Doctoral Thesis

Carbon assimilation in Arabidopsis thaliana rosettes and its partitioning in mature and developing leaves

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Publication Date:
2012

Permanent Link:
https://doi.org/10.3929/ethz-a-007608582

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CARBON ASSIMILATION IN ARABIDOPSIS THALIANA ROSETTES AND ITS PARTITIONING IN MATURE AND DEVELOPING LEAVES

A dissertation submitted to the

ETH ZÜRICH

For the degree of Doctor of Sciences

Presented by

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2012
Acknowledgements

First of all, I would like to thank Prof. Sam Zeeman for giving me the opportunity to do my PhD in his group. Thank you very much for all your inspiring ideas, for the time you spend with me to discuss my project and for giving me the freedom to follow up my own ideas.

I would also like to thank Prof. Willi Gruissem, Dr. Nicola Zamboni and Prof. Pierdomenico Perata for being part of my PhD committee and for being co-referees of my PhD thesis.

Special thanks go to Roland Künzli for helping me to set up our gas exchange system, for answering all my questions and solving all problems I had with the machine. Many thanks also go to Patrick Flütsch for constructing the plant and labelling chambers I needed during my project, and for all the little things which made working in the lab much easier and more effective. I’m also very grateful for the IT support I got from Matthias-Hirsch-Hoffman, Svenson Cornehls and Rolf Joho. Thank you for keeping my computer running smoothly during my whole PhD life.

A big “Thank you” goes to Antonia Müller for her enthusiasm and for optimizing work in the lab, and to Matthias Thalmann for processing a huge amount of samples without getting too mad at me. I wouldn’t have managed so much without you.

I want to express my gratitude to Sabine Klarer, Sara Bangerter, Andrea Gronau and everybody else who took care of my plants during the past four years. Big thanks to all the members of the Zeeman group for the help in the lab and for sharing so many delicious cakes. Special thanks go to Stefan Herwig. We started the project together and went through many ups and downs in the past years. I wish you all the best for finishing your PhD work.

Special thanks to everyone who made my time in Zürich so exceptional. I’m very grateful for all the fun we had while enjoying beers, dinner and BBQ, while exploring the mountains and while watching movies together.

Many thanks to my parents, my brother, my grandparents and all my friends for supporting me on my way. It is good to know you are always there for me no matter how successful my experiments are.

Finally, I would like to thank Oliver Kötting, my most important discovery during my PhD outside of the lab. Thanks for your love, your motivating words and your belief in me.
# Table of Contents

**Acknowledgements**

**Summary**

**Zusammenfassung**

**Abbreviations**

## 1 Introduction

1.1 Leaf development

1.2 Carbon assimilation

1.2.1 Light dependent reactions

1.2.2 Carbon fixation reactions

1.2.3 Regulation of photosynthesis

1.3 Carbon partitioning

1.3.1 Carbon partitioning in autotrophic tissue

1.3.2 Carbon transport

1.3.3 Carbon partitioning in sink leaves

1.4 Regulation of carbon partitioning

1.4.1 Regulation of starch and sucrose synthesis

1.4.2 Regulation of sucrose transport

1.4.3 Linkage of carbon supply and growth

1.5 Aim of the study

## 2 Materials and Methods

2.1 Plant material

2.2 Growth conditions

2.2.1 Growth conditions on soil

2.2.2 Growth conditions in hydroponics

2.3 Enzymes and Chemicals
### 2.4 Arabidopsis gas exchange chambers

26

### 2.5 The gas exchange measuring unit (GDU-26)

27

### 2.6 Setup of gas exchange measurements

28

### 2.7 Determination of leaf area

28

### 2.8 Gas exchange data analysis

28

### 2.9 Measuring photosynthetic parameters with the Licor6400

29

### 2.10 Isotopic labeling chambers

30

### 2.11 Isotopic labeling

31

### 2.12 Autoradiography

32

### 2.13 Ethanol extraction of $^{14}$C labeled plant material

32

### 2.14 Fractionation of $^{14}$C labeled plant material

33

### 2.15 Quantitative measurement of $^{14}$C-labeled plant material – Scintillation counting

36

### 2.16 Establishing a growth curve

36

### 2.17 Perchloric acid extraction and carbohydrate measurements

37

#### 2.17.1 Starch measurement

37

#### 2.17.2 Measurement of glucose, sucrose and fructose

38

### 2.18 Statistical analysis

39

### 3 A method to measure gas exchange of whole Arabidopsis plants

41

#### 3.1 Introduction

41

#### 3.2 Results

43

##### 3.2.1 Design of the GDU-26 gas exchange system

43

##### 3.2.2 Optimizing the setup of the GDU-26 gas exchange system

45

##### 3.2.3 Consistency of the measurements gained with the GDU-26

47

##### 3.2.4 Statistical analysis of the GDU-26 measurements

50

##### 3.2.5 Long term measurements performed with the GDU-26

53

##### 3.2.6 Setup and system limitations of the GDU-26

54

##### 3.2.7 Measuring photosynthetic rate in different environments

55
3.2.8 Measuring photosynthetic rate under stress conditions 57
3.3 Discussion 59

4 Carbon allocation and carbon transport in Arabidopsis thaliana 63
4.1 Introduction 63
4.2 Results 66
  4.2.1 Control experiment 66
  4.2.2 Export of carbon to neighboring leaves and the root 68
  4.2.3 Time course of carbon export to the root and shoot 73
  4.2.4 Time course of carbon partitioning into major metabolic pools 77
  4.2.5 Carbon export in different developmental stages 80
4.3 Discussion 81

5 Carbon partitioning and growth in rosettes of Arabidopsis thaliana 89
5.1 Introduction 89
5.2 Results 91
  5.2.1 Carbon partitioning in sink and source leaves 91
  5.2.2 Leaf growth and carbon partitioning over the diurnal cycle 94
  5.2.3 Growth and carbon partitioning in mutants altered in starch metabolism 103
5.3 Discussion 113

6 General discussion 123

References 131

APPENDIX 141

CURRICULUM VITAE Error! Bookmark not defined.
Summary

Plant growth involves the assimilation of carbon and its utilization for maintenance, storage and growth.

The assimilation of carbon takes place in green tissues of the plants in a process called photosynthesis. In photosynthesis, light energy from the sun is used to generate chemical energy equivalents, which are subsequently used for the fixation of carbon dioxide in the Calvin-Benson cycle. The generated organic carbon compounds are either retained in the chloroplast to produce starch, a transient storage compound, or they are transported into the cytosol where they are used to fuel different metabolic pathways. The majority of carbon reaching the cytosol is used to build up sucrose, which is the major transport form of carbon in Arabidopsis thaliana. Sucrose is transported within the phloem from tissues of high carbon availability, such as mature leaves, to tissues of low carbon availability, such as developing leaves, roots and seeds. The assimilation of carbon during the day and the release of carbon from starch during the night provide a continuous carbon supply for the plant’s metabolism. Despite this, metabolic and physiological processes such as plant growth vary over the diurnal cycle.

In this work, I investigated the assimilation and allocation of carbon in the model species Arabidopsis thaliana. A gas exchange system - GDU 26 - was developed in the course of my project. The system allows carbon assimilation in up to eight Arabidopsis plants to be measured simultaneously for several days. Test measurements revealed stable and reproducible measurements of plants with a wide range in age. It enables the comparison of plants grown in different environmental conditions as well as plants undergoing specific experimental treatments.

I also studied carbon allocation in Arabidopsis thaliana in feeding experiments with radioactively labeled CO₂. Labeling of single Arabidopsis leaves revealed a defined pattern of carbon export from a mature leaf to neighboring developing leaves, following one of the vascular models described for Arabidopsis thaliana. Carbon export, as well as the partitioning of carbon within the tissue, varied over the light period. At dawn, wild-type plants utilize a high amount of assimilated carbon for sugar synthesis and export, as well as the synthesis of structural components. However, later during the day, more carbon is
channeled into the transient storage starch. Plants altered in starch synthesis and degradation experience carbon starvation during the dark period which leads to a reallocation of carbon at the beginning of the successive day. The more severe the alteration is, the stronger the changes in carbon allocation. This reallocation includes increased carbon partitioning into sugars, higher carbon export and reduced carbon flow into structural compounds important for growth. Later during the light period, when the starvation response is overcome, the pattern of carbon partitioning in starch degradation mutants is similar to wild-type. Starch synthesis mutants, on the other hand, which cannot use carbon to build up starch sufficiently, distribute excess carbon equally towards all other compounds, leading to a relative increase of carbon partitioning into structural components compared to the morning. The labeling experiments showed that times of high leaf expansion growth correlate with times of increased carbon partitioning into structural components, especially cell wall and lipids.
Zusammenfassung

Pflanzenwachstum umfasst die Assimilierung von Kohlenstoff und dessen Verwendung für die Versorgung der Pflanze, als Speicher und für Wachstumsprozesse.


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>3PGA</td>
<td>3-Phosphoglycerate</td>
</tr>
<tr>
<td>A</td>
<td>Photosynthetic rate</td>
</tr>
<tr>
<td>ADG2</td>
<td>ADP glucose pyrophosphorylase 2</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ADPGlc</td>
<td>ADP-glucose</td>
</tr>
<tr>
<td>AGPase</td>
<td>ADP-glucose pyrophosphorylase</td>
</tr>
<tr>
<td>AMY3</td>
<td>α-amylase 3</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAM</td>
<td>β-amylase</td>
</tr>
<tr>
<td>BE</td>
<td>Branching enzyme</td>
</tr>
<tr>
<td>BS</td>
<td>Bundle sheet cell</td>
</tr>
<tr>
<td>CC</td>
<td>Companion cell</td>
</tr>
<tr>
<td>Col</td>
<td>Columbia Ecotype</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>cyt</td>
<td>Cytosolic</td>
</tr>
<tr>
<td>d</td>
<td>Days</td>
</tr>
<tr>
<td>DBE</td>
<td>De-branching enzyme</td>
</tr>
<tr>
<td>DPE</td>
<td>Disproportionating enzyme</td>
</tr>
<tr>
<td>E</td>
<td>Transpiration rate</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FBPase</td>
<td>Fructose-1,6-bisphosphatase</td>
</tr>
<tr>
<td>Fru</td>
<td>Fructose</td>
</tr>
<tr>
<td>Fru6P</td>
<td>Fructose-6-phosphate</td>
</tr>
<tr>
<td>Fru1,6BP</td>
<td>Fructose-1,6-bisphosphate</td>
</tr>
<tr>
<td>GA</td>
<td>Gibberellic acid</td>
</tr>
<tr>
<td>GBSS</td>
<td>Granule-bound starch synthase</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>Glc1P</td>
<td>Glucose-1-phosphate</td>
</tr>
<tr>
<td>Glc6P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GWD</td>
<td>Glucan, water-dikinase</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>INV</td>
<td>Invertase</td>
</tr>
<tr>
<td>IRGA</td>
<td>Infrared gas analyzer</td>
</tr>
<tr>
<td>ISA</td>
<td>Isoamylase</td>
</tr>
<tr>
<td>LSF</td>
<td>Like-Sex-Four</td>
</tr>
<tr>
<td>m</td>
<td>Milli</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>µ</td>
<td>Micro</td>
</tr>
<tr>
<td>MEX1</td>
<td>Maltose-excess 1, Maltose transporter</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>PC</td>
<td>Plastocyanin</td>
</tr>
<tr>
<td>pGlcT</td>
<td>Plastidic glucose translocator</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>PHS2</td>
<td>α-glucan phosphorylase 2</td>
</tr>
<tr>
<td>PGI1</td>
<td>Phosphoglucoisomerase 1</td>
</tr>
<tr>
<td>PGM1</td>
<td>Phosphoglucomutase 1</td>
</tr>
<tr>
<td>P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PP</td>
<td>Phloem parenchyma cell</td>
</tr>
<tr>
<td>PQ</td>
<td>Plastoquinon</td>
</tr>
<tr>
<td>PS</td>
<td>Photosystem</td>
</tr>
<tr>
<td>PWD</td>
<td>Phosphoglucon, water dikinase</td>
</tr>
<tr>
<td>RGR</td>
<td>Relative growth rate</td>
</tr>
<tr>
<td>RH</td>
<td>Relative humidity</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature (23°C)</td>
</tr>
<tr>
<td>RuBisCO</td>
<td>Ribulose-1,5-bisphosphate carboxylase oxygenase</td>
</tr>
<tr>
<td>sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>SEX</td>
<td>Starch-excess</td>
</tr>
<tr>
<td>SPP</td>
<td>Sucrose phosphatase</td>
</tr>
<tr>
<td>SPS</td>
<td>Sucrose phosphate synthase</td>
</tr>
<tr>
<td>SS</td>
<td>Starch synthase</td>
</tr>
<tr>
<td>SUS</td>
<td>Sucrose synthase</td>
</tr>
<tr>
<td>Suc</td>
<td>Sucrose</td>
</tr>
<tr>
<td>SUC2</td>
<td>Sucrose transporter</td>
</tr>
<tr>
<td>T&lt;sub&gt;d&lt;/sub&gt;</td>
<td>Dewpoint temperature</td>
</tr>
<tr>
<td>T</td>
<td>Temperature</td>
</tr>
<tr>
<td>TP</td>
<td>Triose phosphate</td>
</tr>
<tr>
<td>TPT</td>
<td>Triose-phosphate/ phosphate translocator</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>UDPase</td>
<td>UTPGlc pyrophosphorylase</td>
</tr>
<tr>
<td>UDPGlc</td>
<td>UDP glucose</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine triphosphate</td>
</tr>
</tbody>
</table>
1 Introduction

Life on earth depends to a large extent on the ability of plants to use energy from sun light to build up organic carbon compounds and to produce oxygen. The synthesized carbohydrates are used by mankind as a direct food resource, as feedstock for farm animals, for the production of biofuel and for many other industrial purposes. With a growing population on earth, the importance of plants as an essential resource is becoming more and more evident. Analyzing carbon metabolism of plants and understanding plant growth is hence of fundamental scientific and public interest.

1.1 Leaf development

The main organs for carbon assimilation and the production of carbohydrates are leaves. Leaves show a high variability in morphological parameters like, size, shape and thickness, which are very important for adaptation to specific environmental conditions and for plant growth.

*Arabidopsis thaliana* leaves form a rosette. Its leaves differ anatomically and morphologically according to the developmental stage at which they were initiated. This phenomenon is known as heteroblasty. In Arabidopsis, almost round juvenile leaves with trichomes only at the adaxial site can be distinguished from elongated, adult leaves, displaying trichomes at the adaxial and abaxial site of the leaf. After the transition from the vegetative stage to the reproductive stage, a third type of leaves, cauline leaves, are produced at the base of each inflorescence.

Leaves are initiated at the periphery of the shoot apical meristem, where primordia are formed. The primordia of the first two leaves appear simultaneously on opposite sides of the shoot apical meristem. Subsequent leaves are initiated in a single phyllotaxis with approximately 137° in between successive leaves (Telfer and Poething, 1994). Leaves develop from a primordium into a mature leaf in a succession of five overlapping and interconnected phases (Gonzalez et al., 2012). During the first 24- to 48-h-long initiation phase, primordia are initiated and grow out as rod-shaped structures. In these protrusions,
an abaxial and adaxial domain is established. During the following cell division phase, cells in the entire primordium divide to generate new cells. The produced cells are small in size and remain like this until the expansion phase. A transition phase marks the beginning of the cell expansion. Cell division ceases first at the tip of the leaf and progresses further on towards the base. This process is also called basipetal maturation. Likewise, cell elongation starts at the tip of the leaf, whereupon it progresses in direction of the base. After transition, all cells are in the elongation phase, in which they expand up to their final size. This elongation is concurrent with cycles of endoreduplication, a process in which cycles of genome duplication take place without cell division.

Leaf maturation is accompanied by a switch from carbon import (sink) to carbon export (source). Young and still developing sink leaves are not able to assimilate sufficient carbon and they partially depend on carbon imported from other regions. Mature source leaves, on the other hand, produce an excess of carbon and can thereby support tissues with a negative carbon balance. The trigger for the sink-source transition is still not clear, but it is unlikely that the carbon balance within the leaf itself plays a crucial role. Albino tobacco shoots grafted onto green tobacco plants show that developing albino leaves first import carbon from the stock and subsequently undergo sink-source transition like green leaves. The leaves stop carbon import without ever reaching a positive carbon balance and die soon afterwards (Turgeon, 1984). Probably, import into developing leaves is terminated by restrictions of phloem unloading. Since leaves predominantly import sugars via a symplastic unloading process (see section 1.3.2), sugar uptake can be restricted by changes in plasmodesmata number or morphology. In higher plants, two types of plasmodesmata have been characterized, so-called simple plasmodesmata and branched plasmodesmata. Simple plasmodesmata form a single pore spanning the cell wall, whereas branched plasmodesmata consist of two or more channels through the cell wall, which are connected by a central cavity. In tobacco plants, the number of simple plasmodesmata dramatically decreases in epidermis and mesophyll cells with progressive sink-source transition (Roberts et al., 2001). Moreover, simple plasmodesmata are converted into branched plasmodesmata by the formation of bridges between adjacent pores during sink-source transition (Oparka et al., 1999). The size exclusion limit of plasmodesmata varies with the morphology and can be modified additionally by interactions with endogenous proteins,
viral proteins or other endogenous molecules (Roberts and Oparka, 2003). Simple plasmodesmata have a relatively high size exclusion limit compared to branched plasmodesmata, thereby allowing higher carbon flux. Free green fluorescent protein (GFP) expressed in the phloem of tobacco plants could be unloaded only from veins in sink leaves. The unloading of GFP from veins was absent in source regions of leaves undergoing the sink-source transition and in mature leaves, illustrating the loss of import ability, probably caused by the lower size exclusion limit of branched plasmodesmata (Imlau et al., 1999). Simultaneously with the changes restricting carbon unloading into sink tissues, modifications in the mature leaves lead to an increased active loading of sucrose into the phloem and, therefore, to an increase in export of carbohydrates (see section 1.4 regulation of carbon transport). Ultimately, the exchange of carbon compounds between net producers and consumers of carbon and the adaptation in carbon flow with the maturation of the tissue facilitates a consistent supply with carbon according to the demand of the tissue.

1.2 Carbon assimilation

Leaves are the major autotrophic tissue and assimilate most of the carbon and produce the majority of carbon compounds. The energy to assimilate carbon is derived from sunlight in a process called photosynthesis. Captured light energy is used to oxidize water, releasing oxygen, and to reduce carbon dioxide, building up carbon compounds. Both processes are the result of numerous complex reactions, which can be divided into light dependent reactions and carbon fixation reactions.

1.2.1 Light dependent reactions

The light dependent reactions take place in the thylakoid membranes of the chloroplast, which contain the photosystems, the light harvesting complexes and electron carrier proteins. In the first of two photochemical reactions operating in series, light is captured by the light harvesting complexes of photosystem II (PSII). The captured energy is transferred to the reaction center of PSII (P680), where a special chlorophyll molecule acts as a reductant transferring its electron to the acceptor plastoquinone (PQ). Water serves as
electron donor for reduction of the oxidized reaction center. Thereby, molecular oxygen is released and protons are generated in the lumen, contributing to a pH and electrical potential difference across the thylakoid membrane. From PQ, the electron is transferred via the cytochrome b$_{6f}$ complex to plastocyanin (PC).

In the second photochemical reaction, energy is captured by photosystem I (PSI) driving the oxidation of PC. Electrons from the reaction center of PSI (P700) are used to reduce ferredoxin which itself reduces NADP$^+$ to NADPH. NADP$^+$ marks the final electron acceptor of the light reactions. The reducing power stored in NADPH is used in the carbon fixation reactions.

Electron flow between the photosystems, through the cytochrome b$_{6f}$ complex, also leads to the transport of protons from the stroma to the thylakoid lumen, further increasing the pH and electrical potential difference between stroma and lumen. Another process involved in the establishment of the electrochemical gradient is a cyclic electron flow from PSI via ferredoxin and cytochrome b$_{6f}$ back to PSI (Bendall and Manasse, 1995; Johnson, 2005). The energy stored in these pH and electrical potential differences serves as the driving force for ATP production by the ATP synthase.

Ferredoxin not only transfers electrons to NADP$^+$, but also to a variety of other electron acceptors, which are for instance involved in nitrogen metabolism, sulfur metabolism and the regulation of carbon metabolism (Knaff, 1996). The redox-regulation of enzymes participating in photosynthetic carbon metabolism is mediated by thioredoxins, which is reduced by ferredoxin. When reduced, thioredoxin can activate several enzymes, like fructose-1,6-bisphosphatase (FBPase), RuBisCO activase and sedoheptulose-1,7-bisphosphatase, by reducing disulfide bonds within the proteins (Buchanan and Balmer, 2005). Under light-limiting conditions, overall around 88% of the electrons transferred from PSII via the electron transport chain to ferredoxin are utilized for the assimilation of CO$_2$ by RuBisCO (see next section), about 12% of the energy is channeled towards the other mentioned processes (Cornic and Baker, 2012).
1.2.2 Carbon fixation reactions

Carbon fixation takes place within the chloroplast stroma in a process named the Calvin-Benson cycle. The carboxylating enzyme is Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO), which apart from its carboxylating activity also has oxygenating activity. In plants, RuBisCO forms a multimeric complex comprising eight large and eight small subunits (L₈S₈). The large subunits contain the active site. The function of the small subunits is not fully revealed yet, but they might be involved in stabilizing and concentrating the enzyme or in improving enzyme kinetics.

In the carboxylation reaction catalyzed by RuBisCO, gaseous CO₂ is added to ribulose-1,5-bisphosphate (RuBP). The derived product is split immediately into two molecules of 3-phosphoglycerate (3-PGA) and is converted to 1,3-bisphosphoglycerate by the enzyme phosphoglycerate kinase. In the next step 1,3-bisphosphoglycerate is reduced to glyceraldehyde-3-phosphate (GAP) by GAP dehydrogenase. These reactions consume 2/3 of the ATP and all of the reducing equivalents (NADPH) required in the Calvin-Benson cycle. Triose phosphate isomerase interconverts GAP to dihydroxyacetone phosphate (DHAP). The equilibrium of the reaction lies on the side of DHAP, which accounts for 95% of this pool. The triose phosphates (TP) formed during the described reactions are either transported out of the chloroplast via the triose phosphate/ phosphate translocator (TPT) or they are converted via fructose-1,6-bisphosphate (Fru1,6BP) to fructose-6-phosphate (Fru6P) for use in the starch biosynthesis pathway. However, most TPs are converted in a series of reactions to regenerate RuBP. Most of these reactions are reversible, except the dephosphorylation of Fru1,6BP to Fru6P and sedoheptulose-1,7-bisphosphate to sedoheptulose-7-phosphate. These irreversible steps drive the reactions towards RuBP regeneration (Sharkey and Weise, 2012).

Besides the carboxylation of RuBP, RuBisCO catalyzes also the oxygenation of RuBP (Maurino and Peterhansel, 2010). During RuBP oxygenation one molecule 3-PGA and one molecule 2-phosphoglycolate are formed. In a process called photorespiration, accumulation of the potentially toxic 2-phosphoglycolate is prevented by its conversion into 3-PGA in a series of reactions taking place in chloroplast, peroxisome and mitochondria. During photorespiration, ATP and reducing equivalents are consumed. Additionally, one-quarter of carbon and an equimolar amount of nitrogen are released as CO₂ and NH₄⁺.
respectively which need to be reassimilated. Although photorespiration significantly decreases photosynthetic efficiency of the plant, it is considered to be important to prevent photoinactivation and damage of PS II at saturating light conditions. Under these conditions, photorespiration consumes excess energy and reducing equivalents produced by the light reactions, thereby maintaining linear electron transport (Cornic and Baker, 2012).

1.2.3 Regulation of photosynthesis

Photosynthesis is mainly regulated by two factors; the amount of light available to drive electron transport and the demand for end-products produced in the Calvin-Benson cycle. Regulation by light is a feedforward control mechanism to ensure optimal usage of light energy (Shikanai, 2010). While under low-light conditions maximal efficiency is favorable, photo-damage has to be avoided under conditions of excess light. Light fluctuates on different timescales in the environment and plants have evolved various adaptation mechanisms. As an immediate response, plants can dissipate excess light energy absorbed by PSII as heat. The amount of absorbed light energy is monitored indirectly via the pH of the thylakoid lumen. Under excess light, the pH decreases and thermal dissipation is induced (Horton and Hague, 1988; Rees and Horton, 1990). Another rapid mechanism for optimal light utilization is state transition, a process balancing excitation energy between the two photosystems. Association of the light-harvesting complex II (LHCII) to either PSII or PSI coordinates light absorption of the two photosystems. If PSII absorbs more light relative to PSI, the PQ pool is reduced. PQ reduction activates a kinase, STN7, which phosphorylates LHCII, leading to the movement of LHCII from PSII to PSI (Bellaﬁore et al., 2005). Conversely, excess absorption of light by PSI leads to oxidation of PQ. This results in dephosphorylation of LHCII by the phosphatase PPH1 and transfer of the complex from PSI to PSII (Shapiguzov et al., 2010). Acclimation of photosynthetic activity under low light intensities is mediated by phototropins, at a timescale of minutes to hours. The mediated modifications include chloroplast movement and opening of stomata (Jarillo et al., 2001; Kinoshita et al., 2001). Two blue-light receptors were identified in Arabidopsis, PHOT1 and PHOT2. Both are serine/threonine protein kinases, which are autophosphorylated upon excitation resulting in the initiation of a phototropin signal (Takemiya et al., 2005). On even longer timescales, for
instance to adapt to seasonal changes, light conditions are monitored over long periods and expression of relevant genes is adjusted accordingly.

The consumption of end-products of the Calvin-Benson cycle acts as a feedback control mechanism of photosynthesis. Several metabolites were identified in the last decades that are involved in this feedback regulation (Paul and Pellny, 2003). Inorganic phosphate (P_i) is introduced into phosphorylated intermediates during the Calvin-Benson cycle and thereby sequestered if Calvin-Benson cycle activity exceeds the carbon demand. P_i is therefore an important metabolite to control not only the synthesis of end-products like starch and sucrose (see section 1.4), but also directly influences photosynthetic reactions. P_i is needed in photophosphorylation and for the activity of ATPase. Moreover, low P_i levels increase the pH gradient of the thylakoid membrane thereby triggering heat dissipation from PSII (Paul and Foyer, 2001).

Limitation of photosynthesis by high sugar levels was shown for many species. This sugar-induced feedback inhibition is proposed to override light and developmental signals in order to adjust photosynthesis to the carbohydrate demand of the plant. Inhibition of photosynthesis occurs by the repression of genes belonging to the photosynthetic pathway, but also by decreased expression of RuBisCO and other Calvin-Benson cycle enzymes (Smeekens, 2000). In transient expression assays using maize protoplasts, hexokinase substrates, like glucose, were demonstrated to affect the promoter activity of genes involved in photosynthesis (Jang and Sheen, 1994). Moreover, glucose treatment (6%) of Arabidopsis seedlings represses expression of photosynthetic genes and inhibits hypocotyl growth and chloroplast synthesis. Glucose signaling is transmitted by hexokinase, the metabolic role of which is the phosphorylation of Glc to Glc6P. The involvement of hexokinase in repression of photosynthesis was shown by overexpression and antisense repression of two of the six hexokinase genes (HXX1, HXX2) found in Arabidopsis. Reduced expression resulted in Glc insensitivity after treatment with 6% Glc while overexpression of the HXXs enhanced Glc sensitivity (Jang et al., 1997).

For plants, it is not only essential to regulate photosynthesis according to the carbohydrate demand, but also with respect to supply of other elements, like nitrogen. Nitrogen is crucial for the synthesis of amino acids and proteins. Therefore, carbohydrate production has to be balanced according to nitrogen supply to achieve coordinated biosynthetic processes. Low
nitrogen availability enables only low rates of amino acid and protein synthesis and hence should have similar effects on photosynthesis as high carbon availability. Indeed, nitrogen deficiency leads to inhibition of growth and photosynthesis and to lower levels of RuBisCO, which is the major nitrogen sink in leaves because of its high abundance relative to other proteins (Paul and Stitt, 1993; Paul and Driscoll, 1997). Carbon skeletons for amino acid synthesis are derived from intermediates of glycolysis such as 3-PGA, phosphoenolpyruvate and pyruvate. Low nitrogen availability causes the down-regulation of central enzymes of the glycolytic pathway, resulting in the accumulation of 3-PGA and hexose phosphates. Similar intermediates accumulate in situations with sugar excess, e.g. if sink tissues are saturated and transport decreases. Thus an analogous metabolic signaling, e.g. via hexokinase, can be assumed in response to high carbon supply and low nitrogen supply (Paul and Pellny, 2003).

1.3 Carbon partitioning

Carbon assimilation is only possible during the light period. In the dark, plants become net consumers of carbon. Therefore, plants need to store carbon during the day in order to endure the dark period. For optimal plant growth, it is essential to balance carbon allocation into storage, growth and maintenance and adapt the allocation to changes in the environmental conditions.

1.3.1 Carbon partitioning in autotrophic tissue

In leaves, carbon assimilated in the Calvin-Benson cycle is either retained in the chloroplast and used to build up the transitory storage compound starch, or exported in the form of triose phosphates to the cytosol where it is mostly converted to sucrose (Figure 1). Sucrose can be imported into the vacuole for storage or it is transported to tissues with low carbon availability (sink tissues). Triose phosphates exported from the chloroplast are furthermore utilized for the synthesis of several other compounds such as amino acids, organic acids and cell walls.
Figure 1: Scheme of photosynthetic carbon metabolism in C₃ plants.
In photosynthetic tissue, RuBisCO assimilates CO₂ during the light period via the Calvin-Benson cycle. This leads to triose phosphates, which are either used for the regeneration of the carbon acceptor or for the synthesis of end-products like starch, sucrose, amino acids, organic acids, proteins or lipids. Sucrose is the major transport form of carbon. It can be imported into the phloem and subsequently distributed to the whole plant. RuBisCO can also oxygenate its substrate to generate 2-phosphoglycolate, which is recycled via photorespiration to form glycerate. RuBP, Ribulose-1,5-bisphosphate; 3-PGA, 3-phosphoglycerate; GAP, glyceraldehyde-3-phosphate; F₁,6BP, Fructose-1,6-bisphosphate; F₆P, Fructose-6-phosphate; TP, triose phosphates. After Stitt et al. (2010).

It is still not fully elucidated how carbon flow is balanced. Two models were proposed to explain the partitioning of carbon between starch and sucrose (Sun et al., 1999; Zhou and Quebedeaux, 2003). According to the “overflow model”, starch is produced when photosynthesis is high. With increasing photosynthetic rates, first sucrose synthesis saturates, before the starch synthesis pathway is activated maximally (Preiss, 1982; Stitt, 1996; Lunn and Hatch, 1997). A model by Eichelmann and Laisk (1994) suggests that under high CO₂ conditions, when photorespiration is inhibited, starch synthesis releases inorganic phosphate from triose phosphates, thus allowing for a continuous ATP synthesis. The “overflow model” is also supported by findings in C₄ plants, where an increase in light intensity results in a decreased ratio of ¹⁴C incorporation into starch relative to sucrose (Lunn and Hatch, 1997). Nevertheless, this model cannot explain starch synthesis under low photosynthetic rates (Lin et al., 1988a; Schulze et al., 1991). This is accounted for in the
second model, the so-called “programmed model”. The “programmed model” describes starch synthesis as a process programmed in order to meet the carbon demand of a plant (Silvius et al., 1979; Schulze et al., 1991; Huber and Hanson, 1992). Thus, more carbon is stored into starch in short days, followed by a longer night, compared to long days, followed by shorter nights. The carbon demand during the night seems to be a crucial factor for the amount of synthesized starch. It was suggested that both models might be active in leaves simultaneously (Sun et al., 1999), with the “overflow model” acting at high photosynthetic rates and the “programmed model” facilitating the adaptation to light-dark cycles.

The synthesis of starch
Starch is composed of the glucose polymers amylopectin and amylose, which form a semi-crystalline granule inside plastids. The major component of starch is amylopectin, which accounts for more than 70% of the starch granule. In amylopectin, glucose residues are linked via α-1,4-bonds to form chains between six and several hundred units long. The glucose chains are branched via α-1,6-bonds to form a discontinuous tree-like structure (Buléon et al., 1998). Within this structure, higher-order structures can form amongst clusters of unbranched. Two neighboring unbranched chains can form double helices which arrange in a regular pattern, establishing crystalline layers, while the regions containing the branch points form amorphous layers of the starch granule.

Amylose, the second component of starch, also consists of α-1,4-linked glucose residues, but is less branched compared to amylopectin. It is thought to be integrated into the amorphous regions of amylopectin (Kozlov et al., 2007), but it is not essential for the formation of semi-crystalline starch granules (Tsai, 1974).

