Comparison of the metabolites profiles between a Graft Chimera ‘Hongrou Huyou’ (Citrus changshan-huyou + C. unshiu Marc.) and Two Donors

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Abstract

Background: Chimeras synthesized artificially by grafting are crucial to breeding of perennial woody plants. ‘Hongrou Huyou’ (Citrus changshan-huyou + C. unshiu Marc.) was a new grafting chimera originated from the junction where a scion Citrus changshan-huyou ("C") was top-grafted onto a stock Satsuma mandarin ‘Owari’ (C. unshiu Marc., “O”). The chimera was simplified as OCC because the cell layer constitutions were O for L1 and C for L2 & L3, respectively. In this study, profiles of primary metabolites, volatiles and carotenoids derived from different tissues were investigated between OCC and two donors, aiming to figure out the relationship between layer donor and metabolites.

Results: Comparison of the metabolite profiles showed that the amount and composition of metabolites were different between the peels and the juice sacs, as well as between OCC and two donors. Metabolites (such as violaxanthin and β-cryptoxanthin in carotenoids, germacrene D in volatiles, citric acid and sorbose in primary metabolites) specifically present or absent in the certain tissue were identified in three phenotypes. According to the principal component analysis (PCA), the total metabolites of chimeric peel properties were derived from the donor C, whereas those of chimeric juice sac properties came from the donor O. Conclusion: Profiles of primary metabolites, volatiles and carotenoids derived from the peels and the juice sacs were systematically compared between OCC and two donors. The content and composition of metabolites differed in tissues as well as between OCC and two donors. Donor dominant metabolite inheritance was dramatic in the different tissues of OCC and basically consistent with the layer origin that the chimeric peels were derived from C and the chimeric juice sacs came from O. These profiles provide potentially chemical markers for genotype differentiation and citrus breeding assessment, more than that, for donor selection during chimera synthesis artificially.
Background

Plant chimeras refer to those plants composed of more than two genotype cells. According to the theory of ‘Tunica-Corpus’, the shoot apical meristem (SAM) of dicotyledonous plants are composed of three cell layers, that is L1, L2 and L3, from the outermost layer [1]. For citrus fruits, the juice sacs and epidermal pericarps are derived from cell layer L1; the color and aroma of the fruit rind, seeds and segment walls are developed from L2; L3 produces vascular bundles, and fruit shape was determined by L2 and/or L3 [2]. Up to now, there have been some reports on the discovery and identification of citrus chimeras. Zhou et al. found that the interaction between cells derived from different genotypes caused the mutation of DNA levels in periclinaly chimeric fruits NFF (L1-L2-L3=N-F-F) and FNN [2]; Wu et al. found that the fruit characteristics of chimera Ekuliku were inconsistent with source donor and cross-sectional structure of the blade was quite different from two donors [3]. Zhang and his colleagues investigated two citrus chimeras named ‘Zaohong’ navel orange [4] and ‘Hongrou Taoye’ orange [5], both two chimeras are from the donors sweet orange (Citrus sinensis) and Satsuma mandarin. The stomatal density and the flesh aroma of the chimera fruits in their studies were not consistent with the source donor, but combined the characteristics of both two donors [4, 5]. Since these variations of morphology and DNA level were occurred in plant chimeras, the accumulation patterns of metabolites oriented to the chimeric tissue and/or cell interaction in chimeras deserved to study.

Citrus fruits are in great value of their nutrient components, and many studies have been concerned the metabolites in oranges (C. sinensis L. Osbeck), mandarins (C. reticulata), pummelos (C. grandis Osbeck) and grapefruits (C. paradisi Macf.) [6-9]. Primary metabolites, such as sugars and organic acids, are a diverse class of organic compounds which are essential for plant growth and internal quality [10]. For example, high content
citric acid coinciding with high level of free amino acids (especially of proline) may be the part of reason that lemon possesses a longer shelf life than other citrus [11]. Volatiles refer to a number of important secondary metabolites and have received extensively attention due to their massive healthiness and commodity value. The d-limonene is a dominant volatile in citrus and has special efficacy with clinicopathologic significance that contributes to breast and rectal cancer [12]. It was reported that linalool and linalyl acetate had been used as anti-inflammatory agents [13], and the rearrangements of Germacrene D had eventually produced some natural compounds [14]. Carotenoids are complex and abundant in citrus fruits [15]. Some carotenoids containing β-ring moieties are precursors of vitamin A which are highly beneficial to chronic disease and cancer prevention [16]. The carotenoids biosynthesis and their regulation in citrus fruits have been extensively reported [17-19], which were helpful for our analysis on carotenoids expression of the chimeras.

Graft chimera is derived from an adventitious shoot at the graft junction and comprised of two distinct genotypes or different species [20-23]. A new graft chimera ‘Hongrou Hyou’ (Citrus changshan-hyou + C. unshiu Marc.) was reported previously that originated at the junction of the scion ‘Changshan Hyou’ (C. changshan-hyou, abbreviated as C) and the stock ‘Owari’ Satsuma mandarin (C. unshiu Marc. abbreviated as O). This chimera remained yellow-skinned like C, but was substituted for the dark orange juice sacs like O (Table 1, Figure 1). Additionally, the chimera combined the specific DNA bands of two donors in nuclear, chloroplast and mitochondrial genome through simple sequence repeat (SSR) amplification, respectively. Therefore, the chimera was assumed as OCC due to that L1 was derived from O while L2/ L3 were from C (Data not shown).

In this study, profiles of primary metabolites, volatiles and carotenoids were investigated at maturation period according to peels and juice sacs in the chimera and two donors, as
well as the correlation of metabolite accumulation between the chimera and each donor plant was analyzed to reveal the contributions of donor plant in different layers.

