RESEARCH PAPER

Differential expression of \textit{SIKLUH} controlling fruit and seed weight is associated with changes in lipid metabolism and photosynthesis-related genes

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Abstract

The sizes of plant organs such as fruit and seed are crucial yield components. Tomato KLUH underlies the locus \textit{fw3.2}, an important regulator of fruit and seed weight. However, the mechanism by which the expression levels of \textit{KLUH} affect organ size is poorly understood. We found that higher expression of \textit{SIKLUH} increased cell proliferation in the pericarp within 5 d post-anthesis in tomato near-isogenic lines. Differential gene expression analyses showed that lower expression of \textit{SIKLUH} was associated with increased expression of genes involved in lipid metabolism. Lipidomic analysis revealed that repression of \textit{SIKLUH} mainly increased the contents of certain non-phosphorus glycerolipids and phospholipids and decreased the contents of four unknown lipids. Co-expression network analyses revealed that lipid metabolism was possibly associated with but not directly controlled by \textit{SIKLUH}, and that this gene instead controls photosynthesis-related processes. In addition, many transcription factors putatively involved in the KLUH pathway were identified. Collectively, we show that SIKLUH regulates fruit and seed weight which is associated with altered lipid metabolism. The results expand our understanding of fruit and seed weight regulation and offer a valuable resource for functional studies of candidate genes putatively involved in regulation of organ size in tomato and other crops.

Keywords: Fruit weight, KLUH, lipid metabolism, seed weight, tomato; transcription factor.
Introduction

The weight/size of plant organs is critically important for the survival of the species (Gonzalez et al., 2009). The final weight of a plant organ is influenced by the combined effect of genetic and environmental signals during growth and development of the plant (Tsukaya, 2003; Horiguchi et al., 2005). The weight of organs such as seed, fruit, root, tuber, and leaf is of importance to plant yield as this is one of the most critical agronomic traits in crop breeding (Ge et al., 2016).

The regulation of seed and leaf size has been studied extensively in rice and Arabidopsis, respectively. These studies have led to the discovery of at least 88 key organ size regulators (Gonzalez et al., 2009; Li and Li, 2016; Vercruysse et al., 2020). The pathways that control seed size include KLUH, ubiquitin–proteasome, G-protein signaling, mitogen–activated protein kinase (MAPK), and plant hormones (Gonzalez et al., 2009; Li and Li, 2016; Li et al., 2019). For leaf size, in addition to KLUH, the pathways that control this trait are DA1–enhancer of DA1 (EOD1), growth regulating factor (GRF)–GRF-interacting factor (GIF), SWITCH/sucrose non-fermenting (SWI/SNF), and plant hormones (Vercruysse et al., 2020). Fruit weight is most extensively studied in tomato (van der Knaap et al., 2017). The pathways regulating fruit weight are also KLUH, as well as cell number regulator (CNR), cell size regulator (CSK), members of the WUS–CLV3 pathway, and plant hormones (van der Knaap and Ostergaard, 2018; Rothan et al., 2019). Remarkably, one of the shared components in seed, leaf, and fruit size regulation is KLUH. However, the role of KLUH and its relationship to other organ size regulatory pathways is not well understood.

KLUH is the founding member of the CYP78A subfamily that was first identified in Arabidopsis to stimulate organ size by promoting cell proliferation (Anastasiou et al., 2007; Adamski et al., 2009). KLUH is proposed to be involved in the production of an unknown signaling molecule that non-cell-autonomously regulates cell proliferation (Anastasiou et al., 2007; Adamski et al., 2009; Eriksson et al., 2010). However, the exact molecular and biochemical nature of the mobile signal remains unknown. Notably, other members of the CYP78A subfamily are also associated with controlling organ size in Arabidopsis (Wang et al., 2008; Fang et al., 2012; Sotelo-Silveira et al., 2013; Yang et al., 2013) as well as in other plant species (Ma et al., 2013; Nagasawa et al., 2013; Yang et al., 2013; Ma et al., 2015a, b; Wang et al., 2015a; Zhao et al., 2016; X. Sun et al., 2017; Qi et al., 2017; Maeda et al., 2019). In rice, GIANT EMBRYO (GE; CYP78A13) plays an important role in controlling the size balance of the embryo and endosperm. This gene is essential for embryo development and grain yield (Nagasawa et al., 2013; Yang et al., 2013). The rice CYP78A OsBSR2 (BROAD-SPECTRUM RESISTANCE2) is associated with seed weight and disease resistance (Maeda et al., 2019). The maize CYP78A PLASTOCHRON1 (ZmPLA1) extends the duration of cell division, leading to increased seed yield and stover biomass (X. Sun et al., 2017). In soybean, wheat, sweet cherry, and pepper, GmCYP78A10, GmCYP78A72, TaCYP78A3, TaCYP78A5, PaCYP78A9, and CaKLUH, respectively, play important roles in or are strongly associated with regulating seed and fruit weight (Chakrabarti et al., 2013; Ma et al., 2013; Ma et al., 2015a, b; Wang et al., 2015a; Zhao et al., 2016). Combined, these studies demonstrate the importance of CYP78A as a critical component of organ size regulation in plants.

The domestication-related CYP78A gene was cloned from tomato a few years ago and considered the ortholog of Arabidopsis KLUH (Zhang, 2012; Chakrabarti et al., 2013). Tomato KLUH underlies the fruit weight locus fu3.2 and is a positive regulator of fruit weight by increasing the number of cell layers in the pericarp (Chakrabarti et al., 2013). We recently demonstrated that the duplication of SIKLUH is the causative variant at the fu3.2 locus, accounting for differential expression that is correlated to gene copy number (Alonge et al., 2020). Given that SIKLUH does not affect cell size (Chakrabarti et al., 2013), it is likely to function in the cell proliferation phase in pericarp at the early stages of fruit development. However, further cellular analyses at different fruit developmental stages are needed to determine when changes in the number of cell layers become evident.

In this study, we performed histological comparisons of fu3.2 near-isogenic lines (NILs) to investigate the changes in the number of cell layers in the pericarp at six developmental time points. We analyzed the RNA sequencing (RNA-seq) data from developing pericarp and seed in fu3.2 NILs that only differ for the allele at the locus as well as lines that are transgenically down-regulating the expression of SIKLUH by RNAi (RNAi-2Q1). The results showed many differentially expressed and co-regulated genes that have been implicated in organ size, lipid metabolism, and photosynthesis. We also analyzed the lipid profiles of 5 days post-anthesis (DPA) fruits from the NILs and RNAi-2Q1 and identified several lipid composition categories that were differentially accumulating. Moreover, the overexpression of a transcription factor (TF) gene SHINE1 (SISHN1), that affects lipid metabolism, resulted in a significant decrease in fruit and seed weight. Combined, our findings imply a tight relationship between SIKLUH-mediated regulation of organ weight and lipid metabolism as well as photosynthesis-related processes.

Materials and methods

Plant materials and growth conditions

NILs with the cultivated and wild-type allele of fu3.2, named fu3.2(p) and fu3.2(w), respectively, RNAi lines down-regulating the expression of SIKLUH (RNAi-2Q1 and RNAi-2G2), and SISHN1-overexpressing transgenic lines were described previously (Zhang, 2012; Chakrabarti et al., 2013; Al-Abdalla et al., 2014). The seeds of
the cd2 mutant and Ailsa Craig (AC) control were obtained from Dr Cornelius Barry, Michigan State University (Nadakuduti et al., 2012). The plants were grown in the greenhouse under a 16 h light/8 h dark photoperiod in Athens, GA, USA.

