

Effects of Water-deficit Stress and Gibberellic Acid on Floral Gene Expression and Floral Determinacy in ‘Washington’ Navel Orange

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ABSTRACT. Effects of water-deficit stress and foliar-applied gibberellic acid (GA₃) on ‘Washington’ navel orange (*Citrus sinensis*) floral gene expression and inflorescence number were quantified. Trees subjected to 8 weeks of water-deficit stress [average stem water potential (SWP) -2.86 MPa] followed by 3 weeks of re-irrigation (SWP recovered to > -1.00 MPa) produced more inflorescences in week 11 than trees well-irrigated (SWP > -1.00 MPa) for the full 11 weeks ($P < 0.001$). After 8 weeks of water-deficit stress, bud expression of *flowering locus t* (*FT*), *suppressor of overexpression of constans1* (*SOC1*), *leafy* (*LFY*), *apetala1* (*API*), *apetala2* (*AP2*), *sepallata1* (*SEPI*), *pistillata* (*PI*), and *agamous* (*AG*) increased during the re-irrigation period (weeks 9 and 10), but only *API*, *AP2*, *SEPI*, *PI*, and *AG* expression increased to levels significantly greater than that of well-irrigated trees. Foliar-applied GA₃ (50 mg·L⁻¹) in weeks 2 through 8 of the water-deficit stress treatment did not reduce bud *FT*, *SOC1*, or *LFY* expression, but prevented the upregulation *API*, *AP2*, *SEPI*, *PI*, and *AG* expression that occurred during re-irrigation in water-deficit stressed trees not treated with GA₃. Applications of GA₃ to water-deficit stressed trees reduced inflorescence number 95% compared with stressed trees without GA₃. Thus, GA₃ inhibited citrus (*Citrus* sp.) floral development in response to water-deficit stress through downregulating *API* and *AP2* expression, which likely led to the failed activation of the downstream floral organ identity genes. The results reported herein suggest that bud determinacy and subsequent floral development in response to water-deficit stress in ‘Washington’ navel orange 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. The water-deficit stress floral-induction pathway provides an alternative to low-temperature induction that increases the potential for successful flowering in citrus trees grown in areas experiencing warmer, drier winters due to global climate change.

Water-deficit stress, as well as low-temperature stress, is documented to induce flowering in citrus [*Citrus* sp. (Barbera et al., 1981, 1985; Chica and Albrigo, 2013a, 2013b; Li et al., 2017; Lovatt et al., 1988a, 1988b; Moss, 1969; Nakajima et al., 1992; Southwick and Davenport, 1986; Tang and Lovatt, 2019)]. The water-deficit floral-induction pathway in citrus is of particular interest because drought is increasingly pervasive due to climate change (Easterling et al., 2000), and drought stress can be regulated in irrigated orchards. With additional knowledge, it might be possible to improve irrigation practices, such as deficit irrigation, to limit the negative effects of drought while deriving a benefit from water deficit that increases flowering and yield in growing areas where winter low temperatures have become insufficient for flower induction due to climate change. Water-deficit stress substitutes for low temperature in citrus-growing regions near the equator that have distinct wet and dry seasons (Reuther, 1973) and is used to supplement low

temperature to increase flowering in the tropical-subtropical citrus-producing areas of Florida and Brazil (Albrigo et al., 2002, 2006). In addition, water-deficit stress-induced flowering is the basis of the ancient technique of *forzatura di limone* developed in Sicily to produce summer (*verdelli*) lemons [*Citrus limon* (Barbera et al., 1981, 1985)]. The technique “forces” trees to flower by imposing 8 weeks of water-deficit stress during the hot summer months. At the end of the stress period, trees are re-irrigated and flower within 3 to 4 weeks. The “*forzatura*” crop is harvested the following summer, when lemons are in demand by consumers. For both ‘Frost Lisbon’ lemon (Lovatt et al., 1988a, 1988b) and ‘Tahiti’ lime [*Citrus latifolia* (Southwick and Davenport, 1986)], inflorescence and flower numbers increased in parallel with the increasing severity or duration of the water deficit imposed, providing evidence of a quantitative effect of water-deficit stress on citrus flowering.

Despite the application of water deficit in commercial citrus production to augment spring bloom (Albrigo et al., 2006) or to induce out-of-season flowering (Maranto and Hake, 1985), research investigating the role of water deficit in regulating floral development at the level of gene transcription is limited (Chica and Albrigo, 2013b; Li et al., 2017). Imposing 6 weeks of moderate water-deficit stress (SWP -2.00 MPa) to induce flowering in ‘Washington’ navel orange (*C. sinensis*) provided evidence that leaf expression of *FT* increased during the stress period and decreased to pre-stress levels on re-irrigation, whereas bud expression of *SOC1*, *LFY*, and *API* increased only after the trees were re-irrigated; bud expression of *FT* was not

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quantified (Chica and Albrigo, 2013b). In a second experiment with 2-year-old ‘Femminello’ lemon trees on trifoliolate orange (*Poncirus trifoliata*) rootstock that flowered after only 2 weeks of minimal water-deficit stress (−1.5 MPa) vs. well-watered control trees (−1.0 MPa), which flowered poorly, leaf *FT* transcripts increased during water-deficit stress and decreased following re-irrigation (Li et al., 2017). Based on RNA sequencing results, Li et al. (2017) hypothesized that activation of a leaf *FT* contributed to the upregulation of *API* in buds, either directly or indirectly through *SOC1*, and ultimately resulted in the activation of the downstream floral organ identity genes. Roles for bud *FT* and *LFY* were not identified (Li et al., 2017). Whereas the results of these two studies suggest that floral induction is regulated by a leaf *FT* in response to water deficit, the role of bud *FT* and downstream genetic control of bud determinacy and floral organ identity in response to drought have yet to be delineated. Furthermore, it is currently not known whether water-deficit stress-induced flowering in citrus proceeds by the same floral development pathway as low-temperature-induced flowering or entails unique regulatory events. Having this information might prove valuable for improving the use of water deficit alone or in conjunction with low temperature to increase citrus flowering and yield.

Regulation of citrus floral development at the level of gene transcription is also of scientific interest and practical importance because, in contrast to *Arabidopsis thaliana* (Wilson et al., 1992), GA_3 inhibits citrus flowering (Guardiola et al., 1982; Lord and Eckard, 1987; Tang and Lovatt, 2019). Exogenous applications of GA_3 to citrus result in continued vegetative development of the shoot apical meristem (SAM) if applied before the SAM is determined (irreversibly committed to floral development) (Lord and Eckard, 1987). As a result, care must be taken to properly time citrus cultural management practices involving foliar-applied GA_3 to avoid negative effects on spring bloom (Lovatt and Coggins, 2019; Tang et al., 2021; Vashisth et al., 2020). It is important to note that thus far, the inhibitory effect of GA_3 on citrus floral development has been demonstrated only under conditions in which low temperature was the stimulus for flowering and that results observed at the level of gene transcription in these experiments have been variable. For example, in the Northern Hemisphere, flowering was reduced in ‘Salustiana’ sweet orange (*C. sinensis*) by a single spray of GA_3 (40 mg·L^{−1}) in early December, which reduced *FT* expression in leaves and *FT* and *API* expression in buds, with no effect on bud expression of *SOC1* or *LFY* (Goldberg-Moeller et al., 2013). In contrast, for ‘Orri’ mandarin (*Citrus reticulata* × *Citrus temple*), four foliar applications of GA_3 (150 mg·L^{−1}) made every 2 weeks starting in mid-November through the end of December reduced bud expression of *FT*, but had no effect on bud *SOC1* or *API* expression, despite reducing flowering (Muñoz-Fambuena et al., 2012a). In a third experiment, seven weekly applications of GA_3 (50 mg·L^{−1}) to ‘Washington’ navel orange trees during weeks 2 through 8 of an 8-week low-temperature floral-induction treatment had no effect on bud expression of *FT*, *SOC1*, or *LFY*, but reduced bud expression of *API* and *AP2*, totally repressed *SEP1*, *PI*, and *AG* expression, and reduced inflorescence number to that of control trees under warm-temperature well-irrigated conditions (Tang and Lovatt, 2019). Taken together, the results published to date fail to establish whether inhibition of citrus floral development by GA_3 is mediated at *FT* and/or by *API* and *AP2*.

Water-deficit stress and well-irrigated treatments provide a second, independent system from low and warm temperatures for promoting and preventing flowering in citrus, respectively, that has been underused in research investigating the roles played by specific genes in regulating floral initiation, floral meristem determinacy, and floral organ specification. Water-deficit stress also provides an additional tool for identifying the genes mediating the inhibitory effect of GA_3 on citrus floral development. Whether the effects of water deficit or GA_3 on citrus flowering are initiated in the leaf or in the bud, each treatment must result in a sequence of events that takes place in the bud to promote or inhibit flowering.

Herein, we report the results of comparative analyses of the temporal patterns of expression of core genes demonstrated to function in classic networks regulating floral development across plant species (Benlloch et al., 2007), and likely regulating floral timing [induction (*FT*, *SOC1*, and *LFY*)], floral meristem identity [determinacy (*LFY* and *API*)], and floral organ identity [flower formation (*AP2*, *SEP1*, *PI*, and *AG*)] in buds of ‘Washington’ navel orange trees in response to water-deficit stress conditions known to promote significant flowering and well-irrigated conditions known to sustain vegetative shoot growth (Lovatt et al., 1988a; Southwick and Davenport, 1986). This research was undertaken with the following objectives: 1) to profile the expression patterns of key genes regulating floral development from induction to floral organ specification in citrus buds in response to water-deficit stress; 2) to identify the genes conferring floral meristem determinacy when water deficit is the stimulus for flowering; and 3) using water-deficit stress as the positive flowering control, to identify the genes mediating the inhibitory effect of GA_3 on citrus flowering. The developmental fate of buds (floral, vegetative, or quiescent) on all trees was determined. To the authors’ knowledge, this study is the first to use water deficit to increase floral intensity to quantify the inhibitory effect of GA_3 on floral gene expression to identify the genes mediating the inhibition of flowering. Thus, this research is the first to quantify changes in citrus *AP2*, *SEP1*, *PI*, and *AG* transcript levels in relation to successful and unsuccessful flowering in response to water-deficit stress, with and without foliar-applied GA_3 , and well-irrigated conditions, respectively. The results provide the first evidence in citrus buds of the temporal relationship between changes in both *API* and *AP2* transcript accumulation with the upstream expression of *FT* and the downstream activation of the floral organ identity genes when flower formation was successful under water-deficit stress.

