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Carbohydrate partitioning and photosynthesis in Arabidopsis thaliana

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Carbohydrate partitioning and photosynthesis in *Arabidopsis thaliana*

by

Dan Joseph Stessman

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Plant Physiology

Program of Study Committee:
Steven Rodermel, Major Professor
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Iowa State University
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2004

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For the Major Program
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CHAPTER 1. GENERAL INTRODUCTION

This dissertation is organized into six chapters. Chapter 1 serves to describe the format of the dissertation and describe the general goal, approach, and expectations of the different projects undertaken. Chapter 2 is a review article submitted for publication that provides background information describing sink/source interactions and mechanisms of short-term and long-term control of photosynthesis. Sinks are considered those tissues that require import of photosynthate, primarily in the form of sucrose, to maintain growth and metabolism. Examples would include roots, fruits, and young expanding leaves. Sources are leaves that have made the transition from importing to exporting photosynthate, and source strength is a measure of the capacity of leaves to assimilate carbon and export sucrose. Past studies in our lab have examined photosynthesis in tobacco leaves and the effects of source strength on leaf development. We have turned to Arabidopsis as a model system to study due to the wealth of genetic information now available and mutant lines that can be easily obtained from the Arabidopsis Biological Resource Center (ABRC). As a basis for studies for sink/source control of photosynthesis, we studied several basic photosynthetic parameters in a single leaf of wild type Arabidopsis. Chapter 3 is a journal paper describing those experiments. To study the effects of increased sink demand on photosynthesis, we studied the immutans variegation mutant of Arabidopsis. These plants provided a resource for studying the effects of increased sink demand on photosynthesis, as green and white sectoring results in a reduction in total photosynthetic leaf area. Chapter 4 is a journal paper in preparation for publication which describes photosynthetic measurements and carbohydrate partitioning in leaves of the immutans. Chapter 5 describes
the characterization of *Arabidopsis* vacuolar acid invertase mutants. The goal of this study was to reduce partitioning into hexose pools by reducing vacuolar acid invertase activity. By reducing the hexose/sucrose ratio in leaves, it was expected that leaves would respond with greater photosynthesis and sucrose export. Chapters 3, 4, and 5 provide an overall view at basic photosynthetic parameters under varying sink and source strength conditions. Chapter 6 presents summarizes the research presented in the entire dissertation.
CHAPTER 2: SHORT-TERM AND LONG-TERM REGULATION OF PHOTOSYNTHESIS DURING LEAF DEVELOPMENT

A book chapter submitted to the Handbook of Plant and Crop Science for publication

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Introduction

Normal growth of plants depends on the coordinated regulation of sink and source metabolism. The growth of new sinks, such as fruits or new leaves, and the demand of non-photosynthetic tissues, such as roots, must be balanced with the source acquisition of nutrients, such as carbon assimilation during photosynthesis in fully-expanded leaves. This balance ensures efficient use of all nutrients by all parts of the plant. Short-term changes in this balance can result in regulation of enzyme activities by metabolic intermediates, while perturbing long-term growth conditions can result in regulation of gene expression and a reallocation of nutrients. For example, C3 photosynthesis is thought to be limited by CO2 at atmospheric concentrations (1). Therefore, C3 plants usually have a large excess of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), presumably to enable the plant to respond to rapidly fluctuating irradiance (2) and as a storage form of nitrogen (3). Typically, when C3 plants are grown at elevated CO2 levels there is an initial increase in
photosynthetic rate as a response to the increased substrate availability. However, after a short period the photosynthetic rate decreases to lower levels than plants grown at normal CO₂ levels. This acclimation response is controlled at the level of gene expression, with decreased expression of photosynthetic genes, such as \textit{RbcS} transcripts and decreases in Rubisco protein (4, 5, 6, 7). This response also ensures reallocation of nitrogen from Rubisco to other parts of the plant. Changes in growth conditions that lower sink demand usually result in down-regulation of photosynthesis (8), while increases in sink demand result in up-regulation of photosynthesis (9, 10). These changes are controlled at the level of enzyme activities and gene transcription.

\textbf{Short-term Control of Photosynthesis}

Leaf development involves a balance between sink demand and source strength that can be affected by a variety of factors (11). Source photosynthesis and sink utilization occur in a coordinated fashion with fine control of enzyme activity by metabolic intermediates and coarse control by changes in photosynthetic gene expression (12). During photosynthesis in source leaves, Rubisco incorporates CO₂ into ribulose-1,5-bisphosphate (RuBP) to form 2 molecules of 3-phosphoglycerate (3-PGA) and ultimately triose phosphate (TP) (Figure 1). While some of this TP is used to regenerate RuBP, the remaining TP is exported from the chloroplast to the cytosol via the triose phosphate translocator with a mandatory exchange of inorganic phosphate (Pi) (13). TP is then synthesized into sucrose in the cytosol and ultimately exported to developing and non-photosynthesizing parts of the plant, e.g. roots, fruits, and young, emerging leaves.
When sink demand declines, the need for source photosynthetic capacity decreases in a coordinated fashion. This sink regulation of source strength is thought to be triggered by a buildup of carbohydrates, namely sucrose, in the source tissues. In the short term, the accumulation of sucrose causes a down-regulation of sucrose phosphate synthase (SPS) (14) and/or an increase in glucose and fructose as the accumulated sucrose is hydrolyzed by apoplastic or vacuolar invertases. Hexose phosphates begin to accumulate due to the decreased SPS activity and possibly the phosphorylation of free hexoses by hexokinase (15). An increase in fructose-6-phosphate stimulates fructose-6-phosphate, 2-kinase (Fru-6-P,2K) to form fructose-2,6-bisphosphate (F-2,6-BP), which in turn inhibits the cytosolic fructose-1,5-bisphosphatase (cytFBPase) (16). The overall result is an accumulation of TP and a decrease in free Pi in the cytosol, which limits the export of TP from the chloroplast via the triose phosphate translocator. In the chloroplast the increased ratio of 3-PGA/Pi stimulates ADP glucose pyrophosphorylase activity, the controlling step in starch synthesis (17, 18), resulting in increased starch in the source leaf. Hence, the overall short-term result of limiting sink utilization of sucrose is an increase in starch accumulation in the source leaf. Starch synthesis thus provides a buffer to a short-term decrease in demand for photosynthate, and provides a temporary “sink” for TP to maintain a high rate of photosynthetic activity in the leaf (12).

**Long-term Control of Photosynthesis**

The long-term adjustment to decreased sink demand involves a coarse control of photosynthesis via a down-regulation of gene expression for photosynthetic genes such as
RbcS and LhcB (5, 19, 20, 21). This down-regulation appears to be linked to the accumulation of carbohydrates, but the mechanism is not well understood. Studies of plants grown in enriched CO₂ environments demonstrate this kind of regulation (22, 23 24). For examples, in similar studies where plants have been transferred from ambient to elevated CO₂ environments (25), there is an initial short-term burst of enhanced photosynthetic activity followed by a down-regulation of photosynthesis, or acclimation response, in which CER values are lower than the ambient control. This decrease in photosynthesis is a result of decreased Rubisco activity, which is a result of decreased amounts of Rubisco protein as well as transcript levels for the nuclear-encoded gene for the Rubisco small subunit (rbcS) and the plastid-encoded gene for the Rubisco large subunit (rbcL). This down-regulation appears to be in response to an accumulation of all carbohydrates in the source leaf (24, 25).

To study the acclimation response we examined several photosynthetic parameters in a single leaf of tobacco grown at either ambient or elevated CO₂ concentrations (22). We selected leaf 10 (in order of emergence) because of its large size at full expansion. When tobacco plants are grown at ambient CO₂ (350 µL/L), individual leaves show a phase of increasing photosynthetic activity, followed by a brief period of maximal photosynthetic activity at day 12 (coincident with full leaf expansion), and ending with a long senescent phase of photosynthetic decline, during which time leaf yellowing occurs and nutrients are reallocated to the rest of the plant (Figure 2) (26, 27, 28). This pattern is similar for chlorophyll concentrations, total soluble proteins, and Rubisco protein contents and activities. To examine photosynthesis at elevated levels of CO₂, ambient-grown tobacco plants were transferred to controlled- CO₂ growth chambers (350 µL/L) when leaf 10 had reached 1 cm in length. This leaf initially shows an accelerated increase in photosynthesis
when compared to ambient conditions (Figure 2). However, the photosynthetic rate reaches an earlier photosynthetic maximum and begins to decline even before the leaf is fully-expanded. The senescent decline appears to proceed at the same rate as in ambient-grown tobacco, but with the carbon exchange rate (CER) reaching zero at approximately day 25 as opposed to day 35 in the tobacco grown at ambient CO2. This pattern is similar for chlorophyll concentrations and for Rubisco contents and activities.

Based on these results, we proposed a ‘temporal shift model’ to explain the acclimation response in plants grown at high CO2 (22) (Figure 3). In this model, there is a shift in timing of leaf development in high-CO2 grown plants with an earlier peak photosynthetic rate and a shift to an earlier onset of senescence. The lower CER observed in high-CO2 grown plants is due to a further progression of the senescent phase when compared to ambient-grown plants examined at the same time point.

If increased source strength results in an earlier onset of senescence, then decreased source strength might be expected to delay the onset of senescence. To test this hypothesis, we examined tobacco plants transformed with the RbcS gene in the antisense orientation (29). These plants have reduced amounts of Rubisco protein and decreased leaf carbohydrates. These plants also have an early slow-growth phenotype with a retarded leaf emergence and a greater number of smaller leaves. This is followed by a normal phase of growth similar at flowering to wild type plants. To study ‘developmentally similar’ leaves, we examined leaf 13 of antisense plants, as opposed to leaf 10 of wild type, due to similar expansion and size of the leaves. Photosynthetic rates of the antisense plants were lower than wild type and reached a peak at day 20, as opposed to day 12 in wild type (Figure 2). A prolonged senescent phase was also observed in antisense plants during which CER did not
fall below zero before day 55, compared to wild type around day 35. A similar pattern was observed with chlorophyll concentrations, Rubisco contents, and Rubisco activities. Taken together with the studies of high-CO₂ grown plants, we hypothesize that there is a threshold level of carbohydrate production which initiates the senescent phase of leaf development. This threshold level perhaps represents the point at which leaf source strength exceeds sink demand for photosynthate. When this occurs, an accumulation of carbohydrates may signal a down-regulation of photosynthesis and a progression into the senescent phase of leaf development.

**Carbohydrate Control of Gene Expression**

Carbohydrates have been shown to modulate the expression of genes in plants, including photosynthetic genes (20, 30, 31, 32, 33). Gene expression appears to be controlled by a “feast versus famine” condition that may exist in the plant. Feast gene expression favors those genes that involve storage and utilization of carbohydrates, while famine favors expression of genes for photosynthesis, reserve mobilization, and export processes (30). The coordination between the two ensures a balance of utilization and acquisition of resources.

Much of the attention about gene expression responses to carbohydrates has focused on hexokinase-mediated signaling. It is known that yeast hexokinase has a dual function of catalysis and signaling. Jang and Sheen have proposed that hexokinase plays a similar role in plants, with a catalytic activity and a hexose-sensing or signaling activity (21, 34). The hexose sensing ability appears to be dependent on metabolism of hexoses rather than on the concentrations of the substrates or products. This was demonstrated in a maize protoplast
expression system, in which sugars that are substrates for HXK were able to repress expression of photosynthetic genes (20, 34). Glucose analogs that are transported across the plasma membrane but are not phosphorylated by HXK did not repress photosynthetic gene expression. The same is true of hexose phosphates that were delivered by electroporation into the protoplasts. 2-deoxyglucose and mannose, which are phosphorylated by HXK but not further metabolized, were able to repress gene expression. Mannoheptulose, an inhibitor of HXK, is able to block the repression caused by mannose (35).

Experiments with transgenic Arabidopsis also demonstrate the signaling effects of hexokinase. Arabidopsis contains two genes encoding hexokinase, AtHXK1 and AtHXK2. Plants that overexpress AtHXK1 or AtHXK2 displayed a hypersensitive response when grown on glucose media, with enhanced repression of RBCS gene expression (21). Antisense hexokinase plants had a hyposensitive response with reduced repression of RBCS expression. Mutants overexpressing the sense yeast HXK2 gene also showed a hyposensitive response even though they had an elevated phosphorylating activity. This occurred presumably because the yeast signaling activity is not recognized by the plant system, and the increased catalytic activity reduced the available substrates for the endogenous enzyme. Overexpression of the Arabidopsis AtHXK1 in tomato shows a decrease in photosynthesis and an accelerated senescence (36).

Invertase, Sucrose Cycling, and a Possible Control Point of Photosynthesis

Invertase is an enzyme that catalyzes the irreversible reaction converting sucrose to glucose and fructose. In plants there are two forms of the enzyme, an acid and a neutral/alkaline form, each reflecting the pH optimum of the enzyme (37, 38). The neutral
form exists in the cytosol while the acid form is located in both the apoplast and the vacuole. The acid invertases are encoded by a small gene family, with separate and multiple genes for both the apoplastic and vacuolar forms. Both forms of invertase, along with sucrose synthase, are associated with sink tissues that require a constant influx of sucrose for metabolism (39). Invertase maintains this flux by hydrolyzing sucrose and thus maintaining a gradient of sucrose from source to sink.

A perplexing observation is that source leaves have acid invertase activity (40). This would seem to confound the ability of the source leaf to export sucrose if it is hydrolyzed. However, it has been proposed that acid invertase is part of a sensing system that initiates sink regulation of photosynthesis (23, 41, 42). According to this proposal, limited utilization of sucrose by sinks would cause an accumulation of sucrose in source leaves. This sucrose would then be hydrolyzed into hexoses by either the apoplastic or vacuolar acid invertases, and the free hexoses would enter the cytosol and be phosphorylated by hexokinase. The hexose-phosphates would be re-synthesized into sucrose. As stated previously, this would ultimately shift partitioning toward starch synthesis. Indeed, species that have high activities of vacuolar acid invertase tend to store higher amounts of starch (43). Also, the net effect of sucrose hydrolysis by invertase and synthesis through hexokinase would generate a signal to down-regulate photosynthesis (Figure 1).

Experiments with tobacco that overexpress a yeast invertase provide an extreme example of the possible effects of source invertase activity. These plants develop pale sectors in the leaves that contain low levels of chlorophyll and accumulate large amounts of all carbohydrates due to an inhibition of sucrose export (15). These leaves also have lower rates of photosynthesis with decreased levels of Calvin cycle enzymes. Interestingly, SPS
activity is increased in these plants, this may be indicative of an increased cycling of sucrose synthesis and hydrolysis. Also interesting is that the pale sectors do not appear until the leaf has made the sink to source transition. This enhances the role of carbohydrates in regulating photosynthesis, and possibly a role for acid invertase in generating the signal by sucrose hydrolysis.

Antisense experiments with acid invertase, however, have not yielded the opposite effect, that being an increase in photosynthesis by decreased generation of hexoses or an increased sucrose/hexose ratio. Rather, these experiments have demonstrated that vacuolar acid invertase controls the ratio of sucrose to hexose stored in tomato fruits and tomato leaves (44, 45), and that there appears to be a threshold level of activity above which hexoses rather than sucrose accumulate (41, 44). This also occurs over a range of species, with those that have low vacuolar acid invertase storing sucrose (23).

Moore et. al. (23) have shown that the acclimation response to high CO₂ is more pronounced in species with high vacuolar acid invertase. In a wide range of species examined, there was no correlation between the photosynthetic decline and the amount of carbohydrate that accumulated in leaves, nor was it correlated with any particular sugar. Rather, high acid vacuolar acid invertase activity was always associated with a decrease in photosynthetic capacity, and those species that lacked an acclimation response had low invertase activity. These data also showed a threshold response.

The evidence for a role of invertase and sucrose cycling in the regulation of photosynthesis is indirect at best. To address this question, we pulse-fed ¹⁴CO₂ to Arabidopsis during different time points of development (46). We chose Arabidopsis
because of its wide use as a model organism, with a wealth of genetic information available. The feeding experiments had two goals: 1) to characterize photosynthesis during development of a single leaf, and 2) to examine the short-term partitioning or flux of newly-synthesized carbohydrates which may correlate with regulation of photosynthesis. For this experiment, we exposed a whole *Arabidopsis* plant to $^{14}$CO$_2$ in an enclosed chamber for a 10 minutes followed by a chase in unlabeled air for 10 minutes to allow time for partitioning of label into the different carbohydrate fractions. After the chase, leaf 8 was harvested and evaluated for partitioning of $^{14}$C into ethanol-soluble and ethanol-insoluble fractions. The soluble fraction was further fractionated into neutral, anionic, and cationic fractions, with the neutral fraction containing sucrose, glucose, and fructose. Partitioning into sucrose, glucose, and fructose was analyzed using TLC plates.

