High-spatiotemporal-resolution transcriptomes provide insights into fruit development and ripening in Citrus sinensis

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Summary

Citrus fruit has a unique structure with soft leathery peel and pulp containing vascular bundles and several segments with many juice sacs. The function and morphology of each fruit tissue are different. Therefore, analysis at the organ-wide or mixed-tissue level inevitably obscures many tissue-specific phenomena. High-throughput RNA sequencing was used to profile Citrus sinensis fruit development based on four fruit tissue types and six development stages from young fruits to ripe fruits. Using a coexpression network analysis, modules of coexpressed genes and hub genes of tissue-specific networks were identified. Of particular, importance is the discovery of the regulatory network of phytohormones during citrus fruit development and ripening. A model was proposed to illustrate how ABA2P mediates the ABA signalling involved in sucrose transport, chlorophyll degradation, auxin homoeostasis, carotenoid and ABA biosynthesis, and cell wall metabolism during citrus fruit development. Moreover, we depicted the detailed spatiotemporal expression patterns of the genes involved in sucrose and citric acid metabolism in citrus fruit and identified several key genes that may play crucial roles in sucrose and citric acid accumulation in the juice sac, such as SWEET15 and CsPH8. The high spatial and temporal resolution of our data provides important insights into the molecular networks underlying citrus fruit development and ripening.

Introduction

Fruits are generally morphologically classified into silique (e.g. Arabidopsis), berry (e.g. tomato), pome (e.g. pear) and hesperidium (e.g. citrus) types among others. The mechanisms underlying fruit development and ripening are unique among different fruit types (Ding et al., 2015). Citrus fruits are classified as hesperidium and have non-climacteric fruit maturation characteristics (Paul et al., 2012). The development and ripening of citrus fruits can be divided into three stages: cell division stage; expansion stage, which involves cell enlargement, water accumulation, and sugar accumulation; and ripening stage (Bain, 1958). At the ripening stage, pigments, sugars and other soluble solids are accumulated, chlorophyll and organic acid are reduced, and the cell wall is extensively modified (Katz et al., 2011; Yu et al., 2012).

Understanding the hormonal regulation of citrus fruit development and ripening is of considerable academic and agronomic value. Previous studies showed that auxin plays a pivotal role in the regulation of fruit set and growth (Goetz et al., 2007; de Jong et al., 2009; Kang et al., 2013; Pattison et al., 2015). The strict spatiotemporal control of auxin distribution and signalling during fruit development was discovered in Arabidopsis (Sundberg and Ostergaard, 2009), tomato (Pattison et al., 2014) and strawberry (Kang et al., 2013). However, a comprehensive investigation of tissue-specific, auxin-related gene expression to illustrate the action of auxin in citrus fruit is currently lacking. The ripening mechanisms of climacteric fruits, especially the mechanism related to ethylene (ETH), have been well studied (Alexander and Grierson, 2002; Klee and Giovannoni, 2011). However, the mechanisms underlying the ripening of non-climacteric fruits remain unclear. Abscisic acid (ABA) was considered to be a ripening control factor for non-climacteric fruits. The levels of ABA sharply increased during the onset of fruit ripening and ripening processes in citrus (Wu et al., 2014b), strawberry (Jia et al., 2011) and cucumber (Wang et al., 2012). The peak ABA level of a late-ripening sweet orange mutant was obtained later than that of the wild-type fruit (Wu et al., 2014b). Similarly, the degreening stage was delayed in a fruit-specific ABA-deficient mutant of sweet orange (Rodrigo et al., 2003; Romero et al., 2012). Moreover, ABA promoted fruit ripening and played an important role in the regulation of fruit development and ripening in strawberry (Ji et al., 2012; Jia et al., 2011; Jia et al., 2013) and cucumber (Wang et al., 2013). Although considerable progress has been made in describing the role of ABA in the regulation of fleshy fruit ripening, the molecular mechanisms remain to be elucidated. To uncover the mechanisms of ABA action at the molecular level, it is necessary to identify all of the components involved in ABA homoeostasis, spatiotemporal distribution and regulatory network.

Sucrose directly accumulates in citrus fruit and is stored in the vacuoles of juice sacs (JSs) cells and mainly translocated from leaves (Sadka et al., 2019). The sugar post-phloem transport is the rate-limiting step (Koch and Avigne, 1990). In citrus fruit, apoplastic and symplastic pathways both occur in post-phloem transport (Koch et al., 1986; Nii and CooMBE, 1988). In the apoplastic pathway, sugar transport is dependent on transport
proteins, including SUTs, SWEETs and TMTs. SWEET proteins function in the efflux of sucrose to the apoplast (Chen et al., 2012). The plasma membrane SUT sucrose/proton cotransporters and TMT sucrose/proton antiporters function in transporting sugar into the cytoplasm and vacuole, respectively (Schneider et al., 2012; Schulz et al., 2011). In citrus, sucrose is delivered to the fruit by the funicular phloem, which is connected to the segment epidermis. Following phloem unloading, sucrose moves into the segment epidermis and parenchymatous, which are hair-like stalks of individual JSs (Koch, 1983; Koch and Avigne, 1990). However, the molecular mechanism underlying sucrose transport into JS cells remains unclear.

Acidity is important for fruit taste. In citrus, acidity is generally dependent on citrate accumulation in the vacuole of JS cells, where citrate contributes more than 90% of the total organic acids. Citrate accumulation in the vacuole depends on the balance of citrate synthesis, transport and degradation or utilization (Cercos et al., 2006a; Sadka et al., 2000). Previous studies (Aprile et al., 2011; Muller and Taiz, 2002; Shi et al., 2015) indicated that vacuolar-type and p-type ATPases play important roles in citrate uptake into the vacuole, the citrate/ H + symporter CsCit1 (Shimada et al., 2006) mediates citrate efflux from the vacuole into the cytoplasm, and citrate is utilized through the Aco-GABA and/or ACL-degradation pathways (Cercos et al., 2006b; Guo et al., 2016; Hu et al., 2015).

Generally, seedless citrus fruit can be divided into four tissues: epicarp (EP; referred to as the flavado), albedo (AL; the spongy white part of the peel), segment membrane (SM; referred to as the segment epidermis, which covers the carpel and JS) and JS (stores a large number of nutritional components beneficial to health) (Sadka et al., 2019). In this study, we chose a navel orange variety (seedless) ‘Fengjie 72-1’ (Citrus sinensis) as the research material, and the EP, AL, SM and JS tissues of this variety can be divided into four tissues at six developmental stages. Overall, this study aimed to uncover new molecular insights into citrus fruit development and ripening and to reveal the specific non-climacteric characteristics of citrus fruit.

Results

Global analysis of the transcriptomes of dissected fruit tissues

To uncover the underlying molecular network that regulates citrus fruit development and ripening, we used RNA-seq to quantify the transcriptomes of 24 fruit tissue samples, including four tissue types at six developmental stages (50–220 DAF; Figure 1a; Table S1). The fruits were sampled after the second physiological fruit-falling period (50 DAF), during the expansion period (80, 120 and 155 DAF), at the colouring period (180 DAF) and at the full-ripening period (220 DAF). The correlation dendrogram illustrates the global relative relationships among the 24 tissues (Figure 1b). All three biological replicates of each sample clustered together, with the exception of AL_2_1 and AL_3_1. The EP samples were clustered together (Figure 1b). For the AL, SM and JS tissues, the following samples were clustered together: stages 1 and 2; stages 3 and 4; stages 5 and 6 (Figure 1b). At stages 1 and 2, the differences among the tissues were less than the differences between the stages, while at stage 3 to stage 6, the differences among the tissues were larger than those between the two stages (Figure 1b).

