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

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Running title:

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

(Li et al., 2003a,b,c; Nebauer et al., 2011). 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

148 fruit abscission, when the maximum rate of accumulation by the fruit occurred;
149 September and December, in the middle and final period of fruit development,
150 respectively; January and February, just before and after fruit harvest, respectively; and
151 March, after the beginning of Spring bud sprouting. Plant material was sampled
152 between 10:00 h and 11:00 h on all six dates. The mature leaves (4th leaf from the apex)
153 from vegetative shoots formed last Spring and the fibrous roots (1.5-2.5 mm in
154 diameter) bearing new formed feeder roots were used in the study.
155
156 *2.2. Carbohydrate analysis*
157 The determination of total soluble sugars and starch (as mg per g of dry weight) was
158 performed as described by García-Luis et al. (2002). Three independent extracts, each
159 obtained from nine different trees (five leaves per tree and three trees per extract), were
160 assayed for each treatment in all the determinations. Sucrose was determined by HPLC,
161 as described by Iglesias et al. (2002).
162
163 *2.3. Gene expression analysis*
164 The expression of sucrose transporters SUT1 and SUT2 (Li et al., 2003c), sucrose
165 synthases SUS1 and SUSA, sucrose-phosphate synthase (SPS, EC 2.4.1.14), α-amylase
166 (AMY, EC 3.2.1.1) and ADP-glucose pyrophosphorylase (AGPP) genes (Li et al.
167 2003a), involved in carbohydrate metabolism, were studied (Table 1). Leaf tissue was
168 finely ground in liquid nitrogen and total RNA was extracted using the TRIzol reagent
169 (Invitrogen), purified using the RNEasy Mini Kit (Quiagen) and treated with RNase-
170 free DNase (Quiagen), according to the manufacturer's instructions. RNA was
171 quantified with a UV/VIS spectrophotometer, and first-strand cDNA was synthesized

172 from 1.2 μg of total RNA with the First Strand cDNA Synthesis Kit AMV (Roche) for
173 real-time PCR (RT-PCR).
174 The oligonucleotide primers used have been described in a previous work (Nebauer et
175 al. 2011). During the year, *Citrus sinensis* glyceraldehyde-3-phosphate dehydrogenase
176 (GAPDH)(Nebauer et al., 2011) exhibited a stable expression among the studied organs
177 and was used as the reference gene. The optimum concentration and amplification
178 efficiency were tested for all pairs of oligonucleotides (Livak and Schmittgen, 2001).
179 Diluted cDNA (2 μg) was used as a template for the semi-quantitative RT-PCR
180 amplification in the 20-μL reactions containing 0.3 μM of each primer (0.15 μM
181 GAPDH) and 10 μL of the SYBR Green PCR master mixture (Power SYBR®Green
182 PCR Master Mix; Applied Biosystems). The PCR mixtures were preheated at 50°C for
183 2 min and then at 95°C for 10 min, followed by 40 amplification cycles (95°C for 15 s;
184 60°C for 1 min). Amplification specificity was verified by a final dissociation (95°C for
185 15 s, 60°C for 20 s and 95°C for 15 s) of the PCR products. The levels of the PCR
186 products were monitored with an ABI PRISM 7000 sequence detection system and
187 were analyzed with the ABI PRISM 7000 SDS software (Applied Biosystems). At least
188 three independent biological replicates per sample and three technical replicates of each
189 biological replicate were used for the RT-PCR analysis. The relative expression levels
190 of the target genes were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).
191 For each gene and organ, the expression was related to the minimal value of the
192 measured dates.
193
194 *2.4. Enzyme assays*
195 One gram of frozen powder was resuspended at 4°C in 5 mL of 100 mM HEPES (pH
196 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

