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Effect of Fruit on Floral Gene Expression and Floral Intensity in Alternate Bearing 'Nules Clementine' and 'Pixie' Mandarin

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Abstract. For alternate bearing mandarin trees (*Citrus reticulata*), high ON-crop yields alternate almost annually with low OFF-crop yields. In this study, effects of crop load on floral gene expression during the 6 months before full bloom (MBFB) and inflorescence number the following spring in early-maturing 'Nules Clementine' and late-maturing 'Pixie' mandarin were compared. The OFF-crop trees of both cultivars flowered profusely in April. In these trees, bud *FLOWERING LOCUS T* (*FT*) expression was first detected only when the air temperatures decreased to the floral-inductive low temperature (LT) range (4 °C < minimum temperature ≦10 °C) at 4 and 6 MBFB for 'Nules Clementine' and 'Pixie' mandarin, respectively. Despite occurring on different calendar dates, maximum *FT* transcript levels corresponded to a similar number of accumulated LT degree-days (56 days at 1 MBFB and 52 days at 3 MBFB) in respective orchards. *LEAFY* (*LFY*) and *APETALA1* (*API*) were upregulated to maximum levels at a similar number of accumulated warm temperature (WT) degree-days (2 days and 5 days with a maximum temperature ≥24 °C following sufficient LT accumulation) at 1 MBFB for 'Nules Clementine' and at 2 MBFB for 'Pixie' mandarin. Subsequent maximum expression of downstream *APETALA2* (*AP2*), *SEPALLATA1* (*SEP1*), *PISTILLATA* (*PI*), and *AGAMOUS* (*AG*) occurred at 1 MBFB for both cultivars, consistent with the initiation of floral organogenesis just before bloom as a result of successful meristem determinacy. The results also provided evidence supporting that the regulation of *FT* and *LFY*/*AP1* in citrus buds is quantitatively dependent on LT and WT accumulation under the OFF-crop condition. In contrast, ON-crop trees of both cultivars did not produce any inflorescences the following spring. Buds of these trees never expressed *FT* during the 6 MBFB, *LFY* expression dropped below the detection limit at 3 MBFB through bloom, and upregulation of *AP1* expression did not occur. Consequently, downstream floral organ identity gene expression was significantly lower in ON-crop trees than in OFF-crop trees at 1 MBFB for both cultivars. Early harvest of ON-crop fruit at 3 MBFB or 2 MBFB failed to restore floral gene expression or spring flowering; instead, it increased vegetative shoot number during return bloom, indicating buds were not determined as late as 2 MBFB (February). Collectively, the results suggest that repression of *FT* during 4 MBFB by the ON crop prevented successful completion of the induction process in floral meristem determinacy by inhibiting *LFY* and *API* upregulation, thereby preventing activation of the floral organ identity genes *AP2*, *SEP1*, *PI*, and *AG* and flower formation.

Introduction

Floral induction in *Citrus* sp. begins in fall or winter in citrus-growing areas where flowering is initiated by low temperatures (LTs) (García-Luís et al. 1992; Lovatt et al. 1988; Moss 1969; Nishikawa et al. 2007; Southwick and Davenport 1986). Floral organogenesis and flowering occur significantly later with the return of warm temperatures (WTs) in spring (Tang and Lovatt 2019). Whether the inductive signal is first perceived by leaves or directly by the bud, the series of developmental events that successfully result in flowering must proceed in the bud to confer floral meristem determinacy and activate the downstream genes essential for floral organ specification and successful flower formation.

The presence of fruit on a shoot (bearing shoot) reduces the number of buds on the shoot that produce inflorescences during return bloom the following spring (Agustí et al. 2022; Martínez-Fuentes et al. 2010; Verreynne and Lovatt 2009). For high-yielding ON-crop trees in an alternate bearing citrus orchard, bearing shoots are the majority and produce few inflorescences the following spring, resulting in an OFF bloom, which subsequently sets a low-yield OFF crop. In contrast, for OFF-crop trees, nonbearing shoots predominate and flower profusely the next spring, producing an ON bloom that results in a high-yield ON crop. Repeating cycles of alternating high and low yields have a negative economic impact on citrus growers and industry. In the ON-crop year, the large number of fruit set per tree reduces fruit size and percentage of marketable crop. Although fruit size is larger in the OFF-crop year, and sometimes too large, there are too few fruit to provide a net profit. In addition, the lack of fruit in the OFF-crop year has negative consequences on every step in the production chain from farm to consumer, including orchard management, harvesting, packinghouse operations, manufacture of value-added products, marketing, and consumer prices, thus jeopardizing the economic stability and sustainability of citrus commodity-based industries.

The inverse relationship between crop load and inflorescence number the following spring and the positive effect of early removal of ON-crop fruit (before mid-August in the Northern Hemisphere) on floral intensity at return bloom in citrus are well-documented (Martínez-Fuentes et al. 2010; Muñoz-Fambuena et al. 2011; Verreynne and Lovatt 2009). In addition,

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the effect of the ON crop on reducing bud expression of key genes in the citrus floral development pathway relative to OFF-crop trees has been demonstrated; these key genes include *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *LEAFY* (*LFY*), and *APETALA1* (*AP1*) (Muñoz-Fambuena et al. 2011, 2012; Shalom et al. 2012, 2014). Whereas *FT*, *LFY*, and *AP1* were proposed as regulators of floral initiation and annual phase transition in citrus (Muñoz-Fambuena et al. 2011; Shalom et al. 2012), more recently, they have been proposed for floral determinacy, which refers to irreversible commitment of the meristem to floral development (Lord and Eckard 1987). In citrus, this appears to be regulated in buds by the expression of sufficient levels of *AP1* alone or together with *APETALA2* (*AP2*), to attain subsequent upregulation of the downstream floral organ identity genes and successful flower formation (Tang and Lovatt 2019, 2022). Importantly, recent results have suggested that floral induction and determinacy in citrus are regulated independently. For example, foliar applications of gibberellic acid (GA_3) during either LT or water-deficit stress induction period resulted in inhibition of flowering by repressing the expression of *AP1* and *AP2* and the downstream floral organ identity genes in the absence of an inhibitory effect on *FT*, *SOC1*, or *LFY* (Tang and Lovatt 2019, 2022). The roles of ON yields and OFF yields and timing of the removal of the ON crop in regulating the expression of floral genes downstream from *AP1* have not been investigated. Thus, it is not known whether the failure of buds on bearing shoots of ON-yield trees to produce flowers the following spring is regulated solely at *FT* and/or at *AP1* alone or together with *AP2*.

The first objective of this research was to determine whether the timing of key floral developmental events during the 6 months before flowering was similar or distinctly different across *C. reticulata* cultivars. Thus, the temporal patterns of expression of core genes demonstrated to function in classic networks regulating floral development across plant species (Benlloch et al. 2007), including induction (floral timing genes *FT*, *SOC1*, and *LFY*), determinacy (floral meristem identity genes *LFY*, *AP1*, and *AP2*), and organogenesis [floral organ identity genes *AP2*, *SEPALLATA1* (*SEP1*), *PISTILLATA* (*PI*), and *AGAMOUS* (*AG*)], were analyzed in buds of OFF-crop trees of early-maturing 'Nules Clementine' and late-maturing 'Pixie' mandarin that flowered at return bloom the following spring. Having two mandarin cultivars in commercial orchards located in two different citrus-production areas made it possible to further explore the relationship between ambient air temperature and the activity of key floral regulatory genes in comparison with results obtained under controlled temperature conditions (Tang and Lovatt 2019). Gene expression data were plotted as months before full bloom (MBFB), not by calendar date, to facilitate comparisons of the results across cultivars and orchards to meet the objectives of the research as well as to improve the utility of the results to end-users across the hemispheres of the global citrus industry.