Normalized read counts (fragments per kilobase of transcript sequence per million base pairs sequenced, FPKM) for each gene were calculated. Between 16 621 (JS6) and 19 455 (EP1) genes with FPKM values more than 0.3 remained in each sample, and more than 85% of which were in the 1 to 100 FPKM range (Figure 1c, Table S2). The genes from all six stages of the same tissue were combined, and a Venn diagram was used to reveal unique or commonly expressed genes among the fruit tissues (Figure 1d). A total of 18 156 genes were common among all four fruit tissues. Notably, of the four tissues, the EP had the largest number of tissue-specific genes.

To identify different gene expression profiles across citrus fruit development in each tissue, STEM (Short Time-series Expression Miner; Ernst and Bar-Joseph, 2006) was used to perform clustering. The results showed that 11, 13, 11 and 7 statistically significant model profiles (coloured profiles) were identified in EP, AL, SM and JS, respectively (Figure 2a; Table S3). In these four tissues, two special model profiles (profile 8, down-regulated profile; profile 39, up-regulated profile) were identified (Figure 2a). Venn diagrams revealed that the number of unique expressed genes was more than that of commonly expressed genes among the four fruit tissues in both profile 39 and profile 8 (Figure 2b), suggesting that different regulation networks occur in each fruit tissue during fruit development. A similar result was also revealed in the Gene Ontology (GO) term enrichment analysis (Figure S1; Table S4). In addition, the eight gene sets of profile 8 and profile 39 were subjected to a pathway enrichment analysis (Table S5). As shown in Figure 2c, more genes associated in the citrate cycle, proteasome, pyruvate metabolism and carotenoid biosynthesis pathways were up-regulated than down-regulated. Notably, in profile 39_JS, the prevalence of enriched genes in the citrate cycle pathway reached 51% (23/45) of all citrate cycle pathway genes, indicating that the TCA cycle was continuously enhanced in JS with citrus fruit development.

Coexpression network analysis reveals distinct regulatory programmes among C. sinensis fruit tissue development

To reveal the functions of networks instead of individual genes, the system biology approach weighted gene coexpression network analysis (WGCNA) (Langfelder and Horvath, 2008) was used to construct coexpression networks. This analysis resulted in 15 distinct modules and one module in grey colour reserved for genes outside of all modules (Figure S2; Table S6). Three tissue-specific modules (blue, 2462 genes; purple, 313 genes; and black, 442 genes) were further analysed (Figure 3a, d and g). The three gene sets of the blue, purple and black modules were subjected to a GO term enrichment analysis (Figure 3b, e and h; Table S7). The genes coexpressed in the blue module (the EP-specific module) were significantly enriched in the ‘lipid metabolism’, ‘response to cadmium ion’ and ‘flavonoid metabolism’ biological processes (Figure 3b). In the purple module (the AL- and SM-specific module), genes were significantly enriched in the ‘xylem or xylem histogenesis’, ‘transport’ and ‘response to toxic substance’ biological processes (Figure 3e). The genes of the black module (the SM- and JS-specific module) were mainly involved in the ‘translation’, ‘ribosome biogenesis’ and ‘protein transport’ biological processes (Figure 3h). To further identify the key
regulatory genes in these modules, WGCNA and Cytoscape (Shannon et al., 2003) were employed to construct and visualize gene networks, in which each node represented a gene and the connecting lines (edges) between genes represented coexpression correlations. The hub genes, which have the most connections in the network, may be the key regulatory genes. In the blue, purple and black module networks, 20 of 512 genes, 18 of 152 genes, and 12 of 134 genes encoded transcription factors (TFs), respectively (Figure 3c, f and i; Table S8). For instance, CsAPL (orange1.1t03247) is the hub gene with the highest number of edges (98 edges) in the purple module network (Figure 3f). In other module networks, the highly connected hub TFs included CsKAN2 (Cs3g09270), CsERF13 (Cs1g03290), CsMYB5 (Cs9g03070), and CsTT8 (Cs5g31400). (Figure 3c, f and i).

In previous studies (Karlova et al., 2014; Leng et al., 2014; Zhang et al., 2015), ABA, jasmonic acid (JA) and indole-3-acetic acid (IAA) were shown to play important roles in fruit development and ripening. We measured the ABA, IAA and JA contents in each fruit tissue across the six developmental stages (Figure 5a, c and e). The associations among modules and plant hormones were determined via the WGCNA. As shown in Figure 4a, ABA was highly correlated with the green module ($r^2 = 0.74$, $P = 3e-05$) and JA was highly correlated with the yellow module ($r^2 = 0.88$, $P = 1e-08$). None of the modules were highly correlated with IAA. The genes coexpressed in the green module were significantly enriched in ‘cellular macromolecule catabolism’, ‘response to osmotic stress’ and ‘intracellular transport’ (Figure 4b, c; Table S7). In the yellow module, the genes were significantly enriched in ‘response to wounding’, ‘organic
Figure 2  Gene expression profile analysis and functional enrichment analysis in each fruit tissue. (a) Module profiles of the genes in each fruit tissue across six developmental stages. (b) Venn diagrams showing the number of shared and uniquely expressed genes among the four fruit tissues in profiles 8 and 39. (c) KEGG pathway enrichment analysis results for the genes clustered in profiles 8 and 39 among the four fruit tissues. The x-axis represents the pathways, and the y-axis represents the percentage of the number of enriched genes relative to the total number of genes in the pathway.
substance metabolism’ and ‘response to stimulus’ (Figure 4d, e; Table S7). Moreover, the gene networks of the green and yellow modules were also constructed and the hub genes were highlighted (Figure S3; Table S8). In addition, in the brown module, the genes were continually down-regulated or up-regulated in all four tissues across the six developmental stages and mainly involved in the ‘response to chemical’, ‘cellulose metabolism’, ‘cell death’ and ‘cell wall organization’ biological processes (Figure 4f, g; Table S7). Notably, five Expansin genes were identified in the brown module (Table S6), and they were largely up-regulated during the expansion period (Figure S4). For example, Expansin-A1 (Cs5g10820 and Cs7g03630) contained ABA-, gibberellin (GA)- and JA-responsive elements (Table S9).

**Figure 3** Expression profiles, functions and networks of tissue-specific genes. (a, d, g), Eigengene expression profiles and heat map showing the FPKM of each gene in the blue, purple and black modules. The y-axis indicates the value of the module eigengene or the gene; the x-axis indicates the sample type and developmental stage. The number of genes in each module is indicated at the top. (b, e, h) GO term enrichment analysis results of the blue, purple and black module genes visualized by the ‘TreeMap’ view of REVIGO. Each rectangle is a single cluster representative. The representatives are joined into ‘superclusters’ of loosely related terms, visualized with different colours. The size of the rectangles is adjusted to reflect the P-value. (c, f, i) Correlation networks of the blue, purple and black modules are visualized by Cytoscape. The transcription factors are shown by large circles.

