Characteristics of Flower Bud Differentiation and Endogenous Hormone Changes in Single-Flower and Lateral-Flower Peony Cultivars (Post-Print)

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Abstract

To compare the flower bud differentiation processes, morphological characteristic differences, and endogenous hormone changes during the differentiation period among the single-flower peony cultivar 'Lihuafen' and the lateral-flower cultivars 'Zimeiyouchun' and 'Yun' efen', and to provide a reference basis for breeding peonies with extended flowering periods. This study observed the flower bud differentiation processes of 'Lihuafen', 'Zimeiyouchun', and 'Yun' efen' through anatomical and paraffin sectioning, and determined the variation patterns of auxin (IAA), abscisic acid (ABA), gibberellin (GA3), and zeatin riboside (ZR) contents at different differentiation stages in 'Lihuafen' and 'Zimeiyouchun' using enzyme-linked immunosorbent assay (ELISA). The results showed that: (1) The flower bud differentiation of 'Lihuafen' comprised only 6 stages; the lateral-flower cultivars 'Zimeiyouchun' and 'Yun' efen' underwent 11 stages of flower bud differentiation, with the terminal flower primordium differentiating first, followed by the lateral flower primordia. (2) From the leaf primordium differentiation stage to the bract primordium differentiation stage, the contents of ABA, GA3, and ZR in the flower buds of 'Zimeiyouchun' and 'Lihuafen' all increased, while IAA decreased; at the initial stage of lateral flower primordium differentiation in 'Zimeiyouchun', the contents of ABA and GA3 decreased, while ZR and IAA increased. (3) The ABA/IAA ratio in 'Lihuafen' was the lowest during the morphological differentiation period. These results indicate that lateral-flower cultivars initiate flower bud differentiation earlier than single-flower cultivars, with a longer differentiation duration and overlapping differentiation periods of some floral organs between terminal and lateral flowers. Higher levels of ZR and IAA, along with lower levels of ABA and GA3, are conducive to initiating lateral flower primordium differentiation.

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Flower Bud Differentiation and Endogenous Hormone Changes in Single and Lateral-Flowered Tree Peony (*Paeonia* Sect. *Moutan*) Cultivars

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Abstract

This study compared the flower bud differentiation process, morphological features, and endogenous hormone dynamics during differentiation in the single-flowered tree peony cultivar 'Lihua Fen' and the lateral-flowered cultivars 'Zimei Youchun' and 'Yun' e Fen', providing a reference for breeding long-flowering tree peony varieties. Flower bud differentiation was observed through anatomical dissection and paraffin sectioning, while enzyme-linked immunosorbent assay (ELISA) was used to determine changes in auxin (IAA), abscisic acid (ABA), gibberellin (GA_3), and zeatin riboside (ZR) contents at different differentiation stages in 'Lihua Fen' and 'Zimei Youchun'. The results showed: (1) 'Lihua Fen' underwent only six differentiation stages, whereas the lateral-flowered cultivars 'Zimei Youchun' and 'Yun' e Fen' completed 11 stages, with the apical flower primordium differentiating earlier than the lateral flower primordium. (2) From the leaf primordium differentiation stage to the bract primordium differentiation stage, ABA, GA_3, and ZR contents increased while IAA decreased in both 'Zimei Youchun' and 'Lihua Fen'; during the initial stage of lateral flower primordium differentiation in 'Zimei Youchun', ABA and GA_3 contents decreased while ZR and IAA increased. (3) The ABA/IAA ratio in 'Lihua Fen' was lowest during the morphological differentiation period. These findings demonstrate that lateral-flowered cultivars exhibit earlier and longer flower bud differentiation compared with single-flowered cultivars, with overlapping differentiation periods for some floral organs between apical and lateral flowers. Higher levels of ZR and IAA, combined with lower levels of ABA and GA_3, facilitate the initiation of lateral flower primordium differentiation.