Our experiments demonstrated that in *Arabidopsis*, the photosynthetic rate declines from the first time point measured (Figure 4B), as do chlorophyll concentrations and total protein levels. This decline occurs even as the leaf is still expanding and increasing in fresh weight. This is similar to results obtained from studies of photosynthetic rates using the entire rosette of *Arabidopsis* (28), but is in contrast to the previously mentioned tobacco experiments which initially have a period of increasing photosynthetic rate followed by a senescent decline (22). The expression of *LhcB* declines slightly during development, with a dramatic decline after full leaf expansion is attained (Figure 6). The expression of the senescence associated gene, *SAG12*, dramatically increased at the last time point measured (Figure 6).
The majority of $^{14}$C incorporation was into the neutral soluble sugar fraction at each time point measured. Further analysis of this fraction revealed that the majority of the label was present in the sucrose fraction (Figure 5B). However, there was an increasing amount of label incorporated into the hexose fraction until full leaf expansion was attained, after which the partitioning of label into hexoses declined (Figure 5B). This increase was not reflected in the total pool sizes of hexoses in the leaf. Rather, the maximum flux of label into hexoses coincided with the strong decrease in $LhcB$ expression. At this time the leaf may have reached a threshold level of hexose metabolism or sucrose cycling, which would support the hypothesis that flux of carbohydrate through invertase and hexokinase signals a decrease in photosynthetic gene expression, since it is the short-term partitioning into hexoses and not a change in total pool size that correlates with gene expression changes. However, this evidence is indirect and cannot account for the decline in photosynthetic rate that occurs from the beginning of Arabidopsis leaf development.

Interestingly, these experiments showed no change in acid invertase or hexokinase activities early in leaf development when partitioning of labeled $^{14}$C into hexoses increases, indicating there is probably some other factor controlling partitioning between sucrose and hexose at this time. However, others have shown that the source leaf acid invertase activity may serve as a mechanism to detect accumulation of carbohydrates under conditions of stress rather than the normal developmental process. It is known that acid invertase expression is induced by pathogen infection or wounding (47, 48, 49, 50, 51). This may serve as a signal to down-regulate photosynthesis to shift metabolic needs towards defense mechanisms.
Conclusions

The balance between sink utilization of photosynthate and source strength is carefully controlled by both a short-term control of sucrose/starch partitioning by intermediate metabolites and by a long-term regulation by gene expression. Perturbing the sink-source balance can interrupt the partitioning of carbohydrates and lead to an accumulation of carbohydrates in source leaves. The accumulation of carbohydrates, specifically sucrose, may generate a signal to down-regulate photosynthesis through metabolism by acid invertase and hexokinase (23). There are also non-hexokinase mediated signaling pathways and sucrose signaling pathways that may also contribute to the regulation of carbohydrate production and its mobilization to sinks (30, 33, 52). To date these pathways are not well characterized. Several mutants have been identified that display a sugar-responsive phenotype, but very few have linked the signal with changes in gene expression. Rather, most have identified points of cross-talk with hormone signaling pathways. The existence of multiple gene families and redundancy of metabolic pathways probably makes direct identification of mutants involved in signaling difficult to obtain or characterize. The combination of activation-tagging, selection of sugar-responsive mutants, T-DNA knockout lines, and information from genome sequencing projects will continue to aid in the dissection of the pathways relating carbohydrate signaling and gene expression responses.

References


Figure Legends

Figure 1. Schematic of fine and coarse control points of carbon partitioning in a source leaf cell. CO₂ is fixed by Rubisco to form triose-phosphate (TP), which can either be converted to starch in the chloroplast, or exit the chloroplast via the triose-phosphate translocator. In the cytosol TP is converted to sucrose involving several steps, with controlling reactions catalyzed by FBPase and SPS. The sucrose is then exported to sink tissues. If sucrose accumulates, it is hydrolyzed by the cell wall or vacuole form of acid invertase and re-enters the cytosol where it is phosphorylated by hexokinase. The phosphorylation of hexoses by hexokinase sends a signal which results in down-regulation of genes involved in photosynthesis (5, 12, 19, 21). Abbreviations: RuBP, ribulose-1,5-bisphosphate; TP, triose-phosphate; ADP-Glc, adenine-5-diphosphate glucose; AGPase, adenine-5-diphosphate glucose pyrophosphorylase; Pi, inorganic phosphate; PPI, Pyrophosphate; F-1,5-BP, fructose-1,5-bisphosphate; FBPase, fructose-1,5-bisphosphatase; F6P, fructose-6-phosphate; F-2,6-BP, fructose-2,6-bisphosphate; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate; SPS, sucrose phosphate synthase; Suc, sucrose; Glc, glucose; Fru, fructose.

Figure 2. Photosynthetic rates (CERs) of tobacco grown at ambient and elevated CO₂ levels, and Rubisco antisense tobacco grown at ambient CO₂. For comparisons of wild type and antisense, plants were maintained under identical grown conditions. For tests at high CO₂, plants were transferred to elevated CO₂ when leaf 10 had reached 1 cm in length. CER measurements were taken from developmentally similar leaves of the different treatments throughout leaf ontogeny. Leaf 10 was used for the ambient and high CO₂-grown wild type
plants, and leaf 13 for the antisense plants. Each point represents the average (±SD) of multiple measurements from at least four different plants. (Adapted from 22, 29).

**Figure 3.** Comparison of temporal shift model as compared to the down-regulation model of acclimation to high CO₂. The inner arrows represent the perceived shift in photosynthetic rate observed under elevated CO₂ concentrations. The down-regulation model explains acclimation as an overall decrease in photosynthesis at all time points. The temporal shift model explains acclimation as a shift to an accelerated leaf development, with the decrease in photosynthesis due to an earlier peak in photosynthetic rate (from 22).

**Figure 4.** Changes in leaf expansion and photosynthetic rates during the development of leaf 8 of *Arabidopsis* grown under constant light conditions. Leaf expansion measurements were initiated when the leaves were 5 mm wide (day 1). Each point is the average of 31 plants. Photosynthetic rates were determined using 5 or 6 leaves per time point (from 46).

**Figure 5.** Carbon partitioning during the ontogeny of Arabidopsis leaf 8 grown under constant light conditions. (A) Incorporation of ^14^CO₂ into ethanol-insoluble and ethanol-soluble fractions. The soluble fraction was further fractionated into neutral, cationic, and anionic fractions. Each time point represents 5 leaf samples. Error bars are the standard error for each point. (B) The neutral fraction was further analyzed for sucrose, glucose, and fructose (from 46).
Figure 6. Northern blot of total RNA from leaf 8 of Arabidopsis grown under constant light. Blots were probed with the photosynthetic gene, LhcB, and the senescence-associated gene, SAG12. Each lane contains 5 µg of total RNA (from 46).
Figure 1
Figure 2
Figure 3
Figure 4

A: Graph showing the increase in leaf width (mm) over days.
B: Graph showing the decrease in photosynthetic rate (nmol CO₂/min/g FW) with leaf age (days).
Figure 5

14C Label Incorporation (nmol/min/g FW)

Leaf Age (days)

A

Fructose
Glucose

Soluble
Insoluble
Calcium
Anion
Figure 6

Leaf Age (days)

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Figure 6
CHAPTER 3: REGULATION OF PHOTOSYNTHESIS DURING ARABIDOPSIS LEAF DEVELOPMENT IN CONTINUOUS LIGHT


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Abstract

Previous investigations in our laboratory have shown that leaf developmental programming in tobacco is regulated by source strength. One hypothesis to explain how source strength is perceived is that hexokinase acts as a sensor of carbohydrate flux to regulate the expression of photosynthetic genes, possibly as a result of sucrose cycling through acid invertase and hexokinase. We have turned to \textit{Arabidopsis} as a model system to study leaf development and have examined various photosynthetic parameters during the ontogeny of a single leaf on the \textit{Arabidopsis} rosette grown in continuous light. We found that photosynthetic rates, photosynthetic gene expression, pigment contents and total protein amounts attain peak levels early in the expansion phase of development, then decline progressively as development proceeds. In contrast, the flux of $^{14}\text{CO}_2$ into hexoses increases modestly until full expansion is attained, then falls in the fully-expanded leaf. Partitioning of carbon into hexoses versus sucrose increases until full expansion is attained, then falls.
The in vitro activities of hexokinase, vacuolar acid invertase, and cell wall acid invertase do not change until the late stages of senescence, when they increase markedly. At this time there are also dramatic increases in hexose pool sizes and in senescence-associated gene (SAG) expression. Taken together, our results suggest that invertase and hexokinase activities do not control the partitioning of label into hexoses during development. We conclude that our data are not readily compatible with a simple model of leaf development whereby alterations in photosynthetic rates are mediated directly by hexose flux or by hexose pool sizes. Yet, these factors might contribute to the control of gene expression.

Introduction

Dicot leaf development is characterized by a phase of increasing photosynthetic rates, usually coincident with leaf expansion, a phase of maximal rates, and finally by a prolonged senescence phase of declining photosynthetic rates (reviewed by Gepstein 1988). During senescence, chloroplasts differentiate into gerontoplasts and resources are reallocated to other parts of the plant (Matile 1992). Previous investigations in our laboratory have revealed that source strength (the capacity to produce and export carbohydrates) plays a central role in regulating tobacco leaf developmental programming. In leaves from plants grown in elevated CO$_2$ (increased source strength), there is an acceleration in the onset of the photosynthetic decline associated with senescence, but the progression and duration of this phase is unperturbed (the “temporal shift” model of acclimation to elevated CO$_2$) (Miller et al. 1997); this model has received recent support (Ludewig and Sonnewald 2000). By contrast, leaves from rbcS antisense mutants have a significantly prolonged senescence phase and are longer-lived than normal (Tsai et al. 1997;
Miller et al. 2000). These mutants express rbcS antisense mRNAs and have reduced carbohydrate production (decreased source strength) because of reduced levels of the Rubisco holoenzyme (Rodermel et al. 1988; Quick et al. 1991a; Quick et al. 1991b).

The tobacco experiments indicate that leaf development is capable of being modulated in diverse ways to respond to a wide range of source strength conditions. Our working hypothesis is that source strength serves as a signal to regulate photosynthetic gene expression, and that changes in photosynthetic gene expression provide a coarse control of photosynthetic rates during leaf development. In support of this hypothesis, it is well-established that sugars exert feedback regulation on photosynthesis (sink regulation of photosynthesis) and that sugars regulate the expression of both photosynthetic and non-photosynthetic genes (reviewed by Koch 1996; Smeekens and Rook 1997; Moore et al. 1999). It has been hypothesized that carbohydrate-dependent control of photosynthetic gene transcription occurs by a signaling mechanism involving cytosolic hexokinase as a flux sensor (Jang and Sheen 1994; Jang et al., 1997). It is possible that a similar mechanism modulates leaf developmental programming, inasmuch as leaves of transgenic tomato plants that overexpress an Arabidopsis hexokinase gene (HXK1) appear to undergo accelerated senescence (Dai et al. 1999).

According to the hexokinase mechanism of sugar-sensing, alterations in cytosolic hexose levels would be anticipated to alter the amplitude of the hexokinase signal (Koch 1996). Yet, sugar levels are not always correlated with changes in photosynthetic gene expression (e.g., Moore et al. 1997; Gesch et al. 1998; Sun et al. 1999). For instance, cytosolic hexose pool sizes are not altered in leaves of tobacco, snapdragon and parsley exposed to elevated CO₂, despite a pronounced down-regulation of photosynthetic gene
expression (acclimation) in these plants (Moore et al. 1997). To account for this lack of correspondence, Seemann and colleagues proposed that sucrose cycling is a major source of hexose signals (Moore et al. 1999). In mature leaves, sucrose is hydrolyzed to glucose and fructose, primarily by vacuolar and cell wall acid invertases. To re-enter metabolism, glucose and fructose must be phosphorylated by hexokinase; sucrose cycling occurs when the phosphorylated sugars are used to re-synthesize sucrose (Huber 1989; Nguyen-Quoc and Foyer 2001). This cycle is energetically costly because one ATP is required per sucrose that is synthesized. Moore et al. (1999) suggested that if sufficient hydrolytic activity exists, alterations in the rate of sucrose cycling through invertase and hexokinase (e.g., by altering the growth CO2 conditions) could enhance signaling through hexokinase, even if there were little measurable affect on cytosolic hexose levels. This would depend on the catalytic activity of hexokinase relative to the rate of hexose transport to the cytosol from the vacuole or apoplas.

Most of the evidence for sucrose cycling is indirect. One of the earliest pieces of evidence was that sucrose cycling offers a mechanism to explain why some species, such as soybean and tobacco, do not accumulate sucrose in their leaves (Huber 1989). Huber showed that such species have high acid invertase activities that prevent the accumulation of vacuolar sucrose, and he suggested that the hexose products of invertase-catalyzed sucrose hydrolysis can serve as substrates for sucrose (futile) cycling. It was later shown that sucrose cycling plays a role in the end-product inhibition of photosynthesis (Goldschmidt and Huber 1992), and that futile cycling might occur when sucrose levels rise due to sink limitations (Geigenberger and Stitt 1993). It is also possible that futile cycling contributes to the regulation of gene expression in transgenic plants that overexpress yeast acid invertase.
(von Schaewen et al. 1990; Sonnewald et al. 1991). More recently, Moore et al. (1998) found that species with high soluble acid invertase activities (and presumably with high capacities for sucrose cycling) have significant acclimation responses to elevated CO₂. This occurs at the level of photosynthetic gene expression. Taken together, these data are consistent with the notion that alterations in photosynthesis and in photosynthetic gene expression are due, in part, to a sugar sensing mechanism that involves cycling between sucrose and hexoses.

The relationship between cytosolic sugar levels and leaf development, like that between sugar levels and photosynthetic gene expression, cannot be generalized (Quirino et al. 2000). For instance, Ludewig and Sonnewald (2000) reported that there is a lack of correlation between sugar levels and tobacco leaf age. Hence, the sucrose cycling hypothesis offers an appealing alternative to explain how source strength is perceived by a leaf. In particular, it opens the possibility that carbohydrate flux and/or the activities of invertase and hexokinase mediate source strength regulation of leaf development. We have recently turned our attention to *Arabidopsis* as a model system to examine this question. One purpose of the present studies was to examine the patterns of change in fundamental photosynthetic parameters during the ontogeny of a single leaf on the *Arabidopsis* rosette. We chose to grow plants in continuous light to avoid the problem of time-of-day sampling. This information is a prerequisite for understanding the molecular mechanisms by which source strength exerts its effect. Our second goal was to test whether developmental-dependent alterations in photosynthesis and gene expression are correlated with alterations in carbon partitioning, carbohydrate pool sizes, flux into hexoses, or with the *in vitro* activities of hexokinase, vacuolar acid invertase, or cell wall acid invertase.
Materials and methods

Plant Material and Growth Conditions

Wild type *Arabidopsis thaliana* (Col-0) seeds were sown on soil saturated with *Arabidopsis* nutrient solution (Lehle Seeds) and stratified in the dark for two days at 4°C. The flats were transferred to growth rooms (continuous illumination, 100 μmol photons m⁻² s⁻¹ at 22°C) and sub-irrigated with water daily or when the topsoil became dry. Emergence of leaf 8 was noted and marked with a small loop of colored thread, and sample leaves were removed from the plant at various days after germination. The fresh weight of each leaf was measured then snap-frozen in liquid N₂ and stored at -80°C for future analysis.

Pigment, Protein and RNA Analyses

Pigment extractions and calculations of pigment concentrations were performed essentially as described by Lichtenthaler (1987). Leaf tissues were extracted with several changes of 95% ethanol in the dark at 4°C, and absorbance measurements were made at 664nm, 649nm and 470nm. Total soluble protein concentrations were determined using the Bradford method. The composition of the protein extraction buffer was as follows: 100mM Tris-HCl (pH 7.5); 20mM KCl; 5mM EDTA; 1mM PMSF; 5mM DTT and 10mM β-mercaptoethanol. Following homogenization, the samples were incubated on ice for 10 min, then centrifuged at 14,000 rpm for 10 min; the supernatant was transferred to a clean tube for storage at -20°C. The absorbances of several aliquots were measured at 595nm and compared against a standard BSA curve according to the microassay procedure (Bio-Rad).

Total RNA was isolated from leaf samples using the Purescript® RNA Isolation Kit (Gentra Systems). RNA gel blot analyses were conducted as described previously (Jiang
and Rodermel 1995). In brief, equal amounts (5 μg) of total cell RNA were electrophoresed through MOPS-formaldehyde gels and transferred to GeneScreen Plus membranes. The filters were hybridized overnight at 65°C, then washed and subjected to PhosphorImage analysis. Probes included *Arabidopsis Lhcb* (for the light-harvesting chlorophyll a/b binding proteins of PSII) (Meehan et al. 1996) and *SAG12* (Lohman et al. 1994).