Spatiotemporal distribution of hormone-related gene expression

We identified *C. sinensis* gene families involved in the synthesis, catabolism, transport, conjugation and signalling of phytohormones (ABA, auxin, JA, GA, ETH, cytokinin, brassinosteroid and salicylic acid) by BLAST using *Arabidopsis* protein sequences as queries (Table S10). The log2 FPKM values for these hormone-related genes were subjected to a hierarchical clustering analysis (Figure 5b, d and f; Figure S5). In addition, the levels of ABA, IAA and JA in each fruit tissue across the six developmental stages were also determined (Figure 5a, c and e; Table S11).
As shown in Figure 5a, the levels of ABA in the four fruit tissues increased with fruit development and the contents of ABA were varied among these four fruit tissues. Notably, the ABA content in the SM was increased by approximately 3-fold at the stage of the expansion period (120–155 DAF) and remained stable during the colouring and ripening periods. The ABA level in the EP was increased by 3.95-fold to 45-fold after 120 DAF and peaked at 220 DAF. The contents of total carotenoids in the four fruit tissues across the six developmental stages were measured (Figure S5f), and no relationship was observed between the content of ABA and that of total carotenoids in the four fruit tissues. In accordance with the ABA levels, the ABA biosynthesis genes NCED12/3/4, AAO3 and AAO3-like and the ABA transport genes ABCG40 and NRT1.2 were increasingly up-regulated in the four fruit tissues with fruit development (Figure 5b). However, ABA catabolism genes showed different expression patterns among the four fruit tissues. For example, CYP70A1 was predominantly expressed in the EP and highly expressed during the early developmental stages in all fruit tissues (Figure 5b). Therefore, the genes involved in biosynthesis, transport, catabolism and conjugation co-regulated the content of ABA in each fruit tissue. Moreover, the expression patterns of several genes in the ABA signalling transduction model ‘PYR/PYL/RCAR-PP2Cs-SnRK2s-SnRK3s’ were in accordance with the ABA levels, including PP2CB/51, SnRK2.12/42.8 and AFB2 (Figure 5b). This finding indicates that these genes may respond to ABA during citrus fruit development. Importantly, AFB2 (Cs3g23480) is the last gene in the ABA signalling transduction model.

The trend curves of IAA content in the four fruit tissues are shown as inverse V shapes (Figure 5c). The levels of IAA in the SM and JS peaked at 120 DAF, while those in the EP and AL peaked at 155 DAF (Figure 5c). Auxin biosynthesis genes and efflux and influx transporters exhibited marked tissue or stage specificity (Figure 5d). The auxin biosynthesis genes YUCCA5 and YUCCA10 were specifically highly expressed in the EP, while YUC3 was specifically highly expressed in the JS. These genes were up-regulated with the progression of fruit development. The auxin efflux transporters were mainly expressed in the EP, AL and SM or at 50 DAF in the four fruit tissues, with PLS1, PLS6, PILS7 and PIN3 highly expressed in the EP and PIN1, PIN3, PIN5 and PIN6 highly expressed in the AL and SM. An exception to this pattern was PILS2, which was highly expressed in the SM and JS at the ripening stages (Figure 5d). Auxin influx transporters such as LAX1, LAX2 and LAX3 were mainly expressed in the AL, SM and JS (Figure 5d). Taken together, these data suggest that the EP and JS may be the synthesis sites of auxin, which is then transported to other fruit tissues.

In the auxin signalling transduction pathway, the auxin receptor genes AFB3 and TIR1 were expressed in all fruit tissues but showed up-regulation at 155–220 DAF in the EP and AL (Figure 5d). The AUX/IAA, ARF and SAUR genes exhibited clear tissue or stage specificity. For instance, AUX2 and IAA4 were mainly expressed in the EP, and SAUR42 was specifically expressed in the JS at the first three developmental stages (Figure 5d). GH3 proteins catalyse the conjugation of IAA to amino acids, thereby reducing free IAA (Staswick et al., 2005) and forming a negative feedback loop to control auxin homeostasis. In this study, six GH3 genes were identified, namely DFL1, DFL2, two GH3.1 genes, GH3.6 and GH3.9. Among these six GH3 genes, only GH3.1 (Cs1g22140) was dramatically up-regulated...
from 5.3-fold to 177-fold) after 155 DAF in all four fruit tissues, at which stage the IAA content declined by 1.46-fold to 3.75-fold in all four fruit tissues (Figure 5c, d). Hence, we hypothesized that GH3.1 might play an important role in IAA homoeostasis.

As shown in Figure 5e, our data indicate that JA was mainly contained in the EP and AL tissues except at 50 DAF and that the JA contents in the SM and JS were much lower than those in the EP and AL from 80–155 DAF. In the EP and AL, the JA levels were moderately increased at 80 or 120 DAF, peaked at 155 DAF, tapered off at 180 DAF, and reached almost zero at 220 DAF. This result was in accordance with the expression patterns of the JA synthesis and signalling genes. As shown in Figure 5f, JA synthesis and signalling genes were highly expressed in the EP at all stages and highly expressed in all fruit tissues at 50 DAF. Together, these results suggest that JA mainly plays roles in the EP and AL during citrus fruit development and ripening.

The expression profiles of the genes related to the other five phytohormones also showed significant tissue-specific or stage-specific characteristics (Figure S5; Table S10). Most of the ETH-related genes, such as ETH biosynthesis genes (ACS1, ACS6, ACS9, ACO1 and ACO4) and signalling genes (ERF1 and ERF2), were highly expressed in fruit at the early developmental stage (50–80 DAF); moreover, several genes were highly expressed in the EP and AL, including ACO2, ACO4, EIN3 and EBF1/2 (Figure S5a). Most of the GA-related genes gradually decreased after 120 or 155 DAF, while the expression of the GID1s and PIF3 genes dramatically increased from 155 or 180 DAF (Figure S5b). The expression of salicylic acid-related genes gradually increased in all fruit tissues with development (Figure S5c). There were not clear patterns in the cytokinin and brassinosteroid genes, which indicated the complicated regulatory networks of these two phytohormones during citrus fruit development. (Figure S5d, e).

Excavation of key genes involved in sugar and acid accumulation of C. sinensis fruit

The taste of citrus fruit is determined by the content of soluble sugars and organic acids. In ripe citrus fruit, sucrose, fructose and glucose are the main soluble sugars, and citric acid and malic acid are the main organic acids. In this study, the soluble sugars in the fruit pulp rapidly accumulated from 80 DAF and moderately accumulated from 155 DAF (Figure 6a). For organic acids, the content of citric acid in the fruit pulp increased dramatically at the
early developmental stages, peaked at 80 DAF and tapered off from 80–220 DAF, whereas the content of malic acid peaked at 80 DAF, declined at 120 DAF, and moderately increased from 155 DAF. The content of quinic acid was constantly decreased and reached almost zero at 220 DAF (Figure 7a).

Citrus fruit primarily directly accumulates sucrose. Hence, the regulation of source-to-sink sucrose transport is vital for sugar accumulation in citrus fruit JSs. In this study, we identified 13 SWEET, 3 SUT and 2 TMT sucrose transporters (Table S10). As shown in Figure 6b, these sucrose transporters were differentially spatiotemporally expressed in C. sinensis fruit during the six developmental stages. For example, SUT4, TMT1, TMT2 and SWEET16 were expressed in all four fruit tissues and their expression levels increased with fruit development. Interestingly, we noted that SWEET15 was specifically highly expressed in the SM and JS, and SWEET10 was specifically highly expressed in the SM. Moreover, the expression levels of SWEET10 and SWEET15 were increasingly up-regulated with fruit development. A phylogeny analysis showed that SWEET10, SWEET12 and SWEET15 were clustered together (Figure S6b), which indicated that these genes have similar functions. Moreover, the expression level of SWEET15 was positively correlated ($R^2 = 0.8763$) with the content of fruit sugar in ‘Anliu’ orange and its high sugar bud sport ‘Honganliu’ orange (Figure 6c; Figure S6d). This relationship and the tissue-specific expression pattern were further verified in different citrus varieties, including sweet oranges ($R^2 = 0.7758$), loose-skin mandarins ($R^2 = 0.8254$) and pummels/grapefruits ($R^2 = 0.7293$; Figure S6c, d and e). Furthermore, a fluorescence in situ hybridization assay was used to characterize the expression pattern of SWEET15 in the JS. As shown in Figure 6d, SWEET15 was expressed in the epidermal cells of the JS. In addition, the genes involved in sucrose biosynthesis and catabolism were also identified (Table S10), and the expression patterns of most of these genes in the four fruit tissues were similar except CWINV5 and SBPase (Figure S6a). Most of the sucrose biosynthesis genes, such as SPS1/2, SPP1 and CCR4A, were up-regulated during the ripening stages in the four fruit tissues, while most of the sucrose synthase genes, such as SUS1/3, hydrolysed sucrose either to fructose and UDP-glucose, were increasingly down-regulated with fruit development.

