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Network Analysis of Differentially Expressed Genes across Four Sweet Orange Varieties Reveals a Conserved Role of Gibberellin and Ethylene Responses and Transcriptional Regulation in Expanding Citrus Fruits

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Abstract

Citrus represents the most important non-climacteric fruits and thus understanding transcriptional control during fruit development is important for improving fruit yield and quality. Compared to relatively intensive transcriptomic studies of ripening citrus fruits, much less is known regarding expanding fruits. To provide a systems view of hormone response and transcriptional regulation in citrus fruit development from Stage I (slow fruit growth) to Stage II (rapid growth), we re-analyzed the fruit transcriptomes which we previously collected from the sweet orange varieties, Newhall, Xinhui, Bingtang, and Succari (*Citrus sinensis* L. Osbeck). A total of 3145 genes were differentially expressed across all four varieties, indicating that they likely have conserved functions in orange fruit development. Using a gene coexpression network-based systems approach, we constructed the subnetworks respectively for gibberellin response, ethylene response, transcription factors and chromatin modifications. Analysis of these subnetworks has led to the identification of more than a dozen major hub genes, such as *EXPA1*, *GASA1/14*, *ERF13*, *HB22*, *ATK1*, and *TOPII*, which represent the most promising candidates for future functional characterization.

Keywords Citrus · Fruit development · Gene coexpression network · Hormone response · Transcription and chromatin modification

Abbreviations

DPA	Days post anthesis
ERF	Ethylene response factor
EXPA	Expansin
FDR	False discovery rate
GA	Gibberellin
GASA	GA-Stimulated in Arabidopsis
GO	Gene ontology
WGCNA	Weighted gene coexpression network analysis

Minghao Cao and Jian Zheng contributed equally to this work.

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Introduction

In general, fruits across species undergo three distinct developmental stages: Stage I (cell division, characterized by slow fruit growth), Stage II (cell expansion, manifested by rapid fruit growth), and Stage III (fruit ripening, marked by cessation of fruit growth and peak of active biochemical reactions for maximizing flavor and taste). According to the respiration pattern and ethylene biosynthesis at Stage III, fruits can be classified into two physiological categories, climacteric fruits such as tomato, apple, peach, and banana, and non-climacteric fruits including citrus, strawberry, grape, and pepper (Gapper et al. 2013; Osorio et al. 2013). Interestingly, although development of non-climacteric fruits does not seem to involve ethylene biosynthesis, accumulating evidence indicates an

important role of ethylene response in fruit ripening or even at the early stage of fruit development in non-climacteric fruits such as pepper and citrus (Fujii et al. 2007; Katz et al. 2004; Lee et al. 2010a; Osorio et al. 2012, 2013). This is in agreement with the general perception that fruits from different species or varieties have evolved some conserved regulatory mechanisms during fruit growth and development.

The genus *Citrus* contains different types of fruits predominantly grown in subtropical and tropical regions. As a worldwide major fruit crop, the citrus fruit not only provides rich nutrition to humans and has significant economic impacts worldwide, but also becomes an important model organism for studying development of non-climacteric perennial fruits due to its available genome sequences (Wu et al. 2014a; Xu et al. 2013), ease of genetic transformation, and identification of an early-flowering orange (Pinheiro et al. 2014). Although classified as non-climacteric fruits, citrus fruits at Stage I (called fruitlets) accumulate a considerable amount of ethylene which declines to a low level at both Stages II and III (Katz et al. 2004). While the pattern of ethylene production at Stage III is consistent with its non-climacteric feature, arguments have been made that citrus fruits probably possess the ethylene production pathway at the early stage and may respond to exogenous ethylene treatments by showing, for example, peel degreening (Katz et al. 2004). In addition to ethylene, other hormones are also involved. For example, gibberellin (GA) has been reported to increase upon pollination and during Stages I and II in order to promote cell expansional growth (Ben-Cheikh et al. 1997; Talón et al. 1990).