**Photosynthetic Rates, Carbon Partitioning Assays, and Carbohydrate Pool Size Determinations**

Photosynthetic rate and carbon partitioning measurements were performed essentially as described by Sun et al. (1999). A closed system (see Figure 1) was constructed for radiolabeled CO₂ feeding. A NaH¹⁴CO₃ solution (Amersham) was injected through a serum stopper into a double-armed flask containing 85% phosphoric acid. This acidified the labeled bicarbonate and released ¹⁴CO₂ to give a specific activity of ~0.1 Ci mol⁻¹. For equilibration, the ¹⁴CO₂ was pumped through the system for at least 3 hours. An entire *Arabidopsis* plant was placed into the cylindrical chamber and exposed for 10 min to the ¹⁴CO₂ under constant light conditions (~125 μmol photons m⁻² s⁻¹, filtered through a 5% CuSO₄ solution to absorb IR radiation). After a 10 min chase in unlabeled air, leaf 8 was removed from the plant, weighed, and snap-frozen in liquid N₂ for future analysis. Before and after exposure to the plant, 5 ml gas samples were removed from the flask with a syringe and injected into sealed vials containing ScintiGest tissue solubilizer to absorb ¹⁴CO₂ during an overnight incubation. Label incorporation was then measured in a scintillation counter to determine the actual specific activity of the system and whether the CO₂ concentration declined significantly during the measurement. Labeled leaves were homogenized in warm
80% ethanol, and incorporation of radioactivity was measured in ethanol-soluble and insoluble fractions as described by Sun et al. (1999). Photosynthetic rates were estimated as the sum of label incorporation into the soluble and insoluble fractions. For some experiments, the soluble fraction was separated into neutral, cationic and anionic fractions by ion exchange chromatography (Sun et al. 1999).

Soluble sugar partitioning was determined by Thin Layer Chromatography (TLC). A 10 µl aliquot was removed from the ethanol-soluble fraction and spotted onto a TLC plate (K5 Silica Gel 150Å) (Whatman). The samples were separated using 85% acetonitrile as the solvent for three separate ascensions. The plates were then dried and exposed to X-ray film for several weeks. The film was developed, and spots corresponding to the migration of glucose, fructose and sucrose were quantified using a densitometer (sugar identifications were based on comparison to several sugar standards run on a separate plate).

For carbohydrate pool size determinations, samples were ground into a powder in liquid nitrogen, extracted with 1 mL of 80% ethanol containing 4 mM Hepes-KOH (pH=7.5), and boiled for 20 min (Strand et al. 1999). After centrifugation at 14,000 rpm for 10 min, the supernatant was transferred to a new tube and the pellet was re-extracted by boiling for 20 minutes with 1 mL of 80% ethanol-Hepes (pH=7.5). After centrifugation, the pellet was used for starch determination according to Klann et al. (1993), while the supernatants were combined, vacuum-evaporated, dissolved in 250 µL of H₂O and extracted with chloroform (Klann et al. 1993). The aqueous fraction was then analyzed for the concentrations of sucrose, glucose, and fructose using kits from Sigma (Sigma No. SCA-20, GAHK-20, FA-20).
Enzyme Activity Assays

Hexokinase activity assays were performed essentially as described by Huber (1989) and Renz et al. (1993). Leaf tissues were homogenized in 500 \( \mu l \) of hexokinase extraction buffer (50 mM MOPS-NaOH, pH 7.5; 5 mM \( \text{MgCl}_2 \); 1 mM EDTA; 0.1\% Triton X-100; 2 mM benzamidine; 2 mM \( \epsilon \)-amino-\( n \)-caproic acid; 10\% glycerol; 5 mM DTT; 0.5 mM PMSF; 2\% PVPP) and centrifuged at 14,000 rpm for 10 min at 4°C. A 200 \( \mu l \) aliquot of the supernatant was desalted through a 1 ml Sephadex G-25 column equilibrated with elution buffer (50 mM MOPS-NaOH, pH 8.0; 5 mM \( \text{MgCl}_2 \); 15 mM KCl; 5 mM DTT) and the eluate was stored at 4°C. A 5 \( \mu l \) aliquot was removed and the protein concentration was determined using the Bradford method. The remainder of the eluate was assayed for hexokinase activity in assay buffer (final concentration of 50 mM MOPS, pH 8.0; 5 mM \( \text{MgCl}_2 \); 15 mM KCl; 5 mM ATP; 0.5 mM NADP\(^+\); 2 \( \mu \) G-6-P dehydrogenase) in a total volume of 1 ml. Glucose was added to a final concentration of 1 mM to start the reaction and NADPH production was monitored at 340 nm.

Soluble (vacuolar) and insoluble (cell wall) invertase activities were determined using procedures described by Huber (1989) for soluble invertase and by Greiner et al. (1999) for insoluble invertase. Glucose amounts were measured spectrophotometrically using a glucose determination kit (Sigma Diagnostics).

Results

As a first step to examine the impact of source strength on Arabidopsis leaf development, we measured several photosynthetic parameters that have been used as diagnostic indicators of the progression of leaf ontogeny in other species (e.g., Jiang et al.)
We decided to follow the course of development of leaf 8 (the eighth true leaf to emerge) because of its large size at full expansion. Visible initiation was difficult to determine, so the first measurements were taken when the leaves were 5 mm wide (“day 1”) (Figure 2A). Full expansion was attained at approximately day 12. Figure 2B shows that the leaves gained fresh weight during most of their development, reaching a peak at 20 days. Fresh weights then fell sharply. As in other systems, this decrease was due primarily to loss of water as the leaf began to dry, but also to a loss of other factors, including carbohydrate and protein (see later, Figures 2D and 6A).

Figure 2C shows that photosynthetic rates declined progressively from the first time point very early in development. Photosynthetic rates were monitored by measuring the total incorporation of $^{14}$CO$_2$ during a 10 min pulse; rates were estimated on a fresh weight basis. Conversion of the highest rates in Figure 2C to a leaf area basis gives numbers reasonably similar to published values for whole plant photosynthesis in *Arabidopsis*, when measured on a leaf area basis at similar light levels (Donahue et al. 1997). Figure 2D shows that the change in total soluble protein levels resembles that of photosynthetic rates, i.e., protein concentrations dropped steeply early in leaf ontogeny then more modestly as development proceeded.

Figure 3A reveals that chlorophyll contents declined moderately until day 20, then fell more sharply. The latter decline was coincident with visible leaf yellowing. Carotenoid contents also declined progressively with leaf ontogeny (Figure 3B) in apparent coordination with chlorophyll (Figure 3C). This suggests that the pathways of chlorophyll and carotenoid biosynthesis, both of which occur in the plastid (von Wettstein et al. 1995; Bartley and
Scolnik 1995), are coordinately regulated during Arabidopsis leaf development. This also has been observed in other systems (e.g., Härtel and Grimm 1998). Chlorophyll a/b ratios (Figure 3D) remained fairly constant until the very end of development at day 25, when they might have decreased slightly. Changes in chlorophyll a/b ratios are indicative of altered stoichiometries between light harvesting and reaction center complexes and/or between PSII and PSI. We conclude that the composition of the thylakoid membrane is relatively constant during leaf development, despite marked changes in most other photosynthetic parameters.

Amasino and co-workers found that photosynthetic genes, such as Lhcb, were down-regulated during the yellowing of fully-expanded Arabidopsis leaves, while senescence-associated genes (SAGs) were up-regulated (Lohman et al. 1994). Figure 4 shows that Lhcb mRNA levels declined progressively during the development of leaf 8, consistent with the other photosynthetic parameters we measured. Conversely, SAG12 mRNA levels increased dramatically, especially during late senescence. SAG12 codes for a cysteine protease and is expressed abundantly in yellow leaves (Lohman et al. 1994).

The patterns of carbon partitioning during Arabidopsis leaf ontogeny were analyzed by separating the total label incorporated into leaves during a 10 min pulse with $^{14}$CO$_2$ (as in Figure 2C) into ethanol-insoluble (primarily starch) and ethanol-soluble fractions. The soluble fraction was further separated into neutral (sugars), anionic (e.g., phosphorylated sugars, organic acids), and cationic (mainly amino acids) fractions. Due to the brevity of the pulse, we assume that these measurements most closely reflect flux into the various pools, rather than pool sizes per se. This assumption is supported by the lack of correlation between the label partitioning measurements and the sugar pool size measurements (see below, Figures 5 and 6). Figure 5A shows that incorporation into the insoluble fraction
declined during early development, plateaued, then fell sharply sometime after full expansion was attained at day 12. Incorporation into the cationic and anionic fractions also decreased markedly sometime after day 12, but did not change appreciably until this time. By contrast, incorporation of $^{14}\text{CO}_2$ into the neutral fraction declined steadily from the first time point at day 5. Further separation of this fraction into sucrose, glucose and fructose revealed that flux into the hexose fraction increased slightly until full expansion was attained at day 12, then declined significantly (Figure 5B). Accompanying the small increases in hexose partitioning during early ontogeny, an increasing proportion of label in the neutral fraction was partitioned into hexoses (versus sucrose) up to day 12 (Figure 5C). This trend was subsequently reversed, with late senescence resembling early development inasmuch as greater than 90% of the label incorporated into the neutral fraction was partitioned into sucrose.

The total pool sizes of starch, sucrose, glucose and fructose during leaf 8 development were also measured (Figure 6). The starch pool reached a peak early in development, then fell progressively (Figure 6A). By contrast, the sucrose pool slightly increased during ontogeny, with the beginning of the increase commencing just before the attainment of full leaf expansion (Figure 6B). Glucose and fructose levels were lower than sucrose until after full leaf expansion was reached, and did not change until late in senescence, when they increased dramatically (Figure 6B).

Moore et al. (1999) suggested that the cycling of sucrose synthesis and hydrolysis through invertase and hexokinase provides a mechanism for the generation of hexose signals to feedback inhibit photosynthetic gene expression. As a first approach to examine the role of invertase and hexokinase in source strength regulation of leaf developmental
programming, we measured the \textit{in vitro} activities of these enzymes during the ontogeny of \textit{Arabidopsis} leaf 8. Figures 7A-C reveal that glucose phosphorylation activity and the activities of vacuolar and cell wall acid invertase changed little during much of development, but that they increased sharply during the terminal stages of senescence.

\section*{Discussion}

Previous investigations of leaf development in \textit{Arabidopsis} have shown that chlorophyll and the expression of representative photosynthetic genes (including \textit{Lhcb}) decline progressively in fully-expanded, yellowing leaves (Lohman et al. 1994). Although photosynthetic rates were not measured in the \textit{Arabidopsis} studies, experiments in tobacco have revealed that photosynthetic rates fall in concert with other photosynthetic parameters as one progresses down the canopy from the youngest to the oldest fully-expanded leaves (Jiang and Rodermel 1995). The changes in photosynthesis in tobacco track very closely with alterations in the content and activity of Rubisco, and also with \textit{rbcS} and \textit{rbcL} mRNA accumulation. This is consistent with the idea that coordinated, transcriptional control of chloroplast and nuclear genes for photosynthetic components is an important mechanism of coarse control of photosynthetic rates throughout leaf development. Canopy-level studies are a useful approach to examine leaf ontogeny, but these sorts of analyses do not provide information about the early events of leaf development because only fully-expanded leaves are examined.

In this report we found that photosynthetic rates, protein contents and photosynthetic gene expression fell dramatically from the first time point we were able to measure, i.e., early in ontogeny when the leaves were still rapidly expanding. Pigment contents also fell
from this time point, although the rates of decrease were less severe. The phase of
increasing photosynthetic rates, which is a defining characteristic of early dicot leaf
ontogeny (Gepstein 1988), occurs coincident with leaf expansion in species like soybean
(Jiang et al. 1993) and tobacco (Miller et al. 1997; Miller et al. 2000), where photosynthetic
maxima are attained at full expansion. By contrast, this phase appears to be relatively short-
lived in Arabidopsis, and must have concluded sometime before our first measurement, well
before the attainment of full expansion. A similar situation may prevail in tomato (Van
Oosten and Besford 1996).

One reason why species differ in the timing of their peak photosynthetic rates might
be that they have different sink/source relationships. Alternatively, there might be species-
specific differences in rates of cell enlargement and plastid division during expansion
(Mullet 1998). Because photosynthetic rates were measured on a fresh weight basis, an
earlier peak than expected might occur if the rate of cell enlargement lags behind the rate of
plastid division. This could give the illusion, as in Figure 1, that senescence commences
early in development, rather than later. A similar phenomenon might underlie differences in
the timing of photosynthetic maxima that appear to be due to growth conditions. For
instance, we have observed that tobacco leaves exposed to elevated CO$_2$ reach their
photosynthetic maxima while they are still expanding (the “temporal shift” model of
acclimation to elevated CO$_2$), versus ambient CO$_2$-grown plants, where the maxima are
attained at full expansion (Miller et al. 1997). Detailed studies of plastid division and cell
enlargement will need to be performed to test these hypotheses. Functional assays will also
have to be conducted to determine the timing of the conversion of individual chloroplasts
into gerontoplasts during leaf ontogeny, i.e., when senescence is initiated on a per plastid basis.

The overall goal of our research is to determine how leaf developmental programming is regulated by source strength. One attractive hypothesis is that sucrose cycling through invertase and hexokinase plays a role in providing hexose substrates for hexokinase-signaling of photosynthetic gene expression, and that this serves as a coarse control on photosynthetic rates (Moore et al., 1999). If this mechanism operates during leaf ontogeny, one might expect to see a correlation between the changes that occur in photosynthesis and photosynthetic gene expression and those that occur in flux into hexoses, hexose pool sizes, invertase activities and/or hexokinase activities. Our measurements suggested that both flux into the hexose pool and partitioning of label into hexoses increased modestly during leaf expansion up to day 12, but that both subsequently declined significantly through days 20 and 25 (Figures 5B and 5C). In contrast with the flux and partitioning data, hexose pool sizes remained relatively constant until late in leaf development, when they increased dramatically. Interestingly, this increase in hexose abundance correlates well with the activities of hexokinase and of both vacuolar and apoplastic acid invertase, as well as with the mRNA abundance of the late senescence marker, SAG12. Although the coincident increases in invertase activities and hexose pool sizes are consistent with an increased conversion of sucrose to hexose, the sucrose serving as a source of this additional hexose appears to be metabolically disconnected from photosynthetic carbon metabolism since the increased sucrose hydrolysis is not reflected in the flux or partitioning data.
The dramatic increases in hexose pool sizes during late senescence are reminiscent of increased hexose pool sizes in the senescing leaves of other species (Crafts-Brandner et al. 1984; Dunphy and Hanway 1976; Quirino et al. 2001). Although the reasons for these increases are not well understood, one hypothesis is that they are required to serve the energetic needs of the cell in the face of declining photosynthetic rates; mitochondria do not degrade in senescent cells. Alternatively, an increase in hexoses could be indicative of a compensation mechanism to adjust the osmotic potential of the senescing cell as other osmotica are removed and reallocated to sinks. This would be consistent with the inverse relationship that has been observed between the levels of nitrate and hexose as osmotica in various plants (e.g., Blom-Zandstra and Lampe 1985; McCall and Willumsen 1999).

In addition to the correlation between the dramatic increases in SAG12 expression and hexose pool size, the timing of large decreases in Lhcb mRNA abundance and in photosynthetic rate between days 12 and 20 are coincident with the timing of the change in flux/partitioning into hexoses between days 12 and 20. Our data are thus consistent with the idea that flux into the hexose pools, or hexose concentrations, might be involved in mediating alterations in the expression of photosynthetic genes and/or senescence-associated genes during leaf development. On the other hand, alterations in neither hexose pools nor in flux or partitioning correlated with the early decline in photosynthetic rates and other photosynthetic parameters (e.g., Lhcb mRNA abundance, pigment contents, etc.). Whereas this suggests that photosynthesis is not regulated by these parameters early in leaf ontogeny, we were not able to determine whether flux into hexoses or changes in hexose pool sizes act as a trigger of the decline in photosynthetic rates, because the peak rate likely occurred before our first measurement.
The lack of change in the in vitro activities of hexokinase, vacuolar acid invertase and cell wall acid invertase during most of leaf development stands in contrast to species, such as soybean and spinach, where acid invertase activities decrease during expansion as the leaves convert from sinks to sources (Huber 1989). Importantly, our data show that the invertase activities do not appear to control flux into hexoses, or the partitioning between sucrose and hexose, because alterations in flux and partitioning occurred in the absence of changes in their activities. This would be consistent with the findings of others (Kingston-Smith et al. 1999), and suggests that during leaf development other factors regulate the access of invertase to sucrose, and thus the access of hexokinase to hexose, such as tonoplast and plasmalemma sucrose transport. The lack of change in hexokinase and invertase activities until late in leaf development also suggests that source strength regulation of leaf development is not mediated by alterations in the activities of hexokinase or acid invertase, because changes in gene expression and photosynthetic rates occurred in the absence of changes in the activities of these enzymes. In this context it is worth pointing out that Huber (1989) and Moore et al. (1999) suggested that high rates of sucrose cycling are associated with high levels of acid invertase. One could infer from this that sucrose cycling should correlate with invertase activity, which, based on our data, would indicate that cycling is constant during much of Arabidopsis leaf development, and thus that futile cycling does not likely play a direct role in regulating photosynthetic rates during development. However, since the data of Huber (1989) and Moore et al. (1999) both demonstrated more of a threshold effect than a correlation, we cannot infer much about the potential role of sucrose cycling in control of photosynthesis based on the lack of correlation between photosynthesis and the change in invertase activity. In fact, the observed lack of correlation between
invertase activities and changes in flux and partitioning into hexoses, along with the lack of correlation between changes in sugar pool sizes and changes in flux into hexoses, suggests that physical and metabolic compartmentation of different sugar pools may confound any efforts to demonstrate a clear role for sucrose cycling in mediating source strength effects on leaf development.

