

Morphological and metabolic changes in Changshan Huyou (*Citrus aurantium*) following natural tetraploidization

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Abstract

Background

Polyploids in citrus are generally used to improve crop varieties. Changshan Huyou (*Citrus aurantium*) is a native citrus species in China that is highly adaptable and has pharmaceutical value. However, the influence in Changshan Huyou following polyploidization remains unclear. Here we evaluated the phenotypic variations and metabolic alterations following autotetraploidization of Changshan Huyou seedlings and fruits and analyzed the associated transcriptomic changes.

Result

The tetraploid seedlings had rounder and thicker leaves, larger floral organs and fruits, and satisfactory viability of pollen grains and ovules. The tetraploid fruits accumulated lower levels of soluble solids but similar levels of organic acids. Metabolic profiling of three tissues of fruits revealed that most of 2064 differentially accumulated metabolites (DAMs), including flavonoids, lignans, and coumarins, were downregulated. In contrast, the upregulated DAMs mainly included alkaloids (clausine K and 2-(1-pentenyl)quinoline), amino acids (L-asparagine and L-ornithine), and terpenoids (deacetylnomilin and evodol) in tetraploid peels, as well as, flavonoids (neohesperidin and quercetin-5-O- β -D-glucoside) and organic acids (2-methylsuccinic acid and dimethylmalonic acid) in juice sacs. The upregulated genes were associated with phenylpropanoid biosynthesis, secondary metabolite biosynthesis, and the biosynthesis of various alkaloid pathways. The results of Pearson correlation analysis showed that the upregulated genes that encoded peroxidase and cytochrome P450 were closely related to a higher accumulation of amino acids and alkaloids in tetraploid peels, and neohesperidin and quercetin glucoside were increased by *ferulate-5-hydroxylase*, *CYP450 81Q32*, *flavonoid 3'-monooxygenase*, *4-coumarate-CoA ligase 1*, and *UDP-glucose flavonoid 3-O-glucosyltransferase*, as well as, some transcription factors in tetraploid juice sacs.

Conclusion

The tetraploid Changshan Huyou investigated here may be used in breeding triploid citrus, especially to produce seedless varieties, and for pharmaceutical purposes in fruit processing, as they influence metabolites following polyploidization.

Introduction

A polyploid is an organism with more than two sets of complete chromosomes, and polyploidy is ubiquitous in plants in the wild [1–4]. Polyploidy is important for ecology and evolution and is a frequently used speciation approach [5]. There are usually two types of polyploids, including allopolyploids and autopolyploids. Allopolyploids help manage evolutionary alterations and enhance species survival due to their dual effects of hybridization and polyploidy [6–8], whereas, autopolyploids usually show greater adaptability to short-term changes caused by changes in the metabolic phenotype [9] mostly subjected to whole-genome duplication [10].

Tetraploidy-mediated gene expression regulation and polyploid genome epigenetic remodeling induce morphological and physiological alterations in citrus plants, such as an increase in organ size, a decrease in plant height, and diverse anatomical characteristics [11–13]. These alterations were found to increase plant growth in autotetraploid *Poncirus trifoliata*, Carrizo citrange, *Citrus wilsonii*, and *C. junos* 'Ziyang' due to their improved tolerance to environmental stress, such as drought, salinity, and heavy metals [14–19] and even biotic stress, such as Huanglongbing (HLB) infection [20], which is a fatal disease that affects various citrus species worldwide, and promising potential applications in citrus rootstocks that are adaptive to increasingly poor planting circumstances. Additionally, tetraploid citrus can significantly modify the essential oil composition of colchicine-induced tetraploid *Citrus limon* [21] and tetraploid sweet orange (*Citrus sinensis*), suggesting that ploidy levels may contribute to the profiling of aromatic flavors in citrus [22].

Autopolyploidization may contribute to an increase in plant reproduction, which can subsequently improve distant hybridization and colonization of plants [23] and is extensively used in citrus breeding through spontaneous doubling or mutation [12, 17, 24–26]. Additionally, autotetraploid germplasm can occur spontaneously or synthetically and is crucial to triploid breeding in citrus. Doubled diploid citrus plants frequently generate pollen grains that are less fertile than the initial diploid genotypes [27, 28]; however, the viability of pollen grains is adequate for applying such types as male parents during sexual interploidy hybridization. Polyploidization can restore fertility among emerging hybrids [29]. The mechanism underlying the formation of the 2n megagametophyte contributes to optimizing sexual polyploid hybridization in citrus [30].

Changshan Huyou (*Citrus aurantium*, diploid) is a native citrus species cultured for many years in local China and is characterized by vigorous growth, frost tolerance, strong adaptability, and storage resistance [31, 32]. Changshan Huyou is a type of sour orange (*Citrus aurantium*) formed by the hybridization of *Citrus grandis* and mandarin [33]; the juice of the mature fruit is a good source of bioactive polyphenols, vitamin C, and folic acid [31, 32]. Moreover, the dried immature fruits of Changshan Huyou, known as Quzhou Aurantii Fructus (QAAF) or *Qu zhi ke* (QZK), were recorded in the Chinese Pharmacopoeia as medicinal materials in 2015 [32, 34] due to the presence of bioactive components, such as flavonoids, volatile oils, coumarins, terpenes (especially limonoids), and steroid glycosides [31, 32, 34–37]. They also exert pharmacological effects, such as antioxidative, antibacterial, anti-inflammatory [34, 36], antitumor [38], hypoglycemic [39], and hypotensive effects, along with their ability to treat non-alcoholic fatty liver [40, 41].

In this study, an autotetraploid seedling (tetraploid) was identified from the diploid Changshan Huyou, which had been bearing fruits for five years. The morphological features, reproductivity, and fruit quality of tetraploids in Changshan Huyou remain undescribed. To obtain comprehensive data on the tetraploids, we investigated the morphological characteristics and metabolic profiles of diploids compared to those of tetraploids and examined the underlying changes in the transcriptome of Changshan Huyou subjected to polyploidization. Our findings showed the features of the seedlings and fruits of

the autotetraploid Changshan Huyou and predicted its superiority as the male and female parents in ploidy hybridization in citrus breeding, as well as, its applications as pharmaceutical alternatives to diploid fruits in the citrus industry.

Results

Ploidy confirmation and genetic identification of tetraploid Changshan Huyou

The tetraploid ploidy level was verified through flow cytometry (FCM); and the result indicated that the fluorescence intensity of the tetraploid cells peaked at about 50, a value that was double of that in the diploid control, suggesting a tetraploid genome. (Fig. 1A).

The genomes of the tetraploid offspring and the diploid progenitors were resequenced to characterize the genetic fidelity and genomic composition of tetraploids. As the Changshan Huyou genome sequence was unavailable, we used the Wanbaiyou genome as a reference to identify SNPs in diploids and tetraploids, including three pummelo genomes and three mandarin genomes. The genome sequences were aligned, which revealed that diploid and tetraploid plants contained 225,450 common SNPs, representing 73.96% sequence identity (Fig. 1B). Based on the results of the phylogenetic analysis of SNPs obtained from diploids and tetraploids, we also identified three pummelo genotypes and three mandarin genotypes. Thus, the tetraploids presented the greatest relationship with the diploids (Fig. 1C). These findings suggested that the tetraploid was an autotetraploid that originated from natural diploid genome doubling.

