INTRODUCTION

To develop varieties with improved characteristics, breeders exploit the genetic variation of important agronomic traits such as fruit weight, grain yield, and overall plant architecture. With the advent of Quantitative Trait Loci (QTL) mapping and cloning methods in past decades, many genes contributing to the increase in fruit weight and crop yield have been identified (Bommert, Nagasawa, & Jackson, 2013; Chakrabarti et al., 2013; Frary et al., 2000; Li et al., 2011; Song et al., 2007). The weight of fruits and vegetables is genetically controlled as early as inflorescence and floral meristem development (van der Knaap & Østergaard, 2017). Specifically, the organization of floral meristems is important in determining the final number of carpels, which collectively become the fruit after fertilization of the ovule.
ovules (Fletcher, Brand, Running, Simon, & Meyerowitz, 1999; Mayer et al., 1998; Xu et al., 2015). Not only is fruit weight affected, the differential regulation of floral meristem development also accounts for variances in rice grain size and maize kernel number (Bommer et al., 2013; Suzuki et al., 2009). Together, these findings indicate that meristem-regulated processes are essential and conserved features for reproductive organ development in higher plants.

Meristems maintain the balance between cell differentiation and self-renewal in a coordinated manner through intercellular communication mediated by WUSCHEL (WUS) and CLAVATA3 (CLV3) proteins (Fletcher et al., 1999; Laux, Mayer, Berger, & Jurgens, 1996; Mayer et al., 1998). In Arabidopsis, CLV3 and WUS orchestrate meristem function via a negative feedback regulatory loop (Brand, Fletcher, Hobe, Meyerowitz, & Simon, 2000; Schoof et al., 2000). CLV3 signals are perceived directly or indirectly by different receptor complexes, principally CLV1, CLV2, CORYNE (CRN), BARELY ANY MERISTEM 1 (BAM1) and RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2) to restrict WUS expression (Shinohara & Matsubayashi, 2015; Somssich, Je, Simon, & Jackson, 2016). WUS activates CLV3 expression at the meristem by binding to the CLV3 cis-regulatory regions in its promoter (Perales et al., 2016; Yadav et al., 2011). CLV3 belongs to the CLE small peptide family and it acts in a non-cell autonomous manner (Cock and McCormick 2001; Lenhard and Laux 2003). CLE family proteins typically share a putative N-terminal signal peptide and the conserved CLE motif known to interact with CLV receptors (Ni & Clark, 2006; Rojo, Sharma, Kovaleva, Raikhel, & Fletcher, 2002; Shinohara & Matsubayashi, 2015). In addition, post-translational modification of CLV3 and certain CLE peptides with a tri-arabinoside chain is required to activate their functions through conformational changes (Shinohara & Matsubayashi, 2013; Xu et al., 2015). The null mutation in cvl3 leads to enlarged floral meristems due to the increase of the central zone, which contributes to the development of supernumerary floral organs (Brand et al., 2000; Fletcher et al., 1999). WUS encodes a homeodomain transcription factor required for specifying stem cell identity (Laux et al., 1996; Mayer et al., 1998). In Arabidopsis, the wus null mutant is characterized by aberrant meristem structure and premature termination of shoot apical meristems (SAMS) and floral meristems (FMs). Premature termination of FM leads to a restriction in stamen and carpel development (Laux et al., 1996). Principally, WUS positively regulates CLV3 expression which in turn leads to downregulation of WUS expression through the interactions of CLV3 with membrane-localized receptors and phosphorylation-dependent downstream effectors (Betsuyaku, Takahashi et al., 2011; Brand et al., 2000; Schoof et al., 2000; Somssich et al., 2016; Song, Lee, & Clark, 2006). In addition to the CLV3-WUS feedback loop, WUS also positively regulates the carpel identity gene, AGAMOUS (AG), during early floral development (Lenhard, Bohnert, Jürgens, & Laux, 2001; Lohmann et al., 2001). Subsequently, AG suppresses WUS expression by binding to the CArG box located downstream of the gene and the recruitment of polycomb group proteins, known to trigger transcriptional repression by enhancing histone methylation (Liu et al., 2011). An ag loss-of-function mutation completely abolishes carpel development in Arabidopsis (Yanofsky et al., 1990).

LC and FAS are two important genes contributing to enlarged fruits with many locules in tomato. Both mutants were selected among different genetic subgroups during tomato domestication because of their positive effects on fruit weight (Blanca et al., 2015; Rodríguez et al., 2011). However, a mutation in FAS often results in unacceptable fruits that are unevenly shaped and therefore, the allele is not commonly found in conventional and commercially grown tomatoes. lc is a mutation near SIWUS, and is expected to cause increased expression by abolishing the binding site of its suppressor AGAMOUS. The causative mutation is comprised of two SNPs that are downstream of the 3’ UTR of SIWUS (Muños et al., 2011). fas on the other hand is caused by a ~294 kb inversion with a breakpoint in the promoter region of SICLV3 (Huang & van der Knaap, 2011; Xu et al., 2015). Although the role of CLV3-WUS in meristem maintenance has been investigated in Arabidopsis and other plant species, it remains unclear whether novel factors are involved in FM regulation in tomato. To understand the molecular mechanism underpinning LC-FAS mediated developmental processes, we conducted a series of genetic and gene expression analyses using backcross populations (Figure S1). We show that lc is a gain-of-function mutation of SIWUS, whereas fas is a partial loss-of-function mutation of SICLV3. Importantly, our RNA-seq results led to the identification of a number of co-expressed gene clusters associated with various developmental processes that might act downstream of LC and FAS.

2 | RESULTS

2.1 | The effects of lc and fas on fruit morphology and reproductive traits

The natural mutations at the lc and fas loci underlie the orthologs of the Arabidopsis meristem organization genes WUS and CLV3, and were originally identified due to their strong effects on locule

