Tomato AUXIN RESPONSE FACTOR 5 regulates fruit set and development via the mediation of auxin and gibberellin signaling

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Auxin response factors (ARFs) encode transcriptional factors that function in the regulation of plant development processes. A tomato ARF gene, SlARF5, was observed to be expressed at high levels in emasculated ovaries but maintained low expression levels in pollinated ovaries. The amiRNA SlARF5 lines exhibited ovary growth and formed seedless fruits following emasculation. These parthenocarpic fruits developed fewer locular tissues, and the fruit size and weight were decreased in transgenic lines compared to those of wild-type fruits. Gene expression analysis demonstrated that several genes involved in the auxin-signaling pathway were downregulated, whereas some genes involved in the gibberellin-signaling pathway were enhanced by the decreased SlARF5 mRNA levels in transgenic plants, indicating that SlARF5 may play an important role in regulating both the auxin- and gibberellin-signaling pathways during fruit set and development.

The plant hormone auxin, indole-3-acetic acid (IAA), plays an important role in various aspects of plant development, such as cell extension, division, and differentiation, as well as in organ and tissue development, and tropism. Auxin response factors (ARFs) bind specifically to a TGTCTC motif found in auxin-responsive promoter elements (AuxREs) and mediate auxin responses. A typical ARF protein contains three parts: an N-terminal DNA-binding domain (DBD), a carboxyl-terminal dimerization domain (CTD) that is similar to motifs III and IV of Aux/IAA proteins, and a middle region (MR) that may activate or repress the expression of early auxin response genes, including small auxin up RNA (SAUR), Gretchen Hagen-3 (GH3) and lateral organ boundaries-domain (LBD)1.

Among the components of the auxin signal transduction pathway, ARF proteins participate in transcriptional regulation of a variety of biological processes related to plant growth and development. Studies of arf mutants have indicated that different ARFs possess diverse functions, which is due to the differences in temporal and spatial expression and affinities with promoters of target genes. Recently, functional studies have demonstrated that ARF genes play an essential role in signal transduction during plant organ development. In Arabidopsis thaliana, ARF2 regulates leaf senescence2, root formation and flower organ senescence2. Moreover, heterologous expression of mango MiARF2 in Arabidopsis inhibits root and hypocotyl growth3. ARF3 plays an important role in lateral root development of Arabidopsis and regulates epidermal cells and trichome formation in tomato4,5. SIARF4 is involved in hypocotyl development, cotyledon growth and fruit pericarp and sugar metabolism during tomato fruit development6,7. Inhibition of ARF2, ARF3, and ARF4 expression by some tasiRNAs may release the repression of Arabidopsis lateral root growth8. Arabidopsis ARF10 and ARF16 have essential roles in the process of root cap development, and the auxin signal cannot bypass them to initiate columella cell production9. Furthermore, ARF7 and ARF19 may regulate Arabidopsis LR formation initiation by interacting with IAA14.

Several ARFs also play an essential role in reproductive development. Tomato SIARF2 is involved in the regulatory mechanism of the fruit ripening process10. In Arabidopsis, ARF6 and ARF8 promote jasmonic acid production and flower maturation11 and influence the developing floral organs12. Downregulation of SIARF6 and

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Results

Expression pattern of \( \text{SlARF5} \) in tomato. \( \text{SlARF5} \) transcripts in different organs and tissues. (B) Expression levels of \( \text{SlARF5} \) at different stages during flower and fruit development. Flower bud samples were at lengths of 3.0–4.5 mm, 4.5–6.0 mm, and 6.0–7.5 mm. Anthesis represents fully opening flowers. Three DAA-U, 6 DAA-U, and 9 DAA-U represent flower samples collected at 3, 6 and 9 d after anthesis, respectively, which were emasculated 2 days before opening. Three DAA-P, 6 DAA-P, and 9 DAA-P represent tomato flowers collected 3, 6, and 9 d after anthesis with hand pollination. ANOVA statistical analyses were performed using SPSS 15.0. Significant differences (\( p < 0.05 \)) between treatments, as determined by Tukey’s tests, are indicated with different letters. Data are expressed as the mean ± standard errors for three replicates.

\( \text{SlARF8} \) in transgenic tomato plants by the overexpression of \( \text{MIR167a} \) may lead to floral development defects and female sterility\(^{15} \). Furthermore, \( \text{ARF8} \) is a negative regulator of fruit initiation and development in \( \text{Arabidopsis} \), tomato and eggplant\(^{16,17} \). Similarly, \( \text{ARF7} \) functions as a negative regulator of fruit set until pollination and fertilization have occurred\(^{18} \). Models have been proposed for the effects of \( \text{ARF8} \), \( \text{ARF7} \) and \( \text{ARF9} \) on plant fruit development through the interactions between the auxin- and GA-signaling pathways during tomato fruit initiation and development.

Tomato—a fleshy climacteric fruit—is a model plant for studying fruit development and ripening\(^{21} \). In general, tomato fruit development is divided into four major phases: fruit setting, cell division, cell expansion, and a fruit ripening period. Phytohormones have been demonstrated to participate in the regulation of every aspect of fruit development; however, each hormone plays a specific role in the fruit set, expansion, and ripening stages. Biochemical analyses have identified two peaks in auxin levels during tomato fruit development: the first at 8 d after pollination at the end of active cell division, and the other at 30 d after pollination\(^{22} \), corresponding to cell expansion (stage III) and the fruit maturation stage (stage IV), thereby suggesting that auxin has an important role in promoting fruit cell expansion\(^{22–24} \) and initiating and enhancing climacteric ripening\(^{25} \). On the other hand, the levels of endogenous GAs peak in two stages: first at 8 d after flowering and then approximately 15 d before ripening\(^{22,24} \), which corresponds to cell division (stage II) and the cell expansion stage (stage III) of fruit development, thereby indicating that GA is an important factor in fruit cell cycle and expansion\(^{22,24} \). GA activity regulates the expression of cell division and the expansion genes in tomato\(^{27} \). Furthermore, previous studies have demonstrated that the expansion of tomato locular cells coinciding with the expression of genes encoding for water flow, organic acid synthesis, sugar storage, and photosynthesis is regulated by auxin and GA\(^{30} \). \( \text{SIIA17} \) in the auxin-signaling pathway may affect fruit cell size in tomato\(^{31} \). Additionally, \( \text{Matsuo et al.} \) suggested that cytokinins regulate fruit development by modulation of auxin biosynthesis and/or polar auxin transport. Certain cell cycle genes, such as cyclin-dependent kinases (CDKs)\(^{32} \) and cell cycle-associated protein kinase \( \text{WEEl} \)\(^{34} \), affect tomato fruit cell division. Meanwhile, certain other genes, including \( \text{TAGLI} \) (\( \text{SHATTERPROOF} \))\(^{35} \), MYB factors\(^{36} \), and \( \text{SIPPC2} \) (phosphoenolpyruvate carboxylase)\(^{37} \), may regulate fruit cell expansion in tomato.

In a previous study, we identified 21 \( \text{SlARFs} \) in the tomato genome; their structures, chromosome locations, and phylogeny were predicted\(^{38} \). However, to date, their functions in fruit development have not been studied (beyond \( \text{SlARF6, SlARF7, SlARF8 and SlARF9} \)). Our data indicated that certain other ARFs also exhibited preferential expression in young ovaries and their mRNA levels were significantly altered during fruit development. Their roles in fruit development remain unclear. To explore the function of \( \text{SlARF5} \) during fruit development, \( \text{SlARF5} \)-silenced tomato plants were generated by the \( \text{amiRNA} \) method. \( \text{SlARF5} \) \( \text{amiRNA} \) lines form seedless fruits without pollination after emascula- tion. These parthenocarpic fruits developed fewer locular tissues with reduced fruit size and weight compared to those of wild-type plants. Downregulation of \( \text{SlARF5} \) resulted in reduced expression of auxin-related genes and increased the expression levels of certain GA-signaling pathway genes. According to these data, we hypothesized that \( \text{SlARF5} \) may participate in the regulation of early fruit set and development by mediating the crosstalk between IAA and GA hormones during tomato fruit development.

