Decreased number of locules and pericarp cell layers underlie smaller and ovoid fruit in tomato smaller fruit (sf) mutant

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Title: Decreased number of locules and pericarp cell layers underlie smaller and ovoid fruit in tomato smaller fruit (sf) mutant

Short title: Characterization of the tomato sf mutant

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Abstract

Fruit size and shape are the prime criteria for domestication and improvement of tomato. The varying sizes and shapes of tomato fruits further signify their importance as agronomic traits. Here, we characterized a tomato mutant, smaller fruit (sf), which bears relatively small and ovoid fruits compared with large and flat fruits of wild-type (WT). Phenotypic measurements and histological analyses revealed that fruit diameter but not fruit length of sf mutant decreased compared with that of WT. This phenotypic change was attributed to significant decreases in locule number and pericarp cell layers in transverse direction, which resulted in the transition of fruit shape from flat in WT to ovoid in sf. Comparison of transcriptomes of ovaries between sf and WT by RNA-Seq identified 2,596 differentially expressed genes, in which 1,737 genes significantly up-regulated and 859 genes dramatically down-regulated in the sf ovary. Further analyses confirmed that some genes, such as CRCa, CNRs, CYCs, WUS, SUNs, OFRs, CDKs, participate in regulation of fruit size and shape of sf mutant. Thus, our study adds a new genetic resource regarding fruit size and shape of tomato, and provides a valuable basis for dissecting molecular regulation of small and ovoid fruit of sf mutant.

Keywords: Cell number; locule number; smaller and ovoid fruit; tomato; transcriptome
Introduction

Fruit, a plant organ in angiosperms, develops from the mature ovary. This seed-bearing structure not only safeguards seed development, but also acts as an agent for seed dispersal (Azzi et al. 2015). Moreover, as a source of food, fruits provide humans a wide range of essential nutrients and minerals. Tomato (*Solanum lycopersicum*) has been cultivated worldwide as an important vegetable due to its high nutritional value and unique taste. In addition, tomato is considered as an ideal model plant for studying fruit development, shape, size, and quality (Chusreeaeom et al. 2014a; de Jong et al. 2015). As the key agronomic traits, fruit size and shape greatly affect the domestication and varietal improvement of tomato (Paran and van der Knaap 2007; Czerednik et al. 2015; Li and He 2015). Moreover, these traits greatly influence the preference of the consumers for tomato fruits.

In plants, cell number and cell size are attributed to cell division and cell expansion, respectively. Although these processes act as crucial determinants of fruit size and shape, the ultimate development is determined by their combined effects (Czerednik et al. 2015). Fruit development can be divided into two broad phases, including early fruit development and fruit ripening (Gillaspy et al. 1993). The early development of tomato fruit comprises three distinct phases (Gillaspy et al. 1993; Bisbis et al. 2006; Mathieu-Rivet et al. 2010; Azzi et al. 2015). In phase I, ovary development, fertilization, and fruit set occur. While in phase II, the ovary further develops
by profound cell division for 7-10 days after anthesis, resulting in an increased cell number. During phase III, the fruit continues to grow through cell expansion until ripening towards almost its final size and shape. At the end of the phase III, endoreduplication causes a remarkable increase in DNA ploidy and cell size, leading to the development of a fleshy pericarp tissue (Cheniclet et al. 2005; Nafati et al. 2011).

Cyclins and cyclin-dependent protein kinases are the vital components in the cell cycle that regulate cell division and expansion in plants (Inzé and De Veylder 2006; Jiang et al. 2015). Cyclins serve as the key regulators of mitotic activity and include 10 types (Wang et al. 2004), in which, A- and B-type cyclins help drive the cells into the M phase, whereas C-, D-, and E-type cyclins are responsible for the G1 to S phase transition (Potuschak and Doerner 2001; Zhang et al. 2014). In tomato, the expression of various classes of cyclins increases during the early fruit development (Joubes et al. 2000). Among the three D-type cyclin genes, D3 cyclin is involved in young fruit growth through cell division (Kvarnheden et al. 2000). Notably, the activity of cyclin-dependent kinases is controlled by the interaction with cyclins (Inzé and De Veylder 2006). Several studies reported that the cyclin-dependent kinase genes can modulate cell division and expansion in tomato fruits (Czerednik et al. 2012; Azzi et al. 2015; Czerednik et al. 2015).

Tomato fruit size is controlled by many genetic loci and a series of genes underlying these loci (Monforte et al. 2014). The first quantitative trait locus (QTL) cloned from tomato is *fruit weight2.2* (*fw2.2*) that encodes the Cell Number Regulator (CNR), a repressor of cell division (Frary et al. 2000; Guo et al. 2010; Guo and Simmons 2011). *FW2.2* gene regulates fruit size at
least in part by controlling the cell number of carpel before anthesis, and the mutation in FW2.2 accelerates research in the domestication of increased tomato fruit size (Frary et al. 2000). Moreover, up to 30% of tomato fruit weight variation is controlled by FW2.2 (Frary et al. 2000). Additionally, FW2.2-like genes have been reported in some other plant species, such as maize (Zea mays), rice (Oryza sativa), soybean (Glycine max), avocado (Persea americana), Prunus avium, and Physalis floridana (Dahan et al. 2010; Guo et al. 2010; Libault et al. 2010; Guo and Simmons 2011; De Franceschi et al. 2013; Xu et al. 2013; Li and He 2015), indicating a conservative role for FW2.2-like genes in cell division across various plant species. Recently, another QTL controlling tomato fruit weight, fw11.3, was identified, and shown to regulate mesocarp cell size but not number of cell layers (Mu et al. 2017).

