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Artigo/Article

Identification of *cis*-regulatory elements related to water-deficit and low-temperature stress within the promoter of *Citrus sinensis APETALA1*

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SUMMARY

Citrus flowering is promoted by water-deficit and low-temperature stress and inhibited by gibberellins, providing a unique system to investigate regulation of floral development. *In silico* analysis, using plant *cis*-acting regulatory element databases, PLACE and PlantCARE, was conducted to compare transcriptional regulation of *LEAFY* (*LFY*), *APETALA1* (*AP1*), and *TERMINAL FLOWER (TFL)* by water deficit, low temperature, abscisic acid and gibberellins in *C. sinensis*, *Arabidopsis thaliana* and *Populus trichocarpa*. A striking enrichment of response elements upregulated by water deficit was found in the *C. sinensis AP1* (*CsAP1)* promoter, but neither the *A. thaliana AP1* (*AtAP1*), nor *P. trichocarpa AP1-1 (PtAP1-1)* promoter. Notably, a tandem array of three response elements, each containing a LFY binding site, coupling element 3 site (CE3), and dehydration-responsive element (DRE), was found within a 100-bp region of the *CsAP1* promoter. The CE3 and DRE sites are associated, respectively, with abscisic acid (ABA)-dependent and ABA-independent signaling pathways induced by water deficit. The CE3 sequence is present in the *AtAP1*, but not *PtAP1-1*, promoter. The DRE site is lacking in both *AtAP1* and *PtAP1-1*. Three LFY binding sites are located in the *CsAP1* and *AtAP1* promoters, with only one in *PtAP1-1*. Multiple C-repeat binding factor (CBF) response elements associated with low-temperature responses through an ABA-independent signaling pathway are present in the *CsAP1* and *AtAP1* promoters, but not *PtAP1-1*. The unique 100-bp regulon of the *CsAP1* promoter suggests that flower formation in *C. sinensis* in response to water-deficit and low-temperature stress is mediated at *AP1* through ABA-dependent and ABA-independent pathways.

Index terms: abscisic acid, flowering, gibberellins, *LEAFY*, *TERMINAL FLOWER*.

Identificação de elementos cis-reguladores relacionados ao déficit hídrico e ao estresse de baixa temperatura dentro do promotor de *Citrus sinensis APETALA1*

RESUMO

A floração de citros é estimulada pelo déficit hídrico e pelo estresse de baixa temperatura e inibida por giberelinas, proporcionando um sistema único para investigar a regulação do desenvolvimento floral. Uma análise *in silico,* utilizando bases de dados de elementos regulatórios

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em *cis* de plantas, PLACE e PlantCARE, foi conduzida para comparar a regulação transcricional de *LEAFY (LFY), APETALA1 (AP1)* e *TERMINAL FLOWER (TFL)* por déficit hídrico, baixa temperatura, ácido abscísico e giberelinas em *Citrus sinensis*, *Arabidopsis thaliana* e *Populus trichocarpa*. Um enriquecimento impressionante na resposta dos elementos regulados por déficit hídrico foi encontrado no promotor *AP1 (CsAP1)* de *C. sinensis*, mas não nos promotores *AP1 (AtAP1)* de *A. thaliana* e *AP1-1 (PtAP1-1) de P. trichocarpa*. Particularmente, um arranjo de três elementos em sequência foi encontrado dentro de uma região de 100 pb do promotor *CsAP1*, cada um contendo o local de ligação ao LFY, o sítio de acoplamento do elemento 3 (CE3) e o elemento responsivo à desidratação (DRE). Os sítios CE3 e DRE estão associados, respectivamente, com vias de sinalização dependentes de ácido abscísico (ABA) e independentes de ABA induzidos por déficit hídrico. A sequência CE3 está presente no promotor *AtAP1*, mas não em *PtAP1-1*. O sítio DRE está faltando em ambos *AtAP1* e *PtAP1-1*. Três sítios de ligação LFY estão localizados nos promotores *CsAP1* e *AtAP1*, com apenas um em *PtAP1-1*. Vários elementos de resposta ao fator de ligação de repetição C (CBF) associados a respostas de baixa temperatura, através de uma via de sinalização independente de ABA, estão presentes nos promotores *CsAP1* e *AtAP1*, mas não em *PtAP1-1*. A região regulatória de 100 pb exclusiva do promotor *CsAP1* sugere que a formação de flor em *C. sinensis* em resposta ao estresse hídrico e a baixa temperatura é mediada por *AP1* através de vias dependentes e independentes de ABA.

