Different flowering response to various fruit loads in apple cultivars correlates with degree of transcript reaccumulation of a TFL1-encoding gene

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Running title: Alternate bearing in apple

Keywords: flowering, *Malus domestica*, alternate bearing, genetic variation, TERMINAL FLOWER 1, fruit load.

Summary

In many perennial fruit trees, flowering in the year following a year with heavy fruit load can be quite limited. This biennial cycle of fruiting, termed alternate bearing, was described 170 years ago in apple (*Malus domestica*). Apple inflorescences are mainly found on short branches (spurs). Bourse shoots (BS) develop from the leaf axils of the spur. BS apices may terminate ~100 days after flowering, with formation of next year's inflorescences. We sought to determine how developing fruit on the spur prevent the adjacent BS apex from forming an inflorescence. The presence of adjacent fruit correlated with reaccumulation of transcript encoding a potential flowering inhibitor, MdTFL1-2, in BS apices prior to inflorescence initiation. BS apices without adjacent fruit that did not flower due to late fruitlet removal, neighboring fruit on the tree, or leaf removal, also reaccumulated *MdTFL1-2* transcript. Fruit load and gibberellin (GA) application had similar effects on the expression of *MdTFL1-2* and genes involved in GA biosynthesis and metabolism. Some apple cultivars are less prone to alternate bearing. We show that the response of a BS apex to different numbers of adjacent fruit differs among cultivars in both *MdTFL1-2* accumulation and return flowering. These results provide a working model for the further study of alternate bearing, and help clarify the need for cultivar-specific approaches to reach stable fruit production.

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INTRODUCTION

In many plants, environmental cues perceived by leaves promote a flowering response termed 'flower induction'. The output is a mobile signal, termed 'Florigen', traveling from the leaf to the apical meristem and changing the latter's fate from leaf to inflorescence formation (Lang *et al.*, 1977). A protein component of this signal is termed FLOWERING LOCUS T (FT) in *Arabidopsis thaliana* (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Corbesier *et al.*, 2007; Tamaki *et al.*, 2007). A similar protein with an opposite function, named CENTRORADIALIS in *Antirrhinum majus* (Bradley *et al.*, 1996) and TERMINAL FLOWER1 (TFL1) in *Arabidopsis* (Shannon and Meeks-Wagner, 1991; Bradley *et al.*, 1997), seems to maintain indeterminacy of the inflorescence meristem as well as delay the floral transition in *Arabidopsis*. Abnormally high levels of FT or reduced function/levels of TFL1 cause the inflorescence meristem to transform quite rapidly into a flower primordium, thus terminating the meristem. The function of TFL1-like proteins has been shown to be important in many other annuals (Tamaki *et al.*, 2007; Putterill *et al.*, 2013) and perennials (Lifschitz *et al.*, 2006; Hsu *et al.*, 2011; Freiman *et al.*, 2012; Iwata *et al.*, 2012; Koskela *et al.*, 2012; Higuchi *et al.*, 2013).

The apple genome contains two FT and two TFL1-encoding genes (Mimida *et al.*, 2009; Kotoda *et al.*, 2010). Transgenic knockdown of TFL1-encoding genes results in precocious flowering (no juvenile phase), terminal single flowers and continuous flowering during the year (Kotoda *et al.*, 2006; Sasaki *et al.*, 2011; Flachowsky *et al.*, 2012). Ectopic overexpression of *MdFT1* (Kotoda *et al.*, 2010), *MdFT2* (Traekner *et al.*, 2010) or *Arabidopsis FT* (Yamagishi *et al.*, 2011) in apple also causes precocious flowering.

Perennials that flower once a year require a mechanism to time flower induction and initiation to a specific season (Bergonzi and Albani, 2011; Samach and Smith, 2013). In some cases, inductive environmental cues may act through an increase in FT, such as in mango (*Mangifera indica*; Nakagawa *et al.*, 2012), or through a reduction in TFL1 as shown in rose (Iwata *et al.*, 2012) and strawberry (*Fragaria vesca*; Koskela *et al.*, 2012). In apple, the formation of inflorescence meristems occurs approximately 100 days after full bloom (DAFB) (Foster *et al.*, 2003). Following anthesis, expression of one of the TFL1-encoding genes, *MdTFL1-1*, decreases within the apices, plateauing well before initiation of the following year's flowers (Hattasch *et al.*, 2008; Mimida *et al.*, 2009, 2011; Kotoda *et al.*, 2010).

In many perennial fruit trees, flowering is quite limited after a year with heavy fruit load (HFL), while flowering is abundant after a year with few fruit (Monselise and Goldschmidt, 1982; Smith and Samach, 2013). This biennial cycle of fruiting, termed 'alternate bearing', is often detrimental in commercial orchards because fruit size in HFL can be below commercial standards. Without farmer intervention (thinning of HFL trees), most apple cultivars eventually enter this cycle of flowering and fruiting (Durand *et al.*, 2013; Figure 1a,b). Among many other traits, apple breeders try to select for cultivars with cycles of lower amplitude (Guitton *et al.*, 2012; Durand *et al.*, 2013).

