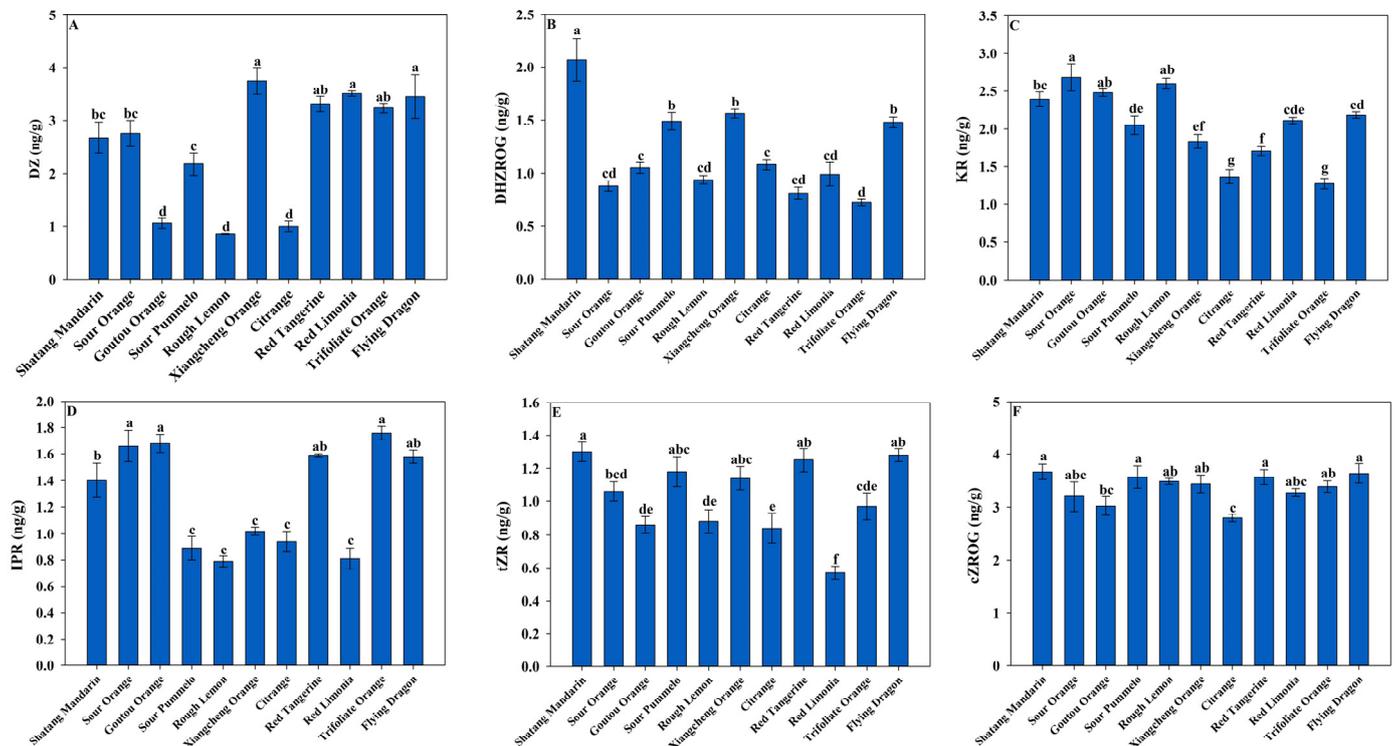


Figure 4. Endogenous cytokinin concentrations in leaf tissues of ‘Shatangju’ scion cultivar grafted onto different rootstocks. DZ = dihydrozeatin (A), DHZROG = dihydrozeatin-O-glucoside riboside (B), KR = Kinetin riboside (C), IPR = Isopentenyl adenine riboside (D), tZR = Trans-zeatin riboside (E), cZROG = cis-zeatin-O-glucoside riboside (F). Error bars indicate the standard error of three biological replicates, and lowercase letters indicate significant differences between rootstocks by LSD ($p \leq 0.05$).

