Variation of Endogenous Hormones during Flower and Leaf Buds Development in ‘Tianhong 2’ Apple

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Additional index words. flower bud differentiation, hormone changes, *Malus domestica* Borkh., spur terminal buds

Abstract. Hormones have an important role in apple flower bud differentiation; therefore, it is necessary to systematically explore the dynamic changes of endogenous hormones during flower and leaf bud development to elucidate the potential hormone regulation mechanism. In this study, we first observed the buds of ‘Tianhong 2’ apple during their differentiation stage using an anatomical method and divided them into physiologically differentiated stages of spur terminal buds, flower buds, and leaf buds. Then, we determined the contents of zeatin riboside (ZR), abscisic acid (ABA), auxin (IAA), and gibberellin (GA₃) in these various types of buds using an enzyme-linked immunosorbent assay. The results showed that the content of ZR and the ratio of ZR to IAA in spur terminal buds decreased significantly during physiological differentiation. The contents of ZR, IAA, and GA₃ in leaf buds culminated at the initial differentiation stage. The content of ZR in flower buds was significantly higher than that in leaf buds after formation of the inflorescence primordium and sepal primordium. Before the appearance of stamen primordium, the content of GA₃ in flower buds was remarkably lower than that in leaf buds. The ratios of ABA/IAA and ZR/IAA in flower buds were significantly higher than those in leaf buds before the appearance of flower organ primordium. Moreover, ABA content, ABA/ZR, and ABA/GA₃ in flower buds were higher than those in leaf buds throughout the whole flower bud morphological differentiation process. Therefore, the reduced ZR content was beneficial to floral induction. The low content of GA₃, and high ratios of ABA/IAA and ZR/IAA were conducive to early morphological differentiation. In addition, high ratios of ABA/GA₃ and ABA/ZR were beneficial to the morphological differentiation of flower buds. Moreover, the high ABA content was beneficial to floral induction and morphological differentiation of flower buds. Our results shed light on the mechanisms of hormonal regulation of apple flower bud differentiation and could potentially strengthen the theoretical basis for artificial regulation of apple flower bud development using exogenous plant hormones.

Received for publication 7 July 2020. Accepted for publication 1 Sept. 2020.

Published online 5 October 2020.

The endogenous hormones were extracted and measured at the Biotechnology Laboratory of the Agricultural College of China Agricultural University. This work was financially supported by China Apple Research System (CARS-27) and Hebei Natural Science Foundation (C2007000448, C2016204144).

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Apple (*Malus domestica* Borkh.) is one of the most important fruit trees in the world. However, due to the infrequent formation of flower buds during its growth, apple fruit production is significantly affected; therefore, the mechanism of apple flower bud differentiation has been widely studied. There are four stages of flowering: flower induction, flower initiation, flower differentiation, and blooming (Hanke et al., 2007), which is a highly complex physiological, biochemical, and morphogenetic process.

Endogenous hormones have important regulatory roles in all stages of flower development (Chandler, 2011). Kondo et al. (1999) suggested that the content of IAA

might be the key factor influencing apple flower bud differentiation. However, IAA has been shown to have both promotional (Li et al., 1996) and inhibitory (Cao et al., 2000) effects on flower initiation, depending on the content of IAA. Cytokinin promotes the induction of flower formation (Hoad, 1984). Spraying 6-BA could induce a higher expression of *MdTFL1*, which promotes apple flower formation (Li et al., 2016, 2017). Sanyal and Bangerth (1998) showed that the decreasing content of ZR increased the proportion of flower buds. In addition, GA₃ inhibited the flowering of woody plants (Pharis and King, 1985), and a low level of GA₃ was beneficial to flower induction (Xing et al., 2014, 2016); however, spraying GA₃ could break the ZR/GA ratio to suppress the expression of *MdSPL* genes and inhibit flower bud formation (Zhang et al., 2016). Chang and Huang (2018) found that GA₃ could promote flowering. Moreover, a high content of ABA promoted apple flower formation (Cao et al., 2000). Spraying uniconazole increased the ABA content in spur terminal buds and accelerated flower bud differentiation (Cao et al., 2003). Furthermore, the balance of endogenous hormones was proposed as the key factor for flower bud differentiation (Wang, 2010; Yang, 2010). Therefore, there are different views on the effect of endogenous hormones on apple flower formation.