FIGURE 1 The effect of natural lc and fas mutant alleles on floral organs and inflorescence development. (a) Tomato varieties containing lc and/or fas mutant alleles carry multinocular fruits. The wild type tomato (Solanum pimpinellifolium LA1589) typically contains only two locules. Size bar = 3 cm. (b) Genomic sequence changes in lc and fas. The genomic sequence underlying the lc mutation shares similarity to the CArG box of Arabidopsis AP3. The two SNPs underlying lc are marked in red and the putative CArG box is highlighted in gray. The fas mutation is caused by a ~294 kb inversion with a breakpoint in the promoter region of SICLV3. (c–e) Inflorescences, flowers and fruits of lc, fas, lc/fas NILs and SICLV3-RNAi lines. Bar = 1 cm. (f–i) The ratio of branched inflorescences and floral organ number in NILs and various transgenic lines. RNAi-CR4 and RNAi-CR9 represent two independent SICLV3-RNAi transgenic lines. pHc2-6-2 and pHc2-7-2 represent two independent transgenic lines that were transformed with SICLV3 genomic sequence driven by a 5.5 kb promoter construct. pHc4 contained a shorter SICLV3 promoter that served as a negative control for the complementation test. Pairwise comparisons were made between the genotypes using ANOVA and means were separated with Tukey’s HSD test with p < 0.05.
number (Figure 1a) (Barrero, Cong, Wu, & Tanksley, 2006; Lippman & Tanksley, 2001; Muños et al., 2011; Rodríguez et al., 2011; Xu et al., 2015). The associated nucleotide polymorphisms are 3’ of SIWUS (Muños et al., 2011; van der Knaap et al., 2014) and the promoter region of SICLV3 (Huang & van der Knaap, 2011; Xu et al., 2015), respectively (Figure 1b). The lc mutations may correspond to the CArG box, which is critical to suppress WUS expression in Arabidopsis (Liu et al., 2011). We determined the effects of lc and fas on fruit morphology and reproductive traits by creating near-isogenic lines (NILs) in the wild species Solanum pimpinellifolium accession LA1589 background that differed only for the alleles at these two loci. In addition to more locules, the expectation is that these meristem organization mutants would lead to increased inflorescence branching (Park, Jiang, Schatz, & Lippman, 2012). Wild type tomato typically develop a single-branch inflorescence with a reiterating pattern of an IM terminating into an FM and the formation of a new IM along the flank of the FM (Figure S1b), whereas fas and especially lc/fas NILs show a significant increase in inflorescence branching (Figure 1c,f). For example, the first inflorescence in the fas and lc/fas NILs nearly always forms a branched architecture — as two IMs appear to emerge from a single terminating SAM (Figure S2). lc/fas also resulted in the highest floral organ number among the NILs especially for locule number (Figure 1d–e,g–i). Overall, lc alone had no effect on inflorescence branching and floral organ number unless in combination with fas (Table S1). Regardless, the most dramatic floral organ number change was for locule number in the natural lc and fas mutants.

SICLV3 has been demonstrated to underlie fas (Xu et al., 2015), which implies that the fas inversion has compromised the promoter of this gene. We also downregulated SICLV3 expression by expressing part of the coding region as an RNAi construct in stably transformed tomato. As expected, the tomato plants transformed with the pHC2 construct, which contained approximately 5 kb of the wild type promoter and the entire coding region of the gene, rescued the bi-locular fruit phenotype in the fas background, whereas the shorter promoter construct pHC4 did not (Figure 1i). On the other hand, downregulation of SICLV3 led to an increase in all floral organs as well as inflorescence branching (Figure 1c–i). Additional phenotypes associated with the severely reduced expression of SICLV3 included nearly seedless fruits, indeterminate meristematic activity as evidenced by the development of ovaries within the initial ovary, widened leaflets with smooth margins, decreased number of secondary leaflets, and reduced complexity of the compound leaf (Figure 2a–b). Extreme phenotypes in the flowers were also occasionally observed, such as an inflorescence that was reinitiated inside a flower (Figure 2c).

To determine whether the selection of lc and fas during domestication might have been due to the associated increases in fruit weight, we evaluated the effect of the natural mutations on this trait. In the wild species background, fruit weight was only significantly increased in the lc/fas double NIL (Table 1a). Fruit area was significantly increased in fas and even more in the lc/fas double NIL. This suggested that the fruits were wider but flatter and thus the increase in locule number did not yield much heavier fruits in the LA1589 background. To determine whether lc and fas exerted a synergistic effect on locule number, we evaluated the genetic effect between these two loci (Table 1b). The epistatic analysis was performed using two-way ANOVA and showed a significant interaction between lc and fas for locule number (p-value < 0.001) but not for other traits (Table 1b). The degree of dominance of each locus showed that lc affected locule number in a mostly additive manner. On the other hand, the fas mutation was nearly completely recessive over the wild type with a d/a value of −0.88 (Table S2).
The effects of lc and fas mutations on fruit size, weight and locule number

(a) Comparisons of fruit perimeter, area and weight between the wild type, lc, fas and lc/fas NILs. The fruit weight represents the average value of 20 ripe fruits from 5 to 6 individual plants per genotype. The average fruit perimeter and area were measured from 8 to 10 ripe fruits. Pairwise comparisons between the NILs were performed using ANOVA and means were separated using Tukey’s HSD test. (b) Effects and interactions of lc and fas on the traits of mature fruits. Significant effects and interactions were shown by the p-values computed from the F ratio in ANOVA.

2.2 | lc and fas produce fasciated inflorescences and enlarged meristems

To determine whether higher locule number was associated with increased size of the floral meristem, we compared their widths in young floral buds prior to the emergence of the carpel primordia. At 3 days post floral initiation (dpi), when the floral meristem was not yet enclosed by sepal primordia, FMs of single and double mutants were significantly wider than that in the wild type (Figure 3a,c). At 4 dpi after the initiation of petal primordia, the FM enlargement in fas was even more pronounced than at 3 dpi, especially in the lc/ fas double mutant background (Figure 3b,d). For example, while wild type meristem stayed around the same width of approximately 147 μm, the lc/fas double NIL increased from 176 to 207 μm from 3 to 4 dpi. Notably, the synergistic effect between lc and fas on meristem size was not observed in floral buds at 3–4 dpi, suggesting that the interaction might happen at a later developmental stage. The data showed a positive trend between the floral meristem size and locule number resulting from the effects of lc and fas. In addition, fas allowed an extended period of meristem expansion compared to the wild type and lc.

2.3 | lc and fas affect the expression of SICLV3, SIWUS, SIYABBY2, and TAG1

To reveal the effect of the lc and fas mutations on gene expression, we performed expression analyses using meristems and young floral buds from the wild type and mutant NILs. RNA was isolated from sympodial shoot apical meristem (SYM), the combined floral and inflorescence meristem (F&IM), and floral buds at 2, 4, and 6 dpi (Figure S1b). As expected, the expression of SICLV3 was significantly lower in the SYMs and F&IMs in lines carrying fas compared to that in the wild type (Figure 4a). On the other hand, a significant increase in SICLV3 expression, specifically in SYMs, was detected in lc. This supported the notion that the two SNPs at CArG box in lc (Figure 1b) abolish the binding of a suppressor, resulting in increased expression of SIWUS and SICLV3 in SYMs. After 2 dpi, expression of SICLV3 was similar among the genotypes. SIWUS expression was significantly increased in the single and double mutants in the SYM but not in the F&IM. At the relatively late developmental stage of 6 dpi, SIWUS expression was higher in all mutants implying a delayed termination of gene expression (Figure 4b).

A significant reduction in SICLV3 expression resulting from the SICLV3 RNAi construct was also found in F&IM and 2 dpi floral bud tissues compared to the wild (Figure 4c). The residual SICLV3 expression was likely due to incomplete RNAi or the transcripts derived from the hairpin RNAi construct. Severe downregulation of SICLV3 also led to higher expression of SIWUS but only at 4 and 6 dpi (Figure 4d). Together, these results showed that the reduction in SICLV3 expression could lead to a delay in the suppression of SIWUS.