To better understand transcriptional regulation during citrus fruit growth and development, transcriptome-based studies via either DNA microarray or RNA sequencing (RNA-Seq) have been performed. Several transcriptomic studies used the citrus fruit samples from Stage II to Stage III, which led to the identification of hundreds to thousands of genes related to fruit maturation (Aprile et al. 2011; Fujii et al. 2007; Wang et al. 2017; Wu et al. 2014b; Yu et al. 2012). In contrast to these studies, several groups used slightly different designs. For example, a very early DNA microarray study revealed transcriptomic changes during three stages of mandarin fruit growth and development including maturation (Cercos et al. 2006). Two recent studies reported transcriptomes within Stage I (Lu et al. 2016; Zhang et al. 2017). Our group specifically compared fruit transcriptomes between Stages I and II (Huang et al. 2016; Qiao et al. 2017). Taken together, these studies demonstrate a dramatic change in transcriptome landscape throughout citrus fruit development. Some of these studies have reported transcriptional regulation involved in responses to hormones, such as GA (Zhang et al. 2017), ethylene (Fujii et al. 2007; Wang et al. 2017; Yu et al. 2012), and cytokinin (Qiao et al. 2017).

Gene co-expression network analysis has become a powerful tool to provide a systems view of gene-gene interactions in model plants and recently in citrus disease response (Du et al. 2015; Rawat et al. 2015; Zheng and Zhao 2013) or fruit biology (Huang et al. 2016; Qiao et al. 2017; Wong et al. 2014). In the current work, we re-analyzed the transcriptome data which we previously collected from sweet orange fruits at two developmental stages, I (45 days post anthesis/DPA) and II (142 DPA) (Huang et al. 2016), with an aim of dissecting hormonal regulation and transcriptional control at the network level. In our prior study, fruits from four varieties, Newhall, Xinhui, Bingtang, and Succari, which have different sugar and acid contents and other characteristics, were collected for RNA Seq analysis. Thus, we reason that those genes that are differentially expressed in all four varieties would represent the most conserved transcriptional landscape when fruits develop from Stage I to Stage II. Among a total of 7430 differentially expressed genes in any of the four varieties, we found that 3145 genes are common across all four varieties. These commonly regulated genes were enriched in several Gene Ontology (GO) terms, and we focused our analysis on GA and ethylene responses and transcriptional regulation. Using the gene co-expression network approach, we constructed GA and ethylene response subnetworks, as well as subnetworks of transcription factors and chromatin modification. These network analyses provide a systems view of hormonal regulation and transcriptional control in expanding citrus fruits.

Materials and Methods

Plant Materials and Transcriptome Data Collection

Four varieties of sweet orange (*C. sinensis* L. Osbeck), Newhall, Xinhui, Bingtang and Succari, were grown in China National Citrus Germplasm Repository managed in Citrus Research Institute of Chinese Academy of Agricultural Sciences/Southwest University, Chongqing, China. Representative fruits at 45 and 142 DPA were harvested. Exocarp (flavedo) and mesocarp (albedo) were carefully removed. The remaining pulp tissues (endocarp) were dissected into small segments, weighted, and then quickly frozen with liquid nitrogen before RNA extraction, as described in our prior work (Huang et al. 2016). The experiment included three independent biological replicates for each stage and each variety.

Analysis of Citrus Homologs in Arabidopsis and GO Enrichment

The RNA sequencing raw data used in the current study has been deposited in the NCBI GEO database (Accession number GSE78046). To analyze the citrus homologs in Arabidopsis, the sweet orange proteins for the whole genome (Wu et al. 2014a; Xu et al. 2013) was input into the functional annotation website Mercator (Lohse et al. 2014) for prediction

of the most closely related proteins in Arabidopsis, using the TAIR10 release of Arabidopsis proteome and a BLAST-Cutoff of 0.8. As described elsewhere (Huang et al. 2016), GO terms were assigned to the citrus genes using the GO terms for their Arabidopsis orthologs (GAF version 2.0 updated on September 5, 2014). GO enrichment analysis was performed in the Gene Ontology Enrichment Analysis Software Toolkit (Zheng and Wang 2008), using the hypergeometric test with the Yekutieli-based adjustment for multiple testing (False Discovery Rate/FDR under dependency) and a cutoff of FDR at 0.05.

Gene Co-Expression Subnetwork Construction and Visualization

The sweet orange gene coexpression network involving 7430 differentially expressed genes was constructed using the Weighted Gene Coexpression Network Analysis (WGCNA) package in R (Langfelder and Horvath 2008). To construct various transcriptional regulation and hormone response-related subnetworks, the genes belonging to specific GO terms were used as seed nodes to extract the sweet orange gene coexpression network, and the resulting gene-gene interactions using an edge weight larger than 0.7 were imported into Cytoscape for visualization and construction of the subnetworks.

