



Primary metabolism changes in transgenic apple plants with reduced activity of sorbitol dehydrogenase

F. Martinelli¹, S. Uratsu², S. Yousefi³, G. Teo² and A.M. Dandekar²

¹ Department of Biology, University of Florence, Sesto Fiorentino, Florence, Italy

² Department of Plant Sciences, University of California, Davis, U.S.A.

³ Department of Horticultural Science, Faculty of Agriculture, Bu-Ali Sina University, Hamedan, Iran

Summary

Background – Sorbitol is the main translocated carbohydrate in species belonging to Rosaceae. It is thought to be a key molecule for the measurement of sink strength in apple. The quantity of sorbitol in plant tissues depends on the different factors such as species, development and physiological status, and type of tissues. **Objective** – An analysis of metabolic changes in primary metabolism was conducted in vegetative sinks of apple transgenic plants with altered sorbitol dehydrogenase (SDH) activity. The aim of this study was to determine the effects of the transgenic modulation of the SDH gene in carbohydrate partitioning and primary metabolism. **Methods** – Three antisenses and one sense transgenic line of cv. ‘Greensleaves’ were obtained for sorbitol dehydrogenase and grown in pots in a controlled environment. **Results** – The two antisense lines A041703/H and A111802/25 showed a reduced SDH activity similarly to antisense lines previously characterized. The three antisense lines showed alterations of three different carbohydrates, one for each plant. A090802-1 showed an increase in sorbitol while A041703/H showed an increase in fructose. The last one, A111802/25, presented an increase in glucose. In vegetative sink tissues of A090802-1 a higher level of amino acids was observed such as lysine, threonine, serine, glutamic acid, and glycine. These metabolic changes were associated with loss of apical dominance, reduced growth of leaf and whole plant. While threonine was also enhanced in A041703/H, ornithine was reduced in A111802/25 and consequently linked with the decrease in citrulline. Stearic acid was reduced in all three antisense transgenic lines. **Conclusions** – These results highlight the key role of sorbitol dehydrogenase in the establishment of sink-source relationships.

Keywords

carbohydrates, leaves, metabolism, metabolomics, source-sink relationship, vegetative sinks

Introduction

Sorbitol is the primary product of photosynthesis and the major translocated form of carbohydrates in many species of the Rosaceae including peach, pear, and apple (Cheng et al., 2005; Daie, 1993). Sorbitol comprises 80% or more of the total soluble carbohydrates translocated in the phloem of

Significance of this study

What is already known on this subject?

- The hypothesis is that SDH could serve as an indicator of sink strength in apple. Seasonal changes in NAD-SDH were found primarily in sink tissues. In previous work, transgenic apple lines for sorbitol-dehydrogenase have been analyzed for carbohydrate partitioning between immature and mature leaves.

What are the new findings?

- The leaf development stage has been correlated to SDH activity in apple and peach. We analyzed the sugar partitioning of additional transgenic lines in the same tissues and the metabolome to clarify the effects of a transgenic modulation of sorbitol-dehydrogenase on the levels of key primary metabolism.

What is the expected impact on horticulture?

- The results highlight the key role of sorbitol dehydrogenase in the establishment of sink-source relationships

apple; 60–80% of soluble carbohydrates in photosynthetic tissues such as leaves and 15% in tissues that are actively utilizing carbohydrates, such as germinating seeds, roots, or callus tissue (Loescher, 1987). In fruits, the quantity of sorbitol depends on the species, variety, state of development, and physiological status. Sorbitol is widely distributed in nature. In fact, it can be found in species of bacteria, insects, animals, yeasts, algae, fungi, and higher plants (Bielecki and Redgwell, 1985). However, certain woody members of the Rosaceae family, including *Malus*, *Pyrus*, *Prunus*, and *Sorbus*, appear to be unique in the entire plant kingdom with respect to their ability to synthesize, accumulate and degrade sorbitol. Sorbitol synthesis in mature leaves is essential for loading products of photosynthesis in plants which translocate sorbitol from leaves to sink tissue. It is estimated that sorbitol synthesis accounts for more than half of newly-fixed CO₂ found in soluble sugars (Moing et al., 1994). However, the amount synthesized depends upon the net photosynthetic rate (NAR). Generally, at low NAR, synthesis of sorbitol increases and sucrose decreases, at high NAR more sucrose and starch are produced (Escobar-Gutierrez and Gaudillere, 1997). In mature leaves of the higher plants, the biosynthesis of sorbitol is accomplished by an NADPH-dependent aldose-6-p reductase (S6PDH) catalyzing the conversion of glucose-6-p to sorbitol-6-p, which is then converted to sorbitol by a specific sorbitol-6-p phosphatase (Grant and Rees,

1981). Sorbitol utilization seems to be restricted to sink tissues and mediated by a NAD-dependent sorbitol-dehydrogenase (SDH), which oxidizes sorbitol to fructose (Loescher, 1987). Confirming this suggestion, a marked increase in S6PDH activity and a decrement in SDH activity have been observed during the conversion from sink to source of apple leaves (Loescher, 1987). SDH has been purified for the first time by Yamaguchi et al. (1994) from a plant source, and the gene was cloned by Yamada et al. (1998). In general, the strength of a sink is defined as the ability to attract products of photosynthesis and is determined not only by the sink, but also by the source, pathway, and other sinks (Minchin and Thorpe, 1996). It is clear that factors such as sink size, time of initiation relative to other sinks, location and distance from the source are strength in the whole plant (Wardlaw, 1990).

Photosynthetic rate, net sugar synthesis, and export capacity increase during leaf development, whereas the ability to import, assimilates decreases progressively as leaves mature. Changes in the amount, partitioning, and activity of enzymes associated with carbohydrate metabolism may play an important role in sink-to-source transitions of leaves. Enzymatic changes often correlate with the accumulation of transport sugars and onset of export in expanding leaves. In fact, in the sorbitol synthesizing species it is shown a marked increase in SPDH activity and a decrement in SDH activity have been observed during the conversion from sink to source of apple leaves (Fang et al., 2020).

It is known that an increasing amount of transport compounds were often associated with the achievement of a positive carbon balance and with the onset of export in expanding leaves (Baïram et al., 2019). In peach leaves, the increase in the amount of soluble carbohydrates and starch is accompanied by the development of the leaves but the ratio of soluble sugars/starch decreases because the increase of the starch is higher than soluble sugars. It is known that two enzymes are related to the levels of starch: the ADPG-PPase and the amylase. The increase of activity of the first enzyme in developing leaves is expected because the enzyme is a control point in starch biosynthesis. On the other hand, the positive correlation between amylase and starch levels suggests that starch accumulation is not related to a decreasing capacity of mature peach leaves to degrade this compound (Sutton et al., 2020). A possible explanation is a contribution of cytosolic forms to the total *in vitro* activity and that amylase could be inhibited by the light and active only at night for starch mobilization and export.