Morphological features of tetraploid leaves, flowers, and fruits

Under normal greenhouse conditions, the tetraploids presented morphological differences in their leaves, floral organs, and pollen grains compared to the corresponding diploid progenitors (Table 1). The tetraploid leaves were significantly rounder (6.47 cm ± 0.60 cm) and had a lower leaf index (1.57 ± 0.13) (Fig. 2A; Table 1). Microscopic analysis suggested distinct anatomical heterogeneities in tetraploids versus diploids, as determined by the thick epidermis and larger palisade tissue cells in tetraploids (Fig. 2B-E; Table 1). The tetraploids had a thicker upper epidermis (16.07 µm ± 2.72 µm), larger palisade parenchyma (79.58 µm ± 5.49 µm), and spongy parenchyma (254.36 µm ± 8.09 µm) (Table 1, Fig. 2B and 2C). The diameter of the leaf midrib was greater in tetraploids (1386.69 µm ± 37.28 µm) than in diploids (864.17 µm ± 17.37 µm) (Table 1, Fig. 2D and 2E). Additionally, the tetraploids presented a significantly lower stomatal density (Fig. 2F and 2G) and significantly longer and wider guard cells than their diploid counterparts (Table 1).

Table 1

Comparison of morphological characteristics of leaves in the tetraploid and the diploid

Strains	Leaf Blade (cm)		Shape Index of Leaf	Blade Thickness (µm)	Guard Cell (µm)		Guard Cell Density (No./mm²)	Epidermis (µm)		Palisade Parenchyma (µm)	Spongy Parenchyma (µm)	Midribs (µm)
	Length	Width			Length	Width		Upper	Lower			
HY	11.31 ± 1.09	6.01 ± 0.69	1.9 ± 0.21	344.15 ± 8.77	21.25 ± 2.11	18.27 ± 1.07	432.45 ± 36.32	11.04 ± 2.01	9.29 ± 1.86	70.59 ± 3.96	241.10 ± 8.47	864.17 ± 17.37
HD	10.13 ± 0.75	6.47 ± 0.60	1.57 ± 0.13	376.70 ± 7.80	25.91 ± 2.44	22.49 ± 2.16	371.14 ± 32.94	16.07 ± 2.72	9.91 ± 1.71	79.58 ± 5.49	254.36 ± 8.09	1386.69 ± 37.28
t-test	NS	**	**	**	**	**	**	**	NS	**	**	**
Different letters within the same column differ significantly (Tukey HSD Test; NS stands for no significantly different, * stands for <i>p</i> < 0.05, ** stands for <i>p</i> < 0.01). The data are presented as mean ± standard deviation (n = 20). HY: diploid, HD: tetraploid.												

Compared to diploid progenitors, tetraploids were larger but had a similar number of floral organs, such as petals, stamens, and ovaries (Table 2, Fig. 2H-2K). The length and width of the tetraploid petals were significantly greater than those of the diploid petals (Table 2, Fig. 2H), resulting in longer flower buds in the tetraploid petals (Table 1, Fig. 2I). The number of stamens in the tetraploid was similar to that in the diploid, but they were considerably longer than the stamens of the diploid (Table 2, Fig. 2J). Compared to those of diploids, the vertical and transverse diameters of tetraploid ovules were greater (Table 2, Fig. 2K). The tetraploid did not have a considerably different pistil from the diploid. The tetraploid had larger pollen grains (Fig. 2L, Table 2) and a little lower viability in the staining and germination of pollen grains (Fig. 2N-2Q, Table 2).

Table 2
Comparison of morphological characteristics of flowers and pollen in the tetraploid and the diploid

Strains	Flower Bud (cm)		No. of	Petal (cm)		Length of stamen (cm)	Length of Pistil (cm)	Ovary (cm)		No. of Stamens	Pollen Grain (μm)		Shape Index of Pollen Grain	Pollen Stability Rate (%)
	Length	Width		Length	Width			Diameter	Height		Length	Width		
HY	2.00 ± 0.21	1.04 ± 0.12	5.00 ± 0.00	1.98 ± 0.15	0.84 ± 0.12	1.24 ± 0.14	1.28 ± 0.26	0.43 ± 0.04	0.56 ± 0.05	24.08 ± 2.22	25.68 ± 1.55	23.85 ± 1.76	1.08 ± 0.09	86.4 ± 9.42
HD	2.11 ± 0.22	1.13 ± 0.07	5.00 ± 0.47	2.28 ± 0.14	1.04 ± 0.13	1.33 ± 0.13	1.41 ± 0.16	0.49 ± 0.07	0.61 ± 0.10	24.20 ± 1.92	29.16 ± 1.99	26.57 ± 2.84	1.11 ± 0.10	85.03 ± 12.43
t-test	*	NS	NS	NS	NS	*	NS	*	*	NS	**	**	NS	NS

Different letters within the same column differ significantly (Tukey HSD Test; NS stands for no significantly different, * stands for $p < 0.05$, ** stands for $p < 0.01$). The data are presented as mean ± standard deviation (n = 20). HY: diploid, HD: tetraploid.

Tetraploid fruits were much larger than diploid fruits (Table 3, Fig. 2R). The edible rate of tetraploids was not significantly different from that of diploids; however, the average fruit weight of tetraploids was double that of diploids because of the larger fruit diameter and height of tetraploids. No significant difference was recorded in the TA of juice; however, the SSC of juice was significantly lower in tetraploids than in diploids. Compared to diploids, tetraploids presented significantly fewer developed seeds, whereas the number of undeveloped seeds in tetraploids was significantly greater than that in diploids.

Table 3
Comparison of the morphological characteristics of fruits in the diploid and the tetraploid

Strains	Fruit Weight (g)	Fruit (cm)		Shape Index of Fruit	Juice Sac Weight (g)	Edible Rate (%)	No. of Segments	No. of Developed Seeds/Fruit	No. of Undeveloped Seeds/Fruit	Rate of developed Seeds/Fruit	SSC of Juice Sac (%)	TA of Juice Sac (%)
		Diameter	Height									
HY	318.90 ± 31.36	9.70 ± 0.42	9.17 ± 1.01	0.95 ± 0.10	227.98 ± 1.72	72.15 ± 5.23	10.00 ± 0.82	21.17 ± 12.84	2.17 ± 1.47	0.84 ± 0.12	10.77 ± 0.15	1.41 ± 0.15
HD	676.46 ± 70.65	13.05 ± 0.48	11.32 ± 0.69	0.87 ± 0.56	488.07 ± 1.60	71.49 ± 3.47	9.55 ± 1.21	6.50 ± 4.60	8.86 ± 8.16	0.39 ± 0.19	8.91 ± 0.09	1.56 ± 0.16
t-test	**	**	**	**	**	NS	*	*	*	**	**	NS

Different letters within the same column differ significantly (Tukey HSD Test; NS stands for no significantly different, * stands for $p < 0.05$, ** stands for $p < 0.01$). The data are presented as mean ± standard deviation (n = 20). HY: diploid, HD: tetraploid.

Differentially accumulated metabolites (DAMs) between tetraploid and diploid fruits

Fruits are crucial products of Changshan Huyou cultivation. Thus, we analyzed whether tetraploids presented an improved metabolic profile than diploids. Comprehensive metabolites were investigated in tetraploids and diploids, focusing on peels, juice sacs, and segment membranes of fruits harvested at 210 DAF by broadly targeted metabolomics using a UPLC-MS/MS system. The differences in the metabolic profiles of three tissues from the fruits of different ploidies were assessed by PCA, and 53.78% of the variables among the three tissues could be explained by the first component, whereas 20.76% could be explained by the second component (Fig. 3A).

In total, 2064 metabolites were grouped into 13 primary classes, such as amino acids or their derivatives, nucleotides or their derivatives, phenolic acids, flavonoids, etc., and identified in three parts of the fruits (Table S1). The total contents of flavonoids and organic acids did not change significantly in tetraploid juice sacs (Fig. S1A), whereas the contents of flavonoids, lignans, coumarins, and vitamins decreased significantly in tetraploid peels (Fig. S1B), and the contents of quinones, saccharides, alkaloids, and lipids decreased significantly in the tetraploid segment membranes (Fig. S1C).