Results

GO Analysis of 3145 Differentially Expressed Genes Common in Four Varieties of Sweet Orange

Because the 3145 differentially expressed genes from 45 to 142 DPA are common in all of four orange varieties analyzed in our prior transcriptome study (Huang et al. 2016), we expect that analysis of these 3145 genes would reveal some important information on the transcriptional architecture which is well conserved in expanding orange fruits. We performed a GO enrichment analysis for those genes and found that that 284 GO terms are overrepresented (FDR > =0.05) compared to the whole orange genome (Supplemental Table S1). Several overrepresented GO terms are regulation of primary metabolic processes (484 genes, FDR = 1.28E-13), developmental process (660 genes, FDR = 1.41E-11), hormone responses (282 genes, FDR = 3.47E-04), transcription factors (257 genes, FDR = 1.19E-10), and chromatin modification (137 genes, FDR = 1.61E-17). For hormone response, we found that only the GA response category is overrepresented (39 genes, FDR = 0.004).

Analysis of GA and Ethylene Response Subnetworks Conserved in Orange Fruits

To provide a systems view of hormone response conserved in sweet orange fruit development, we constructed and analyzed the subnetworks for GA and ethylene hormone responses, respectively, using those 4135 differentially genes common to all four sweet orange varieties (Huang et al. 2016). First, we constructed the GA response subnetwork by using 39 GA response genes as the seed nodes to extract the fruit gene co-expression network constructed previously (Huang et al. 2016). The resulting subnetwork has 423 nodes, which includes 16 of GA response genes, and 590 interactions (Fig. 1, Supplemental Fig. S1 and Table S2). For those interacting genes, cell division (*p* value of 3.0E-18) and cytoskeleton organization (p value of 1.3E-09) are among the most overrepresented GO terms, consistent with the role of GA in regulating cell division, cell expansion and cytoskeleton. Two of the GA response genes, Cs9g16520/*GA20OX1* and orange1.1 t00272/*GA20OX2*, encode GA20 oxidase isoforms 1 and 2, respectively, and are involved in a rate limiting step of GA biosynthesis. Although *GA20OX1* does not show any interaction with those differentially expressed genes, *GA20OX2* interacts with two other uncharacterized genes, Cs2g04700 and Cs2g07010. Furthermore, in this subnetwork, there are eight hub genes with more than 10 interactions. Among these, Cs5g10820/*EXPA1* is the largest hub, with 276 interactions. Arabidopsis EXPA1 is involved in cell wall extension and important for cell growth (Esmon et al. 2006). The second largest hub is Cs6g20220/*GASA14*, interacting with 96 genes. Two other *GASA*-like genes, Cs6g21260/*GASA4* and Cs3g26100/*GASA1*, are also the hubs with slightly fewer interactions, while the fourth GASA gene, Cs5g10680/*GASA6*, has only seven connections. *GASA1* has also been reported by others to be differentially expressed within Stage I in all three citrus species tested, Fallglo hybrid, grapefruit, and Pineapple sweet orange (Zhang et al. 2017). The GASA (GA-Stimulated in Arabidopsis) proteins regulate plant growth through GA-induced and DELLA-dependent signal transduction (Sun et al. 2013). Another major hub is Cs3g22190 (with 42 interactions), which is closely related to Arabidopsis HB22, a homeodomain leucine zipper class I (HD-Zip I) protein that possesses transcription factor activity. Consistent with its expression in orange fruits, this gene is also expressed in Arabidopsis flower and fruit (Tan and Irish 2006). Taken together, these GA response genes are important for cell growth, consistent with the role of GA in cell division and expansion during fruit development from Stage I to Stage II.

Next, we analyzed the subnetwork for ethylene response. Although we did not observe any ethylene biosynthetic genes up- or down-regulated from 45 to 142 DPA in any of the four orange fruits, a total of 23 ethylene response genes were found