Figure 1. Real-time PCR analysis of relative gene expression of \( \text{SIARF5} \) mRNA in tomato. (A) \( \text{SIARF5} \) mRNA expression in tomato. (B) Expression levels of \( \text{SIARF5} \) at different stages during flower and fruit development. Flower bud samples were at lengths of 3.0–4.5 mm, 4.5–6.0 mm, and 6.0–7.5 mm. Anthesis represents fully opening flowers. Three DAA-U, 6 DAA-U, and 9 DAA-U represent flower samples collected at 3, 6 and 9 d after anthesis, respectively, which were emasculated 2 days before opening. Three DAA-P, 6 DAA-P, and 9 DAA-P represent tomato flowers collected 3, 6, and 9 d after anthesis with hand pollination. ANOVA statistical analyses were performed using SPSS 15.0. Significant differences (\( p < 0.05 \)) between treatments, as determined by Tukey’s tests, are indicated with different letters. Data are expressed as the mean ± standard errors for three replicates.
its lowest levels in 6.0–7.5 mm ovaries just before anthesis (Fig. 1B). After pollination and fertilization, SlARF5 expression was maintained at low levels, increasing only slightly at 6 days after anthesis (with hand-pollination) and then decreasing again at 9 days. However, when the flowers were emasculated at 2 days before anthesis, transcription levels increased markedly at anthesis, peaked at 3 days after anthesis (without hand pollination), and then decreased significantly from 6 to 9 days after anthesis. In general, SlARF5 expression was downregulated in ovaries that were pollinated compared to that in ovaries from plants subjected to emasculation. Similar to SlARF5, pollination also suppressed the expression of ARF8 in Arabidopsis and tomato16 and SlARF7 in tomato39.

**Suppression of SIARF5 induces parthenocarpy.** To explore the function of SIARF5 during fruit set and development, amiSlARF5 transgenic plants were generated using A. thaliana miRNA160a as a backbone to express an artificial miRNA (amiRNA) using a 125 bp SIARF5-specific fragment, which was cloned into the pCAMBIA1301-35S vector. The amiRNA binary vector was transferred into tomato using Agrobacterium tumefaciens (Fig. 2A). A total of 11 independent transgenic lines were generated and identified based on PCR and GUS screening (Supplementary Fig. S1). Among them, five lines exhibited significantly lower SlARF5 expression levels compared to those in wild-type plants. In two amiSlARF5 lines, namely, SlARF5-6 and SlARF5-9, the SlARF5 transcription levels were reduced by 67.8% and 68.5%, respectively (Fig. 2B). In addition, although SlARF6, SlARF7, SlARF8, and SlARF19 were closely related with SlARF5 in molecular phylogenetic tree (Supplementary Fig. S2), their expression levels were not evidently affected in these two lines (Supplementary Fig. S3). Thus, these two amiSlARF5 lines were used for further analysis.

No evident differences in vegetative growth or floral morphology were observed between amiSlARF5 and wild-type plants (Supplementary Fig. S4). Furthermore, the fruit set rates in the amiSlARF5-6 and amiSlARF5-9 lines, 65.0% and 68.7%, respectively, were comparable to those of wild-type plants (74.2%) (Table 1). However, the self-pollinated amiSlARF5 fruits were significantly smaller than wild-type fruits (Fig. 3A–F, Table 1). Both the polar and equatorial diameter of the transgenic fruits were much smaller compared to those obtained from wild-type and empty vector plants. Similarly, the weights of pollinated amiSlARF5 fruits (1.83 ± 0.27 g for SlARF5-6 and 1.84 ± 0.28 g for SlARF5-9) were much lower than those of the wild-type and empty vector transgenic fruits, at 3.40 ± 0.97 g and 3.32 ± 0.59 g, respectively.

Emasculation of untransformed ‘Micro-Tom’ plants 2 d before anthesis negatively affected fruit growth. Emasculated ovaries stopped growing 6 d after anthesis, became abortive and ultimately the flowers abscised within 6–9 days (Table 1). However, when the transgenic flowers were emasculated, the parthenocarpic fruit set and growth occurred in SIARF5-6 and SIARF5-9 lines with fruit set rates of approximately 20.5% and 22.4% in these two transgenic lines, respectively. Although the fruit set rate in emasculated transgenic plants was lower than that in self-pollinated plants, suppression of SIARF5 evidently initiated fruit development and enhanced parthenocarpic capability. However, the average size and weight of the parthenocarpic fruits were significantly lower than those of self-pollinated fruits. Consistently, Mapelli et al.22 also revealed that parthenocarpic fruits were generally smaller than seeded fruits. Furthermore, the smaller fruits in the SIARF5 amiRNA transgenic lines formed fewer locular tissues with reduced pulp and enlarged central columella compared to those observed in the...
wild-type fruits, resulting in parts of the locular cavities being empty (Fig. 3J–L). Interestingly, in parthenocarpic SlARF7 RNAi fruits and in the fruits treated with GA$_3$, the locular tissue was barely developed, resulting in almost empty locular cavities.

Unexpectedly, self-pollinated transgenic fruits formed well-developed seeds of normal size and with germination potential; however, each fruit contained much lower numbers of fully developed seeds, 8.0 and 8.5 in SlARF5-6 and SlARF5-9 per fruit, respectively, compared to 20.5 and 18.5 in wild-type and empty-vector fruits, respectively (Table 1). The parthenocarpic fruits in the amiSlARF5 lines had no seeds (Fig. 3K,L).

Suppression of SIARF5 expression affects cell division and expansion during early fruit development. In tomato, the cell division period lasts for 10–14 d. In the next 6–7 weeks, cell division activity is weakened and fruit growth set is predominantly dependent on cell expansion. Our study demonstrated that transgenic plants of the SIARF5-6 and SIARF5-9 lines, in which SIARF5 transcript levels were downregulated, produced smaller fruits than those of wild-type plants after pollination.

| Line                  | Fruit weight (g) | Polar diameter (cm) | Equatorial diameter (cm) | Seeds/fruit | 1000-seed weight (g) | Germination rate (%) | Fruit set rate (%) |
|-----------------------|------------------|---------------------|--------------------------|-------------|----------------------|----------------------|--------------------|
| Wild type             | 3.4 ± 0.97 a     | 1.9 ± 0.12 a        | 2.02 ± 0.27 a            | 20.5 ± 4.9a | 2.40 ± 0.24 a        | 70 a                 | 74.2 (n = 90)      |
| 1301                  | 3.32 ± 0.59 a    | 1.84 ± 0.13 a       | 1.96 ± 0.13 a            | 18.5 ± 2.1a | 2.50 ± 0.23 a        | 68.2a                | 70.1 (n = 75)      |
| ARF5-6                | 1.83 ± 0.27 b    | 1.42 ± 0.33 b       | 1.49 ± 0.18 b            | 8.0 ± 2.8 b | 2.36 ± 0.19 a        | 65.9a                | 65.0 (n = 85)      |
| ARF5-9 (emasculated) | 1.84 ± 0.28 b    | 1.43 ± 0.13 b       | 1.44 ± 0.15 b            | 8.5 ± 0.7 b | 2.37 ± 0.03 a        | 66.7a                | 68.7 (n = 70)      |
| ARF5-6 (emasculated) | 1.54 ± 0.44 c    | 1.34 ± 0.26 bc      | 1.36 ± 0.39 c            | 0 c         | —                    | —                   | 20.5 (n = 75)      |
| ARF5-9 (emasculated) | 1.30 ± 0.42 c    | 1.22 ± 0.25 c       | 1.32 ± 0.27 c            | 0 c         | —                    | —                   | 22.4 (n = 90)      |
| Wild type (emasculated) | —                | —                   | —                        | —           | —                    | —                   | 0 (n = 75)         |

Table 1. Characterization of fruit and seed development in wild-type tomato and amiARF5 transgenic lines. Note: The fruits were from mature fruits of pollinated and emasculated (indicated), approximately corresponding to 45 d after anthesis with pollination and un-pollination, respectively. For each line, 15–30 fruits were sampled for statistical analysis. The total number of flowers for evaluation of fruit set rate is indicated (n).
To understand the cause of different fruit phenotypes, histological cross-sections of wild-type and SlARF5 transgenic plants were analyzed using the ovaries and developing fruits collected at diverse developmental stages, ranging from unpollinated ovaries to fruits 10 mm in diameter (Fig. 4A–O). In general, the pericarps of both the tomato fruits differentiated into the three classic layers: exocarp, mesocarp, and endocarp24. At anthesis, cell size in the pericarps of both wild-type and transgenic ovaries showed no obvious differences (Fig. 4A,F,K). In 3–4 mm fruits, the exocarp cells were at the stage of cell division, whereas the mesocarp and the endocarp cells began to expand. At this stage, the cell size in the mesocarps and endocarps of wild-type and transgenic lines were not significantly different. However, in 5–6 mm fruits, the exocarp and mesocarp cells in transgenic lines were significantly larger than those in wild-type plants. The mean cell area of the exocarp in RNAi fruits was nearly 2-fold greater than that of the wild-type fruits, while mesocarp cells were three times larger than those of wild-type cells (Fig. 4C,H,M, Table 2). The differences in exocarp features of RNAi and wild-type fruits were more clearly observed in the 7–8 mm and 9–10 mm fruits.