3.5. Correlation Analysis of Plant Morphological Traits and Endogenous Hormones

The correlation analysis of endogenous hormone contents and main morphological indexes in 11 rootstocks showed a specific correlation between all indexes (Figure 6). Among them, ST, 5-DS, GA9, DZ, cZ9G, MEIAA, ICA1d, TRP, TRA, IAA-TRP, IAA-Val, IAA-Aal-Me, IAN, and ABA-GE were negatively correlated with plant height, crown width, and rootstock diameter. In addition, GA₂₄, ABA, ACC, DHZROG, KR, IP, 2MeSiPR, 2MeScZR, and IAA-Asp were positively correlated with plant height, canopy width, and scion diameter.

3.6. Expression, Correlation, and PCA Analysis of Differential Metabolites

Custer analysis was performed to understand the distinct expression pattern of identified metabolites between these two groups (Figure 7A). To reveal the relationship between various metabolite classes, a correlation analysis was performed based on the accumulation pattern of identified metabolites (Figure 7B), showing they are closely related to each other, might have similar chemical structures, or might take part in metabolic pathways. To examine the natural variations of metabolic traits among different types of cultivars, the principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) analysis were performed and successfully separated all the varieties. PCA analysis showed significant differences with PC1 (54.89%) and PCA2 (13.27%) (Figure 7C). OPLS-DA model was performed to identify the metabolites responsible for the separation between the samples, where the T-score was (55%) and the orthogonal T-score (11.4%), indicating that

both samples had significant spectral separation, which further indicated that metabolic differences between these samples were statistically significant (Figure 7D).