The increase of soluble carbohydrate content during the development of the leaves in species that make sorbitol synthesis is usually due to an increased accumulation in sorbitol. As different reports showed this increase is due to the appearance of the S6PDH as the leaves are maturing and the decrease in SDH (Merlo and Passera, 1991). These suggestions are strengthened by similar results obtained in apple leaves and in apricot where it is demonstrated that immature leaves could not synthesize sorbitol (Iqbal et al., 2020). Therefore, the inability to form polyols during the early stages of leaf development seems to be a widespread characteristic of woody Rosacea. The enzymatic capacity to synthesize sorbitol appeared at later stages of leaf development and was associated with an increased accumulation of sorbitol, suggesting some relationship(s) to mechanisms controlling partitioning and initiation of export in leaves.

In roots of peach, Lo Bianco et al. (2000) showed that SDH and AI (Acid Invertase) were consistently highest in the meristematic portion but in the shoots, AI was the most

active enzyme in the elongating portion subtending the apex, whereas SDH was primarily associated with meristematic tissues. These authors showed that SDH and AI were the enzymes that correlated with shoot elongation rate and concluded that they were the predominant enzymes for carbohydrate catabolism and the best indicators of sink growth and development in the vegetative sink of peach and in particular in roots. Lo Bianco et al. (1999) suggested that sorbitol and sucrose may play different roles in peach sinks, depending on the development stage of the sink (i.e., young versus mature fruit), on the type of sink (i.e., young versus mature fruit), and on the type of sink (i.e., reproductive versus vegetative organ). Sorbitol seems to have a predominant role in vegetative growth, where SDH activity, but not sucrose cleavage enzyme activities, correlates with shoot growth rate. Transgenic apples with reduced S6PDH activity were obtained and they showed to accumulate sucrose (Kanamaru et al., 2004). However, the amount of sucrose also increased with an increase in S6PDH activity. The increase in S6PDH activity might activate sucrose synthesis by supplying more substrates, by increasing the net use of photosynthates, or by increasing products from starch hydrolysis, but not depriving the substrate for sucrose synthesis. Plants that contained a low amount of sorbitol and contained a high level of sucrose with a very low activity of S6PDH, ceased shoot growth activity in early summer. Since apple uses sorbitol rather than sucrose as the main translocating sugar, it is suggested that the growth of the shoot apex may be stopped by a deficiency of sugar supply to the sink organ. It is observed also that some plants, as Persimmon and tobacco genetically engineered with S6PDH cDNA, developed necrosis or a dwarf shape by accumulating a high level of sorbitol (Kanamaru et al., 2004). Apple plants with antisense inhibition of sorbitol synthesis had lower concentrations of sorbitol but higher concentrations of sucrose and starch in mature leaves at both dusk and predawn (Cheng et al., 2005). The authors demonstrated that the partitioning of the newly fixed carbon to starch was significantly increased, whereas sucrose remained unchanged in the antisense lines with decreased sorbitol production. The increase in starch synthesis was similar to that one observed when sucrose synthesis was decreased in transgenic plants or mutants with reduced activity of TPT (Häusler et al., 2000), of cyto BPase (Zrenner et al., 1996) or SPS (Krause, 1994). These data showed that the photosynthetic system in sorbitol synthesizing species had considerable plasticity.

It is known that sorbitol synthesis is implicated in the response of plants to low temperatures, high salinity, and water stress (Lo Bianco and Rieger, 2002). Lo Bianco et al. (2000) concluded that in peach the increase of the content of sorbitol in source leaves is due not to an up-regulation of its synthesis and a down-regulation of sorbitol utilization and translocation. These conclusions were consolidated by the measurements of SDH activity in sinks that showed decreases and preceded a significant decrease in growth of the immature leaves. As a result of reduced utilization and translocation, sorbitol accumulated and contributed to the osmotic adjustment of both sources and sinks. On the contrary, sucrose metabolism is only marginally reduced, and may therefore support maintenance activities and some growth during drought. Gao et al. (2001) transformed Japanese persimmon (*Dyospiros kaki*) with apple (*Malus × domestica* Borkh.) cDNA encoding NADP-S6PDH and increased levels of sorbitol were detected in some transgenic lines. These plants were also evaluated for salt-stress tolerance under illumina-

tion by monitoring changes in the activity of photosystem II in leaves in terms of the variable (Fv) and maximum (Fm) fluorescence of chlorophyll. Another physiological process that may be influenced by sorbitol content in plants is the boron uptake and its phloem transport that could influence the resistance to boron deficiency. Teo et al. (2006) also studied the effect of a reduced sorbitol production and boron mobilization at the whole plant level and IAA content of internodes and developing leaves. In previous work, two sense and two antisense apple transgenic lines for sorbitol-dehydrogenase have been characterized in relation to soluble sugar partitioning between leaves at different developmental stages (Martinelli et al., 2011). Here, we analyzed the sugar partitioning between the vegetative sink and source tissues as well as the metabolome of immature leaves of three antisense and one sense lines grown in pots in the greenhouse in order to understand the effects of a transgenic modulation of sorbitol-dehydrogenase on the levels of key metabolites of primary metabolism and link them with an abnormal phenotype.

Material and methods

Plant material

Young trees of apple cv. 'Greensleaves' were obtained from genetic transformation, micro-propagated, subjected to acclimatization, planted in pots, and grown in the greenhouse (Martinelli et al., 2011). Immature (close or 1–2 cm of length), middle (light green, not fully expanded but photosynthetically active), mature (green, fully expanded) leaves were harvested and collected with liquid nitrogen. Two types of transgenic trees were in the greenhouse: one contained a gene codifying a sorbitol dehydrogenase (SDH) in sense, the other in the antisense direction. Three antisense lines and one sense line were obtained. Vectors containing SDH cDNA from apple in a sense and antisense orientation were constructed and genetic transformation was performed as previously described (Martinelli et al., 2011). Plants were irrigated with an automatic system on the morning of each day, and fertilized once a week.

