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A transcriptome analysis of two apple (*Malus* × *domestica*) cultivars with different flowering abilities reveals a gene network module associated with floral transitions

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ABSTRACT

Young 'Nagafu No. 2′ apple trees produce fewer flower buds than the trees of other apple cultivars, including 'Yanfu No. 6′ (i.e., 'Fuji' apple tree exhibiting a bud mutation). Thus, we analyzed the flowering characteristics of these two varieties to further characterize apple floral transition. RNA sequencing was used to characterize differentially expressed genes in buds between the two cultivars during floral transition. Differences in several physiological parameters were also analysed. A total of 3,010, 1,139, and 1346 differentially expressed genes were identified at 30, 50, and 70 days after full bloom. These genes combined with the differences in physiological indices indicated that low gibberellic acid levels and high cytokinin levels in 'Yanfu No. 6′ buds during the floral induction stage increased the production of flower buds. High cytokinin levels inhibited gibberellic acid signaling and promoted floral transition. Additionally, low gibberellic acid levels likely inhibited shoot elongation by impairing DELLA degradation. Meanwhile, relatively high indole-3-acetic acid and cytokinin levels synergistically maintained meristem activities and accelerated bud growth. Moreover, the high sucrose content in 'Yanfu No. 6′ buds up-regulated the transcription of *TREHALOSE 6-PHOSPHATE SYNTHASE* and *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE* genes, which ultimately promoted floral induction. The data presented herein indicate that floral transition differences between the two analyzed apple cultivars are linked to sugar and cytokinin signaling pathways.

1. Introduction

Apple is one of the most commercially important tree fruits worldwide. In China, apple cultivar 'Nagafu No. 2′ (i.e., 'Fuji' apple with an altered fruit color) is grown on more than 65% of the total apple-producing area. However, there are several problems associated with 'Nagafu No. 2′ apple orchards. For example, the trees may not produce a sufficient number of flower buds and may exhibit alternate bearing behavior. The insufficient production of flower buds is responsible for unstable and low fruit yields. Apple cultivar 'Yanfu No. 6′, which is a variety that was generated from spur-bearing 'Fuji' apple trees, has a higher proportion of spurs, shorter internodes, larger buds, and more flowers than 'Nagafu No. 2′. Additionally, a previous study indicated that the shoot internode length of an apple spur sport is affected by the gibberellic acid (GA) content (Song et al., 2012).

Flowering is a complex developmental process comprising the floral induction, floral initiation, flower differentiation, and blooming stages (Hanke et al., 2007). During the floral induction period, flowering signals induce the expression of a series of flowering-related genes. In the subsequent floral initiation stage, a series of histological modifications occur, but there are no visible morphological changes. In apple, the floral induction stage is initiated after the cessation of shoot growth (Kotoda et al., 2000), while the fate of buds is determined during the floral initiation period (Hanke et al., 2007). The apple flower differentiation stage is always marked by the appearance of a dome-shaped apex in buds at approximately 12 weeks after full bloom (Abbott, 1997) or 77–127 days after full bloom (DAF) (Foster et al., 2003). Floral organs continue to form, but flowers do not undergo anthesis until the following spring. *Arabidopsis thaliana* comprises six flowering pathways that regulate the floral induction stage (i.e., photoperiod, vernalization, autonomous, GA, ambient temperature, and aging pathways) (Khan et al., 2014). Gibberellic acid promotes floral transitions by activating the transcription of the floral gene *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE* (*SPL*) gene (Porri et al., 2012). Moreover, GA receptors

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(e.g., GIBBERELLIN-INSENSITIVE DWARF1) recognize GA and induce the production of the F-box protein, SLEEPY1, which degrades DELLA proteins (Daviere and Achard, 2013). Five *DELLA* genes [*REPRESSOR OF GA1-3* (*RGA*), *GA-INSENSITIVE* (*GAI*), *RGA-LIKE1* (*RGL1*), *RGL2*, and *RGL3*] encode the major repressors of GA-promoted vegetative growth and floral initiation (Cheng et al., 2004). Exogenous GA_3 inhibits the formation of flower buds in apple, but not in *A. thaliana* (Zhang et al., 2016). Additionally, GA is widely used to inhibit the production of flowers during the following season in many stone fruit tree species (e.g., peach) (Southwick, 2000). However, the underlying molecular mechanism has not been fully characterized.

Sucrose was recently observed to promote floral transitions in *A. thaliana* (Yu et al., 2013). Sucrose-mediated trehalose 6-phosphate (T6P) and TREHALOSE 6-PHOSPHATE SYNTHASE (TPS) regulate *SPL* expression in the shoot apical meristem (Wahl et al., 2013). Cytokinins (CKs) acting in conjunction with other signals (including GA and sugars) are believed to be key regulators of plant growth and development. Cytokinins are reportedly involved in establishing and maintaining shoot and root meristems (Meng et al., 2017; Singh et al., 2017). The antagonistic activities of CKs and GA are associated with the plant development process (Fonouni-Farde et al., 2017). Meanwhile, CKs and indole-3-acetic acid (IAA) exhibit synergistic functions affecting shoot growth (Schaller et al., 2015). Cytokinins also help regulate sugar use and sink strength in apple and *A. thaliana* (Brenner et al., 2005; Karhu, 1997). Although CKs also positively affect apple flower bud formation (Krasniqi et al., 2013), little is known about the associated regulatory mechanism.