‘Tianhong 2’ apple, a dwarf mutant of the ‘Nagano Fuji 2’ apple in China, is characterized by its spur type and higher rate of flower formation (Shao et al., 2008). In apple, the flower buds usually grew at the top of the annual spur (short branches) and the lateral ends of the branches (Buban and Faust, 1982). Both of the vegetative organs and floral structures can be found in the flower buds, which are called mixed-type buds. Several studies have reported the role of plant hormones in flower bud differentiation in apple, but hormone regulating studies of apple flower buds and leaf buds remain limited.

Zhou et al. (1988) studied the changes in endogenous hormone contents in the leaves of flower and leaf buds under normal growth conditions during apple flower bud differentiation stages. However, the changes in endogenous hormones and their potential mechanisms in flower and leaf buds in apple are still unclear. Therefore, in the current study, we investigated the spur terminal buds by anatomical observation. They were distinguished into flower buds with different differentiation stages and leaf buds. The dynamic contents of endogenous hormones during physiological differentiation that spur terminal buds, flower buds, and leaf buds during flower bud differentiation were also determined. The results could provide a theoretical basis for how hormones regulate apple flower bud differentiation.

Materials and Methods

Plant materials and sampling

Ten-year-old ‘Tianhong 2’ apple (*Malus domestica* Borkh. ‘Tianhong 2’) trees grafted

on interstock SH₄₀ and rootstock (*Malus robusta* Rehd.) were grown with routine management (spacing, 2 × 3.5 m) at the comprehensive experimental station in Baoding, Hebei Province of China. Thirty spur terminal buds were randomly collected from 30 trees every 10 d from 5 May (10 d after the spur stopped growing) until the middle of November.

Sample observation and classification

The morphological differentiation of flower buds was classified into seven stages (transformation stage, initial differentiation stage, inflorescence primordium stage, sepal primordium stage, petal primordium stage, stamen primordium stage, and pistil primordium stage) as referring to the previous methods (Cao, 2000; Foster et al., 2003), with some modification. The spur terminal buds were numbered 1 to 30. Each terminal bud was dissected and observed by a stereoscopic anatomical microscope to estimate its differentiation stage and then frozen in liquid nitrogen immediately. The spur terminal buds before morphological differentiation were considered samples of physiological differentiation. At the same time, a part of the spur terminal buds without morphological changes were considered leaf buds, and the rest were flower buds which were classified into different developmental stages according to the observation. Spur terminal buds (physiological differentiation stage, leaf buds, and flower buds) were stored at -80 °C for the measurements of endogenous hormones.

Spurs terminal buds were collected on 5, 15, and 25 May, 25 June, 5 and 25 July, 15 and 25 Aug., 15 Sept., and 5 Nov. These were the concentrated differentiation stages of flower buds (Table 1) used for analyses of hormone quantification.

Determination of endogenous hormone content

Each sample of ≈0.2 g fresh weight (FW) was weighed. The endogenous hormones

(ZR, GA₃, IAA, and ABA) in spur terminal buds were extracted and purified according to Yang et al. (2001). Briefly, the samples were homogenized in cold 80% (v/v) methanol with butyrate hydroxytoluene (1 mmol·L⁻¹) and extracted at 4 °C for 5 h. The extracts were collected after centrifugation at 3500 r/min (4 °C) for 8 min and passed through a C-18 Sep-Pak cartridge (Waters, Milford, MA). Then, they were dried in N₂. The residues were dissolved in phosphate-buffered saline (PBS) to determine the levels of GA₃, ZR, IAA, and ABA. Determination was performed on a 96-well microtitration plate. Each well was filled with 50 μL of either extracts or GA₃, ZR, IAA, and ABA standards and 50 μL antibodies against GA₃, ZR, IAA, and ABA, respectively, and then incubated for 30 min at 37 °C. After washing four times with PBS plus Tween 20 buffer, horseradish peroxidase-labeled goat antirabbit immunoglobulin was added to each well and incubated for 30 min at 37 °C. Then, they were washed as described previously and 100 μL color-appearing solution containing 1.5 mg·mL⁻¹ ophenylenediamine (OPD) and 0.008% (v/v) H₂O₂ was added to each well. The reaction progress was stopped by adding 50 μL 2 mol·L⁻¹ H₂SO₄ per well when the 2000 ng·mL⁻¹ standard had a pale color and the 0 ng·mL⁻¹ standard had a deep color in the wells. Absorbance was recorded at 490 nm. Calculations of the enzyme-immunoassay data were performed as described by Weiler et al. (1981). After obtaining the concentration of the hormone in the sample, the hormone content in the sample was calculated (ng·g⁻¹ FW). Data are shown as the mean ± SE in three replicates.