The second research objective was to assess the effects of ON-crop fruit on important floral developmental processes, especially determinacy, by analyzing floral gene expression in buds of ON-crop trees over the course of the 6 MBFB in comparison with OFF-crop trees as well as ON-crop trees with fruit removed. Removal of the entire ON crop of fruit in August (approximately 8 MBFB) was demonstrated to restore floral intensity to the level of OFF-crop 'Murcott' mandarin (*C. reticulata*) trees the following spring (Shalom et al. 2014). This suggests that thinning of immature fruit (stage II of fruit development) might mitigate alternate bearing, albeit with a reduction in commercial citrus production in the current year. Hence, a subsidiary aim of this study was to test the possibility of early harvest of commercially mature (i.e., marketable) fruit from ON-crop trees, on January (3 MBFB) for 'Nules Clementine' and on February (2 MBFB) for 'Pixie' mandarin, to increase flowering. Notably, these early harvest treatments were implemented both during and after the winter LT induction period that promotes bud *FT* expression (Nishikawa et al. 2009; Tang and Lovatt 2019), but before the return of WTs in spring associated with elevated *AP1* and *AP2* (Tang and Lovatt 2019). Therefore, the analysis of floral gene expression in response to these times for removal of the entire ON crop of fruit was anticipated to provide further insight into the roles of *FT* and *LFY* and/or *API* and *AP2* in the annual regulation of phase transition and bud determinacy (Muñoz-Fambuena et al. 2012; Shalom et al. 2012).

To our knowledge, this study is the first to quantify changes in citrus *AP2*, *SEP1*, *PI*, and *AG* transcript levels in relation to successful and failed flowering in response to OFF-crop and ON-crop yields, respectively. The results provide the first evidence of citrus buds in the temporal relationship between changes in *LFY* and *AP1* transcript accumulation with the upstream expression of *FT* and the downstream activation of the floral organ identity genes when flower formation was successful on nonbearing shoots of OFF-crop trees the following spring but inhibited on bearing shoots of ON-crop trees at return bloom. Furthermore, this study provides the first analysis of the putative role of ambient air temperature in the upregulation of key genes regulating floral determinacy and floral organ identity.

Materials and Methods

Plant materials. Ten-year old 'Nules Clementine' mandarin (*C. reticulata*) trees on 'Carrizo' citrange (*C. sinensis* × *P. trifoliata*) rootstock in a commercial inland orchard in Fillmore, CA, USA (lat. 34°22′N, long. 118°54′W; elevation 136 m above sea level) and 15-year-old 'Pixie' mandarin (*C. reticulata*) trees on 'Troyer' citrange (*C. sinensis* × *P. trifoliata*) rootstock in a commercial orchard in a coastal valley in Ojai, CA, USA (lat. 34°27′N, long. 119°15′W; elevation 298 m above sea level) were used in this research. In each orchard, there were five blocks (replications) (n = 5), and each contained one tree setting an OFF crop and two neighboring trees setting an ON crop. Both orchards reached full bloom in April. For 'Nules' Clementine mandarin trees, the following three treatments (crop loads) were used: the OFF crop harvested in March (standard grower practice; 1 MBFB); the ON crop harvested in March (standard practice; 1 MBFB); and early removal of ON-crop fruit in January (3 MBFB). For 'Pixie' mandarin trees, the three treatments included the following: OFF crop harvested in March (standard grower practice to prevent fruit from becoming too large; 1 MBFB); ON crop harvested in June (standard grower practice to increase fruit size; 2 months after full bloom); and early removal of ON-crop fruit in February (2 MBFB). At each harvest, yield (total fruit weight) for each data tree was recorded in the field. In addition, the total number of fruit harvested was counted for OFF-crop trees. For ON-crop trees, a randomly selected sample of 200 fruit per tree, which represented approximately 5% to 15% of the average total number of fruit per tree, was collected for each tree, and the transverse diameter of each fruit was measured with an electronic caliper. Based on its diameter, each fruit was assigned to one of the following fruit (packing carton) size categories: <64 (<33 mm); <60 (34–36 mm);

56 (37–39 mm); 52 (40–42 mm); 48 (43–45 mm); 44 (46–48); 36 (52–54 mm); 32 (55–57 mm); 28 (58–60 mm); 24 (61–63 mm); 21 (64–67 mm); 18 (68–71 mm); 15 (72–75 mm); 2X (76–79 mm); and 3X (>80 mm). Subsequently, the fruit weight of a specified number of fruit in each fruit size category was collected and used to estimate (extrapolate) the total number of fruit per ON-crop tree.

Bud collection and gene expression analysis. For both cultivars, buds were collected in October (6 MBFB) through March (1 MBFB) from all data trees; however, bud collection for ON-crop trees harvested early (fruit removal treatment) was initiated 1 month after fruit removal. On each sampling date, 20 nonbearing shoots from OFF-crop trees and 20 bearing shoots from ON-crop trees (with each shoot being longer than five nodes with leaves attached) were collected between 1000 and 1100 HR, wrapped with moistened paper towels, and placed in a plastic bag that was sealed and placed in a cooler box for transport. In the laboratory, the five distal buds were immediately excised, frozen in liquid nitrogen, and stored at −80 °C until they were analyzed.

Total RNA for each of the five biological replicates for each treatment on each sampling date was extracted from bud tissue previously ground in liquid nitrogen using a commercial kit (Bioline USA Inc., Taunton, MA, USA). After validation of quality and quantity with a spectrophotometer (NanoDrop 2000; Thermo Scientific, Waltham, MA, USA) and automated electrophoresis (Agilent 2100 Bioanalyzer; Agilent Technologies, Santa Carla, CA, USA), 1 µg RNA was treated with DNase (RQ1 RNase-Free DNase; Promega, Madison, WI, USA) for complementary DNA synthesis using a cDNA synthesis kit with oligo (dT) primer (Tetro cDNA Synthesis Kit; Bioline USA Inc.) in a 30-µL reaction according to the manufacturer's protocol. Expressions of *FT*, *SOC1*, *LFY*, *AP1*, *AP2*, *SEP1*, *PI*, and *AG* were determined using a real-time quantitative polymerase chain reaction (qPCR) detection system (CFX96 Touch; Bio-Rad Laboratories, Hercules, CA, USA). These genes were chosen based on their significant correlation with citrus floral intensity in response to various flowering promoters and inhibitors (Nishikawa et al. 2007, 2009; Pillitteri et al. 2004; Shalom et al. 2012; Song et al. 2010; Tan and Swain 2007; Tang and Lovatt 2019, 2022). Primers used in this study are listed in Table 1. The qPCR reactions with the melt-curve analysis, which confirmed that nonspecific products were not formed, were conducted following the procedures described by Tang and Lovatt (2022).

Using quantification cycle (Cq) values less than 35 obtained from the qPCR (means of three qPCR technical repeats), relative levels of expression (fold change) of the genes of interest were expressed by the normalized relative quantity (NRQ) calculated with the reference gene *BETA-ACTIN* (*ACT*) as the endogenous control (Tang and Lovatt 2019, 2022; Yan et al. 2012) using the Pfaffl method (Hellemans et al. 2007; Pfaffl 2001; Rieu and Powers 2009). For each cultivar, expression of the target genes in buds of OFF-crop trees at 1 MBFB was used as the calibrator control (expression level of 1) for visual presentation of fold changes herein. The data obtained using *ACT* as the primary reference gene were compared with data obtained using *ELONGATION FACTOR 1-ALPHA* (Nishikawa et al. 2009) as a second reference gene. No substantial differences in the results or interpretation of the data reported herein were found (data not shown).

Effects of fruit on bud development at return bloom. At full bloom in April of the following spring, the developmental fate of the distal five buds was determined for each of 20 nonbearing shoots from OFF-crop trees and bearing shoots from ON-crop trees (100 buds/tree). Shoots were randomly selected and tagged in the beginning of the experiment. For 'Pixie' mandarin, the numbers of leafless (one to many flowers with no leaves), leafy (one to many flowers with one to many leaves), and total inflorescences (sum of leafless and leafy inflorescences), vegetative shoots, and quiescent buds (inactive and did not produce a floral or vegetative shoot through 2 months after full bloom) were quantified for each data tree. However, at bloom, 'Nules Clementine' mandarin trees were sequestered under netting to prevent pollen transfer by bees. Thus, floral intensity was evaluated visually in April and estimated

Table 1. Primer sequences for the citrus target and reference genes used in the quantitative real-time polymerase chain reaction (PCR) assays.

