BEDRI KARAKAS
The role of sorbitol synthesis in photosynthesis of peach (*Prunus persica*)
(Under the Direction of MARK W. RIEGER)

This dissertation examines the hypothesis that polyol synthesis enhances photosynthetic capacity in peach and related species. Members of *Prunus* synthesize, translocate, and utilize sorbitol as their main photosynthetic end product whereas most other plants utilize sucrose for those purposes. First, I approached this hypothesis by examining eight genetically diverse *Prunus* species with various sorbitol: sucrose ratios and activities of sorbitol-6-phosphate dehydrogenase (S6PDH), principal sorbitol synthesis enzyme. Leaf photosynthetic capabilities (A), in vitro activity of the S6PDH and sorbitol contents of greenhouse grown plants were measured. I found an inverse relation between A and S6PDH activity of the species. This observation does not support the working hypothesis and that sorbitol synthesis enhances A. Second, I used two peach varieties (i.e., Encore and Nemaguard) to examine the same hypothesis within a single species by source/sink manipulations (i.e., fruiting versus non-fruiting, fruit present versus fruit removed, and shoot tip removal) and existing natural variation (i.e., leaf node position). In all cases, except fruiting versus non-fruiting and fruit present, photosynthesis and S6PDH enzyme activity showed positive correlations. Finally, I analyzed the response of S6PDH gene to shoot tip removal treatment in connection with S6PDH activity and A in potted Nemaguard peach plants. To document hourly changes, leaves were sampled three times during the day (i.e., sunrise, midday, and sunset) and analyzed for S6PDH gene expression and S6PDH activity. Sorbitol-6-phosphate dehydrogenase mRNA transcript levels significantly increased while S6PDH activity decreased 24-hour following shoot tip removal. Gene transcript levels did not change in hourly manner during the day whereas enzyme activity decreased at midday. Response of the enzyme and gene mRNA transcript revealed weak correlations, suggesting that S6PDH regulation was related to posttranslational modifications of the enzyme. Downregulation of the S6PDH following sink removal did not appear to be brought about by a simple feedback

inhibition of the sorbitol on enzyme activity in vitro. These studies suggested that sorbitol or sorbitol forming capacity do not necessarily enhance A across a spectrum of *Prunus* although within a single species, parallel changes in S6PDH activity and A were coordinated through posttranslational changes to the enzyme.

INDEX WORDS: Polyols, Sugar alcohols, Photosynthesis, *Prunus sp*., *Prunus persica*, Sorbitol, Sorbitol-6-phosphate dehydrogenase, Enzyme activity, Gene expression, mRNA.

THE ROLE OF SORBITOL SYNTHESIS IN PHOTOSYNTHESIS OF PEACH (*PRUNUS PERSICA*)

by

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I dedicate this work to the memory of my beloved parents.

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All thanks are for God, the Sustainer and the Cherisher of the Universe and everything in it. Without the Creator and the creation, there would not be any work or activity. So, I thank God for providing us, the creation, all the means and methods to discover.

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CHAPTER 1

INTRODUCTION

In general, higher plants synthesize and translocate sucrose as the main form of carbon from source to sink. However, some plants simultaneously synthesize and translocate both polyols (e.g. sorbitol, mannitol, etc.) and sucrose in various ratios. Polyols (sugar alcohols, alditols) are chemically identified as reduction products of aldoses or ketoses. Sorbitol and mannitol are the two most widely distributed polyols in plants (Bieleski, 1982). Sorbitol is the main photosynthetic product for most members of the Rosaceae, including several economically important species within *Malus* (apples), *Pyrus* (pears), and *Prunus* (stone fruits such as peach, cherry, plum, almond, and apricot). Having the highest sorbitol content of Rosaceous tree fruits, *Prunus* species could be the best model for studying the function of sorbitol in plants.

It is not clear why *Prunus* species synthesize and translocate polyols in addition to sucrose. Polyols are hypothesized to have wide range physiological functions such as enhancement of photosynthesis, storage and translocation of carbon, osmotic adjustment, cold hardiness, stress tolerance, protection of enzymes and membranes, and free radical quenching (Keller, 1989; Loescher, 1987; Shen et al., 1997a; Shen et al., 1997b; Jennings et al., 1998; Nelson et al., 1998), however their function remains speculative until proven otherwise.

Many polyol-forming plants, particularly tree fruits, have high photosynthetic rates compared to plants forming only sucrose (Flore and Lakso, 1989; Stoop et al., 1996). This could result from both increased NADP-NADPH turnover compared to plants that exclusively form sugars (one NADPH used to reduce hexose-P to alditol-P),

and/or less feedback inhibition of photosynthesis due to the additional cytosolic sink for photosynthetically fixed CO_2 used for polyol synthesis (Stoop et al., 1996).

Polyols are undoubtedly involved in carbon storage and translocation in plants. Sorbitol constitutes up to 10% of the fruit fresh weight and more than half of leaf nonstructural carbohydrates in members of the Rosaceae (Loescher, 1987). Also, sorbitol accounts for 9% of leaf dry weight in *Plantago maritima* (Briens and Larher, 1983), and mannitol accounts for 10% of the dry weight of celery leaves (Fellmann and Loescher, 1987). Using polyols for translocation of carbon, instead of sucrose, may confer certain advantages. First, loading of 6-carbon polyols (i.e., sorbitol, mannitol, etc.) into the phloem would cause twice the decrease in osmotic potential of the sieve elements as (12-carbon) sucrose. The concomitant pressure potential, and thus the rate of movement of the phloem sap, would be greater for polyol-forming species. Second, more efficient carbon use in sink tissues may result from transporting polyols instead of sucrose, since the NADH generated at the sink from oxidation of polyol back to hexose would give a higher net ATP yield than the catabolism of an equal amount of sucrose (Escobar-Gutierrez and Gaudillere, 1994; Stoop et al., 1996). Thus, species that generate and translocate large amounts of carbon may have higher efficiency in these tasks when using polyols in favor of sucrose (Figure 1).

Polyols are often termed "compatible solutes", since they can accumulate to high concentrations in cells without adversely affecting enzyme activity, while inducing osmotic adjustment, and in turn, greater stress tolerance (Stoop and Pharr, 1994; Ranney et al., 1991; Wang et al., 1996). Cryoprotection or increased cold hardiness serve as examples. There is an increase in polyol concentration and a decrease in osmotic potential associated with low temperature exposure (Williams and Rease, 1974). Sakai (1961) found out a close correlation between the levels of polyols and the degree of cold

resistance in 17 woody species. Beileski (1982) proposed that the role of polyols in cryoprotection could be due to freezing point reduction of the sapwood and protection of enzymes at low temperatures.

Plant molecular biology has provided a powerful tool for assessing the functions of polyols. To date, most studies have been limited to assessment of the drought, salt, and low temperature tolerance conferred by polyol accumulation using tobacco as a model system. A gene encoding polyol biosynthesis cloned from *Mesembryanthemum crystallinum* (ice plant), a facultative halophyte, was introduced into tobacco under the control of the CaMV 35S promoter. This gene resulted in the production and accumulation of ononitol, which may function as a compatible solute for osmotic adjustment under salt and low temperature stress (Vernon and Bohnert, 1992). These observations showed that plant carbohydrate metabolism could be altered to allow synthesis and accumulation of non-endogenous metabolites. In a similar experiment, mannitol was synthesized in tobacco by introducing a gene encoding mannitol-1-phosphate dehydrogenase (mtlD), an enzyme responsible for mannitol synthesis in bacteria (Tarczynski et al., 1992). Transformed tobacco plants grew and flowered when grown in solution salinized to a level resulting in death of non-transformed plants (Tarczynski et al., 1993). They speculated that mannitol accumulation caused osmotic adjustment of cells, which in turn allowed turgor maintenance and survival under severe salt stress. Karakas et al. (1997) repeated this experiment with the mtlD gene in tobacco, and found that although mannitol accumulation conferred some salt tolerance, it was not through the mechanism of osmotic adjustment. Furthermore, Karakas et al. (1997) found that transformed tobacco plants were intolerant of drought and freezing stress, which would be expected if the mechanism of mannitol action is through osmotic adjustment. Experiments with other osmolytes such as proline (Kishor et al., 1995), fructans (Pilon-

Smits et al., 1995), and trehalose (Holstrom et al., 1996), all using tobacco, have led to the conclusion that accumulation of foreign osmolytes in plants does not confer stress tolerance through the mechanism of osmotic adjustment. In most cases, osmolyte accumulation is insufficient to affect osmotic potential. The role of mannitol in salt tolerance remains unclear. However it is now thought to involve membrane stabilization or enzyme protection (Shen et al., 1997).

Tobacco plants transformed with sense construct of apple cDNA of sorbitol-6-phosphate dehydrogenase (S6PDH), a synthetic enzyme for sorbitol, were tested as to sorbitol has any function in boron (B) mobility (Tao et al., 1995; Brown and Hu, 1996; Brown et al., 1999). The results suggested that sorbitol had an influence in improving the transport of B and conferred some tolerance to B deficiency in tobacco plants. In 1998, once again, tobacco was transformed with a sense S6PDH gene, and surprisingly, plants with high levels of sorbitol were dwarfed and developed necrotic lesions in their leaves (Sheveleva et al., 1998). Most recently, Gao et al. (2001) produced sorbitol-forming Japanese persimmon (*Diospyros kaki*) plants using the same gene, and found sorbitol accumulation dwarfed plants and may have enhanced tolerance of photosynthetic processes to salt ($NaCl_2$) stress. These results are interesting, yet it remains unclear why *Prunus* species are notably the least salt tolerant of crop plants yet produce more sorbitol than virtually any other higher plant.

Research on the functions of polyols to date has been equivocal for two main reasons. First, most studies have been correlative in nature - i.e., polyol concentration is higher in stressed than non-stressed plants. There are always exceptions to these gross correlations. For example, mannitol accumulates during salt stress in celery (Briens and Larher, 1983), yet much higher levels of sorbitol occur in Rosaceaeous trees while they are among the least salt tolerant crop plants (Maas, 1987).

In addition, the stress tolerance demonstrated in laboratory studies has not translated into crop stress tolerance on an agronomic or horticultural basis. The second point refers to the transgenic plant studies using polyol-forming genes. While tobacco is an ideal species for such research, it does not naturally produce mannitol or sorbitol, and therefore lacks the entire suite of enzymes required for their synthesis, translocation, and degradation. Thus, tobacco is useful only for studying polyol accumulation, but not its other proposed functions. A better model would utilize a species that naturally contains high levels of polyol, and can be transformed readily.

Transformation of *Prunus* species has proven to be very difficult if not impossible with current transformation techniques. Therefore, some preliminary work was undertaken to provide support for alternative hypotheses concerning polyol function in *Prunus*, while simultaneously attempting to transform 'Brompton' plum, which is one of the few *Prunus* to have been successfully transformed (Scorza et al., 1995). This research examined the role of sorbitol metabolism in the regulation of photosynthesis.

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CHAPTER 2

REVIEW OF LITERATURE

A. Introduction

Polyols (sugar alcohols) are the single major carbohydrates present in many organisms such as fungi, algae and lichens (Toustar and Shaw, 1962; Bieleski, 1982; Lewis, 1984). They are classified under two major groups: i) the straight-chained, acyclic polyols or glycotols (i.e. sorbitol, mannitol, etc.), and ii) cyclic polyols or cyclitols (i.e. inositol, pinitol etc.) (Table 2.1). Polyols are chemically, physiologically and biologically very similar to the sugars to such extent that some of them (i.e. sorbitol, mannitol, xylitol, erythritol, maltitol, lactitol) are used as sweeteners in the food industry (Bieleski, 1982; Loesher, 1987).

Sucrose is formed as the first free sugar during photosynthesis in higher plants. Few plants (i.e. sugar cane, sugar beet, etc.) use sucrose as their main storage carbohydrate, exclusively (Keller, 1989). However, some higher plant species (i.e. *Prunus*) produce polyols along with sucrose as photosynthetic end products. Mannitol, sorbitol, galactitol, allitol, iditol, ribitol, xylitol and *myo*-inositol are the common polyols found in plants (Table 2.1).

Mannitol and sorbitol are the two major polyols with wide range of distribution across plant species. Therefore, in this review, we will focus on the occurrence, metabolism, possible functions, main experimental approaches taken so far, and some future prospects in studying the functions of polyols in plants.

B. Sorbitol and Mannitol

Sorbitol and mannitol are structurally quite similar except for the position of one OH group (see Table 2.1).

Sorbitol

Sorbitol was named following the isolation from a berry of mountain ash, a member of Rosaceae, *Sorbus aucuparia* in the late 1800s. Later, it was detected in coconut milk and widely used in plant tissue culture. The widespread usage of sorbitol in tissue culture brought up the idea that sorbitol was protecting the cell by either detoxifying the toxic compounds or maintaining the membrane integrity of the cell in culture (Kao and Michayluk, 1975).

Sorbitol is not detected in lower plants, however it is one of the major photosynthetic products of *Rosaceae* and *Plantaginaceae* families (Table 2.1) (Bieleski, 1982). The level of sorbitol found in higher plants varies greatly. It differs from species to species within a family, organ to organ within a plant, from season to season, and even changes diurnally (Plouvier, 1963, Bieleski, 1982; Escobar-Gutierrez and Gaudillere, 1994; Guitierrez and Gaudillere, 1996; Kleinschmidt et al., 1998; Wang et al., 1997; Abnasan-Bantog et al. 2000). Sorbitol is found in the mature leaves of economically important tree crops such as peach, plum, apricot, cherry, apple, quince, and pear in relatively high concentrations. Further discussion about sorbitol is in section D.

Mannitol

Mannitol is believed to be one of the most abundant polyols in nature (Table 2.1). It was first isolated from the manna (dried exudate) of the ash (*Fraxinus ornus*) in the early 1800s (Plouvier, 1963). Mannitol is widely used as food sweetener for diabetic patients (Toustar and Shaw, 1962). Mannitol is another polyol that constitutes a major product of photosynthesis along with sucrose in members of plant families such as

Oleceae (olive), Umbelliferae (celery) and Rubiaceae (coffee) (Bieleski, 1982; Loescher, 1987). The biosynthesis and catabolism of mannitol is thought to be well understood in plants (Fig. 2.2).

C. Proposed Functions of Polyols in Plants

There are many possible/proposed functions of polyols in plants, animals, humans, and common usages in the food, drug, and chemical industries (Fig. 2.1). The possible functions of polyols range from enzyme or protein stabilization in higher and lower organisms (Lozano et al., 1994; Salvucci, 2000) to the protection of plants from pathogen attack (Stoop et al., 1996). Various polyols (i.e. sorbitol, mannitol, and xylitol) are used as food sweeteners in food industry as well as de-toxicants in tissue culture (Kao and Michayluk, 1975). However, in this section, we will focus on the proposed functions of polyols in higher plants under the following main headings: i) *storage of reducing power*, ii) *translocation and storage,* iii) *osmoregulation,* iv) *cryoprotection* and v) *other possible functions*.

i) *Storage of reducing power*

Almost all polyols use NADPH as a hydrogen donor in order to reduce a sugar precursor to a polyol precursor (Lewis, 1984; Keller, 1989) (Fig. 2.3). The reduced energy by $NADP^+$ in source is later released during breakdown in the sink, as NADH or NADPH (Stoop et al., 1996). This may enhance photosynthesis and prevent photoinhibition via the usage of NADPH produced during the light reactions of photosynthesis in the chloroplast. The usage of "extra" NADPH in the cytosol of source leaf cells to create sorbitol may allow for less feedback of photochemical energy through the light reaction system when photosynthetic carbon reduction cannot proceed. Perhaps this is one of the main reasons that Rosaceous tree species produce sorbitol in excess of sucrose as their photosynthetic product (Lewis and Smith, 1967; Keller, 1989).

ii) *Translocation of carbon and storage*

Polyols have been reported to be the only translocated carbon forms other than sucrose in the phloem of polyol producing plants (Stacey, 1974). However, more recent analyses show small amounts of hexoses and oligosaccharides in the phloem as well. In early experiments with ^{14}C labeling, sorbitol was found to be synthesized in mature leaves of plum (Hensen and Ryugo, 1979) and apricot (Redgwell and Bieleski, 1978; Bieleski and Redgwell, 1985) and later translocated to developing leaves. Bielseki and Redgwell (1985) documented that in phloem sap of apricot, sorbitol comprised up to 75% of total soluble carbohydrates.

Polyols are proposed to function as storage carbohydrates just as sucrose and starch. This conclusion is made because some plant species have polyol content ranging from 5 to 90% of total soluble carbohydrates throughout the growing and dormant seasons (Wallaart, 1980; Loescher, 1987; Yamada et al., 1998). Thus by the sheer amount present, polyols act as temporary storage of carbon.

iii) *Osmoregulatory functions*

The possible osmoregulatory function of polyols is one of the most studied and speculated aspects of lower organisms, plants and animals. The substantial increase in polyol concentration of plants under environmental stresses (Briens and Larher, 1983; Hare et al., 1998; Lewis, 1984; Nguyen and Lamant, 1988; Vernon and Bohnert, 1992; Wang and Stutte, 1992; Kann et al., 1993; Popp and Smirnoff, 1995; Zhu et al., 1997) brought about the conclusion that polyols are compatible solutes and cause osmotic adjustment (Loescher, 1987). Experiments with celery under nutrient stresses showed that stressed plants accumulated mannitol via shutting down the catabolism in sinks, resulting in osmotic adjustment (Stoop and Pharr, 1994). Lo Bianco et al. (2000) documented similar results for sorbitol in drought experiments with ‘Nemaguard’ peach. These

authors found that 80% of the solute involved in osmotic adjustment was sorbitol accumulated due to the loss in activity of Sorbitol dehydrogenase (SDH), catabolic enzyme, in sinks. It has long been hypothesized that osmotic adjustment seems to occur due to passive accumulation of the solutes rather than active response to stress (Munns, 1988).

In recent years, experiments conducted with transgenic plants that artificially produce polyols (mannitol, sorbitol) showed that the osmotic adjustment was not the only mechanism of stress tolerance. It has been further speculated that stress tolerance in transgenic plants was due to the combination of other possible functions of polyols (i.e. protecting proteins/enzymes from harmful ions, stabilizing the membranes, and/or acting as chemical signals) (Karakas, 1997; Shen et al., 1999; Steinitz, 1999) (Fig. 2.1).

iv) *Cryoprotection*

In general, polyols and carbohydrates increase in plants under low temperature environments. This increase is proposed to be due to the low temperature induction of related genes in plants (Thomashow, 1999). In polyol producing plants, there is a significant accumulation of corresponding polyol under sub-freezing and freezing temperatures in winter (Sakai, 1961; Sakai and Yoshida, 1968).

Cryoprotection by polyols is proposed to be via solute accumulation and subsequently reduction in freezing point of water. (Sakai and Yoshida, 1968). Polyols have better freeze protection capabilities than other carbohydrates. However, the cryoprotection mechanism may be a combination of the functions of polyols such as freezing point depression, and stabilization of enzymes and membranes. (Sultanbawa and Li-Chan, 1999; Wimmer et al., 1997; Ford et al., 2000) (Fig. 2.4).

v) *Other proposed functions*

Polyols, undoubtly, have a diverse array of functions compared to some other biological compounds. Polyols could be perceived as chemical signals by the cells (Steinitz, 1999) on one hand, or as free-oxygen species quenchers on the other hand (Shen et al., 1997a; Shen et al., 1997b; Jennings et al., 1998; Nelson et al., 1998). Experiments with insects exposed to high temperatures caused significant increases both in sorbitol and mannitol accumulation (Hendrix and Salvucci, 1998; Salvucci et al., 1999; Salvucci, 2000). In plants under various stresses (salt, drought, even pathogen attack), the level of polyols increases significantly (Gorham and Wyn Jones, 1981; Kelavkar and Chatpar 1993; Stoop et al., 1996). Polyols are proposed to stabilize proteins, nucleic acids, and other biological structures of the cell, as well. (Wang et al., 1993; Wimmer et al., 1997; Kaushik and Bhat, 1998; Del Vecchio, 1999) (Figs. 2.1 and 2.4).

D. Various Approaches toward Studying the Metabolism and Functions of Sorbitol in Plants

1. Classic eco-physiological approaches

Common-garden

In the common garden approach, species with different sorbitol: sucrose ratios are collected from various habitats and grown under the similar environmental conditions (i.e. greenhouse). That is to minimize or eliminate differences due to environmental and document the differences in genotype only (Rieger and Duemmel, 1992; Abrams and Mostoller, 1995; Ogren and Sunding, 1996). In this approach, the various biochemical, physiological and morphological characteristics such as photosynthesis, leaf size and duration, enzyme activities and sorbitol levels are measured and cross correlation of these characteristics are established (Reich, 1997). Any significant correlation with sorbitol

found during these studies could be due to genotype and inferences can be made as to the function of sorbitol.

Pulse-chase labeling of the ^{14}C

In this method, labeled ^{14}C is fed via leaf during photosynthesis and metabolic enzymes of sorbitol and the partition of the labeled ^{14}C to sorbitol and is monitored (Hensen and Ryugo, 1979; Grant and Rees, 1981; Bieleski and Redgwell, 1985; Escobar-Gutierrez and Gaudillere, 1996; Wang et al., 1996; Beruter et al., 1997; Escobar-Gutierrez et al. 1998; Wang et al., 1999). Pulse-chase labeling of ^{14}C is used mostly to identify the metabolites and location of sorbitol rather than the function.

2. Molecular biological approaches

Genetic engineering/metabolic engineering

Plant transformation with foreign genes was established by utilizing the Ti-plasmid of *Agrobacterium tumefaciens* in early 1980s. Following a number of transformation experiments, Horsch et al. (1985) reported the first standard protocol for transforming plants with foreign genes via *Agrobacterium tumefaciens*. Since then, hundreds of plant species have been transformed with foreign genes following the modified protocol of Horsch et al. (1984). In the late 1980s, the first commercial product was engineered to down regulate polygracturonase enzyme via antisense mRNA technology, which was later patented as 'FLAVR SAVR' tomato (Sheey et al., 1988; Smith et al., 1988; Kramer and Redenbaugh, 1994). 'FLAVR SAVR' is the first known commercial product of plant genetic engineering.

Early in the 1990s, a new era of transgenic plant technology evolved, termed metabolic engineering (ap Rees, 1995; Dixon et al., 1996; Herbers and Sonnewald, 1996; Dixon and Arntzen, 1997; DellaPenna, 2001). The main approach in metabolic engineering is to manipulate plant metabolic pathways either to produce novel products

or suppress/overexpress the genes that encode for the key enzymes (Mol et al., 1994; Bourque, 1995; Smeekens, 1997; Taylor, 1998; Brisson et al., 1998; Nuccio et al., 1999; Bohnert and Shen, 1999; Chengappa et al., 1999; Wilke, 1999; Chotani et al., 2000; Rathinasabapathi, 2000; Shanks and Stephanopoulos, 2000; Strohl, 2001; Sato et al., 2001).

Metabolic engineering of sorbitol metabolism in studying functions of sorbitol in plants

Sorbitol metabolism is well defined in plants (see section E). The basic idea is to downregulate/overexpress the genes coding for either S6PDH at the source or SDH at the sink site. In either case, plants genetically engineered with different levels of sorbitol will probably be created as compared to untransformed controls.

This approach has been studied using tobacco as a model system. The experiments with tobacco have produced inconclusive results in explaining the function of sorbitol in plants. Tao et al. (1995) was the first to transform tobacco with sense and antisense cDNA of gene coding for S6PDH from apple. The results of this experiment were that plants transformed with sense construct of S6PDH produced sorbitol in tobacco whereas the ones transformed with antisense had no sorbitol detected. These transformed plants with sense construct were later used to test whether sorbitol has any function in boron (B) mobility (Brown and Hu, 1996; Brown et al., 1999). The results suggested that sorbitol had an influence in improving the transport of B and conferred some tolerance to B deficiency in tobacco plants. In 1998, once again, tobacco was transformed with a sense S6PDH gene, and surprisingly, plants with high level of sorbitol were dwarfed and developed necrotic lesions in their leaves (Sheveleva et al., 1998). Most recently, Gao et al. (2001) produced sorbitol-forming Japanese persimmon (*Diospyros kaki*) plants using the same gene, and found sorbitol accumulation dwarfed plants and may have enhanced tolerance of photosynthetic processes to salt (NaCl) stress. These results are interesting,

yet it remains unclear why *Prunus* species are notably the least salt tolerant of crop plants yet produce more sorbitol than virtually any other higher plant.

