# Regulation of Resource Allocation during Reproductive Growth in *Arabidopsis thaliana* L. Heynh.

A thesis submitted to the Board of the Faculty of Biological Sciences of the University of Oxford for the Degree of Doctor of Philosophy

Hilary Term, 2000

By

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I declare that, unless otherwise stated, the work presented in this thesis has been entirely the result of my own research.

[Signature removed from online version of thesis]

Charles K Robinson

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## Abstract

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The *abi3-1* mutant causes moderate perturbation of seed metabolism relative to wild-type seeds, so offering a distinct and discrete treatment for use in experiments investigating regulation of allocation between sources and sinks. *abi3-1* plants continue to initiate new flowers, and hence siliques, for longer than wild-type plants. Total rates of carbon assimilation in the short-term were the same in both genotypes during early reproductive growth. This rate fell to 50-70 % in wild-type plants during later reproductive growth, but did not change in the mutant, consistent with delayed senescence of cauline leaves in *abi3-1* plants. It was found that restriction of carbon and/or nitrogen availability restricted growth in both genotypes, and abolished the mutant phenotype. Specific leaf areas increased under shading and decreased when nitrate was limiting. Reduction in nitrate limitation from 90 to 80 % was considerably less limiting for wild-type plant growth, but remained grossly limiting for *abi3-1* plants. In previously non-acclimatised plants of both genotypes, no difference was found in assimilation and allocation of $^{14}C$-radiolabel supplied at 200 ppm $CO_2$. 800 ppm $CO_2$ similarly had no effect on the wild-type, but caused abolition or inversion of normal source-sink relationships in *abi3-1* plants. A significantly large amount of radiolabel was initially incorporated into starch in all tissues in *abi3-1* plants, and later moved into the water soluble fraction in each tissue, most likely as sucrose. It is proposed that resource allocation is regulated by competition for resources between sinks maintaining sucrose concentration gradients in the phloem, and that sucrose is both the transport and signalling molecule in the mechanism described. No difference in concentration of sucrose in tissues was found between genotypes, but it was found that mutant siliques imported [U-$^{14}C$]glucose into siliques significantly more slowly from the phloem than wild-type siliques. In conclusion, *abi3-1* seeds may be seen as having reduced capacity for growth which causes stimulation of floral meristem development by feedback of sucrose in the phloem. Silique initiation is thereby prolonged, creating a demand for assimilates that delays cauline leaf senescence.

## Preface

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**In memory of my Father**

**Dr. John Sherborne Robinson**

**1935 – 1999**

# Contents

**Abbreviations**

| | |
|---|---|
| AAT | Aspartate aminotransferase |
| ABA | Abscisic acid |
| *aba* | abscisic acid deficient mutation |
| *abi* | abscisic acid insensitive mutation |
| AS | Asparagine synthase |
| AGPase | ADP-glucose pyrophosphorylase |
| C | Flux control coefficient |
| $C_{Rubisco}$ | Flux control coefficient of Rubisco |
| CaMV | Cauliflower mosaic virus |
| cpFBP | Chloroplastic fructose-1,6-bisphosphatase protein |
| d.i. | de-ionised |
| EGTA | Ethylene Glycol-bis(ß-aminoethyl Ether) Tetraacetic Acid |

| | |
|---|---|
| FBP | Fructose-1,6-bisphosphatase protein |
| GA | Gibberellic acid |
| GOGAT | glutamine-oxoglutarate amidotransferase |
| GPA | Global proliferative arrest |
| GS | Glutamine synthase |
| ICL | Isocitrate lyase |
| J | Flux |
| $K_{cat}$ | Rate of catalysis |
| $K_m$ | Concentration of substrate at $^1/_2$ $V_{max}$ |
| L*er* | Landsberg *erecta* ecotype of *Arabidopsis* |
| MS | Malate synthase |
| NiR | Nitrite reductase |
| NR | Nitrate reductase |
| NU | Nitrate uptake |

| | |
|---|---|
| $P_x$ | Pressure of *x* |
| $P_i$ | Inorganic phosphate |
| $PP_i$ | Inorganic pyrophosphate |
| PCMBS | *para*-chloromecuribenzene sulphonic acid |
| PGM | Phosphoglucomutase |
| PGS | Plant growth substance |
| ppm | Parts per million |
| RGR | Relative growth rate |
| RPP | Reductive Pentose-phosphate |
| rbcS | Rubsico small subunit protein |
| Rubisco | Ribulose-1,5-bisphosphate carboxylase/oxygenase |
| RuBP | Ribulose 1,5-bisphosphate |
| SE | Standard Error |
| SE-CC | Sieve element-companion cell complex |

| | |
|---|---|
| SEL | Size exclusion limit |
| s:r | Shoot: root ratio |
| SS | Sucrose synthase |
| Triose-P | Triose phosphate |
| $V_{max}$ | Maximum rate of catalysed reaction |
| VMP | Viral movement protein |
| v/v | volume by volume |
| w/v | weight by volume |
| $\pi$ | Osmotic potential |
| 3PGA | 3-phosphoglycerate |

# Chapter 1

Introduction

# 1.0 INTRODUCTION

## 1.1 AIM

This thesis addresses the effects of altered seed metabolism on the allocation of resources during reproductive growth in *Arabidopsis thaliana* L. Heynh., and investigates how resource allocation may be regulated.

Plants are autotrophic organisms, that is they are literally 'self feeding'. Carbon dioxide is assimilated into plants and converted into carbohydrates by photosynthesis. In parallel with photosynthesis, inorganic forms of nitrogen from the soil are assimilated and converted into amino acids, and ultimately proteins, using carbon skeletons derived from photosynthesis.

Not all parts of a plant show net autotrophism. Tissues such as roots, flowers and seeds are heterotrophic and receive assimilates from net autotrophic tissues. A tissue that has an excess of metabolites for transport is termed a source tissue. Similarly, a tissue that imports metabolites is termed a sink tissue (Münch, 1930; Black, 1993). Thus a leaf is a prime example of a source, and in a vegetatively growing plant, the shoot apex and the roots may be seen as prime sinks.

Different sinks have different patterns of metabolism, growth and development. Allocation of assimilated resources must therefore be regulated. Evidence for regulation of resource allocation is the phenomenon of allometric growth (Farrar, 1992).

The primary division of plant growth is that of shoot and root. The relative growth rates of each part of a plant stay in constant ratio to one another, so demonstrating that plants have allometric growth (Pearsall, 1927). In plants harvested at intervals the relationship of the shoot weight (S) to the root weight (R) has the form: $S = bR^k$, where $b$ and $k$ are constants.

Allometric growth is confirmed in other relationships between the relative growth rates of tissues and organs. For example, the relationship of increasing leaf area to increasing tissue dry weight is also constant. It is generally accepted that plants have such allometric growth (Farrar, 1992).

Allometric ratios have been shown to be heritable and the ratio $k$ is altered by changes in external stimuli, e.g. availability of nutrients in the soil, and also by developmental switches, e.g. flowering (Farrar, 1992, and references therein). There are adaptive responses of allometric growth and also developmental responses of allometric growth. Allometric growth therefore demonstrates that allocation of a plant's resources is regulated.

How plants regulate resource allocation is not wholly understood. Conveyance of resources from sources to sinks is contingent on both availability of supply from sources, and on demand for resources by sinks. To what extent allocation of resources is determined by interactions of source and sink metabolism (supply and demand) is not known. Similarly, it is not know by what signals sources and sinks communicate. Elucidation of relationships of supply and demand between sources and sinks *in planta* is of obvious use in the beneficial manipulation of crop yields.

In the work presented in this thesis the effects of altered seed metabolism, caused by the *abi3-1* mutation in *Arabidopsis*, are investigated in the context of whole plant growth. Experiments on *abi3-1* plants, designed to elucidate mechanisms underlying the regulation of resource allocation, are reported.

This introduction will first present a disquisition on the regulation of nutrient assimilation and allocation. The means by which allometric growth may be regulated will be discussed. Finally, the research strategy of this thesis will be stated.

## 1.2 CARBON AND NITROGEN ASSIMILATION

The primary products of photosynthetic carbon reduction are sucrose and starch, the most significant product of photosynthesis being sucrose as the major molecule of export of fixed carbon (Huber, *et al.*, 1992). Carbohydrate is also stored in the chloroplast/leaf in the form of starch. Starch is the significant end product of 80% of carbon fixed by photosynthesis that is used to produce carbohydrates (Gordon, 1986; Kruger, 1997). The remaining 20% is used in biosynthesis of organic acids, most significantly amino acids. Carbon skeletons for assimilation of inorganic nitrogen are produced by photosynthesis (Peoples and Gifford, 1997). The major products of photosynthetic tissue reflect the major function of the tissue, which is to assimilate carbon and nitrogen into organic forms (sucrose and certain amino acids) that can be exported to and utilised in the rest of the plant.

### 1.2.1 Photosynthesis

Plants assimilate gaseous atmospheric carbon dioxide ($CO_2$) into their photosynthetic tissues, typically leaves, along a concentration gradient maintained by the processes of assimilation and partitioning within that tissue. Photosynthesis occurs within plastidic subcellular organelles called chloroplasts. Chloroplasts are the site of the Reductive Pentose-Phosphate (RPP) pathway, or Calvin Cycle. $CO_2$ is fixed by the carboxylation of ribulose 1,5-bisphophate (RuBP) to form two

molecules of 3-phosphoglycerate (3PGA). This reaction is catalysed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Stitt, 1997).

Rubisco has a low rate of catalysis ($K_{cat}$) and a poor affinity for $CO_2$ *in vivo* (Andrews and Lorimer, 1987). The carboxylase action of Rubisco is therefore inefficient. This inefficiency is compensated for by there being large quantities of Rubsico in leaves, and also high concentrations of the protein (Woodrow and Berry, 1988). Traditional mechanical models of photosynthesis have ascribed Rubisco as being the 'rate-limiting step' in the acquisition of $CO_2$ according to prevalent conditions of saturating light, and limiting low concentrations of $CO_2$ (Farquhar and von Caemmerer, 1982; Sharkey, 1989; Sage, 1990). In 'non-limiting' conditions (i.e. low light and high [$CO_2$]) Rubisco does not limit photosynthesis, but its $K_{cat}$ must be reduced in order to meet the limitations in other parts of the RPP (Woodrow and Berry, 1988).

Stitt and Schulze (1994) have reviewed the central role of Rubisco in control of photosynthesis and plant growth, focussing especially on a series of experiments in which the impact of decreased Rubisco on photosynthesis, allocation and growth in plants transformed with antisense sequences to the nuclear encoding small sub-unit of Rubisco (*rbcS*) (Rodermel *et al.*, 1988; Quick *et al.*, 1991a). Antisense technology provides a set of plants with a range of levels of expression of *rbcS* (Rodermel *et al.*, 1988) enabling evaluation of the contribution of Rubisco, measured as a Flux Control Coefficient (C) (Kacser and Burns, 1973; Kacser and Porteous, 1987;

Stitt and Schulze, 1994), to a) the control of photosynthetic rate, and b) the impact of changed rates of photosynthesis on whole plant composition, allocation and growth (Stitt and Schulze, 1994, and references therein).

A key feature of the experiments reviewed by Stitt and Schulze (1994) is that the breakdown of the historical model was elucidated by experiments on plants that were previously acclimated to growth conditions different from the experiments (Quick *et al.*, 1992; Lauerer *et al.*, 1993). This not only demonstrates variation in $C_{Rubisco}$, but also draws attention to the fact that, as well as short-term capacity to respond to changes in environmental conditions, plants also show phenotypic plasticity in long-term adaptation: $C_{Rubisco}$ remained in the range 0.3-0.7 when photosynthesis was measured under the same conditions under which the plants were grown, but increased to almost total limitation by Rubisco ($C_{Rubisco} = 0.92$) when experimental irradiance was very high compared to very low irradiance of growth (Lauerer *et al.*, 1993). Large transitory changes in irradiance are one such challenge that exist in nature, but so too are variations in mineral nutrition, particularly nitrogen which is essential for amino acid synthesis.

The reduced carbon product of the RPP is triose-P. Triose-P can either be partitioned into starch synthesis within the stroma of the chloroplast, or exported from the chloroplast into the cytosol via the triose-phosphate translocator (TPT) (Fliege *et al.*, 1978; Flügge and

Heldt, 1991). For each molecule of triose-P translocated from the chloroplast, an inorganic phosphate ($P_i$) must enter from the cytosol. This $P_i$ is released when triose-P is converted to sucrose. Sucrose synthesis must be tightly regulated to photosynthesis to ensure sufficient $P_i$ can be returned to the chloroplast, while ensuring the chloroplast is not depleted of triose-P (Huber *et al.*, 1992). Similarly, sucrose synthesis must also be regulated to ensure the partitioning between export (sucrose) and storage (starch) does not impede the overall rate of $CO_2$ fixation (Huber *et al.*, 1992).

The RPP can be therefore regulated by sucrose synthesis in the short-term. The key enzyme regulating the sucrose biosynthesis pathway is sucrose synthase (SS) (Kruger, 1997), down regulation of which causes feedback through the pathway and across the TPT to cause an increas in the ratio of triose-P to $P_i$. This in turn induces ADP-glucose pyrophosphorylase (AGPase), the key regulatory enzyme of the chloroplastic starch synthesis pathway (Kruger, 1997) and so increases starch synthesis, which yields $2P_i$, and so acts to reduce the triose-P: $P_i$ ratio in the chloroplast. This causes down-regulation of AGPase and so reinstates sucrose synthesis. Output from the RPP remains unimpaired throughout.

Most of the sucrose produced from photosynthesis is exported from leaves during the light period, whereas starch is accumulated in the chloroplast stroma and degraded during the night (Heineke *et al.*, 1994; Hattenbach *et al.*, 1997). The amount of temporary partitioning

into starch varies between species (Hattenbach *et al.*, 1997 and references therein), but across several species the transient storage of reduced carbon in the form of starch during the light period enables export of photoassimilates to continue in the dark period at between 15-60% the rate of photoassimilate export in daytime (Fondy and Geiger, 1982; Hendrix and Huber, 1986; Kalt-Torres *et al.*, 1987; Heineke *et al.*, 1994; Riens *et al.*, 1994).

Having concentrated on the carbohydrate products and regulation of photosynthesis, it is of equal importance to consider amino acid synthesis, the most significant product of the 20% of photoassimilate which is partitioned into organic acids, and not least with reference to the role played by Rubisco as a storage protein (Chapin *et al.*, 1990).

### 1.2.2 Nitrogen Assimilation

Amino acids are synthesised by the assimilation of inorganic forms of nitrogen into carbon skeletons supplied from photosynthesis. Plants gain nitrogen principally from the soil and predominantly in the form of nitrate ($NO_3^-$). Whatever the form in which the plant obtains nitrogen from its environment, it must be reduced to ammonia ($NH_4^+$) for it to be assimilated into amino acids for protein synthesis.

The site of $NO_3^-$ metabolism varies with species, environment and stage of development of the plant. Inhibition of metabolism, and of protein and mRNA synthesis, indicates $NO_3^-$ is taken up by a specific, energy requiring, and inducible transport system (Vance, 1997). It can be metabolised in the roots, but commonly, when there are not limiting conditions of $NO_3^-$ in the soil, it is transported via the xylem to photosynthetic tissue. The reduced form of nitrogen for direct assimilation is $NH_4^+$, $NO_3^-$ being reduced to $NH_4^+$ by the enzymes nitrate reductase (NR) to form $NO_2^-$, which is in turn reduced to $NH_4^+$ by nitrite reductase (NiR). $NH_4^+$ is generally phytotoxic at high concentrations and so it needs to be quickly metabolised when rates of nitrogen fixation and assimilation are high (Vance, 1997).

Commonly, $NH_4^+$ is rapidly metabolised into organic compounds (Vance, 1997) proceeding through two steps of partitioning. Firstly, $NH_4^+$ and glutamate are combined to produce the amide glutamine, a reaction catalysed by the enzyme glutamine synthase (GS). Secondly, glutamine is converted to glutamate by the action of glutamine-oxoglutarate amidotransferase (GOGAT). Nitrogen in the form of glutamate can then be converted into other amino acids by transamination reactions catalysed by aminotransferases which require pyridoxal phosphate (vitamin $B_6$) as cofactor (Vance, 1997).

Nitrogen is a potentially limiting nutrient in plants, and given the demand for nitrogen it is unlikely that regulation of nitrogen assimilation occurs at the point of entry, a point

confirmed by the kinetics of the inducible transporter in the roots (Vance, 1997). As the first step of $NO_3^-$ into organic forms, NR is a potential regulator of nitrogen fixation. NR is regulated by i) transcriptional and translational regulation, ii) post-translational modification, and iii) positive and negative effectors (Vance, 1997).

NiR catalyses the reduction of $NO_2^-$ to $NH_4^+$ with NADPH acting as reductant and 6 electrons donated from ferredoxin via ferredoxin-NADP oxido-reductase (Wray, 1993). NiR mRNA synthesis and enzyme activity can be induced by light via phytochrome (Wray, 1993). NiR mRNA has a short half life of 0.5-2h according to species, whereas the half life of the protein is up to 24h (Vance, 1997). Reduced nitrogen, as amino acids, exerts negative feedback on both NiR mRNA and the protein itself (Wray, 1993; Vance, 1997). In general, regulation of NiR is similar to that of NR, being induced by both $NO_3^-$ and light, with control by transcriptional and translational regulation, post-translational modification, and positive and negative effectors (Vance, 1997).

In all, there is induction at each step of fixation of $NO_3^-$ to $NH_4^+$, sequestration, $NO_3^-$ reduction and $NO_2^-$ reduction, by the fundamental nitrogen source, $NO_3^-$. Light also induces the three steps of this pathway, perhaps via sugars from photosynthesis. This has potential and significant relations to the greater co-ordination of carbon and nitrogen fixation in the plant. Assimilation of $NH_4^+$ integrates nitrogen metabolism with that of carbon, and

involves inter-organellar co-ordination. Regulation of this process may occur in the two reactions catalysed by GS and GOGAT. Another level of control of $NH_4^+$ assimilation is by the enzymes aspartate aminotransferase (AAT) and asparagine synthase (AS), which regulate flux of carbon between organic and amino acids.

GS can comprise up to 2% of the total soluble protein in actively nitrogen assimilating root tissue (Vance, 1997). Activity of the enzyme increases rapidly in roots and leaves of plants grown in $NH_4^+$ or $NO_3^-$, and in etiolated leaves upon illumination (Cullimore and Bennett, 1992; McGrath and Coruzzi, 1991). Kinetically and immunochemically distinct isoforms have been identified in both monocot and dicot species (Tingey *et al.*, 1988). Changes in the environment (or during development) result in differential expression of GS isozymes. In all, the many potential isoforms of GS represent what is a multiplicit and very flexible enzyme (Redinbaugh and Campbell, 1993; Yamaya *et al.*, 1992).

GOGAT catalyses the reductive transfer of the amido groups of glutamine to the α-keto position of 2-oxoglutarate (α -ketoglutarate), producing two molecules of glutamate. In conjunction with GS, GOGAT cycles $NH_4^+$ into glutamine and glutamate, the nitrogen being carried from $NH_4^+$ and used in the several amino-transferase reactions to synthesise amino acids. There are two distinct forms of GOGAT in higher plants: Ferredoxin-GOGAT (Fd-GOGAT) which gains reducing power from ferredoxin, and NADH-GOGAT, which

gains reducing power from NADH. NADH-GOGAT is found in non-photosynthetic tissue, in particular legume root nodules, but also in rice cell-cultures. It is present at about $^{1}/_{10}^{th}$ the concentration of Fd-GOGAT and/or GS (Gregerson *et al.*, 1993; Yamaya *et al.*, 1992). Fd-GOGAT is the plastidic form and assimilates $NH_4^+$ from light dependent reduction of $NO_3^-$, and photorespiration (Sakakibara *et al.*, 1991). Before induction of Fd-GOGAT activity there is an increase in Fd-GOGAT mRNA; induction of Fd-GOGAT is therefore transcriptionally controlled; in maize roots, application of $NO_3^-$ increases transcription of Fd-GOGAT mRNA and activity of the protein (Redinbaugh and Campbell, 1993). The enzyme responds to both light and $NO_3^-$ as environmental signals (Redinbaugh and Campbell, 1993).

Nitrogen is the major essential element needed by plants, and soils are commonly deficient in it. However, there is another major nutrient which may be incorporated with carbon to produce organic forms: phosphorus. Phosphates are essential in photosynthesis and respiration as AMP, ADP and ATP, pyrophosphates and sugar phosphates, as well as biosynthesis of membranes. Phosphate is taken up by the roots, primarily as the mono-phosphate anion $H_2PO_4^-$, being more available in acidic soils, and secondarily as the divalent anion $HPO_4^{2-}$, being more available in alkaline soils. Like $NO_3^-$, phosphate is converted into organic forms upon entry into the root, but it is not reduced, remaining as free or organically bound phosphate. As a result it is easily distributed around the plant,

deficiency therefore becoming first apparent in older tissues, in particular leaves. The ratio of N to P can interact to regulate maturation in many species (Salisbury and Ross, 1992; Taiz and Zeiger, 1991).

