Regulation by crop load of starch metabolism genes in leaves and roots of Citrus.

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CROP LOAD REGULATES CARBOHYDRATE METABOLISM IN CITRUS

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ABSTRACT

The fruit is the main sink organ in *Citrus* and captures almost all available photoassimilates during its development. Consequently, carbohydrate partitioning and starch content depend on the crop load of *Citrus* trees. Nevertheless, little is known about the mechanisms controlling the starch metabolism at the tree level in relation to presence of fruit. The aim of this study was to find the relation between the seasonal variation of expression and activity of the genes involved in carbon metabolism and the partition and allocation of carbohydrates in ‘Salustiana’ sweet orange trees with different crop loads. Metabolizable carbohydrates, and the expression and activity of the enzymes involved in sucrose and starch metabolism, including sucrose transport, were determined during the year in the roots and leaves of 40-year-old trees bearing heavy crop loads (‘on’ trees) and trees with almost no fruits (‘off’ trees).

Fruit altered photoassimilate partitioning in trees. Sucrose content tended to be constant in roots and leaves, and surplus fixed carbon is channeled to starch production. Differences between ‘on’ and ‘off’ trees in starch content can be explained by differences in ADP-glucose pyrophosphorylase (AGPP) expression/activity and α-amylase activity which varies depending on crop load. The observed relation of AGPP and UGPP is noteworthy and suggests a direct link between sucrose and starch synthesis. Furthermore, different roles for SUT2 in leaves and roots have been proposed. Variation in soluble sugars content cannot explain the differences in gene expression between the ‘on’ and ‘off’ trees. A still unknown signal from fruit should be responsible for this control.
1. Introduction

The amounts of carbon partitioned to different sink organs may be limited by both source and sink ability to provide and utilize assimilates, respectively (Wareing and Patrick, 1976). Limitations at the sink depend on organ genetic features and the developmental stage, whereas source limitations may be affected by both whole plant status and environmental conditions.

The major component of carbohydrate partitioning is the translocation of sugars from photosynthetic sources to non-photosynthetic sink tissues (Slewinski and Braun, 2010). In Citrus, and in most plants, sucrose is the main transported sugar (Zimmermann and Ziegler, 1975). Diverse transport proteins and enzymes are involved in this process. Phloem-localized sucrose transporters are essential for phloem loading, for maintenance of phloem flux and for sucrose release in apoplastic unloaders (Sauer 2007). Other enzymes, such as invertases or sucrose-phosphate synthase, allow the fine regulation of sugar accumulation and distribution in the plant (Roitsch, 1999; Li et al., 2012). Another component of carbohydrate partitioning is the mobilization of carbohydrate reserves. Starch is the main reserve carbohydrate in plants and acts as a major integrator in plant growth regulation. Marked regulatory properties have been found for ADP-glucose pyrophosphorylase (AGPP), which are involved in starch biosynthesis and are subjected to multilevel regulation (Geigenberger, 2011). Starch degradation occurs via a network of reactions that includes amylases and debranching enzymes (Stitt and Zeeman, 2012). The distribution of carbon units between starch and sucrose biosynthetic pathways is tightly regulated to respond to carbon demands throughout the day and night, and starch synthesis is a key process in the regulation of photoassimilate partitioning and carbon allocation within the plant (Preiss, 1982; Zeeman et al., 2007).
In perennial plants, the carbohydrate reserves which accumulate during winter are crucial for development as they supply the required energy and carbon skeletons to sustain emergence and growth of new plant organs at the beginning of the growing season (Naschitz et al., 2010). Under subtropical conditions, most Citrus trees accumulate reserves during the winter rest and mobilize them during spring when the main flush of bud sprouting occurs and vegetative sprouts and flowers are formed (Goldschmidt and Koch, 1996). These reserves are stored mainly in roots, although high concentrations can also be found in leaves and bark (Goldschmidt and Golomb, 1982).

After fruit set, most fixed carbon accumulates in the fruit. Both the accumulation and mobilization of reserves and production of photoassimilates have been related to fruit load in Citrus (Monerri et al., 2011).