Materials and Methods

PLANT MATERIALS AND TREATMENT CONDITIONS. Five-year-old mature ‘Washington’ navel orange scions on ‘Carrizo’ citrange rootstocks (*C. sinensis* × *P. trifoliata*) grown in 56-L pots containing steam-sterilized soils composed of 50% plaster sand, 25% bark, and 25% peatmoss [v/v (Soil Mix I; University of California, Riverside Agriculture Operations, Riverside, CA)] were used in this research. The research used a complete randomized design with four ‘Washington’ navel orange trees (replications) per treatment and three treatments: (i) well-irrigated (negative control) trees, for which SWP was maintained at approximately −1.00 MPa for the 11 weeks of the experiment by daily irrigation; (ii) water-deficit stress (positive control) trees, for which

SWP was maintained at less than or equal to -2.40 MPa by deficit irrigation for 8 weeks (day 0 to day 55) (Southwick and Davenport, 1986), followed by 3 weeks of re-irrigation starting on day 55 (with SWP recovering to -1.00 MPa by day 60); and (iii) water-deficit stress trees (subjected to the same stress and re-irrigation conditions described for treatment 2) sprayed weekly with $50 \text{ mg}\cdot\text{L}^{-1}$ GA₃ (ProGibb 40%; Valent BioSciences, Libertyville, IL), containing 0.01% non-ionic surfactant (Silwet L77; Helena Chemical, Collierville, TN), in weeks 2 through 8 (seven applications) (Fig. 1). Applications of GA₃ were made in the morning to the entire tree to runoff to give full canopy coverage using a hand sprayer. The SWP values reported herein were measured according to McCutchan and Shackel (1992). For all trees in all treatments, the evening before the day of SWP measurement, three leaves per tree were randomly selected and each leaf was covered with a plastic zip lock bag and then wrapped with aluminum foil to prevent exposure to light. Between 0800 and 1000 HR the following day, each leaf was detached and SWP was determined, using a pressure chamber (PMS Instrument Company, Albany, OR). Midmorning SWP measured in this manner gives maximum SWP and is predictive of midday (lowest) SWP; both midmorning and midday SWP under and overestimate SWP by 0.3 MPa (Fulton et al., 2001). Thus, a midmorning SWP of less than -2.40 ensured that the trees were stressed. This process was repeated every 2 to 3 d during the 8-week stress period and every 5 d during the 3-week re-irrigation period, a sampling strategy that proved effective in a prior experiment. SWP for the well-watered control trees was determined on the same schedule. Note that day -6 refers to the day irrigation was withheld and day 0 refers to the first day that all water-deficit stress-treated trees reached a SWP less than -2.40 MPa, at which time the experiment was initiated (Fig. 2). For both sets of water-deficit stressed trees, the stress was inadvertently interrupted on days 14 and 15 by overwatering. On day 55, the end of water-deficit stress treatment, both sets of water-deficit stress-treated trees were re-irrigated, and by day 60, tree SWP had recovered to a nonstress level (> -1.00 MPa). After the two sets of trees subjected to water-deficit stress

were re-irrigated, they were maintained under well-irrigated conditions through flowering in week 11 (a period of 3 weeks).

All trees used in this research had been maintained under well-irrigated conditions in a temperature/humidity-controlled glasshouse located in the University of California, Riverside, CA (lat. $33^{\circ}58'N$, long. $117^{\circ}19'W$), with supplemental lighting of high-pressure sodium lamps to maintain a 16-h day ($500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of the PAR spectrum) for the 5 months before 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 eliminate a potential negative effect on floral gene expression and floral intensity (Muñoz-Fambuena et al., 2012a). With the exception of irrigation and GA₃ application, all trees were treated the same, including temperature [$24/19^{\circ}\text{C}$ (day/night)], daylength and light intensity [$16/8$ h (day/night)], fertilization ($12N-1.7P-6.6K$, slow-release, water-soluble fertilizer applied in the beginning of the experiment), and relative humidity ($\approx 80\%$).

SAMPLE COLLECTION AND GENE EXPRESSION ANALYSIS. The distal five buds from 15 nonbearing shoots per tree were collected between 1000 and 1020 HR on each sampling day at weeks 2, 4, 6, 8, 9, and 10 from each of the four trees (four replications) per treatment, with the exception that sample collection for the GA₃-treated trees was delayed until 2 weeks after the first GA₃ application. Collected buds, on shoots longer than five nodes with leaves attached, were placed between moistened paper towels in a plastic bag and placed in a cooler box for immediate transport to the laboratory (≈ 5 min). Leaves were removed and the five distal buds on each shoot 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 spectrophotometer (NanoDrop 2000; Thermo Scientific, Waltham, MA) and automated electrophoresis (2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA). For complementary DNA (cDNA) synthesis, $1 \mu\text{g}$ total RNA was first treated with DNase (RQ1 RNase-Free DNase; Promega, Madison, WI) to

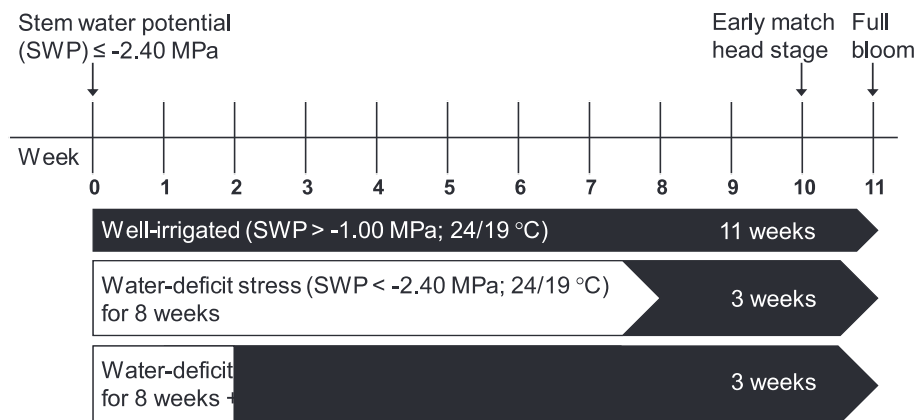


Fig. 1. Experimental design illustrating the different treatment combinations applied over time to ‘Washington’ navel orange trees: (i) 11 weeks of well-irrigated conditions [stem water potential (SWP) > -1.00 MPa (negative control) ■]; (ii) 8 weeks of water-deficit stress (SWP ≤ -2.40 MPa) followed by 3 weeks of re-irrigation [starting on day 55, with SWP recovering to -1.00 MPa by day 60 (positive control) □]; and (iii) 8 weeks of water-deficit stress (SWP ≤ -2.40 MPa) plus weekly foliar applications of $50 \text{ mg}\cdot\text{L}^{-1}$ gibberellic acid (GA₃) in weeks 2 through 8 followed by 3 weeks of re-irrigation (▨). All trees were grown under 16-h day ($500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 24°C and 8-h night at 19°C , with supplemental lighting and relative humidity of $\approx 80\%$. Stages of floral development apply only to 8-week water-deficit stress-treated trees, without GA₃ application: early match head stage, flower buds were small, white balls the size of a match head; and full bloom, maximum number of inflorescences.

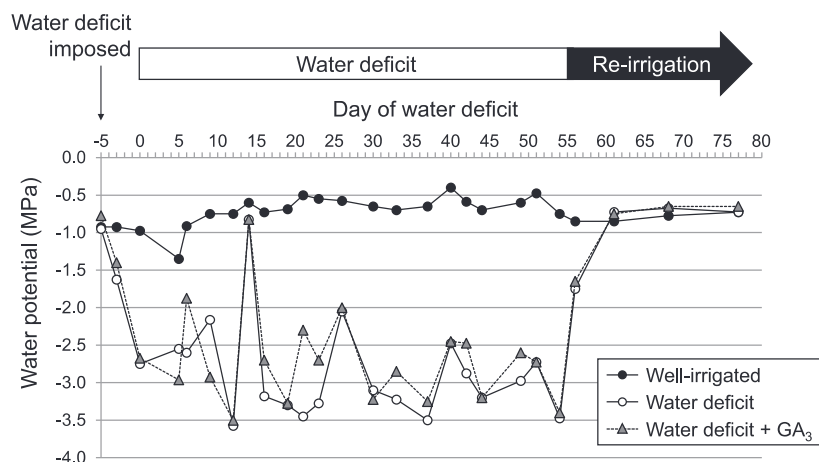


Fig. 2. Stem water potentials (SWP) for ‘Washington’ navel orange trees grown under (i) well-irrigated (SWP > -1.00 MPa) conditions for 11 weeks; (ii) 8 weeks of water-deficit stress (SWP ≤ -2.40 MPa) followed by 3 weeks of re-irrigation; and (iii) 8 weeks of water-deficit stress (SWP ≤ -2.40 MPa) plus weekly foliar applications of 50 mg·L⁻¹ gibberellic acid (GA₃) in weeks 2 through 8 followed by 3 weeks of re-irrigation.

eliminate potential DNA contamination and used in first-strand synthesis using a cDNA synthesis kit with oligo (dT) primer (Tetro cDNA Synthesis Kit, Bioline USA Inc.) in a 30-μL reaction according to the manufacturer’s protocol.

