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Effects of low temperature and gibberellic acid on floral gene expression and floral determinacy in 'Washington' navel orange (*Citrus sinensis* L. Osbeck)

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ABSTRACT

Low temperature (LT) and gibberellic acid (GA_3) were used, respectively, to promote and inhibit flowering in 'Washington' navel orange to identify relationships between floral gene expression and floral intensity. All trees were maintained under warm temperature (WT) (24/19 °C, day/night) from April to September. For trees receiving 11 additional weeks of WT, buds expressed low levels of *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *LEAFY* (*LFY*), *APETALA1* (*AP1*) and *APETALA2* (*AP2*), but never expressed *SEPALLATA1* (*SEP1*), *PISTILLATA* (*PI*) and *AGAMOUS* (*AG*) and did not flower. In contrast, trees transferred to LT (15/10 °C, day/night) for 8 weeks followed by WT through week 11 had significantly greater bud expression of *FT* and *SOC1* in week 2, *AP1* and *AP2* during weeks 8 through 10, and *SEP1*, *PI* and *AG* after transfer to WT; 84% of buds flowered. Foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8 of LT treatment did not affect *FT*, *SOC1* or *LFY* expression, but significantly reduced transcripts of *AP1* by week 8, *AP2*, *SEP1*, *PI* and *AG* after transfer to WT and inflorescence number to values equal to 11-week WT-treated trees. Delaying GA_3 application to weeks 4 through 8 of LT treatment increased *AP1*, *AP2*, *SEP1*, *PI* and *AG* expression equal to LT-treated trees and significantly greater than WT-treated trees; inflorescence number was significantly greater than trees in the WT treatment or receiving seven GA_3 applications, but less than LT-treated trees. The results provide evidence suggesting that in adult citrus buds determinacy and subsequent floral development are controlled by *AP1* and *AP2* transcript levels, which regulate downstream floral organ identity gene activity and the effect of GA_3 on citrus flower formation.

1. Introduction

Flowering in citrus (*Citrus* spp.) is promoted by low temperatures (LT) between 10–18 °C during the day and 5–13 °C at night (Moss, 1969; Southwick and Davenport, 1986; Lovatt et al., 1988; García-Luís et al., 1992; Nishikawa et al., 2007). Two weeks of floral-promoting LT treatment stimulated 'Tahiti' lime (*C. latifolia* Tan.) trees to flower at a low level (Southwick and Davenport, 1986), but 4 weeks of LT were required to significantly increase flowering of 'Washington' navel orange (*C. sinensis* L. Osbeck) (Moss, 1969; Lovatt et al., 1988) and Satsuma mandarin (*C. unshiu* Marc.) (Nishikawa et al., 2007). Floral intensity increased with the duration of the LT treatment, with maximum flowering occurring after 8 weeks of LT for 'Tahiti' lime (Southwick and Davenport, 1986), 'Washington' navel orange (Moss, 1969; Lovatt et al., 1988) and 'Valencia' sweet orange (*C. sinensis*) (Moss, 1976) and 10 weeks for Satsuma mandarin (Nishikawa et al., 2007). For 'Washington' navel orange, maximum inflorescence number was achieved when trees were exposed to 15 °C during the day and 10 °C at night compared to warmer day or night temperatures (Moss, 1969). Using this LT treatment, Moss (1969) demonstrated that photoperiod had no effect on floral intensity of 'Washington' navel orange.

In Satsuma mandarin, low temperature treatments known to increase floral intensity increased the expression of *FLOWERING LOCUS T* (*FT*) in buds and leaves and expression of *LEAFY* (*LFY*) in buds prior to morphological flower development (Nishikawa et al., 2007). In the model plant *Arabidopsis thaliana, FT*, *LFY* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) control flowering time by integrating signals from different floral pathways and subsequently upregulating the genes that establish and maintain floral meristem identity, including *LFY*, which is also classified as a floral meristem identity gene, and *APETALA1* (*AP1*) (Ratcliffe et al., 1999; Moon et al., 2005; Parcy, 2005; Horvath, 2009; Michaels, 2009; Lee and Lee, 2010). The putative homologs of these genes in citrus have been demonstrated to be functionally equivalent to their counterparts in *A. thaliana* (Pillitteri et al., 2004a, b; Endo et al., 2005; Tan and Swain, 2007). Similar to the results in Satsuma mandarin, LT treatments promoting

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flowering in 'Washington' navel orange increased the expression of *FT* in leaves and *SOC1*, *LFY* and *AP1* in buds (Pillitteri et al., 2004a, b; Chica and Albrigo, 2013a, b). The expression of *FT* and *SOC1* increased during the LT period; *LFY* and *AP1* expression occurred only after the trees were transferred to the warm temperature (23–24 °C). Thus, low temperature (10–18 °C day/ 5–13 °C night) is an effective tool for promoting flowering in citrus for the study of floral development.

In contrast, gibberellic acid (GA_3) inhibits flowering in citrus by continuing vegetative development of the shoot apical meristem (SAM) when applied before the SAM is determined (irreversibly committed to floral development) (Lord and Eckard, 1987). Once the citrus bud is determined, coincident with sepal formation, exogenously applied GA_3 no longer has an inhibitory effect on flowering (Lord and Eckard, 1987). The effect of GA_3 on flowering might be mediated by *FT*, since its expression in leaves of 'Salustiana' sweet orange (*C. sinensis*) was reduced 8, 32 and 50 days after a single spray of GA_3 (40 mg L^{-1}), which also reduced inflorescence number. In the same experiment, leaf *FT* expression was increased 8, 32, 50 and 80 days after one application of paclobutrazol (2000 mg L^{-1}), a GA biosynthesis inhibitor, which restored flowering (Muñoz-Fambuena et al., 2012). A single application of GA_3 (40 mg L^{-1}) or four applications of GA_3 (150 mg L^{-1}), which both reduced flowering, did not change the expression of *SOC1* in leaves of 'Salustiana' sweet orange (Muñoz-Fambuena et al., 2012) or buds of 'Orri' mandarin (*C. reticulata* Blanco x *C. temple* Hort. ex Y. Tanaka) (Goldberg-Moeller et al., 2013), respectively. In addition, expression of the floral organ identity genes *LFY* and *AP1* in leaves of 'Salustiana' sweet orange was not affected by a single application of GA_3 (40 mg L^{-1}) in December that reduced flowering in spring (Muñoz-Fambuena et al., 2012). In contrast, four applications of GA_3 (150 mg L^{-1}) made every 2 weeks starting in mid-November increased *LFY* expression but reduced *AP1* expression in the buds of 'Orri' mandarin trees in December and January and reduced flowering (Goldberg-Moeller et al., 2013). Whereas it is clear that foliar-applied GA_3 effectively reduces citrus floral intensity, further research is required to clarify the roles played by *FT*, *SOC1*, *LFY* and *AP1* in mediating GA_3-inhibition of citrus floral development.