Termos para indexação: ácido abscísico, floração, giberelinas, gene *LEAF*Y, gene *TERMINAL FLOWER*

INTRODUCTION

Floral development is the essential first step in fruit production. To be able to regulate floral intensity, it is imperative to understand the underlying processes governing floral induction and bud determinacy (irreversible commitment of the shoot apical meristem [SAM] to floral development). Knowledge of the molecular basis of these two events in floral development in *Citrus* spp. is largely built on results derived from the model plant *Arabidopsis thaliana.* Floral induction begins with endogenous or environmental cues that act on several genetic pathways to upregulate genes that promote flowering (*FLOWERING LOCUS T* [*FT*], *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* [*SOC1*] and *LEAFY* [*LFY*]). These pathways converge on the floral meristem identity genes, which include *LFY* and *APETALA1* (*AP1*) (Weigel & Meyerowitz, 1993). *LEAFY* encodes a plant-specific transcription factor, whereas *AP1* is a member of the MADS-box family of transcription regulators. As the master regulator of floral initiation in *Arabidopsis*, *AP1* activates the downstream floral organ identity genes, *APETALA2* (*AP2*), *SEPALLATA* (*SEP*), *PISTILLATA* (*PI*) and *AGAMOUS* (*AG*) (Wellmer & Riechmann, 2010). In contrast, *TERMINAL FLOWER* (*TFL*), which encodes a homologue of *FT* with opposite function, inhibits flowering and promotes meristem indeterminacy by negatively regulating both *LFY* and *AP1* in *A. thaliana* (Liljegren et al., 1999).

Comparative analyses between *A. thaliana* and perennial woody tree species, such as *Populus* spp. and *Citrus* spp., have demonstrated the extensive conservation of pathways regulating flowering, but also identified features distinct from *A. thaliana*. For example for *Citrus* spp., overexpression studies using the floral meristem identity genes *LFY* and *AP1* from *A. thaliana* were able to promote early flowering when transformed into juvenile citrange rootstocks (*C. sinensis* L. Osbeck x *Poncirus trifoliata* L. Raf.) (Peña et al., 2001). In addition, *CsLFY, CsAP1, CsTFL, C. unshiu FT (CiFT),* and *CsSOC1-like (CsSL1* and *CsSL2*) successfully complemented their respective *A. thaliana* mutants, thus demonstrating functional equivalence (Endo et al., 2005; Pillitteri et al., 2004a, 2004b; Tan & Swain, 2007). Taken together, these results strongly suggest that key components of the *Citrus* floral development pathway are the same as those of *A. thaliana*.

However, distinct differences in the regulation of flowering between the two species have been found. In Washington navel orange (WNO) (*C. sinensis*), flowering is promoted by water-deficit (< –2.1 to –3.0 MPa for > 10 days) and low-temperature stress (day 15-18 ºC, night 10-13 ºC), with inflorescence number positively correlated with the increasing severity or duration of either stress up to 8 weeks (Chica & Albrigo, 2013a, 2013b; Lovatt et al., 1988; Pillitteri et al., 2004a; Tang & Lovatt, in press). In contrast, in *A. thaliana*, flowering time is either advanced or delayed by water deficit as part of the plant's drought escape response (Riboni et al., 2016; Schmalenbach et al., 2014). Recently published floral gene transcription data for WNO provided evidence that *FT*, *SOC1*, *LFY* and *AP1* transcripts were present in buds that subsequently produced inflorescences, as well as in buds that continued vegetative shoot growth, rendering the two bud populations

