The characteristic of Yatu morphogenesis and the efficacy of exogenous hormones on the development of Yatu during fruit development in ‘Yali’ pear (Pyrus bretscheideri Rehd.)

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\textbf{ABSTRACT}

Yatu is a protuberance formed on the base part of ‘Yali’ pear fruit, near the pedicel, causing a shape like a duck head termed Yatu. It is a typical phenotypic trait to evaluate fruit appearance quality. The mechanism for Yatu formation has not been clear yet. Here, 90.8\% of fruits with Yatu generated in outer base part of fruits. Primitive cells of Yatu were found at 10 days after pollination (DAP). There were higher expression levels of PbGA20ox2, PbIPT7a, and PbIPT5a, lower transcription levels of PbGA2ox1, PbNAC1, and PbNAC3 in outer base part of fruits at 10 DAP, accompanied by significantly higher levels of GA\textsubscript{3}, ZR, (GA\textsubscript{3}+ ZR)/ABA, and lower ABA content compared to that in the inner base part of fruits. GA\textsubscript{3} + 6-BA promoted Yatu development by increasing GA\textsubscript{3} content at 10 and 20 DAP, and ZR content at 20 DAP. PAC suppressed Yatu morphogenesis and development by increasing ABA level at 10 DAP. These results suggest that Yatu usually generated in outer base part of fruits, relatively higher GA\textsubscript{3} and ZR contents, lower ABA content promoted Yatu morphogenesis and development.

\textbf{Introduction}

‘Yali’ (Pyrus bretscheideri Rehd.) is native to the Hebei province in China and is one of the superior traditional pear cultivars. The fruits have a very high economy and nutrition value. Yatu is a protuberance that forms on the base of a ‘Yali’ pear and looks like a duck head. It is a typical phenotypic trait to evaluate fruit appearance quality. The phenomenon of fruits without Yatu or with non-typical Yatu often occurs in ‘Yali’ production, although the reason is not yet clear. It is important and necessary to clarify the mechanism of Yatu morphogenesis and development. Previous studies have found the percent of first-order fruits with Yatu and typical Yatu were significantly higher than that of sixth-order fruits. The base part of proximal fruit within an inflorescence possessed relatively higher gibberellin acid (GA\textsubscript{3}) and zeatin riboside (ZR) content, lower abscisic acid (ABA) content, and well-developed vascular bundles, which enhanced fruit sink strength to provide sufficient assimilate for Yatu formation. While the cells in the base part of fruit that tend to develop into Yatu should be found. How the hormones functioned and controlled Yatu formation should be explicit.

In the early phase of fresh fruit development, cell division and cell expansion played a crucial role in the determination of fruit size and shape. The cell division of ‘Yali’ pear fruit started before bloom and ceased at about 25 days after flowering. Cell division intensity and duration affected cell number, which was the determining factor for fruit growth rate. Cell expansion depended on increasing in turgor pressure caused by accumulation of storage products and sugar. In pear fruit development, the specific temporal and spatial pattern of cell division and cell expansion determined the fruit phenotypic trait.

Plant hormones played a critical role in regulating cell division and cell expansion. Cytokinin acted as an inducible factor to exert initiating and promoting functions on cell division. It regulated the assimilate transportation and related protein synthesis to regulate cell expansion. Gibberellins promoted the longitudinal cell expansion by changing the stability of the microtubules via affecting their association with the plasma membrane. ABA counteracted gibberellin efficiency during fruit set and development, which emphasized the importance of hormone homeostasis during fruit development. The objectives of this research were to study the characteristic of Yatu morphogenesis and clarify the regulated mechanism of plant hormones on Yatu development.