Annotation	Accession number	Forward primer (5′ to 3′) Reverse primer (5′ to 3′)	Product size (bp)	PCR product sequence blast against target gene sequence	
				E-value	Identity
FLOWERING LOCUS T (*FT*)	AB027456.1	CCGCGTTGTTGGTGATGTTCTTGA ATTTCAGCCCTAGGCTGGTTCAGA	132	6E-37	95%
SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (*SOC1*)	EU032532.1	TCGACCCAACGGAAAGAAGCTGTA TGCCTAGAAGATTGCAGGAAGCCA	139	5E-46	98%
LEAFY (*LFY*)	AY338976.1	TCTTGGGACAAAGCATCAACAGCG TCAAAGCTGCTGTTAGGGCTGAGA	112	3E-25	92%
APETALA1 (*AP1*)	AY338974.1	ACCGCTCTCAAACACATCAG GCAGCCTTCTCTCTCTCC	137	7E-38	96%
APETALA2 (*AP2*)	EU883665.1	AAATGAAGCTGACTGGCACAACCG AGCGATGATGAAGCTGGTGACTGA	138	9E-18	95%
SEPALLATA1 (*SEP1*)	AB329715.1	TGCTGAGGTGGCTCTCATCATCTT TCTCGAGCTCCTTTGCTGGCTTAT	146	1E-32	90%
PISTILLATA (*PI*)	XM_006472790.1	ATGGCCTTAGAGGATGCCCTTGAA AGCTATCTCCTGTTGCCCAGAACA	144	2E-36	92%
AGAMOUS (*AG*)	HM246523.1	GGGAAGTTGACTTGCACAACAGCA TAGCTCCGGGAATCAAATGGCTGA	142	1E-30	97%
BETA-ACTIN (*ACT*)	GU911361.1	TCACAGCACTTGCTCCAAGCAG TGCTGGAAGGTGCTGAGGGA	130	7E-34	98%

1 month later in May based on the number of young fruit set per shoot, which underestimated the inflorescence number and overestimated the vegetative shoot number.

Relationship between ambient air temperatures and bud *FT*, *LFY/AP1*, and *AP2* expressions. To investigate the relationship between floral induction and LT, the cumulative number of LT degree-days required for maximum expression of *FT* was estimated starting at 1 d after the summer equinox in June, which is the longest day of the year, for each orchard up to the actual date when buds were collected each month. Each day with an average minimum temperature ≤10 °C but >4 °C equaled 1 LT degree-day. This temperature range was based on the research of Tang and Lovatt (2019), who reported that 8 weeks of low night (minimum) temperature of 10 °C (56 LT degree-days) followed by 3 weeks of warm day (maximum) temperature at 24 °C (21 WT degree-days) resulted in maximum flowering in 'Washington' navel orange (*C. sinensis*), and based on evidence that temperatures lower than 4 °C slow citrus metabolism and, thus, were considered to not contribute significantly to floral development (Yelenosky and Wutscher 1985). The relationship between maximum expression of *LFY*, *AP1*, and *AP2* in buds and WT degree-days was estimated as the cumulative number of days with an average maximum temperature ≥24 °C after the accumulation of 56 LT degree-days. The calculations used daily average maximum and minimum air temperatures downloaded from the California Irrigation Management Information System (Sacramento, CA, USA) for the closest station to each orchard. Temperatures did not reach 24 °C before the accumulation of 56 LT degree-days in either orchard.

Statistical analysis. An analysis of variance (ANOVA) was used to test for treatment (crop load) effects on the number of inflorescences, vegetative shoots, and quiescent buds per tree using the general linear model procedure (PROC GLM) of SAS (version 9.4; SAS Institute, Cary, NC, USA). Because buds were collected over time from individual data trees, treatment effects on expression levels of each gene on each sampling day as well as changes in expression levels over time within each treatment were determined using the repeated measures ANOVA with the generalized linear mixed model procedure (PROC GLIMMIX). When the ANOVA indicated significant differences ($P < 0.05$), post hoc comparisons were run using Tukey's honestly significant difference procedure. When the expression level of the target gene in each of the five biological replications was below the threshold value for detection (Cq in qPCR ≥35), the results were reported as not detected (Figs. 1–3; Supplemental Tables S1 and S2).

Results

Effects of fruit on bud development at return bloom. For both mandarin cultivars, there were significant differences in crop load (yield) between the OFF-crop and ON-crops trees. The OFF-crop 'Nules Clementine' mandarin trees produced 23.9 kg (203 fruit) per tree, whereas ON-crop trees produced 86.5 kg (1262 fruit) per tree ($P < 0.001$) (Table 2). At bloom the following year, the low-yielding OFF-crop 'Nules Clementine' mandarin trees produced an average of 43.4 inflorescences (based on the number of fruit set per 100 buds per tree in May, at 1 month after anthesis); this value was significantly greater than that of the

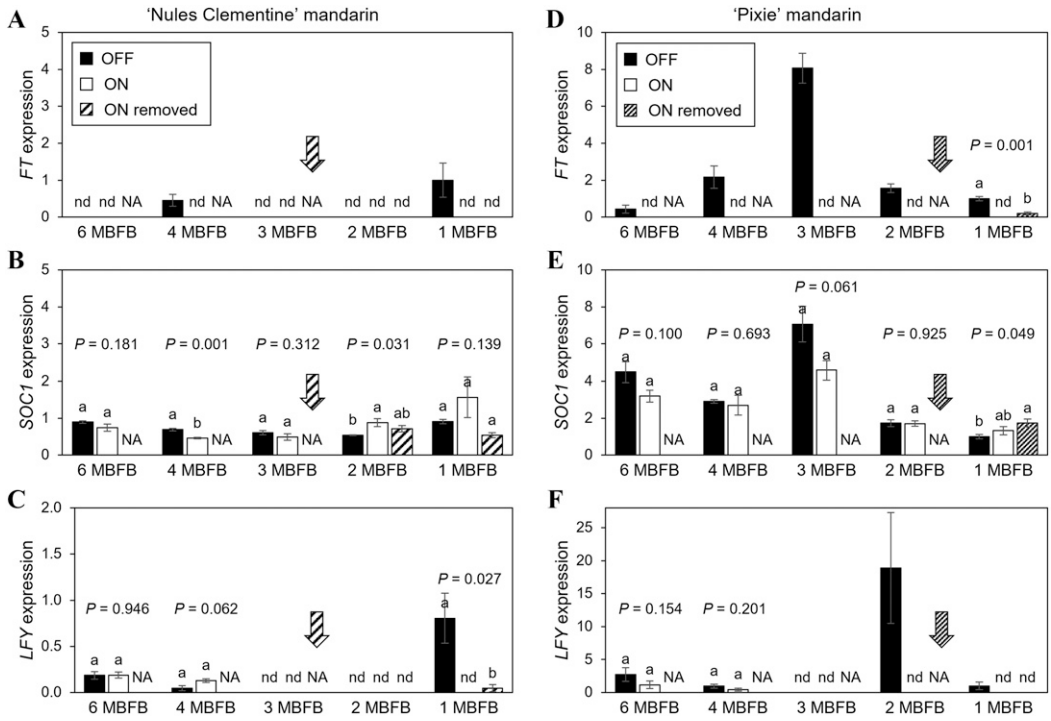

Fig. 1. Relative expression of *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), and *LEAFY* (*LFY*) in buds of OFF-crop trees (OFF), ON-crop trees (ON), and ON-crop trees with the entire ON crop of fruit removed (ON removed) for 'Nules Clementine' (**A**, **B**, and **C**) and 'Pixie' mandarin (**D**, **E**, and **F**). The arrows at 3 and 2 months before full bloom (MBFB) indicate the time of fruit removal treatments for the two cultivars, respectively. Data are means of five trees (replications). Within the same month, different letters are significantly different according to Tukey's honestly significant difference (HSD) test ($P < 0.05$). Here, ND refers to not detected, which indicates that the expression level of the target gene was below the threshold value for detection (quantification cycle in the quantitative polymerase chain reaction >35), and NA indicates no applicable data because trees with the ON crop removed were sampled starting 1 month after fruit removal.