Figure 6  Expression of genes related to sugar accumulation in citrus fruit across tissues and stages. (a) Soluble sugar content in the pulp at each stage. (b) Expression of sucrose transport-related genes across tissues and stages. (c) Expression of SWEET15 and content of total soluble solids (TSSs) in ‘Anliu’ orange and its bud sport ‘Honganliu’ orange. (d) In situ hybridization analysis of SWEET15 in the juice sac at fruit ripening stage. The juice sac (i) was sampled at 150 DAF. SWEET15 expression is detected with green fluorescent probes in the epidermal layers of the juice sac in the transverse section (ii) and vertical section (vi). Whole images of the transverse section and vertical section of the juice sac are indicated by a red frame at the left bottom. (ii, vi) Paraffin sections. Blue colouration represents nuclei stained by DAPI (iv, viii). The SWEET15 expression and nuclear images are merged (v, ix). (i) Scale bar = 1 mm. (ii–ix) Scale bar = 100 µm. Double asterisks represent statistically significant differences ($P < 0.01$) analysed using Student’s t-test. DAF: days after flowering.
However, several invertase genes (which hydrolysed sucrose to glucose and fructose) displayed different expression patterns. Vacuolar invertase (bFrut1) and cell wall invertase (INV4) were increasingly up-regulated with fruit development in the four fruit tissues, while cell wall invertase INV3 was the opposite (Figure S6a).

Citric acid is the main organic acid in citrus fruit. Hence, we identified the genes in the four fruit tissues that were involved in citric acid biosynthesis, catabolism and transport (Table S10). Previous studies showed that the influx and efflux of organic acid from vacuoles and mitochondria were important for organic acid accumulation in citrus fruit (Aprile et al., 2011; Hussain et al., 2017; Shi et al., 2015; Shimada et al., 2006). Therefore, we specifically excavated the genes related to citrate and malate transport (Figure 7b; Table S10). As shown in Figure 7b, most of the H⁺ ATPase genes were increasingly up-regulated with fruit development in all fruit tissues. In particular, CsPH8 (AHA10) was specifically expressed in the SM and JS and highly expressed in the JS.

Then, we analysed the expression of CsPH8 in four different citrus varieties (‘Succari’, ‘Bingtang’, ‘Xinhui’ and ‘Newhall’); the citric acid contents of these varieties were highly variable. We observed that the expression of CsPH8 was highly correlated with the content of citric acid (Figure 7c).

Further, three TFs, MYB5 (Cs9g03070), TT8 (Cs5g31400) and CPC (Cs2g21750), were
highly coexpressed with CsPH8 (Figure S7b, c). In addition, CcCit1, which mediates citrate efflux from vacuoles, and ALMT4 and ALMT8, which mediate malate influx into vacuoles, were up-regulated in all fruit tissues. These findings were consistent with the content of citric acid and malic acid. Moreover, the genes involved in citric acid biosynthesis and catabolism also regulated the accumulation of citric acid. Most of the genes involved in citric acid biosynthesis and catabolism showed the same expression patterns in the four fruit tissues, and their expression was continuously up-regulated with fruit development (Figure S7a).

To further identify the key genes involved in the regulation of expression patterns in the four fruit tissues, and their expression was enriched in different biological processes (Figure 3b, e and h). Notably, the expression of the genes involved in ABA, IAA and JA biosynthesis showed significant tissue specificity, which resulted in differences in the dynamic changes in ABA, IAA and JA content among the EP, AL, SM and JS (Figure 5). In addition, several key sucrose and citric acid accumulation genes, such as SWEET15 and CsPH8, also showed tissue-specific expression patterns (Figures 6b, 7b). Previous studies in strawberry (Hollender et al., 2014; Kang et al., 2013), maize (Zhan et al., 2015), tomato (Pattison et al., 2015) and Populus tremula (Sundell et al., 2017) indicated that tissue-specific transcriptome analyses could reveal a more refined regulation network or novel regulatory mechanisms. Hence, these studies emphasize the importance of tissue-based omics approaches and highlight the potential pitfalls that are inherent in whole fruit or mixed tissues omics research.

**Difference among the transcriptomes of the four fruit tissues**

In this study, many tissue-specific genes were identified in each fruit tissue, which may contribute to the difference among the four fruit tissues. For instance, several coexpressed gene sets were identified by the WGCNA, such as the blue, purple and black modules (Figure 3a, d and g). These tissue-specific gene sets were enriched in different biological processes (Figure 3b, e and h). Notably, the expression of the genes involved in ABA, IAA and JA biosynthesis showed significant tissue specificity, which resulted in differences in the dynamic changes in ABA, IAA and JA content among the EP, AL, SM and JS (Figure 5).

**Exogenous ABA analogue promoted citrus fruit ripening**

According to the results of the transcriptome analysis, ABA plays significant roles in different biological processes during different stages of citrus fruit development. Therefore, we tested the effect of the application of the exogenous ABA analogue AM1 on citrus fruit development in Satsuma mandarin. AM1 significantly promoted citrus fruit colouring, chlorophyll degradation and sugar accumulation (Figure 8a-d), and decreased the content of IAA in both the fruit peel and pulp (Figure 8e); the AM1-mediated promotion of sucrose accumulation was especially notable (Figure 8f). Further, we used qRT-PCR to quantify many genes in different pathways, including the IAA conjugation (Figure 8f), cell wall metabolism (Figure 8g), carotenoid biosynthesis, ABA signalling (Figure 8h), chlorophyll degradation (Figure 8i) and sugar transport pathways (Figure 8j). Notably, the expression of several genes, such as ABF2, RCCR, SWEET15 and GH3.1, was largely up-regulated by AM1 (Figure 8). These results revealed that ABA is crucial for citrus fruit development and ripening.

**ABF2 plays a master role in ABA signalling during C. sinensis fruit ripening**

According to the results above, ABF2 is the key gene that mediates ABA signalling. We identified several genes that were coexpressed with ABF2 and contained at least one ABA-responding cis-element (CACGTG or ACGTG) in the promoter (Figure 9a). These genes included carotenoid and ABA biosynthesis genes (PSY, Z-ISO, NCED1/2/4), carotenoid catabolism genes (HCAR, PAO, RCCR, PP and NYYC1) (CCES), sugar transporter genes (SUT4, SWEET10/15/16), an IAA conjugation gene (GH3.1) and fruit enlargement- and softening-related genes (Expansin-A1/A8). The interaction between ABF2 and several of these genes was further verified by yeast one-hybrid (Y1H) assays or dual-luciferase assays (Figure 9b, c). According to the transcriptome data and verification assays, a model was proposed to illustrate the ABA signalling regulation network mediated by ABF2 in citrus fruit (Figure 10a). In this model, ABF2 plays a master role and is involved in the regulation of sucrose transport, chlorophyll degradation, auxin homoeostasis, carotenoid and ABA biosynthesis, and fruit enlargement and softening.