On the other hand, there are significant differences in the number of cell layers between transgenic amiSlARF5 lines and wild-type plants. In general, the exocarp cells of tomato fruit comprise 4–5 cell layers outside the fruit, and the endocarp cells comprise 1–2 cell layers inside the fruit; therefore, the mesocarp cell number was the major contributor towards the differences in fruit equatorial diameter among the plants with different genotypes. In 3–4 mm fruits, pollinated transgenic fruits formed fewer cell layers (approximately 16 cell layers) than those in the wild-type plants (at least 23 cell layers), a reduction of 30% compared to wild-type plants. Similarly, in the 5–6 mm and 7–8 mm fruits, the pollinated transgenic fruit formed fewer cell layers than those in the wild-type fruit, with levels being 32% and 25–31% lower, respectively (Fig. 5A–I, Table 3). This indicates that inhibition of SIARF5 leads to a significant reduction in the number of cell layers. The small size of mature transgenic fruits may be primarily attributed to the reduced number of cell layers in transgenic fruits, probably as a result of a reduction in the period of cell division that promotes cell layer formation.

Figure 4. Microscopic analysis of the pericarp in the fruits of wild-type and transgenic lines during early tomato fruit development. (A–E) Wild-type fruit; (F–J) Transgenic line amiRNA SIARF5-6 fruit; (K–O) Transgenic line amiRNA SIARF5-9 fruit. (A,F,K) Unpollinated ovary at the anthesis stage; (B,G,L) Pericarp of a 3–4 mm diameter fruit; (C,H,M) Pericarp of a 5–6 mm diameter fruit; (D,I,N) Pericarp of a 7–8 mm diameter fruit; (E,J,O) Pericarp of a 9–10 mm diameter fruit. Bars = 50 μm.
amiSlARF5 and expansion is negatively affected in the transgenic lines compared to the levels observed in wild-type plants, suggesting that both fruit cell division and expansion were downregulated in endoglucanase EXP2, EXP8, xyloglucan, pectinacetylesterase gene. Regarding the biological processes, a high proportion of DEGs was associated with oxidation-reduction activity (60%), which is consistent with kinase genes associated with clusters that are highly expressed in young fruit. Functional enrichment analysis was performed. GO analysis indicated that these DEGs were primarily enriched in ATP, protein, or DNA binding (383), protein kinase activity (147), oxidoreductase activity (131), and catalytic functional enrichment analysis was performed. GO analysis indicated that these DEGs were primarily enriched in the membrane, nucleus, or the cell wall (Fig. 6), which is consistent with reports demonstrating that the cell wall plays essential roles in fruit set and fruit size. Similarly, previous transcriptome analysis indicated that the cell-cycle-related genes were associated with clusters that are highly expressed in young fruit. Regarding the biological processes, a high proportion of DEGs was associated with oxidation-reduction processes, regulation of transcription, protein phosphorylation and metabolic metabolism. Among them, transcription regulation, signal transduction, and metabolic processes accounted for more than half of all annotated DEGs, implying that signal transduction pathways and metabolic activities are strongly affected by inhibition of the SlARF5 gene. In addition, in terms of the cellular components, most DEGs were primarily enriched in the membrane, nucleus, or the cell wall. KEGG analysis further indicated that most of the downregulated genes are involved in the cell cycle (14%), the cell cycle of yeast (12%), and DNA replication (10%) in amiARF5 transgenic fruits, suggesting that SlARF5 is positively involved in cell division. The upregulated genes were associated with phenylalanine metabolism (12%), phenylpropanoid biosynthesis (16%), plant-pathogen interactions (13%), and starch and sucrose metabolism (7%) (Fig. 6). Among these DEGs, the expression of several genes related to fruit development was significantly altered in transgenic plants, including those involved in cell division, cell expansion, and hormone-signaling pathways (Tables 4 and 5 and Fig. 6).

### Cell division- and cell expansion-related genes

| Line     | Fruit size in diameter (mm) | Exocarp Mean area (μm²) | Mesocarp Mean area (μm²) |
|----------|-----------------------------|-------------------------|--------------------------|
| Wild type| 5–6                         | 163.0 ± 9.2 d (n = 1015) | 1601.0 ± 148.8 e (n = 1145) |
| ARF5-6   | 5–6                         | 322.9 ± 14.6 c (n = 1177) | 4968.7 ± 287.7 cd (n = 735) |
| ARF5-9   | 5–6                         | 376.1 ± 11.4 bc (n = 1425) | 4415.0 ± 14.8 d (n = 929) |
| Wild type| 7–8                         | 385.4 ± 36.2 bc (n = 1200) | 4410 ± 237.2 d (n = 1100) |
| ARF5-6   | 7–8                         | 403 ± 34.2 bc (n = 1342) | 5470.5 ± 186.9 bc (n = 720) |
| ARF5-9   | 7–8                         | 536.5 ± 37.9 a (n = 630) | 5975 ± 635.8 ab (n = 510) |
| Wild type| 9–10                        | 468.8 ± 61.4 ab (n = 645) | 5213.6 ± 235.0 bcd (n = 460) |
| ARF5-6   | 9–10                        | 554.6 ± 54.4 a (n = 922) | 6792 ± 315.9 a (n = 475) |
| ARF5-9   | 9–10                        | 552.9 ± 32 a (n = 810) | 6533.8 ± 546.8 a (n = 532) |

Table 2. Cell mean area of the pericarp of wild-type and transgenic fruits. Wild-type and transgenic fruits of 5–6 mm, 7–8 mm, and 9–10 mm diameter, were used for the measurement of cell area (μm²). Approximately 100 cells for exocarp or mesocarp per fruit were analyzed at early stage, whereas more than 30 cells at late stage. The total number of cells each line for analysis is indicated (n). The Data represent the means ± standard error of five fruits from each transgenic at each development stage with three replicates. The different letters indicate the significant differences between wild-type and transgenic fruits (p < 0.05, Student’s t test).

Transcriptomic analysis of the amiSlARF5 and WT fruits. To evaluate expression changes during fruit development in amiSlARF5-9 transgenic and wild-type plants, massively parallel RNA sequencing (RNA-Seq) analyses were carried out using the RNA samples from 3–4 mm young fruits with two replicates. At this stage, SlARF5 was highly expressed in the wild type and no phenotypic differences were observed between amiSlARF5 and wild-type fruits. We generated four cDNA libraries from each genotype for sequencing using the Illumina Hiseq, 2000/2500 system at LC Sciences (Hangzhou, China). Each cDNA library yielded more than 45 million sequence reads (Supplementary Table S1), representing ≥4 Gb sequence data per sample. RNA data from the two biological replicates exhibited good correlations and were used for further analysis (Supplementary Fig. S5). A summary of RNA sequencing, mapping, assembly and annotation is provided in Supplementary Table S1.

Compared to wild-type fruits, a total of 3152 genes were differentially expressed in amiARF5 transgenic plants, with 1719 genes upregulated and 1433 genes downregulated (p < 0.05, |log2(fold changes)| ≥2) (Fig. 6). As expected, SlARF5 was downregulated by approximately 16-fold in transgenic plants. Seventeen genes from the RNA-seq-derived results were validated by qRT-PCR. The results demonstrated that almost all tested genes were in agreement with those from the RNA-seq data (Supplementary Fig. S6), confirming the reliability of RNA-seq analysis.

