Histological, hormonal and transcriptomic reveal the changes upon gibberellin-induced parthenocarpy in pear fruit

Lulu Liu1, Zhigang Wang1, Jianlong Liu1, Fengxia Liu1, Rui Zhai1, Chunqin Zhu1, Huibin Wang1, Fengwang Ma1 and Lingfei Xu1

Abstract

Phytohormones play crucial roles in fruit set regulation and development. Here, gibberellins (GA4+7), but not GA3, induced pear parthenocarpy. To systematically investigate the changes upon GA4+7 induced pear parthenocarpy, dynamic changes in histology, hormone and transcript levels were observed and identified in unpollinated, pollinated and GA4+7-treated ovaries. Mesocarp cells continued developing in both GA4+7-treated and pollinated ovaries. In unpollinated ovaries, mesocarp cells stopped developing 14 days after anthesis. During fruit set process, GA4+7, but not GA1+3, increased after pollination. Abscisic acid (ABA) accumulation was significantly repressed by GA4+7 or pollination, but under unpollinated conditions, ABA was produced in large quantities. Moreover, indole-3-acetic acid biosynthesis was not induced by GA4+7 or pollination treatments. Details of this GA–auxin–ABA cross-linked gene network were determined by a comparative transcriptome analysis. The indole-3-acetic acid transport-related genes, mainly auxin efflux carrier component genes, were induced in both GA4+7-treated and pollinated ovaries. ABA biosynthetic genes of the 9-cis-epoxycarotenoid dioxygenase family were repressed by GA4+7 and pollination. Moreover, directly related genes in the downstream parthenocarpy network involved in cell division and expansion (upregulated), and MADS-box family genes (downregulated), were also identified. Thus, a model of GA-induced hormonal balance and its effects on parthenocarpy were established.

Introduction

For most plants, fruit set and development are triggered by pollination and fertilisation, which are highly sensitive to environmental conditions1, and unsuitable temperatures, rain and high wind speeds negatively affect pollination5. Most fruit species, including pear, apple and cherry, exhibit natural self-incompatibility, and at least two inter-compatible cultivars are necessary to guarantee successful pollination in an orchard, which wastes land and resources. Parthenocarpic fruit set is independent of pollination and, therefore, does not require pollinizers. Previous studies showed that phytohormones, such as auxins and gibberellins (GAs), can promote parthenocarpy in several species3–7. Mesejo et al.8 showed that GA promotes cell division in ovary walls, causing fruit set. In addition to these hormones, other phytohormones, such as cytokinins, ethylene and abscisic acid (ABA), also take part in fruit set9. However, the precise roles of these hormones in regulating parthenocarpic fruit set and development are still poorly understood.

At present, the molecular mechanisms of parthenocarpic fruit set are still unclear in woody fruit trees, with only a few components related to this process having been uncovered. Two auxin response factors (ARFs), namely ARF7 and ARF8 had been shown associate with parthenocarpy. Transgenic tomato with decreased SIARF7 levels formed parthenocarpic fruit10, and a natural parthenocarpic eggplant mutant showed that ARF8...
is downregulated in buds compared with in wild-type plants. Thus, both ARF7 and ARF8 transcription factors are important negative regulators during fruit set. Auxin/indole-3-acetic acid (Aux/IAA) proteins are transcriptional regulators involved in auxin regulated plant development, and a loss-of-function IAA9 mutant tomato formed parthenocarpic fruit, suggesting that IAA9 is also a negative regulator of auxin involved in regulating parthenocarpic fruit set. GA signalling in particular plays an important role in regulating parthenocarpic fruit set because the increased auxin content in ovaries produced by parthenocarpic tomato is mediated through GA. Moreover, tomato mutants pat and pat-2 increase the expression levels of GA biosynthetic genes, increasing the GA content, which forms parthenocarpic fruit. The GA 20-oxidase (GA20ox) family of genes catalyze inactive GA to active GA, and overexpressing the citrus gene CcGA20ox1 in tomato led to the development of parthenocarpic fruits. In addition, GA20xox are a type of catabolic enzyme that deactivate active GA. The silencing of all five GA2ox genes in transgenic tomato plants led to a significant increase in the GA4 content, and the plants exhibited the ability to develop parthenocarpically. DELLA proteins are important negative regulators in the GA signalling pathway that restrict growth and development. Silencing of SDELLA in tomato can induce parthenocarpic fruits. GA depletes the DELLA protein and releases the repression of GA-response genes.

In addition, silencing of a tomato floral organ-specification gene, TM29, a SEPPELLATA-like MCM1, AGAMOUS, DEFICIENCY and SRF (MADS) box gene, also caused seedless fruits. The loss-of-function mutation of MdPIL, a MADS-box transcription factor, produces parthenocarpic fruit in apple. Recently, the MADS box gene AGL6 was shown to regulate parthenocarpic in tomato, and loss function of SIAGAMOUS-LIKE 6 result in tomato facultative parthenocarpy.

Most cultivated pears are self-incompatible; therefore, understanding the mechanisms of GA-induced parthenocarpic fruit would have great benefits for both production practices and theoretical studies. Here we studied changes upon gibberellins (GA4+7) induced parthenocarpic pear fruit set in 'Dangshansuli' (Pyrus bretschneideri Rehd.) to determine whether GA4+7, or GA3, could induce parthenocarpic in pear; (2) to observe the ovaries of unpollinated, pollinated and a 75 mg L⁻¹ GA4+7 treatment of 'Dangshansuli' during early stages using morphological and histological methods to explore the similarities and differences among these treatments; (3) to measure gibberellins, auxin and abscisic acid levels to study the hormone balance effects in fruit set; and (4) to perform an RNA sequencing (RNA-Seq) analysis of the ovary transcriptomes at three stages, 3, 9 and 14 days after anthesis (DAA), to study the molecular regulatory mechanism of GA4+7-induced parthenocarpy in pear.