**Discussion**

Citrus fruits are classified as hesperidium fruits with a unique structure. Using manual dissection, four different types of C. sinensis fruit tissues were isolated across the whole fruit development and ripening period, and RNA-seq was performed. The comprehensive two-dimensional tissue and stage collection and in-depth RNA-seq data set enable genome-scale analyses at a high resolution.
Moreover, the AM1 treatment could decrease the contents of IAA in both the citrus fruit peel and pulp (Figure 8e). Indeed, we also observed that the auxin conjugation gene GH3.1 was highly expressed, which was promoted by ABF2 (Figure 9c), and accompanied by a decrease in IAA levels (Figure 5c, d). Hence, we propose that ABA is involved in regulating auxin homoeostasis through ABF2 and GH3.1. In addition, we found that ABA could promote the expression of the key ABA biosynthesis genes NCEDs and key carotenoid metabolism gene PSY (Figure 8h), the promoters of which could interact with ABF2 (Figure 9). These results indicate that an auto-induction of ABA production (inducing its own production) pathway like ETH production (Paul et al., 2012) is involved in citrus fruit ripening and represents a feedback regulator of carotenoid metabolism. Based on our data, we propose that ABF2 is the magnifier of ABA signalling and plays diverse roles in different metabolism processes during citrus fruit development and ripening (Figure 10a).
these SUSYs and invertases may play roles in controlling sink strength.

Phloem unloading is the key translocation point for sugar accumulation in sink tissue, which is regulated by symplasmic and apoplasmic pathways (Sadka et al., 2019). In the apoplasmic pathway, the transmembrane transport of sucrose is catalysed by various sugar transporters (Aoki et al., 2003). TMTs are proton-coupled antiporters capable of loading a large amount of glucose, fructose and sucrose into the vacuole, and they are localized on the tonoplast (Aluri and Buttner, 2007; Schulz et al., 2011). SUTs (synonym SUCs) are another type of sucrose transporter that is classified into four distinct groups (Payyavula et al., 2011). SUT4 (Group IV) is well separated from the three other groups. The SUT4 proteins from monocots and dicots were reported to be localized on either the tonoplast or plasma membranes and acted as sucrose\textsuperscript{-}\textit{H}\textsuperscript{+} symporters to uptake extracellular sucrose and release sucrose from the vacuole (Schneider et al., 2012; Schulz et al., 2011). Islam et al. (2015) reported that SUTs might play important roles in the transport of sucrose into the citrus fruit JS. Together, TMTs and SUTs modulate soluble sugar accumulation in the vacuole (Reuscher et al., 2014). In this study, we identified two TMTs and three SUTs (Figure 6b). The expression patterns of TMT1/2 and SUT4 were correlated with the level of sucrose, and they were expressed in all four fruit tissues (Figure 6b). However, sucrose only accumulates in the JS tissue in citrus. Hence, the TMT and SUT sucrose transporters are not the key genes. Then, we identified 13 SWEET sucrose transporters (Figure 6b). Notably, SWEET10 and SWEET15 were specifically expressed in the JS and/or SM (Figure 6b). The SWEET sugar transporters are divided into four clades (Chen et al., 2012; Xu et al., 2014). Clade III SWEETs appear to transport sucrose in an \textit{H}\textsuperscript{+}-independent manner and are typically involved in cellular efflux processes (Chen et al., 2015). In our study, SWEET10, SWEET12 and SWEET15 were clade III SWEETs (Figure S6b). Based on the results of the \textit{in situ} hybridization and tissue-specific expression assays, we propose a functional model for sucrose translocation from vascular tissue to JS cells (Figure 10b). In this model, SWEET15 plays a crucial role in the sucrose accumulation of citrus
between symplasmic and apoplastic pathways in citrus fruit should be further researched.

**Identification of key genes involved in citrate accumulation in citrus fruit**

The comprehensive regulation of citrate synthesis, transmembrane transport and degradation/utilization determines citrate accumulation (Cercos et al., 2006b; Sadka et al., 2000). In this study, we found that the expression levels of almost all genes involved in citrate metabolism were continuously up-regulated from 80 DAF (Figure 7d; Figure S7a). This result suggests that the decrease in citrate content during the late stages of fruit development was due to the up-regulation of citrate degradation or utilization pathways, including the Aco-GABA pathway (Cercos et al., 2006b) and ACL pathway (Hu et al., 2015). Several studies have demonstrated the important roles of ACOs in citrate accumulation (Degu et al., 2011; Li et al., 2017; Shilizerman et al., 2007). Transient overexpression of CitAco3 significantly reduced the citric acid content of citrus leaves and fruits (Li et al., 2017). The studies on different citrus cultivars show that GAD (Liu et al., 2014) and ACL (Hu et al., 2015) are also involved in citrate accumulation. Moreover, a report has suggested that the low citrate of Satsuma mandarin is attributed to citrate degradation (catalysed by CitAco3–CitGS2–CitGDU1) and transport (mediated by CitCHX and CitDIC) (Lin et al., 2015a). GABA pathway is reported to contribute to the significant citrate decrease in citrus fruit, which is associated with the expressions of ACO, GS, GAD and IDH (Lin et al., 2015b; Sheng et al., 2017). In this study, the spatial–temporal expression pattern of the genes involved in citrate metabolism showed significant changes during citrus fruit development, including ACLs, ACOs, NAD-IDHs, GSSs, GADs, CsDIC1 and CsDIT1 (Figure 7d). This result suggests that each gene in this network might influence citrate accumulation.

The acidity of citrus fruit is dependent on citrate accumulation in the JS cell vacuole. When citrate is transported to the cytoplasm, the uptake of citrate into the vacuole depends on the activity of the proton pumps (Brune et al., 2002; Muller et al., 1996). Here, we found that the p-type proton pump gene CsPH8 (synonym AHA10) was specifically highly expressed in the JS tissue (Figure 7b). Further, we analysed the expression of citrate metabolism-related genes in four different citrus varieties, the acidity of which ranged from non-acidic to normal acidity. The CsPH8 gene was differentially expressed among the four varieties of fruit, and its expression was positively related to acidity (Figure 7c). Aprire et al. (2011) found that the AHA10 gene was not expressed in no-acid lemon but highly expressed in the wild-type sour lemon. Shi et al. (2015) also found that the expression of CsPH8 in the ‘Honganliu’ orange (low acid) was lower than that in the ‘Anliu’ orange (normal acid), and overexpression of CsPH8 in acidless pomelo JJs, strawberry fruit, and tomato fruit significantly increased the titratable acid or citric acid content (Shi et al., 2019). Moreover, Strazzer et al. (2019) reported that the expression of CitPH1 and CitPH5 (AHA10) was strongly reduced in acidless citrus varieties, and down-regulation of CitPH1 and CitPH5 was associated with mutations that disrupt the expression of MYB, HLH and/or WRKY transcription factors. In this study, three TFs, MYB5 (Cs5g031400) and CPC (Cs2g21750), were highly coexpressed with CsPH8 (Figure S6e, f). Hence, these genes may be involved in regulating the expression of CsPH8 and could play important roles in citrate accumulation. Taken together, we propose that CsPH8 and its regulation genes may play significant roles in citrate transport or
storage in vacuoles of citrus JS cells and could have a dramatic influence on citrate accumulation.

Conclusion

In summary, the high spatial and temporal resolution of transcriptome data provides significant insights into citrus fruit development and ripening, including the identification of several tissue-specific modules and hub TFs, the discovery of the ABA signalling regulatory network during citrus fruit development and ripening, the interaction between ABA and auxin signalling, and the identification of key genes involved in sucrose accumulation and citrate metabolism.