An apple inflorescence contains 4–6 flowers; the number of fruitlets reaching maturity within an inflorescence depends, among other things, on pollination and genotype (Eccher *et al.*, 2013; Celton *et al.*, 2014). Fruit of important cultivars develop mostly on inflorescences formed on spurs (Butler, 1917; Forshey and Elfving, 1989). Spurs are short branches (having limited internode elongation) formed during 2 or more years of sympodial growth (Smith and Samach, 2013). Dormant inflorescence-containing spur buds are released in the spring. After anthesis, one or two shoots (termed 'bourse shoots'; BS) develop from

lateral meristems formed the previous summer in the axils of the first expanded leaves, below the terminal inflorescence (Mehri and Crabbe, 2002; Figure 1c–e). BS contain a few expanded leaves separated by short internodes, followed by bud scales and leaf primordia (first leaves of the following year's flush), and will either terminate with an inflorescence or remain vegetative. The number of primordia formed below the terminal flower is relatively uniform (16–21), with slight differences between genotypes (McLaughlin and Greene, 1991; Costes, 2003). Together with a set plastochron (Fulford, 1966), the time between anthesis and initiation of the following year's inflorescence seems to be programmed and less influenced by the environment (Wilkie *et al.*, 2008). Nevertheless, the presence of developing fruit adjacent to BS severely inhibits inflorescence formation (Figure S1). When most spurs on a tree contain developing fruit, this will lead to alternate bearing (Auchter and Schrader, 1923; Jonkers, 1979). In annuals such as *Arabidopsis*, although time to floral transition can be delayed or hastened, it eventually occurs. In apple, time of inflorescence initiation (TII) is defined more or less by DAFB, but the decision every year is binary: some meristems will go through the transition while others will not.

Here, our aim was to reveal how fruit load interferes with the molecular events leading to inflorescence formation in apple, and whether these events vary with genotype. We report that the presence of adjacent fruit leads to the reaccumulation in BS apices of transcript encoding MdTFL1-2, a flowering repressor. Variations in alternate bearing amplitude among cultivars correspond to differences in *MdTFL1-2* reaccumulation. Taken together, our results provide a working model for the further study of alternate bearing, and suggests points of intervention in apple breeding and management.

RESULTS

Apple inflorescence initiation in response to different levels of crop load

In the spring, heavy-flowering cultivar 'Top Red' trees (Figure 1a) were selected in an experimental orchard and all flowers were removed at full bloom (complete flower/fruitlet removal, designated CFR at 0 DAFB). The following spring, released buds were recorded on the spurs of those trees and close to 100% contained inflorescences (Figure 2a). Flower initiation was also followed in samples of BS apices from those trees during the summer by scanning electron microscopy (SEM; Figures 3 and S2). Initially, the relatively flat meristem produced leaf primordia (Figure 3a). The first noticeable change in the apical meristem occurred when no new leaf primordia formed, causing its enlargement and bulging (doming, Figures 3b and S2a–c). Later stages of inflorescence development are shown in Figures 3c and S2d–l. In the summer, doming meristems were identified at 98–113 DAFB. The timing of these events, relative to day of full bloom, was similar to that described for 'Royal Gala' in New Zealand (Foster *et al.*, 2003), with slight differences in morphology or its interpretation (Figure S2).

The level of transcript encoding the regulator of flower meristem identity APETALA1 (*MdAP1a*, *MdAP1b*; Mimida *et al.*, 2011) and the regulator of flower organ identity PISTILLATA (*MdPI*; Yao *et al.*, 2001) increased in BS apices taken from CFR at 0 DAFB trees (Figure 4a–f). This increase occurred during the period of inflorescence formation (based on SEM monitoring; Figures 3 and S2). Thus, BS apices of 'Top Red' begin forming inflorescences under local conditions at ~94–100 DAFB.

The fate of BS apices, specifically those subtending a spur with at least two fruit reaching commercial harvest, was measured in trees with heavy flowering and no fruit thinning, and thus with HFL. Here, the percentage of BS that formed inflorescences reaching anthesis the following spring was 0 (Figure 2a). A tree that was previously under HFL still formed several inflorescences the following year (Figures 1b and S3a), likely on spurs that developed no fruit. When studying SEM micrographs from random BS taken from HFL trees at the end of the summer, most remained vegetative (Figure 3d). BS apices randomly collected from HFL trees expressed much lower levels of *MdAPIa*, *MdAPIb* and *MdPI* transcript when their expression was increasing in BS apices of trees with no fruit (Figure 4a–f).

For a limited period, young fruitlet removal allows return bloom on the branch (Harley *et al.*, 1935). To determine the time at which the fate of the BS apex is influenced by HFL, the last day on which CFR could still lead to a heavy return bloom was roughly determined. In addition to CFR at 0 DAFB, heavy blooming trees in 2013 were subjected to CFR at a later date, 36 or 80 DAFB, and return bloom was measured the following spring (Figures 2a and S3a). The fate of BS apices was not set before 36 DAFB, since CFR on this date completely reversed the fate of their meristems: almost all (96%) of the BS had a return bloom (Figure 2a). In contrast, CFR at 80 DAFB only partially reversed the fate of the meristems (Figure 2a). This suggests that at least some irreversible events that determine meristem fate occurred before this time.

Expression of FT-encoding genes is not affected by fruit load

In several species, an increase in FT-encoding transcript in leaves is associated with flower induction, an event preceding inflorescence initiation (Andres and Coupland, 2012). If this event can be detected in apples, does a similar event occur in trees under HFL? The

expression of two genes encoding proteins similar to FT, *MdFT1* and *MdFT2*, was determined in three different tissues (see Experimental Procedures): two types of leaves, and BS apices (Figure 1c–e); and under two different regimes of fruit load: HFL and CFR on 0 DAFB. For both genes, in both treatments, highest expression was detected in developing BS apices and not in leaves (Figures S4a,b and S5a,b). In BS apices, expression of *MdFT1* increased gradually and substantially with time in trees with or without fruit, peaking 68–83 DAFB (Figure S4c). The rise in *MdFT2* expression in BS occurred only on the last sampling date, 126 DAFB, in trees with or without fruit, in both years tested (Figure S5c,f). Neither gene's expression increased significantly in the leaves during the measurement period (Figures S4d,e and S5d,e). An event in the leaves preceding TII could therefore not be defined, and a clear effect of fruit load was not detected on the expression of either FT-encoding gene. Thus, additional markers were sought.