Tomato fruits show a great diversity in shapes, such as flat, round, oxheart, ellipsoid, long, obovoid, heart, and rectangular fruits (Monforte et al. 2014; Azzi et al. 2015). This diversity is largely caused by mutations in four genes, namely OVATE, SUN, FASCIATED (FAS), and LOCULE NUMBER (LC), in which, OVATE and SUN regulate fruit elongation, FAS and LC mediate locule number of the fruit (Rodriguez et al. 2011; van der Knaap et al. 2014). Nonetheless, locule number has a pleiotropic effect on fruit size and shape. OVATE, which encodes a protein from the Ovate Family Protein (OFP), functions as a negative regulator of growth, leading to reduced cell elongation and fruit length (Liu et al. 2002; Hackbusch et al. 2005; Wang et al. 2007). Near-isogenic lines (NILs) of tomato carrying the ovate mutation show an increase in fruit elongation as a result of ovary cell proliferation in the proximal region,
whereas the ovary shape remains elongated at the anthesis stage but consequently grows with a slower rate of elongation during fruit development, suggesting that the regulation of *OVATE* on fruit shape is established before anthesis in tomato (van der Knaap and Tanksley 2001; Monforte et al. 2014; van der Knaap et al. 2014). *SUN* encodes a member of the IQD family of calmodulin-binding proteins and positively regulates the development of elongated fruit (Abel et al. 2005; Xiao et al. 2008). The effect of *SUN* on tomato fruit elongation is much more evident than that of *OVATE* (van der Knaap et al. 2014). Overexpression of *SUN* increases cell number in the longitudinal direction but reduces cell number in the transverse direction of the fruit, resulting in an elongated fruit of tomato (Wu et al. 2011). Moreover, the dramatic change in fruit shape controlled by *SUN* occurs at the cell division stage during fruit development (van der Knaap and Tanksley 2001).

The locule number originates from the carpel number within the flower. The increase in locule number usually results in a flat and large tomato fruit (Tanksley 2004; Rodriguez et al. 2011). A mutation in *FAS* leads to an increase in locule number from two to more than seven in tomato fruit (Lippman and Tanksley 2001). The *fas* mutant was previously demonstrated to be caused by mutation in a *YABBY* transcription factor (Cong et al. 2008). However, recent studies reported that the partial loss of *CLV3* (*CLAVATA3*) expression underlies the *fas* mutation (Xu et al. 2015). *LC* functions as a key regulator for the number of carpel primordia, and the *lc* mutation shows a weaker effect on increases of locule number than *fas* (Barrero et al. 2006; Muños et al. 2011). Several studies have reported that tomato *WUSCHEL* (*WUS*), a transcription factor belonging to
the WUS homeobox (WOX) family, is the most likely candidate for lc locus (Monforte et al. 2014; van der Knaap et al. 2014; Azzi et al. 2015; Li et al. 2017). Silencing of SlWUS decreases the number of tomato fruit locules (Li et al. 2017). Moreover, up-regulation of AtWUS gives rise to an increase in the number of floral organs in Arabidopsis, similar to the lc phenotype (Mayer et al. 1998; Clark 2001; Muños et al. 2011).

In this study, we identified a tomato mutant based on fruit size and shape, smaller fruit (sf), through ethyl methanesulfonate (EMS) mutagenesis. To explore the anatomical and genetic bases for the variation of fruits size and shape in sf mutants, a histological study and comparative transcriptome profiling analyses were performed.

**Materials and Methods**

**Plant materials**

The tomato sf mutant was developed by EMS mutagenesis in the background of the inbred line TTD302A following Saito’s method (Saito et al. 2011). In brief, 2,200 TTD302A seeds were treated with 0.7% EMS for 8 h, and followed by rinsing with water. Through germination and cultivation, 663 plants survived, of which 182 individual plants displayed variations in various organs including stems, leaves, flowers, and fruits. The sf mutant was chosen for studying fruit development, and then advanced upto six generations by selfing prior to this work. In this study, the plants of WT TTD302A and sf mutant were grown in a standard greenhouse which located in the experimental field of the Northwest A&F University. Environmental management and pest control were carried out according to standard practices (Feng 2016).
Histological analysis

The ovaries or fruits at -3, 0, 3, 5, 10, and 15 days after flowering (DAF) were collected and fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer with pH 7.2, vacuum-infiltrated for 30 min, and left overnight. The fixed samples were dehydrated in ethanol, embedded in paraplast (Sigma-Aldrich, Steinheim, Germany), and sectioned using a RM2235 rotary microtome (Leica, Solms, Germany) and Leica 819 blades (Solms, Germany) into 5 μm thick ribbons. After removal of paraplast with xylene, the sections were stained with 0.1% toluidine blue in sodium phosphate buffer (pH 7.0), and observed using an Olympus BX 51 microscope (Tokyo, Japan) equipped with an Olympus DP72 digital camera (Tokyo, Japan).