\textbf{Materials and methods}

\textit{Plant materials and treatment}

The experiment was carried out in 2018 in a commercial pear orchard in Botou, Hebei province, China (116.53E, 38.15 N). ‘Yali’ (Pyrus bretscheideri Rehd.) trees on...
*P. betulaefolia* rootstock were planted in 1988 with a spacing at 4 m × 6 m and a north-to-south row orientation. At day 2 before bloom (DBB), the first-order flower was bagged, and the others within the same inflorescence were thinned (Figure 1a). During bloom period, a controlled pollination was made using the prepared pollen of ‘Cuili’, which is a native pear variety. As pollinating, a marker was made on inner side surface of the peduncle that closed to the base part of fruit to help us easily recognize inner and outer base part of fruit while the fruit growing and further drooping (Figure 1a). The percent of fruits with Yatu that generated in the inner and outer base parts of fruits was counted. Samples that were collected from inner and outer base part of fruits were named INN1 and OUT1, respectively (Figure 1b). They were collected at 2 DBB and 10, 20, and 35 days after pollination (DAP) for determining GA3, ZR, and ABA content. Samples selected at 10 DAP were used for preparing paraffin section to observe cell morphology and determine the relative expression levels of genes related to hormone biosynthesis and metabolism. The experiment was designed as a randomized complete block with three replications of three trees each.

**Classification criteria for Yatu**

The difference of a fruit height between two opposite sides was used as a grading standard for Yatu. The height of one side with Yatu was showed by H, the height of opposing side was showed by h. The classification of Yatu was determined by the difference between H and h (e. g. H-h) (Figure 2).

**Plant growth regulators treatment**

GA3+6-BA and PAC were separately applied on two groups of fruits to observe the function on Yatu morphogenesis and development. The application concentrations were 300 mg/L for GA3 and 6-BA, 4000 mg/L for PAC, which have been testified as optimal concentrations. The base part of fruit, approximately a circle of 1/5 the length of a fruit, was treated by hormones. GA3 + 6-BA and PAC were both applied at 2 DBB and 10 DAP, respectively. GA3 and PAC were dissolved in ethanol to make stock solution, 6-BA was dissolved in 1 M NaOH to make stock solution. The control was distilled water containing the same concentration of ethanol and NaOH as that used in treatment groups. One hundred pears of each treatment were randomly selected for observing Yatu development. INN1 and OUT1 that sampled at 2 DBB, 10, 20, and 35 DAP were used for determining GA3, ZR, and ABA contents. INN1 and OUT1 that sampled at 10 DAP were used for determining the relative expression levels of genes related to
Difference that reached 10 mm or more was defined as typical Yatu. Difference no less than 5 mm and lower than 10 mm was defined as non-typical Yatu.

**Investigation of fruits with different types of Yatu**

Mature fruits were harvested at 162 DAP. One hundred mature fruits of each treatment were randomly selected and examined. The test was replicated three times.

**Preparation for paraffin section**

Samples were kept in a formaldehyde acetic acid alcohol (FAA) fixative first. The paraffin sections were prepared as described by 18. They were dehydrated by an ascending concentration of ethanol, cleaned by dimethylbenzene, embedded in paraffin, sectioned on a microtome, double stained by sarranine and fast green, rehydrated, and observed and photographed under a microscope.

**Hormone extraction, purification, and quantification**

The method of hormone determination was done as described by 3. Samples were ground to powder in liquid nitrogen. Hormones were extracted in 80% methanol containing 0.2 g butylated hydroxytoluene under ultrasonic wave, concentrated under nitrogen gas, redissolved in methanol, and filtrated through 0.22 μm filter membrane. High-performance liquid chromatography (HPLC) was used to determine the concentration of GA3, ZR, andABA.