Materials and methods

Plant materials, sample collection and tissue isolation

Fruit samples of the ‘Fengjie 72-1’ navel orange (C. sinensis L. Osbeck) were harvested at 50, 80, 120, 150, 180 and 220 DAF. Three trees were used as one biological replicate. Three biological replicates (three trees per replicate) were harvested at each developmental stage. Twelve representative fruits were sampled from each tree at each developmental stage. After isolating the four fruit tissues (EP, AL, SM and JS) by manual dissection, the samples were rapidly frozen in liquid nitrogen and stored at −80°C. A portion of the samples was used for extracting the total RNA as described previously (Liu et al., 2006), and the other portion of the sample was used for the determination of phytohormones, soluble sugar and organic acid.

RNA-seq and data processing

Twenty-four samples of the four fruit tissues across six developmental stages were harvested, and three biological replicates were harvested for each sample. A total of 72 transcriptome profiles were obtained by RNA-seq using the Illumina HiSeq™ 4000 sequencing platform at Novogene (Beijing, China). The sequencing raw data have been submitted to the NCBI Gene Expression Omnibus (GEO) under the accession number GSE125726. The raw transcriptomic data of 24 pulp samples were used to perform the coexpression network analysis.

Quantification of plant hormones

The samples used for the ABA, JA and IAA quantification were prepared according to the method described by Pan et al. (2010) with a few modifications. In brief, 200 mg of powdered fresh sample was transferred to a 10 mL screw-cap tube. A total of 50 l working solution of internal standards and 2 mL extraction solvent, 2-propanol/H2O/concentrated HCl (2:1:0.002, vol/vol/vol), were added to each tube. The tubes were shaken for 30 min at 4 °C and 200 rpm. Dichloromethane (4 mL) was added to each sample, and the samples were centrifuged for 30 min at 4 °C. Then, the samples were centrifuged for 5 min at 13 000g and 4 °C. A total of 4 mL of the solvent was transferred from the lower phase into a screw-cap vial, and the solvent mixture was concentrated using nitrogen flow. The samples were redissolved in 0.2 mL methanol and filtered with 0.22 μm organic membrane filters. The extracted solution was injected into the reverse-phase column (C18 Gemini 5 μ, 150 × 2.00 mm) for a HPLC–ESI–MS/MS (ABI 4000 QTRAP, Applied Biosystems) analysis. The internal standard working solutions included d5-ABA (Isotopes, cat. no. ID1001), d9-IAA (Aldrich, cat. no. 492817), and H2JA (dihydrojasmonic acid, OIChemim, cat. no. 0145324) as the internal standards for ABA, IAA and JA, respectively. Each sample was characterized based on three replicates.

Measurement of physiological data

Approximately 0.5 g of peel material was used to determine the total chlorophyll content according to the method described by Li (2000). Each sample was assayed with three replicates. The colour index (CI) of the fruit peel was measured using the CiELAB colour system (CR-400, MINOLTA) according to the method described by Zhang et al. (2015). The gas chromatography (GC) method was used to determine the soluble sugar and organic acid concentrations of the fruit pulps as described previously (Yu et al., 2012). The total soluble sugars (TSS) and total acid (TA) of the fruit pulps were measured by a portable refractometer (PAL-1, ATAGO) and an automatic potentiometric titrator (916i-Touch, Metrohm).

Hormonal treatment of citrus trees

A small-molecule ABA mimic, AM1 (Cao et al., 2013), was used to treat the Satsuma mandarin (Citrus unshiu) trees. A total of 12 trees of equal vigour were selected for this experiment. A stock solution of 100 mL AM1 was made with DMSO and diluted with water before application. We set up three treatment concentrations (500, 800 and 1500 μM). The control group was treated with water. Each group consisted of three trees. One litre of solution was instilled into the main stem of the trees, which was expended in approximately three days. The treatments were administered at 135 and 175 DAF after samples collection. And the 135 DAF was set as control stage. The fruit samples were collected at 135, 160, 175 and 195 DAF.

Quantitative analysis of gene expression

The gene expression analysis via qRT-PCR was performed with three biological replicates in the QuantStudio™ 6 Flex real-time PCR system (Applied Biosystems) according to the method described in our previous study (Wu et al., 2014b), and CsActin
was used as the endogenous reference gene (Wu et al., 2014a). The primers are listed in Table S12.

**Fluorescence in situ hybridization (FISH) assay**

Three specific sequences of SWEET15 (Cs7g02970) were used as templates to synthesize three specific fluorescent oligonucleotide probes (5′-GCTCTCGTCTTGCAATGGCATGTGATGATACTCAG GTAT-3′; 5′-AGCTTCTGAAATGCTGACCCGGTGTTAATTATCTCTAG GTAT-3′ and 5′-GCTTAAAGTCTACTGGTCTCCAGCTGACG CAGCATAAA-3′). For in situ hybridization, JS slices (sampled at 150 DAF from C. sinensis fruit) were made and processed as described by Sunde et al. (2003).

**Yeast one-hybrid assays**

The promoter fragments containing ABRE cis-elements (CACGTG or ACGTG) were amplified by PCR using specific primers (Table S12). The amplified promotor fragments were cloned into pAbAi vector as the baits, and the full-length CDS of C. sinensis or ACGTG) were amplified by PCR using specific primers and templates to synthesize three specific fluorescent oligonucleotide primers (Table S12) were ligated to the pGreenII-0800-LUC vector to construct the prey. Y1H assays were performed using the Matchmaker Gold Y1H Library Screening System (Clontech, Mountain View, CA) according to the manufacturer’s protocol. The bait plasmids were transferred into Y1H yeast and screened for resistance concentrations using S/UD-ura with different concentrations of aureobasidin (A/AbA). The protein–DNA interaction was determined based on growth ability of the cotransformed yeast cells on S/UD-Ura-Leu medium supplemented with the corresponding concentration of AbA.

**Dual-luciferase assays**

The full-length CDS of CsABF2 was ligated to the pGreenII-62-SK-LUC vector, and the promoter fragments amplified by PCR using specific primers (Table S12) were ligated to the pGreenII-0800-LUC vector. Subsequently, the two recombinant plasmids were cotransferred into GV3101 (pSoup-p19). The pGreenII-62-SK-LUC vector without the CsABF2 gene was used as a negative control. Activated Agrobacterium was used to infect tobacco (Nicotiana benthamiana) leaves, and the experimental group and the negative control were injected into the same tobacco leaf, with 4–5 leaves used per gene. Subsequently, the tobacco plants were cultured in a dark environment of 25 °C for 24 h, and then cultured for 3 days in an environment of 25 °C under light for 16 h and 22 °C under darkness for 8 h. LUC assays were measured using the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer’s instructions.

**Acknowledgements**

This research was supported by the National Key Research and Development Project (2019YFD1001400), Natural Science Foundation of China (NSFC) (32072541 31601729) and National Modern Citrus Industry System (CARS-26).

**Conflicts of interest**

The authors declare that they have no conflicts of interest.

**Author contributions**

H.Y. and J.W. conceived the project and designed the research. G.F., Y.X. and L.L. carried out samples collection and field experiments. J.W. and G.F. performed the bioinformatics analysis. G.F. performed most of the experiments. J.W., G.F. and H.Y. wrote the paper. All authors read and approved the manuscript.

**References**

Alexander, L. and Grierson, D. (2002) Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. J. Exp. Bot. 53, 2039–2055.

Alferez, F. and Zacarias, L. (1999) Interaction between ethylene and abscisic acid in the regulation of citrus fruit maturation. In Biology and Biotechnology of the Plant Hormone Ethylene. (Kanellis, A.K., Chang, C., Klee, H., Bleecker, A.B., Pech, J.C. and Grierson, D., eds), pp. 183–184. Dordrecht: Springer, Netherlands.

Aluri, S. and Buttner, M. (2007) Identification and functional expression of the Arabidopsis thaliana vacuolar glucose transporter 1 and its role in seed germination and flowering. Proc. Natl Acad. Sci. USA, 104, 2537–2542.