indistinguishable at the level of gene transcription during early development (Chica & Albrigo, 2013a; Tang & Lovatt, in press). However, under low-temperature and water-deficit conditions that resulted in intense flowering in WNO, bud determinacy correlated with increasing *AP1* and *AP2* transcript levels in response to the stress treatments, but only after the stress was alleviated (Tang & Lovatt, in press). This observation is consistent with the critical role of the class A genes, *AP1* and *AP2,* in sepal formation in the ABC model of floral organ specification in *A. thaliana* (Coen & Meyerowitz, 1991). In WNO, sepal formation is the developmental marker indicating bud determinacy (Lord & Eckard, 1987). In fact, in WNO, profuse flowering only occurred under conditions that increased *AP1* and *AP2* transcript accumulation to a level sufficient to result in full *SEP*, *PI* and *AG* expression (Tang & Lovatt, in press).

The role of *TFL* in *Arabidopsis* and *Citrus* may be similar. In *A. thaliana TFL*, maintains shoot indeterminacy by downregulating *LFY* and *AP1* (Liljegren et al., 1999). In WNO, *TFL* was highly expressed in buds of seedling (juvenile) trees, which did not express *LFY* or *AP1* and did not flower under low temperature; whereas *TFL* was expressed only at extremely low levels in buds of adult trees before, during and after a low-temperature treatment that increased *LFY* and *AP1* expression and resulted in intense flowering (Pillitteri et al., 2004a).

A second difference in the regulation of floral development between *Arabidopsis* and *Citrus* is that gibberellins (GA) promote floral development in *A. thaliana* by upregulating *SOC1* and *LFY* (Wellmer & Riechmann, 2010). In contrast, GA, in particular GA_3, inhibits citrus flowering (Lord & Eckard, 1987; Goldberg-Moeller et al., 2013; Tang & Lovatt, in press) by inhibiting *AP1* (Goldberg-Moeller et al., 2013) and *AP2* expression under low-temperature and water-deficit floral-promoting conditions (Tang & Lovatt, in press).

Exposure of subtropical evergreen species, such as *Citrus* spp., to water deficit and low temperature constitutes a stress that elicits a range of biochemical, physiological, and molecular responses (Nakashima & Yamaguchi-Shinozaki, 2006). In *A. thaliana*, differential gene expression induced by water deficit occurs through the binding of upstream transcription regulators to response elements in the promoters of downstream target genes. This action occurs through ABA-dependent and ABA-independent signaling pathways (Yamaguchi-Shinozaki & Shinozaki, 1994; Yamaguchi-Shinozaki & Shinozaki, 2005). In order to identify potential molecular mechanisms by which *Citrus* flowering is regulated by water deficit, low temperature, ABA and GA, *in silico* analyses were utilized to assess key regulatory elements within the promoter regions of *CsLFY*, *CsAP1* and *CsTFL* in comparison with their respective *A. thaliana* and *P. trichocarpa* homologues.

MATERIALS AND METHODS

Promoter sequences

Approximately 1.6 kb, 1.8 kb, and 1.6 kb of the 5' upstream regions of the *CsAP1 CsLFY*, and *CsTFL* genes, respectively, were isolated from WNO (*C. sinensis*) and used as the promoter sequences in this analysis (Pillitteri, 2002). The *AtAP1 (At1g69210)*, *AtLFY (At5g68150)*, and *AtTFL (At5g03840)* genomic sequences were identified from publically available data (Huala et al., 2001; Swarbreck et al., 2007). The promoter lengths of *AtAP1*, *AtLFY* and *AtTFL* corresponded to 2.1 kb, 2.3 kb, and 2.5 kb, respectively, upstream of the translational start site. Promoters of *Populus trichocarpa AP1-1* (*PtAP1-1,* AY616522) and *PtLF* (U93196) were publicly available from GenBank. The coding sequence of the *P. trichocarpa TFL* homologue *PCENL1* (AY383600) was used in a BLAST search against the entire genome of *P. trichocarpa*, release v1.1 (Nordberg, et al., 2014). The subsequent analyses employed the region 2.5 kb upstream from the ATG translational start site as the *PCENL1* promoter.