The genomic inversion in fas also resulted in a breakpoint in the first intron of SIYABBY2b (Figure 1b). As a result, SIYABBY2b expression persisted at low levels in fas and lc/fas at all stages, suggesting that the mutation did not fully block SIYABBY2b transcription (Figure 4e). However, almost all detected transcripts mapped to the 5’ region of SIYABBY2b comprising the first exon and before the breakpoint of the fas inversion (Figure S3). This indicated that the mutation led to the production of truncated RNAs that are likely non-functional.
We wanted to evaluate whether the WUS-dependent AG expression found in Arabidopsis is conserved in tomato. The expression of tomato AG homolog TAG1 was significantly increased in lc, fas, lc/fas floral buds at stage 4 dpi (Figure 4f). These differences were also observed in mutants at 6 dpi albeit to a lesser extent than at 4 dpi. Notably, the synergistic effect of lc and fas was not observed from TAG1 expression, suggesting that SlWUS solely controls TAG1 expression. It is conceivable that TAG1 leads to downregulation of SlWUS though its binding to the CArG box located 3′ of SlWUS. However, the increased expression of TAG1 in lc, fas and lc/fas NILs was not associated with reduced SlWUS expression suggesting that TAG1 might not directly involved in SlWUS repression.

2.4 Pronounced temporal-spatial expression changes of SICLV3 and SIWUS in the lc and fas NILs

To determine whether temporal-spatial expression patterns of SICLV3 and SIWUS were altered in the NILs, we performed RNA in situ hybridization (Figure 5). In FM, the spatial expression of SIWUS revealed no dramatic changes in expression level among the genotypes (Figure 5a), a finding that was consistent with results from the RNA-seq analyses (Figure 4). At 2–3 dpi, we observed an expansion of the SIWUS expression domains in the single and double NILs compared to that in the wild type (Figure 5b). At 4–7 dpi, SIWUS expression in the NILs was constrained again to the center and appeared similar to the pattern found in the wild type (Figure 5c). The most substantial change in SIWUS expression pattern was found in the SICLV3-RNAi lines (Figure 5a–c). Contrary to the single and double NILs, the SICLV3-RNAi led to a lateral expansion of the SIWUS expression domain in the floral buds especially at the later stages of bud development.

Expression of SICLV3 in the FM showed a similar pattern as SIWUS (Figure 5d). While SICLV3 exhibited a similar expression pattern between lc and the wild type in FMs and 3–4 dpi tissues, it is expressed at a much higher level in lc at 7–8 dpi (Figure 5d–f). By contrast, the fas and lc/fas mutants led to a weaker expression of SICLV3 in the floral buds at 3–4 or 7–8 dpi (Figure 5d–f). These RNA in situ hybridization results are complementary with the RNA-seq results in supporting the expression changes of SICLV3 and SIWUS in NILs and RNAi plants.

2.5 Differentially expressed genes in lc and fas

To decipher the genome-wide gene expression changes resulting from mutations in lc and fas, we applied linear factorial analysis on the RNA-seq results across the five developmental stages. Differentially expressed genes (DEGs) that were consistently up- or down-regulated across different stages were identified as genes with significant genotype effect, while DEGs responding differently to genotypic and developmental variations (ex: showing dynamic expression patterns across stages) were considered as genes with significant genotype by developmental
A total of 669 and 13 DEGs were identified with significant genotype (Padj < 0.1) and G × D interaction effects (p < 0.001), respectively (Data S1), which is a relatively low threshold level. The low number of genes that were identified under the model implied that very few differentially expressed genes resulted from one mutation or the other. This finding is consistent with the notion that *lc* and *fas* are not null mutations, and therefore have a relatively weak impact on downstream genes of either mutation. With seven genes shared by the two categories (669 DEGs in the “genotype effect” and 13 DEGs in the “G × D effect”), a total of 675 unique DEGs were used for the downstream data analyses. The 13 DEGs that showed G × D interaction included the known SICL3V (Solyc11g071380), SIWUS (Solyc02g083950) and SIYABBY2b (Solyc11g071810) (Figure 6). Genes involved in nutrient and hormone transport included the sugar transporter SISWEET1b (Solyc04g064620) and the auxin efflux carrier SISoPIN1a (Solyc10g078370). The SISWEET1b is a homolog of Arabidopsis AtSWEET1 that encodes a transmembrane sugar transporter (Chen et al., 2010; Feng, Han, Han, & Jiang, 2015). Sugar transporters are known to affect meristem development by regulating sugar accumulation and distribution in the meristem (Francis & Halford, 2006; Lastdrager, Hanson, & Smeekens, 2014). The *slpin1a* loss-of-function mutation, also known as entire-2, causes aberrant organ positioning in the shoot, inflorescence and floral meristems by disrupting directional auxin transport (Martinez, Koenig, Chitwood, & Sinha, 2016). We also identified genes encoding metabolic enzymes such as glucose-6-phosphate isomerase (Solyc04g076090), ALCOHOL DEHYDROGENASE 2/SIADH2 (Solyc06g059740) involved in fatty acid degradation and the production of volatiles during fruit ripening (Speirs et al., 1998), and a close homolog of Arabidopsis ANTHOCYANINLESS 2 (ANL2) in Arabidopsis (Solyc03g026070),
which affects anthocyanin accumulation (Kubo, Peeters, Aarts, Pereira, & Koornneef, 1999). DEGs with G × D interaction effects also included a NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3) protein (Solyc11 g040040) that is involved in phototropic responses and protein ubiquitination in Arabidopsis (Gingerich et al., 2005; Pedmale & Liscum, 2007).
2.6 | Cluster analysis and DEGs co-expressed with SIWUS

The linear factorial modeling identified a large collection of DEGs that were consistently up- or down-regulated in all developmental stages as well as those showing genotype- and developmental stage- dependent differential expressions. To identify groups of DEGs with similar expression dynamics among the 675 DEGs dataset, we conducted fuzzy C-means clustering using corresponding genes with normalized expression levels in the WT. This led to the identification of eight co-expressed clusters with cluster 1 representing SIWUS and SICLV3 (Figure 7a). Clusters with DEGs that
showed higher expression at early developmental stages (cluster 1, 2, 6) were highly enriched in GO terms for “stem cell population maintenance” and “microtubule motor activity” (Table 2). DEGs with higher expression at the later developmental stages (cluster 5 and 8) were specifically enriched in “steroid biosynthetic processes”. Cluster 1, 5 and 6 were selected as they contained the GO terms that were most significantly overrepresented in any of the clusters (adj. p-value < 1e-05).

Cluster 1 was strongly enriched with genes involved in stem cell population maintenance, consistent with the notion that their expression patterns were negatively correlated with the developmental gradient. Genes enriched for meristem maintenance included SLOC13, SIWUS, putative homologs of the Arabidopsis SHOOT MERISTEMLESS (STM) (SOLyc02_g081120), AGAMOUS-like 20 (AGL20) (SOLyc01_g092950), AINTEGUMENTA (ANT) (SOLyc04_g077490) and LONESOME HIGHWAY (LHW) (SOLyc06_g074110).