**Materials and methods**

**Plant materials and experimental treatments**

Experiments were carried out in a pear orchard located in Meixian, Shaanxi Province, China (34.29°N, 107.76°E, and 514 m above sea level). The average annual precipitation is 574.6 mm, and the average annual temperature is 12.7 °C.

Eight treatments, the unpollinated (control), 25, 50 and 75 mg L⁻¹ GA3, 25, 50 and 75 mg L⁻¹ GA4+7, and hand pollinated, were applied to 15-year-old 'Dangshansuli' trees, which were grafted onto *P. betulifolia* Bge rootstocks. Three branches of each treatment were used as three replicates. Two days before anthesis, all of the treatments and the control were bagged to prohibit pollination. Water was sprayed on unpollinated flowers as the unpollinated treatment. Solutions of GA3 alone at 25, 50 and 75 mg L⁻¹, and GA4+7 alone at 25, 50 and 75 mg L⁻¹, were sprayed on unpollinated flowers of “Dangshansuli” at anthesis. Hand pollination was carried out at the same time. Then, 30 fruits per treatment were randomly sampled for the determination of hormone levels, morphological and cytological observations, and RNA sequencing at each sampling time point. Flowers/fruits were collected at 0, 3, 9, 14 and 153 DAA.

A part of each sample was immediately fixed in formaldehyde-acetic acid-alcohol for histological observation, while the rest of the samples were frozen in liquid nitrogen and then stored at −80°C for further assays.

**Determination of fruit set rate**

A total of 30 blooms on each branch were labelled and bagged immediately after treatments. At 20 DAA, the bags were removed. The formula used to calculate the fruit set rate was as follows:

\[
\text{Fruit set rate} = \frac{(\text{Number of fruitlets remaining}/30) \times 100}{\text{Number of flowers as provide}}
\]

**Phytohormone analysis**

Samples of 0.2 g were ground in an ice-cold mortar with 8 mL of 80% (v/v) methanol extraction medium that contained 1 mM butylated hydroxytoluene as an antioxidant. The extracts were incubated at 4°C for 4 h and then centrifuged at 3500 r.p.m. for 8 min at 4°C. The supernatants were passed through Chromosep C18 columns (C18 Sep-Pak Cartridge; Waters Corporation, Millford, MA, USA), which were washed with 10 mL of
100% and 5 mL of 80% (v/v) methanol. The hormone sediments were dried under nitrogen gas and then dissolved in 2 mL of phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20 and 0.1% (w/v) gelatine (pH 7.5).

Phytohormones were separately analysed using GA$_{1+3}$, GA$_{4+7}$, IAA and ABA ELISA Kits produced by the Phytohormones Research Institute, China Agricultural University, China. Yang’s method for quantitative phytohormone determination was used.

Paraffin sectioning

To conduct the histological observation, fruit samples of unpollinated, pollinated and GA$_{4+7}$ treatments were collected at 3, 9 and 14 DAA, immediately in formaldehyde-acetic-alcohol fixative and stored at 4°C. The ovaries were dehydrated in an ethanol/xylene series and embedded in paraffin, 10 s into 8-μm-thick slices, dried and stained with safranin and fast green. The anatomical images were observed using a microscopical imaging system (BX51 + PD72 + IX71, OLYMPUS, Japan).

Transcriptome analysis

The total isolated RNAs were used for RNA-Seq on an Illumina HiSeq 2500. The sequencing library is prepared by random fragmentation of the cDNA sample, followed by 5’ and 3’ adapter ligation. Adapter-ligated fragments are then PCR amplified and gel purified. Sequences were screened for primer concatemers, weak signal and poly A/T tails. Low-quality reads were eliminated based on the score value (reads with >30% of bases with quality score (Q value) of <20) and the remaining high quality reads were filtered for short reads below 50 bp. Adaptors were first trimmed, and then reads were further assembled by GS de novo assembler (v2.6). Singletons cleaning using Seqclean and lucy with a parameter of minimum length 100 bp Illumina hiseq reads produced in paired-end formats (101 bp) were also assembled using the Trinity software package. Reads were filtered and trimmed, and then mapped onto ‘Dangshansuli’ (P. bretschneideri Rehd.) coding sequences using the SOAP aligner.

Clean reads were mapped using bowtie 1.1.2 to generate read alignments for each sample. The gene expression level was calculated by fragments per kilobase of exon model per million mapped reads. The RNA-Seq data of unpollinated ovaries were used as the controls. A false discovery rate of <0.001 and an absolute value of $\log_2$ ratio > 1 were used as the thresholds for the significance of differentially expressed genes (DEGs). Genes were annotated using the ‘Dangshansuli’ database (http://www.ncbi.nlm.nih.gov/genome/?term=pyrus) as a reference.

Three independent biological replications were sequenced and analysed.

