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ORIGINAL PAPER

Stress-inducible *Arabidopsis thaliana RD29A* promoter constitutively drives *Citrus sinensis APETALA1* and *LEAFY* expression and precocious flowering in transgenic *Citrus* spp.

Vladimir Orbović · Seyed Ali Ravanfar · Yosvanis Acanda · Javier Narvaez · Benjamin A. Merritt · Amit Levy · Carol J. Lovatt

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Abstract Transgenic 'Duncan' grapefruit (*Citrus paradisi* Macf.) and 'Valencia' sweet orange (*Citrus sinensis* [L.] Osbeck) plants ectopically expressing *C. sinensis* (cv. Washington navel orange) *APETALA1* (*CsAP1*) or *LEAFY* (*CsLFY*) genes under control of the *Arabidopsis thaliana* stress-inducible promoter *AtRD29A* flowered under non-inductive (warm temperature, well-watered) greenhouse conditions, whereas their wild-type (WT) counterparts did not. The transgenic plants that flowered exhibited no altered morphological features, except the lack of thorns characteristic of juvenile WT plants. The most precocious T0 line, 'Duncan' grapefruit (Dun134-3) expressing the *CsAP1* gene, flowered and fruited when it was 4.5 years old and the T1 siblings from this line flowered and fruited when they were just over 18 months old. In contrast, T1 seedlings from three lines of 'Duncan' grapefruit expressing the *CsLFY* gene flowered within 3 months after germination, but were unable to support fruit development. Transcript levels of corresponding transgenes in leaves were not correlated with earliness of flowering. To further study the activity of *AtRD29A*, leaves from three 'Carrizo' citrange (*C. sinensis* × *Poncirus trifoliata*) rootstock seedlings transformed with the green fluorescent protein (*GFP*) gene under regulation of the *AtRD29A* promoter were subjected to drought stress or well-watered conditions. Expression of *GFP* was not stress-dependent, consistent with the observation of flowering of *CsAP1* and *CsLFY* transgenic plants under non-inductive conditions. Taken together, the results suggest that *AtRD29A* is constitutively expressed in a citrus background. Despite the loss of control over flowering time, transgenic citrus lines ectopically expressing *C. sinensis AP1* or *LFY* genes under control of the *A. thaliana RD29A* promoter exhibit precocious flowering, fruit development and viable transgenic seed formation. These transformed lines can be useful tools to reduce the time between generations to accelerate breeding.

Keywords *Citrus paradisi* cv. duncan · *Citrus sinensis* cv. valencia · Drought stress · Temperature stress

V. Orbović (✉) · S. A. Ravanfar · Y. Acanda · B. A. Merritt · A. Levy
Citrus Research and Education Center, University of Florida/IFAS, Lake Alfred, FL 33850, USA
e-mail: orbovic@ufl.edu

J. Narvaez · C. J. Lovatt
Department of Botany and Plant Sciences, University of California, Riverside, CA 92521, USA

Introduction

Citrus spp., hybrids and relatives are evergreen woody perennials with a juvenile phase that ranges from 5 to 13 years (Davies and Albrigo 1994). Moreover, genetic control of the process of floral induction

resulting in formation of floral meristems and final development of individual flowers to produce fruit is complex and protracted (Ma 1994). A long juvenile phase and complex floral developmental process impedes conventional breeding based on crossing. Thus, a major goal of commercial tree crop improvement is to reduce the juvenile phase and favor precocious flowering to shorten the time between generations (Pillitteri et al. 2004a). As the worldwide citrus industry fights to survive huanglongbing (HLB), citrus canker, and other lingering and emerging diseases and pests, as well as the negative effects of climate change, rapid cultivar improvement has become even more important. Genetic transformation offers the fastest and most direct method for introduction of desired traits into elite citrus cultivars, with newer technologies (e.g., horizontal gene transfer, constitutive expression of chimeric proteins, and gene stacking) showing promise (Salonia et al. 2020; Sinn et al. 2020). Having plants with a short juvenile phase would facilitate citrus crop improvement.

The "FasTrack" breeding system for plum (*Prunus domestica*) utilizes plants overexpressing *Populus tremuloides FLOWERING LOCUS T* (*PtFT*), a floral timing gene, to shorten the juvenile phase and accelerate flowering (Petri et al. 2018). In apple (*Malus* × *domestica*), overexpression of a birch (*Betula pendula*) floral meristem identity gene was used to produce early-flowering plants to introgress disease resistance (Flachowksy et al. 2007; Le Roux et al. 2012). For citrus, creation of early-flowering phenotypes, the first step required for application of "Fast Track" breeding, has been achieved through ectopic expression of *A. thaliana AP1* (*AtAP1*) and *LFY* (*AtLFY*) in two citrus rootstock cultivars, 'Carrizo' citrange and trifoliate orange (*Poncirus trifoliata*) (Pena et al. 2001; Endo et al. 2005; Cervera et al. 2009) and the scion cultivar 'Meiwa' kumquat (*Fortunella crassifolia*) (Duan et al. 2010); and also with the expression of *C. unshiu FT* (*CiFT*) in trifoliate orange (Endo et al. 2005). In addition, the potential for gene-stacking of selected traits was demonstrated with 'Carrizo' citrange rootstocks overexpressing the *AtAP1* transgene (Cervera et al. 2009). However, to date, there is no early-flowering phenotype of a scion cultivar of commercial importance to the global citrus industry for use in "Fast Track" breeding.

