

**FINAL PROJECT REPORT****WTFRC Project Number:** AH-04-416**Project Title:** Role of Sorbitol in Sugar and Acid Accumulation in Apple Fruit

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**Budget History:**

<b>Item</b>	<b>Year 1: 2004</b>	<b>Year 2: 2005</b>	<b>Year 3: 2006</b>
<b>Salaries</b>	\$21,364	\$22,342	\$23,554
<b>Benefits</b>	\$ 3,843	\$ 4,035	\$ 4,237
<b>Wages</b>			
<b>Benefits</b>			
<b>Equipment</b>			
<b>Supplies</b>	\$12,493	\$12,233	\$13,409
<b>Travel</b>	\$ 800	\$ 800	\$ 800
<b>Miscellaneous</b>			
<b>Total</b>	\$38,500	\$39,500	\$42,000

## Objectives:

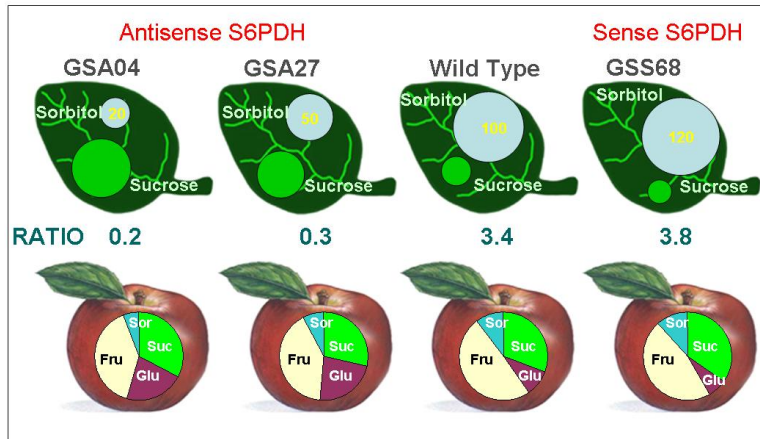


Fig. 1: Metabolic phenotypes of major transgenic genotypes with alterations in sorbitol metabolism due to antisense/sense expression of cDNA encoding S6PDH.

Unfortunately, defining the role of sorbitol in normal apple fruit is complicated by the presence of sucrose, which is partitioned simultaneously with sorbitol. Both sorbitol and sucrose are synthesized from the same products of photosynthesis, creating two pathways of carbon flow into the fruit for conversion to common sugars and other products that determine fruit quality. For example, fructose in fruit can be derived from either sorbitol or sucrose. We have solved this problem by creating transgenic apple trees specifically altered in the ability to make and translocate sorbitol. These plants are in essence specific mutants, and they provide a unique opportunity to evaluate the role of sorbitol in fruit quality. Figure 1 lists the phenotypes of transgenic lines that are currently under study. The sorbitol to sucrose ratio (So/Su) indicates important differences among these lines. Untransformed apple trees have a ratio around 3; we have lines with a ratio more than ten times lower (Fig. 1). The ratio of sorbitol to sucrose decreased from 3.4 to 0.3 and 0.2 in leaves of antisense clones GSA27 and GSA04. In sense clone GSS68, it increased to 3.8. The ratio in fruit pedicel phloem exudates is similar to the ratio found in leaves. As a result, some quality-determining factors such as fruit growth, size, and firmness do not change, but others, such as sugar composition, acid content, and starch content, do change (Fig. 1). Thus, sorbitol plays an important role in the quality of fruit flavor. This research seeks to determine key enzymes which regulate fruit quality, especially flavor, through analyses of activity and expression of encoding genes. We examined one transgenic line in detail and demonstrated that sorbitol plays a key role in the sugar composition, starch distribution and malic acid accumulation in fruit tissues. In this study we extend our analysis of sorbitol metabolism to identify critical proteins/enzymes and genes that regulate sugar and acid composition and starch accumulation (Fig. 2).

