Effects of gibberellin treatment during flowering induction period on global gene expression and the transcription of flowering-control genes in Citrus buds

Ravit Goldberg-Moeller, Liron Shalom, Lyudmila Shlizerman, Sivan Samuels, Naftali Zur, Ron Ophir, Eduardo Blumwald, Avi Sadka

A R T I C L E   I N F O

Article history:
Received 28 June 2012
Received in revised form 26 September 2012
Accepted 28 September 2012
Available online 4 October 2012

Keywords:
Bud
Citrus
Flowering
FT
Gibberellin
Global gene expression

A B S T R A C T

Gibberellins (GAs) affect flowering in a species-dependent manner: in long-day and biennial plants they promote flowering, whereas in other plants, including fruit trees, they inhibit it. The mechanism by which GAs promote flowering in Arabidopsis is not fully understood, although there is increasing evidence that they may act through more than one pathway. In citrus, GA treatment during the flowering induction period reduces the number of flowers; the mechanism of flowering inhibition is not clear; the hormone may act directly in the bud to determine its fate toward vegetative growth, generate a mobile signal, or both. However, bud metabolic and regulatory pathways are expected to be altered upon GA treatment.

We investigated the effect of GA treatments on global gene expression in the bud during the induction period, and on the expression of key flowering genes. Overall, about 2000 unigenes showed altered expression, with about 300 showing at least a two-fold change. Changes in flavonoids and trehalose metabolic pathways were validated, and among other altered pathways, such as cell-wall components, were discussed in light of GA’s inhibition of flowering. Among flowering-control genes, GA treatment resulted in reduced mRNA levels of FT, AP1 and a few flower-organ-identity genes. mRNA levels of PLK-like and SOC1 were not altered by the treatment, whereas LEAFY mRNA was induced in GA-treated buds. Surprisingly, FT expression was higher in buds than leaves. Overall, our results shed light on changes taking place in the bud during flowering induction in response to GA treatment.

© 2012 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Gibberellins (GAs) comprise hundreds of compounds, some of which regulate different aspects of plant growth and development, including seed germination, stem elongation, leaf expansion and flower and seed development. They may act alone or in association with other hormones [1]. In recent years, much progress has been made in understanding GA metabolism and signaling pathways, mainly through the identification and analysis of GA-response mutants (reviewed in [2–6]). These studies revealed that the GA growth-regulatory mechanism is dependent on DELLA proteins, identified as growth inhibitors that act in two ways: they interfere with the activity of growth-promoting transcription factors and they activate the promoters of several genes that upregulate the abscisic acid (ABA) pathway, which is antagonistic to GA. When present, the GA molecule binds to its cellular receptor, GID1, which facilitates conformational changes allowing the binding of the N-terminal region of DELLA. The GA-GID1-DELLA complex inactivates DELLA, mainly by promoting DELLA proteosomal degradation via interaction of DELLA with SCF351/GID2 E3 ubiquitin ligase, which allows its polyubiquitination. The effect of GA on flowering induction is complex as it is species-specific: in long-day, biennial plants, it usually promotes bolting and flowering initiation under non-permissive conditions, whereas in many perennials it inhibits flowering [7,8]. The effects of GA, positive or negative, might be operational at several developmental stages, such as during acquisition of floral competence, floral induction and/or reproductive meristem formation [9].

The floral induction period in citrus starts in mid November and lasts until approximately the end of December–mid January in the Northern Hemisphere [10]. Following induction, the shoot apical meristem (SAM) differentiates into a floral bud [11,12]. Not all SAMs become floral; some continue the vegetative growth of the tree. Thus, in parallel to the floral shoot flush, there is a flush of vegetative shoot growth, which continues through June. Flowering in citrus is induced by low temperature and water stress, while day length has a relatively minor effect [10]. Other factors, such as crop load, bud age and its position along the shoot affect flowering intensity [13,14]. There is extensive cross-talk between autonomous and vernalization flowering pathways and ample
evidence that genes associated with flowering regulation are highly conserved across species [15]. Indeed, citrus genes homologous to Arabidopsis flowering control genes possess most likely similar functions. For instance, overexpression of the citrus Flowering Locus T (FT), Arabidopsis Leafy (L FY) and Arabidopsis Apetal fr (API) genes in citrus greatly reduced the juvenile period, allowing flowering at the seedling stage [16–18]. FT was induced during the annual transition to floral development [19]. In addition, FT and API transcripts accumulated in trees subjected to low-temperature floral-inductive conditions [15,18,20]. Overexpression of the citrus genes LF Y, API and Suppressor of Overexpression of Constans1 (SOC1) in Arabidopsis resulted in phenotypes similar to those observed when the endogenous genes were overexpressed, and LF Y and API rescued Arabidopsis mutants in their respective genes [15,18,20]. Similar findings were demonstrated for citrus Terminal Flower (TFL) [21]. Inverse relationships were found between fruit load and the expression of FT, API and SOC1 in the leaves and buds, especially during flowering induction period [22–24]. The inhibitory effect of GA on perennial flowering has been studied mostly in fruit trees, where its external application during the flowering induction period inhibits flowering [7,8]. Successive applications of the hormone in citrus from the beginning of November until the beginning of January significantly reduced the number of flowers, thus allowing a determination of the flowering induction period [10,25]. However, whether GA acts endogenously to inhibit flowering is still an open question. It has been suggested that seeds are the source of the GA, which moves into the buds and inhibits flowering [8]. In addition, or alternatively, indole acetic acid (IAA), also produced in the seeds, might induce GA biosynthesis [8]. The mechanism of action of GA on floral transition in Arabidopsis and other model plants is thought to follow a complex, and as yet not fully elucidated, mode of regulation. It has been suggested that GA acts directly to induce floral transition at the shoot apex by inducing the expression of SOC1 and LFY via DELLA-mediated pathways [9]. In addition, GA might also act by promoting formation of FT in the leaf in a photoperiod-independent pathway [9].