Results

**Primary metabolites in OCC and its donors**

As shown in Table 2, twenty-one primary metabolites were identified in peels. Based on the significant analysis of statistics, the peels of OCC (OCP) shared more similarities with that of C (CP) in these profiles. Among them, the 4-aminobutanoic acid, shikimic acid and palmitic acid were exclusively detected in OCP and CP, suggesting that these 4 compounds of OCP were only produced by CP. In the contrary, sorbose was specific to the peels of O (OP), with no detectable evidence in OCP and CP. There were higher concentration of the acids (except 2-ketoglutaric acid) and alcohols detected in OCP and CP, whereas there were lower content of sugars (except fucose) detected in OCP and OP. Notably, some particular chemical characteristics were observed in OCP. Among those identical profiles detected in three samples, carbamic acid and fucose exhibited the highest level in OCP; but in total metabolites, OCP showed a significantly lower value than that in any of the donors.

For the juice sacs, there were 18 primary substances listed in Table 3 among the juice sacs of O (OJ), OC (OCJ) and C (CJ). In the present study, no-significant differences were found according to the total primary metabolites between OCJ and the two donors. Interestingly, there were 5 profiles (quininic acid, xylose, arabinose, turanose and scyllo-inositol) in OCJ were significantly different from two donors. Among them, arabinose and quinic acid in OCJ were the highest and the lowest of three cultivars, respectively. The remaining 3 profiles in OCJ were significantly different between two donors. In addition, there were 10 profiles in OCJ consistent with one or two donors. However, these profiles actually showed more similarities with O, for example, 4-aminobutanoic acid, palmitic acid
and allose were common to OCJ and OJ, but were not presented in CJ. Conversely, the sorbose was only specifically existed in CJ, but undetectable in OCJ and OJ.

Interestingly, there were 3 compounds showed some hereditary differences in OCJ (oxalic acid, sorbose and rhamnose). Among them, either oxalic acid or rhamnose were undetectable only in OCJ, which caused the obvious discrepancies between OCC and its donors. However, the sorbose was missing in both OCJ and its layer source donor O.

**Volatile compositions between OCC and its donors**

With regards to the volatiles in the peels of three cultivars, there were 36 substances listed in Table 4, including monoterpenes, sesquiterpenes, alcohols, aldehydes, phenol and others. The monoterpenes were the most abundant of profiles quantified, with d-limonene as the dominant compound, accounting for 88.65%, 81.23% and 80.77% of the total volatiles in OP, OCP and CP, respectively. After d-limonene, followed by γ-Terpinene, β-Myrcene and α-Pinene, they were the main compounds for three samples had in common.

The result showed that OCP had a stronger correlation with CP than with OP. Firstly, according to the significance analysis, 14 volatiles had no significant difference between OCP and CP, but only 3 volatiles between that of OCP and OP. This indicated, CP had the dominant position in the regulation of chemical profiles in OCP and more chemical traits in OCP were inherited from CP. Secondly, the main volatiles of OCP were completely consistent with that of CP, including d-limonene, γ-terpinene, Germacrene D, β-myrcene and α-pinene (sort from high to low concentration), but the main volatiles order in OP were divergent (d-limonene, γ-terpinene, β-myrcene, α-pinene, β-elemene). This was mainly because the Germacrene D was significantly higher in OCP and CP than OP, and so strongly suggest that Germacrene D was mainly originated from CP and OP had less impact on the development of OCP. Thirdly, it is worth noting that 2,4-di-t-butylphenol
was really unique, which was only detected in OP, and this is the only one volatile that OCC and C exclusively possessed in common.

In addition, most of volatiles in OCP were either inclined to one donor, or maintained some degree between two donors. However, only \((E)-3\text{-Hexen-1-ol}\) and 3-Hexenal in OCP exceeded significantly that in two donors.

For the edible juice sacs, the volatiles contained up to 19 constituents (Table 5). OCJ was highly correlated with OJ in total volatiles and monoterpenes (the leading volatiles). Especially in dominant substances, \(d\text{-limonene}\), its concentrations of OJ and OCJ were significantly higher than CJ, occupied 78.07%, 72.64% of the total volatiles in OJ and OCJ, respectively, but only 60.03% in that of CJ. Meanwhile, besides \(d\text{-limonene}\), there were also significant similarities between OJ and OCJ in methyl nonanoate, copaene and octanal, and we considered that all these compounds in OCJ were originated from O to a great extent.

Moreover, typical volatiles quantitative inheritance traits were observed in OCJ. For example, nootkatone and pentadecanal were presented the largest amounts in OCJ. Instead, \(\gamma\text{-terpinene}\) in OCJ was significantly lower than that in any of the donors. Furthermore, what we were particularly interested in was \(\alpha\text{-ylangene}\), which was only detected in OCJ but not in two donors, and this volatile was hardly ever been reported in any citrus species.

**Carotenoid constituents between OCC and its donors**

As it shown in Table 6, a total of 9 carotenoids were detected among OCC and two donors. Generally, the contents and types of carotenoids in OCC were very close to C in peels, and inclined to O in juice sacs.

Obviously, the donor O had the highest contents of all carotenoid components in both peels and juice sacs among three genotypes. In the peels, the carotenoids other than
violaxanthin and Lutein in OCP were all significantly consistent with the donor CP.

According to the juice sacs, the carotenoids in OCJ were intermediate between two donors. Actually, all of the carotenoids detected in OJ and OCJ were particularly higher than those in CJ. It is remarkable that α-carotene accumulated much less than other carotenoids both in the peels and juice sacs.

The dominant components were different between peels and juice sacs in OCC and two donors. Violaxanthin was primary in peels, and β-cryptoxanthin was dominant in juice sacs. The main carotenoids in OCJ, such as β-cryptoxanthin, phytoene and phytofluene, changed much more than those in OCP, which maintained the color of flesh in OCC compared with layer donor O.