222 The carbohydrate content in leaves from the vegetative sprouts formed during spring in
223 study year 1 and in roots were examined during the fruit development period, from June
224 to January, which ended in March just after the beginning of the spring flush of study
225 year 2 (Fig. 1).
226 Starch content was significantly higher in the leaves of vegetative sprouts in the 'off'
227 tress than in the 'on' trees (Fig. 1A). Differences were maximal in June. Afterwards,
228 this content decreased gradually to a common minimum level in both the 'on' and 'off'
229 trees during December. From this time point, starch accumulated until the beginning of
230 bud sprouting in the two tree types to lower again in the 'on' trees by June.
231 Almost no differences in leaves between the 'on' and 'off' trees were observed in either
232 sucrose content or total soluble sugars (Fig. 1C and 1E), which remained nearly
233 constant during the study period. However, a significant increase in total soluble sugars
234 and sucrose occurred in January.
235 No differences in the starch content of the roots between the 'on' and 'off' trees were
236 observed until November (Fig. 1B). Afterwards, starch accumulated in the roots until
237 the beginning of bud sprouting. The accumulation rate was higher in the 'off' trees. The
238 soluble sugar and sucrose content in roots showed a similar behaviour as in leaves (Fig.
239 1C and 1D).
240
241 *3.2. Effect of crop load on gene expression*
242 The expression pattern of the genes involved in starch metabolism, sucrose transport
243 and sucrose metabolism in the 'on' and 'off' trees is shown in Figures 2, 3 and 4,
244 respectively.
245 In leaves, the expression of AGPP decreased from June to November in the 'off' trees,
246 but rose from December to February (Fig. 2A). After spring flush had started, the

247 expression levels fell again. In the ‘on’ trees, AGPP showed a similar behaviour,
248 although the starting level in June was significantly lower, while the winter raise was
249 observed in November, earlier than in the ‘off’ trees (Fig. 2A). In addition, almost no
250 changes were observed in the expression of the AMY gene from June to January in the
251 ‘off’ leaves (Fig. 2C). From that time onwards, a sharp increase occurred until March. A
252 similar trend was observed in the ‘on’ trees despite the higher expression value in June.
253 Very few or no differences were observed between the ‘on’ and ‘off’ trees in the
254 expressions of AGPP and AMY in roots (Fig. 2B and D). The expression of AGPP
255 remained low and nearly constant until December, and a slight increase was observed
256 afterwards. The AMY expression progressively decreased from June to December,
257 followed by a slight increase from February. This increase was more pronounced in the
258 ‘off’ trees (these being the ‘on’ trees in the previous year) than in the ‘on’ ones (Fig.
259 2D).
260 Sucrose transporters SUT1 and SUT2 showed different expression profiles during the
261 year (Fig. 3). The expression of SUT1 fell from June to September in leaves (Fig. 3A).
262 From then onwards, it remained virtually unchanged in the ‘off’ trees, although a slight
263 increase was observed from March. In the ‘on’ trees, a transient increase was observed
264 in January. The SUT2 expression in leaves was significantly higher in the ‘off’ trees in
265 June. Both these levels in the ‘on’ and ‘off’ trees decreased to a minimum in September,
266 and no changes were observed until January, when an increase took place (Fig. 3C).
267 In roots, the SUT1 expression differed between both tree types. Practically no changes
268 were seen in the expression of this gene from June to December in the ‘off’ trees, which
269 fell from this time onwards. However, its expression was lower in the ‘on’ trees during
270 September. The SUT2 levels did not change until December, and a slight increase was
271 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