Analyses of 'Fuji' apple bud mutations have included investigations of a yellow fruit somatic mutation (El-Sharkawy et al., 2015) and a mutation resulting in a columnar architecture (Krost et al., 2013). However, there are relatively few published studies regarding apple flowering mechanisms, although the mutation in 'Yanfu No. 6' resulted in a significant difference in flowering.

In the present study, we applied high-throughput, next-generation sequencing technology to identify genes that are differentially expressed in 'Yanfu No. 6' and 'Nagafu No. 2' buds at 30, 50, and 70 DAF. An initial examination of the cessation of shoot growth revealed that 30 DAF corresponded to the beginning of the apple floral induction stage, which is consistent with the observations of an earlier study that confirmed that the apple floral induction stage occurs 3–6 weeks after full bloom (Buban and Faust, 1982). Additionally, the flower differentiation stage starts at 77–127 DAF (stage 2) (Foster et al., 2003) or 12 weeks after full bloom (Abbott, 1997). Thus, 30–50 DAF corresponded to the floral induction stage, while 70 and 100–130 DAF corresponded to the floral initiation and flower differentiation stages, respectively. Floral induction and floral initiation are prerequisite periods for floral transitions. In this study, 30–70 DAF represented the floral transition period. Furthermore, we developed a model explaining the differences between the two analyzed apple varieties regarding their floral transitions. The data presented herein may be useful for further characterizing the mechanism underlying apple floral transitions.

2. Materials and methods

2.1. Plant materials and sample collection

Samples were collected from 6-year-old 'Nagafu No. 2' /M26/ *Malus robusta* Rehd and 'Yanfu No. 6' /M26/ *M. robusta* Rehd trees cultivated at the Apple Demonstration Nursery at the Yangling Modern Agriculture Technology Park, Shaanxi, China (108°04′ E, 34°16′ N). For each variety, 15 trees were randomly selected and grouped into three blocks of five trees. We previously determined that the application of 300 mg L^{-1} 6-BA or 300 mg L^{-1} GA_3 can significantly affect the flowering rate of the short shoots of 'Nagafu No. 2' trees (Li et al., 2016, 2017; Zhang et al., 2016). To analyze gene responses to 6-BA or GA_3 treatments, 18 'Nagafu No. 2' trees were divided into two groups of nine trees. On a clear morning at 30 DAF, the trees of one group were sprayed with 300 mg L^{-1} 6-BA, while the trees of the other group were sprayed with 300 mg L^{-1} GA_3. Terminal buds on short shoots (< 5 cm) were collected from untreated 'Yanfu No. 6' and 'Nagafu No. 2' trees as well as 6-BA- or GA_3-treated 'Nagafu No. 2' trees at 30, 50, 70, 100, and 130 DAF. Samples were immediately frozen in liquid nitrogen and stored at −80 °C for subsequent sugar and hormone analyses and an RNA extraction.

2.2. Analysis of physiological parameters

Because the effects of 6-BA and GA_3 treatments on the following physiological parameters were determined in previous studies (Li et al., 2016, 2017; Zhang et al., 2016), we analyzed the physiological characteristics of only untreated 'Nagafu No. 2' and 'Yanfu No. 6' trees. For both varieties, 30 current-year shoots at cluster bases (Kotoda et al., 2000) were marked in each block. The shoot length was periodically measured starting from the full bloom stage (0 DAF) to 45 DAF. Additionally, for the two varieties in each block, 20 terminal buds from short shoots (< 5 cm) were periodically collected from 30 to 80 DAF to measure their size and fresh weight.

After the abscission of leaves, the current-year shoots were divided into the following three groups: short (< 5 cm), medium (≥ 5 cm and ≤ 15), and long (> 15 cm) shoots. The proportion of each shoot type among 15 major branches was calculated for both varieties in each block. Moreover, the internode and shoot lengths as well as the number of nodes were determined for 15 long shoots in each block.

In the following year, the percentage of buds producing flowers was calculated for all three shoot types in both varieties. Flowering rates were determined as previously described (Li et al., 2016).

2.3. Identification of sugars, starch, and hormones

2.3.1. Sugars and starch

0.8 g Frozen buds were ground to a powder in liquid nitrogen for a subsequent extraction of soluble sugars and starch, which were then analyzed as previously described (Rosa et al., 2009).

2.3.2. Hormones

0.5 g Frozen buds were ground to a powder in liquid nitrogen. Zeatin riboside (ZR), IAA, GA_{1+3}, GA_{4+7}, and abscisic acid (ABA) extracted from the resulting powder were purified. The hormone concentrations were calculated with an indirect enzyme-linked immunosorbent assay by the Phytohormones Research Institute (China Agricultural University) as described by Yang et al. (2001). Each sample was evaluated three times, with three replicates.

2.4. RNA extraction, cDNA library construction, and RNA-sequencing

We used a cetyltrimethylammonium bromide-based method to extract total RNA from terminal buds collected at 30, 50, and 70 DAF (Li et al., 2016). The integrity of the extracted RNA was verified in an agarose gel, while RNA concentrations were determined using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The subsequent library construction and RNA-sequencing (RNA-seq) were completed by the Beijing Genomics Institute (Shenzheng, China). The prepared libraries were sequenced with an Illumina HiSeq 2000 platform to generate 100-bp paired-end reads.

2.5. Mapping to the reference genome and quantification of gene expression levels

The raw sequencing reads were subjected to a quality control step to ensure that the base ratios were mostly higher than 20 and that the A curve overlapped the T curve and the G curve overlapped the C curve.