The inhibitory effect of GA on flowering in citrus has been well documented, also at the anatomical level [12], but the molecular mechanisms underlying its effect are still unknown. Recently, it has been shown that GA, applied during the flowering induction period, reduced the expression of CiFT and CsLFY in the leaves while paclolbutrazol reversed the effect [26]. Previous works have shown that the bud might receive external flowering stimuli independently of leaves presence [10,27,28]. Therefore, it is reasonable to assume that externally applied GA is directed into the bud where it induces its flowering-inhibitory action, although an indirect effect cannot be excluded. Regardless of whether the effect is direct or indirect however, the GA treatment must result in a series of events that take place in the bud itself. To identify metabolic and regulatory pathways that are altered in the buds upon GA treatment during the flowering induction period, we analyzed global gene expression using Affymetrix Citrus DNA array. In addition, we investigated GA’s effect on the expression of key flowering-control genes to identify those with altered expression following the GA treatment.

2. Materials and methods

2.1. Plant material and GA treatment

Ten-year-old Orri mandarin (Citrus reticulata Blanco × Citrus temple Hort. ex Y. Tanaka) trees grafted on Troyer (Citrus sinensis (L.) Osbeck × Poncirus trifoliata (L.) Raf.) rootstock grown in the central coastal region of Israel (31°58’10.58”N: 34°52’11.36”E) were chosen for the experiments based on their uniformity and flowering intensity during the first year of the experiment. The average climatic conditions during 3 years of experiments and during the treatment period are shown in Supp. Table 1. GA and control treatments were performed over the course of 3 years in four randomized blocks, each containing seven trees (three control trees, three treated trees and one border tree). Within the same plot, different trees were treated every year. GA3 (Pro-Gibb, Valent Biosciences, Libertyville, IL, 0.1 M active compound) was sprayed at a concentration of 150 ppm solution containing 0.1% (v/v) phosphoric acid and 0.025% (v/v) Triton X-100 as surfactant. Control trees were sprayed with a solution containing only the acid and surfactant. Four sprays were applied, starting from Nov 15, at 2-week intervals. The numbers of inflorescences and vegetative shoots were determined on all branches splitting from one major 50–60 mm in diameter-branch located at the south-east side of the tree during peak blossom, usually during the first third of April of the consecutive year. About 15 branches from the spring flush (8–10-months-old), collected from the south-east side of the tree, were taken to the laboratory on ice. Samples were collected at the end of November (2 weeks following the first treatment), mid-December (a few days following the third treatment), mid-January (two weeks following the last treatment) and mid-February. At least 10 buds were removed from the 2–3 most distal nodes and immediately frozen in liquid nitrogen. Samples were kept at −80°C until processing.

2.2. RNA isolation and qPCR analyses

Total RNA was extracted from buds by means of the phenol–chloroform procedure as described previously [29], with modifications. For buds, approximately 0.2 g of frozen tissue was ground (using mortar and pestle) in liquid nitrogen and added to 180 μl of grinding buffer containing 60 μl of phenol saturated with TLE (0.2 M Tris–HCl, pH 8.2, 0.1 M LiCl and 5 mM EDTA). Chloroform (60 μl) was then added, followed by incubation for 20 min at 50°C with occasional shaking. The mixture was then centrifuged at 10,000 × g for 30 min at 4°C and the upper phase was re-extracted with 100 μl TLE-saturated phenol and 100 μl chloroform, and re-centrifuged. The upper phase was extracted with 0.2 ml chloroform, and centrifuged (10,000 × g, 30 min, 4°C). LiCl was then added to the upper phase to a final concentration of 2 M (from 8 Mstock solution), followed by overnight incubation at 4°C and centrifugation at 10,000 × g for 40 min at 4°C. When leaves and stems were used, about 2 g of tissue wasgrounded, and the volumes of the above solutions was increased 10-fold, accordingly. The pellet was dissolved in 50 μl H2O, and precipitated by the addition of 0.1 volume of 3 M sodium acetate, pH 5.2, and 2.5 volumes of cold ethanol, followed by 30 min incubation at −80°C. DNA was removed from the samples using RNase-Free DNase (Promega, Madison, WI) according to the manufacturer’s instructions. cDNA synthesis was performed using iScript cDNA synthesis kit (BioRad, Hercules, CA) according to the manufacturer’s instructions. The quality of the RNA was determined using Agilent RNA 6000 Nano Kit and Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Quantitative (q) PCR (real-time PCR) analyses were carried out with a SYBR Green qPCR Kit (Finnzymes, Espoo, Finland) according to the manufacturer’s instructions in a QIagen Rotor-Gene Q (Corbett Rotor-Gene 6000) real-time analyzer (Qiagen, Doncaster VIC, Australia) using gene-specific primers (Supp. Table 2). qPCR analyses were performed using RNA extracted from samples of the Year 3 of the experiment.