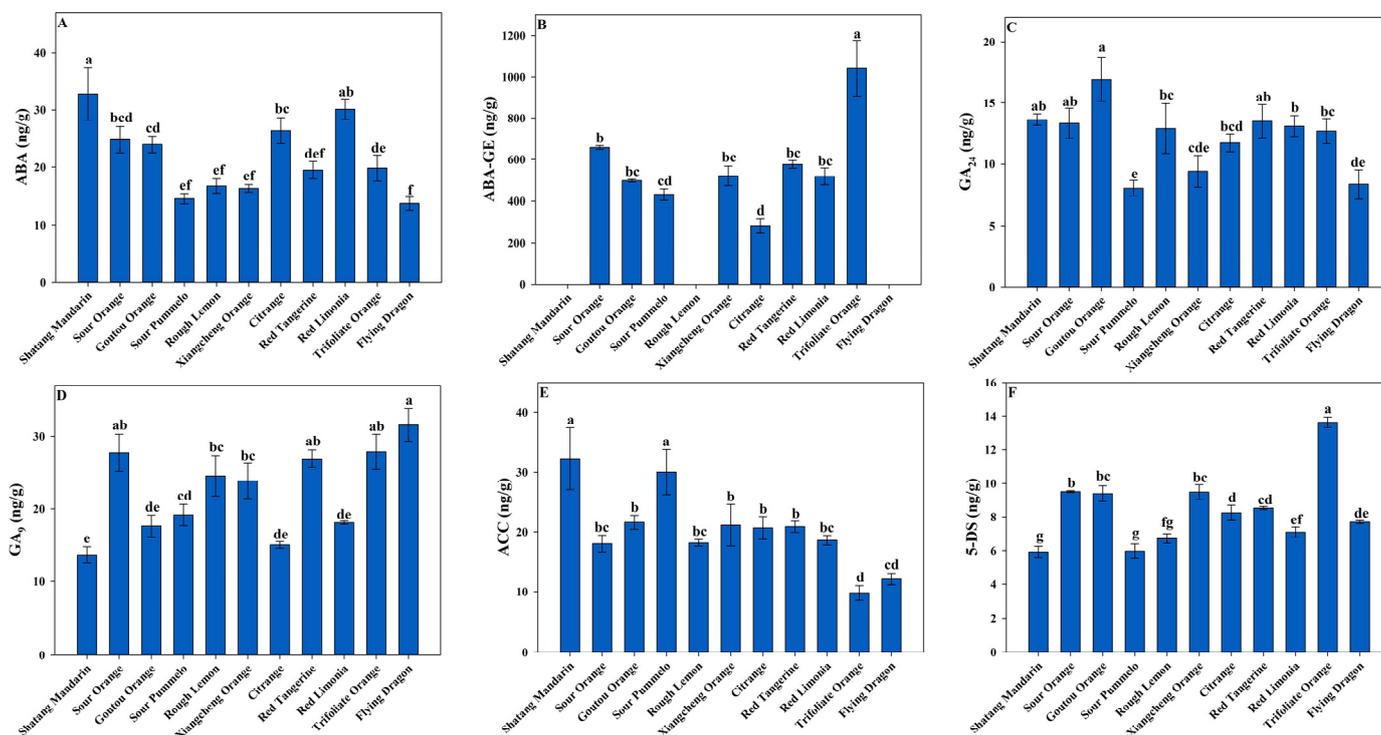


Figure 5. Endogenous abscisic acid, gibberellin, ACC, and 5DS concentrations in leaf tissues of 'Shatangju' scion cultivar grafted onto different rootstocks. ABA = Abscisic acid (A), ABA-GE = ABA glucose ester (B), GA₂₄ (C), GA₉ (D), ACC = 1-aminocyclopropanecarboxylic acid (E) and 5DS = 5-deoxystrigol concentrations (F). Error bars indicate the standard error of three biological replicates, and lowercase letters indicate significant differences between rootstocks by LSD ($p \leq 0.05$).

3.7. Metabolite Profiles of Dwarfing and Vigorous Rootstocks

The different metabolic profiles in the leaves of two grafting combinations ['Shatangju' / 'Flying Dragon' (dwarfing) and 'Shatangju' / 'Shatang Mandarin' (vigorous)] were analyzed (Figure 8). Moreover, 38 metabolites significantly changed between dwarfing and vigorous graft combinations (Figure S1). These differential metabolites mainly include organic acids, amino acids, and their derivatives, flavonoids, nucleotides and their derivatives, alkaloids, phenolic acids, and lipids types. Various organic acid metabolites, including 6-aminocaproic acid, 4-acetylaminobutyric acid, and methyl anthranilate, were found significantly down-regulated in the plants grafted with 'Flying Dragon' rootstocks. In contrast, other organic metabolites were significantly up-regulated in the leaves of 'Flying Dragon' rootstocks (Figure 8A). Among the flavonoid metabolites, while Eriodictyol-7-O-(6''-acetyl)glucoside, 5,6,3',4'-tetrahydroxy-3,7-dimethoxyflavone-6-O-glucoside, kaempferol-3-O-rhamnoside, and kaempferol-7-O-rhamnoside were significantly up-regulated in the leaves of dwarfing 'Flying Dragon' rootstock compared with 'Shatang Mandarin' (Figure 8B). Among the amino acids and their derivatives, several metabolites including, L-Valine, L-Isoleucine, L-Norleucine, L-Histidine, L-Phenylalanine, L-Tryptophan, N'-Formylkynurenine, and L-Saccharopine amino acid were significantly down-regulated in the leaves of plants grafted with 'Flying Dragon' rootstock, while other amino acids presented an up-regulated pattern in 'Shatang Mandarin' rootstock (Figure 8C). Regarding the metabolites of nucleotides and their derivatives, the expression of Guanine and 6-methylmercaptapurine was significantly down-regulated in the leaves of plants grafted with 'Flying Dragon' rootstock. In contrast, the rest of the metabolites were noticeably up-regulated in the leaves of 'Shatang Mandarin' rootstock (Figure 8D). In the case of alkaloid metabolites, Citpressine I, O-Phosphoryl-

acid (ILA), TRP = tryptophan (Trp), IAA-Glc = IAA-glucose, indole-3-carboxaldehyde (ICAld), MEIAA: methyl indole-3-acetate, cZ9G = cis-zeatin N9-glucoside, DHZROG = dihydrozeatin-O-glucoside riboside, cZROG = cis-zeatin-O-glucoside riboside, 2MeScZR = 2-methylthio-cis-zeatin riboside, 2MeSiPR = 2methylthio-isopentenyladenosine, DZ = dihydroze-atin, IP = sopen-tenyl adenine, IPR = Isopentenyl adenine riboside, tZR = Trans-zeatin ri-boside, ACC = 1-aminocyclopropanecarboxylic acid, ACC), 5DS = 5-deoxystrigol.