Comparative studies suggest the genetic network regulating the flowering process is largely conserved among plant species (Benlloch et al. 2007; Jack 2004). The following genes, which are homologous to those found in *A. thaliana*, have been cloned from *Citrus* spp.: *TERMINAL FLOWER1* (*TFL1*), *FT, SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), *LFY*, and *AP1* (Endo et al. 2005; Samach 2012; Pillitteri et al. 2004a, b; Tan and Swain 2007). When ectopically expressed in *A. thaliana*, *CsTFL1* delayed flowering (Pillitteri et al. 2004a), whereas the citrus homologs of *FT, SOC1, LFY* and *AP1* resulted in precocious flowering (Endo et al. 2005; Pillitteri et al. 2004b; Tan and Swain 2007). In addition, overexpression of *CsAP1* or *CsLFY* complemented respective delayed flowering-time mutants of *A. thaliana* (Pillitteri et al. 2004b). Thus, in *A. thaliana*, ectopic expression of either *CsAP1,* or *CsLFY* was sufficient to promote early flowering and convert the vegetative shoot apical meristem (SAM) to an inflorescence and the terminal bud to a flower (Pillitteri et al. 2004b). In *Citrus* spp., expression of *CsLFY* has been documented to regulate floral timing through the integration of floral induction pathways, and both *CsLFY* and *CsAP1* have roles in floral meristem determinacy and subsequent downstream floral organogenesis (Nishikawa 2013). *CsAP1* also controls the response of the SAM to factors that promote or inhibit flowering (Goldberg-Moeller et al. 2013; Tang and Lovatt 2019).

The research reported herein was undertaken to produce transgenic *Citrus* spp. (*C. paradisi* and *C. sinensis*) important to the global citrus industry that have an early-flowering, early-fruiting phenotype with improved control over flowering time than is typically attained with the use of a constitutive promoter, such as 35S CaMV (Behnman et al. 2006; Bihmidine et al. 2012; Qiu et al. 2012). Thus, in the current research, the stress-inducible *AtRD29A* promoter was used to control the expression of the *CsAP1* and *CsLFY* genes and precocious flowering. Since the *AtRD29A* promoter is activated by low temperature, drought and salinity stress (Behnman et al. 2006; Bihmidine et al. 2012; Msanne et al. 2011; Qiu et al. 2012; Yamaguchi-Shinozaki and Shinozaki 2005), in theory flowering should occur in successfully transformed lines only in response to stress and its alleviation, making it possible to upregulate transgene expression and the floral development process when the transgenic plants had reached a size able to support the full development of fruit. The goal of the research was to produce

transgenic lines of commercially important citrus scion cultivars with fast flowering and fruit bearing capabilities that might allow researchers to more rapidly examine phenotypic changes associated with commercially important traits related to fruit quality, disease and pest resistance, abiotic stress tolerance, and others through the use of gene stacking technology.

To meet this goal, the first objective of the current research was to create constructs that placed the expression of *CsAP1* and *CsLFY* under control of the *A. thaliana* stress-inducible *AtRD29A* promoter and use these constructs to produce transgenic lines of 'Duncan' grapefruit (*C. paradisi*) and 'Valencia' sweet orange (*C. sinensis*). The second objective was to use low temperature stress or drought to control levels of transgene expression to regulate the floral development process and induce precocious flowering. Despite the outcome that the *AtRD29A* promoter was not induced by stress, but constitutively expressed in a citrus background, transgenic lines of 'Duncan' grapefruit and 'Valencia' sweet orange ectopically expressing *C. sinensis AP1* or *LFY* exhibited precocious flowering, fruit development, and formation of viable transgenic seeds, more notably in the T1 generation. These transformed lines can be useful tools to reduce the time between generations to accelerate breeding.

Materials and methods

Plant material and growth conditions for transgenic plants

'Duncan' grapefruit (*Citrus paradisi* Macf.) and 'Valencia' sweet orange (*Citrus sinensis* (L.) Osbeck) scion cultivars, and 'Carrizo' citrange rootstock (*Citrus sinensis* [L.] Osbeck × *Poncirus trifoliata* [L.] Raf.) were selected for the research. All three cultivars are commercially important to the worldwide citrus industry. In addition, the high frequency of nucellar embryony in 'Duncan' grapefruit (Holland et al. 1996) and 'Valencia' orange (Koltunow et al. 1995) increased the probability that T1 and T2 generation plants would be derived from nucellar tissue and be genetically identical (clones) to the mother plant. Seeds from fruit collected from each cultivar were peeled, surface-sterilized and planted in tubes with MS medium (Murashige and Skoog 1962). Thirty days after seed germination, the seedlings were transferred to white light (60 µmol m^{-2} s^{-1}) for 3 days to etiolate before being cut into explants used in genetic transformation.