The sequences of target genes, including *FT*, *SOC1*, *LFY*, *API*, *AP2*, *SEP1*, *PI*, and *AG* in citrus were obtained from GenBank and Reference Sequence databases of the National Center for Biotechnology Information (NCBI, Bethesda, MD). These genes were selected based on their demonstrated functional equivalence in their respective *A. thaliana* mutants and the fact that their expression was significantly correlated with floral intensity in response to low temperature in citrus (Nishikawa et al., 2007, 2009; Pillitteri et al., 2004; Shalom et al., 2012; Song et al., 2010; Tan and Swain, 2007; Tang and Lovatt, 2019). Gene-specific primers were designed using the web-based PrimerQuest program (Integrated DNA Technology, San Diego, CA). 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 (NCBI) results of polymerase chain reaction (PCR) product sequence vs. target sequence of each gene of interest are listed in Table 1.

Quantitative real-time PCR (qPCR) was carried out using a real-time detection system (CFX96 Touch; 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 ng), 7.5 μL SYBR green reagent mix [SensiMix SYBR Fluorescein (2X); Bioline USA Inc.], 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 *beta-actin* (*ACT*) as the reference gene (endogenous control) and ‘Washington’ navel orange flowers collected from orchard trees at spring bloom as the calibrator control (expression level of 1). Note that the expression of *LFY* in the ‘Washington’ navel orange flower was low and resulted in the high expression values reported in Fig. 3C. To

verify the results, the data were analyzed using buds collected on day -6 (1 d before irrigation was withdrawn) as the control (expression level of 1). This reduced the expression levels for *LFY* with no substantive change in the results. 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). The data obtained using *ACT* as the primary reference gene were compared with data obtained using *elongation factor 1-alpha* (*EF1-α*) (Nishikawa et al., 2009) as a second reference gene. No substantial differences in the results or interpretation of the data reported herein were found (data not shown). 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.

TREATMENT EFFECTS ON BUD DEVELOPMENT. Maximum bloom occurred at week 11 for trees exposed to 8 weeks of water-deficit stress and re-irrigated for 3 weeks. 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 quiescent buds (inactive, did not produce a floral or vegetative shoot within the duration of the experiment) per tree 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.

STATISTICAL ANALYSIS. Analysis of variance (ANOVA) was used to test for treatment effects on the number of inflorescences, vegetative shoots, and quiescent buds per tree using the general linear model procedure of SAS (version 9.3; SAS Institute Inc., Cary, NC). Because buds were collected over time, treatment effects on expression levels of each gene (after square root transformation of the data to stabilize variance) on each sampling date were determined using the linear mixed model procedure with time as the repeated factor. The changes in gene expression levels over time within each treatment were also determined using the same procedure. When ANOVA testing indicated significant differences, post hoc comparisons were run using Tukey’s honestly significant difference procedure. Data were

Table 1. Primers for the target and reference genes used in the quantitative real-time polymerase chain reactions (PCR).

Annotation	Accession no. (<i>Citrus</i> sp.)	Forward and reverse primer sequence (5' to 3')	Product size (base pairs)	PCR product sequence blast against target gene sequence	
				Expect value	Identity (%)
<i>flowering locus t (FT)</i>	AB027456.1 (<i>C. unshiu</i>)	CCGCGTTGTTGGTGATGTTCTTGA ATTTCAGCCCTAGGCTGGTTCAGA	132	6E-37	95
<i>suppressor of overexpression of constans 1 (SOC1)</i>	EU032532.1 (<i>C. sinensis</i>)	TCGACCCAACGGAAAGAAGCTGTA TGCCTAGAAGATTGCAGGAAGCCA	139	5E-46	98
<i>leafy (LFY)</i>	AY338976.1 (<i>C. sinensis</i>)	TCTTGGGACAAAGCATCAACAGCG TCAAAGCTGCTGTTAGGGCTGAGA	112	3E-25	92
<i>apetala1 (AP1)</i>	AY338974.1 (<i>C. sinensis</i>)	ACCGCTCTCAAACACATCAG GCAGCCTTCTCTCTCTCC	137	7E-38	96
<i>apetala2 (AP2)</i>	EU883665.1 (<i>C. trifoliata</i>)	AAATGAAGCTGACTGGCACAACCG AGCGATGATGAAGCTGGTGACTGA	138	9E-18	95
<i>sepallata1 (SEPI)</i>	AB329715.1 (<i>C. unshiu</i>)	TGCTGAGGTGGCTCTCATCATCTT TCTCGAGTCTCTTGGCTGGCTTAT	146	1E-32	90
<i>pistillata (PI)</i>	XM_006472790.1 (<i>C. sinensis</i>)	ATGGCCTTAGAGGATGCCCTTGAA AGCTATCTCTGTTGCCAGAACA	144	2E-36	92
<i>agamous (AG)</i>	HM246523.1 (<i>C. sinensis</i>)	GGGAAGTTGACTTGCACAACAGCA TAGCTCCGGAATCAAATGGCTGA	142	1E-30	97
<i>beta-actin (ACT)</i>	GU911361.1 (<i>C. sinensis</i>)	TCACAGCACTTGCTCCAAGCAG TGCTGGAAGGTGCTGAGGGA	130	7E-34	98

back-transformed for presentation in Figs. 3–5. When the expression level of the target gene in each of the four biological replications was below the threshold value for detection (C_q in qPCR ≥ 35), the results were reported as not detected (ND) (Figs. 3 and 5). Pearson’s product moment correlation coefficients were calculated to identify significant relationships ($r > 0.5$, $P \leq 0.05$) between average SWP 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.

Results

EFFECTS OF WATER-DEFICIT STRESS AND GA₃ ON FLOWERING IN WEEK 11. For ‘Washington’ navel orange trees grown under well-irrigated conditions for 11 weeks, SWP averaged -0.70 MPa and was greater than -1.00 MPa on all sampling dates except one (Fig. 2), indicating that the trees were not experiencing water-deficit stress (Hake, 1995; Li et al., 2017). For trees in the water-deficit stress treatment, SWP decreased to -2.70 MPa after irrigation had been withheld for 5 d and was subsequently maintained at less than or equal to -2.40 MPa for most of the 54-d treatment period (Fig. 2). Averaged across the 54 d of water-deficit stress, the SWP for the two sets of water-deficit stress-treated trees was not significantly different; compare -2.86 ± 0.65 MPa for stressed trees not treated with GA₃ to -2.70 ± 0.62 MPa for stressed trees treated with GA₃ (Table 2, Fig. 2). The results document that the trees in the two stress treatments were subjected to water deficit of equivalent severity. In addition, when re-irrigated, both sets of trees recovered from water-deficit stress within 5 d [SWP > -1.00 MPa (Fig. 2)]. Thus, differences in floral intensity and gene expression observed for the two sets of trees were solely due to the exclusion or inclusion of GA₃ applications in the treatment. Importantly, both sets of trees subjected to water-deficit stress had SWPs

significantly lower than those of the well-irrigated trees throughout the 54-d water-deficit period and equal to those of well-irrigated trees in weeks 9 through 11 [$P < 0.001$ (Fig. 2)].

Trees that were well-irrigated for 11 weeks (negative control) produced an average of only 0.8 total inflorescence per tree (based in all cases on five buds/15 shoots per tree) (Table 2), indicating that most of the buds collected and analyzed in this research were not committed to floral development at the initiation of the experiment. In contrast, trees subjected to 8 weeks of water-deficit stress (positive control) produced 51 inflorescences per tree, a significantly greater number than that of well-irrigated trees ($P < 0.001$). Average SWP during the first 8 weeks of the experiment was significantly negatively correlated with the number of inflorescences produced per tree at week 11 ($r = -0.97$, $P < 0.001$) and average SWP explained 93% of the variation in inflorescence number. Leafless and leafy inflorescences comprised 57% and 43%, respectively, of the total inflorescences produced by trees subjected to 8 weeks of water-deficit stress. The number of leafless inflorescences produced by water-deficit stressed trees was numerically greater than that of well-irrigated trees (Table 2), but not significantly greater due to the variation in the number of leafless inflorescences produced by the four trees (replications) in the water-deficit stress treatment. The number of leafy inflorescences was significantly increased on trees subjected to 8 weeks of water-deficit stress compared with well-irrigated trees ($P < 0.01$) and was significantly negatively correlated with average SWP of individual trees ($r = -0.80$, $P = 0.018$). Water-deficit stress had no effect on the number of vegetative shoots produced per tree (Table 2).