The number of cells alongside a line through the pericarp perpendicular to the epidermis and endocarp (avoiding vascular tissues) was measured to determine the number of pericarp cell layers (Carrera et al. 2012). Six ovaries or fruits (derived from three individual plants which were grown in the greenhouse) from each of the 6 stages were cut to measure the number of pericarp cell layers and pericarp thickness.

RNA extraction and quality test

The ovaries from WT and sf mutant were collected at -3 DAF at the same time of the day, and immediately frozen in liquid nitrogen. Then the samples were stored at -80°C until isolation of RNA for RNA-Seq analyses. We used a RNA extraction kit to isolate total RNA from the samples (Promega, USA). Then the purity of RNA was examined using the NanoPhotometer spectrophotometer (IMPLEN, CA, USA). Notably, we used RNase-free agarose gel
electrophoresis to get rid of any potential degradation and/or contamination. The concentration of RNA was examined with the Qubit RNA Assay Kit in Qubit 2.0 Fluorometer (Life Technologies, CA, USA) followed by assessment of RNA integrity with RNA Nano 6000 Assay Kit in the Bioanalyzer 2100 system (Agilent Technologies, CA, USA), respectively.

**RNA-Seq library construction and sequencing**

For the construction of RNA-Seq libraries, we used the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB, Ipswich, USA) according to the manufacturer’s instructions. We added six index codes to attribute sequences to various samples (Wang et al. 2009). In brief, we used oligo-dT magnetic beads to isolate poly (A) mRNA from 3 µg total RNA (Life technologies, CA, USA), and then a fragmentation buffer was added to break them into short fragments. After the synthesis of the first-strand cDNA through random hexamer-primed reverse transcription, the second-strand cDNA was synthesized using RNase H and DNA polymerase I. Then the 3’ ends of cDNA fragments were adenylated and the NEBNext adapter oligonucleotides were ligated. We used AMPure XP system to purify the cDNA fragments (Beckman Coulter, Beverly, USA) and the fragments having 150-200 bp in length were preferentially selected. Then we enriched the size-selected and adaptor-ligated cDNA fragments with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index Primer in the PCR reaction. Purification of PCR products was accomplished with an AMPure XP system, whereas the library quality was evaluated with the Agilent Bioanalyzer 2100 system. Finally, we used the paired-end technology to sequence the cDNA libraries on an Illumina HiSeq 4000 platform. For each genotype (WT and sf mutant), two
biological replicates were done, which eventually generated four libraries altogether. All four libraries were then sequenced.

**RNA-Seq data analysis by bioinformatics means**

Through pre-processing of raw reads, low quality sequences as well as reads with more than 5% N bases (bases unknown) and reads containing adaptor sequences were removed. Notably, more than 50% bases were with a quality lower than 20 in each sequence. Then we used TopHat to map the clean reads to the tomato reference genome (Trapnell et al. 2009; Consortium 2012), which allowed no more than one mismatch. Unigenes that were mapped by at least one read, in at least one sample, were used for additional analysis. The R package edgeR was used to identify the differentially expressed genes (Robinson et al. 2010). The level of each gene expression was calculated and normalized to FPKM (Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced). The threshold of the $P$-value in multiple tests was determined using the false discovery rate (FDR). Significant expression differences were considered when the FDR < 0.05 and fold change > 2.

Finally, we deposited the sequencing data to the Short Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI) under the accession number SRS2500645.

**Gene Ontology (GO) enrichment analysis**

The GOseq R package was used for the GO enrichment analysis of differentially expressed genes (Young et al. 2010). Significant enrichment was set with corrected $P$-value < 0.01 of GO terms.
qRT-PCR assay for verification of RNA-seq

To verify RNA-seq data, we separately collected tomato ovaries from both genotypes, which were developmentally similar to the samples of RNA-Seq. We used a RNA extraction kit (Promega, USA) for the isolation of total RNA and the PrimeScript RT reagent Kit (TaKaRa, China) for the synthesis of cDNA, respectively. qRT-PCR assay was performed with SYBR Premix Ex Taq (TaKaRa, China) on an ABI 7500 Real-Time PCR System (Applied Biosystems, USA). For normalization of qRT-PCR data, the tomato EF-1α was used as an internal control gene. Each experiment was replicated with three independent samples. Primers used for qRT-PCR are presented in Table S1.

Results

Phenotypic analysis of sf fruit

The WT TTD302A tomato plant was characterized by large and flat fruits, whereas the sf mutant yielded smaller and ovoid fruits. Notably, these morphological differences of fruits were apparent even during early stages of fruit development (10 and 15 DAF) and continued until the mature red stage (Figure 1A). In addition, we compared some traits of the mature red fruits between sf mutant and WT. As shown in Table 1, while the fruit diameter significantly decreased in the sf mutant, the fruit length remained unchanged compared with that of WT, leading to an elevated fruit shape index (length/diameter ratio) and reduced fruit weight. Moreover, a remarkable difference was found for locule number (Figure 1B). For instance, the sf fruit exhibited 2.6-fold decrease in locule number compared with that of the WT fruit (Table 1). Given that the increase
in locule number usually leads to a large and flat fruit in tomato (Tanksley 2004; Rodriguez et al. 2011), we speculated that the decrease in locule number might be a critical factor determining the smaller and ovoid fruit phenotype in the sf mutant.