In silico identification of response elements and comparative analysis

In silico analyses were performed to identify *cis*-responsive elements in the promoters of genes encoding homologues of *LFY*, *AP1*, and *TFL*. Promoters were queried against two databases of plant *cis*-acting regulatory elements, PLACE and PlantCARE (Higo et al., 1999; Rombauts et al., 1999). Motifs > 6 bp were used in the analysis. Spatial patterning of putative response elements was visualized on respective promoters using Vector NTI Advance 10 software (Invitrogen, Carlsbad, CA). Unless otherwise noted, analyses used a threshold level of 100% maximum homology. In addition, MatInspector, which used position-weighted matrices (PWM), was used to identify transcription factor binding sites (Cartharius et al., 2005). When binding sites identified by MatInspector were redundant within PLACE and PlantCARE at thresholds > 75% core motif similarity

and > 75% matrix similarity, the IUPAC sequence listed in the MatBase transcription factor database was reported (http://www.genomatix.de/ products/portfolio.html). Promoter locations are reported as the distance upstream from ATG start codon.

To discover additional highly conserved regions within homologous promoters, sequences were subjected to Multiple EM for Motif Elicitation (MEME, v3.5.7) analysis with motif width setting > 6 bp and < 50 bp (Bailey & Elkan, 1994). Promoters were then searched for motifs found by MEME using the Motif Alignment and Search Tool (MAST v3.5.7). Multiple sequence alignment was performed with AlignX software bundled with the VectorNTI Advance 10 software package.

RESULTS AND DISCUSSION

In silico identification and comparative analyses of stress-responsive elements

Several classes of putative response elements related to water deficit, low temperature, ABA-dependent, and ABA-independent gene regulation pathways were identified in the promoters of *CsLFY*, *CsAP1* and *CsTFL*. There were more putative stress-responsive elements in the promoter of *CsAP1* than in *CsLFY* or *CsTFL*. In addition, numerous water-deficit and low-temperature stress-responsive elements in the *CsAP1* promoter were common to the promoter regions of one or both *AtAP1* and *PtAP1-1*. Thus, the *CsAP1* promoter region was the primary focus of this investigation. Within a 100-bp region approximately 500 bp upstream from the translation start codon of *CsAP1*, a unique arrangement of three repeating tandem arrays of stress-responsive elements was identified (Figures 1 and 2). Each tandem repeat contains a LFY binding site (CCANTG) (Benllock et al., 2011) and a dehydration-responsive element (DRECRTCOREAT, or simply DRE/CRT; RCCGACA) (Dubouzet et al., 2003) on the forward strand and a coupling element 3 site (CE3) (GCGTGTC) on the reverse-strand (Shen et al., 1996).

The DRE/CRT and CE3 elements are essential for gene induction in response to water-deficit and low-temperature stress through ABA-independent and ABA-dependent pathways, respectively (Liu et al., 1998; Yamaguchi-Shinozaki & Shinozaki, 2005). The DRE/CRT element (RCCGACA) (Figure 1) is essential in the ABA-independent pathway response to dehydration, high salinity and low temperature in *A. thaliana* (Sakuma et al., 2006; Yamaguchi-Shinozaki & Shinozaki, 1994). In addition, the C-repeat (CRT) and low temperature-responsive element (LTR) found within the *CsAP1* promoter each contain the A/GCCGAC motif that conforms to the core of DRE but are specific to low temperature-inducible gene expression (Figure 1) (Sakuma et al., 2006). It is significant that use of a second method of motif discovery analysis, MEME and MAST, also uncovered the 28-bp region that coincides with the tandem repeats found in the promoter of *CsAP1* at positions -382, -351 and -320 (Figures 1 and 3). The three DRE sequences identified are located in close proximity to the LFY binding site within each tandem repeat in the *CsAP1* promoter (Figures 1 and 3). Of interest, a single sequence similar to the DRE core sequence was found near a LFY binding site within the upstream regulatory