The total number of inflorescences produced by trees subjected to 8 weeks of water-deficit stress (51/tree) was reduced 95% by weekly foliar applications of GA₃ during weeks 2 through 8 to only 0.3 inflorescence per tree ($P < 0.001$), a number not statistically different from that of 11-week well-irrigated trees (Table 2). In contrast, GA₃ treatment increased vegetative shoot number

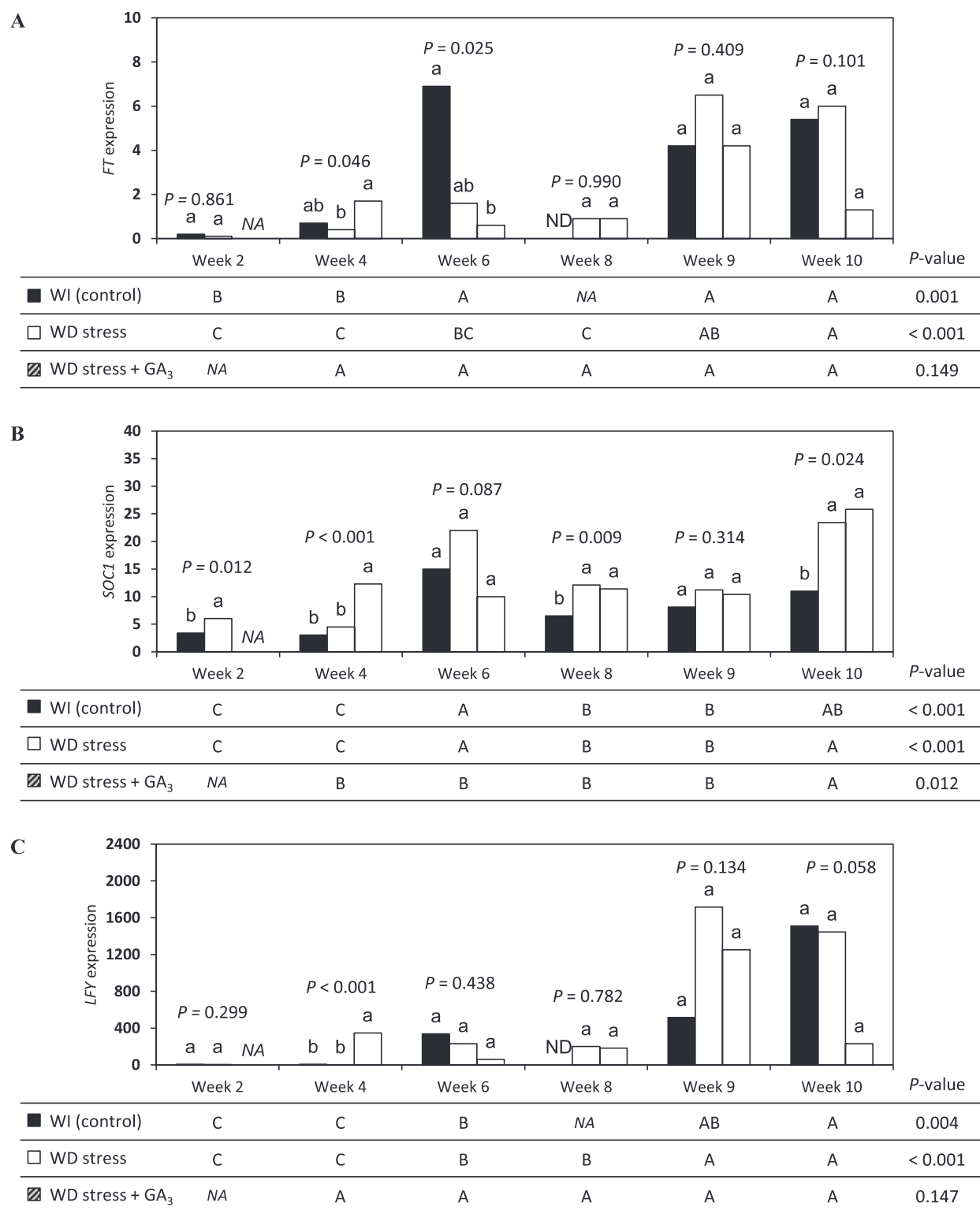


Fig. 3. Relative expression of *flowering locus t* (*FT*) (A), *suppressor of overexpression of constans 1* (*SOC1*) (B), and *leafy* (*LFY*) (C) in buds of 'Washington' navel orange trees grown under (i) well-irrigated {WI [stem water potential (SWP) > -1.00 MPa]} conditions for 11 weeks (■); (ii) 8 weeks of water-deficit (WD) stress (SWP ≤ -2.40 MPa) followed by 3 weeks of re-irrigation (□); and (iii) 8 weeks of WD stress (SWP ≤ -2.40 MPa) plus weekly foliar applications of 50 mg·L⁻¹ gibberellic acid (GA₃) in weeks 2 through 8 followed by 3 weeks of re-irrigation (▨); data are the means of four trees (four biological replicates) (normalized with *beta-actin* expression); means with different lowercase or uppercase letters (given below) are significantly different across treatments within the same week or significantly different across weeks within the same treatment, respectively, at the specified *P* value according to Tukey's honestly significant difference 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 > 35); NA indicates that statistical analysis of the data was not applicable because samples were not collected for gene expression analysis (week 2 when the GA₃ treatment was initiated) or gene expression was not detected.

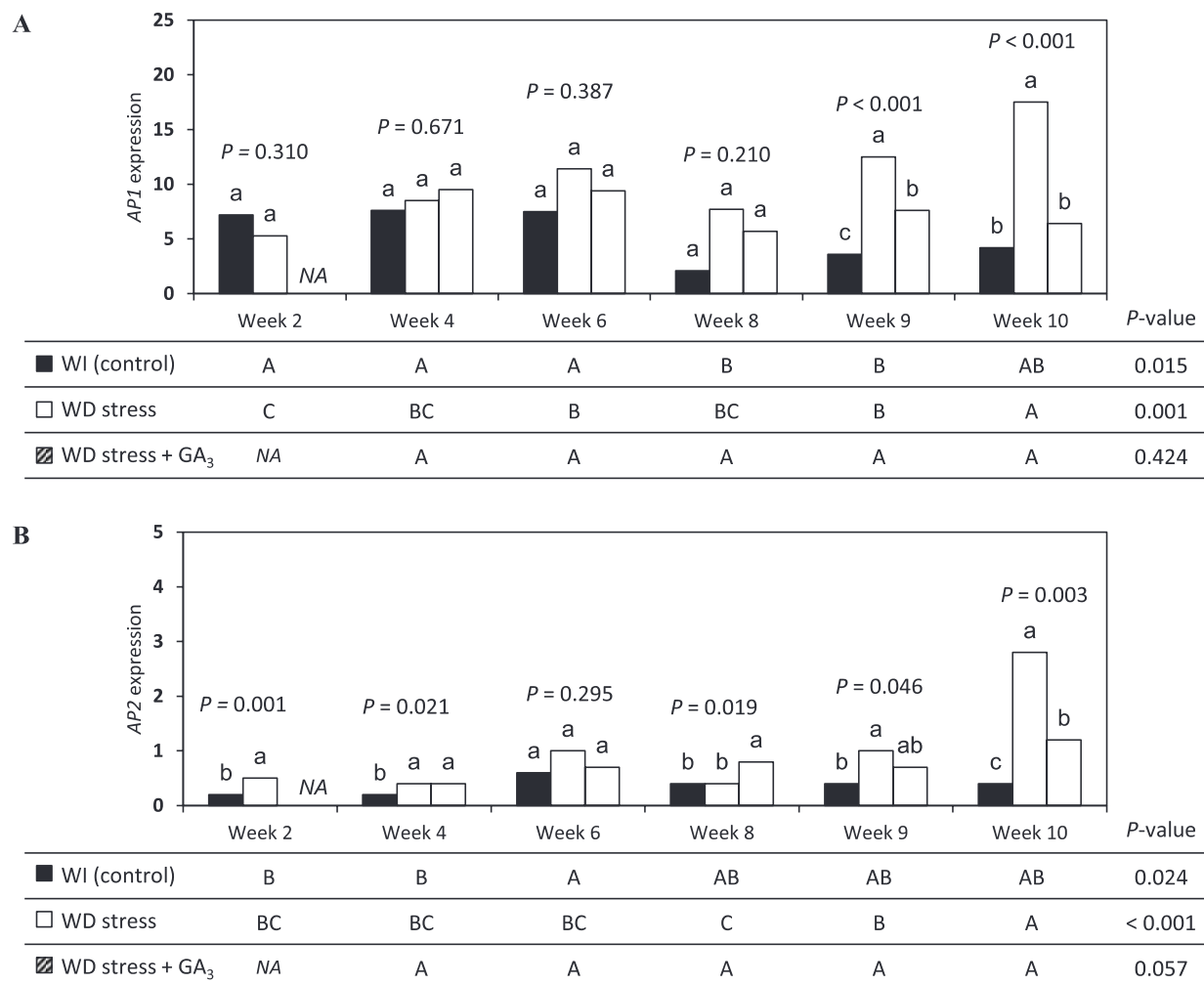


Fig. 4. Relative expression of *apetal1* (*AP1*) (A) and *apetal2* (*AP2*) (B) in buds of ‘Washington’ navel orange trees grown under (i) well-irrigated {WI [stem water potential (SWP) > -1.00 MPa]} conditions for 11 weeks (■); (ii) 8 weeks of water-deficit (WD) stress (SWP ≤ -2.40 MPa) followed by 3 weeks of re-irrigation (□); and (iii) 8 weeks of WD stress (SWP ≤ -2.40 MPa) plus weekly foliar applications of 50 mg·L⁻¹ gibberellic acid (GA₃) in weeks 2 through 8 followed by 3 weeks of re-irrigation (▨); data are the means of four trees (four biological replicates) (normalized with *beta-actin* expression); means with different lowercase or uppercase letters (given below) are significantly different across treatments within the same week or significantly different across weeks within the same treatment, respectively, at the specified *P* value according to Tukey’s honestly significant difference test; ND refers to not detected, which indicates the expression level of the target gene in each of the four biological replicates was below the threshold value for detection (quantification cycle > 35); NA indicates that statistical analysis of the data was not applicable because samples were not collected for gene expression analysis (week 2 when the GA₃ treatment was initiated) or gene expression was not detected.

(> 2.5-fold) to 23.3 per tree compared with trees subjected to water-deficit stress but not treated with GA₃ ($P < 0.01$). Well-irrigated trees produced even fewer vegetative shoots (0.5 vegetative shoot/tree). For the 11-week well-irrigated trees, most buds (73.8/75 buds/tree) remained quiescent (inactive, producing neither floral, nor vegetative shoots) (Table 2). Eight weeks of water-deficit stress significantly reduced the number of quiescent buds to 15.3 per tree compared with trees that were well-irrigated for 11 weeks ($P < 0.001$). The number of quiescent buds was significantly positively correlated with the average SWP during the 8 weeks of the water-deficit treatment ($r = 0.99$, $P < 0.001$), with average SWP explaining 98% of the variation in the number of quiescent buds. The relationship between SWP and number of quiescent buds was largely due to the positive effect of water-deficit stress on inflorescence development; the number of quiescent buds was significantly negatively correlated with the total number of inflorescences per tree across all three

treatments ($r = -0.96$, $P < 0.001$), but not with vegetative shoot number ($r = -0.42$, $P = 0.299$).