**RNA extraction and quantitative real-time PCR**

Total RNA was isolated with RNAPrep Pure Plant Kit (Tiangen, Beijing, China). Reverse transcription was performed with FastQuant RT Kit (Tiangen, Beijing, China). *Pb*GA*PDH* was used as the internal control.19–21 All specific primers were designed on NCBI website and further confirmed by the corresponding melting curves with a single sharp. All primer sequences used for RT-qPCR are listed in Table 1.

| Gene name  | Primer sequences                      |
|------------|---------------------------------------|
| *Pb*GA*PDH* | 5′-GGTCAGGATTACAAGGCAGGG-3′          |
| *Pb*GA*2ox1* | 5′-ACCGGGAATTACAAGGCAGGG-3′          |
| *Pb*GA*2ox2* | 5′-GCCGTTACGAGCTTATCG-3′             |
| *Pb*GA*3ox1* | 5′-ACGGGAATTACAAGGCAGGG-3′          |
| *Pb*GA*3ox2* | 5′-ACGGGAATTACAAGGCAGGG-3′          |
| *Pb*IPT3    | 5′-GACCTAGGCCACCTGTTC-3′             |
| *Pb*IPT5a   | 5′-GCCGTTACGAGCTTATCG-3′             |
| *Pb*IPT5b   | 5′-TGGTAGGCGGCTGTTC-3′               |
| *Pb*IPT7a   | 5′-GACGACGCTTCATCGTATC-3′            |
| *Pb*NED1    | 5′-TGGACCAAAACATCGGCTTC-3′           |
| *Pb*NED3    | 5′-ATCGATATGCGGCGGAGA-3′             |

Quantitative real-time PCR was performed in three replicates using the TransStart Top Green qPCR SuperMix (Transgen, Beijing, China) on a Bio-Rad CFX 96 Real-time PCR Detection System. The procedure was set as follows: template initial denaturation at 95°C for 30 s, template denaturation at 95°C for 15 s, primer annealing at 60°C for 15 s, 40 cycles extension at 72°C for 30 s, and followed by the melting curve analysis. The relative gene expression levels were calculated and normalized by the 2−ΔΔCt method.22

**Statistical analysis**

Data Processing System (DPS2) software was used to analyze the experimental data. The significance of differences among mean values was determined by t-test (p ≤ .05) and Duncan’s multiple-range test (p ≤ .05) using least significant ranges means.

**Results**

**Yatu usually generated in outer base part of fruit**

The percent of fruits with Yatu generated in outer base part of fruits was 9.9 times higher than that in inner (Figure 3a). A lot of parenchyma cells that were characterized by small cells with more layers gathered in outer base part of fruits were found at 10 DAP. They were called primitive cells of Yatu. The 10 DAP was considered as the strongest cell division stage for Yatu. Around the primitive cells, other parenchyma cells were large and loosely arranged (Figure 3b).

**Hormone contents and proportions differed in inner and outer base part of fruits**

The GA3 content in the outer base part of fruits was significantly higher at 2 DBB, 10 DAP, and 35 DAP, and showed no difference at 20 DAP from the inner base part of fruits (Figure 4a). The contents of ZR in the outer base part of fruits all were significantly higher than that in the inner base part of fruits (Figure 4b). ABA content in outer base part of fruits was significantly lower at 10 DAP and 20 DAP compared to that in inner (Figure 4c). At 2 DBB and 10 DAP, the ratio of GA3/ABA, ZR/ABA, and (GA3+ ZR)/ABA in the outer base part of fruits was significantly higher than that in inner (Figure 4 d–f). Consistent with Yatu usually generated in the outer base part of fruits, the higher content of GA3, ZR, (GA3+ ZR)/ABA, the lower content of ABA in the outer base part of fruits promoted Yatu formation.

**Expression levels of genes related to the biosynthesis and metabolism of GA, cytokinin, and ABA in the inner and outer base part of fruits**

The relative expression level of *Pb*GA2ox1 in the inner base part of fruits was 2.1 times higher than that in the outer base part of fruits. The gene *Pb*GA2ox2 possessed relatively higher expression level in the outer base part of fruits than in the inner base part of fruits. There was no significant difference in *Pb*GA3ox1 expression level between the inner and outer base
parts of fruits (Figure 5a). The relatively lower \( \text{PbGA2ox1} \) transcription level and higher \( \text{PbGA20ox2} \) transcription level in the outer base part of fruits were consistent with higher \( \text{GA}_3 \) accumulation at 10 DAP (Figure 4a).