Aoki, N., Hirose, T., Scolfield, G.N., Whitfield, P.R. and Furhank, R.T. (2003) The sucrose transporter gene family in rice. Plant Cell Physiol. 44, 223–232.

Aprile, A., Federici, C., Close, T.J., De Bellis, L., Cattivelli, L. and Roose, M.L. (2011) Expression of the Hplus-ATPase AHA10 proton pump is associated with citrus acid accumulation in lemon juice sac cells. Funct. Integr. Genomic, 11, 551–563.

Archard, D. and Dennis, F. J. (1984) Quantification of free ABA and free and conjugated IAA in strawberry achene and receptacle tissue during fruit development. J. Am. Soc. Hortic. Sci. 109, 330–335.

Bain, J. (1958) Morphological, anatomical, and physiological changes in the developing fruit of the Valencia orange, Citrus sinensis (L) Osbeck. Australian J. Bot. 6, 1–23.

Beil, F.J., Fanzone, M., Piccoli, P. and Bottini, R. (2011) Solar UV-B and ABA are involved in phenol metabolism of Vitis vinifera L. increasing biosynthesis of berry skin polyphenols. J. Agric. Food Chem. 59, 4874–4884.

Brummell, D.A., Harpster, M.H. and Dunsirur, P. (1999) Differential expression of expansin gene family members during growth and ripening of tomato fruit. Plant Mol. Biol. 39, 161–169.

Brune, A., Muller, M., Taiz, L., Gonzalez, P. and Esteberrina, E. (2002) Vascular acidification in citrus fruit. Comparison between acidline (Citrus aurantifolia) and sweet line (Citrus limon/nut) juice cells. J. Am. Soc. Hortic. Sci. 127, 171–177.

Cao, M.J., Liu, X., Zhang, Y., Xue, X.Q., Zhou, X.E., Melcher, K., Gao, P. et al. (2013) An ABA-mimicking ligand that reduces water loss and promotes drought resistance in plants. Cell Res. 23, 1043–1054.

Cercos, M., Soler, G., Iglesias, D.J., Gadea, J., Forment, J. and Talon, M. (2006a) Global analysis of gene expression during development and ripening of citrus fruit flesh. A proposed mechanism for citrus Acid utilization. Plant Mol. Biol. 62, 513–527.

Cercos, M., Soler, G., Iglesias, D.J., Gadea, J., Forment, J. and Talon, M. (2006b) Global analysis of gene expression during development and ripening of citrus fruit flesh. A proposed mechanism for citrus acid utilization. Plant Mol. Biol. 62, 513–527.

Chen, L.Q., Lin, I.W.N., Qu, X.Q., Sosso, D., McFarlane, H.E., Lodonno, A., Samuels, A.L. et al. (2015) A cascade of sequentially expressed sucrose transporters in the seed coat and endosperm provides nutrition for the arabisopsis embryo. Plant Cell, 27, 607–619.

Chen, L.Q., Qu, X.Q., Hou, B.H., Sosso, D., Osorio, S., Fermie, A.R. and Frommer, W.B. (2012) Sucrose efflux mediated by SWEET proteins as a key step for phloem transport. Science, 335, 207–211.

D’Ascot, M.A., Yelle, S. and Nguyen-Quoc, B. (1999) Antisense inhibition of tomato fruit sucrose synthase decreases fruit setting and the sucrose unloading capacity of young fruit. Plant Cell, 11, 2407–2418.

Degu, A., Hatew, B., Nunes-Nesi, A., Silzhizer, L., Zur, N., Katz, E., Fermie, A.R. et al. (2011) Inhibition of aconitase in citrus fruit cellus results in a metabolic shift towards amino acid biosynthesis. Plant, 234, 501–513.

Ding, Y.D., Chang, J.W., Ma, Q.L., Chen, L.L., Liu, S.Z., Jin, S., Han, J.W. et al. (2015) Network analysis of postharvest senescence process in citrus fruits revealed by transcriptomic and metabolomic profiling. Plant Physiol. 168, 357–364.

Ernst, J. and Bar-Joseph, Z. (2006) STEM: a tool for the analysis of short time series gene expression data. BMC Bioinform. 7, 191.

Fan, J., Wang, H., Li, X., Sui, X. and Zhang, Z. (2019) Down-Regulating Cucumber Sucrose Synthase 4 (CsSUS4) suppresses the growth and development of flowers and fruits. Plant Cell Physiol. 60, 752–764.
Goldschmidt, E. (1998) Ripening of citrus and other non-climacteric fruits: A role for ethylene. *Acta Hortic.* 463, 335–340.

Guo, L.X., Zhao, Y.H., Cao, M.H., Qiao, L. and Zheng, Z.L. (2016) Integrated systems biology analysis of transcriptomes reveals candidate genes for acidity control in developing fruits of sweet orange (*Citrus sinensis* L. Osbeck). *Front. Plant Sci.* 7, 486.

Hussain, S.B., Shi, C.Y., Guo, L.X., Kamran, H.M., Sadka, A. and Liu, Y.Z. (2017) Recent advances in the regulation of citric acid metabolism in citrus. *Crit. Rev. Plant Sci.* 36, 241–256.

Islam, M.Z., Jin, L.-F., Shi, C.-Y., Liu, Y.-Z. and Peng, S.A. (2015) Genome-wide identification of citrus ATP-citrate lyase genes and their transcript analysis in fruits reveals their possible role in citrate utilization. *Mol. Genet. Genom.* 290, 29–38.

Jia, H.F., Chai, Y.M., Li, C.L., Lu, D., Luo, J.J., Qin, L. and Shen, Y.Y. (2011) Abbasic acid plays an important role in the regulation of strawberry fruit ripening. *Plant Physiol.* 157, 188–199.

Jia, H.F., Lu, D., Sun, J.H., Li, C.L., Xing, Y., Qin, L. and Shen, Y.Y. (2013) Type 2C protein phosphatase AB1 is a negative regulator of strawberry fruit ripening. *J. Exp. Bot.* 64, 1677–1687.

Karlova, R., Chapman, N., David, K., Angenent, G.C., Seymour, G.B. and de Vries, M. (2007) Transcriptome analysis in fruits reveals their possible role in citrate utilization. *Front. Plant Sci.* 8, 2934.

Hollender, C.A., Kang, C.Y., Darwish, O., Gestet, A., Matthews, B.F., Slovin, J., Alkharouf, N. et al. (2014) Floral transcriptomes in woodland strawberry uncover developing receptacle and anther gene networks. *Plant Physiol.* 165, 1062–1075.

Hu, X.M., Shi, C.Y., Liu, X., Jin, L.F., Liu, Y.Z. and Peng, S.A. (2015) Genome-wide identification of citrus ATP-citrate lyase genes and their transcript analysis in ripening and ABA-injected fruits. *Tree Genet. Genom.* 11, 97.

Kang, C.Y., Darwish, O., Geretz, A., Shahan, R., Alkharouf, N. and Liu, Z.C. (2013) Genome-scale transcriptomic insights into early-stage fruit development in woodland strawberry *Fragaria vesca.* *Plant Cell,* 57, 160–170.

Kachanovsky, D.E., Filler, S., Isaacson, T. and Hirschberg, J. (2012) Epistasis in tomato color mutations involves regulation of phytoene synthase 1 expression by cis-carotenoids. *Proc. Natl. Acad. Sci. USA,* 109, 19021–19026.

Koltunow, A.M. (2007) Expression of aberrant forms of AUXIN RESPONSE FACTOR8 stimulates parthenocarpy in Arabidopsis and tomato. *Plant Physiol.* 145, 1448–1458.