Quantitative real-time PCR validation of gene expression levels

The qRT-PCR was performed on a Life Technologies (ABI) machine using the SYBR Premix Ex Taq Kit (TaKaRa, Dalian, China). The cDNA templates were reverse transcribed using total RNA extracted from ovaries of unpollinated, pollinated and GA$_{4+7}$ treatments at 3, 9 and 14 DAA. Then, 11 selected genes were used to verify the expression results. Actin was used as the internal reference for the gene expression analysis. The use actin gene as internal reference is Actin 7 gene. All of the primers for selected genes were designed using Primer Premier 5.0 software, and are listed in Supplementary Table S2. The PCR reactions were carried out using an initial incubation at 95°C for 30 s, and then 40 cycles of 95°C for 5 s and 60°C for 30 s. All reactions were performed on three biological and three technical replicates. Relative quantification of specific mRNA levels was performed using the cycle threshold (Ct) $2^{-\Delta\Delta Ct}$ method.

MapMan analysis of DEGs

To study the DEGs involved in carbohydrate and photosynthesis metabolism between pollinated and unpollinated, and GA$_{4+7}$-treated and pollinated, we used the online web software Mercator to obtain the pear protein
annotation mapping file for MapMan with the default parameters\textsuperscript{31}. MapMan was then used to classify the DEGs into functional categories\textsuperscript{32}.

**Statistical methods**

The data were statistically analysed using an analysis of variance and tested for significant ($P < 0.05$) treatment differences using Duncan’s test.

**Results**

**GA$_{4+7}$ induced parthenocarpy in ‘Dangshansuli’**

The fruit set rate reached 86.67\% for hand pollination, 88.24–98.85\% for different concentrations of GA$_{4+7}$ treatments, and no fruit set for unpollinated flowers. Although the highest set rate among the GA$_3$ treatments was 58.89\%, which was lower than those of the GA$_{4+7}$ treatments (Fig. 1), all of the GA$_3$-treated fruits were eventually lost before harvest, while GA$_{4+7}$ induced seedless mature fruits of a normal size. Thus, GA$_{4+7}$, rather than GA$_3$, may play an important role during the pear fruit set process.

**Morphological and histological observations in GA$_{4+7}$-induced parthenocarpic fruits**

Morphological observations showed that unpollination, pollination and GA$_{4+7}$ (From this point of the manuscript, the use of GA$_{4+7}$ will refer to the 75 mg L$^{-1}$ concentration) treated fruits at 3, 9 and 14 DAA, mature fruits developed at 153 DAA. However, the unpollinated ovaries wilted at 14 DAA (Fig. 2). GA$_{4+7}$ induced seedless fruits developed similar size as seeded fruits, but developed smaller core and elongated longitudinal diameter compared to seeded fruits.

Longitudinal sections of mesocarps exhibited GA$_{4+7}$-induced cell expansion from 3 DAA (Fig. 3g) and formed larger cells than pollinated ovaries at 14 DAA (Fig. 3j). Cytological observations showed that apoptosis occurred in the unpollinated ovules and that locular cavities expanded (Fig. 4a), while both pollination and GA$_{4+7}$ induced ovules development (Fig. 4d, g). Cell division process was activated during fruit set as well, transverse sections of mesocarps indicated that pollinated and GA$_{4+7}$-treated ovaries developed thicker external mesocarps (EM, located between the vascular bundles and epidermis) with more cell layers than unpollinated ovaries (Fig. 4). The internal mesocarp (IM, between the vascular bundles and endocarp) did not show any differences among the treatments (Fig. 4). In addition, both pollinated and GA$_{4+7}$-treated fruits developed larger vascular bundles compared with unpollinated ovaries (Fig. 4).

**Hormones involved in GA$_{4+7}$-induced parthenocarpy**

The GA$_{4+7}$ treatment dramatically and significantly increased GA$_{1+3}$ and GA$_{4+7}$ concentrations at 3 DAA. At 14 DAA, GA$_{1+3}$ content of treated samples dropped to the same levels as unpollinated ovaries, while GA$_{4+7}$ amount was still significantly higher. GA$_{1+3}$ levels showed no significant differences between unpollination and pollination at same developmental stages, but pollination significantly increased the GA$_{4+7}$ concentrations at both 3 and 14 DAA (Fig. 5a, c). IAA levels decreased all significantly from 0 to 14 DAA, while both GA$_{4+7}$ and
pollination treatments exhibited higher levels of IAA than unpollination at 3 DAA and 14 DAA (Fig. 5b). ABA levels significantly and dramatically increased in unpollinated flowers at 3 DAA and 14 DAA; however, both pollinated and GA-treated ovaries showed lower levels of ABA at same development points. There is higher level of ABA in GA4+7 treatment than pollination at both 3 and 14 DAA (Fig. 5d).
Transcriptome analysis of pear ovaries during fruit set

Based on our previous experiments, we found that at 14 DAA, the unpollinated flowers began to become withered, so we thought it was a key stage for fruit set. Besides, according to our pre-experiments, we found that hormone levels changed dramatically at 9 DAA, and 3 DAA was the earliest stage for fruit set, so we choose these three stages for further study, in order to more systematically clarify the mechanism of GA4 +7 induced parthenocarpic fruit set. Ovaries for RNA-Seq were collected from unpollinated, pollinated and GA4 +7-treated (without pollination) ‘Dangshansuli’ at 3, 9 and 14 DAA. Three independent biological replications were sequenced and analysed.

To investigate changes in the transcriptome during fruit set, we conducted Illumina HiSeq 2500 125PE sequencing of unpollinated, pollinated and GA4+7-treated ovaries at 3, 9 and 14 DAA. There were at least 2.86 million raw reads for each sample, except for one sample (GA4+7_rep3, 65% of the clean reads were mapped to the reference pear), >75% of the clean reads were mapped to the reference pear (P. bretschneideri Rehd.) genome (Supplementary Table S1). A false discovery rate <0.001, and log2 (ratio) > 1 were used as the thresholds to determine the significance of DEGs. To verify the RNA-Seq results, qRT-PCR was conducted for candidate genes. We choose 16 genes which was with low, average and high number of reads in GA4+7 treated fruits at 14 DAA, and seven important genes involved in gibberellin pathway were also included. The results exhibited in similar expression tendencies as the sequencing results, suggesting that the RNA-Seq data in this study are reliable (Supplementary Fig. S1).