Some citrus cultivars present an intense alternate bearing habit. Trees form a huge number of flowers, resulting in a heavy crop load (‘on’ year), followed by a year with very few flowers formed, or none at all (‘off’ year). Hormonal factors and changes in carbohydrate and mineral status appear to participate in the regulation of these processes (Monselise and Goldschmidt, 1982). In alternate bearing sweet orange ‘Salustiana’, the accumulation of reserves is inversely related to crop load (Monerri et al., 2011), and changes in carbohydrate reserves during the year reflect variations in supply and demand. Fruiting trees accumulate most fixed carbon in fruits, while no accumulation is observed in roots before harvest. In the non-fruiting trees, however, most fixed carbon is transported to roots and utilized in growth processes, and after December, stored as reserves. Reserve carbohydrate accumulation in leaves starts by early December, and the levels in leaves are, until bud sprouting, the same in both the ‘on’ and ‘off’ trees. The heavy flower formation which follows an ‘off’ year causes the rapid mobilization of the stored reserves, which are exhausted at full bloom.
Regulation of photosynthesis by fruit has been studied in *Citrus* (Iglesias et al., 2002; Syvertsen et al., 2003; Nebauer et al., 2011). It is assumed that photoassimilate production in leaves is modulated by the demand of sinks (Goldschmidt and Koch, 1996), but this effect is not always observable (Nebauer et al., 2011). It has been described that the root system is a strong and unsaturable sink under cropping conditions, and no enhanced photosynthetic rate by high sink strength related to fruiting was found by Nebauer et al. (2013). The photosynthetic rate was similar in trees with high and low crop loads in ‘Salustiana’ sweet orange (Monerri et al., 2011; Nebauer et al., 2013) when differences in carbohydrate content were highest.

As foregoing information clearly reveals, photoassimilate production and partitioning are highly integrated processes, and understanding how they are controlled will underpin many targets for plant biotechnologists (Halford, 2010).

There are no studies that analyze the effect of fruit on the seasonal expression of carbohydrate metabolism-related genes. It has been shown that the seasonal expression of flowering genes is regulated by fruit (Muñoz-Fambuena et al., 2011; Shalom et al., 2012), although they do not provide enough information to understand the mechanism by which fruit controls the flowering process.

Soluble sugars, like hormones, can act as primary messengers and regulate signals that control the expression of different genes involved in plant growth and metabolism (Rolland et al., 2006; Rosa et al., 2009).

The aim of this study was to analyze the influence of fruit load on the seasonal expression and activity of the genes involved in carbon metabolism, and the possible role of soluble sugars as signals controlling the starch metabolism gene expression in citrus trees. The studied genes were selected from previous works which reported on the relation between its expression and changes in carbohydrate levels provoked by girdling.
After taking into account that field studies may reveal essential roles of genes which cannot otherwise be observed, this work has been carried out in non-manipulated mature trees under cropping conditions during periods when the tree physiology showed distinctive characteristics. Furthermore, in order to assess the effect of fruit on the regulation of the activity of the studied genes, this work was performed in a citrus cultivar that presents an intense alternate bearing habit.

2. Materials and methods

2.1. Plant material

Experiments were performed on 40-year-old trees of the ‘Salustiana’ cultivar of sweet orange (Citrus sinensis [L.] Osbeck) grafted onto a Troyer citrange (C. sinensis [L.] Osb. × Poncirus trifoliata Raf.) rootstock. Trees were drip-irrigated, and mineral elements were supplied in the irrigation water from February to September. Trees present an alternate-year bearing habit, and flowering intensity depends on the fruit load of the previous year. Trees alternated between years of abundant flowering and fruit set (‘on’ year) and years of almost no flowering (‘off’ year). During each year, the ‘on’ and ‘off’ trees were found in the same orchard. Mature fruits were harvested by early February. The ‘on’ trees averaged 3,119 fruits per tree in the study orchard during the previous season, whereas only 43 fruits per tree formed in the ‘off’ trees (Y. Bordón, personal communication). At the beginning of the study (March), the ‘on’ trees, which entered an ‘off’ year, formed only 1.6 flowers per 100 nodes, unlike the 54.1 flowers formed in the ‘off’ trees that entered an ‘on’ year.

Sampling dates for determinations of carbohydrates, enzymatic activity and gene expression were performed based on previous studies (Monerri et al., 2011): June, after
fruit abscission, when the maximum rate of accumulation by the fruit occurred;
September and December, in the middle and final period of fruit development,
respectively; January and February, just before and after fruit harvest, respectively; and
March, after the beginning of Spring bud sprouting. Plant material was sampled
between 10:00 h and 11:00 h on all six dates. The mature leaves (4th leaf from the apex)
from vegetative shoots formed last Spring and the fibrous roots (1.5-2.5 mm in
diameter) bearing new formed feeder roots were used in the study.