To further evaluate whether the changes in the fruit size and shape of the sf mutant were caused by the developing ovary, we performed a time-course comparison of ovary morphology at -3, 0, 3, and 5 DAF between sf mutant and WT (Figure 2A). As expected, no difference in ovary length was observed between the sf mutant and WT, whereas ovary diameters significantly decreased in the sf mutant, and this phenotype was apparent as early as -3 DAF (Figure 2B). We then carried out histological analyses on the ovary/fruit transverse sections of the sf mutant and WT from -3 to 15 DAF (Figure 3A). Surprisingly, we did not observe any significant changes in both number of pericarp cell layers and pericarp thickness of ovaries at -3, 0, 3, and 5 DAF between the sf mutant and WT (Figure 3B-C). However, at 10 and 15 DAF, the number of cell layers in the pericarp of sf fruits was significantly lower than that in WT fruits, which was also associated with the decrease in pericarp thickness (Figure 3B-C). In addition, no change in cell size was observed in the pericarp of ovaries/fruits in the examined stages between the sf mutant and WT (data not shown). This result indicated that the reduced number of pericarp cell layers in the transverse plane of the fruit also contributed to the fruit phenotype in the sf mutant, but not to ovary development.

**Differentially expressed genes (DEGs) in ovaries of sf mutant and WT**
Genome-wide expression analyses were performed to identify genes that regulate fruit size and shape in the sf mutant. Given that the phenotypic changes occurred before anthesis (Figure 2A), we chose the ovaries at -3 DAF for RNA-Seq analyses. As indicated in Table 2, 41.13-51.56 million raw reads from each RNA-Seq library were generated. When adapter sequences and low-quality reads were removed, 40.36-50.52 million clean reads with a total of 6.05-7.58 G bases were found. Out of these clean reads, Q20 (with the base quality of more than 20) and GC were 97.78%-98.08% and 41.93%-42.34%, respectively. Afterwards, these clean reads were clustered into unique reads and mapped to the tomato genome with TopHat (Trapnell et al. 2009; Consortium 2012). In the four libraries, about 36.27-45.14 million clean reads (88.48%-90.30% of total clean reads) were mapped uniquely into the tomato genome.

Through deep sequencing, 20,733 genes were obtained from all libraries. Based on the FDR < 0.05 and fold change > 2 as the threshold of significance, 2,596 DEGs were identified. Among these DEGs, 1,737 genes were significantly up-regulated and 859 genes were significantly down-regulated in the ovaries of the sf mutant compared with those of the WT (Table S2). To verify the DEGs identified by RNA-Seq, qRT-PCR assays were performed using the independent ovaries of both genotypes, which were developmentally similar to the samples used for the transcriptome sequencing. Moreover, 20 DEGs, consisting of 14 up-regulated genes and 6 down-regulated genes, were randomly selected for qRT-PCR analysis. Despite the differences in the particular values of fold-change, the expression patterns of all the 20 genes in the qRT-PCR assays were similar to those in the RNA-Seq data (Figure 4). The pearson correlation coefficient
between qRT-PCR and RNA-Seq data (0.971, \( P = 1.2E-12 \)) further verified the high reliability of the RNA-Seq data.

**Fruit size- and shape-related genes involved in the development of the sf fruit in tomato**

Given that the smaller and ovoid fruit phenotype was attributed to the numbers of locules and pericarp cell layers at the transverse direction of fruit in the sf mutant (Figure 1, 2, and 3; Table 1), we screened the fruit size- and shape-related genes in the DEGs. The transcript levels of *CRABS CLAW a* (*CRCa*) and two *CNRs*, *CNR2* and *CNR10* significantly increased by 20.27-, 3.78-, and 4.04-fold, respectively, in the sf ovary compared with those in the WT ovary (Table 3), and the qRT-PCR validation showed the same expression pattern (Figure 4). *CRCa*, a *YABBY* gene, is required for carpel polarity, specifically, radial growth and elongation (Alvarez and Smyth 1999; Bowman and Smyth 1999). The tomato *CRCa* displays a close phylogenetic relationship with *SlYABBY2b* (Huang et al. 2013), which was previously identified as the *FAS* gene, a key negative regulator of locule number (Lippman and Tanksley 2001; Cong et al. 2008). Accordingly, we speculated that *CRCa* might positively influence the formation of the sf fruit phenotype in tomato, particularly with a reduced locule number. Moreover, given that the *CNR* gene acts as a cell division repressor (Frary et al. 2000; Guo et al. 2010; Guo and Simmons 2011), we generalized that the decreased cell number in the sf fruit might be caused, at least in part, by the induction of *CNR2* and *CNR10* expression levels. In the sf mutant, we further identified two down-regulated genes, *CYCB2;5* (*Cyclin B2;5*) and *CYCU1;1* (*Cyclin U1;1*) belonging to the cyclin family, which are the key regulators in cell cycle machinery (Zhang et al.
2014) (Table 3), supporting that these two genes might also be associated with fruit size and shape in tomato.