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**Figure Legends**

**Figure 1.** A schematic representation of the apparatus used for photosynthetic rate and carbon partitioning assays. Radiolabeled bicarbonate was injected into a serum cap-sealed double-armed flask containing phosphoric acid. $^{14}$CO$_2$ was liberated and circulated by a pump (see text for details).

**Figure 2.** Changes in leaf expansion, fresh weight, photosynthetic rates, and total soluble protein concentration during the ontogeny of leaf 8 of *Arabidopsis*. Leaf expansion measurements were initiated when the leaves were 5 mm wide (Day 1). Each time point is an average of 31 individual plants. To determine fresh weights (B), leaves were removed at
the petiole attachment position and weighed to the nearest mg; between 29 and 53 individual leaves were examined per time point. Photosynthetic rates (C) were determined using 5 or 6 leaves per time point. Total soluble protein concentrations (D) were measured using between 6 and 11 individual leaves per time point. Error bars represent the standard error for each data point. The plants were maintained under constant growth conditions (continuous illumination, 100μmol photons m\(^{-2}\) s\(^{-1}\), 22°C).

Figure 3. Pigment profiles during the ontogeny of leaf 8. A) Chlorophyll. B) Total carotenoids. (C) Chlorophyll and carotenoid contents expressed as a percent of the maximum. D) Chlorophyll \(a/b\) ratios. Sample numbers ranged from 6 to 11 individual leaves per time point. Error bars are the standard error for each data point.

Figure 4. \(Lhcb\) and \(SAG12\) mRNA accumulation during the development of leaf 8. Equal amounts (5μg) of total cell RNA were electrophoresed through a MOPS-formaldehyde gel; a representative ethidium bromide-stained gel is shown as a loading control. The filters were hybridized with \(Lhcb\) and \(SAG12\) probes.

Figure 5. Carbon partitioning during the ontogeny of leaf 8. A) Incorporation of \(^{14}\)CO\(_2\) into ethanol-insoluble and ethanol-soluble fractions. The insoluble fraction was further separated into neutral, cationic and anionic fractions. Each time point represents a minimum of 5 leaf samples. Error bars are the standard error for each data point. The neutral sugars were separated into sucrose, glucose and fructose subfractions and expressed in B) as the amount of label incorporated into each subfraction or in C) as a percent of the total label in the
neutral fraction incorporated into each of the three subfractions. In B), standard errors ranged from 0.8 to 2.9% for sucrose, 0.6 to 1.4% for glucose and 0.5 to 1.8% for fructose.

**Figure 6.** Carbohydrate pool sizes during leaf 8 development. A) Starch. B) Sucrose, glucose and fructose. Each time point represents between 15 and 17 individual leaves for the starch assays and between 7 and 9 individual leaves for the sucrose, glucose and fructose assays. The data are expressed on a fresh weight basis. Error bars are the standard error for each data point.

**Figure 7.** Enzyme activities during leaf 8 ontogeny. A) Glucose phosphorylating activity. B) Soluble (vacuolar) acid invertase activity. C) Insoluble (cell wall) acid invertase activity. Each time point represents between 4 and 10 individual leaves and is expressed on a protein basis. Error bars are the standard error for each data point.
Reservoir (20 Liters)

Figure 1
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<th>Total Sol. Protein (µg/mg FW)</th>
<th>Photosynthetic Rate (nmolCO2/min/g FW)</th>
<th>Fresh Weight (mg)</th>
<th>Leaf Width (mm)</th>
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Figure 2

Leaf Age (Days)

D

C

B

A
Figure 3

Leaf Age (Days)

Chla/b ratio  % Total  Carotenoid content (μg/mg FW)  Chlorophyll content (μg/mg FW)
Leaf Age (days)

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Figure 4
Figure 6

Soluble Sugars
(μmol/g FW)

Starch
(μmol glucose/g FW)

Leaf Age (Days)
Insoluble Acid Invertase Activity (nmol/µg protein/hr)

Soluble Acid Invertase Activity (nmol/µg protein/hr)

Hexokinase Activity (rate/mg protein/hr)
CHAPTER 4. HIGH SINK DEMAND INCREASES PHOTOSYNTHESIS IN THE GREEN SECTORS OF THE \textit{immutans} VARIEGATION MUTANT OF \textit{ARABIDOPSIS}

A paper to be submitted to Photosynthesis Research for publication

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Abstract

The Arabidopsis variegation mutant spotty contains a mutation in the \textit{IMMUTANS (IM)} gene. The green sectors of this mutant contain normal-appearing chloroplasts, whereas the white sectors are heteroplastidic with abnormal plastids as well as rare, normal-appearing plastids. Greater than normal rates of photosynthesis have been observed in green sectors while the white sectors do not undergo photosynthesis. We hypothesize that the increased rate of photosynthesis in green sectors results from an increased sink demand by white sectors as well as the other non-photosynthetic parts of the plant. To test this hypothesis, we measured several photosynthetic parameters and carbohydrate levels in spotty green and white sectors. We found that spotty green sectors have a higher rate of O$_2$ evolution when compared to wild type. The green sectors also have a higher Rubisco activation state and a higher rate of $^{14}$C incorporation in $^{14}$CO$_2$ feeding experiments. The feeding experiments also demonstrated that a greater proportion of $^{14}$C was incorporated into the soluble carbohydrate
fraction. Carbohydrate measurements show a greater accumulation of starch in spotty green sectors at the end of the light period (when compared to wild type), which is completely turned over during the dark. The white sectors of spotty did not contain any starch. Sucrose was higher in green sectors than in wild type at all times during the day/night cycle. This elevated level of sucrose is due to a higher total activity and greater activation state of SPS. We conclude that spotty green sectors have higher rates of photosynthesis with greater production and export of photosynthate, which is due to a high sink-to-source ratio arising between white and green leaf sectors.

**Introduction**

Variegation mutants are powerful tools to understand nuclear-organelle interactions and the mechanisms that control chloroplast biogenesis. These mutants have green sectors that contain cells with normal-appearing chloroplasts and white sectors that contain cells with defective plastids lacking pigments and organized lamellar structures. It is intriguing that mutant screens in Arabidopsis have yielded relatively few nuclear gene-induced variegations in which the mutant plant has a uniform (mutant) genetic background (reviewed in Rödermel, 2001). This is in contrast to variegations in which the green sectors have a wild type genotype and only the white sectors are mutant (e.g., as in mutants induced by some transposons or in tissue chimaeras).

*immutans* is one of the oldest Arabidopsis variegation mutants, with independent *immutans* alleles reported by Rédei in the US and Röbbelen in Germany nearly 50 years ago (Rédei 1963, Röbbelen 1968). The variegation phenotype is expressed only in *im/im* plants, and all tissues of the plant have a uniform genetic constitution (Rédei 1967). One of the
striking aspects of *immutans* is that the extent of variegation can be modulated by growth light and temperature, with higher light or higher temperatures promoting a higher ratio of white-to-green sector formation (Rédei 1963, Röbbelen 1968; Wetzel et al., 1994). The white sectors of *immutans* accumulate the non-colored carotenoid precursor, phytoene, suggesting that the plastids in these sectors are defective in the activity of phytoene desaturase (PDS), the plastid enzyme that converts phytoene to zeta carotene (Wetzel et al., 1994). Using the original *immutans* allele isolated by Rédei, we cloned *IMMUTANS* by map-based methods and found that the gene product bears similarity to alternative oxidase (AOX) in the inner mitochondrial membrane (Wu et al., 1999); a T-DNA tagged allele of *immutans* has also been cloned (Carol et al., 1999). AOX serves as a terminal oxidase in respiration, diverting electrons from the quinol pool to reduce molecular oxygen and form water (Carol et al., 1999). Early suggestions that IMMUTANS might serve an analogous function in plastid membranes has been borne out both in vitro and in vivo (reviewed in Aluru and Rodermel, 2004), and it is now generally accepted that IM is a plastid terminal oxidase (termed PTOX) that oxidizes quinone pools in plastid membranes and reduces molecular oxygen. This likely occurs during the desaturation reactions of carotenogenesis, where IM is proposed to be a component of a redox chain in which electrons are transferred from phytoene to the PQ pool via PDS, and thence to molecular oxygen via IM. Consistent with the *im* phenotype, this function of IM appears to be crucial especially early in the process of thylakoid biogenesis. There is also compelling evidence that IM serves as a terminal oxidase of chlororespiration (oxidation of the PQ pool in the dark), and that it plays a role in photoprotection by preventing over-reduction of the thylakoids and the formation of reactive oxygen species (ROS) (reviewed by Aluru and Rodermel, 2004).
IM is expressed early in seedling development, immediately after seed coat breakage, and at this time the pattern of green and white sectoring is established (Röbbelen 1968, Wetzel et al., 1994). Green and white sector formation is irreversible in the sense that green sectors of young to fully-expanded leaves do not bleach when placed under high light (restrictive) conditions, and white sectors do not turn green when placed in low light (permissive) conditions. We have proposed a working hypothesis of the mechanism of im variegation (reviewed in Aluru and Rodermel, 2004), according to which a lack of IM results in a lack of colored carotenoid formation. Colored carotenoids act as photoprotective agents and serve to quench triplet chlorophyll and oxygen radical production, and below a threshold, the contents of the plastid are susceptible to photooxidation. The role of IM in carotenogenesis would thus be especially important early in leaf ontogeny, when chloroplasts develop from undifferentiated proplastids in the mersistem. Consistent with the im phenotype, we assume that once a white plastid is formed early in leaf development, the effects of photooxidation are irreversible, but that white plastids are capable of dividing and giving rise to clones of white plastids and cells (white sectors) because the requisite functions are refractory to photodamage. On the other hand, we propose that early in chloroplast biogenesis some plastids escape photooxidation, giving rise to clones of chloroplasts and green cells (green sectors). This might be because there is a compensating IM activity in these plastids, or because of intrinsic differences in reaction rates of various radical production and/detoxifying mechanisms, with some developing plastids either producing fewer toxic radicals and/or are detoxifying them better than other plastids-- IM being one of the elements in this network. According to this scenario, the im variegation is
light-dependent because at higher light intensities, fewer of the detoxifying elements (primarily carotenoids) are above the threshold to prevent photooxidation.

We have previously studied the development of the spotty allele of immutans, which was generated by ethyl methansulfonate (EMS) mutagenesis. This allele has an early stop codon in the fourth exon of the IM gene, and the mutant produces no detectable IM mRNA or protein (Wetzel et al., 1994, Aluru et al., 2001). Spotty plants have a stunted growth rate, and although they eventually attain a normal stature, the sink tissues of the mutant display characteristics of a limited photosynthate supply. For example, whereas spotty flowers are morphologically normal, the green siliques are smaller than wild type. White siliques of the mutant lack seeds while variegated siliques have fewer seeds than normal. IM also appears to be important for the development of plastids in roots and etiolated seedlings, inasmuch as amyloplasts and etioplasts of im do not have normal sizes or structures. In addition, the amyloplasts lack starch granules and the roots are shorter than normal (Aluru et al., 2001). Interestingly, the green leaf sectors of spotty have significantly higher than normal rates of photosynthetic oxygen evolution under all light conditions. They also have more chlorophyll and higher chlorophyll a/b ratios under normal light conditions (Meehan 96, Aluru et al., 2001).

In this paper we examine the proposition that the green sectors of im leaves act as sources and the white sectors as potent sinks. We hypothesize that the increases in photosynthesis in the green sectors are due to interactions with the white sectors, perhaps to compensate for reductions in total plant source tissue. A consequence of these interactions is that plant growth and reproductive fitness are maximized. As a first step to test this hypothesis we examined various aspects of photosynthesis and photosynthate partitioning in
the green versus white sectors of leaves from *spotty* plants. We conclude that variegations provide a model system to examine sink/source interactions.

**Materials and Methods**

**Plant Material and Growth Conditions**

Seeds from wild-type *Arabidopsis thaliana* (Columbia ecotype) and the *spotty* allele of *immutans* (Wetzel et al., 1994) were sown on soil saturated with nutrient solution and vernalized at 4°C for 2 days, then transferred to a growth rooms kept at 23°C and illuminated with constant low light (10-20 μmoles m⁻² s⁻¹) for 2 weeks. The light intensity was then increased to 70-100 μmoles m⁻² s⁻¹. Leaf samples were collected for further analyses from plants 3-4 weeks after transfer to higher light. For soluble sugar and starch measurements, the plants were grown under a 16/8 photoperiod and leaf samples were collected every 4 hrs during the cycle.

**Gel Electrophoresis and Protein Analysis**

Thylakoid membrane proteins were isolated from chloroplast preparations of wild-type and *im* plants by osmotic lysis. Chlorophyll extractions and calculations of chlorophyll concentrations were essentially according to Lichtenthaler (1987). The proteins were solubilized in the presence of 1 % SDS and 0.1 % 2-mercaptoethanol for 1 hr and fractionated on a Tricine SDS-PAGE system containing 6 M urea in the resolving gel (Xu et al., 1994).

Samples were loaded at 10 μg of chlorophyll per lane. After electrophoresis, the gel was stained with Coomassie blue and MALDI-TOF was performed on select proteins to
identify thylakoid membrane proteins. For separation of pigment-protein complexes, 100 µg of chlorophyll per sample was gently solubilized according to Allen and Staehelin (1991). Approximately 30-40 µg chlorophyll was loaded per lane and the chlorophyll-protein complexes were separated on a native green gel system at a constant current of 10 mA for 2-3 hrs.

Measurement of Photosynthetic Electron Transport

Crude chloroplast preparations from leaves of 3-4 wk old wild-type and im plants were used for measurement of photosynthetic parameters. The chloroplasts were isolated under ice-cold conditions from randomly selected leaves as described by Allen and Holmes (1986) and resuspended in a resuspension buffer containing 0.1 M sorbitol, 5 mM MgCl₂, 5 mM NaCl, 50 mM HEPES-KOH (pH 7.6). The oxygen measurements were conducted at 25°C under varying light intensities using a Clark-type O₂ electrode (Rank Brothers, Bottisham, UK). PSII activities were measured as net oxygen evolution in a 1 mL reaction mixture containing 0.1 M sorbitol, 40 mM HEPES-KOH (pH 7.6), 5 mM NH₄Cl, 5 mM MgCl₂, 1mM KH₂PO₄, 1.5 mM K₃Fe(CN)₆, 0.4 mM p-phenylenediamine and thylakoids equivalent to 25 µg chlorophyll. Whole chain electron transfer activity was measured as oxygen uptake with methyl viologen as an electron acceptor. The net oxygen consumption was recorded using chloroplasts (25 µg chlorophyll) in a reaction mixture consisting of 40 mM sodium phosphate buffer pH 7.4, 1 mM NaCl, 0.6 mM sodium azide, 5 mM NH₄Cl and 0.12 mM methyl viologen. The rate of oxygen uptake by PSI was determined with 10 µg of chlorophyll per sample in the presence of 0.1 M sorbitol, 5 mM MgCl₂, 5 mM NaCl, 50 mM
HEPES-KOH (pH 7.6), 50 μM DCMU, 2 mM methyl viologen, 1 mM ascorbate and 1 mM diaminodurene. The amount of oxygen evolved and/or consumed was calculated as described by Allen and Holmes (1986).

**Enzyme Activity Assays**

Rubisco activity assays were performed according to Miller et al. (1997). In brief, leaf samples from wild-type and *im* plants were ground in liquid nitrogen and resuspended in 0.5 mL of extraction buffer. Following centrifugation, the soluble fraction was immediately assayed for initial and total Rubisco activities with CO₂ from 10 mM ¹⁴C-labeled NaHCO₃. The reactions were terminated by the addition of formic acid, and incorporation of ¹⁴C into acid-stable products was determined using a liquid scintillation counter.

Sucrose phosphate synthase (SPS) activities were determined as described by Huber et al., 1991. Leaf tissues were ground in liquid nitrogen and resuspended in 400 μL of extraction buffer containing 50 mM Mops-NaOH (pH 7.5), 15 mM MgCl₂, 1 mM EDTA, 2.5 mM DTT, and 0.1% Triton X-100. The soluble fraction was then desalted using Sephadex G-25 equilibrated with extraction buffer. Activity was measured by mixing 45 μL of eluted protein with 25 μL of a substrate solution for measuring activity under maximal conditions (Vₘₐₓ) or limiting conditions (Vₛₑᵢ). The substrate solution for measuring Vₘₐₓ contained 10 mM UDP-glucose, 10mM fructose-6-phosphate, and 40 mM glucose-6-phosphate, 50 mM Mops-NaOH (pH 7.5), 15 mM MgCl₂, and 2.5 mM DTT. The substrate solution for measuring Vₛₑᵢ contained 10 mM UDP-glucose, 10 mM inorganic phosphate, 3
mM fructose-6-phosphate, 12 mM glucose-6-phosphate, 50 mM Mops-NaOH (pH 7.5), 15 mM MgCl₂, and 2.5 mM DTT.