To further evaluate the potential functions of differentially expressed genes (DEGs) in the transgenic fruits, functional enrichment analysis was performed. GO analysis indicated that these DEGs were primarily enriched in ATP, protein, or DNA binding (383), protein kinase activity (147), oxidoreductase activity (131), and catalytic activity (60), which is consistent with kinase genes associated with clusters that are highly expressed in young fruit. Regarding the biological processes, a high proportion of DEGs was associated with oxidation-reduction processes, regulation of transcription, protein phosphorylation and metabolic metabolism. Among them, transcription regulation, signal transduction, and metabolic processes accounted for more than half of all annotated DEGs, implying that signal transduction pathways and metabolic activities are strongly affected by inhibition of the SlARF5 gene. In addition, in terms of the cellular components, most DEGs were primarily enriched in the membrane, nucleus, or the cell wall. KEGG analysis further indicated that most of the downregulated genes are involved in the cell cycle (14%), the cell cycle of yeast (12%), and DNA replication (10%) in amiARF5 transgenic fruits, suggesting that SlARF5 is positively involved in cell division. The upregulated genes were associated with phenylalanine metabolism (12%), phenylpropanoid biosynthesis (16%), plant-pathogen interactions (13%), and starch and sucrose metabolism (7%) (Fig. 6). Among these DEGs, the expression of several genes related to fruit development was significantly altered in transgenic plants, including those involved in cell division, cell expansion, and hormone-signaling pathways (Tables 4 and 5 and Fig. 6).
observed in wild-type fruits (Fig. 7A). In the later stages of fruit development, i.e., 7–8 mm fruits and above, there were no significant differences in the expression patterns of the cell cycle genes between the transgenic and wild-type fruits.

Similarly, in wild-type fruits, the expression of cell expansion-related genes (i.e., \( \text{SlEXPA5} \), \( \text{SlPEC} \), and \( \text{SlXTH1} \)) increased significantly following anthesis during early fruit development. In the 3–4 mm and 5–6 mm fruits, the expression of \( \text{SlEXPA5} \) in transgenic plants was significantly lower than that in wild-type fruits, and in
Figure 6. Analysis of genes regulated by SIARF5 during fruit development. (A) The numbers of differentially expressed genes in tomato fruit after SIARF5 expression was inhibited. (B) Analysis of biological functions of GO-enriched genes. (C) The primarily enriched KEGG pathway for up- and downregulated differentially expressed genes.

Table 3. Number of cell layers in wild-type and SIARF5 transgenic fruits. Wild-type and transgenic fruits of 3–4 mm, 5–6 mm, and 7–8 mm diameter were used for estimating the number of cell layers. Data represent the means ± standard error of five fruits per transgenic line at each development stage with three replicates. Different letters indicate the significant differences between wild-type and transgenic fruits ($P < 0.05$, Student’s t test).
maintained at similar levels during early fruit development (Fig. 7A). These results suggest that the microscopic characteristics of the transgenic lines are primarily due to reduced levels of cell division-related genes.

Auxin-signaling pathway genes. Previous studies have confirmed that auxin stimulates cell division. As shown in Table 5, 15 genes involved in auxin homeostasis, transport and signaling, including LAX2, ARF5, IAA1, IAA2, and IAA14, were downregulated in the transgenic lines compared to the levels observed in wild-type plants, suggesting that IAA-related genes are negatively affected in amRNA SIARF5 transgenic plants. In wild-type plants, the mRNA levels of some auxin response genes (e.g., SIARF9, SIAA1, SIAA2, and SIAA14) increased in 3–4 mm fruits and then decreased to significantly low levels during later stages. However, in transgenic plants, the mRNA levels of SIARF9, SIAA2, and SIAA14 were downregulated compared to those in wild-type plants, whereas SIAA1 was maintained at similar mRNA levels as those in wild-type plants. As expected, the expression of SIARF5 in transgenic plants was significantly lower than that in wild-type plants in the early stages, up to the 5–6 mm fruit stage. Similarly, all detected auxin-responsive genes, including SIARF9, SIGH3, SIAA1, and SIAA2, were significantly downregulated in the early development stage compared to the levels observed in wild-type plants, with the exception of a GH3-like gene, which was significantly upregulated in the 3–4 mm and 5–6 mm fruit stages (Fig. 7B); these results are consistent with the RNA-seq data, thereby suggesting that suppression of SIARF5 strongly affects several genes related to the auxin-signaling pathway. Unlike previous microarray data, in which the expression levels of most auxin-related genes were increased in the pollinated fruit, our RNA-Seq results demonstrated that a majority of the genes involved in the auxin signaling pathway were downregulated, in alignment with the microarray data of GA-treated fruit.

Gibberellin-related genes. The GA-biosynthesis gene, GA20ox1, was significantly downregulated in the transgenic lines compared to levels observed in wild-type plants (Fig. 7C), whereas four GA-related genes, including SLY1, GID1B, Cell wall protein (GA-induced), and GA-regulated family protein, were upregulated in the transgenic lines compared to the levels in wild-type plants (Table 5), thereby suggesting that GA-biosynthesis genes are negatively affected in amRNA SIARF5 transgenic plants, whereas certain GA-signaling genes are upregulated. Similarly, qRT-PCR analysis demonstrated that in wild-type plants, the expression levels of SIGA20ox1 and SIGAST1 markedly increased following pollination, and progressively decreased thereafter. However, SIGA2ox2, SIGA3ox1, SIGA2ox4, and SIGD1 continued to decline following anthesis during fruit development. In the amRNA SIARF5 lines, SIGA3ox1, SIGD1, and SIGAST1 exhibited similar expression patterns as those in wild-type plants, with no significant differences between each successive stage of early fruit development, with

| Gene ID       | Gene Description                                  | T_fpk  | WT_fpk  | log2 (Fold change) | p-value |
|---------------|---------------------------------------------------|--------|---------|--------------------|---------|
| Solyc11g055090.1 | Cyclin A1 (CycA1)                                 | 10.36  | 33.01   | −1.67              | 0.02    |
| Solyc06g065680.2 | Cyclin A2 (CycA2)                                 | 12.02  | 23.7    | −0.98              | 0.02    |
| Solyc10g078330.1 | B-type cyclin cyclid1 (cyclid1)                    | 11.08  | 26.09   | −1.23              | 0.01    |
| Solyc10g009404.0 | CyclinB1-4 (CycB1-4)                              | 12.93  | 29.42   | −1.19              | 0.01    |
| Solyc04g082430.0 | CyclinB2-4 (CycB2-4)                              | 12.94  | 25.64   | −0.99              | 0.02    |
| Solyc04g089580.0 | CyclinU4-1 (CycU4-1)                              | 9.97   | 57.04   | −2.52              | SE-05   |
| Solyc04g107738.0 | D-type cyclin-2 (CycD3-2)                         | 34.34  | 73.59   | −1.1               | 0.01    |
| Solyc04g078470.0 | D-type cyclin-3 (CycD3-3)                         | 6.08   | 30.49   | −2.33              | SE-05   |
| Solyc10g074270.1 | B1-type cyclin dependent kinase (CDKB1)           | 18.59  | 44.79   | −1.27              | 0.01    |
| Solyc04g107840.0 | B2-type cyclin dependent kinase (CDKB2)           | 44.87  | 90.11   | −1.01              | 0.02    |
| Solyc06g076660.0 | Proliferating cell nuclear antigen gene (PCNA)    | 32.97  | 94.27   | −1.53              | 0       |
| Solyc03g115760.0 | E2F transcription factor-like protein (E2FE)       | 3.75   | 9.04    | −1.27              | 0.02    |
| Solyc06g008000.2 | phytochrome interacting factor 3-like 5 (PIF1)    | 13.01  | 3.98    | 1.71               | 0.05    |

Table 4. Differentially expressed genes involved in cell cycle and cell wall development.
the exception of SIGAST1 levels at the 3–4 mm stage. However, the expression level of SIGA20ox1 was significantly downregulated in all successive stages following anthesis, whereas SIGA20ox2 and SIGA20ox4 were significantly downregulated at the anthesis, 3–4 mm and 5–6 mm stages. During later stages, the differences in expression levels became non-significant in both transgenic lines and wild-type plants (Fig. 7C). Previous microarray data 43 demonstrates increased the expression of most GA-signaling genes in GA 3-treated ovaries.

It is worth noting that although these genes belong to the same subfamily, their expression patterns during fruit development were quite different.