Transgene copy number quantification

Approximately 100 mg of leaf tissue was used for DNA extraction using a DNeasy Plant Mini kit (Qiagen Inc., U.S.A.). For real-time PCR analysis, 10 ng of DNA was used for each line. Plasmid DNA (pDU02.0908) was isolated by using a QiaPrep Mini-prep kit (Qiagen Inc., U.S.A.). The DNA concentration was measured by using a Nanodrop 1000 spectrophotometer (Thermo Scientific, U.S.A.). Primers and probes were designed as indicated by Martinelli et al. (2011). Taqman Real-Time PCR analysis was performed in 12 u and all components have been previously indicated (Martinelli et al., 2011). Copy number was determined with the following formula: Weight in Daltons (g mol^{-1}) = (bp size of plasmid + insert) ($330 \text{ da} \times 2 \text{ nucleotides bp}^{-1}$). Number of molecules was determined: Concentration of plasmid ($\text{g } \mu\text{L}^{-1}$)/copy nr = ($178 \times 10^{-9} \text{ g } \mu\text{L}^{-1}$)/($2.27 \times 10^{-17} \text{ g mol}^{-1}$) = $0.78 \times 10^6 \text{ mol } \mu\text{L}^{-1}$. A standard curve was done using ten serial dilutions (dilution factor: 1:10).

Protein extraction, concentration, and activity

Protein content was extracted using a protocol described by Martinelli et al. (2011). The protein concentration of the extracts was determined using the Bradford method (Bradford, 1976) Protein activity was determined

by measuring the signal of band intensity using an assay as described (Martinelli et al., 2011). The statistical analysis was performed measuring intensity (arbitrary unit) of the band on gel electrophoresis with Alphamager software and ANOVA univariate was performed using Duncan's test as post hoc test ($P=0.05$).

Carbohydrate analysis

Immature, middle, and mature leaves (as described previously) were harvested from apple cv. 'Greensleaves' trees (grown as previously described) and immediately put on ice (4°C) until analysis. Three biological replicates (leaves) were used for each stage for each transgenic and wild-type plant. Each sample was weighed; 100 mg of tissues were ground. 1 mL of ethanol solution and 100 μL of inositol were added. Samples were mixed for 1 min at max speed (30 Hz^{-1}), kept on ice for 30 min, and centrifuged for 5–10 min at room temperature. The supernatant was collected four times (a total of 4 mL) and maintained at -20°C until needed.

Samples were dried under nitrogen (or air) stream on a water bath at $40\text{--}50^{\circ}\text{C}$, re-suspended in 1 mL of 0.01 M Tris-HCl buffer, and loaded onto Sep-pack cartridges. Soluble sugars were eluted with 4 mL of water and filtered onto autosampler vials. 1 mL together with sugar standards (glucose, fructose, sucrose, sorbitol) were analyzed by HPLC-MS analysis. Results were expressed by peak height (mm) and sugar concentration (mg g^{-1} of fresh tissue) was determined using the following formula:

$$\text{Sugar concentration} =$$

$$(10 * \text{Height}_{\text{sugar}} * \text{Standard}_{\text{inositol}}) /$$

$$(\text{Standard}_{\text{sugar}} * \text{Height}_{\text{inositol}} * \text{Fresh tissue (g)})$$

Ethanol solution $\text{pH} > 7$ was composed by 80 mL EtOH and 15 mL Tris-HCl 0.1 M.

Metabolomic analysis

Immature and fully expanded leaves were harvested, immediately frozen with liquid nitrogen, preserved at -80°C , and used for the analyses. 4–6 biological replicates for each line were included in the experimental design. One leaf of different plants was used for the analysis of each line analyzed (A090802, A041703/H, A111802/25, S091202/C, Control (wild-type)).

Leaves (20–50 mg) were picked up from -80°C , put in Eppendorf, and grounded with a bead mixer at maximum speed ($F=30 \text{ s}^{-1}$). 2 mL of pre-chilled extraction solvent (MetOH:CHCl₃:1:1) (v/v) was added to 20 mg of ground tissue for each sample and agitated at 4°C per 5 min. After vortexing and centrifugation (6,000 rpm for 2 min), 20 μL of supernatant where completely dried in a SpeedVac concentrator. Methoxyamine and N-methyl-N-(trimethylsilyl)-trifluoro-acetamide were added, and analysis was performed using the Agilent GC-quadrupole MS using a by $10^{\circ}\text{C min}^{-1}$ from 60°C (1 min initial time) to 325°C (10 min final time). 1 μL was injected into the Agilent split/splitless injector at 250°C . 1 pre-injection wash and 2 post-injection washes were performed. Both splitless and split conditions were used. A helium purge flow of 10.5 mL min^{-1} was applied for 1 min (8.2 psi) in the first case. 1 mL min^{-1} helium was used as carrier gas. The source temperature was 230°C and the quadrupole temperature was 150°C . In split conditions, a split ratio of 1:10 and a split-flow rate of 10.3 mL min^{-1} were used.

TABLE 1. Transgene quantification using Real-Time PCR. Ct values and copy g plant⁻¹ were indicated for all transgenic lines (data for S091202 and A090802-1 were previously determined (Martinelli et al., 2011)).

Transgenic lines	Ct Value	Copy 5 μL^{-1}	Copy g plant ⁻¹
S091202	35.5	1.233E+00	5.01E+01
A090802-1	20.3	6.75E+04	2.7E+06
A041703/H	22.2	5.9E+04	2.4E+06
A111802/25	21.2	6.2E+04	2.5E+06

Data acquisition and statistical analysis

Missing values in the data matrix were replaced with "XX". Internal standards were used for each sample. Relative concentrations were determined by peak area (mm^2) and peaks were manually checked for false positive and false negative assignments. Identification was performed using Agilent Fiehn GC/MS Metabolomics RTL Library (Martinelli et al., 2012, 2013). Data were statistically analyzed using Agilent Mass Profiler Professional Software with default parameters for noise reduction, normalization, mass spectral, and compound identification (Allegra et al., 2018; Tosetti et al., 2012). The relative amounts of metabolites were analyzed with one-way ANOVAs ($P \leq 0.05$) with the Duncan post hoc test to determine differences among control and transgenic lines.

Results

Three antisense transgenic lines (A111802/25, A041703/H, A090802-1) and one sense transgenic line were obtained (S091202/C). All lines were confirmed to be transgenic determining copy number using Real-Time PCR (Table 1).

Based on the results of the standard curve obtained using 50 ng of plant genomic DNA, the sense line showed to have a single transgenic SDH copy while the three antisense lines possess 6 copies of the transgene. At the beginning of the vegetative season, the SDH (Sorbitol-Dehydrogenase) activity has been measured in each transgenic and control line using a technique known as gel-activity electrophoresis. This technique is particularly easy to carry out as soon as the conditions for the enzymatic reaction are optimized and it has been used for qualitative and quantitative study of the activity of several ADH proteins. Variations in the values were observed among the different sense and antisense lines comparing with the control. All three antisense lines showed a significantly lower level of activity in comparison to control (Table 2).