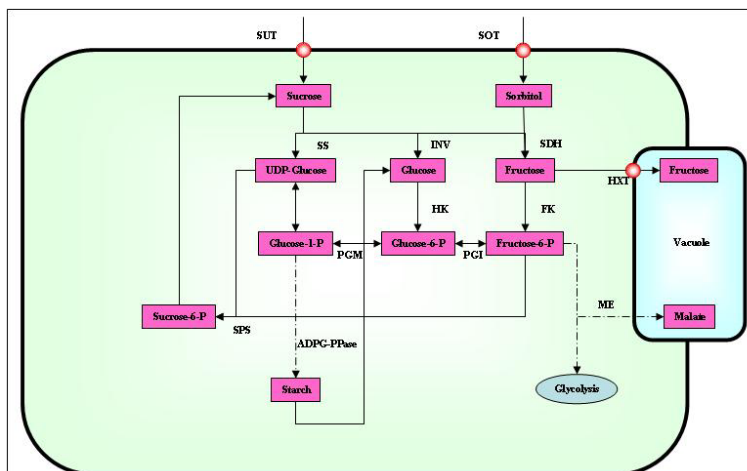


Fig. 2: Pathways and key enzymes regulating carbohydrate metabolism of apple fruit. SS – Sucrose Synthase; INV – Invertase; SDH – Sorbitol Dehydrogenase; HXT – Vacuolar Hexose Transporter; SUT – Sucrose Transporter; SOT – Sorbitol Transporter; HK – Hexokinase; FK – Fructokinase; PGM – Phosphoglucomutase; PGI – Phosphoglucosomerase; SPS – Sucrose Phosphate Synthase; ADPG-PPase – ADPG Pyrophosphorylase; ME – NADP<sup>+</sup>-Malic Enzyme.

This project focuses on understanding biochemical mechanisms responsible for metabolism and partitioning of sorbitol in apple fruit. We have outlined the key role of sorbitol in: 1) sugar composition, 2) starch accumulation, 3) malic acid metabolism, and 4) development of the watercore disorder. For these reasons, understanding metabolism and partitioning of sorbitol in fruit tissues should remain a priority for an industry dependent on production of high

1 Define and validate the role of sorbitol-related enzymes in accumulation of sugar and acid in apple fruit.

2 Determine sorbitol's role in development of water core in apple fruit.

## Significant Findings:

1. The Sorbitol/Sucrose ratio regulates malic acid accumulation and thus acidity levels in fruit.
2. Sorbitol partitioned into fruit regulates accumulation of nonstructural carbohydrates but does not significantly affect firmness.
3. High sorbitol concentration is responsible for water core development.
4. The sorbitol concentration supplied to fruit regulates activity and gene expression of sorbitol related enzymes and thus metabolism in fruit tissues during development and in the final product.

## Methods:

**Field planting of transgenic apple plants:** Transgenic plant samples for this project were collected from two fields:

**Field A:** Source planting of all transgenic apple tree clones expressing sense/antisense S6PDH. This orchard has trees planted in a randomized design on their own roots in a 15x15 ft. spacing. The plot has 354 apple trees, of which 53 are control trees of golden delicious apple cultivars 'Greensleeves' (GS; 26) and 'Ginger Gold' (GG; 27). The other trees are transgenic, of which 106 express anti-sense S6PDH, 111 express sense-S6PDH, and the remaining 74 express other constructs not relevant to this study.

**Field B:** This transgenic production orchard planted in 2001 has 5 clones (all GS): 1026-8 (GSS68), 1026-27(GSA27), 0701-10, 0601-4, and 1020-4(GSA04). These five clones, including a GS control, were bud grafted onto M26 rootstock and planted in 5-tree replicates; each replicate was repeated 5 times in a randomized complete block design in a one acre block. 'Gala' grafted to M26 was planted as a pollinator in between each replicate of 5 trees. This block has micro-sprinkler irrigation and is managed with standard horticultural practices.