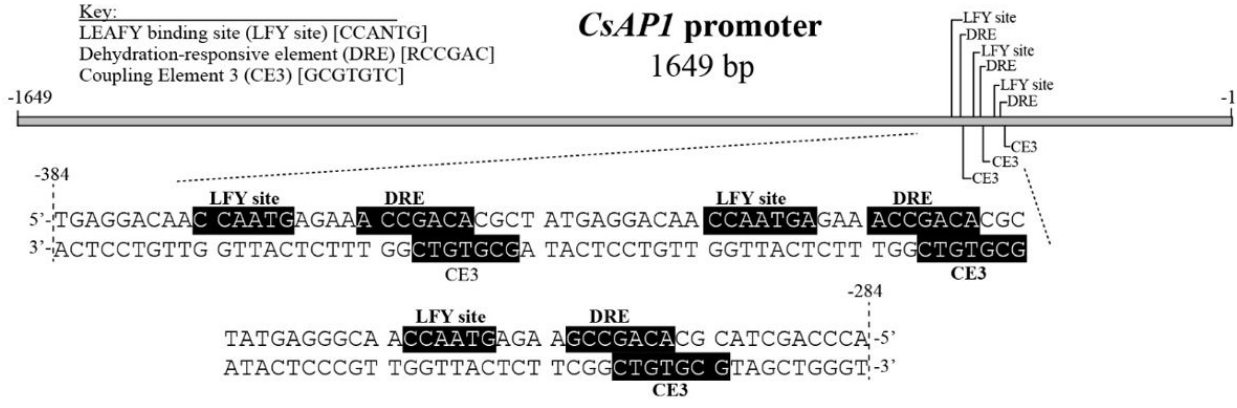

Figure 1. Motif location in the 100-bp region of the *CsAP1* promoter with tandem repeats of water-deficit stress-related response elements and LFY binding sites (black boxes).

regions of both *AtAP1* and *PtAP1-1* at positions -296 and -335, respectively. However, in the promoters of *AtAP1* and *PtAP1-1*, the putative DRE sequences in close proximity to the LFY binding site do not fully conform to the A/GCCGAC core sequence, being GTCGACA and TTCGACA, respectively (Figure 3). Consequently, *A. thaliana* dehydration-responsive element-binding (DREB) proteins may fail to bind (Dubouzet et al., 2003). Consistent with this interpretation, promoter sequence analysis by MatInspector, which takes into account adjacent nucleotide similarity to minimize false positives (Cartharius et al., 2005), failed to detect DRE in the promoter of either *AtAP1* or *PtAP1-1*. Taken together, these results suggest that the DRE site for the ABA-independent pathway present in the *CsAP1* is absent from the promoter region of both *AtAP1* and *PtAP1-1* (Figures 2 and 3).