Currently, there are striking omissions in the evaluation of the floral development pathway in citrus. Notably, with the exception of a *SEPALLATA1* (*SEP1*) gene in Satsuma mandarin (Nishikawa et al., 2009), no expression data have been published for the floral organ identity genes downstream from *AP1*. It is of relevance to this research that the activity of both class A organ identity genes *AP1* and *APETALA2* (*AP2*) is necessary for sepal formation in *A. thaliana* (Bowman et al., 1991; Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005) and that sepal formation was identified as the developmental marker that coincided with irreversible commitment to floral development in 'Washington' navel orange (Lord and Eckard, 1987). However, the potential role of *AP1* and *AP2* in bud determinacy in citrus has not been confirmed. Further, the expression pattern of *A. thaliana* homologs of floral timing, floral meristem identity and floral organ identity genes under conditions that promote or prevent flowering in citrus remain unknown. Warm and low temperature treatments and GA_3 provide valuable tools to promote and prevent flowering for comparative analysis of floral gene expression to increase our understanding of the roles played by these genes in floral induction, bud determinacy and flower formation in citrus.

Thus, the research presented herein was undertaken to compare the effects of a floral-promoting LT treatment of increasing duration (15/10 °C, day/night for 2, 4 and 8 weeks followed by 24/19 °C, day/night for 9, 7 and 3 weeks, respectively) with those of an 11-week WT treatment (24/19 °C, day/night), which did not result in flowering, on the expression sequence of eight classic genes putatively regulating floral timing (induction), floral meristem identity (determinacy), and floral organ identity (flower formation) in the buds of 'Washington' navel orange trees in relation to differences in floral intensity. Research was also conducted to quantify the effects of weekly foliar applications of GA_3 in weeks 2 through 8 compared to GA_3 applications delayed to weeks 4 through 8 of the floral-promoting LT treatment on the expression of each of the eight floral genes and inflorescence number in order to identify the genes associated with floral inhibition caused by GA_3 in citrus. Herein we report the first demonstration of the up-regulation of citrus *AP2*, *SEP1*, *PISTILLATA* (*PI*) and *AGAMOUS* (*AG*) associated with successful flower formation in response to LT treatment and subsequent down-regulation of these same genes when flowering was inhibited in buds of LT-treated trees also treated with GA_3.

2. Materials and methods

2.1. Plant material and treatment conditions

Five-year-old mature 'Washington' navel orange scions on 'Carrizo' citrange rootstock (*C. sinensis* L. Osbeck x *Poncirus trifoliata* L. Raf.) grown in 56-liter pots containing steam-sterilized University of California soil mix I (Baker, 1957) were used in this research. The research used a complete randomized design with four 'Washington' navel orange trees (replications) per treatment and six treatments. In treatments 1 through 3, trees were exposed to LT (16-hr day [500 µmol m^{-2} s^{-1}] at 15 °C/8-hr night at 10 °C) (Percival PGW growth chamber; 2.3 × 1.5 x 2.0 m; Percival, Boone, IA) for 2, 4 and 8 weeks, respectively, and then transferred to the WT (16-hr day [500 µmol m^{-2} s^{-1}] at 24 °C/8-hr night at 19 °C) for the remainder of the experiment culminating with bloom in week 11 (Moss, 1969; Lovatt et al., 1988). In treatments 4 and 5, two sets of 8-week LT-treated trees were sprayed weekly with 50 mg L^{-1} GA_3 (ProGibb 40%, Valent BioScience Corporation, Libertyville, IL), containing 0.01% Silwet L77 surfactant (Helena Chemical Company, Collierville, TN), in weeks 2 through 8 (7 applications) and weeks 4 through 8 (5 applications), respectively. For these two treatments, GA_3 was sprayed on the entire tree to give full canopy coverage. In treatment 6, trees were maintained in the WT for 11 weeks (Fig. 1). All trees used in this research had been maintained under WT conditions in a temperature/humidity-controlled glasshouse, with supplemental lighting to maintain a 16-hr day, for the five months prior to the start of the experiment in September, at the end of the second flush of vegetative shoot growth. All fruit were removed from the trees during this period to prevent a potential negative effect on floral gene expression and floral intensity (Muñoz-Fambuena et al., 2012; Shalom et al., 2012). With the exception of temperature and GA_3 applications, all trees were treated the same, including irrigation time and amount, fertilization, and relative humidity (~80%). Under this irrigation regime, average midday stem water potential for the WT-

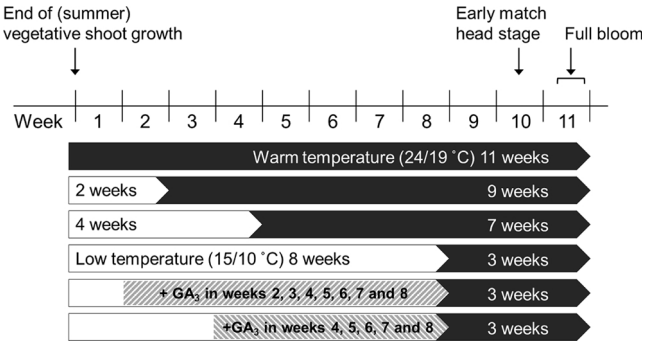

Fig. 1. Experimental design illustrating the different treatment combinations applied over time to 'Washington' navel orange trees: 2, 4 and 8 weeks of low temperature (LT) (15/10 °C, day/night) (□), 8 weeks of LT plus weekly foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8 (▨) or weeks 4 through 8 (▧), or 11 weeks of warm temperature (WT) (24/19 °C, day/night) (■). Stages of floral development apply to 8-week LT-treated trees only: early match head stage, flower buds were small, white balls the size of a match head; and full bloom, maximum number of inflorescences.

treated trees during the 11 weeks was -0.70 MPa and was never less than -1.50 MPa, indicating that the trees were not stressed.

2.2. Sample collection and gene expression analysis

The distal five buds from 15 nonbearing shoots per tree were collected at weeks 2, 4, 6, 8, 9, and 10 from each of the four trees (four replications) in treatments 3 through 6, with the exception that sample collection for the two sets of GA_3-treated trees (treatments 4 and 5) was delayed until 2 weeks after the first GA_3 application, respectively. No samples were collected from trees in treatments 1 and 2, which were used only to evaluate floral intensity. Collected buds were placed between moistened paper towels in a plastic bag and placed in a cooler box for immediate transport to the lab (~5 min). Bud samples were quickly frozen in liquid nitrogen and stored at −80 °C until analyzed. Total RNA was extracted from bud tissue, previously ground in liquid nitrogen, using Isolate Plant RNA Mini Kit (Bioline USA Inc., Taunton, MA) with quality and quantity of RNA evaluated by spectrophotometry using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Carla, CA). For cDNA synthesis, 1 µg total RNA was first treated with RQ1 RNase-Free DNase (Promega, Madison, WI), and used in first-strand synthesis using a Tetro cDNA Synthesis Kit (Bioline USA Inc., Taunton, MA) with oligo (dT) primer in a 30-µL reaction according to the manufacturer's protocol.