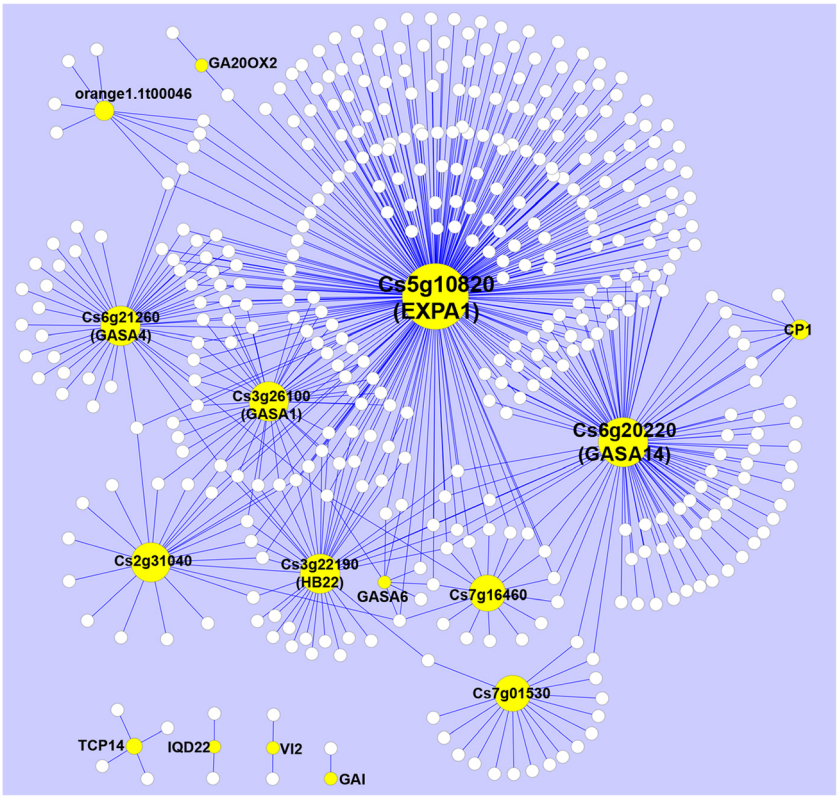

Fig. 1 GA response subnetwork. The GA response subnetwork was constructed using 39 GA response genes as the seed nodes, with the genes involved in GA response coded in yellow

to be differentially regulated. This result supports the notion that ethylene response but not biosynthesis has been increasingly recognized to play a role in fruit ripening or early stage of fruit development in non-climacteric fruits such as pepper and citrus (Fujii et al. 2007; Katz et al. 2004; Lee et al. 2010a; Osorio et al. 2012; Osorio et al. 2013). Therefore, although the presence of 23 ethylene response genes (Supplemental Table S2) does not show overrepresentation of this GO term, we decided to analyze the ethylene response subnetwork with an aim of providing a systems view of ethylene response in early fruit development conserved in four sweet orange varieties. This subnetwork contains 528 connections involving 496 genes (Fig. 2, Supplemental Table S2 and Fig. S2), among which nine are the ethylene response seed node genes (yellow coded in Fig. 2). Cs1g03290/*ERF13* is the largest hub having interactions with 402 genes which belong to several overrepresented GO terms, such as microtubule cytoskeleton, cell proliferation and histone methylation. The other ERF homolog in orange, Cs2g05640/*ERF12*, has only one interaction (Fig. 2). Other mid-sized hubs include Cs4g07410/*ALP* (with 57 interactions) and Cs8g12680/*MYB14* (47 interactions). *ALP* in Arabidopsis encodes an aleurain-like protease involved in senescence in response to ethylene. There are several MYB genes that are also responsive to ethylene, and only Cs8g12680/*MYB14* is present as a seed node in the subnetwork. In addition, three other small hub genes, Cs6g07990/*AUX1*, Cs4g20440/*ABCG40*, and Cs6g17530/*GAI*, have much fewer interactions. Overall, this gene co-expression subnetwork analysis indicates that ethylene response factors (such as *ERF13*) and other ethylene response genes (such as *ALP* and *MYB14*) play a potential role in early fruit development of sweet oranges.

Subnetwork Analysis of Transcriptional Regulation Conserved in Expanding Sweet Orange Fruits

We first analyzed 257 transcription factor genes conserved in orange fruit development. Venn diagram analysis shows that 93 are involved in regulation of primary metabolic process, 72 in both primary metabolic process regulation and developmental process, 37 in both primary metabolic process regulation and hormone response, and 24 in all of the three biological processes (Fig. 3a). Among a total of 69 transcription factor genes which can also be grouped into the hormone response GO term (Fig. 3a), 16 genes belong to ethylene response and 18 to GA response (Supplemental Table S2). Among 110 transcription factor genes that also belong to developmental process (Fig. 3a), 29 are grouped into the GO category of fruit development (Supplemental Table S2), indicating the importance of transcription factor gene expression in the regulation of fruit development. The transcription factor subnetwork contains 451 interactions involving 243 genes (Fig. 3b, Supplemental Table S2 and Fig. S3). We found that 35 out of 257 transcription factor genes are present in this