LFY binding sites (CCANTG) (Benllock et al., 2011) were found in the promoter region of *AP1* for all three species (Figure 3) at similar relative positions from the translational start site. The promoters of both *CsAP1* and *AtAP1* have three LFY binding sites, whereas *PtAP1-1* has only one (Figure 2). The sequences of the three *CsAP1* LFY bindings sites are identical (Figure 3), whereas the sequences of three LFY binding sites of *AtAP1* are uniquely different from each other (Benllock et al., 2011). The three *CsAP1* LFY binding sites are identical

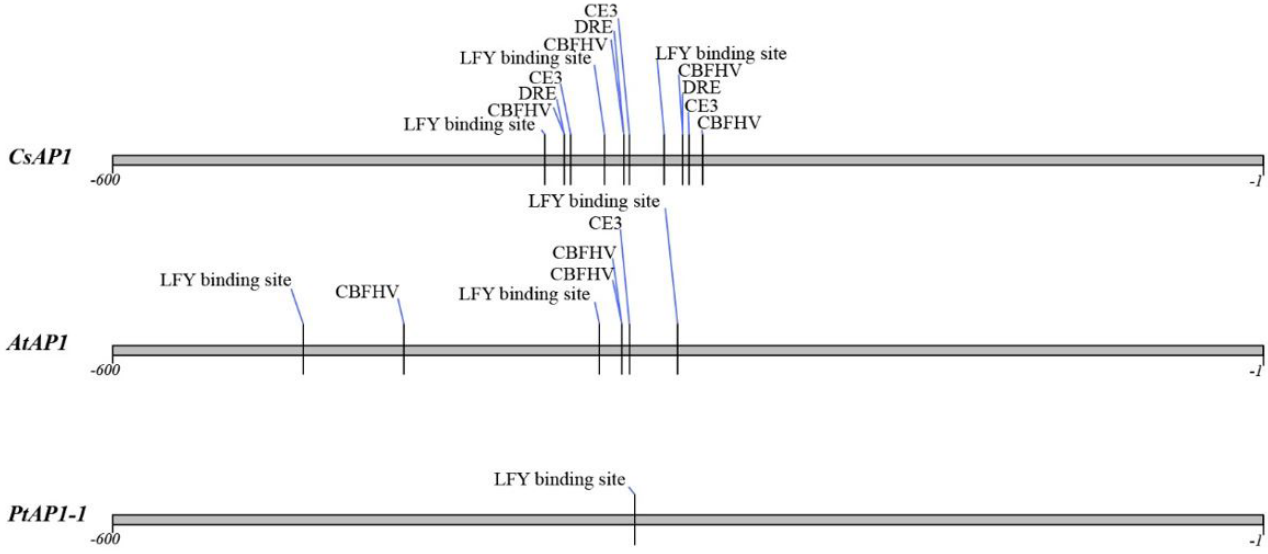

Figure 2. Comparison of motif locations within the promoters of *CsAP1, AtAP1* and *PtAP1-1* indicating the enrichment of *cis*-regulatory elements associated with water-deficit and low-temperature stress in relationship to LFY binding sites in the *CsAP1* promoter in comparison to the promoters of *AtAP1* and *PtAP1-1*.

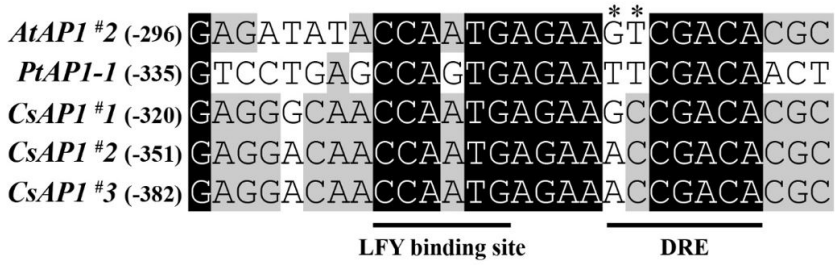

Figure 3. Alignment of the conserved region within the promoters of *CsAP1*, *AtAP1*, and *PtAP1-1*. *AtAP1* #2 refers to LFY binding site 2 (Benllock et al., 2011); *CsAP1* #1-3 refers to the three tandem repeats within the *CsAP1* promoter. Black boxes indicate 100% conservation; gray boxes indicate moderate conservation (50-99%). Positions of the LFY binding site and DRE are underlined. Two asterisks denote the first two nucleotides of the DRE motif. Note that *AtAP1* and *PtAP1-1* do not conform to the consensus RCCGACA.