The sequences of *A. thaliana* homologs *FT*, SOC1, *LFY*, *AP1*, *AP2*, SEP1, *PI* and *AG* in *Citrus* spp. were obtained from GenBank and Reference Sequence databases (National Center for Biotechnology Information [NCBI] http://www.ncbi.nlm.nih.gov). Citrus *FT*, SOC1, *LFY* and *AP1* genes analyzed in this research were total *CiFT* (*CiFT1, CiFT2* and *CiFT3*) (Nishikawa et al., 2007), *CsSOC-like2* (*CsSL2*) (Tan and Swain, 2007), *CsLFY* and *CsAP1* (Pillitteri et al., 2004a), *PtAP2* (Song et al., 2010), *Cu*SEP1 (Nishikawa et al., 2009), respectively; each gene was selected based on its demonstrated functional equivalence in its respective *A. thaliana* mutant. In addition, expression of total *CiFT* (including individual *CiFT1, CiFT2*, *CiFT3* and total *CiFT*) was related to floral intensity in response to low temperature in *C. unshiu* (Nishikawa et al., 2007); *CsSL2* expression was also related to flowering in field-grown *C. reticulata* (Shalom et al., 2012). The sequences of *PI* and *AG* chosen in this research share high identity with *A. thaliana PI* and *AG*, respectively; the predicted protein sequences for the putative *PI* and *AG* were confirmed to be the most similar to those encoded by the *A. thaliana* genes, respectively, using the methods of Samach (2013). Gene-specific primers were designed using the web-based Integrated DNA Technology PrimerQuest program (http://www.idtdna.com/primerquest/Home/Index) with the filter of product size at the range of 100 bp to 200 bp. Annealing temperature and concentration for each primer set were optimized to the efficiency within the range of 90% to 110%. The sequences and the product sizes of the primer pairs used in this study as well as the BLAST results of PCR product sequence versus target sequence of each gene of interest are listed in Table 1.

Quantitative real-time PCR (qPCR) was carried out using the CFX96 Touch™ real-time PCR detection system with C1000 Touch™ thermal cycler (Bio-rad Laboratories, Hercules, CA) in a 15-µL reaction volume containing 1.2 µL cDNA (about 40 ng of input RNA), 0.6 µL gene-specific forward and reverse primer mix (10 nM), 7.5 µL SensiMix™ SYBR & Fluorescein (2X) (Bioline USA Inc., Taunton, MA), and 5.7 µL PCR-grade water. Each reaction was run at 95 °C for 10 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min. Melt-curve analysis ranging from 60 to 95 °C was performed at the end of each qPCR run to confirm that nonspecific products were not formed. Using quantification cycle (Cq) values less than 35 obtained from qPCR, relative levels of expression (fold change) of the genes of interest were calculated using the Pfaffl method (Pfaffl, 2001), with 'Washington' navel orange flowers collected from orchard trees at spring bloom as the control (expression level of 1) and *β-ACTIN* (*ACT*) as the primary reference gene (endogenous control), and reported herein. The selection of *ACT* as the primary reference gene was based on its stability in qPCR analysis across citrus genotypes and tissues (Yan et al., 2012). Reporting the floral gene expression data relative to the expression of each target gene in the 'Washington' navel orange flower was an important comparison made in this research to assess whether the level of floral gene expression increased to that associated with successful flower formation. However, in some cases, the level of target gene expression in the citrus flower resulted in the calculation of high or low relative expression values in the sampled buds despite their Cq values being between 20 and 31. Results based on a second reference gene, *ELONGATION FACTOR 1-ALPHA* (*EF1-α*) (Nishikawa et al., 2009), were similar for all target genes to those using *ACT* as the reference gene (data not shown). The expression pattern of each floral gene with *ACT* as the endogenous control was strongly correlated with its expression pattern when *EF1-α* served as the reference gene ($r = 0.71$ to 0.99, $P < 0.001$ for all genes), confirming the consistency and reliability of the results. Gene expression data for each treatment and sample date were the mean of four biological replicates; each biological replicate was the mean of three qPCR technical replicates.

2.3. Treatment effects on bud development

Maximum bloom occurred in trees exposed to 8 weeks of LT at week 11. At this time, the fate of the distal five buds on each of the 15 nonbearing shoots randomly selected from 100 to 120 shoots per tree was determined as the number 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 plus leafy inflorescences), vegetative shoots, and inactive (dormant) buds for trees in all treatments. Results for the five distal buds on the 15 shoots per tree were averaged for the four individual trees (replications) per treatment and reported as the average value per tree.

2.4. Statistical analysis

Analysis of variance (ANOVA) was used to test for treatment effects on the number of inflorescences, vegetative shoots and inactive buds per tree and the relative expression levels of genes (after square root transformation of the data to stabilize the variance), using the General Linear Model procedure of SAS (version 9.3; SAS Institute, Cary, NC). When ANOVA testing indicated significant differences, post-hoc comparisons were run utilizing Tukey's (HSD) procedure. Relative gene expression data were back transformed for presentation in all figures. Pearson's correlation coefficients were calculated to identify significant relationships ($r > 0.5$, $P \leq 0.05$) between the duration of the low temperature treatment and the developmental fate of buds and between gene expression level and inflorescence number, respectively. Significant correlations were subjected to regression analyses, using the least squares method for the generalized linear model.

3. Results

3.1. Effects of low temperature and GA_3 on flowering in week 11

'Washington' navel orange trees maintained in WT for 11 weeks produced an average of only 0.8 total inflorescence per tree (based in all cases on 5 buds/15 shoots/4 trees/treatment) (Table 2). Thus, the majority of the buds collected and analyzed in this research were not committed to floral development at the initiation of the experiment. Two weeks of LT treatment resulted in a non-significant increase to 2.3 total inflorescences per tree. After 4 weeks of LT treatment, inflorescence number increased to 17 per tree, which was not significantly greater than trees receiving 0 or 2 weeks of LT treatment. Trees exposed to 8 weeks of LT produced significantly more inflorescences (63 inflorescences/tree) than trees in all other treatments

Table 1
Forward and reverse primers for the citrus target and reference genes used in the quantitative real-time PCR (qPCR) assays.

Annotation	Accession number (*Citrus spp.*)	Forward primer (5' to 3') Reverse primer (5' to 3')	Product size (bp)	PCR product sequence blast against target gene sequence	
				E-value	Identity
SOC1	EU032532.1 (*C. sinensis*)	TCGACCCAACGGAAAGAAGCTGTA TGCCTAGAAGATTGCAGGAAGCCA	139	5E-46	98%
FT	AB027456.1 (*C. unshiu*)	CCGCGTTGTTGGTGATGTTCTTGA ATTTCAGCCCTAGGCTGGTTCAGA	132	6E-37	95%
LFY	AY338976.1 (*C. sinensis*)	TCTTGGGACAAAGCATCAACAGCG TCAAAGCTGCTGTTAGGGCTGAGA	112	3E-25	92%
AP1	AY338974.1 (*C. sinensis*)	ACCGCTCTCAAACACATCAG GCAGCCTTCTCTCTCTCC	137	7E-38	96%
AP2	EU883665.1 (*C. trifoliata*)	AAATGAAGCTGACTGGCACAACCG AGCGATGATGAAGCTGGTGACTGA	138	9E-18	95%
SEP	AB329715.1 (*C. unshiu*)	TGCTGAGGTGGCTCTCATCATCTT TCTCGAGCTCCTTTGCTGGCTTAT	146	1E-32	90%
PI	XM_006472790.1 (*C. sinensis*)	ATGGCCTTAGAGGATGCCCTTGAA AGCTATCTCCTGTTGCCCAGAACA	144	2E-36	92%
AG	HM246523.1 (*C. sinensis*)	GGGAAGTTGACTTGCACAACAGCA TAGCTCCGGGAATCAAATGGCTGA	142	1E-30	97%
ACT	GU911361.1 (*C. sinensis*)	TCACAGCACTTGCTCCAAGCAG TGCTGGAAGGTGCTGAGGGA	130	7E-34	98%

The database sources for the accession numbers: NCBI GenBank and Reference Sequence databases (http://www.ncbi.nlm.nih.gov).