To identify genes that might work in concert with SIWUS, the Pearson’s correlation coefficients (PCCs) between gene pairs in cluster 1 were calculated using the normalized expression levels from mutants and WT. As shown in Figure 7b,c, a set of genes were identified with a high correlation to SIWUS expression. The subset of 29 genes was highly expressed in the F&M and, then their expression diminished during the termination of the meristematic potential of the remaining FMs as the floral organ primordia arose (Figure 7c). These genes showed higher expression in fas and lc/fas compared to WT, suggesting that the upregulation might be related to the expansion of SIWUS expression domain (Figure 5). The identification of co-expressed tomato SHOOT MERISTEMLESS (STM) with SIWUS suggested that some genes in this cluster might be related to stem cell function. Indeed, six of the 29 genes highlighted in red (Figure 7c) are involved in meristem or floral development based on previous studies in Arabidopsis (Irish & Sussex, 1990; Krizek, 2011; Lee et al., 2000; Lenhard, Jürgens, & Laux, 2002; Omidbakhshfard, Proost, Fujikura, & Mueller-Roeber, 2015).

Cluster 5 was enriched with genes encoding enzymes in sterol biosynthesis. They included putative orthologs of DWF1 (SOLyc02_g069490), DWF5 (SOLyc06_g074090), DWF7 (STE1) (SOLyc02_g086180) and DET2 (SOLyc10_g086500) (Figure 7d). In contrast to genes co-expressed with the expression of the sterol biosynthesis pathway genes increased with the floral development stages. In addition, these genes were expressed at lower levels in fas and lc/fas compared to WT across different developmental stages, implying the negative roles of these genes in meristem maintenance. As sterols are precursors of brassinosteroids (BRs), membrane components, and signaling molecules during plant development (Vriet, Russinova, & Reuzeau, 2013), these results have provided new insights into the potential roles of sterols/BRs in FM regulation.

Cluster 6 was enriched with genes encoding microtubule motor proteins, including putative homologs of Arabidopsis KINESIN 1 (ATK1) (SOLyc12_g005250), PHAGMOPLAST-ASSOCIATED KINESIN-RELATED PROTEIN 1 (PAKRP1) (SOLyc09_g097860), TETRASPORINE (TES) (SOLyc07_g042560) and ZWICHEL (ZWI) (SOLyc12_g082730). These genes were expressed at higher levels at 2 dpi, indicating that their expression might be positively regulated by genes mediating outer whorl initiation. Phylogenetic analysis revealed that these kinesin genes belonged to different subfamilies (Figure S4), and the co-expression signature of these genes suggested that they might act cooperatively in cell division and cell growth during floral development. In addition to the genes encoding kinesin proteins, the genes closely related to Cyclin B2:3 (SOLyc03_g032190) and Cellulose Synthase-Like D5 (SOLyc09_g075550) were also found in cluster 6. Although kinesins might also play a role in organelle movement (Zhu & Dixit, 2012), the co-expressed pattern of these kinesin genes with putative tomato Cyclin B2:3 and Cellulose Synthase-Like gene suggested that they were more likely involved in cytokinesis (Hunter et al., 2012; Tank & Thaker, 2011).

3 | DISCUSSION

3.1 | Conserved regulatory mechanism between LC-FAS and WUS-CLV3

In the recent years, a number of genes associated with the control of tomato fruit shape and size have been identified (Mu et al., 2017; van der Knaap & Østergaard, 2017; van der Knaap et al., 2014). The key genes that contribute to the flat shape and multi-loculed tomato fruits are SIWUS and SLOC13 (Muños et al., 2011; Xu et al., 2015). The origin and distribution of the mutant alleles of these genes, including their effect on tomato fruit morphology was investigated at the population level (Blanca et al., 2015; Rodríguez et al., 2011). While lc appears to have arisen in wild relatives, fas arose later during domestication (Blanca et al., 2015). Although fas appears to be more effective in producing fasciated fruits than lc (Rodríguez et al., 2011), it is not well understood how each locus alone or in combination controls fruit development and gene expression due to differences in the genetic background of the tomato accessions studied.

Our findings indicate a close conservation between tomato LC-FAS and Arabidopsis WUS-CLV3 regulatory loops. Results of

FIGURE 7 Expression profiles of the co-expressed gene clusters. (a) Eight co-expressed clusters identified from normalized expression values (z-scores) of the wild type samples are clustered using K-mean algorithm in Mfuzz (R package). The dark blue lines represent the average of expression values, whereas the light blue regions represent the maximum and minimum expression values. (b) Pearson Correlation Coefficient matrix based on the expression of genes in WT and mutants in cluster 1. The core genes co-expressed with SIWUS are marked by the red square. (c) Heatmap of core genes co-expressed with SIWUS in cluster 1. Normalized expression values were used for hierarchical clustering. (d) Cluster 5 enriched with genes involved in sterol biosynthesis. Although SOLyc10_g086500 was not significantly differentially expressed below the adj. p < 0.1 threshold, it was included in this analysis based on p-value < 0.01 and its GO signature. (e) Cluster 6 enriched with genes involved in microtubule motor activity and cell cycle processes in cluster 6. Genes highlighted in red are putatively involved in microtubule binding activity and cytokinesis.
expression analysis revealed that SICLV3 was significantly downregulated and SIWUS was significantly upregulated in the SYM of fas. On the other hand, as SIWUS was upregulated in lc, SICLV3 was accordingly upregulated in SYM in this mutant background. These results support our hypothesis that lc is caused by a gain-of-function mutation of SIWUS, while fas is caused by a partial loss-of-function
mutation of SICLVL3. Although the LC-FAS negative feedback regulatory loop was conserved in the SYMs, the lower expression of SICLVL3 in the FM of fas, lc/fas and RNAi-SICLVL3 did not result in an increase of SIWUS expression in F&IMs (Figure 6). This raised a possibility that other factors could compensate the effect of SICLVL3 and maintain the suppression of SIWUS in FM. Interestingly, the SICLE9 gene, a member of the CLV3/EMBRYO-SURROUNDING REGION (CLE) gene family, was identified as one of the top candidates that showed a significant genotypic effect (Figure S5). SICLE9 expression was five-fold higher in the F&IMs of fas and lc/fas NILs as compared to the wild type, while being ten-fold higher in RNAi-SICLVL3 lines. In addition, a previous study showed that the application of SICLE9 and SICLVL3 peptide together onto tomato meristem mutants effectively rescued the phenotype of enlarged meristems (Xu et al., 2015). This finding suggested a possible role of SICLE9 in tomato CLV-WUS pathway and SICLE9 might compensate SICLVL3 function in regulating SIWUS expression.

In addition, we found a moderate upregulation of SICLVL3 expression in F&IMs and a significant increase of SIWUS expression in floral buds at 6 dpi in lc mutant (Figure 6). The results were consistent with our hypothesis that the two SNPs present downstream of the 3′ UTR of SIWUS might abolish the suppression imposed by tomato AGAMOUS. Intriguingly, SIWUS expression was also significantly upregulated in the SYMs of lc, which suggested that other unknown mechanisms were involved in the control of SIWUS expression through the CArG box in the SYM.