to LFY binding site 2 of the *AtAP1* promoter (Figure 3). The sequence of the *PtAP1-1* LFY binding site, although not identical to LFY binding sites 1, 2 or 3 of *AtAP1*, conforms to the core sequence CCANTG (Figure 3). The presence of a LFY binding site located within the promoters of *AP1* for all three species is indicative of the positive regulation of *AP1* by *LFY* originally described in *A. thaliana* (Wagner et al., 1999). Research has determined that mutations in the *AtAP1* LFY binding site 1 prevent *AP1* activation and flowering under long day conditions, whereas mutations in LFY binding site 2 do not (Benllock et al., 2011). The results suggest that *AtLFY* binding site 2 may trigger flower development using a cue other than photoperiod. Given that all three *CsAP1* LFY binding sites are identical to LFY binding site 2 in *A. thaliana*, it raises the question of whether day- neutral plants like WNO broadly use this specific consensus sequence over others.

In silico identification and comparative analyses of ABA-responsive elements

Cis-acting ABA response elements (ABREs) mediate ABA-induced transcription. In a promoter, an ABRE functions with a coupling element in an ABA responsive *cis*-element complex (ABRC) (Shen & Ho, 1995; Shen et al., 1996). Two distinct coupling elements have been identified, coupling element 1 (CE1), having the core sequence CACC (Shen & Ho, 1995), and CE3 with the core sequence GCGTGTC (Shen et al., 1996). The most common ABREs have an ACGT core, but non-ACGT ABREs, including CE3, have been demonstrated to function in ABA-dependent pathways (Hobo et al., 1999; Liu et al., 1998; Yamaguchi-Shinozaki & Shinozaki, 2005). The *CsAP1* promoter contained a putative non-ACGT ABRE, the CE1 core sequence CACC, and notably, the CE3 core sequence GCGTGTC in the reverse strand within each tandem repeat (Figure 1), suggesting regulation by ABA. The CE3 sequence was also found in the reverse strand of the *AtAP1* promoter, but the sequence was not present in the promoter of *PtAP1-1* (Figure 2). It is only recently that ABA was demonstrated to have a positive role in flowering in *A. thaliana*, specifically under water-deficit stress as part of the drought-escape response (Riboni et al., 2016). However, as of yet, only ABA-dependent activation of *AtFT* under water-deficit has been documented (Riboni et al., 2016).

The presence of the CE3 core sequence, a functionally equivalent ABRE, within the upstream region of *CsAP1* suggests a role for ABA in regulating floral development in *C. sinensis*. This is reinforced by the isolation of a gene encoding a C-repeat binding factor (CBF) isolated from *C. sinensis* that was demonstrated to be upregulated by low temperature (< 20 °C), high salinity and ABA (He et al., 2016). Regulation of the *C. sinensis* CBF by ABA is unusual. To date CBF has been reported to be exclusively involved in ABA-independent pathway stress responses in other plants (He et al., 2012). The core motif for the CBF (CBFHV) response element (RYCGAC) (Gu & Cheng, 2014) was found in multiple positions within the promoter region of *CsAP1*, as well as the *AtAP1* promoter, but was not detected in *PtAP1-1* (Figure 2).