Correlation of total carotenoids between OCC and two donors was analyzed to make out the source donor in tissue coloration. It was suggested that the carotenoids accumulation of OCC had obvious donor bias and was different between peels and juice sacs (Table 7). In peels, total of carotenoids of OCP were significantly correlated with CP and OP, respectively. In the juice sacs, only the correlation coefficient between OCJ and donor OJ was statistically significant (0.957). This donor bias of carotenoids in the peel and juice sac of OCC maturation can partly explain why the peel of OCC is light yellow, similar to donor C, whereas the juice sac is dark orange, similar to donor O.

**PCA analysis of metabolites in peels and juice sacs of OCC and two donors**

In terms of three categories of metabolites, principal component analysis (PCA) was performed according to different tissues between OCC and two donors.

In the PC1 direction of the score map, there was a clear distinction between the donor O and the other genotypes (OCC and donor C) in primary metabolites (Figure 2A-1), volatiles (Figure 2A-2) and carotenoids (Figure 2A-3) according to the peels.

In the juice sacs, the donor C was clearly distinguished in the primary metabolites (Figure
2B-1), volatiles (Figure 2B-2) and carotenoids (Figure 2B-3) from OCC and donor O according to the PC1 direction of the score map. However, OCJ was separated from OJ in the PC1 direction (Figure 2B-3), indicating a novel profiling of accumulation pattern in carotenoids in the chimera.

Discussion

Researches focused on phenotypes, fruit qualities and genome compositions [2-5] had contributed to the knowledge of chimeric plants, however, the mechanism of metabolites accumulation between genetically different cells remained unknown. In this work, profiles of the primary metabolites and the secondary metabolites were systematically compared between a novel citrus chimera OCC and its donor plants, which may provide valuable insight into the genetic contributions and inheritance patterns from grafting donors to the chimera.

Donor dominant metabolites analysis in OCC

In citrus chimeras, the juice sacs were developed from L1 cell layer, and the peels were from L2 cell layers [1-2]. In this study, the chimera OCC contained the metabolites more similar to C in peels and closer to O in juice sacs, which seems conforming to the speculation of layer origin. Carotenoids were primary nutrients in citrus, and their content and composition varied greatly among citrus varieties [24]. Several reports have been concerned on the differentiation of citrus genotypes through difference in carotenoid profiles. For example, thirty-two citrus fruits were clearly separated by the difference in their β-cryptoxanthin of juice [16]. Similarly, twenty-five citrus genotypes were classified in the basis of cis-violaxanthin and β-cryptoxanthin in juice [25]. Furthermore, violaxanthin and β-cryptoxanthin were successfully used to recognize 39 citrus genotypes from each other in the flavedo and juice sac [26]. Herein, three groups were classified according to the amount of specific type of carotenoids in citrus. Satsuma mandarin
represented mandarin cultivars and processed abundant β-cryptoxanthin in both the flavedo and juice sac. Oranges were rich of violaxanthin in both the flavedo and juice sacs. Pummelo (C. grandis (L.) Osbeck) was separated from oranges and mandarins lacking of β-cryptoxanthin and violaxanthin [15]. In this study, the donor C was documented to be the hybrids of pummelo, orange and / or other citrus species [27-29]. Obviously, the primary cell lineage of C includes pummelo and orange that of low level of β-cryptoxanthin. Actually, in this study, the OCP contained β-cryptoxanthin as low as CP, while the OCJ accumulated much more β-cryptoxanthin than CJ. Likewise, the previous study on citrus chimera Ekuliku revealed that its juice sac was developed from L1 donor Nankan (C. unshiu), and the peel was developed from L2&L3 donor Hamlin (C. sinensis) [3]. Similarly, the leaf morphology variation of Brassica chimeras, the variation was only controlled by red cabbage, and was reproducible and directional in progenies [30]. Metabolites were firstly biosynthesized in vivo. Three key genes (CitPds, CitZds and CitCrt) in the upstream of the carotenoids biosynthesis pathway were reported to express at low level in a somatic hybrid between C. reticulata and C. limon, which were biased towards parent lemon, resulting that the carotenoids content in the hybrid were pretty low [31]. Similarly, a somatic hybrid between ‘Bonnaza’ naval orange (C. sinensis Osbeck[])and rough lemon (C. jambhiri Lush) showed the comparative carotenoid content with the rough lemon, whose expression patterns of the lycopene ε-cyclase gene (LCYE) and the zeaxanthin epoxidase gene (ZEP) were more closer to the rough lemon [32]. These scientists believed that the expression of carotenoid genes were not a simple additive effect between parents, rather express a certain genomic imprinting, that is the expression of homologous genes in polyploids biased toward one parent [31, 32]. Herein, it was interesting that how the chimera OCC chooses and balances carotenogenesis between two sets of genetic cells. It was assumed that, in the newly produced chimera
OCC, the homologous genes derived from distinct layer may express selectively in the same metabolic pathway because of changes in DNA methylation that were speculated to be induced during grafting [33], and finally produce the coordinate on expression patterns in each fruit tissue to achieve the coexistence of two sets of genetic cells.

**Characteristic metabolites analysis in OCC**

However, the accumulation of a number of metabolites (including primary metabolites and volatiles) in OCC were not “loyalty” to layer source donor, some metabolites deviated far from both two donors (i.e., significantly higher or lower than two donors). This observation was similar to two citrus hybrids exhibited 56 of the 113 volatile profiles in the hybrids that were significantly higher or lower than in parents [34]. In this study, Germacrene D (Table 5) in OCJ was 6-17 times higher than that in CJ and OJ, respectively. The quantities of arabinose was over 3-9 times higher than in CJ and OJ (Table 3), and this profile have been reported as a good source of dietary fiber and could be available for juice production [35]. Taken together, it was assumed that genes altered their expressions may due to some reasons of layer displacement.