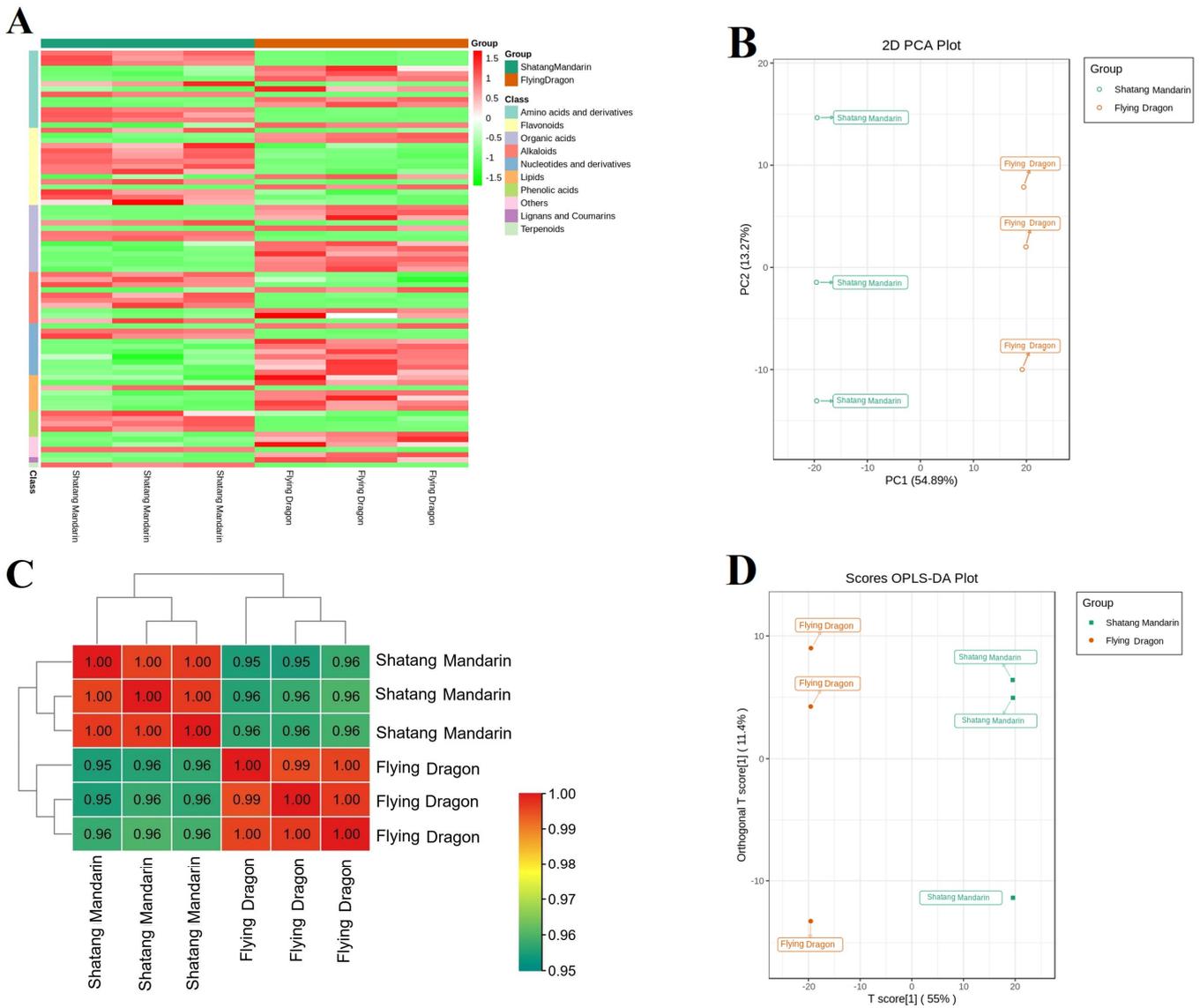


Figure 7. Clustering heat map of all metabolites (A). The horizontal axis indicates the name of the samples, whereas the vertical axis displays all metabolites. The red shade shows that the metabolites content in the samples was higher; green shows the lower content of metabolites. (B) Score plots of principal component analysis. (C) Correlation analysis. (D) Score plots of partial least squares discriminant analysis.