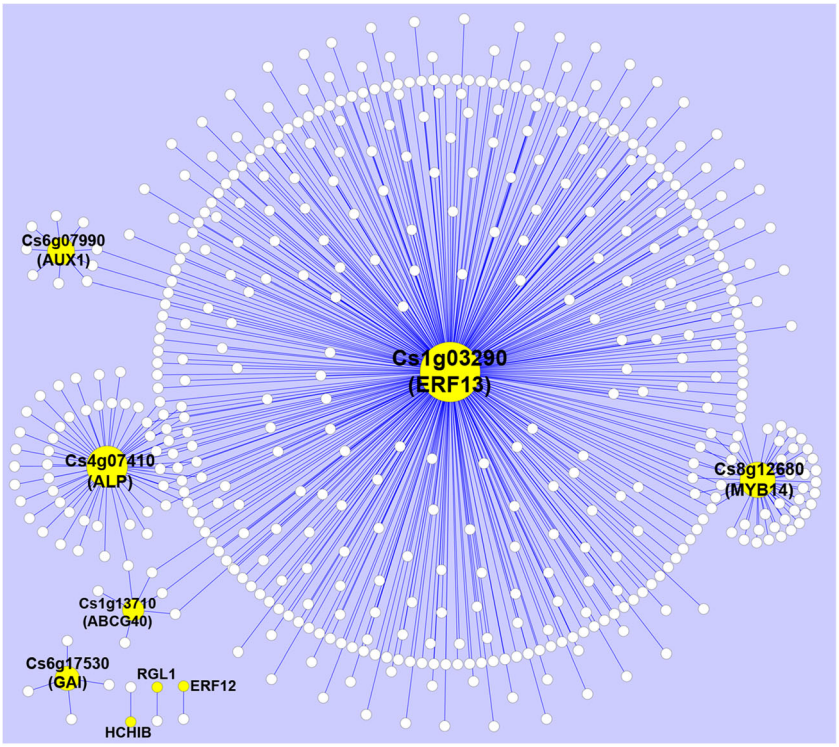

Fig. 2 Ethylene response subnetwork. The ethylene response subnetwork was constructed using 23 ethylene response genes as the seed nodes. The genes involved in ethylene response are coded in yellow

subnetwork. Among those transcription factor nodes, 13 are hub genes with at least 10 connections, and most of them are interconnected to each other (Fig. 3b). Some of the hub genes have their Arabidopsis counterparts having been functionally characterized, such as Cs1g20880/*IAA9*, Cs5g26130/*NAC047*, Cs7g03980/*TCP15*, orange1.1 t02597/*SPL13A*, and orange1.1 t04599/*BLH1*. Arabidopsis *IAA9* is annotated as a negative regulator of auxin response, and the physiological function of Arabidopsis *IAA9* remains unknown; however, role of the *IAA9* counterparts in grape (Fujita et al. 2012) and in particular in tomato fruit development (Wang et al. 2005) has been reported. NAC047 (also called SPEEDY HYPONASTIC GROWTH/SHYG) mediates hyponastic Arabidopsis leaf growth by directly regulating the expression of ACC oxidase isoform 5 gene (*ACO5*) involved in ethylene biosynthesis or controls leaf senescence by acting downstream of EIN2 (Kim et al. 2014; Rauf et al. 2013), although we found that no ethylene biosynthesis genes were differentially regulated in orange fruits. TCP15 is a member of a small family of plant-specific bHLH-containing transcription factor and shown to act in cell proliferation in leaf and floral tissues (Kieffer et al. 2011). SPL13A is a squamosa promoter-binding (SBP) protein-like transcription factor involved in postgerminative development (Martin et al. 2010). BLH1, a member of BEL1-like homeodomain family protein, has been shown to act in early megagametophyte development (Pagnussat et al. 2007).