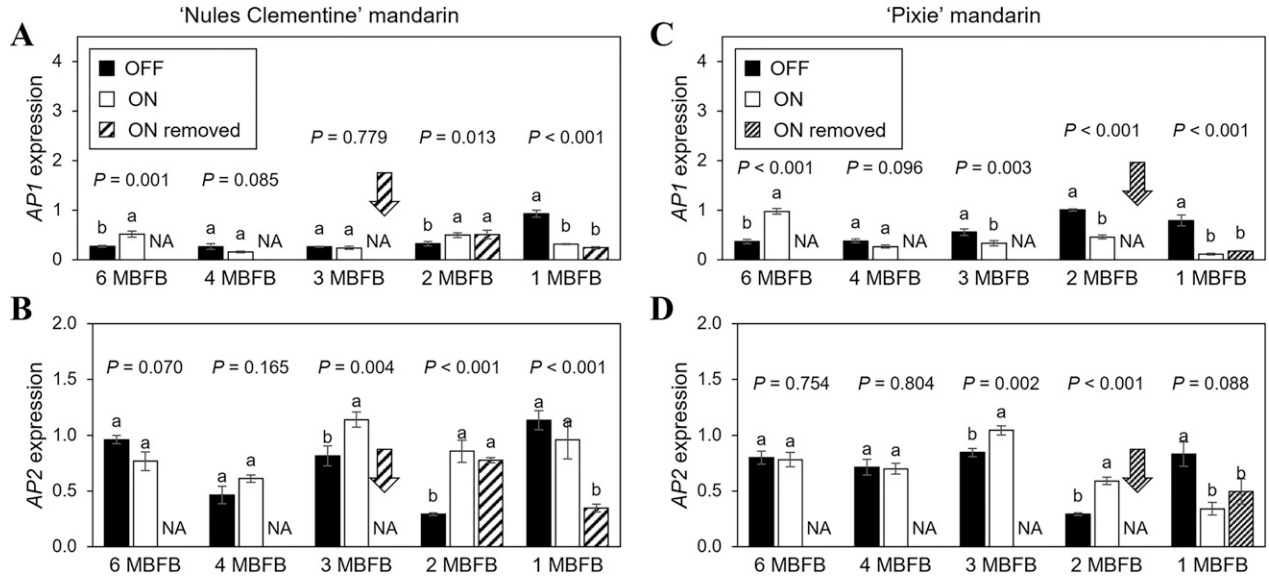

Fig. 2. Relative expression of *APETALA1* (*AP1*) and *APETALA2* (*AP2*) in buds of OFF-crop trees (OFF), ON-crop trees (ON), and ON-crop trees with the entire ON crop of fruit removed (ON removed) for 'Nules Clementine' (**A** and **B**) and 'Pixie' mandarin (**C** and **D**). The arrows at 3 and 2 months before full bloom (MBFB) indicate the time of fruit removal treatments for the two cultivars, respectively. Data are means of five trees (replications). Within the same month, different letters are significantly different according to Tukey's honestly significant difference (HSD) test ($P < 0.05$). Here, ND refers to not detected, which indicates that the expression level of the target gene was below the threshold value for detection (quantification cycle in the quantitative polymerase chain reaction >35), and NA indicates no applicable data because trees with the ON crop removed were sampled starting 1 month after fruit removal.

heavy-yielding ON-crop trees at return bloom (0 inflorescence), consistent with visual observations that the trees did not flower. Similarly, OFF-crop 'Pixie' mandarin trees produced 19.8 kg (185 fruit) per tree, whereas ON-crop trees produced 12-fold and 18-fold more fruit by weight (235.9 kg/tree) and number per tree (3381 fruit/tree), respectively, the following year ($P < 0.001$ for both) (Table 3). In April the following year, OFF-crop 'Pixie' mandarin trees produced an average of 96.4 inflorescences (of

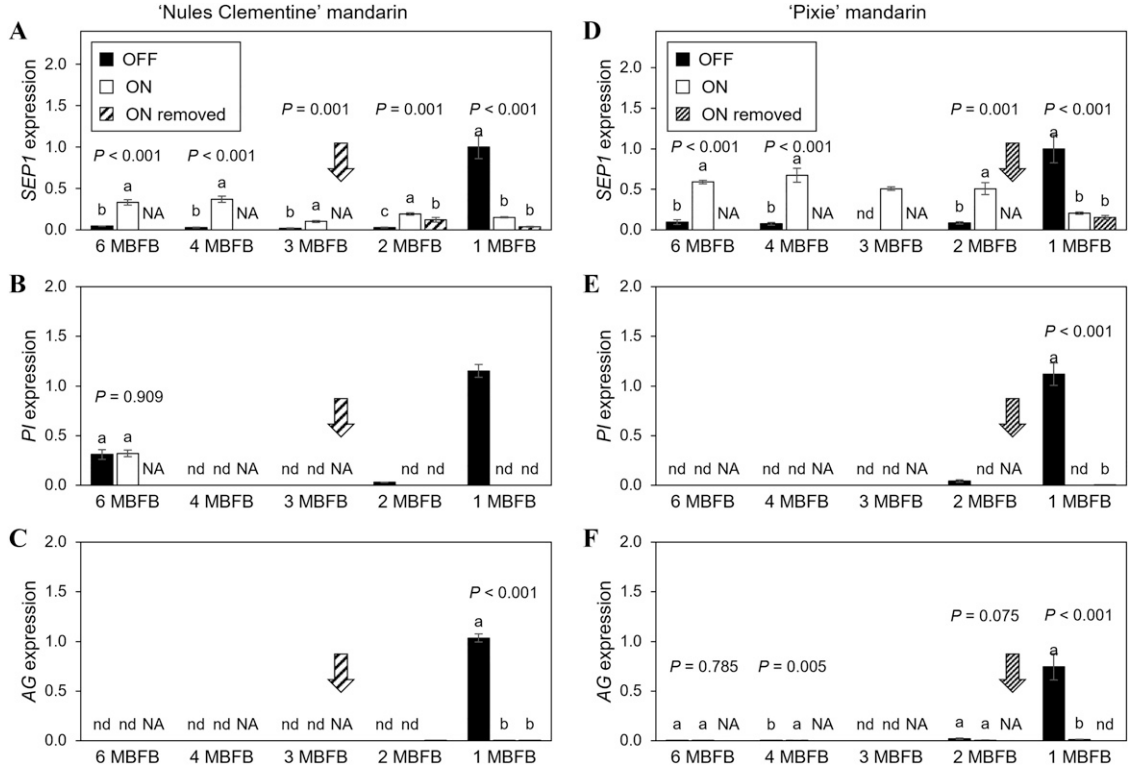

Fig. 3. Relative expression OF *SEPALLATA1* (*SEP1*), *PISTILLATA* (*PI*), and *AGAMOUS* (*AG*) in buds of OFF-crop trees (OFF), ON-crop trees (ON), and ON-crop trees with the entire ON crop of fruit removed (ON removed) for 'Nules Clementine' (**A**, **B**, and **C**) and 'Pixie' mandarin (**D**, **E**, and **F**). The arrows at 3 and 2 months before full bloom (MBFB) indicate the time of fruit removal treatments for the two cultivars, respectively. Data are means of five trees (replications). Within the same month, different letters are significantly different according to Tukey's honestly significant difference (HSD) test ($P < 0.05$). Here, ND refers to not detected, which indicates that the expression level of the target gene was below the threshold value for detection (quantification cycle in quantitative polymerase chain reaction >35), and NA indicates no applicable data because trees with the ON crop removed were sampled starting 1 month after fruit removal.