EFFECTS OF WATER-DEFICIT STRESS AND GA₃ ON THE EXPRESSION OF CITRUS FLORAL TIMING GENES. Transcripts of *FT*, *SOCI*, and *LFY* were detected in buds of trees that were well-irrigated for 11 weeks at the beginning of the experiment, and fluctuated significantly over time ($P = 0.001$, $P < 0.001$, and $P = 0.004$, respectively) (Fig. 3A–C). For trees subjected to 8 weeks of water deficit, bud *FT*, *SOCI*, and *LFY* expression levels during the stress period were not significantly different from those of well-irrigated control trees, except in week 8 when transcripts of *FT* and *LFY* were not detected and transcripts of *SOCI* were reduced 50% in well-irrigated trees, whereas they were continuously expressed in buds of water-deficit stressed trees. After the stressed trees were re-irrigated and SWP recovered to greater than -1.00 MPa, the expression of the three floral timing genes increased significantly in week 9 (*FT* and *LFY*) or week 10 (*SOCI*)

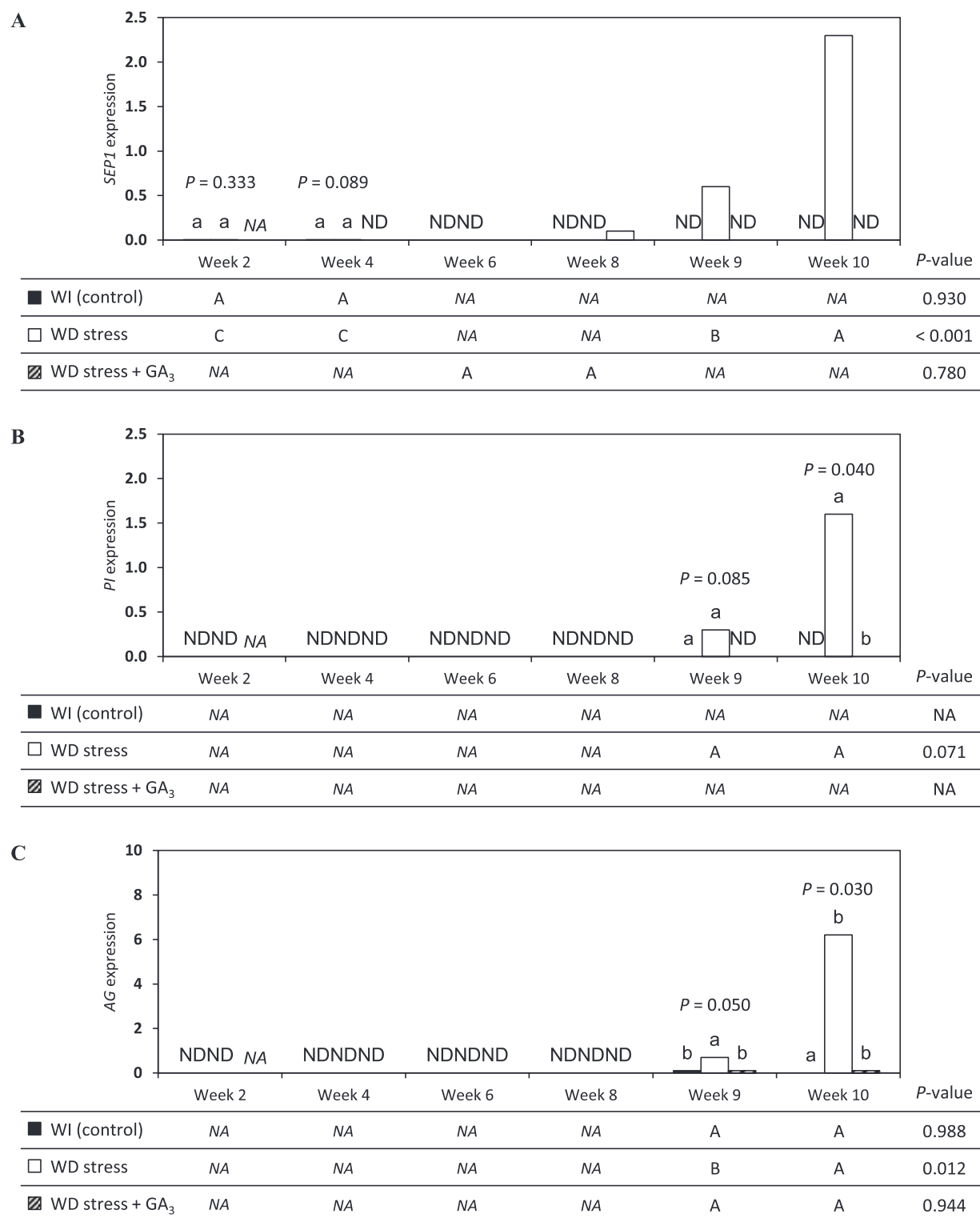


Fig. 5. Relative expression of *sepallata1* (*SEPI*) (A), *pistillata* (*PI*) (B) and *agamous* (*AG*) (C) in buds of 'Washington' navel orange trees grown under (i) well-irrigated {WI [stem water potential (SWP) > -1.00 MPa]} conditions for 11 weeks (■); (ii) 8 weeks of water-deficit (WD) stress (SWP ≤ -2.40 MPa) followed by 3 weeks of re-irrigation (□); and (iii) 8 weeks of WD stress (SWP ≤ -2.40 MPa) plus weekly foliar applications of 50 mg·L⁻¹ gibberellic acid (GA₃) in weeks 2 through 8 followed by 3 weeks of re-irrigation (▨); data are the means of four trees (four biological replicates) (normalized with *beta-actin* expression); means with different lowercase or uppercase letters (given below) are significantly different across treatments within the same week or significantly different across weeks within the same treatment, respectively, at the specified P value according to Tukey's honestly significant difference 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 > 35); NA indicates that statistical analysis of the data was not applicable because samples were not collected for gene expression analysis (week 2 when the GA₃ treatment was initiated) or gene expression was not detected.

Table 2. Average stem water potential (SWP) during the first 8 weeks of treatment (from day 0 to day 54) and the developmental fate of buds of ‘Washington’ navel orange trees grown under (i) well-irrigated [WI (SWP > -1.00 MPa)] conditions for 11 weeks; (ii) 8 weeks of water-deficit (WD) stress (SWP ≤ -2.40 MPa) followed by 3 weeks of WI; and (iii) 8 weeks of WD stress (SWP ≤ -2.40 MPa) plus weekly foliar applications of 50 mg·L⁻¹ gibberellic acid (GA₃) in weeks 2 through 8 followed by 3 weeks of WI.

WD (weeks)	WI (weeks)	GA ₃	Avg SWP (MPa)	Total inflorescences ^a (no./tree)	Leafless inflorescences (no./tree)	Leafy inflorescences (no./tree)	Vegetative shoots (no./tree)	Quiescent buds (no./tree)
0	11	No GA ₃	-0.70 a ^b	0.8 b	0.3 a	0.5 b	0.5 b	73.8 a
8	3	No GA ₃	-2.86 b	51.0 a	29.0 a	22.0 a	8.8 b	15.3 c
8	3	Week 2 to 8	-2.70 b	0.3 b	0.3 a	0.0 b	23.3 a	51.5 b
<i>P</i> value			****	***	NS	**	**	****

^aBud developmental fate was determined at full (maximum) bloom, which occurred at the end of week 11.

^bData are the means of four trees (five distal buds/15 shoots per tree) per treatment. Means followed by different letters within a column are significantly different according to Tukey’s honestly significant difference test in which ** refers to a significant effect at $P < 0.01$, *** at $P < 0.001$, **** $P < 0.0001$; NS = not significant.

compared with week 8 ($P < 0.001$ for all). However, in comparison with 11-week well-irrigated trees, only *SOCI* expression was greater in stressed trees and only in week 10 ($P = 0.024$); there was no significant difference in *FT* and *LFY* expression between the two treatments during the re-irrigation period. It should be noted that *FT*, *SOCI*, and *LFY* transcripts were present in buds of all ‘Washington’ navel orange trees before withdrawing irrigation, despite the trees being maintained for the previous 5 months under well-irrigated warm-temperature conditions that did not result in flowering. The developmental significance of the apparent synchronous decline in bud *FT* and *LFY* transcript levels to below the limit of detection (ND) and 50% decrease in *SOCI* transcript levels observed in week 8 for well-irrigated trees is unclear (Fig. 3A–C).

For trees receiving seven weekly applications of GA₃ starting in week 2 of the 8-week water-deficit stress treatment, bud expression levels for *FT* ($P = 0.046$), *SOCI* ($P < 0.001$), and *LFY* ($P < 0.001$) were significantly greater by week 4 compared with water-deficit stressed trees not receiving GA₃ and well-irrigated trees (Fig. 3A–C). For weeks 6 through 10, there were no significant differences in bud expression of *FT*, *SOCI*, or *LFY* among the three treatments, with the exception of week 8, when the expression of all three genes in well-irrigated trees decreased dramatically as discussed previously, resulting in greater expression of *FT*, *SOCI*, or *LFY* in both sets of water-deficit stressed trees (Fig. 3A–C). Most noteworthy, GA₃ had no inhibitory effect on *FT*, *SOCI*, or *LFY* expression in buds of ‘Washington’ navel orange trees subjected to 8 weeks of water-deficit stress on any sampling date, despite reducing total inflorescence number 95% (Fig. 3A–C, Table 2). Of additional importance, total inflorescence number was not significantly related to the expression level of any floral timing gene across all treatments and sampling dates.