Soluble and insoluble acid invertase activities were measured following procedures described by Huber (1989) for soluble invertase and Greiner et al. (1999) for insoluble invertase. Assays were performed by mixing invertase extracts with a substrate solution containing 50mM sucrose and 100mM sodium citrate-phosphate (pH 5.5) for soluble invertase and the same solution but pH 4.5 for insoluble invertase.

**Photosynthetic Carbon Flux**

Photosynthetic carbon flux measurements were performed essentially as described by Stessman et al. (2002). Briefly, 3-4 week old wild-type and im plants were exposed for 10 min to ¹⁴CO₂ in a closed chamber. The plants were then removed from the chamber and incubated for another 10 min in unlabeled air; leaf samples were removed from the plants, weighed and frozen in liquid nitrogen for further analysis. To measure flux, the frozen, labeled leaves were ground to a powder in liquid nitrogen and separated into ethanol-soluble and ethanol-insoluble fractions as described by Sun et al. (1999). Photosynthetic rates were calculated as the sum of label incorporation into the ethanol-soluble and insoluble fractions.

**Determination of Carbohydrate Content**

Leaf samples were collected from wild-type and im plants at various times during the photoperiod and frozen in liquid nitrogen. For im plants, green and white sectors that were large in size and uniform in coloration were dissected from variegated leaves using a razor blade, and carbohydrate pool size determinations were conducted on each of the tissue types.
Starch, sucrose, glucose and fructose concentrations were determined in ethanol-insoluble and soluble fractions according to Strand et al. (1999): the insoluble fraction was analyzed for starch content according to Klann et al. (1993), and the aqueous fraction was analyzed for sucrose, glucose and fructose using kits from Sigma (Sigma No. SCA-20, GAHK-20 and FA-20).

Results

We have previously reported that green sectors of *im* leaves have significantly higher rates of whole chain oxygen evolution in a CO₂-saturated environment than wild-type *Arabidopsis* leaves (Aluru et al. 2001). These results suggested that the green sectors have higher than normal rates of photosynthesis. To examine this question in greater detail, we measured whole chain, PSI and PSII light-dependent oxygen evolution rates using isolated thylakoid membranes and the O₂ electrode. As shown in Table 1, the green sectors of *im* had higher than normal whole chain photochemical activities (~25-30% higher at each light intensity), confirming our previous data. The rate of PSII-mediated electron transfer (from water to potassium ferricyanide) was also higher than normal (approximately 50-60%). By adding DCMU to the whole chain reaction mixture to inhibit PSII activities and diaminodurene and ascorbate as electron donors, the rate of oxygen uptake via PSI was measured to be ~25-30% higher in *im*, similar to the whole chain rate. We conclude that O₂ evolution rates are significantly higher than normal in *im* green sectors, suggesting that these sectors have elevated photosynthetic electron transport rates.

To test whether the higher than normal photosynthetic rates in the *im* green sectors are due to alterations in protein abundance, we first performed one dimensional (for
membrane proteins) and two dimensional SDS-PAGE gels (for soluble and peripheral proteins) using isolated chloroplasts from *im* green versus wild type leaves. Although these gels did not reveal any major quantitative or qualitative differences between the two tissue-types (data not shown), small changes in abundance (less than two-fold) would have been difficult to quantify (see Lonosky et al., 2004).

Photosynthetic electron transport is tightly coupled to the activity of Calvin cycle enzymes to efficiently provide reducing power for fixing CO₂ and converting it into carbohydrates. Therefore, we turned our attention to the carbon assimilation pathway to examine whether it might provide some clues about the photosynthetic differences between the *im* green and wild type leaves. Rubisco is frequently a regulatory point of carbon fixation, and thus we first measured initial and total activities of Rubisco in the two tissue types. The initial activity is an estimate of the *in vivo* activated state of Rubisco, whereas the total activity is a measure of the total amount of Rubisco that can be activated. Figure 2 shows that the initial activity of Rubisco in *im* green sectors was 1.5-fold higher than normal, while the total Rubisco activity was 1.25-fold higher than normal. These differences are statistically significant. The activation state of Rubisco (the ratio of initial to total activity) was 25% higher for *im* green than wild-type *Arabidopsis*. Considered together, these data suggest that enhance activation of Rubisco is directly correlated with the elevated rates of O₂ evolution in the *im* green sectors.

To further examine carbon fixation, we measured photosynthetic carbon flux of leaves by pulse-labeling with ^14^CO₂. The leaves were from 3-4 week-old *im* and wild type plants grown under normal light conditions (100 μmoles m⁻² s⁻¹) with a pulse-chase time of 10 minutes each. Figure 3 reveals that *im* green sectors have a two-fold increase in ^14^C
incorporation (on a chlorophyll basis). To examine partitioning of this fixed carbon, the $^{14}$C-labeled extracts were separated into ethanol-insoluble (containing starch) and ethanol-soluble fractions (containing soluble sugars -- primarily sucrose-- organic acids, amino acids, and phosphorylated intermediates). Whereas wild-type Arabidopsis allocates approximately 75% of the fixed carbon into the insoluble fraction and 25% into the ethanol-soluble fraction, the green sectors of im allocate approximately 60% into the insoluble fraction and 40% into the soluble fraction. These data suggest that a greater proportion of fixed carbon is incorporated into sucrose as opposed to starch in green sectors of im plants.

Figure 4 shows the total pool sizes of starch and sucrose under a 16/8 hour photoperiod. The wild-type accumulated significant amounts of starch during the light period (Figure 4A), which was progressively mobilized during the night. The im green sectors accumulated more starch by the end of the light period, but this was rapidly mobilized during the dark period at a rate greater than wild type. Although total starch accumulation in the im green sectors was similar to wild-type on a fresh weight basis, it was approximately 1.5-fold higher on a chlorophyll basis (data not shown). This correlates well with the pulse-label data (Figure 3). As expected, the im white sectors did not accumulate any starch.

The diurnal turnover of sucrose in the im green sectors followed the same general pattern as the wild-type, i.e., an increase early in the day, followed by a decrease, then another small increase at the end of the dark period. The decrease in the light period correlates with starch mobilization and also with an increase in sucrose appearance in the white sectors. On the whole, larger pools of sucrose were maintained in the im green sectors versus wild-type during the entire cycle, both on a fresh weight (Figure 4B) and on a
chlorophyll basis (data not shown). Glucose and fructose amounts were also examined during the day/night cycle, and no significant differences were observed when comparing \textit{im} green sectors and wild type leaves (data not shown). Considered together with Figure 3, the data in Figure 4 support the notion that there is increased partitioning of carbon into soluble sugars in the \textit{im} green sectors, which may be used to maintain growth and maintenance of the white leaf sectors.

If the \textit{im} green sectors have enhanced sucrose partitioning and steady state sucrose amounts, it might be predicted that sucrose synthesis is up-regulated in these sectors. To test this hypothesis, we measured the activity of sucrose phosphate synthase (SPS), which is the key regulatory enzyme of sucrose synthesis. SPS activity is controlled by allosteric effectors and is inactivated by phosphorylation (Winter and Huber, 2000), and is typically tested under rate-limiting conditions ($V_{sel}$) and saturating substrate conditions ($V_{max}$). The activation state of the enzyme is expressed as a percentage of $V_{sel}$ activity to $V_{max}$ activity. Figure 5 shows that \textit{im} green sectors have both a higher maximal SPS activity ($V_{max}$) and a higher selective activity ($V_{sel}$) when compared to wild type. This results in a significantly higher activation state of SPS (63% activation in \textit{im} green versus 27% in wild type). These data are consistent with the observations in Figures 3 and 4, and indicate that sucrose synthesis is upregulated in the \textit{im} green sectors. This suggests that these sectors have more sucrose available for export to sink tissues. Interestingly, \textit{im} white sectors have a higher $V_{max}$ but lower activation state than wild type.

Figure 6 shows that \textit{im} white sectors have a significantly higher cell wall invertase activity than green sectors. This would seem to indicate movement of sucrose from green sectors to white sectors. It may also indicate a reduction in cell wall activity in green sectors.
to prevent hydrolysis in the apoplastic space, allowing the movement of sucrose to tissues directly adjacent to green sectors. It is somewhat puzzling that wild type cell wall invertase activity is higher than both green and white sector activity. While im cell wall invertase activity may be associated with movement of sucrose apoplastically, wild type activity may be associated closely with vascular tissues. Vacuolar acid invertase activity was also examined but showed no difference among im green, im white, or wild type leaves (data not shown). This could indicate that cell wall invertase is more important for the import of sucrose into sinks whereas vacuolar invertase has a more general activity that does not contribute to sink strength. Taken together with the SPS activity, we conclude that green sectors produce greater amounts of sucrose, via SPS, to accommodate more sink tissues, and the white tissues are actively importing sucrose to maintain metabolism due to a lack of photosynthetic activity caused by lack of IM in chloroplasts.

Discussion

Feedback regulation of photosynthesis is important to maintain the balance between sink demand for photosynthate and source assimilation rate. The concept of sink regulation of photosynthesis states that as sink demand decreases carbohydrates accumulate in source leaves, causing a decrease in photosynthetic rate initially at the level of intermediate metabolites (short-term regulation) and finally at the level of gene expression (long-term regulation) (Sonnewald 2001, Paul and Foyer. 2001). Conditions that cause an accumulation of carbohydrates cause a dramatic decrease in photosynthesis, while conditions that increase sink demand tend to maintain or increase the photosynthetic rates presumably because of the increased demand for photosynthate (Miller et al., 1997, Stitt et
al. 1990, Diethelm and Shibles, 1989). Regulation of gene expression by carbohydrate sensing mechanisms in plants follows a “feast or famine” pattern of expression. In general, an abundance of carbohydrates upregulates genes for storage and utilization while a depletion of carbohydrates upregulates genes for acquisition (photosynthesis) and mobilization (Koch 1996).

With the green/white sectoring pattern established early in leaf development, *im* plants have a dramatic increase in sink demand from a decreased total source area. The result is a “famine” response with an increase in the photosynthetic rate of the existing green sectors, as observed with increases in both O₂ evolution and ¹⁴C uptake by green sectors of *im*. However, the increase is not reflective of upregulated gene expression, as previous studies demonstrate that expression of photosynthetic genes, such as rbcS and LhcB, is similar when comparing *im* green sectors to wild type (Wetzel et al., 1994). Also, protein gels do not indicate significant changes in protein concentrations. Instead, it appears that increased photosynthesis is due to increases in Rubisco initial and total activity as well as the activation state, which generally reflects carbon assimilation rates, (Miller et al., 1997). Along with Rubisco, correlations between SPS activity and photosynthesis are also frequently observed with higher SPS activity associated with higher rates of photosynthesis (Battistelli et al., 1991). Green sectors of *im* also have both a higher SPS activity and produce more sucrose. Higher amounts of starch accumulate in green sectors of *im* at the end of the light period, but are rapidly mobilized and completely turned over before the end of the dark period. We conclude that the increased demand by sinks leads to increased activities of enzymes for carbohydrate synthesis, and that the increased activity of the Calvin cycle results in an increased activation of the electron transport system (PSI and PSII).
Import of sucrose is vital to maintain metabolism in sink tissues. Plant invertases hydrolyze sucrose into glucose and fructose and are considered to contribute to sink strength by maintaining a gradient of sucrose from source to sink tissues (for reviews see Sturm 1999, Tymowska-Lalanne and Kreis, 1998). Plant acid invertases are localized to both the plant vacuole and cell wall. In general, cell wall invertases are associated with import into newly growing tissues such as young seeds, whereas vacuole invertases are associated with control of the sucrose/hexose content of organs such as fruit. The im plants have a higher cell wall invertase activity in white as opposed to green sectors which is indicative of movement of sucrose from source to sink. The lower cell wall invertase activity in green sectors serves to allow a greater movement of sucrose through the apoplastic space, while the higher activity in white sectors indicates active uptake and hydrolysis of sucrose. The lack of difference in vacuole invertase activity may indicate a diminished role for this enzyme in the uptake and metabolism of sucrose in sink tissues. Taken together we conclude that im green sectors compensate for the increased sink demand, or “famine” state, with a higher photosynthetic rate, greater production of carbohydrates, and increased movement of sucrose to white sectors.

It would be interesting to follow the photosynthetic rate of im green sectors during leaf ontogeny to reveal if there is a prolonged maximum photosynthetic rate or a delay in senescence similar to antisense Rubisco small subunit tobacco plants. The stunted growth rate of im is similar to plants have reduced source strength due to reduced amounts of Rubisco holoenzyme, resulting in an impaired ability to fix carbon and produce carbohydrates suitable for export (Quick et al., 1991a, 1991b, Jiang and Rodermel, 1995, Miller et al., 2000, Tsai et al., 1997). Several other mutants with reduced source strength
have a stunted or slow growth phenotype, including antisense mutants of Rubisco activase, aldolase, chloroplast fructose-1,6-bisphosphatase, phosphoribulokinase, and sedoheptulose-1,7-bisphosphatase (refs). The difference is that all of these mutants have limited carbon assimilation or conversion to photosynthate, whereas \textit{im} is limited by the actual physical photosynthetic area but still has fully functional carbon assimilation pathways, i.e. reduced source strength versus increased sink strength.

Also similar to \textit{im} are transgenic tobacco plants overexpressing a yeast invertase (Stitt et al., 1990). Those plants also have leaves with green and pale sectors. The pale sectors are characterized by low photosynthetic rates, higher Rubisco activity, and high levels of carbohydrate due to inhibition of sucrose export caused by rapid hydrolysis by the invertase transgene. However, green sectors have high rates of photosynthesis and lower carbohydrate levels, but sucrose and starch levels are still higher than wild type tobacco. Similar to \textit{im} white sectors, the reduction of photosynthesis of pale sectors appears to increase the sink demand resulting in higher photosynthesis in green sectors.

While there have been many attempts to increase photosynthetic output, there has been very little success. Plants over-expressing SPS displayed some success, with increased photosynthesis in tomato and increased shoot biomass in tobacco (refs). Plants with decreased activity of fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphate, a regulatory enzyme in sucrose biosynthetic pathway, had increased photosynthesis but only under lower light intensity (ref). Tobacco plants overexpressing a cyanobacterial fructose-1,6-/sedoheptulose-1,7-bisphosphatase had a 1.24-1.5 fold increase in photosynthetic rate by redirecting more carbon away from starch biosynthesis and into regenerative portion of the
Calvin cycle (ref). All of three of the mutant line appear to shift carbohydrate partitioning toward increased sucrose production.

The phenotype of \textit{im} is such that it can be controlled by light intensity, but lack of IM does not appear to affect photosynthesis after green sectors have developed. It would be interesting to investigate the correlation between the degree of variegation and the changes in photosynthetic parameters in the green sectors. One might suspect that as the degree of white sectoring increases that there is a corresponding increase in photosynthesis in green sectors. While \textit{Arabidopsis} can be a difficult system in which to perform physiological studies, the \textit{ghost} mutant of tomato also carries a mutation in a homolog of \textit{IM} and could provide a better system to study this type of sink/source interaction. It would also be interesting to investigate other variegation mutants to reveal if the same relationship exists between green and white sectors.

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involves a decrease of the Calvin-cycle enzymes and an increase of glycolytic enzymes. Planta 183: 40-50


Figure Legends

Figure 1. A pair of spotty plants, the variegated plants used during these experiments. Spotty is an allele of immutans generated by EMS mutagenesis (Wetzel et al., 1994). These plants were initially illuminated with approximately 10 µmol m\(^{-2}\) s\(^{-1}\) for 2 weeks, then with 80-100 µmol m\(^{-2}\) s\(^{-1}\) for 4 weeks.

Table 1. Photosynthetic electron transport of isolated thylakoid membranes of im green sectors or wild type leaves under varying light intensities (50-500 µmol m\(^{-2}\) s\(^{-1}\) or µE). Whole chain electron transfer was measured as oxygen uptake with methyl viologen as an electron acceptor. PSII activity was measured as the rate of oxygen evolution with the transfer of electrons from water to potassium ferricyanide. DCMU was added to the reaction to inhibit PSII, allowing PSI activity to be measured as the rate of oxygen uptake with diaminodurene and ascorbate as electron donors.

Figure 2. Rubisco initial and total activities of im green leaf sectors and wild type Arabidopsis leaves. %Activation is calculated as the ratio of initial to total activity and represents the activation state of the Rubisco enzyme. The blue bars represent wild type and the green bars represent im green sectors. Each bar represents the average ± standard error of 3-6 samples.
Figure 3. Partitioning of newly fixed $^{14}$C into 80% ethanol-soluble or insoluble fractions. Total $^{14}$C uptake represents a relative comparison of assimilation rates between im green leaf sectors (green bars) and wild type leaves (blue bars). Insoluble represents the flux of $^{14}$C into starch, while the soluble represents flux into soluble sugars. Each bar is the average ± standard error of 3-6 samples.