Table 2. Crop load, including total fruit fresh weight and quantity per tree, and developmental fate of buds at return bloom, (as the estimated number of total inflorescences, vegetative shoots, or quiescent buds of 100 buds per tree) of OFF-crop trees (OFF), ON-crop trees (ON), and ON-crop trees with the entire crop of fruit removed 2 months before full bloom (ON removed) for 'Nules Clementine' mandarin. Data are means ± standard error. Within each parameter, different letters indicate significant differences according to Tukey's honestly significant difference test ($P < 0.05$).

	Crop load per tree		Development of 100 buds per tree at return bloom		
	Total wt (kg)	Fruit quantity (no.)	Total inflorescences[i] (no.)	Vegetative shoots (no.)	Quiescent buds (no.)
OFF	23.9 ± 6.8 b	203 ± 60 b	43.4 ± 3.8 a	9.4 ± 2.6 b	47.2 ± 3.6 c
ON	85.5 ± 3.1 a	1262 ± 110 a	0.0 ± 0.0 b	0.4 ± 0.2 b	99.6 ± 0.2 a
ON removed	85.5 ± 7.1 a	1262 ± 172 a	0.4 ± 0.4 b	33.2 ± 4.7 a	66.4 ± 4.4 b

[i]Because the inaccessibility of experimental trees in the 'Nules Clementine' orchard, spring floral intensity, and development fate of buds were estimated 1 month after full bloom based on the number of young fruit set on previously tagged shoots, which underestimated inflorescence number and overestimated vegetative shoot number.

100 buds/tree). Return bloom was dominated by leafless inflorescences, which constituted 91% of total inflorescences produced by OFF-crop trees. The ON-crop 'Pixie' mandarin trees did not produce any inflorescences, consistent with the effect of the ON-crop of fruit on return bloom of 'Nules Clementine'. For ON-crop trees of both cultivars, all 100 distal buds remained quiescent (inactive) the following spring, producing neither inflorescences nor vegetative shoots, through June. This indicated that flowering was inhibited, rather than delayed, in ON-crop trees. Floral intensity (inflorescence number) at return bloom was significantly (negatively) correlated with the previous year's crop load for both 'Nules Clementine' and 'Pixie' mandarin as kilograms per tree ($r = -0.93$ and $P < 0.001$ and $r = -0.95$ and $P < 0.001$, respectively) and fruit number per tree ($r = -0.90$ and $P < 0.001$ and $r = -0.99$ and $P < 0.001$, respectively).

Early harvest of ON-crop 'Nules Clementine' mandarin trees 3 MBFB did not improve flowering (<1 inflorescence/100 buds), but it increased the number of vegetative shoots (33.2); this value was significantly greater than that of both OFF-crop and ON-crop 'Nules Clementine' mandarin trees ($P < 0.001$) (Table 2). Likewise, for 'Pixie' mandarin, removal of the entire ON-crop of fruit 2 MBFB resulted in no change in flowering (<1 inflorescence/100 buds) but an increase in vegetative shoots to a number that was significantly greater than that of both OFF-crop and ON-crop trees ($P < 0.001$) (Table 3). Consequently, ON-crop trees harvested 3 MBFB or 2 MBFB had significantly reduced numbers of quiescent buds compared with ON-crop trees of the two cultivars (Tables 2 and 3), indicating inhibition of spring budbreak of both floral and vegetative buds by the presence of fruit.

Effects of fruit on the expression of citrus floral timing genes. For 'Nules Clementine' mandarin, expression of *FT* was first detected in buds of OFF-crop trees on the second sampling date 4 MBFB, with maximum expression of *FT* just 1 MBFB (Fig. 1A), and bud *FT* expression was below the limit of detection at 6, 3, and 2 MBFB (Supplemental Table S1). For buds of ON-crop trees or ON-crop trees harvested 3 MBFB, transcripts of *FT* were below the limits of detection on all sampling dates. In contrast, for 'Pixie' mandarin, *FT* was expressed in buds of OFF-crop trees as early as 6 MBFB (first sampling data) and on all subsequent sampling dates, with maximum *FT* expression at 3 MBFB (Fig. 1D; Supplemental Table S2). Buds of ON-crop trees did not express *FT* above the limit of detection on any sampling dates. Removal of the ON crop of fruit 2 MBFB upregulated bud *FT*, but the transcript level was still significantly lower than that of OFF-crop trees at 1 MBFB ($P = 0.001$). Thus, for 'Pixie' mandarin, bud *FT* expression for OFF-crop trees was greater than that of ON-crop trees with or without early harvest from 6 MBFB through 1 MBFB.

Buds of OFF-crop and ON-crop 'Nules Clementine' mandarin trees continuously expressed *SOC1* on all sampling dates (Fig. 1B). There was no difference in *SOC1* expression between OFF-crop and ON-crop trees, except that the transcript level in OFF-crop trees was greater at 4 MBFB ($P = 0.001$) and lower at 2 MBFB ($P = 0.031$) compared with that of ON-crop trees. Early harvest of the ON crop 3 MBFB did not result in significant changes in *SOC1* expression compared with ON-crop and OFF-crop trees during the period after fruit removal. Similarly, for 'Pixie' mandarin, *SOC1* was expressed in buds of OFF-crop and ON-crop trees during the 6 months before flowering (Fig. 1E). In OFF-crop trees, despite the sharp increase from 6 MBFB to a maximum value at 3 MBFB ($P < 0.001$) (Supplemental Table S2), *SOC1* expression was never significantly different from that of ON-crop trees. Removing the entire ON crop of 'Pixie' mandarin fruit 2 MBFB had no effect on bud *SOC1* expression at 1 MBFB. For both 'Nules Clementine' and 'Pixie' mandarin, bud expression of *SOC1* was not related to floral intensity at return bloom.

Table 3. Crop load, including total fruit fresh weight and quantity per tree, and developmental fate of buds at return bloom (as the number of total, leafless, and leafy inflorescences, vegetative shoots, or quiescent buds) of OFF-crop trees (OFF), ON-crop trees (ON), and ON-crop trees with the entire crop of fruit removed 2 months before full bloom (ON removed) for 'Pixie' mandarin. Data are means ± standard error. Within each parameter, different letters indicate significant differences according to Tukey's honestly significant difference test ($P < 0.05$).

	Crop load per tree		Development of 100 buds per tree at return bloom				
			Inflorescences				
	Total wt (kg)	Fruit quantity (no.)	Total (no.)	Leafless (no.)	Leafy (no.)	Vegetative shoots (no.)	Quiescent buds (no.)
OFF	19.7 ± 9.0 c	185 ± 80 b	96.4 ± 1.7 a	87.6 ± 2.4 a	8.8 ± 1.1 a	0.2 ± 0.2 b	3.4 ± 1.6 c
ON	235.9 ± 11.5 a	3381 ± 86 a	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	100.0 ± 0.0 a
ON removed	177.9 ± 7.9 b	3633 ± 134 a	0.2 ± 0.2 b	0.0 ± 0.0 b	0.2 ± 0.2 b	41.8 ± 4.2 a	58.0 ± 4.1 b

Bud *LFY* expression was not significantly different between OFF-crop and ON-crop 'Nules Clementine' mandarin trees at 6 or 4 MBFB (Fig. 1C). At 3 and 2 MBFB, *LFY* transcripts were below the limit of detection in both OFF-crop and ON-crop trees. Maximum expression of *LFY* occurred in OFF-crop trees 1 MBFB ($P = 0.004$) (Supplemental Table S1) but remained undetectable in ON-crop trees. Removal of the entire ON crop of fruit 3 MBFB had no effect on *LFY* expression until 1 MBFB, at which time *LFY* was expressed, but at a significantly lower level than that of OFF-crop trees ($P = 0.027$). For both cultivars, the expression pattern of *LFY* in OFF-crop trees was similar, except that *LFY* expression in OFF-crop 'Pixie' mandarin trees was below the limit of detection on only one sampling date (3 MBFB) and had a maximum value at 2 MBFB ($P = 0.005$) (Table S2). Expression of *LFY* in ON-crop 'Pixie' mandarin trees was below the limit of detection 3, 2, and 1 MBFB. Early harvest of ON-crop trees 2 MBFB did not upregulate *LFY* expression, which remained not detectable at 1 MBFB.