Other major plant nutrients are potassium, calcium, magnesium and sulphur, each of which may be deficient in the soil. However, none is immediately incorporated into metabolic pathways at the same scale as nitrogen or phosphorus and these nutrients do not represent a significant part of metabolism in the context of carbon partitioning. The remaining plant nutrients, termed micronutrients, are chlorine, iron, manganese, boron, zinc, copper and molybdenum. These are very rarely limiting (Salisbury and Ross, 1992; Taiz and Zeiger, 1991).

### 1.2.3 Respiration

A most significant metabolic pathway into which fixed carbon is partitioned is that of respiration. Respiration is as vital a part of plant metabolism as photosynthesis, and yet respiration and photosynthesis are apparently futile processes: In photosynthesis, gaseous $CO_2$ is fixed into carbohydrate in an energy requiring reductive process. In respiration, organic substrates are converted into $CO_2$ in an energy producing, oxidative process. There is no apparent futile cycling between the two processes, implying a co-ordinating

regulatory mechanism. Further, for growth, carbon fixation and nitrogen assimilation must exceed respiration, and so there must be integration of these processes in order that a plant may grow.

Energy and carbon skeletons from photosynthesis and respiration are required for N-assimilation. High rates of respiration are required for high rates of biosynthesis, the rate of respiration being modulated by the biosynthetic demand for photosynthesised C-skeletons and respiratory substrates for glycolysis. Hence, it is so that N-fixation can be prioritised by increasing respiratory rate with concomitant reduction in the rate of C-fixation. Indeed, it has been suggested that photosynthesis is modulated by respiration, as is theoretically possible. However, N-assimilation is ultimately limited by the carbon sequestration.

## 1.3 RESOURCE ALLOCATION - TRANSLOCATION

Carbon partitioning within photosynthetic tissues creates a metabolic buffer between the environmental influences on photosynthesis and effective investment of photosynthate (Gifford *et al.*, 1984; Chapin *et al.*, 1990; Stitt and Schulze, 1994a). As has been discussed, the two major resources that are exported from the photosynthetic cell are sucrose and amino acids.

In higher plants the tissue through which resources are transported is the phloem. Metabolites may be distributed locally from the cell of production by diffusive symplastic processes through inter-cellular plasmodesmata, but for transport between organs, from sources to sinks, metabolites must be loaded into the phloem (van Bel, 1992).

### 1.3.1 Phloem Loading

There has been considerable debate as to the pathways and mechanisms of phloem loading (van Bel, 1992). The historical consensus of a symplastic pathway of transfer of photoassimilates from the leaf mesophyll to the sieve element/companion cell (SE-CC) complex of the phloem was challenged by microscopic evidence of a marked decrease in plasmodesmatal density in the vicinity of the SE-CC complex (Geiger *et al.*, 1973; Gunning *et al.*, 1974). Further to electron microscopy, studies have demonstrated a significantly lower osmotic potential within the phloem

relative to the mesophyll (Geiger *et al.*, 1973; Evert *et al.*, 1978; Fisher and Evert, 1982). Experiments with leaves stripped of their dermal layers showed sucrose being taken up preferentially into leaf veins (Geiger *et al.*, 1974; Delrot and Bonnemain, 1978). But perhaps the most compelling evidence was the discovery of sucrose-proton co-transport (Giaquinta, 1976; 1977), the first molecular evidence of likely apoplastic loading. Universality of apoplastic loading into the SE-CC complex was assumed in all Angiosperms (with the exception of Curcurbits), until it was proposed that phloem loading was multiprogrammed (van Bel and Gamalei, 1991).

The mode and regulation of sucrose-proton co-transport has been investigated (Riesmeier *et al.*, 1994). Antisense constructs of a putative sucrose-proton co-transporter cDNA were expressed under control of the CaMV 35S promoter in potato leaves (Riesmeier *et al.*, 1994; Truernit and Sauer, 1995). Transformants with lowered levels of the sucrose transporter mRNA showed local bleaching, indicating reduced levels of photosynthetic activity, and curling of leaves, indicating greater cell expansion in the top side of the leaves (Riesmeier *et al.*, 1994). Further analysis showed more than 20 fold greater concentrations of soluble carbohydrates in these leaves, and a 5 fold greater starch content, compared to controls, suggesting a block in export of sucrose from the leaf. Photosynthesis was reduced in the strongest antisense lines and development of the roots and tubers was decreased considerably (Riesmeier *et al.*, 1994). The local (Truernit and Sauer, 1995) and global (Riesemeier *et al.*, 1994) effects of this transformation provide strong evidence

in support of apoplastic unloading, at least in potato, and not only identifies the transport of sucrose as a key step in distribution of photoassimilate, but also illustrates the effects of poor export in both the leaf and the whole plant.

A similar phenotype to that found in sucrose-proton co-transporter antisense plants was found in transgenic plants expressing a yeast-derived invertase in the cytosol (von Schaewen *et al.*, 1990; Sonnewald *et al.*, 1991). Again, leaves were pale and curved (Sonnewald *et al.*, 1991). Overall growth was stunted, root formation was reduced, and starch and soluble sugars accumulated in the leaves (Sonnewald *et al.*, 1991). Similar studies in tomato and potato concur that sucrose is translocated via the apoplast (Dickinson *et al.*, 1991; Heineke *et al.*, 1992; Sonnewald and Willmitzer, 1992). Analysis of phloem contents in potato show a reduction in the amino acid: sucrose ratio (Heineke *et al.*, 1992), indicating decreased loading of sucrose as a result of expression of yeast-derived invertase in the apoplast (Sonnewald and Willmitzer, 1992).

The similarities in the phenotypes resulting from these two very different transformations are due to the similarity in the effects of perturbing sucrose transport from source leaves. Riesmeier *et al.* (1994) effectively prevented sucrose being loaded into the transport system, and Sonnewald *et al.*, (1991) achieved a comparable effect by preventing export of sucrose from the mesophyll. Little is known about the regulation of sucrose transport into the phloem beyond these studies,

but it is likely that changes in concentration of sucrose and changes in turgor are essential factors in regulation (Stitt, 1996).

### 1.3.2 Phloem Transport

Phloem transport occurs along a symplastic continuity in what are highly developmentally sophisticated tissues in terms of the actual conduits of long-distance transport, the sieve elements (Stitt, 1996). It may be viewed as "transcellular, vectorialized and accelerated cytoplasmic streaming" (Stitt, 1996).

The prevalent view of phloem transport is that it occurs by a process of mass flow driven by a gradient of turgor pressure, a hypothesis originally proposed by Münch (1930) (Farrar, 1992). The sieve elements are considered as pressurised vessels connecting source and sink. Pressures at the source, $P_{source}$, and sink, $P_{sink}$, being determined by the difference in osmotic potentials, $\pi_i$-$\pi_o$, across the boundary membranes at source and sink.

The flux of sucrose, $J_{suc}$, through the phloem is described by the equation:

$$J_{suc} = \Delta P \,.\, C \,.\, k,$$

where $\Delta P$ is the difference in turgor pressure between source and sink, C the concentration of sucrose in phloem, and k a coefficient of conductivity where transport is described by the Hagen-Poisseuille relationship:

$$k = A \cdot r^2 / 8\eta \cdot l,$$

where A is the cross-sectional area of the phloem with radius r, $\eta$ is the viscosity, and l the length. With great variety in r being unlikely in a plant, the key terms are $\Delta P$, C and l (Farrar, 1992).

The coefficient of conductivity, k, may be expected to be affected by the plasmodesmata that provide the continuity of 'cytoplasm' in the highly modified sieve element cells. Plasmodesmata are intercellular supramolecular complexes that form cytoplasmic continuity between cells (Wolf and Lucas, 1994). Experiments in which membrane impermeable fluorescent dyes were injected into cells under normal physiological conditions have confirmed estimates from electron microscopy that plasmodesmata are channels of cytoplasmic continuity between cells in the order of 2.5 nm channel size with a size exclusion limit (SEL) of macromolecules in the size range of 0.8-1 kD (Wolf and Lucas, 1994). Plant viruses encode movement proteins that facilitate passage of viral infection through plant tissues. Expression of viral movement proteins (VMPs) in transgenic plants have demonstrated that these proteins are targeted to plasmodesmata and effect significant increases in the SEL (Wolf and Lucas, 1994). Exogenous introductions of VMPs,

alone or with viral DNA or RNA encoding VMPs, have demonstrated the rapid movement of both proteins and transcripts between cells, further supporting plasmodesmatal function as intercellular conduits (Wolf and Lucas, 1994). Plasmodesmata represent resistance to mass-flow between the mesophyll and the phloem, and so the differential increase of plasmodesmatal SEL caused by VMPs should enhance flux into the phloem. However, experiments testing this hypothesis actually found a negative effect (Lucas *et al.*, 1993), at least in the photoperiod. Control plants were found to have lower rates of carbon export from leaves in the dark relative to the VMP-expressing plants. Young leaves that were not yet expressing VMP showed phenotypic differences to controls, indicating possible pleiotropic effects of the VMP (Lucas *et al.*, 1993; Wolf and Lucas, 1994). Sadly, the only conclusion on the effect of VMPs that can be drawn from these experiments is that they have an effect.

Purely physiological models are somwhat naïve in the context of the phloem not actually being pressurised vessels in a passive sense. The phloem transport pathway is leaky with passive unloading of photoassimilate into the apoplast and active reloading from the apoplast (Minchin and Thorpe, 1987). Biochemical characterisation of companion cells has shown metabolic ability to achieve active reloading including a fully operational glycolytic cycle (Geigenberger *et al.*, 1994) and the presence of sucrose synthase (Nolte and Koch, 1990; Martin *et al.*, 1993). Companion cells also have some of the highest densities of mitochondria in plants (Stitt, 1996). The mechanism of the sucrose-proton co-transporter that loads and reloads sucrose from

apoplasm into the SE-CC complex (Truernit and Sauer, 1995; Riesmeier *et al.*, 1994; Stitt, 1996) requires that, for each molecule of sucrose transported, one molecule of ATP must be produced, from respiration in the mitochondria, for the action of the $H^+$-ATPase in the plasmalemma of the companion cell to produce the proton for the sucrose-proton co-transport. Subsequently, the ADP and $P_i$ resulting from the action of the $H^+$-ATPase must be returned to the mitochondria, without being taken away by the process of mass flow in the SE-CC complex (Stitt, 1996). The concentration of ATP in phloem sap is between 300-500 fold less than the concentration of sucrose, indicating very effective segregation of mass flow from the metabolism of energy required for loading and reloading (Stitt, 1996).

Constitutive expression of the *Escherichia coli ppa* gene, encoding inorganic pyrophosphatase, caused accumulation of sucrose in source leaves of transgenic tobacco, due to prevention of glycolysis by cytosolic hydrolysis of pyrophosphate (Jelitto *et al.*, 1992; Sonnewald, 1992). An unanticipated phenotype was the overall stunting of plant growth, suggesting an effect of the transgene on export of photoassimilates (Sonnewald, 1992). Phloem specific expression of the *E.coli ppa* gene allowed specific investigation of the putative role of $PP_i$ in long-distance transport (Lerchl *et al.*, 1995). Starch accumulated in leaves of transgenic tobacco plants in which cytosolic $PP_i$ was removed from the phloem, and there was an accumulation of UDP-glucose concomitant with a lowered ratio of ATP to ADP in the midribs of leaves, indicating a lowered energy status within the phloem of the transgenic plants relative to wild-type (Lerchl *et al.*,

1995). The transgenic plants showed the same phenotype as those over expressing yeast invertase (Sonnewald, 1992); stunted growth compared to wild-type, with evidence of bleaching of more mature source leaves (Lerchl *et al.*, 1995), confirming a role of $PP_i$ in long distance transport (Sonnewald, 1992; Lerchl *et al.*, 1995). The phenotype is ascribed to impairment of sucrose hydrolysis via sucrose synthase in *ppa*-expressing plants (Lerchl *et al.*, 1995).

The wild-type phenotype was successfully restored in transgenic plants expressing the *rolC ppa* construct by insertion of phloem-specific yeast-derived invertase in the plants (Lerchl *et al.*, 1995). In singly transformed plants, phloem-specific hydrolysis of sucrose by yeast-derived invertase was shown to have no negative effects on plant growth (Lerchl *et al.*, 1995). Having shown altered allocation in *ppa*-expressing plants due to impairment of sucrose hydrolysis via sucrose synthase, it was found that crosses resulting in low levels of yeast-derived invertase activity in the cytosol of the phloem, the wild-type phenotype was almost fully restored in *ppa*-expressing plants (Lerchl *et al.*, 1995), suggesting low levels of invertase can bypass the effects of $PP_i$-dependent sucrose degradation via sucrose synthase by providing an alternative pathway of sucrose hydrolysis. Crosses resulting in higher levels of transgenic invertase expression produced only partial restoration of the wild-type phenotype, and higher levels still caused severe growth retardation, suggesting that sucrose hydrolysis must be tightly controlled (Lerchl *et al.*, 1995).

Further investigation of the effects of transgenic expression of phloem-specific pyrophosphatase showed inhibition of transport of not only carbohydrates, but also amino acids (Geigenberger *et al.*, 1996). This further confirms $PP_i$, and energy metabolism, as essential in long-distance transport through the phloem, and that such transport is an active process (Geigenberger *et al.*, 1996; Minchin and Thorpe, 1987).

### 1.3.3 Phloem Unloading

Processes of phloem unloading remain relatively poorly understood (Sonnewald *et al.*, 1994). They can be divided into two types: Phloem unloading can occur by symplastic routes, or it may involve an apoplastic step that may require hydrolysis of sucrose in cell wall interstices (Sonnewald *et al.*, 1994; Stitt, 1996). The concentration of assimilates is higher in the phloem relative to surrounding cells. Unloading can occur along a concentration gradient. Metabolism and subcellular compartmentation in sink tissue maintains the concentration gradient required for mass transport and suggests symplastic unloading (Sonnewald *et al.*, 1994). This model is supported by high densities of plasmodesmata in certain sink tissues such as potato tubers. Alternatively, mass flow may be maintained by unloading out of the phloem into the apoplast through a sucrose transporter. Subsequent loading of sink tissue may then be mediated by a second sucrose transporter, or by hydrolysis of sucrose in the apoplast by an acid invertase before subsequent loading into the sink by hexose transporters (Sonnewald *et al.*, 1994). Low level

expression of sucrose transporters has been found in potato tubers (Riesmeier *et al.*, 1993) and sink-specific expression of hexose transporter genes has been found in *Arabidopsis* and tobacco (Sauer *et al.*, 1994).

The most comprehensive understanding of phloem unloading is in legume seeds. Seeds are anatomically, genetically and temporally distinct sinks in relation to the maternal plant and these factors have been exploited in experiments to elucidate apoplastic phloem unloading. Microautoradiographical investigation of *Phaseolus* seed coats, loaded with $^{14}C$-photosynthate and treated with *para*-chloromecuribenzene sulphonic acid (PCMBS) to inhibit sucrose transport, demonstrated a 75% increase in silver grain density in the layer of the vascular parenchyma of seed coats over controls (Offler and Patrick, 1984), indicating unloading occurs in this tissue. Microscopical estimates of the surface area of vascular parenchymal plasmalemmæ concur with estimated surface areas required for observed sucrose transfer, and electron microscopical observation of this tissue showed that these cells resemble known secretory cells (Offler and Patrick, 1984). The lack of plasmodesmatal connections between maternal and embryonic tissue has been exploited by removal of embryonic tissue to allow demonstration of active unloading of photosynthate into the apoplastic space between the seed coat and the embryo (Wolswinkel and Ammerlaan, 1983; Patrick, 1983; Thorne and Rainbird, 1983). Use of 'trap' solutions has allowed study of the kinetics of phloem unloading in empty seed coats. Inhibition of unloading in

the presence of KCN, indicating an active component to the process, and sensitivity to organic and inorganic osmotica have been reported. Presence of plant growth substances in trap solutions had modest effects (Gifford and Thorne, 1986; Offler and Patrick, 1986). However, *in vivo* studies of unloading using $^{11}C$ radiolabelling question the accuracy of long-term measurements of seed coat unloading achieved with the surgical techniques (Minchin and Thorpe, 1989).

A more specific approach was applied to determining the contribution, if any, of an apoplastic route of sucrose unloading in potato tubers by over-expression of a yeast-derived invertase (Heineke *et al.*, 1992). Apoplastic localisation of invertase protein was achieved by fusion of the proteinase inhibitor II signal peptide to the coding region of the mature invertase protein (von Schaewen *et al.*, 1990), and control was provided by the coding region of the mature invertase protein without additional targetting, giving cytosolic expression of the protein (Sonnewald *et al.*, 1991). Tuber-specific expression of invertase was achieved by the patatin class I promoter B33 (Rocha-Sosa *et al.*, 1989). Plants expressing apoplastic yeast-derived invertase accumulated glucose and fructose and contained three times less sucrose than controls, indicating that not only is the apoplastic space involved in unloading into the tuber, but that a majority of sucrose imported into the tuber enters via the apoplasm. Starch content was unchanged and overall yield was increased by up to 30% (Heineke *et al.*, 1992; Sonnewald *et al.*, 1994). In the case of plants expressing apoplastic yeast-derived invertase it is notable that some sucrose is imported symplastically, although not as much as might be suspected by the high density of

plasmodesmata found in potato tubers (Sonnewald *et al.*, 1994), and so there is, in the case of potato tubers, no distinction between the two modes of unloading as originally suggested (Sonnewald *et al.*, 1994). In plants expressing increased cytosolic invertase, cytosolic hydrolysis of sucrose caused longer tubers to develop. Starch content was decreased, as was overall yield, relative to untransformed controls (Heineke *et al.*, 1992; Sonnewald *et al.*, 1994). The effects of cytosolic expression of yeast-derived invertase are indicative that the fate of translocated photosynthate within the sink tissue not only impinges on the unloading of the phloem, but that resource partitioning within sinks has potentially great ramifications on the growth of the whole plant.

Invertases associated with the testa, hexose, sucrose and starch concentrations have all been positively correlated with seed development and size (Miller and Chourey, 1992; Weber *et al.*, 1996). Using two genotypes of *Vicia faba*, differing only in the development and size of seeds, it was found that the larger seeds matured later and had more parenchyma cells (Weber *et al.*, 1996) of the type associated with secretion into the apoplastic space between the testa and developing cotyledons (Offler and Patrick, 1984; Offler *et al.*, 1989). Further to this anatomical difference in the testa, it was found that there was greater expression of cell-wall bound invertase, of a type associated with this unloading zone, expressed per cell (Weber *et al.*, 1996) leading to higher hexose concentrations in the zone, which correlated with the mitotic activity of the cotyledons (Weber *et al.*, 1996). The later maturation of the large-seeded genotype, relative to the small-

seeded genotype, was found to correspond with a switch between hexose accumulation and sucrose synthesis, with increasing sucrose concentration apparently causing the maturation in the seeds (Weber *et al.*, 1996). This was tested by addition of sucrose to mitotically active cotyledons *in vitro*, and it was found that sucrose did initiate the maturation programme observed *in vivo* (Weber *et al.*, 1996). It is concluded that development of testa and the embryo are coordinated and that the former controls the phase of cell division in the embryo by metabolic signals, and so controls seed size (Weber *et al.*, 1996).

In a comparable study of *miniature-1* (*mn*) Maize seed, seed size was found to be dependent on segregation of individual genotypes on heterozygous ears (Miller and Chourey, 1992). The *mn* phenotype was found to be closely associated with vanishingly low expression of wall-bound invertase activities, and expression was found to be significantly increased in the heterozygous *Mn:mn* seeds. Presence of the *mn* allele in heterozygotic seeds caused separation of maternal tissue from the base of the endosperm of developing seeds, and invertase expression was histochemically localised to the pedicel-endosperm interface region of developing homozygous wild-type and heterozygous seeds (Miller and Chourey, 1992).

As has been discussed above, sucrose and amino acids are actively loaded into, and transported via the SE-CC complex that constitutes the transport tissue, phloem. Arrival of transported photosynthate in sink tissues requires unloading of the phloem before further partitioning can

occur within the sink tissue. Three factors predominate the efficient unloading of photosynthate into sink tissues: The area of interface between the transport tissue and the sink tissue, the efficiency of transfer across this interface, and the spatial and/or biochemical isolation of assimilates between the transport system and the sink tissue (Wardlaw, 1990). All three of these factors combine to affect the efficiency of unloading according to the type of sink in question and, moreover, the timing of organ initiation and growth (Wardlaw, 1990).