Data analysis and statistics

The experimental data analysis and statistics were processed using SPSS 17.0 software and plotted by Origin 8.0. The contents of endogenous hormones in spur terminal buds (physiological differentiation stage, leaf buds, and flower buds) at different

developmental stages were analyzed by one-way analysis of variance. Differences among developmental stages were detected by Duncan's multiple range test applied to compare the means at a significance level of *P* < 0.05.

Results

Flower buds proportion and differentiation process of 'Tianhong 2' apple

The transformation stage of 'Tianhong 2' apple flower buds appeared during early June (≈40 d after the spur stopped growing). The proportion of flower buds increased along with bud development, which reached 60% to 83.3% at the later stage of flower bud differentiation (Table 1).

The observation results (Table 1) showed that the transformation stage began on 5 June and was concentrated during the middle and last 10 d of June. Initial differentiation appeared on 15 June and was concentrated during the first and middle days of July. Inflorescence primordium occurred on 5 July and was concentrated during the last 10 d of July. Sepal primordium first appeared on 15 July and was concentrated during the last 10 d of July to the middle of August. The petal primordium occurred on 5 Aug. and was concentrated during the middle and last 10 d of August. The stamen primordium appeared slightly on 25 Aug. and was concentrated in September. The pistil primordium occurred on 25 Sept., and a large number of flower buds entered the pistil primordium stage in October.

Dynamic changes in endogenous hormones during bud differentiation

Content of ZR. The ZR content in spur terminal buds decreased significantly during the physiological differentiation stage from 5 May to 25 May. The content of ZR in leaf buds continued to decline after an increase from 25 June to 5 July. There was no

Table 1. Flower bud differentiation process of 'Tianhong 2' apple and relative flower bud percentage (%).

Sample date	Transformation stage	Initial differentiation	Inflorescence primordium	Sepal primordium	Petal primordium	Stamen primordium	Pistil primordium	Total
5 May	0	0	0	0	0	0	0	0.0
15 May	0	0	0	0	0	0	0	0.0
25 May	0	0	0	0	0	0	0	0.0
5 June	51.9	0	0	0	0	0	0	51.9
15 June	20.0	20.0	0	0	0	0	0	40.0
25 June	46.4	10.7	0	0	0	0	0	57.1
5 July	13.8	31.0	13.8	0	0	0	0	58.6
15 July	10.0	20.0	13.3	6.7	0	0	0	50.0
25 July	10.0	6.7	20.0	26.7	0	0	0	63.3
5 Aug.	16.7	16.7	10.0	13.3	20.0	0	0	76.7
15 Aug.	6.7	0	20.0	16.7	33.3	0	0	76.7
25 Aug.	3.3	0	20.0	10.0	33.3	13.3	0	80.0
5 Sept.	0	3.3	0	10.0	6.7	50.0	0	70.0
15 Sept.	0	0	0	13.8	0	62.1	0	75.9
25 Sept.	0	0	3.3	3.3	0	43.3	16.7	66.7
5 Oct.	0	0	0	6.7	0	20.0	53.3	80.0
15 Oct.	0	0	3.3	10.0	0	0	70.0	83.3
25 Oct.	0	0	0	3.1	0	3.1	62.5	68.8
5 Nov.	0	0	0	0	0	6.7	53.3	60.0
15 Nov.	0	0	0	0	0	15.0	55.0	70.0

The numbers in each row indicate the proportions of each differentiation stage. The last column shows the proportion of total flower buds.

significant difference in the content of ZR in flower buds from 25 June to 25 July, but it increased from 25 July to 15 Aug. and then decreased until 15 Sept., which was at the stage of stamen concentrated differentiation.