Effects of fruit on the expression of citrus genes with class a activity. For the two cultivars, *AP1* was constantly expressed in buds of OFF-crop and ON-crop trees from 6 to 1 MBFB (Fig. 2A and 2C). For 'Nules Clementine' mandarin, *AP1* expression was lower in OFF-crop than ON-crop trees at 6 MBFB ($P < 0.001$) and 2 MBFB ($P = 0.013$) and not significantly different at 4 and 3 MBFB (Fig. 2A). However, from 2 to 1 MBFB, bud *AP1* expression significantly increased in OFF-crop trees ($P < 0.001$) and decreased in ON-crop trees ($P < 0.001$) and trees with all fruit removed 3 MBFB ($P = 0.001$) (Supplemental Table S1). Thus, at 1 MBFB, OFF-crop 'Nules Clementine' mandarin trees expressed *AP1* at a significantly greater level than ON-crop trees with and without early harvest ($P < 0.001$) (Fig. 2A). Similarly, in OFF-crop 'Pixie' mandarin trees, *AP1* expression was significantly lower than that in ON-crop trees at 6 MBFB ($P < 0.001$) and not significantly different at 4 MBFB (Fig. 2C). In OFF-crop trees, bud *AP1* expression significantly and progressively increased from 4 to 2 MBFB, when transcript accumulation reached the maximum level ($P < 0.001$) (Supplemental Table S2). This resulted in significantly greater expression of *AP1* in OFF-crop than in ON-crop trees at 3 MBFB ($P = 0.003$), 2 MBFB ($P < 0.001$) and 1 MBFB ($P < 0.001$). Removal of all fruit from ON-crop 'Pixie' mandarin trees 2 MBFB had no effect on *AP1* expression.

For both 'Nules Clementine' and 'Pixie' mandarin, *AP2* expression was not significantly different in buds of OFF- and ON-crop trees at the first two sampling dates, but was significantly lower in buds of OFF-crop than ON-crop trees at 3 MBFB ($P = 0.004$ and $P = 0.002$, respectively) and 2 MBFB ($P < 0.001$ for both) (Fig. 2B and 2D). In OFF-crop trees, there was a significant increase in bud *AP2* expression from 2 to 1 MBFB ($P < 0.001$ for both cultivars), while ON-crop trees expressed *AP2* at a constant level during this period (Supplemental Tables S1 and S2). This resulted in greater bud *AP2* expression in OFF-crop trees compared with that in ON-crop trees of 'Pixie' mandarin at 1 MBFB. There was a similar trend in *AP2* expression associated with crop for 'Nules Clementine' mandarin despite the difference between OFF-crop and ON-crop trees not being statistically significant. Removal of the entire ON crop did not increase *AP2* transcript accumulation in either mandarin cultivar.

Effects of fruit on the expression of citrus floral organ identity genes downstream from *AP2*. In OFF-crop trees of the two mandarin cultivars, *SEP1* expression was significantly lower than that in ON-crop trees from 6 through 2 MBFB (Fig. 3A and 3D). From 2 to 1 MBFB, there was a significant increase in *SEP1* transcript accumulation in OFF-crop trees ($P < 0.001$ for both cultivars), whereas *SEP1* expression in ON-crop trees at 1 MBFB remained at a similar or lower level compared with the previous month (Supplemental Tables S1 and S2). Early harvest of ON-crop trees 3 or 2 MBFB did not increase *SEP1* expression following fruit removal in either cultivar. As a result, bud *SEP1* expression was significantly greater in OFF-crop than in ON-crop trees with and without early fruit removal at 1 MBFB for 'Nules Clementine' and 'Pixie' mandarin ($P < 0.001$ for both).

As early as 6 MBFB, *PI* transcript levels were not significantly different in buds of OFF-crop and ON-crop 'Nules Clementine' mandarin trees, but they dropped below the limit of detection at 4 and 3 MBFB (Fig. 3B). At 2 MBFB, *PI* transcripts were again detectable and increased to the maximum level at 1 MBFB in OFF-crop trees ($P < 0.001$) (Supplemental Table S1), whereas bud *PI* expression in ON-crop trees remained undetected. Removal of the ON crop of fruit 3 MBFB did not upregulate *PI* expression at 2 or 1 MBFB. For 'Pixie' mandarin, *PI* transcripts were not detected in OFF-crop trees from 6 through 3 MBFB, but they increased to a quantifiable level at 2 MBFB and maximum expression at 1 MBFB (Fig. 3E) (Supplemental Table S2). Expression of *PI* was never detected in buds of ON-crop trees. Although removing the ON-crop fruit at 2 MBFB increased *PI* transcripts to a detectable level at 1 MBFB, the expression was significantly lower than that of OFF-crop 'Pixie' mandarin trees ($P < 0.001$).

For 'Nules Clementine' mandarin, bud expression of *AG* remained below the limit of detection for all trees on all sampling dates until 1 MBFB, when OFF-crop trees expressed *AG* at a high level that was significantly greater than that expressed by ON-crop trees with or without fruit removed 3 MBFB ($P < 0.001$) (Fig. 3C). For 'Pixie' mandarin, *AG* was expressed at extremely low levels in both OFF-crop and ON-crop trees at 6, 4, and 2 MBFB, and it was not detected at 3 MBFB (Fig. 3F). From 2 to 1 MBFB, *AG* expression significantly increased to a maximum level in OFF-crop trees ($P < 0.001$) (Supplemental Table S2) but remained at a very low value in ON-crop trees. Fruit removal did not result in *AG* transcription at 1 MBFB. For both mandarin cultivars, only buds of OFF-crop trees accumulated significant levels of *PI* and *AG* transcripts, and only these trees flowered at return bloom.

Relationship between ambient air temperatures and bud *FT*, *LFY/AP1*, *AP2*, and downstream floral organ identity gene expression. For OFF-crop trees that flowered profusely during return bloom, bud *FT* expression was first detected at 4 MBFB in 'Nules Clementine' and 6 MBFB in 'Pixie' mandarin, which corresponded to 9 and 12 LT degree-days, respectively (Fig. 4A and 4B). Maximum *FT* expression occurred when trees had been exposed to an accumulated 56 and 52 LT degree-days for 'Nules Clementine' and 'Pixie' mandarin, respectively, with a difference of only 4 LT degree-days for the two cultivars. It should be noted that the cumulative LT degree-days for both cultivars (Supplemental Table S3) approximated the 8 weeks (56 d) of 10 °C night temperatures, resulting in both maximum bud *FT* expression and floral intensity of 'Washington' navel