The area of interface between transport and sink tissue is distinguishable from the increase in size in a growing sink tissue, contigent on the manner by which growth is measured. For example, it has been calculated that maximum import of photosynthate in a leaf occurs when the leaf is 15-20% of its final size (Fellows and Geiger, 1974; Turgeon and Webb, 1975; Ho and Shaw, 1977). The relative growth rate, RGR, i.e. the increase of plant material per unit of material per unit of time (Hunt, 1978), explains why maximum import occurs at such a relatively early stage of growth. In the case of the leaf, the rate of increase in size is greatest when the whole leaf is relatively small compared to its final size, though the net rate of import into the leaf may be the same throughout growth. The gross gain in leaf size diminishes as a proportion of the overall size of the leaf. This bears relation to the area of interface as this would proportionally decrease with increasing size of a growing sink tissue.

## 1.4 RESOURCE ALLOCATION - SINKS

If sources are easily recognised as having an excess of metabolites for transport, sinks are, by comparison, very complex. There are a multiplicity of sinks (Farrar, 1993a). For example, meristematic sinks are the sites of plant growth, as in root tips and leaf apices, and most foodstuffs represent storage sinks, such as root crops and grains. Respiration in tissues, most notably roots as non-photosynthetic tissues, represents another demand for photosynthate as an energy source. Besides respiration in tissues, there is also demand for metabolites for maintenance of tissues.

### 1.4.1 Sink Strength

Beyond the definition of sink organs as net importers of assimilate, opinions on the nature of sinks diverge considerably (Brouwer, 1983; Ho, 1988; Wardlaw, 1990; Farrar, 1993; Farrar, 1996). The property of a sink which determines the rate at which assimilate is imported into that sink is termed "sink strength" (Farrar, 1993a; Farrar, 1996). There is consensus that sinks compete with one another for resources, but the manner of competition and the means by which it may be characterised remain the subject of great debate (Farrar, 1993a, and following articles). Specific enzyme activities that correlate well with sink activities have been suggested as quantitative indicators of sink strength (Sung *et al.*, 1989; Black, 1993; Jenner and Hawker,

1993). However, single enzyme activities as measures of sink strength have been strongly criticised in the face of the shared nature of metabolic control (Stitt, 1993).

Many observers have noted an apparent hierarchy of sinks in a growing plant (Brouwer, 1983; Ho, 1988; Wardlaw, 1990 for reviews), summarized by the order of priority: Seeds > fleshy fruit parts = shoot apices and leaves > cambium > roots > storage (Minchin and Thorpe, 1996). The general conclusion is that growth of fruits and seeds dominates vegetative growth, with the coda that flowers are less successful than established fruits in competition for assimilates when availability of assimilates is short (Ho, 1984; Ho, 1988; Wardlaw, 1990; Minchin and Thorpe, 1996).

This conclusion, drawn from the many empirical observations (see Brouwer, 1983; Ho, 1988; Wardlaw, 1990 for reviews), effectively separates plant growth into the two distinct phases of vegetative and reproductive growth, with the latter being prioritised over the former for the supposedly teleological reason of reproductive success. Vegetative growth has been modelled with respect to sink competition and maintenance of the allometric ratios, starting with the ratio of shoot: root (s:r) (Thornley, 1972a & b; Reynolds and Thornley, 1982). The models are based on the theory of a functional equilibrium extant between the shoot, as the source of carbohydrate, and the root, as the source of water and essential nutrients (Reynolds and Thornley, 1982; Brouwer, 1983). Essentially, shoots and roots can be considered as different systems within the intact plant, yet with complementary functions (Brouwer, 1983). The constancy of the shoot:root

ratio is argued to be maintained by three universal observations: Firstly, when growth is limited by an essential substance to be absorbed by the roots, root growth is relatively favoured. Conversely, when the limiting factor has to be absorbed by the shoot, shoot growth is favoured. Secondly, disturbance of the s:r that exists in any condition, by either defoliation or root cutting, leads to such changes in the growth pattern that the original ratio rapidly restored. Thirdly, transfer of plants from one condition to another gives rise to changes in the distribution pattern so that the ratio characteristic of the new situation is reached in time (Brouwer, 1983). The concept of functional equilibrium makes sense of vegetative growth in the long term. As much as empirical observations do identify prioritisation of sinks (Ho, 1984; Ho, 1988; Wardlaw, 1990; Minchin and Thorpe, 1996), the concept of functional equilibrium underestimates levels of complexity in source-sink interactions (Farrar, 1992; Farrar, 1996), and does not translate readily into other allometric relationships where there is no functional interdependence (Marcelis, 1996).

The lack of mechanistic understanding of the body of data on source-sink interactions (Wardlaw, 1990; Minchin *et al.*, 1993) has yielded to proposal of models (Thornley and Johnson, 1990; Minchin *et al.*, 1993) based on mechanistic understanding of mass flow as proposed by Münch (1930) as the means of translocation of photoassimilate via the phloem. An original model, based on convective flow induced by an osmotically generated hydrostatic pressure gradient, dealing with a single source and single sink (Thornley and Johnson, 1990), was extended to encompass two or more sinks, with flow in a specific sink set by the interaction between the source,

alternative sinks and the sink in question (Minchin *et al.*, 1993). This model describes bulk flow through the resistance of the phloem sieve elements, driven by osmotically generated pressure gradient and unloading described by Michaelis-Menten kinetics (Minchin *et al.*, 1993; Minchin and Thorpe, 1996). The model demonstrates interactions between sinks and the close linking of source supply and partitioning between sinks, and predicts sink priority and changes in sink priority (Minchin *et al.*, 1993; Minchin and Thorpe, 1993) in short-term situations using experimental observations in a barley split root system (Farrar and Minchin, 1991; Williams *et al.*, 1991).

Theories on the control of carbon allocation have been addressed in the model system of divided barley roots by $^{11}C$ radioisotope feeding experiments (Minchin and Thorpe, 1987) to investigate regulation of allocation by water relations (Williams *et al.*, 1991). Allocation of $^{11}C$, supplied to the second leaf, between two root halves of a vegetatively growing barley, was followed continuously over a 3 h period and response of one root half to treatments with mannitol or sorbitol was assessed. Applied osmotica rapidly increased allocation of $^{11}C$ into the treated root half. The experiment was replicated over at 3 h and 24 h using a pulse of $^{14}C$. The same short-term effect was noted with the 3 h experiment, but after a chase of 3 h allocation between the root halves was found to be no longer affected (Williams *et al.*, 1991). Similarly, slow application of osmotica to a root half did not alter allocation between root halves. It was concluded that altered osmotic potential can have a major but transient effect on carbon allocation, but that it was not of

likely importance *in vivo* as osmoregulation in the sink tissue overcame external challenges (Williams *et al.*, 1991).

Theories relating metabolic activity in sinks to control of allocation have also been investigated (Ho, 1988; Farrar and Minchin, 1991; Farrar *et al.*, 1994; Minchin *et al.*, 1994). Changes observed in the allocation of $^{11}C$ between shoot and both parts of a split root in barley have been applied to the simple mechanistic model of carbon allocation in the whole plant described by Minchin and Thorpe (1993; 1996). The model predicts that in a situation when two sinks have similar $K_m$ values, but different values of $V_{max}$, the sink with the reduced $V_{max}$ value receives a reduced proportion of assimilate when supply is reduced (Minchin *et al.*, 1993).Within 20 minutes of shading the shoot, and thereby reducing the amount of $^{11}C$ fixed by photosynthesis, reduced allocation of newly fixed photoassimilate was observed in the root (Minchin and Thorpe, 1996), giving clear evidence that supply from the source can influence allocation between shoot and root. The model was further tested by cooling the root to reduce the $V_{max}$ value (without gross change to the $K_m$ value). This had the predicted effect of reversing the priorities of the shoot and roots: cooled roots, as expected, received reduced allocation of assimilate, which was reversed when the shoot was shaded (Minchin and Thorpe, 1996). The interval between cooling the roots and shading the shoots led to almost total diminuition of the effect, and it is put forward that this is due to a process of acclimitisation to the lowered root temperature, most likely by modulation of sink enzyme activity (Minchin and Thorpe, 1996). In response to the many previous

experiments on source-sink interactions that have used pruning and other surgical interventions as treatments, it is pointed out that removal, in this instance, of part of the root will increase the resistance to mass flow in the phloem system (Minchin and Thorpe, 1996). However, simulation of such pruning by reducing $V_{max}$ by a factor of 5 (thereby increasing resistance to flow by a similar amount) followed by decrease of supply (by shading) does reverse priority of sinks (Minchin and Thorpe, 1996).

Mechanistic explanation establishes carbon flux as the property of the whole plant and demonstrates measurement of the kinetics of source supply, phloem transport and sink assimilation as pre-requisite to any attempt to predict carbon fluxes (Farrar, 1996; Minchin and Thorpe, 1996). Environmentally and surgically altered capacities of source and sink have been shown to alter allocation of carbon in the whole plant (Minchin and Thorpe, 1996). In the context of the whole plant model described and allowing for adequate functional vasculature in the phloem, debate on the control of carbon allocation has concentrated on whether rate of plant growth as import into sinks is source or sink-limited (Farrar, 1996). Experimental approaches have investigated metabolic regulation in sources and sinks by means of molecular manipulation of metabolism (Sonnewald and Willmitzer, 1992; Stitt, 1993; Sonnewald *et al.*, 1994).

That correlation of single sink specific enzymes with sink strength is no indication of causality (Stitt, 1993), and it has been proposed that integrated approaches using transgenic (or mutant)

plants with deleted or severly reduced levels of enzymes and introduced heterologous proteins, in conjunction with control analyses, can elucidate where control of carbon allocation is exerted in the plant (Stitt, 1993). Further, use of plant pathogens, parasites and symbionts (which can be presumed to harness extant machinery within the plant to ensure allocation of carbon for their own ends) are suggested for experiments which avoid the innately non-physiological approaches of environmental and surgical manipulation of plants (Stitt, 1993).

### 1.4.2 Sink regulation of growth?

The suggestion that plant pathogens, parasites and symbionts as useful treatments in experiments to elucidate regulation of resource allocation (Stitt, 1993) underlines that plant growth can be sink regulated. Analysis of phloem contents in potato transformed with yeast-derived invertase expressed in the apoplast show a reduction in the amino acid: sucrose ratio (Heineke *et al.*, 1992), showing decreased loading of sucrose as a result of expression of yeast-derived invertase in the apoplast (Sonnewald and Willmitzer, 1992).These plants exhibited reduced sink development indicating sucrose as the main export form of carbon from source to sink (Sonnewald and Willmitzer, 1992), in common with similar studies in *Arabidopsis*, tobacco and tomato (von Schaewen *et al.*, 1990; Dickinson *et al.*, 1991; Sonnewald *et al.*, 1991). Resulting from the inhibition of sucrose export was that photosynthesis was decreased in source leaves (and

increased in developing leaves) indicating sink limiting of photosynthesis (Sonnewald and Willmitzer, 1992).

A candidate enzyme for the cleavage of sucrose in potato tubers, and therefore the regulation of sink metabolic activity, is sucrose synthase (SS) (Zrenner *et al.*, 1995). T-type SS isoform DNA was linked in antisense orientation to the global 35S CaMVpromoter resulting in expression of antisense RNA of the same form expressed in the whole plant (Zrenner *et al.*, 1995). However, inhibition of SS activity was found to be tuber specific, indicating independent SS isoforms are functional in different organs in potato (Zrenner *et al.*, 1995). Inhibition of SS did not change the sucrose content of the tubers, however, glucose and fructose content increased from nearly zero to 7 and 2%, respectively, of total tuber dry weight in most strongly inhibited transgenic lines (Zrenner *et al.*, 1995). Starch accumulation was strongly inhibited, although this was not apparently due to inhibition of the starch biosynthesis pathway. The reduced carbohydrate accumulation was accompanied by a decrease in total dry weight of tubers and reduced total soluble proteins in the tubers (mainly the major storage proteins patatin, proteinase inhibitors and the 22 kD proteins) (Zrenner *et al.*, 1995). Availability of free amino acids was not altered in the transgenic plants. These data indicate sucrose synthase as a major determinant of sink strength of the potato tuber. Number of tubers per plant was found to be unaltered, belying the hypothesis that sink strength is inversely proportional to number of sinks in the potato model (Zrenner *et al.*, 1995)

Studies with enzymes of sucrose cleavage point towards source limitation of whole plant carbon allocation in potato. The extent of source limitation has been investigated using top-down control analysis (Sweetlove *et al.*, 1998). Whole plant metabolism was divided into source and sink block of reactions either side of the sucrose pool in the leaf apoplast. Measurements of flux of $^{14}C$ (from $CO_2$) were made in a range of plants at varying light intensities, or transgenic plants specifically altered in either of the two blocks, in order to manipulate flux and apoplastic concentrations of sucrose. The transgenic plants selected for this experiment were two lines of chloroplast fructose-1,6-bisphosphatase (cpFBP-12 and cpFBP-16) (Kossman *et al.*, 1994) and two lines of the phloem associated sucrose transporter (αSUT-13 and αSUT-17) (Riesmeier *et al.*, 1994). All lines were selected for having minimal pleiotropic effects of the transgenes (Sweetlove *et al.*, 1998). Sucrose was confirmed as the sole communicating metabolite between source and sink blocks by applying two different manipulations to the same block and testing whether the relationship between flux and apoplastic sucrose content was the same in each case (Brown *et al.*, 1990; Sweetlove *et al.*, 1998). The relationship was tested in the cpFBP lines, and with varying light intensity applied to wild-type plants. It was found that there was a positive correlation between flux and apoplastic sucrose concentration, and that in each manipulation there was no significant difference between each treatment (Sweetlove *et al.*, 1998). Elasticity coefficients (see section 1.1.1) of the source and sink blocks were calculated by measurement of flux and apoplastic sucrose concentration in the cpFBP and αSUT lines, and in wild-type plants subjected to variations in light intensity (Sweetlove *et al.*, 1998). Calculated elasticity coefficients are relative

values (Fell, 1997) and were expressed over fresh weights of leaves (Sweetlove *et al.*, 1998). From the elasticity coefficients, flux control coefficients for each block were calculated to be 0.81±0.01 for the source block and 0.19±0.01 for the sink block, with confidence limits were calculated from standard errors of the elasticity coefficients (Small and Fell, 1990; Sweetlove *et al.*, 1998).

The flux control coefficients calculated, 0.8 and 0.2 for source and sink, respectively, indicate that carbon allocation within the whole plant of potato is source limited. Indeed, from the experiments of Sonnewald *et al.* (1991), Müller-Röber *et al.* (1992) and Heineke *et al.* (1992), described above (sections 1.2.1 and this section), this is an expected result. Yet approximately 20% of control may reside in the sink block (Sweetlove *et al.*, 1998).

The potato tuber is a perennating organ that stores resources predominately as starch. The effects of altered starch synthesis have been investigated by introduction of a chimeric gene containing subunits of ADP-glucose pyrophosphorylase (AGPase) linked to the 35S CaMV promoter (Müller-Röber *et al.*, 1992). Only partial inhibition of AGPase resulted in the leaves in transformed plants (Müller-Röber *et al.*, 1992). However, almost complete inhibition of AGPase was effected in the tuber with the result of abolition of starch synthesis in the tubers, so proving AGPase as having a unique role in starch biosynthesis in plants (Müller-Röber *et al.*, 1992). This effect enables assessment of sink control of resource allocation, but it was found that the dry

weight yield per plant was reduced by only 30% (Müller-Röber *et al.*, 1992; Sweetlove *et al.*, 1998), indicating that the rate of translocation of carbohydrate into the tubers is not dramatically affected. A similar conclusion was made in a study of tuber specific, apoplastic expression of a yeast-derived invertase (Sonnewald *et al.*, 1997) which caused a major increase in tuber size and freshweight yield per plant. However, expression of yeast-derived invertase in the tuber apoplast does not consistently change the dry weight yield of tubers per plant (Sonnewald *et al.*, 1997). Further, the conclusions of Riesmeier *et al.* (1994) (see section 1.3.1) suggest that the control first ascribed to the sink block by Sweetlove *et al.* (1998) may actually reside predominately in the phloem transport system rather than the tubers themselves (Sweetlove *et al.*, 1998).

The experiments described by Sweetlove *et al.* (1998) are specific to the conditions stated and, therefore, cannot be extended to other conditions. Similarly, potato tuberisation is a teleologically different system to seed production. Although both represent storage sinks, seeds are anatomically, temporally and genetically distinct tissues to the maternal plant (see section 1.3.3). There is therefore, at the very least, potential for greater control of resource allocation in seeds as sinks compared to the perennating system in potato tubers.

The difference between fresh and dry weights of tubers has been used to clarify that growth, in terms of increase of resources in a sink, is little different between the treatments and controls in these experiments. Allowing that water relations appear to have little bearing on sink priority,

other than in the very short term (Williams *et al.*, 1991), dry weight gain is a certain measure of sink growth in this context. However, growth can mean increase in size, shape and number of an organism (Hunt, 1981). The AGPase antisense plants used by Müller-Röber *et al.* (1992) produced significantly more tubers both per plant and per stolon. Obviously, the lack of starch in these tubers and the corresponding increase in sucrose concentration, with up to 30% of tuber dry weight being sucrose, has in some way induced tuberisation in the stolon.

The general conclusion is that growth of fruits and seeds dominates vegetative growth, with the coda that flowers are less successful than established fruits in competition for assimilates when availability of assimilates is short (Ho, 1984; Ho, 1988; Wardlaw, 1990; Minchin and Thorpe, 1996). From a teleological stand point, this suggests that fruits supersede flowers in exhibition of control of allocation. Inflorescence meristems are deactivated, or aborted (Hensel and Bleecker, 1992; Hensel *et al.*, 1994), according to growth in the prioritised fruit sinks. Goal-driven reproduction is essential for the monocarpic habit in which the plant senesces and dies after a single reproductive effort. Sink control of resource allocation is therefore most likely going to be apparent in monocarpic plants, which include the majority of crop species. This has been investigated in a model system, *Arabidopsis thaliana* (Hensel and Bleecker, 1992; Hensel *et al.*, 1994).

*Arabidopsis* can produce more than 20,000 seeds within 10 weeks of germination, a very high level of fecundity achieved at the expense of the soma of the mother plant which is quite senescent at the end of the reproductive effort in the wild-type. In the wild-type Landsberg *erecta* (Ler) line, induction of inflorescence meristems and flower buds ceases in a coordinated, global manner over 2 d, a phenomenon termed "Global Proliferative Arrest" (GPA) (Hensel *et al.*, 1994). Microscopical studies have shown retention of structural characteristics of inflorescence meristems in the face of GPA in wild-type Ler (Hensel *et al.*, 1994). GPA does not happen in wild-type Ler when fruits are pruned from the plant, nor does it occur in the male-sterile (*ms1-1*) line in the Ler background (Hensel *et al.*, 1994). In both these case, inflorescence meristems continue to proliferate and termination occurs by "terminal differentiation" (Hensel *et al.*, 1994) in which histological disruption is observed at the apices, with ensuing loss of the inflorescence meristem (Hensel *et al.*, 1994).

A model is suggested, based on data showing a decreasing number of flower buds at each stage of the arresting proceess, in which arrest occurs firstly in the inflorescence meristems and progresses through to the flower buds on the plant, including those which are post-anthesis (Hensel *et al.*, 1994). There is evidence of a capacity for reinstatement of an arrested meristem, but it is lost to floral buds (Hensel *et al.*, 1994). This difference in capacity for resinstatement is characterised as essentially reversible and irreversible cessation of cell expansion and differentiation in these apices (Hensel *et al.*, 1994). The absence of wild-type GPA in plants

pruned of developing fruits and in the *ms1-1* line indicate that the developing seeds propagate a signal that induces GPA (Hensel *et al.*, 1994). Similar phenotypes are observed in other reduced-fertility mutants, including other male-sterile lines (Melissa Spielman, University of Oxford, UK, personal communication; Chaudhury, 1993; Scott *et al.*, 1998), as well as female-sterile mutants (Modrusan *et al.*, 1994) and mutants of floral development (Schultz and Haughn, 1991; Hensel *et al.*, 1994), showing GPA to be specifically dependent on seed development (Hensel *et al.*, 1994).

## 1.5 INTEGRATING SIGNALS

The results of Hensel *et al.* (1994) show that the fate of inflorescence meristems is controlled by the developing fruit, providing strong, yet circumstantial, evidence that sinks do control allocation of resources in the monocarpic model plant *Arabidopsis*. The monocarpic habit is defined by the coupling of whole-plant senescene to reproduction (Hildebrand, 1881; Hensel *et al.*, 1993). The ecological classification of plants exhibiting the monocarpic habit as ruderals is strongly correlated to adaptation to growth in disturbed environments with high mortality risks (Hensel *et al.*, 1993, and references therein).