The transcription levels of \( \text{PbIPT7a} \) and \( \text{PbIPT5a} \) in the outer base part of fruits were significantly higher than that in the inner base part of fruits (Figure 5b). It was consistent with higher ZR content in the outer base part of fruits at 10 DAP.
(Figure 4b). Both PbIPT5b and PbIPT3 expression showed no significant differences between the inner and outer base parts of fruits (Figure 5b).

As shown in Figure 5c, the genes PbNCED1 and PbNCED3 possessed relatively lower expression levels in the outer base part of fruits than that in the inner base part of fruits. It was consistent with the relatively lower ABA content in the outer base part of fruits (Figure 4c).

**Exogenously applied GA$_3$ + 6-BA and PAC changed the ability of fruits to form Yatu**

At 10 DAP, Yatu could not be seen on the base part of all treated and untreated fruits (Figure 6a). At 20 DAP, the phenotype of Yatu obviously appeared in GA$_3$ + 6-BA-treated fruits and control fruits. The size of Yatu was much bigger in GA$_3$ + 6-BA-treated fruits than control fruits. There was still no Yatu generated in fruits treated by PAC (Figure 6b). At 35 DAP, the size of Yatu in GA$_3$ + 6-BA treated fruits and control fruits increased with time extension. There was still no Yatu appearing in PAC-treated fruits (Figure 6c). At 162 DAP (Figure 6d), the size of Yatu treated by GA$_3$ + 6-BA was significantly higher than that of control fruits, a small bump appeared on the base part of fruit treated by PAC.

After harvest, the percent of fruits with different types of Yatu was counted (Table 2). There was no significant difference between GA$_3$ + 6-BA-treated and control fruits on total percent of fruits with Yatu. GA$_3$ + 6-BA significantly increased the percent of fruits with typical Yatu. Yatu did not appear in PAC-treated fruits. Altogether, the results strongly suggest that GA$_3$ + 6-BA promoted the development of Yatu and PAC showed the opposite efficacy.

**Endogenous hormone contents in base part of fruits treated by GA$_3$ + 6-BA and PAC**

GA$_3$ + 6-BA highly increased GA$_3$ content in the base part of fruits at 10 DAP and 20 DAP. Strangely, the fruits treated by PAC showed a similar pattern with GA$_3$ + 6-BA application at 10 DAP and 20 DAP. The content of GA$_3$ in fruits treated by GA$_3$ + 6-BA was higher than that of fruits treated by PAC. At 35 DAP, there was no significant difference in GA$_3$ content between GA$_3$ + 6-BA-treated and control fruits, while PAC significantly reduced GA$_3$ content (Figure 7a). The content of ZR in the base part of fruits at 20 DAP was significantly increased by GA$_3$ + 6-BA application. PAC showed no significant influence on ZR content from control fruits at 10 DAP, 20 DAP, and 35 DAP. ZR content in the outer base part of fruits mostly was higher than that in the inner base part of fruits (Figure 7b).

PAC increased ABA content in the base part of fruits at 10 DAP. ABA content was significantly higher than GA$_3$ + 6-BA-treated and control fruits at 10 DAP. At 20 DAP, the ABA content in the outer base part of fruits all exhibited lower values than that in the inner base part of fruits. At 35 DAP, three groups all showed a rapidly drop in ABA level (Figure 7c).