It would be more useful to up- or down-regulate sorbitol production in plants that produce sorbitol naturally, since several enzymes are involved in their synthesis, degradation, and translocation. *Prunus* species could be the perfect candidates, for those species have high levels of sorbitol compared to sucrose. However, plants of these species have very low efficiencies of foreign gene transformation. To date, no plants of any *Prunus* species have been transformed successfully to study sorbitol metabolism. We had several attempts to transform plum (*Prunus domestica*) with antisense and sense constructs of S6PDH cDNA from apple, however we have failed to produce transgenic plants due to low transformation efficiency, and recalcitrance in tissue culture, as have been reported with *Prunus* (Mante et al., 1991; Scorza et al., 1995; Birch, 1997).

E. Metabolism of Sorbitol in Plants

Sorbitol biosynthesis and catabolism is well established (Fig. 2.5) from early pulse/chase experiments. Sorbitol is synthesized mainly in mature leaves and transported to sinks via phloem by sugar carriers (Loescher et al. 1982; Gutierrez and Gaudillere, 1996; Marquat et al., 1997; Lalonde et al., 1999) (Fig. 2.5). Sorbitol metabolism in plants has two key enzymes; i) the synthetic one in source leaves, NADP+-dependent S6PDH, and ii) the catabolic one in sinks, NAD+ dependent SDH (Fig. 2.5). The S6PDH was first isolated from the loquat fruit (Hirai, 1979) and leaves (Hirai, 1981) and the gene that codes for the enzyme was later cloned from the apple fruit by Kanayama et al. (1992). The gene expression of S6PDH along with enzyme activities is higher in mature leaves (source) than in folded leaves (sink) of peach (Yamaki, 1980; Sakanishi et al., 1998). The activity of S6PDH was reported to be the highest in reducing glucose-6-phoshpahte to sorbitol-6-phosphate at pH range of 7.0 to 11.0 (Kanayama and Yamaki, 1993). NAD+

dependent SDH was first detected in apple callus (Negm and Loescher 1979) and later purified by Yamaguchi et al. (1994). NAD+ dependent SDH has optimum activity at narrower a range of pH, 9.0 to 9.5 as compared to a range of 7.0 to 11.0 for S6PDH. These two key enzymes are thought to be in close contact that is when there is little or no growth at the sink, activity of SDH dramatically decreases and sorbitol level builds up at the source, causing a decrease in S6PDH activity probably due to change in gene expression of corresponding genes (Jang and Sheen, 1997; Gibson and Graham, 1999; Sheen et al., 1999; Lo Bianco et al., 2000; Smeekens, 2000).

Sorbitol metabolism is quite dynamic in plants (Raese et al., 1978; Yamaki and and Ishikawa, 1986; Yamaki and Moriguchi, 1989; Yamguchi et al., 1996; Kanayama, 1998; Sakanishi et al., 1998). The level of sorbitol varies greatly depending upon the plant species, the time of the day/year and even the researcher. It is usually expressed as sorbitol: sucrose ratio. This ratio ranges from 1.5: 1.0 up to 95.0: 1.0 in *Prunus* species (Escobar-Gutierrez and Gaudillere, 1994; Moing et al., 1997).

F. Some Future Prospects

There have been several attempts in studying the role of polyols in plants using tobacco or yeast as model systems in single gene level (Tao et al., 1995; Sheveleva et al., 1997; Shen et al., 1997; Shen, et al., 1999; Brown et al., 1999). However, as stated above, transformation of organisms that do not produce sorbitol only reveals information on sorbitol accumulation. As previously mentioned, *Prunus* produces high amounts of polyol naturally. However, creating a transgenic model in *Prunus* will not be easy.

Making use of inducible promoters or engineering, existing promoters of the native genes via chimeriplasty (Yoon et al, 1996; Bartlett, 1998) technique could be another way of studying the polyol metabolism. Using inducible promoters via chemical

induction system can be used to study the function of the sorbitol at the gene expression level (Gatz, 1997; Zuo and Chua, 2000).

Sorbitol biosynthesis and catabolism could be studied at the level of gene expression via monitoring the amount of the message (mRNA) by genes that are critical in sorbitol and sucrose pathways. The messages of hundreds of different genes can be monitored simultaneously by using DNA microarray (chip) technology (Lemieux et al., 1998; Bird and Ray, 1991; Schena et al., 1995; Schaffer et al., 2000; Richmond and Somerville, 2000).

Most, if not all, of the studies have focused on the hypothesis that polyols confer protection against plant stresses. A few studies speculated that plants with high polyol levels have higher photosynthesis rates. However, the level of polyols and the photosynthetic correlations have never been thoroughly studied. In the following study, we analyzed photosynthesis in connection with sorbitol metabolism, gene regulation and enzyme activities. These could provide the backdrop for future studies of sorbitol on photosynthesis, photoinhibition, and movement of reducing power from source to sink.

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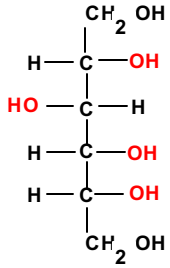
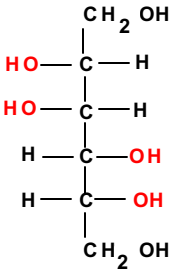
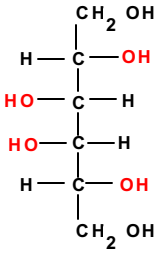
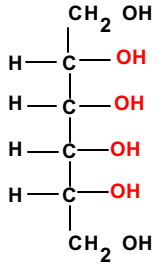
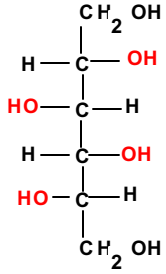
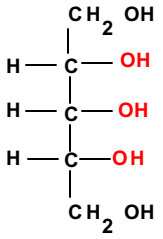
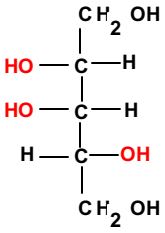
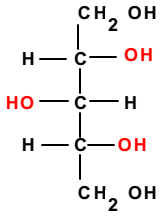
Table 2.1. The most common polyols found in nature.

	Polyol	Chemical structure	Sugar precursor	Natural source	Proposed function(s)	Reference
Acyclic Polyols (*Hexitols*)	**Sorbitol** (D-glucitol)		Glucose-6-P	○ Algae ○ Fungi (yeast) ○ Many higher plants esp. in *Rosaceae* ○ Silkworm ○ Animals	○ Storage and translocation of carbons ○ Osmoregulation ○ Cryoprotection ○ Protecting enzymes and enzyme integrity ○ Increase in Boron uptake	Bieleski, 1982 Lewis and Smith, 1967 Loecher, 1987 Touster and Shaw, 1962 Taylor et al., 1995 Storey, 1997 Ranney et al., 1991 Brown et al., 1999
	Mannitol (D-mannitol)		Fructose-6-P	○ Most abundant polyol in nature ○ Bacteria (*L. arabinosus*) ○ Algae ○ Grasses ○ Lichens ○ Many higher plants	○ Short term storage translocation of carbons ○ Osmoregulation ○ Stress tolerance in plants (i.e. salt, freezing, and drought) ○ Pathogen resistance in plants	Bieleski, 1982 Tarczynski et al., 1993 Karakas et al., 1997 Loescher, 1987 Stacey, 1974 Stoop et al., 1996 Touster and Shaw, 1962 Lewis and Smith, 1967

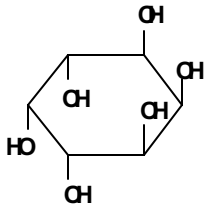
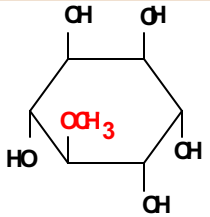
(Table 2.1. continued)

Acyclic Polyols *Hexitols*) (*Hexitols*)	**Galactitol** (Dulcitol)		Galactose	○ Two red algae ○ Fungi (yeast) ○ Higher plants ○ Honeydew from insects living on *Melampyrum* species	○ Osmoregulation ○ Storage and translocation of carbons ○ Cryoprotection?	Bieleski, 1982 Stacey, 1974 Touster and Shaw, 1962 Plouver, 1963 Lewis and Smith, 1967 Taylor et al., 1995
	Allitol (allodulcitol)		D-Allulose	○ Higher plants *Sacrifragaceae* (*Itea ilicifolia* and *Itea virginiana*	○ Osmoregulation? ○ Cryoprotection?	Bieleski, 1982 Plouver, 1963 Stacey, 1974 Touster and Shaw, 1962 Lewis and Smith, 1967
	Iditol (sorbierite)		Sorbose	○ Higher plants Berry of mountain ash *Rosaceae* (*Sorbus aucoparia*)	○ Osmoregulation? ○ Cryoprotection?	Bieleski, 1982 Plouver, 1963 Stacey, 1974 Touster and Shaw, 1962 Lewis and Smith, 1967

(Table 2.1. continued)

Acyclic Polyols (*Pentitols*)	**Ribitol** (Adonitol)		Ribose-5-P	○ Algae ○ Lichens ○ Major pentitols in higher plants	○ Constituent of riboflavin and related enzymes ○ Cryoprotection? ○ Osmoregulation	Lewis and Smith,1967 Bielseki, 1982 Keller, 1989 Touster and Shaw, 1962 Stacey, 1974
	D-Arabitol (D-Arabinitol)		D-Xylulose	○ Fungi (yeast) ○ Lichens ○ Urine of human	○ Long term carbon storage in fungi ○ Osmoregulation ○ Cryoprotection	Bieleski, 1982 Lewis and Smith, 1967 West, 1994
	Xylitol		Xylulose	○ Fungi ○ Intermediate for metabolism of *myo*-inositol in animals ○ Rat lens	○ Osmoregulation ○ Cryoprotection?	Bielseki, 1982 Lewis and Smith, 1967 Stacey, 1974

(Table 2.1. continued)

Cyclic Polyols	***myo*-Inositol**		Glucose-6-P	○ Almost all of the plant species	○ Osmoregulation ○ Stress tolerance in plants ○ Storage and translocation of carbons in plants ○ Cryoprotection?	Plouver, 1963 Stacey, 1974 Leowus and Dickinson, 1982 Nelson et al., 2000 Klages et al., 1999
	Pinitol		Glucose-6-P	○ Most abundant cyclitols in plants ○ Six families of Gymnosperms ○ Thirteen families of Angiosperms	○ Osmoregulation ○ Stress tolerance in plants ○ Cryoprotection?	Plouver, 1963 Vernon et al., 1993 Leowus and Dickinson, 1982 Nguyen and Lamat, 1988 McManus et al., 2000 Nelson et al., 2000 Guo and Oosterhuis, 1995 Popp and Smirnoff, 1995

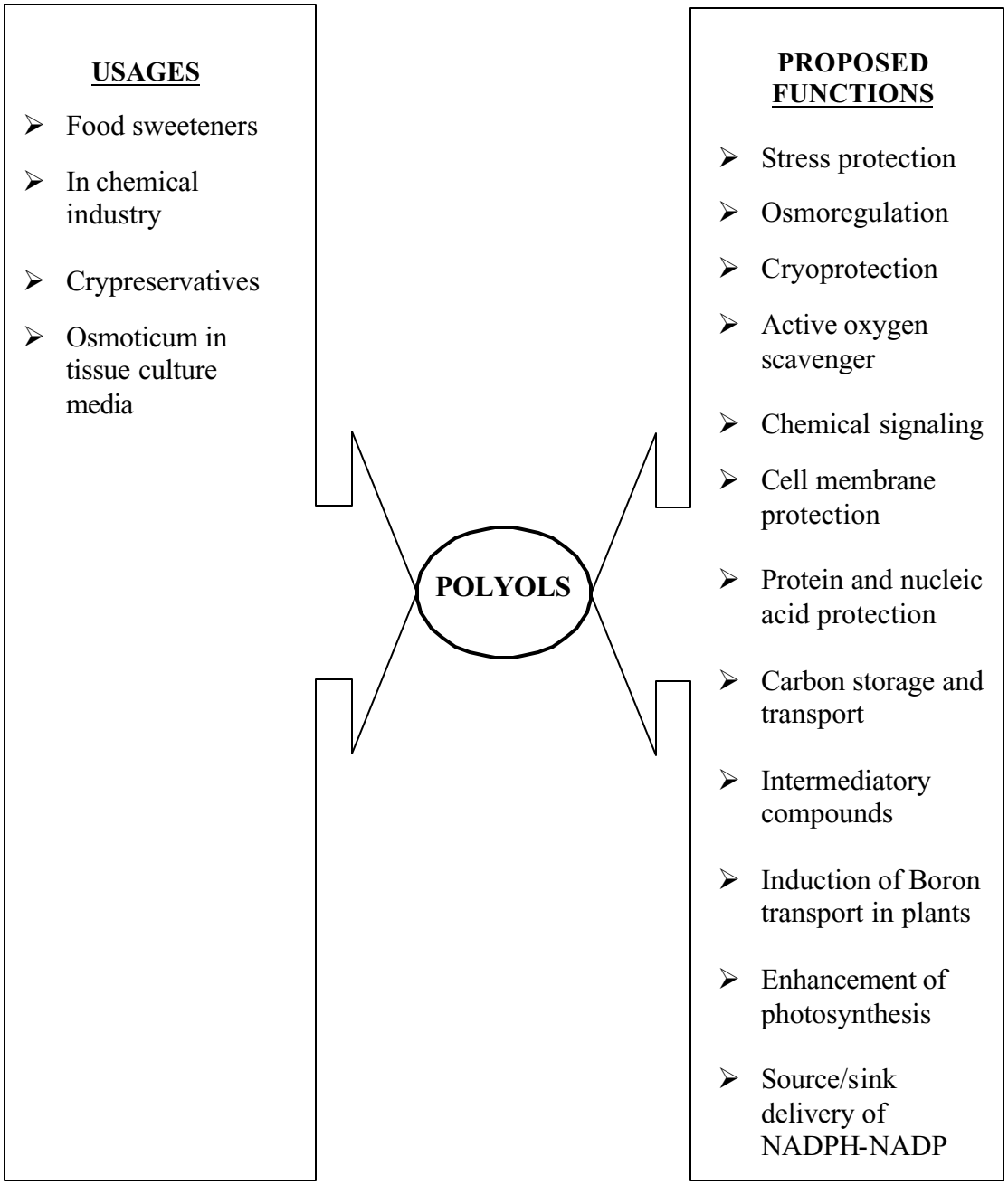
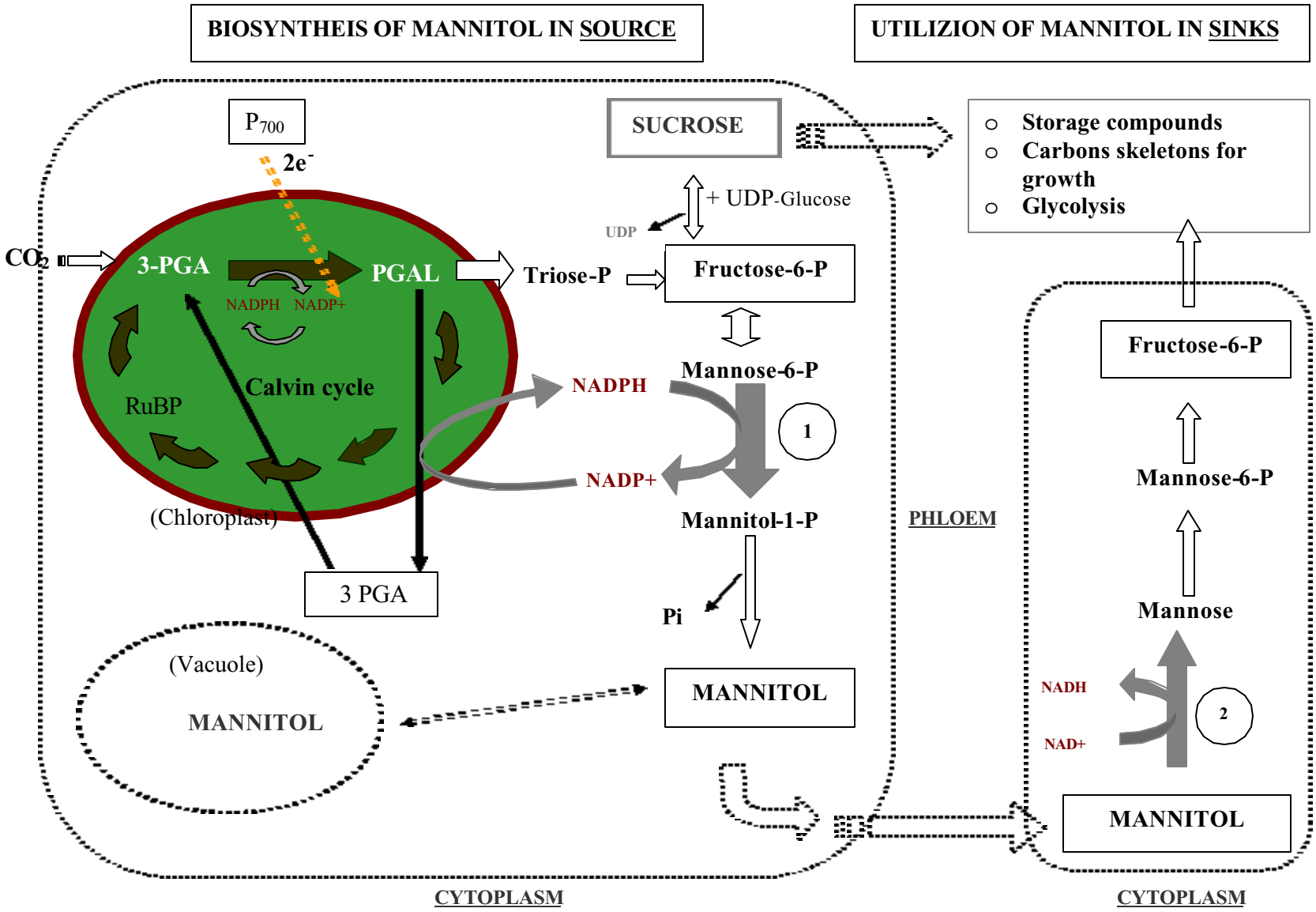


Figure 2.1. A list of usages and proposed functions of polyols across different science disciplines.

Figure 2.2. Mannitol synthesis, transport and catabolism in plants. Two major reactions and their respective enzymes that are critical at source and sink sites: (1) mannose-6-P reductase (M6PR), (2) mannitol dehydrogenase (MDH).


BIOSYNTHEIS OF MANNITOL IN SOURCE
UTILIZION OF MANNITOL IN SINKS
P700
2e-
CO2
3-PGA
PGAL
NADPH NADP+
RuBP
Calvin cycle
(Chloroplast)
3 PGA
SUCROSE
+ UDP-Glucose
UDP
Triose-P
Fructose-6-P
Mannose-6-P
NADPH
NADP+
1
Mannitol-1-P
Pi
MANNITOL
(Vacuole)
MANNITOL
PHLOEM
Storage compounds
Carbons skeletons for growth
Glycolysis
Fructose-6-P
Mannose-6-P
Mannose
NADH
NAD+
2
MANNITOL
CYTOPLASM
CYTOPLASM

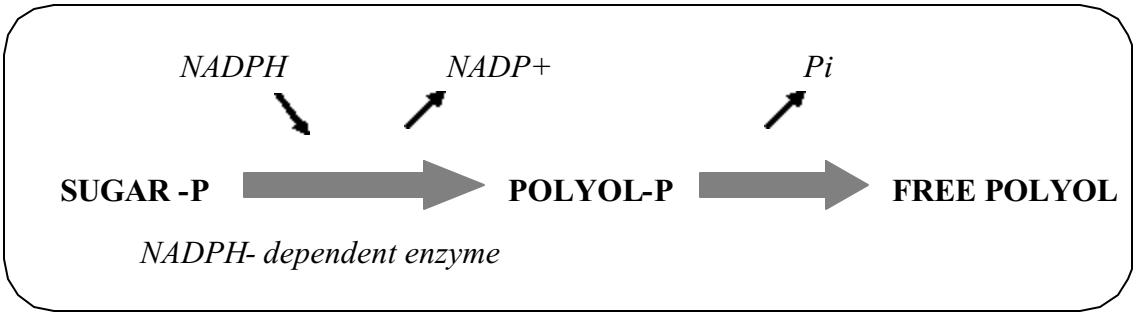


Figure 2.3. The general description of polyol metabolism in plants.