297 from September, decreased until February and increased afterwards. Susy activity in
298 leaves was very low during the study period (Fig. 6C). Nevertheless, a transient strong
299 increase was observed in February in the 'off' trees. INV activity increased from
300 September to January, and then progressively decreased in both the 'on' and 'off' trees
301 (Fig. 6E).
302 In roots, UGPP activity increased at the beginning of the study period (Fig. 6B), and
303 decreased from January in the 'on' trees and from February in the 'off' trees. Susy
304 activity progressively increased with time to peak in February (Fig. 6D). In the 'off'
305 trees, a transient decrease was observed in September. INV activity remained nearly
306 constant and at low rates (Fig. 6F), despite a transient maximum in recorded September
307 in the 'off' trees.
308
309 *3.4. Relations among carbohydrate content, enzyme activities and gene expression*
310 The relations between carbohydrate contents in leaves and roots and the expression and
311 activity of related enzymes and transporters were studied. The main significant relations
312 are schematically illustrated in Figure 7. The higher starch levels in leaves during
313 summer and in roots during winter observed in the 'off' trees (Fig. 1A and 1B)
314 correlated with a higher AGPP expression (r^2 = 0.80; P = 0.01) and greater activity (r^2 =
315 0.62, P = 0.03). Furthermore, AGPP and UGPP activities were highly related in leaves
316 (Fig. 7A). The high correlation between starch content and the expression of both
317 sucrose transporters SUT1 (r^2 = 0.84; P = 0.04) and SUT2 (r^2 = 0.89; P = 0.02) is
318 noteworthy. Leaf INV activity related negatively to starch (r^2 = - 0.81; P = 0.04), but
319 positively to soluble sugar (r^2 = 0.67; P = 0.02) content. Soluble sugar content related
320 negatively to the SPS (r^2 = - 0.77; P = 0.01) and SUSA (r^2 = - 0.60; P = 0.04)
321 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 (r^2 = 0.97; *P* = 0.00) and SUSA (r^2 = 0.70; *P* = 0.02) expression levels. Sucrose synthase and invertase activities related to AGPP activity (r^2 = 0.58; *P* = 0.05, and r^2 = - 0.59; *P* = 0.05, respectively) (Fig. 7). Soluble sugars related positively to the SUT2 expression (r^2 = 0.64; *P* = 0.03) and negatively to AMY expression (r^2 = - 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,

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

Table 1. Nomenclature and reactions catalyzed by the studied enzymes.

Starch metabolism		
AGPP	ADP-glucose pyrophosphorylase	glucose-1-P + ATP → ADP-glucose + PPi
AMY	α-amylase	$[glucose]_n \rightarrow [glucose]_{n-m} + [glucose]_m$
SP	starch phosphorylase	$[glucose]_n$ + Pi ↔ glucose-1-P + $[glucose]_{n-1}$
Sucrose metabolism		
UGPP	UDP-glucose pyrophosphorylase	glucose-1-P + UTP → UDP-glucose + PPi
SUS/SuSy	Sucrose synthase	sucrose + ADP ↔ ADP-glucose + fructose
INV	Invertase	sucrose → glucose + fructose
SPS	Sucrose-phosphate synthase	UDP-glucose + fructose-6-P → UDP + sucrose-6-P
SUT	Sucrose transporter	H^+/sucrose simporter

Abbreviations: fructose-6-P: fructose-6-phosphate; glucose-1-P: glucose-1-phosphate; Pi: phosphate; PPi: pyrophosphate; sucrose-6-P: sucrose-6-phosphate

