

Effect and Functional Mechanism of the Action of Exogenous Gibberellin on Flowering of Peach

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Abstract

This study was conducted to assess the effect of gibberellin and its possible mechanism of action on peach flower formation. At flower induction, 100 mg L⁻¹ of gibberellic acid 3 (GA₃) was sprayed on the leaves of peach [*Prunus persica* (L.) Batsch.] cv. Bayuecui. Using anatomy, immunohistochemistry, and semi-quantitation, the *in situ* distribution of GAs and the expression of the key genes involved in peach flower formation in the apical meristem were studied during flowering differentiation. The results showed that induction of flowering in the Bayuecui peach occurred prior to 10 July in Beijing, China. Flower induction and further differentiation of the peach flower organs were significantly inhibited by leaf-spraying of GA₃ at a concentration of 100 mg L⁻¹ during the induction stage. The flowering rate was only 11.67% after treatment. The distribution of GA₁ in the apical meristem varied during the process of flower bud differentiation. From 13 June to 25 July, the GA₁ signal from control plants was detected mainly in the vascular bundles at the base of the flower buds. No GA₁ signal was detected in the apical meristem. After treatment with GA₃, the distribution was similar to that of the control from 13 June to 3 July. On 13 July, a GA₁ signal was detected in the apical meristem accompanied by an increase in the GA₁ signal in the vascular bundles at the base of the flower buds. The GA₁ signal weakened significantly in both the vascular bundles and the apical meristem on 25 July. The expression of the genes *PpLEAFY* and *MADS6* in flower buds could be detected only on 10 October in the GA₃-treated plants. The critical period for flower induction of Bayuecui peach in Beijing was in early July, during which time, leaf-spraying with 100 mg L⁻¹ GA₃ could effectively inhibit flower induction and further differentiation of the flower buds. GA₁ in the gibberellin family was the suppressor for flower induction in peach. Its action was affected by the stage of flower bud differentiation. Expression of the key genes *PpLEAFY* and *MADS6* involved in flower formation was inhibited by GA₃ treatment.

Key words: peach [*Prunus persica* (L.) Batsch.], gibberellin, flowering induction, anatomy, immunohistochemistry, semi-quantitation RT-PCR

INTRODUCTION

Flower bud differentiation of fruit trees is closely related to agricultural production and is the primary factor affecting yield and quality. In addition to environmental conditions, gibberellins (GAs) are the most important effectors of flowering in higher plants and serve

as an effective method of artificial control of plant flowering. The study of the effects of GAs on flowering plants has become a focus of research. In recent years, understanding of the functional mechanism of GAs in flowering of woody fruit trees has been greatly enhanced owing to widespread use of immunological and molecular technologies. We used these technologies as well as anatomy, immunohistochemistry, and

semi-quantitation to detect morphological changes, *in situ* distribution of GAs in plant tissues, and the expression patterns of the key flowering genes *PpLEAFY* and *MADS6* in shoot apical meristems (SAMs) during peach flower bud differentiation.

Luckwill (1970) first proposed that flowering is regulated by endogenous plant hormones. This report investigated how the balance between GA/cytokinin (CTK) or CTK/GA affected flower bud differentiation. The formation of flower buds in the short shoots of apple was controlled by the flowering initiation hormone CTK from leaves and the flowering suppression hormone GA from seeds. This hypothesis was confirmed by other researchers working in other plant systems (Su *et al.* 1997; Koshita *et al.* 1999; Cao *et al.* 2000, 2001). The results of subsequent studies showed that the effects on the flowering of spraying GA₃ during flower induction differed, depending upon the particular species. The flowering of some long-day herbaceous plants was promoted after GA₃ spraying during the flower-induction period while the plants were still in non-induction conditions (Metzger 1987). The concentration of GAs that promoted flowering increased rapidly, accompanied by the upregulated expression of the key GA response genes related to flowering (Gocal *et al.* 1999, 2001; King *et al.* 2001, 2003; Eriksson *et al.* 2006). For woody fruit trees, flower bud differentiation was significantly inhibited when sprayed with GA₃ during the flower-induction period (McArthey and Li 1998; Zhou *et al.* 1981; Cao *et al.* 2001); however, the types and distribution of GAs as well as the expression patterns of the key flowering genes were not measured in these studies.

