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Additional Information

The flower to fruit transition in *Citrus* is partially sustained by autonomous carbohydrate synthesis in the ovary

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ABSTRACT

Why evergreen fruit tree species accumulate starch in the ovary during flower bud differentiation in spring, as deciduous species do during flower bud dormancy, is not fully understood. This is because in evergreen species carbon supply is assured by leaves during flower development. We suggest the existence of an autonomous mechanism in the flowers which counteracts the competition for photoassimilates with new leaves, until they become source organs. Our hypothesis is that starch accumulated during *Citrus* ovary ontogeny originates from 1) its own photosynthetic capacity and 2) the mobilization of reserves.

Through defoliation experiments, we found that ovaries accumulate starch during flower ontogeny using a dual mechanism: 1) the autotrophic route of source organs activating Rubisco (*RbcS*) genes expression, and 2) the heterotrophic route of sink organs that hydrolyze sucrose in the cytosol. Defoliation 40 days before anthesis did not significantly reduce ovary growth, flower abscission or starch concentration up to 20 days after anthesis (i.e. 60 days later). Control flowers activated the energy depletion signaling system (i.e. SnRK1) and *RbcS* gene expression around anthesis. Defoliation accelerated and boosted both activities, increasing SPS gene expression (sucrose synthesis), and SUS1, SUS3 and cwINV (sucrose hydrolysis) to maintain a glucose threshold which satisfied its need to avoid abscission.

Key words: *Citrus*, parthenocarpy, starch, sucrose, Rubisco, SnRK1

1. Introduction

Most fruit tree species set flowers in a very low proportion. In particular, it is a common phenomenon of self-incompatible hermaphroditic species to have a low fruit/flower ratio (i.e. fruit set), which limits fruit yield [1]. In general, fruit tree species have three waves of flower/fruit abscission, at 1) pre-anthesis, in trees with a very high flowering intensity, 2) at post-anthesis, and 3) in newly set fruitlets. They vary in length and intensity depending on the species, as well as exogenous and endogenous factors [2].

Physiological flower abscission has been attributed to a shortage of carbohydrate (CH) status in both deciduous and evergreen fruit tree species [3,4], although there are serious doubts regarding this hypothesis [5]. In some temperate deciduous species, such as *Prunus sp.* or *Malus sp.*, floral bud differentiation occurs in late summer-early autumn before dormancy, whereas blooming takes place in late winter-early spring. Besides, floral buds sprout earlier than do vegetative buds, and hence flower growth from budbreak to early fruiting should not depend solely on the current photosynthesis [6]. Accordingly, the onset of ovary growth has been suggested as an autonomous process that correlates ovary starch depletion until leaves export CH [4,6].

On the other hand, in evergreen woody species (citrus, avocado, olive, etc.) floral buds do not differentiate before dormancy but rather during budbreak in spring. Thus, blooming and vegetative growth occur simultaneously. Therefore, flowers and new leaves compete for CH produced by the mobilization of starch reserves and the photosynthates from the mature leaves [7,8]. Given that the sink-to-source transition of young leaves occurs around 4 weeks after full bloom [8], competition for CH lasts until the beginning of the first fruitlet abscission, with many ovaries on the tree having high rates of cell division and respiration [9]. Despite this, flowers accumulate starch in the ovary during the development of the inflorescence, as observed in citrus [10,11], avocado [12,13] and olive [14]. This content has been related to flower quality [4, 14] and initial ovary growth and retention [12,13].

The fact that this phenomenon occurs in both deciduous and evergreen fruit tree species suggests the existence of an autonomous mechanism of the flowers to counteract the competition with the new leaves [15], allowing the flower-to-fruit transition. Metabolic pathways involved in the starch biosynthesis are different between source organs (mainly leaves) and sink organs (mainly seeds). In source organs, starch

is directly synthesized as a final product of photosynthesis in the chloroplast by means of the enzyme ADP glucose-pyrophosphorylase (ADPGP) and starch synthase (SS), when there is an excess of triose phosphate. In this case, carbon is derived from fructose-6-P [16-18]. In the case of sink organs, the ADP-glucose needed for starch synthesis is derived from sucrose hydrolysis in the cytosol by the action of sucrose synthase (SUS), and in the apoplast by the action of the cell wall invertase (cwINV) [16, 19].