There was no difference in the ZR content in leaf buds between 25 June and 25 May (late stage of physiological differentiation); however, the content of ZR in flower buds was significantly lower than that during the physiological differentiation stage (5 May–25 May). The content of ZR in leaf buds was 1.3-times to 1.5-times higher than that of flower buds from 25 June to 5 July. The content of ZR in flower buds was significantly higher than that in leaf buds from 15 Aug. to 5 Nov. (except 25 Aug.). These results imply that the reduced ZR content was beneficial to floral induction, and that a certain amount of ZR was required for flower bud morphological differentiation (Fig. 1A).

Content of ABA. The content of ABA first decreased and then increased from 5 May to 25 May, which was during the physiological differentiation stage. The content of ABA was not different in flower buds between 25 June and 25 May (the later stage of physiological differentiation), but it increased significantly on 5 July (when the most flower buds were experiencing initial differentiation), decreased from 5 July to 15 Sept., and then increased sharply. The ABA content in the leaf buds on 25 June was significantly lower than that on 25 May (late physiological

differentiation period), reached its peak on 5 July, decreased to the lowest point from 5 July to 25 Aug., and then increased significantly.

The content of ABA in flower buds was significantly higher than that in leaf buds during the whole morphological differentiation stage, indicating that the high ABA content was beneficial for floral induction and flower bud morphological differentiation (Fig. 1B).

Content of IAA. The content of IAA increased significantly from 5 May to 25 May, which was the physiological differentiation stage. The content of IAA in leaf buds decreased sharply after reaching its peak point on 5 July, slowly decreased from 25 July to 25 Aug., and increased significantly from 25 Aug. to 5 Nov. The content of IAA in flower buds was lower on 25 June than on 25 May, and it increased significantly from 25 June to 5 July, which was at the stage of concentrated differentiation during the initial differentiation and emergence stage of inflorescence primordium. It decreased significantly from 5 July to 25 July; then, it increased significantly, which was the differentiation stage of floral organ primordium.

The content of IAA in leaf buds was 1.7-times to 1.9-times higher than that in flower buds from 25 June to 5 July, and it was significantly lower than that in the flower buds during the same period from 25 July to 5 Nov., which indicated that the high content of

IAA was beneficial to flower organ primordium differentiation (Fig. 1C).

Content of GA₃. The content of GA₃ decreased significantly from 5 May to 15 May; then, it increased significantly, which was the physiological differentiation stage. The content of GA₃ in leaf buds was lower on 25 June than on 25 May (the later stage of physiological differentiation), and it reached its peak point on 5 July (1.3-times higher than that in flower buds), which was not significantly different compared to 25 May, but it decreased significantly from 5 July to 5 Nov. The content of GA₃ in flower buds decreased to the lowest point on 25 June, when a large number of flower buds were in the transformation stage, and it increased significantly from 25 June to 5 July, when a large number of flower buds entered initial differentiation. The content of GA₃ in flower buds increased in volatility from 25 July to 5 Nov.

From 25 June to 15 Aug., before the appearance of the stamen primordium, the content of GA₃ in flower buds was significantly lower than that during the same period of leaf buds and physiological differentiation stage. Furthermore, it was not significantly different than the GA₃ content in leaf buds and physiological differentiation stage, which indicated that the low GA₃ content was beneficial to early morphological differentiation (Fig. 1D).

Dynamic changes in the endogenous hormone ratio during bud differentiation

Ratio of ABA to IAA. The ratio of ABA to IAA was significantly higher on 5 May than on 15 May and 25 May. ABA/IAA in flower buds was significantly higher than that during the physiological differentiation stage (15 May–25 May) and in leaf buds during the same period from 25 June to 25 July. This suggested that a high ratio of ABA/IAA was conducive to early morphological differentiation (Fig. 2A).

Ratio of ABA to GA₃. The ratio of ABA to GA₃ was significantly higher on 5 May than on 15 May and 25 May, and ABA/GA₃ in flower buds was significantly higher than that in leaf buds during the whole morphological differentiation stage (except 15 Sept.). This implied that the high ratio of ABA/GA₃ was beneficial to flower bud morphological differentiation (Fig. 2B).