The impact of GAs on flowering has been extensively studied, but most of the research has concentrated on model herbaceous plants. Studies on the effects of GAs on fruit trees have remained at the level of exogenous spraying and over all organ detection, and although a great deal of basic information has been accumulated regarding the function of GAs, the functional mechanisms have not been clarified. Here we report the possible mechanisms of action of GA on peach flowering and provide a theoretical basis for the artificial control of flowering in fruit trees by observing the morphological changes, the types and distribution of the endogenous GAs, and the expression patterns of

key flowering genes in the SAM using anatomy, immunolocalization, and semi-quantitation.

MATERIALS AND METHODS

Collection and treatment of materials

The experiments were carried out in the orchard of the PLA (People's Liberation Army) (Beijing, China) in 2004 and 2005. The materials tested were robust 8-year-old specimens of the peach cultivar Bayuecui. In 2004, the trees were sprayed with 100 mg L⁻¹ GA₃ at 10-d intervals from 20 May to 21 June, and the controls were sprayed with the same volume of distilled water for a total of four times. The treatments were repeated three times. Short shoots were cut, packed in ice, brought to the laboratory, and used to gather statistics on the flower bud differentiation procedure, scanning electron microscopic observations, and semi-quantitation of gene activity during the time period from 30 May to 10 July at 5-d intervals and from 10 July to 10 October at 10-d intervals. In 2005, the treatments were applied between 3 June and 3 July for a total of four times, and samples were collected for immunolocalization from 8 June to 25 July at 5-d intervals using the same methods as in 2004.

Observation of flower bud anatomy

The axillary buds borne on the shoots were cut for paraffin sectioning and scanning electron microscopy (SEM) preparation according to Yang (2006) and Mi (1999). The images were photographed using an OLYMPUS BH-2 microscope and a HITACHI S-570 SEM, respectively. The flower bud differentiation process was statistically validated using 30 randomly selected buds.

Immunohistolocalization experiment

The materials were treated as described above. Paraffin section preparation and staining followed the methods of Hou and Huang (2005) and Hasegawa *et al.* (1995), respectively. The sections were dewaxed,

rehydrated, and incubated in blocking solution (PBS buffer with 1% BSA) for 1 h at 4°C followed by incubation with the diluted primary antibody GA₁-Me (diluted in an isopyknic mixture of blocking solution and PBST) at 4°C. The sections were briefly rinsed with PBST buffer (PBS buffer with 0.1% Tween-20) three times at 10-min intervals. After a final rinse with PBST buffer, bound antibodies were incubated with goat anti-rabbit alkaline phosphatase labeled immunoglobulin G second antibody (IgG AP; Sigma, USA) in dilution buffer for 1 h at 4°C. The slides were again rinsed with PBST buffer three times. Detection of the amplified signal was carried out using the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT). The stained sections were washed with distilled water for 5 min and then dehydrated and mounted using standard protocols for photomicroscopy (OLYMPUS BH-2). As negative controls in the staining procedures, some samples were not pre-fixed with the carbodiimide fixative EDAC, or the primary antibody GA₁-Me was replaced with PBST in the staining procedure. BSA and BCIP/NBT were from Sigma Co, Ltd., USA, the rabbit anti-goat IgG AP was from Friendship Biotechnology Co, Ltd., and other chemicals were from the Beijing Shiji Factory.

Semi-quantitation analysis

Total RNA was extracted from the axillary buds at different developmental stages using cetyl trimethyl ammonium bromide (CTAB). First-strand cDNA synthesis was performed using 1.5 µg total RNA and M-MLV reverse transcriptase. The expression patterns of *PpLEAFY* and *MADS6* were detected using 18S rRNA as an internal standard. The primers used were LFYF: 5'-GGTGGGATTTGCTTGTGGGTGA-3', LFYR: 5'-AGCTAGAGGCGGTGGCATTGTT-3', 18SF: 5'-GCCTGAGAAACGGCTACCAC-3', and 18SR: 5'-AACCAGACAAATCGCTCCAC-3'. PCR was performed using PrimeSTAR HS DNA polymerase (TaKaRa, Japan) as follows: an initial cycle at 94°C for 5 min followed by a stepped program (94°C, 45 s; 55°C, 40 s; 72°C, 50 s) for 30 cycles. All primers used were synthesized at Shanghai Sangon Ltd., and the PCR-related products were purchased from TaKaRa Co, Ltd., Japan.

