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Research article

Expression of genes in the potential regulatory pathways controlling alternate bearing in 'Fuji' (*Malus domestica* Borkh.) apple trees during flower induction

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ABSTRACT

Most perennial fruit trees have an alternate bearing problem where a heavy fruit load is produced one year (ON year) but few flowers and fruits produced the next year (OFF year), resulting in a significant fluctuation in production. In the present study, comparative transcriptome analysis of terminal buds of apple (*Malus domestica* Borkh., cv. Nagafu No. 2) trees was conducted during the floral induction period in the ON and OFF years to identify the potential regulatory pathways controlling alternate bearing. A total of 1027 differentially expressed genes (DEGs), most of which were involved in secondary metabolism, sugar metabolism, plant hormone pathways, were identified. The analysis focused on differences in sugar content and hormone levels between the ON and OFF trees. Sucrose content, zeatin-riboside (ZR), and abscisic acid (ABA) levels were lower in ON-year buds than in OFF-year buds. ON buds also had elevated levels of gibberellins (GAs), with a higher expression of GA20 oxidase *(GA20ox)* and a significant lower level of *RGA*-like*2 (RGL2)*. Expression analyses also revealed a significantly higher level of *SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE* genes (*MdSPL1*, *MdSPL6* and *MdSPL12*) transcripts levels in buds of OFF trees at 45 days after full bloom (DAFB). *LEAFY (LFY)* expression increased significantly prior to flower induction in OFF buds. These findings provide new information of the role of hormones in alternate bearing, as well as other processes, and provide new insights into the molecular mechanisms regulating alternate bearing in perennial fruit trees.

1. Introduction

Some perennial fruit trees are prone to alternate bearing, which is defined as an agronomical phenomenon where a tree bears high and low fruit loads in alternating years. In other words, there is a heavy fruit load one year (ON year), but little or no yield the next year (OFF year). The OFF year is also characterized by low flower numbers in spring and more vegetative buds, whereas the opposite occurs in the ON year. This biennial cycle of fruiting is widespread in different species of fruit trees (Smith and Samach, 2013; Haberman et al., 2016). For example, some apple varieties like Fuji, Kanzi, Honeycrisp and Elstar are susceptible to alternate bearing, others like Golden Delicious, Gala, Rosy Glow and Pink Lady are not (Krasniqi et al., 2013). It has been widely reported that heavy fruit load reduces flowering in the following year (Monselise and Goldschmidt, 1982), and represents a major problem in commercial orchards causing significant fluctuations in production, which has a negative impact on financial returns. Alternate bearing is a major problem for apple production worldwide.

Although researchers have studied alternate bearing in fruit crops for several decades, many questions pertaining the underlying mechanisms still remain unanswered (Munoz-Fambuena et al., 2013; Fan et al., 2016). High fruit load has been reported to inhibit floral induction and initiation (Samach and Smith, 2013). Physiological aspects of the impact of alternate bearing on flowering have been investigated, especially in regards to plant nutrition, hormones, and carbohydrate and/or mineral levels in alternate bearing apple trees in the ON and OFF years (Monselise and Goldschmidt, 1982). Sugars are critical to plant growth and are also involved in the regulation of flowering (Du et al., 2017). Plant hormones and carbohydrate reserves also play an important role in either repressing or inducing flowering in perennials (Bangerth, 2009). A regulation scheme has been proposed for alternate bearing by Krasniqi et al. (2013) including the relations of light induced

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carbohydrate deprivation, promotion by root-derived, cytokinins (CTKs) and inhibition by seed originating gibberellic acid (GA7). GAs promote vegetative growth and inhibit floral development in fruit trees, and application of exogenous GAs prevent floral bud formation (Haberman et al., 2016; Zhang et al., 2016). This effect, however, is influenced by the timing and type of GAs that are applied. The active GA_1 is derived from the inactive precursor GA_{20} and inhibited floral initiation in *Prunus avium* (Oliveira and Browning, 1993). In addition, studies reported that GA_7 was synthesized in young apple seeds (Luckwill, 1970) and exported from the seeds (Hirst P., 2011, pers. comm), which is responsible for the inhibition of flowering. These conclusions also confirmed the regulatory scheme proposed by Krasniqi et al. (2013). A model of alternate bearing suggests that seed-derived GAs are the transmissible signal that are responsible for flower inhibition (Luckwill, 1970). In contrast to GA, CTK derived in the roots promotes floral bud formation in apple trees (Ramírez et al., 2000). Auxin stimulates GA synthesis in the meristem, and auxin is somehow involved in repressing floral induction (Shalom et al., 2014). Interestingly, application of GAs increased the export of auxin from developing apple fruit (Callejas and Bangerth, 1997). Therefore, the floral-inhibitory action of gibberellin may be partially mediated through auxin. ABA levels are lower in buds of citrus trees in OFF years than that they are in ON years (Shalom et al., 2014). To summarize, correlations have been reported between a reduction in flower development due to heavy fruit load and levels of chemicals and hormones in diverse species. Previous study found that the sugar levels were increased up to 3-fold in apple leaves in OFF years, indicating that sugars, as an energy source, regulate growth and flowering in apple (Krasniqi et al., 2013; Du et al., 2017). Despite these findings, the role of carbohydrates and hormones in alternate bearing remain unclear at the present time.