Construction of T-DNA regions of binary vectors

All three binary vectors were derivatives of pCAMBIA2301 (Fig. 1). The *A. thaliana RD29A* gene promoter fragment (750 bp) was amplified and cloned in the BamHI/PstI sites of pGEM-T Easy vector (Promega) for sequencing. Once the sequence was verified, the *AtRD29A* promoter was digested with BamHI/Pst, and ligated at the 5′-end of the *CsLFY* cDNA (Pillitteri et al. 2004b) cloned in pBS-SK. The NOS terminator (300 bp) was amplified from the *Agrobacterium* binary vector pCAMBIA 2301 and ligated at the 3′-end of the *CsLFY* cDNA (Pillitteri et al. 2004b) in pBS-SK. The *AtRD29A-CsLEAFY-NOS* gene cassette was finally digested out of pBS-SK with BamHI/SalI and cloned into pCAMBIA 2301 for plant transformation. The same strategy was applied for construction of the gene cassette containing the *AtRD29A* promoter, *CsAP1* cDNA (Pillitteri et al. 2004b) and the *NOS* terminator. This gene cassette was also inserted into BamHI/SalI sites of pCAMBIA 2301. For the production of the *AtRD29A*-e*GFP-NOS* gene cassette, *eGFP* was amplified from the pLMNC95 plasmid (Mankin and Thompson 2001). After completion, the cassette was inserted XbaI/HindIII sites of pCAMBIA 2301.

Genetic transformation

For production of transgenic plants, we followed a protocol routinely used for citrus transformation (Orbović and Grosser 2006). Etiolated seedlings were used as the starting material. The stems of seedlings were cut into 15 to 20 mm-long explants that were placed into liquid co-cultivation medium (CCM) (Orbović and Grosser 2006) for 2 to 3 h prior to co-cultivation with *Agrobacterium tumefaciens*. Explants were soaked in freshly prepared *Agrobacterium* suspension for 3 min, then incubated on plates with solid CCM medium for 2 days. Explants were then transferred to regeneration medium (RM) (Orbović and Grosser 2006) with the antibiotic Cefotaxime (330 mg L^{-1}) to eliminate *Agrobacterium* and

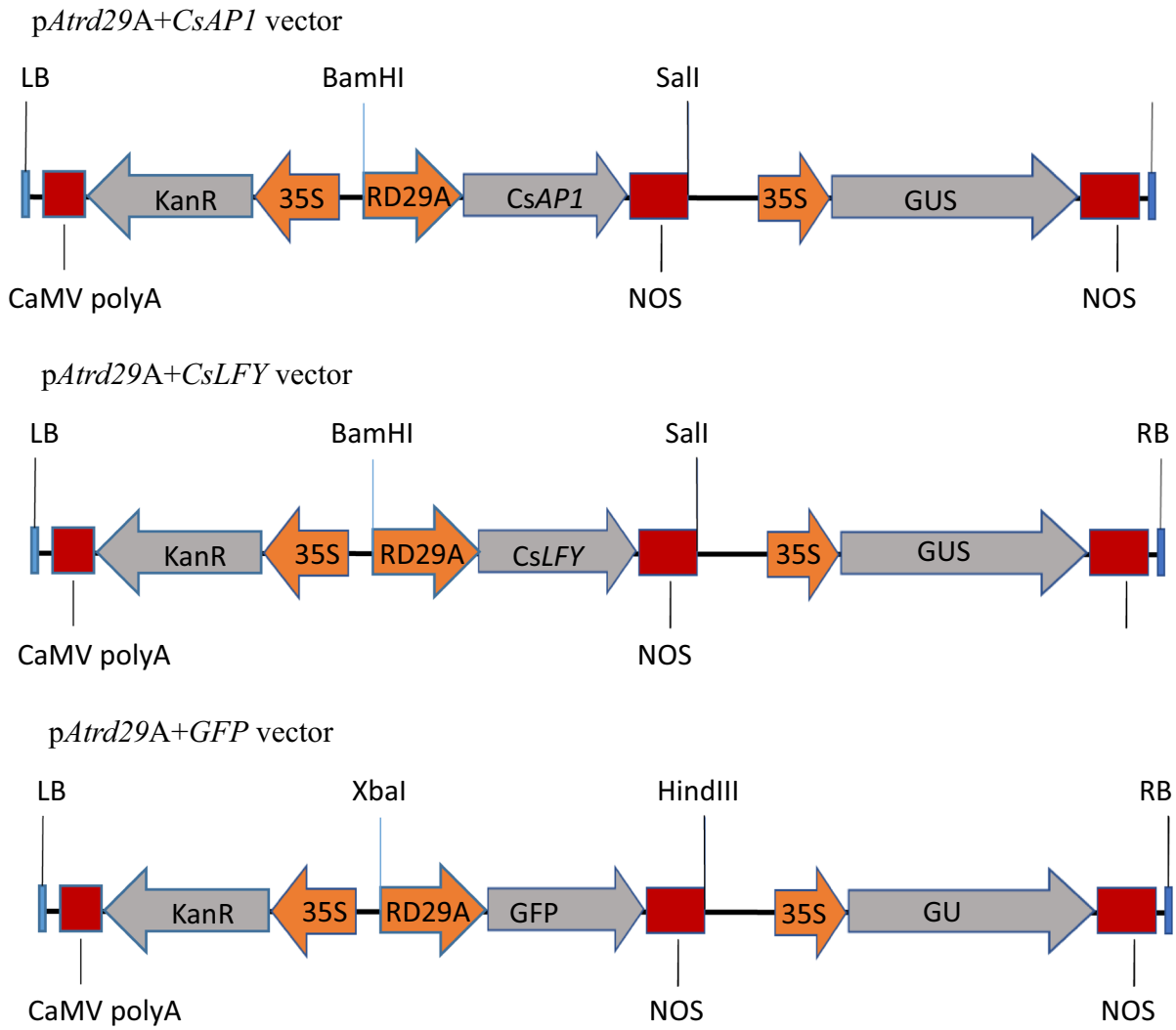

Fig. 1 Schematic presentation of T-DNA regions of binary vectors *AtRD29A+CsAP1*, *AtRD29A+CsLFY*, and *AtRD29A+GFP* used for transformation of citrus plants. All these vectors were derivatives of pCAMBIA2301. LB-left border, RB-right border, CsAP1-*C. sinensis APETALA1* gene, *CsLFY*-*C. sinensis LEAFY* gene, *GFP*-green fluorescent protein gene, *GUS*-beta glucuronidase gene, KanR-*nptII* kanamycin resistance gene, 35S-Cauliflower mosaic virus promoter, RD29A-*AtRD29* gene promoter, NOS-*nopaline synthase* gene terminator, CaMV polyA- Cauliflower mosaic virus polyadenylation sequence