On the contrary, the transgenic sense line showed similar SDH activity compared to the control. A090802-1 has been previously characterized and it showed symptoms of stress such as small, curly, and bleached leaves, the small size of leaves and reduced tree height, loss of apical dominance.

TABLE 2. Values of signal intensity of the band related to SDH (sorbitol dehydrogenase) activity. Data relating to control (wild-type), A090802-1, S091202 were previously reported (Martinelli et al., 2011). The values are the means of two different replicates. Letters indicate different groups basing on ANOVA univariate and Duncan's post hoc test ($P = 0.05$).

'Greensleaves' lines	Signal intensity (Arbitrary unit)
Control	81 (4.3) b
A111802/25	63.5 (1.5) a
S091202	88 (3.9) b
A041703/H	57 (2.9) a
A090802-1	55.5 (3.3) a

In addition to this phenotype, here we report for A041703/H shorter growth, smaller leaves than the control plants, and also loss of apical dominance (Figures 1–3). On the other hand, S091202/C did not show any differences in plant growth and other morphological traits such as control.

The analysis of the main soluble sugars (glucose, fructose, sucrose, sorbitol) was performed in three different developmental stages of leaves (immature, middle, and mature leaves) of transgenic and wild-type plants (Table 3).

As previously reported (Martinelli et al., 2011), the transgenic sense line showed a higher ratio of fructose/sorbitol in immature leaves than control while the four sugar levels were similar to the wild-type control. In middle and mature leaves no significant differences were observed between wild-type control and transgenic sense lines. Antisense transgenic lines showed significant alterations of some carbohydrates compared to control in immature leaves. In A090802-1 sorbitol was enhanced and consequently, the fructose/sorbitol ratio was enhanced. In A041703/H a reduction of glucose was observed while in A111802/25 an increase of fructose was reported (Figure 4).

In middle leaves, much fewer sugar imbalances were observed. Only A111802/25 showed an increase in fructose in comparison to control wild-type plants. This increase was

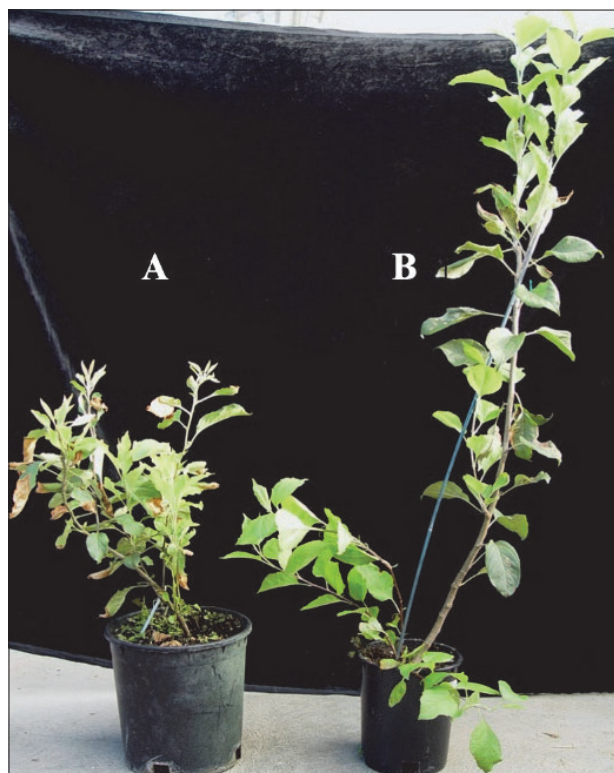
**FIGURE 1.** Apple plants of 'Greensleaves' grown in the greenhouse. Line: (a) transgenic A041703/H, and (b) wild-type control. The transgenic antisense plant showed a shorter growth than control and loss of apical dominance.



FIGURE 2. Transgenic antisense lines A041703/H (left) and A111802/25 of cv. 'Greensleaves' grown in the greenhouse in pots, fertilized once a week, and automatically irrigated every morning.

also observed in mature leaves affecting the fructose/sorbitol ratio. The metabolomic effects of transgenic integration of sorbitol dehydrogenase in antisense and sense lines were investigated. The relative amounts of 6 organic acids were determined (Tables 4 and 5).

Citramalic acid was higher in abundance than all other transgenic and wild-type lines. All transgenic plants showed a significant reduction of citric acid compared to control. S091202/C showed an increase of fumaric acid compared to



FIGURE 3. Mature leaves of apple cv. 'Greensleaves'. Line: (a) Control, (b) A041703/H.

control while antisense line A041703/H showed higher content of lignoceric acid than control.

Relating to amino acids, there were no significant differences between wild-type and all the rest of transgenic plants while threonine was accumulated in antisense lines A041703/H and A090802-1 in comparison to the wild-type control. Interestingly, in A090802-1, a significant rise of isoleucine, aspartic acid, glutamic acid, and glycine was observed in comparison to wild-type plants (Table 6). In addition, lysine was significantly enhanced in A090802 (Table 7). Relating to fatty acids, antisense line A041793/H showed a reduction of palmitic, oleic acid, and stearic acid compared

TABLE 3. Analyses of carbohydrates of immature, middle, and mature leaves for different transgenic lines of 'Greensleaves'. (A090802-1, A111803/A, A111802/25, S091202/C) in comparison to the wild-type control. The values are means of three different measurements (mg g^{-1} of fresh tissue) and they were analyzed with ANOVA univariate using Duncan's post hoc *t*-test ($P=0.05$).

