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Research article

Effect of exogenous GA_3 and its inhibitor paclobutrazol on floral formation, endogenous hormones, and flowering-associated genes in 'Fuji' apple (*Malus domestica* Borkh.)

Songwen Zhang [1], Dong Zhang [1], Sheng Fan, Lisha Du, Yawen Shen, Libo Xing, Youmei Li, Juanjuan Ma, Mingyu Han*

College of Horticulture, Northwest Agriculture and Forestry University, Yangling 712100, China

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ABSTRACT

Gibberellins (GAs) reduce apple (*Malus domestica*) flowering rates; however, the mechanism of their action is not fully understood. To gain a better insight into gibberellin-regulated flowering, here, 5 year-old 'Fuji' apple trees were used to explore the responses of hormones [GA_{1+3}, GA_{4+7}, indole-3-acetic acid (IAA), zeatin-riboside (ZR), and abscisic acid (ABA)], and gibberellin- and flowering-associated genes, to applications of gibberellin acid (GA_3) and paclobutrazol (PAC). Results showed that GA_3 relatively stimulated vegetative growth and delayed floral induction. Moreover, GA_3 spraying significantly affected contents of all endogenous hormones and all the genes tested in at least one time points: the content of endogenous GAs was increased instantly and that of ZR was reduced at 44 days after fullbloom (DAF), which might constitute an unfavorable factor for flower formation; *MdKO* (*ent-kaurene oxidase gene*) and *MdGA20ox* (*GA20 oxidase gene*) were significantly repressed by a high level of GAs through the negative feedback regulation of GA; additionally, the *MdSPLs* (*SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE*) in this study were all significantly repressed by GA_3 but promoted by PAC; the expression of *MdFT1/2* (*FLOWERING LOCUS T*), *MdSOC1* (*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*) and *MdAP1* (*APETALA1*) in GA_3-treated buds changed in the same way, and they were repressed at 44 DAF. We suppose that GA_3 spraying disrupts the balance between ZR and GAs, and inhibits floral induction, probably by suppressing *MdSPLs* and the floral integrators in flower induction, which ultimately contributed to inhibiting flower formation.

1. Introduction

Fuji, the dominant apple cultivar in China, has difficulty in flower bud formation, which usually results in unstable and low fruit production. As a consequence, to ensure regular and abundant flower bud formation of apple, it is critical to explore the underlying mechanism of floral induction. There are several stages included in flowering: floral induction, floral initiation, floral differentiation, and anthesis (Hanke et al., 2007). The first stage, floral induction, is characterized by the active changes in endogenous hormones, induced flowering genes, and other flowering signals, all of which finally cause a transition in the meristem development from the vegetative to the reproductive phase. Floral induction is often associated with shoot growth. Growth cessation of short shoots is considered as a gauge of the beginning of floral induction as well as a prerequisite for floral initiation (Huang et al., 1986). The second stage, floral initiation, is defined as the irreversible morphological transformation of the meristem from an induced growing point to an inflorescence meristem, followed by further floral developments in the production of macroscopic flowers (Hanke et al., 2007).

Abbreviations: DAF, days after fullbloom; IAA, indole-3-acetic acid; CTK, cytokinin; ZR, zeatin-riboside; ABA, abscisic acid; GA_3, gibberellic acid; PAC, paclobutrazol; *FT*, *FLOWERING LOCUS T*; *SPLs*, *SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE* (*SPL*) family of transcription factors; *AP1*, *APETALA1*; *SOC1*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*; *LFY*, *LEAFY*; *KO*, gene encoding ent-kaurene oxidase; *GA20ox*, gene encoding GA20 oxidase; *RGL*, *REPRESSOR OF* GA_{1-3} gene; *SPY*, *SPINDLY*; PBS, phosphate buffer saline; CTAB, cetyltrimethyl ammonium bromide.