We then examined 137 genes classified as the GO term of chromatin modification (Supplemental Table S2). Among these genes, we found that four are involved in the processes of developmental regulation, metabolic regulation and hormone response, Cs3g25860/*IAA24* (auxin response), Cs5g06680/*REF6* (brassionsteriod response), Cs7g15220 and orange1.1 t00172 (the latter two both being closely related to *GRF5* involved in ABA response in Arabidopsis). Using 137 chromatin modification-related genes as the seed nodes, a chromatin modification subnetwork was constructed (Fig. 4). This subnetwork contains 177 genes (50 being the chromatin modification seed nodes) with 560 interactions (Supplemental Table S2 and Fig. S4). Sixteen of those 50 chromatin modification genes present in the subnetwork are major hub genes and most of them are interconnected to each other. Furthermore, except for the three genes involved in histone modifications, Cs5g12860/*HIK* in histone phosphorylation and Cs9g16910/*PLE1* and Cs4g18970/*AUR1* in histone methylation, the other 13 major hub genes are involved in both histone and DNA methylation. Physiological functions for Arabidopsis orthologs of several major hub genes have been reported, such as Cs7g01560/*TOPII*, Cs4g18970/*AUR1*, CS9g16910/*PLE*, Cs1g07430/*DRP5A*, Cs3g16980/*FU*, and Cs2g07340/*ATK1*. TOPII is a type II DNA topoisomerase and has been suggested to function in floral development (Hu et al. 2003). AUR1 is a member of Aurora kinase which can phosphorylate histone and is involved in maintenance of meristematic activity and control of endoreduplication (Petrovska et al. 2012). PLE, also called MAP65–3, is involved in organ-specific cytokinesis (Muller et al. 2002). DRP5A, a member of dynamin protein involved in

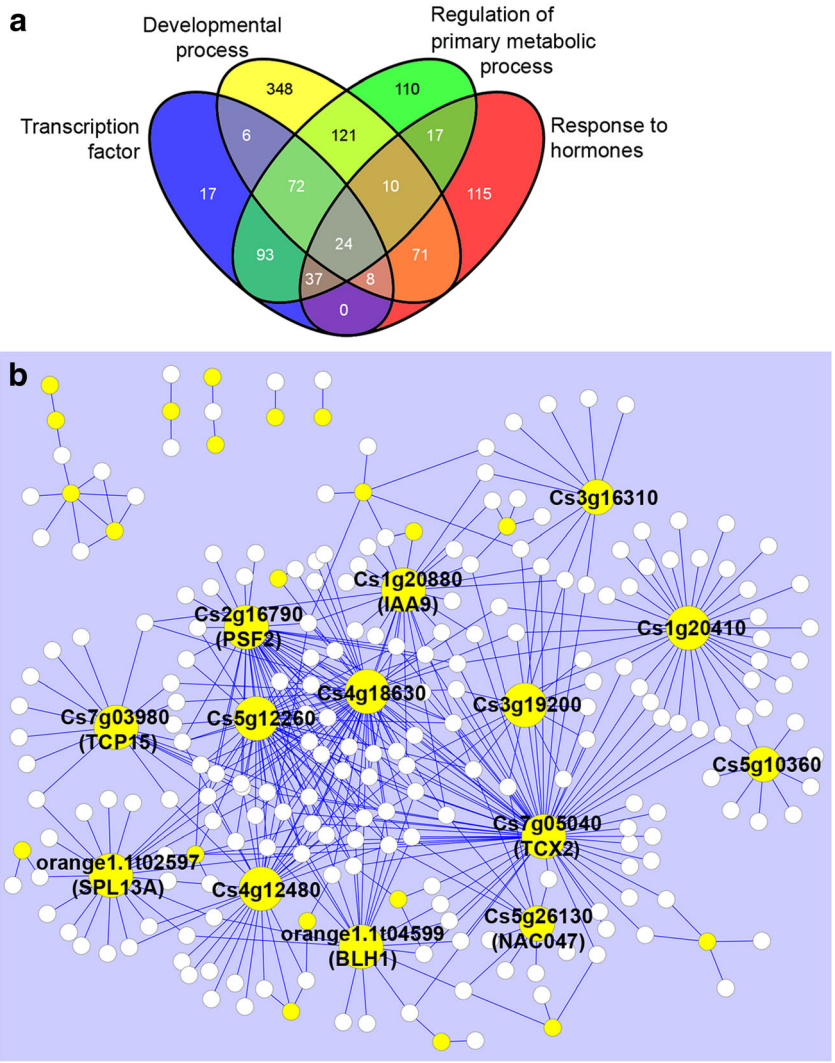

Fig. 3 Transcription factor gene subnetwork. (**a**) Venn diagram showing the distribution of transcription factor genes in hormone response, primary metabolic regulation and developmental process. (**b**) The transcription factor gene co-expression network was constructed using the transcription factor genes as the seed nodes (coded in yellow)