Developing fruit analyses for fw3.2(ys) and fw3.2(wt)

Individual flowers were tagged at anthesis every morning. Developing fruits were collected at anthesis, 5, 7, 10, and 20 DPA, and breaker stage. Developing fruits were bisected equatorially. One half of each fruit was scanned for fruit length and width measurement using ImageJ, and the other half was used for histological analysis.

For the histological analysis of ovaries and developing fruits at 5 and 7 DPA, the samples were fixed overnight in 75% ethanol and 25% acetic anhydride. Samples were then incubated in 80% ethanol at 80 °C and rehydrated in 50% and 30% ethanol for 10 min. Samples were rinsed with ddH2O for 10 min, followed by clearing at room temperature in 0.2 M NaOH/1% SDS while shaking at 30–40 rpm. After 24 h, the samples were further cleared with ClearSee solution (10% xylitol, 15% sodium deoxycholate, 25% urea; VWR International) for 3 d at the same shaking speed and temperature. The samples were rinsed with ddH2O for 5 min and stained for 30 min in calcofluor (0.25% Fluorescent Brightener 28; Sigma) in the dark. Lastly, the samples were rinsed in water and mounted in mounting medium CitiFlour (Electron Microscopy Supplies). The sections were imaged using a Zeiss LSM 880 upright confocal microscope and samples were excited at 405 nm with an emission band of 410–550 nm.

For developing fruits at 10 DPA, 20 DPA, and breaker stage, hand sections were stained with a solution containing one part 0.5% toluidine blue and two parts distilled water for a few seconds. Sections were then rinsed with ddH2O. Images of the stained sections were taken using an Olympus DP70 camera that was mounted on an OLYMPUS MXV10 optical microscope using an Olympus MVX-TVO.63XCC adapter. The generated pictures were used for pericarp cell layer, maximum cell size, and thickness measurements with ImageJ software as previously described (Ramos, 2018). All phenotypic evaluations were performed with two biological replications, each with at least four plants per genotype. For each time point, at least two fruits per plant were analyzed.

Phenotypic evaluations of SISHN1-overexpressing transgenic lines

For fruit weight analysis, 10 fruits at breaker or turning stage from each plant were weighed individually. For seed weight analysis, 50 seeds from each plant were counted and weighed. Three fruits at breaker stage from each plant were weighed individually. For seed weight analysis, 50 seeds from each plant were counted and weighed. Three fruits at breaker stage from each plant were used for pericarp cell layer and thickness analysis as previously described (Ramos, 2018). All phenotypic evaluations were performed independently with two biological replications, each with at least three plants per genotype. For each time point, at least two fruits per plant were analyzed.

Tissue collection and data processing of RNA-seq data

Tissues for RNA extraction were collected with four replicates from pericarp and seed at 5, 7, and 10 DPA in fu3.2 NILs and three replicates from pericarp and seed at 7 DPA of the RNAi-2Q1 and RNAi-2G2 lines down-regulating SIKLUH. RNA-seq library preparation and sequencing were previously described (Chakrabarti et al., 2013). All clean reads for samples from fu3.2 NILs and the RNAi lines of SIKLUH are available in the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) under the accession numbers SRA068200 and SRA068201 (Chakrabarti et al., 2013).

The read mapping was performed using the latest version of the Tuxedo protocol with HISAT2 and StringTie (Pertea et al., 2016). After filtering out adaptor sequences, low-quality reads, and ribosomal reads, the clean reads from each library were mapped to the Heinz 1706 tomato genome version SL3.0 using HISAT2. To quantify all the genes in ITAG (International Tomato Annotation Group) version 3.20, the mapping results were normalized via Stringtie to obtain RPKM (reads per kilobase per million mapped reads). Summary statistics for each of the RNA-seq libraries are shown in Supplementary Table S1. Correlations between samples were determined by using the Spearman rank correlation coefficient (SCC) to check the reproducibility among replicates. For principal component analysis (PCA) of sample replicates, the count data were log transformed using DESeq2 and the PCA plot was generated using the ggplot2 R package.

Differential gene expression analysis

Differential gene expression analysis was performed using the DESeq2 R package (Love et al., 2014) with the count data which were extracted with a Python script included in Stringtie (http://ccb.jhu.edu/software/strungtie/dl/prepDE.py). The genes that were significantly differentially expressed in pericarp and seed at each developmental time point between the NILs as well as in 7 DPA pericarp and seed between fu3.2(ys) and RNAi-2Q1 were identified by Wald test. Genes with |log2ratio|>2 and a false discovery rate (FDR) significance score <0.05 were determined to be significantly differentially expressed genes (DEGs). A differential expression analysis of RNA-seq data from the NILs was also performed using linear factorial modeling to further assess the effects of genotype, the interaction between genotype and developmental stage (G×D), and the interaction between genotype and tissue (G×T) on the gene expression patterns. The likelihood ratio test was used to assess three separate null hypotheses. Null hypothesis 1 was tested to identify genes with significant genotype effects with full model=genotype+issue+developmental stages and reduced model=genotype+developmental stages; Null hypothesis 2 was tested to identify genes significantly affected by G×D with full model=genotype+issue+developmental stages+genotype:developmental stages and reduced model=genotype+issue+developmental stages; Null hypothesis 3 tested whether each gene was affected by G×T with full model=genotype+issue+developmental stages+genotype:issue and reduced model=genotype+issue+developmental stages. The P-values were corrected using the Benjamini–Hochberg method, and the threshold of corrected P-value <0.05 was used for selecting DEGs in the three null hypotheses. A further filtration was performed to eliminate the genes expressed at a low level. Genes with average RPKM >1 among pericarp and seed samples were considered as DEGs. Additionally, the linear factorial modeling can only be applied to sufficiently large data sets with multiple treatments or time points. Since there is only one developmental time point in RNAi-2Q1, we cannot perform the linear factorial modeling with that specific dataset.

Lipid profiling

Lipid profiling was done at RIKEN, Japan, using LC–quadrupole time-of-flight–MS (LC–Q-TOF-MS) as described before (Okazaki et al., 2013; Okazaki and Saito, 2018). Briefly, 5 DPA whole fruit samples from fu3.2(ys), fu3.2(wt), and RNAi-2Q1 were pooled from four plants each, and each sample was replicated five times. These samples were lyophilized and milled to a fine powder. The sample powder was extracted with a mixture of chloroform, methanol, and water by the method of Bligh and Dyer (Bligh and Dyer, 1959; Okazaki and Saito, 2018). The crude lipid extract was finally reconstituted in ethanol and subjected to LC–MS analysis (Okazaki and Saito, 2018). Electrospray ionization was employed for sample ionization. The lipidome dataset obtained in the negative ion mode was subjected to multivariate analysis, orthogonal projection to a latent structure-discriminant analysis (OPLS-DA) (Wiklund et al., 2008), to find the discriminative metabolites among tested samples.
Construction and visualization of the co-expression network

The co-expression network analysis was performed in R using the Weighted Correlation Network Analysis (WGCNA) package (Langfelder and Horvath, 2008). The co-expression network was constructed using RNA-seq data from each NIL independently. For each co-expression network, the genes (cumulative RPKM >6 and variance >1) used for the network were from 24 samples of three time points (5, 7, and 10 DPA), using each biological replicate as an individual dataset (total of 24 samples for each network). To show an approximate scale-free topology, the soft thresholding power of $\beta=17$ was chosen for both networks by the pickSoftThreshold function in the WGCNA package. The modules were obtained using the one-step network construction function (blockwiseModules) with default parameters. The top 50 genes with the highest $k_{ME}$ values were regarded as intramodular hub genes in this study. The networks were visualized using Cytoscape _v.3.7.1.