2.3. nCounter analysis

The RNA levels of trehalose biosynthetic genes and flavonoid biosynthesis genes, and the reference genes, βActin, Cyclophilin and Polyubiquitin 2, were determined using nCounter analysis (NanoString Technologies, Seattle, WA, USA) in VIB MicroArrays Facility
Fig. 1. Effect of GA treatment on the number of flower and vegetative buds. GA was applied during the fall of three consecutive years. The number of generative, mixed and vegetative buds was counted during the following spring in control and GA-treated trees. Values are the mean ± SE (n = 4). Stars denote a significant difference (P ≤ 0.05).

(Leuven, Belgium), according to manufacturer’s instructions using RNA extracted from samples of the Year 3 of the experiment [30]. Probes design was carried out based on genomic sequences (http://www.phytozome.net/, Supp. Table 3).

2.4. Statistical analyses

Analysis of variance was performed on data, and means were separated using Duncan’s new multiple-range test using JMP (SAS Institute, Cary, NC, USA).

2.5. Affymetrix GeneChip® hybridization and data-processing analyses

For global gene expression, the citrus GeneChip (Affymetrix, Inc., Santa Clara, CA) carrying 30,171 probes was used. The array is estimated to represent about 15,500 genes. RNA samples pooled from the end of November–mid-January of Year 1 (GA treatment) and Year 2 (control) were processed as recommended by Affymetrix (Affymetrix GeneChip Expression Analysis Technical Manual) at the Center for Genomic Technologies of the Hebrew University of Jerusalem. Total RNA was quantified and then adjusted to a final concentration of 1 μg/μL. Single-stranded and then double-stranded cDNA was synthesized from total RNA (0.5 μg total RNA for each reaction) using oligo-dT primer and the Affymetrix One-Cycle Labeling kit and control reagents. The resulting double-stranded cDNA was column-purified and then used as a template to generate biotin-tagged cRNA from an in vitro transcription reaction performed with the Affymetrix GeneChip IVT Labeling Kit. The resulting biotin-tagged cRNA (15 μg) was fragmented to strands of 35–200 bases in length following published protocols (Affymetrix GeneChip Expression Analysis Technical Manual) and then hybridized at 45 °C with rotation for 16 h (Affymetrix GeneChip Hybridization Oven 320) with the Affymetrix Citrus Genome array. The arrays were washed and then stained (EukGE-WS2v5 protocol, p 2.3.11), using SAPE and biotinylated anti-SA
Fig. 2. Effect of GA treatment on general metabolism. Differentially expressed probes were analyzed by MapMan. Blue squares represent genes with induced expression, while red squares represent genes with reduced expression. Genes encoding trehalose enzymes are marked. Full gene description is given in Supp. Table 6.

in Affymetrix Fluidics Station 450 followed by scanning in a GeneChip Scanner 3000. Hybridizations were carried out in triplicate, each replicate representing one experimental block. Data processing, including signal analyses, normalization and background subtraction were carried out using Robust Chip Analysis (RMA), as described previously [31]. Statistical test for significantly differentially expressed probes was carried out with the Linear Model for Microarray (limmaGUI) as described previously [32].

Gene ontology analysis was performed using AgriGo (http://bioinfo.cau.edu.cn/agriGO/index.php). Two analysis tools were used: singular enrichment analysis (SEA) which lists enriched Gene Ontology (GO) terms, and parametric analysis of gene set