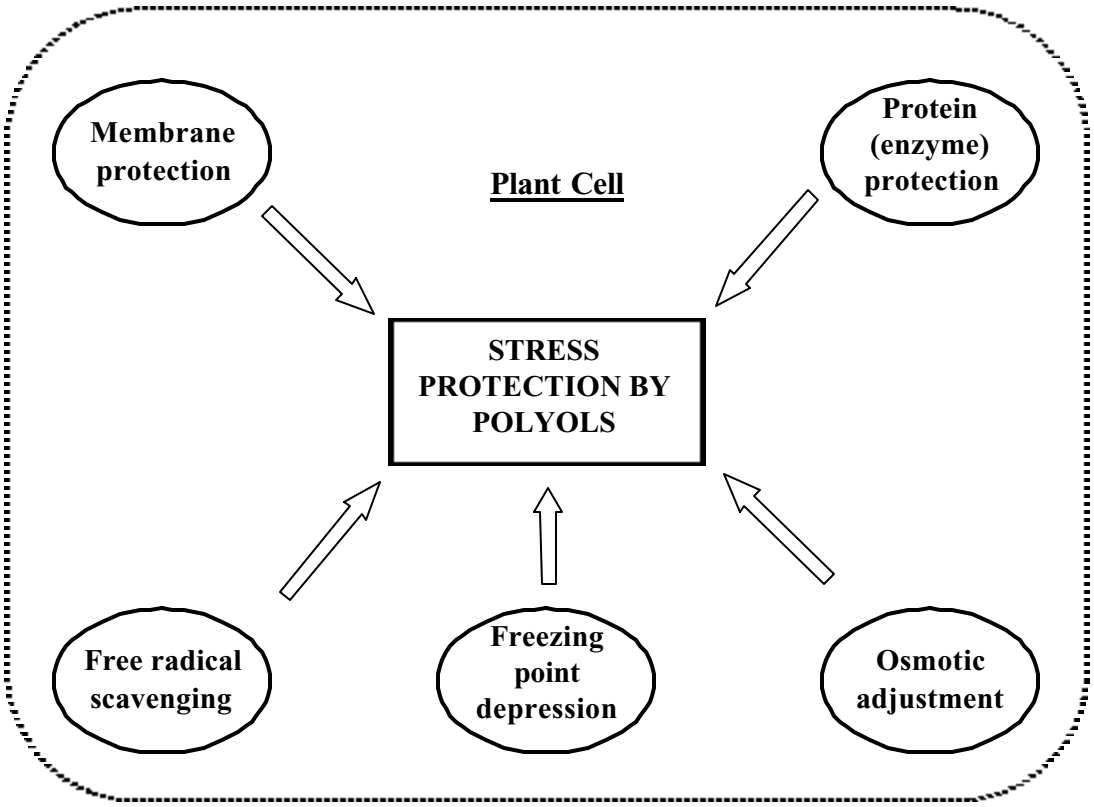
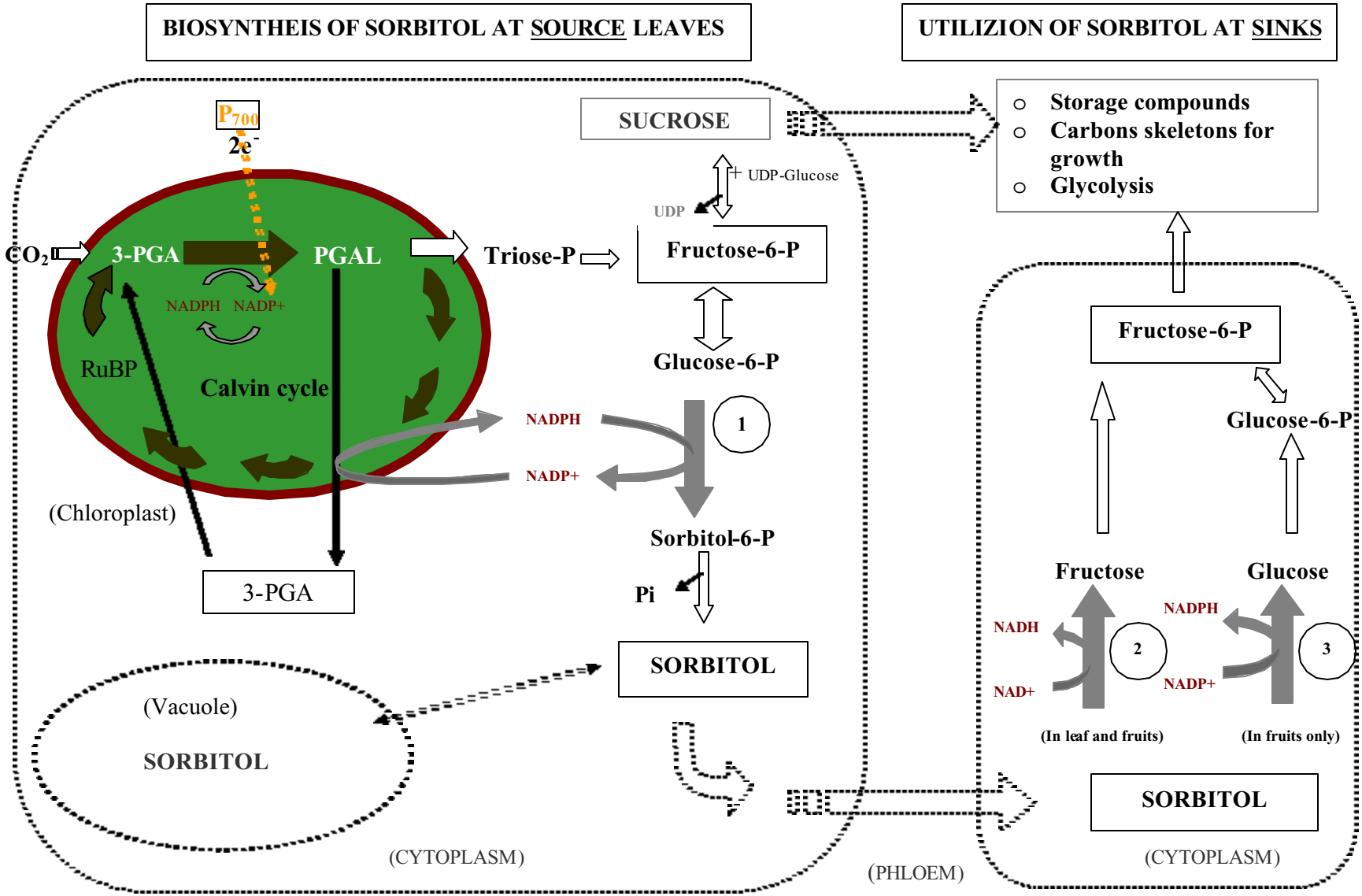


Figure 2.4. The possible protection mechanism by polyols to plant biotic and abiotic stresses.

Figure 2.5. Sorbitol synthesis, transport and catabolism in *Rosaceae* tree species. Three major reactions and their respective enzymes that are critical at source and sink sites: (1) $NADP^+$-dependent sorbitol-6-P dehydrogenase (S6PDH), (2) NAD^+-dependent sorbitol dehydrogenase (SDH) and (3) sorbitol dehydrogenase (SDH) or sorbitol oxidase (SOX).


BIOSYNTHEIS OF SORBITOL AT SOURCE LEAVES
UTILIZION OF SORBITOL AT SINKS
P700
2e-
CO2
3-PGA
PGAL
NADPH NADP+
RuBP
Calvin cycle
(Chloroplast)
3-PGA
Triose-P
SUCROSE
UDP-Glucose
UDP
Fructose-6-P
Glucose-6-P
NADPH
NADP+
1
Sorbitol-6-P
Pi
SORBITOL
(Vacuole)
SORBITOL
(CYTOPLASM)
(PHLOEM)
Storage compounds
Carbons skeletons for growth
Glycolysis
Fructose-6-P
Glucose-6-P
Fructose
Glucose
NADH
NAD+
2
NADPH
NADP+
3
(In leaf and fruits)
(In fruits only)
SORBITOL
(CYTOPLASM)

CHAPTER 3

PHOTOSYNTHETIC CHARACTERISTICS OF EIGHT PRUNUS SPECIES AND NADP–DEPENDENT SORBITOL-6-PHOSPHATE DEHYDROGENASE[1]

[1]Karakas, B., R. Lo Bianco, and M. Rieger. To be submitted to *Tree Physiology*.

Abstract

It has been hypothesized that formation of sorbitol, a major polyol, in mature, exporting leaves of Rosaceous tree fruits may be partially responsible for the relatively high photosynthetic capacity observed in these species compared to trees that do not form polyols. To test this hypothesis, greenhouse-grown plants of eight *Prunus* species (*P. fasciculata, P. persica, P. maritima, P. fremontii, P. cerasifera x P. munsoniana* 'Marianna', *P. havardii, P. mexicana,* and *P. ilicifolia*) having various sorbitol: sucrose ratios and photosynthetic rates were studied to determine whether a systematic relationship between photosynthetic potential and sorbitol content exists. Photosynthesis was measured at varying CO_2 levels under laboratory conditions, along with leaf sorbitol content and sorbitol forming capacity, i.e., the specific activity of the primary sorbitol forming enzyme, sorbitol-6-phosphate dehydrogenase (S6PDH). We found a negative correlation between photosynthesis, at both ambient and elevated CO_2, and S6PDH enzyme activity among the eight species. Similarly, carboxylation efficiencies and CO_2 compensation points also were negatively correlated with S6PDH activity. However, there was no correlation between sorbitol content and photosynthetic parameters or S6PDH enzyme activity. Average leaf sizes of these species were positively correlated with the aridity indexes (precipitation minus evapotranspiration) of the species original habitats. The negative relationship between S6PDH activity and photosynthetic potential, and the lack of relationship between sorbitol content or sorbitol: sucrose ratio and photosynthetic rate do not support the theory that sorbitol production enhances photosynthesis in *Prunus*.

INDEX WORDS: *Prunus* species, sorbitol, polyols, photosynthesis, sorbitol-6-phosphate dehydrogenase.

Introduction

Sucrose is the major end product of photosynthesis in higher plants, and the predominant sugar used to transport carbon throughout the plant. However, Rosaceous tree fruits are rather unique in that the polyol sorbitol is the main photosynthetic end-product and form of carbon translocated, and is often present at concentrations several-fold higher than sucrose. The Rosaceae contains several economically important species, including apple (*Malus*), pear (*Pyrus*), and stone fruits such as peach (*Prunus*). Within this family, *Prunus* species have the highest sorbitol contents, and can be a good model for studying the function of sorbitol in photosynthesis and carbon translocation. Polyols are undoubtedly involved in carbon storage and translocation in plants, although the link between polyols and photosynthesis has not been studied thoroughly. Sorbitol constitutes up to 10% of the fruit fresh weight and more than half of leaf nonstructural carbohydrates in members of the Rosaceae (Loescher 1987). Also, sorbitol accounts for 9% of leaf dry weight in *Plantago maritima* (Briens and Larher 1983), and mannitol accounts for 10% of the dry weight of celery leaves (Fellmann and Loescher 1987).

Using polyols for translocation of carbon instead of sucrose may confer certain advantages. First, the loading of 6-carbon polyols into the phloem would cause the same decrease in osmotic potential of the sieve elements as loading twice as much (12-carbon) sucrose. The concomitant pressure gradient in the phloem, and thus the potential rate of movement of the phloem sap could be greater for polyol-forming species. Efficient phloem transport of photoassimilate may be important for Rosaceous trees which partition a large part of their annual carbon gained toward fruit (Miller and Walsh, 1988). Second, more efficient carbon use in sink tissues may result from transporting polyols instead of sucrose, since a NADH molecule is generated at the sink from oxidation of

polyol back to hexose, giving a higher net ATP yield than the catabolism of an equal amount of sucrose (Escobar-Gutierrez and Gaudillere, 1996; Stoop et al., 1996).

Thus, species that generate and translocate large amounts of carbon may have higher efficiency in these tasks when using polyols instead of sucrose. Many polyol-forming plants, particularly tree fruits, have high photosynthetic rates compared to plants forming only sucrose (Flore and Lakso, 1989; Stoop et al., 1996). One theory suggests that polyol synthesis in source leaves increases NADP-NADPH turnover compared to plants that exclusively form sugars. This stems from the extra NADPH derived from the photosynthetic light reactions that is used in the cytosol to reduce hexose-P to alditol-P (Stoop et al., 1996). In addition, polyol synthesis may provide an additional cytosolic sink for photosynthetically fixed CO_2 in mesophyll cells, reducing the potential for feedback inhibition of photosynthesis by end-product accumulation in chloroplasts (Fig. 3.1). In either case, we might expect a positive correlation between the capacity to form polyols and photosynthetic characteristics, if polyols play a role in the photosynthetic process. Photosynthesis is known to vary among *Prunus* species (Rieger and Duemmel, 1992), as does the ratio of sorbitol: sucrose in exporting leaves (Escobar-Gutierrez and Gaudillere, 1994; Moing et al., 1997). However, it is unknown whether sorbitol content and/or the capacity to produce it correlate with photosynthesis as theory might predict. Here, we performed a survey of the photosynthetic characteristics of eight *Prunus* species and correlated these with sorbitol content and the activity of the primary sorbitol-forming enzyme sorbitol-6-phosphate dehydrogenase (S6PDH) in exporting leaves. The objective was to determine whether photosynthetic characteristics vary systematically with preference for sorbitol as the photosynthetic end product.

Materials and Methods

Plant Material and experimental design

Seeds of six *Prunus* species native to North America were collected in 1995, and plants were grown in 10-liter containers under greenhouse conditions for four years in Athens, Ga (34°N, 85°W). Additionally, two commercially important species, 'Nemaguard' peach and 'Marianna' plum were included with the six native species to obtain a range of plant adaptation and morphology. Nomenclature and some characteristics of the eight species are presented in Table 3.1. 'Nemaguard' and 'Marianna' plants were grown from rooted cuttings for one year, since these plants are fast growing and retain mature characteristics when propagated in this manner. All plants displayed mature leaf characteristics and were comparable in size when measurements were made in the spring of 2000. Only those plants exhibiting active growth (i.e., shoot extension) were used for measurements, since preliminary observations suggested that photosynthesis was strongly affected by sink activity in all plants. Five to seven plants per species were used for measurements, depending on availability. A measure of aridity was calculated for all species except 'Marianna' plum by subtracting annual evapotranspiration (mm) from annual precipitation (mm) for the sites where seed were collected or plants are native (climatic data reported in Kahrl, 1979 and Korzoun et al., 1977). This could not be done for 'Marianna' since it is an interspecific hybrid derived from European and North American parentage.

CO_2 assimilation characteristics of leaves

Carbon dioxide assimilation (A) was measured under laboratory conditions using a LI-6200 portable photosynthesis device outfitted with a ¼-liter chamber (LI-COR, Lincoln, NE). One leaf per plant per species was used for photosynthesis measurement. Measurement conditions were: saturated photon flux density (1800-2200 μmol m^{-2} sec^{-1}) from a 400 W metal halide lamp, temperature of 22-24°C, and relative humidity of 40-60%. A-C_i curves were constructed by measuring A at several different CO_2

concentrations ranging from 100-1200 ul/l. Leaves or portions thereof enclosed in the ¼-liter chamber were located on shoots exhibiting active growth, and were selected from among the most recently fully expanded and matured foliage on the shoot. Plants were acclimated to the laboratory environment for a day prior to measurement of A. Curves were fit to data using an asymptotic function: $A_{360} = A_0+Amax*(1-exp(-b* C_i))$, which in turn was used to calculate assimilation rate at ambient (A_{360}) and saturating CO_2 (A_{max}) for each individual plant. In this equation, A_{360} represents photosynthesis at ambient CO_2 (360 ppm), A_0 is the initial photosynthesis, b is the degree of curvilinearity of the A-C_i curve, and C_i is intercellular CO_2 at time of the measurement. Carboxylation efficiency was estimated as the slope of the first 5 points of the A-Ci curves where the relationship was linear. Carbon dioxide assimilation rate was calculated on a leaf area basis and expressed in µmol CO_2 m^{-2} s^{-1}.

S6PDH Enzyme assay

The activity of the main enzyme of sorbitol synthesis in exporting leaves, S6PDH, was measured to correlate photosynthetic performance with the in-vitro propensity for sorbitol synthesis. Activity was assayed following the protocol developed for sorbitol dehydrogenase (SDH) by Lo Bianco et al. (1998). Three leaves similar to ones used for photosynthesis measurements per species were sampled in early morning for enzyme activity analysis. Approximately 30 to 50 mg of fresh leaf tissue was collected from leaves of similar age and position as those used to measure A. Tissue was homogenized in 2.0 ml of extraction buffer (100 mM Tris at pH 9.0, 8% v/v glycerol, and 20 mM 2-mercaptoethenol) using a pre-chilled mortar and pestle in the presence of washed sand. Tween-20 (0.1% v/v) and polyvinylpolypyrolidine (PVPP, 1% w/v) were also added during homogenization of the tissue. The enzyme was assayed in a total volume of 1ml assay buffer (50 µl of extract, 100 mM Tris at pH 9.0, 0.11 mM NADPH and 50 mM

glucose-6-phosphate) by measuring the change in optical density at 340 nm at 25 ^{o}C on a spectrophotometer, Spectronic 2-D (Milton Roy, Rochester, NY). Tissue protein content was determined by following the method by Bradford (1975) and enzyme specific activity was calculated and expressed as nanomoles of NADPH oxidized per minute per mg protein extracted.

Sorbitol and non-structural carbohydrates

Non-structural carbohydrates were quantified in leaf tissue using gas chromatography (Rieger and Marra, 1994). Leaves similar to those used for enzyme activity assay were sampled at four different times of day -7:30 AM (sunrise), 10:30AM, 2:00 PM, and 7:00 PM (sunset)- within a few days of returning plants to the greenhouse following A measurement. Sampling at different times of the day was done to document the diurnal variation in sorbitol and non-structural carbohydrates. Five leaves per time per plant per species were sampled for carbohydrate analysis. Leaves were stored at –20 ^{o}C until analysis. Frozen leaf tissue of 0.1-0.2 g was ground in a mortar and pestle in the presence of 1.5-3.0 ml of 80 % (v/v) methanol and 0.22 mg of phenyl-β−D-glucopyranose as an internal standard. The homogenate was centrifuged for 3 min at 3900 g, and a 1 ml supernatant was collected and stored at 4 ^{o}C for soluble carbohydrate and sorbitol analysis. The remainder of the supernatant was discarded, and the pellet was washed and centrifuged again with 80% methanol; this supernatant was also discarded. Three ml of deionized water were added, and tubes were then placed in a water bath at 100^{o}C for 1 h to gelatinize the starch. One ml of water, 1 ml of acetate buffer (pH 4.8), and 0.1 ml of amyloglucosidase enzyme solution (7 U) were added, and tubes were placed at 55^{o}C for 24 h. Afterwards, 5ml of internal standard was added (xylitol, 0.2 mg in 1 L of H_2O) and 1.0 ml of the supernatant was stored at –20 ^{o}C until analyzed for the glucose resulting from starch hydrolysis. Samples (100µl) were dried in GC vials and

derivatized with 70 µl BSTFA / DMF mix (1:1, v/v) for injection on a Hewlett Packard 5890A gas chromatograph (Avondale, Pa).

Specific Leaf Area and area per leaf

Specific leaf area (SLA), the area per unit leaf dry weight, was measured in order to document the variation in leaf characteristics and correlate with photosynthesis, S6PDH enzyme activity, and sorbitol content. For each species, 45-50 leaves were sampled and the area was measured using an LI-3000 area meter (LI-COR, Lincoln, NE). Leaves were then transferred to an oven, dried to constant weight at 70°C, and weighed. Area per leaf for each species was also quantified from this same leaf sample.

Drought Experiment

Two of the 8 *Prunus* species, *P. persica* and *P. havardii* were selected for drought study. For each species, 7 greenhouse-grown plants were divided into 2 groups of which three plants well-watered and other 4 plants were supplied with 50% of pot evapotranspiration from well-watered plants. The evapotranspiration was determined by weighing well-watered plants every day and subtracting subsequent daily measurements from each other. The total sample size was for *P. persica,* 8 well watered shoots, 8 stressed shoots and for *P. havardii* 6 well watered shoots and 6 stressed shoots. The drought experiment was undertaken starting May 8, 2001 and lasted for 18 days. These two species were selected for drought study because they are adapted to contrasting habitats (hydric and xeric, respectively) (Table 3.1). The objective of this study was to measure the photosynthesis and S6PDH enzyme activities of these two species under drought stress. Leaf photosynthesis measurements were conducted in the greenhouse at 20-25 ^{o}C, 50-60% relative humidity, and full sunlight (1500-1800 µmol m^{-2} sec^{-1} photon flux densities). Mature leaves at the 5^{th} to 8^{th} visible node from the apex were used for photosynthesis and S6PDH enzyme activity measurements. The same leaf was used for

photosynthesis and S6PDH enzyme activity assays. Unless stated otherwise, the plant material and measurement techniques used for this experiment were the same as the other experiments in this work.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and simple correlation using Sigma Stat (SPSS Inc., Chicago, IL). Tukey's studentized comparison was used to separate means when ANOVA results were significant. When data were not normally distributed, non-parametric methods were used to perform means separation or data were transformed.

Results

Photosynthetic characteristics

Maximum CO_2 assimilation rate at saturating CO_2 (A_{max}) and ambient CO_2 (A_{360}) of the eight species fell into two groups (Table 3.2), with *P. fasciculata, P. persica,* and *P. maritima* having higher rates than 'Marianna', *P. havardii, P. mexicana,* and *P. ilicifolia* (Table 3.2). Ambient and A_{max} of *P. fremontii* was statistically similar to all other species. There were no correlations between assimilation rates and SLA(Table 3.3), leaf size (area per leaf) or water balance of the native environment (precipitation – evapotranspiration, ET), indicating that photosynthetic capacity was not linked to leaf morphology or climatic adaptation.

Carbon dioxide compensation points ranged between 75 and 115 (µl CO_2 per l air) for all species and were not statistically different among species (Table 3.2). However, carboxylation efficiencies varied over two-fold across the species tested, and were proportional to assimilation capacities with substantial overlap occurring among species (Table 3.2).

Area per leaf and specific leaf area (SLA)

Area per leaf differed over 30-fold among species, with peach having among the largest leaf size and the desert species *P. fasciculata, P. fremontii,* and *P. havardii* having the smallest leaves (Table 3.3). However, SLA, a measure of leaf thickness, was less variable, with *P. persica*, 'Marianna' plum, and *P. fasciculata* having the thinnest leaves and *P. ilicifolia* the thickest (Table 3.3). However, only area per leaf was positively correlated with water balance (precipitation-ET) (Fig. 3.2).

Carbohydrates and sorbitol content

The species grouped in two in terms of daily average leaf sorbitol contents whereas the average leaf sucrose tended to be more variable across the species (Table 3.4). Only trace amounts of sucrose were detected (i.e., 0.0-0.23 mg/g FW) in *P. mexicana* leaves. *P. maritima*, *P. havardii, P. fremontii, and P. ilicifolia* had significantly higher average leaf sorbitol content than *P. fasciculata, P. persica* and *P. mexicana* (Table 3.4). Species were grouped in to three in their leaf starch contents (Table 3.4).

Having almost no sucrose in its leaves, *P. mexicana* had the highest sorbitol: sucrose ratio (94.0) among the species studied (Table 3.4). Sorbitol: sucrose ratios across the species ranged from 1.7 in *P. havardii* to 94.0 in *P. mexicana*. The leaf sorbitol and sucrose contents were usually low early in the morning and increased with time until sunset (Fig. 3.3a and b).

There was a statistically significant diurnal change in sorbitol contents and sorbitol: sucrose ratios as well (Fig. 3.4). Sorbitol: sucrose ratios were lowest in the morning and the highest around sunset for almost all the species studied (Fig. 3.4).

S6PDH enzyme activity

There was a 11-fold difference in the specific activity of S6PDH, the main enzyme of sorbitol synthesis in source leaves among the eight species studied (Fig. 3.5).

However, activities were highly variable within a given species, thus few significant differences were noted. *P. mexicana* and *P. ilicifolia* had higher activities than *P. maritima,* yet all other species were similar.

A_{max} and A_{360} were negatively correlated with S6PDH enzyme activity (Fig. 3.6). Similarly, carboxylation efficiencies and CO_2 compensation points also correlated negatively with S6PDH activity (Fig. 3.7), which was expected since assimilation rates were generally correlated with carboxylation efficiency and compensation points. The r^2 values were rather low in all cases, indicating that only 55-59% of the variation in photosynthetic characteristics could be accounted for by S6PDH activity. These negative correlations suggest that photosynthesis may be negatively affected by the capacity to form sorbitol.

There was no significant correlation between leaf sorbitol or sucrose contents and A_{max} or A_{360}, or between leaf sorbitol or sucrose contents and S6PDH activity across species (data not shown). Nor was sorbitol: sucrose ratio correlated with S6PDH enzyme activity (data not shown).

Drought Experiment

P. persica and *P. havardii* watered at 50% the rate of well-watered plants had a significant drop in their photosynthetic capacities (Table 3.5). All photosynthetic measurements were performed at similar Ci levels, eliminating possible stomatal limitation effect. However, only the S6PDH enzyme activity of the *P. persica* decreased significantly while that of *P. havardii* had high variation and did not decrease under drought stress (Table 3.5).

Discussion

We used eight *Prunus* species to provide a spectrum of leaf morphologies, climatic adaptation, photosynthetic characteristics, sorbitol and sucrose contents (ratios),

and in vitro S6PDH enzyme activities. Our preliminary observations of leaf longevity showed that leaves of species such as *P. fasciculata* leaves which have high photosynthetic rate were relatively short-lived and quick to abscise under drought stress, whereas *P. ilicifolia* (low A) survived for well over a year (data not shown). This supports the idea that these species are eco-physiologically diverse (Reich, 1997). In terms of sorbitol: sucrose ratios (1.7 to 94.0) the species studied were quite variable as well (Fig 4). Thus, if sorbitol synthesis were positively related to photosynthesis as hypothesized (Loescher, 1987), this data set should reveal such a correlation. However, photosynthesis and S6PDH activities were negatively correlated across these species, suggesting the opposite of what has been hypothesized (Flore and Lakso, 1989; Loescher, 1987; Stoop et al., 1996).