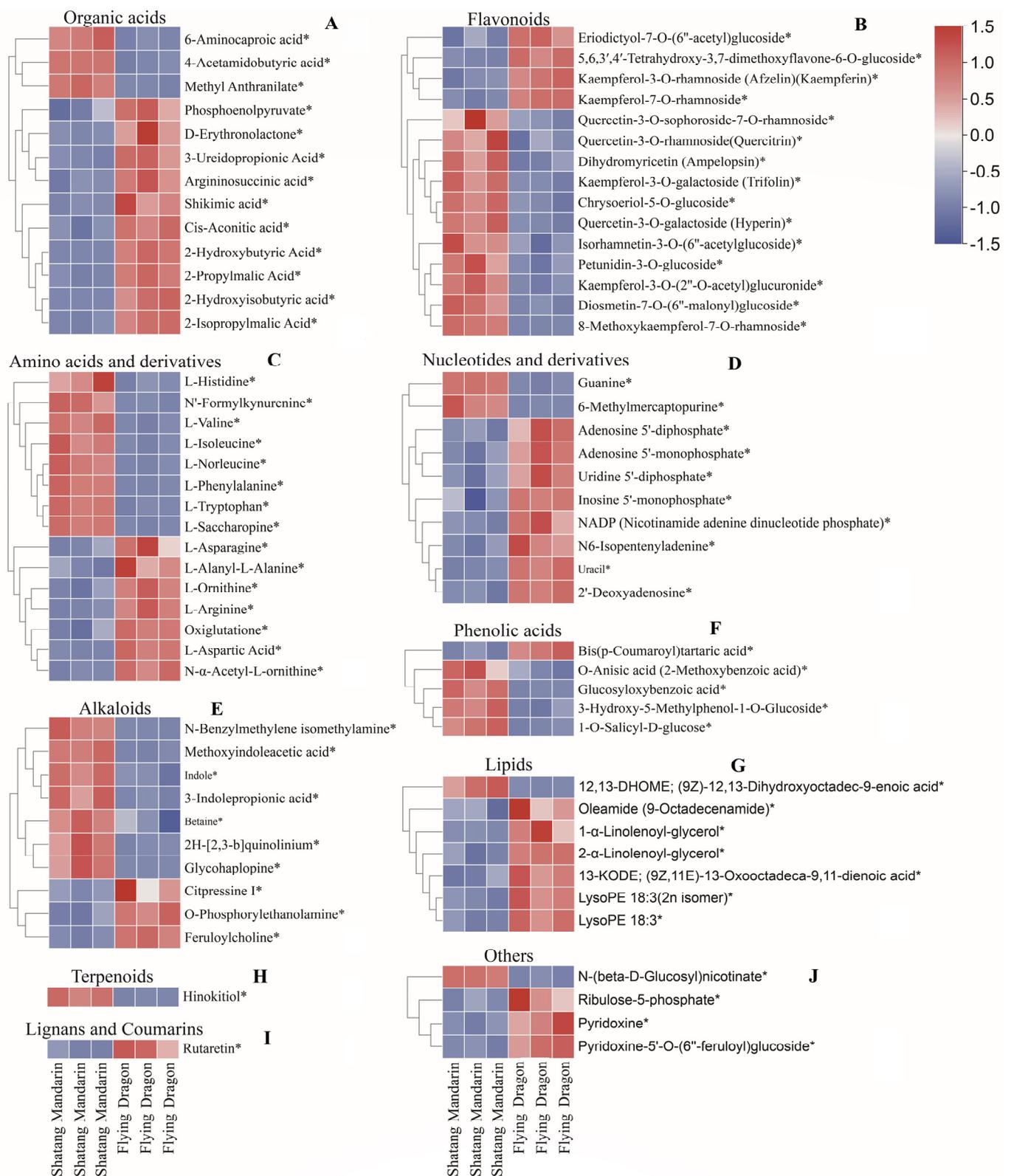


Figure 8. Heatmap of different metabolites in the leaves of 'Shatangju' mandarin scion cultivar grafted with 'Flying Dragon' (dwarfing) and 'Shatang Mandarin' vigorous rootstocks. Organic acids (A), Flavonoids (B), Amino acids and their derivatives (C), Nucleotides and their derivatives (D), Alkaloids (E), Phenolic acids (F), Lipids (G), Terpenoids (H), Lignans and Coumarins (I), and Others (J).