The in situ hybridization results confirmed the expression changes of lc and fas at the tissue level. SICLVL3 signal was weaker in lines carrying fas after 3 dpi, while the SICLVL3 signal was stronger and more persistent in lines carrying lc. In addition, the SIWUS expression domain expanded laterally in lines carrying one or both mutations at 2–3 dpi, when sepal primordia appeared. This correlated with the enlarged FM size at 3 and 4 dpi in lc, fas and lc/fas NILs (Figure 3). Nevertheless, the expansion of SIWUS signals was not observed in single and double mutants after 4–5 dpi, when petal and stamen primordia were initiated. This might be due to the presence of unknown suppressors that function on or before 4 dpi. Since the expression of TAG1 was not likely to lead to downregulation of SIWUS at the earliest timepoints, it was conceivable that Solyco5 g015750 (TMS closely related to SEP3) and Solyco2 g0889210 (closely related to API1) led to downregulation of SIWUS expression. The latter two MADS box proteins were expressed early in tomato floral development and were upregulated in the mutants. In SICLVL3 RNAi lines, SIWUS expression domain was strongly expanded and the expansion persisted to the late stages of floral development, consistent with the phenotype observed in Arabidopsis clv3 null mutant (Brand et al., 2000; Schoof et al., 2000).

Although a slight expansion of SIWUS expression domain in floral buds was observed in all mutants at 2–3 dpi (Figure 5), differential SIWUS expression was not detected between genotypes at 2 dpi from the RNA-seq results. This could be due to a similar rate of increase in FM enlargement and the expansion of SIWUS expression domain in mutants. Furthermore, although we found a relatively weak spatiotemporal SICLVL3 expression signal at 3–4 dpi in fas and lc/fas NILs in comparison to the wild type (Figure 5), the expression of SICLVL3 in fas and lc/fas NILs did not show a corresponding decrease at 4 dpi based on the RNA-seq results. This again might be due to the increase of total cells that expressed SICLVL3, albeit the expression level was lower on a per cell basis.

Unlike Arabidopsis, in which CLV3 is expressed in the L1, L2 and L3 layer (Fletcher et al., 1999), we found that SICLVL3 was absent from L1 and its expression was above and partially overlapped with the SIWUS expression domain in tomato. Similar results were also observed in soybean, in which GmCLV3 was absent in L1-L3 layer and its expression domain overlapped with GmWUS below the L5 layer in the SAM (Wong, Singh, & Bhalla, 2013). Together, these findings raise the possibility that the CLV-WUS meristem regulatory mechanism has somewhat diverged across different species.

In summary, our results imply that lc and fas mutations cause an expansion of the SIWUS expression domain and delay the termination of SIWUS, resulting in the production of larger FMs and more floral primordia.

3.2 Genes responding to LC and FAS expression dynamics during early floral development

Compared to a previous report using a DEX inducible system to identify WUS target genes in Arabidopsis (Busch et al., 2010), 91 out of 675 DEGs were found in both studies, including STM and AINTEGUMENTA (ANT) (Data S2). In addition, 133 out of the same 675 DEGs were differentially expressed in the RNAi-SICLVL3 line (Data S3). These common DEGs were potential candidates to study conserved mechanisms involved in organogenesis and floral development across different plant species. Identifying DEGs shared by lc, fas and lc/fas could potentially help to narrow down genes acting downstream of SIWUS and SICLVL3. It is hypothesized that the lower expression of SICLVL3 in fas and the higher expression of SIWUS in lc would result in a significant number of common DEGs. To our surprise, the percentage of overlapping DEGs between lc and fas was low (5% ~ 36%) (Figure S6, Data S4). This might be because lc is a weak allele, having only a minimal impact on locule number in the wild type tomato background. Therefore, the detection of DEGs associated with floral development in lc might be limited. Or it could be due to the pleiotropic role of CLV3. CLV3 belongs to the CLE small peptide family, which is also involved in plant-microbe interaction, vascular development and long-distance signal transduction (Betsuyaku, Sawa, & Yamada, 2011; Kucukoglu & Nilsson, 2015). Strabala et al. (2006) showed that the ectopic expression of CLV3 causes anthocyanin accumulation in Arabidopsis. Our results also show that the DEGs overlapping between fas and lc/fas (fas-secondary) were enriched in lignin metabolism, flavonoid biosynthesis, and response to environmental stimuli (Figure S6). Together, these results indicated that SICLVL3, in addition to its involvement in maintaining the meristem cell population with SIWUS, might be involved in other developmental processes as well.
We also observed a trend of decrease in the number of overlapping genes as floral buds developed further (Figure S6). Because whole flower buds were used in this study (Figure S1b), the growing mass of sepals, petals and stamens might dilute the meristem-specific transcripts and therefore hinder the detection of DEGs between genotypes.

### 3.3 Novel mechanisms underlying LC-FAS mediated control of meristem development revealed by co-expressed gene clusters

The transcriptional network controlling tomato meristem development is not fully unexplored. To reveal potential players...
participating in LC- and FAS- mediated meristem and floral development programs, a time-course RNA-seq gene expression profiling was conducted. From the cluster analysis, we uncovered a cluster enriched with genes related to microtubule motor activity (Figure 7, Data S1). This group of genes only showed higher expression in lc compared to the wild type and fas, especially in the SYM (Figure S7, Data S1). It is possible that this group of genes is activated by CLV-mediated MAPK signaling cascade (Betsuyaku, Takahashi et al., 2011), as CLV activity is elevated in lc. In Arabidopsis, MAPK cascade targets various TFs involved in a plethora of developmental, defense, and stress responses (Popescu et al., 2009). The tobacco MAPK cascade, positively regulates cytokinesis by phosphorylating NtMAP65-1, a microtubule-associated protein (Sasabe et al., 2006). In our results, we also found an elevated expression of a tomato microtubule-associated protein gene MAP65-1a (Soly21 g072280) in lc.

The identification of a cluster of genes involved in phytosterols and BRs synthesis indicates their putative roles in meristem function during tomato floral development. Genes in this cluster were expressed at lower levels in fas and lc/fas, indicating that the BR level might also be lower in the meristem of fas and lc/fas (Figure 7, Data S1). Phytosterols are the precursors of BRs and the homeostasis of BRs is controlled by a feedback regulatory loops (Vriet et al., 2013). A previous study demonstrates that phytosterols have a BR-independent role in controlling and activating signals for plant development (He et al., 2003). The sterol biosynthesis activities appear to be highly localized to the meristematic region. For example, the tobacco squalene synthase (SQS) enzyme activity is predominantly detected in SAM (De Varencere, Ghosh, & Chappell, 2002). Arabidopsis 3-hydroxy-3-methylglutaryl coenzyme A reductase 2 (HMG2) gene is expressed in SAM and floral tissues (Enjuto, Lumbreras, Marín, & Boronat, 1995). In addition, Arabidopsis FACKEL gene is set involved in embryonic patterning and meristem programming (Jang et al., 2000). Although we propose that sterol biosynthesis in meristematic regions is affected in fas, we cannot rule out the possibility that the linked genes located within the introgressed fas inversion are the cause of the differential expression.