In spite of this, the mechanism of starch accumulation in the ovary, and its physiological role, is still not fully understood. Flowers and fruits are sink organs, but they are also able to fix carbon temporarily in amounts which are highly variable among species, such as peach [20], apple [21] and grape [22]. Accordingly, we may ask up to what extent the starch accumulated in the ovary walls contributes to the early development of flower/fruit. If starch accumulation is an autonomous mechanism of the flower to facilitate fruit set, the ovary of evergreen trees might accumulate starch to counteract competition for CH with the leaves, as occurs in deciduous trees. Therefore, we propose the hypothesis that the starch accumulated during ovary ontogeny is crucial to set the fruit, maintaining an adequate level of glucose that temporarily releases the ovary from the competition with young leaves until they are photo-synthetically active. Starch in the ovary originates from 1) its own photosynthetic capacity, and 2) the mobilization of the reserves. To this end, we study the time-course of CH concentrations in the flowers, the activation of the SnRK1 (SNF1-related kinase 1) sugar deficiency sensor, which is activated by diverse energy-depleting stress conditions and maintains energy homeostasis by modification of key metabolic enzymes [23], and the starch and sucrose synthesis and hydrolysis with regard to flower abscission. We used the *Citrus clementina* tree as a model system because it is a self-incompatible hermaphroditic species that flowers profusely and sets a very low percentage of fruits, ranging from 0.1 to 10% depending on the cultivars, and there is a wide body of knowledge about the nutritional regulation of fruit set [3, 8, 10, 24-27].

2. Materials and Methods

2.1. Plant material, experimental design and defoliation treatments

The experiments involved 15-year-old *Citrus clementina* trees (cv. Marisol) grafted onto ‘Carrizo’ citrange (*Citrus sinensis* x *Poncirus trifoliata*) rootstock, planted 5 m x 5 m apart in a loamy clay soil, with drip irrigation in the Cooperativa de Lliria Research Orchard (Casinos, Valencia, Spain). Eight trees were selected for the experiments. Of these, 4 trees were only used to study the effect of defoliation on the time-course of flower/fruit development and abscission, and the other 4 trees were only used for tissue sampling so as not to interfere in the fruit abscission rate. Treatments were as follows: two of the four main branches in each tree were fully defoliated and the other two were left as control. Branches were considered as individual replicates given that woody branches fix carbon and are completely autonomous for carbohydrates [28]. Defoliation of mature and young leaves was performed forty days before anthesis, i.e. when new buds (either floral and vegetative) were at their early differentiation stage. The experiment was performed with a minimum of 400 flowers per branch [11], and repeated in two consecutive seasons.

2.2. Fruit development evaluation and sampling

Fruit set was recorded weekly for 12 weeks after defoliation. Flowers and fruits were sampled at random every week, weighed, and frozen until analysis. For CH analysis, three replicates of 15 flowers/fruits per treatment and date were collected, frozen in liquid nitrogen, stored at -28°C, and then lyophilized. For gene expression analysis, three replicates of 10 flowers/fruits per treatment and date were collected, frozen in liquid nitrogen and stored as powder at -80°C. Woody samples (branches and new shoots) were harvested in control and defoliated branches at fruit set (i.e. twenty days after anthesis), and were prepared for CH analysis as described below.

2.3. Carbohydrates extraction and analysis

The procedure used for CH determination is that described in our recent studies [10, 11]. Briefly, 100 mg of powdered samples were extracted with 1 ml of 800 ml l⁻¹ ethanol and purified sequentially using cation and anion exchange columns. The eluates were then passed through a C18 Sep-Pak cartridge (Waters-Millipore, Billerica, MA) and analyzed in a Spectra high performance liquid chromatography (HPLC) System (Spectra, San Jose, CA) equipped with a vacuum pump (Spectra P2000, Spectra) and a