Keywords: *Paeonia suffruticosa*, lateral-flowered cultivar, flower bud differentiation, morphology, endogenous hormones

Introduction

Tree peony (*Paeonia* Sect. *Moutan*), belonging to the family Paeoniaceae and genus *Paeonia*, is celebrated as the "king of flowers" for its magnificent blooms and rich colors, yet suffers from an extremely short flowering period—often described as "ten days of bloom after a year of cultivation." Traditional cultivars produce only one flower per annual shoot (single-flower type), with individual plants flowering for merely 5–7 days (extending to 10 days under low temperature conditions) (Liu, 2010; Wang, 2011). However, within the Zhongyuan cultivar group, a few varieties such as 'Zimei Youchun' and 'Zimei Xichun' develop one terminal bud and 1–2 lateral buds within each mixed bud upon completion of differentiation. In spring, these produce one terminal flower and 1–2 sequentially opening lateral flowers on each annual shoot, resulting in greater flower numbers per plant and extended flowering periods exceeding two weeks. These are referred to as lateral-flowered tree peony cultivars, representing a promising pathway for addressing the limitation of short flowering duration in peony breeding.

Flower bud differentiation constitutes a critical developmental phase in ornamental plants, encompassing both morphological and physiological-biochemical processes (Li et al., 2011). This process has been extensively studied across numerous species, including the endangered *Camellia changii* (Zhu et al., 2011), *Lilium formolongi* (Liu et al., 2012), and *Barnardia japonica* (Chen et al., 2020). Among the various internal and external factors influencing flower bud differentiation, endogenous hormones play a pivotal role. Research indicates that auxin (IAA) and gibberellin (GA) often exhibit interactive relationships, with IAA promoting flowering by regulating GA content (Zou et al., 2020), while appropriate levels of IAA and GA_3 facilitate flower bud differentiation in *Ricinus communis* (Chen et al., 2011). Abscisic acid (ABA) demonstrates dual functions in either promoting or inhibiting flowering (Bai et al., 2002), whereas zeatin riboside (ZR) is considered a flowering-promoting hormone that antagonizes GA by impeding GA production and promoting its degradation, while GA simultaneously inhibits ZR response (Li et al., 2019).

Flower bud differentiation has long been a key research focus in *Paeonia* species, though existing studies have exclusively examined single-flower terminal types, covering morphological aspects, differentiation stages, and nutrient dynamics during the process (Yang and Zhang, 1986; Mo et al., 2008; Huang et al., 2009; Lü et al., 2009; Zhang et al., 2019). To date, no investigations have reported on flower bud differentiation and endogenous hormone profiles in lateral-flowered cultivars. As lateral-flowered varieties offer a viable approach for extending the flowering period in tree peony breeding, this study comparatively analyzed the morphological characteristics at each differentiation stage between single-flower and lateral-flowered cultivars, clarified the timing and location of lateral bud initiation, and elucidated the differentiation patterns and features of lateral-flowered cultivars. By determining the dynamic changes in endogenous hormones during differentiation in both cultivar types, we explored the relationship between hormonal profiles and lateral flower bud differentiation, providing a theoretical foundation for field management strategies aimed at prolonging flowering duration and increasing flower numbers.

Materials and Methods

1.1 Materials and Sampling Sites

The experimental materials included two lateral-flowered cultivars, 'Zimei Youchun' and 'Yun' e Fen' , with the single-flower cultivar 'Lihua Fen' serving as the control (Table 1 , Plate I). Both 'Zimei Youchun' and 'Lihua Fen' belong to the Zhongyuan cultivar group and were selected from seedlings at the Zhaolou Peony Garden in Heze, Shandong Province, in 1967 and 1982, respectively. 'Zimei Youchun' consistently produces one lateral flower (occasionally two) in addition to the terminal flower on each flowering branch, with stable flower color and form, and is commonly observed in peony gardens in both Luoyang and Heze. 'Yun' e Fen' , bred by the Gansu Provincial Forestry Science and Technology Extension Station, is a hybrid offspring of *Paeonia qiui* (male parent) and *P. rockii* (female parent) with new cultivar rights number 20170139. Temperature variations at the sampling sites are detailed in Figure 1 [Figure 1: see original paper], and phenological stages were recorded for each material.

Table 1 Main ornamental characteristics and sampling sites of experimental materials

Cultivar	Flowering habit	Flower type and color	Sampling site
'Zimei Youchun'	Terminal + lateral flowers	Rose type, pink	Luoyang Sui & Tang Dynasties Relics Botanic Garden (two cultivars planted adjacent)
'Lihua Fen'	Single flower	Rose type, pink-yellowish	Same as above
'Yun' e Fen'	Terminal + lateral flowers	Lotus type, pink	Peony Breeding Nursery, Gansu Provincial Forestry Research Institution, Lanzhou

Plate I Tested materials. A and B respectively show lateral-flowered cultivars 'Zimei Youchun' and 'Yun' e Fen' (red arrows indicate lateral flowers); C shows single-flowered cultivar 'Lihua Fen'.