Floral initiation in apple occurs in early summer of the previous year, and directly determines the yield of apple that will be potentially produced in the autumn of the following year. Understanding the molecular mechanisms regulating floral induction is important for improving breeding efficiency and maintaining a high crop yield. Complex regulatory networks involving endogenous and multiple environmental cues can mediate flower bud formation in perennial plants (Smith and Samach, 2013). High GA levels in Arabidopsis promote the expression of *SPLs*, which is favorable for flower formation. In contrast, *MdSPLs* are strongly repressed by exogenous GA3 in apple buds during floral induction (Zhang et al., 2016). This suggests that *SPLs* may play an important role in the regulation of flowering in response to a GA signal. *SPLs* also activate important MADS-box genes *LEAFY (LFY)*, *FRUITFUL (FUL)* and *APETALA1 (AP1)* by directly binding to the promoters of these genes (Shalom et al., 2015). Inverse relationships between fruit load and the expression of *FLOWEING LOCUS T (FT)*, *SUPPRESSOR OF OVER- EXPRESSION OF CONSTANS1 (SOC1)* and *AP1* in the leaves of ‘Moncada’ mandarin have been reported, especially during the floral induction period (Munoz-Fambuena et al., 2013). The level of *FT*, whose protein product is synthesized in the veins of the leaves and then transported to the buds, has also been reported to be up-regulated during floral induction in the apical bud (Mimida et al., 2011). Additionally, the transcript level of *MdAP1*, encoding the regulator of flower meristem identity, increased in shoot apices of apple that were sampled from trees in which fruit had been removed during the period of floral formation (Haberman et al., 2016). These genes are involved in the regulation of flower induction.

‘Nagafu No. 2’, as a sport of 'Fuji' variety, is the dominant apple cultivar in China. Alternate bearing in this cultivar, however, is a significant problem and results in unstable and low fruit production. The regulation of flowering during alternate bearing remains unclear and more research is needed to identify factors affecting flowering during an ON or OFF year. Therefore, transcriptome sequencing was used to analyze gene expression and to identify metabolic and regulatory pathways associated with alternate bearing, including secondary metabolism, and specifically carbohydrate and hormone signals. Comprehensive changes in hormone and sugar levels, as well as related gene expression during bud development, were analyzed in ON and OFF trees. This study will provide a better understanding of the regulatory mechanisms controlling alternate bearing.

2. Materials and methods

2.1. Plant materials and sample collection

A total of 12 fourteen-year-old ‘Nagafu No.2’ apple trees were selected from a commercial orchard in Tiandu (107°57′ E, 34°28′ N), Shaanxi, China. Among the 12 selected trees, six had a high fruit load, and were defined as ON trees. In contrast, six of the trees had a very low fruit load, and were defined as OFF trees (Fig. S1). The trees were randomly selected within a group of three blocks containing ON and OFF trees. Terminal buds on current-year spurs (< 5 cm) were collected at 30, 45, 75, 105, 135, and 165 days after full bloom (DAFB) from both ON and OFF year trees, and stored at -80 °C for subsequent RNA extraction and RNA-seq library construction. Total RNA was extracted from terminal buds of spurs using a cetyltrimethyl ammonium bromide (CTAB)-based method with slight modification as described in our previous study (Li et al., 2016).

2.2. Analysis of flowering rate

Two large branches on each ON and OFF trees were tagged and used to determine flower intensity of terminal buds on short shoots (< 5 cm). The total number of terminal buds on short shoots and the number of floral terminal buds on the tagged branches were counted at full bloom for two years (April 8, 2014 and April 7, 2015). Flowering rate (number of floral buds/number of total buds) was calculated from these data.

2.3. Measurement of sugar and starch levels in buds

A total of 0.5 g fresh weight of buds collected during the floral development stages were used for soluble sugar and starch determinations using extraction methods as previously described by Rosa et al. (2009). Sugar levels were measured using high performance liquid chromatography (Waters, USA). The insoluble fraction remaining after soluble sugar extraction was used for starch determination.

2.4. Measurement of endogenous hormone levels in terminal spur buds

GAs (GA_{1+3} and GA_{4+7}), ZR, indole-3-acetic acid (IAA), and ABA were extracted from approximately 0.5 g fresh weight of buds collected during the flower induction period and levels were determined using an indirect ELISA technique which was conducted at the Phytohormones Research Institute (China Agricultural University) as described by Yang et al. (2001). Detailed descriptions of the extraction and quantification methods are provided in our previous research (Zhang et al., 2016). Results are presented as the mean ± SE of three replicates.

2.5. RNA extraction, RNA-seq library construction, and high-throughput sequencing

Total RNA was extracted from buds of ON and OFF year trees collected at 45 DAFB, which was during the period of floral induction. Total RNA was extracted from three replicates and used to prepare cDNA libraries. Six RNA-seq libraries were constructed and sequenced by the Biomarker Biotechnology Corporation (Beijing, China). The protocol used for library construction was previously described in detail by Lou et al. (2014). The libraries were sequenced on an Illumina HiSeqTM 2000 (BGI, Shenzhen, China) platform. Resulting raw reads were filtered into clean reads and aligned to the apple (*Malus domestica* Borkh.) genome (http://www.nature.com/ng/journal/v42/n10/full/