<table>
<thead>
<tr>
<th>GO term</th>
<th>Description</th>
<th>Number in input list</th>
<th>Number in Ref a</th>
<th>P-value</th>
<th>FDR b</th>
<th>% in list</th>
<th>% in Ref</th>
<th>Enrichment fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0016103</td>
<td>Diterpenoid catabolic process</td>
<td>2</td>
<td>2</td>
<td>0.0012</td>
<td>0.048</td>
<td>0.27</td>
<td>0.01</td>
<td>28.5</td>
</tr>
<tr>
<td>GO:0005273</td>
<td>beta-Glucan metabolic process</td>
<td>2</td>
<td>2</td>
<td>0.0012</td>
<td>0.048</td>
<td>0.27</td>
<td>0.01</td>
<td>28.5</td>
</tr>
<tr>
<td>GO:0005274</td>
<td>beta-Glucan biosynthetic process</td>
<td>2</td>
<td>2</td>
<td>0.0012</td>
<td>0.048</td>
<td>0.27</td>
<td>0.01</td>
<td>28.5</td>
</tr>
<tr>
<td>GO:0006075</td>
<td>1,3-beta-Glucan biosynthetic process</td>
<td>2</td>
<td>2</td>
<td>0.0012</td>
<td>0.048</td>
<td>0.27</td>
<td>0.01</td>
<td>28.5</td>
</tr>
<tr>
<td>GO:0045487</td>
<td>Gibberellin catabolic process</td>
<td>2</td>
<td>2</td>
<td>0.0012</td>
<td>0.048</td>
<td>0.27</td>
<td>0.01</td>
<td>28.5</td>
</tr>
<tr>
<td>GO:0016129</td>
<td>Phytosteroid biosynthetic process</td>
<td>3</td>
<td>7</td>
<td>0.0014</td>
<td>0.048</td>
<td>0.40</td>
<td>0.03</td>
<td>12.2</td>
</tr>
<tr>
<td>GO:0009250</td>
<td>Glucan biosynthetic process</td>
<td>7</td>
<td>44</td>
<td>0.0008</td>
<td>0.048</td>
<td>0.94</td>
<td>0.21</td>
<td>4.5</td>
</tr>
<tr>
<td>GO:0006694</td>
<td>Steroid biosynthetic process</td>
<td>9</td>
<td>75</td>
<td>0.0013</td>
<td>0.048</td>
<td>1.20</td>
<td>0.35</td>
<td>3.4</td>
</tr>
<tr>
<td>GO:0008202</td>
<td>Steroid metabolic process</td>
<td>11</td>
<td>94</td>
<td>0.0005</td>
<td>0.048</td>
<td>1.47</td>
<td>0.44</td>
<td>3.3</td>
</tr>
<tr>
<td>GO:0009733</td>
<td>Response to auxin stimulus</td>
<td>15</td>
<td>157</td>
<td>0.0005</td>
<td>0.048</td>
<td>2.01</td>
<td>0.74</td>
<td>2.7</td>
</tr>
<tr>
<td>GO:0006952</td>
<td>Defense response</td>
<td>30</td>
<td>412</td>
<td>0.0002</td>
<td>0.048</td>
<td>4.02</td>
<td>1.94</td>
<td>2.1</td>
</tr>
<tr>
<td>GO:0006118</td>
<td>Electron transport</td>
<td>30</td>
<td>454</td>
<td>0.001</td>
<td>0.048</td>
<td>4.02</td>
<td>2.13</td>
<td>1.9</td>
</tr>
<tr>
<td>GO:0006468</td>
<td>Protein amino acid phosphorylation</td>
<td>36</td>
<td>550</td>
<td>0.0004</td>
<td>0.048</td>
<td>4.82</td>
<td>2.58</td>
<td>1.9</td>
</tr>
</tbody>
</table>

a Gene ontology.  
b Background.  
c Reference.  
d False discovery rate.
enrichment (PAGE), which uses central limit theorem in statistics, and takes expression level into account. Differentially expressed probe sets were displayed on diagrams of metabolic and other processes using MapMan [33].

3. Results

3.1. GA treatment results in a bud population with reduced probability of flowering

GA was applied several times to ‘Orri’ mandarin trees from November to December of three consecutive years. Buds were collected 2 weeks after each treatment, and the blossom was counted the following spring to assess treatment effectiveness. The citrus trees bore three types of buds: generative (leafless flower buds), mixed (leafy, flowers and leaves in various ratios) and vegetative (Fig. 1, lower panel). As expected, the most dramatic effect of the treatment was seen on the generative buds: during the first year, their numbers was reduced by about two orders of magnitude, during the second year they were reduced about sevenfold, and during the third year they were reduced by 100% (Fig. 1, left panels). A less dramatic effect was detected on the mixed blossom: a three- to four-fold reduction in bud number during the first and third years, whereas there was no treatment effect during the second year (Fig. 1, left panels). While reducing flowering, GA is known to induce vegetative growth; however, there was a relatively minor effect on the number of vegetative buds during the second and third years (Fig. 1, left panels). Unlike other alternate-bearing citrus varieties characterized by a clear biennial cycle of consecutive “On” and “Off” years, the alternate bearing in ‘Orri’ was not characterized by a classical biennial cycle: whereas the first year was characterized by a moderate number of inflorescences in control trees (about 550 generative and mixed-type per branch), inflorescence number in the second year was very high (about 2200 generative and mixed-type per branch), and dropped sharply to about 50 in the third year. Overall, the GA treatment resulted in a variable reduction in the percentage of flowering buds out of total buds during the 3 years: from 57% to 23%, from 95% to 84% and from 69% to 17% during the first, second and third years, respectively (Fig. 1, right panels). Buds collected during the 3-year experiment therefore contained populations with various probabilities of flowering, allowing comparative analyses. The effectiveness of the GA treatment was also evident at the yield level (Supp. Fig. 1).