Figure 1 Temperature from June to November 2019 at sampling sites. A: Luolong District, Luoyang City, Henan Province; B: Chengguan District, Lanzhou City, Gansu Province.

From June to November 2019, at 7-10 day intervals between 9:00-11:00 AM, 15-20 well-developed plump buds were collected from the leaf axils of flowering shoots on healthy plants over five years old for each of the three cultivars. Among these, 7-10 buds were dissected to remove outer bud scales, immediately fixed in FAA solution (formalin:glacial acetic acid:70% ethanol = 5:5:90 by volume), and stored at 4 °C for paraffin sectioning and stereomicroscopic observation. Both 'Lihua Fen' and 'Zimei Youchun' underwent paraffin sectioning and stereomicroscopic observation, while 'Yun' e Fen' was observed only under stereomicroscope. The remaining buds from 'Lihua Fen' and 'Zimei Youchun' were washed with distilled water, dried, wrapped in aluminum foil, rapidly frozen in liquid nitrogen for 2-5 minutes, and stored at -80 °C for endogenous hormone determination.

1.3 Observation of Flower Bud Differentiation Process

Paraffin sectioning followed the methods of Zeng et al. (1989) and Wang (2017) with minor modifications. Samples were sectioned at 8-10 m thickness using a Leica RM2235 microtome, stained with safranin-fast green double staining, and observed and photographed under an SDPTOP CX40-RFL optical biological microscope. Simultaneously, following Zhang et al. (2019), samples fixed in FAA were sequentially soaked in absolute ethanol and distilled water for 30 minutes each, then naturally dehydrated on absorbent paper at room temperature for 10-15 minutes. Under a Leica DFC500 stereomicroscope, buds were dissected layer by layer until the apical meristem was exposed, then photographed and recorded. The progression of flower bud differentiation and floral organ development was monitored in conjunction with phenological observations.

1.4 Endogenous Hormone Content Determination

Endogenous hormone contents (ABA, GA_3, ZR, and IAA) were determined by enzyme-linked immunosorbent assay (ELISA) (Zhao et al., 2006) with minor modifications, using three biological replicates per sample.

1.5 Data Analysis and Image Processing

Data were statistically analyzed and charts prepared using Excel 2010 and SPSS Statistics 23.0 software. Images were processed using Adobe Photoshop 2020 software.

Results

2.1.1 Single-Flowered Cultivar 'Lihua Fen'

The flower bud differentiation process comprised six distinct stages: leaf primordium differentiation stage (Le), bract primordium differentiation stage (Br), sepal primordium differentiation stage (Se), petal primordium differentiation stage (Pe), stamen primordium differentiation stage (St), and pistil primordium differentiation stage (Pi), spanning 88 days. The corresponding dates and morphological characteristics for each stage are shown in Plate II and Figure 2 [Figure 2: see original paper].

From early to mid-July, 'Lihua Fen' entered the initial differentiation stage, with the apical growing point protruding upward to differentiate into leaf primordia Le (Plate II: A1, A2). On July 18, the cultivar progressed to the bract primordium differentiation stage, where the apical growing point began to flatten and widen transversely, increasing its surface area with a relatively concave central region, while peripheral cells gradually differentiated to form 凸起 bract primordia Br (Plate II: B1, B2), marking the initiation of flower bud differentiation. By July 28, 'Lihua Fen' entered the sepal primordium differentiation stage, with the apical growing point continuing to invaginate into a shallow disc shape, forming sepal primordia Se inside the bract primordia (Plate II: C1, C2). Following sepal primordium differentiation, a new round of protrusions gradually emerged at the inner edge of the floral disc, forming petal primordia Pe (Plate II: D1, D2). On September 23, 'Lihua Fen' entered the stamen primordium differentiation stage, with the bud enlarging overall and the apical growing point becoming a deep, wide cup shape. As petal primordia continued to develop, numerous new granular protrusions emerged inside them, differentiating into stamen primordia St (Plate II: E1, E2). By October 13, with the differentiation and growth of stamen primordia, the apical growing point further invaginated, the floral disc became prominent, and large protrusions formed at its base, representing pistil primordia Pi (Plate II: F1, F2). At this point, flower bud differentiation in 'Lihua Fen' was essentially complete.