Line	Glucose (mg g^{-1})	Sucrose (mg g^{-1})	Fructose (mg g^{-1})	Sorbitol (mg g^{-1})	Ratio fructose/sorbitol	Ratio sorbitol/sucrose
Immature leaves						
Control	5.18 abc	3.81 abc	17.38 ab	11.54 a	1.50 b	3.02 bc
A090802-1	4.93 bcd	6.44 c	18.10 abc	20.76 b	0.82 a	3.22 bc
A041703/H	9.90 e	4.45 abc	17.55 abc	11.17 a	1.57 bc	2.50 ab
A111802/25	6.64 cd	4.88 bc	30.74 c	12.54 a	2.45 def	2.57 abc
S091202/C	4.10 ab	3.04 ab	25.96 bc	9.04 a	2.87 f	2.97 bc
ANOVA	*	*	*	*	*	*
Middle leaves						
Control	4.50 a	7.84 ab	20.73 ab	11.55 ab	1.79 abcd	1.47 a
A090802-1	2.79 a	7.57 ab	10.76 a	21.24 b	0.51 a	2.81 b
A041703/H	6.87 a	10.12 ab	23.90 abcd	19.58 ab	1.22 abc	1.93 ab
A111802/25	6.87 a	10.37 ab	51.36 d	19.80 ab	2.59 cde	1.90 ab
S091202/C	4.55 a	6.18 a	27.02 abcd	11.01 ab	2.45 bcde	1.78 ab
ANOVA	*	*	*	*	*	*
Mature leaves						
Control	4.63 ab	10.39 abc	31.54 a	16.35 ab	1.93 a	1.57 ab
A090802-1	6.78 b	12.83 abc	33.00 a	18.27 ab	1.81 a	1.42 b
A041703/H	6.22 ab	17.14 c	56.86 ab	19.26 b	2.95 ab	1.12 a
A111802/25	5.17 ab	15.01 bc	74.39 b	19.36 b	3.44 b	1.29 ab
S091202/C	4.03 ab	7.59 a	28.14 a	9.86 a	2.85 ab	1.29 ab
ANOVA	*	*	*	*	*	*

to control (Table 8). All transgenic immature leaves showed a lower amount of stearic acid compared to wild-type control ones. A11802/25, A09082, and S091202/C presented a lower amount of lyxitol (Table 9). A11802/25 and S091202/C showed a reduction of ornithine and citrulline compared to the control wild-type. In sense transgenic line there was a rise of stigmastrol.

Discussion

Sorbitol dehydrogenase (SDH) is an enzyme involved in sorbitol utilization in sink tissues (immature leaves and fruits) catalyzing the production of fructose from sorbitol translocated from source tissues (mature leaves). Transgenic apple plants (cv. 'Greensleaves') have been obtained: three contain SDH transgenic genes in the antisense direction and

TABLE 4. The relative amount of four amino acids was determined by the peak area (mm²) correspondent to each metabolite in immature leaves for each transgenic and wild-type control 'Greensleaves' line. Data were analyzed using ANOVA univariate (p=0.05). Differences in the letters in the same column indicated significant differences between treatments using Duncan's test (P=0.05) as a post hoc test.

Line	Stage	Glyceric acid	Citramalic acid	α-Ketoglutaric acid	Citric acid
Control	Immature	12,160.6 ab	2,900.6 a	12,188.8 a	231,834.4 c
A041703/H	Immature	7,239 a	4,078.6 a	11,048.7 a	107,856.8 ab
A090802	Immature	11,657 ab	7,825.2 c	29,965.6 a	132,199 ab
A11802/25	Immature	10,189.5 ab	5,216 ab	9,442.3 a	200,263 b
S091202/C	Immature	14,733.2 a	6,061.7 ab	5,577.2 a	82,819.5 a
ANOVA		*	*	*	*

TABLE 5. The relative amount of organic acids and amino acids was determined by the peak area (mm²) correspondent to the metabolite in immature leaves for each 'Greensleaves' line. The development stage of the leaves is also indicated. Data were analyzed using ANOVA univariate (p=0.05). Differences in the letters in the same column indicated significant differences between treatments using Duncan's test (P=0.05) as a post hoc test.

Line	Stage	Fumaric acid	Lignoceric acid	Serine	Threonine
Control	Immature	33,252.7 a	949.6 a	92,240.60 abc	45,479.00 a
A041703/H	Immature	172,070.8 ab	2,344.2 b	121,404.40 bc	126,051.00 b
A090802	Immature	39,835 a	829.5 a	170,219.50 c	154,352.40 b
A11802/25	Immature	69,408.1 a	866 a	38,004.83 ab	13,801.33 a
S091202/C	Immature	267,100.7 b	1,480.5 ab	79,718.00 ab	21,241.40 a
ANOVA		*	*	*	*

TABLE 6. The relative amount of four amino acids was determined by the peak area (mm²) correspondent to the metabolite in immature leaves for each 'Greensleaves' line. The development stage of the leaves is also indicated. Data were analyzed using ANOVA univariate (p=0.05). Differences in the letters in the same column indicated significant differences between treatments using Duncan's test (P=0.05) as a post hoc test.

Line	Stage	Isoleucine	Aspartic acid	Glutamic acid	Glycine
Control	Immature	6,370.80 ab	57,376.20 ab	31,116.20 a	28,481.40 ab
A041703/H	Immature	7,217.80 abc	92,257.80 b	37,678.60 a	49,398.20 bc
A090802	Immature	12,803.00 c	180,041.40 c	101,064.40 b	79,007.20 c
A11802/25	Immature	4,998.17 ab	29,874.83 ab	25,044.00 a	11,371.17 ab
S091202/C	Immature	3,508.20 a	53,921.60 ab	36,132.60 a	8,770.40 a
ANOVA		*	*	*	*

TABLE 7. The relative amount of four amino acids was determined by the peak area (mm²) correspondent to the metabolite in immature leaves for each 'Greensleaves' line. The development stage of the leaves is also indicated. Data were analyzed using ANOVA univariate (p=0.05). Differences in the letters in the same column indicated significant differences between treatments using Duncan's test (P=0.05) as a post hoc test.

Line	Stage	Alanine	Lysine
Control	Immature	29,512.00 ab	73,722.50 a
A041703/H	Immature	18,891.20 a	69,180.40 a
A090802	Immature	56,429.33 bc	162,552.00 b
A11802/25	Immature	22,175.00 a	16,243.17 a
S091202/C	Immature	14,898.40 a	61,094.80 a
ANOVA		*	*

TABLE 8. The relative amount of four amino acids was determined by the peak area (mm²) correspondent to the metabolite in immature leaves for each ‘Greensleaves’ line. The development stage of the leaves is also indicated. Data were analyzed using ANOVA univariate (p = 0.05). Differences in the letters in the same column indicated significant differences between treatments using Duncan’s test (P = 0.05) as a post hoc test.

Line	Stage	Palmitic acid	Oleic acid	Stearic acid	Linoleic acid
Control	Immature	38,523.00 bc	2,700.40 b	16,625.00 c	5,479.75 a
A041703/H	Immature	18,842.20 a	603.00 a	10,769.80 ab	3,038.61 a
A090802	Immature	32,017.40 ab	3,241.50 b	10,101.00 ab	3,126.13 a
A11802/25	Immature	29,324.33 ab	1,321.67 b	8,600.33 a	4,420.23 a
S091202/C	Immature	42,123.25 bc	1,891.75 b	10,101.00 ab	3,979.18 a
ANOVA		*	n.s.	n.s.	n.s.