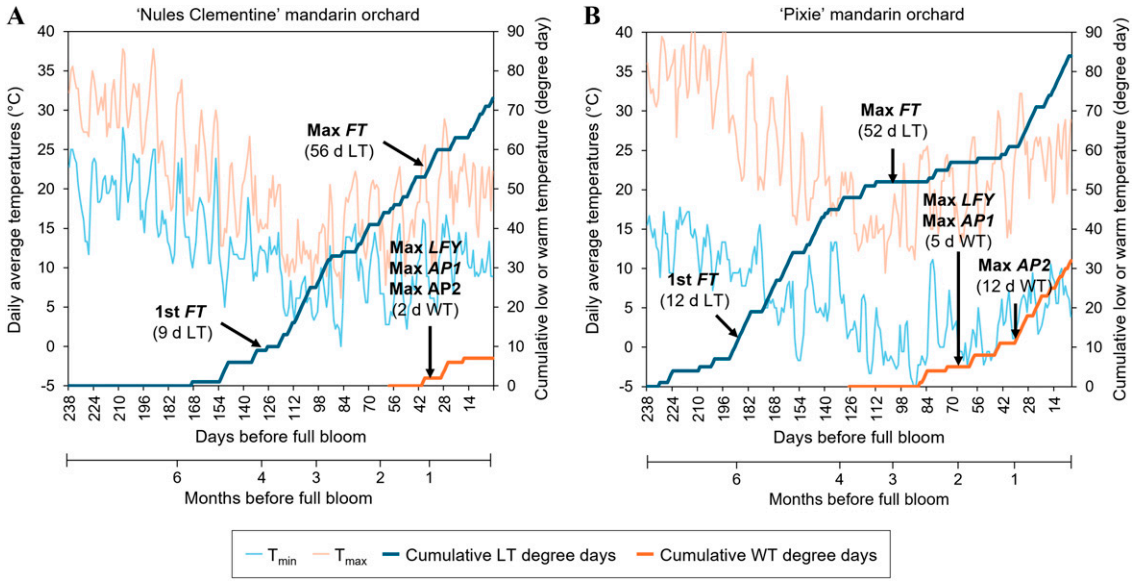

Fig. 4. Daily average minimum temperature (T_{min}) and maximum temperature (T_{max}) and cumulative low-temperature (LT) and warm-temperature (WT) degree-days at the 'Nules Clementine' and 'Pixie' mandarin orchards. Here, 1 LT degree-day indicates a day with a minimum temperature ≤10 °C but >4 °C, and 1 WT degree-day indicates a day with maximum temperature ≥24 °C after the accumulation of a minimum 56 LT degree-days. First detection and maximum of *FLOWERING LOCUS T* (*FT*) expression as well as maximum *LEAFY* (*LFY*), *APETALA1* (*AP1*), and *APETALA2* (*AP2*) in buds of OFF-crop trees of both cultivars are denoted by the arrows with cumulative LT or WT degree-days in the respective orchards.

orange trees under controlled environmental conditions (Tang and Lovatt 2019). In addition, buds of OFF-crop 'Nules Clementine' accumulated *LFY* and *AP1* transcripts to maximum levels at 1 MBFB, when 2 WT degree-days were accumulated after sufficient LT accumulation (56 LT degree-days) (Fig. 4A), which corresponded to maximum *LFY* and *AP1* expressions in OFF-crop 'Pixie' mandarin trees at 5 WT degree-days at 2 MBFB (Fig. 4B). Under controlled environmental conditions, maximum *LFY* and *AP1* expressions both occurred after 7 d of WT treatment following 8 weeks of LT treatment for 'Washington' navel orange (Tang and Lovatt 2019). For both mandarin cultivars, maximum bud expression of *AP2* and downstream floral organ identity genes all occurred at 1 MBFB, which corresponded to 2 WT degree-days for 'Nules Clementine' mandarin but 12 WT degree-days for 'Pixie' mandarin. The number of additional days of WT required by 'Pixie' mandarin relative to 'Nules Clementine' mandarin for maximum expression of *AP2*, *SEP1*, *PI*, and *AG* is consistent with the 7 additional days of WT treatment required for maximum expression of these genes, relative to maximum expression of *AP1*, in buds of 'Washington' navel orange trees, which occurred 2 weeks after the trees were transferred from the 8-week LT treatment to the WT conditions (Tang and Lovatt 2019).

Discussion

Once alternate bearing is initiated, cycles of light OFF and heavy ON crops are perpetuated by the inverse effect of the crop load (fruit number) borne on the tree on inflorescence number the following spring (Goldschmidt and Golomb 1982; Hield and Hilgeman 1969; Monselise and Goldschmidt 1982; Verreynne and Lovatt 2009). In this study, the temporal patterns of floral gene expression, including the putative floral organ identity genes for the first time and genes controlling floral timing and meristem identity, are reported in buds of OFF-crop and ON-crop mandarin trees, which flowered profusely or failed to flower the following spring, respectively. The effect of removal of the ON crop on the genes regulating determinacy, floral organ specification, and flowering was also quantified. For OFF-crop 'Nules Clementine' and 'Pixie' mandarin trees located in two different orchards, the patterns of gene expression were compared relative to ambient air temperature to detect potential differences in the timing of gene expression likely attributable to prevailing temperatures in the two different growing areas rather than the ON crop.

In buds of OFF-crop trees of both cultivars, expression of floral timing genes (*FT*, *SOC1*, and *LFY*), floral meristem identity genes (*LFY* and *AP1*) and floral organ identity genes (*AP2*, *SEP1*) at 6 MBFB (October) indicated the potential initiation of the floral induction process as early as October in *C. reticulata*. Although the activation of *FT* transcription may seem to be "delayed" in 'Nules Clementine' (4 MBFB) compared with 'Pixie' mandarin (6 MBFB), it is noteworthy that only when the daily minimum temperature in the orchards decreased to the floral-inductive range (≤10 °C and >4 °C) were *FT* transcripts first detected in buds of both cultivars (cumulative 9 and 12 LT degree-days, respectively) (Fig. 4, Supplemental Table S3). Consistently, maximum expression of *FT*, commonly associated with signal integration during the induction process (Wigge et al. 2005), occurred in buds of 'Nules Clementine' and 'Pixie' mandarin after a similar amount of LT (≤10 °C and >4 °C) accumulated in the two orchards (56 and 52 LT degree-days, respectively) on different sampling dates (1 and 3 MBFB, respectively). The cumulative LT degree-days that corresponded to maximum *FT* expression in field-grown trees was similar to the LT treatment (8 weeks, or 56 d, at 10 °C night temperature) that significantly increased *FT* transcripts and floral intensity in trees under controlled environmental conditions (Tang and Lovatt 2019). These results support the *FT*-mediated vernalization (LT) flower signaling pathway in citrus buds (Nishikawa et al. 2007; Tang and Lovatt 2019) but leave open the possibility that the environmental cue may be initially perceived by the leaf rather than the bud

(Chica and Albrigo 2013; Muñoz-Fambuena et al. 2012; Nishikawa et al. 2007).

Despite continuous expression during 6 to 4 MBFB in both mandarin cultivars, *LFY* transcripts were undetectable during the winter period at 3 MBFB (daily maximum temperature <24 °C) in buds of OFF-crop trees. As spring temperatures increased in the 'Nules Clementine' and 'Pixie' mandarin orchards where trees were exposed to cumulative 2 and 5 WT degree-days at 1 and 2 MBFB, respectively, *LFY* expression was upregulated to the maximum level. This temperature-related control of *LFY* transcription in field-grown trees is consistent with the findings of a previous study of 'Washington' navel orange trees under controlled temperature conditions; maximum *LFY* expression occurred only after trees were subjected to 7 d of WT treatment following 8 weeks of LT treatment (Tang and Lovatt 2019). In addition, under the controlled environmental conditions used to induce flowering in 'Washington' navel orange, maximum *AP1* expression also occurred 1 week after transfer from the LT conditions to the WT conditions. Similarly, maximum *AP1* expression also occurred at 2 and 5 WT degree-days for 'Nules Clementine' (1 MBFB) and 'Pixie' mandarin (2 MBFB), respectively. In *A. thaliana*, *FT* targets both *LFY* and *AP1*; additionally, *LFY* directly activates *AP1* during floral induction (Abe et al. 2005; Hempel et al. 2000; Liu et al. 2007; Wagner et al. 1999; Wigge et al. 2005; Müller-Xing et al. 2014), suggesting that *LFY* specifies floral meristem identity in part through *AP1* (Liu et al. 2007; William et al. 2004). Thus, in OFF-crop 'Nules Clementine' and 'Pixie' mandarin trees, floral meristem identity, which signifies floral meristem determinacy, likely occurred at 1 and 2 MBFB (in March and February), respectively, when *LFY* and *AP1* reached maximum transcript accumulation. For both cultivars, maximum expression of *AP2*, the second class A gene necessary for sepal formation in the ABCE model for floral organ specification (Bowman et al. 1991; Coen and Meyerowitz 1991; Krizek and Fletcher 2005), occurred at 1 MBFB in OFF-crop trees. This was accompanied by increased expression of the downstream floral organ identity genes *SEP1*, *PI*, and *AG* from below or just above the limit of detection at 2 MBFB to the maximum level at 1 MBFB, followed by profuse flowering during return bloom in April. Upregulation of *AP1* and *AP*2 has been reported to activate the downstream floral organ identity genes in citrus buds that successfully flowered (Tang and Lovatt 2019, 2022). Activation of floral organ specification when temperatures were sufficiently warm in both orchards is consistent with a failsafe mechanism to prevent flowering under adverse (abiotic stress) environments (Tang and Lovatt 2019, 2022).