2.2. Carbohydrate analysis
The determination of total soluble sugars and starch (as mg per g of dry weight) was
performed as described by García-Luis et al. (2002). Three independent extracts, each
obtained from nine different trees (five leaves per tree and three trees per extract), were
assayed for each treatment in all the determinations. Sucrose was determined by HPLC,
as described by Iglesias et al. (2002).

2.3. Gene expression analysis
The expression of sucrose transporters SUT1 and SUT2 (Li et al., 2003c), sucrose
synthases SUS1 and SUSA, sucrose-phosphate synthase (SPS, EC 2.4.1.14), α-amylase
(AMY, EC 3.2.1.1) and ADP-glucose pyrophosphorylase (AGPP) genes (Li et al.
2003a), involved in carbohydrate metabolism, were studied (Table 1). Leaf tissue was
finely ground in liquid nitrogen and total RNA was extracted using the TRIzol reagent
(Invitrogen), purified using the RNEasy Mini Kit (Quiagen) and treated with RNase-
free DNase (Quiagen), according to the manufacturer’s instructions. RNA was
quantified with a UV/VIS spectrophotometer, and first-strand cDNA was synthesized
from 1.2 μg of total RNA with the First Strand cDNA Synthesis Kit AMV (Roche) for real-time PCR (RT-PCR).

The oligonucleotide primers used have been described in a previous work (Nebauer et al. 2011). During the year, *Citrus sinensis* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Nebauer et al., 2011) exhibited a stable expression among the studied organs and was used as the reference gene. The optimum concentration and amplification efficiency were tested for all pairs of oligonucleotides (Livak and Schmittgen, 2001).

Diluted cDNA (2 μg) was used as a template for the semi-quantitative RT-PCR amplification in the 20-μL reactions containing 0.3 μM of each primer (0.15 μM GAPDH) and 10 μL of the SYBR Green PCR master mixture (Power SYBR® Green PCR Master Mix; Applied Biosystems). The PCR mixtures were preheated at 50°C for 2 min and then at 95°C for 10 min, followed by 40 amplification cycles (95°C for 15 s; 60°C for 1 min). Amplification specificity was verified by a final dissociation (95°C for 15 s, 60°C for 20 s and 95°C for 15 s) of the PCR products. The levels of the PCR products were monitored with an ABI PRISM 7000 sequence detection system and were analyzed with the ABI PRISM 7000 SDS software (Applied Biosystems). At least three independent biological replicates per sample and three technical replicates of each biological replicate were used for the RT-PCR analysis. The relative expression levels of the target genes were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). For each gene and organ, the expression was related to the minimal value of the measured dates.

2.4. Enzyme assays

One gram of frozen powder was resuspended at 4°C in 5 mL of 100 mM HEPES (pH 7.5), 2 mM EDTA and 5 mM dithiothreitol. The suspension was desalted (IVSS
Vivaspin 500, Sartorius Biolab, Germany) following the manufacturer’s instructions and assayed for enzymatic activity. The ADPG pyrophosphorylase (AGPP, EC 2.4.1.18), starch phosphorylase (SP, EC 3.6.1.1), UDPG pyrophosphorylase (UGPP, EC 2.7.7.9), sucrose synthase (SuSy, EC 2.4.1.13) and acid invertase (INV, EC 3.2.1.26) activities were assayed (Table 1) as described by Baroja-Fernández et al. (2004) and Muñoz et al. (2005). For the detection of the AGPP and UGPP activities, the production of glucose-1-phosphate from ADP-glucose and UDP-glucose was determined, respectively, in an NAD-linked glucose-6-phosphate dehydrogenase system (Müller-Roeber et al., 1992). NAD reduction was measured spectrophotometrically at 340 nm. Starch phosphorylase activity was assayed by measuring the glucose-1-phosphate released from glycogen in a similar assay. The sucrose synthase and invertase activities were measured in the sucrose breakdown direction. Fructose content was determined spectrophotometrically at 340 nm by the NAD-linked hexokinase/phosphoglucoisomerase/glucose-6-phosphate dehydrogenase coupling method. All the enzymatic reactions were performed at 37°C. One unit (U) is defined as the amount of enzyme that catalyzes the production of 1 µmol of product per min.

2.5. Statistical analysis

Treatment comparison analyses were performed by ANOVA (Statgraphics Plus 5.1 for Windows, Statistical Graphics Corp.). Mean separations were made with the Tukey multiple range test. A linear regression analysis was used to evaluate the relationships between parameters.

3. Results

3.1. Carbohydrate content in leaves and roots
The carbohydrate content in leaves from the vegetative sprouts formed during spring in study year 1 and in roots were examined during the fruit development period, from June to January, which ended in March just after the beginning of the spring flush of study year 2 (Fig. 1).

