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 (GA₃) 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 (API) 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, API and AP2 during weeks 8 through 10, and SEP1, PI and AG after transfer to WT; 84% of buds flowered. Foliar-applied GA₃ (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 API 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 GA₃ application to weeks 4 through 8 of LT treatment increased API, 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 GA₃ 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 API and AP2 transcript levels, which regulate downstream floral organ identity gene activity and the effect of GA₃ 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 OVER-EXPRESSION OF CONSTANS 1 (SOC1) control flowering time by integrating signals from different floral pathways and subsequently up-regulating the genes that establish and maintain floral meristem identity, including LFY, which is also classified as a floral meristem identity gene, and APETALA1 (API) (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
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 (GA3) 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 GA3 no longer has an inhibitory effect on flowering (Lord and Eckard, 1987). The effect of GA3 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 GA3 (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 GA3 (40 mg L^{-1}) or four applications of GA3 (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 ‘Ori’ 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 GA3 (40 mg L^{-1}) in December that reduced flowering in spring (Muñoz-Fambuena et al., 2012). In contrast, four applications of GA3 (150 mg L^{-1}) made every 2 weeks starting in mid-November increased LFY expression but reduced AP1 expression in the buds of ‘Ori’ mandarin trees in December and January and reduced flowering (Goldberg-Moeller et al., 2013). Whereas it is clear that foliar-applied GA3 effectively reduces citrus floral intensity, further research is required to clarify the roles played by FT, SOC1, LFY and AP1 in mediating GA3-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 flowering in spring (Muñoz-Fambuena et al., 2012) or four applications of GA3 (150 mg L^{-1}) in December that reduced flowering in spring (Muñoz-Fambuena et al., 2012). In contrast, four applications of GA3 (150 mg L^{-1}) made every 2 weeks starting in mid-November increased LFY expression but reduced AP1 expression in the buds of ‘Ori’ mandarin trees in December and January and reduced flowering (Goldberg-Moeller et al., 2013). Whereas it is clear that foliar-applied GA3 effectively reduces citrus floral intensity, further research is required to clarify the roles played by FT, SOC1, LFY and AP1 in mediating GA3-inhibition of citrus floral development.

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Thus, the research presented herein was undertaken to compare the effect 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 GA3 in weeks 2 through 8 compared to GA3 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 GA3 in citrus. Herein we report the first demonstration of the up-regulation of citrus AP2, SEPI, 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 GA3.

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; 23 × 1.5 × 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} GA3 (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, GA3 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 GA3 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 GA3 (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.](https://example.com/fig1)
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 GA3-treated trees (treatments 4 and 5) was delayed until 2 weeks after the first GA3 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 and 260/280 nm ratio. RNA quality was determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, 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, SOCI, 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, SOCI, LFY and AP1 genes analyzed in this research were total 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), CsSEP1 (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* ≤ 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 GA3 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
response to the effect of LT without and with seven weekly applications of GA3. 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 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 GA3 in weeks 2 through 8, resulting in only 2.3 in- florescences per tree, a value intermediate to trees exposed to 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 GA3 during LT treatment significantly increased the number of vegetative shoots produced per tree (P < 0.0001) (Table 2). Seven applications of GA3 starting in week 2 of the LT treatment resulted in the greatest number of vegetative shoots (24) produced per tree.

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

<table>
<thead>
<tr>
<th>Annotation</th>
<th>Accession number (Citrus spp.)</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
<th>Product size (bp)</th>
<th>PCR product sequence blast against target gene sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>SOC1</em></td>
<td>EU032532.1 (C. sinensis)</td>
<td>TGACCCAGAGCAGGGAGAAAGGCTGA</td>
<td>ATGATGAGATAAAGTACTATGGGC</td>
<td>139</td>
<td>5E-46</td>
</tr>
<tr>
<td><em>FT</em></td>
<td>AB024766.1 (C. sinensis)</td>
<td>TGCTGAGAACTGGGAGAAGGAGC</td>
<td>ATGGTCCTTTCAGAGGCTTCTCGA</td>
<td>132</td>
<td>6E-37</td>
</tr>
<tr>
<td><em>LFY</em></td>
<td>AY338976.1 (C. sinensis)</td>
<td>GGCTCCTGTTGCTGAGTTCCTCCG</td>
<td>ATTTCATCTGGGAGGAGCTTCTCGA</td>
<td>112</td>
<td>3E-25</td>
</tr>
<tr>
<td><em>AP1</em></td>
<td>AY338974.1 (C. sinensis)</td>
<td>AACGCTCTCAAAACAGCAAGAGC</td>
<td>TGTTAGAGAGAGAGAGAGAGAGAG</td>
<td>137</td>
<td>7E-38</td>
</tr>
<tr>
<td><em>AP2</em></td>
<td>EU883665.1 (C. sinensis)</td>
<td>AACGCTCTCAAAACAGCAAGAGC</td>
<td>TGTTAGAGAGAGAGAGAGAGAGAG</td>
<td>138</td>
<td>9E-18</td>
</tr>
<tr>
<td><em>SEP</em></td>
<td>AB329715.1 (C. sinensis)</td>
<td>TGCGCTGTAACTGGCATTTTAACTCA</td>
<td>AGCTACTTCTTCCTGCGTCCATG</td>
<td>146</td>
<td>1E-32</td>
</tr>
<tr>
<td><em>PI</em></td>
<td>XM_00647290.1 (C. sinensis)</td>
<td>TGCGCTGTAACTGGCATTTTAACTCA</td>
<td>AGCTACTTCTTCCTGCGTCCATG</td>
<td>144</td>
<td>2E-36</td>
</tr>
<tr>
<td><em>AG</em></td>
<td>HM246523.1 (C. sinensis)</td>
<td>GGGAAGCTTGATCGCAAACACGCA</td>
<td>AGAGAGAGAGAGAGAGAGAGAGAG</td>
<td>142</td>
<td>1E-30</td>
</tr>
<tr>
<td><em>ACT</em></td>
<td>GU911361.1 (C. sinensis)</td>
<td>TAGTCCGGGATTCAATAGTGCTGAA</td>
<td>AGAGAGAGAGAGAGAGAGAGAGAG</td>
<td>130</td>
<td>7E-34</td>
</tr>
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</table>