However, in the case of failed flowering for both cultivars, the presence of the heavy ON crop suppressed *FT* expression during the 6-month period before bloom and inhibited *LFY* transcripts from recovering to a detectable level from 3 MBFB through 1 MBFB, despite spring temperatures having returned to >24 °C at 1 and 2 MBFB, respectively. Moreover, the increase in *AP1* expression coinciding with *LFY* upregulation in OFF-crop trees did not occur in ON-crop trees of both cultivars. For alternate bearing 'Moncada' and 'Murcott' mandarin, negative effects of the ON-crop fruit were observed on floral gene expression, including *FT* as early as 5 Nov (5 MBFB) and *LFY* and *AP1* in January (3 MBFB), with a concomitant decrease in inflorescence number at spring bloom compared with OFF-crop trees (Muñoz-Fambuena et al. 2012; Shalom et al. 2012). The results of the current research further documented that the ON crop downregulated the genes downstream from *AP1* at 1 MBFB for both cultivars, indicating that determinacy and floral organ specification failed to occur in buds of ON-crop trees. The results suggest that transcript levels of *AP1*, *AP2*, and *SEP1*, which were continuously expressed during the 6 MBFB in ON-crop trees, were insufficient to lead to flower formation when *FT* and/or *LFY* were not expressed. The transcript levels of the floral timing gene *SOC1* at 2 and 1 MBFB, which were not influenced by crop load, were also insufficient to promote successful floral induction in ON-crop trees.

This interpretation is supported by the results obtained when ON-crop trees were harvested early. Removing the ON crop of 'Nules Clementine' fruit 3 MBFB and 'Pixie' mandarin fruit 2 MBFB did not restore the expression of any floral gene of interest to the level of OFF-crop trees and did not lead to the production of any inflorescences. The results indicated that the earlier negative effects of the ON crop on *FT* transcription in the months before the early harvest treatment were not reversible for the two cultivars even when the source of inhibition (fruit) was removed. Although the early harvests of the ON-crop trees did not result in flowering, the number of vegetative shoots that developed was significantly increased during return bloom, with a concomitant decrease in the number of quiescent buds, indicating an increase in budbreak. This result provides additional evidence that the ON crop inhibits spring budbreak (Martínez-Fuente et al. 2010; Verreynne and Lovatt 2009). The development of vegetative shoots instead of inflorescences in response to fruit removal treatments also establishes that the persistence of the ON crop of fruit through 3 or 2 MBFB prevented at least 33% to 42% of buds from undergoing floral determinacy (i.e., irreversible commitment to floral development) for 'Nules Clementine' and 'Pixie' mandarin, respectively.

Because of the timing of *FT* downregulation by the ON crop, it is logical to propose that to restore bud *FT* expression and flowering in ON-crop trees of both cultivars, the fruit would need to be harvested minimally before 4 MBFB (December). However, how much earlier in the season has not yet been determined for 'Nules Clementine' and 'Pixie' mandarin. For 'Moncada' mandarin, the results of fruit removal treatments at various months before bloom indicated that the negative effect of the ON crop on spring floral intensity became irreversible in November (5 MBFB) or later for this cultivar (Muñoz-Fambuena et al. 2011). Harvest of the ON crop before this date, while not commercially viable for some citrus cultivars or growing areas, would be an excellent tool for manipulating floral gene expression not only to increase our understanding of alternate bearing but also to resolve the intriguing questions of when floral induction is initiated in *Citrus* sp. and whether the signal is environmental or developmental and first perceived by the leaf or bud. In *A. thaliana*, a complex network of genetic pathways regulates the transition from a vegetative to an inflorescence meristem in response to endogenous (developmental) and exogenous (environmental) flowering time signals, which are integrated by *FT*, *SOC1*, and *LFY*, and eventually upregulate *AP1*, resulting in floral determinacy (Pajoro et al. 2014; Srikanth and Schmid 2011).

Until chemical sprays that can upregulate specific floral genes essential to determinacy and floral organ specification are developed, strategies that reduce the ON bloom initiating an ON-crop year will remain common tools used to increase flowering and yield the following year. The practices include foliar-applied GA_3 in December before the ON bloom to reduce floral development, foliar-applied naphthaleneacetic acid during bloom to thin

flower number, and pruning to reduce bloom and stimulate summer vegetative shoot growth (Fake 2012; Haim et al. 2025; Lord and Eckard 1987; Stover et al. 2002). Additionally, in warmer, drier citrus production areas in which the requisite cumulative number of LT degree-days is not routinely reached in an orchard, the presence of the water-deficit stress floral-induction pathway in citrus (Tang and Lovatt 2022) provides commercial citrus growers with a tool to increase flowering and yield.

Conclusion

Although it is well known that LT promotes flowering in citrus, the results of this research are the first to document that, in the absence of the floral-inhibiting ON crop, upregulation of bud *FT* was dependent on a similar amount of ambient LT ($\leq$10 °C and >4 °C) accumulation for two different mandarin cultivars, Nules Clementine and Pixie (56 and 52 LT degree-days, respectively). Subsequently, expression of downstream *LFY*/*AP1* was upregulated to a maximum level in both cultivars after accumulating 2 and 5 WT degree-days (>24 °C) at 1 and 2 MBFB, respectively, following the accumulation the LT degree-days. It is striking that the nearly equal requirements for LT and WT in the two cultivars were observed under distinctly different ambient air temperatures during winter and spring in the two orchards located in different citrus-growing areas and closely corresponded to the effects of LT or WT treatments on floral gene expression and flowering under controlled environmental conditions (Tang and Lovatt 2019). Thus, the results presented here provided the first data approximating the ambient air temperature requirements of citrus buds to attain maximum expression of *FT* and *LFY*/*AP1* for flowering under OFF-crop conditions. Further refinement is anticipated with the collection of data for additional cultivars and growing areas. Notably, LT requirements presented herein could be potentially developed into a model for determining the need to impose water-deficit stress in a given year to ensure successful flowering in warmer citrus-growing areas. Additionally, the results suggest that the degree-day approach, as used herein, may be more appropriate than calendar date for describing the temporal expression patterns of genes controlling plant developmental processes responding to ambient temperatures.

In the sequence of events inhibiting spring flowering following an ON-crop year, the presence of fruit exerted an overriding effect repressing *FT* expression from 4 to 1 MBFB (December through March) in buds of both *C. reticulata* cultivars. This prevented the successful completion of the induction process in floral meristem determinacy by reducing *LFY*/*AP1* expression, thereby preventing activation of the downstream floral organ identity genes in buds of ON-crop trees. The results indicated that the proper time to remove fruit from ON-crop trees to mitigate the severity of alternate bearing is likely cultivar-dependent and would need to be implemented more than 6 MBFB for some cultivars, which has heuristic, but not commercial, value.

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