In the first steps of starch synthesis, fructose-6-phosphate (Fru6P) a Calvin-Benson cycle intermediate is converted to ADP-glucose (ADPGlc; Figure 2). This conversion is catalyzed by chloroplastic phosphoglucoisomerase (PGI) and chloroplastic phosphoglucomutase (PGM), which convert F6P via glucose-6-phosphate (Glc6P) into glucose-1-phosphate (Glc1P). ADP-glucose pyrophosphorylase (AGPase) then uses G1P and adenosyl-triphosphate (ATP) to generate ADP-glucose and pyrophosphate. Abolishing any of the three chloroplastic enzyme
activities results in almost complete loss of starch (Caspar et al., 1985; Lin et al., 1988a; Yu et al., 2000; Kunz et al., 2010).

ADPGlc is the substrate for starch synthases, which transfer the glucose moiety to the non-reducing end of an already existing glucan chain, thereby elongating it. The Arabidopsis genome contains five genes encoding different isoforms of starch synthases; granule-bound starch synthase (GBSS) and starch synthases I-IV (SSI-SSIV). GBSS elongates amylose chains, while SSI, SSII and SSIII are involved in the formation of amyllopectin chains (Zeeman et al., 2010). Analyses of the chain length distribution in mutants of the different isoforms suggested that SSI-SSIII preferentially elongate chains with different lengths. While SSI was found to act predominantly on short chains (Delvallé et al., 2005; Fujita et al., 2006), SSII prefers to elongate intermediate chains (Craig et al., 1998; Morell et al., 2003) and SSIII...
utilizes long chains (Zhang et al., 2005). Arabidopsis mutants lacking any one of the three starch synthases are still able to synthesize starch, but starch content and starch structure is altered, pointing towards an overlapping function of the enzymes. The most severe disruption in starch synthesis can be seen for Arabidopsis plants lacking SSIV. Ssiv mutants have only one or two big starch granules per chloroplast and show a severely decreased growth rate. (Roldán et al., 2007) Furthermore, it was observed that plants lacking simultaneously SSIII and SSIV do not form starch granules although they have remaining starch synthase activity (Szydlowski et al., 2009). These observations led to the idea that either SSII or SSIV is necessary for granule initiation and to determine the correct number of granules per chloroplast. The mechanism for starch granule initiation, however, remains unclear to date.

Branching of the glucan chains is catalyzed by branching enzymes (BE) and progresses simultaneously with the elongation of chains. The BEs cut existing α-1,4-glucan chains and transfer the cut segment to the C6 position of a glucose unit of another chain (Takeda et al., 1993). Thereby, a α-1,6-branch point is formed. The transferred glucan chains comprise of six or more glucose units. In Arabidopsis, two BEs exist (BE2 and BE3) that can compensate for each other. Only loss of both isoforms results in plants unable to synthesize starch (Dumez et al., 2006). BEs create an excess of branch points on the glucan chain, which need to be partially removed again to give rise to a semi-crystalline granule. This is done by debranching enzymes which hydrolyze α-1,6-branch points. Two types of debranching enzymes were identified, namely isoamylase and limit dextrinase (LDA). In Arabidopsis, three isoamylases (ISA1-ISA3) and one LDA were identified. While LDA and ISA3 are reported to function predominantly in starch degradation, ISA1 and ISA2 seem to be involved in starch synthesis (Wattebled et al., 2005; Delatte et al., 2006). Mutation of either of the respective genes leads to reduced starch levels and to the accumulation of a soluble glucan referred to as phytoglycogen (Zeeman et al., 1998; Delatte et al., 2005).

The degradation of starch
During the dark, when no photosynthesis is possible, the accumulated starch is broken down at near linear rate to facilitate continuous carbon supply (Figure 3). As the glucan chains within the crystalline starch granule are densely packed, a disruption of the structure
is needed for hydrolytic breakdown to occur. This is accomplished by phosphorylation of the starch granule by two glucan dikinases; glucan, water dikinase (GWD, also referred to as starch-excess-1 (SEX1), Yu et al., 2001) and phosphoglu- can, water dikinase (PWD, Baunsgaard et al., 2005; Kötting et al., 2005). GWD phosphorylates C6 positions and PWD C3 positions of the glucose residues in amylopectin, thereby partially solubilizing the glucan chains and making them accessible to degrading enzymes. Mutants lacking either GWD or PWD activity have starch-excess phenotypes as they cannot break down their starch efficiently during the night. They also have reduced growth rates. For complete starch degradation, the phosphate groups added by GWD and PWD need to be removed again by the activity of phosphatases. In Arabidopsis, two glucan phosphatases (starch-excess 4 (SEX4), Like-SEX4-2 (LSF2), Kötting et al., 2009; Santelia et al., 2011) and one glucan phosphatase-like proteins (Like-SEX4-1 (LSF1), Comparot-Moss et al., 2010) were identified. SEX4 dephosphorylates glucans at the C6 and C3 position, while LSF2 specifically acts on C3-bound phosphate. Even though no glucan dephosphorylating activity could be detected so far for LSF1, it seems to be involved in starch degradation. Like loss of SEX4, loss of LSF1 also leads to a starch-excess phenotype, but the function of this protein still has to be fully revealed.

At the solubilized granule surface, several enzymes act together to degrade the glucan chains. The major activity is attributed to β-amylases; exoamylases that release maltose from the non-reducing end of a linear glucan chain. Nine genes encoding β-amylases or β-amylase-like proteins exist in Arabidopsis (BAM1-9), five of the encoded proteins (BAM1-4, BAM9) are located to the chloroplast (Fulton et al., 2008, Lee S.K and Zeeman S. C., unpublished data). The highest of the chloroplastic β-amylase activities was observed so far for BAM1 and BAM3, which act partially redundantly. Loss of BAM1 or of BAM3 leads to wild-type or a mild starch-excess phenotype, respectively, but loss of both to a severe starch-excess phenotype (Fulton et al., 2008). β-amylases are not able to hydrolyze branch points (α-1,6-linkages) or α-1,4-bonds close to them. Therefore, DBEs are needed in addition. As mentioned, the DBEs involved in starch breakdown are LDA and ISA3. Both release short branches, but their function seems to be only partially redundant. Loss of LDA activity does not lead to a starch phenotype, loss of ISA3 leads to accumulation of excess
starch, but the double loss of LDA and ISA3 leads to a stronger starch excess than the isa3 mutant alone. This illustrates the importance of LDA in starch breakdown, at least when ISA3 is missing (Delatte et al., 2006).

**Figure 3: Pathway of starch degradation and sucrose synthesis during the night.**

During the dark, carbon reserves stored in starch granules within the chloroplast are degraded and the degradation products are used for the synthesis of sugar intermediates and sucrose to fuel metabolism. The enzymes catalyzing starch degradation are: (1) glucan, water dikinase and phosphoglucon, water dikinase; (2) phosphoglucon phosphatase (SEX4 and LSF2); (3) β-amylase; (4) isoamylase; (5) α-amylase; (6) limit dextrinase; (7) plastidic starch phosphorylase; (8) plastidic disproportionating enzyme; (9) maltose transporter; (10) glucose transporter; (11) hexokinase; (12) cytosolic disproportionating enzyme; (13) cytosolic glucan phosphorylase. After Zeeman et al. (2007)

The minimal substrate for β-amylases are glucan chains with four glucose residues. Thus, the final products of β-amylolytic degradation are maltose and small amounts of maltotriose, which is too short to be further degraded. Maltotriose is metabolized by disproportionating enzyme, which transfers two of the glucose residues to another glucan chain, thereby providing new substrate for the β-amylases, and releasing glucose (Critchley et al., 2001).

Three genes coding for α-amylases (AMY1-3) are present in *Arabidopsis thaliana*, with AMY3 being the only one localized to the chloroplast. α-amylases are endoamylases that hydrolyze α-1,4-bonds and thereby release linear and branched glucan fragments. In contrast to β-amylases, α-amylases can act directly on the granule surface. However, loss of AMY3 does not result in an altered phenotype. Only when mutated in combination with other genes
encoding enzymes important for starch degradation (e.g. SEX4) an effect is seen. The double mutant sex4 amy3, for instance, displays significantly higher starch levels than the sex4 single mutant (Kötting et al., 2009). Still, more work needs to be done to define the exact role of this α-amylase in starch degradation.

Maltose and glucose, the final products of starch degradation, are both transported out of the chloroplast via specific transporters, the chloroplast-envelope glucose transporter (pGlcT, Weber et al., 2000) and the maltose transporter (MEX1, Niittylä et al., 2004; Weise et al., 2004). The pGlcT is integrated in the inner chloroplast membrane and it is assumed that Glc is passively transported through the inner membrane and diffuses through the outer membrane; which is permeable for molecules up to a size of several kilodaltons (Heldt and Sauer, 1971; Weber et al., 2000). A hexokinase isoform is found to be attached to the outer membrane which converts Glc to Glc6P thereby maintaining a high Glc concentration gradient between chloroplast and cytosol (Wiese et al., 1999). Maltose is more abundant as a degradation product (Weise et al., 2004). Consistent with this, the lack of pGlcT does not result in any apparent change in phenotype compared to the wild type, while a lack of MEX1 results in high starch and maltose levels at the end of the night, pale green leaves and reduced growth (Niittylä et al., 2004; Cho et al., 2011).

*The synthesis of sucrose*

Sucrose is synthesized in the cytosol from triose phosphates (TP) resulting from photosynthetic carbon assimilation in the Calvin-Benson cycle (Figure 2; reviewed in MacRea and Lunn, 2006). The TP are transported out of the chloroplast via the triose-phosphate/phosphate translocator (TPT) in strict counter exchange for Pi, which is released from TPs during sucrose synthesis (Fliege et al., 1978). In the cytosol, TPs are condensed to form fructose-1,6-bisphosphate (Fru1,6BP) by aldolase. Subsequently, Fru1,6BP is dephosphorylated by cytosolic fructose-1,6-bisphosphatase (cytFBPase) to Fru6P, in the first irreversible reaction of the pathway. Fru6P is converted via Glc6P to Glc1P by cytosolic phosphoglucoisomerase (cytPGI) and phosphoglucomutase (cytPGM). Further on, UDPGlc pyrophosphorylase (UDPase) catalyzes the synthesis of UDPGlc and PP, from Glc1P and UTP.
Even though the majority of carbon compounds are used to synthesize sucrose, they are not committed to the pathway of sucrose synthesis at this point. Intermediates of this pathway additionally fuel glycolysis, the oxidative pentose phosphate pathway and cell wall synthesis. The last two steps are dedicated to sucrose synthesis. First, UDPGlc and Fru6P are used to form sucrose-6-phosphate (Suc6P) and UDP in a reaction catalyzed by sucrose-phosphate synthase (SPS). Second, Suc6P is hydrolyzed to sucrose and inorganic phosphate (Pi) by sucrose-phosphate phosphatase (SPP). This reaction is irreversible, thereby pulling the previous reactions in the direction of sucrose production.

In the dark, maltose and glucose, the products from starch degradation, serve as precursors for sucrose synthesis rather than Calvin-Benson cycle intermediates. Maltose is metabolized in the cytosol via cytosolic disproportionating enzyme (DPE2, Chia et al., 2004; Lu and Sharkey, 2004), which transfers one of the glycosyl units to an as-yet poorly defined heteroglycan, releasing the other as glucose. A transitory heteroglycan is formed thereby within the cytosol, from which hexose phosphates can be released by cytosolic phosphorylase (PHS2, Lu et al., 2006). The produced glucose and hexose phosphates are then channeled towards the synthesis of sucrose via the same reactions as in the light.

1.3.2 Carbon transport

Sucrose accumulates within the mesophyll cells, the site of photosynthesis. From there it moves symplastically, via plasmodesmata, to the bundle sheath cells (BS) and to phloem parenchyma cells (PP) of minor veins. A high density of plasmodesmata has been demonstrated in the relevant cell types, but still it is not fully resolved if the degree of connectivity is sufficient to explain the observed photoassimilate movement (Lalonde et al., 2003). From the BS/PP, sucrose is proposed to be actively imported into the companion cells (CC) of the phloem, creating a high sugar gradient between sites of high photosynthetic activity (sources tissues) and sites of low photosynthetic activity (sink tissues) where sucrose is unloaded and metabolized. The high sugar concentration in the source causes a decrease of the internal solute potential, followed by the entry of water into the source phloem. In the sink tissues, sucrose is depleted from the phloem. This increases the solute potential in the phloem and consequentially leads to an outflow of water. Thus a hydrostatic pressure
gradient between source and sink tissue is established, which marks the major driving force of sucrose movement (Slewinski and Braun, 2010).

Three mechanisms have been described for phloem loading of sugars; apoplastic loading, symplastic loading and polymer trapping. The most common mechanism is apoplastic loading, which is that found in Arabidopsis. In apoplastic loading species, the phloem cells are symplastically isolated from each other. Therefore, no movement via plasmodesmata is possible. Sucrose has to be transported out of the PP into the apoplast and from there imported again into the phloem sieve element cells. Recently, the transporter facilitating sucrose export into the apoplast was identified (SWEET, Chen et al., 2012). The SWEET transporters are most probably expressed in the PP where they secrete sucrose by a uniport mechanism. Subsequently, sucrose is imported into the CC or sieve elements by the sucrose-H\(^+\) cotransporter (SUC2, Stadler and Sauer, 1996; Sauer, 2007). The required proton gradient is maintained by the plasma membrane H\(^+\)/ATPases (Chen et al., 2012). In symplastic loading, sucrose moves passively along its concentration gradient from the mesophyll cells to the sieve elements without any concentrating step in between. The driving force is solely the carbon demand in sink tissues. Symplastic loading was identified as the dominant mechanism in many woody species (Rennie and Turgeon, 2009). The third loading mechanism is polymer trapping, a symplastic loading strategy in which sucrose is converted to raffinose or stachyose. This conversion takes place in specialized companion cells, so-called intermediary cells. Sugars are concentrated within the intermediary cells by the conversion as they are too big to diffuse back into the PP (Slewinski and Braun, 2010). In most sink organs, sucrose is exported symplastically out of the phloem sieve elements via plasmodesmata. For export into some sinks tissues, like pollen or developing seeds, where symplastic continuity is missing, an apoplastic step is involved.

1.3.3 Carbon partitioning in sink leaves

Developing leaves do not rely exclusively on photosynthetic assimilation of carbon, as they are also supplied with carbon compounds from source tissues in the form of sucrose (Figure 4). Two enzyme classes are known to degrade sucrose. Invertases (INV) hydrolyze sucrose
irreversibly to Glc and Fru, while sucrose synthases (SUS) catalyze the reversible reaction from sucrose and UDP to UDP-Glc and Fru (Sturm and Tang, 1999; Koch, 2004).

**Figure 4: Pathways of carbon metabolism in sink leaves.**
Sink leaves have two sources for carbon, they assimilate carbon by photosynthesis and they import carbon from source tissues. Sucrose is transported within plants via the phloem, from where it enters the sink through plasmodesmata or the cell wall, followed by cleavage into fructose, glucose and UDP-glucose. The enzymes cleaving sucrose are: (1) cell wall invertase; (2) cytoplasmic invertase; (3) sucrose synthase and (4) vacuolar invertase.

Several different isoforms of invertase are found in Arabidopsis. According to their biochemical properties they are classified as acidic INV, comprising cell-wall and vacuolar INV, and as neutral/alkaline INV, comprising cytosolic, plastidic and mitochondrial INV. Cell-wall and vacuolar invertases were found to be expressed organ-specifically and at certain developmental stages (Tymowska-Lalanne and Kreis, 1998). In Arabidopsis, 9 isoforms of cytosolic INV exist; simultaneous loss of two of them (CINV1 and CINV2) causes a reduction in shoot growth and an even more pronounced decrease in root biomass (Barratt et al., 2009) pointing towards their importance for plant growth.

All isoforms of SUS are reported to be localized to the cytosol. Different functions in development of non-photosynthesizing organs, determining sink strength and cellulose
synthesis are attributed to SUS, but the simultaneous loss of four (SUS1, SUS2, SUS3 and SUS4) of the six existing isoforms does not lead to any visible change in phenotype compared to wild-type plants, despite the fact that the mutants have no detectable soluble or membrane-bound SUS activity. The importance of SUS in Arabidopsis is thus still debatable.

1.4 Regulation of carbon partitioning

The carbon supply of plants needs to be fine-tuned between maintenance, growth and storage processes. The importance of carbon investment, for instance into storage, can be seen impressively in mutants lacking transitory starch. These mutants grow like wild type in continuous light, but show decreased growth rates when the length of the dark period is increased (Caspar et al., 1985; Huber and Hanson, 1992).

To balance carbon investment, plants need to sense the amount of carbon assimilated or stored to generate a signal (sugar signal or hormonal signal) in order to regulate carbon flux into different tissues and pathways. Sensing and regulation of carbon supply takes place on multiple levels of central carbon metabolism. Some of these will be discussed in more detail in this section.

1.4.1 Regulation of starch and sucrose synthesis

The first committed step of starch synthesis, the conversion of Glc1P and ATP to ADPGlc and PP\_i by AGPase is the key regulatory step used to control carbon flux into the pathway. AGPase is allosterically regulated via 3-phosphoglycerate (3PGA) which is an activator and P\_i which is an inhibitor. Flux into starch synthesis is increased when 3PGA accumulates and the level of P\_i decreases (Ghosh and Preiss, 1965; Sanwal et al., 1968). The ratio between these two metabolites in leaves reflects the supply of the cell with carbon from photosynthesis and the demand for carbon. When the supply of the cell with photoassimilates exceeds the demand, the 3PGA/P\_i ratio in the chloroplast stroma is high, stimulating the activity of AGPase. Consequently, more carbon is stored in form of starch. In conditions when the demand for carbon is higher, the 3PGA/P\_i ratio is lower and less carbon is channeled into starch synthesis. In this case, more carbon is used for sucrose synthesis and for export
processes. Moreover, AGPase was found to be redox-regulated by the ferredoxin-thioredoxin system. It forms a heterotetrameric complex, comprising of two small and two large subunits. Under oxidizing conditions a disulfide bridge forms between the two small subunits, leading to an enzyme with low activity. Reduction leads to cleavage of the disulfide bond resulting in a more active enzyme (Fu et al., 1998). The redox activation of AGPase is triggered by light or sugars, such as sucrose. Trehalose-6-phosphate (T6P) is proposed to sense the sucrose status of the plant, trigger redox-activation of AGPase and therefore enhance starch synthesis. It was observed, that increasing sugar levels (for instance after the transition from dark to light) are accompanied by increases of T6P, likewise did the T6P levels rise after feeding of sucrose to starved seedlings (Lunn et al., 2006). Increased T6P levels, achieved by overexpression of trehalose phosphate synthase, on the other hand, were shown to promote AGPase activity, as did feeding of isolated chloroplasts with T6P. Other sugars, as trehalose or sucrose, did not show activating function (Kolbe et al., 2005). However, the mechanism how T6P stimulates redox-activation of AGPase is still not clear.

Likewise, the synthesis of sucrose is regulated in many ways. Gene expression of different enzymes in the pathway is regulated transcriptionally and post-transcriptionally. CytFBPase, which catalyzes the dephosphorylation of Fru1,6BP to F6P, is one of the highly regulated enzymes. It is inhibited by fructose-2,6-bisphosphate (Fru2,6BP), whose synthesis and degradation itself is highly controlled. Fru2,6BP is synthesized and degraded by a bifunctional enzyme containing kinase and phosphatase activity (fructose-6-phosphate,2-kinase and fructose-2,6-bisphosphatase, Larondelle et al., 1986). Both activities are controlled via Pi, several triose phosphates, as 3PGA and phosphoenolpyruvate (PEP), and F6P. A high 3PGA/Pi ratio, for instance, inhibits the synthesis and activates the degradation of Fru2,6BP, thereby stimulating cytFBPase activity. High F6P levels, on the other hand, activate synthesis of Fru2,6BP and inhibit its degradation, resulting in the inhibition of cytFBPase (Stitt, 1990; MacRea and Lunn, 2006). From the two enzymes committed to sucrose synthesis, SPS seems to be the major regulatory target. It is reported to be one of the most highly regulated proteins in plant metabolism. In photosynthetic tissue, SPS is allosterically activated by Glc6P and inhibited by Pi (Doehlert and Huber, 1983). Additionally, it is post-translationally phosphorylated. In the dark, phosphorylation of SPS takes place,
which deactivates the enzyme, while dephosphorylation in the light activates the enzyme (Huber et al., 1989).

### 1.4.2 Regulation of sucrose transport

The apoplastic phloem loading mechanism has the advantage over other loading strategies that by regulation of transporter activity a short- and long-term adjustment of sucrose transport can be achieved. Evidence for both transcriptional and post-transcriptional regulation exists for the sucrose transporters. The Arabidopsis sucrose transporter, SUC2, is expressed in the CC of mature leaves and leaves undergoing the sink-source transition. SUC2 was not found to be expressed in developing leaves. This was demonstrated in transgenic tobacco lines in which GFP is expressed under the control of the AtSUC2 promoter. If GFP in these lines is directed to the endoplasmatic reticulum, which prevents movement into the sieve elements, a signal is retained exclusively from CC of source leaves (Wright et al., 2003). A 126-basepair long fragment, present in the SUC2 promoter, was identified as specific for gene expression in mature companion cells, but the exact nature of the element and associated transcription factors have not been identified so far (Schneidereit et al., 2008).

For the potato sucrose transporter, SUT1, post-translational modification by redox-regulation was demonstrated. The redox-state of the transporter influences its multimerization, localization and activity. Oxidation of the transporter leads to the formation of a dimer, which shows increased sucrose transport activity compared to the reduced monomer. Moreover, the oxidation causes a shift in the localization of the protein. While the reduced form was found in the plasma membrane and in intercellular membranes, oxidation of SUT1 targeted the transporter exclusively to the plasma membrane when expressed in yeast cells (Krügel et al., 2008).

### 1.4.3 Linkage of carbon supply and growth

While many studies were performed to reveal regulatory steps within the pathways of central carbon metabolism in the last 20 years, only little is known about the mechanisms coordinating growth processes and metabolism. To maintain and optimize growth, plants must detect the level of available carbon and channel it into the synthesis of structural
components like proteins and cell walls. Lately, two studies found interesting connections between the plant’s carbon status and growth.

Under carbon limiting conditions, e.g. in short days and low light or elongated dark periods, EXORDIUM-LIKE1 (EXL1) is proposed to be part of a pathway controlling growth and development. After treatment with brassinosteroids, exl1-mutants show a significantly higher increase in leaf elongation when grown under low carbon conditions compared to wild-type plants. This suggests that EXL1 restricts brassinosteroid-promoted growth when carbon availability is low. The exact mode of action of EXL1 is thereby still not known (Schröder et al., 2011).

Apart from brassinosteroids, also gibberellins (GA) are known to promote and control growth. Arabidopsis plants depleted of carbon resources during the night, e.g. starch synthesis mutants, show a decreased expression of enzymes involved in GA synthesis during the following light period. This is accompanied by lower levels of GA and decreased growth compared to wild-type plants. However, when treated with GA, carbon partitioning of starch mutants changes, restoring growth to a certain extent. Via this GA signaling pathway, the carbon status during the dark period seems to be reflected in order to trigger growth during the following light-dark cycle (Paparelli et al., unpublished manuscript).

However, still more studies are needed to understand the interaction between carbon metabolism and growth and how these two processes are regulated to facilitate optimal plant growth.

1.5 Aim of the study

Leaf growth was studied intensively on the basis of biomass accumulation and several methods were developed to measure precisely the elongation growth in leaves (Walter et al., 2002a; Walter et al., 2009). Furthermore, leaf development has been studied for several decades already and only recently, a detailed analysis of changes in transcript and protein levels with the development of the leaf was performed (Baerenfaller et al., 2012). Yet, the pathways of carbon metabolism in actively proliferating and actively expanding leaves and their dynamic regulation is not well understood so far. Therefore, the main objective of this
thesis is, to analyze carbon metabolism in growing leaves of *Arabidopsis thaliana* and reveal how it is interlinked with growth.

During my work I focused mainly on two methods to investigate carbon assimilation and carbon allocation in Arabidopsis plants. I studied carbon assimilation by measuring gas exchange of single leaves or whole plants. Several commercial and non-commercial systems have been developed previously, but none allows continuous measurements over a longer time period and of several plants in parallel. Therefore, we designed a gas exchange system to measure carbon and water exchange of whole Arabidopsis rosettes. My work included setting up and optimizing the gas exchange system to give robust and consistent measurements. Moreover, I challenged the system with a variety of different tests to reveal advantages and new possible applications of whole-plant gas exchange measurements.

The second part of my work focused on the study of carbon allocation. I improved existing methods and developed new approaches for isotopic labeling strategies allowing me to study carbon partitioning in whole plants and single Arabidopsis leaves. I used the labeling system to analyze carbon export and source-sink interactions in whole Arabidopsis plants. Labeling approaches have been used previously already to study carbon allocation in several different species, such as sugar beet, squash and tobacco, but comprehensive studies on Arabidopsis, which opens the possibility to combine isotopic labeling with numerous available molecular tools, have not been performed.

Finally, I used the established labeling system to study carbon flux in growing and non-growing tissues to reveal interrelations between carbon metabolism and growth. For this I analyzed and compared carbon partitioning both in rapidly and slowly growing tissues, in phases of high and low plant growth. Additionally, I demonstrated the effect of disturbances in different steps of starch metabolism on carbon partitioning and growth. Some studies have analyzed already carbon partitioning in Arabidopsis, mainly focusing on mutants altered in central carbon metabolism (Zeeman and ap Rees, 1999; Nielsen, 2002; Schneider et al., 2002; Zeeman et al., 2002). However, the effects of these alterations on the temporal and special distribution of carbon were not investigated so far.
2 Materials and Methods

2.1 Plant material

Mutant lines used in this study are listed in the table below (Table 1). All lines are in the Columbia (Col-0) background. Col-0 was used as a control for all experiments.

<table>
<thead>
<tr>
<th>Mutant alleles</th>
<th>Line identifier</th>
<th>Reference</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADG2</td>
<td>Atadg2-1</td>
<td>CS3095</td>
<td>Lin et al. (1988a)</td>
</tr>
<tr>
<td>ISA1/ISA2</td>
<td>Atisa1-1/Atisa2-1</td>
<td>SALK_042704/-</td>
<td>Streb et al. (2008) [51]</td>
</tr>
<tr>
<td>PGI</td>
<td>Atpgi1-1</td>
<td></td>
<td>Yu et al. (2000)</td>
</tr>
<tr>
<td>PGM</td>
<td>Atpgm1-1</td>
<td>TC75</td>
<td>Caspar et al. (1985)</td>
</tr>
<tr>
<td>PWD</td>
<td>Atpwd</td>
<td>SALK_110814</td>
<td>Kötting et al. (2005)</td>
</tr>
<tr>
<td>SEX1</td>
<td>Atsex1-3</td>
<td>TC265</td>
<td>Yu et al. (2001)</td>
</tr>
<tr>
<td>SEX4</td>
<td>Atsex4-3</td>
<td>SALK_102567</td>
<td>Niittylä et al. (2006)</td>
</tr>
<tr>
<td>SSIV</td>
<td>AtssIV</td>
<td>SALK_096130</td>
<td>Roldan et al. (2007)</td>
</tr>
</tbody>
</table>

2.2 Growth conditions

2.2.1 Growth conditions on soil

*Arabidopsis thaliana* seeds were sown by hand in square 200 ml pots (PF 306, gyz-rossat, Otelfingen, Switzerland) on Einheitserde Typ ED 73 (Einheitserde- und Humuswerke Gebr. Patzer GmbH & Co. KG, Sinntal, Germany). The seeds were stratified for three days at 4°C before they were transferred to a Percival AR95 growth cabinet (CLF Plant Climatics, Emersacker, Germany) with a 12-hour photoperiod set to a light intensity of 150 μmol quanta m⁻² s⁻¹, a constant temperature of 20°C and a relative humidity of 65%. After seedling establishment (9-10 days after sowing), individual plants were pricked out and planted in a new pot.
2.2.2 Growth conditions in hydroponics

One-ml tubes filled with 0.65% Plant agar (Duchefa, Netherlands) were used to support plants grown in a hydroponic solution. The bottom of the tubes was cut off and the tubes were placed in a rack with the cut ends immersed in Cramer solution (Gibeaut et al., 1997) containing 0.015M Ca(NO₃)₂, 0.0125M KNO₃, 1mM (NH₄)₂SO₄, 0.75mM Mg(SO₄), 0.5mM KH₂PO₄, 0.1mM Na₂O₃Si, 0.072mM C₁₀H₁₂FeN₂NaO₈, 50µM KCl, 50µM H₃BO₃, 10µM MnSO₄, 2µM ZnSO₄, 1.5µM CuSO₄, 0.075µM (NH₄)₆Mo₇O₂₄. To avoid exposing the solution to light, the bottom of the rack was covered with aluminium foil. One to three seeds were sown per tube and the 0.5l-box containing the rack and the nutrient solution was covered with a lid. After stratification for 3 days at 4°C the seeds were transferred to a Percival growth cabinet with the same conditions specified above (see. 2.2.1). After one week, the lid was removed and the number of seedlings per tube was reduced to one. The nutrient solution was exchanged on a weekly basis. To grow plants with a higher biomass the nutrient solution was reduced to ½ Cramer solution after two weeks and the tubes containing the plants were transferred to plastic boxes with a volume of 40 l.

2.3 Enzymes and Chemicals

Unless otherwise stated, all chemicals were ordered from Sigma (Buchs, Switzerland), including enzymes like invertase and protease. Hexokinase, α-amylase, amyloglucosidase, glucose-6-phosphate dehydrogenase and phosphoglucomutase were ordered from Roche (Rotkreuz, Switzerland).

2.4 Arabidopsis gas exchange chambers

The chambers to measure gas exchange of Arabidopsis rosettes were designed and built in-house (Figure 5). Each of the eight chambers comprises two stabilizing units made of aluminium, corrosion resistant steel and unplasticized PVC, which can be assembled by two long screws. A Plexiglas lid can be screwed on top to create a gas-tight chamber (volume = 25 ml, 8 cm diameter). A rubber lip (nitrile butadiene rubber) seals the gap between the two stabilizing units and foam gasket (ethylene-propylene-diene rubber) seals the connection.
between the stabilizing parts and the Plexiglass lid. Each chamber is connected to an infrared gas analyzer (IRGA) via an inlet and an outlet. Two sensors are mounted into one of the chambers to measure air temperature and light intensity (GaAsp Sensor, Licor, Bad Homburg, Germany).

Figure 5: Arabidopsis gas exchange chamber. (A) Schematic overview of the chamber containing two stabilizing units (1), and a rubber lip (2) and foam gasket (3) for an air-tight assembly of the two units. The two units are connected via screws (4) and closed by a lid (5). (B) Picture of the gas exchange chamber filled with an Arabidopsis plant.

2.5 The gas exchange measuring unit (GDU-26)

The custom-designed system, GDU-26 (DMP Ltd, Switzerland), was built to measure gas exchange. It comprises a control unit, an infrared gas analyzer (LI-7000, Licor, Bad Homburg, Germany) and a Linux computer for data acquisition.

The control unit (Phoenix Mecano AG, Stein am Rhein, Switzerland) includes all the electrical devices; an interface USB-6008 (National Instruments, Ennetbaden, Switzerland), a converter TCC-100 (Moxa, Unterschleissheim, Germany) and eight mass-flow controllers (red-y smart Serie, Vögtlin Instruments, Aesch, Switzerland). Additionally two sensors, to measure light intensity and temperature within the growth cabinet or inside the gas exchange chambers, are connected to the control unit. PTFE tubes (Maagtechnic, Switzerland) are used to connect the control unit, the chambers and the infrared gas analyzer (IRGA). The tubing is attached via one-touch fittings (SMC Pneumatik AG, Switzerland).

All data from the IRGA as well as from the additional sensors is transferred to a Linux computer. A LabView application (National Instruments, Ennetbaden, Switzerland) was programmed to collect the data and to operate the control unit.
A more detailed explanation of the operation of the gas exchange unit is given in chapter 3.1.

2.6 Setup of gas exchange measurements

The gas exchange measurements were performed, unless otherwise stated, with a flow of 200 µmol s\(^{-1}\) within a Percival growth cabinet set to an air temperature of 20°C and a light intensity of 150 µmol quanta m\(^{-2}\) s\(^{-1}\). The incoming air was adjusted to a CO\(_2\) concentration of 360-420 ppm and a relative humidity of 65%. The outgoing air of each chamber was measured for 360 sec before the system switched to the next chamber. A dead time of 90 sec was introduced after each switch to allow stabilization of the measurements. After the dead time, measurements were made every 2.5 sec; every 30 sec the average value was stored by the LabView application.