Figure 4. (A) Starch and (B) sucrose pools sizes in im green (•), im white (▲), and wild type (■) leaf samples during a 16 hour light / 8 hour dark cycle. The light period lasts from 0-16 hours, followed by the dark from 16-24 hours. Each point is the average ± standard error of 3-6 points.

Figure 5. Sucrose phosphate synthase (SPS) activity in im green (•), im white (▲), and wild type (■) leaf samples. $V_{max}$ represents activity under substrate saturating conditions, $V_{sel}$ represents activity under limiting conditions, and % Activation is a ratio of $V_{sel}$ to $V_{max}$. % Activation represents the activation state of the enzyme. Each bar is the average ± standard error of 3-6 samples.

Figure 6. Insoluble acid invertase activity in im green (■), im white (▲), and wild type (■) leaf samples. Each bar is the average ± standard error of 3-6 samples.
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<th>PS I</th>
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<tr>
<td></td>
<td>Wild Type</td>
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Table 1
Figure 2
Figure 3

Carbon Partitioning (umoles $^{14}$CO$_2$ / $\mu$g Chl / hr)

- Total
- Insoluble
- Soluble

[Graph showing data for WT and im G]
Figure 4

(A) Starch content (μg glucose/mg FW) over time (hours) for WT, im G, and im W.

(B) Sucrose content (μg sucrose/mg FW) over time (hours) for WT, im G, and im W.

Figure 4
Figure 5
Insoluble Acid Invertase Activity

(µmol glucose/mg protein/hr)

Figure 6
CHAPTER 5. CHARACTERIZATION OF T-DNA KNOCKOUT MUTANTS OF *ARABIDOPSIS* VACUOLAR ACID INVERTASE

Abstract

To investigate the role of vacuolar acid invertase in the partitioning and sensing of carbohydrates, T-DNA tagged mutants of the two *Arabidopsis* vacuolar invertase genes (*At*β*fruct*3 and *At*β*fruct*4) were obtained from the *Arabidopsis* Biological Resource Center from Ohio State University. Mutant lines were confirmed to have T-DNA mutations by Southern blot analysis. Northern blots and invertase assays revealed that the mutant lines had reduced levels of RNA while only the *At*β*fruct*4 mutant had reduced activity. Double mutants of *At*β*fruct*3 and *At*β*fruct*4 were examined under constant light under varying intensities and ambient and high CO₂. Carbohydrate analysis revealed only a small change in sucrose partitioning with no differences in hexose or starch content. Chlorophyll levels of leaf 7 were found to be nearly identical to wild type when plants were grown in normal or high light, and ambient or high CO₂. Expression of RbcS was also found to be similar to wild type in all growth conditions. We conclude that vacuolar invertase has only a limited influence on partitioning of sucrose and no effect on gene expression during acclimation.

Introduction

Acclimation of plants grown in high CO₂ leads to an accumulation of carbohydrates which results in a down regulation of photosynthesis controlled at the level of gene expression. For example, tobacco plants grown in an enriched CO₂ environment have a
down-regulation of photosynthetic rate when compared to plants grown in ambient CO₂ (Miller et al., 1997). Also, growth of Arabidopsis seedlings on media containing high levels of glucose respond with an inhibition of photosynthetic gene expression, such as the Rubisco small subunit, \textit{RbcS}, and light harvesting chlorophyll, \textit{LhcB} (Jang et al., 1997). The mechanisms by which carbohydrates are sensed and transmitted into altered gene expression are not well understood. Much of the attention has been focused on the role of hexokinase in sensing and signaling changes in photosynthetic gene expression. Hexokinase has been shown to have both a catalytic and signaling function in yeast, and a similar function has been hypothesized in plants (Jang and Sheen, 1994). Studies of Arabidopsis plants over-expressing hexokinase demonstrate a hypersensitive response to glucose in the growth media, with a greater reduction in the expression of \textit{RbcS} when compared to wild type (Jang et al., 1997). The inverse, a reduction in hexokinase, causes insensitivity to glucose levels, with a higher level of \textit{RbcS} expression on glucose media when compared to wild type. Tomato plants that overexpress the Arabidopsis hexokinase also have reduced photosynthesis and accelerated senescence (Dai et al., 1999).

While hexokinase may be involved in the sensing of carbohydrate levels, the source of the carbohydrate has still not been fully characterized. One hypothesis put forth is that sucrose cycling through invertase and hexokinase generates a signal to down-regulate photosynthesis (Huber, 1989, Moore et al. 1998). According to this model, sucrose accumulates in source leaves when utilization becomes limited. This sucrose is then converted by invertase into glucose and fructose which, in turn, are phosphorylated by hexokinase and reenter the pathway of sucrose synthesis. The net result is a cycling of
sucrose synthesis and hydrolysis with a signal generated by hexokinase to down-regulate photosynthetic gene expression.

Most studies have only been able to provide indirect evidence of sucrose cycling (Goldschmidt and Huber, 1992, Moore et al., 1998). For example, transgenic tobacco plants that over-express a yeast invertase have been generated to study sink regulation of photosynthesis. The elevated invertase activity causes a large accumulation of all carbohydrates in source leaves due to the hydrolysis of sucrose before it can be exported. The response is an almost complete inhibition of photosynthesis and gene expression. However, this is an extreme example of carbohydrate accumulation and does not closely reflect normal interactions between sinks and sources. Moore et al. (1998) investigated the growth of several different plant species under an enriched CO₂ environment in an attempt to gain insight into the mechanisms that control photosynthesis under conditions of increased source strength. Growth of some plant species in high CO₂ (1000 ppm) caused an acclimation response resulting in a decrease in photosynthesis when compared to control plants grown at ambient CO₂ (350-400 ppm). However, the acclimation response could not be directly correlated to any carbohydrate pool sizes. Rather, Moore et al. demonstrated a correlation between the acclimation response and the vacuolar acid invertase activity of the different species examined, with a threshold level of activity above which there was a decrease in Rubisco protein content and RbcS expression. They also found a threshold relationship between the invertase activity and the hexose/sucrose ratio. They concluded that the acclimation response involved the cycling of sucrose through invertase and hexokinase. While these results are indicative of effects of vacuolar invertase activity, species-specific differences only provides indirect evidence of the acclimation mechanism.
To further investigate the role of vacuolar acid invertase in the partitioning and sensing of carbohydrates, mutants of the two Arabidopsis vacuolar invertase genes (AtPfruct3 and AtPfruct4) were obtained from the Arabidopsis Biological Resource Center from Ohio State University. To investigate the effects of vacuolar invertase on photosynthetic gene expression, the mutants were be examined under varying light and CO₂ environments. If the acclimation response is dependent on cycling of sucrose synthesis through vacuolar invertase and hexokinase, then mutants lacking vacuole invertase activity would not be expected to exhibit inhibited RbcS expression under saturating photosynthetic conditions.

Materials and Methods

Plant Material and Growth Conditions

Arabidopsis T-DNA insertion lines from the Salk Institute Genomic Analysis Library (SIGnAL) were screened for tagged mutations in the vacuolar acid invertase genes, AtPfruct3 and AtPfruct4, using “T-DNA Express” at the website http://signal.salk.edu/cgi-bin/tdnaexpress (Alonso et al., 2003). Several lines were identified and ordered from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University. Seeds of the Arabidopsis thaliana mutant lines and wild type (Columbia ecotype) were planted on Sunshine LC1 Growing Mix from SUNGRO (Bellevue, Washington) saturated with nutrient solution and vernalized at 4°C for 2 days, then transferred to growth chambers illuminated at different light conditions at 23°C. Several seeds were germinated in individual pots (2 5/8 inches long x 2 5/16 inches wide x 2 3/8 inches deep) arranged in flats arranged in 9 rows and
4 columns (20 9/16 inches long x 10 3/16 inches wide x 2 3/8 inches deep), then thinned to 1 plant per pot after 5-7 days.

For root growth experiments, seeds were vapor sterilized for 10 hours in an enclosed chamber containing 100 mL of bleach acidified with 3 mL of concentrated hydrochloric acid. Seeds were suspended in 0.1% agarose, spread evenly on MS plates containing no sucrose or 2% sucrose, placed in 4°C for 2 days, then transferred to a growth room maintained at 22°C with constant illumination provided by fluorescent lights (70-100 µmol m⁻² s⁻¹).

**DNA Southern Blot Analysis**

T-DNA tagged lines were confirmed to have inserts in the vacuole acid invertase genes by Southern blot analysis. DNA was isolated from *Arabidopsis* leaves using an extraction buffer containing 7M urea, 2% sarkosyl, 35mM sodium chloride, 50 mM EDTA, and 10 mM Tris-HCl (pH=7.6). Approximately 5 µg of DNA were digested with the appropriate restriction enzymes for 4 hours and loaded onto 0.8% agarose gels for electrophoresis at 40 volts for 12-16 hours. The gels were then washed with 0.25 M hydrochloric acid for 5 minutes, followed by washing with 0.2 M sodium hydroxide for 30 minutes and blotted onto GeneScreen Plus nylon membranes by the capillary transfer method. Blots were probed with ³²P-labeled probes under high stringency conditions (65°C; 7% SDS, 0.5 M NaPO₄, pH 7.2, 1 mM EDTA, 1 mg/mL salmon testes DNA) (Wetzel et al., 1994). For a *Atfruct3* gene-specific probe, the cDNA clone (pQAI2) was digested with PstI and EcoRV, releasing a 300 bp fragment from the 3' end of the gene. For a *Atfruct4*
specific probe, the EST 122D15T7 (obtained from the ABRC) was digested with XbaI and SstI, releasing a 450 bp fragment from the 5’ end of the gene.

**RNA Northern Blot Analysis**

RNA was isolated from *Arabidopsis* leaves using the Concert™ Plant RNA Reagent (Invitrogen™) according to instructions provided with the reagent. 5 mg of total RNA were loaded onto 1% agarose/3% formaldehyde gels for electrophoresis at 70 volts for 4 hours. Gels were then washed 5 times with de-ionized water, followed by one wash with 0.1 M sodium phosphate buffer (pH 6.8). RNA was then blotted onto Genescreen Plus nylon membranes by the capillary transfer method using 0.1 M sodium phosphate buffer as the transfer solution. RNA blots were probed in the same way as previously described for Southern blots.

**Carbohydrate Analysis**

Leaf samples were homogenized in 1 mL of extraction solution (80% ethanol, 4 mM Hepes-NaOH pH 7.5) and placed in a boiling water bath for 20 minutes. Samples were then cooled briefly and centrifuged at 14,000 rpm for 5 minutes. The soluble fraction was transferred to a new tube and saved, and 1 mL of extraction solution was added to the insoluble fraction, vortexed briefly, and placed in boiling water bath for 20 minutes. After centrifuging, the soluble fractions from individual samples were pooled together, and both the soluble and insoluble fractions were dried in a vacuum evaporator. The soluble fraction was then dissolved in 250 μL of water and extracted with 250 μL of chloroform to remove chlorophyll. After centrifuging at 14,000 rpm for 5 minutes, the aqueous fraction was
transferred to a new tube and analyzed for sucrose, glucose, and fructose. Sucrose content was determined using 0.15% anthrone dissolved in 13.7 M sulfuric acid (Van Handel, 1968). Glucose and fructose contents were analyzed using Sigma Kits GAHK-20 and FA-20, respectively.

To determine the starch content, the dried insoluble fraction was dissolved in 200 μL of 0.2 M KOH and placed in a boiling water bath for 20 minutes. After briefly cooling, the pH of the sample was neutralized by the addition of 50 μL of 1.0 M acetic acid (final pH 5.5). 20 units of amyloglucosidase and 30 units of α-amylase were added to each sample, and each sample placed in a 37°C water bath overnight (12-16 hours) (Klann et al., 1993). The glucose content of each sample was analyzed by the Nelson-Somogyi method for determination of reducing sugars (Marais et al., 1966).

**Chlorophyll Analysis**

Chlorophyll concentrations were determined essentially as described by Lichtenthatler (1987). Leaf samples were ground in liquid nitrogen and then extracted at 4°C with 1 mL of 95% ethanol for six hours. After centrifuging at 14,000 rpm, the ethanol-soluble fraction was transferred to a new tube. An additional 1 mL of 95% ethanol was added to each sample and placed in 4°C for 6 hours. The two extractions were then combined and absorbance measurements were made at 664 nm, 649 nm, and 470 nm.
Soluble Acid Invertase Assay

Soluble acid invertase (vacuolar) assays were performed similarly to that described by Huber (1989). Leaf samples were homogenized in 400 μL of extraction buffer (50mM Mops-NaOH (pH 7.5), 5mM MgCl₂, 1mM EDTA, 0.05% (w/v) Triton X-100, 2.5mM DTT, 0.1mM PMSF, 1% PVPP) and centrifuged at 14,000 rpm for 10 minutes at 4°C. A 200 μL aliquot of the supernatant was desalted through 1 mL Sephadex G-25 equilibrated with elution buffer (50mM sodium citrate-phosphate, pH 5.0). To analyze invertase activity, 180 μL of eluted protein was added to 420 μL substrate solution (50mM sodium citrate-phosphate (pH 5.0), 50mM sucrose) and placed in 25°C for 30 minutes. The reaction was stopped by boiling for 5 minutes. The resulting hexose concentrations were determined by the Nelson-Somogyi method for determination of reducing sugars (Marais et al., 1966). A 5 μL aliquot of the eluted protein was used for protein determination using the Bradford method ((Bradford 1976).

Results

The *Arabidopsis* genome contains two genes that code for the vacuolar form of acid invertase, *Atbfruct3* and *Atbfruct4*, both of which have been cloned and characterized (Haouazine-Takvorian et al., 1997, Tymowska-Lalanne and Kreis, 1998b), no other putative vacuolar invertase genes have been detected in *Arabidopsis*. Previous northern blot analyses using a non-specific probe which detects both genes revealed the highest expression of vacuolar invertases in roots, stems, and flowers but very little in young leaves. However, gene-specific analysis of expression in leaves by RT-PCR was inconclusive (Tymowska-Lalanne and Kreis, 1998b).
To better examine leaf expression gene-specific probes for both \textit{Atbfruct3} and \textit{Atbfruct4} were used to probe Northern blots of RNA isolated from leaf 8 at different stages of its development. Figure 1 reveals that both genes are expressed in a similar pattern, with steady transcript levels during leaf development, increasing later in development (day 26). This parallels previous assays of soluble acid invertase activity performed during leaf development (Chapter 2, Stessman et al., 2002). Realtime PCR studies by Karen Koch at the University of Florida-Gainesville have also shown that \textit{Atbfruct4} is expressed from 2.5 to 5 times the level of \textit{Atbfruct3} (personal communication).

The goal of this study was to utilize mutants of \textit{Atbfruct3} and \textit{Atbfruct4} to examine the effect of altered vacuole acid invertase activity on carbohydrate partitioning and photosynthetic gene expression. Mutants of both \textit{Atbfruct3} and \textit{Atbfruct4} were identified from the Salk T-DNA Insertion lines of \textit{Arabidopsis} by using the T-DNA Express Gene Mapping Tool located at Salk Institute Genomic Analysis Laboratory (SIGnAL) website (http://signal.salk.edu/cgi-bin/tdnaexpress, Alonso et al., 2003). Screening for invertase mutants identified several possible mutant lines for both \textit{Atbfruct3} and \textit{Atbfruct4} (Figure 2), and seed stocks for Salk lines 6934, 16136, and 100813 were obtained from the Arabidopsis Biological Resource Center (ABRC). Salk 6934 contains a mutation, or T-DNA insertion, in the second intron of \textit{Atbfruct3}. Salk 100813 and 16136 have mutations in the first and sixth exons of \textit{Atbfruct4}, respectively.