3.2. Effect of GA treatment on global gene expression in buds

As first and second year treatments resulted in bud populations with relatively marginal differences in their probability to flower, the effect of GA on global gene expression was analyzed using non-treated buds (95% of probability of flowering, buds from Year 1) and treated buds (25% probability of flowering, buds from Year 1). RNA of GA-treated or control buds, pooled from November to January from the same biological replicate (i.e., experimental block), was hybridized to Affymetrix Citrus DNA array. Overall, 2124 probes were reduced under GA treatment, with 297 showing an at least two-fold reduction, while 2505 probes were induced, with 341 showing at least two-fold induction at a P-value (BH) ≤ 0.005 (Supp. Table 4). Overall, 21,286 out of 30,395 probes on the microarray were GO-annotated by AgriGo: 1809 and 1741 reduced and induced genes, respectively, were GO-annotated (Biological Processes) (Supp. Table 5). Induced and reduced genes were analyzed by SEA as well as with MapMan. Interestingly, reduced genes were classified into more biological processes by SEA than induced genes, i.e., 59 vs. 17 (Tables 1 and 2 and Supp. Table 5). This was also evident from the general metabolism, which showed more reduced genes than induced ones, especially secondary metabolism, amino acid metabolism, glycolysis, fermentation and minor carbohydrate metabolism (Fig. 2, Supp. Table 6). Among the induced genes, two probes belonging to the GA catabolic process were present in GA-treated buds (Table 1). Four probes on the DNA microarray showed homology to gibberellin-2-oxidase (GA2-Ox1), the first enzyme of GA catabolism. Two of these probes showed three- and four-fold induction under GA treatment (Fig. 3, lower panel). The induction in the expression of these genes was confirmed by nCounter analyses (Fig. 3, upper panel). Changes in some of the major pathways are described below.

3.2.1. Secondary metabolism

There was a mixed trend for the expression of genes associated with secondary metabolism (Supp. Fig. 2 and Supp. Table 7); whereas chalcone metabolism genes were usually induced, those of the phenylpropanoid, lignin, flavonols and isoflavonoid pathways were generally reduced. Five genes associated with flavonoids metabolism: 4-coumarate-CoA ligase (4CL), cinamate-4-hydroxylase (C4H), flavone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (AS) showed induction by GA treatment, and were validated by nCounter technology (Fig. 4). Overall, changes in the relative expression of 4 genes, AS, 4CL, C4H and DFR, were in a good agreement with the fold change of their corresponding probes in GA-treated and control buds. Only F3H showed about 1.5-fold increase in GA treated buds, while its corresponding probe showed about 2.7-fold induction. Two side-branch enzymes of the pathway, isoflavone reductase (IFR) that leads to isoflavonoid biosynthesis, and UF3GT that leads to anthocyanin glucoside biosynthesis, were reduced by the GA treatment. In addition, a side branch of the pathway leading to the cell-wall component lignin seemed to be reduced in GA-treated buds, as three genes, including caffeoyl-CoA O-methyltransferase (CCoAOMT), caffeate...
O-methyltransferase (COMT) and hydroxycinnamoyl:CoA transferase (C3H) showed reduced expression (Supp. Fig. 3). In contrast to lignin biosynthesis, there seemed to be induction of genes involved in the synthesis of β-glucan, another cell-wall component composed of cellulose and callose. Three cellulose synthase and two callose synthase genes were reduced in GA-treated buds (Supp. Table 4).

### 3.2.2. Defense response and protein amino acid phosphorylation

Many induced genes belong to the defense-response pathways (Supp. Table 4 and Supp. Table 5), including a few chitinase genes, genes for heat-shock and pathogenesis-related (PR) proteins, and some genes encoding resistance proteins (Supp. Table 5). MapMan revealed a similar picture, with a clear increase in the expression of pathogenesis-related (PR) proteins, and glutathione-S-transferase metabolism and peroxidase genes (Supp. Fig. 4 and Supp. Table 8). In addition, there seemed to be an increase in the signaling pathways of biotic and abiotic stress responses. Many of the genes involved in the stress responses were associated with amino acid phosphorylation, as also evidenced by the SEA results (Table 2).

### 3.2.3. Trehalose

Trehalose metabolism is composed of two enzymes, trehalose phosphate synthase (TPS) which synthesize trehalose phosphate...
and trehalose phosphate phosphatase (TPP) that converts trehalose phosphate into trehalose. Probes for TPS were induced upon GA treatment, while these of TPP were reduced. Therefore, the expression of the genes was investigated from November until January in control- and GA-treated buds (Fig. 5). The transcript level of TPS remained relatively constant in control buds during the tested period, while in the GA treatment induced its levels by about 2.5-fold from November until January, with a reduction to the basal level from January to February. TAA mRNA levels were also constant from November until February in control buds, but in GA-treated buds they were gradually reduced from December to February by about 4-fold.

3.3. Effect of GA treatment on flowering gene expression in buds

As many flowering-control genes contain a MADS box, we searched for these in the microarray (Supp. Table 9). About 45 MADS-box probes were identified, belonging to 28 genes in Citrus clementina (http://www.phytozome.net/). Interestingly, more than 50% of these genes’ expression was reduced in the GA-treated buds (Fig. 6A). In fact, only two MADS-box genes showed slight induction in the GA-treated buds. The expression of 5 genes, 3 reduced and the 2 induced was validated, and these results were in agreement with the microarray results (Fig. 6A). The effect of the GA treatment was examined on the expression of probes homologous to flowering genes from Arabidopsis. These genes were selected based on their functionality, or based on homology with their Arabidopsis homologues (at least 60% identity). Models describing flowering control in Arabidopsis assume the involvement of many genes in four different pathways. Most of their homologous genes from citrus did not show altered expression following the GA treatment (Fig. 6B). FT-like and AP1-like genes showed a five- and two-fold reduction in expression, respectively, following GA treatment. The expression of PIE1-like, ELF7-like and ELF8-like was induced twofold, by about 30% and by about 40%, respectively, in treated buds. In contrast, most of the genes homologous to those involved in flower development (flower-organ-identity genes) showed a clear reduction in GA-treated buds (Fig. 6C): PI-like with a four-fold reduction, STK-like with a two-fold reduction, SEP3(1/2)-like with a five-fold reduction and AP1 with a two-fold reduction. SHP1/2-like showed a smaller, albeit significant reduction, and AP2-like and AG showed no reduction with GA treatment.