In total, 678 (32.8% of total metabolites of 2064), 235 (11.4%), and 472 (22.9%) DAMs were screened from the peels (2X-F_vs_4X-F), juice sacs (4X-P_vs_2X-P), and segment membranes (4X-CW_vs_2X-CW), respectively (Table S2, Fig. 3B). Different patterns of metabolite accumulation were found in different tissues. However, the number of upregulated DAMs (up-DAMs) was considerably lower than that of downregulated DAMs (down-DAMs) (Fig. 3C, Table S3). In the three tissues, the major categories of down-DAMs were flavonoids, lignans, and coumarins; however, the dominant categories of up-DAMs were different between the tissues. In peels, the up-DAMs mainly consisted of alkaloids, terpenoids, and amino acids or their derivatives (Fig. 3D), represented by 2-quinoline (Wagp010286, with a 10.2-fold change), L-asparagine (mws0001, 4.1), and deacetylominilin (Cmpp004617, 2.84) (Fig. 3E). In the juice sacs, flavonoids were the top category in the up-DAMs, followed by terpenoids and organic acids (Fig. 3D), and the representative flavonoids included neohesperidin (pme0001, 2.45), hyperin (MWSHY0113, 2.46), and rhamnetin-3-O-glucoside (Lmjp002906, 2.53) (Fig. 3F). In segment membranes, besides flavonoids, alkaloids and organic acids were the dominant categories in up-DAMs (Fig. 3D), and the representatives included flavonoids such as naringenin-4'-O-glucoside (HJN087, 2.44), butin-7-O-glucoside (HJN090, 2.29), and neohesperidin (pme0001, 2.04), alkaloids such as betaine (MWSmce548, 2.55) and 4-methoxy-3-(3-methylbut-2-en-1-yl)-2-quinolone-8-O-b-D-glucopyranoside (Lwhp011001, 3.22), and organic acids such as shikimic acid (mws0154, 2.30) and citric acid diglucoside (WaYn000716, 2.06) (Fig. 3G).

Differentially expressed genes (DEGs) between tetraploid and diploid fruits

To assess the underlying alterations driven by ploidy in fruits, the global transcriptomic profiles were analyzed using the corresponding tissues of tetraploid and diploid fruits. We acquired 125.18 Gb of clean data from 18 cDNA libraries of quality-filtered sequence data, and > 89.22% of the bases had a quality score of $\geq$ Q30 (Table S4). PCA of gene expression via FPKM showed that three tissues of fruits were clustered separately, and the first two components explained 55.50% of the variance (Fig. 4A). In total, 700, 422, and 514 DEGs were filtered from pairwise comparisons of 2X-F_vs_4X-F, 2X-P_vs_4X-P, and 2X-CW_vs_4X-CW, respectively (Table S5, Fig. 4B). Along with the 2X-P_vs_4X-P comparison, there were more downregulated DEGs (down-DEGs) than upregulated DEGs (up-DEGs). Gene Ontology (GO) analysis revealed that DEGs from different fruit tissues were related to cellular processing and biological metabolic processes, as well as, to the molecular functions of catalytic activity and binding (Fig. S2). According to the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, the most enriched pathway was associated with the biosynthesis of secondary metabolites in all three tissues (Fig. S3). The results of K-means analysis revealed that the DEGs were clustered into seven groups, and the DEGs upregulated only in tetraploid juice sacs, segment membranes, and peels were grouped into subclasses 2, 6, and 7, respectively (Table S6, Fig. 4C). The DEGs that were only upregulated in tetraploid peels (subclass 7) were enriched in the biosynthesis of secondary metabolites; phenylpropanoid biosynthesis; the biosynthesis of various alkaloids; cutin, suberin, and wax; amino sugar and nucleotide sugar metabolism; ubiquinone and other terpenoid-quinone biosynthesis (Table S7, Fig. 4D). However, the number of upregulated DEGs was lower in KEGG enrichment analysis of 4X-P (Table S8, Fig. S4A) and 4X-CW (Table S9, Fig. S4B) groups than that of 4X-F group.

Apart from *Tyrosine aminotransferase 2* (*TAT2*, Cg2g013120) and *Chalcone synthase 1* (*CHS1*, Cg9g011580), DEGs related to alkaloid biosynthesis (ko00996) were expressed mainly in peels and were upregulated in tetraploids (Fig. 4E). Amino acid biosynthesis (ko01230)-related DEGs showed two gene expression patterns that were expressed mainly in peels and whole fruits (Fig. 4F). The former, which included *branched-chain amino acid aminotransferase* (*BCAT*, Cg3g013250 and Cg8g002380), *ornithine dehydrogenase 1* (*TYRA*, Cg4g019760), and *acetylactate synthase I* (*ALS*, Cg2g036730), were expressed at relatively high levels in diploid peels. The latter included *cysteine synthase 2* (*CAS2*, Cg5g041840) and *threonine aldolase* (*THA1*, Cg8g010040), whose levels were relatively high in diploids; in contrast, the levels of *asparagine synthase* (*ASNS*, Cg2g021180 and Cg2g041840) and *pyruvate kinase isozyme A* (*PK*, Cg5g034730) were higher in tetraploid juice sacs. Flavonoid biosynthesis was associated with the greatest number of DEGs expressed in peels and whole fruits, among which *ferulate-5-hydroxylase* (*F5H*, Cg1g005130), *flavonoid 3'-hydroxylase* (*F3'H*, Cg2g002460), *4-coumarate-CoA ligase 1* (*4CL1*, Cg2g026340), and *ECERIFERUM 26* (*CER26*, Cg9g015220) were specifically upregulated in tetraploid juice sacs (Fig. 4G).

Correlations reveal significant interactions between up-DAMs and DEGs enriched in tetraploid fruits

To determine the relationships between metabolic alterations and changes in the transcriptome, correlations were analyzed between up-DAMs and several DEG groups by Pearson's correlation analysis with screening criteria of $|PCC| > 0.85$ and $P < 0.05$ (Tables S10-12). In total, 43 up-DEGs, specifically in 4X-F, were subjected to the top five up-DAMs of each category in the comparison of 2X-F_vs_4X-F (Fig. 5A, Table S10). In general, 18 of the 31 upregulated DEGs encoded oxidases among the correlated genes in peels (Table S10). Alkaloids were closely and positively correlated with seven peroxidases (Cg2g001430, Cg2g001440, Cg2g001470, Cg3g005370, Cg2g018020, Cg2g006540, and Cg2g008860); however, only two genes were negatively correlated with terpenoids. Two amino acids, L-asparagine and L-ornithine, were positively co-expressed with oxidase genes, such as *cytochrome P450* (Cg7g003610 and Cg4g015240) and *tropinone reductase* (Cg6g005140). In the juice sacs, the upregulated flavonoids, including neohesperidin (pme0001), Quercetin-5-O- β -D-glucoside (Smgp004575), and 5,4'-dihydroxy-6,7,8,3'-tetramethoxyflavone (Wagp007122), were positively correlated with upregulated DEGs, such as *F5H* (Cg1g005130), *Cytochrome P450* 81Q32 (Cg1g006040), *Flavonoid 3'-monooxygenase* (*F3'H*, Cg2g002460), *4-coumarate-CoA ligase 1* (*4CL1*, Cg2g026340), *UDP-glucose flavonoid 3-O-glucosyltransferase* (*UFG*, Cg5g029170), and *Protein ECERIFERUM 26* (*CER26*, Cg9g015220), which are involved in phenylpropanoid biosynthesis (ko00940), flavonoid biosynthesis (ko00941), anthocyanin biosynthesis (ko00942), isoflavonoid biosynthesis (ko00943), and flavone and flavonol biosynthesis (ko00944) pathways. These flavonoids were closely correlated with 19 transcription factors (TFs), such as *ethylene-responsive transcription factors* (*ERF014*, Cg3g016320), *NAC domain-containing protein 62* (*NAC62*, Cg5g019820), and *MYB16* (Cg6g025140) (Fig. 5B, Table S11).