RESULTS

Peach flower bud differentiation process

Axillary buds on the short shoots of peach were primarily arranged as syllepsis triads with a central bud surrounded by two lateral ones. Under normal conditions, the central bud will differentiate into a leaf bud, and the lateral ones will become flower buds. The development of the lateral buds borne on the same nodes was determined by free-hand sections in 2004. The results showed that before 10 July, the growth points of the buds were small, narrow, and conical. The cells in the original meristem were small and well organized. On 10 July, the growth point became semi-circular, demonstrating that the flower-induction period was complete and that the buds were beginning the process of differentiation. The apical meristems of the buds became broader and flatter on 20 July, and the differentiation rate was 75%. By 10 August, all buds examined had begun morphological differentiation, and some had progressed to sepal primordium differentiation with a differentiation rate of 100%. On 20 August, all buds had begun sepal primordium differentiation, and 40% demonstrated petal and stamen primordium differentiation. On 30 August, the buds began stamen primordium differentiation, and 22% of them had begun pistil primordium differentiation. At the end of sampling on 10 October, all materials examined were at the stage of pistil primordium differentiation. Based on these observations, the flower-induction period of the Bayuecui peach was identified as occurring prior to 10 July.

Peach flower bud anatomy following GA₃ treatment

The typical arrangement of the floral organs in peach was rotiform. From outer to inner, the outermost layer comprised sepal primordia; the petal primordia were differentiated inward of the sepal primordia followed by two rings of stamen primordia inward from the petal primordia; finally, the growth point differentiated into the pistil primordium of the innermost layer (Fig. 1-A, B, C). The arrangement of the leaf buds at the same stage of development was different from that of flower buds. The developed leaf primordia were relaxed and had al-

veolar edges (Fig.1-D, E, F). After GA₃ treatment, the flowering rate dropped to 11.67%, and the morphology of the lateral buds that would normally be differentiated into flower buds exhibited two forms: One of which was formed during the treatment period whereby flower induction was inhibited and the arrangement of each primordium was similar to that of leaf buds (Fig.1-G), and the other formed after treatment in which the arrangement of each primordium was similar to normal flower buds, but developed abnormally (Fig.1-H, I).

Immunohistolocalization of GA₁ during peach flower bud differentiation

Experiments with Arabidopsis have demonstrated that the proper types and concentration of the GAs in the SAM play important roles in flower transitions (Eriksson *et al.* 2006). To further explore the effects of GA₃ treatment on peach flowering, the GA in peach flower buds was immunohistolocalized using the GA₁-Me antibody, and the results are shown in Fig.2.