![Figure 6](https://example.com/figure6.png)

Figure 6. The influence of GA$_3$ + 6-BA and PAC on Yatu morphogenesis and development, including 10 (a), 20 (b), 35 (c), and 162 days after pollination (d), respectively. In (a–c), the left and right fruit were treated by GA$_3$ + 6-BA and PAC, respectively, the middle fruit was control. In (d), the left fruit was control, the middle and right fruits were treated by GA$_3$ + 6-BA and PAC, respectively. The red arrows indicate Yatu in different developmental stages.
Table 2. The influence of GA3 + 6-BA and PAC on constitution of different types of Yatu.

| Application time | Treatment | Total percent of fruits with Yatu | Non-typical Yatu | Typical Yatu |
|------------------|-----------|-----------------------------------|-----------------|-------------|
| 2 DDB+10 DAP     | GA3 + 6-BA| 92.22 a                           | 26.56 b         | 65.66 a     |
| PAC              | 0 b       | 0 c                              | 0 c             |             |
| Control          | 92.15 a   | 44.69 a                          | 47.46 b         |             |

The different letters following the values in the same column indicate a significant difference (ps .05).

Expression levels of genes related to biosynthesis and metabolism of GA, cytokinin, and ABA in the base part of fruits treated by GA3 + 6-BA and PAC

The gene PbGA2ox1 expression level in the outer base part of fruits all showed significantly lower values than that in inner. GA3 + 6-BA greatly promoted PbGA2ox1 expression level both in the inner and outer base parts of fruits. PAC significantly suppressed PbGA2ox1 expression level in the outer base part of fruits (Figure 8a). The gene PbGA2ox02 possessed relatively higher expression level in the outer base part of fruits than in inner both at treated and control fruits. The PbGA2ox02 expression level was obviously suppressed by GA3 + 6-BA, while it was promoted by PAC (Figure 8b). PbGA3ox1 weakly expressed both in GA3 + 6-BA treated fruits and in control fruits. PAC application greatly increased PbGA3ox1 expression level, which was significantly higher in the outer base part of fruits than that in inner (Figure 8c).

The gene PbIPT7a (Figure 8d) possessed significantly higher expression levels in the outer base part of fruits than that in inner both in treated and control fruits. GA3 + 6-BA and PAC showed no similar influence on the gene expression patterns of PbIPT7a, PbIPT5a (Figure 8e), PbIPT5b (Figure 8f), and PbIPT3 (Figure 8g). Most of them in the outer base part of fruits showed higher expression level than that in inner.

The genes PbNCED1 and PbNCED3 possessed a similar expression pattern both in treated and control fruits. PAC application significantly increased the expression levels of PbNCED1 (Figure 8h) and PbNCED3 (Figure 8i). It was consistent with the elevated ABA content in the base part of fruits treated by PAC at 10 DAP [Figure 7c]. GA3 + 6-BA showed no significant influence on PbNCED1 and PbNCED3 transcription levels.

Discussion

Yatu usually generated in the outer base part of fruit. Reference 3 found the similar results to us. In this study, the primitive cells of Yatu were found at 10 DAP in the outer base part of fruit. They were parenchyma cells that were characterized by small cells with more layers gathered in the outer base part of fruit compared to the surrounding cells. The 10 DAP was considered as the strongest cell division stage, which was an important time point for Yatu formation. The accelerated cell division of primitive cells with subsequent cell expansion promoted Yatu formation.
The highest content of GA3 and ZR was both observed at 2 DBB. There was a time lag between the high accumulation of them and the rapid primitive cells division. Reference 23 found that during fruit development period, cytokinin played a crucial role in stimulating cell division. Carlos et al. (2016) worked with citrus and found that GA triggered and maintained ovary-wall cell division. Therefore, we proposed the activation of primitive cells division of Yatu based on the accumulation of the relative high levels of GA3 and ZR during balloon period. They played an early proliferation-inducing role in the process of Yatu morphogenesis.