Interestingly, a volatile named α-ylangene was exclusively appeared in the juice sac of the chimera OCC (Table 5). α-ylangene was an unique compound barely reported in any citrus volatiles, which was a main sesquiterpenoid at the post-maturation stage in grapes [36]. Similarly, the previous study has reported that the citrus chimeras NFF and FNN had their own new bands, aside from than the specific bands of two donor plants by RAPD analysis, suggesting that the chimeras interacted at DNA level [2]. Therefore, it was speculated that genetic mutation involved in intercellular movement may be responsible for α-ylangene synthesis exclusively in the chimera OCC during the chimeras’ development.

Recently, the genetic mutations were proved due to the traffic of transcription factors (TFs) within a plant and maintain its biological activity [37, 38]. In addition, the heritable
variations caused by intercellular trafficking and gene mutation were extensively studied in chimeras. A grape periclinal chimera ‘Malian’ was derived from cell invasion into L2 to give rise to a spontaneous mutation with borone flesh [39]. And there were some studies reported that berry color variants in grape Pinot can be mapped back to the mutation on a single locus named “berry color locus”, which is consist of four tandem MYB transcription factors on chromosome 2 [40-42]. Fernandez and his colleagues investigated the weight reduction in the berry of a grape chimera which was caused by unusual Vvpl gene expression in L1, L2 or in both cell layers, which lead to phenotypic variation (fleshless) in progeny [43]. In peach mutant, the mutation cell carried a PRUPE.6G281100 allele entered L2, causing a phenotype change of peach from flat to round [44].

Speculation of genetic laws in chimeras’ metabolites

Up to now, there is limited knowledge available with regards to the inheritance regularity of chemical compounds in plant chimeras. The donor bias was a compelling issue on artificial synthesis of chimera and plant breeding. Arguments on correspondence of chimeric phenotype and traits of grafting donor have been put forward. It seemed that the stock donor Satsuma mandarin was liable to act as the inner layer (L1) with focus on the carotenoid synthesis [4, 5, 45]. Therefore, several novel phenotypes with “red-flesh”, including OCC in this study, were discovered after grafting. Coinciding with these reports, the coloration in peel and juice sac of OCC was orient to the layer source donor, however, the ingredients of primary metabolites (such as organic acids and sugars) as well as volatiles (such as γ-terpinene) were partly different from the layer donor and displayed possible “recombination” between layers. Recently, small RNAs and DNA methylation have been considered to be involved in stock-scion interaction to describe genetic variations in graft chimeras. For instance, researchers have found some conserved miRNAs were differentially expressed in graft chimera (Brassica juncea + B. oleracea) progeny rTTT
(sexual self-crossing of the chimera) and donor plant TTT (*B. juncea*), which may contributed to the changes in expression of their target genes [30]. Furthermore, in the grafting chimeras *Brassica juncea* and *B.oleracea*, sequencing analysis revealed that DNA methylation will affect the flowering time- and gibberellin response-related genes expressions, and may lead to the phenotypic variations in progenies [46]. Therefore, OCC possessed metabolites preferring to one donor or intermediated may suffer from delivery factors that modulate the genes involved in metabolites production, transport and accumulation.

**Conclusions**

The genetic pattern and accumulation of primary metabolites, volatiles and carotenoids derived from the peels and the juice sacs were systematically investigated and compared between OCC and two donors. The content and composition of metabolites were different among phenotypes as well as tissues. Metabolites specifically present or absent in the certain tissue (α-carotene and phytoene) were identified in three phenotypes. Donor dominant metabolite inheritance was dramatic in the different tissues of OCC, indicating that the metabolites derived from chimeric juice sacs were closer to L1 donor O and that from chimeric peels were closer to L2/L3 donor C. These profiles provide potentially chemical markers for genotype differentiation and citrus breeding assessment, more than that, for donor selection during chimera synthesis artificially.

**Methods**

**Plant materials and sampling**

The chimera OCC was discovered in an orchard in Changshan county of Zhejiang province of China during a bud mutation investigation in 2001. OCC and the donors (O and C) were separately grafted onto the *Poncirus trifoliata* in 2005 for production and maintained
stable morphologies for 12 years under regular management. Three individual trees were picked out for each genotype, and 10 fruits were harvested from each tree based on the uniform size, peel color and location on the tree at full ripening stage. Peels including epidermis, flavedo and albedo were separated carefully and quickly from juice sacs of each genotype by girdling. Peels and juice sacs obtained from one tree were blended and ground into powder in liquid nitrogen, respectively. Finally, the samples were preserved at -80°C for later research.

**Primary metabolites and volatiles extraction**

The primary and volatile substances were determined using a modification of the procedure originally developed [47]. To determine the primary contents, we first ground 0.2 g tissue into powder using liquid nitrogen, and then added 2.7 ml of pure, precooled (-20°C) pure methanol. These components were mixed and 0.3 ml ribitol (0.2 g/ml) was added as the internal standards. The procedure was later applied to the volatiles samples. For volatiles, samples were freeze-dried with a vacuum freeze-drier (Labconco FreeZoneR, USA) and fully ground in liquid nitrogen. A 0.2 g sample of powder was poured into the centrifuge tube (2 ml volume), which was homogenized with 500 μl double distilled water (DDW) and 500 μl MTBE (containing 0.02 μl/ml methyl pelargonate), followed by gentle shaking. Samples were vibrated using an ultrasonic bath (model FS60, Fisher Scientific, Pittsburgh, PA) maintained at 4°C for 40 min and were centrifuged at 12000 g for 10 min at 4°C. The 200 μl supernatants were then transferred into another tube. Finally, 1 μl sample was injected with a syringe and filtered through a 0.22 μm membrane (SCAA-104, ANPEL, Shanghai, China) for Gas Chromatography-Mass Spectrometry (GS-MS).

**Primary metabolites and volatiles analysis**
The compounds were determined by use of TRACE GC Ultra GC coupled with a DSQ II mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with a TRACE TR-5 MS column (30 m × 0.25 mm × 0.25μm; Thermo Scientific, Bellefonte, PA, USA). With pure helium as a carrier gas, the peels (flavedo and albedo) and juice sacs of samples were identified at 1.0 ml/min with a split ratio of 50:1 and 1:1, respectively. The concentrations of the primary and volatile substances were calculated in μg/g FW. Three replications were used for each sample.