Ratio of ABA to ZR. The ratio of ABA to ZR was significantly higher on 25 May than on 5 May and 15 May. During the whole morphological differentiation stage, ABA/ZR in flower buds was significantly higher than that in leaf buds during the same period (except 5 Nov.) and during the physiological differentiation stage. The results implied that a high ratio of ABA/ZR was beneficial to flower bud morphological differentiation (Fig. 2C).

Ratio of ZR to IAA. The ratio of ZR to IAA decreased significantly from 5 May to 25 May. ZR/IAA in flower buds was significantly lower than that during the physiological differentiation stage. From 25 June to 5 July, when a large number of flower buds was

Fig. 1. (A) Zeatin riboside (ZR), (B) abscisic acid (ABA), (C) indole-3-acetic acid (IAA), and (D) gibberellin 3 (GA₃) contents during the physiological differentiation stage of spur terminal buds, spur leaf buds, and spur flower buds. The different letters indicate significant differences in development during the physiological differentiation stage of spur terminal buds, spur leaf buds, and spur flower buds according to Duncan's multiple range tests ($P < 0.05$). PDSSTB = physiological differentiation stage of spur terminal buds; SLB = spur leaf buds; SFB = spur flower buds.

Fig. 2. (A) Ratio of ABA to IAA, (B) ratio of ABA to GA₃, (C) ratio of ABA to ZR, and (D) ratio of ZR to IAA during the physiological differentiation stage of spur terminal buds, spur leaf buds, and spur flower buds. The different letters indicate significant differences in development during the physiological differentiation stage of spur terminal buds, spur leaf buds, and spur flower buds according to Duncan's multiple range tests ($P < 0.05$). PDSSTB = physiological differentiation stage of spur terminal buds; SLB = spur leaf buds; SFB = spur flower buds.

in the initial differentiation stage, ZR/IAA in flower buds was significantly higher than that in leaf buds during the same period. From 25 July to 15 Sept., ZR/IAA in flower buds was significantly lower than that in leaf buds during the same period. These results indicated that the high ratio of ZR/IAA might be beneficial to early morphological differentiation (Fig. 2D).

Discussion

Flower bud differentiation was a complex physiological process. Previous studies have shown that the increased content of IAA in spur buds promoted flower bud induction (Li et al., 1996). However, the low level of IAA was beneficial to flower formation (Cao et al., 2000; Wang et al., 1989). Our results showed that the content of IAA increased slowly during the physiological differentiation stage and that the content of IAA in flower buds was significantly lower than that in leaf buds before sepal primordium (5 July), thus supporting the view that the low IAA content was beneficial to flower induction. The content of IAA in flower buds was significantly higher than that in leaf buds after 25 July, indicating that the high level of IAA was conducive to flower organ primordium differentiation. Similar conclusions were obtained for Chinese Jujube (Niu et al., 2015) and Crabapple (Wu et al., 2013).

ABA is another important hormone that promotes flowering. The ABA content in flower buds of 'Redchief' apple increased significantly during the physiological differentiation stage of flower buds and remained at a higher level for a long time; however, the content in leaf buds was lower (Cao et al., 2000). In this study, the ABA content in leaf buds during the transformation stage was significantly lower than that in terminal buds during the late physiological differentiation stage; however, there was no difference between the flower buds during the transformation stage and the terminal buds during the late physiological differentiation stage. The content of ABA in flower buds was significantly higher than that in leaf buds during the same period during the whole morphological differentiation stage. This indicated that the high ABA content was beneficial to floral induction and morphological differentiation of apple flower buds. The content of ABA in the lateral buds of off-year olive trees was significantly higher than that of on-year trees during the critical period of flower bud induction, which further confirmed the effect of ABA on flower bud differentiation (Zhu et al., 2015).

The content of GA in the flower buds of 'Redchief' apple decreased sharply during physiological differentiation, but the change in GA in leaf buds was not significant. The content was higher than that in flower buds during different periods, which showed that

the high concentration of GA inhibited flower bud formation (Cao et al., 2000). Our results showed that the content of GA₃ in flower buds was significantly lower than that in leaf buds and in spur terminal buds during the physiological differentiation stage before the stamen primordium stage (25 Aug.), indicating that a high level of GA₃ inhibited flower bud differentiation. Exogenously spraying GA delayed the beginning of flower bud differentiation and reduced the percentage of flower buds (Bertelsen et al., 2002).