Validation of the results with qRT-PCR was performed for each month separately, using RNA extracted from year 3. Five key flowering genes were analyzed in control and GA-treated buds (Fig. 7). As described in the Introduction, while there are some functional
analyses which demonstrate the function of FT, LFY, AP1 and SOC1 in citrus flowering control, the function of FLC in citrus was not demonstrated. We analyzed a gene that showed 47% identity and 69% homology with the Arabidopsis FLC gene, At5g10140.1. FT showed a four-fold induction in mRNA level from November to January, and a sharp decrease toward February, at bud brake. In agreement with the results obtained from the microarray analyses, GA treatment clearly inhibited the induction of FT mRNA levels. The pattern of FLC-like gene expression was similar in control and GA-treated buds, showing a two-fold induction from November to December and then a reduction toward flowering time. The expression of SOC1 barely changed during the induction period, with a slight reduction in February, and no remarkable difference detected between control and GA-treated buds. The expression of LFY remained constant from November to January, and showed a three- to four-fold induction from January to February. Interestingly, unlike the other examined genes, expression of this gene in December and January was higher in GA-treated buds than in controls, but was almost unchanged later on. As LFY was not present on the microarray, but showed an exceptional expression pattern, its expression was investigated in RNA extracted from 2 years, with similar results. The relatively high expression of FT in the buds was somewhat surprising considering the accepted model of FT transcription in the leaf, and its translocation as a protein into the apical meristem where it induces flowering. Therefore, we examined the relative expression of FT in leaves, stems and buds from trees during the flowering induction period. We found that during this 3-month period, FT mRNA levels were highest in the buds and stems, while relatively low levels were detected in the leaves [Fig. 8]. However, in the three organs, transcript levels were induced from November to December.

4. Discussion

The inhibitory effect of GA on flowering induction has been thoroughly investigated in citrus, and has been described in a number of studies [reviewed in 10,34]. The timing of effective treatment is probably dependent on the local environmental conditions, which determine the exact point at which flowering induction initiates and terminates, as well as on the cultivar. Moreover, it is highly likely that the impact of GA treatment differs according to the developmental stage at which it is applied. Early winter application (usually until the end of December) has been suggested to reduce flowering by inhibiting flowering induction [13]. Late application (mid January until February), when bud differentiation can already be observed under a microscope (with sepal initiation), results in inhibition of bud sprouting [12]. Other effects of GA might also exist, such as, suppression of axillary buds development, which might induce the proportion of terminal buds but overall reduces floral intensity. In the current work, treatments were applied from mid November until the end of December, presumably before the onset of bud sprouting, thus testing its effect on flowering induction. However, as for the individual bud flowering induction period might be as short as two weeks [25], and the induction is not necessarily synchronized among all the buds, other effects of the GA treatment cannot be excluded. In all 3 years, the most dramatic effect of GA treatment was the evident reduction in generative buds. In the first and third years, there was also a significant effect on mixed-type buds, with ca. three- and six-fold decreases, respectively, in their numbers. Apart from a slight increase in their number during the third year, there was no effect of GA on the vegetative buds. While there is general agreement on the effects of GA treatments on the reduction of generative shoot production, there are contrasting reports on the treatment’s effects on vegetative and mixed-type shoots [13,35–37]. In most studies, the number of vegetative shoots was not affected by the treatment, but a few studies reported that GA increased vegetative shoot production. Similarly, no effect on the number of mixed-type shoots or a reduction in their number was also reported. It is possible that these discrepancies are due to differences in local environment and/or growth conditions, as well as to cultivar-dependent differential responses. Moreover, it has been suggested that the applied concentration and the method of application greatly affect results [8]. When applied at relatively high concentrations, such as those used in the current work, a high proportion of the hormone is conjugated or metabolized. Indeed, GA application resulted in an increase in the mRNA levels of two GA2-Ox genes, whose products deactivate various GAs. Although GA2-OX is not known to catabolize GA3, the induction of its mRNA levels might indicate GA perception [38].

As mentioned above, control- and GA-treated buds of Year 1 and 2 presented relatively marginal differences in their probability to flower, which could result in a small number of differentially expressed probes (DEPs), if at all. Therefore, for the genomic analysis we used buds from two years, displaying the highest difference in their probabilities to flower. Validation of these results was performed using RNA extracted from the same year. Although we cannot exclude the possibility that some of the DEPs result from year-to-year variations, it should be considered that there were no significant climatic variations between the tested years (Supp Table 1). Moreover, validations by qPCR or nCounter analyses using RNA from the third year of the experiment confirmed the microarray data. Although, as discussed below, alternations in a few metabolic and regulatory pathways, observed here, were also
a consequence of the On- and Off-alternation [24], changes in gene expression between time points in the same year could also contributed to the differences in expression between treatments.

