Molecular Basis of Flower Formation in Apple Caused by Defoliation and Gibberellins

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ABSTRACT. Adequate flower formation limits dependable apple (*Malus ×domestica*) production and is a major challenge for apple industries around the world. ‘Honeycrisp’ is a high value apple cultivar, but consistent flowering is difficult to achieve. Apple flower formation is affected by factors including defoliation, girdling, and gibberellin (GA₄₊₇) and 1-naphthaleneacetic acid (NAA) applications. However, the molecular mechanisms that regulate the effects of these factors are not well understood. We studied the effect of local spur defoliation, GA₄₊₇ and NAA applications on ‘Honeycrisp’ flower formation. Furthermore, we investigated the effect of local defoliation and local GA₄₊₇ application on the transcript levels of two major flower formation genes in the meristems of apple spurs. The floral inhibition gene *terminal flower1-1* (*MdTFL1-1*) and floral promoting genes *flowering locus T* (*MdFT1* and *MdFT2*) of apple. Local application of GA₄₊₇ and defoliation treatments inhibited flower formation, but NAA applications were without effect. Defoliation treatments were accompanied by a significant reduction in *MdFT1, 2* transcript levels compared with controls early in the growing season. Conversely, GA₄₊₇ application was accompanied by a significant increase in *MdTFL1-1* transcripts compared with controls throughout the growing season. These results indicate that GA₄₊₇ inhibits flower formation by upregulating the inhibitory *MdTFL1-1*, and defoliation acts by downregulating transcript levels of *MdFT1, 2* early in the growing season. We also provide evidence that defoliated bourse buds may receive flowering promotion signals from other parts of the tree in the absence of their local leaves.

Flowering is a key developmental stage of apple biology and one of the primary aspects that affects apple production. Flower formation results from the complex interaction of endogenous pathways and environmental conditions. Many apple fruit production problems are associated with flower formation, the most commercially important being biennial bearing, which is “the fluctuation in cropping from year-to-year caused by irregular flowering” (Hirst, 2017). ‘Honeycrisp’ is a newer apple cultivar that is popular with consumers but has a severe tendency for biennial bearing. Therefore, the elucidation of the molecular mechanisms that regulate flower formation is not only important for the commercial production of ‘Honeycrisp’ apples but also makes this cultivar a good model for studying these mechanisms.

Plant growth regulators have been implicated to play a major role in controlling flower initiation and biennial bearing in apple. Exogenous application of gibberellins (GAs) soon after full bloom can promote vegetative growth and inhibit flower formation (Bangerth, 2009; Marino and Greene, 1981; McArtney and Li, 1998; McLaughlin and Greene, 1984). The identification of GA activity in apple seeds during the early stages of

fruit development (Dennis and Nitsch, 1966) led to the assumption that seed-derived GAs inhibit flower formation in apple (Chan and Cain, 1967). Dennis and Neilsen (1999) suggested that the inhibitory effect of GAs from the seeds on flower formation is not ultimate and that further research may implicate GAs, auxins, or both as important regulators for biennial bearing. Mapping a quantitative trait locus in the genome of apple associated with biennial bearing showed four main genomic regions containing floral genes, gibberellin oxidase genes, and meristem identity genes (Guitton et al., 2012). This finding supported the hypothesis that GAs influence flower formation, and further research on the interaction between growth regulators and molecular floral initiation pathways will likely increase our understanding of apple flowering and biennial bearing.

Leaves are the main source of flower formation signals (Hanke et al., 2007). Not only are they the main receptor for environmental signals but they also produce carbohydrates and plant growth regulators. Recently, we showed that flowering did not appear to be related to factors associated with spur carbohydrate status, suggesting a direct role of genes regulating flowering and plant growth regulators (Elsysy et al., 2019). Flower induction genes have been shown to be active in the leaves (Hanke et al., 2007). Flower formation is affected by leaf area (Sahulka, 1967), and defoliation decreased flower formation (Elsysy and Hirst, 2017; Fulford, 1960; Harley et al., 1942). Flower formation was inhibited with partial and whole tree defoliation in ‘Braeburn’, ‘Golden Delicious’, ‘Ramey York’, and ‘Fuji’ (Davis, 2002). There is a clear relationship between flower formation and defoliation time and severity, as flower

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formation increases as defoliation time was delayed (Davis, 1957; Liewelyn, 1968), and as defoliation severity was reduced (Harley et al., 1942; Tustin et al., 1997). In addition to leaf area, the types of leaves are also important for flower formation. Our recent work showed that bourse leaves, but not spur leaves, were important in the regulation of flower formation in apple (Elsysy and Hirst, 2017). Moreover, the effect of local inhibitions such as fruiting and defoliation could be affected by the availability of flowering promotion signals in the whole tree (Elsysy and Hirst, 2017).