The monocarpic habit of *Arabidopsis* is teleologically in diametric contrast to the perennating habit in potato. Nonetheless, in both cases there is considerable evidence of there being some integrating signal, or signals, between sources and sinks. The nature of this mechanism is undetermined (Hensel *et al.*, 1994). However, candidate mechanisms have been identified relating to the physiological signals that induce flowering as the transition between vegetative and reproductive growth (Bernier *et al.*, 1993). In essence, these are plant growth substances and nutrient status, but can be more usefully divided into one or a combination of plant growth substances, carbohydrate status, and nitrate.

### 1.5.1 Plant Growth Substances

The idea that some hormone-like molecules regulate plant growth has dominated much of the history of research on plant growth. In principle, PGSs or precursor forms can be exported from one organ and transported to another where they affect growth. Regulation by PGSs may occur by increase or decrease of production (i.e. positive and negative regulation), stimulation or inhibition, and/or by antagonism between PGSs, the combined effect of which is the coordination of growth (Jackson, 1993). However, there are no complete mechanistic explanations of how PGSs might regulate s: r.

A major hurdle in elucidation of PGS function has been the apparent misunderstanding of the very nature of PGSs in the face of the regenerative, organisational and developmental plasticity of plants, and the fault of comparison of PGS systems with endocrine systems in animals (Trewavas, 1981). Particular criticism has been levied against classical approaches to PGS physiology for neglecting the question of changing sensitivity to PGSs. This results from concepts of plant "hormones" that effect growth responses by changing concentration (Trewavas, 1981; 1982; 1991), yet there has been discrimination between developmental and environmental responses to PGSs, notably the different roles played by abscisic acid (ABA) in a) processes of seed development and dormancy, and b) in stomatal function (Hetherington and Quatrano, 1991). The question of sensitivity belies underlying secondary message processes in cells, including

possible signalling cascades that combine short-term direct responses to environmental stresses, with long-term metabolic changes that may be characterised as developmental responses. Examples of such a PGS are cytokinins.

Cytokinins appear to be the major senescence-retarding PGS in plants (Noodén *et al.*, 1990; Faiss *et al.*, 1997). Leaf senescence has been widely shown to correlate with decreasing levels of cytokinin activity, and it has similarly been widely implicated that roots are the site of cytokinin synthesis and that the PGS is transported via the xylem (Noodén *et al.*, 1990, and references therein). Declining levels of cytokinin may therefore be important in regulation of monocarpic senescence. This has been investigated in Soybean (Noodén *et al.*, 1990). Levels of cytokinins in xylem sap were found to fall sharply with the onset of reproductive growth (Noodén *et al.*, 1990), and this decline was found to be reversible by surgical pod removal (Noodén *et al.*, 1990). This reversal effect was limited to the stages of seed filling, as opposed to seed maturation, on the plant (Noodén *et al.*, 1990), indicating that developing pods can influence senescence processes through the action of root-produced cytokinins carried in the xylem sap. That early pod development appeared to cause rapid decline in cytokinin levels is proposed to mean that pod-development triggers the beginning of monocarpic senescence (Noodén *et al.*, 1990). This correlation is underpinned by the identification of two specific cytokinins (zeatin riboside and dihydrozeatin riboside) of the seven assayed in total (isopentenyladenine, zeatin, dihydrozeatin, and 3 *O*-glucosides) as responding to pod presence (Noodén *et al.*, 1990).

Cytokinin function was further demonstrated in paracrine signalling in whole tobacco plants (Faiss *et al.*, 1997). Isopentanyltransferase is the rate-limiting enzyme of cytokinin biosynthesis. Transgenic tobacco plants, containing the isopentanyltransferase gene (*ipt*) under control of a modified 35S promoter that is repressed under high levels of tetracycline repressor protein, were used to investigate effects of enhanced cytokinin synthesis upon tetracycline application. Specific increases in products of cytokinin biosynthesis, in particular zeatin riboside, were found in the transpiration streams of plants treated with local applications of tetracycline, but growth effects were found only in the locality of tetracycline application. This was also found to be the case in wild-type plants with *ipt* transgenic grafts. These results challenge the role of long-distance signalling by cytokinins, but support cytokinin function in paracrine signalling (Faiss *et al.*, 1997).

These results are not as contradictory as they first appear. Faiss *et al.* (1997) point out that their results do not disprove the work of Noodén *et al.* (1990) and that, in the case of monocarpic senescence, cytokinins may well act as the integrating signal of leaf senescence upon seed development. They also suggest that some of the phenomenology of cytokinins may be due to interactions and antagonistic effects with other PGSs, such as auxin and abscisic acid.

There is a vast literature concerning PGS biology (Jackson, 1983), but little of a conclusive nature (Trewavas, 1981; 1991). The question of sensitivity to PGSs, as opposed to the flaws in

the approach of correlation of dose and response (Strickland and Loeb, 1981; Trewavas, 1983; Cleland, 1983; Trewavas, 1991), has remained largely unaddressed. However, PGS mutants conferring insensitivity to GA and ABA, deficiency in GA, or resistance to auxin were found not to interfere with correlative interactions leading to GPA (Hensel *et al.*, 1994). This is not to say that PGSs are not candidates for long-distance integrating signals, as there are many examples of their implication in such responses, but rather that they are unlikely to act as singly identifiable mediators of plant growth regulation. More fundamental integrating signals may be identified in nutrient metabolism, in particular sugars.

### 1.5.2 Sugars

As has been discussed (see Section 1.1), sucrose is the main product and molecule of export and transport, from photosynthetic tissues into the phloem. Feedback through the pathway of sucrose synthesis via the TPT and $P_i$ has been shown to induce chloroplastic starch synthesis, so maintaining homœostasis of the RPP. The phloem as transport system of sucrose has been most credibly described as functioning by Münchian pressure-flow (see section 1.2), and, by means of active reuptake of leakage of contents, can for most intents and purposes be envisaged as a closed system. It may be viewed as "transcellular, vectorialized and accelerated cytoplasmic streaming" (Stitt, 1996). Sucrose may be utilised directly from uptake from unloading phloem, e.g. in wheat (Patrick, 1997; Miller and Chourey, 1992), or it may be hydrolysed in apoplast prior to use, e.g.

in *Zea mays* (Miller and Chourey, 1992) or *Vicia faba* (Weber *et al.*, 1996) (see Section 1.3). Whichever route, uptake of sucrose from the phloem is, at least in part, contingent on demand from the sink (see Section 1.3). Similarly, induction of new sinks, e.g. floral meristems has been demonstrated to be contingent, at least in part, on adequate availability of resources for new growth (Bernier *et al.*, 1993).

Between production, transport and utilisation, sucrose is the preferred form of photosynthate for long-distance transport (Miller and Chourey, 1992). Within the system summarised above, source and sink activities are inextricably linked via the phloem system to one another. Removal of sinks, or exogenous supply of sucrose, both cause substantial increases in leaf sucrose content and significant decreases in photosynthetic capacity (Foyer, 1988; Huber, 1989; Sheen, 1990). Further, transgenic over-expression of yeast invertase has shown that carbohydrate accumulation inhibits photosynthesis (von Schaewen *et al.*, 1990; Dickinson *et al.*, 1991; Sonnewald *et al.*, 1991). Inhibition of photosynthesis in such plants has been attributed to decreased levels of RPP enzymes and increased levels of glycolytic enzymes (Stitt *et al.*, 1991) and to increased osmotic pressure in leaf cells (Heineke *et al.*, 1992). However, neither model fully and satisfactorily describes feedback regulation of photosynthesis, and an alternative underlying mechanism of feedback regulation has been proposed as being global repression of photosynthetic gene transcription by carbohydrates (Jang and Sheen, 1994).

Evidence for direct photosynthetic gene regulation by carbohydrates underlying direct metabolic regulation of photosynthesis includes the discovery that the activity of seven photosynthetic gene promoters are specifically and coordinately repressed by glucose and sucrose in maize protoplasts (Sheen, 1990). By deletion, mutation and hybridisation of specific promoters, inhibition effects of sucrose and glucose were shown to be the result of specific inhibition of protoplast transcriptional activity (Sheen, 1990). Further, it was found that exogenous application of acetate to maize protoplasts repressed specific photosynthetic gene promoter activity, indicating acetate repression is evolutionarily conserved in the plants (acetate being an unknown metabolite outside of algal photosynthesis) (Sheen, 1990). Gene repression was not found at normal physiological concentrations of sugars, rather between 100-300 mM concentrations of glucose and sucrose (Sheen, 1990), indicating such repression may occur *in vivo* in response to rising local concentrations of sugars.

Similar repression has been found for other photosynthetic genes: steady state transcript levels of *rbcS*, chlorophyll *a/b* binding protein and the δ-subunit of thylakoid ATPase genes were significantly reduced within 5 h by addition of 50 mM glucose to autotrophic cell suspension cultures of *Chenopodium rubrum* (Krapp *et al.*, 1993). And 2 % sucrose or glucose caused repression of light-inducible accumulation of *rbcS* transcript in *Arabidopsis* within 2 h (Cheng *et al.*, 1992). Similarly, accumulation of mRNA of light dependent chlorophyll *a/b* binding protein

was significantly reduced by addition of 2 % sucrose to *Brassica napus* cell culture (Harter *et al.*, 1993).

These results show that metabolic repression of photosynthetic genes overrides developmental and environmental regulatory factors (Sheen, 1990; Jang and Sheen, 1994). Such repression has been termed "anabolic" because it is repression by products. There are examples of catabolic gene repression by carbohydrates, one such being re-repression of malate synthase (MS) and isocitrate lyase (ICL) gene expression upon the return of sucrose to previously starving cucumber cell cultures (Graham *et al.*, 1994). MS and ICL gene expression was correlated closely with the drop of intracellular concentrations of sucrose, glucose and fructose below threshold concentrations (Graham *et al.*, 1994), but no change in respiration rate was detected. Glucose, fructose, raffinose, 2-deoxyglucose and mannose also caused repression of MS and ICL gene expression, and it was noted that all repressive species can be phosphorylated by hexokinase. This observation is supported by 3-methylglucose, which is not phosphorylated, having no repressive effect (Graham *et al.*, 1994). The focus on hexokinase-mediated phosphorylation led the authors to propose that the signal causing change in gene expression originates from either flux of hexose sugars into glycolysis, or intracellular concentration of hexose sugars. Little more is known about secondary signalling involved in carbohydrate mediated repression of gene expression.

Sugars can induce as well as repress genes. ß-amylase activity and levels of ß-amylase mRNA increased significantly in rosette leaves of *Arabidopsis*, with concomitant accumulation of starch, when either whole plants, or excised mature rosette leaves were supplied with sucrose, glucose or fructose (Mita *et al.*, 1995). It was found that darkness strongly repressed sugar induced expression of ß-amaylase (Mita *et al.*, 1995). In the case of the whole plant, ß-amaylase gene expression was found in roots, racemes and cauline leaves; it is posited that this is due to carbon partitioning and sink-source interactions in the whole plant (Mita *et al.*, 1995). These results suggest that the gene for ß-amylase is subject to regulation by carbohydrate metabolic signal. Selective inhibition of electron transport in photosystem II did not inhibit sugar inducible expression in the light, though cycloheximide did cause inhibition. Transgenic *Arabidopsis* expressing the promoter section of the ß-amylase gene linked to ß-glucuronidase coding sequence showed light-dependent sugar-inducible expression in rosette leaves (Mita *et al.* 1995). This suggests a light induced signal and *de novo* protein synthesis in the cytoplasm are involved in the regulation of the gene expression (Mita *et al.* 1995). Further work on ß-amaylase has further elucidated the transduction pathway of sugar inducement of the gene (Takeda *et al.*, 1994). Three inhibitors of protein phosphatases strongly inhibited sucrose-inducible accumulation of mRNAs for ß-amylase, as well as sporamin and the small subunit of ADP-glucose pyrophosphorylase in petioles of sweet potato (Takeda *et al.*, 1994). These results suggest that continuous dephosphorylation of proteins is required for the transduction of carbohydrate metabolic signals of at least some sugar-inducible genes (Takeda *et al.*, 1994).

In all, sugar-inducible gene expression is a relatively nascent area of research. At the most fundamental level, the molecular aspects of sucrose sensing support not only the biochemistry of carbon partitioning in plant tissues, particularly leaves, but, in the context of sucrose transport, describe a discrete system by which resource allocation may be regulated at a fundamental level. However, as has been discussed above (see Section 1.3), there is considerable evidence that nitrate vies with carbon as a regulator of plant growth.

### 1.5.3 Nitrate

The theory of Functional Equilibrium (Brouwer, 1983) is supported by many observations supporting regulation of plant growth by balance of carbon and nitrogen metabolism. Carbon and nitrogen metabolism are closely intertwined, with nitrogen assimilation being dependent on a supply of carbon skeletons from photosynthesis (Peoples and Gifford, 1997), and photosynthesis being potentially limited by nitrogen via Rubisco (Lauerer *et al.*, 1993). However, studies of plants under regimes of nitrate deprivation, and of nitrate-uptake deficient plants, have shown more fundamental levels of interaction between carbon and nitrogen metabolism (Rufty *et al.*, 1988; Scheible *et al.*, 1997a, b & c).

Nitrate deprivation causes increased starch accumulation in the short-term in Soybean, with apparent reallocation of carbon from amino acid synthesis (Rufty *et al.*, 1988). The 'extra'

accumulation of starch in the light was not fully degraded in the ensuing dark period, and so there was an overall increase in starch accumulation in N-deprived plants over a 2 d period after nitrate withdrawal (Rufty *et al.*, 1988). Levels of sucrose were found to accumulate in leaves during the first day after nitrate withdrawal, and activities of FBP and SS remained high for 2 and 3 d after nitrate withdrawal respectively (Rufty *et al.*, 1988). Levels of FBP declined rapidly after 1 day and there was evidence of restricted growth of sink leaves by the second day after withdrawal. These results indicate a biochemical mechanism of nitrate mediated change in shoot-root resource allocation by showing that severe nitrate limitation limits sucrose synthesis and so causes reduced growth of sink leaves in the short-term, with resultant diversion of carbon from shoot to root.

Studies of nitrate-uptake deficient plants have suggested that nitrate may have a yet more fundamental role in regulation of resource allocation (Scheible *et al.*, 1997a, b & c). Tobacco transformants with very low activity of NR were used to screen for processes that are regulated by nitrate. The phenotypes of such plants resembled wild-type plants grown in low nitrate in growth rate and amino acid and protein contents (Scheible *et al.*, 1997a). When grown with high availability of nitrate, such plants accumulated large amounts of nitrate in the shoot, and root growth was inhibited, causing an increase in shoot: root ratio. S:r correlated with leaf nitrate content (Scheible *et al.*, 1997a). Split-root experiments showed root growth was inhibited by accumulation of nitrate in the shoot (Scheible *et al.*, 1997a). Inhibition was overcome by

phosphate limitation, indicating nitrate-depended changes in allocation can be overridden by other signals that increase allocation to root growth (Scheible *et al.*, 1997a). In the absence of organic nitrogen species, high levels of nitrate were found to repress starch metabolism and decreased root sugar content (Scheible *et al.*, 1997a; Scheible *et al.*, 1997c). Sugar content of roots correlated with rate of root growth (Scheible *et al.*, 1997a).

Metabolic effects of high shoot concentrations of nitrate in NR deficient plants were increase of transcription of the enzymes of nitrogen assimilation and organic acid synthesis in the roots and leaves, and accumulation of enzymes of organic acid synthesis and their products (Scheible *et al.*, 1997c). There was notable decrease in ADP-glucose pyrophosphorylase transcription, and activity and levels of starch decreased in leaves and roots (Scheible *et al.*, 1997c). Increased availability of nitrate caused further decrease in ADP-glucose pyrophosphorylase transcription and starch was remobilised at rates approaching those of wild-type plants (Scheible *et al.*, 1997c). It has been shown that such mutant and transgenic NR-deficient tobacco plants compensate for reduced function by modification of diurnal regulation of transcription, post-translational modification and turnover of what NR is expressed (Scheible *et al.*, 1997b), underlining NR as having plastic expression and being essential for plant metabolism.

The effects of accumulation of large amounts of nitrate in roots and leaves of NR-deficient plant (Scheible *et al.*, 1997a, b & c) show that nitrate-derived signals stimulate organic acid

metabolism, repress starch synthesis, and inhibit root growth in nitrate limited NR-deficient plants, leading to the proposal of nitrate as a signal intitiating coordinated changes in carbon and nitrogen metabolism (Scheible *et al.*, 1997c). That NR and NiR genes are induced by nitrate (Vance, 1997) and the work described above (Scheible *et al.*, 1997a, b & c) show nitrate initiates an extended program of gene expression (Scheible *et al.*, 1997c), coordinating several major pathways of further nitrogen metabolism and repressing starch synthesis (Scheible *et al.*, 1997c) and having great bearing on shoot-root resource allocation (Scheible *et al.*, 1997a).

Mechanisms of nitrate sensing have not yet been identified (Scheible *et al.*, 1997c) and it may be that there is interaction with secondary signalling and mechanisms of gene regulation by other compounds of carbon and nitrogen metabolism (Scheible *et al.*, 1997c).

### 1.5.4 Conclusions

Other than those already stated, few conclusions can be made on the role of PGSs in regulation of resource allocation in plants. Future work clarifying the question of tissues' sensitivities to PGSs will lead to a clearer picture of how and when PGSs may act in regulating plant development and growth.

Regulation of resource allocation by carbohydrate status is attractive in its simplicity and discrete bounds within the source-phloem-sink system of allocation. However, carbohydrate metabolism is inextricably linked with that of nitrogen, and nitrogen in itself can act in the manner of a PGS when not assimilated into organic forms. Underlying this observation is increasing evidence that both sugars and nitrate can directly regulate gene expression. With secondary signalling pathways in each model as yet unresolved there is enormous scope for interaction between these key resources at a submetabolic, supramolecular level. A great deal of research on signalling within individual cells is technology led, and until such technologies of single cell 4D imaging can be developed, progress in elucidating such interactions will be slow.

However, successful attempts have been made to investigate interactions between carbon and nitrogen metabolism in plants by exploiting exogenous treatments, of nitrate limitation and shading, or surgery on leaves, in the context of internal treatments in the form of experimenting with near-isogenic mutant lines variously perturbed in starch formation (phosphoglucomutase, or PGM mutant) or nitrate uptake (reduced nitrate uptake, or NU mutant) (Schulze *et al.*, 1994).

*Arabidopsis* plants grown on high levels of nitrogen have high concentrations of carbohydrate and nitrate in rosette leaves during reproductive growth, but these were not found to contribute to bulk flow of assimilated resources to developing seeds, and indeed, were of insufficient size to significantly contribute (Schulze *et al.*, 1994). Shading or removal of rosette leaves during

flowering caused low seed yields, indicating rosette leaves are important in maintaining continuing supply of resources. Nitrate limitation, such as is effectively the case in the NU mutant, caused remobilisation of resources stored in rosette leaves and plants achieved greater seed yield (Schulze *et al.*, 1994). Altering nutrition at the start of flowering, from high to low nitrogen supply, reversed this response causing yields similar to plants grown on continuously low nitrate. Change from a low nutrient input to high nutrient input upon flowering caused yields similar to plants grown at continuously high nutrient availability. Resource assimilation determines seed production during reproductive growth (Schulze *et al.*, 1994).

The starchless PGM mutant grew more slowly and achieved smaller biomass during vegetative growth, compared with wild-type plants, due to high turnover of soluble sugars causing increased respiration (Caspar *et al.*, 1985; Schulze *et al.*, 1991). Reproductive growth rate in this mutant increased to exceed wild-type, and at the end of the lifecycle both genotypes achieved similar biomass. However, growth rate of vegetative tissue (rosette leaves) constituted a large proportion of this overall increase, and rate of seed production was concomitantly lower than wild-type in PGM plants (Schulze *et al.*, 1994). Starch storage is therefore not only important in during vegetative growth, but is of considerable importance in terms of whole plant resource allocation during reproductive growth (Schulze *et al.*, 1994). Comparison of NR activity in all lines showed positive correlation against leaf carbohydrate concentration, and negative correlation against leaf

nitrate concentration, indicating concentrations of carbon and nitrogen stores *in planta* are involved in regulating allocation (Schulze *et al.*, 1994).

In conclusion, candidate integrating signals are not very far beyond essential definition and modelling. Underlying interactions of source supply and sink demand for assimilated resources in plants are very complex. Regulation of gene expression by metabolites such as sucrose is a nascent area of research limited by technology. Recent successful research has used comparative analyses of plants experimentally treated with environmental or surgical manipulations, and use of transgenic and mutant lines with specific and discrete 'internal manipulations' of metabolism. In light of what has been discussed above, the research strategy of this thesis will now be stated.