**Fruit:** Fruit samples were collected at 30, 54, 70, 91, and 118 days after flowering (DAF). Fresh samples were peeled, frozen in liquid nitrogen and kept at -80°C until analyses. For the SDH and ME enzyme assays, fresh samples were used.

**Enzyme Extraction and Assays:** NAD-sorbitol dehydrogenase (SDH) and NADP-malic enzyme (ME) were extracted according to Yamaki and Ishikawa (1986) with slight modifications. Sucrose synthase (SS), sucrose phosphate synthase (SPS), soluble acid invertase (sAI), neutral invertase (NI), fructokinase (FK), hexokinase (HK), and ADP-glucose pyrophosphorylase (AGPase) were extracted according to Tanase and Yamaki (2000) with slight modifications.

SDH activity was assayed by modifying the method described by Yamaki and Ishikawa (1986). The reaction mixture contained 100 mM M Tris-HCl buffer (pH 9.5), 1 mM NAD<sup>+</sup>, 300 mM sorbitol, and the enzyme extract. Enzyme activities were determined as changes in absorbance at 340 nm at 25°C. ME activity was assayed according to Yoshioka et al. (1989). The reaction mixture contained 80 mM M Tris-HCl buffer (pH 7.5), 0.3 mM NADP<sup>+</sup>, 1 mM MnSO<sub>4</sub>, 6 mM malate, and the enzyme extract. Enzyme activities were determined as changes in absorbance at 340 nm at 25°C. Soluble and cell-wall bound acid invertase activities were assayed according to Tanase and Yamaki (2000). The assay mixture contained 30 mM K acetate (pH 4.5), 200 mM sucrose, and enzyme solution. The mixture was incubated for 1 h at 30°C, and the reaction was stopped by boiling before adding 0.75 M Tris-HCl buffer (pH 8.5). The activities were measured as glucose production from sucrose by the enzyme coupling method described earlier (Yamaki, 1980). For NI activity, the assay mixture was identical to that of acid invertase except that 30 mM HEPES-KOH (pH 7.0) was substituted for 30 mM K acetate (pH 4.5). The reaction was stopped by heating the mixture in boiling water for 3 min. The activities were determined as glucose produced from sucrose by the enzyme coupling method of Yamaki (1980). SS and SPS activities were assayed according to Tanase and Yamaki (2000) with a slight modification. For SPS activity, the reaction mixture contained 15 mM HEPES-KOH (pH 8.5), 15 mM fructose-6-phosphate, 2 mM UDP-glucose, 5 mM MgCl<sub>2</sub>, 50 mM NaF, 1 mM sodium orthovanadate, and the enzyme extract. For SS activity, the reaction mixture contained 15 mM

HEPES-KOH buffer (pH 8.5), 15 mM fructose, 2 mM UDP-glucose, 5 mM MgCl<sub>2</sub>, and the enzyme extract. The mixture was incubated for 30 min at 30°C, and the reaction was stopped by addition of 2.5 N NaOH. Sucrose production was determined by Roe's method (1934). For the sucrose cleavage activity of SS, the reaction mixture contained 30 mM HEPES-KOH (pH 7.0), 200 mM sucrose, 5 mM UDP, and the enzyme solution. The reaction was stopped by heating the mixture in boiling water for 3 min. The production of fructose was determined by the enzyme coupling method using ATP, NAD<sup>+</sup>, hexokinase, phosphoglucose isomerase, and NAD-glucose-6-phosphate dehydrogenase (G6PDH) (Morell and Copeland, 1985). FK and HK activities were assayed according to Kanayama et al. (1997) with a slight modification. For HK activity, the reaction mixture contained 30 mM HEPES-NaOH (pH 7.5), 1 mM MgCl<sub>2</sub>, 0.6 mM EDTA, 9 mM KCl, 1 mM NAD<sup>+</sup>, 1 mM ATP, 2 units of G6PDH, 30 mM glucose, and the reaction mixture. For FK activity, 2 units of phosphoglucose isomerase and 30 mM fructose were added. Enzyme activities were determined as changes in absorbance at 340 nm at 25°C. ADPGase activities were assayed according to Smith (1990) with a slight modification. The reaction mixture contained 100 mM Hepes-NaOH (pH 7.8), 5 mM MgCl<sub>2</sub>, 10 mM NaF, 2 mM ADP-Glu, 1 mM NAD, 5 units of G6PDH, 2 units of phosphoglucomutase, and the reaction mixture. Enzyme activities were determined without (control) and with 2 mM sodium pyrophosphate as changes in absorbance at 340 nm at 25°C.