Gene Ontology (GO) enrichment analysis

GO enrichment analysis of the DEGs was performed using the topGO R package (Alexa et al., 2006; Alexa and Rahnenfuhrer, 2020). The reference GO annotation list was downloaded from Plant Transcriptional Regulatory Map (http://plantregmap.gao-lab.org/go.php). The significantly enriched GO terms were determined by FDR-adjusted P-value <0.05. The heatmaps of DEGs and GO terms were generated using the pheatmap R package (Kolde, 2012).

Statistical analyses

Normality, Student’s t-test, and Duncan’s test were calculated for each trait using R software. The data of all the investigated traits follow a normal distribution as determined by Lilliefors test (Abdi and Molin, 2007).

Results

Fruit growth and histological comparisons of fw3.2 NILs

Phylogenetic analysis revealed that SIKLUH was clustered into the same clade with Arabidopsis CYP78A5/KLUH and CYP78A10, wheat CYP78A5, and soybean CYP78A10 and CYP78A12 (Fig. 1) that act as key regulators of organ size (Anastasiou et al., 2007; Adamski et al., 2009; Yang et al., 2013; Ma et al., 2015b; Wang et al., 2015a; Zhao et al., 2016). Of these, AtCYP78A5 and TaCYP78A5 have been demonstrated to stimulate cell proliferation at the early stages of fruit development (5–7 DPA). To gain more insight into the function of SIKLUH in regulating fruit development, we explored fruit growth at six developmental time points from anthesis to breaker stage (Supplementary Fig. S1). Both fruit length and width started to show differences between 10 and 20 DPA (Supplementary Figs S1, S2A, B). This change in length and width was preceded by a significant change in the number of cell layers as early as 5 DPA (Fig. 2A–C; Supplementary Fig. S2C). The maximum cell size showed no significant difference between the NILs at breaker stage (Fig. 2D; Supplementary Fig. S2D) which is consistent with previous findings (Chakrabarti et al., 2013). The pericarp thickness started to show a significant difference between 10 and 20 DPA which corresponded well to the increase in fruit size (Fig. 2E; Supplementary Fig. S2E). The significant difference in cell layers of the pericarp (Fig. 2C; Supplementary Fig. S2C) did not lead to a significant difference in pericarp thickness at 5, 7, and 10 DPA (Fig. 2E; Supplementary Fig. S2E), which was probably due to the small cell size (Fig. 2D; Supplementary Fig. S2D). These results indicate that SIKLUH stimulates pericarp cell proliferation during the early stages of fruit development (5–7 DPA).

Differential gene expression between the NILs during pericarp and seed development

To gain further insights into the molecular mechanisms of SIKLUH governing fruit and seed weight in tomato, a gene expression analysis was performed using RNA isolated from the NIL tissues corresponding to developing pericarp and seed at 5, 7, and 10 DPA. The SCC analysis showed high reproducibility between the four replicates, ranging from 0.97 to 0.98 (Supplementary Fig. S3). Moreover, the PCA showed that the samples clustered based on tissue type and developmental time point but less based on genotype (Fig. 3A). This suggests that the overall transcriptome profiles did not differ dramatically between the NILs.

RNA expression analyses showed that SIKLUH (Soly03g114940) was significantly more highly expressed in fw3.2(y) than in fw3.2(wt) in most of the samples analyzed (Fig. 3B). DEGs between the NILs were identified by six
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pairwise comparisons of pericarp and seed at each developmental time point (Fig. 3C; Supplementary Dataset S1). In the small-fruited NIL fw3.2 (wt), 48 unique DEGs exhibited significantly lower expression and 61 unique DEGs exhibited significantly higher expression at different time points in the developing pericarp and seed compared with the large-fruited NIL fw3.2 (ys). Notably, fewer DEGs were found in seed (16 unique genes) than in the pericarp (97 unique genes) (Fig. 3C; Supplementary Dataset S1), demonstrating more changes in gene expression during the development of the pericarp than during development of the seed as a consequence of differential expression of SlKLUH. This was despite the fact that SlKLUH itself was much more highly expressed in the seed (Fig. 3B). GO enrichment analysis of up-regulated DEGs in the pericarp of fw3.2 (wt) indicated that the DEGs are enriched for three processes related to lipid metabolism, namely ‘Fatty acid metabolic process’ (six genes), ‘Cutin biosynthetic process’ (three genes), and ‘Monocarboxylic acid metabolic process’ (seven genes) (Fig. 3D). However, no significantly enriched biological processes were identified for the down-regulated DEGs in pericarp and in seed of fw3.2 (wt). Consequently, this finding implied that lower expression of SIKLUH led to up-regulation of lipid metabolism-related processes.

To systematically explore the RNA-seq data, linear factorial modeling was applied to identify DEGs significantly affected by genotype, genotype by tissue interaction (G×T), and genotype by developmental stage interaction (G×D). A total of 72 DEGs, which were consistently up- or down-regulated in fw3.2 (wt) across all samples, were identified with significant genotype effects (Supplementary Dataset S2). As expected, SIKLUH was a DEG significantly affected by genotype, with lower expression in pericarp and seed at all developmental stages in fw3.2 (wt) compared with fw3.2 (ys) (Fig. 3B; Supplementary Dataset S2). No DEGs were found with significant G×T and G×D effects.

RNA-seq analysis of the RNAi lines of SIKLUH

Even though the natural fw3.2 NILs show changes in SIKLUH expression, further down-regulation of the gene may lead to the identification of additional DEGs in the SIKLUH pathway. RNAi-2Q1 and RNAi-2G2 were two independent transgenic lines that down-regulated the expression of SIKLUH in the

Fig. 2. Histological analyses of the pericarp at six developmental time points in the fw3.2 NILs. (A) Representative sections of fw3.2(ys) pericarp. (B) Representative sections of fw3.2(wt) pericarp. Scale bars=100 µm (0, 5, 7, and 10 DPA) and 1 mm (20 DPA and breaker stage). (C–E) Cell layer (C), maximum cell size (D), and pericarp thickness (E) comparisons of the NILs. For the cell layer numbers at 10 DPA, 20 DPA, and breaker stage, the endoderm layer and several cell layers below the exoderm were not counted because they were difficult to discern in these sections, hence a decrease in cell layers from 7 to 10 DPA. Asterisks denote significant differences (*P<0.05; **P<0.01; ***P<0.001) as determined by Student’s t-tests. DPA, days post-anthesis. NS, non-significant difference.
Fig. 3. Differential gene expression analyses in developing pericarp and seed between the *fw3.2* NILs. (A) PCA plot showing the clustering of transcriptomes from pericarp and seed tissues at different time points in the *fw3.2* NILs. Each data point represents a biological replicate. (B) Expression of SIKLUH in pericarp and seed tissues at different time points in the *fw3.2* NILs. Asterisks denote significant differences (**P<0.01; ***P<0.001) as determined by Student’s t-tests. NS, non-significant difference. (C) DEGs at different developmental time points of pericarp and seed. (D) Different expression patterns (left panel) and GO enrichment (right panel) of up-regulated DEGs in the pericarp of *fw3.2*wt. The size of the circles indicates the number of DEGs in the given GO term. The color coding indicates the gene ratio calculated as the number of DEGs in the given GO term divided by the total number of genes in the term. The numbers 5, 7, and 10 indicate 5 DPA, 7 DPA, and 10 DPA, respectively. P, pericarp; S, seed.