* Corresponding author.
E-mail addresses: zhangsongwen686@163.com (S. Zhang), afant@nwsuaf.edu.cn (D. Zhang), likessd@126.com (S. Fan), lishadu1992@163.com (L. Du), 15136236336@163.com (Y. Shen), xinglibo1986@live.com (L. Xing), lym19890525@163.com (Y. Li), m13659181256@163.com (J. Ma), hanmy@nwsuaf.edu.cn (M. Han).

[1] These authors contributed equally to this study.

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Floral induction is the most important and investigated stage in apple flowering, in which endogenous hormones and flowering-associated genes become actively involved in determination of bud fate. Active plant hormones include auxins, cytokinins (CTKs), abscisic acid (ABA), gibberellins (GAs) and so on, which constitute a complicated crosstalk network in regulating floral induction. These hormones function differently in flower formation, and they can be either synergistic or antagonistic (Hanke et al., 2007). However, to our best knowledge, GAs are most strongly associated with reproductive competence (Pharis and King, 1985). In *Arabidopsis*, It is reported that GAs promote the transition from vegetative to inflorescence development but inhibit flower differentiation (Yamaguchi et al., 2014). Among the identified GAs, GA_1, gibberellic acid (GA_3), GA_4, and GA_7 are regarded as the most common biologically active forms. The GA signaling pathway is recognized as one of the four flowering pathways in plants. DELLAs restrain plant growth by repressing downstream genes (Davière et al., 2008), whereas GAs promote growth by targeting the DELLAs, leading to their degradation (Mutasa-Göttgens and Hedden, 2009). In *Arabidopsis*, GAs promote flowering by activating floral integrators, including *FT*, *SOC1* and *LFY* (Achard et al., 2007). It is reported that *FT* protein was transported from leaf (Corbesier et al., 2007) and activates downstream genes (*SOC1*, *LFY* and *AP1*) in the shoot apex, leading to floral induction.

It is reported that *SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE* genes (*SPLs*) are also involved in the complicated regulation network of flowering in Arabidopsis: *SPL3*, *SPL4*, and *SPL5* regulate vegetative phase changes (Jung et al., 2011); *SPL3* activates *LEAFY* (*LFY*), *FRUITFUL* (*FUL*), and *APETALA1* (*AP1*) by directly binding their promoter regions, and evidence suggests that *AtSPL4* and *AtSPL5* have overlapping functions (Yamaguchi et al., 2009); additionally, *AtSPL10*, *AtSPL11*, and *AtSPL2* are redundantly involved in the reproductive phase (Shikata et al., 2009). A previous phylogenetic analysis of *Malus domestica* (Li et al., 2013) showed that *MdSPL1/9* are close orthologous genes of *AtSPL3/4/5*, and that *MdSPL3* and *MdSPL10/11* are orthologous of *AtSPL2/10/11* and *AtSPL6*, respectively, having similar structural characteristics and, therefore, potentially similar functions.

In contrast with Arabidopsis, exogenous GAs inhibit flower bud formation in apple (http://www.sciencedirect.com/science/article/pii/S030442389800209X, Marcelle and Sironval, 1963). In many stone fruit trees and in peach, gibberellins are used as a chemical technique during the inductive period to reduce flower formation for the following season (Southwick and Glozer, 2000). The underlying molecular mechanism is still not fully understood. Most recently, Shalom et al. (2015) reported that the expression of *SPLs* in citrus buds was significantly suppressed by heavy fruit load. Since it has been suggested that GAs in bourse buds were derived from seeds of nearby fruits (Luckwill et al., 1969), we suspected that *SPLs* may respond to the level of GAs and GA_3 treatment would inhibit the expression of *SPLs*, which might help to explain low flowering rate caused by exogenous GA_3.