Figure 1

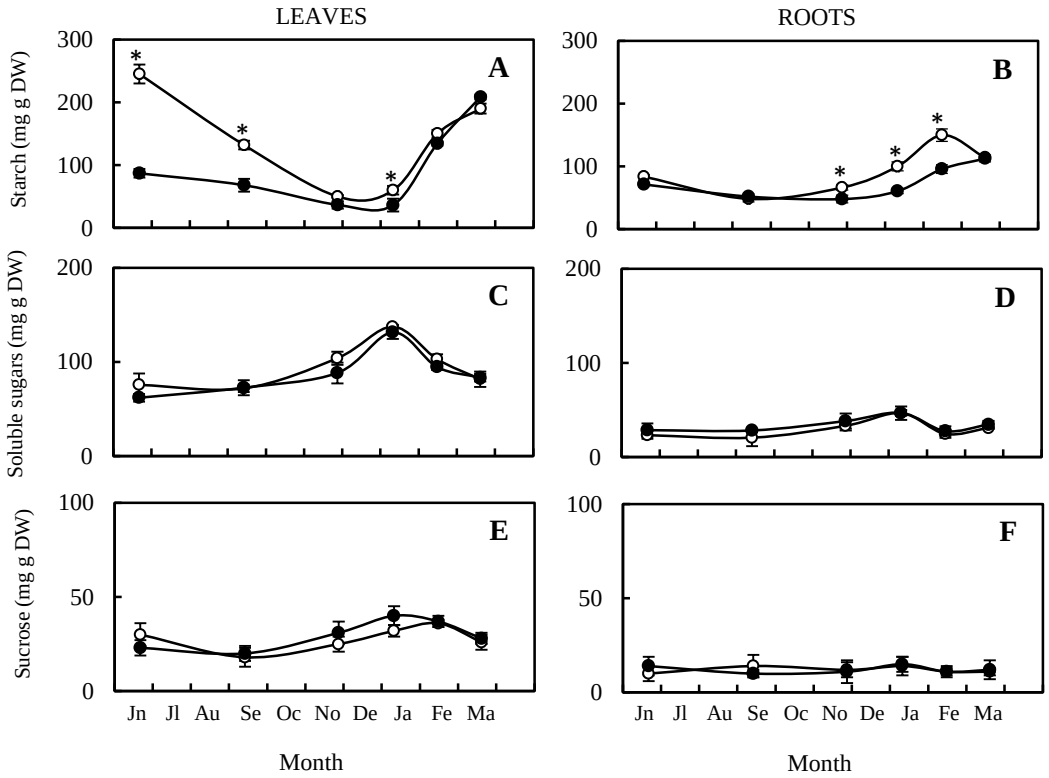

LEAVES
ROOTS
Starch (mg g DW)
Soluble sugars (mg g DW)
Sucrose (mg g DW)
A
B
C
D
E
F
300
200
100
0
50
Jn Jl Au Se Oc No De Ja Fe Ma
Month

Figure 2

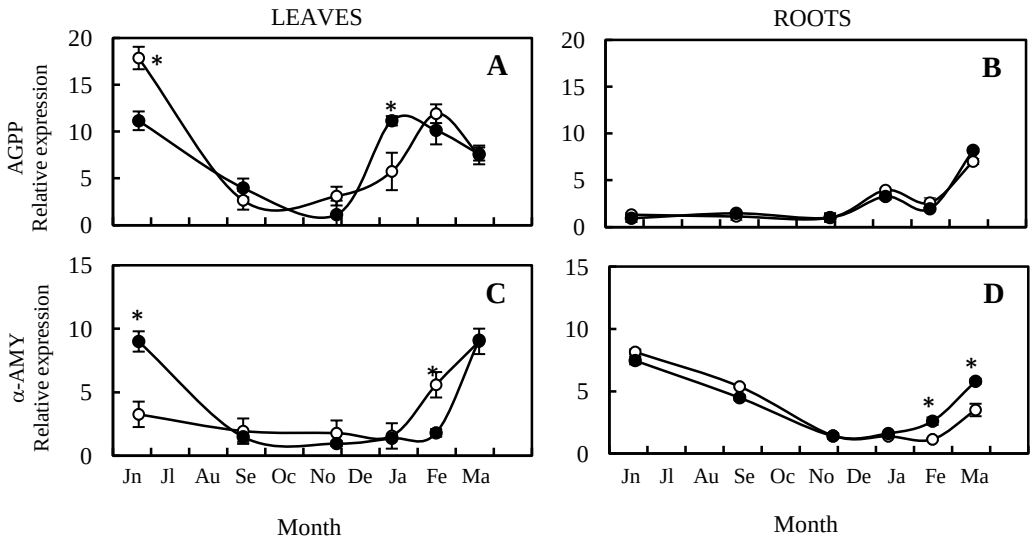

LEAVES
ROOTS
AGPP
Relative expression
α-AMY
Relative expression
A
B
C
D
0
5
10
15
20
Jn Jl Au Se Oc No De Ja Fe Ma
Month
Month