differential refractometer (Spectra R150, Spectra). Sucrose, glucose and fructose were identified according to their retention times. Results were expressed as milligram per gram dry weight (DW). Starch levels were determined in the pellets remaining after the extraction of soluble sugars. The residue was dissolved in 5 ml water, gelatinized by autoclaving for 2 hours at 130°C, centrifuged for 10 min and 4 ml of supernatant removed. The sample was incubated by shaking for 2 h at 55° C with 0.1 ml fucose (internal standard) (Sigma Chemica Co. Inc., Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 0.5 ml of sodium acetate (pH 4.5) and 1 ml of 1218 U amyloglucosidase from *Rhizopus* (Sigma Chemica Co. Inc.). Samples were centrifuged for 30 min and 27000 g, and the supernatant was dried in vacuo and redissolved in 1 ml water. The glucose released was determined by HPLC as above. Results were expressed as milligram glucose released per gram DW. Three independent biological samples under each experimental condition were evaluated in technical triplicates

2.4. Gene expression analysis by qRT-PCR

RNA isolation, quantification, and quality analysis were performed as described in a recent work [29]. Total RNA was extracted from frozen tissues and subsequently treated with DNase I (RNase-Free DNase Set, Qiagen, USA). The amount of RNA was measured by spectrophotometric analysis (NanoDrop ND-1000 spectrophotometer, Thermo Fisher, USA). The absence of DNA contamination was checked by performing a no-reverse transcription assay which consisted of a PCR with each RNA sample using the *Citrus* actin primers. No amplified products were detected, which confirmed the purity of the RNA extracts. The transcripts present in 1 µg of total RNA were reverse-transcribed using the QuantiTect® Reverse Transcription Kit (Qiagen, USA) in a total volume of 20 µl. A 2.5 µl aliquot of a 4-time diluted first-strand cDNA was used for each amplification reaction. Quantitative real-time PCR was carried out on a Rotor Gene Q 5-Plex (Qiagen, USA) using the QuantiTect® SYBR® Green PCR Kit (Qiagen, USA). The reaction mix and conditions followed the manufacturer's instructions with certain modifications. The PCR mix contained 2.5 µl of diluted cDNA, 12.5 µl of QuantiTect® SYBR Green PCR Master Mix (Qiagen, USA), 1.5 µl of 0.3 µM primer F, and 1.5 µl of 0.3 µM primer R, the final volume being 25 µl. The cycling protocol for the amplification consisted of 15 min at 95° C for pre-incubation, then 40 cycles of 15 s

at 94° C for denaturation, 30 s at 60° C for annealing and 30s at 72° C for extension. RT-PCR reactions were repeated three times for each gene and monitored in real time with the Rotor Gene Detector. After amplification, melting-curve analysis excluded artefactual amplifications. The relative expression of RNA transcripts was quantified with the threshold cycle values (Ct) obtained from each sample using the $2^{-\Delta\Delta C_t}$ method. Expression levels were calculated relative to the constitutively expressed β -act gene [29]. The relative gene expression level is given by $2^{-\Delta\Delta C_t}$. Normalization was performed to the lowest Ct value. Three independent biological samples under each experimental condition were evaluated in technical triplicates. Primers used for gene expression analysis are listed in Table S1.

2.5. Statistical analysis

Data were subjected to ANOVA analysis and Student's t-test for mean separation using STATGRAPHICS Centurion software. When required, percentages were arcsin-transformed to homogenize the variance.

3. Results

3.1. Fruit growth and abscission

The ovary-to-fruit transition occurred between 1 and 2 weeks after anthesis, and the cell division stage of fruit growth lasted for 4-5 weeks (Figs. 1A-D). In our experiment, the preanthesis flower abscission did not occur, but the post-anthesis flower abscission reached almost 20% of the flowers initially formed, and the physiological fruitlet drop reached up to 80% in the control branches (Figs 1E, F), coinciding with the onset of the rapid fruit growth stage (Fig. 1A-D).

Surprisingly, leaf removal 40 days before anthesis did not significantly reduce ovary growth or abscission up to the onset of the cell division stage 28 days after anthesis (DAA) (Figs. 1A-D). Thus, the effect of defoliation was postponed for more than 2 months. At that time, the abscission rate was significantly increased during the physiological fruitlet drop (Fig. 1F) and 15 days later (i.e. 58 days after anthesis) all the fruitlets from defoliated branches (DEF) abscised (Fig. 1E).