### Table 5. Differentially expressed genes involved in auxin and gibberellin signaling.

| Gene ID          | Gene Description                                           | T_fpkm | WT_fpkm | log (Fold changes) | p-value |
|------------------|------------------------------------------------------------|--------|---------|--------------------|---------|
| Solyc01g113110.2 | Auxin Efflux Facilitator (LAX2)                            | 11.01  | 25.92   | −1.24              | 0       |
| Solyc02g07550.2 | Auxin efflux carrier family protein                         | 12.14  | 36.84   | −1.6               | 0       |
| Solyc12g04500.1 | IAA carbonyltransferase 1 (IAMT1)                           | 0.4    | 10.02   | −4.65              | 0.01    |
| Solyc09g090910.1 | Auxin-induced protein 13 (IAA13)                            | 8.74   | 25.32   | −1.53              | 0       |
| Solyc09g083290.2 | Indole-3-acetic acid inducible 14 (IAA14)                  | 4.85   | 11.4    | −1.23              | 0.04    |
| Solyc09g083280.2 | AUX/IAA transcriptional regulator family protein (IAA1)    | 10.35  | 24.45   | −1.24              | 0.01    |
| Solyc09g064530.2 | Auxin-induced protein 12 (IAA12)                           | 6.26   | 29.27   | −2.22              | 0       |
| Solyc07g008020.2 | Indole-3-acetic acid inducible 35 (IAA35)                  | 5.61   | 15.42   | −1.46              | 0.02    |
| Solyc06g008590.2 | Indoleacetic acid-induced protein 17 (IAA17)               | 6.84   | 43.15   | −2.66              | 0       |
| Solyc06g008580.2 | AUX/IAA transcriptional regulator family protein (IAA22)   | 1.03   | 11.55   | −3.49              | 0.01    |
| Solyc04g081240.2 | Auxin Response Factor 5 (SIARF5)                           | 0.95   | 14.26   | −3.9               | 5.00E-05|
| Solyc06g084070.2 | AUX/IAA gene family protein (IAA2)                         | 0.81   | 14.07   | −4.12              | 0       |
| Solyc09g083290.2 | AUX/IAA gene family protein (IAA24)                        | 4.85   | 11.4    | −1.23              | 0.04    |
| Solyc03g121060.2 | AUX/IAA gene family protein (IAA18)                        | 40.95  | 20.48   | 1                  | 0.03    |
| Solyc08g082630.2 | Auxin Response Factor 9 A (SIARFP9A)                       | 5.54   | 33.12   | −2.58              | 5.00E-05|
| Solyc09g083290.2 | AUX/IAA gene family protein (SIAA14)                      | 4.85   | 11.4    | −1.23              | 0.04    |
| Solyc01g107390.2 | Auxin- and ethylene-responsive GH3-like protein (GH3-1)   | 3.48   | 1.42    | 1.3                | 0.05    |
| Solyc06g062920.2 | Auxin-regulated dual specificity cytosolic kinase           | 23.67  | 2.69    | 3.14               | 5.00E-05|
| Solyc06g075690.2 | Auxin-regulated protein AF416289                          | 49.05  | 2.97    | 4.05               | 5.00E-05|

### Discussion

In plants, ARFs encode important transcription factors and regulate gene expression in response to auxin by binding specifically to the TGTCTC sequence in the auxin response elements found in the promoters of primary/early auxin response genes. Recently, all ARF family genes were identified in tomato44, an ideal model plant for studying fruit development and ripening. However, only SIARF6, SIARF7, SIARF8, and SIARF9 have been characterized to date and have been demonstrated to be essential for tomato fruits generated through the regulation of auxin signal transduction. In this study, transgenic plants with reduced SIARF5 expression were developed using artificial miRNA silencing methods in order to examine the role of SIARF5 in fruit initiation and development by affecting the IAA and GA signaling pathways.

**Suppressing expression of SIARF5 in tomato induces parthenocarpic fruit.** Previous studies have demonstrated that SIARF8 is a negative regulator of fruit initiation in Arabidopsis46. SIARF7 and SIARF8 affect fruit initiation and lead to parthenocarpy46,48. SIARF7 functions as a negative regulator of fruit set until pollination and fertilization18. In addition, SIARF9, which is enriched with serine, was reported to be negatively regulated cell division during early fruit development46. Interestingly, these ARFs are typical tomato auxin response factors with a glutamine-rich middle region belonging to the class II subfamily, and the others being SIARF5, SIARF6-1, SIARF8-1, SIARF19, and SIARF19-1. ARFs enriched with glutamine are likely to function as transcriptional activators48. It is worth noting that although these genes belong to the same subfamily, their expression patterns during fruit development were quite different. SIARF5 mRNA levels were evidently higher in emasculated fruits than those in self-fertilized fruits. These results suggest that SIARF5 plays a role in fruit initiation when pollination occurs.
In Arabidopsis, ARF5 affects lateral organ development, primary root initiation, flower primordium initiation, and cotyledon development in embryos. Our data demonstrated that amiRNA SIARF5 transgenic fruits were significantly smaller than wild-type fruits, and their average weight and equatorial and longitudinal
diameter were significantly decreased compared to that of wild-type fruits (Table 1). Interestingly, the number of seeds in the self-fertilized transgenic lines was significantly lower than that in wild-type plants. However, when the transgenic flowers were emasculated, the amiSlARF5 plants developed parthenocarpic fruits, and the parthenocarpic fruit set rate was approximately 20.5% and 22.4% in two independent transgenic lines. The average size and weight of the parthenocarpic fruits were significantly lower than those in self-pollinated fruits. In previous studies, downregulation of SIDELLA49, TM2990, CHS51 and AUCSLA gene52, mutations of pat53 and hydra54 and transformation of aberrant ARF819 led to the formation of smaller parthenocarpic fruits, implying that these genes are positive regulators of fruit development in tomato. However, the parthenocarpic tomato fruits developed in rolB55, SIARF7 RNAiplant18, SIAA9 knockout56 and STTIR1 over-expression transgenic plants57 were similar in size to those of wild-type plants. Interestingly, overexpression and inhibition of SIARF9 mRNA levels led to smaller fruits and larger fruits, respectively20, suggesting different functions of ARFs in parthenocarpic fruit development.

SIARF5 modulates the expression of cell division- and expansion-related genes and the cross-talk between auxin- and GA-signaling. The signal transduction mechanism by which auxin regulates cell division and cell expansion during fruit set and growth is largely unknown. Previous studies have demonstrated that suppression of SIARF7 reduces cell division ability and enhances cell expansion ability46, whereas ARF6 may affect epidermal cell differentiation of petals in Arabidopsis44. In the present study, the inhibition of SIARF5 reduced fruit cell division and expansion. RNA-Seq data demonstrated that the expression levels of most cell cycle genes were downregulated in the 3–4 mm diameter fruit stage. GO annotation and KEGG analyses indicated that the cellular components of cell walls and pathways of the cell cycle-related to fruit development were observed to be enriched. The cell cycle genes, cylcB1.1 and CDKB2.1, which are significantly downregulated in amiSIARF5 plants during early fruit development, compared to the levels in wild-type fruits, indicating that cell division activity in amiSIARF5 fruits was decreased. However, in transgenic tomato plants with inhibition of SIARF7 or SIAA9, the expression of cell cycle-related genes was lower and higher than that in wild-type plants, respectively39,58.

Cell expansion genes are induced by pollination and GA treatment45. Expression of the cell expansion gene EXP5, which is induced by pollination rather than by GA application, is downregulated during early fruit development, whereas XTH1 and PEC are upregulated by GA application46. In our study, RNA-Seq data demonstrated that certain cell expansion genes (e.g., EXP2/EXP12, AGPS1, CEL5, and TPX1) in the amiSIARF5 line were downregulated compared to the levels in wild-type plants, suggesting that cell expansion abilities in the amiSIARF5 line were weakened. In a previous study, the mRNA levels of two cell expansion-related genes, namely, SIEPEC and SIXTH1 in the SIARF7 RNAi line, were similar at anthesis but became higher than those in the wild-type plants following anthesis during early fruit development49.

Plant hormones, such as auxin and gibberellins, play pivotal roles in tomato fruit development. The IAA and GA pathways regulate the cell cycle and expansion genes and determine the final fruit size26,40,41. In normal fruit, there is a rapid increase in the GA levels induced by successful pollination and fertilization, following which GA may induce an increase in auxin levels during the first 10 DPA42,55. However, in amiSIARF5 plants, a majority of the IAA-related genes were significantly downregulated, as demonstrated by the RNA-Seq data and further verified by qRT-PCR analysis. IAA-related genes, SIARF9, IAA2 and IAA14, which are induced by pollination and auxin treatment43, were downregulated during early fruit development in amiSIARF5 lines. However, expression of GH3, which is induced by auxin application to unpollinated ovaries51, increased in transgenic plants, as demonstrated by the qRT-PCR and RNA-Seq results, thereby suggesting that at least part of the IAA pathway was enhanced.