Figure 3

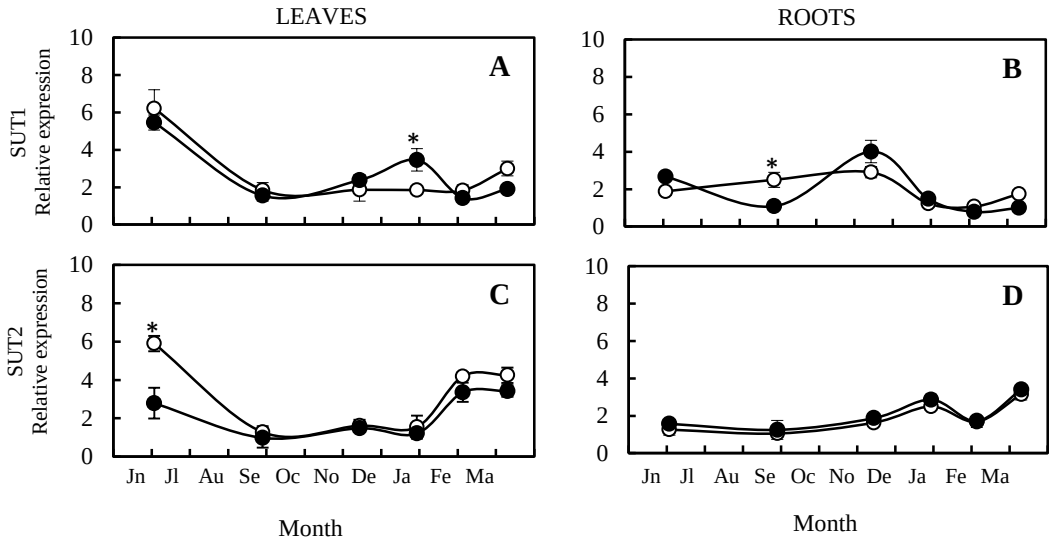

LEAVES
ROOTS
SUT1
Relative expression
SUT2
Relative expression
A
B
C
D
*
Jn Jl Au Se Oc No De Ja Fe Ma
Month

Figure 4

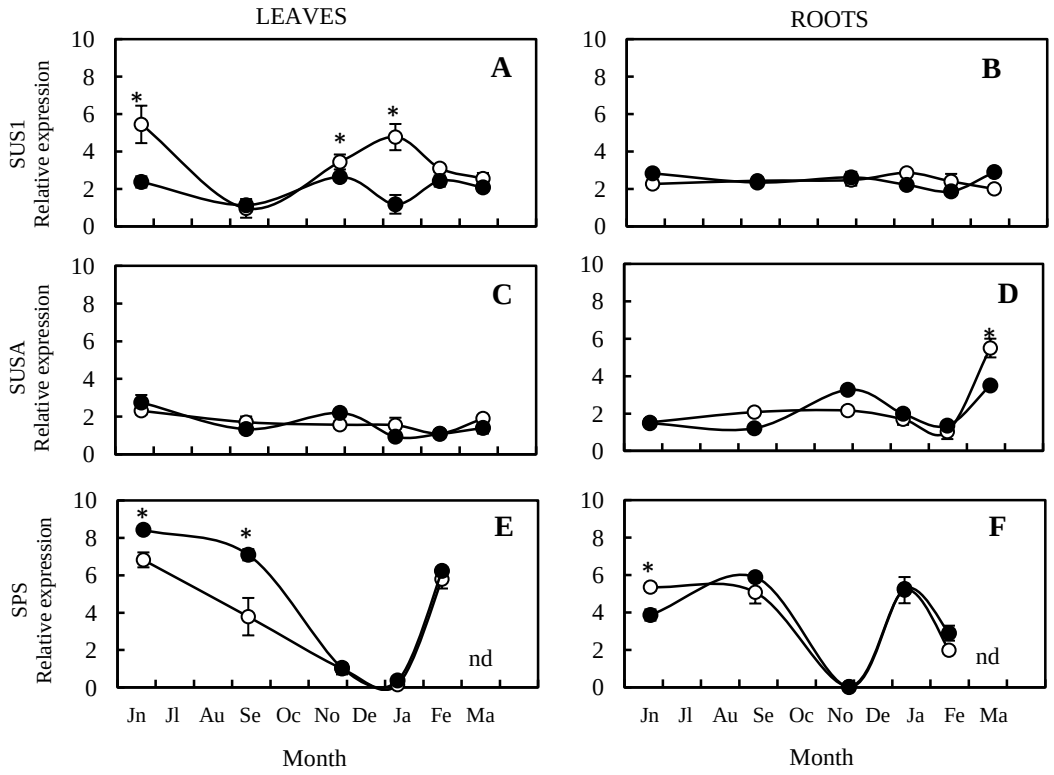

LEAVES
ROOTS
SUS1
Relative expression
SUSA
Relative expression
SPS
Relative expression
A
B
C
D
E
F
nd
nd
Jn Jl Au Se Oc No De Ja Fe Ma
Month
Jn Jl Au Se Oc No De Ja Fe Ma
Month