In fruit-bearing shoots, flower formation is affected by several environmental and internal cues. For example, sugar, temperature, and daylength are positive effectors, whereas GAs are well-known negative regulators (Mimida et al., 2013). Bioactive GAs may play a role in the control of key flowering genes in apple (Guitton et al., 2016). The crosstalk between sugars and plant growth regulators mediates bud development and flower formation in apple (Xing et al., 2015). *Flowering locus T* (*MdFT1*), *apetala1* (*MdAPI*), and the apple ortholog of *leafy* (*MdAFL*) are flowering promoters, but *terminal flower1* (*MdTFL1*) is a flowering repressor (Mimida et al., 2013; Xing et al., 2015). Two flowering regulator gene families, *FT* and *TFL*, have been characterized and isolated in various plant species, with *FT* generally promoting flowering and *TFL* inhibiting flowering (Hanke et al., 2007). In addition, homologs of these two genes have been characterized in several fruit species, including apple (Kotoda and Wada, 2005; Kotoda et al., 2006; Mimida et al., 2013), Japanese pear (*Pyrus pyrifolia*), European pear (*Pyrus communis*), quince (*Cydonia oblonga*), Chinese quince (*Chaenomeles sinensis*), loquat (*Eriobotrya japonica*) (Esumi et al., 2005), and sweet orange [*Citrus sinensis* (Pillitteri et al., 2004)]. It has been well established in apple that *MdFT1* is the main flower formation promoter (Kotoda et al., 2010; Mimida et al., 2011), and *MdTFL1* is a major flower repressor (Flachowsky et al., 2012; Kotoda et al., 2006).

Over the last 10 years, much of the research to explain the molecular mechanisms of flower formation in apple has focused on two main genes, *MdTFL1* and *MdFT1*. Several transgenic apples with antisense *MdTFL1* RNA transgene flower very early compared with control plants (Kotoda et al., 2006). In addition, *MdTFL1* plays an important role in the vegetative growth of apple, and silencing this gene is a critical step toward forming the flowering bud in apple (Flachowsky et al., 2012; Kotoda et al., 2006). Exogenous application of gibberellins led to a significant increase in the *MdTFL1* transcript level in apple (Haberman et al., 2016).

Two flowering genes of apple, *MdFT1* and *MdFT2*, induced early flower formation in transgenic *Arabidopsis thaliana* (Kotoda et al., 2010). The transcript levels of *MdFT1* were higher in meristematic buds, but *MdFT2* showed higher transcript levels in the reproductive organs (Kotoda et al., 2010). *MdTFL1* transcripts were identified in the meristem of apple buds before budbreak (Hättasch et al., 2009; Mimida et al., 2011). In early summer during the time of flower initiation, *MdTFL1* is barely detectable and *MdFT1* and some other flowering genes are upregulated (Hättasch et al., 2009; Mimida et al., 2011).

We hypothesize that local flower formation regulators affect the expression patterns of flower promotion and inhibition genes. The objective of our study was to determine the local effects of gibberellins, auxins (NAA), and defoliation on flower

formation in ‘Honeycrisp’ apple and to investigate the relationships between flower formation and expression patterns and levels of *MdTFL1-1* and *MdFT1, 2* in meristematic buds.

Methods

Plant material

Mature ‘Honeycrisp’ trees planted in 2003 and growing on ‘M.7 EMLA’ (M.7) rootstock were used for the present investigation. Trees were planted at a spacing of 3 × 5 m, trained to a vertical axis form, and managed according to standard commercial practices. The orchard was located at the Samuel G. Meigs Horticulture Facility of Purdue University, Lafayette, IN. Crop load was adjusted manually by hand thinning at petal fall to achieve light crop loads of 60–80 fruit per tree. In 2012, the year preceding the experiment, a series of severe spring frosts resulted in almost total fruit loss, which provided a uniformly low previous crop load for all trees.

Experimental procedure

The experiment was arranged as a randomized complete block design, with 24 trees arranged in six blocks, with four trees per block, treated as a single experimental unit. The experiment was conducted over two consecutive seasons, Spring 2013 and 2014, on the same set of trees.