Starch content was significantly higher in the leaves of vegetative sprouts in the ‘off’ tree than in the ‘on’ trees (Fig. 1A). Differences were maximal in June. Afterwards, this content decreased gradually to a common minimum level in both the ‘on’ and ‘off’ trees during December. From this time point, starch accumulated until the beginning of bud sprouting in the two tree types to lower again in the ‘on’ trees by June.

Almost no differences in leaves between the ‘on’ and ‘off’ trees were observed in either sucrose content or total soluble sugars (Fig. 1C and 1E), which remained nearly constant during the study period. However, a significant increase in total soluble sugars and sucrose occurred in January.

No differences in the starch content of the roots between the ‘on’ and ‘off’ trees were observed until November (Fig. 1B). Afterwards, starch accumulated in the roots until the beginning of bud sprouting. The accumulation rate was higher in the ‘off’ trees. The soluble sugar and sucrose content in roots showed a similar behaviour as in leaves (Fig. 1C and 1D).

3.2. Effect of crop load on gene expression

The expression pattern of the genes involved in starch metabolism, sucrose transport and sucrose metabolism in the ‘on’ and ‘off’ trees is shown in Figures 2, 3 and 4, respectively.

In leaves, the expression of AGPP decreased from June to November in the ‘off’ trees, but rose from December to February (Fig. 2A). After spring flush had started, the
expression levels fell again. In the ‘on’ trees, AGPP showed a similar behaviour, although the starting level in June was significantly lower, while the winter raise was observed in November, earlier than in the ‘off’ trees (Fig. 2A). In addition, almost no changes were observed in the expression of the AMY gene from June to January in the ‘off’ leaves (Fig. 2C). From that time onwards, a sharp increase occurred until March. A similar trend was observed in the ‘on’ trees despite the higher expression value in June. Very few or no differences were observed between the ‘on’ and ‘off’ trees in the expressions of AGPP and AMY in roots (Fig. 2B and D). The expression of AGPP remained low and nearly constant until December, and a slight increase was observed afterwards. The AMY expression progressively decreased from June to December, followed by a slight increase from February. This increase was more pronounced in the ‘off’ trees (these being the ‘on’ trees in the previous year) than in the ‘on’ ones (Fig. 2D).

Sucrose transporters SUT1 and SUT2 showed different expression profiles during the year (Fig. 3). The expression of SUT1 fell from June to September in leaves (Fig. 3A). From then onwards, it remained virtually unchanged in the ‘off’ trees, although a slight increase was observed from March. In the ‘on’ trees, a transient increase was observed in January. The SUT2 expression in leaves was significantly higher in the ‘off’ trees in June. Both these levels in the ‘on’ and ‘off’ trees decreased to a minimum in September, and no changes were observed until January, when an increase took place (Fig. 3C). In roots, the SUT1 expression differed between both tree types. Practically no changes were seen in the expression of this gene from June to December in the ‘off’ trees, which fell from this time onwards. However, its expression was lower in the ‘on’ trees during September. The SUT2 levels did not change until December, and a slight increase was observed in both the ‘on’ and ‘off’ trees from January onwards (Fig. 3D).
The expression of the SUS1 gene in leaves oscillated during fruit development with differences found between the tree types (Fig. 4A). These changes were more pronounced in the ‘off’ trees, with a higher expression in early summer and January. In contrast, almost no changes were noted in SUSA (Fig. 4C). Despite being higher in the ‘on’ leaves until September, the SPS expression in both the ‘on’ and ‘off’ trees decreased until January to rise afterwards at the same level in both trees (Fig. 4E). The SUS1 expression in roots did not change in the ‘on’ and ‘off’ trees during the study period (Fig. 4B). Practically no changes were observed in the SUSA expression until February, when it increased in both the ‘on’ and ‘off’ trees (Fig. 4D). The SPS expression in roots fell in November, recovered in January, and decreased after the fruit harvest in February (Fig. 4F). Except for June, no differences were observed between the ‘on’ and ‘off’ trees.