2.7 Determination of leaf area

The projected leaf area was used for determination of the photosynthetic rate and the transpiration rate. For this purpose a digital picture of the plants (including a scale) was taken once per day. The pictures were converted with Photoshop (Adobe Photoshop CS4 Extended, Adobe Systems, USA) to a black-and-white image, with the rosette being black and the background being white. ImageJ (ImageJ 1.42q, National Institutes of Health, USA) was used to calculate the leaf area, based on the scale, from the black-and-white picture.

2.8 Gas exchange data analysis

Prior to data acquisition, parameters like relative humidity, light intensity and CO\(_2\) content of the incoming air were checked. Data which were not in the specified range were not used for further calculations. The values for ΔCO\(_2\), ΔH\(_2\)O, flow rate and the projected leaf area were used to calculate photosynthetic parameters according to van Caemmerer and Farquhar (1981).

The following formulas were applied to calculate the transpiration rate E and the photosynthetic rate A:
Materials and Methods

\[ E \text{ in mmol} \frac{H_2O}{m^2 \cdot s} = \text{Flow} \cdot \frac{(H_2OB - H_2OA)}{\text{Leaf area}} \cdot \frac{1}{1 - \left(\frac{(H_2OB)}{1000}\right)} \]

\[ A \text{ in } \mu\text{mol CO}_2/(m^2 \cdot s) = \text{Flow} \cdot \frac{(CO_2A - CO_2B)}{\text{Leaf area}} - \left(\frac{CO_2B \cdot E}{1000}\right) \]

- **Flow** = flow rate of air entering the chamber in µmol s\(^{-1}\)
- **CO\(_2A\)** = CO\(_2\) content of the incoming air in µmol CO\(_2\) mol air\(^{-1}\)
- **H\(_2OA\)** = H\(_2O\) content of the incoming air in mmol H\(_2O\) mol air\(^{-1}\)
- **CO\(_2B\)** = CO\(_2\) content of the outgoing in µmol CO\(_2\) mol air\(^{-1}\)
- **H\(_2OB\)** = H\(_2O\) content of the outgoing air in mmol H\(_2O\) mol air\(^{-1}\)
- **Leaf area** = projected leaf area in m\(^2\)

The relative humidity (RH) was calculated on the basis of the dewpoint temperature \(T_d\) (calculated by the Licor7000) and the air temperature \(T\) (measured by a sensor mounted within the chamber) using the August-Roche-Magnus approximation according to the following formula:

\[ RH \text{ in } \% = 100 \cdot \frac{\exp\left(\frac{17.271 \cdot T_d}{237.7 + T_d}\right)}{\exp\left(\frac{17.271 \cdot T}{237.7 + T}\right)} \]

2.9 Measuring photosynthetic parameters with the Licor6400

To complement whole plant measurements performed with our system, photosynthesis measurements on single leaves were performed with a Licor6400 connected to a 6400-40 leaf chamber fluorometer (Licor Environmental GmbH, Bad Homburg, Germany). The area enclosed in this chamber is 2 cm\(^2\). Calibration of the device, and all preliminary tests have been performed as described in the user manual prior to every measurement series. A new CO\(_2\) cylinder (Licor Environmental GmbH, Bad Homburg, Germany) was installed on a daily basis and the soda lime as well as the desiccant was exchanged if necessary. All
measurements have been performed with a stomatal ratio of 0.5 and an air flow of 100 μmol s⁻¹.

For photosynthesis measurements under standard growth conditions CO₂ was set to 400 ppm and light intensity to 150 μmol quanta m⁻² s⁻¹. After stabilization of the CO₂ parameters the data were recorded three times every 10 sec. 3 leaves per plant were measured and 5 plants per genotype.

2.10 Isotopic labeling chambers

Three different labeling chambers were designed and constructed in house. All chambers are made of Plexiglas with foam gaskets to seal between the different components.

For isotopic labeling of whole rosettes a square chamber with a side length of 24 cm and a height of 30 cm was used (Figure 6). The chamber comprises a lower part made of black Plexiglas and a lid made of transparent Plexiglas. In the lower part the plants are placed on a laboratory table, adjustable in height. In the lid, a holder and a fan are mounted. On the holder, a petri dish containing the isotopically labeled reagent (sodium bicarbonate) can be placed. The fan allows an even distribution of the isotopically labeled CO₂. The chamber is closed via four clamps and is sealed with foam gasket in between the upper and lower parts. The chamber has two inlets and two outlets which can be used to connect it to other devices.

Figure 6: Chamber for isotopic labeling of whole rosettes. (A) Picture of the isotopic labeling chamber for whole rosettes. (B) Schematic overview of the labeling chamber. The chamber comprises of an upper (1) and lower (2) Plexiglas part, sealed by foam gasket (3). An adjustable lab table (4) is used to position the plants (5) in relation to the external light source (6). The isotopically labeled sodium-bicarbonate is placed in a petri dish (7) and CO₂ is released via adding an access of lactic acid (8). A fan (9) is mounted within the chamber to allow an even distribution of labeled CO₂.
Two leaf chambers (small - 7 cm x 3 cm and miniature - 0.8 cm diameter) were constructed to label individual leaves of an Arabidopsis plant. With the small chamber (Figure 7, A and B) mature leaves with a minimum length of 2 cm can be labeled, while with the miniature chamber (Figure 7, C and D) developing leaves with a minimum length of 0.5 cm can be labeled.

![Figure 7: Chambers for isotopic labeling of individual leaves. (A/B) Small leaf chamber for isotopic labeling of mature leaves. (C/D) Mini leaf chamber for isotopic labeling of small, developing leaves.](image)

Both leaf chambers consist of a lower and an upper part made of transparent Plexiglas which can be clamped over single leaves. The Plexiglas parts are surrounded by foam gasket to enable an air-tight sealing, yet avoid damage of the leaf. In the lower part of the chambers is an inlet and an outlet for connecting it to the whole plant chamber containing the isotopically labeled gas. A battery driven pump within the whole plant chamber (Nitto Kohki, Germany) provides an even air-flow through the single leaf chambers.

### 2.11 Isotopic labeling

For isotopic labeling experiments the described labeling chambers were placed under fluorescent bulbs in a fume hood. The height of the bulbs was adjusted to achieve a light intensity similar to the standard growth conditions (150 µmol quanta m⁻² s⁻¹, unless stated otherwise). For labeling whole rosettes, the plants were grown under standard conditions on soil or in the hydroponic system and placed in the whole plant chamber at the indicated time points. Isotopically labeled carbon in form of sodium $^{14}$C-bicarbonate (Hartmann Analytic GmbH, Braunschweig, Germany) was placed in a petri dish on the holder, the chamber was closed and the fan was started to allow an even air-distribution within the chamber. $^{14}$CO₂ with a specific activity of 60-120 µCi mmol⁻¹ was released from sodium
bicarbonate by acidification with lactic acid. After the pulse period the chamber was opened and the plants were placed in normal air for the following chase period.

Labeling experiments on individual leaves were performed in either the small or the miniature leaf chamber depending on the size of the leaf. Isotopically labeled carbon was first released in the whole plant chamber as described above. The air was circulated between the chambers via tubes attached to the inlet and outlet of the whole plant chamber and the leaf chambers. A pump attached to the outlet of the whole plant chamber supported the air-flow towards the leaf chambers. The air-flow was controlled with a valve mounted in between the two chambers. Before starting the labeling, the air was circulating for 10 min within the system. Then, a single leaf was mounted in the leaf chamber and the valve was opened to allow gas flow to the leaf chamber for a certain pulse. After the pulse the valve was closed, and the leaf was quickly removed from the chamber and kept under normal atmosphere for the following chase period.

2.12 Autoradiography
To visualize transport of carbon within the Arabidopsis rosette, autoradiograms were made after $^{14}\text{C}$ labeling of single leaves. Following the chase period, either the rosette was removed from the root (soil-grown plants) or the whole plant was taken (hydroponically-grown plants) and placed between four layers of Whatman No.3 paper. The plant material was immediately dried in a gel drier (Bio Rad GelDrier, Reinach, Switzerland) for 20 min at 80°C. After drying, the Whatman paper was exchanged and the plants were flattened under a weight for 1 week. Afterwards, the plant material was wrapped in transparent cling film and exposed to a Kodak BioMax MR film for between 2 and 13 days.

2.13 Ethanol extraction of $^{14}\text{C}$ labeled plant material
After isotopic labeling, leaf material was harvested, weighed and transferred to 80% (v/v) ethanol at 80°C to stop metabolic processes. Root material was first dried with tissue paper, weighed and transferred to 80% (v/v) ethanol at 80°C. After 10 min extraction, the material was cooled down and stored at -20°C before further processing. The volume of 80% (v/v)
ethanol for harvesting as well as the volume for the following extraction was adjusted according to the amount of leaf material. Single leaves and roots were harvested into 3 ml ethanol and 1 ml was used for the subsequent extraction steps. Whole rosettes were harvested into 20 ml ethanol and extracted in 3 ml for each extraction step.

During the extraction, the plant material was separated into water soluble, ethanol soluble and insoluble fractions. To achieve this, the ethanol was decanted and the tissue was ground in an all-glass homogenizer in 0.5 ml 80% (v/v) ethanol. The homogenized material was transferred to a new tube and the homogenizer was washed twice with 80% (v/v) ethanol. All washes were transferred to the same tube as the homogenate. After centrifugation (RT, 2400 g, 12 min) the supernatant was decanted. Four further extraction steps in 50% (v/v) ethanol, 20% (v/v) ethanol, sterile water and again 80% (v/v) ethanol were performed on the homogenate. All decanted supernatants were collected in one tube and further processed as described in section 2.14.

2.14 Fractionation of $^{14}$C labeled plant material

The collected and pooled supernatants from the extraction were designated as the soluble fraction. The pellet remaining after the extraction was dried briefly at room temperature, resuspended in 1 ml sterile water and designated as the insoluble fraction. The soluble and insoluble fractions were further fractionated to determine the amount of isotopic label in individual compound classes. The fractionation procedure described here was adapted from Avice et al. (1996), Zeeman et al. (1999) and Runquist and Kruger (1999) (Figure 8).
The soluble fraction was dried in a SpeedVac (Savant SPD2010, Thermo Scientific, Wohlen, Switzerland) and redisolved firstly with two 1-ml washes of sterile water to yield the water soluble fraction and then in two washes of 1 ml 98% (v/v) ethanol to give the ethanol soluble fraction. An aliquot of both fractions was taken to measure the amount of incorporated $^{14}$C with liquid scintillation counting (see section 2.15). To measure the amount of $^{14}$C in the insoluble fraction, an aliquot of 20-200 µl was transferred to a new tube, added up with NCS tissue solubilizer (GE Healthcare, Glattbrugg, Switzerland) to 1 ml and incubated at RT overnight. Subsequently, the fraction was measured by liquid scintillation counting (see section 2.15). The sum of all three fractions gave the overall label in the tissue.
The soluble fraction was further fractionated by ion-exchange chromatography into neutral, basic, acidic and nucleotide fractions. To achieve this, an aliquot of the water soluble fraction (0.75-1.5 ml) was loaded on freshly prepared sequential 1.5 ml columns of a cation exchanger (Dowex 50WX4 hydrogen form, 100-200 mesh), followed by an anion exchanger (Dowex 1X8 chloride form, strongly basic, 200-400 mesh). Compounds not binding to either column were eluted with 10 ml of sterile water and collected as the neutral fraction, consisting mainly of sugars (Fowler and ap Rees, 1970; Avice et al., 1996). Afterwards the two columns were separated and the compounds bound to the cation exchanger were eluted with 10 ml 1M NH₄OH yielding the basic fraction, consisting mainly of amino acids. Compounds bound to the anion column were eluted with 5 ml 2M formic acid, followed by 5 ml 8M formic acid yielding the acidic fraction, consisting mainly of organic acids. Material still bound to the anion column was eluted with 5 ml 1M ammonium formate in 4M formic acid yielding the nucleotide fraction. Because of a low amount of radioactive label in the nucleotide fraction it was dried in a SpeedVac before liquid scintillation counting. For all other fractions, an aliquot of 1 ml was taken to measure the amount of ¹⁴C with liquid scintillation counting (see section 2.15).

Aliquots of the insoluble fraction were separately digested to determine the radioactive label in starch and in proteins. To measure ¹⁴C in starch, two aliquots (one sample and one control) of 0.1-0.25 ml were transferred to new tubes and boiled at 95°C for 15 min. The sample was incubated for at least 6 h at 30°C in 0.5 ml reaction mix containing 1 unit of amylglucosidase (EC 3.2.1.3, from Aspergillus niger), 10 units of α-amylase (EC 3.2.1.1, from pig pancreas) and 0.1M sodium acetate buffer (pH 4.8). The control was incubated only in sodium acetate buffer (pH 4.8) with a final concentration of 0.1M. After incubation, undigested material in the sample and control were precipitated with 20 mg KCl and 3 times the volume of methanol (1.5 ml) for 1 h at 4°C. The material was centrifuged (RT, 3000 g, 15 min) and 1 ml of the supernatant was transferred to a new tube and measured by liquid scintillation counting (see section 2.15). The amount of ¹⁴C in the starch fraction was defined as the radioactive label in the sample less the label in the control.

To measure the amount of radioactive label in proteins, two aliquots of the insoluble fraction (0.1-0.25 ml) were transferred to new tubes and filled up to 0.5 ml with sterile water. The sample was incubated overnight at 37°C in a reaction mix containing 1 unit
protease type XIV (EC 3.4.24.4, from *Streptomyces griseus*) with a final concentration of 100 mM sodium phosphate buffer (pH 7.0). The control was incubated alike, but without enzyme. After incubation, both aliquots were centrifuged (RT, 12000 g, 12 min) and 0.5 ml of the supernatant was transferred to a new tube and measured by liquid scintillation counting (see section 2.15). The amount of $^{14}$C in proteins was defined as the radioactive label in the sample less the label in the control. The amount of $^{14}$C in the starch and protein fractions was subtracted from the overall label in the insoluble fraction; the remaining amount of $^{14}$C was ascribed to the cell wall fraction (Avice et al., 1996).

### 2.15 Quantitative measurement of $^{14}$C-labeled plant material – Scintillation counting

To determine the amount of radioactive label in the fractions and digests described above, an aliquot was taken, adjusted to 1 ml with sterile water and 4 ml liquid scintillation cocktail were added. After an incubation period of 30 min in the dark, the samples were measured with a scintillation counter LS1801 (Beckman, Nyon, Switzerland). The water soluble, insoluble, neutral, basic and protein fractions were measured with the Ulitma Gold Scintillation Cocktail. For fractions with a high concentration of organic solvents or salts, like the ethanol soluble, acidic, nucleotide and starch fraction, Ultima Gold LLT Cocktail was used (both from Perkin Elmer, Schwerzenbach, Switzerland).

### 2.16 Establishing a growth curve

To establish a growth curve of single leaves, the plants were grown as described above. The leaves were counted on a daily basis and the day of leaf initiation was noted (day 0 = leaf reached 1 mm in length). Leaves were harvested every two days from day 0 onwards. The developmental stage after Boyes (2001) was noted, the fresh weight was measured and the leaf was immediately fixed on a sheet of paper together with a scale. The paper was scanned and the leaf area was determined using ImageJ.
For growth curves of whole rosettes, pictures of soil-grown or hydroponically-grown plants were taken every two to three days from day 7 onwards using a fixed camera. The pictures were analyzed with a macro for the image processing software Fiji (Schindelin, 2008). The macro was written by Michael Mielewczik (Group of Crop Science, ETH Zürich). It automatically segmented the pictures to gain only the green leaf area. Subsequently, the leaf area could be determined with ImageJ.

2.17 Perchloric acid extraction and carbohydrate measurements

To measure starch and sugar levels, plant material was harvested during the last 30 min of the light period (the last 30 min) or the last 30 min of the dark period. After harvesting, the plant material was weighed and immediately frozen in liquid nitrogen. The frozen material was homogenized in a Genogrinder (Spex Sample Prep, Metuchen, USA) twice for 1 min each time and extracted in ice-cold 0.7 M perchloric acid (1 ml per 100 mg plant material) for 5 min. After centrifugation (4°C, 3000 g, 10 min), the supernatant was used to determine levels of soluble sugars, the pellet was used to measure the amount of starch.

2.17.1 Starch measurement

The insoluble pellet was washed with 12 ml of sterile water followed by 5 washes with 12 ml 80% (v/v) ethanol, to ensure the removal of all residual sugars and perchloric acid. After each washing step the insoluble material was collected via centrifugation (4°C, 3000 g, 10 min). Subsequently the pellet was dried at RT and resuspended in 1.5 ml sterile water. Two 100-µl aliquots (sample and control) were taken and transferred to a new tube to perform starch measurements.

Both aliquots were boiled at 95°C for 15 min, cooled to RT and subsequently the sample was incubated with 100 µl reaction mix for 4 h at 37°C. The reaction mix contained 0.5 units of amylglucosidase (EC 3.2.1.3, from Aspergillus niger), 5 units of α-amylase (EC 3.2.1.1, from pig pancreas) and 0.2 M sodium acetate buffer (pH 4.8). The control was incubated as the sample in a mixture containing 0.2 M sodium acetate buffer (pH 4.8) and sterile water instead of the enzymes. After the incubation time, the insoluble material was collected by centrifugation (RT, 3000 g, 10 min) and 3 times 25 µl of the supernatant were measured.
Glucose released from starch was measured in an enzymatic assay according to Jones et al. (1977). In this assay, glucose and ATP is converted to glucose-6-phosphate (Glc6P) and ADP by hexokinase. Subsequently, Glc-6P is oxidized to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. This reaction is NAD\(^+\)-dependent and the reduced NADH is measured spectrophotometrically at 340 nm.

The assay was performed in a 96-well plate in a reaction mix containing 25 \(\mu\)l sample or control supernatant, 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, pH 7.5); 1 mM MgCl\(_2\); 0.5 mM ATP; 0.4 mM NAD and 1.4 units of hexokinase (from yeast, EC 2.7.1.1) added up to a volume of 200 \(\mu\)l with sterile water. After measuring the initial absorbance at 340 nm, 1 unit of glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides, EC 1.1.1.49) was added and the absorption at 340 nm was monitored using a plate reader (Infinite M1000, Tecan, Männedorf, Switzerland) until the maximal absorption was reached. As each glucose molecule leads to the formation of one NADH, the glucose content can be inferred from the \(\Delta OD\)\(_{340}\). Based on a standard curve established with glucose on the plate reader a \(\Delta OD\)\(_{340}\) = 1 corresponds to 53.87 nmol glucose.

### 2.17.2 Measurement of glucose, sucrose and fructose

Following extraction in perchloric acid, 1 ml supernatant was immediately adjusted to pH 6 with neutralizing solution containing 2M KOH and 0.4M 2-(N-morpholino)ethanesulfonic acid (MES). After centrifugation (4°C, 10000 g, 10 min) 1 ml of the supernatant was used to measure soluble sugars using the NADH assay described above (see section 2.17.1). All measurements were performed in triplicates using 25 \(\mu\)l supernatant each. The assay was performed in a 96-well plate in a reaction mix containing besides the sample 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, pH 7.5); 1 mM MgCl\(_2\); 0.5 mM ATP; 0.4 mM NAD and 1.4 units of hexokinase (from yeast, EC 2.7.1.1) added up to a volume of 200 \(\mu\)l with sterile water. After measuring the initial absorbance at 340 nm, 1 unit of glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides, EC 1.1.1.49) was added and the absorption at 340 nm was monitored in a plate reader until the end point of the reaction was reached. To measure the amount of fructose, 1 unit of phosphoglucoisomerase (from yeast, EC 5.3.1.9) was added and the change in absorption at 340 nm was monitored again. Fructose is converted to fructose-6-phosphate by hexokinase,
which can then be converted to Glc6P by phosphoglucoisomerase. Increase of Glc6P leads to a further reduction of NAD. Additionally, the sucrose content was measured by adding 5 units invertase (from *Saccharomyces cerevisiae*, EC 3.2.1.26) to the reaction mix and monitoring the change in absorption at 340 nm for a 3rd time. Invertase hydrolyzes sucrose to fructose and glucose which are further converted by hexokinase and glucose-6-phosphate dehydrogenase leading to a further increase in NADH.

**2.18 Statistical analysis**

For simple statistical analysis, Microsoft Excel functions were used. For the correlation analysis, the variance analysis and plotting of the corresponding figures (see section 3.2.4) the software package R was used (R Development Core Team, http://www.r-project.org/).
3 A method to measure gas exchange of whole Arabidopsis plants

3.1 Introduction

Plants can capture energy from the sun to perform photosynthesis, during which light energy is converted to chemical energy equivalents which can be used to assimilate carbon dioxide. The assimilated carbon is subsequently utilized to form all the building blocks from which a plant is made. Due to the overall importance of photosynthesis, accurate measurement of photosynthetic efficiency is needed. Such measurements can reveal important information about the overall performance of a plant and its ability to build up biomass.

A non-destructive, non-invasive method to monitor photosynthetic parameters is via gas exchange measurements. Measuring gas exchange is a rapid method that can be used in the laboratory as well as in the field to investigate the photosynthetic rate of plants under different environmental conditions (Sun et al., 1999; Valentini et al., 2000), but also to compare plant lines with altered photosynthetic capacity.

Several methods to measure gas exchange have been developed in the last decades. Most of the commercially available systems are based on a leaf chamber connected to an infrared-gas analyzer (IRGA). The leaf chamber is clamped over a single leaf and the gas exchange of a small area of the leaf blade (2-10 cm²) is measured. This kind of measurements bring along a range of downsides. First of all, photosynthetic rate can vary over the leaf surface (e.g. due to differences in stomatal distribution; Cheeseman, 1991). Measuring multiple replicates at different sites of the leaf is needed to get a useful average photosynthetic rate. Additionally, despite contradictory reports, it can be assumed that the photosynthetic rate partly depends upon the developmental stage of the plant. For apple leaves it was shown that in the early phase of development leaves show an increasing photosynthetic rate, reaching maximum rates at the final expansion. In later stages, during leaf senescence, a decrease in photosynthetic rate was observed (Kennedy and Johnson, 1981; Gepstein, 1988). In a study performed on Arabidopsis, on the other hand, it was argued that gas exchange is uniform between leaves in a mature rosette (Lake, 2004).
However, in this experiment only leaf 4 to leaf 12 was measured, thereby excluding old and still developing leaves, presumably due to leaf size restrictions. This clearly shows that estimating the overall photosynthetic performance of a whole plant is difficult from individual leaf measurements.

Gaining photosynthetic data for single leaves is certainly valuable in many fields of research e.g., leaf development and senescence, but to improve our understanding of whole-plant physiology and biomass accumulation, photosynthetic measurements on a whole-plant level are required. Therefore, chamber systems to measure whole shoots have been developed for different species (van Iersel and Bugbee, 2000; Burkart et al., 2007). For *Arabidopsis thaliana* commercially available systems as well as custom-made systems have been established (Donahue et al., 1997; Tocquin and Périlleux, 2004). All enable the assembly of a gas-tight chamber around the shoot of an Arabidopsis plant allowing gas exchange measurements of the whole leaf area. Nevertheless, photosynthetic rates gained from these systems are still variable. For whole plant measurements of Col-0, published values range from 3.5 to 9 µmol m⁻² s⁻¹ (Dodd et al., 2004; Tocquin and Périlleux, 2004), probably due to differences in analytical aspects of the gas exchange measurements, but also due to differences in growth conditions (Lake, 2004). Most of these systems have in common, that they only measure one plant at a time. As photosynthetic parameters are highly sensitive to growth conditions (e.g., temperature, relative humidity and light intensity) it is essential to grow the plants under the exact same conditions to allow comparison of independent measurements or to measure plants in parallel. A multiple chamber system was used by Dodd et al. (2004) but a detailed description of such a system has not been published so far.

To be able to measure the photosynthetic performance of Arabidopsis plants and to overcome the aforementioned downsides of previous systems we constructed a multiple chamber system to measure gas exchange of whole rosettes (Figure 9). Our system allows up to eight plants to be simultaneously and continuously monitored. Here the design and the performance of our gas exchange system are described.
3.2 Results

3.2.1 Design of the GDU-26 gas exchange system

As already mentioned (see section 2.5), the system consists of eight Arabidopsis gas exchange chambers, a control unit, an IRGA and a computer for data acquisition. A schematic overview is shown in Figure 9.

The air running through the system is pressurized air, reduced to near-ambient air-pressure. The air is first channeled, if necessary, through soda lime to control the CO$_2$ content and a humidification unit to increase the relative humidity. After humidification, the air is split into 9 channels, eight of them to supply the chambers with air and the ninth to obtain a reference for the CO$_2$ and H$_2$O content in the incoming air. The flow to the chambers is regulated by eight mass-flow controllers, one preceding each Arabidopsis gas exchange chamber, allowing the flow to be individually regulated. Subsequently, the air runs through the respective chamber and back to the control unit. The control unit uses a gas-switching system to channel the air coming from the chambers such that gas exchange of only one chamber at a time is measured via the IRGA. The outflowing air of all other chambers is set free, when not being measured, so flow through all eight chambers is continuous. The IRGA
is measuring CO$_2$ and H$_2$O content of the air before it is introduced to the control unit (reference air) and after it has passed through a chamber. In this way, it is possible to calculate the change in CO$_2$ and H$_2$O content of the air for each chamber. Additionally, the IRGA monitors system parameters to check the stability of the measurements (e.g., air temperature, pressure). All data are recorded every 1-2 sec and collected in an averaged form by a LabView application. Using the LabView application, the gas exchange system is easy to operate, the measurements can be followed in real-time and the data are automatically stored (Figure 10).

Figure 10: LabView application to control the GDU-26 gas exchange unit. With the program it is possible to control the gas flow, monitor the gas exchange and environmental parameters and set the parameters for data acquisition.

We designed our Arabidopsis gas exchange chambers to allow a fast and easy assembly of the system. The hypocotyl of the plant is placed between the foam gaskets of the two stabilizing units which are screwed together subsequently (see section 2.5). The system can be used to measure plants grown on soil, in hydroponics, or on virtually any other substrate. Additionally, attention was paid to keep the chamber volume minimal (25 ml). This allows rapid stabilization of the measurements after switching chambers and gives more accurate
measurements compared to bigger chambers as the risk of dead volume within the chamber is minimized.

We decided to design a system in which eight plants are measured together. This allows on the one hand a rather continuous measurement of the photosynthetic parameters (at least once per hour) and on the other hand a reasonably high throughput. Increasing the number of plants would increase the throughput, but would lead to less measurements per time point (stability of the measurements cannot be controlled sufficient) or to less measurements over the day (changes in photosynthetic parameters might remain undetected). A reduction of the plants measured is possible any time, as the chambers can be excluded from the measurements easily via the LabVIEW application. This offers the possibility to monitor plants precisely and follow rapid changes e.g. after the application of stresses.

Our system can be easily introduced into any growth cabinet, enabling precise control of growth conditions. The system can be used to monitor photosynthetic performance over the course of several days. Thereby diurnal changes of photosynthetic parameters as well as the change in photosynthetic performance of developing plants or plants undergoing a specific treatment can be investigated. Measuring several plants in parallel has the additional advantage that results can be expressed relative to a control or wild-type plant, thereby minimizing the errors that can occur during gas exchange measurements (e.g., differences in environmental conditions).

3.2.2 Optimizing the setup of the GDU-26 gas exchange system

Test gas exchange measurements were performed to determine whether only the introduction of plant material into the chamber results in measurable changes of the gas composition. The absence of plant material, vice versa, should not lead to measurable changes of the gas exchange parameters. To prove this, empty chambers were measured for a complete 12-h light period. Some chambers were properly closed while in others a leak was introduced into the chambers (Figure 11, A and B).
When the chambers are closed properly, the $\Delta CO_2$ values as well as the $\Delta H_2O$ values are constantly around 0 with only slight fluctuations over the 12-h measurement period. The $\Delta CO_2$ value in Figure 11 A is varying between -0.16 and -0.33 µmol m$^{-2}$ s$^{-1}$ and the $\Delta H_2O$ value in Figure 11 B is varying between 0 and 0.12 mmol m$^{-2}$ s$^{-1}$. These changes might be a result of a shift between the reference and the sample cell of the IRGA as calibration only takes place once at the beginning of the measurement. Still the observed shift is only minor and can be neglected when a plant is measured.

**Figure 11: Test and optimization runs of the gas exchange system.** Measurements of gas exchange, $\Delta CO_2$ (A) and $\Delta H_2O$ (B), of a properly closed, empty chamber and an empty chamber with an artificially introduced leak. (C) $\Delta CO_2$ and (D) $\Delta H_2O$ values without a dead time before data acquisition. The gray shaded area indicates the data that is not stored if a dead time of 90 sec is applied. Measurements were repeated 8 times and representative data of one plant is shown.
Introduction of a leak into the gas exchange chambers leads to a considerable increase in the \( \Delta CO_2 \) and \( \Delta H_2O \) values and to unstable measurements. In the depicted example in Figure 7, \( \Delta CO_2 \) is varying between -2.86 and 2.77 \( \mu mol \ m^{-2} \ s^{-1} \) and \( \Delta H_2O \) values are varying between 0.25 and 0.53 mmol \( m^{-2} \ s^{-1} \). Both parameters increased two to five times compared to a properly assembled system. Moreover, it can be seen that a leak within the gas exchange system leads to unstable measurements visible as a shift in the measured values over one measurement period of 360 sec. This shift in the measured values is a good and easy to detect quality parameter to check for a correct chamber assembly and was further on used to evaluate every measurement.

When the control unit switches from one chamber to the next, air from the previous chamber is still in the tubing. This air needs to be removed from the tubing systems before correct measurements can be performed. To account for this, a “dead time” is introduced into the data acquisition. In this period, air is running through the system, but no measurements are recorded. The length of the required dead time was determined experimentally (Figure 11, C and D) for a given flow rate. For this, the chambers were filled with plants, an air flow of 200 \( \mu mol \ s^{-1} \) was applied and data were recorded without any dead time in between chamber switches as an average every 15 sec. A typical pattern can be seen for \( \Delta CO_2 \) and \( \Delta H_2O \) for each measurement period. For \( \Delta CO_2 \) (Figure 11, C), the first value is relatively low with about -30 \( \mu mol \) (the \( \Delta CO_2 \) value of the previous chamber was -5 \( \mu mol \)), the second measurement point in each period is around -35 \( \mu mol \) and the third at -36.8 \( \mu mol \). The \( \Delta CO_2 \) value is further decreasing and from the sixth measurement point on, this is after 90sec, the measurements are stable, reaching in average -37.3 \( \mu mol \). Similar results were observed for \( \Delta H_2O \) after switching the chamber (Figure 11, D). Therefore, a “dead time” of 90 sec was introduced to the system as a default. This means that, within the first 90 sec after each chamber switch, no measurements are recorded. If necessary, the dead time can be reduced, e.g. if a higher flow rate is applied, or prolonged in case of lower flow rates.

**3.2.3 Consistency of the measurements gained with the GDU-26**

To check whether measurements are consistent and stable when all chambers are filled, eight Col-0 plants were measured for a complete 24 h period (12 h light/ 12 h dark). The
plants were 32 days old and an air flow of 200 µmol s⁻¹ was applied to perform the measurements. Every 360 sec the chamber was changed, a dead time of 90 sec was applied and the following 270 sec the values were recorded as an average of 30 sec. Therefore, for each measurement period 9 measurements are recorded. As shown before (section 3.2.2), multiple measurements during one measurement period allow for an easy quality control. Fluctuations occurring during the measurement, e.g. caused by leakage, are revealed quickly and the affected measurements can be discarded.

All data points gained during the measurement are plotted against a time axis. As the measurements of one measurement period are stable they are all plotted over each other and appear as only one point in Figure 12.

Generally, for all eight chambers we observed stable values with only slight changes during the course of the light period. In the beginning of the light period, following the switching on of the lights in the growth cabinet, there was a rapid increase of photosynthetic rate (A) as well as an increase in transpiration rate (E, Figure 12, A, inset). Both values stabilize after 20 min and increase only slightly thereafter, with a maximum reached around 8 h into the light. Subsequently, both A and E remain stable or slightly decrease until the end of the light period. Overall, this leads to a variation in A over the light period of ±11% and a variation in E of ±15% in relation to the average. The pattern with a maximum of A and E around 8 h into the light was observed in many of the subsequent measurements. So far, stable photosynthetic rates over the day have been reported (Leymarie et al., 1998; Dodd et al., 2004), but these measurements were not analyzed in detail for changes over the light and dark period. Hennessey and Field (1991) on the other hand describe circadian rhythms for A and E in Phaseolus vulgaris. When they transfer plants grown under normal day-night rhythms to continuous light, an oscillation of A and E can be seen with a maximum at the subjective noon and a minimum at the subjective midnight. These oscillations are attributed to be a result of circadian control over photosynthesis. The observed changes in a 12-h light period might also be due to circadian regulation of photosynthetic processes.
A method to measure gas exchange of whole Arabidopsis plants

Figure 12: Comparison of gas exchange measurements recorded in parallel with the GDU-26. The measurements for photosynthetic rate (A) and transpiration rate (B) of each of the eight chambers (Ch1 to Ch 8) are shown over a complete one day-night cycle. The insets illustrate photosynthetic rate and transpiration rate in the first 30 min after the light was switched on. Open bars indicate the light period, solid bars the dark period.