**Real time quantitative TaqMan PCR:** PCR primers and compatible TaqMan® probes were designed using Primer Express (Applied Biosystems, Foster City, CA). To prevent co-amplification of contaminating genomic DNA (gDNA), TaqMan PCR primers were designed to cover exon-exon junctions where possible (Leutenegger et al., 1999).

Total cellular RNA was isolated by the hot borate method (Wan and Wilkins, 1994). gDNA contamination in the total RNA fraction was digested with RNase-free DNase I (Invitrogen, Carlsbad, CA) for 15 min at 37°C and inactivated at 95°C for 5 min followed by chilling on ice. Absence of gDNA contamination was confirmed using a universal 18S TaqMan PCR system on digested total RNA. Complementary DNA (cDNA) was synthesized using 50 units of SuperScript III reverse transcriptase, 600 ng random hexadeoxyribonucleotide (pd(N)<sub>6</sub>) primers, 10 U RNaseOut (RNase inhibitor), and 1 mM dNTPs (all Invitrogen) in a final volume of 40 µl. Reverse transcription proceeded for 120 min at 50°C. After addition of 60 µl water, the reaction was terminated by heating for 5 min to 95°C and cooling on ice.

Each PCR reaction contained 20x Assay-on-Demand primer, probes for the respective TaqMan system, and commercially available PCR mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems) with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl<sub>2</sub>, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase, 0.25 U AmpErase UNG, and 5 µl diluted cDNA sample in a final volume of 12 µl. The samples were placed in 96 well plates and amplified in an automated fluorometer (ABI PRISM 7700 Sequence Detection System, Applied Biosystems). AB's standard amplification conditions were used: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C. Fluorescent signals were collected during the annealing phase and CT values extracted with a threshold of 0.04 and baseline values of 3-10.

**Housekeeping gene validation experiment:** To determine the most stably transcribed housekeeping gene, a housekeeping gene validation experiment was run on representative samples from all tissue types. Three commonly used housekeeping genes were selected: a TaqMan PCR system recognizing plant 18S rRNA (ssrRNA), apple glyceraldehyde 3-phosphate dehydrogenase, and apple ribosomal protein S19. 18S rRNA was transcribed most stably, and therefore had least standard deviation across all tissues. 18S rRNA CT values were used to normalize the target gene CT values.