To gain a better insight into the regulation of GA in apple flowering and the relationship between GA and *MdSPLs*, we explored the expression profiles of *MdSPLs* and the floral integrators in apple terminal buds under the exogenous GA_3 treatments at 27, 44 and 74 DAF, with the GA biosynthetic inhibitor paclobutrazol (PAC) as a contrast. It is reported that floral induction in apple occurred between 39 and 53 days after fullbloom (stage 1) (Foster et al., 2003) or 3–6 weeks after fullbloom (Buban and Faust, 1982), when a narrow, flat vegetative meristem starts becoming a broad, flat one (reviewed by Hanke et al., 2007). According to the previous research on shoot apex development, here, the time point of 27 DAF is comparable to stage 0, a vegetative stage; 44 DAF and 74 DAF correspond to stage 1, which includes floral induction and floral initiation and is the key period to determine the fate of bud. To our best knowledge, there are few previous reports associating the effects of GA on *MdSPLs* in fruit trees. Additionally, the negative GA regulation and the effect of PAC on two GA biosynthetic oxidases (*MdKO* and *MdGA20ox*) were also examined. Moreover, to provide fundamental knowledge of hormonal responses to the exogenous treatments, a time course of plant hormone levels was plotted to help explain the effects of GA on apple flowering.

2. Materials and methods

2.1. Plant materials, growth conditions, and treatment

In this study, 27 uniform 5-year-old 'Fuji'/T337/*Malus robusta* Rehd. apple trees, grown at the experimental orchard of Northwest Agriculture and Forestry University in Yangling (108°04′ E, 34°16′ N), China, were used and randomly divided into three groups (nine treated with GA_3, nine with PAC, and the other nine trees were sprayed with water as controls) in this study (these Fuji apple trees had not shown alternate bearing phenomenon). Each group had three blocks, as three replicates, with three trees in each block. This experiment was conducted from 14 days after full bloom (DAF) till 166 DAF (before leaf abscission) in 2014 and hormonal treatments were performed as described by Cao et al. (2000) with a slight modification: 300 mg L^{-1} GA_3 (Sigma Chemical Co., Deisenhofen, Germany) was sprayed three times on clear mornings at 14, 22, and 34 DAF (April 22, April 30, and May 12, respectively). These treatments were all performed with a low-pressure hand-wand sprayer. As contrast, 300 mg L^{-1} PAC (dissolved in methanol) (Solarbio Life Science, Beijing, China) was sprayed at 14 DAF. To ensure efficient absorption by trees, 1 g m^{-2} PAC was later applied to the soil at 22 DAF (See Table 1 for application details). Fullbloom is defined as the date when 80% of king flowers on terminal buds are open.

2.2. Investigation of physiological indicators and sampling

To determine the effect of GA on shoot growth and its cessation time, 20 current-year short shoots (<5 cm) of each block were tagged randomly, as described by Cao et al. (2000) at 13 DAF in 2014, before any treatment, and their lengths were measured at 1-week intervals.

Additionally, 100 terminal buds on short shoots (<5 cm; with no fruit on them) (see supplemental pictures) in each block were tagged randomly at 13 DAF and the number of flowers in full bloom among the tagged buds was counted in the following year (April 8, 2015).

Terminal buds on current-year short shoots (<5 cm; with no fruit on them) were collected into liquid nitrogen at 27, 44, 74, 104, 134 and 166 DAF, then stored at −80 °C and used for analyses of hormone quantification and gene expression.

2.3. Quantitative analysis of endogenous hormones in terminal spur buds

The extraction, purification, and determination of endogenous levels of GAs (GA_{1+3} and GA_{4+7}), ZR, IAA, and ABA by an indirect ELISA technique were performed at the Phytohormones Research Institute (China Agricultural University) as described by Yang et al. (2001). The samples were homogenized in liquid nitrogen and hormones were extracted by cold 80% (v/v) methanol with butylated hydroxytoluene (1 mmol L^{-1}) overnight at 4 °C. The extracts were collected after centrifugation at 10,000 × g (4 °C) for 20 min and passed through a C_{18} Sep-Pak cartridge (Waters, Milford, MA, USA), then dried in N_2. The residues were dissolved in PBS (0.01 mol L^{-1}, pH 7.4) to determine the levels of GAs (GA_{1+3} and