Given that the overall transcriptome profiles of 7 DPA pericarp and seed were similar between RNAi-2Q1 and RNAi-2G2 (Fig. 4A; Supplementary Fig. S4), we focused on RNAi-2Q1 expression data for further analysis. A total of 899 and 247 DEGs were identified in 7 DPA pericarp and seed of the RNAi-2Q1 line, respectively (Fig. 4B; Supplementary Dataset S3). As expected, the total number of DEGs in the *fu3.2*(ys)–RNAi-2Q1 dataset was much higher than that of the *fu3.2*(ys)–*fu3.2*(wt) dataset (Figs 3C, 4B), possibly resulting from the more extensive down-regulation of SIKLUH by RNAi compared with the NILs (Chakrabarti et al., 2013). In 7 DPA pericarp, six down-regulated and 33 up-regulated genes were shared in both datasets (Supplementary Fig. S5; Supplementary Table S2). Interestingly, SIKLUH was the only common DEG that was down-regulated in both RNA-seq datasets in 7 DPA seed (Supplementary Fig. S5; Supplementary Table S2).

Similar to the GO term enrichment of the up-regulated DEGs in pericarp of the *fu3.2*(wt), the DEGs that were up-regulated in RNAi-2Q1 pericarp were also enriched for ‘Fatty acid metabolic process’, ‘Cutin biosynthetic process’, and ‘Monocarboxylic acid metabolic process’ (Figs 3D, 4C). Again, the reduced expression of SIKLUH led to enhanced expression of lipid metabolism-related genes. The enriched GO terms of the down-regulated genes in 7 DPA pericarp of RNAi-2Q1 included terms related to cellular processes, such as ‘Microtubule-based process’, ‘Cell cycle process’, and ‘Microtubule cytoskeleton organization’. Genes involved in these processes included putative orthologs of the Arabidopsis ATAURORA1 (AUR1) (Solyc08g066050), ARABIDOPSIS NPK1-ACTIVATING KINESIN 1 (ATNACK1) (Solyc03g119220), MICROTUBULE-ASSOCIATED PROTEIN 65-3 (MAP65-3) (Solyc03g007130), and TETRASPORE (Solyc07g042560). In addition, down-regulated DEGs in the RNAi-2Q1 developing seeds were primarily associated with processes related to transport and homeostasis, whereas up-regulated DEGs in the seeds were not enriched for any biological processes (Fig. 4C). Collectively, the GO enrichment analyses of DEGs from both expression studies suggested that decreased expression of SIKLUH in *fu3.2*(wt) and the RNAi-2Q1 results in smaller sizes of fruit and seed possibly by increasing lipid metabolism.

Characterization of DEGs involved in lipid metabolism pathways

The DEGs were mapped onto pathways using the ACYL-LIPID METABOLISM database (http://aralip.plantbiology.msu.edu/pathways/pathways). We detected 23 and 101 lipid metabolism-related DEGs in the *fu3.2*(ys)–*fu3.2*(wt) dataset and in the *fu3.2*(ys)–RNAi-2Q1 dataset (Supplementary Table S3), respectively. ‘Cutin synthesis and transport’ and ‘Fatty acid elongation and wax biosynthesis’ were the two most abundant lipid metabolism pathways shared by the *fu3.2*(ys)–*fu3.2*(wt) and the *fu3.2*(ys)–RNAi-2Q1 datasets (Supplementary Fig. S6). We found 16 DEGs (seven DEGs were shared between the two datasets) and 31 DEGs (six DEGs were shared between the two datasets) involved primarily in ‘Cutin synthesis and...
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Differential accumulation of lipids

The gene expression data implied that changes in lipid metabolism were a consequence of the differential expression of SlKLUH. To further investigate the effects of SlKLUH on lipid metabolism, we performed lipid profiling of 5 DPA fruits from the NILs and RNAi-2Q1. A total of 425 metabolites were detected and 58 were annotated as signals derived from known lipids (Supplementary Fig. S7). An OPLS-DA (Wiklund et al., 2008) was performed to identify the major difference in the lipid profile between the different genotypes. The OPLS-DA (Fig. 7A) and OPLS loading S-plot (Fig. 7B) revealed that the discriminative metabolites with significantly increased levels in fw3.2(wt) and RNAi-2Q1 were non-phosphorus glycerolipids, including monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), and phospholipids, including phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and four unknown lipids (Table 1). Only four unknown lipids were significantly decreased in fw3.2(wt) and the RNAi-2Q1 line (Table 2). This result provides information about the effects of SlKLUH expression levels on lipid metabolites. However, details of the biosynthesis of the discriminative lipids and the mechanisms by which SlKLUH affects their accumulation remain unknown.

Identification of gene co-expression modules in fw3.2(ys) and fw3.2(wt) by WGCNA

To identify pathways that are consistently associated with SlKLUH expression, WGCNA was performed using the RNA-seq data from either fw3.2(ys) or fw3.2(wt). The networks identified from this analysis might be directly linked to the function of SlKLUH in regulating organ size. A total of 11 modules (comprised of 43–4199 genes) were identified in fw3.2(ys) (Fig. 8A; Supplementary Dataset S4), and 10 modules (comprised of 48–4845 genes) were recognized in fw3.2(wt) (Fig. 8B; Supplementary Dataset S5). SlKLUH was assigned to the yellow module (YYM) in fw3.2(ys) containing 1676 genes (Supplementary Dataset S4), whereas this gene was assigned to the green module (WGM) in fw3.2(wt) containing 1245 genes (Supplementary Dataset S5). The eigengenes of fw3.2(ys) YYM (Fig. 8C) and fw3.2(wt) WGM (Fig. 8D) showed consistently higher expression in seeds compared with pericarp, which mirrored the expression levels of SlKLUH.
Of the 1676 genes in YYM, 421 (~25.1%) were shared with the co-expressed genes in WGM (Supplementary Fig. S8). This relatively low overlap suggested that the module-specific transcriptome profile was noticeably changed as a result of higher SIKLUH expression. Genes with the highest $k_{ME}$ values are referred to as intramodular hub genes and are thought to play critical roles in maintaining network structure and function (Barabási et al., 2011; Langfelder et al., 2013). We found that SIKLUH ($k_{ME}=0.971$) ranked 32nd and formed a hub gene in YYM (Fig. 9A; Supplementary Dataset S4). This suggested that SIKLUH acted to maintain the network structure and function for this module. In contrast, SIKLUH ($k_{ME}=0.885$) in the fw3.2 (wt) dataset ranked 366th, and was therefore not a hub gene (Fig. 9B; Supplementary Dataset S5).

GO term enrichment analysis identified genes in YYM related to ‘Photosynthesis’, ‘Response to stimulus’, and ‘mRNA metabolic process’ (Fig. 10). Even though no common enriched GO terms were identified, many enriched GO term categories relate to photosynthesis, chloroplast organization, and chlorophyll biosynthesis. These results suggested a common theme of SIKLUH-co-expressed genes in fw3.2 (ys) and fw3.2 (wt) that impact chloroplast functioning such as in carbon fixation which might possibly be directly related to organ growth. In addition, no GO terms were enriched in the shared set of 421 genes.