The negative correlation between photosynthesis (both A_{360} and A_{max}) and S6PDH enzyme activity (Fig. 3.6) indicates that *Prunus* species with higher photosynthetic rate tend to have relatively low sorbitol-forming capacity. While correlation does not imply a cause-effect relationship, one possible explanation might involve NADPH production during light reactions of photosynthesis and utilization in Calvin cycle and sorbitol synthesis reaction. The proposed link between polyol synthesis and photosynthesis centers on NADPH produced by the light reactions. In the Calvin cycle, conversion of 1,3-bisphosphoglycerate to glyceraldehyde 3-phosphate normally utilizes NADPH. Polyol synthesis also requires NADPH in the cytosol, thus provides a release for excess NADPH by oxidation of glyceraldehydes 3-phosphate to 1,3-bisphosphoglycerate in the cytoplasm (Fig. 3.1). This may be useful under high light conditions that could lead to photoinhibition unless NADPH is utilized in some way other than photosynthetic carbon reduction. Thus, polyol synthesis could prevent down-regulation of photosynthesis by photoinhibition by consuming excess NADPH. Our

results might suggest that there is a competition for, not an excess of, NADPH, such that high S6PDH activity reduces the pool of available NADPH in the mesophyll cells cytoplasm thereby depleting substrate (i.e. glyceraldehyde 3-phosphate) available to the Calvin cycle (Fig. 3.1). The second way in which polyol synthesis is proposed to enhance photosynthesis is by preventing feedback inhibition of photosynthesis by starch accumulation (Stoop et al., 1996). Sorbitol synthesis would provide an additional cytosolic sink for photosynthetically fixed CO_2 and therefore reduce the temporary storage of photosynthate as starch in chloroplasts. However, our data revealed no negative correlation of A with photosynthetic end-product and carbohydrate contents (sucrose, sorbitol, or starch) across species, indicating that feedback inhibition of photosynthesis did not occur under the conditions of this study.

In the experiment with two species, we found behavior during water stress to be different than that of well-watered conditions. It is possible that these species in their original habitats are subject to drought stress during the course of their growing season and have higher S6PDH enzyme activities as compared to the normal greenhouse environment. Under mild drought stress, the photosynthesis of both *P. persica* and *P. havardii* significantly dropped (Table 3.5). Unlike *P. persica*, S6PDH activity of *P. havardii* was not affected.

Lack of correlation between sorbitol content and A also does not support the theory that sorbitol production enhances photosynthesis. However, sorbitol content is often not correlated or poorly correlated with activities of its synthetic or catabolic enzymes (Lo Bianco et al. 2000). Lack of correlation between sorbitol content and S6PDH activity was not surprising, since the amount of sorbitol in leaves at any given time depends not only on synthesis rate, but also on the rate of export and the extent of sink utilization of sorbitol.

Using the "common garden" approach to this question, we do not find evidence to support Loescher's (1987) theory. However, correlation does not imply cause-effect, and large variations of measured parameters within a species prevent firm conclusions from being made. Future studies should focus on a single species to eliminate genetic variation, and therefore utilize methods of imposing variation in photosynthesis and consequently enzyme activity rather than surveying natural variation in a plant population.

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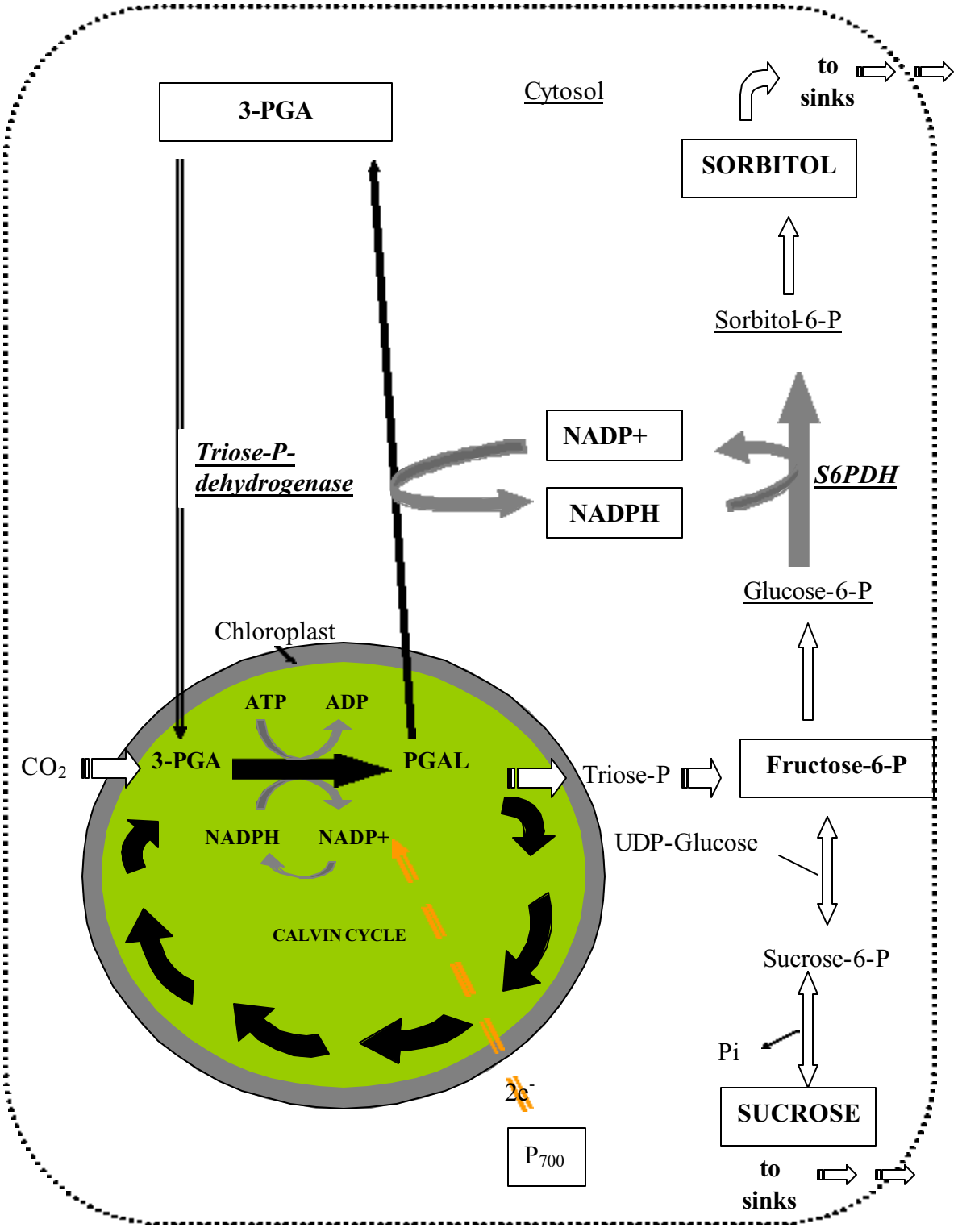

Figure 3.1. Schematic drawing of NADP-NADPH turnover during sorbitol biosynthesis.

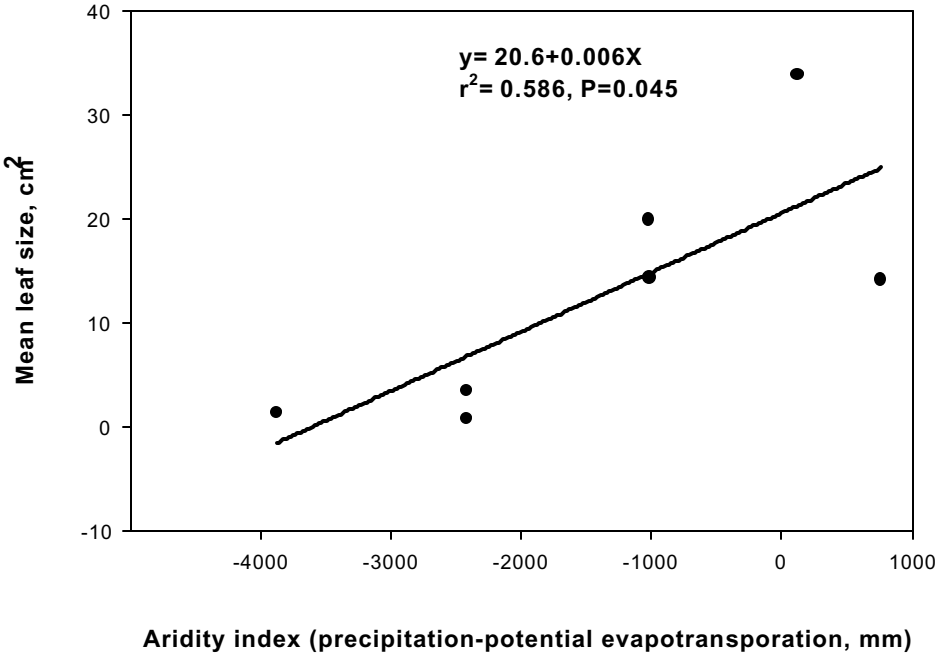

Figure 3.2. Correlation between leaf size (cm^2) and aridity index (precipitation – evapotranspiration) of various *Prunus* species native habitats.

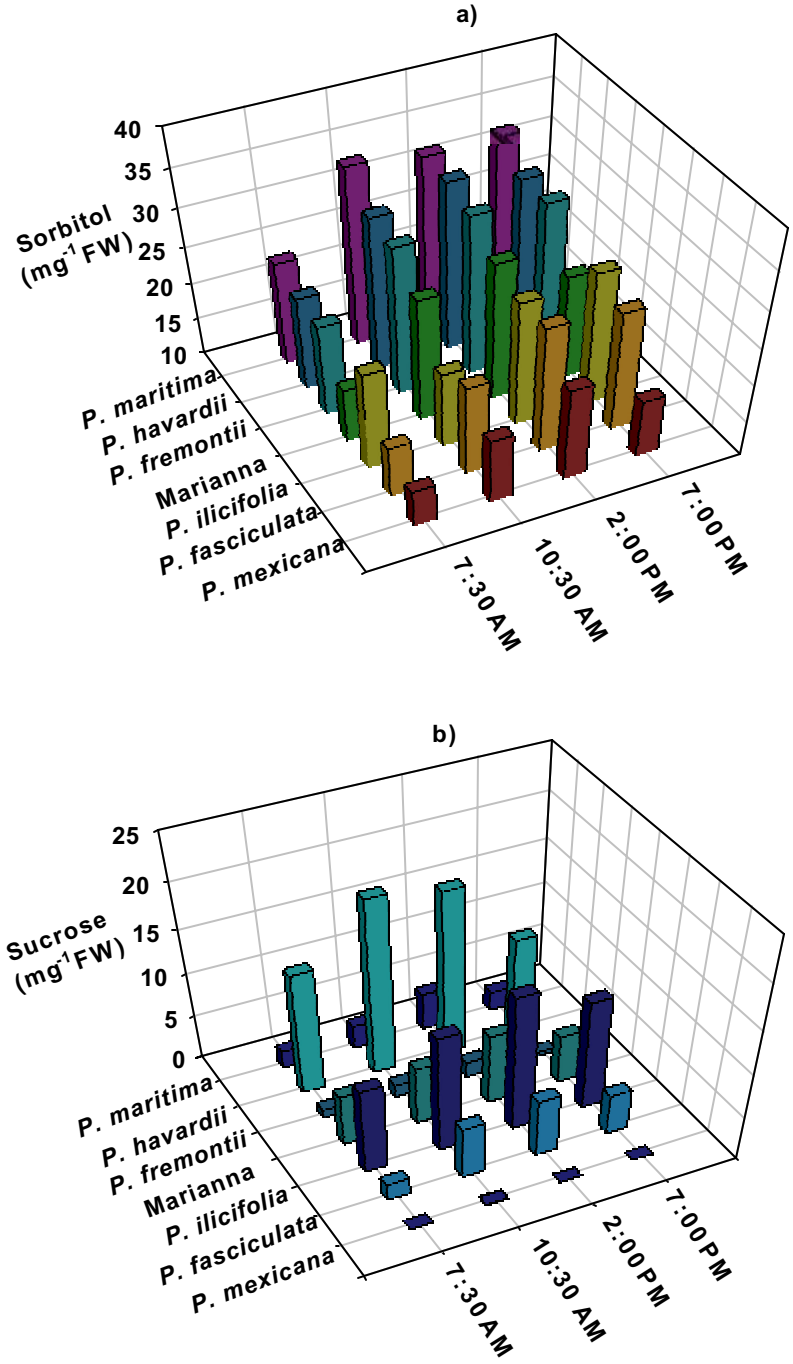

Figure 3.3. Diurnal change in sorbitol (a) and sucrose (b) contents across the *Prunus* species studied. Values for *P. persica* are not available due to loss of its samples.

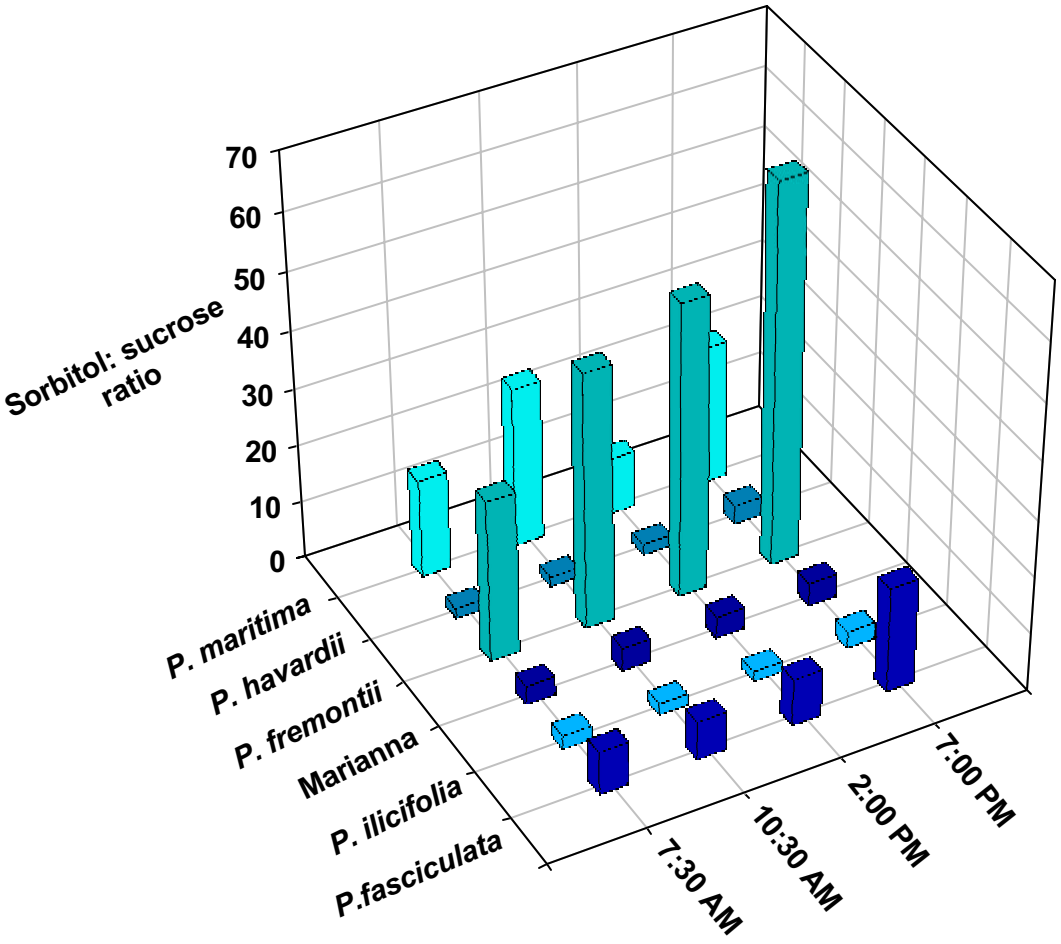

Figure 3.4. Diurnal change in sorbitol: sucrose ratios of the various *Prunus* species. It was not possible to show diurnal sorbitol: sucrose ratios for *P. mexicana* due to it's very low or zero leaf sucrose values.

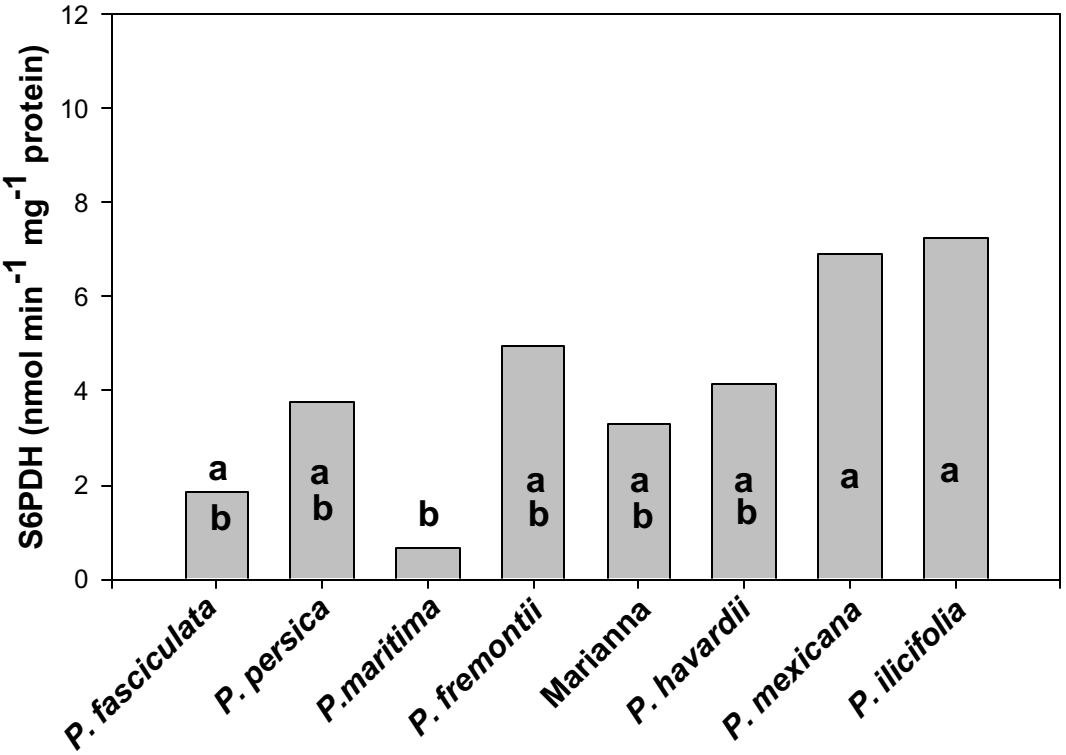

Figure 3.5. Sorbitol-6-phosphate dehydrogenase activity of various *Prunus* species.

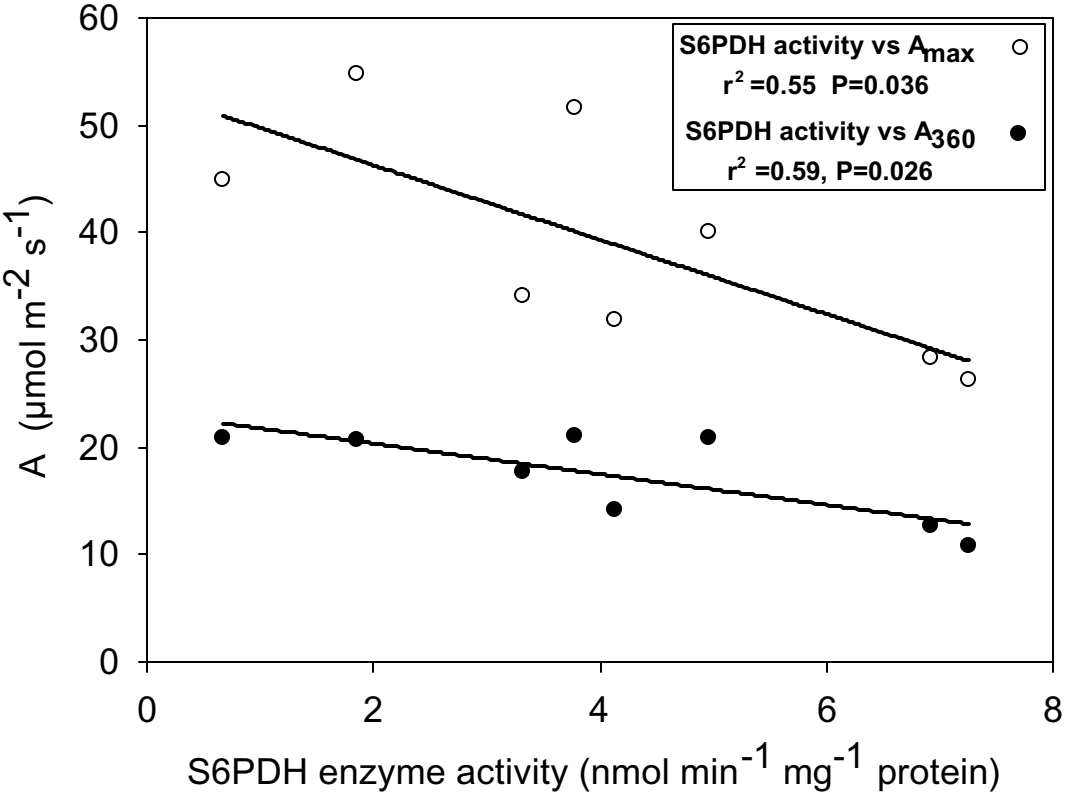

Figure 3.6. The correlation between A_{max} (µmol m^{-2} sec^{-1}), A_{360} (µmol m^{-2} sec^{-1}) and S6PDH enzyme activity (nmol min^{-1} mg protein) of *Prunus* species studied.

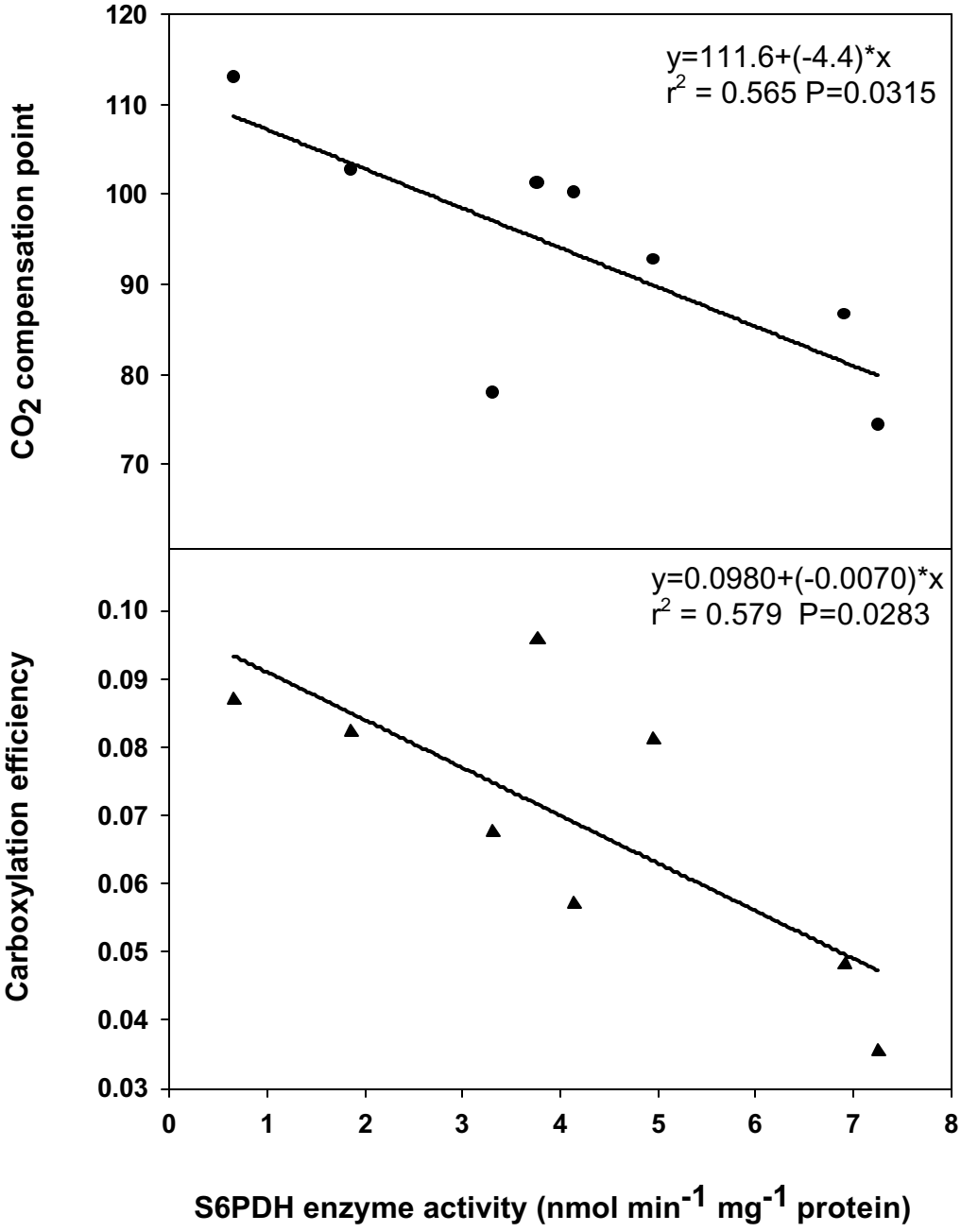

Figure 3.7. The correlation between CO_2 compensation point, carboxylation efficiency and S6PDH enzyme activity (nmol min^{-1} mg^{-1} protein) of *Prunus* species studied.