GA20ox1, an enzyme involved in GA biosynthesis, catalyzed the conversion of GA3 into GA4 and GA30, respectively, which are precursors of bioactive GAs46. The expression of GA20ox1 was significantly decreased in amiSIARF5 lines during early fruit development compared to that in wild-type fruits, which is consistent with the reduction in parthenocarpic fruit size of SIARF7 RNAi plants46. Similarly, the genes coding GA biosynthetic enzymes, GA2ox2 and GA2ox4, which catalyze active GA1 and GA3 conversion into inactive GA34 and GA30, were significantly downregulated in amiSIARF5 lines. However, GA3ox1, involved in the conversion of GA30 into GA3 and GA4, exhibited similar expression levels in transgenic and wild-type plants. On the other hand, GA-signaling transcription genes, such as GID1, were positively induced by GA treatment59. In our RNA-Seq data, the expression levels of GID1 and certain other known GA-signaling and response genes in amiSIARF5 lines increased compared to those in wild-type plants. These results suggest that levels of certain GA-signaling-related genes increased. This may be attributed to the feedback regulation on GA-signaling, which is similar with a previous study in PitGID1 over-expression lines56. The more pronounced responses to GA compared to those of wild-type plants were found in over-expression lines, while PitGA2ox1 expression was reduced51. Although three GA-regulated family proteins were downregulated in the amiSIARF5 lines, their functions were undefined. Two of these genes were probably cell cycle family genes in Arabidopsis (Table 5), which may function as the downstream genes of GA signaling pathway. Consistently, in our RNA-Seq results, mRNA levels of most cell cycle genes were also downregulated (Table 4).

The DEGs between amiSIARF5 transgenic and wild-type plants, identified by RNA-seq, were compared with the data from microarray analyses of GA3-treated and pollinated fruits (RNA from emasculated flowers)43. Our data demonstrated that the expression levels of most cell cycle and expansion genes were downregulated in transgenic plants, unlike their increased expression in GA3-treated and pollinated plants43. Furthermore, the alterations in the expression of some GA and IAA pathway genes in our study were in contrast to those observed by microarray analysis in pollinated fruits43; however, the altered levels of certain GA-related genes, such as SIGA3ox1 and cell wall protein precursor in GA-treated fruits43, were similar with our data. These results suggest that the inhibition of SIARF5 enhanced part of the GA pathway, similar to the results involving GA-treated fruits43.
Previous studies have indicated that the expression of GA metabolism genes is regulated by Aux/IAA and ARF proteins, and changes in GA metabolism partially mediate auxin action during development. ARF7 and ARF19 serve as crosstalk between auxin signaling and in ethylene responses in Arabidopsis. The RNA-seq results of SIARF3 RNAi lines indicated that the SIARF3 gene regulates the auxin-dependent transcription of trichome formation by mediating auxin, ethylene, and GA signaling in tomato. Recently, Breitel et al. demonstrated that SIARF2 links the signals of auxin, ethylene, and other hormones and affects the ripening capacity of fruit tissue.

In pollination-dependent and parthenocarpic fruit set, ARF2 and IAA9 mediate the crosstalk between auxin and GA during this development process. In obligatory parthenocarpy caused by SIMIR159-overexpressing tomato cv. Micro-Tom plants, SIGAMYBs potentially contribute to fruit initiation and regulate auxin and gibberellin responses.

In the amiRNA SIARF5 lines, when the SIARF5 mRNA was silenced, fruit set was evidently different from that observed in previous studies, in which the fruit set may be due to elevation of hormones (especially auxin) by expression of auxin biosynthesis genes in ovaries and ovules. However, in our transgenic lines, GA signaling was potentially regulated by GA biosynthesis pathways (downregulated gene expression) through negative feedback, as a result, some genes involved in GA-signaling pathway were upregulated. SIARF5 may function as SIARF7, which was suggested by De Jong et al. When SIARF5 was inhibited, the special downstream genes, such as auxin attenuating genes might fail to form transcripts; therefore, the inhibition imposed on ovary development was relieved, thereby enabling the ovary to expand and form the fruit. Meanwhile, in our transgenic plants, most of the auxin-related genes were not regulated and only GH3 was increased in the amiSIARF5 transgenic plants. The hormone-related gene expression was consistent with the ARF7 RNAi results. As a result, once SIARF5 was inhibited, increased GA signaling gene expression may facilitate fruit development. On the other hand, the smaller fruit size in amiSIARF5 plants may be attributed to the weakened auxin signaling pathway.

In conclusion, SIARF5 is an essential component of auxin signaling during tomato fruit development. SIARF5 may be critical for the crosstalk between auxin and GA during fruit development. In normal-pollination ovaries of wild-type plants, the mRNA level of SIARF5 was decreased, and the auxin- and GA-signaling pathways converged at fruit set and development. However, in emasculated flowers of wild-type plants, the SIARF5 mRNA levels were increased. Therefore, the SIARF5 mRNA levels potentially negatively regulate the GA-signaling pathway. A partially enhanced GA-signaling pathway may lead to the formation of parthenocarpic fruit.

Materials and Methods

**Plant materials and growth conditions.** Tomato (*S. lycopersicum* L. cv. Micro-Tom) plants were grown in a standardized greenhouse at the experimental farm at Zhejiang University, under 14/10 h light/dark periods with 200 μmol m⁻² s⁻¹ photo flux and 27/22 °C (day/night) temperatures.

For the measurement of early fruit development parameters, the flowers were emasculated 2 days before opening, then ovaries were collected at 0, 3, 6, and 9 DAA-U and DAA-R and early developing fruits were collected in the following stages: pollinated ovaries at anthesis and 3–4 mm, 5–6 mm, 7–8 mm, and 9–10 mm diameter fruit stages, corresponding to approximately 6, 8, 10, and 12 DAA-P stages of the wild-type, respectively.

**Construction of amiSIARF5 vectors.** amiRNAs were engineered into a 128 bp fragment containing the miRNA160a stem-loop, synthesized by Invitrogen, and then cloned into the BamHI and Hind III sites of a modified pCAMBIA1301 vector with a 35 S promoter inserted using Kpn I and Sma I, which was used for plant transformation. The detailed protocol for generating amiRNAs is provided in Supplementary Data S1. The pCAMBIA1301 plasmid with a 35S CaMV promoter was used as a control for plant transformation. Transgenic tomato plants were generated using the amiSIARF5 vectors by the Agrobacterium-mediated leaf disk method, as described by Sun et al., with the exception that the antibiotics were replaced with 6 μg/mL hygromycin and 300 mg/L Timentin.

For the detection of amiSIARF5 lines to select positive transgenic plants, root materials of the transgenics were assayed for GUS activity. To confirm that the T-DNA sequence of amiRNA SIARF5 had been transformed into tomato plants, a region of 285 bp DNA from leaf materials was amplified by PCR detection (TaKaRa). The specific amiRNA fragment sense primer, (5'-AGAGAGCGGTATCTGTTGTTT-3') was used in combination with a primer from the pCAMBIA1301 vector (5'-CGACAGTAGTGGTCCCCAAGAT-3') to amplify the DNA fragment. PCR procedures included an initial denaturation at 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min plus a final extension at 72 °C for 7 min.

**Microscopy.** To estimate the cell area, cell numbers and cell layers in the ovaries and developing fruits of both wild-type and transgenic SIARF5 plants, five fruits per line in each stage were sampled for analysis using fluorescence microscopy. Ovaries and young fruits were fixed with 2.5% glutaraldehyde overnight, rinsed with 0.1 M sodium cacodylate buffer (pH 7.4), and dehydrated through a graded series of ethanol solutions. Samples were embedded in Spurr’s resin, and semithin sections were obtained and stained with 1% methylene blue and viewed using fluorescence microscopy (DMLB, Leica, Germany). The micrographs were captured with a Leica DFC 420 C camera using the Leica Application Suite software (Leica Microsystems, Germany).