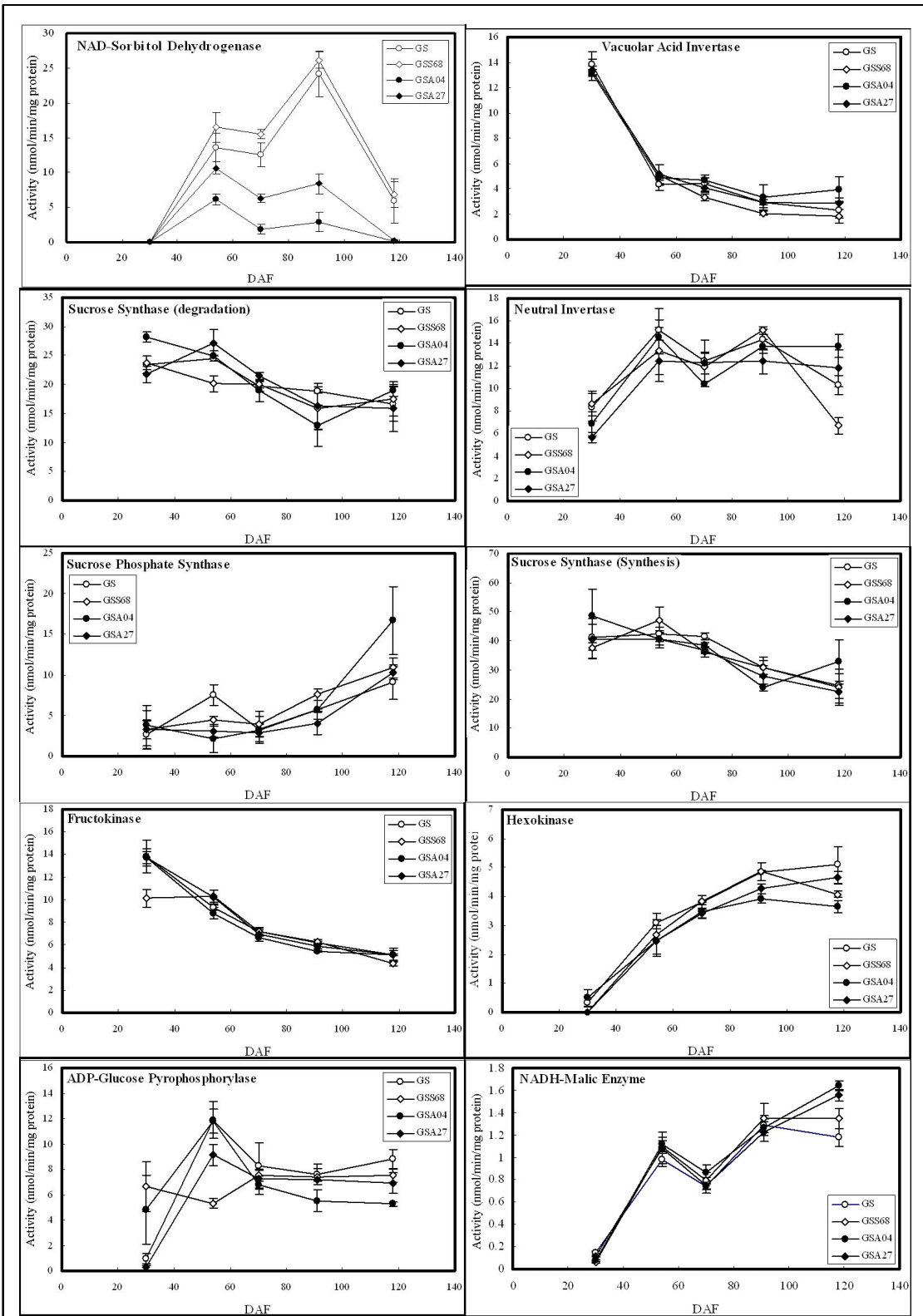
**Relative quantitation of gene transcription:** Final quantitation was done using the comparative CT method (User Bulletin #2, Applied Biosystems) and was reported as relative transcription, or the n-fold difference relative to a calibrator cDNA (i.e. lowest target gene transcription). In brief, the housekeeping gene, 18S rRNA, was used to normalize the CT values of the target genes ( $\Delta$ CT). The  $\Delta$ CT was calibrated against the weakest signal within each target gene. The linear concentration of

target molecules relative to the calibrator was calculated by  $2^{-\Delta Ct}$ . Therefore, all gene transcription were expressed as an n-fold difference relative to the calibrator.