(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 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 GA3 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 GA3 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 GA3. The floral response to the five applications of GA3 was equal to that of trees exposed to 4 weeks of LT without GA3 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.

### 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 GA3 (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

<table>
<thead>
<tr>
<th>LT</th>
<th>WC</th>
<th>GA3</th>
<th>Total inflorescences No. per tree</th>
<th>Leafy inflorescences</th>
<th>Leafless inflorescences</th>
<th>Vegetative shoots</th>
<th>Inactive buds</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 wks</td>
<td>11 wks</td>
<td>No GA3</td>
<td>0.8 c</td>
<td>0.5 b</td>
<td>0.3 b</td>
<td>0.5 b</td>
<td>73.8 a</td>
</tr>
<tr>
<td>2 wks</td>
<td>9 wks</td>
<td>No GA3</td>
<td>2.3 c</td>
<td>2.0 b</td>
<td>0.3 b</td>
<td>2.8 b</td>
<td>70.0 a</td>
</tr>
<tr>
<td>4 wks</td>
<td>7 wks</td>
<td>No GA3</td>
<td>17.0 bc</td>
<td>12.5 ab</td>
<td>4.5 b</td>
<td>2.8 b</td>
<td>55.3 ab</td>
</tr>
<tr>
<td>8 wks</td>
<td>3 wks</td>
<td>No GA3</td>
<td>63.0 a</td>
<td>31.5 a</td>
<td>31.5 a</td>
<td>1.0 b</td>
<td>11.0 c</td>
</tr>
<tr>
<td>8 wks</td>
<td>3 wks</td>
<td>Wk 2 to 8</td>
<td>22.5 b</td>
<td>18.8 ab</td>
<td>3.8 b</td>
<td>11.8 b</td>
<td>40.8 b</td>
</tr>
<tr>
<td>8 wks</td>
<td>3 wks</td>
<td>Wk 4 to 8</td>
<td>22.5 b</td>
<td>18.8 ab</td>
<td>3.8 b</td>
<td>11.8 b</td>
<td>40.8 b</td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td></td>
<td>&lt; 0.0001</td>
<td>0.0033</td>
<td>0.0005</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

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 GA3 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 \((73.8 \text{ and } 70.0, \text{ respectively})\) 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 GA3 \((r = -0.99, P < 0.0001)\), but not with vegetative shoot number \((r = -0.20, P = 0.464)\).

3.2. Effects of warm and low temperatures and GA3 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 GA3 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 different across all treatments on any sampling date.

3.3. Effects of warm and low temperatures and GA3 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 8 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 to 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 GA3 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 GA3 (Fig. 4a).

Reducing the GA3 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 GA3 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 GA3 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 GA3 (Fig. 4b). Five applications of GA3 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 GA3 \((P = 0.0003)\). 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 \((r = 0.94, P < 0.001)\).

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 GA3 applications during weeks 2 through 8 (Fig. 5a). In contrast, for buds of LT-treated trees receiving only five GA3 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 GA3 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 GA3 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 GA3 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 GA3 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 GA3 in weeks 4 through 8 \((P < 0.001)\).
Expression of AG in buds of trees receiving 8 weeks of LT with seven weekly applications of GA3 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 GA3 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 GA3 and significantly greater than that of 11-week WT-treated trees and trees treated with GA3 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), FT 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 in in fluorescence per 75 buds per tree with no LT treatment (11 weeks of WT) to a high of 63 in-fl orescences 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 in-fl orescence 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 in-fl orescence/75 buds/tree), it is of interest that FT, SOCI, LFY, API1 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, SOCI and LFY expression patterns were variable over
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, GA3 inhibits flowering when applied before buds become determined (before sepal formation) (Lord and Eckard, 1987). Both GA3 treatments significantly reduced inflorescence number (96% and 60% when applied in weeks 2–8 and 4–8 of the LT treatment, respectively), but neither GA3 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 GA3 treatments applied to 8-week LT treated trees. Weekly foliar applications of GA3 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 GA3 applications to weeks 4 through 8 of the LT treatment resulted in greater flowering than observed when GA3 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 GA3 is independent of an effect on FT, SOC1 and LFY expression, but instead the result of down-regulated AP1 and AP2 transcription by GA3. 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 GA3 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 GA3 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
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 GA₃ 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 GA₃ 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 and 9 and 10, and *AP2*, *SEP1*, *PI* and *AG* expression was equal to that of the WT-treated trees exposed to 8 weeks of low temperature (LT) (15/10 °C, day/night) (□), 8 weeks of LT plus weekly foliar-applied GA₃ (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*) (*Pfaff, 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 GA₃ in weeks 2 through 8 or 4 through 8, respectively.

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 GA₃ 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 dormancy, 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.

References