($P < 0.0001$). There was a significant positive correlation between the duration of the LT period and the number of inflorescences produced per tree ($r = 0.95$, $P < 0.0001$). The duration of the LT period explained 91% of the variation in inflorescence number (Fig. 2). The number of leafy inflorescences was also significantly (positively) correlated with the duration of the LT treatment ($r = 0.82$, $P < 0.001$). Moreover, leafy inflorescences dominated bloom, comprising 89% and 74% of total inflorescences produced by trees exposed to 2 and 4 weeks of LT, respectively (Table 2). In contrast, leafless inflorescences were only produced in significant number by trees receiving 8 weeks of LT. Thus, the number of leafless inflorescences was not as strongly correlated with the duration of the LT treatment ($r = 0.75$, $P < 0.001$) as leafy inflorescences ($r = 0.82$, $P < 0.001$). The total number of inflorescences produced by trees receiving 8 weeks of LT treatment was reduced 96% when trees were treated with seven foliar applications of GA_3 in weeks 2 through 8, resulting in only 2.3 inflorescences per tree, a number equal to that of trees maintained in WT for 11 weeks ($P < 0.0001$) (Table 2). When the GA_3 treatment was restricted to five applications in weeks 4 through 8, floral intensity was 22.5 inflorescences per tree, a value intermediate to trees exposed to 8 weeks of LT without and with seven weekly applications of GA_3. The floral response to the five applications of GA_3 was equal to that of trees exposed to 4 weeks of LT without GA_3 treatment but significantly greater than trees maintained in WT for 11 weeks ($P < 0.0001$). The results suggest that 4 weeks of LT treatment is sufficient for a proportion (30%) of the buds to become committed to floral development.

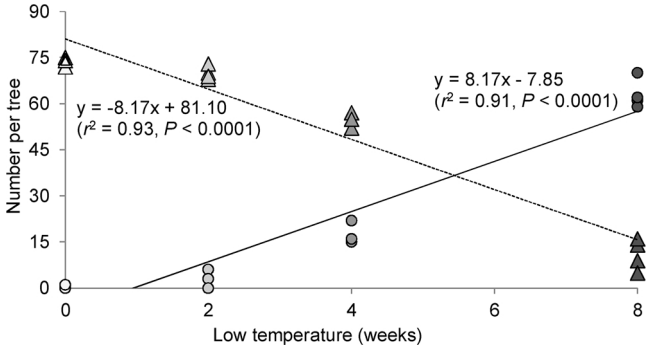

Fig. 2. Effect of low temperature (LT) (15/10 °C, day/night) on the number of inflorescences (– ●–) and inactive buds (– ▲–) of 'Washington' navel orange trees exposed to 2, 4, 6 and 8 weeks of LT and transferred to warm temperature (WT) (24/19 °C, day/night) for 9, 7, 5 and 3 weeks, respectively; trees receiving no LT treatment remained in the WT for 11 weeks. Data are the means of five distal buds/15 shoots/tree averaged across four trees per treatment.

The length of the LT treatment had no effect on the number of vegetative shoots produced per tree (Table 2). Trees exposed to 8 weeks of LT or 11 weeks of WT produced an equivalent number of vegetative shoots. In contrast, application of GA_3 during LT treatment significantly increased the number of vegetative shoots produced per tree ($P < 0.0001$) (Table 2). Seven applications of GA_3 starting in week 2 of the LT treatment resulted in the greatest number of vegetative shoots (24)

Table 2
Developmental fate of buds of 'Washington' navel orange trees exposed to 2, 4 and 8 weeks of low temperature (LT) (15/10 °C, day/night), 8 weeks of LT plus weekly foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8 or weeks 4 through 8, or 11 weeks of warm temperature (WT) (24/19 °C, day/night)[a].

LT	WC	GA_3	Total inflorescences No. per tree	Leafy inflorescences	Leafless inflorescences	Vegetative shoots	Inactive buds
0 wks	11 wks	No GA_3	0.8 c	0.5 b	0.3 b	0.5 b	73.8 a
2 wks	9 wks	No GA_3	2.3 c	2.0 b	0.3 b	2.8 b	70.0 a
4 wks	7 wks	No GA_3	17.0 bc	12.5 ab	4.5 b	2.8 b	55.3 ab
8 wks	3 wks	No GA_3	63.0 a	31.5 a	31.5 a	1.0 b	11.0 c
8 wks	3 wks	Wk 2 to 8	2.3 c	2.0 b	0.3 b	24.0 a	48.8 b
8 wks	3 wks	Wk 4 to 8	22.5 b	18.8 ab	3.8 b	11.8 b	40.8 b
P-value			< 0.0001	0.0033	0.0005	< 0.0001	< 0.0001

[a] Data are the means for four trees (5 apical buds/15 shoots/tree) per treatment. Data were collected in week 11. Means followed by different letters within a vertical column are significantly different at the specified *P*-value according to Tukey's honestly significant difference (HSD) test.

per tree ($P < 0.0001$). When GA_3 application was delayed to weeks 4 through 8, vegetative shoot number per tree was reduced by 50% ($P < 0.0001$), with a concomitant increase in inflorescence number. These results are consistent with a proportion of the bud population being committed to floral development by week 4 of LT treatment.

For trees receiving 11 weeks of WT or only 2 weeks of LT, the majority (73.8 and 70.0, respectively) of the 75 buds observed per tree remained inactive (dormant) (Table 2). There was a progressive and significant decrease in the number of inactive buds per tree related to the increase in the number of weeks at LT, e.g., 55.3 and 11.0 buds remained inactive per tree after 4 and 8 weeks of LT treatment, respectively ($P < 0.0001$) (Table 2). The number of inactive buds was significantly (negatively) correlated with the duration of the LT treatment ($r = -0.96$, $P < 0.001$) (Fig. 2), with the duration of the LT treatment explaining 93% of the variation in the number of inactive buds per tree. The negative relationship between LT and the number of inactive buds was largely due to the positive effect of LT on inflorescence development. The number of inactive buds was significantly (negatively) correlated with the total number of inflorescences per tree across all treatments, including GA_3 ($r = -0.99$, $P < 0.0001$), but not with vegetative shoot number ($r = -0.20$, $P = 0.464$).