## **Results and Discussion:**

**Comparison of activities of key enzymes regulating sugar metabolism:** The most important enzyme in apple fruit is SDH, which catabolizes sorbitol, the principal translocated sugar in fruit. SDH activity is highest late in fruit development, when sugar content in fruit drastically increases. In control trees, SDH activity was not detected in immature fruit at 30 DAF. Then, activity increased and peaked in premature fruit at 91 DAF. At harvest, activity was detected at very low levels (Fig. 3). In GSS68, SDH activity was also not detected at 30 DAF. The pattern of SDH activity was the same as in the control, but the activity tended to be higher throughout fruit development. In antisense clones GSA04 and GSA27, activities were lower than the control, especially at 91 DAF when the activity was highest in control fruit, and were not detected at harvest. Earlier work showed interruption of assimilate by girdling led to decreased SDH activity in apple fruit (Beruter and Feusi 1997). Low SDH activity of girdled apple fruit was recovered by *in vitro* sorbitol (Archbold, 1999). These reports suggest that sorbitol supply regulates SDH activity in fruit. Our results with transgenic apple fruit support this hypothesis with direct genetic evidence. In cultured celery cells, expression of the mannitol dehydrogenase (MDH) gene was repressed by hexose, and it was suggested that HK and sugar phosphorylation are involved in signaling its repression (Prata et al., 1997).

SS and invertases play are critical to sink organs since they catabolize sucrose, the other translocated sugar in apple. In general, these enzymes contribute to sequential stages of sink initiation, expansion, and storage/maturation (Koch, 2004). Girdling during active starch synthesis as mentioned above also led to decreased neutral invertase activity (Beruter and Feusi 1997), which suggests neutral invertase is important at this stage. In this study, no differences in these enzymes were detected between control and transgenic fruit, though sucrose contents in phloem exudates were different. Fructokinase and hexokinase are crucial enzymes for further metabolizing hexose. Sucrose synthase (synthesis) and sucrose phosphate synthase re-synthesize sucrose in fruit. ADP-glucose pyrophosphorylase synthesizes starch. We did not detect prominent differences of these enzymes among control and transgenic fruit (Fig. 3), though sugar and starch concentrations were different. The difference in sugar concentration may be too small to overcome homeostasis and change enzyme activities in fruit, unlike SDH, which is directly affected by phloem sap with altered sugar composition.



**Fig. 3: Analysis of key enzymes regulating carbohydrate metabolism in normal and transgenic apple fruit at various stages of development.**

It has been suggested that ME is associated with decreased malic acid in apple fruit (Yoshioka et al. 1989). ME activities increased during development (Fig. 3). They were similar in control and transgenic plants until 91 DAF, but at harvest activities in antisense clones GSA04 and GSA27 were higher than those of the control and sense clone GSS 68. There is less acid in the antisense clones than in the control and sense clone (previous report). This suggests low acid causes high ME activity.

**Comparison of gene expression of key proteins regulating sugar metabolism:** To characterize sorbitol transport (SOT) and metabolism (SDH) proteins, we analyzed individual alleles with real time quantitative TaqMan® PCR. For each target gene, there was more than one sequence representing allelic forms; we analyzed 21 sequences corresponding to each of the 8 alleles used in this analysis (4 *SOTs* and 4 *SDHs*). PCR primers and TaqMan® probes were designed based on information available for these sequences in the apple unigene set on the NCBI web site (<http://www.ncbi.nlm.nih.gov/UniGene/UGOrg.cgi?TAXID=3750>). Figure 4 shows data for 4 alleles for each of the 2 genes (*SOTs* and *SDHs*). Expression of *SDH2*, *SDH3*, and *SDH4* were higher than that of *SDH5* (Fig. 4). Expression of *SDH2* in control and GSS68 lines was generally higher than that observed for GSA04 and GSA27, except at 54 and 70 DAFB. At 30 DAFB, expression of the *SDH2* gene was detected, though enzyme activity was not detected (Fig. 3 & 4). Expression of *SDH3* in control and GSS68 was higher than in GSA04 and GSA27 except at 30 DAFB. Low expression of *SDH3* in lines GSA04 and GSA27 may result from low SDH activities through fruit development. In addition, low expression of *SDH2* in GSA04 and GSA27 after 91 DAFB may partially be a result of low SDH activities at the later stages of fruit development.

Expression of *SOT1*, *SOT3*, and *SOT4* in control and GSS68 lines at 30 DAFB was higher than that in GSA04 and GSA27, and though expression of *SOT2* in GSA04 and GSA27 was higher at 54 DAFB (Fig. 4), we did not detect prominent differences in *SOTs* expression among control/sense and antisense clones.