Table 3.1. Nomenclature, leaf size, origin, and index of aridity for the eight *Prunus* species studied.

Species	**Common name**	**Mean leaf size, (cm^2)**	**Seed source and climatic adaptation**	**Precipitation - potential evapotranspiration (mm)**
P. fasciculata	Desert almond	0.9	California deserts, arid temperate	-2413
P. persica[1]	Peach	34.5	China, humid temperate	+125
P. maritima	Beach plum	13.6	Coastal Massachusetts, humid temperate	+762
P. fremontii	Desert apricot	3.6	Southern California desert, subtropical arid	-2413
P. cerasifera x P. munsoniana 'Marianna'[2]	Marianna plum	26.0	Hybrid of humid temperate adaptation	-
P. havardii	Harvard plum	1.5	Southwest Texas, 1200-2000 m elevation, arid temperate	-3875
P. mexicana	Big-tree plum	15.1	Texas, subhumid temperate	-1016
P. ilicifolia	Holly-leaved cherry	21.4	Coastal southern California, arid temperate	-1020

[1]Along the Yangtze river, Jiangsu, Hunan, Sichuan, Jaingxi provinces
[2]Hybrid of European and N. American species; could not estimate Precip-PET

Table 3.2. Mean photosynthesis (A360, Amax), CO_2 compensation point and carboxylation efficiencies of various *Prunus* species.

***Prunus* species**	**Photosynthesis** (μmol m^{-2} s^{-1})		**CO_2 compensation point** (μl CO_2 per l air)	**Carboxylation efficiency**
	A_{360}	**A_{max}**		
P. fasciculata	20.6 a[1]	54.8 a	102.7 a[1]	0.082 ab[1]
P. persica	21.1 a	51.6 a	101.4 a	0.096 a
P. maritima	20.8 a	44.8 a	113.0 a	0.087 ab
P. fremontii	21.0 ab	39.9 ab	92.7 a	0.081 ab
Marianna	17.6 b	34.0 b	78.0 a	0.068 ab
P. havardii	14.2 b	31.8 b	100.1 a	0.057 b
P. mexicana	12.7 b	28.2 b	86.6 a	0.048 bc
P. ilicifolia	10.7 b	26.3 b	74.3 a	0.036 c
Species effect[2]	0.001	0.001	0.065	0.001

[1] Means followed by the same lowercase letter within each column are not significantly different, P<0.05, Dunn's or Tukey's Test.

[2]Prob.> F value for the species effect of a given variable.

Table 3.3. Specific leaf area (SLA) and area per leaf of various *Prunus* species. Means contain the same lowercase letters for each figure is not significantly different at P ≤ 0.05 levels.

***Prunus* species**	**Average area per leaf** (cm^2)	**Specific leaf area** (cm^2 g^{-1} dry weight)
P. fasciculata	0.9 d	220.2 a[1]
P. persica	34.5 a	207.2 ab
P. maritima	13.6 c	166.8 b
P. fremontii	3.6 d	164.7 b
Marianna	26.0 ab	227.6 a
P. havardii	1.5 d	184.9 b
P. mexicana	15.1 c	197.1 ab
P. ilicifolia	21.4 bc	141.8 c
Species effect[2]	0.001	0.001

[1] Means followed by the same lowercase letter within each column are not significantly different, P≤ 0.05, Dunn's Test.

[2]Prob.> F value for the species effect of a given variable.

Table 3.4. Leaf starch, sorbitol, sucrose contents and sorbitol: sucrose ratios of various *Prunus* species. Values for *P. persica* are not available due to loss of samples.

***Prunus* species**	**Starch** (mg g^{-1} FW)	**Sorbitol** (mg g^{-1} FW)	**Sucrose** (mg g^{-1} FW)	**Sorbitol: sucrose ratio**
P. fasciculata	8.2 cb[1]	22.4 b	6.0 b	6.8 b
P. maritima	12.3 ab	34.1 a	2.7 b	28.0 ab
P. fremontii	12.8 a	30.2 ab	1.4 b	44.9 a
Marianna	12.5 ab	26.8 ab	6.6 b	4.1 bc
P. havardii	9.7 b	30.7 ab	19.8 a	1.7 c
P. mexicana	9.1 b	19.1 b	0.23 b	94.0 a
P. ilicifolia	9.6 b	20.4 b	13.3 ab	2.0 bc
Species effect[2]	0.001	0.001	0.001	0.001

[1] Means followed by the same lowercase letter within each column are not significantly different, $P \leq 0.05$, Tukey's Test. The data for sorbitol: sucrose ratios were log-transformed due to failure in normality and equal variance test. Sorbitol: sucrose ratios are the mean of all individual sample ratios.

[2] Prob.> F value for the species effect of a given variable.

Table 3.5. Leaf ambient photosynthesis (A_{360}) and S6PDH enzyme activity of *Prunus persica* and *Prunus havardii* plants under well watered and drought stressed conditions in the greenhouse.

Species	A_{360} (µmol m^{-2} s^{-1})		S6PDH activity (nmol min^{-1} mg^{-1} protein)	
	well-watered	stressed	well-watered	stressed
P. persica	22.9 a[1]	16.0 b	6.0 a	4.6 b
P. havardii	17.5 a	10.9 b	1.3 a	0.8 a

[1]Means followed by the same lowercase letter within each row for each variable are not significantly different, $P \leq 0.05$, t-test.

CHAPTER 4

RESPONSE OF NADP-DEPENDENT SORBITOL-6-PHOSPHATE DEHYDROGENASE ACTIVITY AND PHOTOSYNTHESIS TO SOURCE/SINK MANIPULATION AND LEAF NODE POSITION[1]

[1]Karakas, B., R. Lo Bianco, and M. Rieger. To be submitted to *HortScience*.

Abstract

Polyol synthesis along with sucrose in source leaves of *Prunus* species has been proposed to be the source of increasing in NADP-NADPH turnover and therefore higher photosynthetic rate compared to other plants that form only sucrose as photosynthate. Peach (*Prunus persica*) has very high levels of sorbitol, a major polyol, in its exporting leaves. Therefore, we used two peach cultivars, 10-year-old "Encore" trees and 1-year-old "Nemaguard" plants to test this hypothesis. Source/sink manipulations and natural variations (e.g., leaf node position) were applied to affect photosynthesis and the concomitant changes in the activity of the primary sorbitol forming enzyme, sorbitol-6-phosphate dehydrogenase (S6PDH) and sorbitol contents in exporting peach leaves. Four main experiments were designed: i) long term fruit effect (i.e., fruiting vs. non-fruiting) in "Encore" trees, ii) short-term fruit effect (24-hour after fruit removal) in "Encore" trees, iii) leaf node position effect in "Nemaguard", and iv) shoot tip removal effect in "Nemaguard", to determine whether sorbitol forming capacity or sorbitol content varied in connection with photosynthesis. Both long and short-term fruit effects in leaf ambient photosynthesis (A_{360}) detected (e.g., A_{360} decreased significantly following fruit removal). However S6PDH activity remained similar regardless of the fruiting in short or long-term study. A_{360} and S6PDH changed in parallel across the leaf node positions in the shoot. Shoot tip removal resulted in reduction of both A_{360} and S6PDH activity. Overall, we found positive correlations between A_{360} and S6PDH activity across experiments (with the exception of the short-term fruit effect). The positive relationship between S6PDH activity and photosynthesis further supports the hypothesis that polyol biosynthesis might increase NADP-NADPH turnover and subsequently photosynthesis in peach.

INDEX WORDS: *Prunus persica*, peach, photosynthesis, sorbitol biosynthesis, sorbitol-6-phosphate dehydrogenase, fruit effect.

Introduction

Polyols are involved in carbon storage and translocation in some higher plant species (i.e., *Prunus*). However, the link between polyols and photosynthesis has not been studied thoroughly. Sorbitol constitutes up to 10% of the fruit fresh weight and more than half of the leaf nonstructural carbohydrates in some members of the Rosaceae family (Loescher 1987). Sorbitol is synthesized in mature leaves and translocated into sinks (i.e. fruits, growing leaves and shoot tips) where it is converted to other sugars or temporarily stored. Fruits are reported to be strong sinks for sorbitol, especially during phase III of growth in peach (Nii, 1997).

Feedback inhibition is defined as a cellular control mechanism in which an enzyme that catalyzes the formation of a particular product in the cell is inhibited when that substance has accumulated to a certain level, thereby balancing the amount provided with the amount needed. Polyol-producing plants, particularly tree fruits (i.e., *Prunus*), have relatively high photosynthetic rates compared to plants forming only sucrose (Flore and Lakso, 1989; Stoop et al., 1996). It has been proposed that polyol synthesis in source leaves increases NADP-NADPH turnover compared to plants that form only sucrose as photosynthetic end product. This is accomplished by preventing feedback inhibition of photosynthesis at the level of energy production phases of photosynthesis, light reaction, by drawing the extra NADPH that is used in the cytosol to reduce hexose-P to polyol-P (Stoop et al., 1996). Polyol synthesis may also prevent feedback inhibition of photosynthesis in chloroplasts by providing an additional cytosolic sink for photosynthetically fixed CO_2 in mesophyll cells. Sorbitol, an important polyol, is the major photosynthate found in very high concentrations in mature peach leaves.

Karakas (2001) found a negative correlation between, the activity of the principal enzyme for sorbitol synthesis, sorbitol-6-phosphate dehydrogenase (S6PDH), and

photosynthesis among eight *Prunus* species originating from contrasting habitats but grown under same conditions. This finding disfavors the hypothesis that sorbitol formation acts to enhance photosynthesis in exporting leaves (Stoop et al., 1996), at least on an evolutionary or plant community or *Prunus* species level. It is possible, however, that sorbitol production may have a different effect on photosynthesis when studied at the individual leaf level, within an individual plant. Thus, the present study was performed using peach as a model because its mature leaves contain high sorbitol levels. Source/sink manipulations were applied to affect photosynthesis and the concomitant changes in S6PDH enzyme activity. In addition, natural variation in photosynthesis due to leaf node position was explored to determine whether sorbitol forming capacity or sorbitol content varied systematically with photosynthesis.

Materials and Methods

Plant material and experimental design

Four experiments were conducted to either induce changes in photosynthesis, or exploit its natural variation, in order to observe concomitant changes in the response of S6PDH. The first two experiments used mature "Encore" peach (*Prunus persica*) trees, and the other two experiments were performed using "Nemaguard" cuttings. All experiments were directed at testing the same hypothesis concerning the relationship between sorbitol production and photosynthesis. Ten-year-old, late maturing "Encore" peach trees growing at the University of Georgia Horticultural Research Farm in Watkinsville, GA and 1-year-old, potted "Nemaguard" plants. Encore trees were trained to a perpendicular V system. "Nemaguard" plants were grown from rooted cuttings for one year in 10-liter containers under greenhouse conditions and later transferred outside in to an open area in Athens, GA (34°N, 85°W). These four experiments were conducted aver a one year: (I) fruiting versus non-fruiting experiment June 26 to Aug 10, 2000, (II)

fruit removal experiment July 26 to Aug 14, 2000, (III) leaf node position experiment Sept 12 to Sept 20, 2000 and (IV) shoot tip removal experiment March 22 to Apr 9, 2001. All four experiments were repeated at least three times. All photosynthesis measurements were conducted under full sunlight on a clear day between 11:00 AM and 12:30 PM.

Experiment I: Long- term effect of fruit removal.

All fruits were removed from one of two scaffold limbs of several "Encore" trees in the early stage of fruit growth. This yielded one scaffold with elongating shoots subtended by fruits, and the other scaffold with shoots only. Fruit removal was intended to increase the source/sink ratio on one half of the tree, thereby reducing the photosynthetic rate of individual leaves (Bruchou and Genard, 1999). Three shoots from each scaffold limb (i.e., 3 shoots with fruiting and three shoots without fruits) of each of three V-shaped trees were tagged and photosynthesis of two recently expanded, matured leaves between node no. 5 to no. 8 from the apex of each shoots were measured. Following the photosynthesis measurement, the leaves were sampled and half of each leaf was used for enzyme activity immediately and the other half was stored at –20 ^{o}C for carbohydrate analysis. These measurements were repeated three times on different days with different trees/shoots.

Experiment II: Short-term effect of fruit removal.

Two similar adjacent leaves between nodes 5 and 8, on three different fruiting shoots were selected and photosynthesis was measured for the first leaf of two tagged leaves from each shoot and sampled for enzyme and carbohydrate analysis as soon as the photosynthesis measurement was performed. Then fruits on these shoots were removed. The next day (24-hours later), the photosynthesis of the second selected leaf for each shoot was measured and these leaves were collected right after the photosynthesis measurement. Half of each sampled leaf was used to assay enzyme activity and the other

half was stored at –20°C for later carbohydrate analysis. These measurements were also repeated three times with different trees and shoots on different days.

Experiment III: Leaf node position.

Three greenhouse-grown potted peach plants were transferred out to an open area. Healthy shoots with at least 20 leaves were selected from potted plants. Photosynthesis and enzyme activities were measured in at least 10–12 leaves from the apex to the base of the shoot. Similarly, all the leaves were collected following the photosynthesis measurements and half of each leaf was used to assay enzyme activity, with the other half for carbohydrate measurement. This experiment was repeated three times with different potted plants on different days.

Experiment IV: shoot tip removal.

This experiment was conducted in the greenhouse. Two similar recently expanded, mature leaves between nodes 5 and 8 were selected for photosynthesis and enzyme activity measurements. Photosynthesis of one of the two selected leaves for each shoot was measured, the leaf was collected and the shoot tips (apex and lateral) and rapidly growing young leaves were excised, immediately after measurement. Twenty-four hours following the first measurement, photosynthesis of the second tagged leaf was measured and this leaf was sampled for enzyme and carbohydrate measurement. The experiment was repeated three times.

Photosynthesis measurements

Carbon dioxide assimilation (A) was measured under field and greenhouse conditions using a LI-6200 portable photosynthesis device outfitted with a ¼-liter chamber (LI-COR, Lincoln, NE).

S6PDH Enzyme assay

S6PDH activity was assayed following the modified protocol developed for sorbitol dehydrogenase (SDH) by Lo Bianco et al. (1998). Approximately 80 to 100 mg of fresh leaf tissue was collected from the leaves used to measure A. Tissue was homogenized in 3.0 ml of extraction buffer (100 mM Tris at pH-9.0, 8% v/v glycerol, and 20 mM 2-mercaptoethenol) using a pre-chilled mortar and pestle in the presence of washed sand. Tween-20 (0.1% v/v) and polyvinylpolypyrolidine (PVP, 1% w/v) were also added during homogenization of the tissue. The homogenate was centrifuged at 3000 *g* for 15 min and the supernatant collected. The supernatant was desalted through a Sephadex G-25 column at 4 °C. The enzyme was assayed in a total volume of 1ml assay buffer (50 µl of extract**,** 100 mM at pH 9.0, 0.11 mM NADPH and 50 mM glucose-6-phosphate) by measuring the change in optical density at 340 nm at 25 ^{o}C using a Spectronic 2-D (Milton Roy, Rochester, NY) spectrophotometer. Enzyme specific activity was calculated and expressed as nanomoles of NADPH oxidized per minute per g fresh weight (FW) of tissue.

Sorbitol and non-structural carbohydrates

Non-structural carbohydrates were quantified in leaf tissue using gas chromatography (Rieger and Marra, 1994). Half of the leaves for which A was measured were stored at –20 ^{o}C until analysis. Frozen leaf tissue of 0.1-0.2 g was ground in a mortar and pestle in the presence of 1.5-3.0 ml of 80 % (v/v) methanol and 0.22 mg of phenyl-β−D-glucopyranose as an internal standard. The homogenate was centrifuged for 3 min at 3900 g, and a 1 ml supernatant was collected and stored at 4 ^{o}C for soluble carbohydrate and sorbitol analysis. Samples (100µl) were dried in GC vials and derivatized with 70 µl BSTFA/DMF mix (1:1, v/v) for injection on a Hewlett Packard 5890A gas chromatograph (Avondale, Pa).

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and simple correlation using Sigma Stat (SPSS Inc., Chicago, IL). Tukey's studentized comparison was used to separate means when ANOVA results were significant. When data were not normally distributed, non-parametric methods were used to perform means separation.

Results

Long- term effect of fruit Encore peach removal experiment

Ambient photosynthesis was lower in scaffold limbs without fruits as compared to the ones with fruits (Table 4.1). However, S6PDH enzyme activity was similar regardless of fruiting condition (Table 4.1). Neither sorbitol nor sucrose content changed with either the presence or the absence of fruit (Table 4.1). Fruiting did not have any effect on sorbitol: sucrose ratios in trees (Table 4.1).

No correlation was observed between photosynthesis and S6PDH enzyme activity in scaffold limbs with fruit (Fig. 4.1a). However, in scaffold limbs without fruit, there was a significant positive correlation between A360 and S6PDH activity (Fig. 4.1b).

Short-term effect of Encore peach fruit removal experiment

A360 dropped significantly within 24 hours following the fruit removal (Table 4.2). However, S6PDH activity did not change in response to fruit removal or reduction in photosynthesis (Table 4.2). Within the 24-hour period of fruit removal, sorbitol increased whereas sucrose decreased significantly (Table 4.2). Leaf sorbitol: sucrose ratios increased in the shoots with following fruit removal (Table 4.2). No significant correlation between photosynthesis and S6PDH activity was observed regardless of fruit presence or removal (Fig. 4.2a and b). This was somewhat in contrast to results in the long-term fruit-removal experiment (Fig. 4.1).

Nemaguard peach leaf node position experiment

A_{360} and S6PDH activity changed with the leaf node position and reached a maximum at nodes between 4 to 10 (Fig. 4.3). The change in A_{360} and S6PDH activity across the leaf position tended to be in parallel (Fig. 4.3).

Overall, in the leaf position experiments, photosynthesis and S6PDH enzyme activities were positively correlated (Fig. 4.4). A significant positive correlation was also observed between A_{360} and leaf sorbitol contents in potted plants, although the r^2 value was quite low (Fig. 4.5). Neither sucrose nor total sugars were correlated to leaf node position.

Nemaguard peach shoot tip removal experiment

Maximum leaf photosynthesis (Amax) and S6PDH enzyme activity decreased significantly within 24-hour following shoot tip removal from the potted plants (Table 4.3). Again, there was a significant positive correlation between maximal Amax and S6PDH activity in the shoot tip removal experiments, although variation was large (Fig. 4.6).

Highly significant positive correlation between A360 and S6PDH activity was observed when all data from experiments with Encore and Nemaguard pooled (Fig. 4.7).

Discussion

We analyzed sorbitol metabolism in mature peach leaves by altering their photosynthetic capacities via removing various sinks (i.e., fruits, shoot tips, etc.). In all experiments in this study, we were able to manipulate leaf photosynthesis by sink removal from the plants. In leaf node position, shoot tip removal, and in 1 of 2 cases in long-term fruit effect experiments, A360 and in vitro S6PDH activity was positively correlated in exporting leaves (Fig. 4.1b, Fig. 4.4, and Fig. 4.6). However, highly significant correlation between A360 and S6PDH was found when data from both Encore and Nemaguard pooled (Fig. 4.7) suggesting that sorbitol may enhance photosynthesis. This response was especially strong with the removal of vegetative sinks using young

"Nemaguard" peach plants (Fig. 4.4, Fig. 4.6). Everard et al. (1997) found similar positive relationships among photosynthetic capacity, mannitol synthesis and mannitol-6-phosphate reductase (M6PR) in experiments with celery. Collectively, these findings suggest that polyol metabolism is positively related to photosynthetic capacity, consistent with the hypothesis that polyols enhance photosynthesis (Loescher, 1987). However, the decrease in A_{360} with the presence or removal of fruits in "Encore" did not give the same clear response among S6PDH activity, photosynthesis, and sorbitol. We also observed cultivar "Encore" to have much lower S6PDH activity than "Nemaguard". The activity of the enzyme tended to be quite variable depending on the growth rate of the shoot as well (Fig. 4.3). High variations in sorbitol and sorbitol: sucrose ratios with in *Prunus,* reported by Moing et al., (1997) may suggest parallel variations in S6PDH activities. As a result of the four experiments with "Encore" and "Nemaguard", the following conclusions may be made: 1) "Nemaguard" could be a better system for correlating these parameters (i.e., photosynthesis, S6PDH, and sorbitol contents) than "Encore", 2) "fruit effect" seems to be more complex than shoot tip removal or leaf position effect, 3) the age of the 'Encore" could make it difficult for clear correlations of these parameters possibly due to some reasons associated fruit effect, and 4) controlled environments (i.e., greenhouse with potted plants) could provide a better environment for testing these parameters compared to the field conditions (with trees). Experiments with peach by Sakanishi et al. (1998) also revealed similar results, in that S6PDH activity was regulated by demand in sinks and the demand could be variable during the day and throughout the growing season.

Leaf sorbitol and sorbitol: sucrose ratios responded quite differently with long-term fruit and short-term fruit removal experiments. In long-term fruit removal experiments, neither sorbitol nor sorbitol: sucrose ratio changed significantly (Table 4.2) however short-term fruit removal caused sorbitol and sorbitol: sucrose ratios to increase significantly within a 24-hour period (Table 4.2). The reason for this increase in sorbitol levels in the short-term fruit removal treatment could be that sudden removal of a strong

sink, such as a fruit, causes sorbitol to accumulate in the mature leaves. However, the plant later may direct and transport this build up of sorbitol to other growing sinks such as shoot tips, or growing leaves, when sufficient time exists for redirection, as was the case with the long-term fruit removal treatment. Thus, the increase in sorbitol and sorbitol: sucrose ratio in the short-term experiments may be a transient response to sink (fruit) removal. Similar observations were reported with root-restricted potted peach trees by Nii (1997). The author examined changes in nonstructural carbohydrate contents of mature leaves before and after fruit removal. Sorbitol contents were increased with removal of fruits. It was concluded that when fruits, major sink organs, were removed from the peach trees, translocation of sorbitol from leaves to fruit ceased which resulted increase in leaf sorbitol content in the leaf and may suggest that fruits are fairly strong sinks for sorbitol during phase III of peach fruit development.

In conclusion, source-sink manipulations tend to produce parallel changes in photosynthesis and sorbitol synthesis, supporting the hypothesis that polyol synthesis may enhance photosynthesis (Loescher, 1987). Positive relations between photosynthesis and S6PDH activity in the current study with a single species may further suggest that contradictory results in the previous study with eight species (Karakas, 2001) could have been due to high genetic variation among species.

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Table 4.1. Leaf ambient photosynthesis (A360), S6PDH activity, sorbitol, sucrose, and sorbitol: sucrose ratios in scaffold Encore peach limbs with fruit versus without fruit.