Plate II Morphological observation of flower bud differentiation in *Paeonia suffruticosa* 'Lihua Fen' . A1-F1: Stereomicroscopic observation; A2-F2: Paraffin sections under optical biological microscopy. A1, A2: Leaf primordium differentiation stage; B1, B2: Bract primordium differentiation stage; C1, C2: Sepal primordium differentiation stage; D1, D2: Petal primordium differentiation stage; E1, E2: Stamen primordium differentiation stage; F1, F2: Pistil primordium differentiation stage. Le: Leaf primordium; Br: Bract primordium; Se: Sepal primordium; Pe: Petal primordium; St: Stamen primordium; Pi: Pistil primordium. The same abbreviations apply below.

2.1.2 Lateral-Flowered Cultivar 'Zimei Youchun'

Flower bud differentiation initiated on July 10 and concluded on November 5, lasting 118 days. The entire process comprised 11 stages: leaf primordium differentiation stage (Le), top flower bract primordium differentiation stage (TBr), top flower sepal primordium differentiation stage (TSe), top flower petal primordium differentiation stage (TPe), top flower stamen primordium differentiation stage (TSt), top flower pistil primordium differentiation stage (TPi), lateral flower bract primordium differentiation stage (LBr), lateral flower sepal primordium differentiation stage (LSe), lateral flower petal primordium differentiation stage (LPe), lateral flower stamen primordium differentiation stage (LSt), and lateral flower pistil primordium differentiation stage (LPi) (Plate III). Notably, TPe, TSt, and TPi overlapped with LBr, while TPi coincided with LSe (Figure 2 [Figure 2: see original paper]).

On July 10, 'Zimei Youchun' entered the top flower bract primordium differentiation stage (Plate III: b1, b2). By July 28, it progressed to the top flower sepal primordium differentiation stage (Plate III: c1, c2). On August 12, the cultivar entered the top flower petal primordium differentiation stage (Plate III: d1, d2). Simultaneously, lateral flower primordia began differentiating at the axils of the third or fourth leaf primordia from the base of the bud, entering the bract primordium differentiation stage (Plate III: B). On September 13, 'Zimei Youchun' entered the top flower stamen primordium differentiation stage (Plate III: e1, e2), while lateral flower bract primordia continued differentiating. By September 23, it entered the top flower pistil primordium differentiation stage (Plate III: f1, f2), essentially completing top flower differentiation, at which point lateral flower bract primordia were still developing. On October 1, after an extended period of bract primordium differentiation, lateral flowers entered the sepal primordium differentiation stage (Plate III: C). Subsequently, on October 10 and October 20, lateral flowers progressed through petal primordium differentiation (Plate III: D) and stamen primordium differentiation stages (Plate III: E), respectively. Finally, on November 5, lateral flowers entered the pistil primordium differentiation stage (Plate III: F), completing the entire differentiation process.

Plate III Morphological observation of flower bud differentiation in *Paeonia suffruticosa* 'Zimei Youchun' . a1–f1: Stereomicroscopic observation; a2–f2: Paraffin sections under optical biological microscopy; A–F: Stereomicroscopic observation. a1, a2: Leaf primordium differentiation stage; b1, b2: Top flower bract primordium differentiation stage; c1, c2: Top flower sepal primordium differentiation stage; d1, d2: Top flower petal primordium differentiation stage; e1, e2: Top flower stamen primordium differentiation stage; f1, f2: Top flower pistil primordium differentiation stage; A: Lateral flower leaf primordium differentiation stage; B: Lateral flower bract primordium differentiation stage; C: Lateral flower sepal primordium differentiation stage; D: Lateral flower petal primordium differentiation stage; E: Lateral flower stamen primordium differentiation stage; F: Lateral flower pistil primordium differentiation stage. Le: Leaf primordium; TBr: Top flower bract primordium; TSe: Top flower sepal primordium; TPe: Top flower petal primordium; TSt: Top flower stamen primordium; TPi: Top flower pistil primordium; LBr: Lateral flower bract primordium; LSe: Lateral flower sepal primordium; LPe: Lateral flower petal primordium; LSt: Lateral flower stamen primordium; LPi: Lateral flower pistil primordium. The same abbreviations apply below.