3.2. Effects of warm and low temperatures and GA_3 on the expression of citrus floral timing genes

Transcripts of *FT* were detected in buds of trees maintained in WT for 11 weeks on all sampling dates, except week 8 (Fig. 3a). Expression of *FT* fluctuated significantly across the six sample dates, with expression significantly greater in week 6 than weeks 2 and 4, but equal to that of weeks 9 and 10 ($P = 0.0037$). For buds of LT-treated trees, *FT* expression did not change significantly over time and was significantly greater only at week 2 of the LT treatment compared to WT treated trees ($P = 0.0190$). Five or seven weekly applications of GA_3 to LT-treated trees had no significant effect on *FT* transcript levels on any sampling data (Fig. 3a). Similar to *FT*, *SOC1* expression occurred in buds of trees maintained under WT on each of the six sample dates and was significantly greater in week 6 than weeks 2 and 4, but equal to that of weeks 9 and 10 ($P = 0.0005$) (Fig. 3b). Buds of LT-treated trees also had significantly greater *SOC1* expression by week 2 compared to WT-treated trees ($P = 0.0290$). Bud *SOC1* expression was not significantly greater during weeks 4 through 10 for LT-treated trees than trees in the 11-week WT treatment. Five or seven weekly applications of GA_3 to LT-treated trees did not significantly affect *SOC1* expression (Fig. 3b). The expression of *LFY* was also detected in buds of trees in WT for 11 weeks on all sampling dates except at week 8, with maximum *LFY* expression in week 11 ($P = 0.0005$) (Fig. 3c). For buds of LT-treated trees, *LFY* expression reached a maximum after transfer to WT in week 9 ($P = 0.0158$), but was never significantly greater than *LFY* expression in buds of trees in the 11-week WT treatment. Neither GA_3 treatment had a significant effect on *LFY* expression (Fig. 3c). The values reported for *LFY* expression in Fig. 3c are high because *LFY* expression was very low in the 'Washington' navel orange flower, which served as the control (expression level of 1). This was not the case for *FT* or *SOC1*. To assess the validity of the results, the data were also analyzed using buds collected from trees at the start of the experiment (time zero). The expression levels for *LFY* were reduced, but no substantive changes in the data and their interpretation resulted. Total inflorescence number was not significantly related to expression of any floral timing gene across all treatments on any sampling date.

3.3. Effects of warm and low temperatures and GA_3 on the expression of citrus genes having class A activity

For trees in the 11-week WT treatment, bud *AP1* expression decreased over time, resulting in significantly greater expression during weeks 2 through 6 than weeks 8 through 10 ($P < 0.0001$) (Fig. 4a). In contrast, *AP1* expression did not change in the buds of LT-treated trees from week 2 through 10. As a result, transcript levels of *AP1* in buds of LT-treated trees were 8-, 6-, 3-fold greater than those of WT-treated trees in weeks 8 ($P = 0.0006$), 9 ($P = 0.0016$) and 10 ($P = 0.0167$), respectively. Seven foliar applications of GA_3 during weeks 2 through 8 of the LT treatment reduced bud *AP1* expression by 54%, 58% and 46% during weeks 8 ($P = 0.0006$), 9 ($P = 0.0016$) and 10 (not significant), respectively, relative to LT-treated trees not receiving GA_3 (Fig. 4a). Reducing the GA_3 treatment to five applications in weeks 4 through 8 resulted in *AP1* transcript levels that were not significantly different from those of trees receiving seven GA_3 applications (Fig. 4a). Bud *AP2* expression was more than 3- to 10-fold lower than *AP1* expression over the six sampling dates for trees receiving 11 weeks of WT (Fig. 4b). For the 8-week LT-treated trees, bud *AP2* expression significantly increased from weeks 2 through 10 ($P < 0.0001$), with maximum expression in week 10, after transfer of the trees to WT. As a result, bud *AP2* expression was significantly greater in LT-treated trees than 11-week WT-treated trees on all sampling dates ($P < 0.01$) except weeks 2 and 6. Seven foliar applications of GA_3 during weeks 2 through 8 of the LT treatment reduced *AP2* expression in week 10 ($P = 0.0003$) compared to LT-treated trees not receiving GA_3 (Fig. 4b). Five applications of GA_3 in weeks 4 through 8 of LT treatment had no effect on *AP2* expression; thus, the expression level in week 10 was greater than LT-treated trees receiving seven applications of GA_3 ($P = 0.0003$). Inflorescence number was strongly correlated across all treatments with expression of *AP1* at weeks 8 ($r = 0.91$, $P < 0.001$), 9 ($r = 0.73$, $P = 0.001$) and 10 ($r = 0.84$, $P < 0.001$) and *AP2* at weeks 8 ($r = 0.64$, $P = 0.015$), 9 ($r = 0.66$, $P = 0.008$) and 10 ($r = 0.94$, $P < 0.001$).

3.4. Effects of warm and low temperatures and GA_3 on the expression of citrus floral organ identity genes downstream from AP2

Transcripts of *SEP1* were at the limit of detection in weeks 2 and 4 and not detected thereafter in the buds of trees receiving 11 weeks of WT (Fig. 5a). For buds of trees exposed to 8 weeks of LT, *SEP1* transcripts were at the limit of detection or below it during the first 8 weeks, with *SEP1* expression increasing significantly after transfer of the trees from LT to WT ($P < 0.0001$). This significant increase in *SEP1* expression after transfer of LT-treated trees to WT failed to occur in buds of LT-treated trees receiving seven weekly GA_3 applications during weeks 2 through 8 (Fig. 5a). In contrast, for buds of LT-treated trees receiving only five GA_3 applications during weeks 4 through 8, *SEP1* was expressed during the 2 weeks after transfer to WT. Transcripts of *PI* were never detected in buds of trees maintained in WT for 11 weeks, except for a very low detectable transcript level in week 9 (Fig. 5b). For 8-week LT-treated trees, *PI* transcripts were only expressed at significant levels in buds after the trees were transferred from the LT to WT, with the value 3-fold greater in week 10 than week 9 ($P = 0.0020$). As observed for *SEP1*, seven applications of GA_3 from week 2 through 8 of the LT treatment blocked the increase in *PI* expression that occurred after LT-treated trees were transferred to the WT, whereas five applications of GA_3 in weeks 4 through 8 of the LT treatment resulted in *PI* expression after the trees were transferred from the LT to the WT. Thus, by week 10, the *PI* expression level in buds of LT-treated trees receiving five GA_3 applications was greater than that of trees in the 11-week WT treatment, but intermediate to that of buds of 8-week LT-treated and 8-week LT-treated trees also treated with GA_3 in weeks 2 through 8 ($P = 0.0075$). Transcripts of *AG* were below the limit of detection in weeks 2 through 8, with *AG* expression occurring at very low levels during weeks 9 and 10 in buds of trees in the 11-week WT treatment (Fig. 5c). For buds of trees exposed to 8 weeks of LT, *AG* was expressed at low levels in weeks 4 and 8 of the LT treatment. Expression of *AG* increased after the trees were transferred from the LT to WT ($P < 0.0001$) to a maximum value at week 10 that was significantly greater than that of trees in all other treatments, except 8-week LT-treated trees also treated with GA_3 in weeks 4 through 8 ($P < 0.001$).