Character	Treatment	
	Fruiting	**Non-fruiting**
A_{360} (μmol m^{-2} s^{-1})	13.9 a[1]	10.1 b
S6PDH activity (nmol min^{-1} g^{-1} FW)	50.2 a	41.2 a
Sorbitol (mg g^{-1} FW)	35.4 a	34.3 a
Sucrose (mg g^{-1} FW)	3.2 a	1.8 a
Sorbitol: sucrose ratio	17.4 a	19.0 a

[1]Means contain the same lowercase letters for each row is not significantly different at $P \leq 0.05$ levels.

Table 4.2. Leaf A360, S6PDH activity, sorbitol, sucrose, and sorbitol: sucrose ratios in scaffold Encore peach limbs with fruit present versus 24-hours after fruit removed.

Character	Treatment	
	Fruit present	**Fruit removed**
A_{360} (μmol m^{-2} s^{-1})	14.0 a[1]	11.9 b
S6PDH activity (nmol min^{-1} g^{-1} FW)	18.3 a	15.0 a
Sorbitol (mg g^{-1} FW)	27.1 b	31.3 a
Sucrose (mg g^{-1} FW)	7.8 a	5.6 b
Sorbitol: sucrose ratio	3.9 b	7.3 a

[1]Means contain the same lowercase letters for each row is not significantly different at $P \leq 0.05$ levels.

Table 4.3. Leaf Amax and S6PDH enzyme activity before and 24-hour after shoot tip removal experiments with Nemaguard peach plants. Means contain the same lowercase letters for each figure is not significantly different at $P \leq 0.05$ level.

Character	Shoot tip removal	
	before	24-hour after
A_{max} (μmol m^{-2} s^{-1})	36.2 a	32.7 b
S6PDH activity (nmol min^{-1} g^{-1} FW)	107.0 a	82.9 b

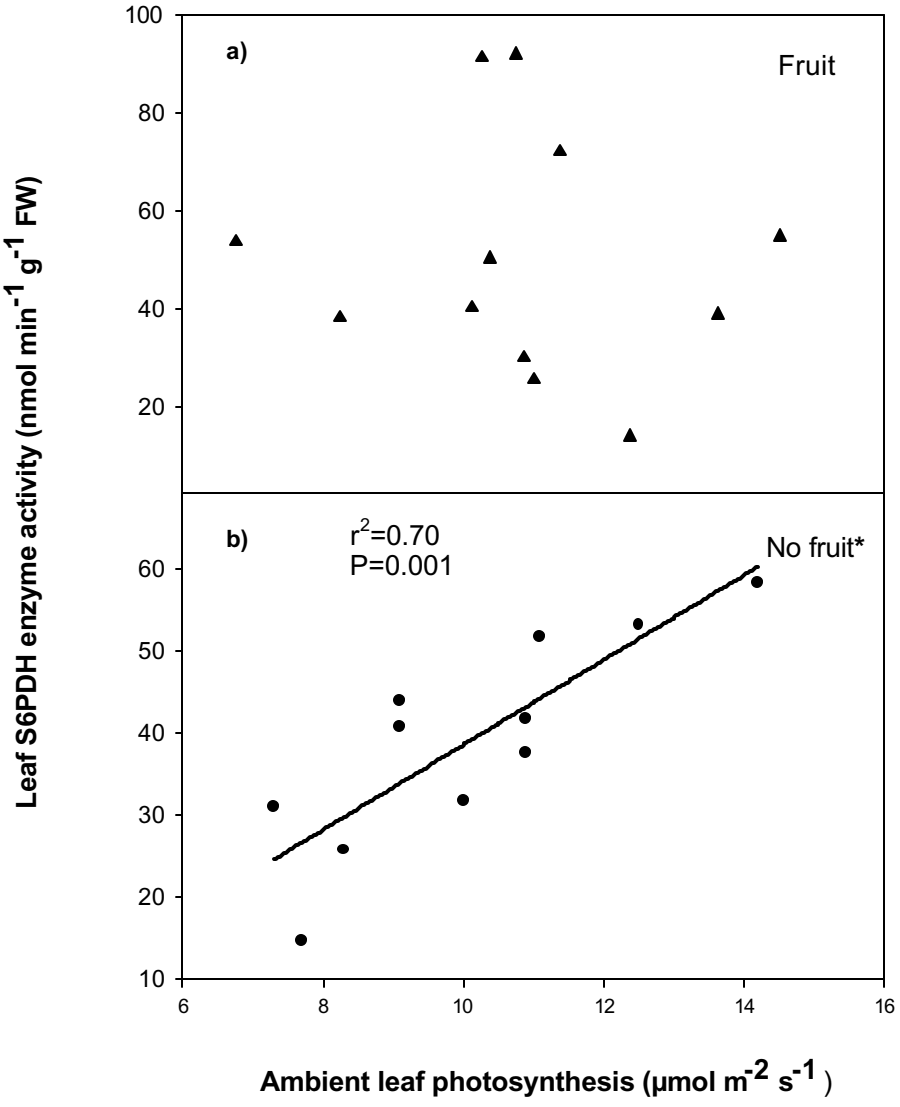

Figure 4.1. Correlation between A360 and S6PDH activity in trees with a) fruiting and b) non-fruiting scaffold limbs of Encore peach tress in long-term fruit effect experiments. Asterisk (*) shows significant correlation at $P \leq 0.05$ level.

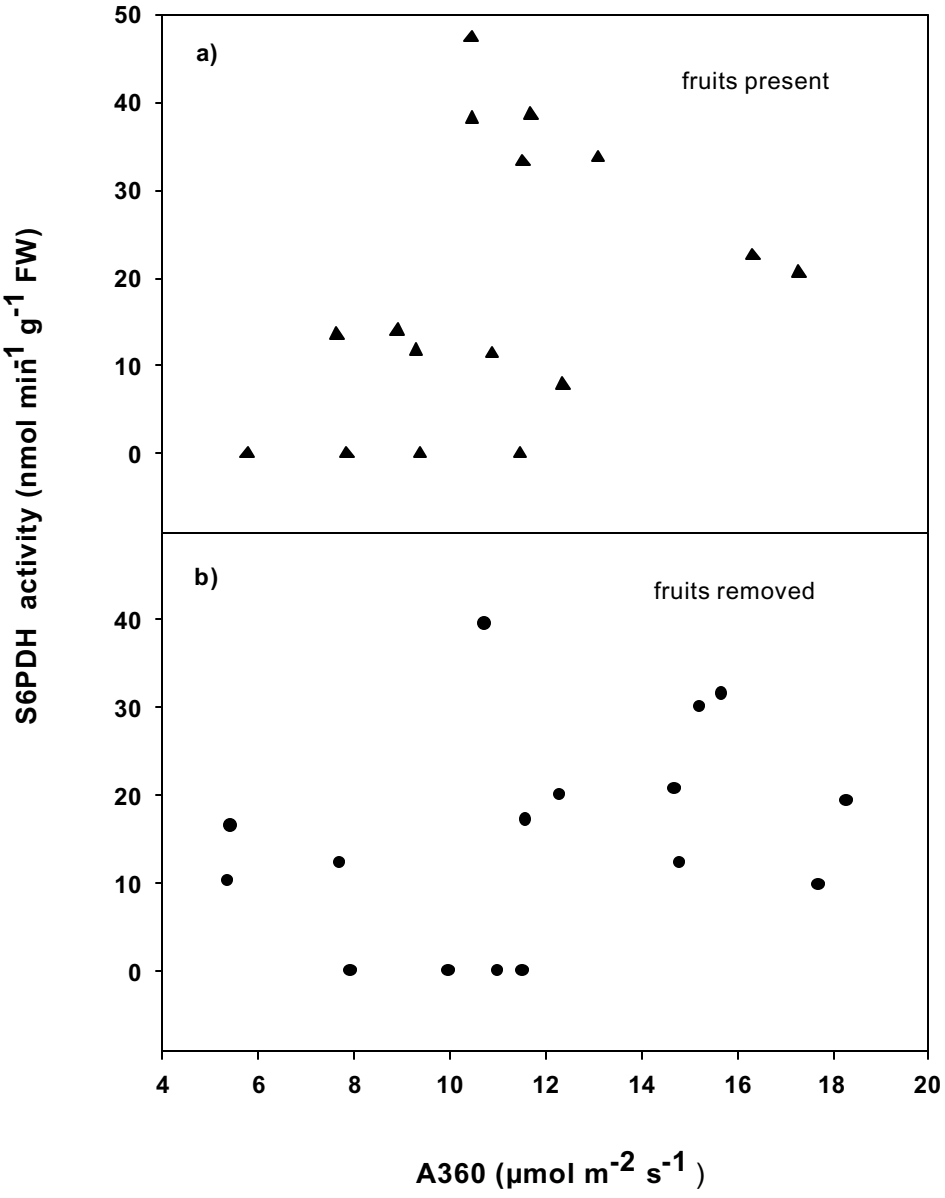

Figure 4.2. Correlation between A360 and S6PDH activity in Encore peach shoots with a) fruit present and b) fruits removed in short-term fruit effect experiments. Asterisk (*) shows significant correlation at $P \leq 0.05$ levels.

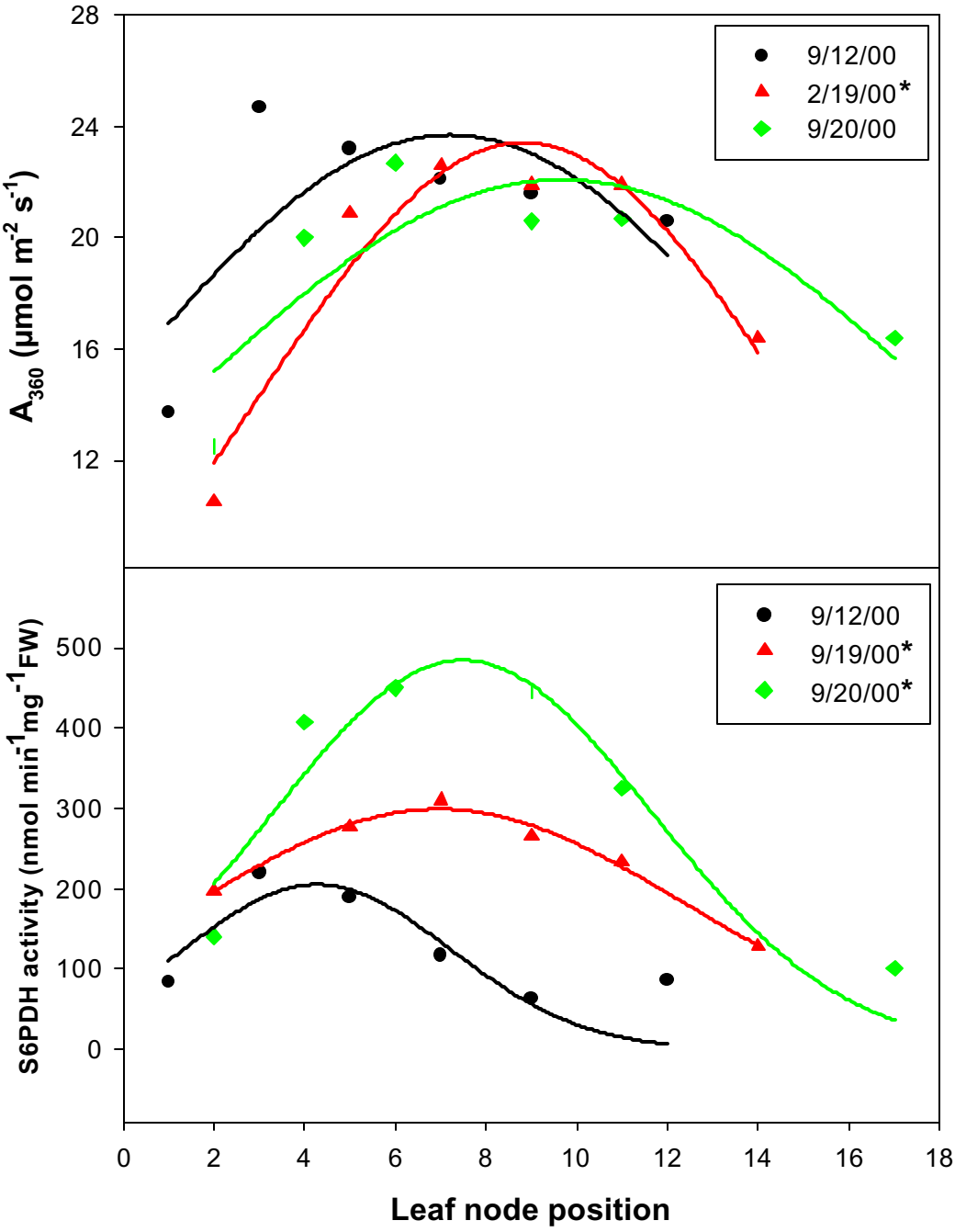

Figure 4.3. Correlation between leaf node position and A360 or S6PDH activity in potted Nemaguard peach plants in leaf node position experiments. Each curve indicates independent measurements at 3 different dates. S6PDH activity = 484.75 x exp (-0.5 x $((X-X_0)/4.19)^2$ Asterisk (*) shows a significant correlation at ≤ 0.05 levels.

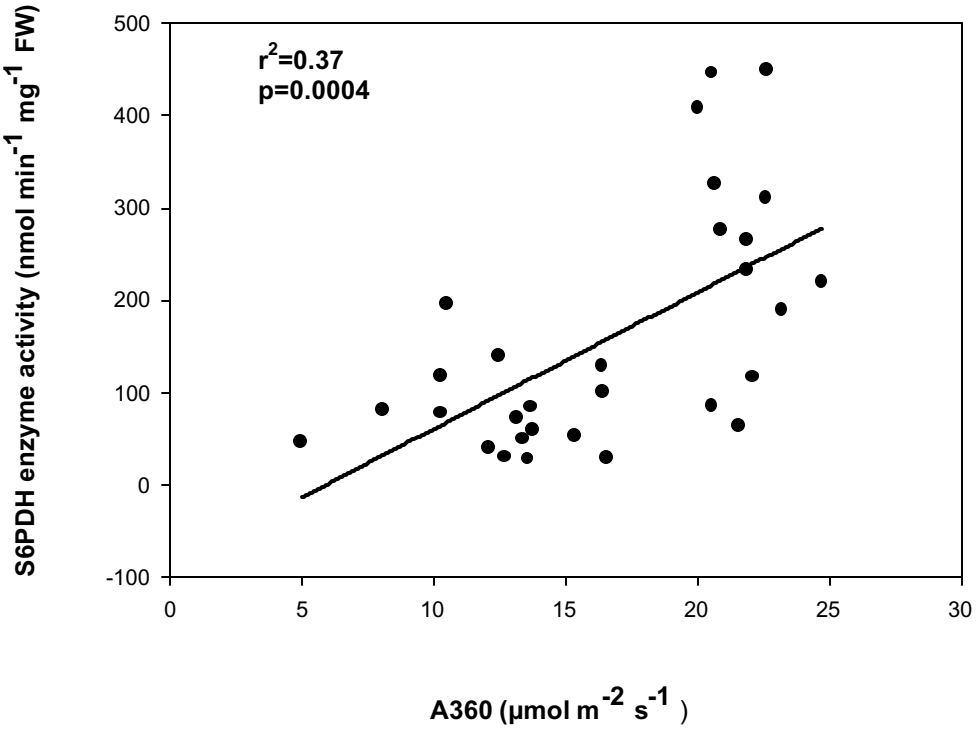

Figure 4.4. Correlation between A360 and S6PDH enzyme activity in potted Nemaguard peach plants in the leaf node position experiments. This is a composite of all data from experiments that were repeated in 3 different dates with 3 different plants.

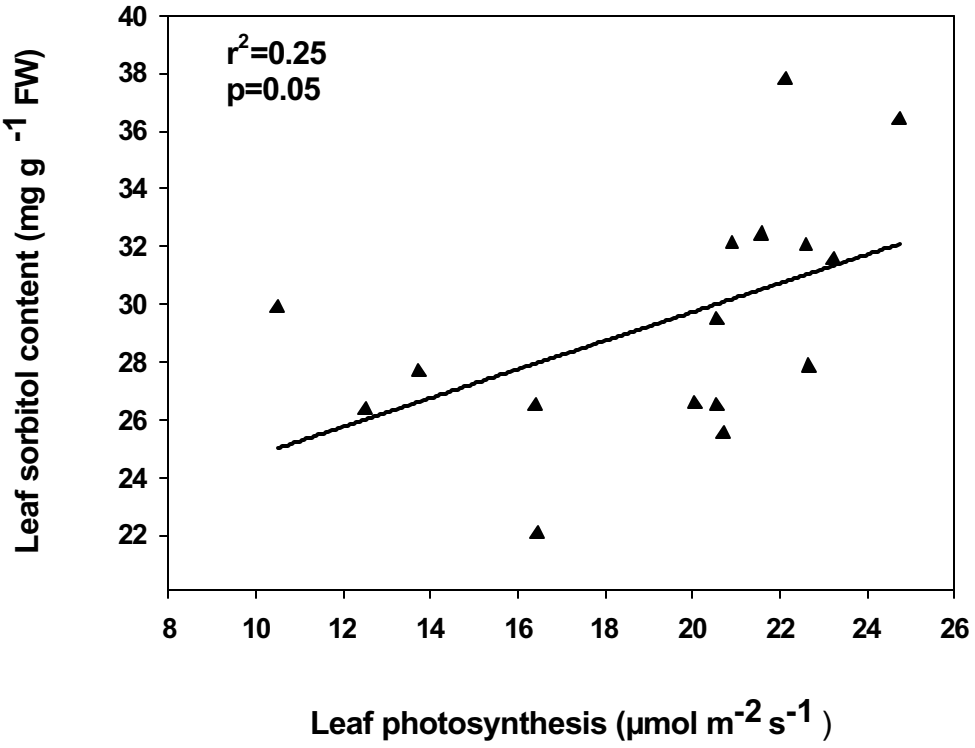

Figure 4.5. Correlation between A360 and leaf sorbitol contents in leaf node position experiments with potted Nemaguard peach plants.

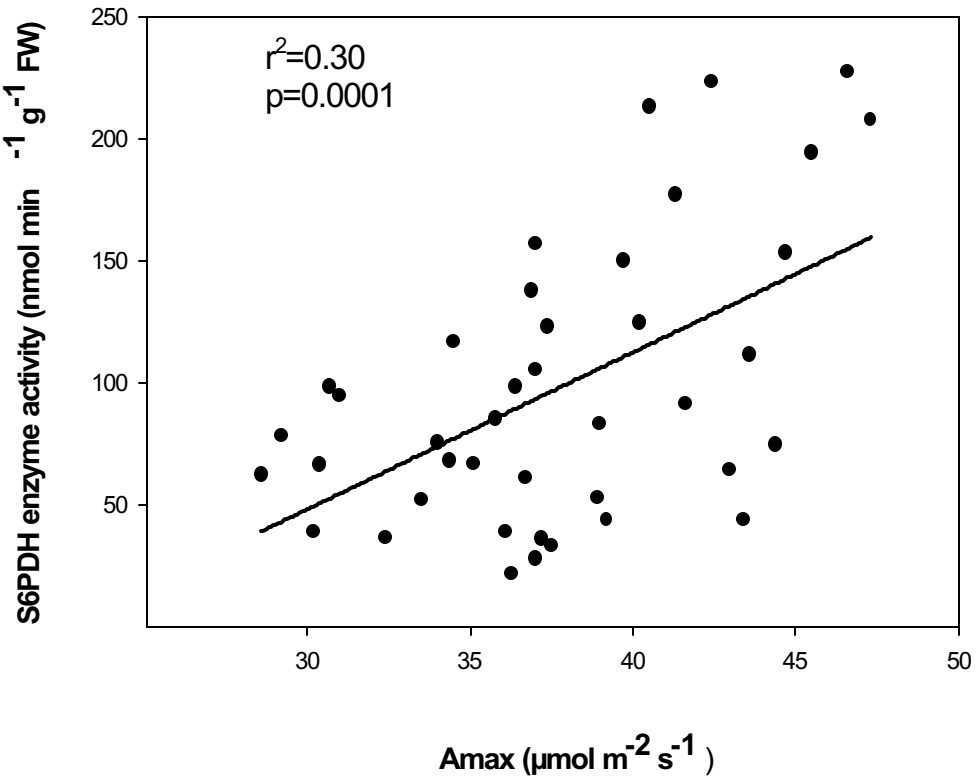

Figure 4.6. Correlation between Amax and S6PDH activity in shoot tip removal experiments with potted Nemaguard peach plants.

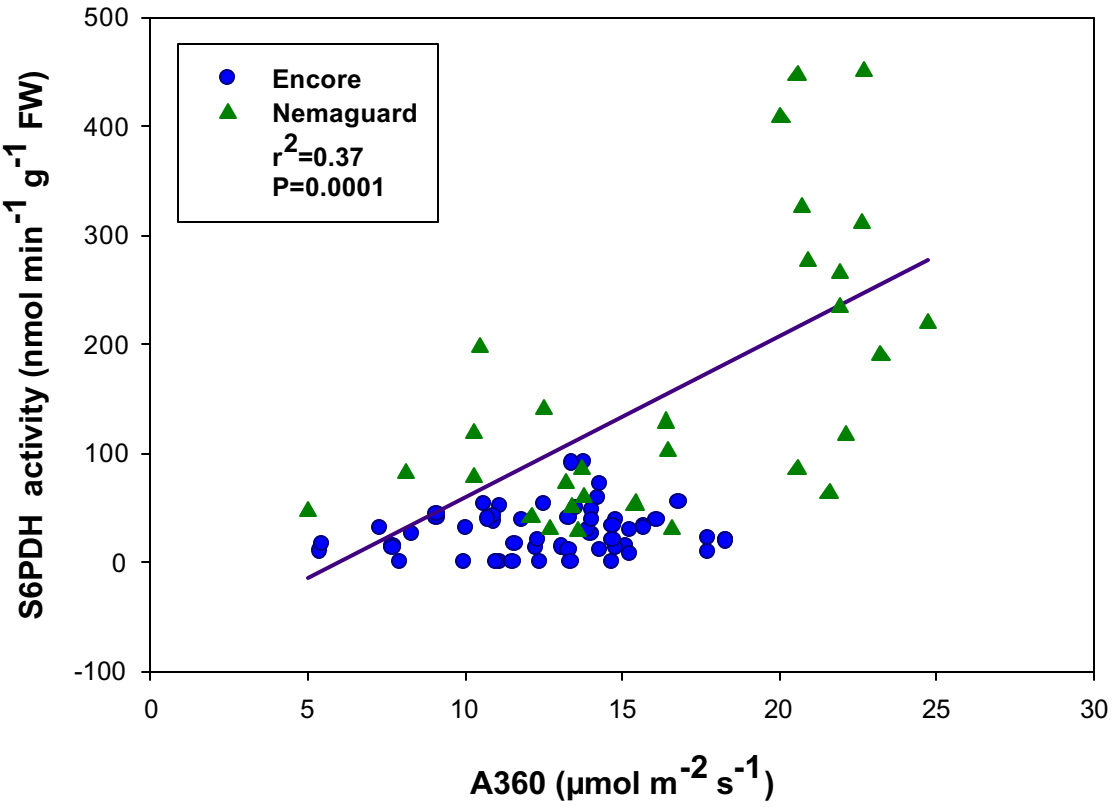

Figure 4.7. Overall correlation between A360 and S6PDH enzyme activity in Encore and Nemaguard peach plants. This is a composite of all data from all experiments except shoot tip removal. In shoot tip removal experiment, photosynthesis was measured at higher CO_2 level (>1000 ppm).

CHAPTER 5

RESPONSE OF THE GENE FOR NADP-DEPENDENT SORBITOL-6-PHOSPHATE DEHYDROGENASE TO VEGETATIVE SINK MANIPULATION AND TIME OF DAY[1]

[1]Karakas, B. and M. Rieger. To be submitted to *Plant and Cell Physiology*.