TABLE 9. The relative amount of “miscellaneous compounds” was determined by the peak area (mm²) correspondent to the metabolite for each ‘Greensleaves’ line. The means for each line were analyzed with ANOVA univariate (p = 0.05) using Duncan’s test as a post hoc test. Differences in the letters for the same column indicated significant differences between treatments.

Line	Stage	Lyxitol	Ornithine	Citrulline	Stigmasterol
Control	Immature	63,855.00 c	62,035.50 bc	86,789.50 c	962.50 a
A041703/H	Immature	42,729.67 bc	34,097.40 ab	37,071.75 ab	1,180.00 a
A090802	Immature	32,093.00 ab	87,883.00 c	72,000.00 bc	1,623.67 a
A11802/25	Immature	17,256.17 a	14,181.50 a	18,862.00 a	1,342.75 a
S091202/C	Immature	21,113.60 ab	7,528.60 a	12,076.20 a	2,645.67 b
ANOVA		*	*	*	*

one in the sense direction. Previous work has shown the altered sugar partitioning between vegetative sinks and source tissues in two antisenses and two sense transgenic lines (Martinelli et al., 2011). Two of them and additional two antisense transgenic levels were characterized at the metabolomic level in this work. Here we aim at focusing on the transgenic effect on other primary metabolism metabolites such as organic acids, amino acids, fatty acids, and others using an untargeted metabolomic approach. The aim was to link these metabolic changes with key phenotypic traits measured here and previously highlighted (Martinelli et al., 2011). Qualitative evaluation of the phenotypes of these plants was performed during the vegetative season to determine differences in their vegetative behavior in comparison to control plants (wild-type ‘Greensleaves’). Although we have identified significant altered vegetative growth, modified leaf and

tree parameters, it is worthy to state that usually phenotypic differences due to altered carbon metabolism should become more relevant once the plants bear fruits. It is when fruits are present that an important sink is present in addition to a vegetative sink that should cause a drastic altered sink-source relationship in plants with low SDH activity such as antisense transgenic lines. On the other hand, it is possible that some effects that we did not see in transgenic lines might be due to the fact that in the juvenile stage, even those plants with low SDH activity should require less sorbitol utilization such as the wild-type control plants.

Anyway, the A041703/H transgenic line showed curled and bleached leaves, a reduction in the tree growth, reduced leaf dimensions, and also a loss of apical dominance compared to the control plant. This evidence might have been partially linked to a possible decrease in root growth that has a negative effect on stem water potential, as was registered for ‘Greensleaves’ transformed to regulate the activity of S-6-PDH (Teo et al., 2006). The phenotype of transgenic line A090802 has been previously highlighted (Martinelli et al., 2011). It showed curled and bleached leaves, a reduction of leaf dimensions and tree height, premature loss of leaves, an elevated growth of axillary shoots at the apical part. In order to link these altered phenotypes with a significant change in SDH activity, we have performed a gel assay analysis in the two new antisense transgenic lines (A11802/25 and A041703/H). The other transgenic antisense and sense lines were already analyzed (Martinelli et al., 2011). Results confirmed the significant decrease of SDH activity in antisense transgenic lines. This should occur when the transduction is inhibited by higher levels of antisense RNA of the transgenic plants hybridizing RNA transcripts produced by SDH genes naturally present in the apple genome. The similar levels of SDH activity in the sense line exclude possible inhibition of gene expression due to co-suppression. It is worthy to notice that SDH genes are usually members of a small family as shown by Park et al. (2002). This family could be divided into

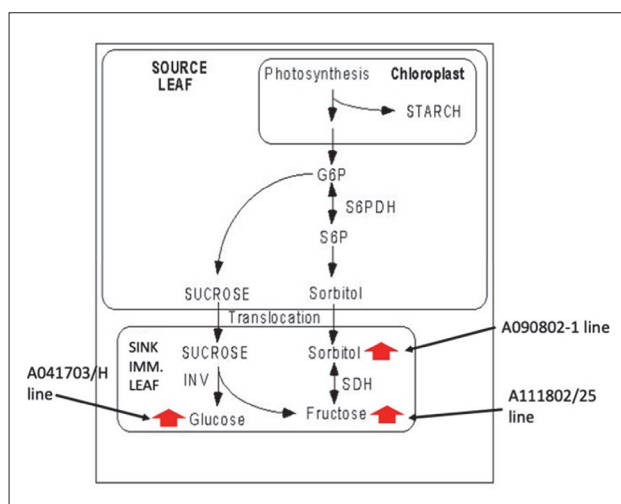


FIGURE 4. Sugar biosynthesis and metabolism in source and sink leaf tissues, key enzymes, and effects of sugar levels in transgenic antisense lines produced in this study.

two groups basing on amino acid identity. One group included MdSDH2, MdSDH3, MdSDH4 with 90–92% identity each other, and the other was represented only by MdSDH1 that has a slightly low identity with this first group (69–71%). These two groups were clearly separated from a third group that included peach SDH (BAA94084) and sour cherry SDH (AAK71492). The sequence used for all our transgenic lines was the same as the one previously indicated. It was provided by Yamada et al. (1998) and only analyzed in fruit tissues, not in vegetative sinks. Sugar partitioning in immature, middle, and mature leaves of transgenic has been determined. Among the transgenic lines, line A041703/H revealed a significant increase of glucose compared to control in immature leaves, whereas the content of sucrose did not significantly vary between transgenic and control lines. It is known that most part of the glucose existing in sink tissues is produced by the conversion of sorbitol to glucose, catalyzed by a sorbitol oxidase (SOX), and also the degradation of sucrose by invertase that produce glucose and fructose. Indeed, the increase of glucose in vegetative sink tissues of this line is a clear consequence of the reduced activity of SDH due to antisense transgenic inhibition. In apple, sucrose is also converted into fructose and glucose but usually in a lower quantity compared to sorbitol (Daie, 1993). It is possible that a decrease of the SDH activity detected in line A041703/H may influence the content of glucose through the activity of sorbitol oxidase. In A090802-1 a significant increase of sorbitol was observed in vegetative sink tissues agreeing with the reduced activity of sorbitol dehydrogenase. Although not significant a trend of increase of sucrose was observed agreeing with this data. A higher amount of fructose was detected in line A111802/25. This could be generated by sucrose through the activity of invertase. It is known that the ratio of fructose/sorbitol could be a valid index of the SDH activity. It has been shown that the sucrose cycle and the sugar transport system are responsible for the stable level of fructose highlighted the roles of sorbitol and sucrose in modulating

sugar metabolism and accumulation in sorbitol-synthesizing species (Li et al., 2018). When sorbitol synthesis is reduced by transgenic inhibition of *A6PR* in the source leaves of apple trees, less sorbitol is moved from the leaves to the sink fruit tissues but on the other hand, more sucrose is transported. Sorbitol metabolism is downregulated in this plant, while sucrose is induced in the transgenic fruit to compensate for the reduced flux of fructose produced from sorbitol. This altered sugar metabolism and transport causes homeostasis of fructose and sucrose and increased glucose and galactose content in the transgenic apple fruit showing the metabolic flexibility of sorbitol-synthesizing plant species (Li et al., 2018).