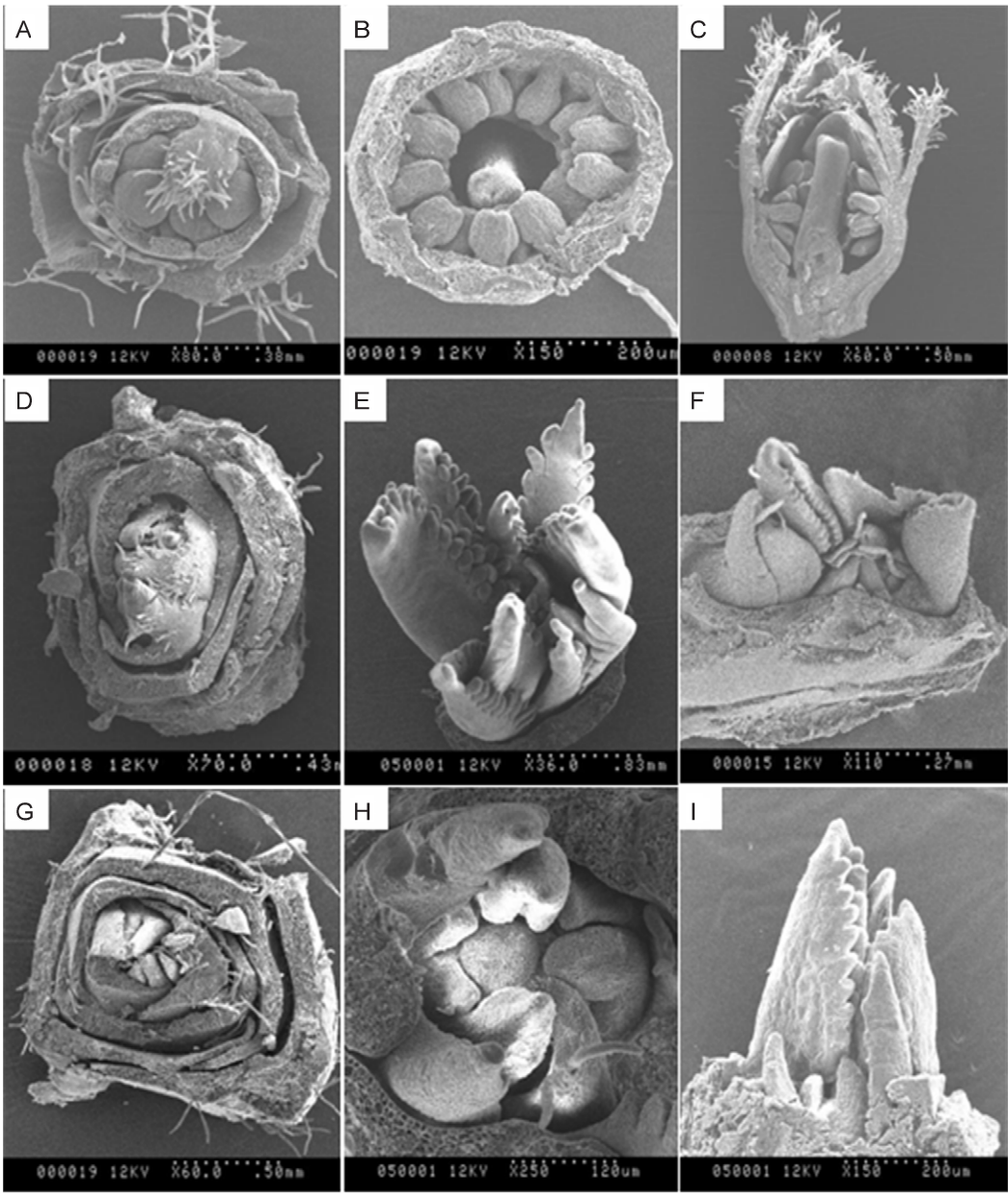


Fig. 1 Scanning electronic microscope observation of normal flower buds, leaf bud and flower bud treated by GA₃. A, B, C, normal flower bud; D, E, F, normal leaf bud; G, H, I, flower bud treated by GA₃. All the materials were sampled on 30, September.

From 13 June to 25 July, the GA_1 signals in untreated buds were found mainly in the vascular bundles at the base of the flower buds, while at the same time, signals were not detected in the SAM (Fig.2-A, B, C, D, E). After GA_3 treatment, the distribution of GA_1 was similar to that of the controls from 13 June to 3 July (Fig.2-F, G, H); however, on 13 July, signals were detected in the SAM accompanied by an increase in the GA_1 signals in the vascular bundles at the base of the flower buds (Fig.2-I). On 25 July, the GA_1 signals in both the vascular bundles and the SAM weakened significantly (Fig.2-J). These results suggested that GA_1 plays a periodic inhibition role in peach flower transitions.

Negative controls followed the same staining procedure except that the GA_1 -Me antibody was replaced by PBS-T (Fig.3-A, B), and the samples were not prefixed with EDAC (Fig.3-C, D). GA_1 signals were not detected in these sections, thereby confirming the reliability of the staining procedure.