Reduction in *MdTFL1-1* expression in BS apices prior to flower initiation

Expression of the two genes encoding TFL1 in apple was studied. In trees in which all fruit had been removed, *MdTFL1-1* was predominantly expressed in BS apices (Figure S6a). The highest expression levels were measured near bloom, followed by a steep decrease with time, plateauing at 68 DAFB (Figure 4g), as shown previously (Mimida *et al.*, 2009; Kotoda *et al.*, 2010). Since *MdTFL1-1* seems to be a flowering inhibitor, reduced expression in BS may serve as an internal clock to time inflorescence initiation in apple. Nevertheless, a similar reduction in *MdTFL1-1* expression occurred in trees with HFL (Figure 4g). Using semi-quantitative RT-PCR, others have reported an effect of fruit load on this gene's expression in apices of 1-year-old shoots (Kittikorn *et al.*, 2011). Here, *MdTFL1-1* levels decreased more rapidly in trees with HFL (Figure 4g); this difference could not explain why these BS apices remain vegetative.

Increased *MdTFL1-2* expression in BS apices adjacent to fruit

In CFR at 0 DAFB trees, *MdTFL1-2* was highly expressed in BS apices during anthesis. At other time points and in all other tissues examined, *MdTFL1-2* expression was basal (Figure S7a). Using semi-quantitative RT-PCR, others have shown an expression pattern more similar to that of *MdTFL1-1*, albeit at lower levels (Mimida *et al.*, 2009). However, in HFL trees preceding TII, transcripts of *MdTFL1-2* reaccumulated in BS apices (Figures 4h,i and S7b). The first date on which expression was significantly higher in trees with fruit compared to trees without fruit varied slightly between sampling years (Figure 4h,i); nevertheless, this striking reaccumulation of *MdTFL1-2* in HFL BS apices was consistent over 3 years of sampling (Figures 2b,d and 4h,i). By 94 DAFB, *MdTFL1-2* levels in BS apices of HFL trees were up to 80-fold higher than those in BS apices of CFR at 0 DAFB trees (Figure 2b).

***MdTFL1-2* accumulation in spurs without fruit**

The presence of fruit inhibits inflorescence initiation in adjacent BS apices and increases *MdTFL1-2* levels in those apices before TII. The two responses could be independent or connected: a fruit-load-dependent increase in *MdTFL1-2* in BS apices toward TII might determine the vegetative fate of those meristems. We looked for cases in which prevention of inflorescence initiation occurs in the absence of adjacent fruit, and asked if in those cases, *MdTFL1-2* accumulation also precedes TII.

As already noted, inflorescence initiation was not inhibited when fruitlets were removed at 0 or 36 DAFB. On the other hand, fruitlet removal at 80 DAFB was too late to allow complete inflorescence initiation (Figure 2a), suggesting that at this time point, the vegetative fate of the meristem carrying adjacent fruit was already partially determined. BS

apices were sampled 94 DAFB from trees in which fruit had been removed at the different time points. At 94 DAFB, only HFL trees carried fruit, but *MdTFL1-2* levels in BS apices were also high in trees from which fruit had been removed at 80 DAFB (Figure 2b). Thus, *MdTFL1-2* levels in BS apices were correlated with meristem fate, and not with concurrent fruit presence (Figure 2a,b).

Previous research (Luckwill, 1970; Li *et al.*, 2003) has suggested that the fate of the BS apex also depends on the amount of developing fruit in neighboring spurs or in general, on overall fruit load per tree. We studied return flowering and *MdTFL1-2* expression in BS apices without fruit in a tree with moderate fruit load. Forty pairs of neighboring spurs were marked; all fruitlets were removed from one of the spurs 8 DAFB, thus generating pairs of adjacent spurs: one with HFL and the other with no fruit. For the spurs in each pair that developed several fruit, return bloom was 4% (Figure 2a), similar to a HFL tree (Figure 2a). For the spurs from which fruitlets were removed at 8 DAFB, return bloom reached 57% (Figure 2a). In the BS apices of the latter spurs at 94 DAFB, *MdTFL1-2* expression was significantly higher than in spurs taken from trees in which all flowers/fruit had been removed at 0 DAFB (Figure 2b). Both expression and flowering results suggest that the fate of the BS apex depends heavily on conditions within the spur, i.e., presence/absence of adjacent fruit and on overall fruit load per tree.

Removing BS leaves at certain times before TII prevents flowering of spurs with no fruit (Harley *et al.*, 1942). In trees with heavy flowering, localized CFR was performed at 0 DAFB in 160 spurs. In 20 of those spurs, leaves were removed from the BS apex at 49 DAFB. Return flowering was 18% in HFL spurs and 66% in spurs in which flowers were removed at 0 DAFB (Figure 2c). On the other hand, removing all leaves from the BS apex in spurs without fruit reduced return bloom from 66% to 1.8% (Figure 2c). Leaf removal also caused a 13-fold increase in *MdTFL1-2* levels in BS apices 62 DAFB, only 13 days after leaf

removal (Figure 2d). In the absence of developing fruit, local leaf removal in a BS will cause an increase in *MdTFL1-2* levels and prevent the floral transition.