Brassinosteroids are growth promoting hormones in general and the BR contents are maintained at a low level in the meristem, particularly in the organ boundary (Hepworth & Pautot, 2015). The KNOX genes, such as STM, maintain the identity of meristem and boundary in the SAM by suppressing the BR levels and directly activating genes involved in boundary formation (Boloudc et al., 2012; Johnston et al., 2014; Spinelli, Martin, Viola, Gonzalez, & Palatnik, 2011; Tsuda, Kurata, Ohyanagi, & Hake, 2014). In Arabidopsis, BR biosynthesis mutant, det2, and BR membrane-bound receptor mutant, bri1, cause extra carpel formation (Gendron et al., 2012). Together, these findings raise the possibility that higher expression of SJWUS and tomato STM in the FM of lc, fas and lc/fas suppressed sterol/BR biosynthesis, thereby triggering the formation of extra boundaries and floral organs.

In summary, our in situ hybridization and RNA-seq analyses have captured the dynamics of gene expression in vegetative meristem, floral meristem, and young floral buds in lc and fas. These results have provided useful information for the future study of important developmental questions, such as the link between the meristem regulation and floral organ determinacy. These results can also be integrated with other large-scale datasets at various levels to decipher the regulatory network in meristem development, and provide predictive models in improving fruit traits.

4 | MATERIALS AND METHODS

4.1 | Plant materials and near-isogenic line (NIL) development

*Solanum pimpinellifolium* accession LA1589 seeds were obtained from Tomato Genetics Resource Center (http://tgrc.ucdavis.edu/). Mature fruits from LA1589 typically contain two locules and are about 1 cm in diameter. Both *Solanum lycopersicum* cv. Orange Strawberry and Yellow Stuffer seeds were obtained from Tomato Growers Supply Company. Orange Strawberry contains the lc and fas mutant alleles and bears large fruits with 14 locules on average. Yellow Stuffer bears large fruits with 3.6 locules on average and only carries the lc allele. The NILs carrying the mutant alleles were derived from repeated backcross to the wild species *S. pimpinellifolium* accession LA1589. After six backcross generations to introgress each locus separately, the lc and the fas lines were crossed to one another to create the double NIL. To further reduce the size of the introgression regions at both loci, we made three more backcrosses to LA1589 and identified close recombination breakpoints, followed by two more self-pollinated generations to generate final BC2F2 population (family 135133) (Figure S8). During this selection, lc and fas loci were maintained in the heterozygous state, while the surrounding loci were selected to be homozygous wild type and selected for recombinants around the genes. Three NILs, lc, fas, lc/fas and the wild type (WT), were created from the BC2F2 population. The primers used to select recombinants are listed in Table S3.

The size of the introgressed segment varied for each locus (Figure S9). For lc, the region was approximately 184 kb, from 47,014 to 47,198 kb on Chr.2 (SL2.50). For fas, the introgression size was about 351 kb, from 54,842 to 55,193 kb on Chr.11 (SL2.50). The fas mutation was caused by a large inversion (294 kb) which limited the ability to narrow down the region further in this NIL.

4.2 | Cloning of the RNAi-SICLV3 constructs

To reduce the expression of *SICLV3* in wild type tomatoes, a hairpin RNAi construct was created using the pKYXL80 vector similar to the method described in Siminszky, Gavilano, Bowen, and Dewey (2005). A gene-specific fragment of 355 bp was amplified from SICLV3 with the following primer pairs: CRF1: 5′-AATTCTAGAAGCTTTCAATCTCTTGTGCTTGCAGTA-3′ and CRR1: 5′-ATGGAGCTCTCGAGATGAAACCATATACTACCCT-3′. The amplified product was digested with HindIII/Xhol and SacI/Xbal to construct sense and antisense fragments flanking the 151 bp region.
of soybean \( \omega-3 \) fatty acid desaturase (FAD3) intron (Figure S10). Next, both digested fragments were inserted into vector pKYXL80. The resulting EcoRI-XbaI fragment from pKYXL80 containing the CaMV35S promoter, SICLV3 sense hairpin-stem, FAD3 intron and SICLV3 antisense hairpin-stem was subcloned into binary vector pKYXL71 between TL border and the RBCS subunit terminator to produce the final RNAi- SICLV3 construct, named pRNAi-CR. The pRNAi-CR was stably transformed into S. pimeloides accession LA1589. We selected two independent T0, pRNAi-CR4 and pRNAi-CR9, which were shown to contain six and one copy of the transgene respectively, and were further evaluated by phenotypic and expression analysis in the T1 generation. Target specificity of this RNAi experiment was examined through BLASTN using Tomato genome cDNA database (SL3.20) with the designed hairpin-stem sequence (Figure S11). Comparisons of the expression level of all the SICLE gene families between the wild type and RNAi-SICLV3 lines in tomato FM and 2dpi floral buds are shown in Figure S12.

4.3 | Morphological analysis

4.3.1 | Inflorescence branching, floral organ number and fruit locule number counts

Five to six plants were selected from the seedlings generated by family 13S133 for each genotypic class (lc, fas, lc/fas and the wild type) and transplanted in 1-gallon pots in the greenhouse. For the transgenic T1 lines, three to seven plants were used. Inflorescence branching was evaluated on 40 inflorescences per plant. In addition, 40 flowers at anthesis were collected per plant to evaluate sepal, petal and stamen number. To evaluate locule number, 40 ripe representative fruits were collected from each plant, and locule number was counted in cut fruits.

4.3.2 | Fruit weight and dimension analysis

For fruit weight analysis, 20 ripe representative fruits per plant were selected and weighted. For fruit dimension analysis, eight to ten fully mature fruits from each of the genotypes were cut horizontally and scanned. Tomato analyzer 3.0 (Rodríguez et al., 2010) was used to analyze the scanned images for fruit perimeter and area following the instructions (http://vanderknaaplab.uga.edu/tomato_analyzer.html).

4.3.3 | Morphological analysis of inflorescence structure and meristem size measurement

The first young inflorescences of lc, fas, lc/fas NILs and the wild type were collected in the greenhouse and immediately placed in ice-cold RNAlater (QIAGEN) to preserve the tissue structure. The inflorescences were imaged using an Olympus SZH10 stereo-microscope and Olympus DP-10 digital camera. For the meristem size measurement, paraffin slide sections were made in the same way as described in in situ hybridization procedures. Paraffin slide sections were rehydrated through ethanol series and stained with 1% Toluidine Blue (Sigma-Aldrich). Stained tissues were further dehydrated through ethanol series and finally mounted with Cytoseal 60 (Thermo Scientific). Images were taken under a fluorescence microscope and meristem size was measured using ImageJ software (NIH). The width of floral meristems was measured along a line between two sepal and petal primordia in floral buds at stage 3 and 4 days post floral initiation (dpi), respectively. For each genotype and time point, at least five meristems were measured. A two-tailed t-test was performed for statistical analysis.