It has been recognized that cytokinin can promote flower formation (Qin et al., 2010; Su et al., 2007; Zeng et al., 2008). The results of this study showed that the content of ZR in the spur terminal buds of 'Tianhong 2' apple decreased during the physiological differentiation stage, and the same trend was found during the studies of 'Redchief' apple (Cao et al., 2000), 'Fuji' apple (Li et al., 2016), 'Starking' apple (Luo et al., 1987), and 'Ezhi 8' and 'Leccino' olive both on-year and off-year (Zhu et al., 2015), thereby showing that the reduction of ZR may be the signal for flower induction. The content of ZR in flower buds was significantly lower than that in leaf buds during the same period before the appearance of petal primordium (July 25), which indicated that the high ZR content may not be beneficial to flower initiation. In 12-year-old *Citrus satsuma* with a flowering rate of more than 90%, the content of ZR was lower than that of 2-year-old seedlings without flowering during the flower bud initiation (Zhang et al., 1990).

Flower bud differentiation in plants is a complex physiological and biochemical process. Endogenous hormones comprise one of the key factors; however, the amount of single hormones cannot fully explain the phenomenon. Therefore, the balance between hormones also has an important role in flower formation. In our studies, ABA/IAA decreased rapidly during the early stage of physiological differentiation; furthermore, the ABA/IAA ratio in the flower buds was higher than that in the leaf buds before the sepal primordium appeared, suggesting that the relatively high ratio of ABA/IAA was beneficial to early morphological differentiation. Cao et al. (2000) also proposed that a high ratio of ABA/IAA was beneficial to the flowering of 'Redchief' apples. Cao et al. (2000) also suggested that a high ratio of ABA/GA was beneficial to flowering. During the whole morphological differentiation stage, we also found that the ABA/GA₃ ratio in flower buds was higher than that in leaf buds during the same period and higher than that during the physiological differentiation stage, indicating that a high ratio ABA/GA₃ is beneficial to morphological differentiation of flower buds in 'Tianhong 2' apple. The conclusion that a high ratio of ABA/GA₃ was beneficial to flower bud formation was consistent with observations of *Phyllagathis fordii* (Cai et al., 2017) and *Chrysanthemum morifolium* (Feng and Yang, 2011). In 'Tianhong 2' apple, the ABA/ZR ratio in flower buds was higher than that in leaf buds during

the whole morphological differentiation stage; furthermore, low ABA/ZR ratio inhibited the transition from vegetative growth to reproductive growth in pear buds (Yang et al., 2015), which indicated that the high ratio of ABA/ZR was beneficial to flower bud morphological differentiation.

A high ratio of ZR/IAA is also beneficial to flower bud differentiation (Cai et al., 2017; Wang et al., 2013; Wu et al., 2013; Zhu et al., 2015). In this study, from the single content changes of ZR and IAA, ZR content was decreased during the physiological differentiation stage and the IAA content increased. The contents of the two hormones in the flower buds during the transformation stage and during initial differentiation were lower than those of the leaf buds (Fig. 1A and C). This indicated that the low contents of ZR and IAA were conducive to flower induction and early morphological differentiation. However, the ratio of ZR to IAA decreased during the physiological differentiation stage, and the ZR/IAA in flower buds was higher than that in leaf bud before the appearance of the flower organ primordium (Fig. 2D). This indicated that the high ratio of ZR/IAA was beneficial to early morphological differentiation. The results showed that the flower induction and early morphological differentiation of flower buds depend not only on the content changes of single-hormone ZR and IAA but also on the balance between the two hormones, which was consistent with the results of Cao et al. (2000).

Conclusions

Our observations indicated that the transformation stage of 'Tianhong 2' apple flower buds appeared 40 d after the spur stopped growing. The reduced ZR content was beneficial to floral induction. The low content of GA₃ and high ratios of ABA/IAA and ZR/IAA were conducive to early morphological differentiation. In addition, high ratios of ABA/GA₃ and ABA/ZR were beneficial to the morphological differentiation of flower buds. Moreover, the high ABA content was beneficial to floral induction and the morphological differentiation of flower buds.

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