## 1.6 RESEARCH STRATEGY

As has been discussed above, plant growth is regulated on many levels. Functional equilibria can be related to interactions between the environment, photosynthesis, respiration and nitrogen assimilation, each of which can be manipulated to a greater or lesser extent. Theories about how growth is regulated centre on the action of plant growth substances, sucrose mediated control and mechanisms of C/N balance. There is a strong argument that whole plant carbon allocation is the result of many partial processes (Farrar, 1992).

The aim of this work was firstly to investigate the effects of altered seed metabolism on allocation of photoassimilate through the whole plant during reproductive growth, and secondly, to elucidate by what means such allocation is regulated.

### 1.6.1 Selection of a mutant with altered seed metabolism

Seeds are anatomically, genetically and temporally distinct sinks in relation to the maternal plant during reproductive growth. Use of mutant or transgenic plants with altered seed metabolism is an obvious strategy for investigating differential carbon allocation with reference to wild-type.

An example of such an approach is afforded by Weber and co-workers. Transgenic plants expressing a legumin B4 promoter yeast invertase construct *in vivo* in the apoplast have lower levels of sucrose and higher levels of hexoses in the embryos, and starch and protein content of the seeds is reduced (Weber *et al.*, 1998a). Fresh weight:dry weight ratio is increased in these plants and seeds are very wrinkled indicating altered osmotic conditions as a result of high concentrations of reducing sugars (Weber *et al.*, 1998a), but there are no data given on whether whole plant carbon allocation is changed as a result of metabolic alterations in these sinks. There is evidence that seed development is largely regulated by metabolic controls (Weber *et al.*, 1998b) and that sucrose has a predominant role in inducing its own uptake system and the induction of differentiating processes associated with storage in seeds (Weber *et al.*, 1998b). This, and evidence from transgenic plants expressing a yeast derived invertase in the apoplast (Weber *et al.*, 1998a, b and c), can be used to support hypotheses that seed development is regulated by transport of sugars from outside of the seeds, and possibly, that transport of sugars may be regulated by seed development (cf. Sweetlove *et al.*, 1998).

Likely candidates for altered seed metabolism at the tissue or organ levels are PGS mutants. For example, Abscisic Acid (ABA) has been implicated in many processes in higher plant seeds, including: vivipary, desiccation tolerance, dry matter accumulation, inception and maintenance of dormancy, and regulation of expression of genes encoding reserve and late-embryogenesis proteins and enzymes (Black, 1991).

Exogenously applied ABA has been shown to alter flux of assimilate to or from the seed (Gifford and Thorne, 1986; Offler and Patrick, 1986 and references therein). However, there are considerable uncertainties in attempts to correlate response with exogenous dose of PGSs, Trewavas (1981, 1991) having argued convincingly that the mechanisms by which PGSs function can be divided into concentration of, and sensitivity to, PGSs. The use of monogenic mutants differing from wild-type in endogenous PGS content, or sensitivity, offers a decisive means of elucidating regulatory functions of PGSs (Kaarsen, 1982). Plants with altered ABA physiology are therefore reasonable candidates for having altered seed metabolism.

There is a large range of PGS mutants available in the model genetic organism *Arabidopsis thaliana* L. Heynh. *Arabidopsis* plants that are both deficient and insensitive to ABA have been identified (Koornneef *et al.*, 1984), of which *abi3* has been determined to be seed specific (Giraudat *et al*,. 1992; Parcy *et al*,. 1994) and as having differential expression of late abundant proteins (Koornneef *et al.*, 1989). *abi3* was therefore selected as a monogenic mutant with potentially altered seed metabolism.

### 1.6.2 ABI3

The *ABI3* gene has been isolated by positional cloning and pinpointed to a 35 kb region of chromosome 3 by RFLP marking and use of overlapping cosmid clones (Giraudat *et al.*, 1992).

An 11 kb subfragment was shown to complement the mutant phenotype in transgenic plants and a candidate *ABI3* gene was identified within the 11 kb fragment as being expressed within developing fruits (Giraudat *et al.*, 1992). The primary structure of the encoded protein was deduced from sequence analysis of a corresponding cDNA clone. The predicted ABI3 protein displays discrete regions of high similarity to the maize *viviparous-1* protein (Robichaud *et al.*, 1986; Giraudat *et al.*, 1992), which is proposed to be a transcriptional activator regulating gene expression during seed development.

Transgenic *Arabidopsis* plants carrying a translational fusion between 5.4 kb of the *ABI3* promoter and the β-glucuronidase (*GUS*) reporter gene have been reported to display no significant *abi3*-driven GUS expression in vegetative tissues (i.e. roots, leaves and stems), indicating the seed specificity of the *ABI3* gene (Parcy *et al.*, 1994). However, *abi3*-driven GUS expression has since been reported in vegetative quiescence processes of dark germinated and dark grown plants (Rohde *et al.*, 1999). *ABI3* is expressed in the seed from 12 days after pollination onwards (Parcy *et al.*, 1994).

There are five mutant alleles of the *abi3* locus: *abi3-1* (Koornneef *et al.*, 1984), *abi3-3* (Nambara *et al.*, 1992), *abi3-4* and *abi3-5* (Ooms *et al.*, 1993), and *abi3-6* (Nambara *et al.*, 1994), each offering a different severity of mutation. For example, in the most severe mutant, *abi3-4*, the size

of the ABI3 gene product (85 kD) is 40% that of the predicted wild-type protein (116 kD), the result of a point mutation that gives rise to a premature stop codon (Parcy *et al.*, 1994).

### 1.6.3 Previous molecular and biochemical characterisation of ABI3

Modified expression of 14 out of 18 mRNAs, grouped according to their co-ordinated temporal expression during silique development in the wild-type, was found in the *abi3-4* mutant allele. This indicates that the ABI3 protein directly participates in the regulation of several developmental programmes and that multiple regulatory pathways can lead to simultaneous expression of distinct mRNA markers. Ectopic expression of *ABI3* conferred ability to accumulate several seed-specific mRNA markers in response to ABA in transgenic plantlets, suggesting that expression of the marker mRNAs might be controlled by an *ABI3*-dependent and ABA-dependent pathways in seed. Characterisation of the *aba* mutation reveals that accumulation of these mRNAs is not correlated with the ABA content of seed (Parcy *et al.*, 1994).

Biochemical studies of *abi3* seeds have demonstrated significant differences between mutant and wild-type seed (Koornneef *et al.*, 1989; Finkelstein and Somerville, 1990; Nambara *et al.*, 1992; Ooms *et al*,. 1993; Parcy *et al.*, 1994). 12S (cruciferin) and 2S (arabin) proteins are absent in the severe alleles *abi3-4* and *abi3-5* and this correlates with levels of dormancy in a range of *aba*

and/or *abi* genotypes (Koornneef *et al.*, 1989; Nambara *et al.*, 1992). There is no difference in the total fatty acid content of mutant compared to wild-type seeds, but in *abi3* seed the major storage form of fatty acid with more than 20 carbon units, eicosenoic acid (20:1), is one third the level found in wild-type seeds. However, precursor forms, 18:1 and 18:2, are correspondingly higher (Finkelstein and Somerville, 1990). Tolerance of desiccation has been correlated with the mono:oligosaccharide ratio in seeds (Ooms *et al.*, 1993): The *abi3-1* homozygote has a mono:oligosaccharide ratio of 1.28, compared to 1.02 in the wild-type. Both *abi3-1* and wild-type have large amounts of raffinose and stachyose relative to more severe mutant alleles. The total sugar content of the *abi3-1* seeds was found to be 2.5 fold higher than in wild-type, percentage sucrose content in *abi3-1* measured at 88.8% compared to 77.9% in the wild-type (Ooms *et al.*, 1993).

The three major metabolic pathways of carbon partitioning in abscisic acid-insensitive *Arabidopsis* seed have all been shown to differ from wild-type. *abi3-1* has been shown to have metabolism least different to wild-type, and therefore best comparable to wild-type.

### 1.6.4 Selection of *abi3-1*

Plants homozygous for the *abi3-1* allele were chosen for this study because they offered the most moderate mutant phenotype of the 5 mutant *abi3* alleles known. In the more severe alleles, less

than 40% of the seeds germinate after 8 days of dry storage, whereas the rate of germination in *abi3-1* is not significantly different from wild-type (Ooms *et al.*, 1993). Because *abi3-1* is a relatively weak allele the effects on carbon partitioning can be seen as distinct from other phenotypic effects which result from the more severe alleles, e.g. precocious germination. The yield of seeds carrying the *abi3* mutation has been reported as being not significantly different from wild-type (de Bruign & Vreugdenhil, 1993). However, these experiments were performed on plants where the number of siliques allowed to develop was considerably less than normal.

Selection of *abi3-1* confers several advantages to this study, the aim of which is to investigate the regulation of resource allocation during reproductive growth. Firstly, it offers altered metabolism specifically in the seed but with a low risk of pleiotropic effects from the mutation which could undermine comparison with wild-type. Secondly, it should enable investigation of all three integrating signals that may regulate resource allocation. Having altered seed metabolism may elucidate source-sink interactions and the centrality of sucrose to the regulatory process. The growth of *abi3-1* is comparable to wild-type and so may allow investigation of aspects of functional equilibrium in respect of possible altered allocation compared to wild-type. Finally, investigation of the growth of this ABA-insensitive mutant may yield evidence on PGSs as regulators of plant growth while avoiding the pitfalls of experimental exogenous application of PGSs.

### 1.6.5 Strategy

The strategy of research was as follows:

1 To conduct an in-depth analysis of growth through the life cycle comparing *abi3-1* and wild-type plants to establish, a) any difference in growth between the two genotypes, and b) to identify any differing traits in *abi3-1* compared to wild-type.

2 To investigate any alterations in carbon allocation in *abi3-1* compared to wild-type in the short term using $^{14}CO_2$ radiolabel feeding experiments.

3 To investigate perturbations of supposed functional equilibrium in the plants by analysing growth of *abi3-1* and wild-type plants in conditions of altered light and nitrogen availability.

4 To investigate alterations in the carbon allocation of *abi3-1* compared to wild-type caused by altered concentration of $CO_2$ in the short term.

5 To attempt to experimentally test sucrose as an integrating signal in the event of positive results in the experiments outlined in 1-4 above.

# Chapter 2

## Materials and Methods

# 2.0 MATERIALS AND METHODS

## 2.1 MATERIALS

### 2.1.1 Reagents

Chemicals were obtained from Boehringer Mannheim UK (Diagnostics and Biochemicals Ltd., Sussex, UK), Sigma-Aldrich Chemical Co. (Poole, Dorset, UK), BDH (Poole, Dorset, UK), and Fisons (Loughborough, Leicestershire, UK).

Na[$^{14}$C]bicarbonate and [U-$^{14}$C]glc were obtained from Amersham International plc, Amersham, Buckinghamshire, UK.

All enzymes were obtained from Boehringer Mannheim UK, except invertase.

### 2.1.2 Plant Material

Seed of the wild type (Landsberg '*erecta*') and the abscisic acid insensitive mutant *abi3-1*, of *Arabidopsis thaliana* (L.) Heynh were supplied by the Nottingham Arabidopsis Stock Centre (NASC), University of Nottingham, UK.

## 2.2 GENERAL METHODS

### 2.2.1 Plant Growth Conditions

Seeds were sown onto Johnson's Medium (Epstein, 1972, see Appendix 1) containing 1.0% agar (w/v) (Bacto-Agar, Difco Laboratories, Detroit, Michigan, USA) and chilled for 48-72 h at 4 °C in the dark. Seeds were then transferred to a growth room at 21 °C in a 16 h light (250 $\mu$mol quanta $m^{-2}$ $s^{-1}$)/ 8 h dark photoperiod for 5-7 days until germinated. Seedlings were sown onto horticultural sand (E. A. Goundrey & Sons Ltd., Duns Tew, Oxon, UK) in 4 x 4 cm vacupots.

Plants were grown at 21°C in a 16 h light/ 8 h dark photoperiod. During illumination light intensity was 250 $\mu$mol quanta $m^{-2}$ $s^{-1}$. Plants were watered daily to field capacity with Johnson's Medium.

When applicable, light availability was modulated by shading with muslin cloth, and nitrate availability was modulated by concoction of Johnson's Medium with 10% and 20% of nitrate components.

### 2.2.2 Harvesting Plant Material

Whole plants were harvested by injecting 20-40 ml of water into the vacupot in which the plant had grown to emulsify the growing medium and prevent root damage. After harvesting excess sand on the roots was removed by immersion in water.

### 2.2.3 Weight Determinations

Fresh weights were determined after plant material had had excess water from harvesting patted off with tissue paper.

To determine dry weight, a known fresh weight of plant material was dried at 70 °C until no further change of weight occurred.

### 2.2.4 Leaf Area Determination

Leaf areas were determined by photocopying intact plants and cutting out the opaque leaf shapes on the photocopy. These were then weighed and weights of paper converted to area by comparison with the weight of a known area of paper.

### 2.2.5 Statistical Analyses

Differences in data were tested for significance using Student's *t*-test. Mentions of difference in the text are significant and qualified at $p < 0.05$.

## 2.3 $^{14}CO_2$ PULSE-CHASE FEEDING EXPERIMENTS

### 2.3.1 Supply of Radiolabel

For the supply of $^{14}CO_2$ plants were placed in a perspex chamber. The aerial portions were contained in a clear perspex partition (volume 8 l), while the roots were contained in a separate gas-tight chamber containing perlite moistened with Johnson's medium. Plants were tranferred into the chamber for a period of at least 8 h prior to the experiment. At the start of the light period 1.48 MBq of $^{14}CO_2$ (200 kBq $\mu$mol$^{-1}$) was released into the chamber. Conditions were as for plant growth. After one hour the remaining $^{14}CO_2$ was removed from the chamber into 10 % (w/v) KOH trap, and plants either harvested or allowed to continue photosynthesising for a further 1 or 3 h.

When applicable, concentration of $CO_2$ was modulated by, firstly, drawing air through the sealed chamber via 10% w/v KOH to remove $CO_2$ from the air in the chamber. The required concentration of $CO_2$ in the chamber was then achieved by addition of aqueous lactic acid to an appropriate quantity of Na($^{12}$C)Bicarbonate at 0, 1, 2 and 4 h time points.

### 2.3.2 Extraction of Plant Material

After dissection of harvested plants into siliques and flowers, cauline leaves, racemes, rosette leaves, and roots, tissue was frozen in liquid $N_2$ and stored at –80 °C.

Frozen tissue was ground in liquid $N_2$ and 2-5 ml of methanol: chloroform: 50 mM $(NH_4)_2CO_3$ (12:5:3) was added to the frozen powder. The mixture was then centrifuged at 12,000 *g*, 5-10°C

for 20 minutes. The pellet was then reextracted three times in methanol: chloroform: 50 mM $(NH_4)_2CO_3$ and the supernatants combined.

### 2.3.3 Fractionation of Tissues

The pellet (water insoluble substances) was resuspended in 5 ml $H_2O$. The combined supernantants were separated into chloroform and aqueous phases by the addition of equal volumes of 50 mM $(NH_4)_2CO_3$. The lower chloroform phase contained lipids and pigments (chloroform soluble substances), the upper aqueous phase contained all other soluble components (water soluble substances). The phases were separated from one another and evaporated to dryness *in vacuo*. The chloroform soluble substances were redissolved directly in 5 ml scintillation fluid (Optiphase 'Hisafe' 3, Fisher Scientific UK, Ltd., Loughborough, Leics.) and the water soluble substances redissolved in 5 ml $H_2O$.

### 2.3.4 Determination of Specific Activity in Tissue Fractions

The $^{14}C$ content of each tissue fraction was determined by liquid scintillation spectrophotometry after mixing with scintillation fluid (Optiphase 'Hisafe' 3, Fisher Scientific UK, Ltd., Loughborough, Leics.) as appropriate.

## 2.4 DETERMINATION OF TISSUE SUCROSE CONCENTRATIONS

### 2.4.1 Harvesting of Plants

Plants were harvested 4 h into the light period and immediately dissected into siliques, cauline leaves, racemes, rosettes and roots. The fresh weight of each tissue was determined and the tissue was then frozen in liquid $N_2$ and stored at -80 °C until used.

### 2.4.2 Killing and Extraction of Tissue

Each sample was killed separately by immersion in boiling 80% (v/v) ethanol for 5 minutes. The 80% ethanol was decanted into round-bottomed flasks and each sample re-extracted three more times. Ethanol-soluble samples were pooled and evaporated to dryness *in vacuo*. Dried samples were redissolved in 5 ml d.i. $H_2O$.

### 2.4.3 Assay of Sugars

Sucrose was assayed according to the method of Sweetlove *et al.* (1996b). The reaction mixture (100 $\mu$l) contained 200 mM Na acetate, pH 5.5, 10 $\mu$l yeast invertase concentrate and 10 $\mu$l extract. Controls contained no invertase. After an hour of incubation at 37 °C samples were assayed for glucose and fructose. To assay for glucose, 10 $\mu$l of the invertase digest was added to a reaction mixture (200 $\mu$l) containing 100 mM Tris, pH 8.1, 5 mM $MgCl_2$, 0.25 mM $NAD^+$, 1 mM ATP and 0.7 units of hexokinase. The change in absorbance at 340 nm was determined after the addition of 0.56 units of glucose 6-phosphate dehydrogenase. To assay for fructose, 0.7 units of phosphoglucose isomerase were added to the reaction mixture following the assay for glucose and the change in absorbance at 340 nm determined.

## 2.5 INCUBATION OF DETACHED SILIQUES WITH [U-$^{14}$C]Glc

### 2.5.1 Preparation of Tissue

Racemes were cut from plants at different times after germination. The bases of the racemes were trimmed under either distilled water or 50 mM EGTA. All but three siliques were removed from each raceme.

### 2.5.2 Supply of Radiolabel

The basal ends of two racemes were placed in 2 ml of 10 mM MES, pH 5.5; 0.3 mM [U-$^{14}$C]glc (148 GBq mol$^{-1}$); 50 mM sucrose, contained in a sealed Magenta tissue culture pot (370 cm$^3$). The chamber was incubated at 21 °C for 5h. The $^{14}CO_2$ released was collected in 10% (w/v) KOH.

### 2.5.3 Ethanol Extraction of $^{14}$C-labelled Tissue

At the end of the incubation period siliques were removed from the racemes. Siliques and recemes from each sample were killed separately by immersion in boiling 80% (v/v) ethanol for 5 minutes. The 80% ethanol was decanted into round-bottomed flasks and each sample extracted three more times.

### 2.5.4 Fractionation of Tissue

The ethanol-insoluble fraction was homogenised using a pestle and mortar and resuspended in 1 ml d.i. $H_2O$. A sample of this suspension was solubilised using 'Sinctran' tissue solubiliser (BDH Chemical Ltd., Poole, Dorset) according to the manufacturer's instructions.

The ethanol-soluble fraction was evaporated to dryness *in vacuo* and redissolved in a 12: 5: 3: mixture of methanol: chloroform: 50 mM $(NH_4)_2CO_3$. On addition of further $(NH_4)_2CO_3$ the system separated into two phases. The lower phase contained lipids and pigments (chloroform soluble substances), the upper phase contained all other soluble components (water soluble substances). The phases were separated from one another and evaporated to dryness *in vacuo*. The chloroform soluble substances were dissolved directly in 5 ml scintillation fluid (Optiphase 'Hisafe' 3, Fisher Scientific UK, Ltd., Loughborough, Leics.) and the water soluble substances dissolved in 5 ml $H_2O$.

### 2.5.5 Determination of $^{14}C$ Specific Activity in Tissue

The $^{14}C$ content of each sample was determined by liquid scintillation spectrophotometry after mixing with scintillation fluid (Optiphase 'Hisafe' 3, Fisher Scientific UK, Ltd., Loughborough, Leics.) as appropriate.

# Chapter 3

## The effect of the *abi3-1* mutation on growth of *Arabidopsis thaliana*

## 3.0 The effect of the *abi3-1* mutation on the growth of *Arabidopsis* plants

The aim of the work described in this chapter was to conduct a detailed analysis of growth between wild-type (Landsberg *erecta*) and *abi3* mutant plants throughout the life cycle to test the hypothesis that perturbations in seed development and maturation in *abi3* would alter resource allocation at the whole plant level.

## 3.1 Introduction

This chapter presents an in-depth growth analysis comparing wild-type *Arabidopsis* and *abi3* mutant plants in order to establish a) any difference in growth between the two genotypes, and b) to particularly identify any notable differences in source and sink tissues between the genotypes. As discussed in Chapter 1, *abi3* mutants were selected because of the seed-specificity of the mutation (Giraudat *et al.*, 1992; Parcy *et al.*, 1994) and the exhibition of general down-regulation of processes of seed maturation in these mutants (Parcy *et al.*, 1994). The weakest allele of *abi3*, *abi3-1* (Ooms *et al.*, 1993), was chosen to minimise any effects on accumulation of storage products and to avoid problems caused by viviparous germination. Perturbed seed development and maturation in *abi3-1* have been correlated with significant molecular and biochemical alterations in this sink tissue compared to wild-type (Ooms *et al.*, 1993; Parcy *et al.*, 1994). This perturbation of seed development and maturation was used as a specific treatment against wild-

type controls in growth analysis to test the hypothesis that such perturbations would alter resource allocation at the whole plant level.