We also detected no prominent differences in expression of genes for *AGPase*, *HXT1*, *HXT2*, *HXT3*, *NIN1*, *NIN2*, *SS1*, and *SS2* in this experiment. No differences in expression of *AGPase* were observed in clones throughout fruit development. Changes corresponded roughly with changes in activities. Expression of *HXT1*, *HXT2*, and *HXT3* in control and GSS68 was only higher than GSA04 and GSA27 at 30 DAFB. No other differences in expression of *HXTs* genes were observed in clones throughout fruit development. We did not observe significant differences in expression of *NIN* genes or their activities, although *NIN1* was expressed more than *NIN2*. Expression of *SS* genes in control and GSS68 fruit was very high at 30 DAFB, then decreased drastically and kept almost constant.

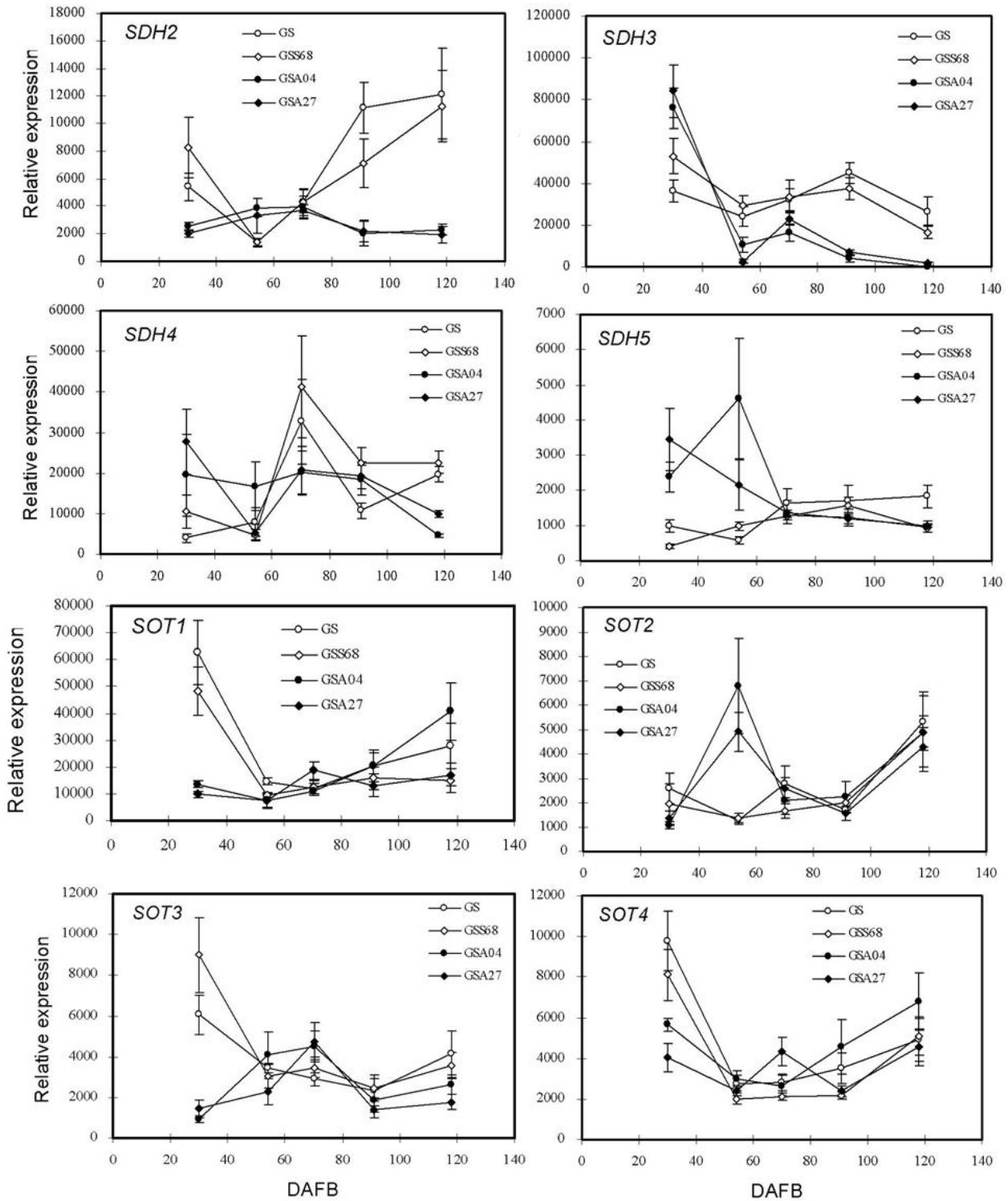


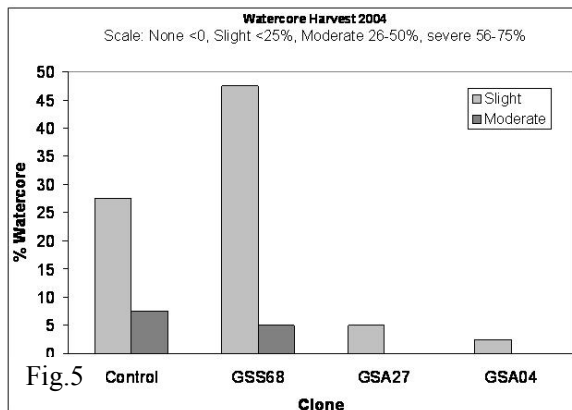
Fig.4. Gene expression of key proteins regulating sugar metabolism in control (□) and transgenic apple fruit, GSS68 (□) GSA04 (●) and GSA27 (◆) at various stages of development as revealed by Real time PCR. Each value is the mean (±SE) of 6 replicates.



Except at 30 DAFB, expression of *SS* genes was almost the same among clones, like their enzymatic activities.

Sugars can play pivotal roles as signaling molecules (Rolland et al. 2006), and thus sorbitol may regulate gene expression. In sliced tissues of Asian pear fruit, the amount of SDH protein, activity, and mRNA increased in the presence of not only sorbitol, but also sucrose, glucose, and mannitol (Iida et al., 2004). In cultured celery cells, gene expression of mannitol dehydrogenase was repressed by hexose, and it was suggested that HK and sugar phosphorylation are involved in signaling its repression (Prata et al., 1997). In this