Discussion

Polyploidization in *Citrus* and its related genera usually leads to genotypes indicated by thick and round leaves, large guard cells, pollen grains, and flowers, along with poor fruit features such as thick and rough rinds and high organic acid contents relative to those of diploid plants [13, 42, 43]. In this study, the tetraploid Changshan Huyou had the representative morphological features (Fig. 2–4, Tables 1–2) and fruit qualities (Table 3) of tetraploid *Citrus* or related plants, along with the organic acid content of juice, which is different from other reported tetraploid fruits in citrus [12, 13]. However, the tetraploid had desirable traits for citrus breeding, such as adequate validity of the pollen grains remaining at 85.03% of staining viability and a germination rate of 33.43%, which is competent in hybridization (Table 2), as well as, ovule viability, as indicated by some developed seeds per fruit under free pollination (Table 3). Thus, the tetraploid Changshan Huyou may be used to directly develop new varieties or can serve as an excellent male and female parent in reciprocal crosses with other diploid citrus or related genera to produce triploid hybrids with seedless characteristics, which is the typical feature of emerging citrus species [12, 44] acquired by sexual hybridization with tetraploid parents ($2x \times 4x$, $4x \times 2x$, or $4x \times 4x$) [45] to yield genetic modifications in pharmaceutical and volatile compounds [26].

The ploidy level is strongly associated with tolerance to several types of biotic/abiotic stress factors in citrus. The use of polyploids, especially homologous tetraploid rootstocks in citrus and related genera, increases resistance to salinity [25, 46–48], heavy metal toxicity [49, 50], and drought [51], especially in greenhouse production. These reports indicated that tetraploid rootstocks with thicker and greener leaves, lower stomatal density, larger stomata, and lower respiration rates mostly showed greater salt tolerance because of lower chloride ions (Cl^-) accumulation in leaves and delayed damage to *Citrus macrophylla* [47], *Poncirus trifoliata* [16, 52], Carrizo citrange, and Cleopatra mandarin [16] plants. The tetraploid rootstocks of *Poncirus trifoliata*, *Citrus limonia* Osbeck, and *Citrus reshni* can sequester higher levels of chromium (Cr) into roots, accompanied by a decrease in leaf transfer rate, thus protecting the photosynthetic apparatus and green pigments from oxidative injuries [50]. Additionally, the tetraploid Swingle citrumelo (*Citrus paradisi* $\times$ *Poncirus trifoliata*) rootstock confers better resistance to HLB than its respective diploid progenitors because of fewer symptoms of HLB, limited oxidative stress, and

less secondary root degradation [20]. Besides having higher metabolic levels, citrus tetraploids have higher levels of expression of stress-related genes that contribute to stress resistance [17, 24]. The tetraploids investigated in this study had good yields of fruits and seeds, which encouraged the application of the rootstock to nucellar seedlings generated from these developed seeds and may contribute to biotic/abiotic stress resistance.

Alterations in metabolites following tetraploidization can influence yield, as well as, the constitution of functional metabolites in seedlings and nutrients in fruits. Polyploidization can affect the constitution of volatile organic compounds (VOCs) in Volkamer lemon (*C. limonia*) leaves [53], increase the content of terpenoids [21], such as limonene and cyclic monoterpenes, and improve the antioxidant activity of essential oils in *Citrus limon* [21] with modified compositions of essential oils [22]. Besides influencing seedlings, autotetraploidization can also influence metabolic alterations in the tetraploid fruits of Ponkan mandarin (*C. reticulata*) [13]. Citrus fruits are rich in primary metabolites, such as sugars, organic acids, amino acids, sugar alcohols, and fatty acids, as well as, different secondary metabolites, including carotenoids, limonoids, and flavonoids. These metabolites can be used to measure the quality of citrus fruits, which are key human dietary nutrients [13, 54–56]. Consequently, evaluating the features of autotetraploid fruits is important for their use in citrus breeding. Flavonoids and carotenoids may accumulate at lower levels in tetraploid Ponkan fruits [13]; however, in a study, the tetraploid Satsuma mandarin had a relatively high content of carotenoids in the flavedo [12], which may be due to the differences in their determinate samples. In this study, the dominant categories of DAMs, especially up-DAMs (Fig. 3E-3G), were significantly different between fruit tissues. Alkaloids, terpenoids, and amino acids or their derivatives were positively affected in tetraploid peels; however, flavonoids and coumarins were negatively affected. Citrus peel extracts, including coumarins, flavonoids, alkaloids, and terpenes, display antibacterial and anti-inflammatory activities, whereas, amino acids exhibit pharmacological activities [54–56]. For example, L-asparagine, a key amino acid associated with the citrus green disease HLB [20], is specifically upregulated in tetraploid peels in this study. A tetraploid rootstock of CH (a somatic hybrid of Changsha mandarin + Benton citrange) can increase the accumulation of sugars, flavonoids, and some specific amino acids (especially asparagine), increasing the resistance or tolerance of CH to HLB [57, 58]. Additionally, L-asparagine is a promising candidate in the fields of medicine and food for reducing the production of acrylamide, which is likely carcinogenic and neurotoxic to humans [59]. Additionally, citrus flavonoids, which mainly include flavanones, flavones, and flavonols, are extremely important for human health. Neohesperidin is a flavonoid glycoside detected in citrus fruits; it acts as an antioxidant, greatly suppressing angiotensin II-mediated vascular remodeling and hypertension *in vitro* and *in vivo* [32]. In this study, tetraploid juice sacs accumulated flavonoids such as neohesperidin at relatively high levels and simultaneously presented higher expression of *F3H* and several TFs, such as WRK40 and MYB16. Among citrus flavonoids, polymethoxyflavones (PMFs) are a class of abundant specialized metabolites with remarkable anticancer properties in citrus [60]. Flavonoid O-methyltransferases (OMTs), flavonoid hydroxylases, and flavone O-demethylases influence the accumulation of citrus PMFs [61–63]. The tetraploid juice sacs investigated in this study showed higher contents of 5,4'-dihydroxy-6,7,8,3'-tetramethoxyflavone (HHWagp007122), and the contents of the upregulated DEGs, such as *F5H*, *CYP450*, *F3H*, and *4CL1*, were simultaneously increased in the tetraploid. Overall, certain flavonoids accumulated at higher levels, and genes related to flavonoid biosynthesis were synchronously overexpressed in mature tetraploid fruits, indicating that an increase in pharmaceutical applications of tetraploid fruits and polyploidization can influence the metabolism of Changshan Huyou.

Conclusions

To summarize, we discovered an autotetraploid Changshan Huyou, and analyzed alterations in morphology, physiology, metabolites, and underlying gene expression compared to its diploid progenitors. We found that the shape, stomata, and anatomical characteristics of the tetraploid leaves changed, the tetraploid pollen grains had satisfactory viability, and the tetraploid fruits included certain developed seeds. Our results also showed that flavonoids, lignans, and coumarins were mainly changed in three tissues of tetraploid fruits; however, amino acids and alkaloids were the major groups enriched with upregulated DAMs in tetraploid peels. Pathway analysis indicated that genes upregulated by tetraploidization were related to phenylpropanoid biosynthesis, secondary metabolite biosynthesis, and various alkaloid biosynthesis pathways. Among them, the genes encoding peroxidase and cytochrome P450 are closely related to the increased accumulation of alkaloids and amino acids in peels, and the accumulation of certain flavonoids, such as neohesperidin, quercetin glucoside, and tetramethoxyflavone, is increased by a set of pathway genes and several TFs in juice sacs. These findings indicated that the tetraploid Changshan Huyou can be used in triploid citrus breeding for seedless varieties and pharmaceutical usage in fruit processing, demonstrating its influence on metabolites following polyploidization.