TREATMENTS. Two hundred flowering spurs were randomly selected in each block (across four trees), tagged using durable plastic tags, and flowers were removed before petal fall. Tagged spurs were at least 20 cm apart from each other to avoid possible interactions among different treatments. Each treatment was randomly assigned to 50 spurs across the four trees in each block according to the tag color. Treatments were a) control, b) defoliation, c) exogenous application of 400 mg·L⁻¹ GA₄₊₇ (Provide; Valent Biosciences, Libertyville, IL), and d) exogenous application of 0.147 mL·L⁻¹ NAA (Fruitone N; AMVAC Chemical Corp., Newport Beach, CA). Both GA₄₊₇ and NAA treatments were applied using small spray bottles to target the application only to specific spurs assigned to these treatments. Defoliation treatments were reapplied on the same spurs 2 weeks and 1 month after the start of the experiment because after defoliation, new leaves emerged on the spurs as bourses continued to grow. Treatments were applied at a time that previous studies indicated to be most effective in manipulating flowering. Defoliation was applied at petal fall (Davis, 1957; Liewelyn, 1968), GA₄₊₇ was applied 2 weeks after full bloom (Marino and Greene, 1981; McArtney and Li, 1998; McLaughlin and Greene, 1984) and NAA was applied four times to the same spurs 55, 65, 75, and 85 d after full bloom (McArtney et al., 2007). Full bloom occurred on 3 May 2013 and 10 May 2014.

PLANT MATERIAL AND SAMPLE COLLECTION. Ten bourse buds were collected from each treatment and each block, and in each sample date, they were immediately immersed in liquid nitrogen then stored in a –80 °C freezer for later RNA extraction. Bourse bud samples were collected on three sample dates, ≈25, 45, and 65 d after full bloom (DAFB) for control, defoliation, and GA₄₊₇ treatments. However, for NAA treatment, bourse buds were collected on 55, 65, and 85 DAFB. During winter, while trees were dormant, 20 tagged spurs were collected from each treatment in each block, and bourse buds were dissected under a dissecting microscope (Olympus Corp., Center Valley, PA) to determine flower formation (Hirst and Ferree, 1995).

RNA EXTRACTION AND QUANTIFICATION. On each sampling date, buds of each treatment in each block were pooled into one sample. Analysis of transcript levels was conducted on three blocks with three technical replications for each block. NAA treatment did not show significant effects on flower formation in either year of the study; therefore, transcript levels for these treatments were not analyzed. Total RNA was extracted from bud samples as previously described (Japelaghi et al., 2011), and cDNA was synthesized as described by Shalom et al. (2012). Primers for the genes encoding for Flowering locus T, MdFT1 (MDP0000132050) and MdFT2 (MDP0000139278), and Terminal flower MdTFL1-1 (MDP0000255437) were designed using Primer 3 software (Untergasser et al., 2012) (Table 1). Real-time PCR was then carried out on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA), with PerfeCTa SYBR Green FastMix (Quantabio, Beverly, MA) in a 15- μ L final volume to determine the transcript levels of the target genes. The *ubiquitin C (UBC)* gene (MDP0000205182) was used as an internal control for the normalization of gene transcript levels (Storch et al., 2015).

Statistical analysis

FLOWER FORMATION. Data were analyzed using flower formation as the response factor. Treatments were compared within each year separately. Data are presented as percentage of flower formation for each treatment in each year. Analysis was conducted using binomial logistic regression with mixed effects, with all treatments compared with control ($P < 0.05$), using the statistical package R (version 3.2.2; R Foundation, Vienna, Austria).

GENE TRANSCRIPT LEVELS. Real-time quantitative reverse transcriptase PCR results were analyzed using the $2\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Comparisons were performed among treatments within dates. Statistical significance was determined using the two-tailed Student's *t* test ($P \leq 0.05$). Data are presented as a fold change in the transcript level. Analyses were also conducted using R.

Results

EFFECT OF TREATMENTS ON FLOWER FORMATION. Flower formation of control spurs was variable between the 2 years of investigation, indicating a high degree of biennial bearing among the trees used for this study. Controls in 2013 exhibited 71% flower formation compared with 11% in 2014 (Fig. 1). Similar trends for the effect of treatments on flower formation of 'Honeycrisp' were observed in 2013 and 2014, despite the difference in overall flower formation between the years. GA₄₊₇ application significantly inhibited flower formation in both years, but defoliation significantly inhibited flower formation only in 2013 (Fig. 1). However, NAA applications did not affect flower formation compared with controls in either year of the experiment (Fig. 1).