The effects of GA on flower induction, and the mechanisms associated with GA action in annual/biennial long-day plants, has been studied in model plants, especially *Arabidopsis*, using mutants defective in GA biosynthesis, and those with altered responses to GA [9]. Results of these studies showed that although *FT* dominates long-day flowering, GA contributes to flowering in *Arabidopsis* under both long-day and short-day conditions. The current model suggests that GA, synthesized in the leaf, acts in two ways: (i) it enhances the expression of *FT*, via *CONSTANS*, and (ii) it is translocated into the shoot apex where it promotes, directly or indirectly, the expression of *SOC1* and *LFY* via DELLTA-mediated pathways [39]. *LFY* has been shown to contain GA-responsive cis-acting elements in its promoter [9]. Because of the lack of practical genetic tools in citrus, monitoring changes in the expression of flowering and other genes can provide a means to understand flowering control by GA. Previously, the effects of GA on the expression of flowering control genes in the leaf was described [26]. Also, gene expression profiles in leaves and buds of On- and Off-crop trees were described [22–24]. However, the current work provides, a study describing the effects of GA on the bud transcriptome and on the expression of flowering control genes within the bud. Since GAs alter flowering, the comparison between the results presented in this work and those detected in buds and leaves of On- and Off-crop trees
displaying different probabilities to flower is valuable. For instance, it could be assumed that GA-treated buds would behave similarly to buds of On-crop trees, and vice versa. A result of the microarray experiments is that the GA treatment resulted in the reduced expression of many MADS-box genes in the bud. Although the functions of most of these genes are not yet clear, it might be assumed that at least some of these genes are involved in floral meristem and floral organ development. Five flowering control genes analyzed in the current work, *FT, LFY, AP1, SOC1* and *FLC*-like, were also analyzed by Muñoz-Fambuena et al. [22,23,26], but it was recently shown that the primers used by the later authors for *AP1, SOC1* and *FLC*-like recognized wrong genes [40]. Therefore, we could only compared the expression of *FT* and *LFY* (Fig. 7 and [24]) and the other published work [22,23,26]. As a result of the GA treatment, there was a reduction in the mRNA levels of *FT* during the flowering induction period both in the buds and in the leaves (Fig. 7 and [26]). The expression of the gene was up-regulated in buds and leaves of Off- versus On-crop trees, demonstrating its importance in flowering induction [22–24]. Similarly to *FT*, the expression of *AP1* during the flowering induction period was also reduced in GA-treated buds and in buds of On-crop trees [24]. The specific effect of the treatment on *FT* and *AP1* expression, with no detectable effect on genes showing homology to upstream *Arabidopsis* genes, suggests that GA acts directly on *FT* and *AP1*. As already noted, in *Arabidopsis* GA induces the expression of *LFY* and *SOC1*. Overall, the above results are compatible with the notion that GA treatment during winter reduces transcript levels of *FT* and *AP1* that in turns does not reach high enough levels to induce *LFY* and promote floral transition. Similar to buds of On- and Off-crop trees, we could not detect any effect on *SOC1* mRNA levels [24]. However, a noticeable result, confirmed in two treatment years, was the induction of *LFY* upon GA treatment during December and January, while during February, control buds displayed higher expression levels of the gene. While compared to previous reports, this result seems to be exceptional, but only in part. During February, close to bud break, the increase in control buds was compatible with what was described previously, i.e., *LFY* expression was higher in leaves and buds of Off- than On-crop trees [22,23]. However, during the winter, December and January, GA induced the expression of the gene specifically in the buds, but not in the leaves, which displayed higher expression levels upon paclobutrazol treatment [26]. In addition, during the winter, buds of Off-crop trees displayed higher expression levels of *LFY* than buds of On-crops [24]. This would suggest that, similarly to the *Arabidopsis* gene, *LFY* expression is induced by GA specifically in the bud. However, the induction in GA treated buds during the winter was too early to induce flowering in the spring. Also, the GA treatment could induce other factors, which mask *LFY* flowering-promoting activity. Using PLACE (http://www.dna.afic.go.jp/PLACE/), we searched for GA-responsive cis elements in the citrus *LFY* promoter. A few elements could be identified, but only partial sequence of the *Arabidopsis* *LFY* promoter element was identified (CAACTC in citrus vs.
CACTGTG in Arabidopsis), questioning its functionality [41]. Whether this or another element(s) is functional, or the mode of regulation of LFY by GA specifically in the bud (and not in the leaf) is different, requires further investigation. Overall, the above results provide support to the idea that, depending on the reproductive status of the tree, the hormonal control of flowering in citrus depends upon the levels of GAs during the winter [8]. Regardless of the mechanism by which GA exerts its inhibitory effect on flowering, a few genes which control floral organ development, including PI-like, AP3-like, STK-like, SHP1/2-like and SEP1/2/3(1/2))-like, exhibited a significant reduction in mRNA levels with GA treatment.