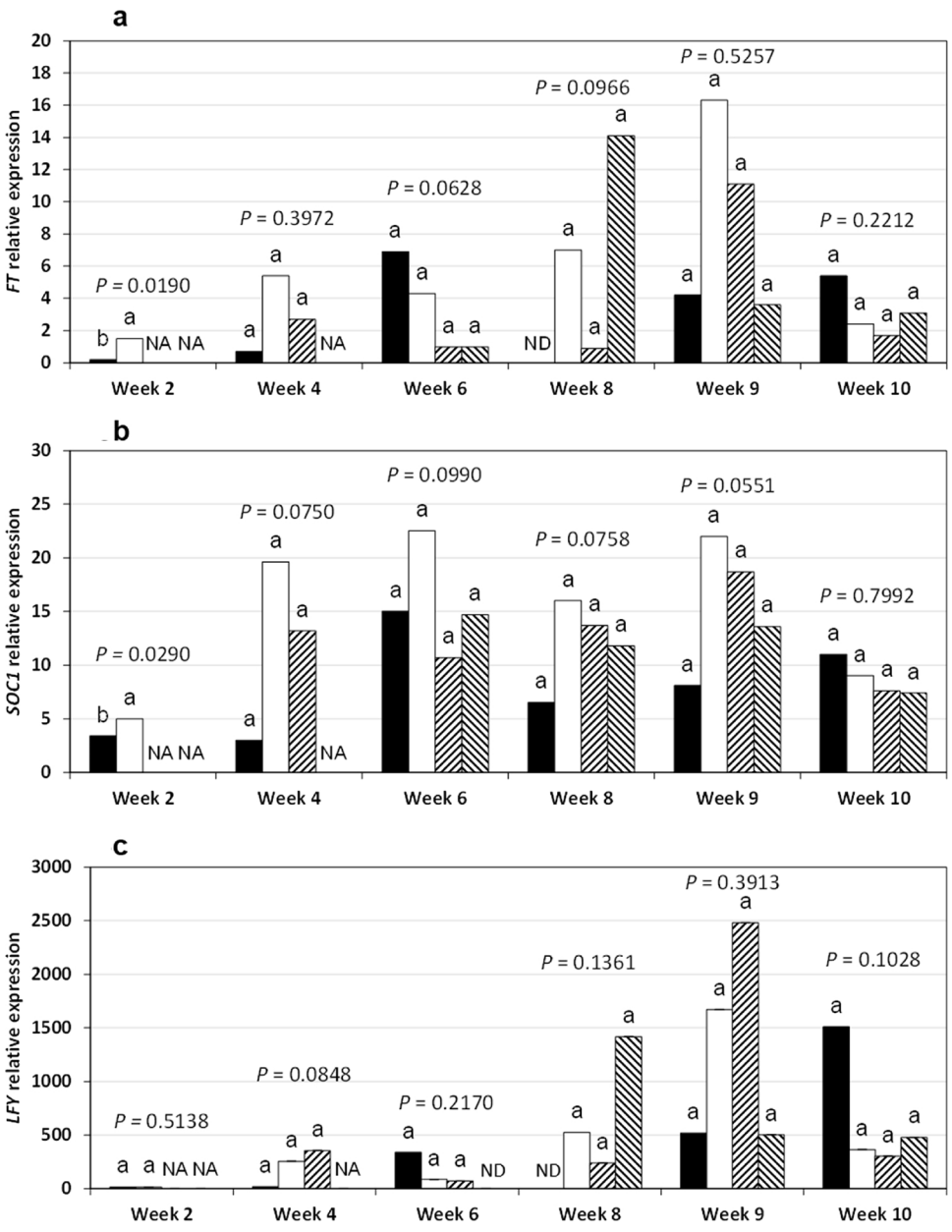

Fig. 3. Relative expression of *FT* (**a**), *SOC1* (**b**) and *LFY* (**c**) in buds of 'Washington' navel orange trees exposed to 8 weeks of low temperature (LT) (15/10 °C, day/night) (□), 8 weeks of LT plus weekly foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8 (▨) or weeks 4 through 8 (▧), or 11 weeks of warm temperature (WT) (24/19 °C, day/night) (■); data are the means for four trees (replications) calculated relative to the expression of each target gene in 'Washington' navel orange flowers (expression level = 1; normalized with *β-ACTIN* expression) (Pfaffl, 2001); for the same week, vertical bars with different lower-case letters are significantly different at the specified *P*-value according to Tukey's honestly significant difference (HSD) test; ND refers to not detected, which indicates the expression level of the target gene in each of the four biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35); NA, indicates that samples were not collected for analysis in week 2 and weeks 2 and 4 for LT-treated trees treated weekly with GA3 in weeks 2 through 8 or 4 through 8, respectively.

Expression of *AG* in buds of trees receiving 8 weeks of LT with seven weekly applications of GA_3 during weeks 2 through 8 was significantly reduced by week 10 to a level less than trees receiving 8 weeks of LT and equal to trees receiving 11 weeks of WT ($P = 0.0001$) (Fig. 5c). In contrast, five GA_3 applications in weeks 4 through 8 of the LT treatment increased *AG* expression by week 10 to a value equal to that of 8-week LT-treated trees not treated with GA_3 and significantly greater than that of 11-week WT-treated trees and trees treated with GA_3 from week 2 through 8 of LT ($P = 0.0001$). Inflorescence number was strongly correlated across all treatments with expression of *SEP1* at weeks 9 ($r = 0.94$, $P < 0.001$) and 10 ($r = 0.85$, $P < 0.001$), *PI* at weeks 9 ($r = 0.77$, $P < 0.001$) and 10 ($r = 0.98$, $P < 0.001$) and *AG* at weeks 9 ($r = 0.63$, $P = 0.012$) and 10 ($r = 0.90$, $P < 0.001$).

4. Discussion

The results presented herein were the first comparing the relative expression pattern of floral timing genes, floral meristem identity genes, and floral organ identity genes in the buds of citrus trees grown for a prolonged period (~eight months) under warm temperature conditions (24 °C day/19 °C night) with that in buds of trees exposed to 8 weeks of low temperature (15/10 °C, day/night). In this research, as previously reported, floral intensity increased with the duration of LT treatment (Moss, 1976; Southwick and Davenport, 1986; Lovatt et al., 1988; Nishikawa et al., 2007), from a low of 0.8 inflorescence per 75 buds per tree with no LT treatment (11 weeks of WT) to a high of 63 inflorescences per 75 buds per tree for 8-week LT-treated 'Washington' navel orange trees. The duration of the LT period explained 91% of the variation in inflorescence number per tree ($P < 0.0001$). The capacity of some buds, but not others, to flower after only 2 or 4 weeks of LT suggests that shoot (bud) age might be a factor in the response of citrus buds to conditions that promote flower formation (Tan and Swain, 2006; Chica and Albrigo, 2011). In light of the fact that the WT-treated trees did not flower (< 1 inflorescence/75 buds/tree), it is of interest that *FT*, *SOC1*, *LFY*, *AP1* and *AP2* (at a low level) were expressed in the buds of these trees at the start of the experiment and throughout the 11 weeks of continued WT treatment. For buds of both WT-treated and LT-treated trees, *FT*, *SOC1* and *LFY* expression patterns were variable over