research, greatly decreased sorbitol in phloem of antisense lines could be responsible for suppression of *SDH2* and *SDH3* expression in antisense fruit, which results in low SDH activities. This suggests sugar composition, especially sorbitol, in phloem sap affects sugar metabolism and thus fruit quality.

Sorbitol transporters were not apparently regulated by sorbitol, although they are an important component of sorbitol metabolism. No prominent differences were observed among different lines (Fig. 4). *SOT* gene expression is low in tissues of watercored fruit (Gao et al., 2005), where there are high concentrations of sorbitol. This suggests *SOT* expression may be regulated by factors other than sugars.



There is a relationship between sorbitol and watercore formation in our transgenic lines that produce different levels of sorbitol (Fig. 5 and 6). GSS68 makes more sorbitol and less sucrose, and these fruit display a very significant incidence of watercore when compared to the control. In contrast, lines with less sorbitol (GSA27 and GSA04) also have very low incidences of watercore. Watercore formation is related to sorbitol metabolism. Thus, we focused on sorbitol dehydrogenase (SDH), which catabolizes sorbitol, and sorbitol transporter (SOT), which translocates sorbitol from apoplast into cytoplasm. We did not detect different levels of SOTs gene expression among clones. Gene expression of SDHs in control and GSS68 were higher than in GSA04 and GSA27. These results show that high sorbitol, not SOT and SDH, are responsible for watercore formation. They also suggest that watercore formation may be caused by low activity of SOT and SDH in comparison to the sorbitol supply.

## Literature review:

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