Moreover, \textit{WUS}, which is presumably a candidate for \textit{lc} locus and a regulator of locule number (Monforte et al. 2014; van der Knaap et al. 2014; Azzi et al. 2015; Li et al. 2017), was up-regulated in the \textit{sf} ovary (Table 3), suggesting that the changed expression of \textit{WUS} might affect locule number of the \textit{sf} fruit. In addition, several \textit{SUN} and \textit{OFP} family members were also differently expressed in the ovaries of the \textit{sf} mutant compared with those of WT. For example, EMS mutation down-regulated the transcripts of \textit{SUN}, \textit{OFP15}, \textit{OFP17}, and \textit{OFP19}, but up-regulated the \textit{SUN19} and \textit{OFP11} expressions in the \textit{sf} ovary (Table 3), indicating that these genes might also participate in the development of the \textit{sf} fruit.

\textbf{Two cell division protein kinase (CDK) genes are associated with the regulation of fruit size and shape of \textit{sf} mutant in tomato}

Gene ontology (GO) term enrichment analysis (Corrected \textit{P}-value < 0.01) enabled us to understand the probable functions of identified DEGs. The up-regulated genes were grouped into two major categories, namely, biological process and molecular function (Figure 5). The most significantly enriched GO terms were “protein phosphorylation” \textit{(P = 2.2E-08)} and “protein kinase activity” \textit{(P = 2.2E-09)} in the biological process and molecular function groups, respectively (Figure 5, Table S3). Further, in these two GO terms, two \textit{CDK} genes, \textit{CDK10} and \textit{CDK12}, were identified to be up-regulated by 2.39- and 2.17-fold, respectively, in the \textit{sf} mutant compared with those in WT (Table 4, Table S3). These data indicated that \textit{CDK10} and \textit{CDK12}
might be associated with the regulation of fruit size and shape in tomato and play positive roles in the formation of the *sf* fruit phenotype. In addition, for genes that were down-regulated in the *sf* mutant, no significantly enriched GO terms were found (Table S4).

**Discussion**

**Fruit phenotype in the *sf* mutant is attributed to significant decreases in locule number and pericarp cell layers of transverse direction**

The natural diversity in size and shape of tomato fruits has facilitated the isolation of a good number of fruit size and shape mutants in tomato (Guo et al. 2010; Carrera et al. 2012; Chusreeaeom et al. 2014a, b). Among the factors determining the size and shape of a fruit, cell division, cell expansion, and locule number play crucial roles in fruit size and shape phenotypes in tomato (Czerednik et al. 2015). In this study, we identify a tomato *sf* mutant in the background of TTD302A, a cultivated variety with flat fruits (Figure 1). Compared with WT TTD302A, the fruits of the *sf* mutant are smaller and ovoid. This phenotype is possibly due to the decreased fruit diameter and unchanged fruit length in the *sf* mutant (Figure 1, Table 1). Interestingly, the observed phenotype remains apparent even in the ovary developmental stage (-3 DAF) (Figure 2). Further observation reveals that the locule number, which originates from the carpel number within the flower, is significantly reduced in the *sf* fruit (Figure 1, Table 1). However, the number of pericarp cell layers in the transverse plane is lower in fruits at 10 and 15 DAF but remains unaffected in ovaries at -3, 0, 3, and 5 DAF (Figure 3). These results suggest that the fruit size and shape of the *sf* mutant are collectively controlled by locule number and transverse cell
division, where the locule number may play a dominant role during ovary development and the very early stages of fruit development. In addition, the cell size is not different between sf and WT fruits (data not shown), indicating that the cell expansion is not involved in the sf phenotype.