In the dark, A (now negative as it represents respiratory loss) changes ±20%. E shows an even more prominent change, increasing in the course of the night by 25-40%. Dodd et al. (2004) also observed an increase in transpiration in the second half of the dark period which they attributed to circadian regulation.

On average, the plants in the eight chambers show a photosynthetic rate of 7.45 µmol m⁻² s⁻¹ during the day with a standard deviation of 0.34 between the chambers. This is a difference of 4.6% between parallel measurements. The transpiration rate is in average 1.38 mmol m⁻² s⁻¹ during the day with a variation of 8.7% between the chambers. The variation during the night is slightly higher with 15% for respiration rate and 10.5% for transpiration.
rate. This increased variation during the night might be explained by the lower $\Delta CO_2$ and $\Delta H_2O$ values compared to the day. Therefore, the measurements get less accurate and so more error prone.

The average photosynthetic rate of 7.45 $\mu$mol m$^{-2}$ s$^{-1}$ gained from these measurements lies in the range of already published photosynthetic rates for Arabidopsis. In the literature, measurements for whole Arabidopsis plants grown under a 12h day/12h night cycle range between 5-10 $\mu$mol m$^{-2}$ s$^{-1}$ (Donahue et al., 1997; Sun et al., 1999; Poulson et al., 2002). The standard deviation of 0.34 is relatively low compared to the value of ~0.6 observed for the gas exchange systems used by Sun et al. (1999) and Tocquin et al. (2004). Measurements with commercially available systems on single leaves also show similar or even higher variation between plants (SE~0.2-1.5, n=5; Lake, 2004). This shows that the GDU-26 delivers reliable data which is comparable or superior to commercially available whole plant systems or other custom made devices.

3.2.4 Statistical analysis of the GDU-26 measurements

To evaluate the effect of external variables on the photosynthetic measurements of the GDU-26, statistical tests were performed. Gas exchange data of more than 70 wild-type plants, grown and measured independently over several months, are chosen randomly. A correlation analysis and a one-way analysis of variance of the data should reveal, which, if any, of these variables are connected to photosynthetic and transpiration rates. The evaluated variables are plant age, leaf area, the chamber in which the plant was measured and relative humidity.

The plant age in the dataset varies between 25 and 36 days and is restricted (due to the increasing size of the plant) by the chamber design and the flow rate of the air through the system (see below for limitations of the system). As plants with a wide age difference were measured, leaf area also differs (ranging from 180 mm$^2$ to 1500 mm$^2$). This might result in an increasing effect of shaded leaves in older plants. Therefore, it was checked if a correlation between leaf area and photosynthetic parameters exists.
As a third parameter, it was analyzed whether there is any effect on photosynthetic performance of the chamber in which the measurement took place. This could be caused by a defective chamber or by the chamber position in the growth cabinet (the gas exchange chambers were always placed in the same formation in the same growth cabinet for all measurements in this data set).

The system does not include an automatic feedback regulation for the relative humidity. Therefore, the water content of the air within the chamber does vary. It was analyzed if this variation in relative humidity correlates with changes in photosynthetic parameters. Relative humidity has a lower limit of 55% - that of the incoming air - and has an upper limit of 95%. Exceeding the upper limit was prevented by increasing the air flow through the system. If this was not sufficient the data was discarded.

The results of this test series are depicted in Figure 13, where all variables are plotted against \( A \) and \( E \). Additionally, each plot shows the correlation coefficient calculated after Spearman (\( \rho \) value) and the corresponding p value. None of the tested variables shows a strong correlation (\( \rho > 0.7 \)) to either \( A \) or \( E \). The lowest correlation can be found between the chamber and \( A \) or \( E \). This shows that the chamber in which the plant was measured, and its position within the growth cabinet, does not influence photosynthetic measurements. Weak correlations (\( 0.2 < \rho < 0.4 \)) exist between the age and \( A \) or \( E \) as well as the leaf area and \( A \) or \( E \). As the correlations are positive, this indicates either an increase in \( A \) and \( E \) with an increase in the age and leaf area of a plant. Alternatively it could reflect an increase in shaded leaf area, and therefore an underestimation of the actual leaf area. As shaded leaf area was shown to be \(~5\%\) of total leaf area for plants grown in similar conditions (Donahue et al., 1997; Sun et al., 1999), this would not be expected to have a strong influence. However, none of the correlations is significant and more data would be necessary to substantiate this correlation and to reveal its source.
A method to measure gas exchange of whole Arabidopsis plants

Figure 13: Correlation analysis of photosynthetic rate and transpiration rate with external variables. (A) Boxplot of A and E against plant age and chamber in which the plant was measured. (B) Plot of A and E against leaf area and relative humidity. In the left upper corner the correlation coefficient (\(\rho\)) and significance of the correlation (\(p\)) between the two variables is highlighted. A, photosynthetic rate; E, transpiration rate; RH, relative humidity of the incoming air.
A common problem in gas-exchange measurements is the change in relative humidity (RH) caused by transpiration. Optimally, each flow rate would be adapted so that the RH of the incoming and outgoing air is comparable between measurements. This is not possible for a whole plant measuring system, therefore either the RH of the outgoing air is kept stable by modulating the RH of the incoming air, or the incoming air is kept stable and the RH of the outgoing air is varying. In our system the latter is the case, while there is still day to day variation of the incoming RH. When the RH of the incoming air is kept stable, a high E is causing a high RH of the outgoing air. A correlation between the two parameters can therefore be expected. However, a high RH of the outgoing air does have only minor effects on A, as only a weak correlation was found between RH and A ($\rho < 0.3$).

Still the data recommends measuring plants in a comparable developmental stage and with comparable leaf area, rather than plants of the same age. In this way differences in RH humidity are kept minimal between different chambers.

3.2.5 Long term measurements performed with the GDU-26

An important advantage of our multiple chamber system is that it is possible to carry out measurements running for several days without major user intervention. Eight plants can be measured continuously in real-time. Limitations are only the rosette size and the air flow through the system as described below. Stable measurements are gained from the system as seen in Figure 14. The mean photosynthetic rate per day is stable at 6.9 $\mu$mol m$^{-2}$ s$^{-1}$, varying only between 6.7 and 7.1 $\mu$mol m$^{-2}$ s$^{-1}$ over the six days of measurements. Similar stable results are obtained for respiration (average= -0.9 $\mu$mol m$^{-2}$ s$^{-1}$, min= -0.7 $\mu$mol m$^{-2}$ s$^{-1}$, max= -1 $\mu$mol m$^{-2}$ and s$^{-1}$). The transpiration rate is less stable over the measurement period and decreases over the light periods from an average of 2.1 to 1.6 mmol m$^{-2}$ s$^{-1}$. In the dark, transpiration fluctuates in the first days, but stabilizes later at around 0.8 mmol m$^{-2}$ s$^{-1}$. The changes in transpiration rate might result from a rise in relative humidity due to the increase of leaf area during the measurement period. However, these small changes in relative humidity do not affect photosynthetic rate.
A method to measure gas exchange of whole Arabidopsis plants

Figure 14: Long term measurement with the GDU-26. Gas exchange of Col-0 plants measured continuously over a period of 6 days. A measurement of one example plant is depicted. Open bars indicate the light period, solid bars the dark period.

3.2.6 Setup and system limitations of the GDU-26

Rosette size and the photosynthetic capacity of the plants to be measured are the most important parameters for setting up the gas exchange system. Theoretically, there is no lower size limitation of the rosette diameter, as long as the plants can be introduced gastight into the chamber, but there is an upper size limitation of the rosette diameter given by the chamber design of 8 cm. For smaller plants, the photosynthetic rate becomes limiting, as the ΔCO₂ value becomes too small to get reliable data. The air flow can be decreased, which leads to an increase in ΔCO₂ and simultaneously to an increase in ΔH₂O. A small flow rate additionally increases the response time of the system and reduces the numbers of measurements that can be taken per measurement period. Therefore the air flow through the system has to be optimized in a way so that ΔCO₂ is maximal, but at the same time a too high RH causing condensation is avoided and a feasible response time of the system is sustained. The air flow of the system can be adjusted in a range between 0 and 200 µmol s⁻¹. For plants with a high transpiration rate, the flow rate might be limiting rather than the size of the plant. If the RH in the outgoing air at the maximal flow rate is too high, thus causing condensation, no reliable measurements can be performed.
3.2.7 Measuring photosynthetic rate in different environments

With the GDU-26 gas exchange system, plants can be monitored under different environmental conditions. To demonstrate the sensitivity of the system Col-0 plants were measured under different light intensities of 60, 160 and 540 µmol quanta m$^{-2}$ s$^{-1}$. All plants were grown under standard conditions for three weeks, before they were transferred to the respective light conditions. After 3 days of adaptation, two sets with eight plants each were measured for each light intensity over a 12-h light period (Figure 15).

![Figure 15: Gas exchange in different light conditions. Photosynthetic rate (A) and transpiration rate (B) of Col-0 plants in different light intensities of 60, 160 and 540 µmol quanta m$^{-2}$ s$^{-1}$. Open bars indicate the light period. Mean ± SE (n=16).](image)

Both photosynthetic rate and transpiration rate differ with the fluence rate. At 540 µmol quanta m$^{-2}$ s$^{-1}$, both data sets show an average photosynthetic rate of 10.6 µmol m$^{-2}$ s$^{-1}$, with standard deviations of 1.0 and 1.3, respectively. Likewise, the data for 160 µmol quanta m$^{-2}$ s$^{-1}$ (7.1 ± 0.6 µmol m$^{-2}$ s$^{-1}$ and 6.2 ± 0.8 µmol m$^{-2}$ s$^{-1}$) and 60 µmol quanta m$^{-2}$ s$^{-1}$ (2.9 ± 0.5 µmol m$^{-2}$ s$^{-1}$ and 2.6 ± 0.7 µmol m$^{-2}$ s$^{-1}$) gave highly reproducible results. The variation obtained for transpiration rate (Figure 15, B) was slightly higher. For 540 µmol quanta m$^{-2}$ s$^{-1}$ the averages for transpiration rate are 2.3 and 2.9 µmol m$^{-2}$ s$^{-1}$ with standard deviations of 0.2 and 0.3, respectively. Again similar data was gained for 160 µmol quanta m$^{-2}$ s$^{-1}$ (1.6 ± 0.2 µmol m$^{-2}$ s$^{-1}$ and 1.9 ± 0.2 µmol m$^{-2}$ s$^{-1}$) and for 60 µmol quanta m$^{-2}$ s$^{-1}$ (1.0 ± 0.2 µmol m$^{-2}$ s$^{-1}$ and 0.6 ± 0.1 µmol m$^{-2}$ s$^{-1}$). This data show that the variation within the dataset and between the datasets is very similar for each light intensity. Thus, comparing data between batches of plants should be possible. Additionally, these data show that environmental factors like light intensity have a big impact on photosynthetic rate and transpiration rate.
which can be readily monitored with this gas exchange system. In a similar manner, other environmental factors and their influence on photosynthetic gas exchange could be analyzed.

Photosynthetic responses can be also monitored on shorter time scales. Inevitably, the faster the rate of change in conditions, the more limited the number of plants that can be measured in parallel. In an LED chamber, light intensity was changed every hour and the effect on one plant at a time was measured. The measurements started with the lowest light intensity of 10 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \) which was increased stepwise to 50, 150 and 250 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \) (Figure 16, A). This cycle was repeated 3 times.

**Figure 16: Gas exchange in changing light conditions.** Gas exchange of Col-0 plants was measured in an LED chamber with light conditions changing every hour from 10 to 50, 150 and 250 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \) (A). This cycle was repeated three times. The curves for photosynthetic rate (B) and transpiration rate (C) of one example plant are depicted.
Photosynthetic rate reacted immediately on light intensity changes, and increased stepwise from 0.6 µmol m\(^{-2}\) s\(^{-1}\) to 2.8 µmol m\(^{-2}\) s\(^{-1}\), 6.6 µmol m\(^{-2}\) s\(^{-1}\) and 8.4 µmol m\(^{-2}\) s\(^{-1}\). These results are stable over all three cycles. The transpiration rate reacted more slowly than the photosynthetic rate. However, distinct differences in transpiration were visible for the different light intensities. On average, transpiration rate increased from 1.1 mmol m\(^{-2}\) s\(^{-1}\) to 1.5 mmol m\(^{-2}\) s\(^{-1}\), 2.1 mmol m\(^{-2}\) s\(^{-1}\) and 2.6 mmol m\(^{-2}\) s\(^{-1}\). The results are slightly higher in the second and third cycle especially for the lower light intensities. While transpiration rate is increasing during the first cycle with 10 µmol m\(^{-2}\) s\(^{-1}\) light, it is steadily decreasing in the second cycle after switching back from high light to the lower light intensity. It seems that, one hour of low light does not seem to be sufficient for the plant to adjust stomatal conductance and to achieve a stable rate of transpiration. Nevertheless, it could be shown that the effect of fast changes in the environmental conditions can be followed precisely via our gas exchange system.

3.2.8 Measuring photosynthetic rate under stress conditions

Another application for gas exchange measurements is monitoring the effect of stress conditions on the plants’ photosynthetic rate. Two experiments were performed to show, on one hand stresses leading to an immediate response and on the other hand stresses with a mild or latent effect on photosynthesis (Figure 17). Both experiments were carried out using hydroponically-grown plants to allow an easier and more uniform stress application.

To simulate a strong stress situation on the plant the root was cut from the shoot (Figure 17, A and B). The initial A was 5.84 µmol m\(^{-2}\) s\(^{-1}\) and started to decline immediately after removing the root. Within 15 min it was reduced by 10%, after 50 min A was reduced to 50% of its original value and decreased further on to 0.5 µmol m\(^{-2}\) s\(^{-1}\) by the end of the light period. In the following dark period, respiration rate was constant during the whole night, but reduced by one third compared to the preceding dark period before cutting. Likewise, transpiration declined quickly after removal of the root. From an initial transpiration rate of 1.07 mmol m\(^{-2}\) s\(^{-1}\), E decreased steadily by 90% within 3 h after cutting.
A method to measure gas exchange of whole Arabidopsis plants

Figure 17: Gas exchange under stress conditions. (A) Photosynthetic rate and (B) transpiration rate of a Col-0 plant from which the roots were cut after 29 h (5 h into the light period). The measurements were repeated for three plants; the data for one representative plant are shown. (C) Photosynthetic rate and (D) transpiration rate of Col-0 plants under salt stress. All plants were measured for 6 h in the light period before treatment with 50 mM NaCl or 200 mM NaCl. Open bars indicate the light period, solid bars the dark period. Mean ± SE (n=4).

Salt stress was used to illustrate that it is possible to gain consistent data for many replicate plants while following stress effects on photosynthetic rate over longer time periods (Figure 17, C and D). In this experiment, plants were grown in ½-strength Cramer solution and measured with the GDU-26. In the middle of the light period of the first day NaCl solution was added to the nutrient solution of the stressed plants to result in a concentration of 50 mM and 200 mM NaCl, respectively. To the nutrient solution of the control plants an equal amount of water was added. When watered with 200 mM NaCl solution the plants responded immediately to the salt stress and photosynthetic rate as well as transpiration
rate were reduced. Within the first two hours photosynthetic rate declined drastically by 50% followed by a more moderate decrease of another 10% until the end of the light period. Respiration rate during the subsequent dark period was slightly increased at the beginning of the night, but was similar to the control later during the dark. Photosynthetic rate did not recover during the next light period; it reached a value of 34% compared to the untreated plant and decreased again during the course of the day. Transpiration rate was reduced considerably by 65% within the first 2 h after the salt stress was applied. Following this, transpiration further declined until the end of the light period and was considerably reduced in the subsequent dark period compared to the control plants. Treatment with 50 mM NaCl solution did not have an instant effect on photosynthetic rate, but transpiration rate declined by 20% steadily until the end of the light period. During the subsequent night transpiration was reduced to half of the control plants. There was no difference to the control plants measurable for photosynthetic rate in the remaining 6 hour of light following the treatment as well as for the respiration rate during the subsequent dark. However, during the following light period photosynthetic rate was decreased by about 2% per hour while photosynthetic rate in the control plants was stable. This trend was visible for every plant, even though the standard error in the groups was quite high. The data shows that it is possible with the GDU-26 to differentiate between factors affecting transpiration and/or photosynthetic rate and that a reduction in transpiration does not necessarily lead to an immediate reduction on photosynthetic rate as well.

3.3 Discussion

We have developed GDU-26; a multiple chamber system to measure gas exchange of up to eight Arabidopsis thaliana rosettes simultaneously and long-term. Our system can easily be integrated into every growth cabinet and can be used with plants grown on versatile substrates. This flexibility makes the GDU-26 applicable for many different purposes. Our test experiments show that the GDU-26 gives accurate and stable measurements, consistent between the different chambers and reproducible for different batches of plants. Furthermore, we could show that the gained measurements are stable over several days. Additionally, our measurements are comparable to measurements obtained from commercially-available single-chamber systems. We could show that the system allows us to
measure and compare plants grown in different environmental conditions; (i.e. different light intensities). This allows us to monitor gas exchange parameters of plants undergoing a specific treatment or to compare plants in stress situations (e.g. salt stress). A variety of other environmental and stress parameters and their effect on photosynthetic performance could be analyzed with this system. Moreover, it could be applied to study the photosynthetic performance of the large number of gene knock-out or overexpression lines available today.

With only small changes in the setup it would be possible to adapt the system to even more applications. For example a gas mixer combined with a pure CO$_2$ supply would firstly enable a more stable CO$_2$ content then with the compressed air provided to our plants. This would lead to even more stable data gained by the system and to very high degree of reproducibility. This improvement would also allow CO$_2$ response curves to be recorded. With CO$_2$ response curves, a variety of photosynthetic parameters like maximum velocity of RuBisCO carboxylation ($V_{\text{cmax}}$), electron transport rate ($J$) and triose phosphate utilization (TPU) can be calculated (Sharkey et al., 2007). Thus, more subtle changes in photosynthetic performance could be revealed.

Another possible extension of the system would be an automatically adjustable flow rate regulated by the relative humidity within each chamber. This would allow to maintain a constant ΔH$_2$O by adapting the flow rate of the air and thus would avoid big differences in relative humidity level between different chambers filled with plants of very different size. In this case the flow rates would vary between the chambers and it would be necessary to check if this is affecting photosynthetic parameters. Certainly, this would allow adaptation of the flow rate only within a narrow range. A lower limitation to air flow is set by ΔCO$_2$ in order to get reliable data as describe before and an upper limit is necessary in order to avoid stress for the plants by too strong air flow through the chambers.

So far all pictures to determine the projected leaf area are taken manually. It would be possible to combine the GDU-26 with a camera system that takes pictures of all chambers automatically. Additionally, this would allow to monitor rosette growth, thus obtaining photosynthetic data as well as growth data for the exact same plants.
The outstanding advantage of our system compared to many others so far described (Donahue et al., 1997; Tocquin and Périlleux, 2004) is the parallel measurement of eight plants. Thus inaccuracies arising from differences in growth conditions and differences in setup and calibration of the gas exchange system can be excluded. This results in more robust and reproducible data.
4 Carbon allocation and carbon transport in *Arabidopsis thaliana*

4.1 Introduction

To optimize growth, carbon is transported within higher plants from regions of high net CO$_2$ assimilation to regions of low or without net CO$_2$ assimilation. Isotopically labeled compounds have been used to study these transport processes already for many decades. In the 1950s Vernon and Aronoff used $^{14}$C to demonstrate that radioactively marked carbon is assimilated as CO$_2$ and subsequently introduced into many different compounds like sugars, starch and amino acids (Vernon and Aronoff, 1952). The majority of label was found in sucrose, which was identified as the primary transported form of carbon in plants. Additionally, other transport compounds have been characterized, like raffinose, stachyose and verbascose, most of them being plant species-dependent (Lalonde et al., 2004). It was shown that after labeling a soybean leaf, radioactive products are transported acropetally (towards the tip of the plant) as well as basipetally (towards the root). Hence, radioactive label accumulates in the root and in young tissues like the shoot apex (Aronoff, 1955). This movement follows a defined pattern, determined by the vascular tissue. Following the vascular connections in tobacco, carbon is transported from the labeled leaf upwards to the leaves n+3, n+5, n+8 and n+10 (Jones et al., 1959; Joy, 1964). Additionally, export occurred down to the root, but not to any leaf lower on the axis. All importing leaves were positioned at the same side of the rosette as the labeled one. In leaves positioned opposite to the labeled one, only traces of label could be detected. In general, carbon import could be seen only for developing leaves. Mature leaves did not show any import.

While carbon import was observed only in developing leaves, carbon export was observed only in mature leaves (Fisher, 1956; Jones et al., 1959). When a leaf turns from a sink leaf into a source leaf, a switch from import of carbon to export of carbon takes place. This switch is a gradual process starting at the tip of the leaf and proceeding further towards the base. It follows the basipetal maturation of the leaf (Avery, 1933). Hence export of carbon first starts in the tip of the leaf, while the base is still importing and proceeds towards the base until the whole leaf is exporting (Jones and Eagles, 1962; Turgeon and Webb, 1973).
In Arabidopsis only a few studies analyzing the vascular connections on a whole plant basis have been performed. Busse and Evert (1999) analyzed microscopically vascular traces in developing rosettes (with up to 8 leaves) and found a closed vascular pattern, in which all the vascular strands are interconnected. Kang et al. (2003) reconstructed the vascular pattern of older vegetative shoots and reproductive shoots using a molecular marker for the procambium (ATHB-8::GUS). They found an open vascular pattern, with free ending veins, in early vegetative shoots which develops into a closed pattern only after formation of leaf 8. According to the model by Busse and Evert (Figure 18, A), from leaf 6 on, each leaf (n) is connected to the leaves two and three positions lower on the rosette (n-3, n-2). According to the model developed by Kang et al. (Figure 18, B) vascular bundles connect the leaves that stand in a vertical line along the stem (along the same orthostichy). From leaf 6 on, each leaf is connected with a leaf trace five positions lower in the rosette (n-5) and from leaf 9 on, each leaf is connected to two leaf traces which are five and eight positions lower on the rosette (n-5, n-8). However, in both studies they found irregularities in the vascular connections being a hint for a stochastic element in the linkage of the vascular bundles. In reports about the long-distance movement of viruses, which spread through the plant via the phloem, the model proposed by Kang et al. was supported. When a leaf was inoculated with a virus, leaves positioned at the rosette in n+5 and n+8 were predominantly infected by the virus (Roberts et al., 2007). Like carbon transport, infection of new leaves was restricted exclusively to sink leaves.

Many carbon translocation studies on the whole plant level have been performed in the past (e.g., sugar beet, tobacco, soybean and tomato). Although carbon translocation appears to be similar in principal, the extent of carbon export and the sink-source relations vary strongly between the species. In Arabidopsis, carbon translocation is not well described so far. $^{14}$C labeling was mainly performed to answer specific questions, e.g. in mutants altered in central carbon metabolism (Zeeman and ap Rees, 1999; Nielsen, 2002; Schneider et al., 2002; Zeeman et al., 2002). In these experiments, either whole plants or detached leaves were labeled and the distribution of carbon was analyzed after certain time periods. To analyze carbon transport between source and sink tissues and to be able to link carbon partitioning with leaf growth, a systematic analysis of the transport processes in Arabidopsis is fundamental.
Therefore I developed an improved labeling system. The system allows isotopic labeling of individual leaves in two leaf chambers - either for mature leaves or for developing leaves. Labeling individual leaves opens the possibility to follow carbon allocation in temporal and spatial dimensions. Furthermore, it allows comparison of carbon partitioning between tissues and for changes of carbon partitioning according to the developmental stage to be monitored. The aim of the work presented in this chapter is to illustrate carbon transport on a whole plant level in Arabidopsis.

Figure 18: Models of the vascular pattern in an Arabidopsis vegetative shoot. (A) Model of the vasculature proposed by Busse and Evert (1999). Numbers mark the leaf traces and lines their interconnections. Leaf 1 and leaf 2 are connected to both vascular traces from the cotyledons, leaf 3 is connected to a cotyledon and leaf 1, leaf 4 is connected to a cotyledon and leaf 2. Leaf 5 marks an exception as it is connected to the same cotyledon trace as leaf 3 and to leaf 2. From leaf 6 on, each leaf (n) is connected to the leaves two and three positions lower on the rosette (n-3, n-2). C, cotyledon trace. (Adapted from Busse and Evert, 1999). (B) Model of the vasculature proposed by Kang et al. (2003). Numbers mark the rosette leaves and solid lines represent the vascular bundles that connect the leaves n+5 and the connections to the first leaves (cotyledons and leaf 1-5). The dashed lines represent the vascular bundles that connect the leaves n+8. The cotyledons and leaf 1-4 are connected directly to the vasculature of the hypocotyl. Leaf 5 is connected via a vascular bundle originating from leaf 2 (n-3). From leaf 6 on, each leaf is connected with a leaf trace five positions lower in the rosette (n-5) and from leaf 9 on, each leaf is connected to two leaf traces which are five and eight positions lower on the rosette (n-5, n-8). C, cotyledon. (Adapted from Kang et al., 2003).

Autoradiograms were performed to demonstrate the pattern of carbon allocation. Quantitative measurements by liquid scintillation counting revealed the allocation of carbon from source to sink tissues and the time course of carbon flux into sink tissues and into
major metabolic compounds. Furthermore the influence of the developmental stage of leaves on carbon allocation is shown.

4.2 Results

4.2.1 Control experiment

Prior to any labeling experiment, the setup of the labeling chambers was tested for airtightness. Only a sealed chamber allows the analysis of transport processes from a labeled leaf to unlabeled tissues. In a first test, leaf 8 was clamped into the chamber while it was still attached to the rosette. Just before opening the valves to allow flow of $^{14}$C-containing air to the small leaf chamber, the petiole of leaf 8 was cut. After 10 min labeling, the rosette was harvested as well as the cut leaf and both were exposed to a film (Figure 19, A and D). The cut leaf gave a strong signal on the film, but signal from the rosette was hardly visible. Small leaves standing next to the labeled one gave a faint signal. This is probably due to $^{14}$CO$_2$ released from the chamber while attaching and removing the leaf. The same experiment was repeated and the labeled leaf as well as the unlabeled rosette were harvested separately and the amount of $^{14}$C incorporated into each part was determined by liquid scintillation counting. In the labeled leaf, around 800'000 counts per minute (cpm) were detected, while in the unlabeled rosette only 1'200 cpm were measured. This is 0.15% of the overall label found in the plant. From these results it can be deduced that if the chamber is properly attached to the leaf, negligible amounts of $^{14}$CO$_2$ escape from the chamber that can be fixed by other leaves of the rosette.

The labeling of the leaves and the chase period took place in the same fume hood under the same fluorescent bulbs. In theory, $^{14}$CO$_2$ can escape during the experiment from the chamber or it can be released from respiring labeled plants. To evaluate the amount of $^{14}$CO$_2$ accumulating within the fume hood a second control experiment was performed. For the test, a plant was positioned next to a labeled plant for a complete pulse and chase period without being labeled itself. Subsequently the plant was harvested and exposed to a film. The autoradiogram shows a faint signal for the leaves orientated to the labeled plant (Figure 19, B and E). The same experiment was repeated and the rosette was harvested in
order to analyze the amount of $^{14}$C incorporated into the tissue by liquid scintillation counting. The plants labeled during this experiment accumulated 80’000 cpm, while the control plant positioned next to the labeled ones accumulated 80 cpm (0.1% compared to labeled plants). These data suggests there is negligible accumulation of $^{14}$C underneath the fume hood during the pulse-chase experiment. Additional assimilation of $^{14}$CO$_2$ during the chase period will therefore not have any influence on the labeling pattern.

![Figure 19: Test of the single leaf labeling chamber](A-C) Pictures and (D-F) autoradiograms of Col-0 rosettes labeled with $^{14}$CO$_2$. (A/D) Leaf 8 was introduced to the chamber when still attached to the rosette. Just before labeling for 5 min with $^{14}$CO$_2$, the petiole of leaf 8 was cut. (B/E) The rosette was placed next to the labeled plants for a whole pulse and chase period. (C/F) Demonstration of a distribution pattern when a leak is introduced during the labeling process. Leaf 8 was labeled in a not properly closed labeling chamber for 5 min followed by a chase period of 1 h. The leaves are numbered according to the sequence of emergence; starting with 1 for the first emerging true leaf. Plant material was exposed to the film for 7 days.

To demonstrate the distribution pattern of an untight chamber, a leak was introduced while labeling a single leaf. Leaf 8 was labeled with $^{14}$CO$_2$ for a pulse period of 5 min in a leaky chamber, followed by a chase period of 1 h. After the chase period the plants were harvested, dried and exposed to a film (Figure 19, C and F).

A strong signal was seen for leaf 8 and for all developing leaves younger than leaf 14. Leaves older than leaf 14 give a weaker signal, which decreased the further away the leaf was
positioned from the labeling chamber. The mature leaves like leaf 3, 5 and 13, which are adjacent to leaf 8, showed a clear signal. Leaves further away from the labeling chamber, like leaf 4, 7 and 12 were only slightly visible. Additionally, an increase in signal was apparent in the younger leaves. This might be due to accumulation of radioactive signal as the young leaves import carbon from several source leaves, in addition to carbon assimilation, while the fully developed leaves export carbon. Two distinct labeling patterns can be seen for single leaves. Some leaves show an even distribution of carbon and some show a stronger signal in the veins. A clear signal in the veins is an indication for transport into the leaf. An evenly distributed signal on the other hand is either a sign of a leak – i.e. the CO₂ was assimilated of the leaf itself - or that the imported compounds are already evenly spread throughout the leaf, which can be seen mostly for young importing leaves. However, the labeling pattern of the rosette after introduction of a leak clearly differed from the pattern derived with a sealed chamber (Figures 20-23).

4.2.2 Export of carbon to neighboring leaves and the root

In the following experiments, the system was used to illustrate the pattern of carbon allocation from source tissues to sink tissues in Arabidopsis either qualitatively by autoradiograms or quantitatively by measuring the amount of incorporated radioactivity via liquid scintillation counting. The first experiment aimed to reveal if a distinct pattern of carbon translocation can be seen in Arabidopsis. Therefore, different mature leaves were labeled, and the translocation of label into rosette leaves was analyzed. Plants used in this experiment were grown on soil under standard conditions. They had 17 to 19 leaves and no inflorescence was visible at the time of the experiment. During the labeling experiment, a single leaf was labeled with ¹⁴CO₂ for 5 min, followed by a 1-h chase in normal air. After the chase, plants were harvested, dried and exposed to a film.

Three mature leaves were chosen for the labeling experiment. Considering the first true emerging leaf as leaf 1, leaf 4, leaf 6 and leaf 8 were labeled with ¹⁴CO₂ (Figure 20). All three labeled leaves showed a strong signal on the autoradiogram, confirming that they assimilated high amounts of radioactive CO₂ during the pulse. Additionally, signals from other leaves were detectable. Labeling leaf 4 (Figure 20, A and D) resulted in a strong signal
Carbon allocation and carbon transport in *Arabidopsis thaliana*

in leaf 17 and a weaker signal in leaf 9 and 12, mainly along the major veins. It appears that the overall signal intensity decreases with leaf age, but this is difficult to evaluate on the basis of autoradiograms alone. In leaf 12, the base of the leaf was labeled strongly, but in the leaf tip only the major veins were labeled. When leaf 6 was labeled (Figure 20, B and E) a strong signal could be seen in leaf 11 and 14. Additionally, much weaker signals could be seen for most of the other rosette leaves, corresponding to background signal. Labeling leaf 8 (Figure 20, C and F) led to a strong signal in leaf 13 and a labeling along the major vein system in leaf 10 and 11. Also here most of the other rosette leaves showed a weak background signal.

![Figure 20: Autoradiograms to visualize carbon transport within an Arabidopsis rosette.](image)

Export from the labeled leaf shows a similar pattern in the three autoradiograms. In each case, carbon import is restricted to young, neighboring leaves. Young leaves on the opposite side of the rosette did not import significant amounts of labeled carbon, nor did leaves older than the labeled leaf, irrespective of their rosette position. Very small developing leaves show a strong signal evenly dispersed over the whole leaf blade, but older, still developing
leaves show a signal mostly restricted to the veins. For several leaves, e.g. leaf 12 in Figure 20 D, an increase of signal towards the leaf base could be seen. This pattern was observed on many autoradiograms in this work and is restricted to leaves of a length of 1.5 cm to 2.4 cm (not all autoradiograms are shown). It could be that the restriction of label to the veins is due to a too short chase period and the label is still moving into the leaf blade or that these leaves are in the process of sink-source transition.