The public database Massbank (http://www.massbank.jp/) and Metlin (https://metlin.scripps.edu/index.pCF) supported to identify tentative metabolite substances; for some other compounds, we obtained information from the published literatures.

**Carotenoids extraction**

The total carotenoids of OCC and its donor parents were extracted according to a previously described method [48] with some modification. Juice sac powder (1 g) and peel powder (0.5-1g) were homogenized in a 50 ml centrifuge tube after lyophilization using a lyophilizer (LABCONCO FreeZone®). Next, 15 ml pigment extraction solvent (n-hexane/acetone/anhydrous ethanol, 2:1:1, v/v/v, containing 0.1‰ BHT) was added. Samples subjected to ultrasonic vibration for 30 min and centrifuged for 10 min at 4000 g at 4°C. Supernatants were transferred to another 50 ml centrifuge tube, and sediment was extracted using 15 ml pigment solvent until it was colourless. Supernatants were combined in 50 ml separating funnel and washed 3 times using a saturated 10% of NaCl solution until neutral, and the underlayer was discarded. Supernatants were separated into a 10 ml centrifuge tube and then concentrated under the vacuum condition. The samples were redissolved with 2 ml of methyl tert-butyl ether (MTBE) and 2 ml of 10%
KOH (containing 0.1‰ BHT), and the residue was dried under nitrogen. Samples were kept in the dark for 10 hours of saponification. Then, 4 ml saturated NaCl and 2 ml MTBE (containing 0.1‰ BHT) were added to better separate layers and wash away the water, and then 5 ml NaCl was added 3 times to wash the solution to neutral. Meanwhile, the supernatant was concentrated by the vacuum concentration and was diluted with 0.6–1 ml MTBE (containing 0.1‰ BHT). The samples were centrifuged at 12000 rpm for 30 min at 4 °C for later determination.

**Carotenoids analysis**

A gradient elution method of OCPLC, composed of A (acetonitrile/methanol, 3:1, v/v, containing 0.1‰ BHT, 0.05% TEA) and B (100% MTBE, containing 0.1‰ BHT) as the mobile phase, was used to determine the carotenoid contents. The flow rate was fixed at 1 ml/min. The gradients were used as followed, 0 min: (95: 5); 0–10 min: A-B (95: 5); 10–19 min: A-B (86: 14); 19–29 min: A-B (75: 25); 29–54 min: A-B (50: 50); 54–66 min: A-B (26: 74); 67 min: A-B (95: 5). The volume of the above gradient solvent was 20 µl and the test adopted an external standard method for quantitation. All carotenoids extraction, saponification and other determinations identified above were conducted under low light levels or in the dark.

**Statistical analysis**

The concentration of each chemical compound was indicated as the means ± standard deviation of three replicates. A statistical analysis was performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). The significant differences were calculated using one-way analysis of variance (ANOVA) followed by Duncan’s multiple-range test at the 5% level (p < 0.05) and was shown in the tables with lowercase letters (a, b, c, etc.) between
cultivars. The substance undetectable was marked with “nd” in all tables of metabolite profiles. Correlation analysis was carried out by Pearson test, and the significant index was marked with “**” (p<0.01). Principal component analysis was carried out by the SIMCA 14.1.

**Abbreviations**

OP: the peel of ‘Owari’ satsuma mandarin; OCP: the peel of OCC; CP: the peel of ‘Changshan Huyou’; OJ: the juice sac of ‘Owari’ satsuma mandarin; OCJ: the juice sac of OCC; CJ: the juice sac of ‘Changshan Huyou’

**Declarations**

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**Availability of data and materials**

All data generated and analyzed in this study is presented in this published article.

**Authors’ contributions**
MZ and CZ contributed to the experimental design. LJ contributed to the data analysis and wrote the manuscript. QW provided experimental materials. KZ performed the experiments. FK and JX provided financial support. SZ and GW contributed to experimental material planting management. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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Tables

Table 1
The cultivars used in this study and their morphological traits. Peel color and juice sac color were captured at full ripening stage (collection period is December 2017)
| No. | Cultivars                  | Scientific name                     | Abbreviation | Peel color |
|-----|----------------------------|-------------------------------------|--------------|------------|
| 1   | 'Owari' satsuma mandarin  | *C. unshiu*                         | O            | Orange     |
| 2   | ‘Hongrou Huyou’           | *C. unshiu* + *C. changshan-huyou*  | OCC          | Yellow     |
| 3   | ‘Changshan Huyou’         | *C. changshan-huyou*                | C            | Yellow     |

Table 2

Primary metabolite profiles (μg g⁻¹ FW) were measured in the peels of OCC and its donor plants.
Primary metabolite profiles (μg g⁻¹ FW) in the peel of OCC and its donor plants