cytokinesis, is important for growth at a low temperature (Miyagishima et al. 2008). FU, a member of the Fused Ser/Thr kinase family, acts as an essential phragmoplast-associated protein that functions in different cell type-specific modes of cytokinesis (Oh et al. 2005). ATK1, also called kinesin 1, plays a critical role in spindle morphogenesis in male meiosis (Chen et al. 2002). Besides the major hub genes described above, some smaller hubs, such as Cs2g01540/*EDE1*, have also been functionally studied. EDE1 has a GO assignment of histone methylation and acts in Arabidopsis fruit development in particular in endosperm development through the control of microtubule and cytokinesis (Pignocchi et al. 2009).

Discussion

In our prior work, we identified 3145 genes which are differentially regulated from Stage I to Stage II in all four sweet orange varieties analyzed. This number represents almost half of 7430 differentially regulated genes in any of the four varieties, indicating that a considerable proportion of fruit development-related genes are conserved in sweet orange fruits. Thus, analysis of these commonly regulated genes may lead to a better understanding of the conserved transcriptional architecture for sweet orange fruit development from Stage I to Stage II. GO enrichment analysis has revealed that many biological processes or molecular functions are overrepresented. Among those overrepresented GO terms, hormone response is of particular importance. Within the hormone response category, only GA response is overrepresented, suggesting that GA-exerted cell division and elongation is critical for the transition from Stage I (cell division) to Stage II (cell expansion responsible for rapid fruit growth).

Results from the network analysis for GA and ethylene response, respectively, have provided novel insights into hormonal regulation during fruit expansion. We have found that in the GA response subnetwork expansin (EXPA1) and

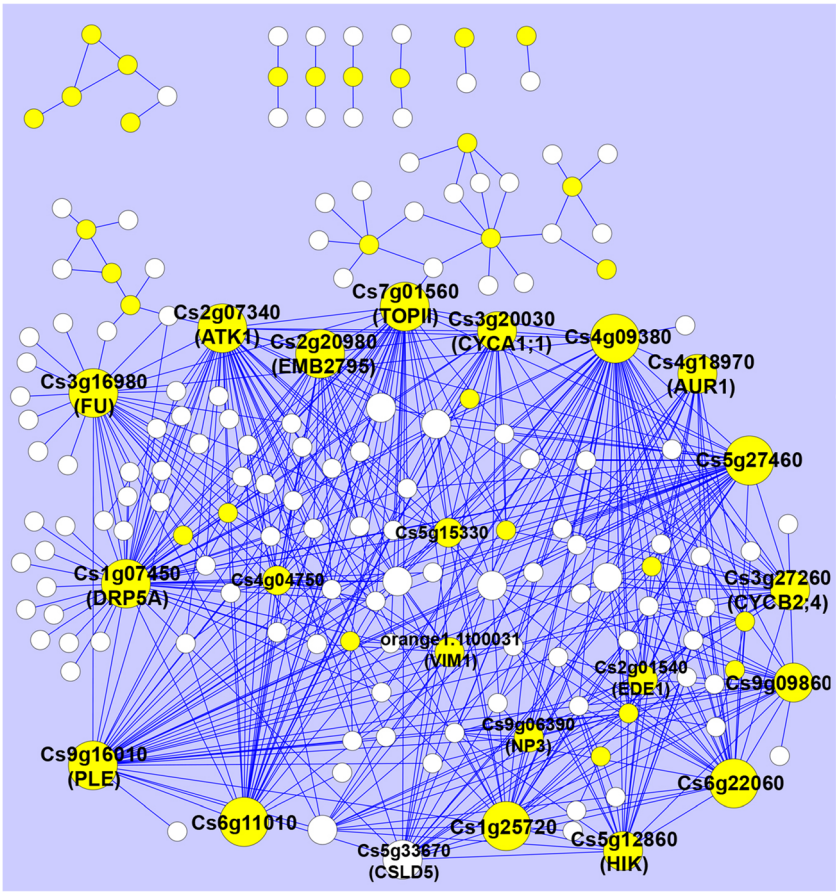

Fig. 4 Chromatin modification gene subnetwork. The subnetwork was constructed by using 137 genes involved in chromatin modification as the seed nodes (coded in yellow)