3.3. Effect of crop load on enzyme activity

The activity of enzymes related to starch and sucrose metabolism are presented in Figures 5 and 6. AGPP activity was higher in ‘off’ tree leaves than in the ‘on’ trees until September (Fig. 5A), after which time it decreased until February, but recovered in March. SP activity was also higher in the leaves of ‘off’ trees in June, but similar from September to March in both tree types (Fig. 5C). In roots, AGPP activity was very low during the study period, although a slight increase occurred from November (Fig. 5B). SP activity increased slowly and progressively in the ‘on’ trees (Fig. 5D). This increase was delayed until September in the ‘off’ trees, although higher levels were reached from November as compared to the ‘on’ trees. A similar trend of UGPP activity was observed in the leaves of both the ‘on’ and ‘off’ trees (Fig. 6A), which was initially higher in June in the ‘off’ trees, and equalled as
from September, decreased until February and increased afterwards. Susy activity in
leaves was very low during the study period (Fig. 6C). Nevertheless, a transient strong
increase was observed in February in the ‘off’ trees. INV activity increased from
September to January, and then progressively decreased in both the ‘on’ and ‘off’ trees
(Fig. 6E).

In roots, UGPP activity increased at the beginning of the study period (Fig. 6B), and
decreased from January in the ‘on’ trees and from February in the ‘off’ trees. Susy
activity progressively increased with time to peak in February (Fig. 6D). In the ‘off’
trees, a transient decrease was observed in September. INV activity remained nearly
constant and at low rates (Fig. 6F), despite a transient maximum in recorded September
in the ‘off’ trees.

3.4. Relations among carbohydrate content, enzyme activities and gene expression

The relations between carbohydrate contents in leaves and roots and the expression and
activity of related enzymes and transporters were studied. The main significant relations
are schematically illustrated in Figure 7. The higher starch levels in leaves during
summer and in roots during winter observed in the ‘off’ trees (Fig. 1A and 1B)
correlated with a higher AGPP expression ($r^2 = 0.80; \ P = 0.01$) and greater activity ($r^2 =
0.62, \ P = 0.03$). Furthermore, AGPP and UGPP activities were highly related in leaves
(Fig. 7A). The high correlation between starch content and the expression of both
sucrose transporters SUT1 ($r^2 = 0.84; \ P = 0.04$) and SUT2 ($r^2 = 0.89; \ P = 0.02$) is
noteworthy. Leaf INV activity related negatively to starch ($r^2 = -0.81; \ P = 0.04$), but
positively to soluble sugar ($r^2 = 0.67; \ P = 0.02$) content. Soluble sugar content related
negatively to the SPS ($r^2 = -0.77; \ P = 0.01$) and SUSA ($r^2 = -0.60; \ P = 0.04$)
expression levels (Fig. 7A), due mainly to sugars other than sucrose (Fig. 1C and 1E).
In roots, similar relations were observed between starch and the AGPP expression and activity (Fig. 7B). Changes in the AGPP expression also related to changes in the SUT2 (r² = 0.97; P = 0.00) and SUSa (r² = 0.70; P = 0.02) expression levels. Sucrose synthase and invertase activities related to AGPP activity (r² = 0.58; P = 0.05, and r² = -0.59; P = 0.05, respectively) (Fig. 7). Soluble sugars related positively to the SUT2 expression (r² = 0.64; P = 0.03) and negatively to AMY expression (r² = -0.57; P = 0.05).

4. Discussion

Crop load is known to affect carbohydrate production and partitioning in several trees, such as apple (Naschitz at el., 2010), olive (Bustan et al., 2011) and citrus (Goldschmidt and Golomb, 1982; Monerri et al., 2011). During its development, citrus fruit is the main sink organ (Monerri et al., 2011), and it captures almost all available photoassimilates. Accordingly, differences in carbohydrate content and related enzyme activities throughout seasons between the ‘on’ and ‘off’ trees are reported in our study. This different behaviour was observed mainly from May to September in leaves, and from December to March in roots, when higher starch levels were found in non-fruiting trees. This finding suggests a role of fruit in the regulation of the genes relating to the metabolism of this reserve carbohydrate.

The higher starch level noted in leaves from June to September in the ‘off’ trees can be explained by a higher gene expression, greater AGPP activity, and a lower expression of the α-amylase and sucrose phosphate synthase genes. Furthermore, the increased leaf starch content correlates with not only AGPP activity, but also with the expression of sucrose transporters. These results, as previously reported (Li et al., 2003c), suggest different physiological roles for these transporters.
SUT1 has been described to drive sucrose loading in sources. Accordingly, the expression of this transporter is enhanced under the high photoassimilate availability and demand conditions of June. The use of dry matter by fruit in the ‘on’ trees and by vegetative growth, mainly the root system, in the ‘off’ trees in June (Goldschmidt and Golomb, 1982; Monerri et al., 2011) could explain this result. However, the less demand in the ‘off’ trees during winter and, to a lesser extent in the ‘on’ trees, provoked increased starch synthesis. Starch content and AGPP expression correlated highly with the SUT2 expression in leaves. These results support the hypothesis that the SUT2 protein may act as a sugar sensor (Barker et al., 2000).