Southern blots analyses were performed to identify homozygous mutant lines and to verify that the seed stocks contained the same mutations as illustrated by the SIGnAL T-DNA Express mapping tool. Genomic DNA for both mutants and wild type were digested with Bgl II, a restriction enzyme that does not cut within either of the \textit{Atbfruct3} and
*Atbfruct4* genes or within the T-DNA borders used for mutagenesis of the Salk lines. Figure 3A shows a shift in the size of the band for *Atbfruct3* indicating a mutation within the endogenous gene. Figure 4A also shows an increase in the size of *Atbfruct4* band, with the change in size equal to that of the T-DNA insert. It is also important to determine the number of T-DNA inserts in the mutant lines, as other mutations may have secondary effects that alter the phenotype of the mutants. The Salk lines contain the kanamycin resistance gene, NPT II, as a selection marker for transformation. Typical transformations result in 1-2 random insertion of the T-DNA in the genome. Probing both invertase mutant lines with NPT II reveals that Salk 6934 contains up to 6-7 T-DNA inserts (Figure 3B), while Salk 100813 contains up to 4 inserts (Figure 4B). However, transformation with *Agrobacterium* typically results in several T-DNA inserts at the same locus. The map of the transformation vector, pROK2, indicates a Bgl II site adjacent to, but outside, of the T-DNA right border, and a second Bgl II very close to the left border. If several T-DNAs were inserted as concatamers, it is possible that the Bgl II sites from the vector were also maintained in the insert locus. Digestion with Bgl II would then cut out several NPT II genes from the same locus, resulting in a multiple banding pattern as shown in Figures 3B and 4B. To retest for insert numbers, genomic DNA was digested with restriction enzymes, Sal I or Not I, which are known to cut less frequently. The resulting Southern blots revealed a single band when probed with NPT II (data not shown). Also, the Salk 6934 mutant line was crossed with wild type in an attempt to segregate out the different inserts. Southern blot analysis of the F₂ generation of this cross did not reveal any segregation of the T-DNAs (data not shown). Therefore, it is assumed that the bands shown in Figures 3B and 4B are located at the same locus or closely linked loci.
Northern blot analyses were performed to examine the effect of the mutations on transcript levels. Figure 5 shows mRNA levels of the single mutants 6934, 100813, 16136, and the cross of Salk 6934 and 100813. Both 6934 and 100813 have mRNA for the corresponding genes, while the double mutant contains no RNA for either \textit{Atbfruct3} or \textit{Atbfruct4}. The Salk 16136 line, which contained a T-DNA in the sixth exon of \textit{Atbfruct4}, produced an mRNA approximately 1-1.2 kb larger than the endogenous gene. This was probably due to a fusion of the endogenous \textit{Atbfruct4} and the T-DNA and a termination of transcription occurring within the T-DNA. A comparison of transcript levels does not indicate a reciprocal increase in RNA of the unmutated gene to compensate for the loss of a single vacuolar acid invertase. That is, there was not an increase in \textit{Atbfruct4} expression in Salk 6934 and there was not an increase in \textit{Atbfruct3} expression in Salk 100813.

Vacuolar invertase activity is believed to be controlled at the level of transcription as changes in mRNA levels reflect changes in activity (Tymowska-Lalanne and Kreis, 1998b, Sturm, 1999). There are also no known regulatory mechanisms for vacuolar invertase activity such as protein modification or inhibitors. To investigate activity, we measured leaf 8 soluble invertase activity of the mutants. The loss of \textit{Atbfruct3} (Salk 6934) resulted in an insignificant decrease in activity, while a mutation in \textit{Atbfruct4} (Salk 100813) caused a 70% decrease in activity (Figure 6). These activities are reflective of the gene-specific expression levels examined in Figure 1. The double mutant, Salk 6934x100813, had a 95% loss of activity, which reflects the loss of both transcripts (Figure 5). The double mutant, Salk 16136x6934, has approximately 30% of the wild type activity despite mutations in both genes. The T-DNA in Salk 16136 is located downstream of the catalytic site, which is
located in exon 3. The resulting fusion protein could possibly contain the active catalytic site and have some invertase activity, albeit somewhat diminished.

Growth and metabolism of sink tissues are dependent on the synthesis and transport of sucrose from source leaves. Because vacuolar acid invertase catalyzes the hydrolysis of sucrose into fructose and glucose, import of sucrose by sinks is dependent on invertase activity in some species to maintain a gradient of sucrose from source to sinks (Tang et al., 1999). Carbohydrate levels were examined in the different mutant lines to reveal if altering soluble invertase activity affects partitioning into the sucrose, hexose, and starch pools of leaves (Figure 7). The double mutant, Salk 6934x100813, was the only line with a significant effect in any carbohydrate pool. This line had a two-fold increase in the sucrose contents of leaves, but no differences in hexose or starch levels. Based on these results it is apparent that the ratio of sucrose/hexose is not affected by even a 67% reduction in vacuole invertase, as seen in the single gene mutants. This is similar to other studies that show a threshold relationship between soluble invertase activities and sucrose accumulation (Moore et al., 1998, Scholes et al., 1996, Zrenner et al., 1996).

For all subsequent experiments, only the double mutant line Salk 6934x100813 was studied, as there were no significant differences in phenotype or carbohydrate levels between the single mutants and wild type. For an easier reference to this line, the double mutant will be referred to as Salk 2X (in abbreviation of vacuolar acid invertase times two). To further examine carbohydrate partitioning, diurnal changes in sucrose and starch were tested. It is possible that differences in sucrose and starch pools may only be evident at transition times between light and dark when sink demand or photosynthetic activity is highest. However, diurnal levels of sucrose changed very little during the cycle with the
mutant accumulating twice as much sucrose at all time points when compared to wild type (Figure 8A). Salk 2X starch accumulation was very similar to that of wild type, with only a small increase at the end of the light period and a similar rate of turnover during the dark period (Figure 8B).

Some simple characteristics of sink growth were examined in the Salk 2X line to determine if growth of the plants was retarded by lack of vacuolar invertase. The leaf width of leaf 7 was measured as an indicator of the rate of leaf expansion, with no differences observed between Salk 2X and wild type (Figure 9A). Root growth was measured on MS agar plates with or without sucrose in the media. Again, no differences were seen when comparing the double mutant and wild type (Figure 9B). However, germination occurred slightly later in the mutant which caused the graph line to appear shifted to the right, but the slope of the line is identical for both Salk 2X and wild type. Root growth on media containing sucrose was accelerated in both Salk 2X and wild type, indicating that uptake of sucrose from the media is not impaired in the mutant line. As a measure of reproductive growth, siliques size and the number of seeds per silique were examined. Again, there was no difference between the double mutant line and wild type. Although the molecular components of roots and seeds were not examined, it can be reasonably concluded that lack of vacuolar acid invertase activity has no severe impact on the import of sucrose or growth of sinks in the double mutant.

The second goal of this study was to examine altered partitioning of carbon into sucrose and examine the effects on gene expression during leaf development. To achieve this, plants were grown under constant light for two purposes; a) to reduce the effect of the day/night cycle, and b) to examine photosynthetic parameters under saturating conditions.
The partitioning of carbohydrates during leaf development was examined in a single leaf (Figure 10). The sucrose content of double mutant was only slightly higher than wild type until full leaf expansion (day 15) at which time sucrose began to accumulate to higher levels (Figure 10A). This was in conjunction with elevated levels of glucose and fructose in the wild type during the same time period (Figure 10B & C). However, the shift in partitioning toward sucrose in the Salk 2X line was not significantly different, indicating that vacuolar acid invertase has only a slight control of partitioning of sucrose in leaves even at the latest stages of development. This result differs from those observed under the day/night cycle (Figure 7A, 8A) in which sucrose accumulated to significant levels in the Salk2X leaves. This suggests that sucrose levels might be moderated in the light, but vacuolar invertase does not influence this partitioning in constant light. Lastly, no differences were observed in starch content (Figure 10D).

It has been proposed that cycling of sucrose synthesis and hydrolysis may be responsible for feedback inhibition of photosynthesis sink limitation (Huber, 1989, Moore et al., 1998). Under this proposal sucrose accumulates in source leaves when utilization by sinks is limiting. The sucrose is hydrolyzed by invertase, phosphorylated by hexokinase, and re-enters the sucrose synthesis pathway. Hexokinase signaling results in a down regulation of photosynthetic genes and a decrease in photosynthesis. The chlorophyll contents of leaves can provide a good indication of photosynthetic activity, as the levels closely parallel carbon exchange rates during *Arabidopsis* leaf development (Miller et al., 1997). The Salk 2X levels of chlorophyll were nearly identical to wild type at all leaf ages (Figure 11). This suggests that there were no major differences in photosynthetic activity even after the leaf stage when sucrose began to accumulate (Figure 10A).
Increasing source strength leads to a down-regulation of photosynthesis, presumably due to the limitation in sink utilization of photosynthate and the accumulation of carbohydrates in source leaves (Paul and Foyer, 2001). The double mutant line was tested under three different environments that have been demonstrated to increase the photosynthetic rate of Arabidopsis: 1) growth in high light (250-300 μmol m\(^{-2}\) s\(^{-1}\)); 2) growth in high light in which plants were transferred to an enriched CO\(_2\) environment (1000 ppm) at the leaf day 1 stage, and 3) growth in high light and enriched CO\(_2\) proceeding from germination through senescence (Walters et al., 1999, Sun et al., 1999). Chlorophyll measurements under these conditions revealed very little difference between the mutant line and wild type (Figure 12). When compared to normal light-grown plants, high light grown-plants have less chlorophyll but maintain a constant amount until day 8, after which time chlorophyll declines quite rapidly (Figure 12A). After transferring high light-grown plants from an ambient CO\(_2\) environment (375 ppm) to an enriched CO\(_2\) environment (1000 ppm), there was an initial decline in chlorophyll after 1 day, a modest decline until day 8, and rapid decline thereafter similar to high light and ambient CO\(_2\) conditions (Figure 12B). Growing plants under high light in a constant enriched CO\(_2\) environment showed a slight increase in chlorophyll amounts from the first few days, followed by a decline after day 4 (Figure 12C). Taken together this data would seem to indicate that lack of vacuolar invertase has no effect on chlorophyll contents and possibly photosynthetic rate, considering that the two are closely regulated.

An interesting finding is the comparison of the different light environments (Figure 13). The lower chlorophyll content of high light-grown plants is an acclimation response to the light condition and not a reflection of the photosynthetic rates, which has been shown to
be significantly higher in studies under similar conditions (Sun et al., 1999, Walters et al., 1999). More important is the timing of senescence under the different light regimes. Senescence is the remobilization of nutrients from source leaves to different parts of the plant and is characterized by a yellowing of the leaves, or loss of chlorophyll, as the leaf ages. The chlorophyll in the high light-grown plants was completely depleted by day 16, while chlorophyll in leaves grown under normal light was not depleted until day 29-30. This seems to indicate that higher photosynthetic rates greatly accelerate the development of Arabidopsis leaves. However, no differences were observed between the Salk 2X invertase mutants and wild type.

Under sink limiting conditions, the accumulation of carbohydrates in source leaves is "sensed" by the cell, generating a signal resulting in decreased expression of photosynthetic genes, such as the nuclear-encoded gene for the small subunit of Rubisco, RbcS. The regulation of gene expression is proposed to involve the phosphorylation of hexoses by hexokinase (Jang et al., 1997). One possible source for hexoses in source leaves is the hydrolysis of sucrose by invertase. To investigate if the Salk 2X lines reduce the source of hexoses for hexokinase signaling, RNA from leaves at different developmental stages was analyzed for RbcS expression. Two different RNA blots were probed with RbcS to investigate photosynthetic gene expression, and again probed with 18S rRNA to correct for unequal loading of the RNA. The Northern blots of Salk 2X and wild type RNA show a steady decline in RbcS expression throughout leaf development (Figure 14A), with a slightly higher transcript level in the double mutant, as indicated by the ratio of RbcS to 18S rRNA (Figure 14B). Similar blots were performed for the plants treated with high light and enriched CO₂ (same conditions as the chlorophyll experiments). High light-treated wild
type plants had a higher level of RbcS expression than the Salk 2X mutant (Figure 15), while plants transferred to the enriched CO₂ environment showed a variable expression pattern (Figure 16). Leaves from plants grown under high light and constant CO₂ enrichment displayed an almost identical expression pattern (Figure 17). Taken together there does not appear to be an altered expression pattern of RbcS in the vacuolar invertase mutants when compared to wild type grown under all conditions tested. RbcS expression in both lines dramatically decreased between day 4 and day 8, compared to approximately day 15-20 in the normal light-grown plants. Expression declined at a slightly greater rate in plants grown in high CO₂. Again, this is similar to the chlorophyll measurements and may be indicative of an accelerated developmental program that is affected more by high light than a high CO₂ environment.

Lastly, there are other carbohydrate signaling pathways independent of the hexokinase-sensing system (Smeekens 2000). For example, sucrose fed to detached sugar beet leaves has been shown to decrease the expression of the gene coding for the proton-sucrose transporter, SUC2 (Chiou and Bush, 1998). Therefore, it seemed logical to examine the expression of SUC2 in the Salk 2X mutants since there is an increase in sucrose levels later in leaf development. However, the RNA blots revealed no difference in SUC2 expression at any time during leaf development (Figure 18A, C). The lack of significant differences between the Salk 2X mutants and wild type may cause an increase in cell wall invertases to compensate for the loss of vacuolar invertase. However, similar to SUC2, no differences were observed in the expression of the predominantly leaf-expressed form of Arabidopsis cell wall invertase, Atffruct1 (Figure 18A, B). While no increase in expression was observed in the Salk 2x line, the existing activity may be enough to compensate for
vacuolar acid invertase, as vacuolar invertase appears to be present in excessive amounts and a 95% loss of activity has very little effect on phenotype. Lastly, the expression of the senescence associated gene, \textit{SAG12}, displayed no change in expression pattern in the mutant (Figure 18A, D). The Salk 2X mutants do not display a phenotype that alters the timing of senescence as evidenced by no differences in the decline in chlorophyll levels, \textit{RbcS} expression, or the timing of \textit{SAG12} expression.

**Discussion**

The goal of this study was to examine the role of vacuolar acid invertase in partitioning of sucrose in source leaves and any possible correlation with an acclimation response to increased source strength. The approach was to examine mutants lacking vacuolar invertase activity with expectations that differences in sucrose levels would lead to altered expression of the photosynthetic gene \textit{RbcS}. For this study, T-DNA insertion mutant lines were obtained for the two Arabidopsis genes, \textit{Atf3fruct3} and \textit{Atf4fruct4}. All mutant lines studied, including the double mutant (Salk 2X), were phenotypically similar to wild type under several different growth conditions. Along with tests of plants grown under constant normal or high light and ambient or high \textit{CO}_{2} concentrations, Salk 2X mutants were also grown under short-day or long-day cycles, treated with cold temperatures (14°C) for 3 weeks, or subjected to water deficit for 10 days and displayed no difference in physical phenotype when compared to wild type control plants (data not shown).

Along with vacuolar invertase, cell wall invertase and sucrose synthase also hydrolyze sucrose and are associated with sink metabolism of sucrose. The different subcellular locations of the three enzymes may contribute to the function of the enzymes and
the use of the products of those reactions. Typically cell wall invertases are associated with newly growing tissues such as rapidly dividing embryos (Weber et al., 1995). Sucrose synthase converts sucrose into fructose and UDP-glucose. Studies indicate it is associated with biosynthetic reactions, such as cell wall and starch synthesis, providing an energetically favorable form of glucose, UDP-glucose, for the corresponding reactions. Vacuolar acid invertase activity in tomato determines the hexose/sucrose content but has little effect on total dry weight accumulation of carbohydrates (Scholes et al., 1996, Ohyama et al. 1995). In carrot tap roots, the concerted activity of both cell wall and vacuolar invertases is required for normal fresh weight accumulation (Tang et al., 1999). Thus, there are species-specific differences in sink metabolism and functions of the different sucrose-metabolizing enzymes.

Growth of sinks in *Arabidopsis* appears unaltered when lacking vacuolar invertase, as evidenced by the growth rates of leaves, roots, or fruits (siliques and seeds). The growth rate of seedlings germinated on MS was more rapid when sucrose was added to the media but apparently does not require metabolism by vacuolar invertase.

The single-gene mutants displayed different levels of expression and activity, with *Atfifruct4* contributing about two-thirds of the total activity in leaves. The lack of both genes resulted in only background activity indicating no other genes contribute to the leaf soluble acid invertase activity. The sucrose content was altered only in the double mutant, although increases did not occur in leaves until later in development. The lack of sucrose hydrolysis resulted in a slight increase in glucose and fructose content, although the difference was not significant. The lack of a significant change in sucrose content and hexose/sucrose ratio may be the reason for the lack of dramatic differences observed in chlorophyll content and expression of *RbcS, SUC2*, and *SAG12*, genes previously shown to
be affected by carbohydrates. In their studies of different species with varying vacuolar invertase activities, Moore et al. (1998) demonstrated that an acclimation response only occurred in those species with a hexose/sucrose ratio <0.25. In the Salk 2X mutants this ratio was reduced to approximately 1.1, compared to 2.5 for wild type.

Differences in signaling may arise from the different subcellular localizations of hexose and its access to hexokinase. Hexokinase is a cytosolic protein which is known to be bound to mitochondria, associated with cellular membranes in maize, and located at the outer envelope of plastids in spinach (da-Silva et al, 2001, Weise et al., 1999). Induction of systemic acquired resistance and repression of photosynthetic gene expression in tobacco expressing a yeast invertase in the apoplast or vacuole also involves transport through the secretory pathway. The hydrolysis of sucrose in the apoplast by cell wall invertase and the subsequent transport of the resulting hexoses by hexose transporters could funnel substrate directly to a form of hexokinase that is directly in contact with the transport complex. The same could be stated for glucose originating from starch degradation that is exported from the plastid by a transporter complexed with hexokinase. Indeed, Sharkey et al., (2004) have recently proposed a mechanism by which the starch-to-sucrose conversion involves maltose metabolism and requires hexokinase activity. Storage of starch would also represent a much larger pool of hexoses and larger potential signal when compared to the soluble sugar pool size.