EFFECTS OF WATER DEFICIT AND GA₃ ON THE EXPRESSION OF CITRUS GENES WITH CLASS A ACTIVITY. In buds of 11-week well-irrigated trees, *API* expression decreased significantly (72%) in week 8 compared with previous weeks (Fig. 4A). The greater transcript levels observed in weeks 2, 4, and 6 were not observed in week 9 ($P = 0.015$). In contrast, for trees subjected to water-deficit stress, despite a 33% decrease in expression in week 8 compared with the first 6 weeks, *API* expression significantly increased (>2-fold) from week 8 to a maximum value in week 10, after 2 weeks of re-irrigation ($P = 0.001$). As a result, transcript levels of *API* in buds of the water-deficit stressed trees were

3- and 4-fold greater than those of well-irrigated trees at weeks 9 and 10 ($P < 0.001$ for both weeks), respectively.

Bud *AP2* expression was significantly lower in weeks 2 and 4 than week 6 but returned to levels similar to week 2 during weeks 8 through 10 for well-irrigated trees [$P = 0.024$ (Fig. 4B)]. For trees subjected to water-deficit stress for 8 weeks, *AP2* expression did not change significantly from weeks 2 through 8, but increased after re-irrigation 7-fold from week 8 to a maximum value at week 10 ($P < 0.001$). Thus, with re-irrigation, trees subjected to 8 weeks of water-deficit stress exhibited significantly greater *AP2* expression by the end of week 9 ($P = 0.046$) and week 10 ($P = 0.003$) than well-irrigated trees.

Seven weekly applications of GA₃ to water-deficit stress-treated trees had no effect on *API* expression during the stress period, but significantly reduced *API* transcript levels during the re-irrigation period by 40% and 63% at the end of weeks 9 and 10 ($P < 0.001$), respectively, relative to water-deficit stress-treated trees not receiving GA₃ (Fig. 4A). As a result, *API* transcript levels in buds of GA₃-treated trees were equal to those of the well-irrigated (negative control) trees by week 10. Similarly, application of GA₃ had no effect or increased *AP2* expression during the 8-week water-deficit stress period, but subsequently prevented the significant increase in *AP2* transcript accumulation that occurred during the re-irrigation period in buds of water-deficit stressed trees not treated with GA₃, reducing *AP2* expression more than 50% by week 10 [$P = 0.003$ (Fig. 4B)]. Inflorescence number was significantly correlated across all treatments with the expression levels of *API* at week 9 ($r = 0.89$, $P < 0.001$) and week 10 ($r = 0.91$, $P < 0.001$), and *AP2* at week 9 ($r = 0.63$, $P = 0.028$) and week 10 ($r = 0.89$, $P < 0.001$).

EFFECTS OF WATER DEFICIT AND GA₃ ON THE EXPRESSION OF CITRUS FLORAL ORGAN IDENTITY GENES DOWNSTREAM FROM AP2. Transcripts of *SEPI* were not detected on any sampling date in buds of trees that were well-irrigated for 11 weeks (Fig. 5A). For water-deficit stress-treated trees, *SEPI* transcripts were first detected after SWP returned to a nonstressed level (> -1.00 MPa) during the first week of the re-irrigation period that followed the 8 weeks of stress. Maximum *SEPI* expression occurred at week 10 ($P < 0.001$). Similarly, *PI* transcripts were not detected in buds of well-irrigated trees for the duration of the 11-week experiment and only detected in water-deficit stressed trees after the trees were re-irrigated (Fig. 5B). For water-deficit stress-treated trees, bud *PI* transcript levels increased 5-fold from week 9 to a maximum value at week 10 (2 weeks after re-irrigation). Transcripts of *AG* were also not detected in buds of well-irrigated

trees at any time during the 11-week experiment (Fig. 5C). Bud *AG* expression was also not detected in water-deficit stressed trees during the 8-week stress period. However, after re-irrigation, *AG* transcript levels increased 9-fold from week 9 to a maximum level at week 10 (2 weeks after re-irrigation).

Weekly application of GA₃ to ‘Washington’ navel orange trees in weeks 2 through 8 of the 8-week water-deficit stress treatment inhibited the accumulation of *SEPI*, *PI*, and *AG* transcripts that occurred during the re-irrigation period in buds of trees subjected to 8 weeks of water-deficit stress but not treated with GA₃ (Fig. 5A–C). Thus, *SEPI*, *PI*, and *AG* all failed to reach the maximum values of expression in week 10 for water-deficit stress-treated trees applied with GA₃. Total inflorescence number was strongly correlated across all treatments with the expression levels of *SEPI* at week 9 ($r = 0.83$, $P = 0.003$) and week 10 ($r = 0.88$, $P < 0.001$), *PI* at week 10 ($r = 0.90$, $P < 0.001$), and *AG* at week 10 ($r = 0.91$, $P < 0.001$).

Discussion

In citrus, as in other woody perennial tree crops, the floral development process is protracted. Successful transition from a vegetative to reproductive SAM occurs many months before floral organogenesis and spring bloom. Environmental conditions at the time of key developmental events are critical to successful flowering and commercial tree crop productivity. Flowering must be timed to environmental conditions that favor flower formation and retention, successful syngamy, including synchrony with pollinizer bloom and pollinator activity in out-crossing species, and promote fruit set of seeded and parthenocarpic fruit, such as the ‘Washington’ navel orange (Hong and Jackson, 2015).

The results of this research are consistent with earlier reports that various drought treatments, independent of photoperiod, promoted flowering in citrus (Barbera et al., 1981, 1985; Chica and Albrigo, 2013b; Li et al., 2017; Lovatt et al., 1988a, 1988b; Moss, 1969; Nakajima et al., 1992; Southwick and Davenport, 1986) and add to the growing body of literature that water deficit (drought) is an important environmental factor inducing flowering across species (Takeno, 2016). For ‘Washington’ navel orange trees subjected to 8 weeks of water-deficit stress (average SWP -2.86 MPa), after being maintained for 5 months under well-irrigated warm-temperature conditions, most (68%) of the buds produced inflorescences, 12% produced vegetative shoots, and 20% remained quiescent. In contrast, 98% of buds on well-irrigated trees remained quiescent, only 1.0% produced inflorescences, and 0.7% produced vegetative shoots, indicating that most buds were not committed to floral development at the start of the experiment. Average SWP during the first 8 weeks of the experiment was significantly negatively correlated with the number of inflorescences produced per tree at week 11, with average SWP explaining 93% of the variation in inflorescence number.

The first results documenting that GA₃ inhibits water-deficit stress-induced flowering in citrus are presented herein. When GA₃ was applied during weeks 2 through 8 of the 8-week water-deficit stress treatment, inflorescence number was reduced 95%, with a concomitant 63% increase in vegetative shoot number compared with trees subjected to 8 weeks of water-deficit stress not treated with GA₃. Although both sets of trees were subjected to water-deficit stress of equal severity, 69% of the buds of water-deficit stress trees treated with GA₃ remained

quiescent. In addition to inducing floral development, 8 weeks of water-deficit stress increased percent budbreak in week 11 to 80% compared with 69% for water-deficit stressed trees treated with GA₃ and 1.6% for well-irrigated trees. Inflorescence number, but not vegetative shoot number, in the present research was strongly negatively correlated with the number of quiescent buds per tree across all treatments. Thus, for ‘Washington’ navel orange, the effect of water-deficit stress on floral induction and floral budbreak was closely related. Whether this constitutes a single response or two separate responses to water deficit is unknown. In ‘Owari’ Satsuma mandarin (*Citrus unshiu*), the parallel increases in floral induction and budbreak due to low temperature were demonstrated to be two distinct responses (García-Luís et al., 1992).

In light of the fact that the well-irrigated trees did not flower (<1 inflorescence/75 buds/tree), it is of interest that *FT*, *SOCI*, *LFY*, *API*, and *AP2* (at a low level) were expressed in the buds of these trees throughout the 11-week well-irrigated treatment. The expression of *SOCI*, *LFY*, and *API* in buds of well-watered control trees was also reported by Chica and Albrigo (2013b). For trees exposed to water deficit, *FT*, *SOCI*, and *LFY* transcripts accumulated in buds during the re-irrigation period following the stress treatment. However, only *SOCI* was expressed at a level greater than that of the well-irrigated control trees at the end of the stress period in week 8 and 2 weeks after re-irrigation in week 10. The lack of significant difference in *FT* expression in buds of water-deficit stressed and well-irrigated trees is consistent with the possibility that floral development in response to water deficit in this study was also initiated through activation of a leaf *FT* (Chica and Albrigo, 2013b; Li et al., 2017). Li et al. (2017) proposed that an activated leaf *FT* might upregulate bud *API* directly or indirectly via a bud *SOCI*. It is tempting to speculate the significantly greater expression of *SOCI* in buds of water-deficit stressed trees at weeks 8 and 10 supports a role for *SOCI* in this hypothetical model. Nonetheless, it is unlikely that bud *FT*, *SOCI*, and *LFY* are the key regulators of bud determinacy given the fact that GA₃ applied during the water-deficit stress treatment resulted in the expression of these genes at levels equal to or greater than those of water-deficit stressed trees not treated with GA₃ on all sampling dates and yet, GA₃ profoundly suppressed the number of inflorescences that developed by 95%. These results compared with results obtained when low temperature was the stimulus for floral induction are consistent with the lack of effect of GA₃ on *SOCI* and *LFY* expression observed in buds of ‘Salustiana’ sweet orange (Muñoz-Fambuena et al., 2012b) and ‘Orri’ mandarin (Goldberg-Moeller et al., 2013), and *FT*, *SOCI*, and *LFY* expression in buds of ‘Washington’ navel orange (Tang and Lovatt, 2019). Although the roles of leaf and bud *FT* in citrus floral development await clarification, the results of this research add to the growing body of evidence that *FT* is transcribed in the citrus bud itself (Goldberg-Moeller et al., 2013; Muñoz-Fambuena et al., 2012a; Tang and Lovatt, 2019; Tang et al., 2021).