In ‘Salustiana’ sweet orange, no differences were observed in the photosynthetic rate between the fruiting and non-fruiting ‘Salustiana’ trees throughout the year (Monerri et al., 2011; Nebauer et al., 2013). Therefore, similar photoassimilate production at the tree level has to be assigned to the ‘on’ and ‘off’ trees as similar total leaf area and photosynthetic capacity have been estimated in both tree types (Monerri et al., 2011). Although photoassimilate synthesis is similar between trees, but with differing demand, our data reveal hat sucrose content tends to be maintained more or less constant in leaves in the ‘off’ trees by channeling the surplus fixed carbon to starch production, and to fruit in the ‘on’ trees. In line with this, a high correlation is seen between AGPP and UGPP activities in leaves, suggesting the connection via hexoses as proposed by Muñoz et al. (2006).

No differences were observed in the soluble sugar content between the ‘on and ‘off’ trees, although an increase took place in January. The highest content of soluble sugars in leaves correlates with the lowest starch accumulation, which is due mainly to an increase in hexoses (data not shown). The higher sink strength of leaves during this period coincides with higher invertase and diminished Susy activity. It has been stated
that their relative activities determine how much carbon enters the storage pathways for starch biosynthesis, and how much enters the glycolytic pathway (Halford, 2010). Some studies have demonstrated that Susy activity is closely related to starch accumulation and invertase is associated with glucose and fructose production, principally for flux into glycolysis (Trethewey et al., 1998). However, the increase in soluble sugars towards mid-winter in Citrus was observed long before (Jones and Steinacker, 1951; Toritaka et al., 1974) and has been related to the role of soluble sugars as an osmotic, cryoprotective strategy against cold injury.

The rise in soluble sugars, other than sucrose mainly, is also observed in roots. Unlike leaves however, this higher content correlates with increased starch content. The accumulation of reserves in roots occurs from December onwards in both the ‘on’ and ‘off’ trees, which coincides with the lower sink strength of the ‘on’ trees fruit. Nevertheless, starch content is higher in the roots of the ‘off’ trees and correlates with root AGPP activity. The soluble sugar level correlates with both sucrose synthase activity and the SUT2 expression (Fig. 1B, 3D and 6D). The role of SUT2 as a transporter in sink organs has been previously described in Citrus (Li et al., 2003b,c).

A significant correlation between the expression levels of a member of a gene family and total activity has been proposed to be related to the transcriptional regulation of the enzyme activity (Li et al., 2012). However, the fact that these correlations are lacking suggests that the post-translational regulation of the protein might regulate its activity or that another family member may play a predominant role in total activity.

The AGPP expression in leaves, which explains the differences in starch accumulation between the ‘on’ and ‘off’ trees, is well-related to AGPP activity, thus indicating its mainly transcriptional regulation. Besides, the differences in root starch content correlate with the activities of those enzymes involved in starch synthesis. Nonetheless,
the AGPP expression shows no differences between the ‘on’ and ‘off’ trees, suggesting additional levels of regulation.

It has been hypothesized that soluble sugars modulate the expression of those genes involved in starch synthesis (Koch, 1996). However, we observed no differences in soluble sugars between the ‘on’ and ‘off’ trees, and sucrose content remained nearly constant throughout the study period. Apparently the absolute levels of sugars do not drive the regulation of the differential gene expression between the ‘on’ and ‘off’ trees. However, this control may also be exerted by different phytohormones produced by fruit, whose participation in the regulation of many carbon metabolism-related activities is well-known (Albacete et al., 2008). GAs enhance sucrose formation, activates SPS activity, phloem loading and unloading, and increases sink strength through activating invertase activity (Iqbal et al., 2011). It has been reported that GAs interacts with other phytohormones, such as ABA or salicylic acid, to regulate carbon allocation and distribution (Moreno et al., 2011). Furthermore, Peng et al. (2011) described that ABA regulates SUT1 activity in apple by stimulating sugar accumulation in fruit. A previous work (Nebauer et al., 2011) found significant differences in the expression of the enzymes analyzed in this manuscript in ‘Salustiana’ sweet orange between the shoots bearing fruit and those without, thus confirming that the signals generated by fruit may regulate the carbohydrate metabolism in trees. It has been recently reported that fruit inhibits flowering by repressing the expression of flowering genes in leaves of alternate bearing Citrus (Muñoz-Fambuena et al., 2011). The specific role of phytohormones in all these regulations has to be further studied.