2.1.3 Lateral-Flowered Cultivar 'Yun' e Fen'

Flower bud differentiation began on July 4 and ended on November 1, lasting 120 days. The process followed the same 11-stage pattern as 'Zimei Youchun' . However, lateral flower differentiation in 'Yun' e Fen' commenced 45 days after top flower initiation, approximately 15 days later than in 'Zimei Youchun' . Morphological characteristics at each differentiation stage are shown in Plate IV. Stage initiation times are presented in Figure 2 [Figure 2: see original paper], where TPe and TSt overlapped with LBr, and TPi coincided with both LSe and LPe.

On July 4, 'Yun' e Fen' entered the top flower bract primordium differentiation stage (Plate IV: B1). By July 14, it progressed to the top flower sepal primordium differentiation stage (Plate IV: C1). On August 2, the cultivar entered the top flower petal primordium differentiation stage (Plate IV: D1). Lateral flower bract primordium differentiation began on August 28 (Plate IV: A2). On September 10, 'Yun' e Fen' entered the top flower stamen primordium differentiation stage (Plate IV: E1). By September 29, it had entered the top flower pistil primordium differentiation stage (Plate IV: F1), essentially completing top flower differentiation. Concurrently, lateral flowers progressed to the sepal primordium differentiation stage after prolonged bract primordium development (Plate IV: B2). Subsequently, on October 13 and October 23, lateral flowers entered petal primordium differentiation (Plate IV: C2, D2) and stamen primordium differentiation stages (Plate IV: E2), respectively. Finally, on November 1, lateral flowers entered the pistil primordium differentiation stage (Plate IV: F2), completing the differentiation process.

Plate IV Morphological observation of flower bud differentiation in *Paeonia suffruticosa* 'Yun' e Fen' (stereomicroscopic observation). A1: Leaf primordium differentiation stage; B1: Top flower bract primordium differentiation stage; C1: Top flower sepal primordium differentiation stage; D1: Top flower petal primordium differentiation stage; E1: Top flower stamen primordium differentiation stage; F1: Top flower pistil primordium differentiation stage; A2: Lateral flower bract primordium differentiation stage; B2: Lateral flower sepal primordium differentiation stage; C2, D2: Lateral flower petal primordium differentiation stage; E2: Lateral flower stamen primordium differentiation stage; F2: Lateral flower pistil primordium differentiation stage.

Figure 2 Initiation times of flower bud differentiation stages in 'Lihua Fen' , 'Zimei Youchun' , and 'Yun' e Fen' . In 'Zimei Youchun' , TPe, TSt, and TPi coincide with LBr, while TPi coincides with LSe. In 'Yun' e Fen' , TPe and TSt coincide with LBr, and TPi coincides with both LSe and LPe.

2.2 Changes in Endogenous Hormones During Flower Bud Differentiation

Changes in endogenous hormone contents during flower bud differentiation in the single-flowered cultivar 'Lihua Fen' are shown in Figure 3A [Figure 3: see original paper]. ABA, GA_3, and ZR contents increased from the Le to Br stages, reaching maximum levels for ABA and ZR at the Br stage, while IAA content decreased. From Br to St stages, ABA and ZR contents gradually declined. GA_3 content showed no significant change from Br to Pe stages but decreased gradually from Pe to Pi stages. GA_3 maintained high levels during bract, sepal, and petal primordium differentiation stages but low levels during stamen and pistil primordium differentiation. IAA content showed no significant change from Br to Se stages but decreased continuously after entering the petal primordium differentiation stage, suggesting that lower IAA levels may be required for floral organ primordium differentiation.

Similar to 'Lihua Fen', the lateral-flowered cultivar 'Zimei Youchun' exhibited distinct hormone content variations during differentiation (Figure 3B [Figure 3: see original paper]). From Le to TBr stages, ABA, GA_3, and ZR contents increased while IAA decreased, indicating that higher ABA, GA_3, and ZR levels combined with lower IAA levels may facilitate flower bud differentiation initiation. During the initial stage of lateral flower primordium differentiation (TSe to TPi/LBr stages), ABA and GA_3 contents decreased significantly, while ZR and IAA showed initial increases followed by decreases, suggesting that lower ABA and GA_3 levels together with higher ZR and IAA levels may promote lateral flower primordium initiation.