Kanamycin (70 mg L^{-1}) to suppress the growth of non-transformed shoots. Control wild type shoots were regenerated from non-inoculated explants on RM medium without antibiotics. Thin cross-sections of shoots that sprouted from explants, which had been co-incubated with *Agrobacterium* strains carrying different binary vectors, were used for GUS histochemical assay to reconfirm the presence of the ß-glucuronidase reporter gene present in the T-DNA of all pCAMBIA 2301 binary vector constructs. Pieces of leaves from transgenic plants were also tested by GUS assay (for details on GUS assay see Orbović and Grosser 2006).

Transgenic T0 shoots were micro-grafted on clonal 'Carrizo' citrange seedling rootstocks. The newly-obtained transgenic plants were kept in the greenhouse where they were watered and fertilized on a regular basis. In the period between mid-March and mid-November, these plants were fertilized two times a week and watered once a week. During the rest of the year, plants were fertilized once a week and watered

once a week. The temperature in the greenhouse was never below 15.5 °C for longer than few minutes, i.e., the time needed for the thermostat to detect the low temperature threshold and turn on the heaters.

Analysis of CsAP1 and CsLFY genes expression levels

Transcript levels of *CsAP1* and *CsLFY* in transgenic plants were estimated with qRT-PCR. Total RNA was extracted from leaves of transgenic 'Duncan' grapefruit and 'Valencia' orange plants with the RNeasy plant mini kit (Qiagen) and subjected to RT-qPCR analysis according to the manufacturer's instructions. cDNA was synthesized from 1 µg of RNA using the iScript cDNA Synthesis kit (Bio-Rad Labs). *C. sinensis* primers were used to amplify the *Actin* housekeeping gene fragment as a reference for normalization of transcripts of the *CsAP1* and *CsLFY* target genes (Table 1). The qPCR validations were carried out using the iTaq Universal SYBR Green mix (Bio-Rad Labs) under the following conditions: 95 °C for 2 min denaturation, 42 cycles at 95 °C for 5 s, 57 °C for 10 s and 72 °C for 15 s. Amplification specificity was verified by melt curve analysis from 55 to 95 °C. The *CsAP1* and *CsLFY* transcript levels in transgenic plants relative to WT plants were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Table 1 Forward (f) and reverse (r) primers used in qRT-PCR reactions to quantify the expression of *C sinensis ACTIN* (*CsACT*), *APETALA1* (*CsAP1*), and *LEAFY* (*CsLFY*) in leaves of transformed and wild-type 'Duncan' grapefruit and 'Valencia' sweet orange

Name	Sequence[a]
ACT (f)	5′-TCACAGCACTTGCTCCAAGCAG-3′
ACT (r)	5′-TGCTGGAAGGTGCTGAGGGA-3′
AP1 (f)	5′-TCTCACAAAGGGAAGCTCTT-3′
AP1 (r)	5′-CACTCCAGGGGCCAGTTA-3′
LFY (f)	5′-TCTTGGGACAAAGCATCAACAGCG-3′
LFY (r)	5′-TCAAAGCTGCTGTTAGGGCTGAGA-3′

[a]Tang and Lovatt (2019), with the exception of the *AP1* forward and reverse primers designed specifically for this research to detect the transgene, but not the native gene

Low temperature stress treatment to induce flowering

Six-month-old seedlings of the T2 generation from the Dun134-3 *CsAP1* transgenic line and WT seedlings of the same age growing in 200-mL cone-tainers (3.8 × 25 cm) of potting mix were subjected to 8 weeks of low temperature floral-induction conditions (11 ± 1 °C continuously during an 8-h day/16-h night) or non-inductive conditions (25 ± 1.5 °C continuously during an 8-h day/16-h night) in walk-in growth chambers. At the end of 8 weeks of treatment, all plants were maintained at 25 ± 1.5 °C with an 8-h day/16-h night. Leaf tissue samples were collected after 4 and 8 weeks of treatment to estimate *CsAP1* transcript accumulation.

Drought stress treatment to induce GFP expression

Whole leaves were cut from three lines of 3-year-old 'Carrizo' citrange plants transformed with the *GFP* gene under control of the *AtRD29A* promoter and left in uncovered Petri dishes for 48 h at 26 ± 3.0 °C. At the end of this period, wilting, a symptom of drought stress, was visually obvious for all leaves. At that time, explants were cut from the drought-stressed leaves and examined for GFP fluorescence under a microscope equipped with a blue (λ 450–490 nm) light source. Control leaves were cut from well-watered WT plants of the same age just before they were inspected for the presence of GFP. Leaves of similar size and age were used for this comparison.