Venn diagrams displayed the distribution of DEGs in pollinated and GA4+7-treated ovaries. At 3 DAA, 177 (8.1% of the total) upregulated and 140 (6.4% of the total) downregulated genes were common to pollinated and GA4+7-treated (without pollination) ‘Dangshansuli’ at 3, 9 and 14 DAA. Three independent biological replications were sequenced and analysed.

DEGs involved in GAs homoeostasis and signalling during fruit set

GA4+7 treatment provided >30 ng/g. FW of exogenous GA4+7 (which is more than other treatments), so no more endogenous biosynthesis is needed, thus only ent-kaurenoic acid oxidases (KAOs) were induced. In addition to KAOs, pollination also induced ent-copalyl diphosphate synthase (CPS) (LOC103957280) and GA20ox2-like (LOC103960493) at 14 and 9 DAA, respectively. Genes encoding GA-inactivating GA20ox showed similar expression trends between GA4+7 and
pollinated treatments. Namely, at 3 DAA, GA2ox1-like (LOC103945984) was upregulated and then from 9 to 14 DAA exhibited downregulation. In addition, GA2ox8 (LOC103931224) in GA4+7-treated ovaries were downregulated at 3 DAA, while the downregulation in pollinated ovaries was delayed to 9 DAA. GA4+7 negatively regulated the expression of the GA receptor GID1 (LOC103938790), as well as pollination. The gene encoding DELLA protein GAI1-like (LOC103943039) was also downregulated in these two treatments at 14 DAA (Fig. 6).

DEGs involved in auxin and ABA metabolism and their signalling pathways

Four auxin efflux carrier components were upregulated in GA4+7-treated and pollinated ovaries. In particular, the auxin efflux carrier component 6 (LOC103951142) was upregulated more than eightfold (log2 fold change). DEGs involved in auxin signal transduction were also modified, and ARF5-like (LOC103930094), ARF6 (LOC103951320), ARF18-like (LOC103962541) and ARF19-like (LOC103959396) were downregulated in GA4+7-induced parthenocarpic fruits, as well as in the pollinated ovaries (Fig. 7a).

Two genes encoding 9-cis-epoxycarotenoid dioxygenase (NCED3) were significantly downregulated in GA4+7-treated and pollinated ovaries. In particular, one NCED3 (LOC103945979) was downregulated more than fivefold (log2 fold change). A variety of DEGs involved in the ABA signalling pathway were repressed, and 40 protein
phosphatase 2Cs were modified during the fruit set process (Fig. 7b). Specific information of genes involved in Fig. 7 was exhibited in Supplementary Tables S6 and S7.

Cell cycle and expansion-related genes were upregulated during fruit set

Cell division-related and cell wall-related genes were upregulated in GA4+7-induced parthenocarpic fruits, as well as in pollinated ovaries. In total, there were 31 upregulated cyclin genes in GA4+7-induced ovaries, most of which were significantly upregulated at 3 DAA. However, the upregulation was delayed to 9 DAA in pollinated ovaries. Among these cyclins, a U4-1-like cyclin (LOC103952770) was upregulated more than sixfold (log2 fold change) in both GA4+7-treated and pollinated ovaries at 14 DAA. Nine G2/mitotic-specific cyclins were upregulated in GA4+7-induced parthenocarpic fruits from 3 DAA, while their upregulation in pollinated ovaries was delayed to 9 DAA. During fruit development, the elevated levels of these G2/mitotic-specific cyclins increased, and at 14 DAA, all of these G2/mitotic-specific cyclin genes were upregulated more than threefold (log2 fold change) in parthenocarpic fruits, as well as in pollinated ovaries. In addition, two cyclin-dependent kinase (CDKs) (LOC103952922 and LOC103961775) genes were upregulated in GA4+7-induced parthenocarpic fruits from 3 DAA, while the upregulation in pollinated ovaries was delayed to 9 DAA. RNA-Seq results showed that 10 expansin genes were upregulated, and except for expansin-like B1 (LOC103960312), in both GA4+7-induced parthenocarpic and pollinated ovaries, especially expansin-A15 (LOC103951053), expansin-B3-like (LOC103934614) and expansin-A6-like (LOC103944903), which were upregulated more than fourfold (log2 fold change) (Fig. 8). In total, 103 cell wall-related DEGs were involved in GA4+7-induced parthenocarpic fruits at 3 DAA, and 72 cell wall-related genes were changed by pollination. At later stages, ~140 cell wall-related genes were significantly modified in parthenocarpic and pollinated ovaries (Supplementary Table S3).