Materials and methods

Plant materials

The tetraploid Changshan Huyou was identified based on several seedlings in 2013. It was top-grafted onto the five-year-old *Poncirus trifoliata* in 2014 and flowered in 2019. The diploid progenitors of the tetraploid Changshan Huyou were used as controls. These plants were planted under consistent conditions for about 10 years at Zhejiang A&F University.

Ploidy analysis

Fresh and young leaves were quickly chopped with a cell lysis buffer for nuclear extraction, stained with 4',6-diamidino-2'-phenylindole (DAPI), and filtered through a 50 µm cell strainer. The diploid leaves of Changshan Huyou were used as controls. Flow cytometry (FCM) was conducted on potential seedlings to determine ploidy levels [18]. The results were plotted on a graph using the Origin 2021 software.

Genetic tetraploid detection through SNP analysis

CTAB was used to extract genomic DNA from one random tetraploid and one diploid [64]. The genomes were resequenced at the Beijing Genomics Institute (BGI) (Shenzhen, China). The clean reads obtained were mapped against the Wanbaiyou (*Citrus grandis*) reference genome (<http://citrus.hzau.edu.cn/download.php>) to identify single nucleotide polymorphisms (SNPs) in diploids and tetraploids. We also obtained genome

resequencing data for three mandarin (*C. reticulata*) (NCBI GenBank: 44977118, 53161798, and 53161808) and three pummelo (*C. grandis*) (NCBI GenBank: 4185428, 42092598, and 44972828) genotypes based on citrus annotation project database [65] to identify SNPs through alignment against the Wanbaiyou reference genome. Next, a phylogenetic tree was constructed based on those SNPs acquired from six genotypes using the online tool iTOL v6 (Interactive Tree Of Life, <http://itol.embl.de/>).

Morphological and microscopic analyses

Autotetraploids and corresponding diploid plants growing in identical environments were evaluated using morphological indicators, including leaf (leaf size and thickness), flower (floral bud, petal, pistil, and ovary sizes; petal and stamen numbers; pollen viability), and fruit (fruit size and seed number) characteristics. Additionally, guard cell size, stomatal density, and pollen size were recorded at the microscopic level. We obtained 20 fully expanded leaf samples from 10 independent diploid and tetraploid plants for each measurement. We also evaluated stomatal size and density through scanning electron microscopy (SEM) (SU-8010; Hitachi, Tokyo, Japan) [66]. Stomatal density (No./mm²) was calculated using ImageJ (V1.8.0) based on 10 viewing fields of each image. Longitudinal resin-embedded leaf cross-sections were obtained following the method described by [15]. Mature leaves were fixed in a 2.5% glutaraldehyde solution at 4°C overnight, followed by gradient ethanol dehydration and Spurr resin embedding. Later, 0.05% toluidine blue O (CI 52040; Merck, Darmstadt, Germany) was added to stain cross-sections (2–3 µm), followed by examination and imaging using a Leica DMLA microscope (Leica Microsystems, Wetzlar, Germany). Blooming flowers were picked to measure the pollen viability and germination rate *in vitro* [67]. Each experiment was independently performed in triplicate, and each observation was repeated in three sets of five fields of view.

Fruit evaluation

Mature fruits from diploids and tetraploids were harvested at 210 days after flowering (DAF) for quality determination. Various characteristics, including fruit size, weight, edible rate, segment number, seed number, titratable acidity (TA), and soluble solids content (SSC) [68], were assessed. All samples were subjected to three replications.

Metabolite extraction and profiling

Metabolites were extracted and quantified following the methods described by Wuhan MetWare Biotechnology Co., Ltd. The whole fruit harvested at 210 DAF was divided into the peel (including the flavedo and albedo), juice sac, and segment membrane, followed by liquid nitrogen freezing. The samples, including the peel of the diploid (2X-F), the peel of the tetraploid (4X-F), the juice sac of the diploid (2X-P), the juice sac of the tetraploid (4X-P), the segment membrane of the diploid (2X-CW), and the segment membrane of the tetraploid (4X-CW), were dried with a vacuum dryer and ground into a powder for quantitative and qualitative metabolic experiments. Each dry sample (50 mg) was extracted with 70% (v/v) methanol (1.2 mL) for 30 min, and this step was conducted five times. Finally, the supernatants were obtained following 3 min of centrifugation (12,000 rpm) and later passed through 0.22-µm filters [69]. MS/MS (tandem mass spectrometry) and UPLC (ultraperformance liquid chromatography, UPLC) (ExionLC™AD, <https://sciex.com.cn/>) were used to analyze the filtered extracts. The R software and AB Sciex 1.6.3 were used to evaluate the UPLC-MS/MS results. The Pearson correlation coefficient (PCC) was determined using the R package (base package; Hmisc). and displayed as heat maps. Differentially abundant metabolite accumulation was assessed through hierarchical clustering heat map analysis (HCA) using the R package, and the normalized metabolite signal intensities (unit variance scaling) were visualized using the color spectrum. We also conducted unsupervised principal component analysis (PCA) using the R software function `prcomp` (www.r-project.org). Differentially accumulated metabolites (DAMs) were identified based on the absolute log₂(fold change) (FC ≥ 2 or FC ≤ 0.5) and VIP (VIP > 1) values extracted from the OPLS-DA results. The KEGG Compound database (<http://www.kegg.jp/kegg/compound/>) was used to annotate those identified metabolites, whereas the KEGG Pathway database (<http://www.kegg.jp/kegg/pathway.html>) was used for mapping. The pathways enriched with markedly differentially abundant metabolites were imported for MSEA (metabolite set enrichment analysis), and p < 0.05 after hypergeometric testing suggested pathway significance.

RNA extraction and transcriptome sequencing

The plant samples were immersed in liquid nitrogen immediately after collection. The RNAprep Pure Plant Kit (Tiangen, Beijing, China) was used to extract RNA. A 1% agarose gel was used to analyze the integrity and quality of RNA. Then, 1 µg of RNA was used for constructing the library. We established and sequenced 18 high-quality RNA libraries. High-throughput sequencing was conducted using an Illumina HiSeq6000 platform (Illumina, San Diego, CA, USA). Moreover, transcriptome sequencing analysis was performed in triplicate to obtain high-quality data. The RNA sequencing data were also aligned against those of the *C. grandis* cv. 'Wanbaiyou' v1.0 reference genome (http://citrus.hzau.edu.cn/data/Genome_info/HWB.v1.0/HWB.v1.0.genome.fa) with HISAT2. Then, StringTie was used to determine fragments per kilobase of transcript per million fragments mapped (FPKM). We identified differentially expressed genes (DEGs) with an FDR < 0.05 and a |log₂FC| ≥ 1. DEGs were analyzed using the R software package. The raw transcriptome data were imported into the NCBI Sequence Read Archive (BioProject: PRJNA1151860).

Correlation analysis was performed and PCCs were calculated using the R software (`cor-function`). The p-value (< 0.05) and correlation coefficient (> 0.85) thresholds were set to filter the results. Heat maps showing correlation clustering and gene expression were constructed using MultiExperiment Viewer and TB tools v2.012, respectively. Correlation networks were constructed using Cytoscape 3.9.1 software.

Statistical analysis

We used SPSS 26.0 (SPSS Inc., Chicago, IL, USA) to conduct one-way ANOVA. Tukey's HSD test was performed to detect among-group differences (P ≤ 0.05 or P ≤ 0.01).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets generated and/or analyzed during the current study are available in the NCBI SRA repository, with accession number PRJNA1151860 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA1151860>). All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Competing interests

The authors declare no competing interests.