Whole plant growth was analysed in depth through the life cycle by destructive harvesting of large cohorts of material ($n$ = 5-10) every 3-4 d. Plants were grown under standard conditions (see Chapter 2, Materials and Methods) in a randomised block design. Harvested plants were dissected into siliques and flowers, cauline leaves, racemes, rosette leaves and roots and fresh weights were determined before drying material to a constant dry weight determination. Leaf areas were determined and numbers of siliques and leaves were counted on each plant (see Chapter 2, Materials and Methods). *Arabidopsis* has a 56-70 day life cycle and can demonstrate significant phenotypic plasticity even when grown in controlled conditions (Somerville and Meyerowitz, 1994); in other words it is a 'weedy' species. The randomised block design, cohort size and frequency of harvesting were intended to determine plant growth with statistical validity and useful resolution through all stages of the life cycle. The gross anatomy of *Arabidopsis* lends itself well to dissection of each plant into source, sink and transport material *in sensu lato* (leaves, roots and siliques, and racemes). Both fresh and dry weights were determined in order that growth as expansion might be discriminated from growth as accretion, with potential correlation with leaf areas. Leaves and siliques were counted as numerical determination of source and sink 'blocks' for correlation with dry weight determination in each harvest.

## 3.2 Results

The effect of the *abi3-1* mutation was investigated by detailed morphometric analysis of wild-type and *abi3-1* plants. Early vegetative development was observed to be very similar in the two genotypes. No difference in rate or frequency of germination was observed (data not shown).

Figure 3.1 a-d shows changes in fresh weight of tissues in wild-type and *abi3-1* plants through the lifecycle. Figure 3.1a shows no significant difference in root fresh weights between wild-type and *abi3-1* plants. Figure 3.1b shows change in fresh weight of rosette leaves. The mean fresh weight of rosette leaves through the lifecycle is significantly less in wild-type compared to *abi3-1*, 8.5 ± 6.4 and 32.3 ± 13.0 mg, respectively; means ± SE ($n$ = 5 for wild-type; $n$ = 10 for *abi3-1*). Figure 3.1c shows mean fresh weights of racemes and siliques in wild-type and *abi3-1* plants through the life cycle. Again, standard errors are relatively large, but there is clear demonstration that *abi3-1* plants achieve greater fresh weight of total reproductive tissue (321.9 ± 35.8 mg, as opposed 64.5 ± 35.2 mg in wild-type at 43 d after germination, means and errors as before). Figure 3.1d shows mean fresh weight of siliques in wild-type and *abi3-1* plants through the life cycle. Flowering occurs in both genotypes at between 21 and 24 d after germination. Fresh weight of siliques in wild-type and *abi3-1* plants are not significantly different up to 28 d after germination. After 28 d after germination, the mean fresh weight of siliques becomes significantly different between wild-type and *abi3-1* plants. The rate of floral initiation in the

**Figure 3.1** Tissue fresh weight change in wild-type and *abi3-1*plants. Wild-type (◆) and *abi3-1* (■) plants were dissected into root, rosette, raceme and, siliques.

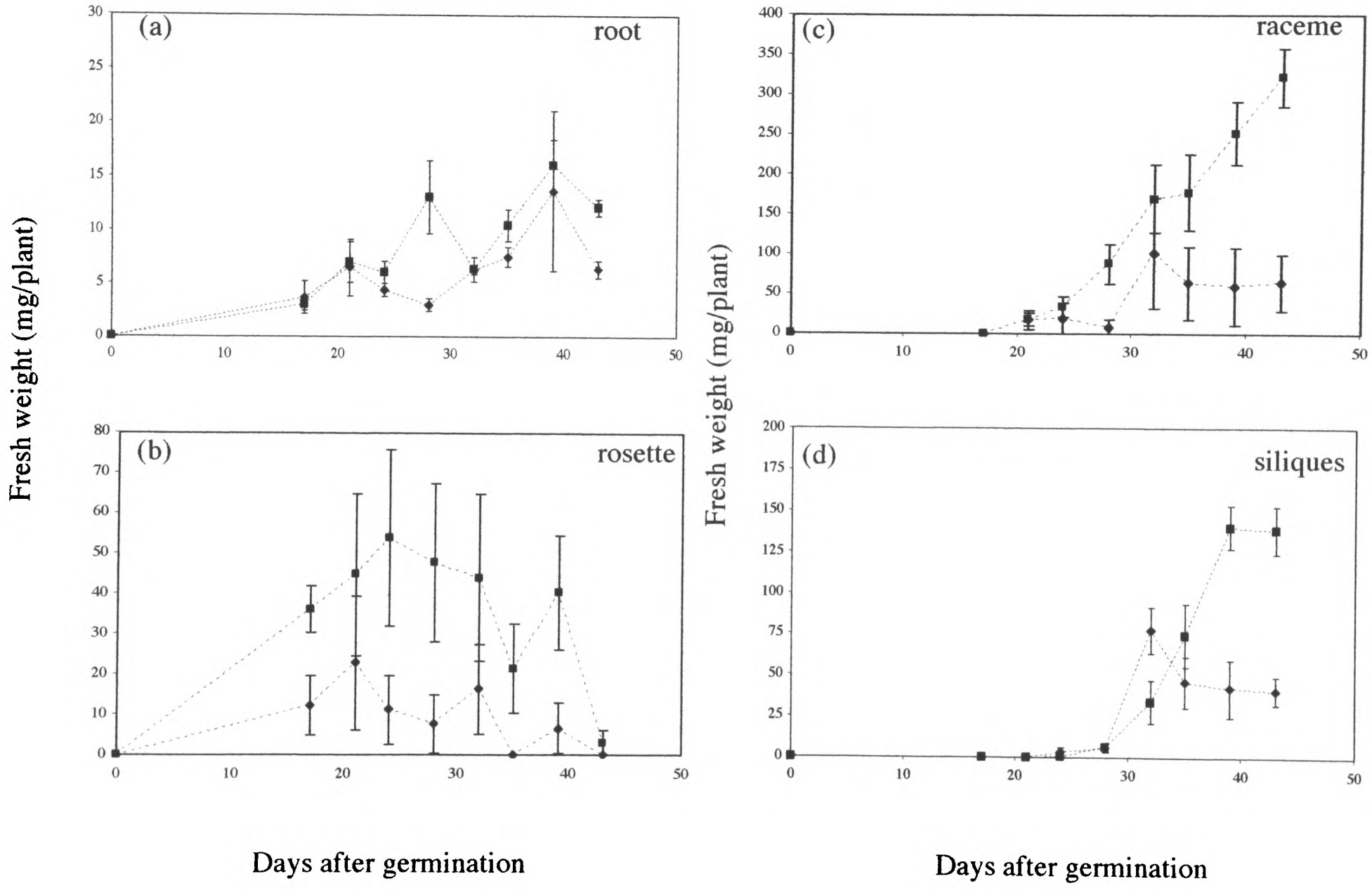


**Figure 3.2** Tissue dry weight change in wild-type and *abi3-1* plants.

Wild-type (◆) and *abi3*-1 (■) plants were dissected into root, rosette, raceme and, siliques. Dry weights determined after incubation at 70 °C until constant weights were achieved.

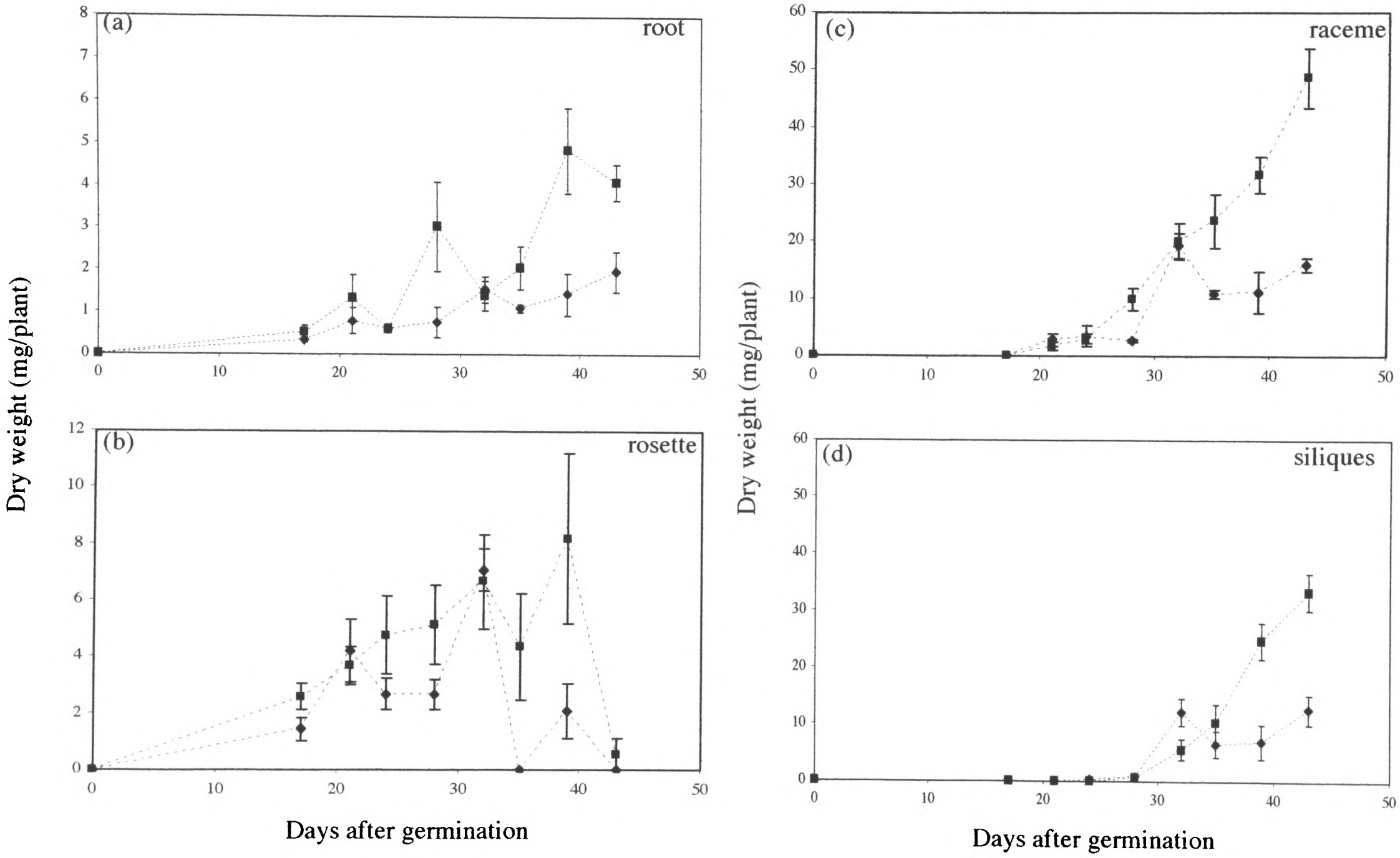


**Figure 3.3** Silique development in wild-type and *abi3-1* plants.

Siliques were harvested from wild-type (◆) and *abi3-1* (■) plants and the dry weight (a) (same as Fig 3.2d) and number (b) determined. Values are the mean ± SE ($n$ = 5 for wild-type, $n$ = 10 for *abi3-1*).

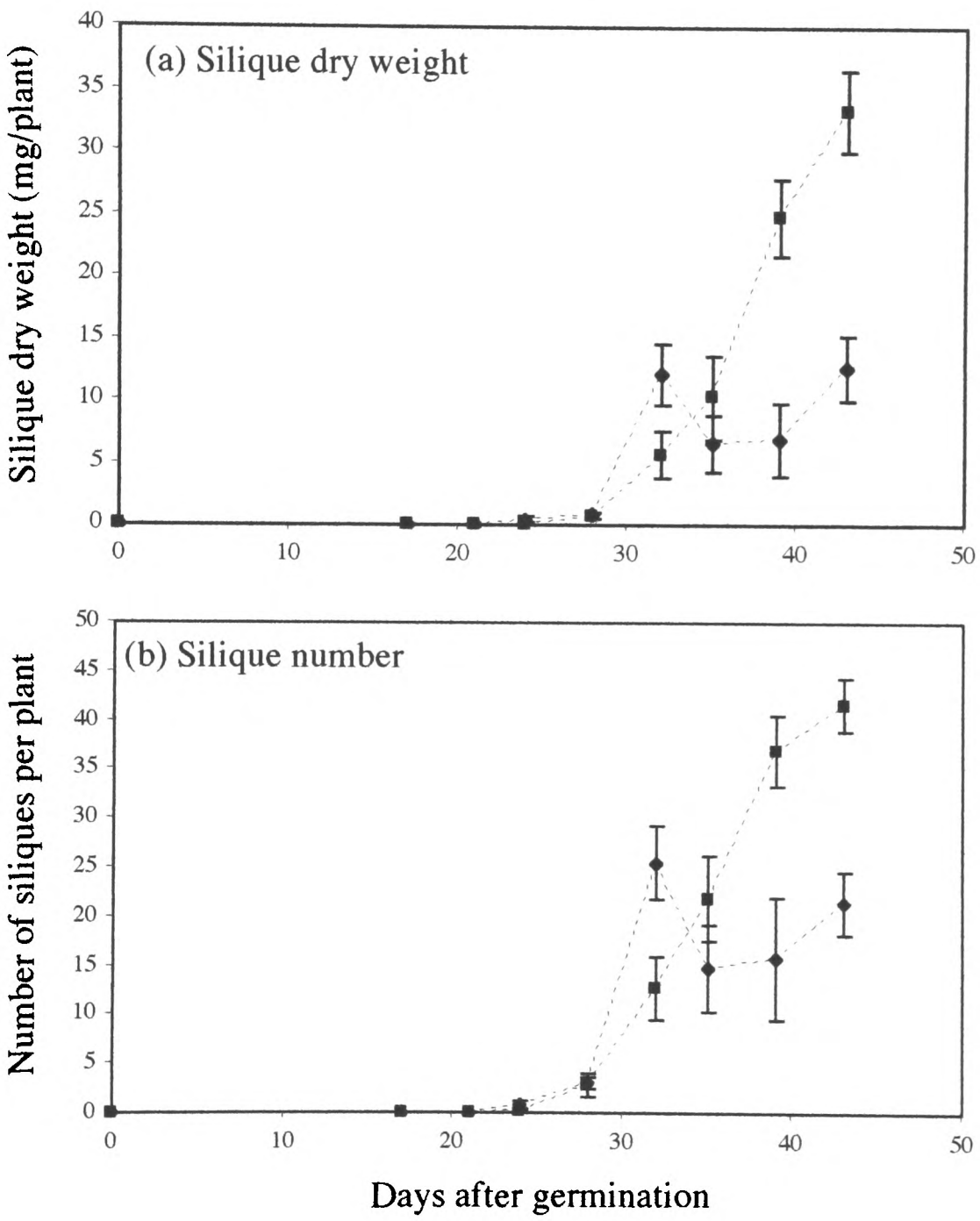
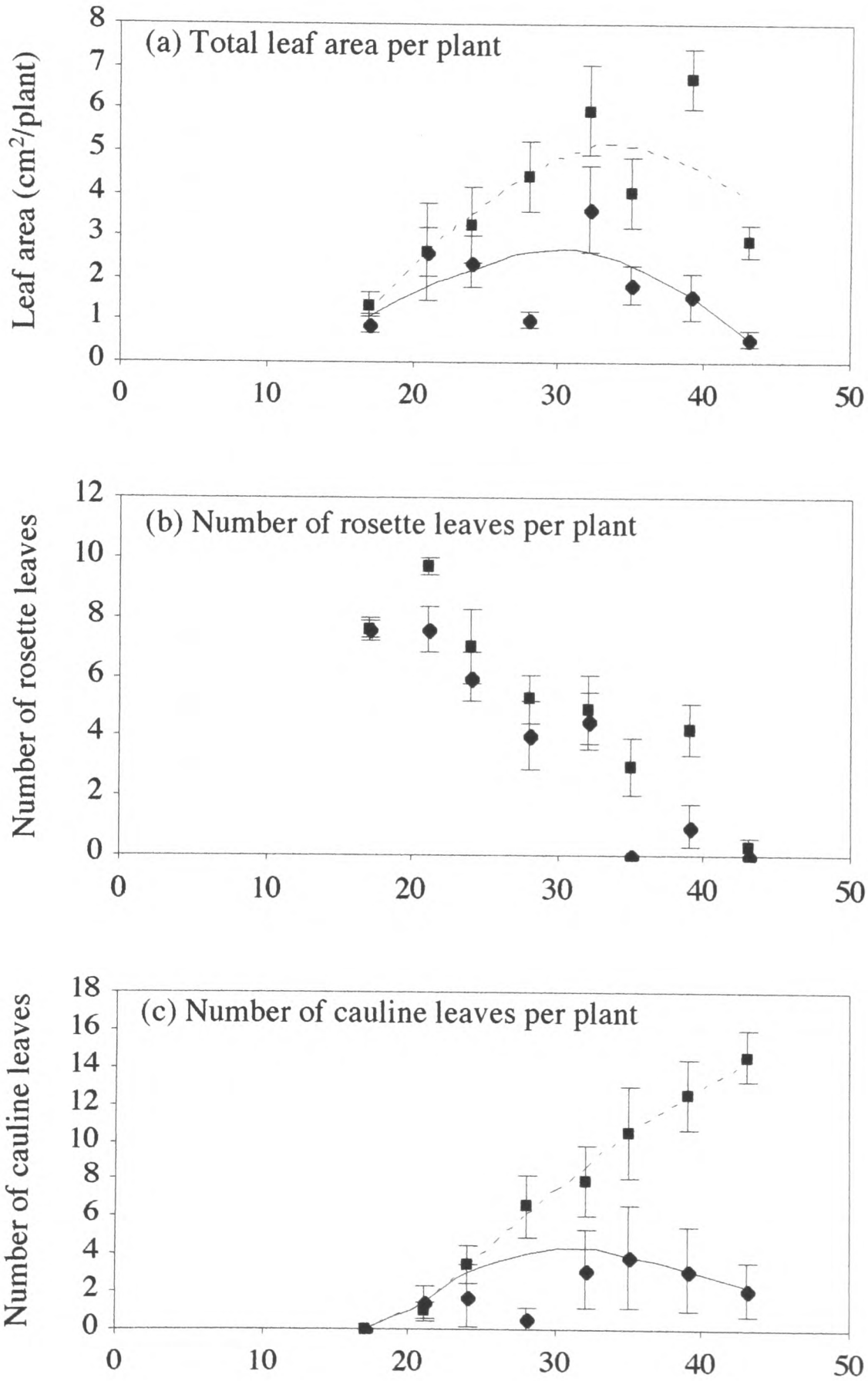


**Figure 3.4** Leaf development in wild-type and *abi3-1* plants.

Leaves were harvested from wild-type (◆) and *abi3-1*(■) plants and the total leaf area (a), number of rosette leaves (b) and, number of cauline leaves (c) determined. Values are the mean ±SE ($n$ = 5 for wild-type, $n$ = 10 for a*bi3-1*). Fitted lines are second order polynomials.



wild-type is initially greater than that of *abi3-1* plants. The difference of mean silique fresh weights, juxtaposed to the data shown in figure 3.1c, suggests that the difference in mean fresh weights of reproductive tissue in the two genotypes is due to a greater weight of siliques in *abi3-1*.

The relative growth rate (RGR) of the aerial portions of each genotype were calculated to be 40.5 ± 22.1 and 37.8 ± 16.5 mg $g^{-1}$ $day^{-1}$ for Landsberg *erecta* and *abi3-1* plants, respectively; means ± SE as before. From these data it is concluded that the *abi3-1* mutation has little effect on vegetative growth, consistent with the reported expression pattern of ABI3 (Parcy *et al.*, 1994).

In the analysis of change in fresh weights, standard errors of the means in both genotypes are large relative to the means. This is likely due to a combination of three factors. Firstly, *Arabidopsis* is a "weedy" species and so there is considerable variation within each harvested cohort. Secondly, *in vivo* water-status is likely to vary in fresh leaf tissue and will increase variation in each cohort of harvested material. Thirdly, the method of harvesting involved injection of the sand, in which plants were grown, with an excess of water to cause emulsification of the medium for ease of clean extraction of roots. In using this method, there was potential for excess water to adhere to the plants, in particular the rosette leaves close to the growing medium. Although excess water was shaken from plants, and tissues were gently patted dry with tissue

paper, there remained some potential for perturbed fresh weights. For this reason it was decided to determine tissue dry weights.