several GASA-related genes, such as GASA1/4/14 (Sun et al. 2013), may act as major hubs. This result supports that GA-stimulated cell elongation is important for fruit development in particular at Stage II (Gapper et al. 2013; Kumar et al. 2014). In the ethylene response subnetwork, ERF13 acts as a super hub interacting with more than 400 genes. As no ethylene biosynthesis-related genes are found differentially regulated from Stage I to Stage II, this finding supports the argument that ethylene action (rather than biosynthesis) is possibly involved in fruit development even in non-climacteric fruits (Fujii et al. 2007; Gapper et al. 2013; Katz et al. 2004; Kumar et al. 2014; Lee et al. 2010a; Osorio et al. 2012, 2013). Given that ERF13 in Arabidopsis is also involved in response to other hormones, such as auxin, ABA, jasmonate and salicylate, and affects glucose response and seedling growth rate (Lee et al. 2010b; Schweizer et al. 2013), it is possible that ethylene may cross-talk with other hormones during fruit expansional growth. Hormone cross-talk is also evident by our finding that the ethylene response subnetwork contains two small hubs involved in both GA and auxin responses, GAI (a GA signaling protein) and AUX1 (an auxin influx transporter).

Our result that 257 transcription factor genes and 137 chromatin modification-related genes are also differentially regulated in fruit development from Stage I to Stage II supports the importance of large-scale transcriptional regulation during this developmental transitioning period. While many studies have focused on transcription factors, epigenetic profiling has implied potential involvement of miRNA or histone modification-related genes during fruit development (Liu et al. 2014; Xu et al. 2015). Thus, identification of the chromatin modification subnetwork and in particular the major hubs in the subnetwork will provide further clues to the complexity of transcriptional control in expanding citrus fruits.

Another interesting finding from our GO enrichment and subnetwork analyses is intertwining of transcription factors and hormone response. More than a quarter of transcription factor genes commonly expressed in all four sweet orange varieties are categorized into the process of hormone response. Among those hormone-related transcription factor genes, almost half are assigned as GA or ethylene response, and the others are related to different hormones, such as auxin and ABA. Three of those, *IAA9*, *HB22* and *ERF13*, are of particular importance. IAA9 orthologs in grape and tomato have bene reported to function in fruit development (Fujita et al. 2012; Wang et al. 2005), indicating an important role of transcriptional regulation in response to auxin during fruit development. HB22, which possesses transcription factor activity

and has been implicated a function in flower and fruit development in Arabidopsis (Tan and Irish 2006), may play an important role in mediating GA response in expanding orange fruits. ERF13 has been demonstrated to act in orange peel degreening due to chlorophyll degradation during fruit maturation (Yin et al. 2016), and thus it is likely to mediate transcriptional control in expanding fruits in response to low levels of ethylene and possibly other hormones. Testing these and other hub genes which are involved in hormone response and transcriptional regulation will provide convincing support for their functions during fruit development from Stage I to Stage II.

In summary, we have found that genes classified to the categories of GA and ethylene responses, transcriptional regulation and chromatin modification are differentially regulated in all four sweet orange varieties. These four orange varieties differ significantly in the contents of acids and slightly in sugars, and they exhibit other phenotypic differences in tree morphologies and fruit sizes. Citrus genus contains many different types of fruits predominantly grown in subtropical and tropical regions. Therefore, those genes that are commonly regulated across four varieties likely have conserved functions in the early stage of fruit development. Using the gene coexpression-based systems approach, we have presented the subnetworks for GA response, ethylene response, transcription factors and chromatin modification, respectively. These subnetworks have provided a systems view of hormonal control and transcriptional regulation, which intertwine to some extent. Furthermore, analysis of these subnetworks has led us to identify more than a dozen major hub genes, such as *EXPA1*, *GASA1/14*, *ERF13*, *HB22*, *ATK1*, and *TOPII*. As the hub genes in Arabidopsis have been found to probably have a causal-effect relationship, these citrus hub genes represent the most promising candidates for functional testing in the future. Findings from this study may provide insights into genetic or molecular mechanisms in the control of growth and development in expanding fruits for citrus and other types of subtropical and tropical plants.

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Author Contributions M.C., J. Z., Z.Z and Z.-L.Z. performed bioinformatic analyses, Y.Z. performed systems biology analysis, and all authors discussed the results. Y.Z. and Z.-L.Z. wrote the article.

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