4. Discussion

Citrus is a valuable fruit, and higher productivity is essential for growers and the economy [40]. Dwarfism is one of the most valuable traits in fruit production for dense cultivation to obtain a maximum harvest index and effective orchard management [8,41]. Rootstocks significantly influence the morphological features of grafted plants, among which the reduction of scion vigor is one of the fascinating phenomena [7,11]. Earlier research has shown that plants grafted on taller rootstocks displayed increased primary shoot lengths and scion trunk diameter [42]. On the other hand, dwarfing rootstocks or interstock modify shoot architecture by reducing the values of morphological traits such as primary shoot length, node number, sylleptic shoot, and internodal length [25,43,44]. In the present experiment, we noticed that the ‘Shatangju’ scion cultivar grafted onto the ‘Flying Dragon’ rootstock encouraged the small stature trees. However, trees grafted with other rootstocks like ‘Shatang Mandarin’, ‘Goutou Orange’, and ‘Sour Orange’ increased plant height, crown width, scion trunk diameter, apex shoot length, internodal length, and the entire plant growth. Compared with other rootstocks, plants grafted on dwarfing rootstocks displayed lower values of morphological traits with higher returns [1], which is consistent with our findings. Similarly, Nasir et al. [45] examined the influence of ‘Kinnow Mandarin’ grafted on three different rootstocks. They reported that plants grown on more vigorous rootstock (‘Rough Lemon’) increased their growth concerning plant height, scion trunk diameter, leaf areas, and internodal length, while plants grafted on ‘Carrizo Citrange’ showed to be a dwarfing rootstock.

Soluble sugar, protein, and enzyme activities (i.e., SOD and POD) widely exist in plants, which protect plants from harmful damage and affect the metabolism and distribution of hormones in plants [46]. Zhao et al. [19] reported the effect of various dwarfing interstocks on the morphological behavior and biochemical and physical parameters of apple plants, indicating that the plant height of various dwarf interstocks was negatively correlated with the soluble sugar content. In addition, more significant dwarfing effects are related to increased leaf enzymatic activities; subsequently, these activities have a negative impact on the growth of the scion part, which leads to tree dwarfing. Our present study established the relationship between soluble sugar, protein, enzyme activity, and rootstock vigor of grafted scion parts. There was a little difference in soluble sugar contents among different scion/rootstock combinations, indicating that different rootstocks had little effect on soluble sugar in the leaves of ‘Shatangju’ mandarin. Moreover, the highest soluble sugar content was observed with trees grafted onto ‘Trifoliolate Orange’ rootstock. Previous studies have also found a substantial increase in soluble sugar content in the scion buds of the ‘Auksis’ scion cultivar grafted with dwarfing (B.36) rootstock. In contrast, no significant differences were observed in glucose accumulation between the semi-dwarfing, dwarfing, and super-dwarfing graft combinations. The differences in soluble sugar accumulation in buds produced by these rootstocks may be related to the different periods required for fruit ripening among ‘Ligol’ and ‘Auksis’ cultivars [28]. Additionally, the soluble protein content of trees grafted with ‘Citrange’ and ‘Flying Dragon’ rootstocks was significantly higher than that of other graft combinations, which could effectively enhance the antioxidant capacity of the ‘Shatangju’ scion to a certain extent.

Plants produce different scavenging enzymes, i.e., SOD and POD, to overcome the negative effects of ROS [46]. The enzyme activities showed significantly different behavior in various rootstocks when grafted with the ‘Shatangju’ scion cultivar. In this current study, we found that the SOD activity of trees grafted with ‘Sour Orange’ rootstock was noticeably higher. In contrast, the lowest SOD activity was obtained with trees grafted with ‘Trifoliolate Orange’ rootstock. In addition, POD is engaged in numerous physiological processes in plants, such as peroxide scavenging, cell wall synthesis, lignification, and IAA metabolism [47]. The POD activity in the current study was found to be minimum in trees grafted with ‘Flying Dragon’ and ‘Trifoliolate Orange’ rootstocks, followed by ‘Red Limonia’ and ‘Shatang Mandarin’ rootstocks. Moreover, the POD and SOD activities of ‘Shatang Mandarin’ rootstocks were higher than ‘Flying Dragon’ rootstocks. The higher

activities of defense antioxidant enzymes in the vigorous graft combination reflected that their scavenging capability for ROS was stronger and thereby accelerated wound healing, allowing plants to recover regular growth earlier [48]. On the other hand, the 'Flying Dragon' presents disadvantages such as low tolerance to drought and graft incompatibility with some scion varieties [49].