Exogenous application of gibberellin (GA) inhibits flowering in apple (Guttridge, 1962). At 30 DAFB (2013) or 34 DAFB (2014), 200 ppm GA₃ was sprayed on spurs with no fruit. Before GA treatment, localized CFR was performed at 22 DAFB in 2013 from trees with moderate flowering, or at 0 DAFB in 2014 from trees with heavy flowering. In the BS apex, the GA₃ treatment caused a relatively rapid change in expression of GA-responsive genes shown to be involved in a negative feedback loop that maintains GA homeostasis (Yamaguchi, 2008): an increase in GA deactivation (GA2 OXIDASE; Figure 5a,b) together with a decrease in GA biosynthesis (GA20 OXIDASE; significant reduction in 2014, Figure 5c,d). The GA₃ treatment seemed to have a long-term effect on the BS apex because it caused a significant increase in *MdTFL1-2* levels at 90–94 DAFB (Figure 5e,f). In addition, this single application caused a reduction (albeit not significant) in flowering (Figure 5g,h).

Others have suggested that fruit load inhibits apple flowering through an increase in active GA levels (Bangerth, 2009). BS apices with or without fruit have different expression levels of the aforementioned GA-responsive genes. The presence of adjacent fruit caused a response similar to that of GA₃ application: an increase in expression of a gene encoding GA2 OXIDASE (Figure 6a,b) and a decrease in expression of two genes encoding GA20 OXIDASE (Figure 6d,e,g,h). In some cases, the initial differences in these GA-responsive genes' expression between treatments occurred earlier than the detected differences in *MdTFL1-2* (Figure 4h,i, Table S1). Thus, fruit load causes the accumulation of *MdTFL1-2* in BS apices, but prior to this accumulation, other fruit-load-related changes in BS apices may be evident.

Genetic variation in response to different fruit loads

The degree of alternate bearing differs among commercial apple cultivars (Atay *et al.*, 2013). Some of this variation was measured using the parameters and treatments developed above in 'Top Red'. A similar effect of fruit load on the expression of *MdTFL1-2* (Figure 4j) and the abovementioned GA metabolism genes (Figure 6c,f,i) was detected in cultivar 'Golden Delicious'.

A previous report on hand thinning of 'Fuji'/'M9' trees over a 5-year period showed that leaving one fruit per spur results in 37–64% return flowering on the spurs; leaving two fruits led to no flowering, whereas leaving no fruit led to 80–90% return flowering (Davis, 2002). When comparing 'Golden Delicious' and 'Top Red' trees in which all spurs were hand-thinned to leave one flower per spur, the expression of *MdTFL1-2* in BS apices was intermediate to high in 'Top Red' (Figure 2b) and very low (similar to BS apices with no adjacent fruit) in 'Golden Delicious' (Figure 7b). Return flowering was 21% in 'Top Red' (Figure 2a) and much higher in 'Golden Delicious' (Figures 7a and S3b). This suggests that natural variation in the response to different levels of fruit load may be a result of differences in *MdTFL1-2* accumulation rate in BS apices.

We further compared cultivar 'Granny Smith' to 'Top Red', this time studying the effect of localized complete or partial (leaving one or two fruitlets) flower/fruit removal from 120 spurs per tree. A change in the local number of fruitlets per inflorescence significantly affected both *MdTFL1-2* levels and return bloom in both genotypes (Figure 7c,d). In all three treatments, *MdTFL1-2* levels in BS apices were significantly lower in 'Granny Smith' than in 'Top Red' (Figure 7d), whereas percent return bloom was significantly higher in the former (Figure 7c). Variation in the *MdTFL1-2* gene is probably not the cause for variation in

flowering response to different fruit loads, since we did not identify any linkage between a particular *MdTFL1-2* allele and regular bearing (Table S2, Figure S10, Data S2, S3).

DISCUSSION

Alternate bearing in apple has been studied for at least 170 years (Cole, 1849; Butler, 1917), as it is a major constraint on fruit production. Links revealed here between fruit load, *MdTFL1-2* accumulation and delay of flowering offer a new working model for the study of this phenomenon (Figure S8).

Recent research in mango, citrus (*Citrus reticulata*) and avocado (*Persea americana*) has suggested that fruit load prevents flowering in the following year by inhibiting the expression of FT-encoding genes in leaves, stems or buds (Muñoz-Fambuena *et al.*, 2012; Nakagawa *et al.*, 2012; Nishikawa *et al.*, 2012; Shalom *et al.*, 2012; Ziv *et al.*, 2014). In these non-deciduous species, transcripts of FT-encoding genes accumulate during the winter, likely in response to cold temperatures (Nakagawa *et al.*, 2012), before the initiation of inflorescences. In mango trees with previous HFL, *FT* expression was at least eightfold lower (Nakagawa *et al.*, 2012).

In apple, as well as most other deciduous trees, the tree enters winter with buds containing flower primordia formed during the summer. These primordia will reach anthesis after winter dormancy, in the subsequent spring. Initial morphological changes in BS apices during the formation of the apple inflorescence (TII) are detected at 98–113 DAFB.

In our experiments, we did not identify any fruit load effect on the expression of the two FT-encoding genes, *MdFT1* and *MdFT2*. Higher levels of *MdFT1* expression were found in BS apices than in the leaf subtending the BS. In these apices, expression increased at least 6.5-fold from 14 to 83 DAFB, in trees with or without fruit. This initial increase, followed by a gradual decrease from the end of July until January, has been described previously (Kotoda

et al., 2010). *MdFT1* could be involved in shoot growth, similar to the FT-encoding gene described in poplar (*Populus* spp., Hsu et al., 2011). On the other hand, it could be necessary for flower induction, as it peaks before TII. A rise in *MdFT2* expression in BS apices occurred in both treatments on the last sampling date, after TII. This pattern is similar to that described for *MdFT2* in apical buds of fruit-bearing shoots (Kotoda et al., 2010). This increase in expression may be seasonal, occurring toward dormancy, and not related to flowering.