To quantify the amount of exported carbon from a single leaf, leaf 8 was labeled as described above. After the chase, the plants were either harvested to perform autoradiograms (Figure 21, A and B) or each leaf was harvested separately to quantify the amount of radioactive label by liquid scintillation counting (Figure 21, C).

![Figure 21: Qualitative and quantitative representation of carbon export within an Arabidopsis rosette. (A) Picture and (B) autoradiograms of a Col-0 rosette labeled with $^{14}$CO$_2$. (C) Quantitative analysis of the amount of radioactive carbon within the different rosette leaves. Mean ± SE (n=4). In the experiment, leaf 8 was labeled for 5 min, followed by a chase period of 1 h. Subsequently the rosette was either dried for autoradiography or the leaves were harvested separately for quantitative analysis. Leaf 1 marks the first emerging true leaf. L1 leaf 1, L2 ..., R remaining leaves and meristem. Plant material was exposed to the film for 9 days.]

Of the total amount of $^{14}$C incorporated within the pulse, 91% remained in the labeled leaf after the chase. Labeled carbon could also be detected in leaf 13 (1.3%), leaf 16 (3%) and the
remaining part containing all leaves smaller than leaf 16 and the shoot apex (4.1%). In all other harvested leaves, 0.02% to 0.2% of label could be measured, accounting in total for less than 0.8% of the exported carbon. These data confirm the results gained from the autoradiograms (Figure 20 and Figure 21, A and B). Most of the exported carbon is allocated to small developing leaves and transport mainly occurs into leaves adjacent to the labeled leaf.

Leaf 13, the biggest leaf importing carbon from leaf 8, was labeled in an additional experiment to elucidate whether it is only importing carbon, or acting as an importing and exporting leaf simultaneously. The experiment was performed exactly as described before, but leaf 13 was labeled instead of leaf 8 (Figure 22, A and C). Leaf 13 gives a clear signal on the autoradiogram suggesting that it incorporated a sufficient amount of $^{14}$C during the pulse. In contrast to autoradiograms performed after labeling leaf 8, no strong signals from other leaves were detected. A slight signal was seen for leaves 15, 16 and 18. Thus leaf 13 seems to import more carbon from leaf 8 (Figure 21, A and B) than it exports to other leaves.

Additionally, we wanted to elucidate if the movement of carbon can be reversed when a mature leaf turns back into a sink leaf. For this, leaf 5 was darkened 48 h prior to the labeling experiment. While leaf 5 was still darkened, leaf 10, a mature leaf standing next to it, was labeled as described above. After the chase the plants were harvested, the aluminum foil used to darken the leaf was removed and the plants were exposed to a film (Figure 22, B and E). Leaf 10 was strongly labeled after the pulse, but no signal was visible in leaf 5. The signal was restricted to leaf 18 and to the vein systems of leaves 13 and 15. Although it can be expected that leaf 5 has a high sugar demand after 48 h in the dark and should act as a sink, carbon was not transported to the leaf, at least from leaf 10.
Carbon allocation and carbon transport in *Arabidopsis thaliana*

Figure 22: Autoradiograms to visualize carbon transport within Arabidopsis rosettes. (A-B) Pictures and (C-D) autoradiograms of Col-0 rosettes labeled with $^{14}$CO$_2$. (A/C) Leaf 13 was labeled for 5 min followed by a chase period of 1 h. (B/D) Leaf 5 was covered 24 h before labeling leaf 10 for 5 min followed by a chase period of 1 h. The leaves are numbered according to the sequence of emergence; starting with 1 for the first emerging true leaf. Plant material was exposed to the film for 2 days.

Carbon transport was only monitored within the rosette so far, but not from rosette leaves into the roots. Therefore, plants were grown in hydroponics and leaf 8 was labeled with $^{14}$CO$_2$ as described above. Subsequently, autoradiograms were performed illustrating carbon transport on a whole plant level (Figure 23).

The same pattern of carbon allocation could be seen as in the previous autoradiograms. Labeling leaf 8 led to a strong signal in leaf 13 and leaf 16 and a signal in the veins of leaf 10. Additionally the root gave a strong signal. This means that carbon from one leaf is simultaneously transported up the rosette to young, developing leaves at the shoot apex and down the rosette into the root system.
Carbon allocation and carbon transport in *Arabidopsis thaliana*

4.2.3 Time course of carbon export to the root and shoot

In all previous labeling experiments, a 1 h chase period was applied before analyzing the plant tissue. To visualize the dynamics of carbon export from a source leaf into sink leaves time course experiments were carried out. In a first experiment, autoradiograms were performed with *Arabidopsis* shoots only. Therefore, leaf 8 was labeled with $^{14}$CO$_2$ for 5 min and the chase period was varied from 0 min to 180 min. After the chase period the plants were harvested, dried and exposed to a film (Figure 24).

The autoradiograms showed a progressive movement of carbon from the labeled source leaf (leaf 8) into the neighboring sink leaves. Although care was taken to label plants of the same size and developmental stage, no consistent movement of label into leaf 13 could be seen, but movement into either leaf 13, leaf 16 or leaf 19.

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Figure 23: Autoradiogram to visualize carbon transport to sink tissues. (A) Picture and (B) autoradiogram of a Col-0 plant labeled with $^{14}$CO$_2$. Leaf 8 was labeled for 5 min followed by a chase period of 1 h. The leaves are numbered according to the sequence of emergence; starting with 1 for the first emerging true leaf. Plant material was exposed to the film for 13 days.
Carbon allocation and carbon transport in *Arabidopsis thaliana*

Figure 24: Autoradiograms to visualize the temporal dynamics of carbon transport within an Arabidopsis rosette. Pictures (A-C, I-K) and autoradiograms (D-H, L-N) of Col-0 rosettes labeled with $^{14}$CO$_2$. In all experiments leaf 8 was labeled for 5 min followed by varying chase periods of 0 min (A, D), 5 min (B, E, F), 15 min (C, G, H), 30 min (I, L), 60 min (J, M) and 180 min (K, N). In (F) and (H) a close-up of the region marked with the red arrow is shown. The leaves are numbered according to the sequence of emergence starting with 1 for the first emerging true leaf. Plant material was exposed to the film for 2 days.
After a 5 min $^{14}$CO$_2$ pulse the labeled leaf gave a clear signal, but no movement of label out of this leaf was visible yet (Figure 24, A and D). However, after just 5 min chase in normal air, a clear signal could be seen in the neighboring leaf (leaf 13; Figure 24, B and E). The signal appears as a precisely separated line which probably marks the major vein (Figure 24, F). Ten min later (15 min chase), the signal in the sink leaf had spread from the major vein into the minor vein system (Figure 24, C, G and H). From 30 min chase on, uniform signal across the whole leaf blade of the sink leaf could be seen (Figure 24, I-N), suggesting the labeled carbon was distributed evenly throughout the leaf. After 180 min chase only a faint signal in the sink leaves was visible at all, but no further spreading of the signal to sink leaves standing on the opposite site of the labeled leaf could be seen after the prolonged chase. The data show that autoradiographs are not the most suitable method for time course labeling experiments, as the amount of export is varying strongly with the size of the sink leaf and the signal strength is varying. The signal strength is differing presumably as a result of different amounts of radioactive carbon assimilated by the leaf. Quantitative measurements are therefore always depicted as relative values based on the overall incorporated signal. This is not possible for autoradiograms, which limits their application.

Because of the limited resolution of autoradiograms, a quantitative analysis of carbon was performed. Arabidopsis plants were grown in hydroponics to be able to quantify the export into the sink leaves and the root system. Leaf 8 of plants with 16 to 20 leaves was labeled in the middle of the light period with $^{14}$CO$_2$ for 5 min, followed by a chase in normal air varying from 0 min to 360 min. The labeled leaf, the unlabeled leaves and the root were harvested separately after the chase periods and the amount of carbon allocated to the different tissues was determined (Figure 25).

Export to the unlabeled rosette leaves (sink leaves) was already seen after the pulse. One percent of the labeled carbon was detected in the sink leaves immediately after the 5 min pulse. After a 15 min chase, 2.5% of label were detected within the sink leaves. Export increased steadily until 180 min after the pulse to a value of 16%. After that, export values remained stable. Export of label to the roots was slightly delayed compared to export into rosette leaves. A significant amount (3%) of labeled carbon could be detected only after
30 min. The amount of imported carbon increased in the following 30 min up to 7.5% (after 1 h chase) and remained at around 7.5% - 9% for the remaining chase period. After 360 min chase even a slight decrease in the amount of labeled carbon was seen.

**Figure 25: Export of \(^{14}\)C to the root and rosette leaves.** Leaf 8 of Col-0 plants was labeled in the middle if the light period for 5 min with \(^{14}\)CO\(_2\), followed by chase periods of 0, 15, 30, 60, 180 or 360 (EoD) min. At each time point, the labeled leaf, the unlabeled (sink) leaves and the root were harvested separately, and the incorporation of \(^{14}\)C was determined. The sum of the label in the labeled leaf, the sink leaves and the root is set to 100%. Mean ± SE (n=5).

CO\(_2\) release from the plants was not measured during the chase period, hence it is possible that labeled carbon was lost in the course of the experiment due to respiration. This would lead to an underestimation of the exported amount of carbon and can be expected especially for the root. Indeed, a decrease in total counts of the whole plants could be seen after chase periods longer than 180 min.

Based on the results of this experiment it was concluded that a 60 min chase period is suitable to analyze carbon export for the following reasons. First, a major part of carbon export takes place within the first hour. From a maximum of 25% of exported carbon, after 1 h 18% were already exported from the labeled leaf. Second, the amount of total carbon within the plants does not change significantly within the first hour after the labeling experiments. This suggests that only a small portion of labeled carbon is lost due to respiration during this time. Third, a 60 min chase period in the light is suitable to perform experiments at different time points over the day without interfering with the day-night cycle of the plant.
4.2.4 Time course of carbon partitioning into major metabolic pools

The previous experiments demonstrated the translocation of carbon from source to sinks. However, $^{14}$CO$_2$ labeling can also be used to analyze the partitioning of carbon into the different major metabolite pools and biosynthetic end-products. Therefore, the plant material was fractionated into soluble and insoluble compounds after isotopic labeling. The soluble compounds were further fractionated by ion-exchange chromatography into neutral, acidic and basic compounds. The neutral fraction mainly contains sugars, like sucrose, glucose and fructose. The acidic fraction contains mainly organic acids and sugar phosphates, and the basic fraction contains amino acids. Likewise, the insoluble fraction was processed by specific enzymatic digestions. Thereby, the amount of carbon introduced into starch, proteins and other insoluble compounds, like cell wall material, was identified. The proportion of the total assimilated label found in each fraction was determined via liquid scintillation counting. Assuming that $^{14}$C labeled carbon is partitioned in the same way as $^{12}$C carbon, this data can reveal the amount of assimilated carbon channeled into different metabolic pathways.

To assess carbon flow in source and sink tissues, a time course experiment was performed. The plants for the experiment were grown on soil under standard conditions. Leaf 8 was labeled with $^{14}$CO$_2$ for 5 min followed by chase periods between 0 min and 180 min. After the chase, the labeled leaf and the unlabeled leaves were harvested separately, and the amount of carbon partitioned to the different compound classes was determined (Table 2).

After the pulse, most of the labeled carbon was found in the soluble fraction of the labeled leaf (~80%). Within the soluble fraction, the basic compounds contained most of the labeled carbon, followed by acidic compounds, neutral compounds and nucleotides. The amount of label decreased tremendously after a chase of 15 min in all fractions, except the neutral fraction, indicating that most of these metabolites are rapidly turned over. These compounds are probably Calvin-Benson cycle or photorespiratory intermediates. With a further increase in chase period, the amount of carbon in the neutral fraction started to decrease from a maximum of 22% after 15 min chase, to 6% after 180 min chase. This
probably represents sucrose, which is rapidly labeled after $^{14}$CO$_2$ application, but is then either metabolized within the tissue or exported to sink tissues.

Table 2: Time course of carbon partitioning into the major carbon compound classes in the labeled leaf and the importing sink leaves. Leaf 8 of Col-0 plants was labeled in the middle of the light period for 5 min with $^{14}$CO$_2$, followed by a chase period of 0, 15, 30, 60 or 180 min. After the chase, the labeled leaf and the unlabeled rosette (sink leaves) were harvested separately and the incorporation of $^{14}$C was determined. The sum of the label in the labeled leaf and the sink leaves is set to 100%. The relative amount of $^{14}$C incorporated into the different compound classes as a percentage of the total label in the plant is shown. The red values indicate the label in each fraction, when the sum of total label in the sink is set to 100%. Mean ± SE (n=5).

<table>
<thead>
<tr>
<th>% of total $^{14}$C recovered in each fraction</th>
<th>After pulse</th>
<th>15 min chase</th>
<th>30 min chase</th>
<th>60 min chase</th>
<th>180 min chase</th>
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</thead>
<tbody>
<tr>
<td><strong>Labeled leaf</strong></td>
<td>97.5 ± 0.7 (100)</td>
<td>96.7 ± 0.9 (100)</td>
<td>93.9 ± 0.7 (100)</td>
<td>91.5 ± 1.0 (100)</td>
<td>89.0 ± 1.0 (100)</td>
</tr>
<tr>
<td>Water soluble compounds</td>
<td>80.2 ± 3.0 (77)</td>
<td>61.7 ± 3.2 (59)</td>
<td>55.7 ± 2.2 (59)</td>
<td>46.7 ± 1.0 (46)</td>
<td>43.6 ± 4.1 (45)</td>
</tr>
<tr>
<td>Neutral</td>
<td>14.8 ± 1.4 (15)</td>
<td>22.3 ± 1.3 (23)</td>
<td>21.4 ± 1.8 (23)</td>
<td>10.6 ± 1.7 (12)</td>
<td>6.5 ± 0.8 (7)</td>
</tr>
<tr>
<td>Acidic</td>
<td>20.6 ± 0.8 (20)</td>
<td>13.7 ± 0.7 (14)</td>
<td>15.3 ± 1.7 (16)</td>
<td>18.0 ± 1.5 (20)</td>
<td>22.6 ± 1.8 (25)</td>
</tr>
<tr>
<td>Nucleotide</td>
<td>7.4 ± 0.6 (7)</td>
<td>0.9 ± 0.1 (1)</td>
<td>0.6 ± 0.1 (1)</td>
<td>0.5 ± 0.0 (1)</td>
<td>0.5 ± 0.0 (1)</td>
</tr>
<tr>
<td>Basic</td>
<td>25.1 ± 1.6 (25)</td>
<td>13.8 ± 1.2 (14)</td>
<td>10.6 ± 1.2 (11)</td>
<td>7.5 ± 0.5 (8)</td>
<td>4.8 ± 0.5 (5)</td>
</tr>
<tr>
<td>Insoluble compounds</td>
<td>17.1 ± 1.7 (17)</td>
<td>30.6 ± 2.8 (31)</td>
<td>34.4 ± 1.7 (37)</td>
<td>41.3 ± 2.4 (43)</td>
<td>40.0 ± 4.2 (45)</td>
</tr>
<tr>
<td>Starch</td>
<td>16.6 ± 1.4 (17)</td>
<td>26.6 ± 2.1 (28)</td>
<td>27.5 ± 2.1 (29)</td>
<td>31.7 ± 2.9 (34)</td>
<td>33.0 ± 2.5 (37)</td>
</tr>
<tr>
<td>Protein</td>
<td>0.8 ± 0.2 (1)</td>
<td>3.0 ± 0.6 (3)</td>
<td>2.9 ± 0.4 (3)</td>
<td>4.3 ± 0.4 (5)</td>
<td>3.8 ± 0.4 (4)</td>
</tr>
<tr>
<td>Remaining insoluble</td>
<td>0.3 ± 0.1 (0)</td>
<td>1.9 ± 0.6 (2)</td>
<td>2.2 ± 0.3 (2)</td>
<td>3.5 ± 0.2 (4)</td>
<td>3.3 ± 0.3 (4)</td>
</tr>
<tr>
<td>$\text{EtOH}$ soluble compounds</td>
<td>1.7 ± 0.1 (2)</td>
<td>3.9 ± 0.6 (4)</td>
<td>3.8 ± 0.3 (4)</td>
<td>5.1 ± 0.2 (6)</td>
<td>5.0 ± 0.2 (6)</td>
</tr>
</tbody>
</table>

| **Sink leaves**                               | 2.1 ± 0.6 (100) | 3.5 ± 0.8 (100) | 6.1 ± 1.7 (100) | 8.5 ± 1.0 (100) | 11.0 ± 1.0 (100) |
| Water soluble compounds                       | 1.9 ± 0.6 (75)  | 2.7 ± 0.5 (79)  | 4.4 ± 0.4 (73)  | 4.2 ± 0.4 (50)  | 4.7 ± 0.4 (43)  |
| Neutral                                       | 0.4 ± 0.1 (11)  | 1.5 ± 0.2 (47)  | 2.2 ± 0.3 (37)  | 1.0 ± 0.2 (13)  | 0.6 ± 0.1 (6)   |
| Acidic                                        | 0.7 ± 0.2 (25)  | 0.5 ± 0.1 (12)  | 0.9 ± 0.1 (14)  | 1.1 ± 0.2 (14)  | 1.3 ± 0.1 (12)  |
| Nucleotide                                    | 0.1 ± 0.0 (1)   | 0.0 ± 0.0 (0)   | 0.0 ± 0.0 (0)   | 0.1 ± 0.0 (1)   | 0.1 ± 0.0 (1)   |
| Basic                                         | 1.0 ± 0.3 (35)  | 0.7 ± 0.2 (19)  | 1.2 ± 0.1 (20)  | 1.8 ± 0.1 (23)  | 2.0 ± 0.2 (21)  |
| Insoluble compounds                           | 0.6 ± 0.1 (22)  | 0.8 ± 0.3 (19)  | 1.5 ± 0.3 (24)  | 4.0 ± 0.9 (44)  | 5.7 ± 0.6 (52)  |
| Starch                                        | 0.5 ± 0.1 (16)  | 0.4 ± 0.2 (9)   | 0.7 ± 0.2 (11)  | 1.2 ± 0.3 (11)  | 1.9 ± 0.4 (17)  |
| Protein                                       | 0.1 ± 0.0 (2)   | 0.1 ± 0.1 (3)   | 0.3 ± 0.1 (4)   | 0.7 ± 0.1 (9)   | 1.3 ± 0.2 (11)  |
| Remaining insoluble                           | 0.1 ± 0.0 (2)   | 0.2 ± 0.1 (4)   | 0.4 ± 0.1 (7)   | 1.1 ± 0.1 (14)  | 2.0 ± 0.3 (18)  |
| $\text{EtOH}$ soluble compounds               | 0.1 ± 0.0 (4)   | 0.1 ± 0.0 (4)   | 0.2 ± 0.0 (3)   | 0.4 ± 0.1 (6)   | 0.6 ± 0.2 (5)   |

After the rapid decrease of label in the acidic fraction from 0 min to 15 min chase, the amount of label increased slightly from 14% to 23% after 180 min chase. Possibly, organic acids like malate, citrate and fumarate are built up to serve as storage compounds within the vacuole. In contrast, label in the basic compounds kept decreasing after the rapid drop
Carbon allocation and carbon transport in *Arabidopsis thaliana* during the first 15 min chase period. From the 25% of label introduced into the basic fraction just after labeling, only on fifth remained after a chase period of 180 min. As basic compounds represent amino acids, it is likely that they are used to build up proteins. Therefore, the label is depleted continuously and would appear more and more in the protein fraction.

While 80% of the label could be found in the soluble fraction right after the labeling pulse, 17% were introduced into insoluble compounds (mainly starch). Carbon partitioning into starch is rapid, as half of the label that could be detected in starch after 180 min chase was already found in starch after the 5-min pulse. The amount of label in starch further increased from 17% to 27% in the following 15 min chase period, followed by a slight increase to 33% over the rest of the chase period. This might be a slight overestimation, as carbon export into the roots is not monitored in this experiment. With the longer chase, more carbon is exported; less label is retained in the rosette and therefore, the relative amount of $^{14}$C in the starch fraction increases. In other insoluble compounds, like proteins and cell wall material, hardly any label could be detected immediately after the labeling, but the amount of label was increasing steadily over the first hour of the chase. Only minor changes could be seen in the additional two hours chase. Similar to the increase in label in proteins and cell wall, carbon partitioning to ethanol soluble compounds, representing lipids, pigments and waxes, increased steadily from 1.7% immediately after labeling, to 5.1% after 1 h chase. No more label was introduced into the ethanol soluble compounds in the subsequent 2 h. After 180 min chase, 11% of the label was exported to the sink leaves, which was a bit less compared to the plants grown on hydroponics (see above).

Numerous changes can be identified in partitioning within the sink leaves, compared to the labeled source leaf. To simplify the interpretation of the results, the amount of radioactive label in each fraction was related to the total label in each tissue (labeled leaf or unlabeled leaves), rather than to the overall label in the plant (red values in Table 2). This allows easy comparison of the partitioning in the labeled source leaf and the unlabeled sink leaves. In the early chase periods, the differences in partitioning are mainly due to the large amount of label in the soluble fraction, specifically from the labeling of neutral compounds. This is
Carbon allocation and carbon transport in *Arabidopsis thaliana*

expected as sucrose, which is found in the neutral fraction, is the major transported form of carbon in Arabidopsis. The amount of label in the neutral fraction (sucrose) peaked 30 min after labeling. Subsequently, label in the neutral fraction decreased as in the source leaf. The amount of label found in the acidic and basic fractions did not correspond to the partitioning within the source leaf. While the amount of label in the acidic fraction was drastically decreased (only half of the label compared to the source leaf), the amount of label in the basic fraction was four times higher than in the labeled leaf. Likewise, the amount of label partitioned into insoluble compounds was higher in the sink leaves than in the source leaf. This is due to three times as much carbon being partitioned into proteins and nearly five times as much carbon being partitioned into the cell walls. Less label was introduced into starch in the unlabeled leaves compared to the labeled leaves. However, it is surprising that imported carbon was incorporated into starch, as so far, no transporter importing starch precursors into the chloroplast is described.

### 4.2.5 Carbon export in different developmental stages

The preceding autoradiograms showed carbon export from mature leaves (Figure 20, Figure 21 and Figure 22), but only minor export from small, still developing leaves (leaf 13, Figure 22, A and C). To elucidate when the switch from an importing leaf to an exporting leaf takes place in Arabidopsis, leaf 8 was labeled at four different time points when the plants had an age between 25 to 35 days. During this period, leaf fresh weight increased from 0.012 g to 0.059 g. According to the growth curve shown in Figure 26 B, this is a range where the leaf has reached 10% to 60% of its final size, and the relative growth rate declines from 62% to 9% growth per day. The plants used in this experiment were grown in hydroponics, and leaf 8 of 25, 28, 32 and 35 days old plants was labeled with $^{14}$CO$_2$. Surprisingly, export from leaf 8 into the sink leaves as well as into the root could be seen at all four time points (Figure 26 A). The relative export to the root increased from 3.4% to 7.4% of total label with the age of the leaf. Export to the sink leaves, on the other hand, did not vary significantly with the age. However, the overall export increased gradually from 9.9% in 25 days old plants, to 10.7%, 12.6% and finally 15.3% in 35 days old plants. Even though this seems to be only a minor increase in carbon export, leaf 8 of 35 days old plants assimilated in average 6.2 times more carbon than leaf 8 of 25 days old plants, accounting for an absolute increase in carbon
export of 9.6 times. The low absolute export of carbon from developing leaves might explain the rather faint signals on the autoradiograms (Figure 22).

Figure 26: Export of $^{14}$C from leaf 8 to the root and rosette leaves at different developmental stages. Leaf 8 of Col-0 plants aged 25, 28, 32 and 35 days was labeled in the middle of the light period for 5 min with $^{14}$CO$_2$, followed by a chase period of 1 h. After the chase the labeled leaf, the unlabeled (sink) leaves and the root were harvested separately. (A) The relative incorporation of $^{14}$C into the sink leaves and the root. The sum of the label in the labeled leaf, the sink leaves and the root is set to 100%. The asterisks indicate a significant difference compared to export at 25 days plant age (t-test; significance levels: *, p <0.05). Mean ± SE (n≥3). (B) Fresh weight and relative growth rate (RGR) of the labeled leaf (leaf 8). The highlighted area marks the size range at which leaf 8 was labeled. Mean (n≥5).

4.3 Discussion

The labeling experiments presented here are dynamic pulse-chase experiments. A short pulse in $^{14}$CO$_2$ labeled air is followed by a chase period in normal atmosphere. Carbon assimilated during the pulse remains in the labeled leaf for biosynthetic processes, storage and for cell maintenance. However, a significant part of the assimilated carbon is exported to sink tissues such as developing leaves and the root. Although it is considered to be very low, respiration from both the shoot and the root might occur during the chase, which was not measured. Zeeman et al. (1999) observed no significant release from the shoots during a 5 h chase period and only 3% of label was released from the roots during the same time. Hence, the chase periods were kept minimal to reduce loss of label due to respiratory processes.
Carbon allocation and carbon transport in *Arabidopsis thaliana*

*Carbon is exported from source leaves mainly to neighboring sink leaves*

The autoradiograms performed with the labeled mature leaves illustrate that carbon export occurs mainly into the neighboring developing leaves. Comparable results were obtained after labeling of different mature leaves (Figure 20, Figure 21 and Figure 23). Quantitative analysis of the incorporated radioactivity into single leaves support these results. The majority of autoradiograms performed after labeling a mature leaf n gave a signal in the leaves n+5, n+8 and, if already emerged, leaf n+13 (Figure 20, Figure 21 and Figure 23). This agrees with the model of vascular connections proposed by Kang et al. (2003). In this model, each true leaf (n) is connected via two leaf traces to leaves standing five (n+5) or eight (n+8) positions higher in the rosette. For leaf 4, this would mean a direct vascular connection to leaf 9 and 12. Further on leaf 9 is directly connected to leaf 14 and 17, and leaf 12 is connected to leaf 17 and 20. The autoradiogram of a rosette after labeling leaf 4 showed export to exactly these leaves. A weaker signal was seen for leaf 9 and 12 and a strong signal for leaf 17. While the directly connected leaves import only little carbon, presumably because they are already in the transition phase from sink to source leaves, carbon is imported predominantly by leaf 17 via the leaf traces of leaf 9 and 12. Infrequently, carbon import into the leaves n+2 and n+3 could be observed, e. g. after labeling leaf 8 (Figure 16 C and F, Figure 19). In these autoradiograms, carbon from leaf 8 was imported into leaf 13 (n+5) and leaf 16 (n+8), but additionally also into leaf 10 (n+2) and leaf 11 (n+3). Connection to the leaves n+2 and n+3 was reported by Busse and Evert (1999), but their model is not applicable to the majority of labeling patterns observed in this study. Both studies, however, reported irregularities in the vascular patterns. While Busse and Evert (1999) found the proposed pattern in four out of nine analyzed seedlings, Kang et al. (2003) found the exact same pattern for three out of five vegetative rosettes. This might be an indication for stochastic events during vascular pattern formation. Another possible explanation for carbon import into leaves n+2 and n+3 could be transport via leaves standing lower in the rosette. Carbon assimilated by leaf 8 is transported towards the root via the vascular connections of leaf 3 and leaf 5, by these connections transport upwards into leaf 10 and leaf 11 could be possible. However, the fine structure and the connections of the vascular strands still need to be resolved to fully explain transport processes within the phloem.
All autoradiograms and the quantitative data indicate that two to three leaves are importing carbon at the same time. While very young leaves show an even distribution of signal all over the leaf blade, older importing leaves show a decrease in signal from the base to the tip. These gradients in carbon import were observed also for other species (Jones and Eagles, 1962; Turgeon and Webb, 1973). The authors propose that changes in carbon translocation follow the basipetal maturation of the leaf. During leaf development, the tip matures first, while the base continuous growing and matures later (Avery, 1933). A gradient in radioactive signal was observed for leaves with a length of 1.0 to 1.8 cm. Considering a final length of 3.5 to 4.0 cm for a mature leaf (data from leaf 13, data not shown here), the transition from import to export takes place when the leaf has reached 25-50% of its final length. This suggests an early transition, in which a leaf is turning from a carbon sink to an exclusive carbon source. In sugar beet and in Curcubitacea, the sink-source transition was analyzed by autoradiography and quantitative measurements, and was shown to occur in a slightly later time period when the leaves had a final length of 30-60% (Turgeon and Webb, 1973; Fellows and Geiger, 1974; Turgeon, 1989). However, the proposed transition phase for Arabidopsis is only based on autoradiograms. More quantitative data for carbon importing and carbon exporting leaves is necessary to estimate the time of the sink-source transition precisely.

**Carbon is exported simultaneously to sink leaves and the root**

The gradual sink-source transition of leaves implies that for this time span, carbon transport within the leaf has to be bidirectional, as carbon is simultaneously imported from the base and exported from the tip of the leaf. Bidirectional transport can not only be seen on a single leaf level, but also on the whole plant level. When a leaf of a whole plant, including the roots, is labeled, carbon is transported into the young, neighboring leaves as well as into the roots. This simultaneous transport towards the tip and the root was seen for all labeled leaves and has been described also in soybean, tobacco and sugar beet (Aronoff, 1955; Crafts, 1956; Jones et al., 1959; Joy, 1964). It suggests that at certain regions within the stem, bidirectional transport occurs. Bidirectional transport was shown to take place in different phloem bundles as well as in different sieve tubes within one bundle in Populus and beans (Biddulph and Cory, 1960; Vogelmann et al., 1982). It was never shown to occur
in the same sieve tube, which would also not agree with our current understanding of phloem function. So far, a difference between acropetally and basipetally transporting sieve tubes or bundles could not be demonstrated. Cell-specific markers for tobacco, however, suggest a more complex structure of the phloem than assumed so far. The expression of galactinol-synthase (GAS) in tobacco phloem shows a high expression restricted to one of two companion cells and its sieve elements. In all analyzed veins, GAS expression was found in the adaxial cells, but never in the abaxial cells (Haritatos et al., 2000). Whether this also reflects a difference in the direction of transport is not resolved so far. It is obvious that separate sieve tubes are necessary to achieve a bidirectional flow, therefore also structural differences can be assumed. Further investigation of the Arabidopsis phloem would be necessary to verify the existence of different phloem strands and to understand how bidirectional flow is accomplished within the plant and the extent to which it occurs.

*Carbon export is occurring already in young leaves*

To elucidate the time point of sink-source transition within Arabidopsis leaves, leaf 8 at different developmental stages was labeled and export rates during the day to the root and shoot were analyzed. The data shows that already young, developing leaves translocate a significant amount of assimilated carbon into other sink tissues. The youngest labeled leaf (10% final size) exported 9.9% overall, 3.9% to the root and 6% to sink leaves. The oldest labeled leaf (60% final size) exported 15.3% overall, 8% to the root and 7.4% to sink leaves. Import of carbon into the leaves was not tested in this experiment, but from the previous data it can be assumed that leaf 8 with a final size of 10-25% (the first two tested time points) is also importing significant amounts of carbon. However, even though the relative amount of exported carbon is not changing significantly with the developmental stage of the plant, the absolute amount of carbon export will change dramatically. As the leaves labeled in this experiment increase 6 times in size, the slight change in relative export from 9.9 to 15.3% is resulting in a 10-fold increase of absolute carbon export. In addition to carbon export during the day, export during the night can be expected as well. A large amount of carbon is used to build up the transient storage starch which is degraded during the night. The released carbon is used to maintain metabolism and is exported into developing tissues were it can be utilized for growth. It can be expected that older leaves export more carbon
reserves during the night compared to younger leaves, which invest these reserves into growth. However, following carbon export during the night is not practicable with the described labeling system. Light is needed for the assimilation of the labeled $^{14}\text{C}$, restricting the labeling period to the day. Any labeling during the day will introduce $^{14}\text{C}$ into different pools and tissues, which makes it difficult to monitor label released from starch during the night. Additionally, respiration will lead to a significant loss of label during the night. To enable calculation of carbon export at night respiratory losses need to be measured separately for the rosette and root.

*Carbon is allocated to the major metabolic pools within the first hour after assimilation*

In the time course experiment, a linear increase of radioactivity could be seen in sink tissues within the first hour after labeling a source leaf. In the subsequent 2 h, a moderate import into sink leaves was visible, but only little import into the root was still taking place. Overall, about 25% of label was exported from the labeled source leaves within the same photoperiod. In the literature, slightly higher export rates were described for sugar beet (Fellows and Geiger, 1974) and *Curculbita pepo* (Webb and Gorham, 1964; Turgeon and Webb, 1973). 24% of carbon was found to be exported to sink leaves in sugar beet after a chase period of 1 h. In *Curculbita pepo*, 50% of label from a single leaf was found to be exported after a chase period of 2 h, 16% of which were transported to the roots. Export processes in the dark have not been measured, but experiments by Zeeman et al. (1999) showed, 6.9% export to the root at the end of the day from a whole rosette and 22.7% at the end of the following night. Therefore, it can be assumed that carbon released from starch is transported to the sinks, significantly increasing the amount of exported carbon after a whole day-night cycle.