Figures 3.2a-d shows tissue dry weight changes in wild-type and *abi3-1* plants. Root dry weight changes, Figure 3.2a, show significant difference between wild-type and *abi3-1* plants after 35 d after germination, in contrast to figure 3.1a. However, Figure 3.2b shows no significant difference between wild-type and *abi3-1* rosette dry weights, again in contrast to fresh weights shown in Figure 3.1b. Figure 3.2c shows *abi3-1* plants achieve a greater total weight of reproductive tissue, i.e. racemes and siliques, and Figure 3.2d shows that this difference occurs as a greater weight of siliques on *abi3-1* plants compared to wild-type. The greater initial rate of floral initiation in the wild-type between 28 and 32 d after germination is confirmed.

There is a notable decrease in standard errors relative to means in the changes in tissue dry weights shown in Figure 3.2 compared to Figure 3.1. However, an apparent anomaly is fluctuations in dry weights during the lifecycle. This is most notable in Figure 3.2b at 35 d after germination in the wild-type, where the mean dry weight of the rosette falls to 0 mg per plant, before increasing to approximately 2 mg per plant at 39 d after germination. A similar phenomenon is observed in Figure 3.2 d, where the mean dry weights of siliques in the wild-type fall at 35 and 39 d after germination, before increasing to approximately 12 mg per plant at 43 d after germination. Such fluctuations are very unlikely in a single plant, but can appear as a result

of variation between randomly harvested cohorts of each genotype. It is conceded that the lower number of individuals harvested for the wild-type cohorts ($n = 5$), relative to that for *abi3-1* ($n = 10$) may have contributed to this apparent anomaly. However, the fluctuations result as a factor of the destructive harvesting of each cohort required to collect the data shown and so the data are valid.

Figure 3.3 shows silique development in wild-type and *abi3-1* plants. Figure 3.3a shows change in silique dry weight in each genotype through the lifecycle, and Figure 3.3b shows change in total silique number in each genotype through the life cycle. Silique growth differs between genotypes. Timing of flowering is unnaffected, but rate of floral initiation is initially greater in wild-type compared with *abi3-1* plants. However, *abi3-1* plants continue floral initiation longer than wild-type plants (Figure 3.3b). The average weight of a silique does not differ between the genotypes (data not shown). The mutant phenotype of continued floral intitiation first becomes apparent after 35 d after germination (Figures 3.2d, Figure 3.3a&b).

Figure 3.4a-c shows leaf development in wild-type and *abi3-1* plants. After 30 d after germination the total leaf area in the wild-type declines as the plant senesces. This decline is delayed in *abi3-1*, and there is a significantly greater leaf area per plant in the mutant after 39 d after germination. There is no significant difference in the number of rosette leaves per plant in each genotype (Figure 3.4b), nor in the dry weight of rosette leaves (Figure 3.2b). The difference

in total leaf area must therefore be accounted for in the cauline leaves. Figure 3.4c shows that the number of cauline leaves begins to decline after 35 d after germination, and the number of cauline leaves in the mutant continues to increase after this point.

## 3.3 Discussion

The aim of the work described in this chapter was to conduct a detailed analysis of growth between wild-type (Landsberg *erecta*) and *abi3* mutant plants in order to establish a) any difference in growth between the two genotypes and, b) to identify any particular differences in source and sink tissues between the genotypes. *abi3* mutants were selected for this study because of the seed-specific nature of the mutation (Giraudat *et al.*, 1992; Parcy *et al.*, 1994), and the weakest allele of *abi3*, *abi3-1* was chosen to minimise any effects of the mutation on accumulation of storage products and to avoid problems caused by viviparous germination (Ooms *et al.*, 1993). Perturbed seed development and maturation in *abi3-1* have been correlated with significant molecular and biochemical differences in this sink tissue compared to wild-type seed (Parcy *et al.*, 1994; Ooms *et al.*, 1993) and this was used as a specific treatment in a growth analysis, against wild-type controls, to test the hypothesis that such perturbations would alter resource allocation at the whole plant level.

The hypothesis was tested by detailed analysis of growth by destructive harvesting of whole plants in cohorts ($n$ = 5-10) grown under standard conditions in a randomised block design. Harvested plants were dissected into roots, rosettes, racemes and siliques and fresh weights were determined. Leaf area per plant was determined and number of rosette and cauline leaves per plant were counted along with number of siliques. Dry weights of tissues were determined.

The detailed analysis of growth of wild-type and mutant plants was successful in identifying differences between the two genotypes arising from the *abi3-1* mutation. Further, the analysis of growth successfully identified differences in source and sink tissue in the mutant compared to the wild type and this can be attributed to the effects of the mutation.

The lack of difference in rate and frequency of germination of the seeds of each genotype, coupled with the similarity in the relative growth rate of the aerial portions of each genotype leads to the conclusion that the *abi3-1* has little effect on vegetative growth. This is consistent with the reported expression pattern of *ABI3* (Parcy *et al.*, 1994). Vegetative expression of the *ABI3* gene has been demonstrated in dark-grown plants (Rohde *et al.*, 1999) and implicated in plastid differentiation pathways in vegetative tissues grown in the dark, or returned to the dark (Rohde *et al.*, 2000). However, these reports are based on dark grown plants and there is no evidence of vegetative expression of ABI3 in plants grown under normal 16 h photoperiod.

There is no significant difference between the genotypes in the timing and initiation of silique development (Figures 3.1d, 3.2d and 3.3), but mutant plants initiate new siliques more slowly and for longer than wild-type, consequently producing more siliques and a greater total mass of siliques per plant (Figures 3.2d and 3.3a&b). The average weight of silique does not differ between the genotypes (Figure 3.3a&b, Table 3.1), in concurrence with *abi3-1* being a weak allele of *abi3* and having only minor effects on storage product accumulation compared to wild-type (Koornneef *et al.*, 1989; Finkelstein and Somerville, 1990). The phenotype of greater mass and number of siliques first becomes apparent after 35 d after germination. After this point there will be significant expression of ABI3 in the mature wild-type siliques (from 12 d after anthesis) (Parcy *et al.*, 1994).

The increased total weight of siliques in *abi3-1* plants implies increased assimilation of carbon in the mutant. The growth analysis revealed greater total leaf area and delayed leaf senescence in the mutant compared to the wild-type from 39 d after germination (Figure 3.4a). This coincides with the continuation of silique initiation in the mutant and the cessation of silique initiation in the wild-type (Figure 3.3). There is no significant difference in dry weights and numbers of rosette leaves in wild-type and mutant, indicating that difference in the total leaf areas is due to difference in the cauline leaves. Analysis of cauline leaf number per plant reveals not only a higher number of cauline leaves per plant in the mutant compared to the wild-type, but also delayed senescence of cauline leaves in *abi3-1* plants.

In conclusion, the absence of functional ABI3 in developing embryos has the secondary effects of increasing silique initiation and growth in mutant plants, and concomitantly increasing initiation and delaying senescence of cauline leaves in mutant plants. The null hypothesis that perturbations in seed development and maturation do not alter resource allocation at the whole plant level can be dismissed. However, correlation does not indicate causation, and it may be that there are phenomena that remain undetected at the level of resolution in the growth analysis presented. On the basis of the results shown in Figures 3.3 and 3.4, the hypothesis that increased allocation of carbon to silique growth results from a higher rate of carbon assimilation in the cauline leaves can be proposed. This is investigated in Chapter 4.

# Chapter 4

Differences in resource allocation between

wild-type and *abi3-1* plants in the short-term

# 4.0 Differences in resource allocation between wild-type and *abi3-1* plants in the short-term using $^{14}CO_2$ radiolabel feeding experiments

The aim of the work described in this chapter was, firstly, to use pulse-chase feeding of $^{14}CO_2$ radiolabel to wild-type and *abi3-1* plants to test the hypothesis, arising from Chapter 3, that increased carbon allocation to silique growth in *abi3-1* plants results from a higher rate of carbon assimilation in the cauline leaves in *abi3-1* during late seed development, and secondly, to examine the relationship between long-term growth, as analysed in Chapter 3, and short-term metabolism.

## 4.1 Introduction

This chapter presents an investigation of the fate of $^{14}C$ supplied as $CO_2$ to photosynthesising wild-type (Landsberg *erecta*) and *abi3-1* plants to test firstly, that increased allocation of carbon to silique growth in *abi3-1* resulted from higher rate of carbon assimilation in the cauline leaves in *abi3-1*, and secondly, to examine whether long-term changes in carbon allocation revealed by morphometric analysis (see Chapter 3) correspond to short-term changes in carbon metabolism.

To investigate the pattern of carbon metabolism in the short term a sealable chamber was designed for supply of a pulse of $^{14}CO_2$ to plants. An infra-red gas analyser (IRGA) was used to

test that the chamber did not restrict photosynthesis of plants sealed into it. $^{14}CO_2$ was supplied to plants in the sealed chamber for 1 h (pulse) followed by continued photosynthesis for up to 3 h in the presence of $^{12}CO_2$ (chase). Plants were harvested at the end of the pulse (1 h time point) and then 1 h and 3 h into the chase (2 h and 4 h time points, respectively) and the amount of $^{14}C$ recovered in water-soluble, chloroform-soluble (lipids and pigments) and insoluble components of siliques and flowers, cauline leaves, racemes, rosettes leaves and roots was determined. Net source tissue is expected to assimilate a large proportion of the label during the pulse and to export label during the chase. A sink tissue is expected to incorporate label throughout the experiment. The total incorporation of label into water-soluble, chloroform-soluble and insoluble components gives a measure of the total rate of assimilation. The experiment was carried out on plants of around 30 d after germination, and on plants of around 38 d after germination, when the phenotype described in Chapter 3 is clearly apparent (see figures 3.3 and 3.4).

## 4.2 Results

Figure 4.1 shows transition from photosynthesis to respiration for 9 plants in the chamber. The Perspex chamber for $^{14}CO_2$ pulse was connected to an Infra-red Gas Analyser and relative $CO_2$ concentration (i.e the difference in concentration of $CO_2$ between in-put and out-put air) was measured every 10 minutes in the chamber for 5 h under experimental conditions to test that the chamber was gas tight. Nine dark-acclimated plants were introduced into the chamber after 5

**Figure 4.1** Photosynthesis of plants in sealed Perspex $^{14}CO_2$ feeding chamber.

The Perspex chamber for $^{14}CO_2$ pulse was connected to an Infra-red Gas Analyser and relative $CO_2$ concentration in the chamber was determined every 10 minutes for 5 h to test that the chamber was gas tight. 9 plants were introduced into the chamber after 5 hours and left for a further 19h to determine photosynthesis and the transition from photosynthesis to respiration.

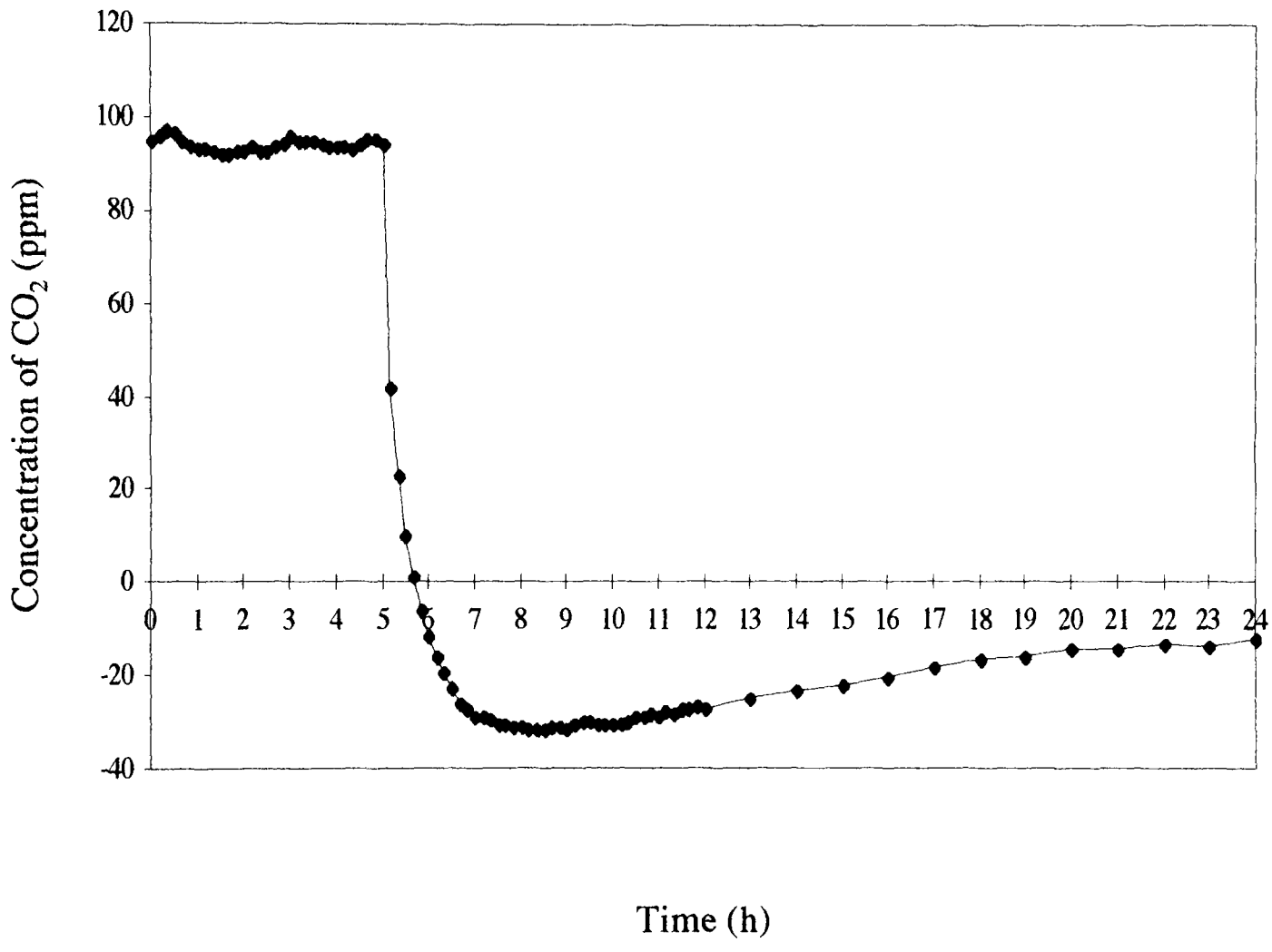


**Figure 4.2** Incorporation of $^{14}C$ from $^{14}CO_2$ by wild-type and *abi3* plants.

Intact plants (30 and 38 d old) were exposed to $^{14}CO_2$ and allowed to photosynthesise for a 1 h pulse (black bar). A subset of plants were harvested and the remainder incubated for a further 1 or 3 h chase (2 and 4 h time points). At each point the total $^{14}C$ incorporated per plant was determined. Values are mean ± SE ($n = 3$). a = wild-type, 30 d after germination, b = *abi3-1*, 30 d after germination, c = wild-type 38 d after germination, d = *abi3-1* 38 d after germination.

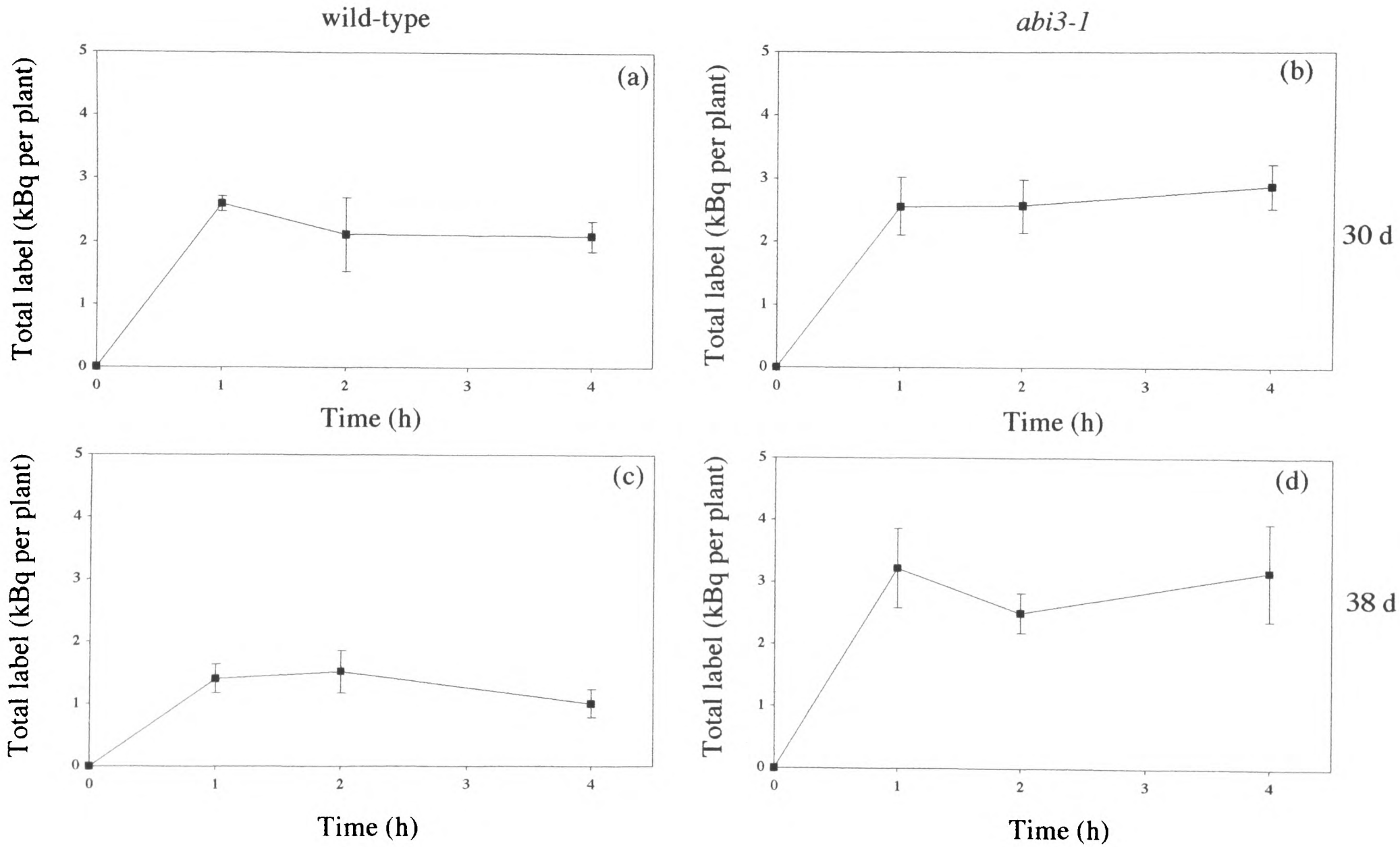


**Figure 4.3** Metabolism of $^{14}CO_2$ by wild-type and *abi3-1* plants at 30 d after germination

Intact plants were exposed to $^{14}CO_2$ and allowed to photosynthesise for a 1 h pulse. A subset of plants were harvested and the remainder incubated for a further 1 or 3 h chase (2 and 4 h time points). At each harvest the plants were dissected into siliques, cauline leaves, racemes, rosette leaves and roots. Each tissue was extracted in methanol: chloroform: $(NH_4)_2CO_3$. Extracts were separated into three fractions: chloroform soluble (solid bars); water soluble (stippled bars); and insoluble (open bars). The $^{14}C$ content of each fraction was determined and summed to give the total $^{14}C$ per tissue. Data is expressed as a percentage of the total $^{14}C$ incorporated (see Figure 4.2) and values are the mean ± SE ($n = 3$). The error bars refer to the error on the total $^{14}C$ per tissue, not the individual fractions. (a) = wild-type siliques, (b) = *abi3-1* siliques, (c) = wild-type cauline leaves, (d) = *abi3-1* cauline leaves, (e) = wild-type racemes, (f) = *abi3-1* racemes, (g) = wild-type rosette leaves, (h) = *abi3-1* rosette leaves, (i) = wild-type roots, (j) = *abi3-1* roots.