A gradual decline in *MdTFL1-1* transcript levels from 0 to 68 DAFB (Figure 4g; Kotoda and Wada, 2005) may serve as the internal clock that determines TII in apples (Mimida et al., 2009; Flachowsky et al., 2012). A seasonal reduction in TFL1 was shown to control time of flowering in rose and strawberry (Iwata et al., 2012; Koskela et al., 2012). Using semi-quantitative RT-PCR, others noticed an effect of fruit load on this gene's expression in apices of 1-year-old shoots at one time point (Kittikorn et al., 2011). However, this effect was not observed here in BS apices, where many time points were sampled over several years using a more sensitive quantitative assay.

Similar to *MdTFL1-1*, expression of *MdTFL1-2* in BS apices is high at anthesis, and then declines. While *MdTFL1-1* expression remains low toward TII and is unaffected by fruit load, we show that *MdTFL1-2* transcripts may reaccumulate in BS apices. Our experiments suggest that there are at least two major fruit-related parameters determining the fate of a specific BS apex: the number of fruit developing on the spur and the overall fruit load per tree. Interestingly, the contribution of each of these parameters to the following year's flowering in apple can be estimated by measuring the reaccumulation of *MdTFL1-2* in BS apices toward TII.

This reaccumulation was also detected in the absence of fruit in the spur due to late fruit removal (80 DAFB), GA₃ application or BS leaf removal at 49 DAFB. Again, in all three cases, the effect on return flowering could be predicted by measuring *MdTFL1-2* in BS apices before TII. A strong negative correlation (Figure 8), based on data from two different years using two different cultivars, was found between *MdTFL1-2* levels at 90–94 DAFB and flowering the following spring.

Evidence that both TFL1-encoding apple genes are expressed in the meristem has been provided by RNA in-situ hybridization (Mimida *et al.*, 2011). The ability of both apple proteins to inhibit flowering in *Arabidopsis* and complement the *tfl1-1* mutation has been previously shown (Kotoda and Wada, 2005; Mimida *et al.*, 2009). Meristems in transgenic apple trees in which both TFL1-encoding genes have been knocked down seem to lose all timing restrictions, forming floral meristems randomly and consistently (Kotoda *et al.*, 2006; Sasaki *et al.*, 2011; Flachowsky *et al.*, 2012). In BS that reaccumulate *MdTFL1-2*, there does not seem to be any period during which expression of both TFL1-encoding genes is low. Thus, our working hypothesis is that fruit load prevents flowering through reaccumulation of *MdTFL1-2* in BS apices (Figure S8).

However, the minute, one-flower "inflorescence" and low fruit production of *MdTFL1* knockdowns prevent the use of these transgenic lines to study the effect of fruit load in the absence of both genes. Our results warrant studying the phenotype of apple trees in which only *MdTFL1-2* is knocked down or out. Since both genes are expressed in juvenile tissue (Mimida *et al.*, 2009), a tree with intact *MdTFL1-1* may maintain a juvenile period (which could last several years) and inflorescence initiation could be restricted to once a year. Perhaps the only extraordinary phenotype would be high levels of flowering in BS apices from spurs with HFL. Even using new approaches, such as the CRISPR-Cas9 system, obtaining a flowering tree with this genotype will likely take several more years.

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Shading apple trees (Auchter *et al.*, 1926) or removing BS leaves at certain times before TII (Harley *et al.*, 1942; Figure 2c) will prevent flowering of spurs with no fruit. Spur leaves begin to export carbohydrates within 10 days of growth onset (Hansen, 1977). Perhaps photosynthetically active leaves export a negative regulator of *MdTFL1-2* expression to the BS apex. This might explain why *MdTFL1-2* transcript levels in BS apices are relatively high at 0 DAFB and decrease thereafter, and why leaf removal at 49 DAFB caused an increase in *MdTFL1-2* levels. This putative leaf-derived negative regulator of *MdTFL1-2* expression might be a carbohydrate. There is some evidence that an artificial increase in sugars increases flowering in apple (Lakso, 1994).

Exogenous application of GA can inhibit flowering in apple (Guttridge, 1962; Marcelle and Sironval, 1963; Tromp, 1982). Here, inhibition of flowering caused by exogenous application of GA₃ was relatively weak, perhaps because it was provided as a single application to specific spurs. Fruit load and exogenous application of GA₃ had a similar effect on expression of certain genes encoding GA2 OXIDASE, GA20 OXIDASE and *MdTFL1-2*, and on inhibition of return flowering. Significant changes in *MdTFL1-2* levels were mostly detected after changes in the GA-related genes. Clearly, changes in *MdTFL1-2* levels are not the first response to the above perturbations. Inhibition of Apple flowering by application of exogenous GA might require changes in expression of other/additional genes.

GA inhibition of rose flowering appears to be mediated by induction of a TFL1-encoding gene (Randoux *et al.*, 2012), and this might also be the mode of GA inhibition of flowering in apple. The observed differences in GA-related gene expression in response to fruit load do not necessarily indicate whether there is more, less or the same amount of active

GA in BS apices of HFL spurs. Although the observed changes should lead to a lower amount of active GA in these apices, they could be a result of higher levels of active GA in these apices triggering a negative feedback loop aimed at maintaining GA homeostasis (Yamaguchi, 2008). A popular model of alternate bearing suggests that seed-derived GAs are the transmissible signal that causes flower inhibition (Luckwill, 1970). Our findings cannot refute or support such a model. If active GAs are indeed accumulating in the BS apices of HFL spurs (as suggested by others), this accumulation might contribute to reaccumulation of *MdTFL1-2* levels.