My time course experiment revealed carbon flow through intermediary metabolite pools towards the synthesis of end products, like cell wall and proteins. While the flow through pools, like nucleotides and basic compounds, was very fast and only visible right after the labeling pulse, carbon flow through neutral compounds, mainly sucrose, was detectable from the end of the labeling up to 30 min after labeling. The flow into end products was
Carbon allocation and carbon transport in *Arabidopsis thaliana*

varying, too. Carbon incorporation into starch was very fast, occurring mainly during the labeling period itself and finishing within 15 min after labeling. Carbon incorporation into proteins seems to take place in two phases; that is within the first 15 min after labeling and between 30 to 60 min after labeling. These two phases might be due to incorporation of amino acids derived from different sources. During photorespiration, glycine and serine are formed which can be used directly for protein synthesis. They might cause the first increase in label within proteins. All other amino acids are synthesized out of triose phosphates exported from the chloroplast. Incorporation of these amino acids into proteins might mark the second increase of label in this fraction.

Incorporation of carbon into the remaining insoluble products was also highest within the first 15 min after labeling, but proceeded until 60 min after the pulse. Only minimal changes could be seen between the 60 min chase period and the 180 min chase period in the labeled leaf. This suggests that most of the labeled carbon reached its final destination after 60 min. Naturally, a time shift was observed for carbon flow within the sink leaves as the carbon is imported progressively over the first hour. In the sink leaves, the maximum incorporation of carbon into proteins and cell wall material could be seen only after a chase of 180 min. However, there are more obvious differences in carbon partitioning between the source leaf and the sink leaf. The source leaf channeled the assimilated carbon towards starch and organic acids, both storage compounds, and exported a significant amount of carbon to sink tissues. The sink tissue on the other hand is supplied with carbon via sucrose from the source tissues. The carbon from sucrose is then channeled into cell wall material, basic compounds and proteins, but also into storage compounds. The carbon flow into cell wall material, basic compounds and proteins was increased 5, 4 and 2.5 times, respectively, compared to the source leaf. Carbon partitioning into storage compounds on the other hand was decreased two-fold. This marks clear differences in carbon partitioning between actively growing tissues and more mature tissues. Growing tissues partition more into amino acids and proteins to increase biosynthetic processes, which accompanies the synthesis of structural components. Simultaneously, less carbon is used to build up storage compounds. Nevertheless, about 17% of labeled carbon from sucrose was introduced into starch. This is surprising as sucrose is imported to the cytosol where it is either cleaved to
fructose and glucose by invertase or to fructose and UDP-glucose by sucrose synthase, or transported to the vacuole. Starch synthesis, on the other hand, takes place in the chloroplast. To accumulate radioactively labeled carbon in starch, precursors of starch have to be imported to the chloroplast (further discussion see section 5.3).

The data gained from the optimized $^{14}$C labeling setup of experiments showed a defined pattern of carbon export from a mature source leaf into neighboring sink leaves (for instance from leaf 8 to leaf 13). This can be used to compare carbon utilization of assimilated and imported carbon, one question that will be investigated in the following chapter. Moreover, the data revealed general differences in carbon partitioning in a mature leaf compared to a developing leaf. This suggests differences in utilization of carbon reserves which might be linked to the developmental stage and growth of the leaf. Whether carbon allocation is changing systematically with phases of high and low growth and how this is influenced by alterations in central carbon metabolism will be another focus of the next chapter.
5 Carbon partitioning and growth in rosettes of *Arabidopsis thaliana*

5.1 Introduction

After fixation of CO$_2$ via the Calvin-Benson Cycle, this carbon is used to build up all structural components of a plant, but also storage and signaling compounds. However, it is still not well understood how metabolic processes and growth are integrated. So far, most studies analyzed metabolism or growth on a whole plant level, without actually focusing on the actively growing tissues. With the isotopic labeling method described in the previous chapter, I wanted to analyze carbon fluxes in developing leaves of *Arabidopsis thaliana* and correlate carbon flow to phases of active growth.

Despite a constant influx of carbon via photosynthesis, leaf growth was demonstrated to vary over the diurnal cycle (Seneweera et al., 1995; Walter et al., 2005; Walter and Schurr, 2005). Nondestructive methods, like measuring leaf expansion rates, revealed growth patterns with a defined phase and shape. *Arabidopsis thaliana* leaves, for example, show highest growth rates at the beginning of the light period, followed by moderate growth over the remaining light period. During the dark, only low growth rates can be observed (Wiese et al., 2007). Several results indicate that these growth patterns are influenced by the metabolic state of the plant, rather than environmental fluctuations. Changes in environmental conditions, like water availability, nutrient availability and air temperature, change only the amplitude of growth but not the pattern (Schurr et al., 2000; Ainsworth et al., 2005). Changes in carbohydrate availability on the other hand influence both growth rate and growth pattern. For example, in the starchless mutant *stf1*, high growth rates are detected at the beginning of the light period, like in wild type, and additionally between the second half of the light period and the beginning of dark. During the night, growth is significantly reduced (Wiese et al., 2007). More evidence for the interplay between carbohydrate metabolism and growth comes from CAM and C3 plants. In contrast to C4 plants, which grow predominantly at dusk or dawn, CAM and C3 plants grow during daytime (Gouws et al., 2005; Poire et al., 2010). This change in growth phases might be a result of differences in CO$_2$ assimilation and carbon availability within the leaves. Roots, on the other
hand, which are constantly supplied with carbon, also show constant growth rates over the day-night cycle (Iijima et al., 1998; Walter et al., 2002b). Whether the expansion of leaves is also correlated to the accumulation of biomass, is not clear, as precise methods to analyze dial patterns of biomass accumulation do not exist. However, it seems inevitable that leaf expansion and the synthesis of new structural components such as cell walls are correlated. Yet, it is still not clear whether carbon metabolism is dictating growth or rather accommodating growth.

In this chapter, three approaches analyzing the interplay between major carbon fluxes and growth are described. The first part focuses on carbon partitioning in sink and source leaves. A source leaf assimilates all carbon necessary to maintain its own metabolism, accumulate storage compounds and facilitate residual growth for itself. Moreover, it can export part of the assimilated carbon to support other tissues. A sink leaf has two carbon sources. It is able to assimilate carbon by itself via photosynthesis and it can import carbon in the form of sucrose, which is distributed via the phloem from source leaves (see chapter 4). Radioactive labeling was used to compare carbon partitioning in sink and source leaves and additionally to analyze into which pathways carbon is channeled depending on whether is was assimilated by the leaf itself or delivered as sucrose. In the second part of this chapter, I correlate carbon partitioning with periods of active leaf expansion growth. I show that changes in leaf expansion rate over the light period are reflected in changes in carbon partitioning in wild-type plants and two mutant lines altered in starch synthesis. In the third part of the chapter, I investigated whether several mutations causing alterations in central carbon metabolism result in similar re-partitioning of carbon and growth changes. A collection of mutant lines altered in starch synthesis and in starch degradation was chosen to provide a gradient from starchless to accumulation of excess starch. The effect of these metabolic disturbances on photosynthesis, growth and carbon partitioning is shown.

Based on the carbon partitioning data gained from these experiments, I try to develop a quantitative, time resolved model of the major carbon fluxes in an Arabidopsis rosette, with a focus on actively growing tissues.
5.2 Results

5.2.1 Carbon partitioning in sink and source leaves

Radioactive labeling of source and sink leaves enables the comparison of carbon partitioning in the leaf at different developmental stages and comparison of carbon partitioning in an unlabeled sink leaf versus a labeled sink leaf. If a sink leaf is labeled exclusively, all labeled compounds must derive from carbon photo-assimilated by the leaf itself. If the sink leaf is not labeled, then all labeled compounds found in this leaf were imported from labeled source leaves, predominantly in the form of sucrose.

To analyze carbon partitioning in sink leaves and source leaves, a radioactive labeling experiment was performed. Plants were grown on soil under standard conditions. For one set of plants, a single mature leaf (leaf 8) was labeled with the small leaf chamber. For the second set of plants, a small developing leaf (leaf 13) was labeled with the help of the mini leaf chamber. A $^{14}$CO$_2$ pulse of 5 min was followed by a chase period of 60 min. After the chase, the labeled leaf was harvested for both plant sets and the unlabeled leaf 13 was also harvested from the first set of plants. Subsequently, the amount of labeled carbon partitioned to the different compound classes was determined.

Leaf 8 serves as a source leaf to feed leaf 13, leaf 16, leaf 18 and so on (Figure 20 and 21). This allows the analysis of carbon partitioning in leaf 13 after import of carbon from leaf 8. Hence, the described experimental setup allows comparison of carbon partitioning in the source versus the sink (labeled leaf 8 vs. unlabeled leaf 13), of carbon assimilated by the source versus the sink (labeled leaf 8 vs. labeled leaf 13) and of assimilated carbon versus imported carbon (labeled leaf 13 vs. unlabeled leaf 13).

Differences in carbon partitioning between the source and the sink within the same plant (labeled leaf 8 vs. unlabeled leaf 13) were already shown before (4.2.5). Similar results were gained in this experiment (Figure 27) and therefore will not be described here.
Figure 27: Carbon partitioning of assimilated and imported carbon. A mature leaf (leaf 8) or a developing leaf (leaf 13) of Col-0 was labeled with \(^{14}\)CO\(_2\) for 5 min in the middle of the light period. Following a chase period of 1 h, the labeled leaves 8 and 13 and the unlabeled leaf 13 (after labeling leaf 8) were harvested separately. The incorporation of \(^{14}\)C into the different metabolic pools was determined. The relative amount of \(^{14}\)C incorporated into the fractions as a percentage of the total label in the leaf is shown. Black asterisks indicate a significant difference between labeled leaf 8 and labeled leaf 13, grey asterisks indicate a significant difference between labeled leaf 13 and unlabeled leaf 13 (t-test; significance levels: *, p <0.05; **, p <0.01; ***, p <0.001). Mean ± SE (n=5).

Clear differences were observed in partitioning of assimilated carbon between mature and developing leaves (Figure 27). In a mature leaf, more than 50% of the assimilated carbon was channeled into soluble compounds, while only 5% of the carbon was used to build up ethanol soluble compounds like, pigments, waxes and lipids (Figure 27 A). In developing leaves, in contrast, carbon was partitioned equally between water soluble and insoluble compounds (each 45%) and 10% were partitioned into ethanol soluble compounds. The
most distinct changes were found within the insoluble fraction (Figure 27 B). Developing leaves partitioned assimilated carbon equally between starch, protein and the remaining fraction (each 15%), but mature leaves invested most carbon into the storage compound starch (25%) and much less into proteins and the remaining fraction (7% and 9%, respectively). No significant change could be observed for partitioning within the soluble fraction (Figure 27 C). Independent of the developmental stage, most of the carbon within the soluble compounds could be found in the acidic fraction, followed by the neutral and the basic fractions. These data show that assimilated carbon is invested into different metabolic processes depending on the developmental stage of the leaf. As expected, a developing leaf invests more carbon resources into structural compounds, while a mature leaf partitions more carbon into storage compounds.

Comparing carbon partitioning in the labeled leaf 13 with partitioning in the unlabeled leaf 13 reveals for which metabolic processes assimilated carbon is used in contrast to imported carbon (Figure 27). Naturally, a time delay in carbon partitioning of imported carbon in respect to assimilated carbon can be assumed which might be reflected in the partitioning data. However, after the 1 h chase, only slight differences in partitioning could be noticed for the two carbon sources.

Carbon ass partitioned equally between soluble and insoluble compounds after assimilation (44% and 45.3%, respectively), while imported carbon was used more for the synthesis of soluble metabolites (53.5% in the soluble fraction, 37.4% in the insoluble fraction, Figure 27 A). For both carbon sources, comparable amounts of carbon were channeled towards ethanol soluble compounds (10.6% and 9.1%, respectively).

However, carbon partitioning within the soluble fraction was not differing significantly with the carbon source (Figure 27 C). Even though significantly more carbon was found in the soluble metabolites when carbon was imported, the proportion of label found in neutral, acidic or basic compounds was the same. All three were slightly increased in the unlabeled leaf 13 relative to the labeled leaf, which can explain together the increase of flux towards soluble compounds.

Partitioning within the insoluble fraction differed considerably (Figure 27 B). The same proportion of carbon was channeled towards proteins and the remaining fraction (13.4%
and 15.4%, 14.5% and 14.8%, respectively), but significantly less carbon was partitioned into starch when carbon was imported into the leaf. Yet, a surprisingly big fraction of imported carbon was still channeled into starch (9.3%, compared with 15.3% of the assimilated carbon).

5.2.2 Leaf growth and carbon partitioning over the diurnal cycle

Carbon export and partitioning over the diurnal cycle

As already described, changes in leaf expansion growth over the diurnal cycle were shown for Arabidopsis plants. Expansion peaks in the early morning and slows in the second half of the day. This growth pattern was modified in mutants altered in starch metabolism. In starchless pgm plants, there was enhanced growth in the second half of the day in addition to the growth peak in the morning. Thus a second peak in growth was observed just after the beginning of the dark period which was not seen in the wild type (Wiese et al., 2007).

To better understand the link between metabolism and active growth phases, I performed $^{14}$C labeling experiments at different time points over the course of the light period with wild-type plants and pgm. Pgm1-1 can be designated as being starchless as it synthesizes less than 1% starch compared to the wild type (Caspar et al., 1985). Additionally, I incorporated the pgi mutant which synthesizes still around 20% of starch compared to wild type and hence has an intermediate starch phenotype (Yu et al., 2000). All three genotypes were labeled just after the onset of light and after 2, 6 and 10 h of light. The plants were grown on soil under standard conditions. A single leaf (leaf 8) was labeled with $^{14}$CO$_2$ for 2 min, followed by a chase period of 60 min. After the chase, the labeled leaf and the unlabeled leaves were harvested separately, and the amount of carbon partitioned to the different tissues and compound classes was determined.

First, the amount of carbon allocated to the sink leaves was analyzed (Figure 28). Carbon export varied over the course of the day in wild type and in the two mutant lines. The highest export rates were observed in the morning, when the plants were just exposed to light. Already after 2 h into the light, export was reduced and decreased further towards the
end of the light period. In the wild type, carbon export was initially 15.8% and reduced to 7.1% after 10 h of light. *Pgm* and *pgi* both showed a significantly higher degree of export in the morning. Within the first two hours of light, 15-22% of carbon was exported in *pgm* and 18-23% in *pgi* compared to 9-16% of export in wild. Later during the light period, export in both mutant lines dropped to values similar to that of the wild type.

**Figure 28:** Carbon export into sink leaves at different time points over the diurnal cycle. Leaf 8 of Col-0, *pgm* and *pgi* plants was labeled with $^{14}$CO$_2$ for 2 min just after the plants were exposed to light (0 h) and after 2 h, 6 h and 10 h into the day. Following a chase period of 1 h the labeled leaf and the unlabeled rosette (sink leaves) were harvested separately and the incorporation of $^{14}$C was determined. The relative amount of $^{14}$C incorporated into the unlabeled leaves is shown as a percentage of the total label in the plant. Asterisks indicate a significant difference to wild type (t-test; significance levels: *, p <0.05; **, p <0.01). Mean ± SE (n≥5).

The complete dataset for carbon partitioning within the source and sink leaves of Col-0, *pgm* and *pgi* can be found in the Appendix (Table 1A - 3A). Here the data is split into three figures, where partitioning into the major fractions of source and sink leaves and partitioning into the insoluble and soluble sub-fractions is shown separately (Figure 29, 30 and 31, respectively). In the following part, the differences in carbon partitioning over the diurnal cycle as well as differences in carbon partitioning between the wild type and the mutants are highlighted.
Carbon partitioning and growth in rosettes of *Arabidopsis thaliana*

Figure 29: Carbon partitioning into the major metabolic fractions at different time points over the diurnal cycle. Leaf 8 of Col-0, *pgm* and *pgi* plants was labeled with $^{14}$CO$_2$ for 2 min just after the plants were exposed to light (0 h) and after 2 h, 6 h and 10 h into the light. Following a chase period of 1 h, the labeled leaf and the unlabeled rosette (sink leaves) were harvested separately and the incorporation of $^{14}$C into the different fractions was determined. The sum of the label in the labeled leaf is set to 100% as well as the sum of the label in the sink leaves. The relative amount of $^{14}$C incorporated into the different fractions as a percentage of the total label in the tissue is shown. Mean ± SE (n≥5).

Like export, carbon partitioning into the major metabolite fractions - the water soluble, ethanol soluble and insoluble fractions – changed dramatically within the first two hours of light (Figure 29). Later in the day, partitioning was rather stable. As a general trend, less carbon was used in the morning to build up insoluble compounds and more was channeled into water soluble and ethanol soluble compounds. This was reversed later during the light period, when more carbon was used to build up insoluble compounds and less was used for the synthesis of water soluble and ethanol soluble compounds.
As pgm and pgi are both altered in starch synthesis, a reduction in carbon partitioning towards the insoluble compounds can be expected. This was indeed the case. Partitioning into the insoluble fraction, which includes starch, proteins and cell wall material, reached only 50% of the wild-type level in pgi and 15 to 25% in pgm. Instead, more carbon was channeled into the soluble compounds. Interestingly, partitioning into the ethanol soluble compounds showed opposite trends. In the wild type, partitioning into ethanol soluble compounds within the source leaf was highest in the morning and decreased steadily over the day. In both starch synthesis mutants, partitioning into ethanol soluble compounds was lowest in the morning and increased thereafter. In the sink leaves of wild-type plants, a clear peak for carbon partitioning into the ethanol soluble compounds was visible 2 h after the onset of light, which could be observed also in pgi, but less pronounced. In pgm sink leaves, partitioning into ethanol soluble compounds increased slightly throughout the light period, as in the source leaves.

More carbon was partitioned into insoluble compounds within the source leaf in the course of the day. The reason for this increase could be identified when the insoluble compounds were further fractionized into starch, proteins and remaining insoluble compounds, like cell wall material (Figure 30). The only increase of labeled carbon within source leaves could be observed for starch. Just after the onset of light, only 14.1% of carbon was used to build up starch in wild-type plants, while at the middle of the light period 32.8% were channeled towards starch. Pgi showed a similar pattern, but with 60-70% less carbon used for starch synthesis compared to the wild type. As expected, carbon flow into starch was negligible in pgm (1-2.3%). In the sink leaves, only small amounts of carbon were partitioned into starch with only slight changes over the light period.
Carbon partitioning and growth in rosettes of *Arabidopsis thaliana*

Figure 30: Carbon partitioning into the insoluble compound classes at different time points over the diurnal cycle. Leaf 8 of Col-0, *pgm* and *pgi* plants was labeled with $^{14}$CO$_2$ for 2 min just after the plants were exposed to light (0 h) and after 2 h, 6 h and 10 h into the day. Following a chase period of 1 h the labeled leaf and the unlabeled rosette (sink leaves) were harvested separately and the incorporation of $^{14}$C into different compound classes was determined. The sum of the label in the labeled leaf is set to 100% as well as the sum of the label in the sink leaves. The relative amount of $^{14}$C incorporated into the different insoluble fractions as a percentage of the total label in the tissue is shown. Mean ± SE (n≥5).

Carbon partitioning into proteins was increased in the mutants compared to the wild type in source and sink leaves. Only at the onset of light, wild-type plants partitioned slightly more carbon into proteins within the source than *pgm* (5.1% and 3.8%, respectively). Apart from this, carbon flow to proteins in the source leaf was slightly increased in *pgm*, but strongly increased in *pgi* (7-12% in *pgi*, 3.5-5% in wild type). In the sink leaf, a strong increase was observed for both mutants over the whole light period (8-13% in *pgm* and 11-15% in *pgi* compared to 4-6% in wild type). Less partitioning towards the remaining insoluble
Carbon partitioning and growth in rosettes of *Arabidopsis thaliana*

compounds was measured in *pgm* and *pgi*, especially in the sink leaves. Partitioning into the remaining fraction was reduced over the whole day from 5-8% in wild type to 3-5% in both mutants. Within the source leaf the decrease was less pronounced. Wild-type plants partitioned 6% of carbon into the remaining fraction over the day with a small peak after 2 h of light. In both mutants, partitioning into the remaining fraction was very low at the onset of light, but increased in *pgi* to reach a maximum of 3.9% after 2 h of light and in *pgm* to 5.7% at the end of the day (more than wild type at this time point).

The soluble sub-fraction is composed of neutral, acidic and basic compounds (Figure 31). In the neutral fraction sugars, such as sucrose, glucose and fructose, can be found. The acidic fraction comprises organic acids and sugar phosphates and the basic fraction contains amino acids. After the onset of light, partitioning into sugars was highly increased compared to partitioning in the remaining light period. In the labeled leaves of wild type, 43.9% of carbon was partitioned into sugars at the beginning of the light period, 2 h later only 16.2% were observed in this pool. At the end of the light period partitioning into sugars was again slightly increased (23.0%). Like partitioning into most insoluble compounds, partitioning into the other soluble compounds was reduced at the beginning of the day. A drastic change could be seen for the acidic compounds. While wild type channeled 12% into these compounds at the beginning of the day, 2 h later 20.0% of the labeled carbon could be found in this fraction. This change was less pronounced for the basic compounds. In the morning, 6.6% of labeled carbon was introduced into basic components, increasing to 9.3% 2 h later.

The described differences of carbon partitioning towards neutral compounds at the beginning of day was not seen within the sink leaves. Here, partitioning was stable over the first three time points; only at the end of the light period partitioning into sugars was highly increased. Carbon flow into acidic compounds of wild-type sink leaves decreased steadily from 19.9% in the morning to 12.8% at the end of the light period. An opposite trend could be seen for carbon partitioning into basic compounds. In the early morning, 11.3% of labeled carbon could be detected in basic compounds, followed by an increase to 19.8% at the middle of the light period.
Carbon partitioning and growth in rosettes of *Arabidopsis thaliana*

Figure 31: Carbon partitioning into the soluble compound classes at different time points over the diurnal cycle. Leaf 8 of Col-0, *pgm* and *pgi* plants was labeled with $^{14}$CO$_2$ for 2 min just after the plants were exposed to light (0 h) and after 2 h, 6 h and 10 h into the day. Following a chase period of 1 h the labeled leaf and the unlabeled rosette (sink leaves) were harvested separately and the incorporation of $^{14}$C into different compound classes was determined. The sum of the label in the labeled leaf is set to 100% as well as the sum of the label in the sink leaves. The relative amount of $^{14}$C incorporated into the different soluble fractions as a percentage of the total label in the tissue is shown. Mean ± SE (n≥5).

In the mutants, carbon flow into sugars was increased dramatically. In *pgm*, partitioning into sugars increased 2 to 2.5-fold and in *pgi* 1.5 to 2-fold. The same pattern could be observed for source and sink leaves. No major differences between the starch synthesis mutants and wild type could be seen for carbon partitioning into the acidic and basic compounds in source leaves, but within the sink leaves partitioning into basic compounds was significantly reduced. While in wild-type plants, partitioning towards basic compounds increased over the day, the partitioning was constant in *pgi* and *pgm*. This implies that from 2 to 10 h into...
the light, 3 to 4 times less carbon was introduced into basic compounds within the sink leaves.

As \textit{pgi} and \textit{pgm} can only synthesize a limited amount of starch, redistribution of fixed carbon is inevitable. The reallocation of carbon to other pathways could be seen for several compound classes. Interestingly, carbon allocation was only increased towards neutral compounds and proteins. Flow into the other compound classes, like structural, ethanol soluble and basic components, was decreased. One hypothesis to explain the strong reallocation towards sugars is a starvation effect. Not only the partitioning into starch is altered in these mutants, but the insufficient starch reserves also affect carbon availability during the night. Both lines lack sugar at the end of the dark period and therefore suffer from starvation. The high amounts of carbon channeled into sugars could be a result of this starvation response.

In order to evaluate the effect of starvation on carbon partitioning, I performed an additional experiment in which wild-type plants were exposed to an extended dark period of 4 h before labeling after transfer back to the light (WT EN). Carbon partitioning after the extended night was compared to wild-type and to \textit{pgm} labeled at the beginning of the light period (Table 3). Carbon export to sink tissues was not changed after an extended night (data not shown), but some similarities in carbon reallocation were distinguishable after an extended night in wild type compared to \textit{pgm}. In both lines, carbon flow into sugars was increased at the expense of carbon flow into nearly all other compounds. The only exceptions were carbon partitioning into starch in source and sink leaves and into proteins within the sink leaves. This indicates that, as a response to starvation, high amounts of sugars are synthesized, while flow through most other metabolic pathways is down-regulated. Surprisingly, carbon partitioning into the remaining insoluble compounds, which is mainly cell wall material, was even less in WT EN than in \textit{pgm}. After the sudden starvation, nearly no carbon is utilized for synthesis of new cell wall material especially within the source leaf (0.2%). Apart from carbon allocation towards cell wall material, the WT EN data is intermediate between the wild type at the beginning of the light period and
pgm, suggesting that the starvation is not as pronounced after a 4 h longer dark period as in pgm.

As a matter of course, carbon partitioning into starch was higher in WT EN than in pgm, but it was also higher than in the wild type at the beginning of the normal light period. The extended night does not only seem to cause a shift in carbon flow towards sugars, but also towards starch.

Table 3: Carbon partitioning into the major carbon compound classes in wild type after an extended night. Leaf 8 of Col-0 plants was labeled with $^{14}$CO$_2$ for 2 min at the onset of light after exposure to a 4 h extended night (WT EN). Following a chase period of 1 h, the labeled leaf and the unlabeled rosette (sink leaves) were harvested separately and the incorporation of $^{14}$C into different compound classes was determined. The sum of the label in the labeled leaf is set to 100% as well as the sum of the label in the sink leaves. The relative amount of $^{14}$C incorporated into the different soluble fractions as a percentage of the total label in the tissue is shown. Mean ± SE (n=5). Data for wild-type plants and pgm labeled after the onset of a normal light period was derived from the previous experiment (WT 0 and pgm 0, respectively).

<table>
<thead>
<tr>
<th>% of total $^{14}$C recovered in each fraction</th>
<th>WT EN</th>
<th>WT 0</th>
<th>pgm 0</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Labeled leaf</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water soluble compounds</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Neutral</td>
<td>72.14 ± 0.66</td>
<td>59.83 ± 0.79</td>
<td>92.34 ± 1.37</td>
</tr>
<tr>
<td>Acidic</td>
<td>62.48 ± 0.74</td>
<td>43.85 ± 5.04</td>
<td>79.71 ± 1.87</td>
</tr>
<tr>
<td>Nucleotide</td>
<td>4.44 ± 0.10</td>
<td>12.13 ± 0.81</td>
<td>7.00 ± 0.60</td>
</tr>
<tr>
<td>Basic</td>
<td>n.d.</td>
<td>0.92 ± 0.10</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>Ethanol soluble compounds</td>
<td>5.75 ± 0.21</td>
<td>6.74 ± 0.37</td>
<td>3.52 ± 0.21</td>
</tr>
<tr>
<td><strong>Insoluble compounds</strong></td>
<td>24.59 ± 0.68</td>
<td>29.78 ± 1.55</td>
<td>4.67 ± 1.01</td>
</tr>
<tr>
<td>Starch</td>
<td>20.3 ± 0.72</td>
<td>14.12 ± 2.05</td>
<td>1.00 ± 0.18</td>
</tr>
<tr>
<td>Protein</td>
<td>4.61 ± 0.12</td>
<td>5.09 ± 0.39</td>
<td>1.57 ± 0.43</td>
</tr>
<tr>
<td>Remaining insoluble</td>
<td>0.20 ± 0.09</td>
<td>6.32 ± 0.37</td>
<td>1.66 ± 0.30</td>
</tr>
<tr>
<td>Ethanol soluble compounds</td>
<td>3.27 ± 0.14</td>
<td>10.40 ± 1.07</td>
<td>2.98 ± 0.39</td>
</tr>
<tr>
<td><strong>Sink leaves</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water soluble compounds</td>
<td>84.28 ± 1.25</td>
<td>60.30 ± 0.88</td>
<td>83.20 ± 3.91</td>
</tr>
<tr>
<td>Neutral</td>
<td>68.38 ± 1.49</td>
<td>20.16 ± 5.22</td>
<td>66.89 ± 3.16</td>
</tr>
<tr>
<td>Acidic</td>
<td>6.63 ± 0.44</td>
<td>19.85 ± 4.83</td>
<td>12.34 ± 0.64</td>
</tr>
<tr>
<td>Nucleotide</td>
<td>n.d.</td>
<td>1.80 ± 0.05</td>
<td>0.44 ± 0.04</td>
</tr>
<tr>
<td>Basic</td>
<td>9.73 ± 0.42</td>
<td>11.29 ± 2.20</td>
<td>3.58 ± 0.29</td>
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<td>Insoluble compounds</td>
<td>13.84 ± 1.06</td>
<td>29.39 ± 1.17</td>
<td>15.74 ± 3.91</td>
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<td>Starch</td>
<td>3.29 ± 1.26</td>
<td>8.81 ± 0.67</td>
<td>6.37 ± 2.12</td>
</tr>
<tr>
<td>Protein</td>
<td>5.82 ± 0.61</td>
<td>5.54 ± 0.08</td>
<td>2.41 ± 0.57</td>
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<tr>
<td>Remaining insoluble</td>
<td>4.85 ± 1.73</td>
<td>12.09 ± 0.82</td>
<td>5.33 ± 1.08</td>
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<td>Ethanol soluble compounds</td>
<td>1.93 ± 0.31</td>
<td>10.31 ± 0.54</td>
<td>1.06 ± 0.06</td>
</tr>
</tbody>
</table>
5.2.3 Growth and carbon partitioning in mutants altered in starch metabolism

For the starch synthesis mutants pgm and pgi, reallocation into soluble compounds takes place as starch synthesis is impaired (5.2.2). This reallocation exceeds the reduction expected due to a lower starch synthesis rate and goes along with numerous other changes in carbon partitioning, which cannot be linked to starch synthesis directly. To reveal general patterns in carbon reallocation, two larger sets of mutants altered in starch metabolism were analyzed.

The first set comprises mutants altered in starch synthesis. As described in detail in the introduction, starch is synthesized from the Calvin-Benson cycle intermediate Fru6P, which is converted to ADPGlc by PGI, PGM and AGPase. AGPase catalyzes the first committed step of starch synthesis. However, loss of function of any of the three enzymes results in decreased rates of starch synthesis. A nearly complete loss of starch synthesis occurs in pgm. Pgm1-1 is synthesizing less than 1% starch compared to the wild type (Caspar et al., 1985). In pgi mutants, some starch can still be synthesized attributed to import of Glc6P, Glc1P or ADPGlc from the cytosol (Kunz et al., 2010). The same mutant (pgi1-1) as in the previous experiment was used. AGPase forms a heteromeric enzyme complex consisting of two large subunits and two small subunits. A mutation in the enzymatically active small subunit APS1 leads to a strong decrease in starch levels (Lin et al., 1988b), while a mutation in the regulatory large subunit APL1 causes a less pronounced reduction in starch amounts (40% of wt, Lin et al., 1988a). The line adg2-1 is carrying a mutation in the APL1 gene.

In the following steps of starch synthesis ADPGlc is used as a substrate by starch synthases (SSI-SSIV and GBSS), which transfer the glucose moiety to an existing glucose chain. With the help of branching (BE2-BE3) and debranching enzymes (ISA1 and ISA2) the typical crystalline structure of starch is achieved. Loss of one of the starch synthases affects not only starch levels, but primarily starch structure and starch granule number. The ssIV mutant included in this experiment has reduced levels of starch (60-65% of wild type, Roldán et al., 2007) and a reduced number of starch granules. Additionally, the isa1/isa2 double mutant is included in the analysis. The loss of one of the two debranching enzymes involved in starch synthesis results in the accumulation of soluble phytoglycogen and therefore reduced starch levels (30% of wild type, Delatte et al., 2005). The identical phenotype of both mutants led to the
idea that both work together in a complex (Delat et al., 2005). The double mutant was used here.

The second set contains mutants with altered starch degradation. To loosen up the semi-crystalline structure of the starch granule and make it accessible for enzymatic degradation during the night, glucan phosphorylation is necessary. The two enzymes, glucan, water dikinase (GWD) and phosphoglucan, water dikinase (PWD) catalyze the phosphorylation of some glycosyl residues of amyllopectin. Lack of GWD or PWD leads to an incomplete degradation of starch during the night and hence to a starch excess phenotype (Yu et al., 2001; Kötting et al., 2005). As many starch degrading enzymes cannot act beyond a phosphorylated site, subsequent dephosphorylation is required for a complete degradation of the granule. Two phospho-glucan phosphatases, SEX4 and LSF2, remove these phosphate groups. Since the function of SEX4 and LSF2 are partially redundant, only the lack of SEX4 leads to a strong starch excess phenotype and only the sex4 mutant was included into the second set (Kötting et al., 2009; Santelia et al., 2011).