Photosynthetic processes and sugar metabolism were modified during fruit set

To analyse carbohydrate and photosynthesis-related metabolisms, DEGs were grouped based upon their biological functions using MapMan. Results showed that the number of DEGs involved in carbohydrate and photosynthesis metabolisms increased with time going (from 3 to 14 DAA) (Fig. 9, Supplementary Fig. S3). Photosynthesis-related genes were activated, with 59 (Supplementary Fig. S3c) and 45 (Fig. 9a) upregulated DEGs at 9 and 14 DAA, respectively, in pollinated ovaries, and 55 (Supplementary Fig. S3d) and 61 (Fig. 9b) upregulated DEGs in parthenocarpic fruit at 9 and 14 DAA,
respectively. At 3 DAA, mainly cell wall-related genes were induced in both pollination and GA$_{4+7}$-treated ovaries. Minor CHO, Lipids, TCA and light reactions processes were activated at 9 DAA, especially for light reactions process, all of DEGs which was involved in this process were upregulated (Supplementary Fig. S3). There were more DEGs involved in these processes at 14 DAA, and most of them were upregulated in both pollination and GA$_{4+7}$-treated fruits.

Four sucrose synthases (LOC103935320, LOC103950289, LOC103950290 and LOC103950291) were upregulated in parthenocarpic and pollinated ovaries at 14 DAA. Genes encoding elements of the sugar degradation process also changed after GA$_{4+7}$-treatment and pollination. Three sucrose hydrolases (LOC103959831, LOC103961960 and LOC103963401) and two alpha amylases (LOC103950868 and LOC103955697) were induced in both parthenocarpic and pollinated ovaries. Two invertases (LOC103961960 and LOC103963401) were upregulated in GA$_{4+7}$-induced parthenocarpic fruits at 9 and 14 DAA, pollination showed similar expression patterns. In particular, the expression of LOC103961960 was upregulated about eightfold in both parthenocarpic and pollinated ovaries at 14 DAA. Genes involved in cell wall synthesis, such as UDP-glucose 4, 6-dehydratase (LOC103966239 and
Our study, exogenous application of GA$_4$, induced parthenocarpic pear fruit, although exogenous applications of GA$_4$ and GA$_3$ were only found in GA$_{4,7}$-induced parthenocarpic fruits at 14 DAA (Supplementary Table S4).

**Transcription factors (TFs) involved in fruit set**

TFs play crucial roles in regulating many developmental processes. There were 124, 240 and 449 DEGs at 3, 9 and 14 DAA, respectively, encoding TFs in GA$_{4,7}$-induced parthenocarpic ovaries, and 59, 199 and 385 DEGs encoding TFs in pollinated ovaries at 3, 9 and 14 DAA, respectively (Supplementary Fig. S4). There were 32, 139 and 329 differentially expressed TFs showed similar expression patterns between GA$_{4,7}$ and pollination treatments at 3 DAA, 9 and 14 DAA, respectively, which shows that the number of TFs increased with time going. Besides, 91, 165 and 177 differentially expressed TFs exhibited differential expression pattern between GA$_{4,7}$ and pollination treated ovaries at 3 DAA, 9 and 14 DAA, respectively. The majority of changed TFs belong to the bHLH, MYB, NAC and WRKY families (Supplementary Fig. S4).

MADS-box genes may play important regulatory roles during fruit development. AGL19 is one of the MADS-box family genes, both GA$_{4,7}$ and pollination treatments repressed the expression of AGL19 (LOC103952886) at 9 DAA. Besides, AGL9 (LOC103961438) was only downregulated in GA$_{4,7}$-treated ovaries at 14 DAA. In our analysis, two DEFs (LOC103938026 and LOC1039666740) were significantly repressed in parthenocarpic ovaries at 14 DAA, while only one DEF (LOC103938026) was significantly downregulated in pollinated ovaries.

**Discussion**

Phytohormones play a crucial role in regulating fruit set, and exogenous applications of plant hormones can induce artificial parthenocarpy. GA$_3$ can induce parthenocarpic fruits in several plants, including tomato and grape. In our study, exogenous application of GA$_{4,7}$, rather than GA$_3$, induced parthenocarpic pear fruit, although exogenous applications of GA$_{4,7}$ could increase the GA$_{1,3}$ content. Pollination increased GA$_{4,7}$ levels, but barely affected the GA$_{1,3}$ content. Thus, GA$_{4,7}$, rather than GA$_3$, may play the main role in regulating 'Danghansuli' fruit set. Pollination promoted the expression of CPS (except for at 9 DAA), KO, KAOs and GA20oxs, which synthesise bioactive GA, while GA$_{4,7}$ only promoted the expression of KAOs. GA20oxs, which function to deactivate bioactive GA, most of which were downregulated in parthenocarpic ovaries, as well as in pollinated ovaries. Both GA$_{4,7}$ and pollination repressed the expression of GID1B-like from 9 DAA. At 14 DAA, the expression levels of GAI1-like were also repressed, and pollination resulted in a similar expression pattern. Thus, GA$_{4,7}$ barely changed the mRNA levels of the encoded biosynthetic GA genes, since GA content was already high to support fruit set processes, while mainly influencing GA-response genes.

Auxins also play key roles during fruit set, and the application of IAA was shown to induce cell division in the tomato pericarp. GA$_{4,7}$ did not increase the IAA concentration, but it promoted auxin efflux carrier components (1c, 1b and 6) expression levels, which was consistent with pollination.

ABA inhibit cell division by inducing ICK1, a CDK inhibitor, and ABA levels were relatively high in unpollinated ovaries and decreased after pollination. An important ABA biosynthesis gene in tomato, LeNCED1 was repressed after pollination. Both GA$_{4,7}$ and pollination treatments decreased ABA accumulation in ovaries compared with unpollination, and NCED3 and NCED5 were significantly downregulated in these two treatments. Thus, GA coordinates with IAA and the ABA balance, promoting cell division and expansion, thus stimulating fruit set and development.