The results shown in Fig. 8, confirmed previous reports that FT transcript levels were induced in the leaves during the period of flowering induction [19,22]. However, among the tested organs, FT expression was highest in the buds, far exceeding that in the leaves. In a recent report, the mRNA levels were analyzed also in leaves and buds but it is not clear whether the analyses of the two organs were performed in a single qPCR experiment [23]. The accepted model from Arabidopsis, which has been confirmed in other plant species as well, is that FT expression is induced in the leaves upon long day conditions, and the FT protein moves to the apical meristem. As mentioned above, citrus is relatively day-length neutral, therefore, other factors, such as hormonal status of the bud might help determining FT expression level. Whether FT is transcribed in the bud itself is not yet clear. In fact, one report claimed that FT mRNA can be detected in the apical meristem of citrus bud using in situ hybridization [42], although the presented results did not allow drawing any firm conclusions. Although the bud also contains vegetative tissues (leaf primordia), our results give some support to the latter work. Previous work demonstrated that while cultured in vitro, isolated buds could flower upon cold treatment [27,28], and defoliation did not affect flowering intensity [10]; these and our results suggest that the bud might receive independent flowering signals, such as hormonal cues, and respond to them.

Regardless of whether GA plays an endogenous role in flowering inhibition, its exogenous application inhibits flowering, thus forcing most of the bud population toward vegetative growth, and the tree to an Off-crop year. In light of this, we propose explanations for some of the changes in specific metabolic pathways.

Flavonoid biosynthesis: The GA treatment resulted in increased expression of genes related to the major flavonoid biosynthetic pathway, accompanied by a reduction in two of its side branches, leading to isoflavonoid and lignin biosynthesis (see also below). Increase in flavonoid biosynthetic pathways and metabolite levels were detected in buds of Off trees [24]. Inhibition of flowering by GA imposes an Off year, which due to the low number of fruits, is characterized by a general increase in storage molecules in various tree organs. In agreement with previous reports, flavonoids might act in the bud as storage molecules [43,44].

Trehalose metabolism: Another noteworthy result is the altered expressions of trehalose biosynthetic genes TPS and TIP. While TPS that condenses glucose-6-P with UDP-glucose to generate trehalose-6P, is induced, TIP that dephosphorylate trehalose-6P to generate trehalose, is reduced by the GA treatment. Although in higher plants it is usually below detection levels, it is present at high levels in resurrection plants, where it serves as an osmoprotectant [45]. Changes in the trehalose biosynthetic genes and/or enzymes, and not necessarily trehalose levels themselves, were postulated to play a signaling or regulatory role in stress–response pathways, in normal vegetative growth and in the transition to the flowering phase [46,47]. The altered pathway in GA-treated buds might, therefore, suggest that the pathway is also involved in maintaining vegetative growth in citrus bud. An increase in trehalose biosynthetic pathway was also detected in buds of ON-crop trees [24].

Cell-wall metabolism: GA treatment resulted in the altered expression of some genes associated with β-glucan and lignin biosynthesis, two cell-wall components. Whereas genes of β-glucan metabolism (cellulose and callose) seemed to be induced, those of lignin metabolism were reduced. β-1,4-glucan (cellulose) comprises the core component of the plant cell wall [48], while β-1,3-glucan (callose) is a component of specialized walls and wall-associated structures at a particular stage of growth and differentiation [49]. Lignin is crucial for the structural integrity of the cell wall, contributes to the stiffness and strength of the stem [50]. Changes in the ratio between cellulose and lignin were recorded in other systems, and in a few cases these changes were induced upon GA treatment [51–53]. Therefore, it is suggested that in the GA-treated buds, which display reduced flowering, the reduced lignin-to-callose ratio is required for bud vegetative growth, as in other cases [54,55].

In summary, the current work sheds light on some of the processes, especially in secondary metabolism, that occur in citrus buds during flowering induction period in response to GA treatment. As expected, GA reduced the mRNA levels of FT, AP1 and some floral-organ-identity genes, but it induced that of LFY. Surprisingly, the expression of FT in the bud far exceeded that in the leaf. Some of these findings warrant further investigation, especially with regard to the phenomenon of alternate bearing, resulting in biennial alternations in yield.

Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2012.09.012.

References


\(\text{CiFT effects, in the other trees, of early flowering Arabidopsis development: of LEAFY confers flowering, in trees, by LEAFY with its Mandarin, in AP3,} \)


\text{F. Tan, S.M. Swain, Genotypes of flower initiation and development in annual and perennial plants, Physiol. Plant. 128 (2006) 8–17.}


\text{L. Pena, M. Martin-Trillo, J. Juarez, J.A. Pina, L. Navarro, J.M. Martinez-Zapater, Constitutive expression of Arabidopsis LEAFY or APA1A1 genes in citrus trees} \(\text{Thevelein, P.}

\text{The Arabidopsis bHLH factor and Arabidopsis} \(\text{AtTPS1}

\text{A. van Dijk, H. Schleuppen, S. Smeekens, Arabidopsis trehalose-6-phosphate synthase 1 is essential for normal vegetative growth and transition to flowering, Plant Physiol. 135 (2004) 969–977.}


\text{K. Kudlicka, R. Brown, Cellulose and callose biosynthesis in higher plants. I. Solubilization and separation of (1–3)- and (1–4)-beta-glucan synthase activities from mung bean, Plant Physiol. 115 (1997) 643–656.}


\text{E. Pau, V. Carocho, C. Marques, Á. de Sousa, N. Borralho, P. Sivadon, J. Grima-Pettenati, Transcript profiling of Eucalyptus xylem genes during tension wood formation, New Phytol. 167 (2005) 89–100.}