Hormone synthesis and transport have been demonstrated in previous studies to restrict tree growth [39]. Auxin and cytokinin encourage tree growth and the development of axillary buds. Gibberellins promote internodal elongation, whereas ABA increases tree aging [50]. Else et al. [51] reported that the dwarfing effect of M.9 rootstock is linked with the reduced capability of IAA transport, along with greater export of ABA than more vigorous rootstocks (MM.106). Reduced IAA transport to the roots influences biomass production and activities of cytokinin and gibberellin [52]. This mechanism limits axillary bud sprouting and internode elongation, resulting in shorter internode length, decreased growth vigor, and dwarfing of the scion [53,54]. Another study reported that [55], the dwarfing of the tree may be due to the blocked IAA transport, which inhibits the synthesis of CTK in the roots, resulting in the weakened growth of the aerial parts. Gibberellins are crucial for plant growth and a controlling factor of plant architecture. GA-related dwarfing can be divided into two categories: a responsive mutant that is linked with GA signaling and a dwarf mutant that is associated with the GA anabolic pathway. The synthetic dwarf mutant is produced by a GA deficiency due to abnormalities in GA synthetase or other GA metabolic enzymes. Our present study found that the trees grafted with 'Flying Dragon' rootstock showed a significantly higher level of GA₉, and lower levels were recorded with trees grafted onto 'Shatang Mandarin' rootstocks.

Strigolactones represent the most recently described group of plant hormones involved in many aspects of plant growth regulation [56]. It is mainly synthesized in the root system and then transported to other parts. 5-Deoxystrigol (5-DS) is ubiquitous in plants, the first product in the strigolactone synthesis pathway, and the rest are derivatives of 5-DS. SLs are closely related to plant growth and development. Moreover, IAA, ABA, GA, CTK, ETH, and other hormones work together to regulate plant shape, branching, and root system.

Anatomical structure, material transport, photosynthesis, plant hormones, and other factors are closely related to dwarfing rootstocks; however, limited studies have shown the relationships between the degree of dwarfing and metabolomics. Earlier studies have shown that small-molecule organic acids can affect plant growth and development [57]. Compared with vigorous ('Shatangju'/'Shatang Mandarin') graft combination, most of the organic acids in the dwarf ('Shatangju'/'Flying Dragon') graft combination was up-regulated, which is consistent with previous research results. Flavonoids may regulate the polar transport of auxin, and phytohormones can regulate secondary metabolites in a concentration-dependent manner [58]. The differential metabolites in flavonoids are mainly down-regulated in trees grafted with dwarfing ('Flying Dragon') rootstock than that of trees grafted with vigorous rootstock. Changes in the external environment will disrupt plants' primary and secondary metabolic profiles, so hormonal changes assume the corresponding regulatory role in adapting plants to environmental changes [59]. In this trial, differential metabolites were mainly enriched in the biosynthetic pathways of secondary metabolites, which is consistent with previous studies and further shows that hormones have an important effect on plant dwarfing.

5. Conclusions

In the present study, we found that citrus rootstocks noticeably affected the morphological traits of grafted 'Shatangju' plants. 'Flying Dragon' rootstock significantly reduced the plant height of the scion cultivar. Leaf POD activity of trees grafted with 'Flying Dragon' rootstock was significantly lower than other graft combinations. Moreover, a low concentration of ABA in 'Shatang Mandarin' was recorded when grafted on the 'Flying Dragon' rootstock. The differential expression of leaf metabolites may be involved in the reduction of scion growth by citrus rootstocks. Overall, these findings indicate that the

‘Flying Dragon’ rootstock may be the best option in the high-density plantation of citrus fruits under net house conditions.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae8070608/s1>, Figure S1: KEGG enrichment analysis (A) and volcano plot (B).

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