Pollination and the following double-fertilisation occurrences in the carpel cause a coordinated sequence of cell division, expansion and differentiation events that result in fruit set and maturity. Exogenous applications of GA$_{4,7}$ promoted the development of unpollinated ovaries, while the unpollinated ovaries ceased to grow and ovules aborted at 14 DAA. Serrani et al. also found that unfertilised ovules degenerated in the former case in tomatoes. GA$_{4,7}$-treatments produced larger cells than pollination in the mesocarp, which was consistent with GA inducing larger cells than pollination in tomato. However, GA$_{4,7}$ promoted external mesocarp division because GA$_{4,7}$-induced parthenocarpic ovaries had more cell layers than those of the unpollinated ovaries, but similar cell layers as the pollinated ovaries.

Transcriptome data showed that the amounts of cell cycle and cell wall-related genes were modified by GA$_{4,7}$ treatments, suggesting the importance of cell division and cell expansion in fruit set and development. The cell cycle is mainly regulated by two key classes of regulators, CDKs and cyclins [8]. A-, B- and D-type cyclins mainly regulate the S and G2/M phases, and B-type CDKs are specifically expressed in G2 and M phase. Our study showed that 33 DEGs, including B-type CDKs, A-, B-, D- and U-cyclins and G2/mitotic-specific cyclins, were positively regulated by GA$_{4,7}$ or pollination during fruit development.
set, suggesting that activated G2/M phase regulators are available for cell proliferation. A previous study showed that 14 cell cycle genes were positively associated with cell proliferation during apple fruit set and development.

The cell wall is comprised of cellulose, hemicellulose and pectin, as well as proteins, which influence cell expansion. Expansin A (EXPA) and B (EXPB) belong to the α- and β-expansin families, respectively, and many of their members have the ability to induce rapid cell expansion. In our study, EXPB3-like and five EXPAs were significantly upregulated in GA4 + 7-induced parthenocarpic fruits. Glucanases, other wall hydrolases, and XETs affect cell enlargement by regulating cell expansion activities. In our analysis, two endo-1,3;1,4-beta-D-glucanase-like genes had decreased expression levels, while four glucomannan 4-beta-mannosyltransferase genes had significantly increased expression levels in parthenocarpic and pollinated ovaries. Xyloglucan endotransglycosylase/hydrolase (XTH) enzymes play crucial roles in promoting cell expansion by disassembling xyloglucan, and the expression of several XTHs was increased, with XTH8, XTH9, XTH31 and XTH32 being significantly upregulated in GA4 + 7-induced parthenocarpic ovaries and pollinated ovaries. A variety of cell cycle and cell wall-related genes were significantly upregulated in our analysis, suggesting that these categories of genes also take part in fruit set.

UDP-glucuronic acid, a nucleotide sugar that is a precursor of the cell wall, is formed through the activity of UDP-glucose dehydrogenase, which catalyses UDP-glucose into UDP-glucuronic acid. Our analysis showed that both UDP-glucose dehydrogenase and UDP-glucuronic acid decarboxylases were significantly increased in parthenocarpic and pollinated ovaries. This result was consistent with pericarps undergoing rapid cell division and expansion periods, and the activation of cell wall synthesis.

Photosynthesis provides necessary nutrition for fruit and seed set, and deficient photosynthesis is a primary driver of flower, fruit and seed abortion. Antisense silencing of SlIAA9 induced parthenocarpic tomatoes, which had upregulated expression levels photosynthesis-associated genes, and our analysis corroborated these results. According to the MapMan analysis, the expression levels of photosynthesis-associated genes were upregulated in GA4 + 7-induced parthenocarpic pear ovaries, and pollinated ovaries showed similar results. Plant growth relies on photosynthesis products, mainly in the form of sucrose in most crop species, and invertase cleaves the sucrose to hexose, which promotes fruit and seed set. Our analysis showed that the DEGs involved in sucrose degradation were upregulated in parthenocarpic ovaries, and pollinated ovaries showed similar results. Plant growth relies on photosynthesis products, mainly in the form of sucrose in most crop species, and invertase cleaves the sucrose to hexose, which promotes fruit and seed set.

The present study clarified that MADs-box family genes play roles in regulating fruit set. Class B MADs-box genes control petal and stamen development, and the loss-of-function of a class B MADs-box results in parthenocarpy in apple, and DEFICIENS (DEF) belongs to class B MADs-box family. SIDEF functions as a repressor in ovules and inhibits the development of

---

**Fig. 10** The mechanistic model of fruit set. Gene names in red font represent related genes were upregulated, instead, those in green font represent related genes were downregulated.
ovaries in unpollinated tomatoes, and it was upregulated before or during anthesis in the wild type, but not in the pat mutants. In our analysis, DEF (LOC103966740) was significantly repressed in parthenocarpic and pollinated ovaries. The class E MADS-box genes are essential for all floral whorls involved in specifying organ identities, and AGL9 is an E class gene. Our studies exhibited that AGL9 was repressed in parthenocarpic fruits.

Conclusions

In summary, our studies found that GA4+7, but not GA3, induced pear parthenocarpy, which was consistent with pollenization increasing the GA4+7 contents but not GA1+3. GA4+7 maintained normal cell growth in mesocarp, as well as pollenization, while unpollinated ovaries ceased growing and then shrunk. Genes involved in cell cycle and cell expansion were upregulated in GA4+7-induced parthenocarpic pear fruit. RNA-Seq results also revealed that the photosynthesis and sugar metabolism processes were activated, suggesting that they may also take part in fruit set. GA4+7 did not increase the IAA level but promoted its transport through gene expression. GA4+7 repressed the ABA biosynthetic gene NCED, resulting in lower ABA levels in parthenocarpic ovaries. GA4+7-repressed genes (MADS-box family genes AGL9 and DEF) were directly related to parthenocarpy. The model presented in Fig. 10 represented that application of GA4+7 to unpollinated ovaries may promote the hormonal balance, photosynthesis, sugar metabolism, the cell cycle and cell expansion, and also repress AGL9 and DEF expressions, resulting in fruit set.