Carbon partitioning was analyzed in both sets of mutants to reveal whether common patterns in carbon partitioning exist if either the synthesis or degradation of starch are disturbed. Moreover, export of carbon from source leaves to sink tissues was analyzed to understand to what extent alterations in starch metabolism effect the sink-source balance in the different genotypes. In addition to carbon allocation, rosette growth and photosynthesis of the described genotypes was examined to link photosynthetic capacity with carbon metabolism and growth.

Several of these parameters were already analyzed in previous studies for some of the genotypes. Yet, a comprehensive analysis of mutants altered in starch metabolism and of the effects of the respective alteration on carbon allocation, growth and photosynthesis has not been performed before. To gain comparable data, plants for all experiments described in this section were grown in hydroponic cultures according to the standard protocol (see section 2.2.2).

To analyze carbon partitioning, 3-week-old plants were labeled with $^{14}$CO$_2$; 4-week-old plants were harvested to measure starch and dry weight and photosynthetic rate was
measured on plants of an age of 4 to 5 weeks. The photosynthetic measurements were performed with a portable Licor system (Licor 6400) under standard conditions with 150 µmol m⁻² s⁻¹ light and 380 ppm CO₂. To calculate the projected leaf area, a picture of the shoot was taken every 2 to 3 days starting at the 7th day after sowing.

**Photosynthesis and growth in mutants altered in starch metabolism**

The majority of published starch values in wild-type plants and mutant lines affected in starch metabolism was gained from experiments performed on soil. To evaluate the hydroponics setup used in this experiment, starch content in the plants at the end of the light and the end of the dark period was measured and compared to available data. Although the starch levels measured in the hydroponically grown plants were slightly lower as the published values, they displayed the same trend (Table 4).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>A (µmol/m² s)</th>
<th>DW shoot (mg)</th>
<th>DW root (mg)</th>
<th>Root/shoot</th>
<th>Starch (mg/ g FW) EOD</th>
<th>Starch (mg/ g FW) EON</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0</td>
<td>4.71 ± 0.21</td>
<td>58.40 ± 3.14</td>
<td>8.90 ± 0.54</td>
<td>0.15</td>
<td>9.30 ± 0.41</td>
<td>0.17 ± 0.04</td>
</tr>
<tr>
<td>adg2</td>
<td>4.00 ± 0.20 *</td>
<td>32.39 ± 4.01</td>
<td>4.93 ± 0.60</td>
<td>0.15</td>
<td>8.05 ± 1.50</td>
<td>0.28 ± 0.02 *</td>
</tr>
<tr>
<td>ssiV</td>
<td>4.47 ± 0.10</td>
<td>15.61 ± 1.23</td>
<td>3.23 ± 0.26</td>
<td>0.21 ***</td>
<td>5.79 ± 0.19 ***</td>
<td>0.38 ± 0.04 **</td>
</tr>
<tr>
<td>pgi</td>
<td>5.20 ± 0.23</td>
<td>14.13 ± 0.89</td>
<td>3.12 ± 0.18</td>
<td>0.22 ***</td>
<td>1.86 ± 0.25 ***</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td>pgm</td>
<td>4.66 ± 0.43</td>
<td>13.92 ± 1.02</td>
<td>2.74 ± 0.26</td>
<td>0.19 ***</td>
<td>0.01 ± 0.00 ***</td>
<td>0.01 ± 0.00 **</td>
</tr>
<tr>
<td>isa1/isa2</td>
<td>5.43 ± 0.17 *</td>
<td>34.13 ± 3.25</td>
<td>5.43 ± 0.71</td>
<td>0.16</td>
<td>1.51 ± 0.12 ***</td>
<td>0.19 ± 0.14</td>
</tr>
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<td>pwd</td>
<td>4.63 ± 0.23</td>
<td>46.11 ± 2.40</td>
<td>7.19 ± 0.48</td>
<td>0.16</td>
<td>9.19 ± 1.55</td>
<td>3.29 ± 0.18 ***</td>
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<td>sex4</td>
<td>4.64 ± 0.38</td>
<td>17.72 ± 1.54</td>
<td>2.18 ± 0.19</td>
<td>0.12 **</td>
<td>20.53 ± 0.74 ***</td>
<td>13.62 ± 1.12 ***</td>
</tr>
<tr>
<td>sex1</td>
<td>5.27 ± 0.16</td>
<td>6.41 ± 0.58</td>
<td>1.01 ± 0.13</td>
<td>0.16</td>
<td>36.49 ± 2.07 ***</td>
<td>29.77 ± 2.25 ***</td>
</tr>
</tbody>
</table>

Just small differences could be observed for photosynthetic rate (Table 4). Only adg2 and isa1/isa2 showed a significantly lower or higher photosynthetic rate than the wild type, respectively. Photosynthetic parameters of adg2 were also analyzed with our whole plant gas exchange unit, GDU-26 (see chapter 3). However, no differences were observed on the whole plant level. The differences measured on a single leaf basis may originate from the fact that only the oldest leaves of Arabidopsis could be measured with the Licor 6400. The
measured leaf area was 2 cm², hence excluding all developing and still expanding leaves. As adg2 flowers earlier compared to the wild type, it might be possible that the measured leaves were already in the senescence stage, during which photosynthetic rate decreases progressively. The increased photosynthetic rate of isa1/isa2 has not been verified on a whole plant level so far. However, the decreased dry weight and leaf area compared to the wild type does speak against an enhanced overall photosynthetic performance.

Wild-type shoots and roots showed the highest dry weight with 58.4 mg and 8.9 mg, respectively. Consequently, all other genotypes showed a significant reduction in shoot and root dry matter. The differences in dry weight of the plants were also reflected by the projected leaf area. The projected leaf area of the plants was used to generate a growth curve (Figure 32). The biggest leaf area was observed for wild-type plants followed by the genotypes pwd, isa1/isa2 and adg2. A significantly smaller leaf area was measured for ssiV, pgi, pgm, sex4 and sex1, with sex1 yielding the smallest plants. This was reflected also in the relative growth rates (RGR) of the different genotypes (Appendix Table A4). Wild type showed the highest average relative growth during the measurement period with 28.2% d⁻¹, followed by pwd and isa1/isa2. The lowest growth rates were measured for pgm and sex1 with 21.8% d⁻¹. A strong correlation was found between projected leaf area and dry weight of the shoot (Pearson correlation: ρ= 0.977) or root (ρ= 0.982). Nevertheless, the ratio of shoot to root dry matter was found to be significantly increased for pgm, ssiV and pgi. The dry weight of the roots of all three genotypes was increased compared to the dry weight of the shoot. While a wild-type root had approximately 15% the weight of the shoot, it was around 20 % in pgm, 21% in ssiV and 22% in pgi. A reduction in root to shoot ratio was observed for sex4, in which the root had only 12% of the weight of the shoot.
Carbon partitioning and growth in rosettes of *Arabidopsis thaliana*

**Figure 32:** Leaf area of wild type and mutants altered in starch metabolism. The projected leaf area was calculated from pictures taken every 2 to 3 days starting at 7 days after sowing until 29 days after sowing. Mean n=10.

*Carbon export and partitioning in mutants altered in starch metabolism*

To analyze carbon partitioning in these lines, $^{14}$C labeling experiments were performed. The plants were grown in hydroponics under standard conditions. At an age of 3 weeks, the whole rosette was labeled with $^{14}$CO$_2$ for 1 h just after the onset of light or in the middle of the light period. Following a chase period of 1 h, the labeled rosettes and the roots were harvested separately and the amount of carbon partitioned to the different tissues and compound classes was determined.

Carbon partitioning at the beginning of the light period is depicted in Figure 33 and in the appendix (Table A5), partitioning in the middle of the light period is shown in Figure 34 and in the appendix (Table A6).

Col-0 showed a similar partitioning as already seen for the single leaf labeling. In the morning, high export rates to the roots could be detected as well as an increased carbon flow towards structural components (remaining insoluble, EtOH soluble) and proteins. In the middle of the day, carbon flow into starch was highly increased at the expense of all other compound classes.
Figure 33: Carbon partitioning at the beginning of the day in Col-0 and mutants altered in starch metabolism. Whole shoots of Col-0, ssv, adg2, pgj, pgm, isa1/isa2, pwd, sex4 and sex1 were labeled with $^{14}$CO$_2$ for 1 h just after the plants were exposed to light. Following a chase period of 1 h, the labeled shoot and the unlabeled root were harvested separately and the incorporation of $^{14}$C into the different fractions was determined. The relative amount of $^{14}$C incorporated into the different fractions as a percentage of the total label in the plant is shown. Asterisks indicate a significant difference to wild type (t-test; significance levels: *, p <0.05; ** p <0.01; ***, p<0.001). Mean ± SE (n≥5).
Figure 34: Carbon partitioning in the middle of the light period in Col-0 and mutants altered in starch metabolism. Whole shoots of Col-0, ssIV, adg2, pg1, pgm, isa1/isa2, pwd, sex4 and sex1 were labeled with $^{14}$CO$_2$ for 1 h just after the plants were taken out of the dark. Following a chase period of 1 h the labeled shoot and the unlabeled root were harvested separately and the incorporation of $^{14}$C into the different fractions was determined. The relative amount of $^{14}$C incorporated into the different fractions as a percentage of the total label in the plant is shown. Asterisks indicate a significant difference to wild type (t-test; significance levels: *, $p<0.05$; ** $p<0.01$; ***, $p<0.001$). Mean ± SE (n≥5).
In all mutant lines, carbon partitioning into the insoluble fraction was lower than in wild type and also lower in the morning than in the afternoon (Figure 33 B and Figure 34 B). These reductions were mainly caused by the lower incorporation of label into starch. All starch synthesis mutant showed a reduction of carbon flow into starch, as expected (Figure 33 C and Figure 34 C). Starch levels and partitioning into starch correlated to a large extent. The lower the starch levels in the mutant at the end of the day, the less carbon was introduced into starch during the labeling experiment. The only exception was ssIV, which partitioned more carbon into starch at the middle of the day than did adg2, but showed lower starch levels than adg2 at the end of the day. Carbon was partitioned almost equally between starch, proteins and the remaining insoluble fraction (cell wall material) in the morning. At midday however, incorporation into starch was strongly increased, while incorporation into proteins and cell wall material was only slightly changed. In the wild type and mutants with a moderate impairment of starch metabolism, less carbon was introduced into these compounds, but in mutants with stronger impairments even more carbon could be found in these fractions at midday compared to the morning.

The decreased carbon flow towards starch was accompanied by numerous other changes in partitioning both between compound classes and in terms of whole-plant allocation. Export of carbon into the root increased at both time points with decreasing starch synthesis capacity (Figure 33 A and Figure 34 A). While the wild type transported 8.7% of carbon to the roots, pgm exports 19.4% at the beginning of day. Carbon export in the other starch synthesis mutants fell somewhere between wild type and pgm.

An increased carbon partitioning into water soluble compounds, mainly sugars, accompanied high export rates (Figure 33 B and Figure 34 B). The wild type partitioned 42.6% or 32.8% into the water soluble phase in the morning and midday, respectively. The starchless pgm partitioned ~60% into water soluble compounds at both time points. Pgi, ssIV and adg2, which are all capable of starch synthesis to a certain extent, partitioned ~50% into the water soluble compounds in the morning, but reduced the partitioning into the water soluble phase at midday (although not to wild type levels). The majority of label found in the water soluble fraction in the mutants could again be attributed to neutral compounds.
(mainly sugars), especially in the morning. The more severely starch synthesis is affected, the more carbon was channeled into sugars, while partitioning into acidic and basic compounds was reduced (Figure 33 D). At midday, partitioning into neutral compounds was still increased relative to the wild type in most of the mutants, although less compared to the morning, but partitioning into acidic compounds was enhanced as well.

For isa1/isa2 an increase of carbon flow into the neutral compounds was detectable from morning to midday. This could be attributed to the increase of label in phytoglycogen (appendix Table A5 and Table A6). In all mutants, except pgm, an increase of label in starch could be observed at midday compared to morning. As isa1/isa2 cannot synthesize starch properly, soluble phytoglycogen is made instead. After the fractionation of the plant material, phytoglycogen can be found in the neutral fraction, therefore increasing the amount of carbon introduced into the water soluble and neutral compounds. By precipitating the phytoglycogen from the neutral fraction, the amount of carbon introduced into phytoglycogen can be determined. Indeed, only 11.2% of label was found in this compound in the morning, but 24.4% at the middle of the day (appendix Table A5 and Table A6). Adding up the carbon partitioned to phytoglycogen and starch in isa1/isa2 was resulting in the same amount of carbon partitioned into starch as in wild-type plants. However, carbon partitioning of isa1/isa2 showed several more differences to wild type, some of which were similar to the other starch synthesis mutants, but not all. Isa1/isa2 partitioned less carbon into the remaining soluble at both labeling time points compared to the wild type. The increased partitioning towards this compound, seen for the other starch mutants at the middle of the day, was not observed for isa1/isa2. Moreover, a strong increase in carbon flow towards acidic compounds could be detected in the middle of the day, which might be a sign, that isa1/isa2 is building up organic acids as alternative storage compounds.

Carbon partitioning into ethanol soluble compounds (pigments, waxes and membranes) was higher in the morning than at midday in wild type. In adg2, slightly more label was incorporated into this fraction at the morning, while all other lines showed a reduced incorporation compared to the wild type. This was reversed at midday. All lines but ss IV showed a higher partitioning into the ethanol soluble compounds than wild type.
Carbon partitioning in mutants with impaired starch degradation was generally more similar to wild type than partitioning in the mutants impaired in starch synthesis (Figure 33 and 34). Yet, some changes were consistent between the two groups, even though they were less pronounced in the mutants altered in starch degradation.

*Pwd, sex4* and *sex1* showed an increase in export at the beginning of the light period, a trend which grew more severe with increasing starch excess. The amount of exported carbon at midday on the other hand resembled that of wild type.

As for the mutants impaired in starch synthesis, an increase in carbon flow to soluble compounds could be seen for lines impaired in starch breakdown at the beginning of day. This was due to an increase in label in the neutral fraction, while label in the acidic and basic compounds was decreased compared to the wild type. The more severe the starch excess, the more carbon was partitioned into sugars. This resulted in 17.0% of labeled carbon found in sugars in *pwd*, 17.7% in *sex4* and 31.2% in *sex1*. At midday, less labeled carbon could be identified in the soluble compounds. Partitioning into the neutral fraction was reduced and the partitioning pattern resembled that of the wild type.

Carbon partitioning towards the insoluble fraction was reduced in the mutant lines compared to the wild type. Surprisingly, carbon flow into starch was increased in the morning. While the wild type partitioned 15.6% into starch, 14.9%, 16.5% and 17.7% of labeled carbon was identified in the starch fraction of *pwd*, *sex4* and *sex1*, respectively. Carbon flow into proteins and the remaining insoluble compounds was strongly reduced. At midday, however, carbon partitioning into starch was decreased in all three mutant lines. Partitioning into proteins and remaining insoluble compounds resembled the wild type.

Partitioning into the ethanol soluble compounds was reduced in the morning in the lines impaired in starch degradation, while it was increased at the middle of the day.

At the beginning of the light period, a similar redistribution of carbon could be seen in lines altered in starch synthesis and in lines altered in starch degradation. The more severe the alteration in starch metabolism the more drastically carbon partitioning was affected. Later during the light period, on the other hand, mutants capable of starch synthesis no longer showed strong differences in partitioning and distributed their carbon normally.
5.3 Discussion

Assimilated and imported carbon is utilized similarly in primary metabolism

One could expect that carbon assimilated within the chloroplast is more frequently used for the synthesis of carbon compounds in that compartment, while carbon imported into the cytosol in form of sucrose is more likely used for biosynthetic processes. Surprisingly, I observed only little differences in carbon partitioning between assimilated carbon and imported carbon after $^{14}$C feeding experiments. When carbon is imported from source tissues, around 10% more can be found in the soluble fraction, whereby carbon flow into all three sub-fractions, neutral, acidic and basic compounds, is equally increased. Carbon partitioning into the insoluble compounds, on the other hand, is decreased, but mainly at the expense of flow into starch. Carbon allocation towards proteins and other insoluble compounds is similar for imported and assimilated carbon.

Again, carbon flow into starch from imported carbon is observed in the sink leaves, as observed for the time course labeling experiment (see section 4.2.5). However, synthesis of starch from imported carbon seems to be restricted to sink leaves. Only low amounts of radioactivity were detected in root starch (<0.1% after 60 min chase) after labeling (data not shown). This indicates that the detection of labeled carbon in starch of sink leaves is unlikely due to the fractionation method, but might indicate exchange of carbon compounds between cytosol and chloroplasts.

Labeled carbon compounds could be imported into the chloroplast to fuel the oxidative pentose phosphate pathway, to refill the Calvin-Benson cycle with intermediates or as direct precursors for starch. A xylulose-5-phosphate/phosphate translocator is known to be located in the inner membrane of the chloroplast, which transports in counter exchange with Pi several pentose phosphates into the chloroplast, such as ribulose-5-phosphate and xylulose-5-phosphate, but also triose phosphates and erythrose-4-phosphate (Eicks et al., 2002). Some of them are direct Calvin-Benson cycle intermediates or can be converted into those. Refilling of the cycle with labeled intermediates could explain the incorporation of labeled carbon in starch of sink leaves.
In order to directly synthesize starch out of imported sucrose, starch precursors need to be imported into the chloroplast. Several pathways are conceivable. After import into sink tissues, sucrose is cleaved either by SUS into Fru and UDP-Glc or by invertase into Glc and Fru. Fru and Glc are then phosphorylated by fructokinase and hexokinase to Fru6P and Glc6P, respectively. Both sugar phosphates can be interconverted into each other by cytosolic PGI. Glc6P could be transported into the chloroplast and subsequently utilized for starch synthesis. A glucose-6-phosphate/phosphate translocator was identified only in heterotrophic tissue (Kammerer et al., 1998), but its expression was found to be up-regulated in photosynthesizing tissues of starch synthesis mutants (Kunz et al., 2010). UDPGlc derived from sucrose cleavage by SUS can be converted by UGPase into Glc1P. Glc1P itself marks another possible starch precursor which could be imported in the chloroplast. Recently, it was shown that Glc1P can be taken up by both heterotrophic tissue and autotrophic protoplasts from leaf mesophyll tissue (Fettke et al., 2011). A third possibility would be the import of ADPGlc, the direct precursor for starch synthesis. In cereal endosperm, an ADPGlc transporter is known to support starch synthesis in amyloplasts. However, Arabidopsis, does not possess a cytosolic AGPase, nor a transporter with a high affinity for ADPGlc (Kirchberger et al., 2007). Baroja-Fernández et al. (2004) claim that SUS can use ADP to convert sucrose into Fru and ADPGlc. They propose this as the predominant pathway for the production of ADPGlc and subsequently the synthesis of starch. However, some controversy exists about this alternative pathway, and it is not supported by several published data (discussed in Streb et al., 2009).

The activity of any of the described transporters in chloroplasts of developing leaves could mediate the import of labeled starch precursors into chloroplasts. This could explain the accumulation of imported radioactive label into the starch fraction of sink leaves and additionally the accumulation of small amounts of starch in mutants altered in starch synthesis, like *pgm*. Indeed, carbon partitioning into starch of *pgm* sink leaves was considerably higher compared to source leaves (Figure 30). While in the labeled leaf maximally 0.13% of the assimilated carbon was introduced into starch, it was 4.67% of the carbon imported into sink leaves.
Carbon partitioning is varying over the light period

Carbon partitioning in plants labeled at different time points over the light period showed striking differences (Figure 28-31, summarized in Figure 35). Even though photosynthesis is rather constant over the day and, therefore, also carbon supply of the plant can assumed to be constant, the flow of carbon into different metabolic pathways is highly variable. Changes in carbon partitioning can be detected within the labeled source leaves, in the unlabeled sink leaves and for carbon export from source to sinks. Changes in carbon fluxes in the first two hours of light exposure were particularly large. Later during the light period changes in partitioning were more subtle.

![Figure 35: Model of carbon partitioning in wild-type plants at the beginning of the light period (A) and at the middle of the light period (B). The thickness of the arrow indicates the amount of carbon channeled into the respective pools.]

Just after the onset of light, carbon flow into sugars was highly increased at the expense of flow into starch, acidic and basic compounds. The increased flow towards sugars was accompanied by higher carbon export to sink leaves and to the root. This implies that even wild-type plants, despite possessing starch as transient carbon storage for night-time metabolism, are depleted in soluble metabolites in the morning at least in sink tissues and need to be replenished. The high export rates in the morning could either be a result of the increased partitioning into sugars, which “pushes” sucrose into the phloem and subsequently to the sink, or they could cause the increased partitioning into sugars, as a
high sink strength is “pulling” sugars towards itself, therefore channeling more carbon into the synthesis of sugars. Additional experiments, for instance on detached leaves, could elucidate if the observed carbon fluxes in the morning are caused by the sink strength or if internal mechanisms cause the channeling of carbon towards sugars. Moreover, data of absolute sugar levels in sink and source leaves at the end of the dark period would reveal the carbon status of the different tissues and could be helpful in the further interpretation of the observed carbon partitioning patterns.

Carbon flow to structural compounds, like proteins, the remaining insoluble fraction comprising cell wall material and ethanol soluble compounds including lipids was also increased in the beginning of the light period. Carbon flow into storage compounds, on the other hand, was decreased in the morning, but was strongly enhanced later during the light period. While only 14% of carbon was introduced into starch at the beginning of the light period, partitioning into this fraction increased to more than 30% later on. The flow of carbon into starch was thereby consistent with the starch amount measured over a 12-h light period (Zeeman et al., 2004; Lee S.K. unpublished data). In the first hour of the light period, the rate of starch synthesis is only ~50%, followed by a steady increase to the maximal synthesis rate in the middle of the light period. During the last quarter of the light period, starch synthesis rates are slightly decreasing again (Figure 29; Zeeman et al., 2004). This trend seems to be even more pronounced in longer light periods. Starch content of plants in a 16 h light/ 8h dark regime is only slightly increasing in the first 8 h of the day. Most of the starch is synthesized in the second half of the light period (Zhang et al., 2005; Zhang et al., 2008). Carbon partitioning in these light conditions shows presumably even more changes than in a 12 h light/ 12 h dark regime.

Alterations in starch synthesis lead to a complex reorganization in carbon partitioning

Similar changes in carbon partitioning could be observed for all studied genotypes affected in starch synthesis. The changes increased gradually with the severity of the mutation. Naturally, carbon flow into starch was reduced in all lines. This was accompanied by changes in carbon allocation to nearly all other compound classes. At the beginning of the light
period, these changes were most obvious (summarized in Figure 36). A high amount of carbon was channeled into sugars at the expense of most other compounds, and more carbon was exported from the rosette into the roots. This is most likely due to a so-called starvation response caused by a lack of sugars in the preceding dark period (Smith and Stitt, 2007). As the plants do not accumulate sufficient amounts of starch, carbon reserves are depleted early during the dark period leading to an inhibition of growth. At the beginning of the following light period, assimilated carbon is not used for biosynthesis and growth, but is channeled into sugars. Gibon et al. (2004) estimated that 75% of the assimilated carbon is accumulating in the form of sugars at the beginning of the light period in pgm. In our labeling experiments of mature pgm leaves, we could confirm that, within the first minutes of light 80% (Figure 31) and within the first hour of light 44% (Figure 33) of the assimilated carbon is introduced into sugar compounds.

![Figure 36: Model of carbon partitioning at the beginning of the light period in wild-type plants (A) and in plants suffering from starvation (B). The thickness of the arrow indicates the amount of carbon channeled into the respective pools.](image)

Despite the increased carbon export and carbon flow into sugars, carbon partitioning into proteins was also increased in starch synthesis mutants. This was observed especially in sink leaves of pgi and pgm, in which more than twice as much carbon was introduced into proteins compared to the wild type at all times during the light period (Figure 30). Interestingly, this was accompanied by a decrease in carbon flow towards basic compounds.
representing amino acids (Figure 31). The increased flow of carbon into proteins might indicate an increased protein turnover. Higher rates of protein synthesis would consequently lead to an increased utilization of amino acids and probably a higher carbon flux through these compounds, which would explain the low amount of labeled carbon found in the basic fraction. The trigger for the increased protein turnover might be the carbon starvation pgm and pgi experience during the dark period. In metabolite and transcript studies of wild-type plants exposed to an extended night amino acid levels and expression of genes involved in protein degradation increased towards the end of the dark period. A similar situation could be seen for pgm after a normal night. (Gibon et al., 2006; Usadel et al., 2008). Therefore, it is likely that the lack of resources in pgm and pgi activates protein degradation to release carbon. During the light period, when sufficient carbon is available, proteins can be synthesized again resulting in a high carbon flow into this compound. Much more energy is consumed for the biosynthesis and degradation of proteins (4.7-7.9 ATP per amino acid, Zerihun et al., 1998) compared to the synthesis and degradation of starch. Thus, the alternative usage of proteins is associated with high costs for the plants. This, together with the decreased utilization for structural components at the beginning of the light period, could explain the decrease in growth rates observed in starch synthesis mutants.

In the middle of the light period, carbon partitioning into starch was increased for all lines except for pgm and isa1/isa2 (isa1/isa2 displays an increased partitioning into phytoglycogen instead; summarized in Figure 37). Excess carbon, which cannot be used for the synthesis of starch due to the respective alteration, was now equally distributed between all other compounds. Carbon flow into alternative storage compounds, for example organic acids, was not observed. Only in pgm, carbon partitioning into sugars was still highly increased in the middle of the light period, which agrees with the accumulation of sugars observed in metabolite studies performed with pgm (Bläsing et al., 2005; Gibon et al., 2006).

The increase of carbon flow towards sugars was accompanied by reduced carbon partitioning into structural components. In pgm, the amount of carbon used to build up
structural compounds was reduced in the beginning of the light period to half of the wild type. However, later during the light period, more carbon was utilized for the synthesis of structural compounds compared to the wild-type plants compensating for the reduced carbon flow in the morning.

![Figure 37: Model of carbon partitioning in the middle of the day in wild-type plants (A), plants altered in starch synthesis (B) and plants altered in starch breakdown (C). The thickness of the arrow indicates the amount of carbon channeled into the respective pool.](image)

Alterations in starch degradation lead to a reduced carbon allocation towards starch

Plants altered in starch degradation display a carbon partitioning phenotype similar to starch synthesis mutants at the beginning of the light period. Despite their ability to synthesis starch properly, these mutants cannot mobilize their carbon reserves during the night, and, consequently, suffer from starvation. The similar partitioning pattern is supporting the idea that the increased carbon flow into sugars and the resulting increase in export is caused by the starvation during the dark and not by the impairments in the starch synthesis mutants per se.

After the onset of light, carbon was channeled into sugars at the expense of carbon flow into acidic, basic and ethanol soluble compounds, as well as proteins and the remaining insoluble compounds (Figure 37 C). This was accompanied by an increased export of carbon
into the roots, as was also seen for starch synthesis mutants. Additionally, an increase in carbon flow into starch was observed in starch degradation mutants compared to the wild type. As a consequence of carbon limitation, starch synthesis was shown to be up-regulated by post-translational regulation of AGPase in wild-type plants after exposing them to an extended night (Gibon et al., 2004). Starch synthesis during the following light period was increased, in order to avoid starch depletion in the subsequent night. However, this up-regulation seems to be only transient in the starch degradation mutants, as in the middle of the light period all lines utilized slightly less carbon for starch synthesis than the wild type. Overall this seems to result in equal amount of newly synthesized starch in wild-type plants and starch degradation mutants over the light period.

Later during the light period, carbon partitioning differed only slightly from the pattern observed in wild-type plants (Figure 37 C). A small increase in partitioning towards ethanol soluble, acidic and basic compounds was found, accompanied by a small decrease in partitioning towards starch, even though it was not significant. Different mechanisms could explain this decrease in partitioning into starch. Although the plants are not altered in starch synthesis per se, there might be a feedback control mechanism signaling to the plant that a sufficient amount of starch was accumulated already. This is unlikely, because high amounts of starch are abundant within the cell after the onset of light, but even more carbon is partitioned towards starch. It is also possible that the chloroplast reaches the maximum amount of starch that it can accumulate. This is consistent with the fact that the amount of carbon introduced into starch is decreasing with the starch excess phenotype of the plants. The mutant, which accumulates the highest amounts of starch, sex1, had the lowest partitioning into this compound in the middle of the day. Yet, sex1 displays a much higher starch excess phenotype than sex4 and pwd suggesting that the maximum amount of starch that can be accumulated is much higher and down-regulation of starch synthesis is not caused by this limitation, at least in sex4 and pwd. A third possible reason would be a compensation for the lower partitioning into acidic, basic and ethanol soluble compounds at the beginning of the light period.
Partitioning into proteins and structural compounds was affected in starch degradation mutants similarly to the starch synthesis mutants. As a result of the carbon starvation, less carbon was used for the synthesis of proteins and structural compounds in the beginning of the day (50% less in sex1 compared to the wild type), but this was compensated for later during the light.

I observed similar carbon export rates in the middle of the day for wild-type plants and starch degradation mutants. In a previous study, higher export rates were shown for sex4 compared to the wild-type (Zeeman and ap Rees, 1999). This experiment was also performed at the middle of a 12 h light period, but partitioning after a 5 h chase, rather than a 1 h chase as in my experiment, was analyzed. After the longer chase, carbon export in sex4 was more than twice as much as in wild type. It might be that in contrast to wild-type plants, which export the majority of carbon within the first hour (Figure 25), sex4 shows different export dynamics. An additional time-course experiment would reveal if this is indeed the case.
6 General discussion

The aim of the project was to investigate growth processes on a whole plant and metabolic level in *Arabidopsis thaliana*. Several metabolite studies exist for this species, but they only report metabolite levels at a given developmental stage of the plant. It is not described for which processes the metabolites are utilized. Absolute values of metabolites are not sufficient to relate growth and metabolism. For this, a detailed knowledge about the amount of assimilated carbon and its utilization for different metabolic pathways has to be obtained.

To achieve this, I developed a system to measure gas exchange of whole Arabidopsis rosettes, in the course of my thesis. This system measures up to eight plants in parallel allowing for reliable and comparable data. With the gas exchange system, a precise knowledge about carbon uptake can be obtained for plants growing in different environmental conditions, for plants exposed to different stresses and for plants modified in their carbon metabolism. This knowledge is an important prerequisite for the interpretation of carbon partitioning data, as data gained from isotopic labeling experiments are mostly relative values. To obtain precise measurements of carbon partitioning on a whole plant and single leaf level, and with a relatively high throughput, I optimized existing isotopic labeling methods.

With these two methods established, I studied carbon assimilation and carbon partitioning to elucidate how these processes are related to growth. Moreover, I analyzed how alterations in central carbon metabolism affect the assimilation and partitioning of carbon, and consequently, the plant’s ability to grow.

*Interplay between carbon partitioning and growth*

Biomass is gained by proliferation and extension of cells. Essential for cell expansion is the generation of new cell wall and membrane material, but also the increase in volume facilitated by influx of water into the vacuole. Water uptake is triggered by the relaxation of
the cell wall, which lowers the water potential within the cell and results in the influx of water. Leaf expansion is thereby limited biophysically by the amount of water influx and also metabolically, be the supply of carbon. Pantin et al. (2011) showed that the expansion of leaves during development is limited first by metabolism, observable as reduced growth rates during the night, probably caused by limited carbon availability in the developing leaves. Later in development (4-5 days after leaf emergence in wild type and later in starch mutants), growth rates during the day are lower than during the night, which is probably caused by limitations of the hydraulic system to provide the expanding cells with water.

On a metabolic level, as already mentioned, growth is characterized by high biosynthetic activity, in particular the synthesis of proteins and of structural components, like lipids for membranes and cell wall material. In isotopic feeding experiments, the rate of protein synthesis is indicated by the amount of carbon channeled into proteins, while the rates of lipid and cell wall synthesis are indicated by carbon flow towards ethanol soluble and the remaining insoluble material, respectively.

In developing leaves, a high biosynthetic activity can be assumed as the cells still proliferate and expand. At times of high cell proliferation, carbon allocation into the proteins and the ethanol soluble fraction can be expected, while a strong cell expansion can be expected to result in carbon incorporation into the remaining insoluble fraction.

Sink leaves generally incorporate more carbon into all three fractions mentioned. While carbon flow into proteins and the remaining insoluble fraction was observed to be high (~5% and 12%, respectively) for all feeding time points, carbon incorporation into ethanol soluble compounds peaked after 2 h of illumination.