| No. | Primary content (μg g⁻¹) | OP      | OCP               | CP      |
|-----|-------------------------|---------|-------------------|---------|
|     |                         | 11.32±2.40c | 97.79±16.79a     | 73.42±0.78b   |
| 1   | Carbamic acid           | 8.23±0.45c    | 205.82±14.44a    | 173.78±9.83b   |
| 2   | Cyclohexanecetic acid   | 0.56±0.09c     | 2.28±0.41b       | 3.35±0.32a     |
| 3   | Malic acid              | 92.87±9.42c    | 211.84±8.73b     | 280.92±12.48a   |
| 4   | Quinic acid             | 273.14±11.78b  | 341.09±25.51a    | 354.57±47.87a   |
| 5   | 2-Ketoglutaric acid     | 64.77±6.22a    | 35.08±2.73b      | 41.24±3.70b     |
| 6   | 4-Aminobutanoic acid    | nd                             | 42.49±3.71a      | 24.26±7.77b     |
| 7   | Shikimic acid           | nd                             | 11.21±1.44a      | 11.16±1.06a     |
| 8   | Palmitic acid           | nd                             | 29.14±2.07a      | 31.97±1.54a     |
|     | Sum                     | 439.57±18.76b  | 830.25±24.62a    | 910.69±71.6a    |
|     | Organic acids           |         |                   |         |
| 9   | Xylose                  | 215.09±3.73a   | 79.35±6.14c      | 112.00±5.65b    |
| 10  | Mannose                 | 24997.79±1538.92a | 11139.04±654.61c | 14008.21±659.76b |
| 11  | Galactose               | 6961.91±512.69a | 2910.33±191.21c  | 3885.98±176.32b |
| 12  | Fucose                  | 5.83±0.60c      | 12.82±1.55a      | 9.50±0.92b      |
| 13  | Fructose                | 20950.50±1276.05a | 9533.23±501.14b  | 10982.41±464.13b |
| 14  | d-Psicose               | 246.44±11.15a   | 47.54±9.82c      | 88.83±6.39b     |
| 15  | Turanose                | 91.76±9.73a     | 25.64±2.07b      | 33.00±2.89b     |
| 16  | Sucrose                 | 16576.74±471.54a | 7352.21±162.93c  | 8777.85±359.66b |
| 17  | Myo-Inositol            | 1102.16±76.96a  | 1035.31±59.25ab  | 965.58±39.84b   |
| 18  | Sorbose                 | 160.32±4.82a    | nd               | nd               |
|     | Sum                     | 71308.53±3772.64a | 32135.47±1437.52b | 38863.34±1677.70b |
|     | Sugars                  |         |                   |         |
|     | Total                   | 71928.66±3809.02a | 33382.56±1449.96c | 40369.88±1645.33b |
|     | Alcohols                |         |                   |         |
| 19  | Glycerol                | 125.80±13.79c   | 247.62±32.8b     | 303.96±32.17a   |
| 20  | Scyllo-Inositol         | 54.75±4.92c     | 169.21±4.08b     | 291.89±14.46a   |
|     | Sum                     | 180.56±18.63c   | 416.84±29.40b    | 595.85±36.82a   |
|     | Total                   | 71928.66±3809.02a | 33382.56±1449.96c | 40369.88±1645.33b |
Table 3

Primary metabolite profiles (μg g⁻¹ FW) were measured in the juice sacs of OCC and its donor plants.

| No. | Primary content (μg g⁻¹) | OJ       | OCJ      | CJ       |
|-----|--------------------------|----------|----------|----------|
|     |                          |          |          |          |
|     | Organic acids            |          |          |          |
| 1   | Oxalic acid              | 134.46±111.75<sup>c</sup> | 352.70±31.67<sup>b</sup> | 569.63±39 |
| 2   | Malic acid               | 206.33±21.44<sup>b</sup> | 172.40±8.68b | 363.95±60 |
| 3   | 4-Aminobutanoic acid     | 10.36±1.00<sup>b</sup> | 16.01±4.16a | nd       |
| 4   | Citric acid              | 1131.33±9.58<sup>ab</sup> | 1213.74±59.98a | 965.81±16 |
| 5   | Quinic acid              | 37.49±4.79<sup>b</sup> | 22.24±1.77<sup>c</sup> | 50.68±3.64<sup>c</sup> |
| 6   | Palmitic acid            | 38.62±2.57<sup>a</sup> | 20.23±0.16b | nd       |
|     | Sum                      | 1446.81±18.13<sup>a</sup> | 1444.61±48.07<sup>a</sup> | 1399.60±2 |
|     | Sugars                   |          |          |          |
| 7   | Xylose                   | 57.18±0.58<sup>a</sup> | 16.76±1.28b | 4.47±0.77<sup>c</sup> |
| 8   | Arabinose                | 5.39±0.10<sup>b</sup> | 15.11±0.81a | 2.36±0.39<sup>c</sup> |
| 9   | Fructose                 | 14259.18±237.19<sup>a</sup> | 12389.95±1357.50b | 11731.42± |
| 10  | Mannose                  | 155.25±17.50a | 139.88±11.24a | 162.82±27 |
| 11  | Sorbose                  | nd       | nd       | 54.88±1.5<sup>c</sup> |
| 12  | Glucose 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme, (1Z)-Rhamnose | 17227.71±687.96a | 17073.88±1494.37a | 15731.50± |
| 13  | Rhamnose                 | 17.51±1.60a | nd       | 10.55±0.8<sup>c</sup> |
| 14  | Myo-Inositol             | 1508.79±45.70a | 1503.50±31.59a | 240.54±9.<sup>3</sup> |
| 15  | Allose                   | 2.69±0.26a | 2.10±0.28b | nd       |
| 16  | Sucrose                  | 21362.29±1880.50a | 21537.81±1705.67a | 22017.88±4 |
| 17  | Turanose                 | 223.49±34.64a | 160.54±14.71b | 17.18±2.0<sup>c</sup> |
|     | Sum                      | 54819.49±2889.60a | 52839.53±3493.60a | 49973.6±2 |
|     | Alcohol                  |          |          |          |
| 18  | Scyllo-Inositol          | 64.63±3.99<sup>c</sup> | 116.81±1.38b | 190.91±9.<sup>c</sup> |
|     | Total                    | 56330.92±2910.91a | 54400.95±3463.03a | 51564.11± |

Table 4
Volatile profiles (μg g⁻¹ FW) were measured in the peels of OCC and its donor plants.