Expression patterns of *PpLEAFY* and *MADS6* at different developmental stages of peach flowering after GA_3 treatment

PpLEAFY was expressed mainly at the beginning of flower bud morphological differentiation and reached its highest level on 20 July (Fig.4-A). The expression of *MADS6* was not detected during the flower-induction period, but rather during sepal primordium differentiation (Fig.4-B). The expression of *MADS6* was up-regulated, accompanied by the differentiation of each floral organ. The expression of both *PpLEAFY* and *MADS6* was inhibited after GA_3 treatment. The expression of these two genes could not be detected during the flower induction and differentiation period except for a low level of expression on 10 October (Fig. 4-C, D).

DISCUSSION

Morphological changes in peach flower buds following GA_3 treatment

The period of flower induction is a very important tran-

sition point that is critical in regulating and controlling flower bud differentiation after embryo development in flowering plants. In addition to environmental conditions, gibberellins (GAs) are the most important molecules affecting the flowering of plants and serve as effective regulators for artificial control of plant flowering. Previous studies have revealed that endogenous GAs play an inhibitory role in the flowering of most fruit trees. Radioimmunoassay results obtained by Prang *et al.* (1997) showed that GA_3 transported from the seeds of the developing apple cultivars Golden Delicious, Star, and Jonathan Gold inhibited flower induction while the seedless Spencer apple, which mainly exported GA_4 , could induce flowering. High concentrations of GA_3 were transported from the apple seeds because of excessive fruiting (Stephan *et al.* 1999), and the concentrations of GA_1 , GA_{19} , and GA_{20} in the leaves of the shoots also exhibited a peak during the flower-induction period (Ramirez 2000). These results showed that the inhibition caused by endogenous GAs in fruit trees depends upon the specific types and transport of GAs.

In our study, we investigated the effects of exogenous GAs on peach flowering. Peach flower bud differentiation was significantly inhibited, with a flowering rate of only 11.67%, by spraying with 100 mg L⁻¹ GA_3 . On 10 October, all control plants had begun pistil differentiation, and each floral organ was arranged rotiformly. During normal development, the size of the lateral buds should be different compared to the size of the leaf bud. However, GA_3 treatment resulted in significantly less difference in size between the central leaf bud and the lateral flower buds borne on the same node. Two morphotypes were observed for the lateral buds after GA_3 treatment: one in which the arrangement of each primordium was similar to the loosely arranged normal leaf bud, and the other that was similar to the normal flower bud except that the primordia were leaf-like with alveolar (pitted) edges. These results implied that the effects of GA_3 on peach flowering may be via two mechanisms: one in which exogenous GA_3 inhibits flower induction (manifested by the loosely arranged "leaf" primordium in the lateral buds), and the other in which high concentrations of GA_3 are accumulated in plants because of exogenous spraying, which then suppresses the normal differentiation of the