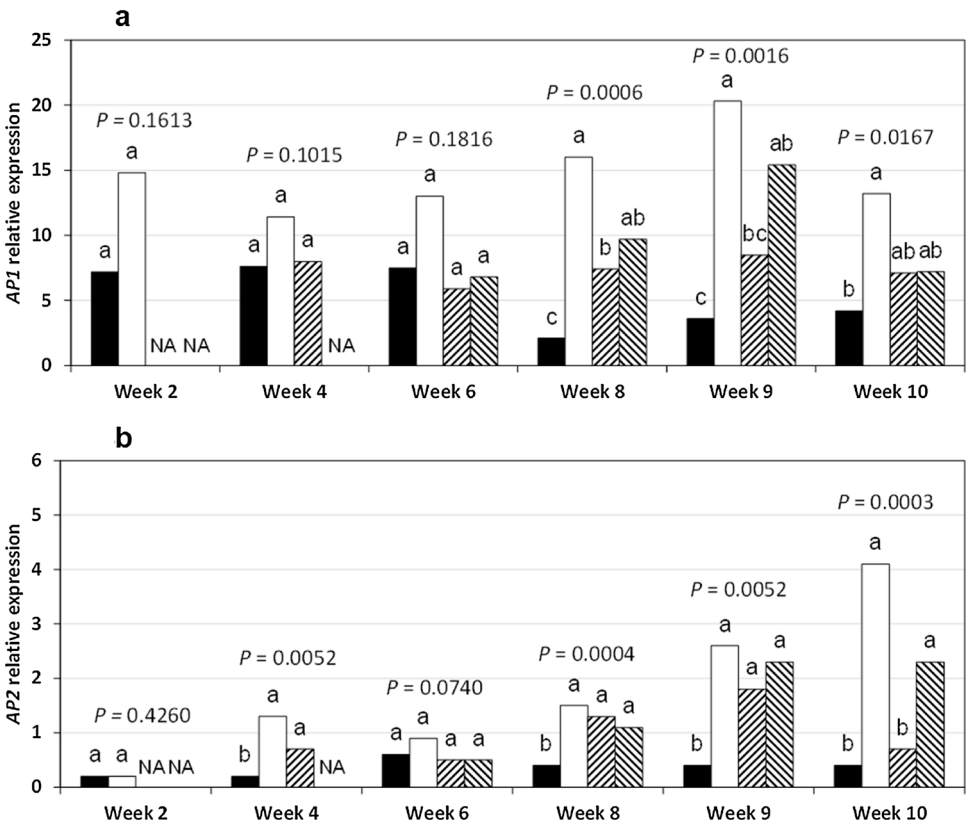

Fig. 4. Relative expression of *AP1* (**a**) and *AP2* (**b**) in buds of 'Washington' navel orange trees exposed to 8 weeks of low temperature (LT) (15/10 °C, day/night) (□), 8 weeks of LT plus weekly foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8 (▨) or weeks 4 through 8 (▧), or 11 weeks of warm temperature (WT) (24/19 °C, day/night) (■); data are the means for four trees (replications) calculated relative to the expression of each target gene in 'Washington' navel orange flowers (expression level = 1; normalized with *β-ACTIN* expression) (Pfaffl, 2001); for the same week, vertical bars with different lower-case letters are significantly different at the specified *P*-value according to Tukey's honestly significant difference (HSD) test; NA, indicates that samples were not collected for analysis in week 2 and weeks 2 and 4 for LT-treated trees also treated weekly with GA_3 in weeks 2 through 8 or 4 through 8, respectively.

time and transcript levels were not significantly different between the two treatments, with two exceptions. In week 2 of the experiment, *FT* and *SOC1* expression was significantly greater in buds of 8-week LT-treated trees than 11-week WT-treated trees. In addition, in week 8, *FT* and *LFY* expression decreased to levels below the limit of detection in the buds the WT-treated trees but were strong in the buds of LT-treated trees. It is unclear whether these early differences in floral timing gene expression had a subsequent impact on *AP1* and *AP2* expression and flowering of LT- and WT-treated trees. Buds of 8-week LT-treated trees expressed significantly greater levels of both *AP1* and *AP2* by week 8 of the LT treatment and during the two weeks after transfer from the LT to the WT conditions compared to buds of the 11-week WT-treated trees. Importantly, only buds of 8-week LT-treated trees expressed *SEP1*, *PI* and *AG* at significant levels, which occurred only after transfer of the trees to WT (weeks 9 and 10) just prior to when the trees flowered. Buds of 11-week WT-treated trees never expressed *SEP1* or *PI* at significant levels, and *AG* expression was present only at a very low level during weeks 9 and 10 and did not flower. In *A. thaliana*, expression of *LFY* and *AP1* is one of the first indications that the SAM has been induced to flower (Melzer et al., 1999). However, since buds of 11-week WT-treated trees never expressed *SEP1* or *PI* and only expressed *AG* at very low levels and did not flower, the results suggest the process of induction had been initiated by the start of the experiment (end of the second flush of vegetative shoot growth in September) but the resulting transcripts levels *LFY* and/or *AP1* were likely insufficient at a critical stage of floral develop to confer determinacy under WT conditions.

For 'Washington' navel orange, GA_3 inhibits flowering when applied before buds become determined (before sepal formation) (Lord and Eckard, 1987). Both GA_3 treatments significantly reduced inflorescence number (96% and 60% when applied in weeks 2–8 and 4–8 of the LT treatment, respectively), but neither GA_3 treatment had an effect on *FT*, *SOC1* or *LFY* expression. Importantly, the results demonstrated that, among the genes analyzed in this study, *AP1* and *AP2* were the earliest genes in the floral development pathway for which bud transcript levels were responsive to GA_3 treatments applied to 8-week LT treated trees. Weekly foliar applications of GA_3 in weeks 2 through 8 of the LT treatment reduced the expression of *AP1* in weeks 8 and 9 and *AP2* in week 10 to levels significantly less that of 8-week LT-treated trees with a concomitant decrease in floral intensity. Delaying the weekly GA_3 applications to weeks 4 through 8 of the LT treatment resulted in greater flowering than observed when GA_3 was applied in weeks 2 through 8 (Table 2) and bud *AP1* expression that was intermediate to that of the 8-week LT-treated and 11-week WT-treated trees in weeks 8, 9 and 10, with bud *AP2* expression equal to that of 8-week LT-treated trees during this period. Inflorescence number was most strongly correlated across all treatments with the expression patterns of *AP1* at week 8 ($r = 0.91$, $P < 0.001$) and *AP2* at week 10 ($r = 0.94$, $P < 0.001$). Taken together, the results provide significant evidence suggesting that floral inhibition in citrus caused by GA_3 is independent of an effect on *FT*, *SOC1* and *LFY* expression, but instead the result of down-regulated *AP1* and *AP2* transcription by GA_3. The results are in sharp contrast to the analysis of *FT* in leaves of 'Salustiana' sweet orange (Muñoz-Fambuena et al., 2012), but are consistent with the results of studies analyzing *SOC1*, and *LFY* expression in buds of 'Orri' mandarin (Goldberg-Moeller et al., 2013) and leaves of 'Salustiana' sweet orange (Muñoz-Fambuena et al., 2012). Further, Goldberg-Moeller et al. (2013) previously reported that GA_3 treatment reduced flowering in 'Orri' mandarin and reduced bud *AP1* expression, with no negative effect on *LFY* expression. The results of this research do not preclude the possibility that GA_3 regulates floral induction through *FT* prior to or during the early initiation of the process.

The results presented herein are the first to demonstrate the relationship between *AP1* and *AP2* transcript levels and the activity of the downstream floral organ identity genes in citrus buds. Bud expression of *AP1* and *AP2* was significantly greater by week 8 in LT-treated trees than that of the WT-treated trees. Removal of the LT stimulus upon transfer of the trees to WT at the end of week 8 did not interfere with the continued accumulation of *AP1* and *AP2* transcripts and resulted in increased expression of the downstream floral organ identity genes, resulting in flowering. The results indicate that 8 weeks of LT are