3.2. Carbohydrate metabolism

In the flowers of control branches, the relative expression of *SPS* (sucrose biosynthesis) was upregulated 7.3-fold from -40 to -20 DAA (Fig. 2A), this correlating with a significant increase in sucrose concentration (Fig. 2E). Then, *SPS* expression progressively downregulated becoming nil at fruit set (i.e. 17 DAA) (Fig. 2A). Sucrose hydrolysis in the cytosol, mediated by *SUS1* and *SUS3*, significantly increased (3-fold from -40 DAA to anthesis), and remained almost constant during the following 6 weeks (Fig 2B, C). On the other hand, the relative expression of the cw-INV, which hydrolyzes sucrose in the apoplast, increased 2-fold at the preanthesis, and remained almost constant until fruit set (+17 DAA), and then downregulated 40 DAA (Fig 2D). The increase in the expression of the enzymes related with sucrose hydrolysis correlated with a decrease in sucrose concentration from -20 DAA onwards (Fig. 2E). Glucose concentration significantly increased along with flower/ovary development (Fig. 2F) whereas fructose concentration decreased (Fig. 2G). Hexoses (glucose + fructose) concentration stayed almost constant until fruit set, when it increased due to the glucose contribution (Fig 2. H).

Defoliation significantly reduced ovary sucrose concentration at anthesis (Fig 2E), although *SPS* relative expression (sucrose biosynthesis) was constantly higher compared to the control flowers (Fig 2A). Given that sucrose hydrolysis was not significantly enhanced in flowers from defoliated branches until +17 DAA, the reduction in ovary sucrose concentration at anthesis might be due to the lack of transport from the leaves, i.e. to leaf removal. Consequently, the concentration of glucose and fructose also dropped (Fig. 2F-H).

Regarding starch metabolism in the control flowers, the relative expression of *ADPGP* (starch biosynthesis) increased 1.3-fold from -40 to 0 DAA, and it was significantly downregulated after fruit set (Fig 3A). On the other hand, the relative expression of α -*AMY* (starch hydrolysis) upregulated 7.5-fold at -20 DAA compared to -40 DAA, and also at fruit set (+17 DAA) (3.3-fold), after a temporary downregulation at anthesis (Fig. 3B). As a result, starch ovary concentration fell significantly from -20 DAA to fruit set (Fig. 3C). Surprisingly, defoliation 40 days before anthesis did not reduce significantly compared to the control starch concentration until fruit set, i.e. 57 days later, just before the increase in fruitlet growth rate (Fig. 1C, D), and due to a significant upregulation (almost 30-fold) in α -*AMY* expression (Fig. 3B). The lack of an effect of

defoliation on starch concentration, during the period from preanthesis to anthesis, might be explained by a simultaneous reduction in both starch synthesis (*ADPGP* expression) and hydrolysis (α -*AMY* expression) (Fig. 3B).

3.3. Activation of energy depletion signaling and carbon fixation

The process of flower development in control branches activated both the energy depletion signaling system, i.e. β *SnRK1* expression, and also the carbon fixation by means of Rubisco. In particular, we selected to analyze the small subunit of Rubisco (*RbcS*) because it is *de novo* synthesized in the nucleus and transported to the chloroplast to fix carbon [30]. Thus, in ovaries from control branches, β *SnRK1* expression was upregulated 2.4-fold -20 DAA and 3-fold at anthesis compared to -40 DAA, whereas *RbcS* upregulated 3-fold during anthesis and fruit set (+17 DAA). At that time, β *SnRK1* was downregulated. In the fruits from DEF branches, both mechanisms were enhanced and activated earlier (Fig 4). Moreover, fruits from DEF branches were able to mobilize CH from the bark tissue of young and mature branches, as seen by the analysis of glucose, fructose, sucrose and starch concentrations 20 DAA, which was significantly reduced compared to the control branches (Fig 5).