Figure 3 Endogenous hormone contents during flower bud differentiation in *Paeonia suffruticosa* 'Lihua Fen' (A) and 'Zimei Youchun' (B). In panel A, Le, Br, Se, Pe, St, and Pi represent leaf primordium, bract primordium, sepal primordium, petal primordium, stamen primordium, and pistil primordium of 'Lihua Fen', respectively. In panel B, Le, TBr, TSe, TPe, TSt, TPi, LBr, LSe, LPe, LSt, and LPi represent leaf primordium, top flower bract primordium, top flower sepal primordium, top flower petal primordium, top flower stamen primordium, top flower pistil primordium, lateral flower bract primordium, lateral flower sepal primordium, lateral flower petal primordium, lateral flower stamen primordium, and lateral flower pistil primordium of 'Zimei Youchun', respectively. TPe/LBr indicates coincidence of TPe and LBr; TSt/LBr indicates coincidence of TSt and LBr; TPi/LBr indicates coincidence of TPi and LBr; TPi/LSe indicates coincidence of TPi and LSe; TPi/LPe indicates coincidence of TPi and LPe. Different lowercase letters indicate significant differences for the same hormone across different stages. The same convention applies below.

2.3 Hormone Balance and Flower Bud Differentiation

Analysis of endogenous hormone balance relationships (Figure 4 [Figure 4: see original paper]) revealed similar changing trends in IAA/GA_3 and

(IAA+ZR)/GA_3 ratios between 'Lihua Fen' and 'Zimei Youchun' . In 'Lihua Fen' , both ratios decreased gradually, reaching maximum values of 8.487 and 7.099, respectively, at the Le stage, then declining progressively. In 'Zimei Youchun' , both ratios were relatively high during the leaf primordium differentiation stage, decreased at the TBr stage, increased significantly during LBr formation, and peaked at 13.678 and 11.194, respectively, during the TSt/LBr stage.

The ABA/IAA ratio in 'Lihua Fen' was lowest at the Le stage (0.865), increased at the Br stage, then decreased gradually from Se to St stages before rising significantly at the Pi stage to reach its maximum (1.537). In 'Zimei Youchun' , the ABA/IAA ratio increased significantly from Le to TSe stages, peaking at the TSe stage (2.305), then decreased gradually during LBr formation, reaching its minimum (0.780) at the TPi/LBr stage before increasing again.

The ZR/GA_3 ratio in 'Lihua Fen' showed an initial increase followed by a decrease, rising from Le to Br stages then declining gradually. Conversely, the ZR/GA_3 ratio in 'Zimei Youchun' decreased significantly from Le to Br stages, then increased gradually, peaking at the TSt/LBr stage before declining.

Figure 4 Changes in endogenous hormone ratios during flower bud differentiation in *Paeonia suffruticosa* 'Lihua Fen' (A) and 'Zimei Youchun' (B).

Discussion

3.1 Characteristics of Flower Bud Differentiation in Single and Lateral-Flowered Peony Cultivars

Although different peony cultivars exhibit variations in differentiation timing and progression, all follow the sequential order of leaf primordium, bract primordium, sepal primordium, petal primordium, stamen primordium, and pistil primordium differentiation. The emergence of bract primordia is generally considered the marker for initiation of peony flower bud differentiation, signaling the transition from vegetative to reproductive growth (Wang, 1986). In this study, the single-flowered cultivar 'Lihua Fen' initiated differentiation in mid-July over a 90-day period, consistent with previous reports (Wang, 1986; Wang and Zhang, 1987; He et al., 2014). The lateral-flowered cultivars 'Zimei Youchun' and 'Yun' e Fen' completed 11 differentiation stages, with the apical flower primordium differentiating prior to the lateral flower primordium. Lateral flower bract primordium differentiation occurred synchronously with apical flower differentiation, and after apical flower differentiation was essentially complete, lateral flowers rapidly completed differentiation of sepal, stamen, and pistil primordia. Each cultivar' s progression conformed to the general pattern of peony flower bud differentiation.

Compared with 'Lihua Fen' , the lateral-flowered cultivars 'Zimei Youchun' and 'Yun' e Fen' began differentiation earlier (on July 18, July 10, and July 4, respectively) and required longer periods (88 days for 'Lihua Fen', 118 days for 'Zimei Youchun', and 120 days for 'Yun'e Fen') due to lateral flower primordium development. 'Yun' e Fen', as a distant hybrid offspring of *Paeonia delavayi* and *P. qiui*, inherits its lateral flowers from the maternal parent, whereas 'Zimei Youchun' originated from natural hybridization within the Zhongyuan cultivar group. The mechanisms underlying lateral flower development in these cultivars warrant further investigation.