Results

CsAP1 transgenic lines

Two transgenic lines of citrus plants expressing *CsAP1* were evaluated: 'Duncan' grapefruit line Dun134-3 and 'Valencia' sweet orange line Val190-2 (Table 2). The transgenic line Dun134-3 was the most precocious line of all transgenic plants produced. The T0 Dun134-3 plant flowered after just 4.5 years, with 56% of its T1 siblings (17/30) flowering after 18 months. Both the T0 plant and the T1 generation plants of the Dun134-3 *CsAP1* line produced fruit from self-pollinated flowers. The harvested T1 fruit had 20 to 49 fertile seeds, from which the 6-month-old

Table 2 Characteristics of the T0, T1 and T2 generations of 'Duncan' grapefruit and 'Valencia' sweet orange transgenic plants expressing *C. sinensis APETALA1* (*CsAP1*) and *C. sinensis LEAFY* (*CsLFY*) genes under control of the *A. thaliana RD29A* promoter, including the result of *GUS* assays, leaf relative transgene expression, flowering age, fruit and viable seed production

Citrus spp.	Transgene	T0 line						T1 generation					T2 generation	
		Line name	GUS assay[a]	Transgene expression[b]	Flowering age (years)	Fruit	Seeds	GUS assay (+):(−) plants	Flowering age (months)	Percent flowering plants	Fruit	Seeds	Transgene expression	Flowering age (months)
C. paradisi ('Duncan' grapefruit)	*CsAP1*	Dun134-3	(+)	99.3	4.5	(+)	(+)	15:0	18	56 (18/32)	(+)	(+)	400–600 at 6 mos	NF at 27
	CsLFY	Dun120-13	(+)	69.4	NF[c] at 11	–	–	–	–	–	–	–	–	–
		Dun120-21C	(+)	78.3	8	(+)	(+)	12:0	3	8 (2/24)	(−)	–	–	–
		Dun120-25	(+)	231.9	8	(+)	(+)	36:0	3	51 (28/55)	(−)	–	–	–
		Dun124-11	(+)	198.6	8	(+)	(+)	7:0	8	57 (17/30)	(−)	–	–	–
		Dun124-13C	(+)	104.9	8	(+)	(+)	8:0	3	2 (1/50)	(−)	–	–	–
C. sinensis ('Valencia' orange)	*CsAP1*	Val190-2	(+)	239.8	9	(+)	(+)	11:0	NF at 24	–	–	–	–	–
	CsLFY	Val178-1	(+)	209.7	NF at 12	–	–	–	–	–	–	–	–	–

[a]Histochemical *GUS* expression assay (see Materials and Methods)

[b]Leaf transgene expression is relative to the expression of *AP1* and *LFY* in leaves of wild-type 'Duncan' grapefruit and 'Valencia' sweet orange plants, which is set at a value of 1.0

[c]NF, Plants have not flowered

T2 generation plants discussed below were derived. The 'Valencia' orange *CsAP1* expressing line, Val190-2, flowered after nine years. Although the Dun134-3 line flowered much earlier than Val190-2, the Val190-2 line expressed the *CsAP1* transgene at a much greater level (a leaf relative expression of 240 for Val190-2 vs. 100 for Dun134-3) (Table 2). Growth of the Val190-2 tree was slowed due to a Chilli thrips (*Scirtothrips dorsalis*) infestation in the greenhouse. When the sixth fruit produced by this tree reached 4–5 cm in diameter, we collected all fruit and their underdeveloped seeds and recovered T1 plants under in vitro conditions (Shen et al. 2011).

CsLFY transgenic lines

Six T0 plants expressing the *CsLFY* gene were evaluated, five 'Duncan' grapefruit lines and one 'Valencia' orange line (Table 2). Four of the five *CsLFY* transgenic 'Duncan' grapefruit lines flowered in the greenhouse after 8 years, but the 'Valencia' orange *CsLFY* line did not flower. Relative expression levels of *CsLFY* gene in leaves from the transgenic plants varied from almost 70 to over 240 (Table 2). There was no clear relationship between the levels of *CsLFY* expression in leaves and the ability of the T0 transgenic plants to flower under non-inductive conditions (Table 2). For example, the Dun120-13 and Dun120-21C lines had relatively similar low levels of *CsLFY* expression (Table 2); Dun120-21C flowered and Dun120-13 did not. The Val178-1 line, which expressed *CsLFY* at a level 2.7 times greater than the flowering Dun120-21C line never flowered. Interestingly, for the T1 siblings, a variable percentage (given in parentheses) of the Dun120-21C (8%), Dun120-25 (51%), Dun124-11 (57%) and Dun124-13C (2%) transgenic lines expressing the *CsLFY* gene flowered within 3–8 months after germination under non-inductive conditions in the greenhouse (Table 2; Fig. 2). However, all flowers of these seedlings abscised before setting fruit.

Low temperature stress treatment to induce flowering

Six-month-old T2 generation seedlings of the Dun134-3 *CsAP1* transgenic line and WT control plants were either treated for 8 weeks with low temperature (11 °C, 8-h day/16-h night) to induce flowering or maintained under non-inductive (control) conditions (25 °C, 8-h day/16-h night). At week 0, relative leaf transcript levels of *CsAP1* were significantly greater in the T2 generation transgenic plants than WT plants (Fig. 3). After 4 weeks, leaf *CsAP1* transcripts increased further in T2 Dun134-3 plants in both the low and warm temperature treatments compared to WT plants. Surprisingly, during the following 4 weeks, expression of *CsAP1* decreased in leaves of the T2 generation transgenic plants in both treatments, but remained significantly greater than *CsAP1* expression in leaves of WT plants (Fig. 3). None of the WT plants or the T2 generation plants of the Dun134-3 *CsAP1* line flowered under either the low or warm temperature conditions. Although the T2 generation plants of the Dun134-3 *CsAP1* line are now 27 months old, they have not flowered yet. In contrast, T1 generation plants of the Dun134-3 *CsAP1* line are now 5 years old and have flowered multiple times under non-inductive (warm, well-watered) conditions (Fig. 4).