A major contribution of the current research to the elucidation of citrus flower development was the analysis of transcript levels of additional core genes downstream from *API*, including *AP2*, *SEPI*, *PI*, and *AG*. Three substantive results were obtained. First, for water-deficit stressed trees, both *API* and *AP2* were upregulated from week 8, the end of the stress period, to week 9, 1 week after re-irrigation, when tree SWP was restored to greater than -1.00 MPa, and from week 9 to week 10, 2 weeks after re-irrigation, when

maximum expression for both genes occurred, resulting in significantly greater transcript levels by weeks 9 and 10 than those of 11-week well-irrigated trees. Second, the floral organ identity genes *SEPI*, *PI*, and *AG* were activated only in buds of water-deficit stress-treated trees after re-irrigation, in parallel with the increased expression of *API* and *AP2*, reaching maximum levels in week 10. In contrast, for well-irrigated control trees, *SEPI* and *PI* transcripts remained undetectable throughout the 11-week experiment; *AG* was only expressed in week 9 and at a marginal level significantly lower than trees subjected to 8 weeks of water-deficit stress. Taken together, the results suggest that *API* and *AP2* were only expressed at levels sufficiently high to upregulate the downstream floral organ identity genes *SEPI*, *PI*, and *AG* in buds of water-deficit stressed trees, which led to flowering, but remained too low in the buds of well-irrigated trees to upregulate floral organ specification and thus, flower development failed. Importantly, according to the ABCE model for floral organ specification, activity of both class A genes, *API* and *AP2*, is necessary for sepal formation (Bowman et al., 1991; Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005). Of specific relevance to this research, sepal formation was identified as the developmental marker indicating irreversible commitment to floral development (bud determinacy) in ‘Washington’ navel orange (Lord and Eckard, 1987), consistent with the roles of *API* and *AP2* in regulating citrus floral meristem determinacy in response to water-deficit stress. The third substantive result was that the upregulation of *API* and *AP2* and concomitant activation of *SEPI*, *PI*, and *AG* in response to water-deficit stress during weeks 1 and 2 of the re-irrigation period were inhibited by GA_3 applied during the water-deficit stress period, and flower production failed. These results, taken together with the absence of a negative effect of foliar-applied GA_3 on bud expression of *FT*, *SOC1*, or *LFY* in response to water-deficit stress, identify *API* and *AP2* as the genes mediating the inhibitory effect of GA_3 on water-deficit stress-induced flowering in citrus. The results provide additional evidence that adequate levels of *API* and *AP2* expression are required for the upregulation of the floral organ identity genes and flowering in response to water-deficit stress in citrus. In all cases, expression of the floral organ identity genes downstream from *AP2* paralleled the expression of *API* and *AP2* and when their activity was repressed, flowering did not occur. It should be noted that bud determinacy might also require expression of at least one or possibly all four *SEP* genes (Ditta et al., 2004; Pelaz et al., 2000). Across all treatments, inflorescence number was not significantly related to the expression of *FT*, *SOC1*, or *LFY*, but was strongly correlated with the expression of *API*, *AP2*, *SEPI*, *PI*, and *AG* in week 10 (2 weeks after re-irrigation).

Striking similarities in the expression patterns of core genes regulating floral development in ‘Washington’ navel orange trees induced to flower by water-deficit in this study and low-temperature stress in a previous study (Tang and Lovatt, 2019) were identified. With the exception of the stimulus used to induce flowering, note that all biotic attributes of the trees in both experiments (such as genotype, age, and size), as well as environmental factors (including the conditions during the 5 months preceding each experiment, the length of each stress treatment and recovery period, daylength, light intensity and quality, and humidity), and GA_3 treatment were the same. Eight weeks of water deficit (SWP < -2.40 MPa followed by re-irrigation to SWP > -1.00 MPa for 3 weeks) and low temperature [15/10 °C (day/night) followed by 3 weeks at 24/19 °C (day/night)]

significantly increased inflorescence number compared with control trees maintained in the well-watered, warm-temperature condition [SWP > -1.00 MPa, 24/19 °C (day/night)]. Seven GA_3 applications during each stress reduced inflorescence number 95% and 96%, respectively, similar to that of control trees. Bud expression of *FT*, *SOC1*, and *LFY* was unaffected in a manner related to inflorescence number by either stress or GA_3 treatment. In contrast, in response to both stresses, expression of *API* and *AP2* significantly increased 1 week after the trees were transferred to the nonstressed condition, followed by maximum *SEPI*, *PI*, and *AG* expression 1 week before flowering (week 10). Applications of GA_3 prevented the upregulation of these genes, which also failed to occur in the buds of control trees; neither set of trees flowered. The results of the two separate experiments provide consistent evidence supporting the role of *API* and *AP2* in regulating citrus floral meristem determinacy and the inhibitory effect of GA_3 on citrus flowering.

Based on the results of the preceding comparison, it is likely that water-deficit and low-temperature stress regulate citrus floral development through overlapping pathways, whereby *API* and *AP2* expression are necessary for bud determinacy and downstream activation of *SEPI*, *PI*, and *AG* for flower formation. This interpretation is consistent with the ability of water-deficit stress to augment low-temperature floral induction in citrus (Albrigo et al., 2002, 2006; Chica and Albrigo, 2013b). With each stress, significant expression of *SEPI*, *PI*, and *AG* only occurred after trees were transferred to the nonstressed (well-irrigated warm-temperature) condition, providing evidence of a possible failsafe mechanism to synchronize flowering with periods of available water and warmer temperature (Tang and Lovatt, 2019). Specifically, activation of *API* and *AP2* would occur under drought conditions or the low temperatures of fall and winter, but expression of *API* and *AP2* to levels sufficient to confer bud determinacy and upregulate downstream floral organ identity genes, *SEPI*, *PI*, and *AG*, would only occur after spring temperatures were sufficiently warm or adequate water was available, thereby preventing flower production under adverse environmental conditions.

Conclusions

Water-deficit stress is an effective floral-induction pathway in citrus. Results presented herein provide the first evidence demonstrating that *API* and *AP2* regulate bud determinacy in response to water-deficit stress in ‘Washington’ navel orange and upregulate the expression of the downstream floral organ identity genes, resulting in successful flowering. The results also provide the first evidence suggesting that the inhibitory effect of GA_3 on water-deficit stress-induced flowering in citrus is mediated by *API* and *AP2*. The presence of the water-deficit stress floral-induction pathway in citrus increases the potential for floral success of the species in nature. It might also provide growers with a tool to mitigate the negative effects of climate change and promote the positive effects of water deficit on citrus flowering and yield of commercial citrus grown in areas experiencing warmer, drier winters.

Literature Cited

Albrigo, L.G., J.I. Valiente, and H.W. Beck. 2002. Flowering expert system development for a phenology based citrus decision support system. *Acta Hort.* 584:247–254. <https://doi.org/10.17660/ActaHortic.2002.584.30>.