In most species, plant hormones were critical to control cell division and expansion.6 Previous studies found the high expression levels of GA20ox and GA3ox significantly promoted bioactive GAs biosynthesis in tomato and strawberry.6,24 The overexpression of GA2ox significantly decreased the content of bioactive GAs and exhibited an obvious dwarf phenotype in solanum and Pyrus rootstock.25,26 In this study, the lower transcription level of PbGA2ox1 and the higher expression level of PbGA20ox2 improved GA3 content in the outer base part of fruits compared to that in inner at 10 DAP. The higher transcription levels of PblPT7a and PblPT5a synergistically promoted the higher accumulation of ZR in the outer base part of fruits at 10 DAP compared to that in inner. Reference 27 found the increased expression levels of PbnCED1 and PbnCED3 increased ABA content in avocado fruits. In this study, the lower expression levels of PbnCED1 and PbnCED3 were consistent with the lower

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**Figure 8.** The influence of GA3 + 6-BA and PAC on the expression patterns of genes related to gibberellin acid (GA) metabolism, cytokinin (CTK) biosynthesis, and abscisic acid (ABA) biosynthesis at 10 days after pollination, including PbGA2ox1 (a), PbGA20ox2 (b), PbGA3ox1 (c), PbIPT7a (d), PbIPT5a (e), PbIPT5b (f), PbIPT3 (g), PbnCED1 (h), and PbnCED2 (i). Different letters above the bars indicate significant difference (Duncan’s multiple-range test, \( p < 0.05 \)). INN1/OUT1 + P, the inner/outer base part of fruits treated by PAC; INN1/OUT1 + G, the inner/outer base part of fruits treated by GA3 + 6-BA.
ABA content in the outer base part of fruits. We speculated that the relatively higher content of GA\textsubscript{3} and ZR at 2 DBB and 10 DAP, the lower ABA content at 10 DAP promoted Yatu morphogenesis and development. \textsuperscript{14} found exogenous GA and BA application regulated cell elongation and expansion through altering the microtubule array organization. Reference \textsuperscript{28} found exogenous GA treatment effectively increased plant height by 20.4\% via increasing epidermis cell length of the petiole. In this study, we found GA\textsubscript{3} + 6-BA application significantly increased GA\textsubscript{3} content at 10 DAP and 20 DAP, and increased ZR content at 20 DAP. We speculated that GA\textsubscript{3} + 6-BA treatment accelerated the development process to form typical Yatu via increasing the endogenous GAs and cytokinin that promoted cell elongation.

PAC was a plant growth retardant by inhibiting GA biosynthesis. Suppressing KO, which acted in the oxidation process of ent-kaurene to ent-kaurenolic acid by the application of PAC, resulted in a reduction of GA concomitant with an inhibition on the plant growth rate,\textsuperscript{29–31} while the reduction could be rescued by GA application.\textsuperscript{32,33} In this study, PAC significantly increased ABA level at 10 DAP and sharply decreased GA\textsubscript{3} content at 35 DAP. We speculated that PAC application suppressed the development process to form Yatu by increasing ABA content and inhibiting GA biosynthesis that suppressed cell elongation.

In this study, we found Yatu usually generated in the outer base part of fruit, which possessed higher contents of GA\textsubscript{3} and ZR at 2 DBB and 10 DAP, the lower ABA content at 10 DAP. The application of GA\textsubscript{3} + BA and PAC showed promotion and suppression on Yatu formation, respectively. In short, plant hormone played an important role in regulating Yatu formation. It could be concluded that the relatively higher content of GA\textsubscript{3} and ZR and lower content of ABA promoted Yatu morphogenesis and development.

Acknowledgments

This research was supported by the Pearl Industry Technology Engineering Research Center of the Ministry of Education.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

Pear Industry Technology Engineering Research Center of the Ministry of Education (2021); Earmarked Fund for the China Agriculture Research System (CARS-28-09).

Author contributions

G.D. and X.Y.Z. designed the experiments. Y.Z. and W.L. contributed equally to this work. Y.Z. and W.L. carried out all of the experiments with technical support from G.D. Y.Z and X.S. analyzed data. Y.Z. and W.L. wrote the manuscript with input from G.D.

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