Interestingly, A090802-1 (protein activity was lower than control) revealed a decrease of the ratio fructose/sorbitol probably due to lower activity of SDH. Anyway, line A041703/H that showed a short growth, a stressed phenotype (see above) and a low SDH activity did not reveal any decrease in the ratio. An explanation of this evidence may be that the content of the active SDH isoform could be sufficient to utilize sorbitol to produce fructose. Sorbitol/sucrose is another ratio usually used to determine the efficiency of sorbitol and sucrose metabolism.

In middle leaves (light green, completely opened, not completely fully expanded but photosynthesizing photosynthesis did not completely occur at a high rate, leaves were in a stage between the sink and source leaves. Probably SDH might still work even if carbon partitioning was affected also by photosynthesis. Although the fructose/sorbitol and sorbitol/sucrose ratio greatly varied among antisense, sense, and control lines, no significant differences were detected (except for line A090802-1 that showed a higher value of sorbitol/sucrose) (Table 3) revealing that in middle leaves SDH activity might have less influence on the partitioning of sugars than other enzymes, such as S6PDH, SPS (sucrose-phosphate-synthase) and FBPase (fructose-1,5-bi-phosphatase). It is known that SDH is specifically expressed in sink tissues (even if the expression was also detected in source leaves of

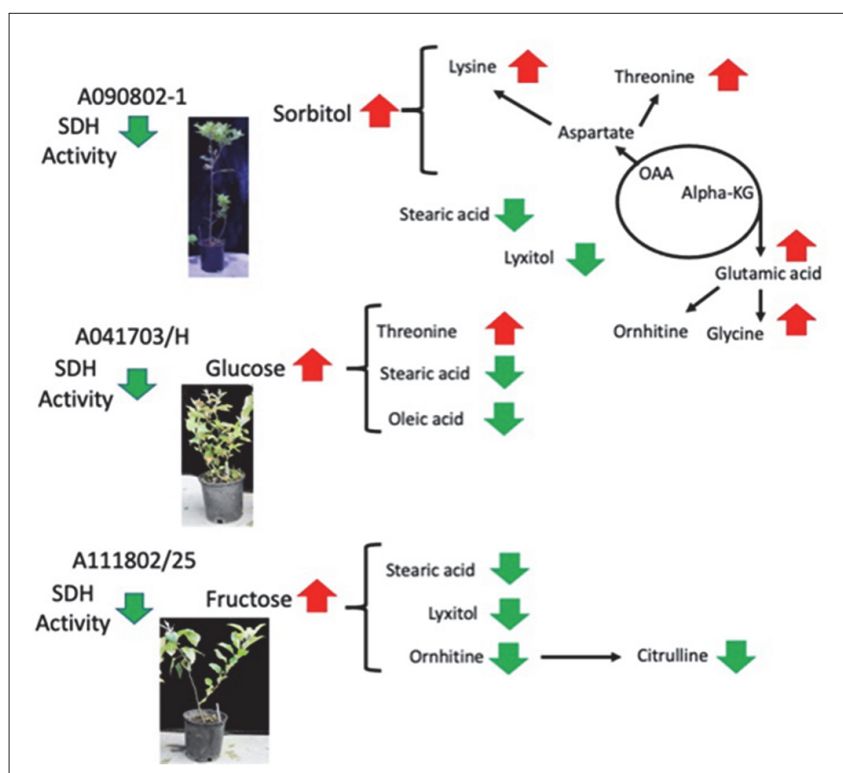


FIGURE 5. Key metabolic effects of antisense inhibition of sorbitol dehydrogenase in the three transgenic lines described in this work. The altered phenotype was linked with key metabolite changes in primary metabolism.

apple by Park et al. (2002). Although the values were highly different, no significant differences were detected in mature leaves because of the high variability of the measurements between the same line. This fact was expected because in source leaves, photosynthesis occurs at a high level and the activity of several enzymes (SPS, FBPase, S-6-PDH) might produce high variability in sugar determination. It is known the activity of S-6-PDH that converts glucose-6-phosphate in sorbitol-6-phosphate in most Rosaceae species (apple included) (Li et al., 2018). Also, fructose-1,5-biphosphatase (CytoFBPase) is active in mature leaves and catalyzes the conversion of fructose 1,6-biphosphate to fructose-phosphate that is utilized with glucose by the sucrose-phosphate-synthase (SPS) to produce sucrose-6-phosphate (Daie, 1993). It is possible that this enzyme is responsible for the higher amount of fructose in antisense line A111802/25. CytoBPase activity is inhibited by the signal metabolite fructose 2,6-bisphosphate (F2,6BP) (Stitt, 1990). On the other hand, complex regulation of SPS activity is also known. The enzyme is subjected to allosteric regulation by metabolites and post-translational modification (Huber and Huber, 1992) and is activated by glucose-6-phosphate (G6P) whereas it is inhibited by inorganic phosphate (Pi) (Doehlert and Huber, 1983).

A metabolomic profile of immature leaves of the three transgenic antisense lines (A111802/25, A090802, A041703/H, S091202/C) was also performed to determine how the modulation of SDH activity may affect the vegetative sink metabolism (mostly primary metabolism). As shown in Figure 5, amino acid biosynthesis and metabolism were mostly affected, especially in antisense line A090802-1.