Differentially and co-expressed transcription factors are implicated in tomato fruit and seed weight control mediated by SIKLUH

Gene expression dynamics of those involved in lipid metabolism and photosynthesis-related processes are regulated directly by TFs. In the DEG analyses, nine TFs were shared in
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the *fw3.2* (*ys*)–*fw3.2* (*wt*) and *fw3.2* (*ys*)–RNAi-2Q1 datasets (Supplementary Table S4), suggesting that these TFs might play important roles in regulating fruit and seed weight in the KLUH pathway. For example, *Solyc04g074990* (ZF–HD), *Solyc08g079800* (GRF), and *Solyc04g077510* (GRF) were down-regulated in 7 DPA pericarp in both *fw3.2* (*wt*) and the RNAi-2Q1 line (Supplementary Figs S5, S9; Supplementary Datasets S1, S3; Supplementary Table S4). GRFs are known as
positive regulators of primary cell proliferation and play important roles in regulating organ size in plants (Horiguchi et al., 2005; Vercruysen et al., 2015; Li et al., 2016; Shimano et al., 2018; Zhang et al., 2018). Solyc04g074990 encodes a ZF-HD TF that is closely related to Arabidopsis HB22, HB25, and HB33. In Arabidopsis, overexpression of ATHB25 results in wider siliques and larger seeds, while simultaneous knockdown of ATHB25, ATHB22, and ATHB31 leads to smaller seeds (Bueso et al., 2014). Therefore, the down-regulation of Solyc04g074990, Solyc08g079800, and Solyc04g077510 was associated with smaller fruit and seed in fw3.2(wt) and RNAi-2Q1, suggesting that they may function as positive regulators of tomato fruit and seed size. However, further study is required to dissect their exact roles in fruit and seed weight regulation mediated by KLUH in tomato.

The DEGs in the TF category are considered to change expression as an indirect consequence of the expression level of SIKLUH. However, these DEGs may or may not be found in the same module as SIKLUH. TF genes that are found in the same module as SIKLUH may be more directly involved in the entire KLUH network. To obtain further insight into the transcriptional regulation of the KLUH pathway, we sought out the TFs in these two modules. The YYM harbored 117 TFs (6.98%) which were classified into 35 families. The 10 most abundant TF families in YYM were bHLH (13), C2H2 (11), MYB (9), MYB-related (6), B3 (6), Trihelix (5), bZIP (5), ERF (5), AP2 (4), and NAC (4) (Supplementary Fig. S10A; Supplementary Table S4). The WGM contained 76 TFs (6.10%) mainly from families classified as bHLH (12), C2H2 (6), HD-ZIP (4), GRAS (4), MYB (3), MYB-related (3), NAC (3), Trihelix (3), bZIP (3), and Dof (3) (Supplementary Fig. S10B; Supplementary Table S4). Thirty-seven TFs were shared by YYM and WGM (Supplementary Table S4). The orthologs of some of common TFs were known from other studies to participate in organ size regulation in plants. For example, VAL1 (AT2G30470) (Soly06g082520), a member of the B3 domain TFs and a negative regulator of oil production, plays a major role in plant embryo development (Tsukagoshi et al., 2005, 2007; Suzuki et al., 2007; Schneider et al., 2016). The putative ortholog of Arabidopsis SUPERMAN (SUP, AT3G23130), Soly09g089590, encodes a zinc-finger protein that in Arabidopsis has been proposed to control cell proliferation by regulating the transcription of genes that affect cell division, thus regulating organ size (Hiratsu et al., 2002; Nibau et al., 2010). Interestingly, three of the 37 TF genes (Soly02g089540, Soly06g060830, and Soly11g072470) were also identified as DEGs in the fw3.2(wt)–RNAi-2Q1 dataset (Supplementary Table S4). The putative tomato HB2 (Soly06g060830) was up-regulated in pericarp of RNAi-2Q1. In Arabidopsis, overexpression of ATHB2 (AT4G16780) significantly affects the α-linolenic acid and total fatty acid contents as well as plant growth and seed dry weight (Vigeolas et al., 2011; Nehlin, 2015; Ivarson et al., 2017). One of the LATERAL ORGAN BOUNDARIES DOMAIN (LBD) family of TF genes, Soly11g072470, was also up-regulated in the pericarp of RNAi-2Q1. Populus LBD1 is involved in the regulation of secondary growth in Populus and an activation-tagged mutant of PatLBD1 showed increased stem diameter and smaller leaves (Yordanov et al., 2010).

Table 1. Discriminative metabolites predicted by OPLS-DA with increased levels in fw3.2(wt) and RNAi-2Q1

| Retention time (min) | m/z     | Annotation                       | Average intensity (mean ±SD) |
|----------------------|---------|----------------------------------|-------------------------------|
|                      |         |                                  | fw3.2(wt)                     | fw3.2(wt)                     |
|                      |         |                                  | RNAi-2Q1                      | RNAi-2Q1                      |
| 4.49                 | 716.522 | PE_34:1 ([M-H]+)                 | 0.40±0.092                    | 0.48±0.068                    | 0.57±0.067                    |
| 4.31                 | 740.522 | PE_36:3 ([M-H]+)                 | 0.75±0.13                     | 1.1±0.14                      | 1.25±0.29                     |
| 4.39                 | 745.556 | Unknown                          | 0.60±0.059                    | 0.72±0.060                    | 0.71±0.11                     |
| 4.40                 | 744.553 | Unknown                          | 1.3±0.11                      | 1.6±0.078                     | 1.6±0.26                      |
| 4.20                 | 768.553 | Unknown                          | 0.82±0.12                     | 1.1±0.10                      | 1.3±0.29                      |
| 4.40                 | 804.575 | PC_34:1 ([M+HCOO]-)              | 4.8±0.50                      | 5.6±0.21                      | 5.7±0.92                      |
| 3.99                 | 819.526 | MGDG_36:6 ([M+HCOO]-)            | 21±1.7                        | 24±2.2                        | 26±1.5                        |
| 4.20                 | 828.575 | PC_36:3 ([M+HCOO]-)              | 3.4±0.33                      | 4.3±0.42                      | 5.3±0.99                      |
| 3.99                 | 835.533 | PI_34:1 ([M-H])                  | 1.3±0.16                      | 1.6±0.099                     | 1.5±0.22                      |
| 4.81                 | 832.606 | PC_36:1 ([M+HCOO]-)              | 0.85±0.14                     | 0.93±0.21                     | 0.93±0.20                     |
| 3.56                 | 935.574 | Unknown                          | 13±0.71                       | 14±0.86                       | 14±0.56                       |
| 3.56                 | 981.579 | DGDG_36:6 ([M+HCOO]-)            | 9.7±0.49                      | 10±0.69                       | 10±0.37                       |

Table 2. Discriminative metabolites predicted by OPLS-DA with decreased levels in fw3.2(wt) and RNAi-2Q1

| Retention time (min) | m/z     | Annotation                       | Average intensity (mean ±SD) |
|----------------------|---------|----------------------------------|-------------------------------|
|                      |         |                                  | fw3.2(wt)                     | fw3.2(wt)                     |
|                      |         |                                  | RNAi-2Q1                      | RNAi-2Q1                      |
| 4.17                 | 786.528 | Unknown                          | 0.58±0.028                    | 0.56±0.10                     | 0.45±0.07                     |
| 4.57                 | 814.559 | Unknown                          | 1.1±0.094                     | 1.0±0.12                      | 0.9±0.11                      |
| 0.27                 | 1068.510| Unknown                          | 5.4±0.80                      | 5.5±1.0                       | 5.1±0.58                      |
| 0.31                 | 1126.515| Unknown                          | 0.74±0.26                     | 0.79±0.33                     | 1.2±0.25                      |

DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.
KLUH regulates tomato fruit and seed weight. Other TF genes previously described as organ size regulators were co-expressed with KLUH in either YYM or WGM. For example, four AP2 TF genes were identified in YYM but not in WGM (Supplementary Figs S10, S11; Supplementary Table S4). Of these, three AP2 TF genes (Solyc02g064960, Solyc10g084340, and Solyc04g049800) were clustered with the APETALA-like subfamily (Supplementary Fig. S11). AT4G36920, a member of the APETALA-like subfamily, plays an important role in determining seed size and oil contents without substantial changes in seed fatty acid composition (Jofuku et al., 2005; Ohto et al., 2009; Yan et al., 2012). In rice, SUPERNUMERARY BRACT (OsSNB) was identified as a negative regulator of seed weight (Jiang et al., 2019). In tomato, AP2a (Solyc03g044300) was identified as a major negative regulator of fruit ripening via regulation of ethylene biosynthesis and signaling (Chung et al., 2010; Karlova et al., 2011). The RNAi lines of SlAP2a showed smaller fruit size than the wild type (Chung et al., 2010). Another AP2 TF gene, Solyc02g030210, is one of the orthologs of WRINKLED1 (WRII) (Supplementary Fig. S11). The positive roles of AtWR1 and its orthologs in regulating lipid metabolism and seed mass have been extensively demonstrated (Baud et al., 2009; Shen et al., 2010; Qu et al., 2012; Ma et al., 2013; Wu et al., 2014; Ivarson et al., 2017). In addition, putative auxin response factor (ARF) genes (Solyc07g043620, Solyc07g043610, and Solyc07g016180) and WUSCHEL-related homeobox (WOX) (Solyc02g077390) were identified as co-expressed genes of SIKLUH in WGM only (Supplementary Table S4). Many ARFs regulate gene expression in response to auxin, and have been identified as important regulators of organ size, including ARF2 in Arabidopsis (Schruff et al., 2006), ARF1 in rice (Aya et al., 2014), ARF18 in rapeseed (Liu et al., 2015), ARF2 in sea buckthorn (Ding et al., 2018), and ARF19 in the woody plant Jatropha curcas (Y. Sun et al., 2017). In addition, several ARFs in cucumber were identified which putatively regulate carpel number variation through interaction with the orthologs of CLV3 and WUS (Che et al., 2020). WOXs are also well known to be associated with organ size. For example, overexpression of STENOFOLIA (STF), a WOX family TF gene, significantly increases plant size, including leaf width and stem thickness, through enhancing cell proliferation (Wang et al., 2017). These data suggest that TFs may play a key role in fruit and seed weight regulation in the KLUH pathway in tomato.
Fig. 9. Network depiction of the SlKLUH-containing modules with hub genes. (A) YYM network in fw3.2(ys); (B) WGM network in fw3.2(wt). Fifty hub genes with the edge weight higher than 0.25 (A) and 0.2 (B), respectively, are visualized by Cytoscape. The pink circles represent TFs. Red lines show the edges of SlKLUH to its neighbor genes. Nodes represent genes, and node size is correlated with connectivity of the gene.

Fig. 10. Significantly enriched GO terms of the YYM (left panel) and WGM (right panel). The size of the circles indicates the number of co-expressed genes in the given GO term. The color coding indicates the gene ratio calculated as the number of co-expressed genes in the given GO term divided by the total number of genes in the term. The x-axis indicates the FDR-adjusted $P$-value.
Overexpression of SISHN1 significantly decreases fruit and seed weight

In tomato, certain lipid metabolism–related genes have been experimentally characterized for their involvement in fruit cutin biosynthesis and fatty acid elongation. To evaluate if genes that impact these two pathways also play roles in fruit and seed weight, we identified the sources of transgenic or natural mutant lines that differ in their lipid metabolism. One example is WAX INDUCER1/SHINE1 (WIN/SHN1) which encodes a transcription factor that regulates the ‘Cutin synthesis and transport’ and ‘Fatty acid elongation and wax biosynthesis’ pathways in Arabidopsis (Aharoni et al., 2004; Broun et al., 2004; Kannangara et al., 2007; Li-Beisson et al., 2010, 2013). In tomato, SHINE1 (SISHN1; Solyc03g116610) and SISHN2 (Solyc12g009490) are putative orthologs of Arabidopsis WIN/SHN1. SISHN2 showed significantly higher expression in the pericarp of fw3.2(wf) (Supplementary Dataset S2) and the RNAi-2Q1 line (Supplementary Dataset S3) compared with fw3.2(yw). On the other hand, SISHN1 showed low or undetectable expression in our datasets (0–0.1 RPKM). Overexpression of SISHN1 increases cuticular wax accumulation, resulting in improved drought tolerance in tomato (Al-Abdallat et al., 2014). To assess whether overexpression of SISHN1 affects fruit and seed weight, we evaluated these traits in the previously described SISHN1–overexpressing lines (Al-Abdallat et al., 2014). The results showed that high and ubiquitous expression of SISHN1 significantly decreased fruit weight by reducing the number of cell layers and pericarp thickness compared with the non-transgenic control (Fig. 11). Seed weight was also reduced in the overexpression lines (Fig. 11). The results imply that fruit and seed weight may be directly affected by changes in lipid metabolism by the paralog of SISHN1, SISHN2.

Tomato CD2 (Solyc01g091630), a HD-Zip TF gene, was found in the WGM, and is involved in cutin biosynthesis and wax deposition (Nadakuduti et al., 2012; Kimbara et al., 2013). We evaluated the fruit and seed weight of the cd2 mutant in the AC background. No significant differences in fruit and seed weight were found between cd2 and the control under greenhouse conditions (Supplementary Fig. S12A). In the field trial, fruit weight in cd2 was significantly higher than in the control, whereas seed weight was significantly lower than in the control (Supplementary Fig. S12B). The inconsistent results between the greenhouse and field trials as well as between the fruit and seed suggested that these traits were not significantly affected by CD2.

Discussion

SIKLUH appears to function in the cell proliferation phase at the early stages of fruit development

CYP78A is a highly conserved plant-specific subclade in the CYP450 family (Nelson, 2006; Mizutani and Ohta, 2010). Members of CYP78A are recognized to positively regulate organ weight and size as well as development in plants such as Arabidopsis (Wang et al., 2008; Fang et al., 2012; Sotelo-Silveira et al., 2013; Yang et al., 2013), rice (Nagasawa et al., 2013; Yang et al., 2013; Maeda et al., 2019), wheat (Ma et al., 2015a, b), maize (X. Sun et al., 2017), soybean (Wang et al., 2015a; Zhao et al., 2016), J. curcas (Tian et al., 2016), sweet cherry (Qi et al., 2017), tomato, and pepper (Chakrabarti et al., 2013). Different CYP78As regulate organ size differently. For example, Arabidopsis KLUH/CYP78A5 appears to affect cell proliferation at the early stages of integument growth, therefore regulating the seed size (Anastasiou et al., 2007; Adamski et al., 2009). In contrast, EOD3/CYP78A6 and CYP78A9 are primarily involved in the regulation of cell expansion phases during the later stages of integument development (Fang et al., 2012). Moreover, the rice and maize PLASTOCHRON1 (PLA1) genes stimulate leaf growth by prolonging the duration of cell division (Miyoshi et al., 2004; Mimura and Itoh, 2014; X. Sun et al., 2017). In this study, we found that SIKLUH affects pericarp cell proliferation in the early stages of fruit development (5–7 DPA). The phylogenetic analysis supported the notion that SIKLUH controls cell proliferation as it is the closest ortholog to AtCYP78A10 and AtCYP78A5 (Fig. 1).