**sf fruit development is regulated by some fruit size- and shape-related genes**

Previous studies showed that locule number of tomato fruit can be regulated by *FAS* and *LC* (Rodriguez et al. 2011; van der Knaap et al. 2014), and *FAS* has a more pronounced effect on locule number than *LC* (Barrero et al. 2006; Muños et al. 2011). *FAS* was previously considered as a *YABBY* transcription factor, *SlYABBY2b* (Lippman and Tanksley 2001; Cong et al. 2008; Huang et al. 2013). Nevertheless, recent studies demonstrated that the *fas* mutation was caused by the down-regulation of *SlCLV3* (Xu et al. 2015). Although the real candidate gene for *fas* locus remains disputed, both *SlYABBY2b* and *SlCLV3* can modulate locule number in tomato, because the introduction of *SlYABBY2b* or *SlCLV3* can both rescue the *fas* phenotype in different extents, leading to a low locule number and small fruit size (Cong et al. 2008; Xu et al. 2015). In the current study, no difference is found in *SlYABBY2b* (*Solyc11g071810*) and *SlCLV3* (*Solyc11g071380*) expression levels between sf and WT ovaries through RNA-Seq analysis (Table S2), suggesting that *SlYABBY2b* and *SlCLV3* do not contribute to the sf fruit phenotype. However, another *YABBY* transcription factor, *CRCa*, was induced by the mutation in the sf ovary (Table 3). In tomato, the *YABBY* family can be divided into five groups: *CRC*, *IND* (*INDEHISCENT*), *YABBY1*, *YABBY2*, and *YABBY5* (Huang et al. 2013). *CRC* acts as a reproductive *YABBY* gene, and is only expressed in floral organs (Alvarez and Smyth 1999;
AtCRC expression in *Arabidopsis* is mostly limited to carpels and nectaries, and the mutation of *AtCRC* leads to a wider but shorter pistil, indicating that *AtCRC* is involved in inhibiting early radial growth and promoting later longitudinal growth of the developing pistil (Alvarez and Smyth 1999; Bowman and Smyth 1999). *CRCa* of tomato shows a close phylogenetic relationship with *SlYABBY2b*, and is specifically expressed in the flower buds during early stages of development (10 days or more before anthesis) (Huang et al. 2013). Taken together, we speculate that *CRCa* may display, at least in part, similar functions with *SlYABBY2b* and *AtCRC*, and serve as a negative regulator of carpel/locule number and radial growth of tomato ovary/fruit, based on its increased expression in the ovary at -3 DAF of the *sf* mutant, which carries a reduced locule number and fruit diameter (Figure 1, Table 1, Table 3). However, the effect of *CRCa* on ovary/fruit elongation remains unclear, owing to the fact that both ovary and fruit lengths are unchanged in the *sf* mutant compared with those in WT (Table 1). Moreover, *WUS* is up-regulated in the *sf* ovary (Table 3). This phenomenon is unusual because *WUS* serves as a positive regulator of locule number, and silencing it decreases fruit locules in tomato (Monforte et al. 2014; van der Knaap et al. 2014; Azzi et al. 2015; Li et al. 2017). Nevertheless, the fold change (*sf* vs WT) in *WUS* is much lower than that of *CRCa* (Table 3), implying that the up-regulation of *CRCa* may be a dominant determinant of decreased locules in the *sf* mutant, although an increased expression of *WUS* might affect locule number of the *sf* fruit, this influence is weak and insufficient for countering the effect of *CRCa* on locule number.
CNR, a repressor of cell division in plants, and FW2.2, an important CNR gene of tomato, can regulate the cell number of carpel before anthesis and subsequently change fruit size (Frary et al. 2000). Some CNR genes in other species have been demonstrated to play conserved roles in cell number control, such as ZmCNR1 in maize (Guo et al. 2010; Guo and Simmons 2011), fruit weight2.2 like3 (OsFWL3) in rice (Xu et al. 2013), fruit weight2.2-like1 (GmFWL1) in soybean (Libault et al. 2010), fruit weight2.2-like (Pafw2.2-like) in avocado (Dahan et al. 2010), PavCNR in Prunus avium (De Franceschi et al. 2013), and PfCNR1 in Physalis floridana (Li and He 2015), and all of these genes are putative homologues of tomato FW2.2. However, our study suggests that FW2.2 is not involved in the regulation of cell division in the sf mutant, based on the data of unchanged expression of FW2.2 between sf and WT ovaries (Table S2). Nevertheless, another two CNR genes, CNR2 and CNR10, may be involved in negative regulation of cell division in tomato fruit, owing to the fact that they are up-regulated in the sf fruit (Table 3), which exhibits a decreased pericarp cell number in transverse sections (Figure 3). Therefore, our results provide a new perspective of the regulation of other CNRs, except FW2.2 and its orthologues, in cell division. Furthermore, previous reports indicated that the CYC gene functions as an important downstream component in the regulation of CNR on cell division (Li and He 2015). Additionally, our data show that the expressions of two CYC genes, CYCB2;5 and CYCU1;1, are negatively correlated with the mRNA levels of CNR2 and CNR10 in the sf mutant (Table 3). Accordingly, we predict that CNRs may decrease the cell number, at least in part, through repressing the CYCs in tomato fruit, which will be confirmed in future studies.
SUN and OVATE can mediate tomato fruit elongation (Rodriguez et al. 2011; van der Knaap et al. 2014). In this study, SUN and OFP family members are also demonstrated to be involved in fruit shape regulation of the tomato sf mutant. However, the expression patterns of the same gene family, such as SUN and SUN19, OFP15, OFP17, OFP19, and OFP11 (Table 3), do not correspond to their roles. Therefore, we speculate that these genes modulate the change in fruit size and shape of the sf mutant, but the individual effect of each gene may be minor. For instance, SUN suppression reduces fruit length, which may be compensated by an increase in SUN19 expression, leading to an unchanged fruit elongation. In addition, cyclin-dependent kinases belong to the cell division protein kinase (CDK) family, and are likely to manipulate the ratio of cell division and expansion in plant development, as a result, the organ size and shape remain unchanged (Hemerly et al. 2000; Imajuku et al. 2001; Boudolf et al. 2004; Verkest et al. 2005; Iwakawa et al. 2006; Roeder et al. 2010; Czerednik et al. 2015). For instance, the overexpression of CDKA1 in tomato increases cell number, which is associated with the decrease in their size, without a significant effect on the final fruit size and shape (Czerednik et al. 2015). Our data suggest that the CDK10 and CDK12 may participate in the regulation of cell division, but they do not influence cell expansion, leading to a change in fruit size and shape in the sf mutant. This evidence shows functional similarities and differences in homologous genes and provides novel insights into the regulatory mechanism of CDKs during fruit development.