The amplitude of alternate bearing (variance in annual yield) differs among apple cultivars (Lauri et al., 1995). Annual yield is affected by additional parameters, such as natural and chemical flower/fruitlet thinning, percentage of bud release from dormancy, number of flowering spurs per tree and fruit size. Our comparison of cultivars was not based on variance in annual yield, but on how a BS apex responds to different numbers of adjacent fruit on the spur, or to overall fruit load per tree. In all three cultivars, an increasing number of fruit on the spur gradually decreased the chances of the BS apex entering the floral transition. While two or more fruit led to a non-flowering fate in 'Top Red' and 'Golden Delicious', some BS apices in 'Granny Smith' still flowered when spurs contained two developing fruit. Indeed 'Granny Smith' is less alternate bearing, based on variance in annual yield (Atay *et al.*, 2013). In practice, to achieve consistent yields in 'Top Red', flowering/fruitlet needs to occur in alternate spurs every year, since a spur with even one fruit has a very low chance of return bloom. In 'Golden Delicious', keeping a single fruit appears to be sufficient for return bloom, and therefore a strong thinning program that leaves no more than one fruit per spur should be enough to provide consistent fruit yields.

For all fruit amounts (none, one, two), each genotype responded differently with respect to both *MdTFL1-2* reaccumulation and return flowering. When comparing 'Top Red' and 'Granny Smith', the presence of additional fruit increased *MdTFL1-2* levels in both genotypes, but levels in 'Top Red' were always higher, matching the lower rate of return bloom. This suggests that the two cultivars do not differ in their response to a certain level of *MdTFL1-2*, but may differ in the level of transcript accumulated. Interestingly, the effect of each additional fruit was not stronger in 'Top Red'. Local removal of all fruit from the spur led to significantly higher levels of *MdTFL1-2* in BS apices of 'Top Red' compared to 'Granny Smith' at 90 DAFB. Thus, differences in basal (with no adjacent fruit) reaccumulation of *MdTFL1-2* in BS apices might explain the different flowering behaviors of these two cultivars. Since this was a local treatment and not uniform removal of all fruitlets from the tree, the difference in basal reaccumulation of *MdTFL1-2* levels may be due to unmeasured differences in overall fruit load per tree. If overall fruit load per tree was similar, the two cultivars may differ in their local interpretation of this parameter. Such a difference is not likely to be caused by a unique *MdTFL1-2* allele. We did not find any *MdTFL1-2* allele linked to regular bearing. In addition, previously identified QTLs that seem to affect the degree of alternate bearing in apple map to regions that do not contain the two TFL1-encoding genes (Guitton *et al.*, 2012).

In summary, variations in alternate bearing amplitude among cultivars may be a result of differences in *MdTFL1-2* reaccumulation in response to different fruit loads or in basal levels of *MdTFL1-2* in apices. Identifying alleles of genes that cause of these differences will likely help guide breeding programs. The knowledge obtained here regarding the response of the cultivars studied may already help improve their performance, by tailoring specific thinning procedures.

EXPERIMENTAL PROCEDURES

Plant material and treatment

The experiments were conducted over three different years (2011, 2013 and 2014) in a commercial apple orchard, planted in 1997, at the 'Matityahu' research station of the Agricultural Research Organization (ARO) in Northern Israel (33°04'04"N, 35°27'04"E, altitude 667 m). We studied 'Top Red' (a clone of 'Red Delicious') grafted on rootstock 'M9' over three seasons (2011, 2013, 2014), 'Golden Delicious', also on 'M9' during two seasons (2013 and 2014), and 'Granny Smith' grafted on 'Hashabi 13-4' (local rootstock) during 2014. All trees were grown and pruned according to commercial practice. A list of experiments is provided in Table S3.

Trees in the different experiments were selected in the spring based on level of flowering. For most experiments, selected trees were with relatively high and uniform flowering (800–1000 spurs with inflorescences). Flowers/fruitlets were not hand or chemically thinned, unless this was the specific treatment. DAFB, specific to the orchard conditions, cultivar and year, was determined as the day with the highest number of flowers at anthesis: for 'Top Red' it was 27, 14 and 8 April in 2011, 2013 and 2014, respectively; for 'Golden Delicious', 8 April 2013, and for 'Granny Smith', 8 April 2014.

At full bloom (0 DAFB), 36 or 80 DAFB, all flowers/fruitlets were removed (CFR) from five trees on each date (five biological repeats). Following the removal, 100 spurs from which flowers/fruitlets were removed were selected from each tree and marked for RNA sampling or measurement of percent flowering spurs the following spring. The same number of similar types of spurs was selected from five additional trees in which no flowers or fruitlets were removed (HFL), each containing at least two fruits until harvest. In five

additional trees, all inflorescences were hand-thinned, leaving one flower (preferably the terminal 'king' flower), so that each fruit-bearing spur contained a single fruit. All treatments were performed on 'Top Red', and CFR, HFL and single-fruit treatment were also performed on 'Golden Delicious'.

In five additional 'Top Red' trees, chosen for moderate flowering (~500–800 spurs with inflorescences), 40 pairs of neighboring spurs, randomly distributed in the tree, were selected. All fruitlets were removed 8 DAFB from one of the spurs in the pair (localized CFR 8; spur without fruit), leaving the neighboring spur with at least two fruits (HFL).

In four 'Top Red' and four 'Granny Smith' trees chosen in 2014 for a similar high rate of flowering, 120 flowering spurs were selected and marked at 0 DAFB. At 26 DAFB, fruitlets in the selected spurs were randomly thinned to maintain either two, one or no fruit (40 spurs per treatment).

The flower/fruit were removed by cutting the pedicel/fruit stalk with secateurs. Chemical thinning is described in Figure S3b.