Abstract

Sorbitol, a major sugar-alcohol is found in high concentrations in mature leaves of economically important *Prunus* species such as apricot, peach, plum, and cherry. It varies greatly with species, variety, season, and even time of day. The functional significance of this variation of sorbitol levels needs to be studied thoroughly. In peach, sorbitol is synthesized via sorbitol-6-phosphate dehydrogenase (S6PDH) in mature leaves and transported to rapidly growing sinks for utilization. Karakas (2001) reported that removing young leaves and shoot tips from growing peach shoots was an effective and quick model system for studying photosynthetic and S6PDH enzyme responses to source-sink manipulation. We used this approach in connection with mRNA levels of the S6PDH gene to test the regulation of sorbitol biosynthesis. S6PDH enzyme activity and gene expression levels were measured following shoot tip removal of actively growing shoots as well as diurnal changes of S6PDH enzyme activity and S6PDH gene expression levels of mature leaves. We observed a significant decrease in S6PDH activity 24-hour following shoot tip removal. However, S6PDH gene expression levels of mature peach leaves increased significantly within 24-hour after shoot tip removal. Activity of S6PDH was lower at midday than at sunrise. In vitro incubation of enzyme extract with sorbitol did not affect S6PDH activity. No significant diurnal changes in S6PDH gene expression levels were detected. The response of S6PDH enzyme and gene to shoot tip removal was in opposite directions. Collectively, the data suggest a posttranslational mode of enzyme regulation in response to short-term source-sink manipulation.

INDEX WORDS: *Prunus persica*, sorbitol, polyols, photosynthesis, enzyme activity, sorbitol-6-phosphate dehydrogenase, gene expression, mRNA.

Introduction

Sorbitol is found at high levels in the mature leaves of economically important *Prunus* species such as apricot, peach, plum, and cherry (Bileski, 1982, Loescher, 1987). The concentration of sorbitol in these plants varies greatly with species, variety, season, and even time of day (Bieleski, 1982; Escobar-Gutierrez and Gaudillere, 1994; Escobar-Guitierrez and Gaudillere, 1996; Kleinschmidt et al., 1998; Wang et al., 1997). The functional significance of sorbitol remains unclear, but the enzymes involved in its synthesis and degradation have been characterized. Specifically, sorbitol is synthesized by the NADPH-requiring enzyme sorbitol-6-phosphate-dehydrogenase (S6PDH), and catabolized by the NAD^+-requiring sorbitol dehydrogenase (SDH). The activities of these two enzymes appear to be regulated by various factors such as photosynthetic rate, sorbitol concentration, leaf position/age and time of year (Kanayama, 1998; Abnason-Bantog et al., 2000; Karakas, 2001).

The metabolism of sorbitol is spatially separated, and it is rare to find both synthesis and degradation in the same tissues at the same time. It is synthesized largely in mature leaves, and then transported to active sinks such as expanding leaves, shoot tips, root tips, and fruits, where it is primarily oxidized to fructose via SDH (Loescher et al. 1982; Gutierrez and Gaudillere, 1996; Marquat et al., 1997; Lalonde et al., 1999). The activity of S6PDH, the key sorbitol synthetic enzyme, may be regulated by demand for sorbitol in developing sinks (Sheen et al., 1999; Lo Bianco et al., 2000). However, the regulation of the gene encoding S6PDH has received little study. In the only known publication on the topic, the expression of the S6PDH gene roughly paralleled the amount of enzyme and its activity when peach leaves of different age were sampled (Sakanishi et al. 1998). However, there appeared to be a lag of several days to two weeks between a change in gene expression and a subsequent change in S6PDH enzyme content or

activity. This suggests that S6PDH turnover is slow and *de-novo* synthesis delayed relative to transcript accumulation. Therefore, short-term (i.e., 24-hr) changes in S6PDH activity induced by source-sink manipulations as shown by Karakas (2001) would seem to result from post-translational effects, rather than changes in gene expression and concomitant enzyme contents.

Karakas (2001) reported that removing young leaves and shoot tips from growing peach shoots was an effective and quick model system for studying photosynthetic and S6PDH enzyme responses to source-sink manipulation. Therefore, we used this approach in connection with northern blotting to better understand how sorbitol synthesis is regulated. Also, we measured S6PDH activity and gene expression at various times throughout the day to describe the nature of short-term (hourly) changes in these parameters.

Materials and Methods

Plant material and experimental design

One-year-old 'Nemaguard' peach (*Prunus persica*) plants were grown from rooted cuttings in 10-liter containers in a greenhouse in Athens, GA (34°N, 85°W). Two shoots on each plant were selected, and two adjacent leaves at 6-8 nodes below the apex were tagged for measurement of photosynthesis and enzyme activity. Measurements were performed in the greenhouse between 10:30 AM and 12:30 PM as described below. Following photosynthesis measurement of both leaves, one of the two leaves was removed, cut lengthwise, and half was used for immediate determination of S6PDH activity while the other half was flash-frozen in liquid nitrogen and stored at -80 °C for subsequent mRNA determination. Actively growing shoot tips were removed from the shoot to reduce sink demand from the remaining leaf, and approximately 24-hr later, photosynthesis, S6PDH activity, and mRNA of the second tagged leaf were measured in

a manner similar to that for the first leaf. This experiment was performed four times on different days, using 5 different plants, for a total sample size of 20 leaves per treatment (before and 24-hour after shoot tip removal). Another experiment was designed to document the change in S6PDH activity and S6PDH gene expression throughout the day. For this experiment, leaves at nodes 6-8 from the apex were sampled three times during the day: at 7:30 AM (sunrise), 12:30, and (7:00 PM) sunset. This experiment was repeated twice with a sample size of six leaves per treatment.

Four representative pairs of samples for shoot tip removal and two samples for the time of day experiments were used for mRNA determination.

Measurement of leaf photosynthesis

Leaf carbon dioxide assimilation (A) was measured using a LI-6200 portable photosynthesis device outfitted with a ¼-liter chamber (LI-COR, Lincoln, NE). Measurement conditions were: light intensity of about 1500-2000 μmol m^{-2} sec^{-1}, temperature of 22-24°C, and relative humidity of 40-60%. Measurements were made at around 1200 ppm CO_2 to reduce the effect of variation in stomatal aperture on photosynthesis. At this level of CO_2, intercellular CO_2 readings were about 1000 ppm, near saturation for most C3 plants. Thus, variation in A should reflect changes in the biochemistry of photosynthesis, and should not be confounded by availability of CO_2 to the photosynthetic apparatus.

Enzyme activity

The activity of S6PDH was measured in order to correlate photosynthetic performance of the plant with the in-vitro propensity for sorbitol synthesis. Enzyme activity was assayed following the modified protocol developed for sorbitol dehydrogenase (SDH) by Lo Bianco et al. (1998). Approximately 80 to 100 mg of fresh leaf tissue was collected from the same leaves used to measure A. Tissue was

homogenized in 3.0 ml of extraction buffer (100 mM Tris at pH-9.0, 8% v/v glycerol, and 10 mM 2-mercaptoethenol) using a pre-chilled mortar and pestle in the presence of washed sand. Tween-20 (0.1% v/v) and polyvinylpolypyrolidine (PVPP, 1% w/v) were also added during homogenization of the tissue. The homogenate was centrifuged at 3000 *g* for 15 min and the supernatant was saved. The supernatant was desalted by chromatography through a Sephadex G-25 column at 4 °C. The enzyme was assayed in a total volume of 1.0 ml assay buffer (50 µl of extract, 50 mM at pH 9.0, 0.18 mM NADPH and 50 mM glucose-6-phosphate) by measuring the change in optical density at 340 nm at 25 ^{o}C on a Spectronic 2-D (Milton Roy, Rochester, NY) spectrophotometer. Enzyme specific activity was calculated and expressed as nanomoles of NADPH oxidized per minute per gram fresh weight of leaf tissue.

Total RNA isolation and Northern Blot Analysis

Total RNA was extracted from the frozen leaf tissue by using a Plant RNA Isolation Kit (RNeasy Plant Mini Kit., Qiagen Inc., Valencia, CA). Extraction was done following the supplier's protocol. Total RNA was visualized under UV light following 2% formaldehyde agarose gel electrophoresis to ensure RNA integrity (Fig. 5.1). Formaldehyde agarose gel electrophoresis was performed by standard procedures using 0.66 to 2.26 µg of total RNA per lane depending on availability, for each pair of treatments. Similar amounts of total RNA were loaded on to the gel for each pair of treatments. Following electrophoresis, the separated total RNA bands were transferred to a positively charged nylon membrane by overnight capillary transfer using 10XSSC transfer buffer at pH 7.0. A S6PDH cDNA from apple (Kanayama et al., 1992) was labeled by random primer incorporation of dUTP linked to digoxigenin [(DIG) DIG-High Prime, Boehringer Mannheim, USA]. Pre-hybridization and hybridizations of the membrane was carried out overnight at 39 ^{o}C in DIG Easy Hyb provided in the kit. The

membrane was washed in 2XSSC, 0.1% SDS for 2x5 min at room temperature and 0.5XSSC, 0.1% SDS for 2x15 min at 56 ^{o}C. The hybridization was detected immunologically with alkaline phosphatase conjugated to antibodies against DIG and the chemiluminescence substrate CSPD (Boehringer Mannheim, USA). Chemiluminescence was recorded by direct detection using a Chemimager (Alpha Innotech Corparation, San Leandro, CA, U.S.A.).

S6PDH activity and mRNA levels versus time of day

In order to document hourly changes in gene and enzyme activity, leaves were sampled three times: at sunrise, 12:30 PM and sunset. Each sampled leaf was used for S6PDH activity and mRNA analysis by dividing it lengthwise into two pieces. This experiment was repeated two times with a sample size of six for each treatment for S6PDH activity measurement. Only two representative samples from each repeat were analyzed for mRNA. Enzyme activity and mRNA determinations were carried out as described above.

Sorbitol incubation of enzyme extract in vitro

A simple experiment was performed to document possible feedback inhibition of S6PDH activity by sorbitol *in vitro*. Sorbitol was incubated with peach leaf enzyme extract following a modified protocol of Archbold (1999). In assay cuvette, 0 mM sorbitol (assay buffer only), 200 mM sorbitol, 200 mM PEG (MW=400), or 200 mM mannitol was added to each leaf enzyme extract and the mix was incubated for at least two hours prior to the activity assay. Polyethylene glycol and mannitol were used to provide osmotic potentials similar to that of the sorbitol treatment to separate substrate and osmotic effects on enzyme activity. Each treatment was replicated eight times.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) or paired or regular t-test and simple correlation using Sigma Stat (SPSS Inc., Chicago, IL). Tukey's studentized comparison was used to separate means when ANOVA results were significant. For mean comparisons or correlations, all data from repeated experiments were pooled.

Results

Correlation of mRNA levels in adjacent leaves.

In shoot tip removal experiments, two adjacent, similar leaves were tagged, with one sampled before shoot tip removal and one 24-hour later. We assumed that they were physiologically similar, since destructive sampling dictated that at least two leaves were used per replicate. There was a high correlation between the levels of mRNA from leaves sampled before, and adjacent leaves sampled 24-hour after shoot tip removal, with data falling just above a 1:1 line (Fig. 5.2). This result suggests that mRNA levels in paired leaf samples were reflective of each other, and supports the assumption that leaves were similar, at least in terms of S6PDH transcript levels. S6PDH activity levels were known to be similar for adjacent leaves at nodes 6-8 (Karakas, 2001) as were photosynthesis measurements (data not shown), further justifying the experimental assumption that adjacent leaves were physiologically similar.

Photosynthesis and enzyme activity response to shoot tip removal

In contrast to previous in shoot tip removal experiment results by Karakas (2001), leaf photosynthetic rate did not change significantly in response to shoot tip removal (Table 5.1). However, S6PDH activity was reduced significantly by shoot tip removal (Table 5.1). In this experiment, we used the same plants one month after the previous shoot tip removal experiments described by Karakas (2001) were performed. During this one-month period, growth rate slowed, which may have reduced the influence of shoot

tip removal on source-sink relationships in the shoot, resulting in a smaller impact on photosynthesis. However, this did not preclude an examination of S6PDH gene expression and enzyme activity, since the later consistently decreased in response to shoot tip removal.

Due to high variation in Amax, another statistical analysis was performed to examine the relationship between Amax and S6PDH. Results from the highest showed a significant positive correlation between leaf photosynthesis and S6PDH enzyme activity measured during the shoot tip removal experiment when all data were pooled (i.e., before and after shoot tip removal) (Fig. 5.3). Thus, although shoot tip removal did not produce significant, parallel changes in photosynthesis and S6PDH activity as determined by paired t-test (Table 5.1) an overall positive trend was revealed when data were pooled. This apparent contradiction stems from a difference in statistical analysis between the data presented in Table 5.1 and Figure 3. In Table 5.1, paired t-tests were used with a relatively small sample size, and in Fig. 5.3, correlation tests with a higher sample size were used. The discrete t-tests do not have the power to resolve the overall trend seen with correlation analysis. We believe that, in general, photosynthesis and S6PDH activity parallel each other, since overall correlations were significant and previous shoot tip removal experiments by Karakas (2001) support this view. While significant, the low r^2 (0.30) indicates that much of the variation in S6PDH activity cannot be explained by variation in photosynthesis.

S6PDH activity, time of day, and sorbitol as a source of feedback inhibition on S6PDH activity

Enzyme activity was also responsive to the time of day, with activity lower at midday than at sunrise (Fig. 5.4). Incubation of peach leaf extracts with 200 mM sorbitol in vitro did not change the activity of S6PDH (Fig. 5.5), indicating that the enzyme does

not exhibit feedback inhibition by sorbitol in vitro. The concentration of 200 mM sorbitol added to enzyme extracts is at least twice as great as the change in concentration of leaf sorbitol between sunrise and midday (Karakas (2001). S6PDH activity responded similarly when incubated with equimolar concentrations of mannitol. However, incubation with polyethylene glycol (PEG) greatly increased S6PDH activity relative to all other treatments (Fig. 5.5). The reason why PEG enhanced activity of this enzyme in vitro is not known to as at this time. PEG concentrations were similar to those of sorbitol and mannitol, thus the enhancement was not an osmotic effect.

Transcription of the sorbitol-6-phosphate dehydrogenase gene (mRNA levels)

S6PDH gene transcripts changed in response to shoot tip removal from the plant (Fig. 5.6). Transcript levels increased significantly 24-hr after shoot tip removal. However, the transcript levels were not significantly different when sampled throughout the day (Fig. 5.7).

Discussion

In this study, we used source-sink manipulation to induce changes in photosynthesis and the activity of the main sorbitol synthetic enzyme, S6PDH. In addition, we reported novel, short-term changes (24-hr) in transcript accumulation for this enzyme. Photosynthesis and S6PDH activity were positively correlated in this study, as found previously by Karakas (2001), although decreases in photosynthesis 24 hr after shoot tip removal were non-significant when evaluated by t-test. Therefore, new information on S6PDH regulation has been presented, yet new questions also have been raised.

Karakas (2001) documented shoot tip removal to be a quick and effective way of manipulating source/sink relationships in potted peach plants. Shoot tip removal was especially effective in changing the activity of S6PDH in both Karakas (2001) and

current study. However, photosynthesis in the slower growing shoots in the current experiment did not respond the same as the faster growing shoots used in Karakas (2001), despite using the same plants. This may have been due to a slower growth rate and root confinement as plants remained in the same pots during the summer. Removal of weak sinks (low growth rate) may not have been as effective as the strong sinks (high growth rate) in terms of impact on photosynthesis. In any event, sink removal consistently caused a decrease in S6PDH activity, which provides a model for studying the source of enzyme regulation.

In addition to the drop in S6PDH activity resulting from source-sink manipulation, a change in activity of S6PDH on an hourly time-period was detected (Fig. 5.4). The apparent drop in S6PDH activity during midday could be due to the decrease in growth or lower photosynthetic rates due to midday depression of photosynthesis and less activity or consequently less sorbitol utilization in sinks. The activity of S6PDH is known to be regulated by demand in sinks; this demand could be variable during the day and throughout the growing season (Sakanishi et al., 1998). The reduction in S6PDH enzyme activity could be also due to feedback inhibition by sorbitol, which builds up in source leaves during the day (Stoop et al., 1996; Lo Bianco et al., 2000). Relatively low sink demand for sorbitol due to root confinement in these plants could have accentuated this effect. Therefore, high levels of sorbitol in source leaves could have caused the S6PDH enzyme activity to drop significantly, similar to the sugar repression reported for mannitol dehydrogenase (MTD) in celery (Stoop et al., 1996). However, we found no evidence for feedback inhibition when the enzyme was incubated with sorbitol *in vitro* (Fig. 5.5). Based on previous measurements of diurnal variation in sorbitol concentration Karakas (2001), 200 mM sorbitol should have been sufficient to cause feedback inhibition, since the change in sorbitol *in vivo* is much less than this concentration.

Therefore, the S6PDH down-regulation reported here does not appear to stem from a simple feedback inhibition mechanism.

Gene expression (mRNA levels) increased within 24-hour of shoot tip removal (Fig. 5.6), although time of day did not affect transcript levels (Fig. 5.7). This increase in mRNA in response to sink removal may be considered a generalized stress response (i.e., biotic and/or abiotic). High levels of various polyols (e.g., sorbitol, mannitol) have been reported in plants under various environmental stresses as well during pathogen invasion (Stoop et al., 1996). Williamson et al. (1995) isolated a gene for MTD from celery, and later found its mRNA to be more abundant when celery cells were cultured on mannitol. They found a pathogenesis-related protein, ELI3, from parsley and *Arabidopsis* to have high homology with MTD from celery, supporting the function of the gene and mannitol in pathogen attack. In fact, pathogen attack caused an increase in expression of the MTD gene in celery, which would lead to greater potential catabolism of mannitol (Stoop et al., 1996). In a more recent study of transgenic tobacco, plants transformed with mannitol dehydrogenase (MTD) showed that when infected with fungus, mRNA levels increased compared to non-infected ones (Jennings et al., 1998). It is possible, although speculative, that our peach plants perceived shoot tip removal as a pathogen attack, and the increased expression the gene encoding S6PDH resulted from a signal relating the need for more polyol under stress conditions. The close relationship between mannitol and sorbitol, and their corresponding metabolic enzymes, supports the idea that sorbitol synthesis potential, through increased mRNA levels, should respond positively to manipulation which mimics stress or pathogen attack.

The increase in mRNA level following shoot tip removal can also be explained on the basis of "feast and famine" theories of sugar-responsive gene regulation (Koch, 1996). Although not on absolute levels, "feast" responses are those that involve up/down-

regulation of genes when a large supply of carbohydrate substrate becomes available. "Famine" responses are the opposite, where scarce supplies of carbohydrate cause genes to up/down regulate. The work by Archbold (1999) on SDH can be considered a feast response, since incubation with high sorbitol treatment up-regulated SDH activity in apple fruit tissue. The response reported here could be a feast response of the S6PDH gene as well, since we would expect a temporary accumulation of sorbitol in source leaves in response to sink removal. Considering sorbitol as a temporary and mobile storage compound, perhaps sorbitol synthesis potential, i.e., transcript level is increased to prepare for new sink development resulting from shoot tip removal and subsequent lateral bud break.

Along with transcript accumulation resulting from shoot tip removal (Fig. 5.6), simultaneously S6PDH enzyme activity dropped significantly (Table 5.1). Time of day experiments also indicated that gene transcription levels do not seem to change on an hourly basis, although enzyme activity does. Thus, S6PDH activity and mRNA levels were related neither in shoot removal nor in time of day experiments. Similar findings were reported by Sakanishi *et al.* (1998) in a similar study with S6PDH gene/enzyme and others with different genes/enzymes (Everard et al., 1994; Yamaguchi et al., 1996). This disagreement between the *in vitro* S6PDH activity and mRNA levels suggests that there is posttranslational, rather than transcriptional regulation of this enzyme. The source of the posttranslational regulation is unclear, but does not seem to be related to feedback inhibition by sorbitol accumulation in the leaf. A novel aspect of this study is the relatively fast response (about 24-hour) of the S6PDH gene to source/sink manipulation, which is much faster than the day- or week-long responses reported by Sakanishi et al. (1998).

The lack of correspondence between transcript accumulation and enzyme activity reported here has been reported for the mannitol system in celery as well. Everard et al. (1994) reported that the extracted enzyme activity of mannose-6-phosphate reductase (M6PR), analogous to S6PDH, did not correspond to the amount of M6PR protein. The slow response of S6PDH enzyme activity or amount to its transcript level in peach also agrees with the findings we report here (Sakanishi et al., 1998).Yamaguchi et al. (1996) also found similar weak correlations between the activity of NAD-dependent sorbitol dehydrogenase (SDH) and its transcript in apple fruits, also suggesting posttranscriptional modification of this important enzyme for sorbitol catabolism. Sequence comparisons made between M6PR and S6PDH (Kanayama et al., 1992) by Everard et al. (1997) showed homology across the complete open reading frames of both genes. The similarity of both genes supports the contention that their regulation may be similar as well.

Collectively, the data support a posttranslational mode of enzyme regulation in response to short-term source-sink manipulation, and although changes in gene expression occur, they do not explain the direction of the regulation. Furthermore, it is clear that the S6PDH gene is more rapidly responsive than previously indicated (Sakanishi et al., 1998). Similar results reported in studies of mannitol gene regulation and enzyme activity support the view of a generalized role for polyols in plants.

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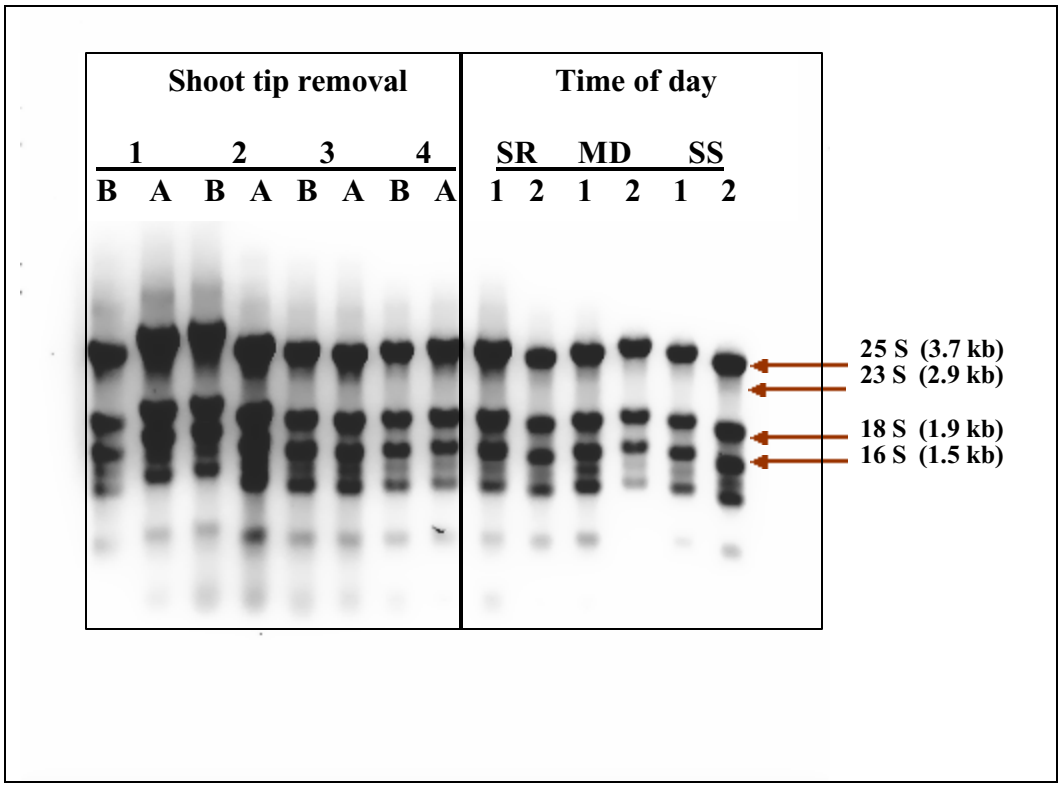


B= before shoot tip removal, A= 24-hour shoot tip removal
SR= sunrise, MD= 12:30, SS= sunset

Figure 5.1. An image of leaf total RNA from peach after 2% formaldehyde agarose gel electrophoresis, stain with ethidium bromide, and UV light exposure. Sharp bands of ribosomal (rRNA) indicate that total RNA was isolated intact. The bands confirm the equal amount of total RNA loaded to gel for each treatment pairs. Note that the shift in bands is due to addition of ethidium bromide to the gel for visualization of total RNA under UV light.