The link between organ weight and lipid metabolism in plants

Arabidopsis KLUH is proposed to produce an unknown signaling molecule that non-cell-autonomously regulates cell proliferation in different organs (Anastasiou et al., 2007; Adamski et al., 2009; Eriksson et al., 2010). It has been hypothesized that the unknown signaling molecule might be fatty acid-derived molecules (Anastasiou et al., 2007; Eriksson et al., 2010; X. Sun et al., 2017) based on the following evidence: (i) Arabidopsis CYP78A5, CYP78A7, and CYP78A10, and maize PLA1 hydroxylate short-chain fatty acids, including lauric acid (C12:0), myristic acid (C14:0), myristoleic acid (C14:1), and palmitic acid (C16:0) (Imaishi et al., 2000; Kai et al., 2009). Similarly, activation of rice CYP78A13 decreases nicotinic acid, shikimic acid, and quinic acid contents and increases the contents of glyceric acid and palmitic acid (Xu et al., 2015). These results suggest that OsCYP78A13 might control organ growth via modification of short-chain fatty acid-derived molecules. Furthermore, OsCYP78A13 rescued the klu-4 mutant, implying that the signals produced by the CYP78A subfamily proteins are identical in rice and Arabidopsis (Yang et al., 2013; Xu et al., 2015). However, the application of exogenous 12-hydroxyalldric acid did not rescue the major phenotype of the cyp78a5/a7 double mutant (Kai et al., 2009), suggesting that the substrates catalyzed by CYP78A subfamily proteins remain elusive in plants. (ii) Cytochrome P450s catalyze various types of oxygenation reactions using fatty acids as substrates (Pinot and Beisson, 2011). Eight cytochrome P450 genes involved in fatty acid modification are transcriptionally regulated by KLUH/CYP78A5.
activity in Arabidopsis (Anastasiou et al., 2007). (iii) Many studies revealed a mechanistic link between lipid metabolism and seed size. For example, overexpression of miRNA167A results in lower α-linolenic acid content and larger seed size via decreased transcription of fatty acid desaturase3 (CsFAD3) in Camelina sativa (Na et al., 2019). Similarly, GmFAD3-silenced plants contain reduced levels of linolenic acid (18:3) and produce significantly larger seeds in soybean (Singh et al., 2011). Down-regulation of BnDof5.6 in canola reduces both embryo size and fatty acids content (Deng et al., 2015). Moreover, reduced expression of HECT E3 ligase in canola results in larger seeds with increased lipid content (Miller et al., 2019). Other studies have also supported a link between lipid metabolism and seed weight (Chen et al., 2012; Liu et al., 2016; Lunn et al., 2018; Meru et al., 2018; Guo et al., 2019). For example, the mutation of Arabidopsis TRANSPARENT TESTA2 (TT2) significantly increased the seed fatty acid content and decreased seed weight (Chen et al., 2012). Overexpression of rice ACYL-CoA-BINDING PROTEIN 2 (OsACBP2) confers an increase in grain size and seed oil content (Guo et al., 2019). Similarly, the increased seed oil is also concomitant with an increase in seed weight in transgenic lines overexpressing Arabidopsis Seipin1 (AtSEI1) (Lunn et al., 2018).

In the present study, the fvi3.2 NILs and the RNAi-2Q1 that down-regulate the expression of SIKLUH offer opportunities to reveal the molecular mechanisms controlling fruit and seed weight by this CYP78A member. DEGs between the two expression datasets, the NILs and the fvi3.2(ys)–RNAi-2Q1, were enriched for genes that were part of several lipid metabolism pathways (Figs 3D, 4C; Supplementary Fig. S6). These results provide an indication of a possible link between SIKLUH-mediated fruit and seed weight regulation and lipid metabolism in tomato such that decreased expression of SIKLUH results in increased expression of many lipid metabolism-related genes. In addition, the transgenic lines overexpressing SISHN1 that show a significant decrease in fruit and seed weight also indicate a correlation between fruit and seed weight and lipid metabolism.

In our study, we found that fruit and seed weight were significantly different between cd2 and the control under field conditions and in opposite directions (Supplementary Fig. S12B). Furthermore, the lack of consistency between field and greenhouse and the differing response suggest that CD2 has no dramatic effect on fruit and seed weight in tomato (Supplementary Fig. S12). Moreover, altered expression of other lipid metabolism-related genes such as GDSL1 (Solyc11g006250) and GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE 6 (SIGPAT6, Solyc09g014350) have no demonstrable effect on fruit weight (Girard et al., 2012; Petit et al., 2016). These results indicate the complicated relationship between lipid metabolism
and fruit and seed weight. Furthermore, co-expression analyses also did not show a tight link between SIKLH and lipid metabolism. In fact, only 37 (~2.2%) and 44 (~3.5%) lipid metabolism–related genes were identified in YYM and WGM (Supplementary Table S3), respectively. Therefore, it is possible that lipid metabolism is associated with but not directly regulated by SIKLH, and that this pathway instead is associated with photosynthesis-related processes. However, little is known about the relationships among KLUH, photosynthesis-related processes, and lipid metabolism, which need to be further studied.

**DEG and co-expression network analyses provide a valuable resource of candidate genes putatively involved in organ weight regulation in tomato and other plants**

The molecular mechanisms underpinning KLUH-mediated fruit and seed weight are poorly understood in tomato. The differential expression and co-expression analyses led to the identification of a number of candidate genes putatively involved in organ weight regulation mediated by SIKLH in tomato.

In addition to the nine TFs which are common DEGs in both the *fw3.2*(*ys*)–*fw3.2*(*wt*) and the *fw3.2*(*ys*)–RNAi-2Q1 datasets (Supplementary Table S4), many DEGs encoding enzymes or transporters in ‘Cutin synthesis and transport’ and ‘Fatty acid elongation and wax biosynthesis’ pathways were also identified (Figs 5, 6). Moreover, some of them are putatively involved in both plant development and lipid metabolism based on previous studies, including HOTHEAD (HTH; *AT1G72970*) (*Soly06g062600*), DEFECTIVE IN CUTICULAR RIDGES (DCR; *AT5G23940*) (*Soly03g025320*), ATP-BINDING CASSETTE G11 (ABCG11; *AT1G17840*) (*Soly01g105450*), and lipid transfer protein (LTP) genes. HTH, catalyzing the biosynthesis of long-chain α,ω-dicarboxylic fatty acids, is required for the prevention of organ fusions in floral organs in Arabidopsis and rice (*Krolkowsi et al.*, 2003; *Kurdyukov et al.*, 2006; *Akiba et al.*, 2013; *Xu et al.*, 2017). DCR is involved in cutin and triacylglycerol biosynthesis (*Panikashvili et al.*, 2009; *Rani et al.*, 2010). The *dcr* mutants had wider and longer seeds than the wild type (*Rani et al.*, 2010). ABCG11 is involved in sterol/lipid homeostasis and vascular development in addition to plant growth (*Panikashvili et al.*, 2010, 2011; *Le Hir et al.*, 2013; *Yadav et al.*, 2014). Seven out of eight LTP genes were down-regulated in RNAi-2Q1 compared with *fw3.2*(*ys*) (Fig. 6). LTPs are known to affect cuticle biosynthesis and transport, as well as seed development (*Kim et al.*, 2012; *Wang et al.*, 2015b; *Deng et al.*, 2016; *Koudri et al.*, 2018). Notably, tomato CUTIN SYNTHASE1 (*SICUS1*; *Soly11g006250*), an important gene involved in the ‘Cutin synthesis and transport’ pathway, was up-regulated in 7 DPA pericarp of *fw3.2*(*wt*) and RNAi-2Q1 (Fig. 5). It encodes GDSL–motif esterase/acyltransferase/lipase protein and has been shown to be associated with both lipid metabolism and epidermal cell development (*Segado et al.*, 2020).