Leaf removal

In 2014, in five 'Top Red' trees chosen for high flowering rate, all flowers were removed from 160 spurs at full bloom (localized CFR 0). From these spurs, 20 were randomly selected and at 49 DAFB, all leaves from the axis of the BS were removed, by cutting the petioles with secateurs.

Tissue sampling

Four spurs were sampled from each tree at every time point from spring until autumn. Three different tissues from these spurs were collected: the leaf that subtends the BS (designated spur leaf), the second-most mature leaf of the developing BS (BS leaf), and the BS apex from which all true leaves were removed (scales and primordial leaves intact; see Figure 1d,e).

Flowering measurements

Following spring/summer treatments, the effect on flowering was determined the following spring, at full bloom. Previously marked spurs in trees (9–28 per treatment) were examined and scored as either flowering (containing an inflorescence) or vegetative (producing only leaves), and percent flowering spurs was calculated. Rate of flowering was also scored at the tree level by visual assessment. Each tree was given a flowering score on a scale of 0–5, '5' representing the highest flowering volume.

Application of GA

In the 2013 experiment, six 'Top Red' trees with moderate bloom (~300–500 spurs with inflorescences) were chosen and 22 DAFB, all fruitlets were removed from 40 spurs per tree. In three trees, these spurs were sprayed with 200 ppm GA₃ and 0.025% (v/v) of the surfactant Triton X-100 (Sigma) on 30 DAFB. In the three remaining trees, these spurs were sprayed with surfactant only. In the 2014 experiment, 'Top Red' trees with a high rate of flowering were chosen and in each tree, on 0 DAFB, all fruitlets were locally removed from 160 spurs (localized CFR 0). In five trees, 50 of these spurs were sprayed with GA₃ solution on 34

DAFB. Five additional trees were not sprayed (control). GA₃ (200 ppm) was prepared by adding 0.5 ml of the commercial product 'Giberlon' (40,000 ppm; Fine Agrochemicals Ltd., Worcester, England) to 100 ml water. Spurs were sprayed at around noon with a hand-held sprayer until they were drenched.

Statistical analysis

Data from qPCR results, and flowering were analyzed by one-way analysis of variance (ANOVA) using JMP version 10 software (SAS Institute, Cary, NC, USA). Differences between treatments were determined by Student's *t* test. In multiple comparisons, Tukey–Kramer HSD was implemented. Statistical significance was determined at $P \leq 0.05$. In cases where the variance was unequal and the data did not show a normal distribution, the statistical tests were conducted on transformation to ranks of the values.

SEM, RNA extraction, expression analysis and allele sequencing

See Methods S1, in which there is reference to Figure S9, Tables S4 and S5 and Data S1.

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Short legends for Supporting Information

Figure S1. Schematic representation of BS development in the spur branch of 'Top Red' during two years of alternate bearing.

Figure S2. Inflorescence development in 'Top Red' based on SEM images of BS apices sampled from June to October.

Figure S3. Flowering in response to different treatments of fruit load.

Figure S4. Expression of *MdFT1* in three different tissues and under two levels of fruit load during the summer of 2011.

Figure S5. Expression of *MdFT2* in three different tissues and under two levels of fruit load during the summers of 2011 and 2013.

Figure S6. Expression of *MdTFL1-1* in three different tissues and under two levels of fruit load during the summer of 2011.

Figure S7. Expression of *MdTFL1-2* in three different tissues and under two levels of fruit load during the summer of 2011.

Figure S8. Model describing the interactions between gene expression, flower induction and fruit load.

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Figure S9. Expression of *MdTFL1-2* in BS apices from HFL trees, measured relative to *histone 3* or *actin 7*.

Figure S10. Alignment of different *MdTFL1-2* alleles.

Table S1. Timing of molecular events in different treatments.

Table S2. *MdTFL1-2* alleles and bearing habits of different cultivars.

Table S3. List of the different treatments.

Table S4. List of primers for cloning, qPCR and allele sequencing.

Table S5. Testing primer specificity for the two AP1-encoding genes.

Data S1. Sequences of genes analyzed and primer positions

Data S2. Sequences of *MdTFL1-2* alleles.

Data S3. Positions of Polymorphism between *MdTFL1-2* alleles

Methods S1. Additional experimental procedures.

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Figure Legends

Figure 1.

(a,b) Pictures of two adjacent 'Top Red' trees taken at the same date (April 27 2011), one with many flowers (a), the other with very few flowers (b).

(c) A spur with an inflorescence at full bloom; a BS with two to three open leaves can be seen.

(d) Spur with three developing fruit (one was removed) and a BS at 43 DAFB.

(e) Sketch of picture in (d). The BS apex will likely remain vegetative due to the presence of adjacent fruit. In the absence of fruit it may terminate with an inflorescence that will reach anthesis in the subsequent spring.

Figure 2. Flowering and *MdTFL1-2* expression in BS apices in response to different treatments.

Experiments were performed in the summer of 2013 (a,b) and 2014 (c,d) on 'Top Red' trees (see Experimental Procedures). Trees with HFL were not thinned, and selected spurs contained at least two fruits. In OFL trees, all flowers clusters in the tree were thinned to one

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flower at 0 DAFB, containing one fruit until harvest. In CFR trees, all flowers/fruit on a tree were removed at full bloom (0 DAFB) or at 36 or 80 DAFB. ‘Pairs of spurs’ – pairs of proximate spurs were chosen, and in one spur from each pair, all fruitlets were removed at 8 DAFB (LCFR 8 spur), while its neighbor kept at least two fruit until harvest (HFL spur). In localized CFR 0 trees (c,d), all flowers were removed from 160 spurs per tree at 0 DAFB. In 20 of these spurs, all leaves from the axis of the BS were removed at 49 DAFB (LCFR 0, LR 49).