Expression of the β-glucuronidase (GUS) gene in transgenic lines

Histochemical analysis of GUS reporter gene expression was conducted on leaves of transgenic T0, T1 and T2 generation lines. All the transgenic plants analyzed showed high constitutive GUS expression in leaves (Table 2); WT plants used as the negative control did not show any GUS expression (data not shown).

Drought stress treatment to induce GFP expression

The placement of the *GFP* gene under control of the *AtRD29A* promoter resulted in constitutive, rather than stress-induced, expression of *GFP* in leaves from three transformed lines of 3-year-old Carrizo' citrange rootstocks. GFP fluorescence observed in wilted leaves after exposure to drought stress for 48 h was equal to that of leaves collected from well-watered plants immediately before visual inspection for GFP (Fig. 5). The presence of GFP was not detected in drought stressed or well-watered leaves from WT plants.

Fig. 2 Photograph of the three month-old 'Duncan' grapefruit seedlings from the Dun124-11 transgenic line. These T1-generation seedlings expressing *CsLFY* gene formed flower-like structures that grew to be 5–6 mm in diameter and abscised before setting fruit

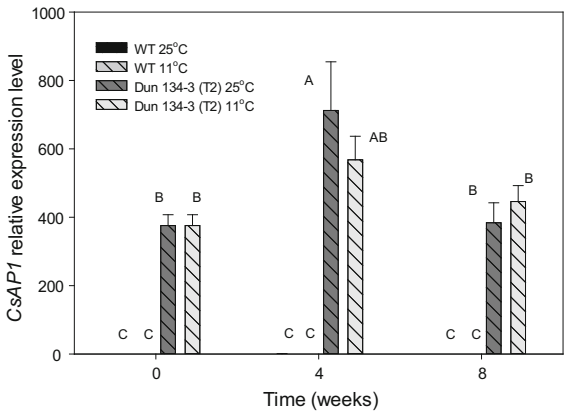

Fig. 3 Relative expression levels of the *APETALA1* (*CsAP1*) transgene under control of the *A. thaliana RD29A* promoter in leaves of the T2 generation 'Duncan' grapefruit Dun134-3 CsAP1 line and wild-type (WT) plants exposed to low (11 °C) and warm (25 °C) temperature treatments for 0, 4 and 8 weeks. In each treatment WT plants served as the control (leaf relative expression equals 1, which cannot be seen on the graph). Data are presented as means + SE (n = 5–20 plants). Means labeled with different capital letters are significantly different by the Duncan Multiple Range Test at $P < 0.05$

Fig. 4 Photograph of the five year-old 'Duncan' grapefruit plant from the Dun134-3 transgenic line. This T1-generation plant expressing *CsAP1* is shown bearing fruit for the third time

Discussion

Eight transgenic lines of citrus plants expressing *CsAP1* or *CsLFY* genes were evaluated in this research. The presence and expression of transgenes in selected, kanamycin-resistant T0 plants and T1 and T2 siblings were reconfirmed by RT-PCR gene expression analyses of the *CsAP1* and *CsLFY* genes, and also by histochemical assays for *GUS* reporter gene expression. Despite the fact that each gene was under control of the *AtRD29A* stress-inducible promoter, in all cases, flowering occurred under non-inductive (warm temperature, well-watered) greenhouse conditions. Further, flowering occurred throughout the year and was not associated with potential seasonal changes in photoperiod or temperature in the greenhouse. The transgenic plants did not exhibit any morphological features that were different from their WT counterparts of the same age. Transgenic plants that flowered stopped producing thorns, a