Authors' contributions

PH wrote the manuscript, under the supervision of and with contributions from MZ and CZ; TX performed the experiments with contributions from PH, GW, LZ, and YY; MZ and CZ conceived and coordinated this project. PH, TX and GW contributed equally to this manuscript. All authors reviewed the manuscript and agreed to the submission.

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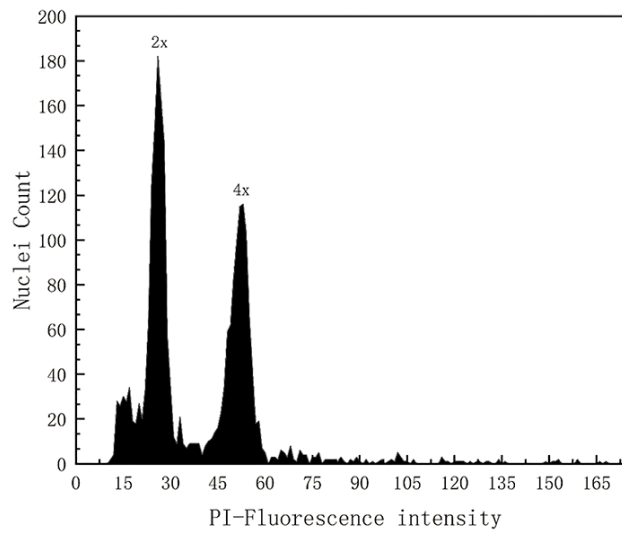
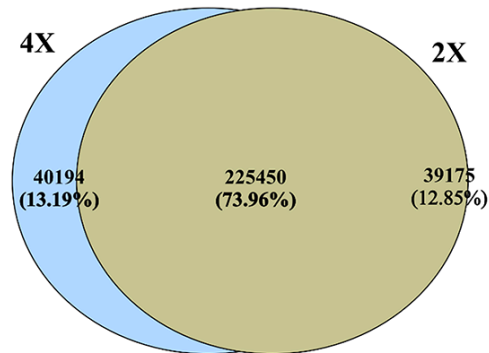
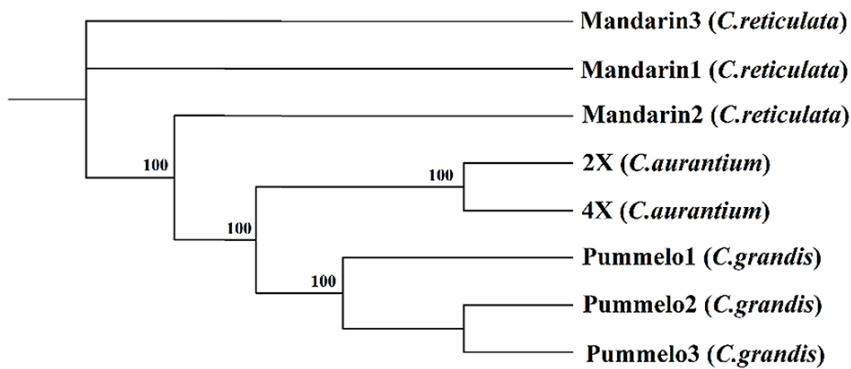
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Figures

A**B****C****Figure 1**

Ploidy levels and genetic constitution analysis of the obtained tetraploid Changshan Huyou. (A) Ploidy level of diploid and tetraploid plants determined by flow cytometry (FCM). (B) Venn diagram of SNP number and proportion between diploid and tetraploid. (C) A phylogenetic tree was constructed using the obtained SNPs among three individual mandarins, three individual pummelos and the diploid and tetraploid Changshan Huyou.

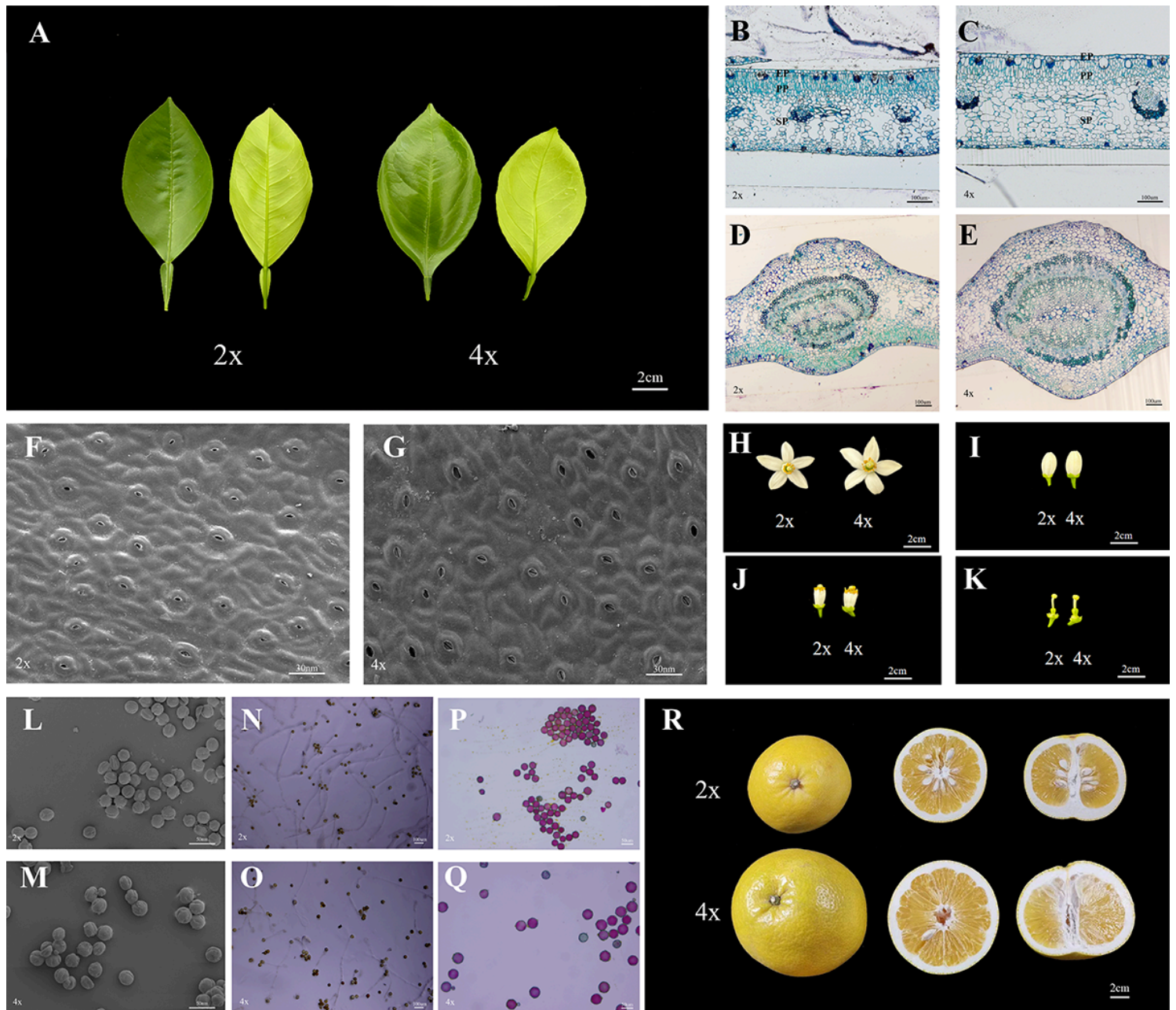


Figure 2

Morphology of the leaves (**A**; Bar = 2 cm), transversal sections of leaves and midribs (**B-E**; Bar=100µm), and scanning electron micrographs of stomatal density (**F, G**; Bar = 30 nm) in the diploid and the tetraploid. EP: Epidermis, PP: Palisade parenchyma, SP: Spongy parenchyma. Morphology of flowers (**H-K**; Bar=2cm), scanning electron micrographs of pollen grains (**L, M**; Bar=50 nm), the pollen germination (**N, O**; Bar=100µm), and pollen stability (**P, Q**; Bar=50 µm). morphological characteristics of fruits in the diploid and the tetraploid. Bar=2cm.