**Quantitative real-time PCR.** Total RNA was extracted from tomato ovaries using the TRIzol reagent (Invitrogen, Germany), according to the manufacturer’s instructions. The first cDNA strand was generated using the iScript™ Reverse Transcription System (Promega, Madison, WI, USA), following the manufacturer’s protocol. The qPCR experiment was carried out using SYBR Premix Ex Taq (Takara) in a BioRad CFX96 Real-time PCR Detection System, as described by Wu et al., with three biological replications and three technical replicates.
RNA sequencing and data analysis. Tissues were collected from pollinated fruits during the 3–4 mm diameter stage from amirNA SIARF3 lines and wild-type plants. Total RNA was extracted using TRIZol reagent (Invitrogen, CA, USA), according to the manufacturer’s instructions with two biological replicates. Four RNA libraries were sequenced using a Hiseq 2000/2500 sequencing system (illumina), following the manufacturer’s protocol. Following the removal of low-quality reads, the sequenced RNA-seq reads were aligned to the tomato genome using TopHat version 2.0.8b with modified parameters. The aligned read files were handled by Cufflinks, and the relative abundances of the transcripts were measured by the normalized RNA-seq fragment counts. Expression levels of each gene in FPKM (fragment per kilobase of exon per million fragments mapped) and the fold change of each gene were calculated. Comparisons with a q-value less than 0.05 were used for screening differentially expressed genes and further analyzed by enrichment of gene ontology (GO) terms and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. GO analysis was performed on differentially expressed genes using the Plant MetGenMAP database. Pathway enrichment analysis was performed using the KEGG database.

Statistical Analysis. ANOVA statistical analyses were performed on all data using SPSS 15.0 (p < 0.01) and data were tested for significant differences (p < 0.05) using Tukey’s test. Means and standard errors were calculated from three biological replicates.