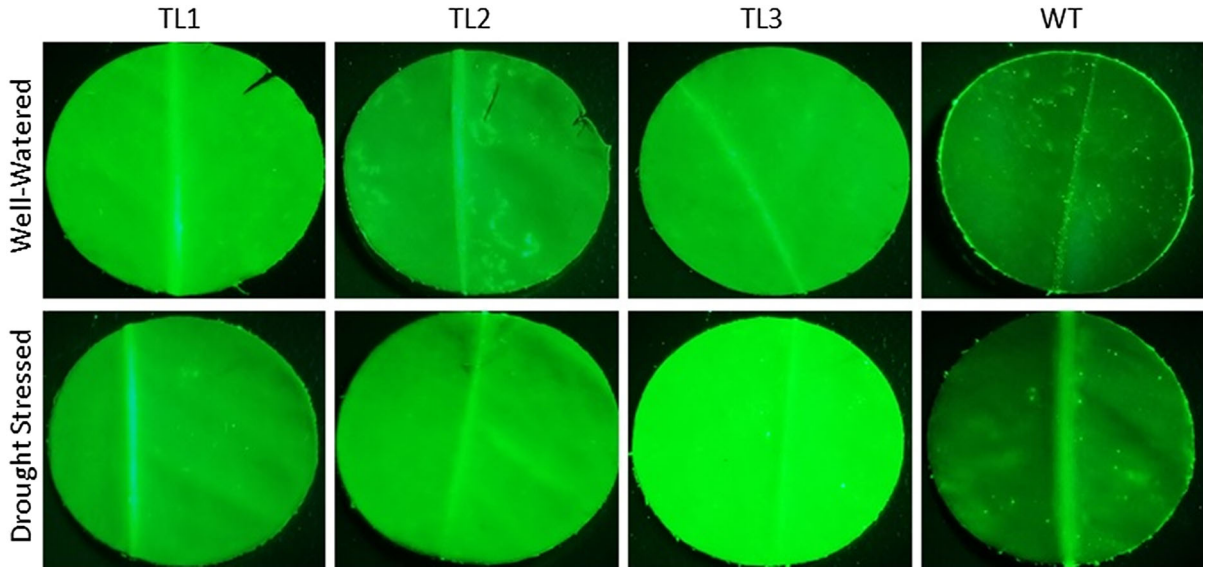

Fig. 5 Photographs of leaf explants from three 'Carrizo' citrange rootstock lines (TL1, TL2, and TL3) transformed with the *A. thaliana RD29A* inducible promoter + the green fluorescent protein gene (*AtRD29A+GFP*) binary vector and wild-type (WT) plants. The photographs were taken with the camera attached to microscope equipped with a blue light (λ 450–490 nm) source to induce GFP fluorescence. GFP fluorescence was present in the leaves of all three transgenic 'Carrizo' citrange lines independent of the treatment imposed. There was no GFP fluorescence in leaves of WT plants

characteristic of juvenility in *Citrus* spp., whereas plants that did not flower continued to develop thorns.

Unexpectedly, the *AtRD29A* promoter appeared to be constitutively expressed in the *Citrus* species and hybrids used in this research. Three lines of evidence support this conclusion. First, all eight T0 *CsAP1* and *CsLFY* transgenic 'Duncan' grapefruit and 'Valencia' orange plants expressed significantly greater levels of the respective transgene compared to WT plants under non-inductive greenhouse conditions (Table 2). Of these eight T0 plants, all flowered under the same non-inductive conditions, except one 'Duncan' grapefruit line and one 'Valencia' line expressing the *CsLFY* transgene, and the WT plants (Table 2). Second, exposing T2 generation Dun134-3 seedlings to either low (11 °C) or warm (25 °C) temperatures for 4 weeks resulted in significant similar increases in leaf expression of the *CsAP1* transgene in plants in both treatments compared to week 0 and WT plants (Fig. 3). In addition, despite an additional 4 weeks of low and warm temperature treatment, respectively, by week 8 leaf *CsAP1* expression in the T2 generation Dun134-3 line decreased to week 0 levels for plants at both temperatures. The scientific basis for these changes in leaf *CsAP1* expression in transgenic plants under both temperature treatments is unknown. Low temperature stress is divided into chilling stress (< 20 °C) and cold stress (< 0 °C) (Ritonga and Chen 2020). The *AtRD29A* promoter is known to be upregulated at both chilling (≤ 19 °C) and cold stress temperatures (≥ − 5 °C) (Ishitani et al. 1998). Taken together, the results of these experiments provided no evidence to support the induction of *AtRD29A* at 11 °C. In contrast, low (chilling stress) temperatures between 10 and 15 °C are known to induce flowering in adult WT *Citrus* spp., independent of photoperiod (Chica and Albrigo 2013). A third line of evidence supporting constitutive expression of *AtRD29A* is that similar levels of GFP fluorescence were observed in both wilted drought-stressed and turgid well-watered leaves of transgenic *AtRD29A-GFP* 'Carrizo' citrange rootstocks; no GFP fluorescence was observed in WT plants (Fig. 5). Visible leaf wilting in response to drought stress is sufficient to upregulate *AtRD29A* in other plant species (Xiao et al. 2015). Although varying degrees of constitutive expression ("leakiness") of target genes regulated by *AtRD29A* in the absence of stress have been documented (Bihmidine et al. 2012; Estrada-Melo et al. 2015; Qiu et al. 2012), the results presented here are the first to provide evidence of this phenomenon in *Citrus* species and hybrids. Altering the orientation and positioning of the

gene cassette within the T-DNA region have been proposed as strategies to reduce "leakiness" of the target gene when *AtRD29A* is used as the promoter (Bihmidine et al. 2012). These factors may underlie the seemingly exclusive constitutive expression of *AtRD29A* and the target genes in the transgenic lines reported here. Other than the loss of control over flowering time, constitutive expression of *AtRD29A* and the *CsAP1* and *CsLFY* target genes resulted in plants with an early-flowering phenotype and none of negative effects on plant morphology or health that have been reported with the use of the 35S CaMV promoter (Behnman et al. 2006; Bihmidine et al. 2012; Qiu et al. 2012; Xiao et al. 2015).