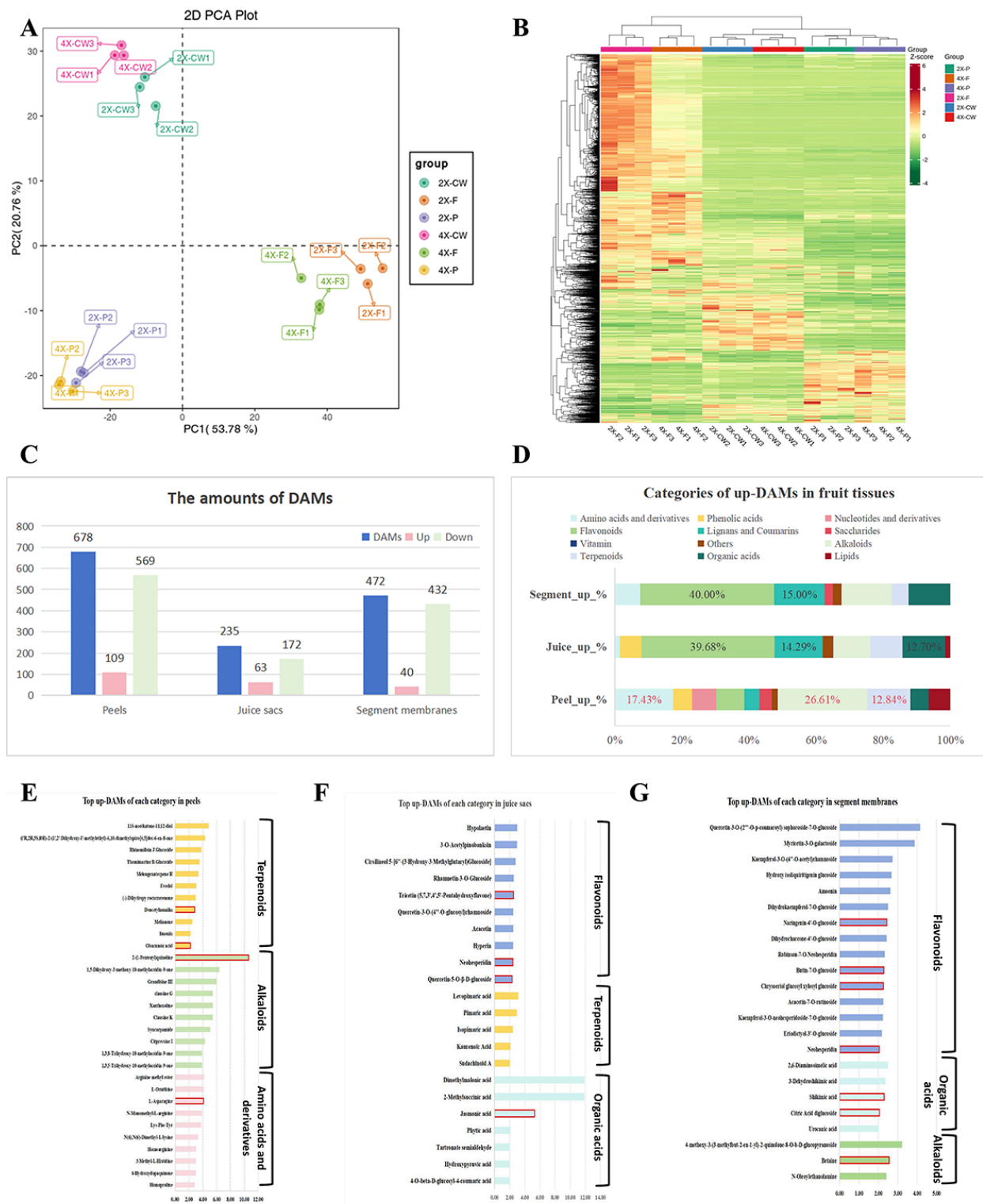


Figure 3

Comparative metabolic profiling in peels, juice sacs and segment membranes between tetraploid and diploid. (A) Principal component analysis (PCA) of all samples from three tissues of fruits (B) Hierarchical cluster analysis (HCA) of all metabolites identified in three tissues of fruits. (C) The amounts of differentially accumulated metabolites (DAMs), up-regulated DAMs and down-regulated DAMs. (D) Categories of up-regulated DAMs in fruit tissues. (E-G) Representatives of up-regulated DAMs in peels, juice sacs, and segment membranes, respectively.

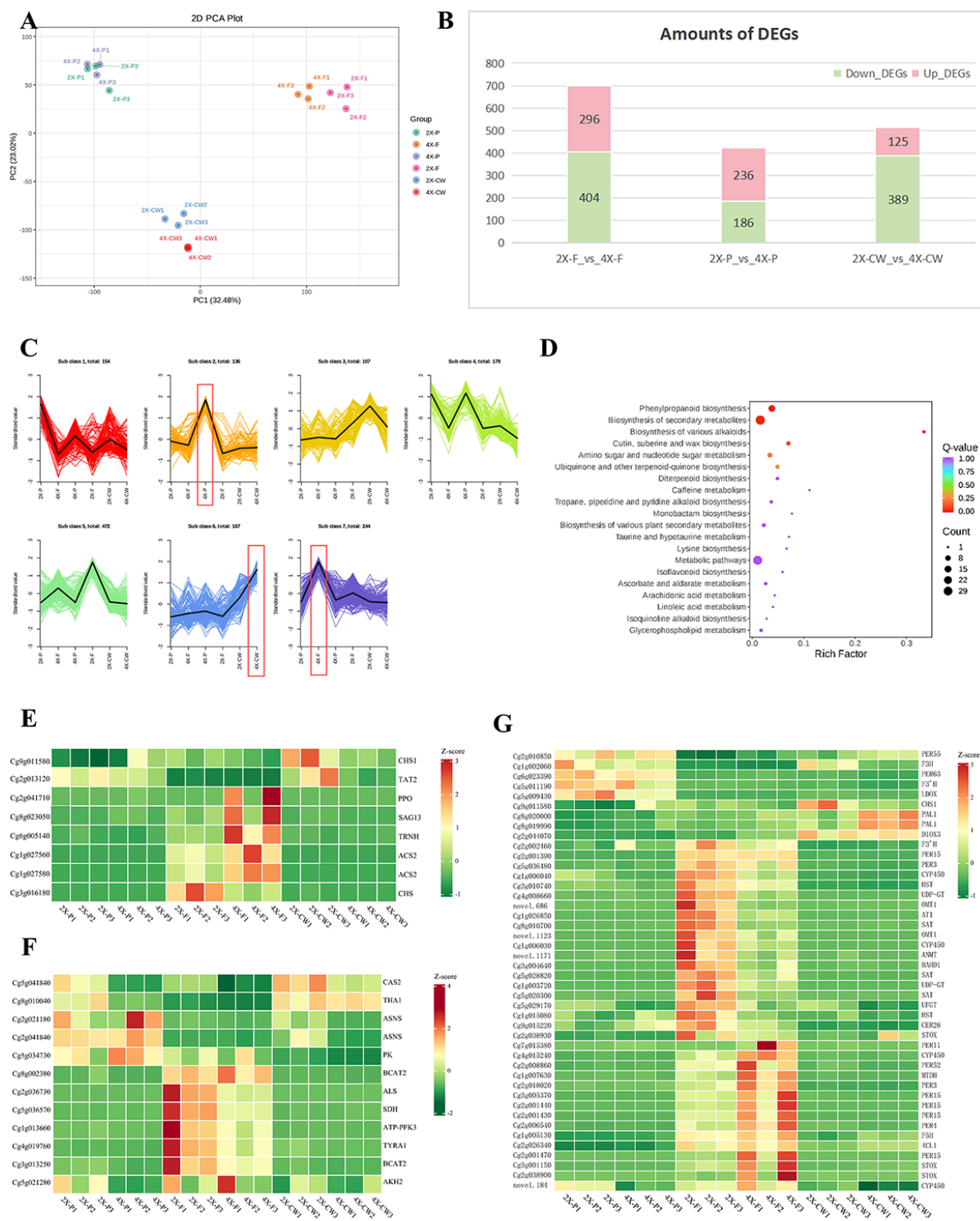
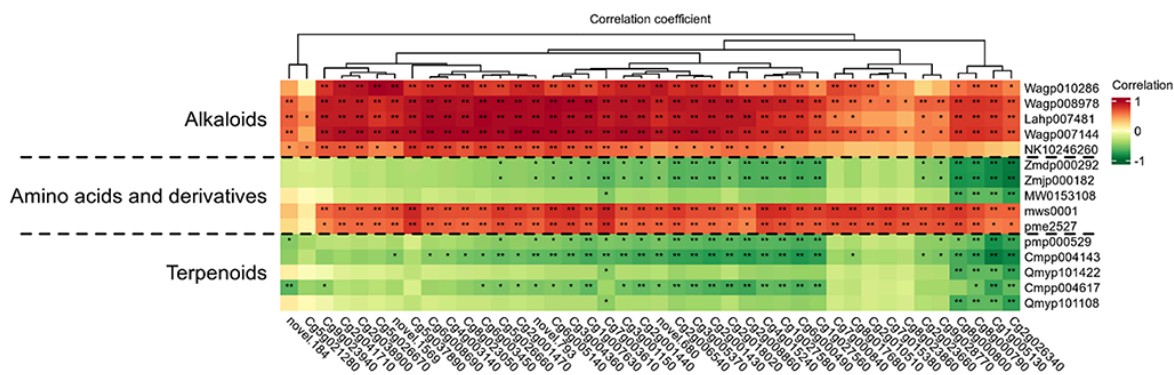