In conclusion, the loss of vacuolar acid invertase in Arabidopsis had little effect on carbohydrate signaling and no effect on gene expression or leaf developmental programming. Future considerations could involve a combination of mutants of vacuolar and cell wall invertase. Arabidopsis has primarily only one expressed gene for cell wall
invertase, *Atβfrcitheructl*. The goal would be to find the combination that reduces the hexose/sucrose ratio below 0.25, the level predicted by Moore et al. (1998) to reduce the acclimation response. Other possibilities are crosses to lines that over-express sucrose phosphate synthase (SPS). There has been some success correlating both increased SPS activity and increased partitioning into sucrose (*Arabidopsis*), increased photosynthetic rate (tomato), or prolonged photosynthesis with increased shoot biomass and flower numbers (tobacco) (Signora et al., 1998, Laporte et al., 1997, Baxter et al., 2003). In general, shifting partitioning to active sucrose synthesis tends to increase export and maintain a higher photosynthetic rate. Reducing sucrose hydrolysis by invertase should only enhance this characteristic.
References


**Figure Legends**

**Figure 1.** Northern blots of vacuole acid invertase expression in leaves of wild type *Arabidopsis* during development. 5 μg of total RNA from leaf 8 were loaded onto each lane of the gel. Blots were probed separately with gene-specific probes. An 18S rRNA probe was used to correct for unequal loading. Blots were exposed to X-ray film for exposure; the *Atβfruct4* blot was exposed for 2 days, the *Atβfruct3* blot was exposed for 5 days, and the 18S rRNA blot was exposed for 1 hour. Day 1 of leaf age was determined as the time when the leaf reached 5 mm in width.

**Figure 2.** Gene maps of the *Arabidopsis* vacuolar acid invertase genes, *Atβfruct3* and *Atβfruct4*. Locations of Salk T-DNA insert lines are displayed for both genes. Mutant lines used for experiments are shown in bold: Salk6934 contains a T-DNA in the second intron of *Atβfruct3*, Salk 100813 contains a T-DNA insert in the first exon of *Atβfruct4*. 
Figure 3. Southern blot analysis of Salk 6934. Each lane represents 5μg of DNA from Salk 6934 or wild type Arabidopsis digested with Bgl II. (A) A Atβfruct3-specific probe shows a shift in the size of the wild type gene. Lane 1 is a homozygous mutant line with a single band at ~5900 bp, lane 2 is a heterozygous mutant with two bands corresponding to the mutant and endogenous form of Atβfruct3, and lane 3 is wild type with one band at ~6300 bp. (B) A blot probed with NPT II shows the number of T-DNA inserts in the mutant lines.

Figure 4. Southern blot analysis of Salk 100813. Each lane represents 5 mg of DNA from Salk 100813 or wild type Arabidopsis digested with Bgl II. (A) A Atβfruct4 probe shows the shift in size of the wild type gene due to the addition of the T-DNA. The size difference is equal to the size of one T-DNA (~4200 bp). Lane one is a homozygous mutant (~4700 bp), lane 2 is heterozygous, and lane 3 is wild type (~8900 bp). The probe also hybridized to the Atβfruct3 gene, as shown by the band at approximately 6300 bp. (B) A blot probed with NPT II shows the number of T-DNA inserts in the mutant lines.

Figure 5. Northern blot analysis of vacuolar acid invertase mutants. Total RNA from leaf 8 of 28-30 day-old plants was loaded onto each lane. Gene-specific probes were used to detect either Atβfruct3 or Atβfruct4. A picture of the ethidium-bromide stained gel is shown to correct for unequal loading. Salk 16136, which contains a T-DNA insert in the sixth exon of Atβfruct4 (Figure 6), produced an mRNA 1200-1400 bp larger than the endogenous gene (2200 bp) and had enzyme activity (Figure 6).
Figure 6. Soluble acid invertase activity of mutant lines. Activity was measured in leaf 8 of 28-30 day-old plants. Sample numbers ranged from 4-8 with bars representing the standard error. (*) indicates significance when compared to wild type. (**) Indicates significance when compared to all other lines.

Figure 7. Carbohydrate levels in vacuolar acid invertase lines. (A) Sucrose. (B) Hexoses. (C) Starch. Leaf 8 samples were taken at midday from 28-30 day-old plants grown under a 14 hr light/8 hour dark diurnal cycle. Sample numbers ranged from 8-15 samples with error bars representing standard error. (*) indicates a significant difference when compared to wild type.

Figure 8. Diurnal levels of sucrose and starch in the vacuolar acid invertase double mutant (Salk 2X, □) and wild type (WT, •). Leaf 8 samples were taken from 28-30 day-old plants grown under a 14 hour light/8 hour dark cycle. Each point represents the average ± standard error of 4 samples.

Figure 9. Growth of leaf 7, roots, or fruits of the double mutant of vacuolar invertase and wild type Arabidopsis. Salk 2X is the homozygous mutant resulting from the cross of Salk6934 and Salk100813. (A) Leaf expansion was measured beginning at day 17 after germination and represents the average of 23 plants. (B) Root growth was measured as root length of seedlings grown on agar plates containing 1X MS salts or 1X MS with 2% sucrose. Each point represents the average of 15-30 seedlings. (C) Silique length and number of seeds per siliques were determined from siliques removed from the middle portion of bolts.
from mature plants (40-45 days old). Each bar represents the average of 52-54 siliques for size measurements and 20 siliques for seed count.

**Figure 10.** Carbohydrate contents during development in leaves of the vacuolar acid invertase double mutant (Salk 2X, ■) and wild type (WT, ∗). (A) Sucrose, (B) Glucose, (C) Fructose, (D) Starch. Leaf 8 samples were taken at different ages from plants grown under constant illumination (70-100 \(\mu\text{mol} \text{ m}^{-2} \text{s}^{-1}\)). Day 1 is defined as the time when the leaf reaches 5 mm in width.

**Figure 11.** Chlorophyll content in leaves of plants grown under normal light conditions. Salk 2X, (■) and wild type (∗) leaf 7 samples were taken at different stages of development from plants grown under constant illumination (70-100 \(\mu\text{mol} \text{ m}^{-2} \text{s}^{-1}\)). Day 1 is defined as the time when leaf 7 reaches 5 mm in width.

**Figure 12.** Chlorophyll content in leaves of plants grown under high light and ambient or enriched CO\(_2\). Salk 2X (■), or wild type (∗) leaf 7 samples were taken at different stages of development from plants grown under (A) constant illumination (250-300 \(\mu\text{mol} \text{ m}^{-2} \text{s}^{-1}\)) and ambient CO\(_2\) (375 ppm), (B) high light (250-300 \(\mu\text{mol} \text{ m}^{-2} \text{s}^{-1}\)) and transferred to enriched CO\(_2\) (1000 ppm) at leaf 7 day 1, or (C) high light and constant CO\(_2\) (1000 ppm). Day 1 is defined as the time when leaf 7 reaches 5 mm in width.

**Figure 13.** A comparison of chlorophyll contents of plants grown under different light conditions. This figure is a combination of Figures 11 and 12A. Normal light (NL) was 70-
100 μmol m$^{-2}$ s$^{-1}$ and high light (HL) was 250-300 μmol m$^{-2}$ s$^{-1}$. Salk 2X-NL(■), wild type-NL(●), Salk 2X-HL ( ), wild type (●).

**Figure 14.** Expression of *RbcS* during leaf development grown under normal light conditions. Salk 2X (■), or wild type (●) leaf 9 samples were taken at different stages of development from plants grown under constant illumination (70-100 μmol m$^{-2}$ s$^{-1}$). Two separate RNA gels were run with 2 μg of total RNA loaded in each lane. *RbcS*-1A was used to probe for Rubisco small subunit expression. *18S rRNA* was used as a probe to adjust samples for unequal loading. (A) Northern blots were exposed to phosphorimaging screens and expression levels were analyzed using the Quantity One (BioRad) computer program. (B) The ratio of *RbcS* to *18S rRNA* expression was averaged for the two blots and plotted on a graph.

**Figure 15.** Expression of *RbcS* during leaf development grown under high light conditions. Salk 2X (■), or wild type (●) leaf 9 samples were taken at different stages of development from plants grown under constant illumination (250-300 μmol m$^{-2}$ s$^{-1}$). (A) Two separate RNA gels were run with 2 μg of total RNA loaded in each lane. (B) The ratio of *RbcS* to *18S rRNA* expression was averaged for the two blots and plotted on a graph.

**Figure 16.** Expression of *RbcS* during leaf development grown under high light conditions and transferred to enriched CO$_2$ at day 1. Salk 2X (■), or wild type (●) leaf 9 samples were taken at different stages of leaf development from plants grown under constant illumination
(250-300 \mu\text{mol m}^{-2} \text{s}^{-1}) and transferred to enriched CO$_2$ at day 1, which is defined as the time when the leaf reaches 5 mm in width. (A) Two separate RNA gels were run with 2 \mu g of total RNA loaded in each lane. (B) The ratio of \textit{RbcS} to \textit{18S rRNA} expression was averaged for the two blots and plotted on a graph.

**Figure 17.** Expression of \textit{RbcS} during leaf development grown under high light conditions and enriched CO$_2$. Salk 2X (■), or wild type (+) leaf 9 samples were taken at different stages of leaf development from plants grown under constant illumination (250-300 \mu\text{mol m}^{-2} \text{s}^{-1}) and enriched CO$_2$ (1000 ppm). (A) Two separate RNA gels were run with 2 \mu g of total RNA loaded in each lane. (B) The ratio of \textit{RbcS} to \textit{18S rRNA} expression was averaged for the two blots and plotted on a graph.

**Figure 18.** Expression of \textit{AtCW1}, \textit{SUC2}, and \textit{SAG12} during leaf development grown under normal light conditions. Salk 2X (■), or wild type (+) leaf 9 samples were taken at different stages of leaf development from plants grown under constant illumination (70-100 \mu\text{mol m}^{-2} \text{s}^{-1}). (A) Two separate RNA gels were run with 2 \mu g of total RNA loaded in each lane. \textit{AtCW1}, \textit{SUC2}, and \textit{SAG12} were used as probes, and \textit{18S rRNA} was used to adjust samples for unequal loading. Northern blots were exposed to phosphorimaging screens and analyzed using the Quantity One (BioRad) computer program. (B) The ratio of \textit{RbcS} to \textit{18S rRNA} expression was averaged for the two blots and plotted on a graph.
Figure 1
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Diurnal Starch Content
(μmol glucose eq./g FW)

Diurnal Sucrose Content
(μmol/g FW)

Figure 8
A

B

C

Figure 9
Figure 10

Starch Content
(μmol glucose eq./g FW)

Fructose Content
(μmol/g FW)

Glucose Content
(μmol/g FW)

Sucrose Content
(μmol/g FW)

Leaf Age (days)
Figure 11
Figure 13
A

Leaf 9 Age (days)

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B

Figure 14
Figure 15
Figure 16
Figure 17
Figure 18

A

18S RNA
SUC2
SAG 12
ACW1

Leaf Age (days)

2X WT
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B

Relative Expression (SUC2/18S rRNA)

C

Relative Expression (CW1/18S rRNA)

D

Relative Expression (SAG 12 / 18S rRNA)
CHAPTER 6. GENERAL CONCLUSIONS

Conclusions

The goal of the research presented in this dissertation was to characterize carbohydrate partitioning and photosynthesis in relation to sink/source interactions in Arabidopsis. Previous studies in our lab focused on tobacco leaf development which demonstrated that growing plants under conditions of increased source strength (high CO₂) resulted in accelerated leaf development and a down-regulation of photosynthesis (Miller et al., 1997). Subsequent studies of tobacco plants with reduced amounts of the Rubisco small subunit displayed prolonged leaf development and a delayed onset of senescence due to a limited photosynthetic capacity (Miller et al., 2000). Together these studies demonstrated that leaf development is directly related to photosynthetic capacity. Other studies have shown that responses to high CO₂ can be directly attributed to accumulations of carbohydrates in source leaves (Moore et al., 1998).

The model of regulation of photosynthesis proposes that limited sink utilization of photosynthate results in an accumulation of carbohydrates in sources leaves which, in turn, are sensed and translated into a down-regulation of photosynthesis. To date, the mechanism by which the signal connecting carbohydrate sensing to changes is gene expression is not well understood. Carbohydrates are known to regulate gene expression in plants by several proposed pathways (Koch 1996). One of the better characterized pathways involves sugar signaling via hexokinase. Experiments with hexokinase mutants of Arabidopsis displayed sensitivity to glucose levels with altered expression of photosynthetic genes such as RbcS and LhcB. Over-expression of hexokinase in tomato also resulted in decreased
photosynthesis and an early onset of senescence, similar to experiments of plants grown in high CO$_2$. The proposed model of carbohydrate sensing states that futile cycling of sucrose synthesis and hydrolysis by invertase generates a signal through hexokinase resulting in the down-regulate photosynthetic gene expression (Foyer, 1988, Huber, 1989, Moore et al., 1998).

To further investigate the role of carbohydrate partitioning during leaf development we turned to *Arabidopsis* as a model system to study leaf development. The first step was to characterize and compare wild type leaf development to our previous studies with tobacco. As shown in chapter 3, *Arabidopsis* leaf photosynthesis declined from the first time point measured as opposed to tobacco which first increased to a peak photosynthetic rate coincident with full leaf expansion, then gradually declined (Miller et al., 1997). Flux of carbohydrates into hexoses reached a maximum point coincident with full leaf expansion in *Arabidopsis* at which point there was a large decline in the expression of the photosynthetic gene LhcB. This change did not correlated to carbohydrate pool sizes, which may indicate that changes in gene expression are not dependent on pool sizes but on active metabolism or cycling of sucrose and hexose concentrations.

Studies of increased sink strength in the variegation mutant *immutans* were presented in chapter 4. The increase in the sink/source ratio, due to the loss of total photosynthetic area, causes a stunted growth phenotype of *immutans* plants, but eventually *im* plants reach a size similar to wild type (Aluru et al., 2001). It was revealed that the green sectors of *im* leaves have a higher photosynthetic rate when compared to wild type leaves. All photosynthetic parameters tested were higher than wild type, including O$_2$ evolution, activation of the photosystems, Rubisco total activity and activation state, and assimilation
of $^{14}$CO$_2$. Pulse-label feeding experiments also showed an increased flux of newly fixed carbon into the soluble carbohydrate fraction. Carbohydrate pool sizes indicated that im green sectors contain elevated levels of sucrose and accumulate more starch at the end of the light period of the day/night cycle. Flux of carbon into the soluble carbohydrate fraction, elevated levels of sucrose, and increased SPS total activity and activation state indicate greater export and utilization of photosynthate. Hydrolysis of sucrose in the apoplastic space by cell wall invertase can prevent sucrose export from source leaves. However, this activity was greatly reduced in im green sectors when compared to white sectors and to wild type leaves. The conclusion is that increased demand for photosynthate leads to an increase in photosynthetic rate in green sectors when compared to wild type. This is manifested by a shift toward sink uptake of sucrose by the non-photosynthetic white sectors and a higher activation of the Calvin cycle and photosystems in green sectors.

Chapter 5 presents studies of Arabidopsis mutants lacking vacuolar acid invertase. A previous study of several different plant species demonstrated that the hexose/sucrose ratio of leaves is determined by vacuolar acid invertase activity which, in turn, determined the extent of an acclimation response to growth in high CO$_2$ and the expression of RbcS (Moore et al., 1998). Plant species with a hexose/sucrose ratio below 0.25 did not respond with a down-regulation of RbcS expression while those above had up to a 20% reduction in expression. By studying vacuolar acid invertase mutants of Arabidopsis, the goal was to observe similar characteristics within a single a species. Results of that study indicate that vacuolar invertase does affect the accumulation of sucrose in source leaves of plants grown under a diurnal cycle. However, leaves of plants grown in constant light do not accumulate significantly more sucrose until after full leaf expansion, indicating a limitation on export
capacity, sink uptake, or combination of both. Plants grown under varying conditions for maximum photosynthetic output did not display any variations in chlorophyll amounts or $RbcS$ gene expression when compared to wild type plants. Expectations were that lack of vacuolar invertase would change either the hexose/sucrose ratio or limit the cycling of sucrose synthesis and hydrolysis, and thereby reduce feedback inhibition of photosynthetic gene expression via hexokinase signaling. However, mutants showed only a slight decrease in hexose/sucrose ratio and displayed no change in gene expression. The lack of change may be related to cell wall invertase activity. As shown in the study of im, cell wall invertase is affected more than vacuolar acid invertase by the carbohydrate status of the plant. Also, transport of hexoses across the plasma membrane may be an additional requirement for signaling. Overall, the redundancy of the system makes it difficult to dissect the carbohydrate signaling mechanism by observing single mutations or modifications in the suspected pathway. Further studies with combined vacuole and cell wall invertase mutants may reduce the hexose/sucrose ratio to a more significant level, allowing for studies of the influence of the two different sugars on gene expression.

References