Figure 5

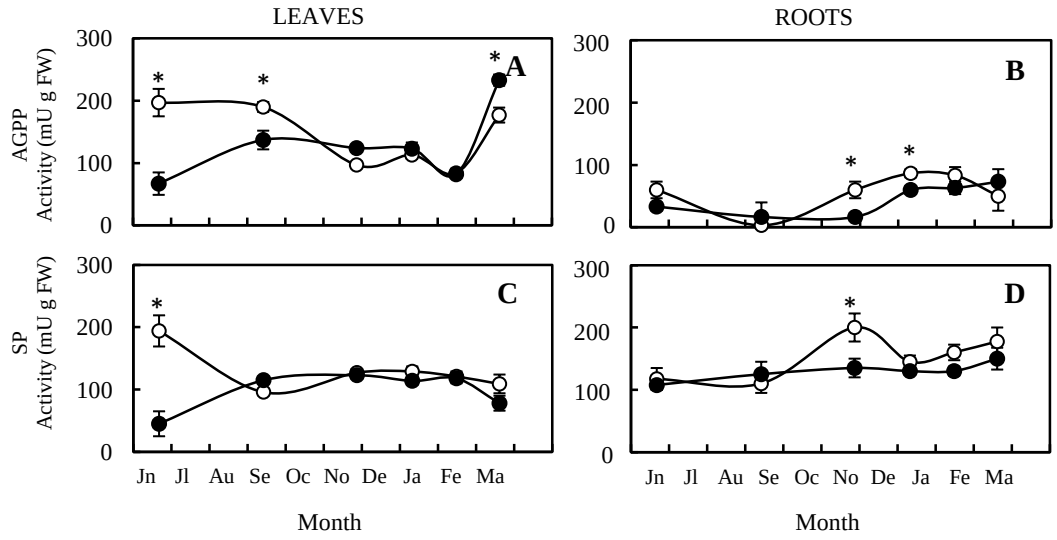

LEAVES
ROOTS
AGPP
Activity (mU g FW)
SP
Activity (mU g FW)
300
200
100
0
A
B
C
D
*
Jn Jl Au Se Oc No De Ja Fe Ma
Month