4.3.4 | Statistical analysis

Analysis of variance (ANOVA) and Tukey's mean separation tests (HSD) were performed using the average of 20–40 measurements from each plant except for fruit size dimension, in which an average of eight fruits was used. Comparisons were made using the average per plant and 3–7 plants per genotype. Epistasis between the two loci was determined using two-way ANOVA with the following model: \( Y_{ijk} = \mu + a_i + b_j + ab_{ij} + \epsilon_{ijk} \), in which \( a \) and \( b \) represented the effect of lc and fas, whereas \( ab \) was the interaction factor. In addition, \( i \) represented the \( i \)th allele at lc, \( j \) represented the \( j \)th allele at fas, while \( k \) represented the number of all plants used in the analysis. To estimate how lc and fas contributed to trait variance, dominance-to-additive variance ratio (d/a) was calculated using the following equation: \( d/a = (2Aa-AA-aa)/(AA-aa) \). AA and aa represented the phenotypic effects caused by homozygous derived and wild type alleles, respectively. Aa represented the phenotypic effect caused by heterozygous alleles.

4.4 | In situ hybridization to determine LC and FAS expression in floral meristems

RNA in situ hybridization was performed with digoxignin-labeled RNA using by Wu, Xiao, Cabrera, Meulia, and van der Knaap (2011) with minor modifications. To generate the RNA probes, full-length SICLV3 (Solycc11 g071380) and SIWUS (Solycc02 g083950) cDNA was amplified from M82 cDNA using Phusion Taq (Invitrogen) and ligated into the pSC-A-amp/kan vector containing T7 and T3 promoter (pro-vided by Lippman’s lab, CSHL). Clones pSL-CLV3-1 and pSL-CLV3-4 were generated to make SIWUS antisense and sense probes, respec-tively. Clone pSL-WUS-4 was used to make both SIWUS sense and antisense probes. Depending on the orientation of the insert, T7 or T3 RNA polymerase was used to transcribe sense or antisense RNA.

Young inflorescences were fixed with ice-cold 4% (w/v) paraformaldehyde and vacuum infiltrated with a pressure of 25–28 in Hg for 20–30 min until the samples had sunk. Samples were dehyd-rate through ethanol series, followed by histoclear replacement. Paraffin wax (Polyscience) was used for sample embedding with at least six fresh exchanges for 3 days. Microtome sections were taken to obtain 10 \( \mu \)m thick ribbons. Slides were rehydrated in an ethanol series followed by Proteinase K digestion and acetylation. The hybridization reactions were conducted at 55°C overnight with
the gene-specific DIG-labeled probes. Excess probes were washed off with saline-sodium citrate buffer (SSC buffer) and slides were blocked with blocking reagent (Roche). To detect the signal, slides were incubated with alkaline phosphatase-conjugated antibody (anti-DIG-AP Fab fragments, Roche) at room temperature for 2 hr. Non-specific binding of antibody was washed three times with BSA buffer for 1.5 hr each. Finally, Western Blue (Promega) was applied to each slide and incubated overnight in dark at room temperature for the color reaction. Images were taken under the fluorescence microscope (Leica) equipped with a digital camera in Molecular and Cellular Image Center in OARDC.

### 4.5 | Tissue collection and RNA extraction

For each genotype, approximately 300 first and second inflorescences with only 3–4 visible floral buds were collected from 5 to 7-week old plants that were sown over a 6-week window. For each replicate, tissues were collected daily between 2 and 3 p.m. During the collection, all inflorescences were immediately immersed in ice-cold RNAlater (QIAGEN) solution at five times the volume of the sample in order to prevent RNA degradation. After sample collection, vacuum infiltration was applied until tissues sunk to the bottom of RNAlater solution. The tissues were then stored in RNAlater at −80°C. Five different developmental stages including sympodial shoot apical meristem (SYM), floral and inflorescence meristems (F&IM), 2 days post floral initiation (dpi), 4 and 6 dpi floral buds were collected from each genotype with three replicates. For the dissection, meristems and buds of different developmental stages were isolated using forceps under a dissection microscope and immediately put into a 1.5 ml eppendorf tube in 1 ml of fresh RNAlater that was kept on ice. The samples were stored at −80°C prior to the RNA extraction. Prior to RNA extraction, the precipitated RNAlater crystals need to be dissolved by occasionally shaking the eppendorf tube at room temperature several times. The RNAlater reagent was further removed using a fine-tip drawn-out glass pipette. RNA extraction was conducted using Trizol® (Invitrogen Inc.) following manufacturer’s recommendation. The quality of RNA in each sample was examined using Agilent Bioanalyzer prior to RNA library preparation. Samples with a total RNA amount of 1–2 μg were used for subsequent RNA library preparation.

### 4.6 | 3′ Tag RNA-seq library preparation

3′ Tag RNA-seq, a low-cost RNA-seq alternative, was adapted to our study. Meyer, Aglyamova, and Matz (2011) presented a 3′ Tag RNA-seq approach that solely focused on sequencing the 3′ end of mRNA. This 3′ Tag RNA-seq method requires only 5 million reads per sample, which can significantly reduce the cost for sequencing per sample by allowing a higher degree of multiplexing. RNA-seq libraries of approximately 300 bp fragments were prepared following the 3′ Tagseq protocol of Meyer et al. (2011) with little modifications as per directions from Dr. Thomas Juenger, The University of Texas at Austin. In total, sixty libraries were made, including our four genotypes at five developmental stages, and each with three biological replicates. The mRNA was enriched using Oligo d(T)25 magnetic beads (NEB) by following the manufacturer’s recommendations. The bound mRNA was eluted from the beads by adding 2× Supertscript II first-strand buffer (Invitrogen) supplemented with 10 mM DTT. Samples containing eluted mRNA, magnetic beads and first-strand buffer were incubated at 94°C for 2 min to fragment the mRNA, and immediately placed on ice. Samples with fragmented RNA were placed on the magnetic rack to remove the Oligo d(T)25 beads. The supernatant containing fragmented mRNA and first-strand buffer was transferred to a new tube for first-strand cDNA synthesis. First-strand cDNA was synthesized using Supertscript II reverse transcriptase in the presence of 3′ Oligo dT primers and 5′ RNA adaptors with GGG at 5′ end at 42°C for 1 hr. To amplify targeted 3′ end cDNA, AccuPrime Taq polymerase (Invitrogen) was used for the 16 cycles of PCR amplifications. To purify the resulting 3′ end cDNA products, the excess primers, nucleotides, salts and enzymes were removed by Agencourt AMPure XP using 1.8 volume of beads solution. cDNA quantity was measured using the Qubit HS and all samples were diluted to 40 ng in 42 μl total volume. Next, library-specific barcodes and Illumina universal adaptors were incorporated to each cDNA by five cycles of PCR in 50 μl total volume using AccuPrime Taq polymerase (Invitrogen). Afterwards, six libraries were pooled together. To size select 300–350 bp fragments, 0.7 volume of Agencourt AMPure XP beads to 1.0 volume of sample was used and the supernatant was collected to remove cDNA size larger than 400 bp. Next, 0.85 volume of Agencourt AMPure XP beads to 1.0 volume of sample was used to target cDNA size around 300–350 bp. The bound cDNA was eluted from the beads by adding distilled water. The fragment size and concentrations of the samples were examined using Agilent Bioanalyzer and Qubit HS. Samples with 30 ng total amount were mixed together to create two pools with 30 3′ Tag RNA-Seq libraries each. The RNA libraries were sent to the Genomic Resources Core Facility at Weill Cornell Medical College for 100 bp single-end sequencing on the Illumina HiSeq2500. The raw data are deposited at NCBI under accession numbers GSE129809 (3′ Tag RNA seq) and GSE129901 (whole mRNA seq).