| No. | Volatiles (μg g⁻¹) | OP          | OCP          | CP            |
|-----|--------------------|-------------|--------------|---------------|
| 1   | α-Thujene          | 144.35±1.27b| 207.23±1.41a | 264.98±1.12a  |
| 2   | α-Pinene           | 804.57±7.00b| 842.83±5.74ab| 1089.03±1.12b |
| 3   | Sabinene           | 128.77±1.12a| 140.56±1.01a | 173.87±1.02a  |
| 4   | β-Pinene           | 236.92±2.06b| 429.98±3.04a | 521.91±2.12a  |
| 5   | β-Myrcene          | 1262.26±11.19a| 1204.28±8.61a| 1506.58±1.12b |
| 6   | α-Phellandrene     | 33.08±0.34c | 55.34±0.30b  | 70.20±0.30a   |
| 7   | α-Terpinene        | 73.05±0.61b | 118.21±0.78a | 143.5±0.78a   |
| 8   | d-limonene         | 60800.80±328.50a| 59857.96±265.43a| 66345.79±328.50a |
| 9   | β-cis-Ocimene      | 46.03±0.37b | 49.36±0.37b  | 68.38±0.37b   |
| 10  | γ-Terpinene        | 3213.21±26.66b| 5430.11±38.18a| 6360.18±26.66b|
| 11  | Terpinolene        | 149.98±1.34b| 246.07±1.77a | 286.81±1.77a  |
| 12  | Linalool           | 152.41±1.01a| 55.37±0.24b  | 54.97±0.24b   |
| 13  | α-Terpineol        | 76.01±0.36a | 64.6±0.26a   | 40.38±1.24a   |
| 14  | Citronellal        | 28.25±0.23a | 19.82±0.15b  | 19.28±0.15b   |
| 15  | Methyl 2-methyloctanoate  | 227.59±0.04a| 225.77±0.11a | 228.57±0.11a  |
| 16  | Citronellol acetate | 5.56±0.02b  | 16.83±0.14a  | 15.44±0.14a   |
| 17  | (R)-lavandulyl acetate | 16.55±0.15c | 68.04±0.47b  | 98.38±0.47b   |

**Sum**

|               | 67399.39±382.27a | 69032.36±328.01a | 77288.25±382.27a |

**Sesquiterpene**
|   | Compound          | Percentage | Sum          | Sum          |
|---|-------------------|------------|--------------|--------------|
| 18| Copaene           | 44.00±0.41b| 60.57±0.48ab | 68.01±0.1b   |
| 19| β-Cubebene        | 35.07±0.34b| 45.92±0.34ab | 52.66±0.2b   |
| 20| β-Elemene         | 21.77±0.17a| 18.42±0.16a  | 25.39±0.1b   |
| 21| Caryophyllene     | 22.68±0.17b| 58.03±0.44a  | 68.36±0.0b   |
| 22| (E)-β-Famesene    | 37.4±0.34b | 117.74±1.05a | 128.65±0.0b  |
| 23| Germacrene D      | 123.72±1.22b| 2772.96±22.34a| 2525.56±0.0 |
| 24| γ-Elemene         | 17.19±0.12b| 196.80±1.61a | 174.66±0.0   |
| 25| (−)-β-Elemene     | 440.45±4.29a| 138.93±1.14b | 171.49±0.0   |
| 26| δ-Cadinene        | 57.02±0.56a| 69.14±0.57a  | 79.21±0.4b   |
| 27| δ-Elemene         | 33.08±0.29c| 155.35±1.22b | 195.32±0.0   |
|   | Sesquiterpene alcohols |          |              |              |
| 28| Nootkatone        | 2.79±0.04c | 28.59±0.17b  | 67.88±0.0b   |
|   | Sum               | 857.75±8.16b | 4232.06±34.13a | 4286.69±0.0 |
|   | Alcohol           |            |              |              |
| 29| (E)-3-Hexen-1-ol  | 8.30±0.05c | 34.14±0.10a  | 15.79±0.1b   |
|   | Aldehydes         |            |              |              |
| 30| 3-Hexenal         | 51.52±0.22c| 72.66±0.05a  | 64.51±0.1b   |
| 31| Hexanal           | 25.21±0.11a| 25.38±0.01a  | 18.56±0.0b   |
| 32| (E)-2-Hexenal     | 5.49±0.05ab| 7.48±0.07a   | 4.41±0.0b    |
| 33| Decanal           | 72.59±0.63a| 80.43±0.70a  | 99.15±0.4b   |
|   | Sum               | 154.81±1.01a| 185.95±0.83a | 186.63±0.0   |
|   | Phenol            |            |              |              |
| 34| 2,4-di-t-butylphenol | nd         | 42.13±0.31a  | 37.71±0.0b   |
|   | Others            |            |              |              |
| 35| o-Cymene          | 103.06±0.90b| 91.13±0.57b  | 156.95±0.0   |
| 36| n-Hexadecanoic acid | 58.71±0.73b| 115.47±1.80b | 207.42±1.0   |
|   | Sum               | 161.77±1.63b| 206.6±2.37b  | 364.37±2.0   |
|   | Total             | 68582.02±393.12a| 73691.11±365.44a | 82141.73±0.0 |