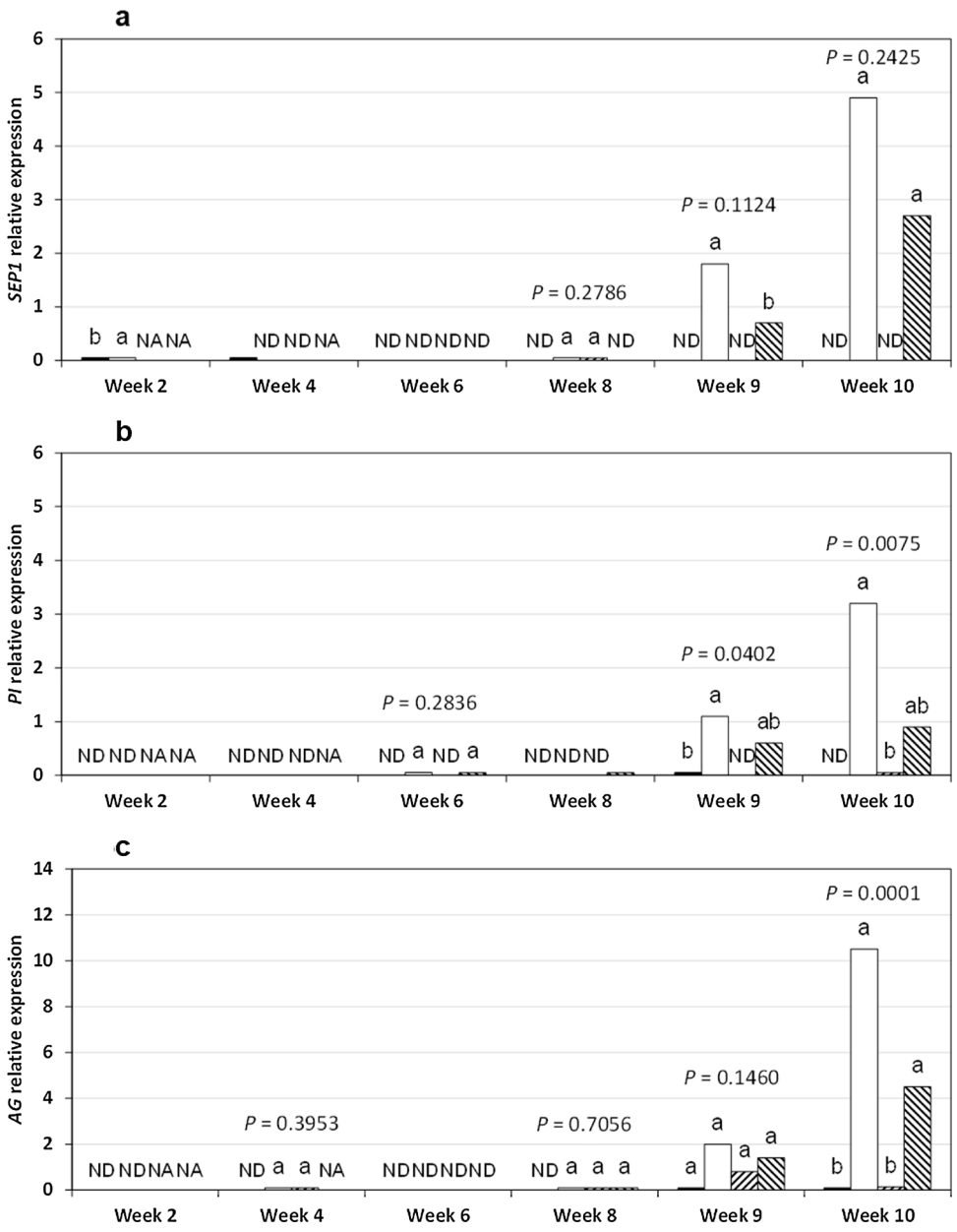

Fig. 5. Relative expression of *SEP1* (**a**), *PI* (**b**) and *AG* (**c**) in buds of 'Washington' navel orange trees exposed to 8 weeks of low temperature (LT) (15/10 °C, day/night) (□), 8 weeks of LT plus weekly foliar-applied GA_3 (50 mg L^{-1}) in weeks 2 through 8 (▨) or weeks 4 through 8 (▧), or 11 weeks of warm temperature (WT) (24/19 °C, day/night) (■); data are the means for four trees (replications) calculated relative to the expression of each target gene in 'Washington' navel orange flowers (expression level = 1; normalized with *β-ACTIN* expression) (Pfaffl, 2001); for the same week, vertical bars with different lower-case letters are significantly different at the specified *P*-value according to Tukey's honestly significant difference (HSD) test; ND refers to not detected, which indicates the expression level of the target gene in each of the four biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR > 35); NA, indicates that samples were not collected for analysis in week 2 and weeks 2 and 4 for LT-treated trees also treated weekly with GA_3 in weeks 2 through 8 or 4 through 8, respectively.

sufficient for floral bud determinacy. If the floral regulatory pathways in *C. sinensis* and *A. thaliana* were conserved, the results reported herein would suggest the low transcript levels of *AP1* and *AP2* observed in buds of 11-week WT-treated trees were apparently insufficient to activate *SEP1* or *PI* expression or to increase *AG* expression and thus, flowering did not occur. Similarly, when flowering was inhibited with seven applications of GA_3 in weeks 2 through 8 of LT treatment, bud *AP1* and *AP2* transcript levels were significantly reduced in weeks 8 and 9 and week 10, respectively, compared to 8-week LT-treated trees; transcripts of *SEP1* were not detected and transcripts of *PI* and *AG* were at the limit of detection in week 10. When GA_3 application was delayed to week 4 (only 5 applications), *AP1* expression was intermediate to that of 8-week LT-treated and 11-week WT-treated trees at weeks 8, 9 and 10, and *AP2, SEP1, PI* and *AG* expression was equal to that of the LT-treated trees and significantly greater than that of the WT-treated trees over the same 3 weeks before bloom; the floral intensity of the trees in this GA_3 treatment was intermediate to that of the 8-week LT-treated and 11-week WT-treated trees. Thus, in all cases where bud *AP1* and *AP2* expression was reduced to levels less than LT-treated trees and equal to WT-treated trees, *SEP1*, *PI* and *AG* activity was repressed and flowering did not occur. Across all treatments, inflorescence number was strongly correlated with the expression of *SEP1*, *PI* and *AG* in weeks 9 and 10 ($r \geq 0.90$, $P < 0.001$ for the three genes).

Taken together, the results of this research suggest that *AP1* and *AP2* transcript levels regulate the expression of downstream floral organ identity genes, *SEP1*, *PI* and *AG* and control the developmental fate of 'Washington' navel orange buds after the initiation of the floral induction process. According to the ABCE model for floral organ specification, expression of both class A genes, *AP1* and *AP2*, is required in *A. thaliana* for sepal formation (Bowman et al., 1991; Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005). For 'Washington' navel orange, sepal formation was the developmental marker coincident with irreversible commitment to floral development after which GA_3 no longer inhibited flowering (Lord and Eckard, 1987) and may be related to the putative role of *AP1* and *AP2* in citrus bud determinacy. This possibility awaits confirmation of the relationship between *AP1* and *AP2* gene expression and sepal formation in the citrus floral bud.

The results presented herein provide the first evidence in citrus that the greater expression of *AP1* and *AP2* in response to LT conferred determinacy and upregulated the expression of the downstream floral

organ identity genes, resulting in maximum flowering. Significant expression of *SEP1*, *PI* and *AG* only occurred after the 8-week LT-treated trees were transferred to WT, suggesting a possible failsafe mechanism to synchronize flowering with the warmer temperatures of spring. Transcription of *AP1* and *AP2* would increase under the low temperatures of fall and winter to a level that confers bud determinacy, but the downstream floral organ identity genes, *SEP1*, *PI* and *AG*, would only be expressed after spring temperatures are sufficiently warm, thereby preventing flower production under adverse temperature conditions.

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