Figure 4

Comparative transcriptome profiling in peels, juice sacs and segment membranes between tetraploid and diploid. **(A)** Principal component analysis (PCA) of all samples from three tissues of fruits. **(B)** The amounts of differentially accumulated metabolites (DAMs), up-regulated DAMs and down-regulated DAMs. **(C)** K-means analysis of all differentially expressed genes (DEGs) identified from three tissues of fruits of tetraploids and diploids. **(D)** KEGG enrichment analysis of DEGs specially up-regulated in tetraploid peels. **(E-G)** Heatmap of DEGs involved in alkaloids, amino acids, and flavonoids biosynthesis pathways, respectively. 4x-p, tetraploid juice sacs; 2x-p, diploid juice sacs; 4x-f, tetraploid peels; 2x-f, diploid juice sacs; 4x-cw, tetraploid segment membranes; 2x-cw, diploid segment membranes.

A



B

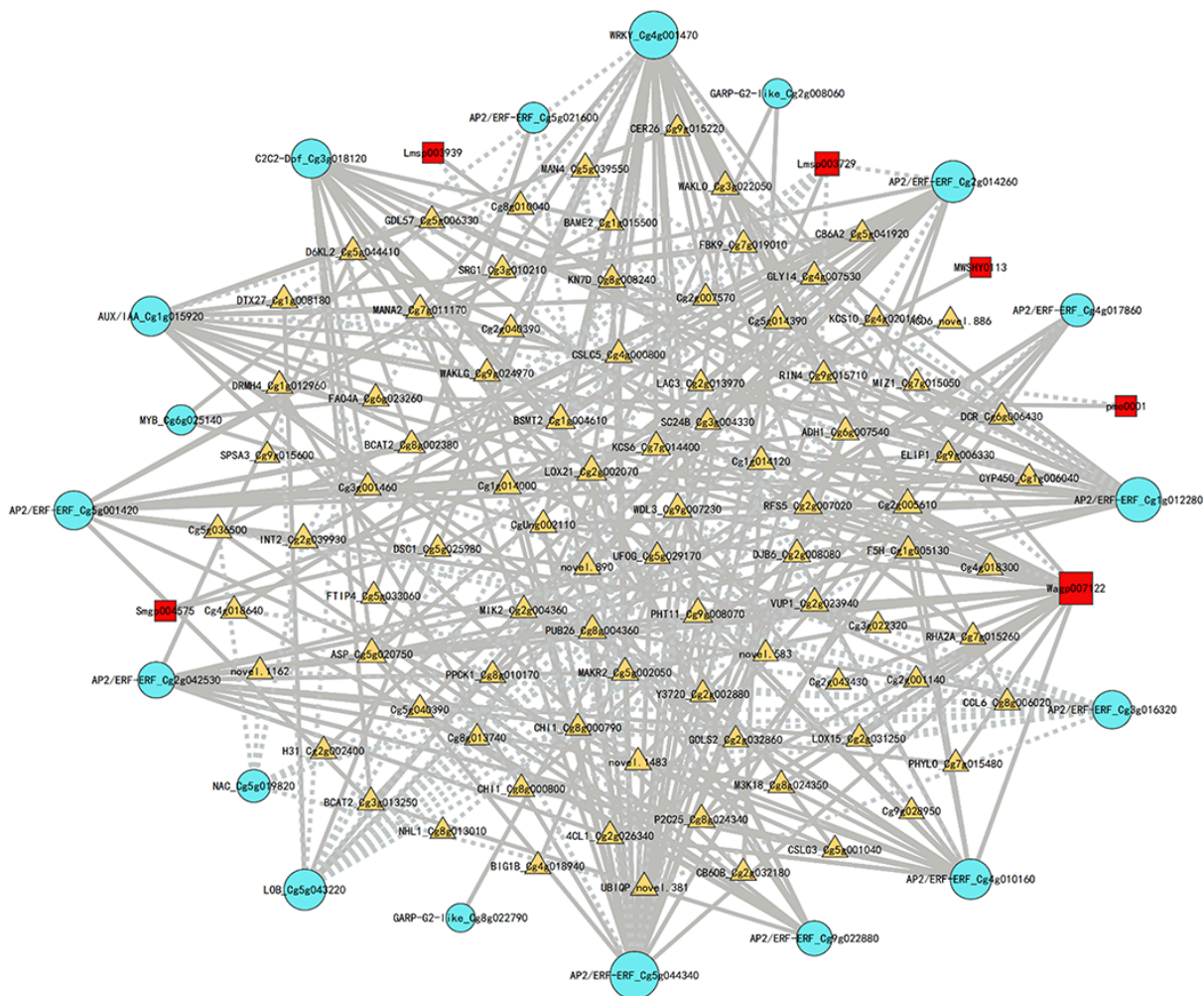


Figure 5

Correlation analysis of up-DAMs and DEGs related to amino acids, alkaloids and flavonoids. (A) Up-regulated amino acids and alkaloids were correlated with the up-regulated DEGs in tetraploid peels. (B) The up-regulated flavonoids were correlated with DEGs involved in pathway of flavonoids biosynthesis in juice sacs. The yellow triangles represents structural genes. The blue circles represents TFs. The red squares represents metabolites. Solid lines indicated negative correlation. Dotted lines indicated positive correlation.

Supplementary Files

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