**Table 5**
Volatile profiles (μg g⁻¹ FW) were measured in the juice sacs of OCC and its donor plants.

| No. | Volatiles (μg g⁻¹) | OJ     | OCJ    | CJ     |
|-----|--------------------|--------|--------|--------|
|     |                    | 0.675±0.013ₐ | 0.357±0.021₇ | 0.415±0.1₈ |
| 1   | Monoterpene        | 46.22±0.38ab | 33.91±0.24b  | 20.53±0.₉ |
| 2   | Linalool           | 20.90±0.17b  | 16.01±0.32c  | 28.08±0.₁ |
| 3   | γ-Terpinene        | 383.05±5.92a | 402.18±4.53a | 298.70±1  |
| 4   | β-Limonene         | 44.59±0.31a  | 36.43±0.36b  | 29.54±0.₁ |
| 5   | β-Myrcene          | 1.96±0.03c   | 2.48±0.01b   | 3.09±0.₀  |
|     | Monoterpene esters |        |        |        |
| 6   | Methyl 2-methyloctanoate | 11.01±0.10b | 18.12±0.10a | 1.80±0.₀  |
| 7   | Methyl nonanoate   | 1.85±0.01b   | 1.87±0.01b   | 3.65±0.₀  |
|     | Sum                | 801.93±3.33a | 633.21±2.97a | 341.75±5  |
|     | Sesquiterpene      |        |        |        |
| 8   | Germacrene D       | 0.17±0.01b   | 2.81±0.09a   | 0.46±0.₀  |
| 9   | Copaene            | 14.31±0.51b  | 19.92±0.36b  | 49.69±0.₁ |
| 10  | α-ylangene         | nd          | 16.05±0.10a  | nd     |
| 11  | Germacrene B       | 11.45±0.39a  | 6.99±0.36ab  | 0.74±0.₀  |
|     | Sesquiterpene alcohols |        |        |        |
| 12  | Nootkatone         | 1.13±0.04b   | 3.62±0.07a   | 0.45±0.₀  |
|     | Sum                | 27.06±0.95c  | 43.14±0.62b  | 57.59±0.₁ |
|     | Aldehydes          |        |        |        |
| 13  | Decanal            | 1.47±0.01c   | 2.47±0.14b   | 3.59±0.₀  |
| 14  | Dodecanal          | 2.25±0.01ab  | 2.83±0.12a   | 1.72±0.₁  |
| 15  | Pentadecanal       | 2.28±0.03b   | 3.63±0.14a   | 2.53±0.₁  |
| 16  | Octanal            | 1.06±0.01a   | 1.13±0.03a   | 0.69±0.₀  |
|     | Sum                | 7.06±0.06b   | 10.06±0.43a  | 8.53±0.₃  |
|     | Phenol             |        |        |        |
| 17  | 2,4-di-t-Butylphenol | 3.53±0.05b | 6.24±0.02a  | 5.42±0.₂  |
| 18  | n-Tridecan-1-ol    | 23.51±1.39a  | 16.12±0.33b  | 12.68±0.₁ |
|     | Sum                | 27.04±1.44a  | 22.36±0.35a  | 18.1±0.₄  |
|     | Others             |        |        |        |
19 n-Hexadecanoic acid 1.39±0.03a 0.83±0.08b 1.52±0.04a
Total 864.48±5.81a 709.60±4.45a 427.49±7

Table 6

Carotenoid content (μg g⁻¹ DW) were measured in the peel and the juice sac of OCC and its donor plants.

| No. | Carotenoid content (μg g⁻¹ DW) | OP         | OCP        | CP          | OJ          | OCJ         |
|-----|-------------------------------|------------|------------|-------------|-------------|-------------|
| 1   | Violaxanthin                  | 941.53±42.97a | 772.11±54.36b | 911.83±37.03a | 5.02±0.75a  | 5.20±1.27a  |
| 2   | Luteoxanthin                  | 67.33±8.93a  | 33.24±3.57b | 29.94±5.62b | 18.04±3.64a | 21.79±3.34a |
| 3   | Lutein                        | 110.04±8.67a | 44.54±5.56b | 24.98±1.27c | 5.67±1.94a  | 4.97±1.55b  |
| 4   | Zeaxanthin                    | 61.40±5.73a  | 3.36±0.84b  | 4.63±1.21b  | 34.39±1.18a | 19.97±2.32ab|
| 5   | β-cryptoxanthin               | 356.81±8.04a | 9.07±1.66b  | 14.65±0.96b | 290.73±4.00a | 132.74±6.43b|
| 6   | α-carotene                    | 12.13±1.52a  | nd         | nd          | 5.27±0.10a  | 1.46±0.03b  |
| 7   | β-carotene                    | 15.37±3.01a  | 5.19±1.23b  | 1.26±0.18b  | 14.31±1.81a | 4.25±0.34b  |
| 8   | Phytoene                      | 383.58±9.23a | 44.71±3.41b | 71.34±6.41b | 123.30±6.02a| 42.01±1.43b |
| 9   | Phytofluene                   | 243.24±13.28a| 26.80±3.15b | 49.58±3.76b | 106.59±0.80a| 18.43±1.27b |
|     | Total                         | 2191.43±101.38a | 939.02±73.78b | 1108.21±56.44b | 603.32±20.24a | 250.82±19.98t |

Table 7

Correlation coefficient of carotenoid content was analyzed between OCC and two donors.

The double star “**” indicates the coefficient index is significant at p< 0.01.
| Tissues   | Metabolites | Cultivars | O     | OCC   | C     |
|-----------|-------------|-----------|-------|-------|-------|
| Peel      | Carotenoids | O         | 1     | OCC   | C     |
|           |             | OCC       | 0.892**| 1     |       |
|           |             | C         | 0.905**| 0.999**| 1     |
| Juice sac | Carotenoids | O         | 1     | OCC   | C     |
|           |             | OCC       | 0.957**| 1     |       |
|           |             | C         | 0.146  | 0.210 | 1     |

**Figures**

![Image A](image1.jpg)

![Image B](image2.jpg)
Fruit morphology of ‘Hongrou Hyou’ (OCC) and its donor plants were harvested in full ripening stage. External appearance (A) and transverse section appearance (B) of ‘Owari’ satsuma mandarin (O), OCC (the chimera) and ‘Changshan Hyou’ (C) were exhibited from left to right. Bars of external and transverse sections are both 5.0 cm.

Figure 2

Contents of metabolites was analyzed by PCA according to the fruit tissues between OCC and its donor plants. Primary metabolites (A-1), volatiles (A-2) and carotenoids (A-3) were analyzed in peels of three cultivars. Primary metabolites (B-1), volatiles (B-2) and carotenoids (B-3) were analyzed in juice sacs of three cultivars. The bold dots colored in red, purple, and green represent O, OCC and C, respectively.
Supplementary Files

This is a list of supplementary files associated with the primary manuscript. Click to download.

Supplementary tables.docx