Figure 6

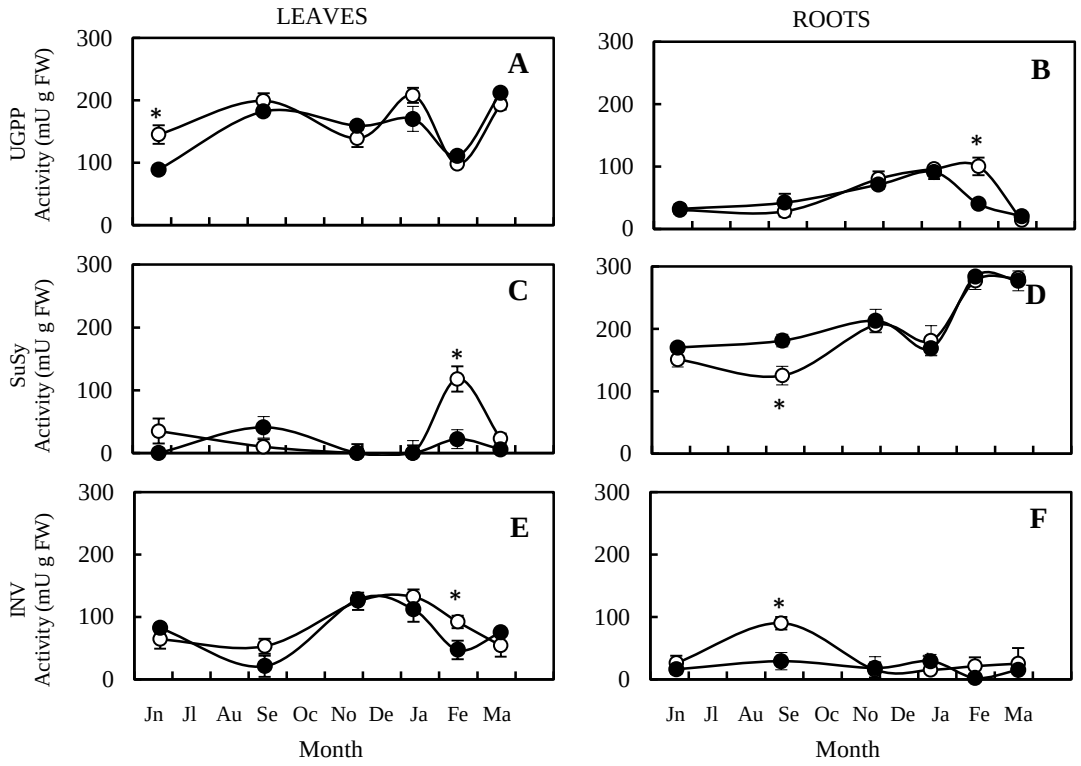
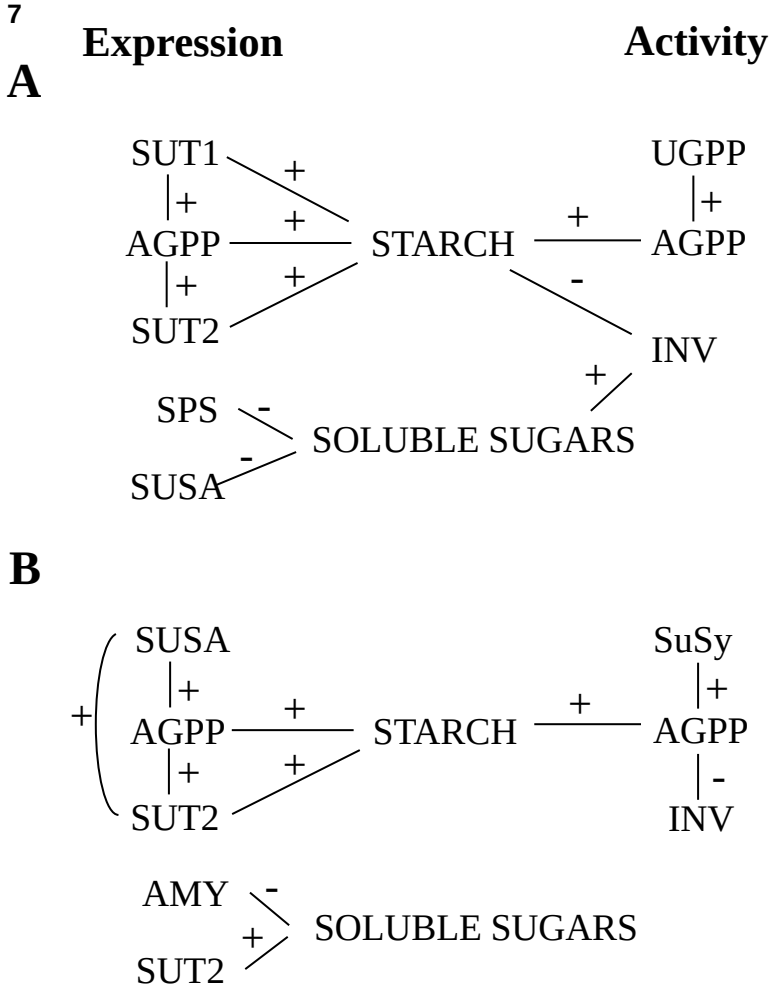

LEAVES
ROOTS
UGPP Activity (mU g FW)
SuSy Activity (mU g FW)
INV Activity (mU g FW)
300
200
100
0
A
B
C
D
E
F
*
Jn Jl Au Se Oc No De Ja Fe Ma
Month

Figure 7


Expression
Activity
A
SUT1
AGPP
SUT2
STARCH
UGPP
AGPP
INV
SPS
SUSA
SOLUBLE SUGARS
B
SUSA
AGPP
SUT2
STARCH
SuSy
AGPP
INV
AMY
SUT2
SOLUBLE SUGARS