4. Discussion

At anthesis, *Citrus* accumulates starch in active chloroplasts and amyloplasts of the ovary walls. By contrast, during the ovary-to-fruit transition starch content progressively diminishes [10,11] to satisfy the energy demanded by ovary cell division [31]. In fact, below a glucose threshold level, the SnRK1 energy sensor is upregulated and the development program is inhibited [23]. Surprisingly, defoliation of old and young leaves 40 days before anthesis did not reduce the starch content significantly in the ovary until fruit set +17 DAA (Fig. 3), i.e. two months later, whereas that of sucrose was significantly reduced during anthesis and fruit set (Fig. 2), in accordance with previous studies [10].

Our results suggest that the ovary regulates both starch biosynthesis and hydrolysis, depending on its sink demand and carbon availability, and this occurs through both the heterotrophic (sink organ) and autotrophic (source organ) routes of starch metabolism.

In the heterotrophic route, CH needed for starch synthesis is provided by the hydrolysis of sucrose translocated from the leaves, a reaction mediated by sucrose synthase (SUS) which converts sucrose into UDP-glucose and ADP-glucose [16,17]. In our experiments, *SUS1* and *SUS3* expression significantly increased together with *ADPGD* expression (starch biosynthesis) during preanthesis (Fig. 2). Moreover, defoliation significantly reduced sucrose content in the ovary from -20 DAA (Fig. 2) with concomitant levels of *ADPGP* expression (Fig. 3). In the autotrophic route, starch and sucrose synthesis in the leaves is coordinated through a feed-back-feed-forward mechanism which depends on tree sink demand [32]. In this process, ADPGP and SPS are the key enzymes regulating starch and sucrose synthesis, respectively [16,17,19]. According to our experiments, this self-regulatory mechanism can be also found in the flower, because in the flowers from control branches, *ADPGP* expression was upregulated and *SPS* was downregulated at anthesis compared to -20 DAA, whereas in the flowers from DEF branches they were downregulated and upregulated, respectively (Figs. 2, 3). The reduced sucrose concentration in the ovary due to defoliation, might explain this contrasting behavior. But the most surprising finding was the activation of 1) CH starvation signaling through upregulation of β *SnRKL* and 2) C fixation through upregulation of *RbcS* in flowers from both control and DEF branches (Fig. 4). During the ovary-to-fruit transition, ovary growth rate is significantly increased due to mesocarp cell division [29] and therefore sink demand. However, the sink-to-source transition of young leaves occurs around 4 weeks after full bloom, and, since they cannot sustain the onset of ovary growth, abscission starts [8]. In control branches, we found a maximum increase in β *SnRKL* expression at anthesis, suggesting a CH deficit signaling [23]. Then, the ovary responded: 1) upregulating *RbcS* expression (C fixation), and 2) maintaining *ADPGP* (starch synthesis) and increasing α *AMY* (starch hydrolysis) expression from anthesis to fruit set, which 3) resulted in a significant increase in glucose concentration at fruit set, and 4) afterwards, β *SnRKL* downregulated in the ovary. These results are supported by the behavior of the ovaries on DEF branches, in which β *SnRKL* upregulation and *RbcS* were significantly enhanced with respect to control branches. In early stages of development, the citrus exocarp presents stomata and photosynthetic capacity [33, 34], and transports C fixed in the form of sucrose to the mesocarp during cell division [33], an ability that decreases as the ovary grows [34]. Ovaries from DEF branches also increased α *AMY* expression (starch

hydrolysis) and *cwINV* expression (sucrose hydrolysis from the apoplast), suggesting a coordinated activation of several mechanisms to obtain C due to the limitation of CH supply imposed by defoliation [10].