(a,c) Percentage of flowering spurs, as measured in the spring of the year following the treatment. Preselected spurs [$n = 22$ in (a), $n = 18$ in (c)] were scored for presence/absence of inflorescence.

(b,d) Relative expression of *MdTFL1-2* in BS apices collected 94 (b) or 62 (d) DAFB for the same treatments and trees as in (a) and (c), respectively. Relative *MdTFL1-2* expression was measured using quantitative real-time RT-PCR (qPCR) and the housekeeping histone 3-encoding gene (see Methods S1). Numbers are mean values of five independent biological repeats (trees) $\pm$ standard error of the mean (SE; bars).

Different letters represent significant differences according to Tukey-Kramer HSD test on ranked data ($P \leq 0.05$).

Figure 3. BS apical meristems based on SEM images from 'Top Red'.

(a–c) Images of BS apices from trees in which all flowers were removed at full bloom (CFR 0): (a) meristem at 55 DAFB, apical meristem is still forming leaf primordia; (b) meristem at 113 DAFB, apical meristem is forming an inflorescence; (c) meristem at 152 DAFB, sepals of the terminal flower have initiated.

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(d) Image of a BS apex from a tree with HFL at 113 DAFB. Unlike the apex in (b), collected on the same date, this apex is still forming leaf primordia. Each image represents several samples examined for the specific treatment. Ap, apex; L, leaf; Tm, terminal flower meristem; B, bract; Tb, terminal bract; Lm, lateral flower meristem; Bl, bractlet; S, sepal. Scale bar is 0.1 mm.

Figure 4. Expression of flowering-related apple genes in BS apices from trees with or without developing fruit.

Heavy flowering trees were either untreated, thus reaching heavy fruit load (HFL), or all flowers were removed at full bloom (CFR 0). All experiments were with the 'Top Red' cultivar except (j), which was with 'Golden Delicious'. Samples were collected during the summer of 2011 (a,c,e,g,i) and 2013 (b,d,f,h,j).

Relative expression of *MdAP1a* (a,b), *MdAP1b* (c,d), *MdPI* (e,f), *MdTFL1-1* (g) and *MdTFL1-2* (h-j), measured as described in Figure 2b. Numbers are mean values of three to five independent biological repeats (trees) $\pm$ SE (bars). Asterisks represent a significant difference between treatments at a specific time point according to Student's t test on ranked data ($P \leq 0.05$).

Figure 5. Gene expression and flowering response of BS apices to GA₃ application.

Experiments were performed in the summer of 2013 (a,c,e,g) and 2014 (b,d,f,h) on 'Top Red' trees. In each tree, flowers/fruitlets were removed from a number of spurs at a certain date (40 spurs on 22 DAFB, 2013, 160 spurs on 0 DAFB, 2014). In some trees the aforementioned spurs were sprayed with 200ppm GA₃ and in others they were not (surfactant in 2013, no

treatment in 2014). The single GA₃ treatment was given on 30 DAFB (2013) or 34 DAFB (2014). Relative expression of *MdGA2ox2b* (a,b), *MdGA20ox1c* (c,d) and *MdTFL1-2* (e,f) measured as described in Figure 2b. (g,h) Percentage of flowering spurs, as measured in spring, the following year of the treatment. Numbers are mean values of three (2013) or five (2014) independent biological repeats (trees) $\pm$ SE (bars). Asterisks represent a significant difference between treatments at a specific time point according to Student's t test on ranked data ($P \leq 0.05$).

Figure 6. Expression of GA-related apple genes in BS apices from trees with or without developing fruit.

RNA samples are the same as those used in Figure 4. Relative expression of *MdGA2ox2b* (a-c), *MdGA20ox1a* (d-f) and *MdGA20ox1c* (g-i) measured as described in Figure 2b. Numbers are mean values of three to five independent biological repeats (trees) $\pm$ SE (bars). Asterisks represent a significant difference between treatments at a specific time point according to Student's t test on ranked data ($P \leq 0.05$).

Figure 7. Variation among cultivars in flowering and *MdTFL1-2* expression in response to different fruit loads.

(a,b) Experiments with 'Golden Delicious' performed in the summer of 2013. Treatments were similar to those performed on 'Top Red' (Figure 2a,b). Different letters indicate a significant difference between treatments according to Student's t test on ranked data ($P \leq 0.05$). (a) Average score of flowering intensity per tree as estimated during bloom of the following year (2014). (b) Relative expression of *MdTFL1-2* in BS apices collected 70 DAFB.

(c,d) A comparison of 'Granny Smith' and 'Top Red' trees in response to different amounts of adjacent fruit on the spur. In each tree, 120 spurs were selected, and at 26 DAFB, fruitlets in the spurs were thinned to maintain two, one or no fruit. All comparisons between cultivars for a specific fruit load were found significantly different according to Student's t test ($P \leq 0.05$). Different letters indicate a significant difference between treatments in the same cultivar according to Tukey-Kramer HSD test ($P \leq 0.05$). (c) Percentage of flowering spurs calculated as in Figure 2a, based on 21 preselected spurs per treatment. (d) Relative expression of *MdTFL1-2* in BS apices collected 90 DAFB, measured as described in Figure 2b.

Numbers are mean values of ten (a), three (b) or four (c,d) biological repeats (trees) $\pm$ SE (bars).

Figure 8. Correlation between *MdTFL1-2* relative expression in BS apices at ~90 DAFB and percentage of flowering spurs. Data are taken from Figure 2a,b and Figure 7c,d. Each dot represents a treatment's mean value of independent biological repeats (trees; $n = 4-5$). Full dots represents data from 'Top Red' and empty dots are data from 'Granny Smith'. Adjusted R^2 is 0.81.

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