Nevertheless, there is a strong relation between variation in the soluble sugar content
throughout the year and the activity of these genes. The changes in soluble sugar content and the AGPP and SUT2 expressions correlate highly in roots, suggesting that the expression of these genes may be modulated by hexoses, as hypothesized by Koch (1996). However, these carbon metabolism-related activities are under complex spatial and temporal regulation (Kleczkowski et al., 2010), and nothing is known about whether there being a common mechanism responsible for differential sugar regulation (Rosa et al. 2009). In fact, distinct relations between gene expressions in accordance with tissues, stress conditions and light rhythms have been reported (Kleczkowski et al., 2009). Accordingly, a negative correlation is found between soluble sugar content and the expression of SUT2 and AGPP in leaves.

Although the expression of the carbon metabolism-related genes has been previously studied in relation to crop load and carbon status in Citrus (Komatsu et al., 2002; Li et al., 2003a,b,c), these works were neither carried out under natural field conditions nor throughout the year to cover all developmental stages of a tree. One important factor is that growing plants in greenhouses or growth chambers may not represent an optimal environment for functional studies (Kleczkowski et al., 2010). The evaluation of the roles of each gene/isozyme should include field trials conducted under natural conditions, as is the case in this work. In addition, the used techniques have allowed the study into the relation between the expression patterns of carbon metabolism genes with variation in carbohydrate content along the year.

5. Conclusion

Our data indicate the complexity of the carbohydrate metabolism network in Citrus by integrating source-sink interactions and environmental conditions, mediated by sugar signals, and probably by hormones as well. Differences in the starch content between
the ‘on’ and ‘off’ trees can be explained by the differential expression/activity of AGPP
and \( \alpha \)-AMY. Different regulation (transcriptional and posttranscriptional) levels for
leaves and roots are revealed for AGPP. Significant linear correlations are found
between the AGPP expression or activity and other starch metabolism-related genes.
The relation with UDPG is of special interest as it links sucrose and starch synthesis,
while the relation with SUT2 transporter suggests that SUT2 may act as a sugar sensor
in leaves and as a sucrose transporter to sink organs in roots. The control exerted by
fruit of the genes related to starch metabolism is not mediated through changes in the
content of soluble sugars as primary messengers, and a hormonal signal should be
responsible for this regulation. Nevertheless, a strong relation exists between variation
in soluble sugar content throughout the year and the AGPP expression. In addition,
differences between sources and sinks are observed. In roots, the soluble sugars
variation pattern runs in parallel with the AGPP and SUT2 expressions. However, a
negative correlation is found between AGPP activity and the SUT2 expression in
leaves.

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Legends for Figures

Fig. 1. Seasonal pattern of starch (A,B), soluble sugars (C,D) and sucrose (E,F) content in the leaves (A,C,E) and roots (B,D,F) in the ‘on’ (●) and ‘off’ (○) Salustiana trees. Values are mean (±SE) of three determinations in nine different trees performed from June (Jn) to March (Ma). Significant differences ($P<0.05$) between trees for each date are indicated by an asterisk.

Fig. 2. Changes in the relative expression of ADP-glucose pyrophosphorylase (A,B) and α-amylase genes (C,D) in the leaves (A,C) and roots (B,D) of the ‘on’ (●) and ‘off’ (○) Salustiana trees. Values are mean (±SE) of three determinations in nine different trees performed from June (Jn) to March (Ma). Significant differences ($P<0.05$) between trees for each date are indicated by an asterisk.

Fig. 3. Changes in the relative expression of SUT1 (A,B) and SUT2 (C,D) sucrose transporter genes in the leaves (A,C) and roots (B,D) of the ‘on’ (●) and ‘off’ (○) Salustiana trees. Values are mean (±SE) of three determinations in nine different trees performed from June (Jn) to March (Ma). Significant differences ($P<0.05$) between trees for each date are indicated by an asterisk.

Fig. 4. Changes in the relative expression of sucrose synthase 1 (A,B), sucrose synthase A (C,D) and sucrose phosphate synthase (E,F) genes in the leaves (A,C,E) and roots (B,D,F) of the ‘on’ (●) and ‘off’ (○) Salustiana trees. Values are mean (±SE) of three determinations in nine different trees performed from June (Jn) to March (Ma).
Significant differences ($P<0.05$) between trees for each date are indicated by an asterisk. nd: not determined.