The difference of pericarp cell number between sf and WT fruits occurs at approximately 10 DAF (Figure 3), but the expression levels of related genes change as early as -3 DAF, suggesting that
the regulatory model of transverse cell division of fruit is established before the anthesis stage in tomato. Although some genes can be implicated in the regulation of fruit size and shape of the sf mutant, the fruit length remains unaffected between sf and WT (Figure 1, Table 1), which may be due to the compensation phenomenon among these regulatory genes, such as SUNs and OFPs.

**Conclusion**

In summary, our study identifies a tomato sf mutant, which produces fruits smaller and more ovoid than those of the WT, and reveals the anatomical consequences and gene resources for the variation of fruits size and shape in this mutant. The sf mutant will add a new and novel genetic resource of fruit size and shape of tomato, and this study will provide a valuable basis for dissecting the molecular mechanism of the sf fruit development and enrich the theory of tomato fruit size and shape regulation. However, as it stands now, our understanding of the regulation of the sf fruit development is based on the bioinformatics data, thus, further elucidation of the specific roles of these regulatory genes in fruit size and shape by tomato transformation (gene knockout and overexpression), and the identification of the relationships among these genes, will provide further insight into the regulatory mechanism of the fruit phenotype control in the tomato sf mutant.

**Supplementary Materials**

Table S1. List of primers used for qRT-PCR verification.

Table S2. Differentially expressed genes in ovaries between sf mutant and WT.

Table S3. Significantly enriched GO terms in the up-regulated genes.
Table S4. Enriched GO terms in the down-regulated genes.

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Author's Contributions

YZ and YL (Yan Liang) planned the experiments. YZ, YL (Yushun Li) and JZ conducted the experiments. YZ, YL (Yushun Li) and TM analyzed the data. YZ wrote the paper with contributions from all authors.

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Table 1. Phenotypic characterization of mature fruits of wild-type (WT) and sf mutant.

<table>
<thead>
<tr>
<th>Phenotypic characters</th>
<th>WT</th>
<th>sf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (mm)</td>
<td>62.39 ± 4.48a</td>
<td>63.55 ± 5.04a</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>72.60 ± 5.49a</td>
<td>50.31 ± 4.50b</td>
</tr>
<tr>
<td>Fruit shape index&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.86 ± 0.08a</td>
<td>1.27 ± 0.16b</td>
</tr>
<tr>
<td>Locule number</td>
<td>8.73 ± 1.45a</td>
<td>3.42 ± 0.51b</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>181.42 ± 16.85a</td>
<td>86.82 ± 9.64b</td>
</tr>
</tbody>
</table>

The values shown are the means ± SD of 20 mature fruits from six independent plants which were grown in the greenhouse. Different letters (a and b) in the same column indicate significant differences ($P < 0.05$) between WT and sf mutant determined by Duncan’s test.

<sup>1</sup> Fruit shape index is a ratio of fruit length to fruit diameter.
Table 2. Summary of the transcriptome assembly.

<table>
<thead>
<tr>
<th>Samples</th>
<th>WT_rep1</th>
<th>WT_rep2</th>
<th>sf_rep1</th>
<th>sf_rep2</th>
</tr>
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<tbody>
<tr>
<td>Raw reads</td>
<td>41,129,292</td>
<td>46,641,556</td>
<td>43,601,968</td>
<td>51,563,912</td>
</tr>
<tr>
<td>Clean reads (%)</td>
<td>40,361,124</td>
<td>45,791,152</td>
<td>42,878,874</td>
<td>50,515,194</td>
</tr>
<tr>
<td></td>
<td>(98.13%)</td>
<td>(98.18%)</td>
<td>(98.34%)</td>
<td>(97.97%)</td>
</tr>
<tr>
<td>Clean bases</td>
<td>6.05G</td>
<td>6.87G</td>
<td>6.43G</td>
<td>7.58G</td>
</tr>
<tr>
<td>Q20 (%)</td>
<td>97.87%</td>
<td>98.08%</td>
<td>97.78%</td>
<td>97.83%</td>
</tr>
<tr>
<td>GC (%)</td>
<td>42.22%</td>
<td>42.34%</td>
<td>42.15%</td>
<td>41.93%</td>
</tr>
<tr>
<td>Mapped clean reads (%)</td>
<td>36,553,395</td>
<td>41,652,230</td>
<td>38,291,004</td>
<td>45,536,513</td>
</tr>
<tr>
<td></td>
<td>(90.57%)</td>
<td>(90.96%)</td>
<td>(89.30%)</td>
<td>(90.14%)</td>
</tr>
<tr>
<td>Unique mapped clean reads (%)</td>
<td>36,267,195</td>
<td>41,347,658</td>
<td>37,941,036</td>
<td>45,142,576</td>
</tr>
<tr>
<td></td>
<td>(89.86%)</td>
<td>(90.30%)</td>
<td>(88.48%)</td>
<td>(89.36%)</td>
</tr>
</tbody>
</table>
Table 3. List of fruit size- and shape-related genes that were differentially expressed in the ovaries of sf mutant and WT.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene annotation</th>
<th>Fold Change (sf/WT)</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solyc01g010240</td>
<td>CRCa (YABBY transcription factor)</td>
<td>20.27</td>
<td>4.57E-02</td>
</tr>
<tr>
<td>Solyc04g007900</td>
<td>CNR2 (Cell number regulator 2)</td>
<td>3.78</td>
<td>4.34E-46</td>
</tr>
<tr>
<td>Solyc06g066590</td>
<td>CNR10 (Cell number regulator 10)</td>
<td>4.04</td>
<td>4.68E-02</td>
</tr>
<tr>
<td>Solyc12g094600</td>
<td>CYCB2;5 (Cyclin B2;5)</td>
<td>-4.65</td>
<td>4.26E-04</td>
</tr>
<tr>
<td>Solyc07g052610</td>
<td>CYCU1;1 (cyclin U1;1)</td>
<td>-2.23</td>
<td>5.56E-05</td>
</tr>
<tr>
<td>Solyc02g083950</td>
<td>WUS (WUSCHEL)</td>
<td>2.84</td>
<td>4.37E-04</td>
</tr>
<tr>
<td>Solyc10g079240</td>
<td>SUN</td>
<td>-5.06</td>
<td>7.02E-05</td>
</tr>
<tr>
<td>Solyc08g007920</td>
<td>SUN19</td>
<td>2.89</td>
<td>3.03E-02</td>
</tr>
<tr>
<td>Solyc06g073040</td>
<td>OFP11 (Ovate family protein 11)</td>
<td>3.69</td>
<td>8.99E-10</td>
</tr>
<tr>
<td>Solyc07g055240</td>
<td>OFP15 (Ovate family protein 15)</td>
<td>-2.92</td>
<td>2.30E-02</td>
</tr>
<tr>
<td>Solyc09g018200</td>
<td>OFP17 (Ovate family protein 17)</td>
<td>-5.95</td>
<td>4.88E-03</td>
</tr>
<tr>
<td>Solyc09g082080</td>
<td>OFP19 (Ovate family protein 19)</td>
<td>-19.90</td>
<td>4.73E-12</td>
</tr>
</tbody>
</table>
**Table 4. List of cell division protein kinase genes in the DEGs with enriched GO terms between the sf and WT ovaries.**