It is of interest that time to flowering of T1 generation siblings of four 'Duncan' grapefruit primary transformants (i.e., from selfed T0 scions) expressing the *CsLFY* gene, and one 'Duncan' grapefruit T1 scion expressing the *CsAP1* gene was significantly reduced compared with the flowering time of the T0 scions (Table 2). Flowering time in the T0 plants could have been influenced by genetic, epigenetic, hormonal, and/or metabolic factors released from the rootstock into the scions (Prassinos et al. 2009; Jensen et al. 2010; Liu et al. 2017). Similarly, epigenetic reprograming and genome structural variations are known to occur during tissue culture and may explain the segregation of flowering time among T1 and T2 siblings (Stroud et al. 2013; Stelpflug et al. 2014; Fossi et al. 2019). Precocious flowering of a trifoliate orange spontaneous mutant has been related to changes in the DNA methylation status of genes controlling the flowering process (Zhang et al. 2014). Only about 20% of the trifoliate orange mutant seedlings showed a short juvenile phase, flowering within 1 and 2 years after germination (Zhang et al. 2014), like the segregation of the early flowering phenotype in T1 and T2 progeny of *CsAP1* and *CsLFY* transgenic lines observed in this work. In contrast, *GUS* gene expression did not segregate in the T1 generation 'Duncan' grapefruit and 'Valencia' sweet orange lines expressing the *CsAP1* gene, nor in the four T1 'Duncan' grapefruit lines expressing the *CsLFY* gene (Table 2). Nucellar polyembryony is found in many *Citrus* groups (Davies and Albrigo 1994) and common in both 'Duncan' grapefruit (Holland et al. 1996) and 'Valencia' orange (Koltunow et al. 1995). The absence of segregation of *GUS* gene expression in T1 seedlings (Table 2) confirms that embryos developing from the 'Duncan' grapefruit and 'Valencia' orange seeds were not the products of sexual hybridization, but originated from nucellar tissue, making the T1 plants genetically identical to the mother plant.

The variable levels of transgene expression observed in leaves of the transformed lines of 'Duncan' grapefruit and 'Valencia' sweet orange may be related to changes in DNA methylation patterns during tissue culture, transgene copy number, T-DNA rearrangements, position effects and/or integration into the heterochromatin (Weinhold et al. 2013; Stroud et al. 2013; Stelpflug et al. 2014; Jupe et al. 2019). Some of these factors could lead to transgene silencing (Weinhold et al. 2013; Jupe et al. 2019). Transgene silencing results when transcription fails due to DNA methylation leading to chromatin compaction, or RNA is degraded post-transcription. Widely reported epigenetic transgene silencing by RNA-directed DNA methylation, which can be seen as a heritable decrease in transgene expression (Weinhold et al. 2013) was not observed in the transgenic 'Duncan' grapefruit line Dun134-3 expressing the *CsAP1* gene under control of the *AtRD29A* the promoter. Relative expression levels in leaves of 6-month-old T2 siblings were 4- to sixfold greater (Fig. 3) than that of the T0 Dun134-3 transgenic plant (leaf relative expression 99.31) (Table 2). For *Citrus*, the goal of transformed plant lines with a stable heritable phenotype is likely facilitated by the frequency of nucellar embryony and parental cloning, but also complicated by the group's genetics. Grapefruit (*C. paradisi*) originated as a hybrid of sweet orange (*C. sinensis*) × pummelo (*C. maxima*) and 'Valencia' orange (*C. sinensis*) as a hybrid of pummelo (*C. maxima*) and common mandarin (*C. reticulata*) (Li et al. 2010). Diploid differences in heterozygosity at loci also exist (Pillitteri et al. 2004a). In addition, spontaneous autotetraploids are found to varying degrees among polyembryonic citrus seeds, including those of 'Duncan' grapefruit (Aleza et al. 2008). Increased ploidy number would impact the factors associated with transgene copy number, expression and silencing.

The lack of correlation between leaf *CsAP1* and *CsLFY* transcript levels and the earliness of flowering in the transgenic lines might also be anticipated due to the disparate floral developmental events that occur in the bud. Flower development requires multiple steps subsequent to successful induction and transition of

the vegetative SAM to a floral meristem, including initiation of floral organ primordia, floral organ specification and development of individual flowers (Benlloch et al. 2007; Ma 1994). Overexpression of *CsAP1* or *CsLFY* transgenes in a juvenile plant may not always be sufficient to successfully downregulate various inhibitors of flowering and upregulate critical flowering pathway genes downstream from *LFY* and *AP1* (i.e., *AP1* and/or *APETALA2* [*AP2*], respectively). Upregulation of *AP1* and *AP2* is essential for floral meristem determinacy (irreversible commitment to flowering) in *Citrus* and subsequent activation of the downstream floral organ identity genes necessary for formation of individual flowers (Tang and Lovatt 2019).

Taken together, the results of this research provide evidence of early-flowering phenotypes in transgenic lines of two *Citrus* scion species, *C. paradisi* and *C. sinensis*, of commercial importance to the global citrus industry. Lines of 'Duncan' grapefruit and 'Valencia' sweet orange expressing the *CsAP1* or *CsLFY* transgene flowered precociously, bore fruit and produced viable seeds through the T1 and T2 generations. Despite the loss of ability to control flowering time using low (chilling) temperature or drought stress due to the constitutive expression of *CsAP1* and *CsLFY* transgenes under regulation of the *AtRD29A* promoter, the transgenic lines exhibit none of the negative effects on plant morphology or health associated with use of the constitutive 35S CaMV promoter. The precocious flowering capabilities of these lines might allow researchers to more rapidly examine phenotypic changes associated with commercially important traits related to fruit quality, disease and pest resistance, abiotic stress tolerance, and others through the use of gene stacking technology.

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Author contribution CJL, VO, and YA designed the experiments, JN and YA constructed binary vectors, VO, SAR, BM, and YA preformed the experiments, all authors wrote the manuscript.

Declarations

Conflict of interest Authors declare no conflict of interest.

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