Acknowledgements

This work was supported by the China Agriculture Research System (CARS-29-40) and Weinan Experimental Station foundation of Northwest A&F University.

Conflict of interest

The authors declare that they have no conflict of interest.

Received: 2 July 2017 Revised: 7 November 2017 Accepted: 28 November 2017
Published online: 03 January 2018

References

1. Ruane, Y. L., Patrick, J. W., Bousayen, M., Oskio, S. & Fernie, A. R. Molecular regulation of seed and fruit set. Trends Plant Sci. 17, 656–665 (2012).
2. Ramirez, F. & Davenport, T. L. Apple pollination: a review. Sci. Hortic. 162, 188–203 (2013).
3. Gillaspy, G., Ben-David, H. & Grussm, W. Fruits: a developmental perspective. Plant Cell 5, 1439–1451 (1993).
4. Ding, J. C. et al. Cytokinins-induced parthenocarpic fruit development in tomato is partly dependent on enhanced gibberellin and auxin biosynthesis. PLoS ONE 8, e70080 (2013).
5. Mesejo, C., Reig, C., Martinez-Fuente, A. & Augusti, M. Parthenocarpic fruit production in loquat Eriobotrya japonica Lindl by using gibberelic acid. Sci. Hortic. 126, 37–41 (2010).
6. Niu, Q. F. et al. Effects of exogenous application of GA3 and N-2-chloro-4-pyridyl-N’-phenyleuca on induced parthenocarp and fruit quality in Pyrus pyrifolia ‘Cuiguan’. Plant Growth Regul. 76, 251–258 (2015).
7. Sugiyama, K., Karm, D. & Muro, T. Induction of parthenocarpic fruit set in watermelon by pollination with boll-gourd Lagenocen scions Molnda. Pollen. Sci. Hortic. 171, 1–5 (2014).
8. Mesejo, C., Yuste, R., Reig, C. & Martinez-Fuente, A. Gibberellin reactivates and maintains ovary-wall cell division causing fruit set in parthenocarpic Citrus species. Plant Sci. 247, 13–24 (2016).
9. Nitsch, J. P. Plant hormones in the development of fruits. Q. Rev. Bot. 27, 33–57 (1952).
10. de Jong, M., Wolters-Arts, M., Feron, R., Mariani, C. & Vriezen, W. H. The Solanum lycopersicum auxin response factor 7 SIARF regulates auxin signaling during tomato fruit set and development. Plant J. 57, 160–170 (2009).
11. Du, L. et al. SmARF8, a transcription factor involved in parthenocarpy in eggplant. Mol. Genet. Genom. 291, 93–105 (2016).
12. Wang, H. et al. The tomato Aux/IAA transcription factor IAA9 is involved in fruit development and leaf morphogenesis. Plant Cell 17, 2676–2692 (2005).
13. Serrani, J. C., Carrera, E., Ruiz-Rivero, O. & Gallego-Giraldo, L. Inhibition of auxin transport from the ovary or from the apical shoot induces parthenocarpic fruit-set in tomato mediated by gibberellins. Plant Physiol. 153, 851–862 (2010).
14. Моск, M., Proaño, K., Nuezc, F. & Garcia-Martínez, J. L. Role of gibberellins in parthenocarpic fruit development induced by the genetic system pat-3-pat-4 in tomato. Plant Physiol. 111, 545–550 (2000).
15. Greco, M., Chiappetta, A., Bruno, L. & Bttm, M. P. bosodiana caudicium induces changes in DNA methylation and chromatin patterning. J. Exp. Bot. 63, 695–709 (2012).
16. Martinez-Bello, L., Mottiz, T. & Lopez-Diaz, I. Silencing C19-GA 2-oxidases induces parthenocarpic development and leaf morphogenesis. Plant Physiol. 170, 37–48 (2015).
17. Marti, C. et al. Silencing of DELLA induces facultative parthenocarpy in tomato. Plant J. 52, 865–876 (2007).
18. Harberd, N. P., Belfield, E. & Yasumura, Y. The angiosperm gibberellin-GD1-DELLA growth regulatory mechanism: how an “inhibitor of an Inhibitor” enables flexible response to fluctuating environments. Plant Cell 21, 1326–1339 (2009).
19. Hirano, K. et al. Characterization of the molecular mechanism underlying gibberellin perception complex formation in rice. Plant Cell 22, 2680–2696 (2010).
20. Ampomah-Dwamena, C. Down-Regulation of TM09, a Tomato SEPALLATA homolog, causes parthenocarpic fruit development and floral reversion. Plant Physiol. 130, 605–617 (2002).
21. Yao, J. L., Dong, Y. H. & Morris, B. A. M. Parthenocarpic apple fruit production conferred by transposon insertion mutations in a MADS-box transcription factor. Proc. Natl Acad. Sci. USA 98, 1306–1311 (2001).
22. Klap, C. et al. Tomato facultative parthenocarpy results from SIAGAMOUS-LIKE 6 loss of function. Plant Biotechnol. J. 15, 634 (2017).
23. Yang, J. C., Zhang, J. H., Wang, J. Q., Zhou, Q. S. & Liu, L. J. Water deficit-induced senescence and its relationship to the remobilization of pre-stored carbon in wheat during grain filling. Agron. J. 93, 196–206 (2001).
24. Henwood, A. Formalin pigment from formaldehyde-acetic acid-alcohol fixed tissues? J. Histotechnol. 33, 109–111 (2010).
25. In, Z. Q. et al. ARDCS regulates the G2 to M transition of the cell cycle and is critical for the function of Arabidopsis shoot apical meristem. Cell Res. 17, 815–828 (2007).
26. Matlla, M. A. & Salmond, G. P. C. Complete genome sequence of seratia plymuthica bacteriofage MAM1. J. Virol. 86, 13872–13873 (2012).
27. Haas, B. J. et al. De novo transcript sequence reconstruction from RNA-Seq reference generation and analysis with Trinity. Nat. Protoc. 8, 1494–1512 (2013).
28. Li, R. et al. SOAP2: an improved ultrafast tool for short read alignment. Bioinformatics 25, 1966–1967 (2009).
29. Langmead, B., Trapnell, C., Pop, M. & Salzberg, S. L. Ultrafast and memoryefficient alignment of short DNA sequences to the human genome. Genome Biol. 10, R25 (2009).
30. Smajstrl, O. L. et al. Hierarchical mechanisms for direct reprogramming of fibroblasts to neurons. Cell 155, 621–635 (2013).
31. Lohse, M. et al. Mercator: a fast and simple web server for genome scale functional annotation of plant sequence data. Plant Cell Environ. 37, 1250–1258 (2014).
32. Thimm, O. et al. Mapman: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J. **37**, 914–939 (2004).