References
1. Guillfoyle, T. J. & Hagen, G. Auxin response factors. Curr. Opin. Plant Biol. 10, 453–460 (2007).
2. Lim, P. O. et al. Auxin response factor 2 (ARF2) plays a major role in regulating auxin-mediated leaf senescence. J. Exp. Bot. 61, 1419–1430 (2010).
3. Ren, Z., Liu, R., Gu, W. & Dong, X. The Solanum lycopersicum auxin response factor SIARF2 participates in regulating lateral root formation and flower organ senescence. Plant Sci. 256, 103–111 (2017).
4. Wu, B. et al. Over-expression of mangifera indica L. MiARF2 inhibits root and hypocotyl growth of Arabidopsis. Mol. Biol. Rep. 38, 3189–94 (2010).
5. Zhang, X. et al. Auxin Response Gene SIARF3 Plays multiple roles in tomato development and is involved in the formation of epidermal cells and trichomes. Plant Cell Physiol. 56, 2110–2124 (2015).
6. Yoon, E. K., Yang, J. H. & Lee, W. S. Auxin and abscisic acid responses of auxin response factor 3 in Arabidopsis lateral root development. J. Plant Biol. 53, 150–154 (2010).
7. Sagar, M. et al. SIARF4, an auxin response factor involved in the control of sugar metabolism during tomato fruit development. Plant Physiol. 161, 1362–1374 (2013).
8. Jones, B. et al. Down-regulation of DR12, an auxin-response-factor homolog, in the tomato results in a pleiotropic phenotype including dark green and blotchy ripening fruit. Plant J. 32, 603–613 (2002).
9. Marin, E. et al. miR390, Arabidopsis TAS3 tasiRNAs, and their Auxin Response Factor targets define an autoregulatory network quantitatively regulating lateral root growth. Plant Cell 22, 1104–1117 (2010).
10. Wang, J. W. et al. Control of root cap formation by microRNA-targeted auxin response factors in Arabidopsis. Plant Cell 17, 2204–2216 (2005).
11. Ito, J. et al. Auxin-dependent compositional change in mediator in ARF7- and ARF19-mediated transcription. Proc. Natl. Acad. Sci. 113, 6562–6567 (2016).
12. Hao, Y. W. et al. Auxin response factor SIARF2 is an essential component of the regulatory mechanism controlling ripening in tomato. PLoS Genet. 11, e1005649 (2015).
13. Punitha, N. et al. Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation. Development 132, 4107–4118 (2005).
14. Tabata, R. et al. Arabidopsis auxin response factor 6 and 8 regulate jasmonic acid biosynthesis and floral organ development via repression of class I KNOX genes. Plant Cell Physiol. 51, 164–175 (2010).
15. Liu, N. et al. Down-regulation of auxin response factors 6 and 8 by microRNA 167 leads to floral development defects and female sterility in tomato. J. Exp. Bot. 65, 2507–2520 (2014).
16. Goetz, M., Vivian-Smith, A., Johnson, S. D. & Kolotunov, A. M. Auxin response factor 9 is a negative regulator of fruit initiation in Arabidopsis. Plant Cell 18, 1873–1886 (2006).
17. Du, L. et al. SmARF8, a transcription factor involved in parthenocarpy in eggplant. Mol. Genet. Genomics 291, 93–105 (2016).
18. De Jong, M., Wolters-Arts, M., Garcia-Martinez, J. L., Mariani, C. & Vriezen, W. H. The Solanum lycopersicum AUXIN RESPONSE FACTOR 7 (SIARF7) mediates cross-talk between auxin and gibberellin signalling during tomato fruit set and development. J. Exp. Bot. 62, 617–26 (2011).
19. Goetz, M. et al. Expression of aberrant forms of auxin response factor 8 stimulates parthenocarpy in Arabidopsis and tomato. Plant Physiol. 145, 351–366 (2007).
20. De Jong, M. et al. Solanum lycopersicum auxin response factor 9 regulates cell division activity during early tomato fruit development. J. Exp. Bot. 66, 3405–16 (2015).
21. Foadal, M. R. Genome mapping and molecular breeding of tomato. Int. J. Plant Genom. 64358, https://doi.org/10.1155/2007/64358 (2007).
22. Mapelli, S. C., Frowse, C., Torti, G. & Soressi, G. P. Relation-ship between set, development and activities of growth regulators in tomato fruits. Plant Cell Physiol. 19, 1281–1288 (1978).
23. Abdel-Rahman, M. Patterns of hormone respiration and ripening enzymes during development maturation and ripening of cherry tomato fruits. Physiol. Plant. 39, 115–118 (1977).
24. Gillaspy, G., Ben-David, H. & Gruissem, W. Fruits: a developmental perspective. Plant Cell 5, 1439–1451 (1993).
25. El-Sharkawy, I. et al. Expression of auxin-binding protein1 during plum fruit ontogeny supports the potential role of auxin in initiating and enhancing climacteric ripening. Plant Cell Rep. 31, 1911–21 (2012).
26. Syu, V. & Bangerth, F. Induced parthenocarpy: a way of changing the levels of endogenous hormones in tomato fruits (Lycopersicon esculentum Mill.). 1. Extractable hormones. Plant Growth Regul. 1, 243–251 (1983).
27. Bohnier, J., Heeden, P., Bora-Haber, E. & Bangerth, F. Identification and quantitation of gibberellins in fruits of Lycopersicon esculentum and their relationship to fruit size in L. esculentum and L. pimpinellifolium. Physiol. Plant. 73, 348–353 (1988).
28. Srivastava, A. & Handa, A. K. Hormonal regulation of tomato fruit development: a molecular perspective. *J. Plant Growth Regul.* **24**, 67–82 (2005).
29. Carrera, E., Ruiz-Rivero, O., Perez, L. E. P., Alejandro, A. & Garcia-Martinez, J. L. Characterization of the *procera* tomato mutant shows novel functions of the SIDELA protein in the control of flower morphology, cell division and expansion, and the auxin-signaling pathway during fruit-set and development. *Plant Physiol.* **160**, 1581–1596 (2012).
30. Lemaire-Chamley, M. et al. Changes in transcriptional profiles are associated with early fruit tissue specialization in tomato. *Plant Physiol.* **139**, 750–769 (2005).
31. Su, L. Y., Audran, C., Bouzayen, M., Roustan, J. P. & Chervin, C. The Aux/IAA, SI-IAA17 regulates quality parameters over tomato fruit development. *Plant Signal Behav.* **10**, e1071001 (2016).
32. Matsuo, S., Kikuchi, K., Fukuda, M., Honda, I. & Inanishi, S. Roles and regulation of cytokinins in tomato fruit development. *J. Exp. Bot.* **63**, 5569–79 (2012).
33. Czerednik, A., Busscher, M., Bielen, B. A. M., Wolters-Arts, M. & de Maagd, R. A. Regulation of tomato fruit pericarp development by an interplay between CDK8 and CDA1 cell cycle genes. *J. Exp. Bot.* **63**, 2605–2617 (2012).
34. Gonzalez, N., Gévaudant, F., Hernould, M., Chevalier, C. & Mouras, A. The cell cycle-associated protein kinase WEE1 regulates cell size in relation to endoreduplication in developing tomato fruit. *Plant J.* **51**, 642–55 (2007).
35. Vrebahol, J. et al. Fleshy fruit expansion and ripening are regulated by the Tomato SHATTERPROOF gene TAGLI. *Plant Cell* **21**, 3041–62 (2009).
36. Machemer, K. et al. Interplay of MYB factors in differential cell expansion, and consequences for tomato fruit development. *Plant J.* **68**, 337–50 (2011).
37. Guillet, C. et al. Regulation of the fruit-specific PEP carboxylase SIPEPC2 promoter at early stages of tomato fruit development. *PLoS One* **7**, e36795 (2012).
38. Wu, J. et al. Identification, isolation and expression analysis of auxin response factor (ARF) genes in *Solanum lycopersicum*. *Plant Cell Rep.* **30**, 2059–2073 (2011).
39. De Jong, M., Wolters-Arts, M., Feron, R., Mariani, C. & Vriezen, W. H. The *Solanum lycopersicum* auxin response factor 7 (SIARF7) regulates auxin signaling during tomato fruit set and development. *Plant J.* **57**, 160–170 (2009).
40. Serrani, J. C., Fos, M., Atarés, A. & Garcia-Martinez, J. L. Effect of gibberellin and auxin on parthenocarpic fruit growth induction in the cv. micro-tom of tomato. *J. Plant Growth Regul.* **26**, 211–221 (2007).
41. Bürger-Kühler, S. & Rangher, F. Relationship between cell number, cell size and fruit size of seeded fruits of tomato (*S. lycopersicum esculentum* Mill.), and those induced parthenocarpically by the application of plant growth regulators. *Plant Growth Regul.* **1**, 143–154 (1982).
42. Pattison, R. J. et al. Comprehensive Tissue-Specific Transcriptome Analysis Reveals Distinct Regulatory Programms during Early Tomato Fruit Development. *Plant Physiol.* **168**, 684–701 (2015).
43. Vriezen, W. H., Feron, R., Maretto, F., Keijman, J. & Mariani, C. Changes in tomato ovary transcriptome demonstrate complex hormonal regulation of fruit set. *New Phytol.* **177**, 60–76 (2008).
44. Kumar, R., Yagi, A. K. & Sharma, A. K. Genome-wide analysis of auxin response factor (ARF) gene family from tomato and analysis of their role in flower and fruit development. *Mol. Genet. Genomics* **285**, 245–260 (2011).
45. Krogan, N. T. & Berleth, T. A dominant mutation reveals asymmetry in MP/ARF5 function along the adaxial-abaxial axis of shoot lateral organs. *Plant Signal Behav.* 7, 940–943 (2012).
46. Herod, O., Weijers, D., Lau, S. & Jürgens, G. Auxin responsiveness of the MONOPTEROS-BODENLOS module in primary root initiation critically depends on the nuclear import kinetics of the Aux/IAA inhibitor BODENLOS. *Plant J.* **85**, 269–277 (2016).
47. Wu, M. F. et al. Auxin-regulated chromatin switch directs acquisition of lower primordium founder fate. *eLife* **4**, e09269 (2015).
48. Cole, M. et al. DORNRÖSCHEN is a direct target of the auxin response factor MONOPTEROS in the Arabidopsis embryo. *Development* **136**, 1643–1651 (2009).
49. Marti, C. et al. Silencing of DELLA induces facultative parthenocarpic in tomato fruits. *Plant J.* **52**, 865–876 (2007).
50. Ampomah-Dwamena, C., Morris, B. A., Sutherl, P., Veit, B. & Yao, J. J. Down-regulation of TM29, a tomato *SEPALATA* homolog, causes parthenocarpic fruit development and floral reversion. *Plant Physiol.* **130**, 605–617 (2002).
51. Schijlen, E. G. et al. RNA interference silencing of chalcone synthase, the first step in the flavonoid biosynthesis pathway, leads to parthenocarpic tomato fruits. *Plant Physiol.* **144**, 1520–1530 (2007).
52. Molessini, B., Pandolfini, T., Rotino, G. L., Dani, V. & Spena, A. *Auscia* gene silencing causes parthenocarpic fruit development in the tomato cv. micro-tom of tomato. *Plant Physiol.* **149**, 534–48 (2009).
53. Olimpieri, L. et al. Tomato fruit set driven by pollination or by the parthenocarpic fruit allele are mediated by transcriptionally regulated gibberellin biosynthesis. *Planta* **226**, 877–887 (2008).
54. Rojas-Gracia, P. et al. The parthenocarpic *hydra* mutant reveals a new function for a *SPOROCYTENESS*-like gene in the control of fruit set in tomato. *New Phytol.* **214**, 1198–1212 (2017).
55. Carmin, N., Salts, Y., Dedivoca, B., Shahbazi, S. & Barg, R. Induction of parthenocarpic in tomato via specific expression of the rolB gene in the ovary. *Plant Cell* **217**, 726–735 (2003).
56. Ueta, R. et al. Rapid breeding of parthenocarpic tomato plants using CRISPR/Cas9. *Sci. Rep.* **7**, 507 (2017).
57. Ren, Z. & Wang, X. *STIR1* is involved in crosstalk of phytohormones, regulates auxin-induced root growth and stimulates stenospermocarpic fruit formation in tomato. *Plant Sci.* **253**, 13–20 (2016).
58. Wang, H. et al. Regulatory features underlyiing pollination-dependent and -independent tomato fruit set revealed by transcript and primary metabolite profiling. *Plant Cell* **21**, 1428–1452 (2009).
59. Serrani, J. C., Ruiz-Rivero, O., Fos, M. & García-Martínez, J. L. Auxin-induced fruit set in tomato is mediated in part by gibberellins. *Plant J.* **56**, 922–934 (2008).
60. Hedden, P. & Phillips, A. L. Gibberellin metabolism: new insights revealed by the genes. *Trends Plant Sci.* **5**, 523–30 (2000).
61. Mauriat, M. & Moritz, T. Analyses of GA20ox- and GID1-over-expressing aspen suggest that gibberellins play two distinct roles in wood formation. *Plant J.* **58**, 989–1003 (2009).
62. Frigerio, M. et al. Transcriptional regulation of gibberellins metabolism genes by auxin signaling in Arabidopsis. *Plant Physiol.* **142**, 553–63 (2006).
63. Li, J., Dai, X. & Zhao, Y. A role for auxin response factor 19 in auxin and ethylene signalling in Arabidopsis. *Plant Physiol.* **140**, 899–908 (2006).
64. Breitel, D. A. et al. Auxin response factor 2 intersects hormonal signals in the regulation of tomato fruit ripening. *PLoS Genet.* **12**, e1005903 (2016).
65. Tang, N., Deng, W., Hu, G., Hu, N. & Li, Z. Transcriptome profiling reveals the regulatory mechanism underlying pollination dependent and parthenocarpic fruit set mediated by auxin and gibberellin. *PLoS One* **10**, e0125355 (2015).
66. da Silva, E. M. et al. microRNA159-targeted SIAGMY transcription factors are required for fruit set in tomato. *Plant J.* **92**, 95–109 (2017).
67. Rotino, G. L., Perri, E., Zottini, M., Sommer, H. & Spena, A. Genetic engineering of parthenocarpic plants. *Nat. Biotechnol.* **15**, 1398–1401 (1997).
68. Mezzetti, B., Landi, L., Pandolfini, T. & Spena, A. The deh9-iaaM auxin-synthesizing gene increases plant fecundity and fruit production in strawberry and raspberry. *BMC Biotechnol.* **4**, 1 (2004).
69. Sun, H. J., Uchii, S., Watanabe, S. & Ezura, H. A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. *Plant Cell Physiol.* 47, 426–31 (2006).
70. Jefferson, R. A., Kavanagh, T. A. & Bevan, M. W. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6, 3901–7 (1987).
71. Schmittgen, T. D. & Livak, K. J. Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* 3, 1101–8 (2008).
72. Trapnell, C., Pachter, L. & Salzberg, S. L. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* 25, 1105–1111 (2009).

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Author Contributions
S.L. performed the experiments, analyzed data and wrote the paper. Q.F. performed the qRT-PCR, Y.Z. and L.Q. helped in experiments, C.P. analyzed the RNA-seq data, A.T.L.S. helped to collect the data and revise the manuscript. G.L. designed the experiments and wrote the paper.

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