**Fig. 5.** Changes in the ADPG pyrophosphorylase (A,B) and starch phosphorylase (C,D) activities in the leaves (A,C) and roots (B,D) of the ‘on’ (●) and ‘off’ (○) Salustiana trees. Values are mean (±SE) of three determinations in nine different trees performed from June (Jn) to March (Ma). Significant differences ($P<0.05$) between trees for each date are indicated by an asterisk.

**Fig. 6.** Changes in the UDPG pyrophosphorylase (A,B), sucrose synthase (C,D) and invertase (E,F) activities in the leaves (A,C,E) and roots (B,D,F) of the ‘on’ (●) and ‘off’ (○) Salustiana trees. Values are mean (±SE) of three determinations in nine different trees performed from June (Jn) to March (Ma). Significant differences ($P<0.05$) between trees for each date are indicated by an asterisk.

**Fig. 7.** Main significant relations ($P<0.05$) among carbohydrates and related enzyme expression and activities in Salustiana leaves (A) and roots (B). +: positive correlations, -: negative correlations.
Table 1. Nomenclature and reactions catalyzed by the studied enzymes.

<table>
<thead>
<tr>
<th>Metabolism</th>
<th>Enzyme</th>
<th>Reaction 1</th>
<th>Reaction 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch metabolism</td>
<td>AGPP</td>
<td>ADP-glucose pyrophosphorylase</td>
<td>glucose-1-P + ATP → ADP-glucose + PPI</td>
</tr>
<tr>
<td></td>
<td>AMY</td>
<td>α-amylase</td>
<td>[glucose]<em>n → [glucose]</em>{n-m} + [glucose]_m</td>
</tr>
<tr>
<td></td>
<td>SP</td>
<td>starch phosphorylase</td>
<td>[glucose]<em>n + Pi ↔ glucose-1-P + [glucose]</em>{n-1}</td>
</tr>
<tr>
<td>Sucrose metabolism</td>
<td>UGPP</td>
<td>UDP-glucose pyrophosphorylase</td>
<td>glucose-1-P + UTP → UDP-glucose + PPI</td>
</tr>
<tr>
<td></td>
<td>SUS/SuSy</td>
<td>Sucrose synthase</td>
<td>sucrose + ADP ↔ ADP-glucose + fructose</td>
</tr>
<tr>
<td></td>
<td>INV</td>
<td>Invertase</td>
<td>sucrose → glucose + fructose</td>
</tr>
<tr>
<td></td>
<td>SPS</td>
<td>Sucrose-phosphate synthase</td>
<td>UDP-glucose + fructose-6-P → UDP + sucrose-6-P</td>
</tr>
<tr>
<td></td>
<td>SUT</td>
<td>Sucrose transporter</td>
<td>H⁺/sucrose importer</td>
</tr>
</tbody>
</table>

Abbreviations: fructose-6-P; fructose-6-phosphate; glucose-1-P; glucose-1-phosphate; Pi: phosphate; PPI: pyrophosphate; sucrose-6-P: sucrose-6-phosphate
Figure 2

LEAVES

AGPP
Relative expression
20
15
10
5
0

Ja  |  Jl  |  Au  |  Se  |  Oc  |  No  |  De  |  Ja  |  Fe  |  Ma

a-AMY
Relative expression
20
15
10
5
0

Ja  |  Jl  |  Au  |  Se  |  Oc  |  No  |  De  |  Ja  |  Fe  |  Ma

ROOTS

A

B

C

D

Month

*
Figure 3
Figure 4
Figure 5

LEAVES

AGPP Activity (mU g FW)

SP Activity (mU g FW)

Month

ROOTS

Month

LEAVES

AGPP Activity (mU g FW)

SP Activity (mU g FW)

Month

ROOTS

Month

* * * * * *

LEAVES

AGPP Activity (mU g FW)

SP Activity (mU g FW)

Month

ROOTS

Month

* * * * * *

LEAVES

AGPP Activity (mU g FW)

SP Activity (mU g FW)

Month

ROOTS

Month

* * * * * *
Expression | Activity

A

\[
\begin{align*}
SUT1 & \quad + \\
AGPP & \quad + \\
SUT2 & \quad + \\
SPS & \quad - \\
SUSA & \quad + \\
\hline
\end{align*}
\]

B

\[
\begin{align*}
SUSA & \quad + \\
AGPP & \quad + \\
SUT2 & \quad + \\
SuSy & \quad + \\
\hline
\end{align*}
\]