33. Maserio, S., Colombo, L., Grini, P. E., Schnittger, A. & Kater, M. M. The emerging importance of type I MADS box transcription factors for plant reproduction. *Plant Cell*** **23**, 865–872 (2011).

34. Serrani, J. C., Fos, M., Atarés, A. & García-Martínez, J. L. Effect of gibberellin and auxin on panthecarpic fruit growth induction in the cv micro-tom of tomato. *J. Plant Growth Regul.* **26**, 211–221 (2007).

35. Jung, C. J. et al. Gibberellin application at pre-bloom in grapevines down-regulates the expressions of VWA9 and VWA7, negative regulators of fruit set initiation, during panthecarpic fruit development. *PLoS ONE* **9**, e95634 (2014).

36. Liu, X. et al. The role of gibberellins and auxin on the tomato cell layers in parthenocarpic tomato genotypes. *PLoS ONE* **9**, 510–510 (1998).

37. Wang, H. et al. iC11, a cyclin-dependent protein kinase inhibitor from Arabidopsis thaliana interacts with both Cdc2a and Cdc3a, and its expression is induced by abscisic acid and its expression is induced by abscisic acid. *Plant J.* **15**, 501–510 (1998).

38. Nitsch, L. M. et al. Abscisic acid levels in tomato ovaries are regulated by Arabidopsis, a key enzyme for matrix polysaccharides in cell walls. *J. Exp. Bot.* **58**, 3609–3621 (2007).

39. Wang, H. et al. Regulatory features underlying pollination-dependent and -independent tomato fruit set revealed by transcript and primary metabolite profiling. *Plant Cell*** **21**, 1428–1452 (2009).

40. Mazzucato, A. et al. Transcriptome analysis during cell division in plants. *PLoS ONE* **9**, e114134 (2014).

41. Malladi, A. & Johnson, L. K. Expression profiling of cell cycle genes reveals key facilitators of cell production during carpel development, fruit set, and fruit growth in apple *Malus domestica* Borkh. *J. Exp. Bot.* **62**, 205–219 (2011).

42. Bashline, L., Lei, L., Li, S. & Gu, Y. Cell wall, cytoskeleton, and cell expansion in higher plants. *Mol. Plant* **7**, 586–600 (2014).

43. Atkinson, R. G., Johnston, S. L., Yauk, Y., Sharma, N. N. & Schröder, R. Analysis of xyloglucan endotransglucosylase/hydrolase XTH gene families in lowfruit and apple. *Postharvest Biol. Tech.* **51**, 149–157 (2009).

44. Beemster, G. T. S. et al. Genome-wide analysis of gene expression profiles associated with cell cycle transitions in growing organs of Arabidopsis. *Plant Physiol.* **138**, 734–743 (2005).

45. InzéD, V. L. D. Cell cycle regulation in plant development. *Annu. Rev. Genet.* **40**, 77–105 (2006).

46. Breyne, P. et al. Transcriptome analysis during cell division in plants. *Planta*** **199**, 14825–14830 (2002).

47. Malin, A. & Johnston, L. K. Expression profiling of cell division genes reveals key facilitators of cell production during carpel development, fruit set, and fruit growth in apple *Malus domestica* Borkh. *J. Exp. Bot.* **62**, 205–219 (2011).

48. Bashline, L., Lei, L., Li, S. & Gu, Y. Cell wall, cytoskeleton, and cell expansion in higher plants. *Mol. Plant* **7**, 586–600 (2014).

49. Beemster, G. T. S. et al. Genome-wide analysis of gene expression profiles associated with cell cycle transitions in growing organs of Arabidopsis. *Plant Physiol.* **138**, 734–743 (2005).

50. InzéD, V. L. D. Cell cycle regulation in plant development. *Annu. Rev. Genet.* **40**, 77–105 (2006).

51. Breyne, P. et al. Transcriptome analysis during cell division in plants. *Planta*** **199**, 14825–14830 (2002).