Ovary growth and flower abscission rates did not differ significantly between fruitlets from control and DEF branches during the +20 DAA, differences starting during the physiological fruit drop (Fig. 1). The concentration of glucose in the developing ovaries of control branches increased significantly during fruit set, which is related to the high contribution generated by the hydrolysis of starch and sucrose. But the concentration of glucose in the developing ovaries of defoliated branches dropped significantly with respect to the control ovaries, and remained constant at 25 mg g⁻¹ DW (Fig. 2), which is similar to previous studies conducted with the same variety [11]. This suggests that there is a glucose threshold value that allows for the initial development of the ovary, regulated by CH signaling [10, 35]. The concentration of glucose in a given tissue can vary depending on [39]: 1) hydrolysis of sucrose; 2) hydrolysis of starch; 3) CO₂ fixation and glucose synthesis; 4) conversion to glucose-6-P by hexokinase to meet the respiratory demand. At the onset of the rapid growth stage fruits do not fix C [34], but hydrolyze sucrose in an extremely active manner. Ovaries from DEF branches that overcame fruit set managed to mobilize the reserves of the wood and hydrolyzed the sucrose, as shown by the reduced content of CH in the bark and the increased *cw-INV* expression. Similar results were reported by [10]. However, in fruits from control branches, it is worth noting the reduction in *cw-INV* gene expression and the upregulation of *SUS1* and *SUS3* genes during fruit set. A possible explanation would be the different products of the reaction: *cw-INV* generates glucose and fructose for respiration, while *SUS* generates fructose and also ADP-glucose, a precursor of cellulose which is needed to form new cell walls because the cell division [17, 19]. Defoliation also reduced the expression of *SUS1* in the ovary significantly, suggesting a main role of *SUS1* compared to *SUS3*. The restriction of the availability and transport of sucrose to the ovary probably reduced the discharge through the symplast, which would diminish the content of sucrose in the cytosol and, hence, the expression of *SUS1* [19].

5. Conclusion

In conclusion, the ability of flowers/fruitlets to mobilize CH reserves will determine their success during the physiological fruitlet drop and, therefore, crop load.

Citrus flowers are able to accumulate starch in the ovary during ontogeny through a double mechanism: 1) their photosynthetic capacity (autotrophic route) and 2) the mobilization of reserves (heterotrophic route). The ovary upregulates the photosynthetic route when the CH disponibility is diminished, which is pointed out through *βSnRK1* upregulation. In accordance with our results, the role of starch in the *Citrus* ovary is to establish a glucose threshold level that allows the ovary-to-fruit transition developmental program (fruit set), regardless of the presence of leaves, until young leaves are photo-synthetically active and become source organs.

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7. Caption to Figures

Figure 1. Early fruit growth and abscission in control (CNT) and defoliated (DEF) branches of *Citrus clementina* cv. Marisol. Each value is the average of 25 fruits randomly sampled from trees (A-D), and the average abscission of 8 branches (E-F). Figures 1B and 1D are the same data as 1A and 1C, respectively, with a reduced axis scale. Standard errors are given as vertical bars. * indicates significant differences.

Figure 2. The time-course of the gene expressions of *SPS*, involved in sucrose synthesis (A) and *SUS1*, *SUS3* and *cwINV* involved in sucrose hydrolysis (B-D), and sucrose (E), glucose (F), fructose (G) and glucose + fructose (hexoses, H) concentrations in the ovary of flowers from control (CNT) and defoliated (DEF) branches of *Citrus*

clementina cv. Marisol. Each value is the average of three biological replicates. Standard errors are given as vertical bars. *indicates significant differences.

Figure 3. The time-course of the gene expressions of *ADPGP* involved in starch synthesis (A) and *αAMY* involved in starch hydrolysis (B), and starch concentration (C) in the ovary of flowers from control (CNT) and defoliated (DEF) branches of *Citrus clementina* cv. Marisol. Each value is the average of three biological replicates. Standard errors are given as vertical bars. *indicates significant differences.

Figure 4. The time-course of the gene expression of *βSnRK1* involved in the CH starvation signaling (A) and the small subunit of Rubisco (*RbcS*) (B) in the ovary of flowers from control (CNT) and defoliated (DEF) branches of *Citrus clementina* cv. Marisol. Each value is the average of three biological replicates. Standard errors are given as vertical bars. *indicates significant differences.

Figure 5. Effect of leaf removal on CH starvation in the bark tissue of branches and current shoots of control (CNT) and defoliated branches (DEF) of *Citrus clementina* cv. Marisol. Each value is the average of three biological replicates. Standard errors are given as vertical bars. *indicates significant differences.