This plant showed enhanced levels of several amino acids which are connected at metabolic levels such as lysine, threonine, glutamic acid, and glycine. Amino acids are the principal long-distance forms of N transported from sources (i.e., mature leaves) to sinks (including young leaves during vegetative growth). Immature leaves require organic N for growth and development. Developing leaves are the major N sinks during the vegetative phase while developing fruits, flowers and seeds are key sinks at the reproductive stage. Transport of N from root to shoot occur in the xylem, while the phloem is the site of N partitioning from source leaves to sinks. Sink organs generally show low xylem import due to low transpiration rates. Typically, amino acid concentrations are higher in young leaves compared with mature leaves, due to photo-respiratory recycling. Transporters responsible for vacuolar ammonium storage still need to be identified. In young tobacco leaves, the most present amino acid is proline, while it is much less present in mature and senescing leaves. During the reproductive phase, the major sink tissues are seeds and developing fruits in annual species, while during vegetative growth and in perennials such as apple, developing leaves are one of the strongest sinks for N. In all sinks such as sink leaves, N release from the phloem is due to symplastic movement using neighboring parenchyma cells (Patrick, 1997). However, amino acid and ureide transporters are highly expressed and work as retrieval of organic N leaking into the apoplast (Tegeger, 2014). Nitrogen partitioning in sink tissues depends on N uptake and metabolism in source vegetative tissues, and the trend of export of nitrogen from source leaves and sinks to import. In *Arabidopsis*, it was shown that amino acid transport in shoot modulates N uptake in roots, mature leaf metabolism, and allocation to sinks. In pea, increased amino acid phloem loading correlated with N root uptake and usage in source and sink (Zhang et al., 2015).

Based on these preliminary findings we may speculate that in the transgenic line A090802-1 the excessive growth of axillary leaves and the presence of many immature leaves with active growth induce a high sink strength of C and N. Especially nitrogen is transported in these vegetative sink tissues because the sink is strengthened by the absence of SDH activity. This explains why several amino acids such as lysine, threonine, glycine, and glutamic acid increased. SDH decrease of activity when there is no enhancement of fructose and glucose seems to be strictly associated with an enhanced N sink. Why the other antisense lines did not show the same significant increase of amino acid? Although not significant, a trend of increase of some amino acids was also observed in A04173/H such as serine, aspartic acid, glutamic acid, and glycine. It is possible that these increases are compensated by higher activity of invertase that enhances fructose and glucose reducing the sink strength compared to the other antisense line A090802-1. Increases in N export from leaves and variations of leaf N concentrations affect shoot-to-root signal triggering induction of N uptake and delivery to leaves. This was confirmed in transgenic pea plants when higher N availability enhances photosynthesis and stimulated C loading in the phloem (Bradford, 1976). The regulatory effect of N in phloem depends on the plant developmental stage (Santiago and Tegeger, 2017). When mature leaves focus on N movement, photosynthesis and N/C assimilation were repressed. Our data support the hypothesis that for normal vegetative growth, increases of both sugar and amino acids should be avoided in sink tissues. Plant growth strictly depends on source-sink relationships and its feed-forward regulatory mechanisms. Both source and sink strengths are affected by N transport. N presence in mature photosynthesizing leaves influences carbon fixation, uptake, and allocation to sinks. Root uptake and transport to shoot are important for vegetative growth. This altered amino acid metabolism may be linked with the phenotype characterized by a short growth, loss of apical dominance, and smaller, curly and bleached leaves. Transgenic *Arabidopsis thaliana* plants over-expressing a gene involved in lysine accumulation showed an abnormal phenotype. These phenotypes were observed and were completely normal and produce seeds (Tzchori et al., 1996). Previous works suggested that the modulation of lysine and threonine metabolism is different among plant species and controlled by many factors at the biochemical, physiological, and environmental levels. The induction of free lysine and threonine to specific tissues, such as seed, may reduce issues linked with abnormal phenotypes and reduced yields. Another possibility will be to allow an increased content of free lysine and threonine into seed proteins causing a stable increase of both amino acids and reducing deleterious effects of enhancement of these amino acids during seed development and germination. Lysine and threonine biosynthesis need carbon, nitrogen, and energy through photosynthesis. Abnormal phenotypes linked to lysine and threonine increases were not observed anymore when plants were grown at a low light intensity and plants do not accumulate any more high content of lysine levels than plants grown at higher light intensity. This might be due to reduced energy obtained from photosynthesis although it is not sure (Galili, 1995). Lysine alteration seemed to have an effect on the levels of other amino acids such as glutamate. This amino acid was shown to be negatively correlated with lysine free level content (Allegra et al., 2018) suggesting that enhanced lysine biosynthesis might trigger the conversion of glutamate to aspartate (Zhu and Galili, 2003). Seedlings with

higher biosynthesis of lysine showed reduced growth and white spots on the cotyledons. Interestingly, a decrease of the amount of palmitic acid was detected in line A041703/H compared to control though no differences were detected for the other fatty acids analyzed. Between mature leaves of control plants and A111802/25, significant differences were observed for two amino acids (isoleucine and alanine) for palmitic acid and other acids (glyceric acid and α -ketoglutaric acid). Anyway, these differences were not within the same class of metabolites and have to be further investigated. Organic acids produced in the TCA cycle is the engine that allows the conversion of alpha-ketoglutarate in glutamic acid and glycine as observed in antisense A090802-1 line. So, although some of the organic acid showed a trend of increase such as alpha-ketoglutarate and citramalic acid. In A041703/H a trend of increase was observed for citramalic acid, fumaric acid. Significant increase was observed for lignoceric acid. Taken together an increase in the total amount of organic acid observed in antisense lines with reduced growth, axillary shoots might contribute to the altered sink strength. If normal source-sink relationship is disrupted this will cause a reduced growth of the whole plant associated to decrease in root growth and architecture. Relating to fatty acid, it has been observed a higher level of them in young immature leaves than in mature ones. Here we observed a reduction of stearic acid in antisense lines that might be linked with an altered metabolism of lipids in leaves with decreased SDH activity. The evidence that stearic acid was reduced showed that these immature leaves have a modification of key components of primary metabolism such as fatty acids. In conclusion, our work demonstrated that the modulation of sorbitol dehydrogenase is a key important factor for a normal growth of the plant at whole level. In addition, it is an important factor to allow a balanced source-sink relationship that affects not only carbohydrate metabolism but other key components of primary metabolism such as amino acids, organic acids and fatty acids. Future studies will focus on the study of molecular and physiological mechanisms involved in the relationship of fruit sinks and mature source leaves with consequences in fruit qualitative parameters.

Data availability

Data supporting the results reported in a published article are freely available to readers without undue qualifications.

Conflicts of interest

The authors declare that this research work has been conducted without any conflict of interest.

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Addresses of authors:

Federico Martinelli^{1,*}, Sandie Uratsu², Sanaz Yousefi³, Gianni Teo² and Abhaya M. Dandekar²¹ Department of Biology, University of Florence, Sesto Fiorentino, Florence, 50019, Italy² Department of Plant Sciences, University of California, One Shields Avenue, Mail Stop 4, Davis, CA 5616, U.S.A.³ Department of Horticultural Science, Faculty of Agriculture, Bu-Ali Sina University, Hamedan, Iran

* Corresponding author;

E-mail: federico.martinelli@unifi.it

Tel.: +393318039998