### 4.7 | Gene expression analysis

Raw reads were checked for their quality in FASTQC. Total raw reads for each sample ranged between 5 to 10 million (Table S4). Filtering steps were performed using fqtrim program (http://ccb.jhu.edu/software/fqtrim/index.shtml) to remove up to 50% low quality reads with QC score < 20 as well as poly-A tail contaminations. About 94% of clean reads mapped to ITAG2.4 Released Tomato Genome through Rsubread 3.4 (Liao, Smyth, & Shi, 2013) and 81% of clean reads mapped to annotated genes. Most of the genes were either not expressed or expressed at low levels (Figure S13). Pearson correlations were performed to check the reproducibility between replicate. An average correlation (r = 0.99) was obtained which showed a high reproducibility between samples of the same stage and genotype in this experiment. For the sequencing coverage, around 55%–60% of 29,324 tomato reference
genes showed at least three mapped reads among all samples. Gene expression levels were normalized using the RPM value (Reads Per Million). The significance of differentially expressed genes (DEGs) was determined by linear factorial modeling in DESeq2, of which likelihood ratio test was applied (Clevenger et al., 2017). To identify genes with significant genotype effects using DESeq2 in R, the full model (Genotype + Time) and reduced model (Time) were used to test whether the observed differences in read counts of a given gene between genotypes were significantly larger than the variations between developmental stages and replicates. Similarly, genes with significant genotype by time point interactions were identified form the full model using (Genotype + Developmental stage + Genotype * Developmental stage) as well as the reduced model (Genotype + Developmental stage). For the genotype category, DEGs with adjusted p-value < 0.1 in at least one comparison were selected, whereas the threshold of p-value < 0.001 was used for selecting DEGs with interaction effects. The p-value was adjusted with the default Benjamini-Hochberg method in DEseq2. The relatively lower stringency (adj. p < 0.1) was used as a cutoff in the genotype category because of the weak cis-regulatory mutant alleles at lc and fas, which leads to 669 DEGs with significant genotype effect. In addition, because very few DEGs (seven genes) were identified in the interaction category with adj. p < 0.1, we objectively selected a p-value cutoff based on SIWUS expression, close to the p-value of <0.001. As a result, 13 DEGs with significant interaction effects were identified. Notably, the model we used for identifying genotype effect contained gene variables with genotype × developmental stage effects. Therefore, we identified seven DEGs genes shared by the two methods.

To obtain an overview of enriched Gene Ontology (GO) terms for the 675 DEGs, Arabidopsis homologs of these DEGs were used as inputs in the Cytoscape plug-in, GlueGO v2.1.6 (Bindea et al., 2009). To identify co-expressed genes, samples were clustered based on the normalized expression (Z-score) of the wild type across four developmental stages (F&M, 2, 4, and 6 dpi). By using the Mfuzz package (Kumar & Futschik, 2007) with fuzzy c-mean algorithm in R, DEGs were grouped into eight clusters. Furthermore, to identify a core of genes showing similar expression dynamics in mutants within each cluster, expression values from lc, fas and lc/fas NILs were Z-score normalized with WT. The normalized expression values were used to calculate PCCs between gene pairs within each cluster. To select the core co-expressed genes, the PCC matrix was hierarchically clustered using Ward’s method and visualized through heatmap3 in R (Zhao, Guo, Sheng, & Shyr, 2014). DEGs clustered within the hierarchical sub-group were objectively selected as a sub-cluster as they were tightly co-expressed among WT and mutants through different developmental stages.

4.8 | Whole mRNA-seq sample preparation, library construction, and data analysis

For the whole mRNA-seq, tissues were collected from the wild type and RNAi-SLCLV3 lines at three developmental stages, each with four replicates. The first stage included inflorescence meristems, floral meristems and the youngest floral buds. The second and third stages included floral buds at 4 and 6 dpi, respectively. Plants were grown in two-gallon pots under natural light supplemented with artificial light (16/8 hr light/dark cycle) in the greenhouse in Wooster, OH, USA, 2013. For each genotype, 100–150 young inflorescences (the largest floral bud was smaller than 0.5 cm) from six individual plants were collected using forceps. For each replicate, tissues were weekly collected during 10 a.m.–12 p.m. in the greenhouse over 4 weeks.

Strand-specific RNA libraries of approximately 250 bp fragments were prepared following the protocol of Zhong et al., 2011. Briefly, six libraries were barcoded and pooled in one lane. The RNA-seq libraries were sent to the Illumina HiSeq2000 at Genomic Resources Core Facility at Well Cornell Medical College for 50 bp single–end sequencing. After filtering low quality reads and de-multiplexing, the quality of 50 bp raw reads were checked through FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The cleaned raw reads were then mapped to ITAG2.3 Released Tomato Genome through the Tophat2 high throughput short read aligner. Total reads from sense strand for each sample are about 20–30 million. Gene expression levels were normalized using RPKM (Reads Per Kilobase per Million) for whole mRNA-seq.

4.9 | Phylogenetic analysis

Phylogenetic tree analysis was performed to assigned nine differentially expressed kinesin genes to one of 10 plant kinesin families. To retrieve the kinesin protein sequences in tomato, full-length protein sequences were downloaded from the International Tomato Annotiation Group release 3.20 predicted proteins (ITAG 3.20) (http://solgenomics.net/). To retrieve the Arabidopsis kinesin proteins, we selected kinesin genes based on Zhu and Dixit (2012) and downloaded their sequence from The Arabidopsis Information Resource (TAIR) database. Sequences of maize kinesin proteins were retrieved from Maize Genetics and Genomics Database (MaizeGDB) website (https://www.maizegdb.org/). Kinesin proteins from other organisms were also selected based on previous analyses reported on the online website (https://labs.cellbio.duke.edu/kinesis/index.html) created by Liz Greene, Steve Henikoff and Sharyn Endow. The Multiple Expectation Maximization for Motif Elicitation (MEME) tool (Bailey & Elkan, 1994) was used to define the conserved motifs in tomato kinesin genes. The multiple sequence comparison by log- expectation (MUSCHEL) algorithm implemented in the Molecular Evolutionary Genetics Analysis program (MEGA, version7.0) was used to perform multiple sequence alignment with default settings (Kumar, Stecher, & Tamura, 2016). Phylogeny tree was constructed using the neighbor-joining method with nucleotide p-distance and 1,000 bootstrap replicates.

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CONFLICT OF INTEREST

The authors declare no conflict of interest associated with the work described in this manuscript.

AUTHOR CONTRIBUTIONS

YHC and ZH performed the research; YHC contributed to new analyses tools and analyzed the data; JCC and EvdK supervised the research and writing; EvdK designed the research; YHC wrote the manuscript with revisions from JCC and EvdK. All authors approved the manuscript.

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SUPPORTING INFORMATION

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

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