EFFECT OF GA₄₊₇ AND DEFOLIATION TREATMENTS ON MdFT1, 2 AND MdTFL1-1 TRANSCRIPT LEVELS. Defoliation

Table 1. Gene-specific primer sets used for quantitative real-time reverse transcription polymerase chain reaction analyses of *MdTFL1-1* and *MdFT1, 2* in cDNA synthesized from extracted RNA from apical buds of 'Honeycrisp' apple. The *Ubiquitin C (UBC)* gene was used as an internal control for gene expression normalization.

Gene	Accession code ^z		Primer sequence 5'-3'	Reference
<i>MdFT1, 2</i>	MDP0000132050	F	TCATGGTGGATCCTGATGCA	Storch et al. (2015)
	MDP0000139278	R	AAGCTTGCCGAGTTGTTG	
<i>MdTFL1-1</i>	MDP0000255437	F	ACACCTTCCTCGAGGGATCA	
		R	TTTCTCTCTGCGCGTTGAAGT	
<i>UBC</i>	MDP0000205182	F	TTGCTGGTGTATCTCTGCATC	
		R	AGACCCACCTACTCCCGTCT	

^zAccession code correspond to unigene numbers from *Malus ×domestica* genome.

Fig. 1. Effect of defoliation and gibberellin (GA₄₊₇) and 1-naphthaleneacetic acid (NAA) applications on 'Honeycrisp' apple flower formation in 2013 (A) and 2014 (B). Data are presented as percentage of flower formation and were analyzed using binomial logistic regression with mixed effects. Different letters indicate significantly different values to the control ($P < 0.05$).

reduced *MdFT1, 2* transcript levels both at 24 DAFB in 2013 and 49 DAFB in 2014, whereas GA₄₊₇ treatment only affected *MdFT1* transcript levels at 49 DAFB in 2014 (Fig. 2A and E). On all other sample dates, *MdFT1, 2* steady state transcript levels were unaffected by the treatments (Fig. 2B–D and F).

MdTFL1-1 transcripts were significantly downregulated by defoliation on all sample dates in 2013 and on the first two sample dates (26 and 49 DAFB) in 2014 (Fig. 3). Application of GA₄₊₇ increased *MdTFL1-1* transcript levels at 46 and 66 DAFB in 2013 and 26 DAFB in 2014 (Fig. 3B–D).

Discussion

Despite hand thinning to reduce crop loads to levels that would be considered "light" by commercial standards; flower formation in 2014 was very low (Fig. 1). This highlights the

Fig. 2. Effect of defoliation and application of gibberellins (GA₄₊₇) to 'Honeycrisp' apple on *MdFT1, 2* (MDP0000132050) and (MDP0000139278) expression patterns in 2013 at 24 d (A), 46 d (B), and 66 d (C) after full bloom and in 2014 at 26 d (D), 49 d (E), and 69 d (F) after full bloom (DAFB). Real-time quantitative reverse transcriptase PCR results were analyzed using the 2^(-ΔΔCT) method using *Ubiquitin C (UBC)* as an internal control. Statistical significance was determined using the two-tailed Student's *t* test. Different letters indicate significantly different values to the control (*P* < 0.05).

very biennial nature of 'Honeycrisp' and may have been due to the time of thinning (petal fall), as earlier blossom thinning has been shown to be more effective at promoting return bloom than later thinning as practiced here (Tromp, 2000).

Two major flower formation inhibitors have been previously described in apple: GAs (Marino and Greene 1981; McCartney and Li, 1998; McLaughlin and Greene, 1984) and defoliation (Elsysy and Hirst 2017; Fulford, 1960; Harley et al., 1942). In most of these previous studies, treatments were applied either on whole trees or to major branches. However, in this study, we applied these two putative flowering inhibitors locally to individual spurs. We demonstrated that these inhibitors gain their effects by affecting the treated spurs, as the local exogenous GA₄₊₇ application significantly inhibited local flower formation compared with the control in both years. Similar trends were seen for local defoliation which also inhibited local flower formation compared with the control, although this was significantly lower only in 2013 (Fig. 1). Local defoliation and GA₄₊₇ application likely act via manipulation of the complicated molecular networks involved in the apple flowering pathway.