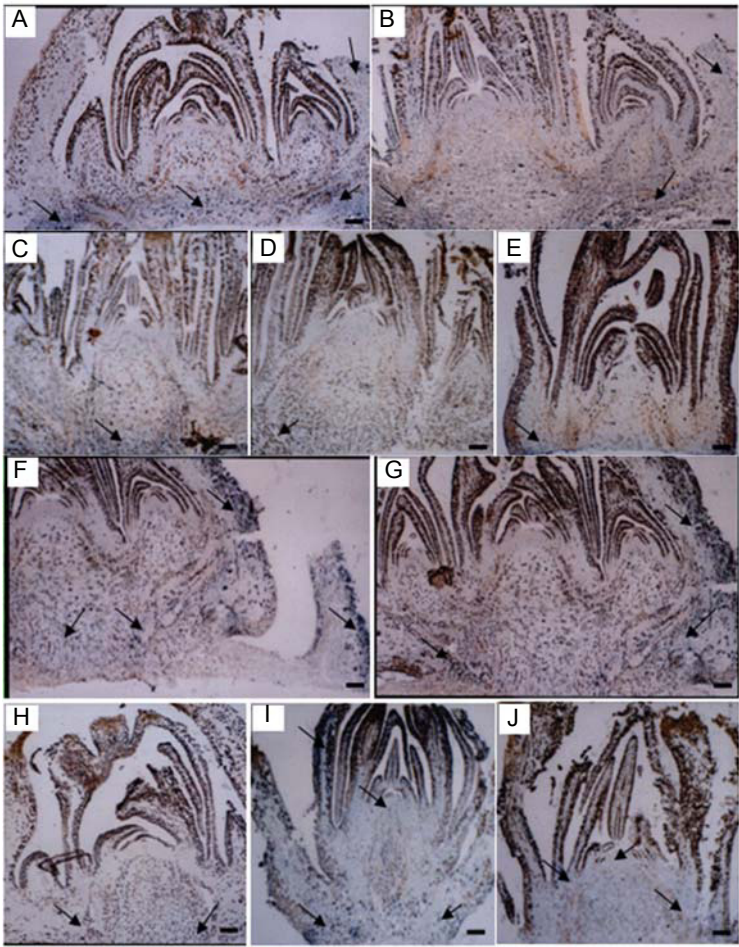


Fig. 2 Immunohistochemical localization of GA₁ in normal flower bud and GA₃-treated of peach. A-E, normal bud sampled (13, June; 23, June; 3, July; 13, July; 25, July) represent different differentiation period; F-J, GA₃ treated bud sampled in the same time with the normal flower bud. All the materials were sampled in 2005. The distribution of detected GA₁ was directed by the black arrows.

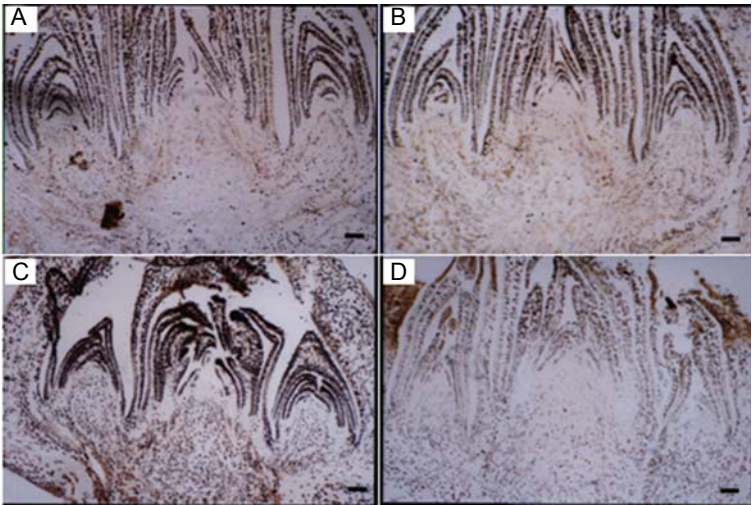


Fig. 3 Controls for immunohistochemical localization. A, B, the GA₁-Me antibody was replaced by PBS-T during the immunostaining procedure; C, D, the materials were not pre-fixed with EDAC.

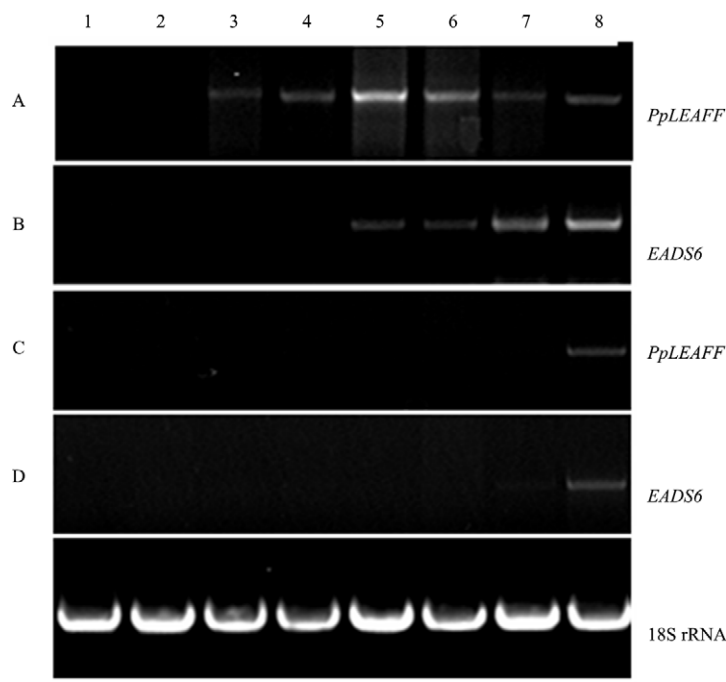


Fig. 4 Semi-quantization RT-PCR analysis of *PpLEAFY* and *MADS6* at different flowering periods of normal and GA₃-treated flowerbud of peach. A,B, normal flower bud; C,D, bud treated by GA₃. The numbers on the top of the picture indicated the flower bud differentiation period: 1-4, the period of flower induction; 5-8, the morphological differentiation period; the numbers at the bottom of the picture represent the date of sampling. All the materials used in the experiment were sampled in 2004.