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene annotation</th>
<th>Fold Change (sf/WT)</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solyc03g115660</td>
<td>CDK12 (<em>Cell division protein kinase 12</em>)</td>
<td>2.17</td>
<td>2.63E-11</td>
</tr>
<tr>
<td>Solyc07g053910</td>
<td>CDK10 (<em>Cell division protein kinase 10</em>)</td>
<td>2.39</td>
<td>1.61E-32</td>
</tr>
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</table>

BP, biological process; MF, molecular functions.
Figure Legends

Figure 1. Fruit phenotype of the tomato sf mutant. (A) Morphology of fruits of wild-type (WT) and sf mutant at 10, 15 days after flowering (DAF), mature green (MG), breaker (Br), turning (T), and mature red (MR) developmental stages. (B) Transverse sections of mature red fruits of WT and sf mutant. Bars = 5 cm.

Figure 2. Phenotypic analysis of pistils from WT and sf mutant. (A) Morphology of pistils at -3, 0, 3, and 5 DAF. Bar = 1 cm. (B) Time-course comparison of ovary length and ovary diameter at -3, 0, 3, and 5 DAF. Values are means ± SD from six ovaries.

Figure 3. Histological analysis of transverse sections of ovaries/fruits from WT and sf mutant. (A) Transverse sections of ovaries at -3, 0, 3, 5, 10, and 15 DAF. Bars = 200 µm. (B-C) Time-course comparison of number of pericarp cell layers (B) and pericarp thickness (C) of ovaries from -3 to 15 DAF. Values represent means ± SD of six ovaries.

Figure 4. qRT-PCR verification of DEGs identified by RNA-Seq. The blue and red bars represent RNA-Seq and qRT-PCR data, respectively. The tomato EF-1α gene was used as an internal control. The values shown represent the means ± SD from three biological replicates.
Figure 5. Gene ontology (GO) terms that were significantly enriched in the up-regulated genes between sf and WT ovaries. The results were summarized in two main categories: biological process (BP, blue) and molecular function (MF, red). GO terms were sorted based on corrected $P$-value, and the corrected $P$-value < 0.01 was used as the significance cut-off.
Figure 1

254x190mm (300 x 300 DPI)
Figure 2

A

WT

sf

-3 0 3 5 DAF

B

Ovary length (mm)

0 1 2 3 4

-3 0 3 5 DAF

Ovary diameter (mm)

0 1 2 3 4

-3 0 3 5 DAF

254x190mm (300 x 300 DPI)
### Figure 3

<table>
<thead>
<tr>
<th></th>
<th>-3 DAF</th>
<th>10 DAF</th>
<th>15 DAF</th>
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<td><img src="WT-3DAF.png" alt="Image" /></td>
<td><img src="WT-10DAF.png" alt="Image" /></td>
<td><img src="WT-15DAF.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>sf</strong></td>
<td><img src="sf-3DAF.png" alt="Image" /></td>
<td><img src="sf-10DAF.png" alt="Image" /></td>
<td><img src="sf-15DAF.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**B**

![Graph](Graph-B.png)

**C**

![Graph](Graph-C.png)

254x190mm (300 x 300 DPI)
Figure 4

![Graphs showing relative expression levels for different gene pairs across WT and sf conditions, with data from RNA-Seq and qRT-PCR](image)

254x190mm (300 x 300 DPI)
Figure 5

190x254mm (300 x 300 DPI)