Flowering locus T is produced in leaves of many species (Mimida et al., 2013), but in apple, it is unclear whether it is produced in apical buds (Kotoda et al., 2010) or leaves (Xing et al., 2015). In this experiment, local spur defoliation reduced flower formation in 2013 compared with the control, and this reduction was accompanied by a reduction in *MdFT1, 2* transcript levels early in the season (24 DAFB) (Fig. 2A). Defoliation removes the source of any potential leaf-produced

Fig. 3. Effect of defoliation and application of gibberellins (GA₄₊₇) to 'Honeycrisp' apple on *MdTFL1-1* (MDP0000255437) expression patterns in 2013 at 24 d (A), 46 d (B), and 66 d (C) after full bloom and in 2014 at 26 d (D), 49 d (E), and 69 d (F) after full bloom (DAFB). Real-time quantitative reverse transcriptase PCR results were analyzed using the 2^(-ΔΔCT) method using *Ubiquitin C (UBC)* as an internal control. Statistical significance was determined using the two-tailed Student's *t* test. Different letters indicate significantly different values to the control (*P* < 0.05).

MdFT1, 2. Therefore, the decline in *MdFT1, 2* transcript levels as a result of spur defoliation suggests that leaves are the major site of *MdFT1, 2* production in apple, although our data suggest there may be other sources. Two observations led us to make this suggestion. First, flower formation occurred in defoliated spurs in both years, and defoliated spurs showed similar flower formation compared with the control in 2014. Second, *MdFT1, 2* transcript levels were only reduced in defoliated spurs on one sample date in 2013 (24 DAFB) and one sample date in 2014 (49 DAFB). On all other sample dates, transcript levels were unaffected (Fig. 2). If *MdFT1, 2* transcripts are necessary for flower formation, then these must have originated from other sources to be present in apical buds of defoliated spurs.

Logically, treatments that reduce flowering, such as defoliation, might be expected to increase transcripts of negative regulators of flowering, such as *MdTFL1*. Previously, defoliation of bourse shoot apices 49 DAFB led to a large increase (13-fold) in *MdTFL1-2* 13 d after leaf removal (Haberman et al., 2016). However, in this study, defoliation at petal fall led to a consistent reduction in *MdTFL1-1* transcripts compared with controls in almost all sample dates in both years (Fig. 3). Therefore, we suggest that local leaves may be more important for the expression of *MdTFL1-1* than the expression of *MdFT1, 2*.

Both the crop load and GA application showed a similar positive effect on *MdTFL1-2* expression level (Haberman et al., 2016). In this experiment, reduction in flower formation in response to local GA₄₊₇ application was observed in both years and was accompanied by significant increases in *MdTFL1-1* transcript levels 46 and 66 DAFB in 2013 and 24

DAFB in 2014 (Fig. 3). On the other hand, in five of the six sample dates across both years, exogenous GA₄₊₇ application did not affect *MdFTL1*, 2 transcript levels compared with the control (Fig. 2). Hence, we suggest that exogenous GA₄₊₇ application inhibits flowering in ‘Honeycrisp’ apple by promoting *MdTFL1-1* expression (on two sample dates in 2013 and one sample date in 2014) and acts in addition to the role of GAs in downregulating the SOC1 transcription factor in apical buds (Xing et al., 2015).

All treatments were located on the same trees. We ensured that treated spurs were at least 20 cm from each other to minimize interactions among them. Although it is possible that one treatment affected another, this would seem unlikely to have played a major role in determining the results for two reasons. First, any effects would be reflected in the controls, and all results were compared with controls. Second, any distant effects would seem to be minor in comparison with the local spur treatment effect.

In conclusion, this study suggests that exogenous GA₄₊₇ application inhibited flowering by increasing the expression levels of a negative regulator of flowering, *MdTFL1-1*, in apical buds 46 and 66 DAFB in 2013 and 24 DAFB in 2014 (Fig. 3). Defoliation, on the other hand, reduced the flowering promoters (*MdFTL1*, 2) 24 DAFB in 2013 and 49 DAFB in 2014 and reduced the flowering inhibitor (*MdTFL1-1*) in all sample dates in 2013 and in two of three sample dates in 2014 (Figs. 2 and 3). We propose that flower-promoting signals are not localized only within the spurs but have different sources within the tree. Moreover, the leaves in the vicinity of flower buds regulate expression pattern of *MdTFL1-1* more than the expression of *MdFTL1*, 2.

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