flower buds, especially floral organ primordium differentiation, providing visual evidence for the effect of exogenous GA₃ on the development of SAMs in peach. This second mechanism is exemplified by the primordia with alveolar edges.

Distribution of GA₁ in peach SAMs after GA₃ treatment

In evolutionarily more recent plant groups, GA precursors can be converted into GA₂₀ and GA₉ via two parallel routes: either through the C-13 hydroxylation pathway or by the non-13-hydroxylation pathway catalyzed by GA₂₀-oxidase followed by further conversion to GA₁ and GA₄ by GA₃-oxidase. The results of studies on the long-day plants *Lolium* and *Arabidopsis* showed that the main effects of GA₁ and GA₄ in *Lolium* were to promote the elongation of the shoot, but had little effect on flower transition (MacMillan *et al.* 2005). In *Arabidopsis*, the concentration of GA₄ was maintained at the highest level during flower transition. At the same time, the level of GA₁ was only 1/40 of that of GA₄, and remained at the lowest level during the

entire transition period (Eriksson *et al.* 2006), demonstrating that GA₁ may be a negative regulator or has no impact on flowering. In our study, the GA₁ distribution in SAMs during the peach flower-induction period was immunolocalized using the GA₁-Me antibody. The results showed that GA₁ signals were significantly increased in the SAM after GA₃ treatment and weakened significantly when the meristem began morphological differentiation. The GA₁ signals were not detected during the entire flowering period in the untreated flower buds, suggesting that the concentration of GA₁ in SAMs may inhibit peach flower differentiation.

Effects of GA₃ treatment on the expression patterns of *PpLEAFY* and *MADS6*

The flower meristem identity genes *LEAFY* and *APETALA1*, which play key roles in plant flowering, are the marker genes for flower transition and floral organ differentiation (Eriksson *et al.* 2006). On short days, the effect of exogenous GA₃ on *Arabidopsis* was related to the expression levels of the flower identity

meristem gene *LEAFY*. The activity of the *LEAFY* promoter increased rapidly after GA₃ treatment, which increased the transcription and expression level of *LEAFY* (Blázquez *et al.* 1998). Here, we used semi-quantitative RT-PCR to detect the expression patterns of *PpLEAFY* and *MADS6* in peach flower buds sampled at different stages of differentiation. The results showed that the expression patterns of *PpLEAFY* and *MADS6* were similar to those reported in the previous studies on untreated flower buds. After GA₃ treatment, the expression of *PpLEAFY* and *MADS6* decreased significantly and was nearly undetectable except on 10 October, when weak expression was detected. These results suggest that GA₃ treatment during flower induction inhibits the normal expression of the peach flower meristem identity genes.

In summary, the effect of GA₃ on peach flowering may be owing to the absorption of GA₃ from leaves, causing changes in the concentration and distribution of GA₁ in SAMs, which in turn inhibits the normal expression of the key flowering genes *PpLEAFY* and *MADS6*. However, the exact way by which the high concentrations of GA₃ from leaves cause the changes in type, distribution, and amount of GAs in SAMs that affect the normal expression of the flower promoting genes remains to be studied.

CONCLUSION

The flower-induction period of the peach cultivar Bayuecui occurred prior to 10 July in Beijing. Flower induction and further differentiation could be significantly inhibited by leaf spraying of 100 mg L⁻¹ GA₃. GA₁ in the GAs family may play a periodic inhibitory role on the flowering process of Bayuecui peach. The normal expression of the flower transition key genes *PpLEAFY* and *MADS6* could be suppressed by exogenous GA₃ treatment.

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