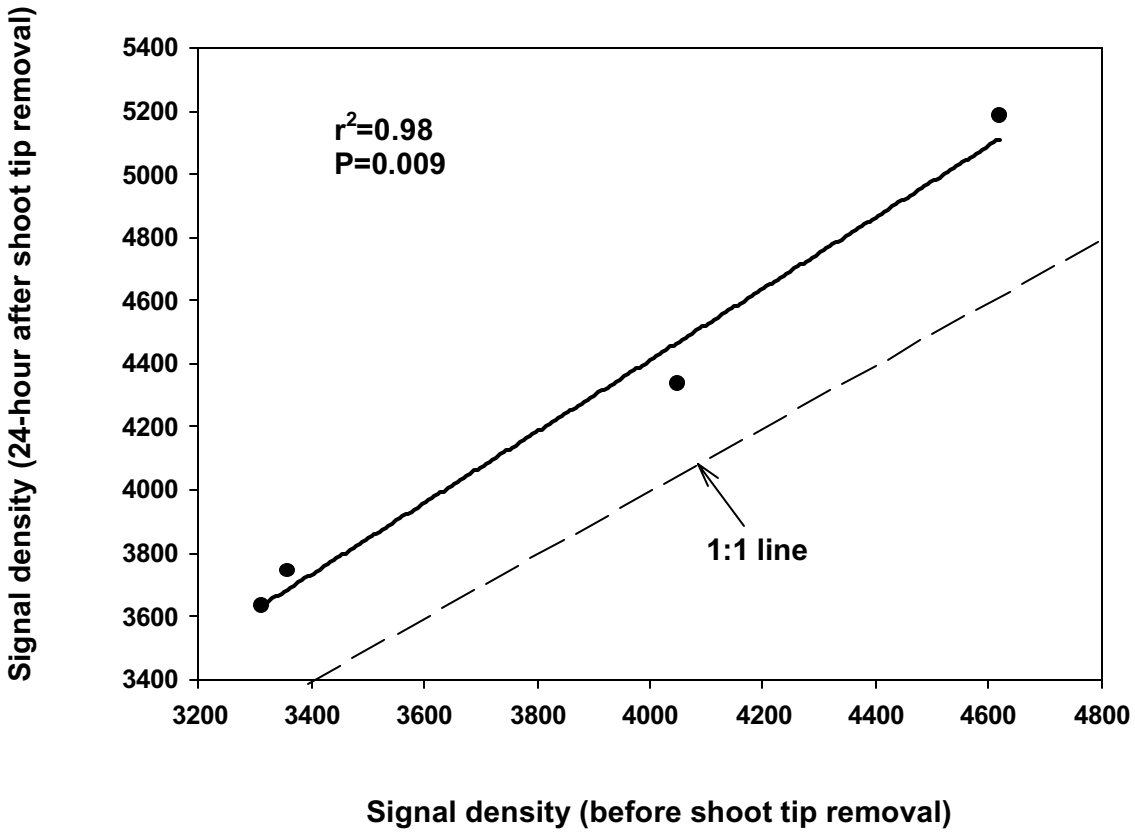


Figure 5.2. Correlation between signal densities of mRNA (chemiluminescence unit) before and 24-hour after shoot tip removal. Note that a 1:1 line of perfect correlation was drawn to show the proximity of data points to this line.

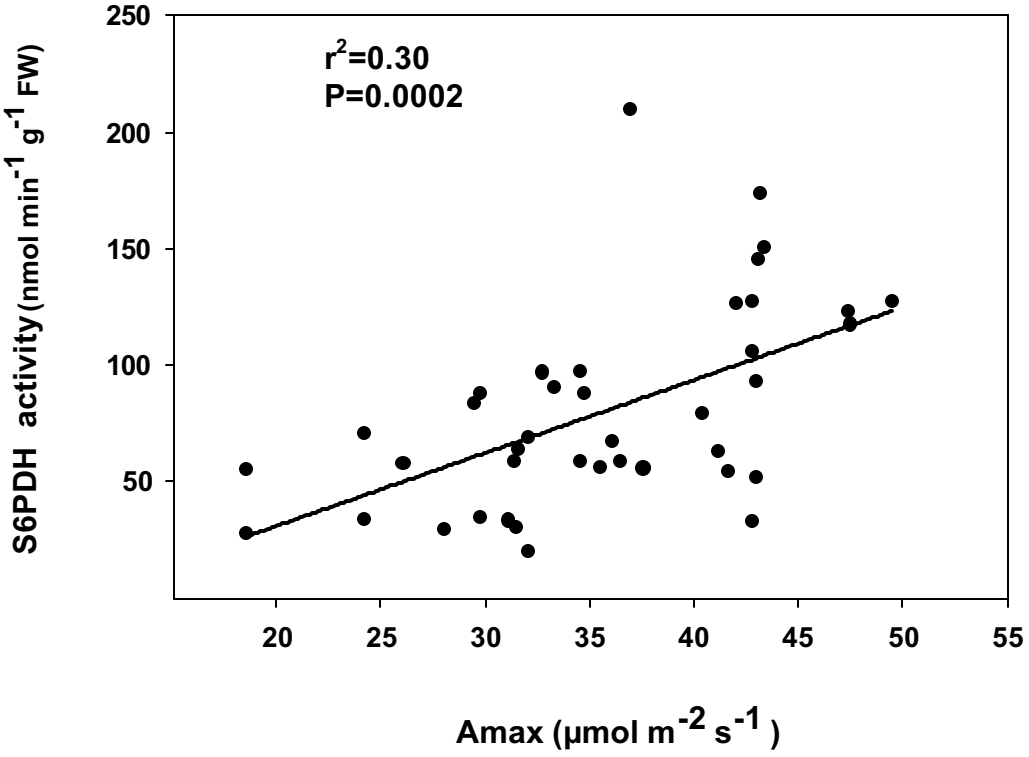


Figure 5.3. Correlation between Amax and S6PDH activity of potted peach plants in greenhouse. This correlation was generated by pooling all the photosynthesis and enzyme activity measurements taken before and after shoot tip removal.

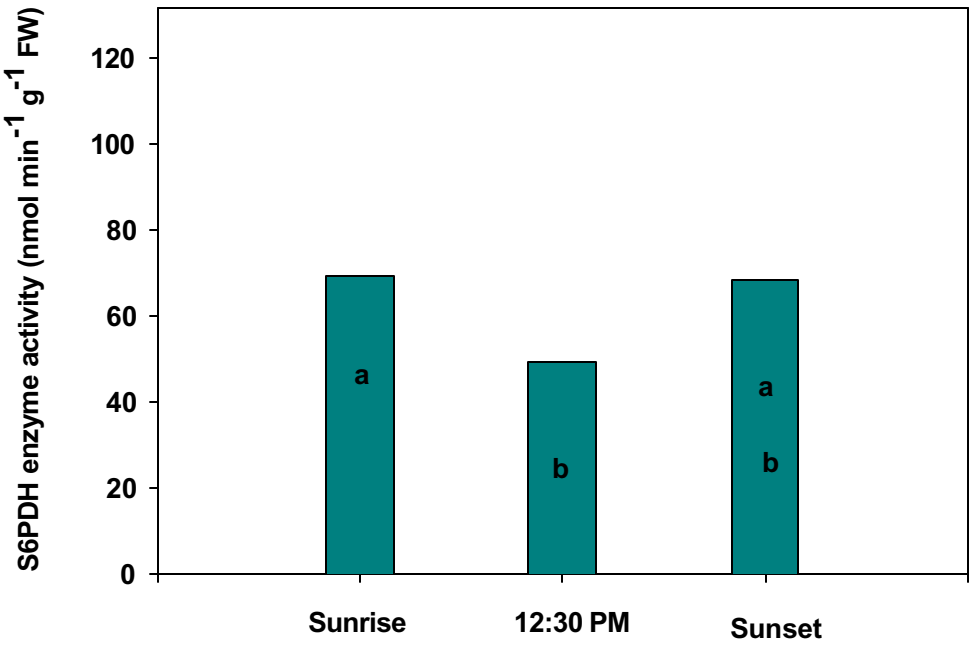


Figure 5.4. Time of day and S6PDH activity. Means contain the same lowercase letters for each figure are not significantly different at P≤ 0.05 levels. Leaves were sampled at sunrise, 12:30, and at sunset. The experiment was performed twice, with each time of day having a total sample size of 12.

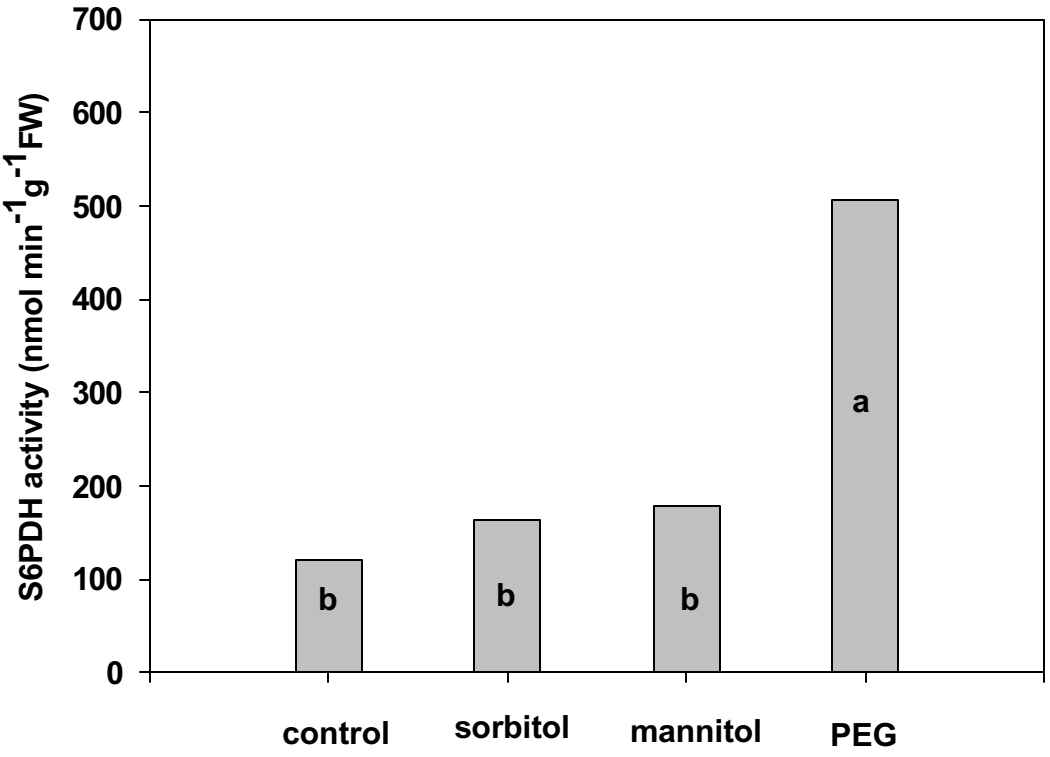
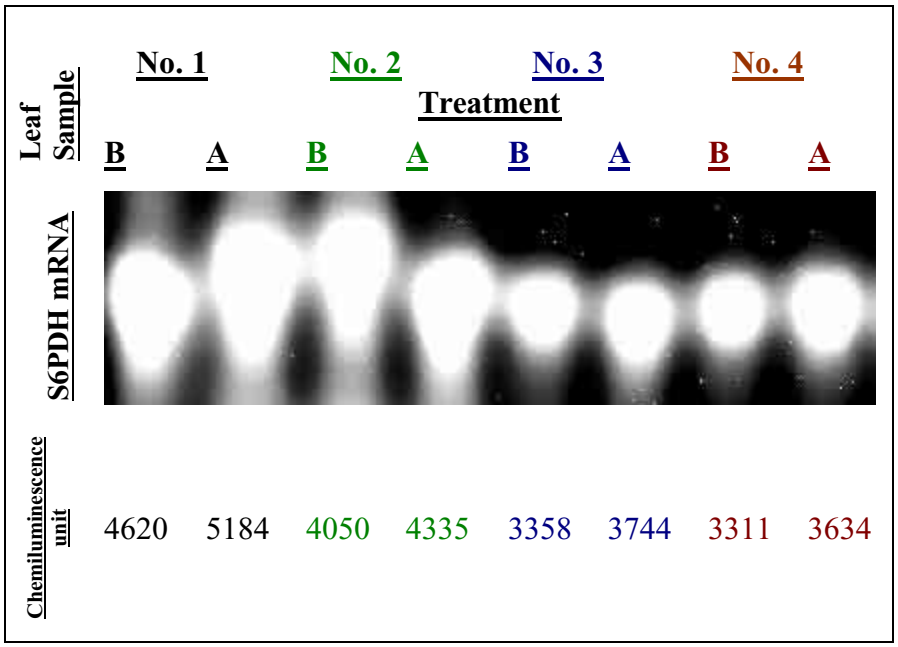


Figure 5.5. The effect of sorbitol incubation on peach leaf S6PDH activity *in vitro*. For each activity measurements control (assay buffer, 50 mM tris), 200 mM sorbitol, 200 mM PEG or 200 mM mannitol is added to enzyme extract and incubated for at last two-hour at room temperature before enzyme assay. Means containing the same lowercase letter are not significantly different at P≤ 0.05 levels.



B=before shoot tip removal, A=24-hr after shoot tip removal.

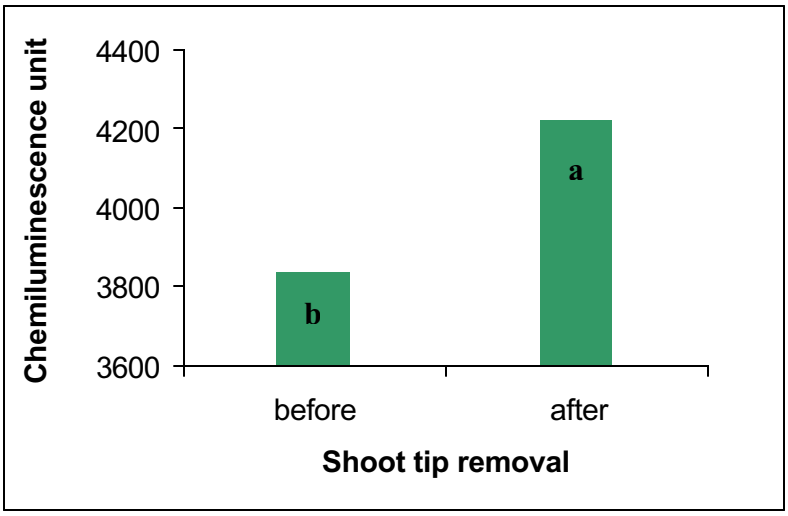
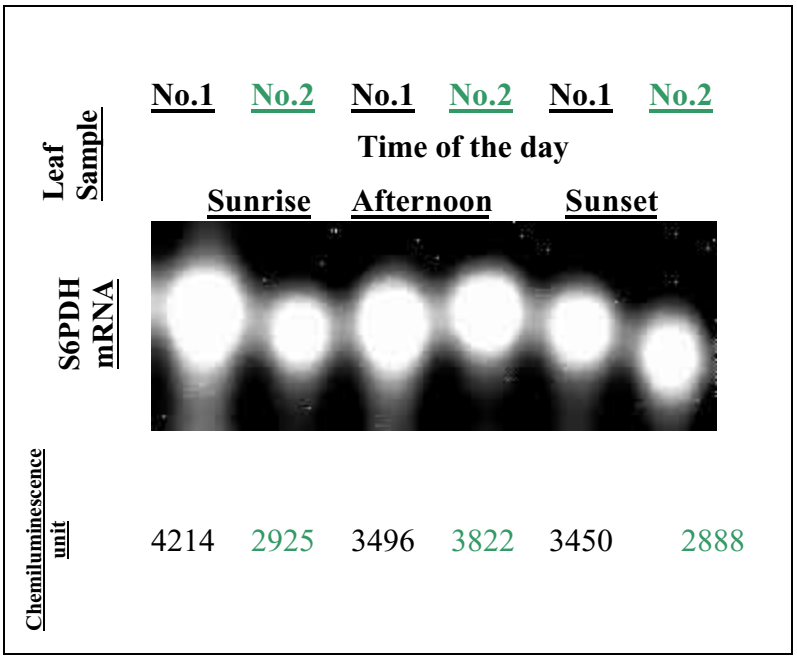


Figure 5.6. Transcript (mRNA) levels and the corresponding signal density readings (chemiluminescence unit) for S6PDH gene before and 24-hour after shoot tip removal of potted peach plants. Chemiluminescence unit containing the same lowercase letters for each sample pairs are not significantly different at $P \leq 0.05$ levels when compared by paired t-test. .



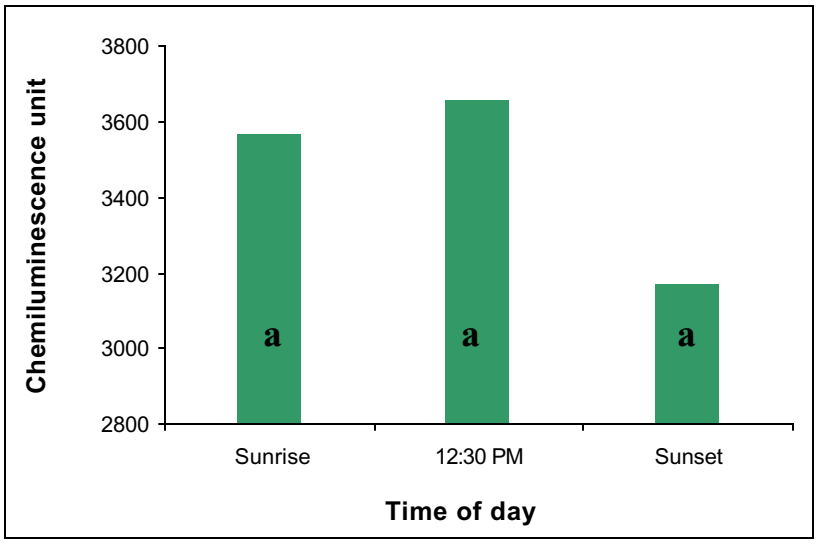


Figure 5.7. Sorbitol-6-phosphate dehydrogenase gene transcripts (mRNA) levels and corresponding density readings at different time of day. Two samples were evaluated at sunrise, 12:30 PM and sunset. Chemiluminescence unit containing the same lowercase letters for each sample at different time of day are not significantly different at $P \leq 0.05$.

Table 5.1. Amax and S6PDH activity before and 24-hour after shoot tip removal of potted peach plants in the greenhouse. Means containing the same lowercase letters for each row are not significantly different at P≤ 0.05 levels.

Character	Shoot tip removal	
	Before	**24-hour after**
Amax ($\mu mol\ m^{-2}\ s^{-1}$)	36.8 a	35.7 a
S6PDH activity ($nmol\ min^{-1}\ g^{-1}$ FW)	91.2 a	68.9 b

CHAPTER 6

CONCLUSIONS AND FUTURE PROSPECTS

Most higher plants exclusively synthesize sucrose and translocate it as the main form of carbon from source to sink. However, members of the Rosaceae family simultaneously synthesize and translocate both sorbitol and sucrose in various ratios. Researchers have been trying to answer the question of why some plants such as *Prunus* species produce sorbitol along with sucrose. Polyols have been associated with various functions, such as storage and translocation of carbon, osmotic adjustment, cold hardiness, plant stress tolerance, protection of enzymes and membranes, and free radical quenching (Keller, 1989; Loescher, 1987; Shen et al., 1997a; Shen et al., 1997b; Jennings et al., 1998; Nelson et al., 1998). *Prunus* species have been reported to have high photosynthetic rates and these high rates are hypothesized to be due to sorbitol synthesis in their source leaves (Flore and Lakso, 1989; Stoop et al., 1996). Specifically, high photosynthetic rates are thought to a result from increased NADP-NADPH turnover compared to plants that exclusively form sugars (one NADPH used to reduce hexose-P to alditol-P), and/or less feedback inhibition of photosynthesis due to the additional cytosolic sink for photosynthetically fixed CO_2 used for polyol synthesis (Stoop et al., 1996). However, this hypothesis has never been studied thoroughly.

My initial plan was to create a transgenic *Prunus* species, plum (*P. domestica*) with low or no sorbitol production via transformation with antisense S6PDH cDNA from apple and measure photosynthesis of transgenic plants. Putative transgenic plums were generated with positive PCR results. However, Southern analysis revealed negative results. Repeated failure to transform plum successfully led to a modified research plan.

The new approach focused on sorbitol biosynthesis, starting with several species from contrasting habitats with natural variations in sorbitol contents. From there, the research focused on progressively more specific experiments involving source/sink manipulation in peach (*Prunus persica*), and finally to sorbitol-6-phosphate dehydrogenase (S6PDH) gene expression. I have carried out three major studies in order to analyze sorbitol synthesis in relation to photosynthesis in various ways. As a result, I concluded the following major results:

- Photosynthesis and S6PDH activity were negatively correlated across eight different species, suggesting that sorbitol synthesis may represent a cost, not a benefit to leaf photosynthetic capacity of these species in greenhouse environments.
- Photosynthesis and S6PDH activity within a single *Prunus* species tended to have positive correlations, which was contradictory to the results with multi-species study. When all data from single species pooled, the correlation was quadratic.
- S6PDH gene expression and S6PDH activities did not change in parallel when source/sink manipulation occurred, suggesting possible post-translational modifications of the S6PDH enzyme during these experiments
- Down-regulation of S6PDH activity in response to sink removal was not related to a simple feedback inhibition of sorbitol on S6PDH activity.
- S6PDH gene might be more rapidly responsive than previously indicated by Sakanishi et al. (1998)

The first two studies presented conflicting data with respect to the role of sorbitol in photosynthesis, which was unexpected. Perhaps the high degree of genetic variation among the 8 species used in the first study made it difficult to assign a cause-effect relationship to the photosynthesis-S6PDH correlations. The more deliberate source/sink

manipulations used in the second study, with genetic variation reduced, may be more sound means of the testing overall hypothesis than a simple correlation study. A study with transgenic tobacco with S6PDH or mannitol-1-phosphate dehydrogenase (MT1D) could more directly reveal differences between transformed and non-transformed plants in NADP-NADPH turnover by measuring chlorophyll fluorescence under photo-inhibiting conditions. If sorbitol or mannitol has a role in enhancing photosynthesis via drawing extra energy (NADPH) from light reactions, plants transformed with S6PDH or MT1D genes should give lower chlorophyll florescence than non-transformed ones.

Further studies can be performed using molecular techniques (e.g., DNA micro-array from *Arabidopsis*) to test gene expression levels of all the genes that are involved in sorbitol and sucrose metabolism under various treatments (Lemieux et al., 1998; Schena et al., 1995; Schaffer et al., 2000; Richmond and Somerville, 2000). This approach might be useful to see simultaneous changes in sorbitol and sucrose metabolism related genes to document the relations between these two pathways. S6PDH or SDH genes with inducible promoters can be transferred to plants with high levels of sorbitol to regulate S6PDH or SDH activities and provide a wide range of sorbitol levels within single species (Gatz, 1997; Zuo and Chua, 2000).

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