3.2 Relationships Between Endogenous Hormones and Peony Flower Bud Differentiation

3.2.1 ABA and Peony Flower Bud Differentiation During the initiation phase of flower bud differentiation (leaf primordium to bract primordium stages), ABA content increased in both 'Zimei Youchun' and 'Lihua Fen', showing an accumulation trend similar to findings in autumn chrysanthemum 'Jinba' (Feng and Yang, 2011). This suggests that ABA may be transported from leaves to flower buds and accumulate there, reaching maximum content at the Br stage. This accumulation appears beneficial for initiating peony flower bud differentiation, as exogenous ABA application increased flower bud differentiation rate in vanilla (*Vanilla planifolia*) (Wang et al., 2016), and high ABA concentrations in scales facilitated differentiation in *Lycoris aurea* (Zhang et al., 2019). Conversely, during lateral flower primordium initiation in 'Zimei Youchun' (the initial stage of lateral flower bract primordium differentiation), ABA showed a decreasing trend, indicating that lower ABA levels may promote lateral flower primordium differentiation in peony.

3.2.2 ZR and Peony Flower Bud Differentiation In some plants, ZR content increases before flower bud differentiation and decreases afterward, as observed in litchi (*Litchi chinensis*) (Chen, 2007). However, other species show low initial ZR content that increases substantially and stabilizes at high levels during later differentiation stages, including fig (*Ficus carica*) (Luo et al., 2007), saffron crocus (*Crocus sativus*) (Zhang et al., 2018), and licorice (*Glycyrrhiza uralensis*) (Yan et al., 2019). Additionally, low ZR concentrations in scales benefited differentiation in *Lycoris chinensis* (Fan et al., 2021), while high ZR levels in shoot apices facilitated floral organ primordium morphogenesis in daylily (*Hemerocallis*) (Li et al., 2021).

In this study, ZR content increased from leaf primordium to bract primordium stages in both 'Zimei Youchun' and 'Lihua Fen', consistent with findings in fig, saffron crocus, and licorice, suggesting that high ZR content in flower buds promotes initiation and development of peony flower bud differentiation. During lateral flower bract primordium initiation in 'Zimei Youchun', ZR content increased, suggesting that higher ZR levels may facilitate lateral flower primordium differentiation in peony.

3.2.3 GA_3 and Peony Flower Bud Differentiation During flower bud differentiation initiation in autumn chrysanthemum 'Jinba', endogenous GA_3 content increased in leaves while showing opposite changes in apical buds (Feng and Yang, 2011). In this study, GA_3 content increased in flower buds of both 'Zimei Youchun'and 'Lihua Fen'from leaf primordium to bract primordium stages. Research has shown that exogenous GA_3 promotes floral development in autumn chrysanthemum 'Jinba' under short-day conditions (Yang et al., 2008). We hypothesize that during the physiological differentiation stage of peony flower buds, GA_3 is transported from leaves to buds, accumulating and promoting differentiation. During lateral flower primordium initiation in 'Zimei Youchun', GA_3 content decreased, suggesting that lower GA_3 levels may facilitate lateral flower primordium differentiation in peony.

3.2.4 IAA and Peony Flower Bud Differentiation During flower bud differentiation initiation, IAA content decreased in both 'Zimei Youchun' and 'Lihua Fen'. Research indicates that low IAA levels in flower buds facilitate differentiation in saffron crocus (Zhang et al., 2018), suggesting that reduced IAA content promotes peony flower bud differentiation initiation. We hypothesize that during early peony flower bud differentiation, apical buds require lower IAA levels, with IAA transported from buds to leaves, resulting in decreased bud IAA content. During lateral flower primordium initiation in 'Zimei Youchun', IAA showed an increasing trend, indicating that higher IAA levels may promote lateral flower primordium differentiation in peony.