- Albrigo, L.G., J.I. Valiente, and C. Van Parys de Wit. 2006. Influence of winter and spring weather on year-to-year citrus fruit set and yield variation in São Paulo, Brazil. *Proc. Intl. Soc. Citricult. X Congr.* 1:263–267.
- Barbera, G., G. Fatta del Bosco, and B. Lo Cascio. 1985. Effects of water stress on lemon summer bloom: The “forzatura” technique in the Sicilian citrus industry. *Acta Hort.* 171:391–397, <https://doi.org/10.17660/ActaHortic.1985.171.36>.
- Barbera, G., G. Fatta del Bosco, and G. Occorso. 1981. Some aspects on water stress physiology of forced lemon (*Citrus limon* Burm) trees. *Proc. Intl. Soc. Citricult. IV Congr.* 2:522–523.
- Benlloch, R., A. Berbel, A. Serrano-Mislata, and F. Madueno. 2007. Floral initiation and inflorescence architecture: A comparative view. *Ann. Bot.* 100:659–676, <https://doi.org/10.1093/aob/mcm146>.
- Bowman, J.L., D.R. Smyth, and E.M. Meyerowitz. 1991. Genetic interactions among floral homeotic genes of *Arabidopsis*. *Development* 112:1–20, <https://doi.org/10.1242/dev.112.1.1>.
- Chica, E.J. and L.G. Albrigo. 2013a. Changes in *CsFT* transcript abundance at the onset of low-temperature floral induction in sweet orange. *J. Amer. Soc. Hort. Sci.* 13:184–189, <https://doi.org/10.21273/JASHS.138.3.184>.
- Chica, E.J. and L.G. Albrigo. 2013b. Expression of flower promoting genes in sweet orange during floral inductive water deficits. *J. Amer. Soc. Hort. Sci.* 138:88–94, <https://doi.org/10.21273/JASHS.138.2.88>.
- Coen, E.S. and E.M. Meyerowitz. 1991. The war of the whorls: Genetic interactions controlling flower development. *Nature* 353:31–37, <https://doi.org/10.1038/353031a0>.
- Ditta, G., A. Pinyopich, P. Robles, S. Pelaz, and M.F. Yanofsky. 2004. The *SEP4* Gene of *Arabidopsis thaliana* functions in floral organ and meristem identity. *Curr. Biol.* 14:1935–1940, <https://doi.org/10.1016/j.cub.2004.10.028>.
- Easterling, D.R., G.A. Meehl, C. Parmesan, S.A. Changnon, T.K. Karl, and L.O. Mearns. 2000. Climate extremes: Observations, modeling, and impacts. *Science* 289:2068–2074, <https://doi.org/10.1126/science.289.5487.2068>.
- Fulton, A.R., R. Bucher, B. Olson, L. Schwankl, C. Gilles, N. Bertagna, J. Walton, and K. Shackel. 2001. Rapid equilibration of leaf and stem water potential under field conditions in almonds, walnuts and prunes. *HortTechnology* 11:609–615, <https://doi.org/10.21273/HORTTECH.11.4.609>.
- García-Luís, A., M. Kanduser, P. Santamarina, and J.L. Guardiola. 1992. Low temperature influence on flowering in *Citrus*. The separation of inductive and bud dormancy releasing effects. *Physiol. Plant.* 86:648–652, <https://doi.org/10.1111/j.1399-3054.1992.tb02182.x>.
- Goldberg-Moeller, R., L. Shalom, L. Shlizerman, S. Samuels, N. Zur, R. Ophir, E. Blumwald, and A. Sadka. 2013. Effects of gibberellin treatment during flowering induction period on global gene expression and the transcription of flowering-control genes in *Citrus* buds. *Plant Sci.* 198:46–57, <https://doi.org/10.1016/j.plantsci.2012.09.012>.
- Guardiola, J.L., C. Monerri, and M. Agustí. 1982. The inhibitory effect of gibberellic acid on flowering in *Citrus*. *Physiol. Plant.* 55(2):136–142, <https://doi.org/10.1111/j.1399-3054.1982.tb02276.x>.
- Hake, K.D. 1995. Regulation of flowering in *Citrus limon* by water-deficit stress and nitrogen compounds. Univ. Calif., Riverside, PhD Diss. 9707046.
- Hong, Y. and S. Jackson. 2015. Floral induction and flower formation – The role and potential applications of miRNAs. *Plant Biotechnol. J.* 13:282–292, <https://doi.org/10.1111/pbi.12340>.
- Krizek, B.A. and J.C. Fletcher. 2005. Molecular mechanisms of flower development: An armchair guide. *Nat. Rev. Genet.* 6:688–698, <https://doi.org/10.1038/nrg1675>.
- Li, J.-X., X.-J. Hou, J. Zhu, J.-J. Zhou, H.-B. Huang, J.-Q. Yue, J.-Y. Gao, Y.-X. Du, C.-X. Hu, C.-G. Hu, and J.-Z. Zhang. 2017. Identification of genes associated with lemon floral transition and flower development during floral inductive water deficits: A hypothetical model. *Front. Plant Sci.* 8:1013, <https://doi.org/10.3389/fpls.2017.01013>.
- Lord, E.M. and K.J. Eckard. 1987. Shoot development in *Citrus sinensis* L. (Washington navel orange). II. Alteration of developmental fate of flowering shoots after GA₃ treatment. *Bot. Gaz.* 148:17–22, <https://doi.org/10.1086/337623>.
- Lovatt, C.J. and C.W. Coggins. 2019. Delaying fruit senescence with gibberellic acid (GA₃). University of California integrated pest management guidelines: Citrus. Univ. Calif. ANR Publ. 3441.
- Lovatt, C.J., Y. Zheng, and K.D. Hake. 1988a. A new look at the Kraus Kraybill hypothesis and flowering in *Citrus*. *Proc. Intl. Citrus VI Congr.* 1:475–483.
- Lovatt, C.J., Y. Zheng, and K.D. Hake. 1988b. Demonstration of a change in nitrogen metabolism influencing flower initiation in citrus. *Isr. J. Bot.* 37:181–188.
- Maranto, J. and K.D. Hake. 1985. Veredelli summer lemons: A new option for California growers. *Calif. Agr.* 39(5):4.
- McCutchan, H. and K.A. Shackel. 1992. Stem water potential as a sensitive indicator of water stress in prune trees (*Prunus domestica* L. cv. French). *J. Amer. Soc. Hort. Sci.* 117:606–611, <https://doi.org/10.21273/JASHS.117.4.607>.
- Moss, G.I. 1969. Influence of temperature and photoperiod on flower induction and inflorescence development in sweet orange (*Citrus sinensis* L. Osbeck). *J. Hort. Sci.* 44:311–320, <https://doi.org/10.1080/00221589.1969.11514314>.
- Muñoz-Fambuena, N., C. Mesejo, M.C. González-Mas, E. Primo-Millo, M. Agustí, and D.J. Iglesias. 2012a. Fruit load modulates flowering-related gene expression in buds of alternate-bearing ‘Moncada’ mandarin. *Ann. Bot.* 110(6):1109–1118, <https://doi.org/10.1093/aob/mcs190>.
- Muñoz-Fambuena, N., C. Mesejo, M.C. González-Mas, D.J. Iglesias, E. Primo-Millo, and M. Agustí. 2012b. Gibberellic acid reduces flowering intensity in sweet orange [*Citrus sinensis* (L) Osbeck] by repressing *CiFT* gene expression. *J. Plant Growth Regul.* 31:529–536, <https://doi.org/10.1007/s00344-012-9263-y>.
- Nakajima, Y., S. Susanto, and K. Hasegawa. 1992. Influence of water stress in autumn on flower induction and fruiting in young pomelo trees (*Citrus grandis* L. Osbeck). *J. Jpn. Soc. Hort. Sci.* 62(1):15–20, <https://doi.org/10.2503/jjshs.62.15>.
- Nishikawa, F., T. Endo, T. Shimada, H. Fujii, T. Shimizu, and M. Omura. 2009. Differences in seasonal expression of flowering genes between deciduous trifoliolate orange and evergreen Satsuma mandarin. *Tree Physiol.* 29:921–926, <https://doi.org/10.1093/treephys/tpp021>.
- Nishikawa, F., T. Endo, T. Shimada, H. Fujii, T. Shimizu, M. Omura, and Y. Ikoma. 2007. Increased *CiFT* abundance in the stem correlates with floral induction by low temperature in Satsuma mandarin (*Citrus unshiu* Marc.). *J. Expt. Bot.* 58:3915–3927, <https://doi.org/10.1093/jxb/erm246>.
- Pelaz, S., G.S. Ditta, E. Baumann, E. Wisman, and M.F. Yanofsky. 2000. B and C floral organ identity functions require *SEPALLATA* MADS-box genes. *Nature* 405:200–203, <https://doi.org/10.1038/35012103>.
- Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:2002–2007, <https://doi.org/10.1093/nar/29.9.e45>.
- Pillitteri, L.J., C.J. Lovatt, and L.L. Walling. 2004. Isolation and characterization of *LEAFY* and *APETALA1* homologues from *Citrus sinensis* L. Osbeck ‘Washington’. *J. Amer. Soc. Hort. Sci.* 129:846–856, <https://doi.org/10.21273/JASHS.129.6.0846>.
- Reuther, W. 1973. Climate and citrus behavior, p. 280–337. In: W. Reuther (ed.). *The citrus industry*. 2nd ed. Vol. 3. Univ. Calif. Press, Berkeley, CA.
- Shalom, L., S. Samuels, N. Zur, L. Shlizerman, H. Zemach, M. Weissberg, R. Ophir, E. Blumwald, and A. Sadka. 2012. Alternate bearing in citrus: Changes in the expression of flowering control genes and in global gene expression in on-versus off-crop trees. *PLoS One* 7:e46930, <https://doi.org/10.1371/journal.pone.0046930>.
- Song, C., Q. Jia, J.G. Fang, F. Li, C. Wang, and Z. Zhang. 2010. Computational identification of citrus microRNAs and target analysis

- in citrus expressed sequence tags. *Plant Biol.* 12:927–934, <https://doi.org/10.1111/j.1438-8677.2009.00300.x>.
- Southwick, S.M. and T.L. Davenport. 1986. Characterization of water stress and low temperature effects on flower induction in *Citrus*. *Plant Physiol.* 81:26–29, <https://doi.org/10.1104/pp.81.1.26>.
- Takeno, K. 2016. Stress-induced flowering: The third category of flowering response. *J. Expt. Bot.* 67:4925–4934, <https://doi.org/10.1093/jxb/erw272>.
- Tan, F.-C. and S.M. Swain. 2007. Functional characterization of *AP3*, *SOCI* and *WUS* homologues from citrus (*Citrus sinensis*). *Physiol. Plant.* 131:481–495, <https://doi.org/10.1111/j.1399-3054.2007.00971.x>.
- Tang, L. and C.J. Lovatt. 2019. Effects of low temperature and gibberellic acid on floral gene expression and floral determinacy in ‘Washington’ navel orange (*Citrus sinensis* L. Osbeck). *Scientia Hort.* 243:92–100, <https://doi.org/10.1016/j.scienta.2018.08.008>.
- Tang, L., G. Singh, M. Dewdney, and T. Vashisth. 2021. Effects of exogenous gibberellic acid in Huanglongbing-affected sweet orange trees under Florida conditions. I. Flower bud emergence and flower formation. *HortScience* 56:1531–1541, <https://doi.org/10.21273/HORTSCI16080-21>.
- Vashisth, T., C. Oswalt, M. Zekri, F. Alferez, and J.D. Burrow. 2020. 2020–2021 Florida citrus production guide: Plant growth regulators. Hort. Sci. Dept., Univ. Florida/IFAS Ext., CMG17.
- Wilson, R.N., J.W. Heckman, and C.R. Somerville. 1992. Gibberellin is required for flowering in *Arabidopsis thaliana* under short days. *Plant Physiol.* 100:403–408, <https://doi.org/10.1104/pp.100.1.403>.
- Yan, J., F. Yuan, G. Long, L. Qin, and Z. Deng. 2012. Selection of reference genes for quantitative real-time RT-PCR analysis in citrus. *Mol. Biol. Rep.* 39:1831–1838, <https://doi.org/10.1007/s11033-011-0925-9>.