In silico identification and comparative analyses of GA-responsive elements

Whereas it is well document that GA_3 inhibits flowering in *Citrus* spp., the mechanism remains unclear. A GA-responsive sequence associated with blocking gene transcription was not identified in the promoters of *CsAP1, CsLFY* or *CsTFL*. In contrast, promoters of the *LFY* homologues of *C. sinensis, A. thaliana,* and *P. trichocarpa* contain a conserved region having a motif similar to a known GA response element (GARE), which was originally found in the *AtLFY* promoter through comparative analyses with the *PtLF* promoter (Blázquez & Weigel, 2000). This motif was not observed in the *CsAP1* or *CsTFL* promoter. The GARE motif (CAACTGTC) in *PtLF* and *AtLFY* differs from the sequence found in the *CsLFY* promoter by 1 bp, (CAAATGTC). Mutation in this region has been shown to abolish GA-responsiveness under long days in *A. thaliana* (Blázquez & Weigel, 2000). In WNO, repeated GA_3 applications during water-deficit and low-temperature floral-promoting treatments dramatically reduced floral intensity. Bud *LFY* expression was not affected. However, *AP1* and *AP2* expression were dramatically reduced and activity of the downstream floral organ identity genes was totally repressed. Mediation of the floral inhibitory effect of GA_3 through the activity of *CsAP1* and *CsAP2*, which are essential for sepal formation, is consistent with the fact that GA_3 can no longer prevent *C. sinensis* flowering once the SAM has initiated sepal formation (Lord & Eckard, 1987).

It must be emphasized that a GARE that results in the repression of floral gene activity has not been identified.

Thus, an alternative possibility is that the inhibitory effect of GA on *Citrus* flowering is not through the direct repression of *LFY* or *AP1*, but indirectly through the upregulation of *TFL*, the antagonist to *LFY* and *AP1* that inhibits flowering and confers meristem indeterminacy in *A. thaliana* (Liljegren et al., 1999) and *C. sinensis* (Pillitteri et al., 2004a). In support of this proposed mechanism, motif discovery analysis using MEME and MAST revealed several GARE sequences in the promoter of *CsTFL*. In particular, the TAACAAA box, first identified in the promoter of a barley (*Hordeum vulgare*) α-amylase gene, controls the upregulation of gene expression by GA and downregulation by ABA (Gubler & Jacobsen, 1992). It is tempting to speculate that the TAACAAA box in the *CsTFL* promoter may be under similar regulation. However, in light of the fact that juvenile buds have high levels of *CsTFL* mRNA, whereas adult (competent) buds have low expression, upregulation of *CsTFL* by GA may be restricted to juvenile buds in WNO (Pillitteri et al., 2004a). Nevertheless, the effect of GA_3 on bud *CsTFL* expression for trees in both developmental phases warrants further investigation.

CONCLUSION

Research results published over the years have established that *AP1* is an essential factor in the network of genes conferring floral meristem identity, floral organ specification, and flower development (Benllock et al., 2011). In WNO, expression of *AP1*, with subsequent expression of *AP2*, the two genes necessary for sepal formation, appears to confer bud determinacy, leading to the upregulation of the downstream floral organ identity genes and flower formation. Given this critical function, it is not surprising that the *CsAP1* promoter contains many different regulatory elements in order to provide for fine-tuning of its expression in response to different developmental and environmental cues. The multiple and combinatorial regulatory elements associated with *CsAP1* may impart a unique failsafe system to citrus floral buds. Whereas transcript accumulation of *AP1* and *AP2* increases in a manner paralleling the duration of the water-deficit and low-temperature stress period, this increase in expression occurs *only* after the stress has been alleviated (Tang & Lovatt, in press). As a result, downstream floral organ identity gene activity increases and flower formation occurs under conditions of adequate water and warm temperature.

In silico comparison analysis of the *CsLFY*, *CsAP1* and *CsTFL* promoters with their *A. thaliana* and *P. trichocarpa* homologues has a clear benefit for elucidating potential mechanisms regulating *Citrus* floral development at the molecular level. However, *in silico* analysis alone is insufficient to define precise regulatory models. To reinforce the results of the *in silico* analyses presented herein, they were evaluated in light of published data to promote a greater understanding of floral development in *Citrus* spp. and to help define the objectives of future research. The heuristic nature of this effort will hopefully lead to further research and to the development of new methods for regulating *Citrus* floral development in order to facilitate optimal floral intensity and improve yield on an annual basis.

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