In Arabidopsis, nine cytochrome P450 genes were transcriptionally affected by mutations in CYP78A5/KLUH (*Anastasiou et al.*, 2007) of which eight were linked to fatty acid modifications. In our datasets, only CYP76C4 (*Soly02g090350*, *AT2G45550*) was identified as a down-regulated DEG in 7 DPA pericarp and seeds in RNAi-2Q1 (Supplementary Dataset S3), but not in the NILs, suggesting a role for CYP76C4 in the KLUH pathway in both tomato and Arabidopsis. Further studies are required to confirm the biological and biochemical functions of CYP76C4 and its relationship to KLUH in Arabidopsis and tomato.

Co-expression network analyses identified common and unique genes sets between YYM and WGM including many TFs putatively associated with fruit and seed weight in tomato (Supplementary Table S4). Co-expressed genes of interest that are not TF genes in YYM and WGM were also found. For example, the RING-type E3 ubiquitin ligase EOD1 (*AT3G63530*) (*Soly01g062260*) was identified as a negative regulator of organ size (*Disch et al.*, 2006; *Li et al.*, 2008; *Li and Li*, 2015; *Vanhaeren et al.*, 2017). The *cod1* mutants had larger organs and increased biomass, while overexpression of EOD1 resulted in reduced organ growth (*Disch et al.*, 2006; *Xia et al.*, 2013). Arabidopsis DWF4 (*AT3G50660*) (*Soly02g093540*) encodes a C-22 hydroxylase that catalyzes a rate-determining step in brassinosteroid biosynthesis. Overexpression of DWF4 significantly increased seed number and weight, thus increasing seed yield in Arabidopsis (*Choe et al.*, 2001), *Brassica napus* (*Sahni et al.*, 2016), and rice (*Wu et al.*, 2008).

Together, we propose many DEGs and co-expressed genes that are putatively involved in the fruit and seed weight regulation mediated by SIKLH. This knowledge is helpful to elucidate the whole picture of the KLUH pathway regulating organ size in tomato and other crops. However, the exact functions of these candidate genes remain to be studied in tomato.

**Conclusion**

Our results reinforce the notion that lipid metabolism is involved in SIKLH-mediated regulation of fruit and seed weight through a possible mechanism as proposed in Fig. 12. The differential expression of SIKLH between the NILs results in different co-expression networks that are associated with fruit and seed development, possibly through modulating photosynthesis-related processes. The TFs identified in YYM and WGM are putative upstream regulators of SIKLH. In the small-fruited NIL *fw3.2*(*wt*) and RNAi-2Q1, lower expression of SIKLH is associated with increased expression of many genes involved in lipid metabolism, especially for genes involved in ‘Cutin synthesis and transport’ and ‘Fatty acid elongation and wax biosynthesis’. Thus, the contents of certain non-phosphorus glycerolipids and phospholipids were...
increased while the contents of the four unknown lipids were decreased. Importantly, a number of lipid-related genes and TFs putatively involved in the regulation of fruit and seed weight in tomato were also identified, providing potential targets for further dissecting the molecular mechanisms underlying fruit and seed weight in tomato and other crops.

**Supplementary data**

The following supplementary data are available at JXB online.

Table S1. Summary of RNA-seq mapping for all samples.

Table S2. Shared DEGs in 7 DPA pericarp and seed in *fw3.2*(ys)–*fw3.2*(wt) and *fw3.2*(ys)–RNAi-2Q1 comparisons.

Table S3. Lipid-related DEGs in the *fw3.2*(ys)–*fw3.2*(wt) and *fw3.2*(ys)–RNAi-2Q1 comparisons.

Table S4. Common transcription factors identified in *fw3.2*(ys)–*fw3.2*(wt) and *fw3.2*(ys)–RNAi-2Q1 comparisons and transcription factors in YYM and WGM.

Table S5. Lipid-related genes in YYM and WGM.

Fig. S1. Developing fruit at six developmental time points in the *fw3.2* NILs.

Fig. S2. Phenotypic evaluations of the NILs in the second replication.

Fig. S3. Spearman correlation coefficient (SCC) analysis of transcriptomic profiles of the 48 replicates from *fw3.2*(ys) and *fw3.2*(wt).

Fig. S4. Spearman correlation coefficient (SCC) of transcriptomic profiles of all 12 replicates from transgenic lines RNAi-2G2 and RNAi-2Q1 that down-regulate *SIKLUH*.

Fig. S5. Up- and down-regulated genes in pericarp and seed at 7 DPA in the RNAi-2Q1 or *fw3.2*(wt) compared with the *fw3.2*(ys).

Fig. S6. Overview of the distribution of the DEGs in lipid metabolism pathways.

Fig. S7 Comparison of known lipids of 5 DPA fruits from *fw3.2*(ys), *fw3.2*(wt), and RNAi-2Q1.

Fig. S8. Comparison of co-expressed genes in YYM and WGM.

Fig. S9. Expression profiles of *Soly04g074990* (ZF-HD), *Soly08g079800* (GRF), and *Soly04g077510* (GRF) in developing pericarp and seeds in the NILs.

Fig. S10. Overview of distribution of TF families that were co-expressed with *SIKLUH* in YYM and WGM.

Fig. S11. Phylogenetic analysis of AP2 transcription factors from tomato, Arabidopsis, and five WRI1 orthologs from other plant species.

Fig. S12. Fruit weight and 50 seeds weight of *cd2* and Ailsa Craig (AC) control from the greenhouse and field trial.

Dataset S1. DEGs at different developmental time points of pericarp and seed in the *fw3.2* NILs.

Dataset S2. DEGs significantly affected by genotype in the *fw3.2* NILs.

Dataset S3. DEGs in 7 DPA pericarp and seed between *fw3.2*(ys) and RNAi-2Q1.

Dataset S4. Co-expressed genes in the 11 modules identified in *fw3.2*(ys) using WGCNA.

Dataset S5. Co-expressed genes in the 10 modules identified in *fw3.2*(wt) using WGCNA.

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Author contributions

Conceptualization, QL and EvdK; Investigation, QL, MC, NKT, YO, KS, AA-A, and EvdK; Software, QL, NKT, and YO; Resources, AA-A and EvdK; Data curation, QL, MC, YO, KS, and EvdK; Formal analysis, QL, MC, and YO; Visualization, QL and YO; Validation, QL, MC, and EvdK; Supervision, EvdK; Project administration, EvdK; Writing—original draft, QL; Writing—review and editing, MC, NKT, YO, KS, AA-A, and EvdK; Funding acquisition, QL and EvdK;

Conflict of interest

The authors declare no competing interests.

Data availability

The data supporting the findings of this study are available from the corresponding author, EvdK, upon request.

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