In contrast to sink leaves, source leaves, if elongating at all, do so without cell proliferation. Leaf 8, the source leaf labeled in most of the experiments performed, was still elongating at the time point of labeling. It is in a similar developmental stage as leaves used for leaf expansion measurements. Leaf expansion measurements are limited to a minimal size of 0.5 mm leaf length and are performed usually on leaves having less than 50% of the size of the fully expanded leaf. At this stage, the leaf is in an intermediary stage of leaf expansion, in which maximal growth rates can be observed (Donnelly et al., 1999; Wiese et al., 2007). Measuring growth of wild-type leaves revealed highest leaf expansion rates at one hour.
after the onset of light, followed by a decrease in expansion during the day. The peak in expansion rates coincides with a high partitioning of carbon into proteins, lipids and cell wall material within the first 2 h of the light period. The partitioning pattern of the sink leaves indicates continuous cell proliferation and synthesis of new membranes and cell walls, possibly accompanied by continuous expansion throughout the light period. Unfortunately, the methods used to measure leaf elongation so far, did not allow to measure structures that small. New camera systems, which allow leaf elongation measurements without fixation of the leaf in a focus plane, could help to overcome this size limitation.

**Carbon starvation during the night and its effects on carbon partitioning and growth**

Plants suffering from carbon starvation during the dark period have overall reduced growth rates, probably caused by the lack of carbon reserves during the dark and by the degradation of proteins used as an alternative carbon reserve during the time of starvation (see Discussion 5.3). In addition, these plants have altered temporal growth patterns, as was shown for pgm (Wiese et al., 2007). Leaf expansion is significantly dampened in the beginning of the light period, but starting from the middle of the day it increased to reach its maximum one hour after dusk. This change in expansion pattern is most likely caused by carbon starvation during the night. As a result of this starvation, plants do not utilize their carbon for biosynthetic process at the beginning of the following light period, but rather build-up sugars (Gibon et al., 2004). This reduced utilization of carbon for biosynthetic processes was reflected in the partitioning pattern of starchless mutants.

However, carbon flow into proteins is still peaking at 2 h after the onset of light; but carbon flow into cell wall material and lipids is low in the morning and increasing over the light period to reach its maximum at the end of the day. In sink leaves of the starchless pgm, even two peaks for carbon partitioning into cell wall material could be observed, the first after 2 h of light and the second at the end of the light period, coinciding with the two peaks in leaf expansion. From this data, it can be deduced that leaf expansion and carbon partitioning into cell wall material and lipids, two processes essential for cell expansion, are accompanied by each other. In times of high leaf growth rates, a bigger amount of carbon is channeled towards cell wall material and lipid synthesis, and less carbon is used for carbon
export and the synthesis of storage compounds, while it is vice versa at times of low leaf growth.

**Regulation of carbon partitioning**

Regulation of carbon partitioning between starch and sucrose takes place at several different levels of metabolism. It is adjusted according to photosynthesis and the supply of carbon, but also to the carbon demand of sink tissues. Especially at the beginning of the light period, a high demand for carbon in the sinks can be expected, which might explain the high carbon flow into sugars and the high carbon export rates observed in feeding experiments. However, this does not fully explain the observed temporal changes in carbon partitioning, especially not the high flow of carbon into structural compounds at the beginning of the day. Other regulatory mechanisms are needed to adjust carbon investment into growth and storage according to the available carbon.

One level of regulation seems to be carbon flow into starch. The carbon partitioning data presented here suggests that starch synthesis is not linear for the whole light period, but is showing a temporal pattern as well. In the beginning of the light period, only 15% of carbon is utilized for starch synthesis, followed by an increase which peaks at 35% around midday. This pattern is present even in starch degradation mutants, like sex1, which suffer from a severe carbon starvation during the night. The starvation response leads to an increased carbon flow into sugars resulting in a decreased flow into all other compounds. Only carbon flow into starch is unaffected. It shows a similar pattern as in wild type.

It seems reasonable that carbon partitioning into starch is the primarily regulated process, as it is important to assure synthesis of sufficient carbon reserves. When less carbon is used for starch synthesis, for instance at the beginning of the light period, the excess carbon can be used for growth. Plants grown in short days, with only 8 h light, need to build up the same amount of starch reserves in less time. As more carbon is channeled into starch, less is available for growth, which is reflected indeed in less growth during the day and higher growth during the night (Sulpice R. and Stitt M., unpublished data). In longer photoperiods, on the other hand, less carbon is used to build up starch, mainly later in the light period (Zhang et al., 2005; Zhang et al., 2008). Therefore, more carbon can be used for growth. I
would expect high growth rates in the beginning of the light period under these conditions, but this has not been shown experimentally so far.

At least starch degradation is described to be under circadian control (Graf et al., 2010). By anticipating the length of the dark period, plants can immediately adjust their starch degradation to an unexpected onset of night, thereby maintaining a near linear breakdown of starch over the whole dark period. It is highly likely that also starch synthesis is affected by the circadian clock although so far no direct link was revealed. However, starch synthesis adapts for instance in response to different photoperiods. In short days relatively more carbon is partitioned into starch compared to long days, so that at the end of the light period a similar amount of starch reserves are available. To achieve this, the plant needs to anticipate the length of the day and coordinate carbon partitioning accordingly. A regulatory mechanism is needed for this and regulation by the circadian clock is highly likely, but so far nothing is known about the nature of the mechanism or the regulatory factors involved.

Other factors might also be involved in the regulation of carbon partitioning. The repartitioning of carbon occurring after carbon starvation is likely controlled not only by carbon demand. Recently, a link between nighttime carbon status and gibberellin signaling was shown (Paparelli et al., unpublished manuscript). Only sufficient carbon reserves during the night lead to the expression of gibberellins, which themselves influence carbon partitioning and activate the transcription of several genes involved in cell expansion. In the case of carbon starvation, gibberellin synthesizing genes are less expressed which results in less growth, potentially being the reason for the high amount of carbon channeled into sugar compounds.

*Carbon flow into a metabolite compared to actual metabolite levels*

Metabolite levels are the result of the amount of metabolite generated minus the amount of metabolite consumed. Their level is determined by the fluxes of reactions producing and consuming the metabolite. An increase in a metabolite level can therefore represent an increase in flux into the metabolite, but also a decrease in the consumption of the metabolite. Considering metabolite levels alone cannot tell about fluxes in a certain pathway. To measure carbon flux through metabolite pools, time course experiments need
to be performed (section 4.2.5), most probably even with more chase periods to achieve a higher resolution. My focus however, lies on the utilization of carbon for export, storage processes and, finally, growth. Therefore, most experiments in this work measure carbon partitioning only at one time point (60 min after the isotopic pulse). Most of the export into the sink tissues and the carbon flow into end-products or quasi-products, like starch, proteins and cell wall material (belonging to the remaining insoluble fraction) is completed after a chase period of 60 min. Flow through intermediary pools, like sucrose, sugar phosphates (belonging to the acidic fraction), nucleotides and amino acids (belonging to the basic fraction), on the other hand, is peaking early after the pulse period and cannot be evaluated correctly from partitioning data after a 60 min chase period.

Even though an increased carbon partitioning into a certain metabolite is not always reflected on the metabolite level, this can be seen for many end-products or quasi-products. For example, carbon flow into starch, which marks a quasi-product, and starch levels at the end of the light period correlate well in starch synthesis mutants, the amount of accumulated starch is solely dependent on the amount of carbon partitioned into it. The increased carbon flow into sugar compounds is reflected in increased sugar levels, especially Glc and Fru, in many starch synthesis mutants. Pgi shows 6-fold increased Glc and Fru levels compared to the wild type, while pgm shows a 10-fold increase. Higher sugar levels were also detected in starch degradation mutants (Bläsing et al., 2005; Kunz et al., 2010). Sex4 displays a 3-fold increase of Glc and Fru in the first half of the light period (Comparot-Moss et al., 2010). This is probably caused by the high carbon flow into sugar compounds after the onset of light, the typical repartitioning of carbon occurring after carbon starvation during the night.

Other isotopic labeling approach needed to analyze carbon partitioning into specific soluble metabolites

With $^{14}$C labeling, export of carbon and carbon partitioning into different compound classes can be studied. The applied method is suitable to study carbon flow into compounds like starch and proteins, but not into specific soluble metabolites. Ion exchange chromatography separates the soluble metabolites only roughly into neutral, acidic and basic compounds.
Changes in partitioning into these pools can be detected with $^{14}$C labeling, but cannot be attributed to a change of one particular metabolite. Therefore, either more refined separation methods for the soluble fraction are needed, for instance HPLC, or a different labeling method needs to be applied.

An alternative method is $^{13}$C labeling coupled to gas-chromatography mass-spectrometry (GC-MS) or liquid-chromatography mass-spectrometry (LC-MS) for the analysis of the plant material. The isotopic labeling of the plant material can be performed as described for $^{14}$C labeling, but the fractionation of the material is performed by gas chromatography or liquid chromatography. Following the chromatographic separation, the metabolites and their labeling pattern (amount of $^{13}$C incorporated) are identified by mass-spectrometry.

$^{13}$C labeling was heavily used in the last 10-15 years to study metabolic fluxes in plants and other organisms (reviewed in Schwender, 2011). Most of these experiments were performed with heterotrophic tissues grown on minimal medium to which one $^{13}$C-labeled carbon source, for instance $1^{-13}$C-glucose, $U^{-13}$C-glucose or $U^{-13}$C-sucrose, is added (Williams et al., 2008; Lonien and Schwender, 2009; Masakapalli et al., 2010). After attaining a steady-state for the labeled metabolites, the plant material is harvested, extracted and the labeling signature of each metabolite is analyzed. Depending on the carbon source and the pathway of synthesis, defined $^{12}$C/$^{13}$C patterns can be observed for certain metabolites. This information, together with a biochemical network model, is used to calculate metabolic fluxes. In order to detect as many metabolites as possible, different detection techniques and extraction methods need to be combined, but complete detection of the incorporated $^{13}$C is unlikely.

However, a combination of $^{14}$CO$_2$ and $^{13}$CO$_2$ labeling would enable to monitor both carbon fluxes into different tissues and macromolecules via $^{14}$C labeling, and carbon flow into certain individual metabolites by $^{13}$C labeling. The calculation of carbon fluxes in central metabolism is practically impossible when labeling with $^{13}$CO$_2$ instead of a labeled sugar, as the carbon of all metabolites is derived from $^{13}$C. However, this setup would allow to measure carbon partitioning into and flux through selected metabolites in photosynthesizing plants under natural growth conditions.
References


References


Table A1: Carbon partitioning in Col-0 at different time points over the diurnal cycle. Leaf 8 of Col-0 plants was labeled with $^{14}$CO$_2$ for 2 min just after the plants were exposed to light (0 h) and after 2 h, 6 h and 10 h of light. Following a chase period of 1 h, the labeled leaf and the unlabeled rosette (sink leaves) were harvested separately and the incorporation of $^{14}$C was determined. The sum of the label in the labeled leaf is set to 100% as well as the sum of the label in the sink leaves. The relative amount of $^{14}$C incorporated into the different compound classes as a percentage of the total label in the tissue is shown. Mean ± SE (n≥5).

<table>
<thead>
<tr>
<th></th>
<th>% of $^{14}$C recovered in each fraction related to the total amount per tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Col-0 0</td>
</tr>
<tr>
<td><strong>Labeled leaf</strong></td>
<td></td>
</tr>
<tr>
<td>Water soluble compounds</td>
<td>59.83 ± 0.79</td>
</tr>
<tr>
<td>Neutral</td>
<td>43.85 ± 5.04</td>
</tr>
<tr>
<td>Acidic</td>
<td>12.13 ± 0.81</td>
</tr>
<tr>
<td>Nucleotide</td>
<td>0.92 ± 0.10</td>
</tr>
<tr>
<td>Basic</td>
<td>6.74 ± 0.37</td>
</tr>
<tr>
<td>Insoluble compounds</td>
<td>29.78 ± 1.55</td>
</tr>
<tr>
<td>Starch</td>
<td>14.12 ± 2.05</td>
</tr>
<tr>
<td>Protein</td>
<td>5.09 ± 0.39</td>
</tr>
<tr>
<td>Remaining insoluble</td>
<td>6.32 ± 0.37</td>
</tr>
<tr>
<td><strong>Ethanol soluble compounds</strong></td>
<td>10.40 ± 1.07</td>
</tr>
<tr>
<td><strong>Sink leaves</strong></td>
<td></td>
</tr>
<tr>
<td>Water soluble compounds</td>
<td>60.30 ± 0.88</td>
</tr>
<tr>
<td>Neutral</td>
<td>20.16 ± 5.22</td>
</tr>
<tr>
<td>Acidic</td>
<td>19.85 ± 4.83</td>
</tr>
<tr>
<td>Nucleotide</td>
<td>1.80 ± 0.05</td>
</tr>
<tr>
<td>Basic</td>
<td>11.29 ± 2.20</td>
</tr>
<tr>
<td>Insoluble compounds</td>
<td>29.39 ± 1.17</td>
</tr>
<tr>
<td>Starch</td>
<td>8.81 ± 0.67</td>
</tr>
<tr>
<td>Protein</td>
<td>5.54 ± 0.08</td>
</tr>
<tr>
<td>Remaining insoluble</td>
<td>12.09 ± 0.82</td>
</tr>
<tr>
<td><strong>Ethanol soluble compounds</strong></td>
<td>10.31 ± 0.54</td>
</tr>
</tbody>
</table>
Table A2: Carbon partitioning in *pgm* at different time points over the diurnal cycle. Leaf 8 of *pgm* plants was labeled with $^{14}$CO$_2$ for 2 min just after the plants were exposed to light (0 h) and after 2 h, 6 h and 10 h of light. Following a chase period of 1 h, the labeled leaf and the unlabeled rosette (sink leaves) were harvested separately and the incorporation of $^{14}$C was determined. The sum of the label in the labeled leaf is set to 100% as well as the sum of the label in the sink leaves. The relative amount of $^{14}$C incorporated into the different compound classes as a percentage of the total label in the tissue is shown. Mean ± SE (n≥5).

<table>
<thead>
<tr>
<th>Labeled leaf</th>
<th>pgm 0</th>
<th>pgm 2</th>
<th>pgm 6</th>
<th>pgm 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water soluble compounds</td>
<td>92.38 ± 0.80</td>
<td>84.51 ± 0.67</td>
<td>83.60 ± 0.49</td>
<td>82.24 ± 1.56</td>
</tr>
<tr>
<td>Neutral</td>
<td>80.01 ± 1.63</td>
<td>59.34 ± 1.50</td>
<td>54.24 ± 1.02</td>
<td>51.29 ± 2.95</td>
</tr>
<tr>
<td>Acidic</td>
<td>6.07 ± 0.66</td>
<td>18.64 ± 1.57</td>
<td>18.69 ± 1.52</td>
<td>19.42 ± 1.20</td>
</tr>
<tr>
<td>Nucleotide</td>
<td>0.23 ± 0.02</td>
<td>0.37 ± 0.04</td>
<td>0.27 ± 0.01</td>
<td>0.35 ± 0.04</td>
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<tr>
<td>Basic</td>
<td>4.68 ± 0.74</td>
<td>3.93 ± 0.62</td>
<td>6.76 ± 1.22</td>
<td>9.22 ± 1.24</td>
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<tr>
<td>Insoluble compounds</td>
<td>4.58 ± 0.57</td>
<td>10.07 ± 0.42</td>
<td>10.29 ± 0.30</td>
<td>11.65 ± 1.40</td>
</tr>
<tr>
<td>Starch</td>
<td>0.13 ± 0.09</td>
<td>0.05 ± 0.02</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Protein</td>
<td>3.86 ± 1.05</td>
<td>6.48 ± 0.22</td>
<td>5.94 ± 0.25</td>
<td>5.91 ± 0.70</td>
</tr>
<tr>
<td>Remaining insoluble</td>
<td>0.93 ± 0.25</td>
<td>3.54 ± 0.47</td>
<td>4.35 ± 0.22</td>
<td>5.74 ± 1.06</td>
</tr>
<tr>
<td>Ethanol soluble compounds</td>
<td>3.04 ± 0.26</td>
<td>5.42 ± 0.33</td>
<td>6.10 ± 0.27</td>
<td>6.11 ± 0.73</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sink leaves</th>
<th>pgm 0</th>
<th>pgm 2</th>
<th>pgm 6</th>
<th>pgm 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water soluble compounds</td>
<td>84.06 ± 2.59</td>
<td>82.86 ± 0.84</td>
<td>76.50 ± 1.96</td>
<td>79.77 ± 2.01</td>
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<tr>
<td>Neutral</td>
<td>67.74 ± 2.12</td>
<td>59.60 ± 3.39</td>
<td>52.79 ± 3.61</td>
<td>58.07 ± 3.35</td>
</tr>
<tr>
<td>Acidic</td>
<td>10.92 ± 0.95</td>
<td>13.49 ± 1.22</td>
<td>11.56 ± 0.44</td>
<td>12.92 ± 1.80</td>
</tr>
<tr>
<td>Nucleotide</td>
<td>0.42 ± 0.03</td>
<td>0.36 ± 0.03</td>
<td>0.40 ± 0.16</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td>Basic</td>
<td>4.68 ± 0.70</td>
<td>4.65 ± 0.50</td>
<td>7.02 ± 1.36</td>
<td>7.84 ± 1.02</td>
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<td>Insoluble compounds</td>
<td>14.83 ± 2.60</td>
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<td>21.12 ± 2.01</td>
<td>18.02 ± 1.98</td>
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<tr>
<td>Starch</td>
<td>2.00 ± 1.33</td>
<td>0.01 ± 0.01</td>
<td>4.67 ± 0.66</td>
<td>1.06 ± 0.65</td>
</tr>
<tr>
<td>Protein</td>
<td>8.12 ± 1.53</td>
<td>8.99 ± 0.36</td>
<td>13.21 ± 1.04</td>
<td>11.65 ± 1.44</td>
</tr>
<tr>
<td>Remaining insoluble</td>
<td>3.15 ± 0.83</td>
<td>5.70 ± 0.36</td>
<td>3.24 ± 0.54</td>
<td>5.31 ± 1.25</td>
</tr>
<tr>
<td>Ethanol soluble compounds</td>
<td>1.11 ± 0.05</td>
<td>2.45 ± 0.46</td>
<td>2.38 ± 0.19</td>
<td>2.21 ± 0.11</td>
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</table>
Table A3: Carbon partitioning in *pgi* at different time points over the diurnal cycle. Leaf 8 of *pgi* plants was labeled with ^14^CO_2 for 2 min just after the plants were exposed to light (0 h) and after 2 h, 6 h and 10 h of light. Following a chase period of 1 h, the labeled leaf and the unlabeled rosette (sink leaves) were harvested separately and the incorporation of ^14^C was determined. The sum of the label in the labeled leaf is set to 100% as well as the sum of the label in the sink leaves. The relative amount of ^14^C incorporated into the different compound classes as a percentage of the total label in the tissue is shown. Mean ± SE (n≥5).

<table>
<thead>
<tr>
<th>% of ^14^C recovered in each fraction related to the total amount per tissue</th>
<th><em>pgi</em> 0</th>
<th><em>pgi</em> 2</th>
<th><em>pgi</em> 6</th>
<th><em>pgi</em> 10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Labeled leaf</strong></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Water soluble compounds</td>
<td>81.70 ± 1.54</td>
<td>68.61 ± 2.97</td>
<td>71.96 ± 2.05</td>
<td>74.08 ± 1.80</td>
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<tr>
<td>Neutral</td>
<td>61.34 ± 2.01</td>
<td>33.49 ± 4.12</td>
<td>43.14 ± 3.47</td>
<td>47.53 ± 4.79</td>
</tr>
<tr>
<td>Acidic</td>
<td>8.49 ± 1.00</td>
<td>23.12 ± 1.77</td>
<td>17.29 ± 2.00</td>
<td>16.54 ± 1.79</td>
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<tr>
<td>Nucleotide</td>
<td>0.32 ± 0.03</td>
<td>0.4 ± 0.03</td>
<td>0.28 ± 0.05</td>
<td>0.30 ± 0.03</td>
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<tr>
<td>Basic</td>
<td>7.72 ± 0.54</td>
<td>8.13 ± 2.22</td>
<td>6.60 ± 1.27</td>
<td>7.23 ± 1.69</td>
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<tr>
<td>Insoluble compounds</td>
<td>12.63 ± 0.98</td>
<td>22.75 ± 1.17</td>
<td>21.34 ± 1.38</td>
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<tr>
<td>Starch</td>
<td>3.37 ± 0.39</td>
<td>6.69 ± 0.38</td>
<td>10.89 ± 0.73</td>
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</tr>
<tr>
<td>Protein</td>
<td>7.61 ± 0.84</td>
<td>12.37 ± 1.00</td>
<td>9.28 ± 1.07</td>
<td>9.09 ± 0.56</td>
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<tr>
<td>Remaining insoluble</td>
<td>1.67 ± 0.51</td>
<td>3.90 ± 1.25</td>
<td>2.16 ± 0.90</td>
<td>2.71 ± 0.96</td>
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<td>Ethanol soluble compounds</td>
<td>5.67 ± 0.57</td>
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<td>6.71 ± 0.72</td>
<td>6.22 ± 0.57</td>
</tr>
<tr>
<td><strong>Sink leaves</strong></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Water soluble compounds</td>
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<tr>
<td>Neutral</td>
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</tr>
<tr>
<td>Acidic</td>
<td>14.05 ± 1.48</td>
<td>15.49 ± 1.37</td>
<td>14.23 ± 1.06</td>
<td>11.89 ± 1.22</td>
</tr>
<tr>
<td>Nucleotide</td>
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<td>0.38 ± 0.05</td>
<td>0.36 ± 0.02</td>
<td>0.58 ± 0.05</td>
</tr>
<tr>
<td>Basic</td>
<td>8.28 ± 0.97</td>
<td>7.04 ± 1.26</td>
<td>8.08 ± 1.26</td>
<td>7.47 ± 1.12</td>
</tr>
<tr>
<td>Insoluble compounds</td>
<td>23.66 ± 2.28</td>
<td>25.20 ± 1.23</td>
<td>24.63 ± 2.03</td>
<td>20.20 ± 1.14</td>
</tr>
<tr>
<td>Starch</td>
<td>4.24 ± 1.22</td>
<td>7.32 ± 0.34</td>
<td>7.14 ± 1.15</td>
<td>4.46 ± 1.73</td>
</tr>
<tr>
<td>Protein</td>
<td>13.45 ± 1.88</td>
<td>14.08 ± 1.08</td>
<td>14.91 ± 1.43</td>
<td>11.31 ± 0.59</td>
</tr>
<tr>
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<td>5.97 ± 0.97</td>
<td>3.80 ± 0.67</td>
<td>2.59 ± 0.68</td>
<td>4.98 ± 1.76</td>
</tr>
<tr>
<td>Ethanol soluble compounds</td>
<td>3.06 ± 0.37</td>
<td>5.27 ± 1.34</td>
<td>3.01 ± 0.40</td>
<td>2.42 ± 0.26</td>
</tr>
</tbody>
</table>
Table A4: Relative growth rate of Col-0 and mutants altered in starch metabolism. Pictures of Col-0 and starch mutant plants grown in hydroponics were taken every 2 to 3 days from 7 days after sowing on. The exposed leaf area was calculated on the basis of the pictures and the leaf areas were used to calculate the relative growth rate per day (RGR d⁻¹). Mean n≥10.

<table>
<thead>
<tr>
<th></th>
<th>7-10 d</th>
<th>10-12 d</th>
<th>15-17 d</th>
<th>17-19 d</th>
<th>19-22 d</th>
<th>22-24 d</th>
<th>24-26 d</th>
<th>26-29 d</th>
<th>29-31 d</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0</td>
<td>0.318</td>
<td>0.422</td>
<td>0.297</td>
<td>0.268</td>
<td>0.287</td>
<td>0.260</td>
<td>0.253</td>
<td>0.223</td>
<td>0.211</td>
<td>0.282</td>
</tr>
<tr>
<td>adg2</td>
<td>0.315</td>
<td>0.356</td>
<td>0.266</td>
<td>0.241</td>
<td>0.291</td>
<td>0.267</td>
<td>0.211</td>
<td>0.191</td>
<td>0.157</td>
<td>0.255</td>
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<tr>
<td>ssiV</td>
<td>0.270</td>
<td>0.404</td>
<td>0.258</td>
<td>0.213</td>
<td>0.237</td>
<td>0.247</td>
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<td>n.d</td>
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<tr>
<td>pgI</td>
<td>0.335</td>
<td>0.200</td>
<td>0.245</td>
<td>0.183</td>
<td>0.253</td>
<td>0.264</td>
<td>0.213</td>
<td>0.207</td>
<td>0.210</td>
<td>0.235</td>
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<tr>
<td>pgm</td>
<td>0.271</td>
<td>0.263</td>
<td>0.217</td>
<td>0.192</td>
<td>0.218</td>
<td>0.215</td>
<td>0.213</td>
<td>0.184</td>
<td>0.185</td>
<td>0.218</td>
</tr>
<tr>
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<td>0.256</td>
<td>0.274</td>
<td>0.245</td>
<td>0.297</td>
<td>0.272</td>
<td>0.227</td>
<td>0.217</td>
<td>0.188</td>
<td>0.259</td>
</tr>
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<td>0.347</td>
<td>0.287</td>
<td>0.250</td>
<td>0.299</td>
<td>0.274</td>
<td>0.247</td>
<td>0.223</td>
<td>0.207</td>
<td>0.269</td>
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<td>0.249</td>
<td>0.240</td>
<td>0.169</td>
<td>0.215</td>
<td>0.224</td>
<td>0.206</td>
<td>0.197</td>
<td>0.207</td>
<td>0.218</td>
</tr>
<tr>
<td>sex4</td>
<td>0.256</td>
<td>0.327</td>
<td>0.269</td>
<td>0.237</td>
<td>0.269</td>
<td>0.263</td>
<td>0.256</td>
<td>0.177</td>
<td>0.212</td>
<td>0.252</td>
</tr>
</tbody>
</table>
Table A5: Carbon partitioning in Col-0 and starch mutants at the beginning of the light period. Col-0 and starch mutant plants grown in hydroponics were labeled with $^{14}$CO$_2$ for 1 h just after the plants have been exposed to light. Following a chase period of 1 h, the rosette and the root were harvested separately and the incorporation of $^{14}$C was determined. The relative amount of $^{14}$C incorporated into the different compound classes as a percentage of the total label in the plant is shown. n.d., not determined. Mean ± SE (n≥5).

<table>
<thead>
<tr>
<th>% of total $^{14}$C recovered in each fraction after labeling at the beginning of the light period</th>
<th>Control</th>
<th>Lines impaired in starch synthesis</th>
<th>Lines impaired in starch breakdown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeled leaf</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Col-0</td>
<td>91.29 ± 0.73</td>
<td>90.18 ± 1.16</td>
<td>90.18 ± 1.16</td>
</tr>
<tr>
<td>adg2</td>
<td>87.25 ± 1.91</td>
<td>85.33 ± 1.83</td>
<td>85.33 ± 1.83</td>
</tr>
<tr>
<td>ssvIV</td>
<td>80.58 ± 2.42</td>
<td>80.58 ± 2.42</td>
<td>80.58 ± 2.42</td>
</tr>
<tr>
<td>pgi</td>
<td>88.99 ± 0.12</td>
<td>88.99 ± 0.12</td>
<td>88.99 ± 0.12</td>
</tr>
<tr>
<td>pgm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>isa1/isa2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pwd</td>
<td>88.84 ± 0.67</td>
<td>88.84 ± 0.67</td>
<td>88.84 ± 0.67</td>
</tr>
<tr>
<td>sex4</td>
<td>88.63 ± 0.87</td>
<td>88.63 ± 0.87</td>
<td>88.63 ± 0.87</td>
</tr>
<tr>
<td>sex1</td>
<td>87.90 ± 0.98</td>
<td>87.90 ± 0.98</td>
<td>87.90 ± 0.98</td>
</tr>
<tr>
<td>Water soluble compounds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>92.52 ± 0.71</td>
<td>97.52 ± 2.26</td>
<td>97.52 ± 2.26</td>
<td></td>
</tr>
<tr>
<td>Neutral</td>
<td>47.52 ± 2.66</td>
<td>52.03 ± 2.13</td>
<td>52.03 ± 2.13</td>
</tr>
<tr>
<td>Phytoglycogen</td>
<td>3.75 ± 0.23</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Acidic</td>
<td>15.15 ± 0.61</td>
<td>14.17 ± 1.04</td>
<td>14.17 ± 1.04</td>
</tr>
<tr>
<td>Nucleotide</td>
<td>0.41 ± 0.24</td>
<td>0.43 ± 0.22</td>
<td>0.43 ± 0.22</td>
</tr>
<tr>
<td>Basic</td>
<td>9.80 ± 0.92</td>
<td>8.50 ± 0.83</td>
<td>8.50 ± 0.83</td>
</tr>
<tr>
<td>Insoluble compounds</td>
<td>41.16 ± 0.88</td>
<td>34.28 ± 1.61</td>
<td>34.28 ± 1.61</td>
</tr>
<tr>
<td>Starch</td>
<td>15.59 ± 1.35</td>
<td>10.15 ± 2.38</td>
<td>10.15 ± 2.38</td>
</tr>
<tr>
<td>Protein</td>
<td>13.39 ± 0.73</td>
<td>11.96 ± 0.54</td>
<td>11.96 ± 0.54</td>
</tr>
<tr>
<td>Remaining insoluble</td>
<td>12.12 ± 1.32</td>
<td>12.18 ± 1.20</td>
<td>12.18 ± 1.20</td>
</tr>
<tr>
<td>Ethanol soluble compounds</td>
<td>7.56 ± 0.42</td>
<td>8.4 ± 0.78</td>
<td>8.4 ± 0.78</td>
</tr>
<tr>
<td>Root</td>
<td>8.71 ± 0.73</td>
<td>9.80 ± 1.16</td>
<td>9.80 ± 1.16</td>
</tr>
</tbody>
</table>
Table A6: Carbon partitioning in Col-0 and starch mutants in the middle of the light period. Col-0 and starch mutant plants grown in hydroponics were labeled with $^{14}$CO$_2$ for 1 h in the middle of the light period (after 6h light). Following a chase period of 1 h, the rosette and the root were harvested separately and the incorporation of $^{14}$C was determined. The relative amount of $^{14}$C incorporated into the different compound classes as a percentage of the total label in the plant is shown. n.d., not determined. Mean ± SE (n≥5).

<table>
<thead>
<tr>
<th>% of total $^{14}$C recovered in each fraction after labeling at the beginning of the light period</th>
<th>Control</th>
<th>Lines impaired in starch synthesis</th>
<th>Lines impaired in starch breakdown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeled leaf</td>
<td>Col-0</td>
<td>adg2</td>
<td>ssiV</td>
</tr>
<tr>
<td>Col-0</td>
<td>93.45 ± 0.78</td>
<td>93.90 ± 0.73</td>
<td>92.05 ± 1.05</td>
</tr>
<tr>
<td>Water soluble compounds</td>
<td>32.75 ± 3.84</td>
<td>43.46 ± 5.25</td>
<td>40.91 ± 1.76</td>
</tr>
<tr>
<td>Neutral</td>
<td>11.56 ± 1.67</td>
<td>13.29 ± 2.03</td>
<td>8.89 ± 0.33</td>
</tr>
<tr>
<td>Phytoglycogen</td>
<td>5.54 ± 0.70</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Acidic</td>
<td>11.61 ± 0.85</td>
<td>15.16 ± 0.75</td>
<td>17.16 ± 1.53</td>
</tr>
<tr>
<td>Nucleotide</td>
<td>0.98 ± 0.13</td>
<td>0.84 ± 0.22</td>
<td>1.09 ± 0.21</td>
</tr>
<tr>
<td>Basic</td>
<td>7.42 ± 1.14</td>
<td>9.97 ± 3.30</td>
<td>12.17 ± 0.26</td>
</tr>
<tr>
<td>Insoluble compounds</td>
<td>54.90 ± 3.16</td>
<td>44.06 ± 4.55</td>
<td>45.52 ± 2.25</td>
</tr>
<tr>
<td>Starch</td>
<td>36.11 ± 1.77</td>
<td>21.07 ± 3.39</td>
<td>27.97 ± 2.73</td>
</tr>
<tr>
<td>Protein</td>
<td>7.78 ± 1.64</td>
<td>7.19 ± 1.53</td>
<td>6.16 ± 0.66</td>
</tr>
<tr>
<td>Remaining insoluble</td>
<td>11.00 ± 2.93</td>
<td>11.55 ± 3.67</td>
<td>11.39 ± 3.35</td>
</tr>
<tr>
<td>Ethanol soluble compounds</td>
<td>5.80 ± 0.56</td>
<td>6.38 ± 0.92</td>
<td>5.61 ± 0.42</td>
</tr>
<tr>
<td>Root</td>
<td>6.55 ± 0.78</td>
<td>6.10 ± 0.73</td>
<td>7.95 ± 1.05</td>
</tr>
</tbody>
</table>