3.2.5 Hormone Balance and Flower Bud Differentiation Plant flower bud differentiation is regulated not only by individual hormones but also by hormonal balance interactions. Higher ABA/GA ratios facilitate morphological differentiation in *Lycoris radiata* (Wang et al., 2008), *Siraitia grosvenorii* (Mo et al., 2015), and hybrid plum-apricot *Prunus domestica* × *armeniaca* 'Fengweimeigui' (Mo et al., 2015). Elevated ABA/IAA ratios benefit differentiation and morphogenesis in saffron crocus, 'Liguang' apricot (*P. armeniaca* var. *glabra*), chrysanthemum, and jujube (Feng and Yang, 2011; Abdul et al., 2021; Zhang et al., 2018; Zhao et al., 2020). Lower IAA/GA_3 ratios favor flower bud formation in jujube (Niu et al., 2015), while higher ZR/GA_3 and (IAA+ZR)/GA_3 ratios promote floral organ primordium development in daylily (Li et al., 2021).

In this study, from leaf primordium to bract primordium stages, both 'Zimei Youchun' (top flower bract primordium stage) and 'Lihua Fen' showed decreased IAA/GA_3, (IAA+ZR)/GA_3, and ZR/GA_3 ratios, along with increased ABA/IAA and ABA/GA_3 ratios. This suggests that lower IAA/GA_3, (IAA+ZR)/GA_3, and ZR/GA_3 ratios combined with higher ABA/IAA and ABA/GA_3 ratios facilitate the transition from vegetative to reproductive growth in peony, consistent with previous research. During lateral flower primordium initiation, 'Zimei Youchun' exhibited increased IAA/GA_3, (IAA+ZR)/GA_3, and ZR/GA_3 ratios, peaking during lateral flower bract primordium formation, while ABA/IAA and ABA/GA_3 ratios decreased gradually, reaching minima at the final stage of lateral flower bract primordium formation before increasing. This indicates that higher IAA/GA_3, (IAA+ZR)/GA_3, and ZR/GA_3 ratios combined with lower ABA/IAA and ABA/GA_3 ratios promote lateral flower primordium differentiation in peony.

3.3 Lateral-Flowered Peony Cultivars and Long-Flowering-Period Breeding

Within China' s Zhongyuan peony group, few stable lateral-flowered cultivars exist. Beyond 'Zimei Youchun' and 'Zimei Xichun' , the new cultivar 'Huanju Yitang' (registration number MD 2021066), bred using 'Zimei Youchun' as a parent, also produces 1–2 lateral flowers on annual shoots. However, lateral flower numbers in cultivars such as 'Taohua Feixue' , 'Zimei Shuangjiao' (registration number MD 2021055), and 'Zimei Tanchun' (registration number MD 2021056) require further observation. Recently, some domestic distant hybridization cultivars also exhibit stable lateral flower characteristics similar to 'Zimei Youchun' , producing at least one lateral flower bud. Examples include 'Huaxia Meiguihong' (purple-red flowers, new cultivar rights number 20090005, parentage *Paeonia delavayi* × *P. rockii*) and 'Xiao Xiangfei' (orange flowers, new cultivar rights number 20140077, parentage *P. lutea* × *P. suffruticosa* 'Cengzhong Xiao') developed by Wang Lianying' s team at Beijing Forestry University, with individual plant flowering periods reaching 20 days and 15 days, respectively. Additionally, 'Yun' e Fen' (powder-blue flowers, new cultivar rights number 20170139, parentage *P. rockii* × *P. qiui*), 'Chenyun' (orange flowers, new cultivar rights number 20150066, parentage *P. delavayi* × *P. rockii*), and 'Cailian' (orange-red bicolor flowers, new cultivar rights number 20150067, same parentage as 'Chenyun'), all developed by the Gansu Provincial Forestry Science and Technology Extension Station, exhibit individual plant flowering periods of up to 20 days (Wang and Yuan, 2015). Unlike seedling-selected Zhongyuan cultivars with unknown parentage, these new cultivars all have maternal parents belonging to the fleshy disc wild species subgroup that produce 1–2 or more lateral flowers with extended flowering periods (averaging over 15 days). Although 'Zimei Youchun' is a Zhongyuan cultivar, its lateral flower development mechanism may be similar to that of distant hybrid offspring like 'Yun' e Fen' derived from fleshy disc wild species. This provides a starting point for investigating the molecular mechanisms underlying lateral flower development and offers fundamental information for peony systematic studies. Natural hybridization breeding is far less efficient than directed artificial hybridization; using peony species and cultivars with lateral flowers as parents can facilitate the development of new varieties with multiple lateral flowers and extended flowering periods, helping to alleviate the problem of short, concentrated flowering periods.

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