Langfelder, P. and Horvath, S. (2008) WGCNA: an R package for weighted correlation network analysis. *BMC Bioinform.* 9, 559.

Leng, P., Yuan, B. and Guo, Y. (2014) The role of abscisic acid in fruit ripening and responses to abiotic stress. *J. Exp. Bot.* 65, 4577–4588.

Li, H.S. (2000) Principles and Techniques of Plant Physiology and Biochemistry Experiment, pp. 134–137. Beijing: Higher Education Pres.

Lin, Q., Wang, C., Dong, W., Jiang, Q., Wang, D., Lin, S. and Chen, M. et al. (2015b) Transcriptome and metabolome analysis of citrus and organic acid metabolism in Ponkan (*Citrus reticulata*), fruit during fruit maturation. *Genome,* 58, 64–74.

Muller, M.L., Irienskieuser, U., Rubinstein, B. and Taiz, L. (1996) On the mechanism of hyperacidification in lemon - Comparison of the vacuolar H+ ATPase activities of fruits and epicotyls. *J. Biol. Chem.* 271, 1916–1924.

Pattison, R.J., Csukasi, F. and Catala, C. (2014) Mechanisms regulating auxin action during fruit development. *Physiol. Plant.* 151, 62–72.

Paul, V., Pandey, R. and Srivastava, G.C. (2012) The fading distinctions between classical patterns of ripening in climacteric and non-climacteric fruit and the ubiquity of ethylene-An overview. *J. Food Sci. Techn.* 49, 1–21.

Pattison, R.J., Csukasi, F., Zheng, Y., Fei, Z., van der Knaap, E. and Catala, C. (2015) Comprehensive tissue-specific transcriptome analysis reveals distinct regulatory programs during early tomato fruit development. *Plant Physiol.* 168, 1684–1701.

Paul, V., Pandey, R. and Srivastava, G.C. (2012) The fading distinctions between classical patterns of ripening in climacteric and non-climacteric fruit and the ubiquity of ethylene-An overview. *J. Food Sci. Techn.* 49, 1–21.

Payyavula, R.S., Tay, K.H., Tsai, C.J. and Harding, S.A. (2011) The sucrose transporter family in Populus: the importance of a tonoplast PtaSUT4 to biomass and carbon partitioning. *Plant J.* 65, 4527–4541.

Katz, E., Boo, K.H., Kim, H.Y., Eigenheer, R.A., Phinney, B.S., Shulaev, V., Negre-Zakharov, F. et al. (2011) Label-free shotgun proteomics and metabolite analysis reveal a significant metabolic shift during citrus fruit development. *J. Exp. Bot.* 62, 5367–5384.

Klee, H.J. and Giovannoni, J.J. (2011) Genetics and control of tomato fruit ripening and quality attributes. *Annu. Rev. Genet.* 45, 41–59.

Koijma, K., Yamada, Y. and Yamamoto, M. (1995) Effects of abscisic-acid injection on sugar and organic-acid contents of citrus fruit. *J. Jpn. Soc. Hortic. Sci.* 64, 17–21.
Insights into citrus fruit regulation networks

Schneider, S., Hulpe, S., Schulz, A., Yaron, I., Holl, J., Imlau, A., Schmitt, B. et al. (2012) Vacuoles release sucrose via tonoplast-localised SUC4-type transporters. Plant Biol. 14, 325–336.

Schulz, A., Beyerl, D., Marten, I., Wormit, A., Neuhaus, E., Poschet, G., Butten, M. et al. (2011) Proton-driven sucrose symport and antiport are provided by the vacuolar transporters SUC4 and TMT1/2. Plant J. 68, 129–136.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N. et al. (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–2504.

Sheng, L., Shen, D., Yang, W., Zhang, M., Zeng, Y., Xu, J., Deng, X. et al. (2017) GABA pathway rate-limit citrate degradation in postharvest citrus fruit evidence from HB Pumelo (Citrus grandis) × Fairchild (Citrus reticulata) hybrid population. J. Agric. Food Chem. 65, 1669–1676.

Shi, C.Y., Hussain, S.B., Yang, H., Bai, Y.X., Khan, M.A. and Liu, Y.Z. (2019) Rice spatial allocation of resources during grain filling. J. Exp. Bot. 70, 529–544.

Shlizerman, L., Marsh, K., Blumwald, E. and Sadka, A. (2007) Iron-shortage-like H⁺-ATPase genes: identification and transcript analysis to investigate their possible relationship with citrate accumulation in fruits. Front. Plant Sci. 6, 135.

Slavack, P.E., Serban, B., Rowe, M., Tiyaki, I., Maldonado, M.T., Maldonado, M.C. and Suza, W. (2005) Characterization of an Arabidopsis enzyme family that conjugates amino acids to indole-3-acetic acid. Plant Cell, 17, 616–627.

Strasser, P., Spelt, C.E., Li, S., Biele, M., Federici, C.T., Roose, M.L., Koes, R. et al. (2019) Hyperacidification of citrus fruits by a vacuolar proton-pumping P-ATPase complex. Nat. Commun. 10, 744.

Sun, L., Sun, Y.F., Zhang, M., Wang, L., Ren, J., Cui, M.M., Wang, Y.P. et al. (2012a) Suppression of 9-cis-epoxycarotenoid dioxygenase, which encodes a key enzyme in abscisic acid biosynthesis, alters fruit texture in transgenic Populus tremula. J. Agric. Food Chem. 60, 3097–3108.

Sunberg, E. and Ostergaard, L. (2009) Distinct and dynamic auxin activities in citrus fruit vesicles and calli. Plant Cell, 21, 531–541.

Sun, L., Yuan, B., Zhang, M., Wang, L., Cui, M., Wang, Q. and Leng, P. (2012b) Transcriptional changes during fruit development and ripening of sweet orange (Citrus sinensis). Bmc Genom. 13, 10.

Zhan, J.P., Thakare, D., Ma, C., Lloyd, A., Nixon, N.M., Arakaki, A.M., Burnett, W.J. et al. (2015) RNA sequencing of laser-capture microdissected compartments of the maize kernel identifies regulatory modules associated with endosperm cell differentiation. Plant Cell, 27, 513–531.

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** Visualization of the GO enrichment analysis of the genes in profiles 8 and 39 in the four fruit tissues by REVIGO.

**Figure S2** Coexpression modules identified by WGCNA.

**Figure S3** Correlation networks of the green (a) and yellow (b) profiles.

**Figure S4** Gene expression heatmap of *Expansins*.

**Figure S5** Expression profiling of hormone-related genes and the contents of total carotenoids in the four fruit tissues at six development stages.

**Figure S6** Sucrose metabolism-related genes.

**Figure S7** Citric acid metabolism-related genes.

**Figure S8** Content of soluble sugars and organic acids of treated Satsuma fruits.

**Table S1** RNA-seq statistics.

**Table S2** Gene expression in the four fruit tissues at six developmental stages.

**Table S3** Data from the STEM analysis.

**Table S4** GO term enrichment analysis of the genes in profiles 8 and 39 in the four fruit tissues.

**Table S5** KEGG pathway enrichment analysis results of the genes in profiles 8 and 39 in the four fruit tissues.

**Table S6** Data from the WGCNA analysis.

**Table S7** GO term enrichment analysis results of the black, blue, purple, green, yellow and brown modules.

**Table S8** Module network file for the blue, black, purple, green and yellow modules.

**Table S9** Number of cis-elements in the promoter of *Expansin* genes.

**Table S10** List of key genes identified by RNA-seq.

**Table S11** Statistically significant difference analysis of the content of ABA, IAA and JA among different stages and tissues.

**Table S12** Primers used in this study.