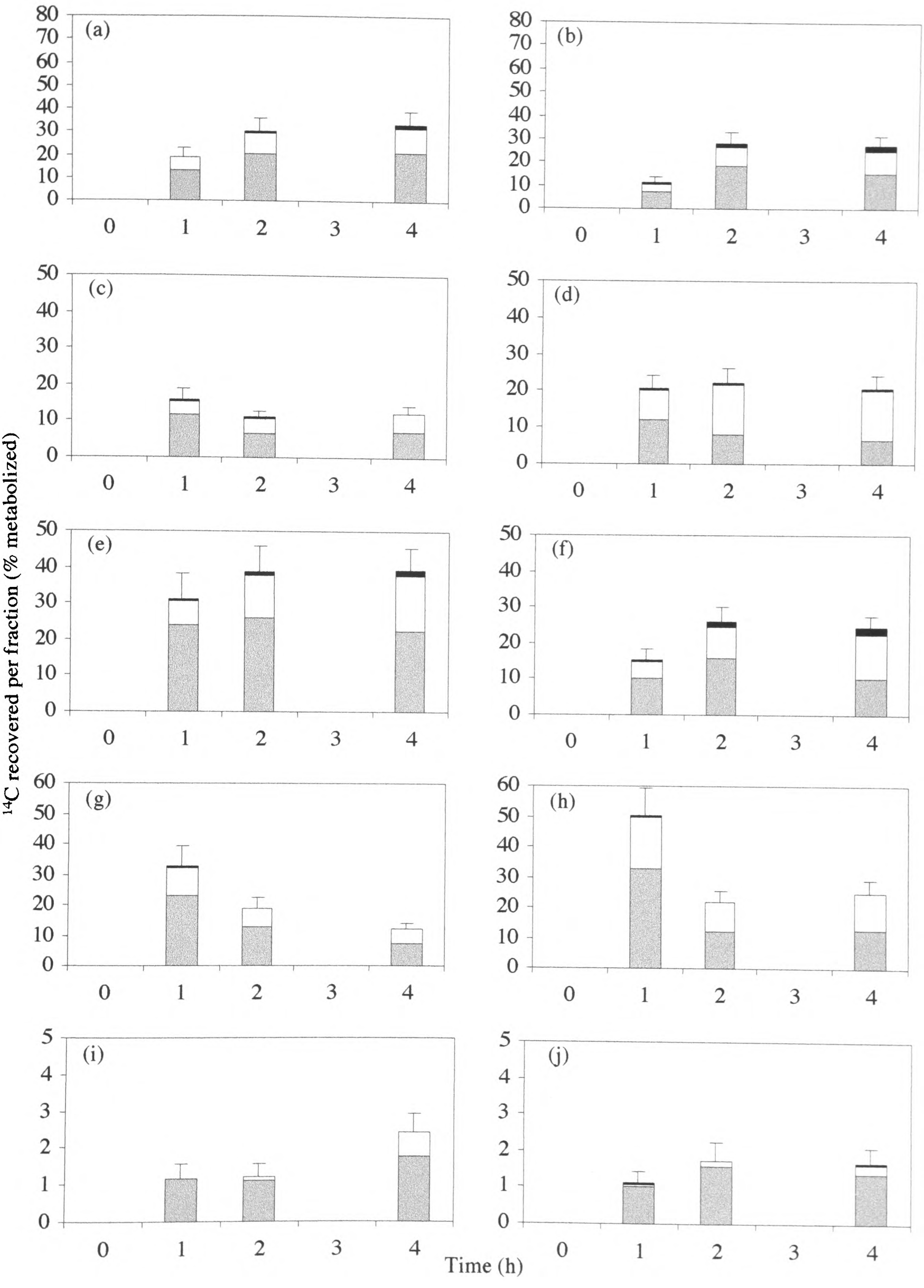

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
$^{14}$C recovered per fraction (% metabolized)
Time (h)

**Figure 4.4** Metabolism of $^{14}CO_2$ by wild-type and *abi3-1* plants at 38 d after germination

Intact plants were exposed to $^{14}CO_2$ and allowed to photosynthesise for a 1 h pulse. A subset of plants were harvested and the remainder incubated for a further 1 or 3 h chase (2 and 4 h time points). At each harvest the plants were dissected into siliques, cauline leaves, racemes, rosette leaves and roots. Each tissue was extracted in methanol: chloroform: $(NH_4)_2CO_3$. Extracts were separated into three fractions: chloroform soluble (solid bars); water soluble (stippled bars); and insoluble (open bars). The $^{14}C$ content of each fraction was determined and summed to give the total $^{14}C$ per tissue. Data is expressed as a percentage of the total $^{14}C$ incorporated (see Figure 4.2) and values are the mean ± SE ($n = 3$). The error bars refer to the error on the total $^{14}C$ per tissue, not the individual fractions. (a) = wild-type siliques, (b) = *abi3-1* siliques, (c) = wild-type cauline leaves, (d) = *abi3-1* cauline leaves, (e) = wild-type racemes, (f) = *abi3-1* racemes, (g) = wild-type rosette leaves, (h) = *abi3-1* rosette leaves, (i) = wild-type roots, (j) = *abi3-1* roots.

$^{14}$C recovered per fraction (% metabolized)

(a)

(b)

(c)

(d)

(e)

(f)

(g)

(h)

(i)

(j)

Time (h)

hours and left for a further 7 h to measure photosynthesis and the transition from photosynthesis to respiration. After 12 h relative $CO_2$ concentration was determined hourly. The figure shows stable measurement of relative $CO_2$ concentration in the chamber over 5 hours, indicating that the chamber was gas tight. On placing 9 plants in the chamber and resealing it, the relative concentration of $CO_2$ in the chamber falls steeply due to $CO_2$ fixation by the plants. Net photosynthesis occurs for 3 h, as indicated by the continuing decline of relative $CO_2$ concentration in the chamber. After 3 h, relative $CO_2$ concentration in the chamber begins to rise, indicating that the plants have begun net respiration. Plants photosynthesise at a maximum rate for 1.5 h.

The total incorporation of $^{14}C$ into water-soluble, chloroform-soluble and, insoluble components is shown in Figure 4.2 and gives a measure of the total rate of assimilation. At 30 d after germination there is significant fixation of $^{14}C$ by both wild-type and *abi3-1* plants (Figure 4.2a & b). There is no change in the amount of label in the chase at any stage (Figure 4.2). The total rate of assimilation from the labelling after the pulse is approximately 2-3 $\mu$mol $CO_2$ $h^{-1}$ per plant. At 38 d after germination the total rate of assimilation in mutant plants remained at 2-3 $\mu$mol $CO_2$ $h^{-1}$ per plant (Figure 4.2d) but, the rate of assimilation in wild-type decreased to 1-2 $\mu$mol $CO_2$ $h^{-1}$ per plant (Figure 4.2c). This is consistent with the decline in leaf area observed in the wild-type plants in the growth analysis (See Chapter 3, Figure 3.4).

The pattern of label distribution through wild-type and mutant plants at 30 d after germination is shown in Figure 4.3a-j. Roots are sink tissues and accumulate less than 2% of total label in each genotype (Figure 4.3i & j). In both genotypes, the rosette show significant assimilation of carbon during the pulse, and export of label during the chase (Figures 4.3g and h). The majority of the reduction of labelling in the chase is in the loss of water soluble components, which is also consistent with export (Figures 4.3g & h). Some label is assimilated by the cauline leaves, but there is no significant export from this tissue during the chase (Figures 4.3c & d). There is no significant changes in the proportion of label in the raceme tissues of either genotype, which remain heavily labelled throughout the experiment (Figures 4.3e & f). This is compatible with its primary role in transport. There is a steady increase through the chase in the portion of label recovered as insoluble and chloroform-soluble components in the raceme tissue (Figures 4.3e & f) which may indicate developing apices in the raceme tissue. In both genotypes, the siliques and flowers show an increase in labelling in the first hour of the chase (Figures 4.3a &b) indicating that they are the major sinks. The portion of label in the insoluble and chloroform-soluble components of the siliques and flowers increases during the chase (Figures 4.3a &b), indicating growth and storage product accumulation. At 30 d after germination, carbon is primarily fixed in the rosette leaves and there is transport to the developing siliques and flowers.

The pattern of label distribution through wild-type and mutant plants at 38 d after germination is shown in Figure 4.4a-j. Less than 2% of total label was recovered in the roots in each genotype

(Figure 4.4i & j). Very little label is assimilated in the wild-type rosette during the pulse (Figure 4.4g) relative to *abi3-1* plants (Figure 4.4h), indicating the latter are still photosynthetic. The proportion of label in the rosette leaves remains the same in both genotypes through the chase, and, is significantly less than the proportion of label recovered in the rosette leaves in each genotype at 30 d after germination (Figures 4.4g & h, cf. Figures 4.3g & h), indicating that there is no longer export of label from the rosette leaves and reflecting the senescence of the rosette leaves at this stage in both genotypes (see Chapter 3, figure 3.4b).

There is no significant change in the proportion of label in the raceme tissue, of either genotype, which remains heavily labelled throughout the experiment (Figures 4.3e & f). The proportion of insoluble and chloroform-soluble components does not increase in the wild-type (Figure 4.4e), reflecting an absence of developing apices consistent with cessation of floral initiation (see Chapter 3, Figure 3.3b). In contrast, the proportion of insoluble and chloroform-soluble components in *abi3-1* raceme tissue increases during the chase (Figure 4.4f), reflecting a continuing presence of developing apices consistent with both continuing floral and cauline leaf initiation (see Chapter 3, Figures 3.3b and 3.4c).

The assimilation of label during the pulse in wild-type cauline leaves is similar at both 30 d and 38 d after germination (Figure 4.4c, cf. Figure 4.3c) with no evidence of export of label. In contrast, in *abi3-1* plants at 38 d after germination most of the assimilation occurs in the cauline

leaves, and the proportion of label decreases during the chase indicating export from the cauline leaves (Figure 4.4 d, cf. Figure 4.3d). These observations are consistent with delayed cauline leaf senescence in *abi3-1* (see Chapter 3, Figure 3.4).

Most of the assimilation of label in the wild-type occurs in the siliques, with the proportion of the chloroform-soluble component increasing during the chase, and, no decline in the amount of label in the siliques during the chase (Figure 4.4a). These observations reflect the slow rate of assimilation in the wild-type leaf tissues at 38 d after germination (Figures 4.4c & d), and indicate both that the silique wall is photosynthetic and is the major source for continuing storage product accumulation in the maturing seeds. In *abi3-1* at 38 d after germination, the siliques and flowers continue to show an increase labelling during the first hour of the chase, and the proportion of label in the chloroform-soluble and insoluble components increases during the chase, indicating that the siliques and flowers continue to be the major sinks and are actively growing and accumulating storage product (Figure 4.4b, cf. Figure 4.3b).

The differing patterns of carbon metabolism of wild-type and *abi3-1* plants at 38 d after germination described above are consistent with the differences in leaf development identified in the earlier growth analysis (see Chapter 3, Figures 3.3b and 3.4c).

## 4.3 Discussion

The aim of the work described in this chapter was, firstly, to use pulse-chase feeding of $^{14}CO_2$ radiolabel to wild-type and *abi3-1* plants to test the hypothesis, arising from Chapter 3, that increased carbon allocation to silique growth in *abi3-1* plants results from a higher rate of carbon assimilation in the cauline leaves in *abi3-1* during late seed development, and secondly, to examine the relationship between long-term growth, as analysed in Chapter 3, and short-term metabolism.

$^{14}CO_2$ was supplied to plants in a sealed chamber for 1 h (pulse) followed by continued photosynthesis for up to 3 h in the presence of $^{12}CO_2$ (chase). Plants were harvested at the end of the pulse (1 h time point) and then 1 h and 3 h into the chase (2 h and 4 h time points, respectively) and the amount of $^{14}C$ recovered in water-soluble, chloroform-soluble (lipids and pigments) and insoluble components of siliques and flowers, cauline leaves, racemes, rosettes leaves and roots was determined. A net source tissue is expected to assimilate a large proportion of the label during the pulse and to export label during the chase. A sink tissue is expected to incorporate label throughout the experiment. The total incorporation of label into water-soluble, chloroform-soluble and insoluble components gives a measure of the total rate of assimilation. The experiment was carried out on plants of around 30 d after germination, and on plants of

around 38 d after germination, when the phenotype described in Chapter 3 is clearly apparent (see figures 3.3 and 3.4).

The incorporation and metabolism of $^{14}CO_2$ by wild-type and *abi3-1* plants at 30 d and 38 d after germination was successfully investigated, and that the source of carbon for silique development varies between wild-type and *abi3-1* plants in the short-term, as well as the long-term, is confirmed.

Figure 4.1 shows that photosynthesis of plants sealed into the experimental chamber is unimpaired by their confinement for at least 1.5 h, and that plants continue in net photosynthesis for up to 3 h, before changing to net respiration. This observation allows confident use of the chamber for a $^{14}CO_2$ pulse of 1 h preceding a chase with $^{12}CO_2$.

The total incorporation of $^{14}C$ into water-soluble, chloroform-soluble and insoluble components (Figure 4.2) gives a measure of the total rate of carbon assimilation in each genotype at each time point. The estimate of the total rate of assimilation of carbon, at 1-2 $\mu$mol $CO_2$ $h^{-1}$ per plant, for wild-type plants at 38 d after germination is in contrast to estimates of 2-3 $\mu$mol $CO_2$ $h^{-1}$ per plant wild-type plants at 30 d after germination and for *abi3-1* plants at both time points. This result is consistent with the decline in leaf area observed in the wild-type in Chapter 3. Confirmation that

this decline in carbon assimilation was looked for in the detailed metabolism of $^{14}CO_2$ in individual tissues in the pulse-chase feeding experiment:

The data in Figures 4.3 and 4.4 demonstrate that, in the wild-type, there is a shift in the source of carbon from rosette leaves to the silique wall during seed development. This has also been observed for other members of the Brassicaceæ (King *et al.*, 1997). In plants carrying the *abi3* mutation the cauline leaves act as the significant source of carbon for silique development when the rosette leaves are no longer net exporters of carbon. It is not clear whether the silique wall becomes the source for silique development later in development. This could be investigated by conducting a further pulse-chase feeding experiment later in the *abi3* lifecycle. By the same token, it is reasonable to suggest that the cauline leaves in the wild-type may also provide photoassimilate for silique growth. There may be concurrence of export from cauline leaves with export from rosette leaves in the wild-type in a short period beyond the temporal resolution of the experiment as carried out. This could be confirmed by conducting a further pulse-chase feeding experiment in the wild-type at, perhaps, 34 d after germination. In contrast, in *abi3* plants, the phase where cauline leaf photosynthesis is the source of carbon for silique growth is prolonged as a result of increased initiation and delayed senescence of cauline leaves.

It is clearly demonstrated that plants with the *abi3* mutation show increased development of siliques and that this is concomitant with increased initiation and delayed senescence of cauline

leaves. It has been shown that, using RNA blot analysis and histochemical staining of transgenic plants carrying an ABI3-promotoer/GUS fusion, the gene product is only expressed in seed tissue from 12 d after anthesis in the flower (Parcy *et al.*, 1994). There is, therefore, a discrepancy in the known site of *abi3* expression and the observed phenotype of the cauline leaves. It is possible that ABI3 is expressed at very low levels in tissues other than the developing seed, and the phenotype may be due to decreased sensitivity to abscisic acid in these tissues. However, the GUS reporter assay is highly sensitive, and so this is unlikely. Furthermore, when ABI3 is ectopically expressed in leaf tissues of transgenic *Arabidopsis* plants, application of abscisic acid induces mRNAs that are normally embryo specific and, require the presence of functional ABI3 for their expression in the embryo (Parcy *et al.*, 1994). This further suggests that ABI3 is normally absent in vegetative tissues. However, vegetative expression of the *ABI3* gene has been demonstrated in dark-grown plants (Rohde *et al.*, 1999) and implicated in plastid differentiation pathways in vegetative tissues grown in the dark, or returned to the dark (Rohde *et al.*, 2000). This has only been demonstrated in light stressed tissue, and in a fraction of plants analysed (Rohde *et al.*, 1999). Such expression is very unlikely to be occurring in unstressed plants grown in a 16 h photoperiod.

A more likely alternative hypothesis is that the perturbation of seed development in *abi3* mutant plants results in the production of long-distance signals that alter either floral initiation, or,

cauline leaf development. These signals may be either negative regulators absent in the mutant, or positive regulators that are present only in the mutant.

Seed derived signals have been previously hypothesised as explanatory of the similar phenotype of increased silique production in male sterile mutants of *Arabidopsis*, and in plants carrying the either the *abi1* or *abi2* mutations (Hensel *et al.*, 1994), but no effect on leaf senescence was reported (Hensel *et al.*, 1993). The phenotype reported in *abi1* mutant plants is consistent with the requirement of the ABI1 gene product for ABI3-mediated signalling in developing embryos (Parcy and Giraudat, 1997). Control of leaf senescence by the developing silique has been suggested to involve cytokinins derived from the roots (Noodén *et al.*, 1990), but this is uncertain in the light of evidence put forward that suggests cytokinins are not involved in long distance signalling (Faiss *et al.*, 1997).

That plant growth substances (PGS) may regulate whole plant growth cannot be discounted. However, alternative hypothesis might be a resource competition model: Pod removal in *Pisum* increases the initiation of new flowers and has been interpreted as evidence for long-distance signals that coordinate reproductive development and growth (Lockhart and Gottschall, 1961). In the G2 variety of pea, this behaviour occurs only under long-day conditions, even though the timing of flowering is unaffected by photoperiod (Reid, 1980), and this has been correlated with differences in the rate of fruit growth (Gianfanga and Davies, 1981). Initiation of new flowers

may therefore be limited by demand for nutrients in already developing seeds, i.e. there is competition for allocation of resources in the plant.

However, an alternative explanation for the behaviour described in pea is that maturing seeds produce a factor that inhibits further floral initiation. These two possibilities are not easily distinguished (Hensel *et al.*, 1994), but that the total storage product accumulation is similar in wild-type and *abi3-1* plants suggests that the phenotype described here does not result from a competition within the plant for resources. It is therefore more likely that some signal from maturing seeds results from abscisic acid signalling within the developing seeds.

The *abi3-1* mutation has little effect on the quantity of lipid and protein storage products accumulated per embryo (Finkelstein and Somerville, 1990; Koornneef *et al.*, 1989). However, in *abi3-1* plants there are changes in the nature of proteins, lipids and sugars that accumulate in the *abi3-1* seed, most notably an increase in the accumulation of sucrose (Ooms *et al.*, 1993).

Sucrose breakdown and phloem unloading have been identified as key determinants of sink growth in maize and bean (Miller and Chourey, 1992; Weber *et al.*, 1996). Sucrose has been implicated in floral (and so silique) initiation (Bernier *et al.*, 1993) and also leaf senescence (Krapp *et al.*, 1993). Control of leaf senescence by sucrose is the result of down-regulation of

genes involved in the production of the photosynthetic apparatus (Jang and Sheen, 1994; Krapp *et al.*, 1993) and it has been established that a reduction in leaf senescence can lead to an increased seed yield in tobacco (Gan and Amasino, 1995). The increased seed development observed in *abi3* could therefore be a secondary effect of altered leaf development. However, it has also been reported that removal of developing siliques from *Brassica* leads to delayed leaf senescence (Noodén *et al.*, 1990), which suggests that altered silique development may alter leaf senescence directly. As ABI3 is expressed specifically in seeds under normal conditions (Parcy *et al.*, 1994; cf. Rohde *et al.*, 1999; 2000) the latter effect is more likely in the system under investigation here. The perturbation of the general sucrose metabolism in *abi3-1* seeds (Ooms *et al.*, 1993) may affect the unloading of sucrose from the phloem, which may, in turn, provide the basis of a long-distance sucrose signal giving rise to the phenotype described.

In summary, that *abi3-1* shows reduced sensitivity to abscisic acid in seeds does not mean that the phenotype of whole plant resource allocation is due to a mechanism involving PGS function. Another candidate PGS as a regulator of resource allocation is cytokinin. However, the role of this PGS in whole plant resource allocation is not likely. Niether have PGSs been found necessary in models that describe plant growth. However, seed specific insensitivity to abscisic acid is the root cause of the phenotype described, even though it does not give mechanistic explanation of it. One mechanistic explanation is that floral initiation continues in the mutant because of some difference in competition for resources in wild-type and mutant seeds. An

alternative mechanism is the production of a factor by developing seeds that acts as a long-distance signal between tissues and causes the phenotype. Sucrose is a likely candidate signal in light of altered general sucrose metabolism in *abi3-1* seed relative to wild-type, and that is is the major molecule of carbon transport in phloem. Although the mechanism of sucrose as a molecule of transport and long-distance signalling is well evidenced in the literature, and is apparently favourable on the basis of the data presented in Chapter 3 and this chapter, the resource competition model cannot be discounted. Both models will be tested in Chapter 5.

# Chapter 5

The effect of the *abi3-1* mutation on the growth of *Arabidopsis* plants grown under conditions of reduced light and availability of soil nitrogen

## 5.0 The effect of the *abi3-1* mutation on the growth of *Arabidopsis* plants grown under conditions of reduced light and availability of soil nitrogen.

The aim of the work described in this chapter was to investigate the effects of the *abi3-1* mutation on whole plant resource allocation in plants grown under reduced light and/or availability of soil nitrogen in order to test the resource competition hypothesis put forward in Chapter 4.

## 5.1 Introduction

This chapter presents an investigation into the resource competition hypothesis in explanation of phenotype of *abi3-1* plants described in chapters 3 & 4. Vegetative growth has been modelled with respect to sink competition and maintenance of the allometric ratios, starting with the ratio of shoot: root (s:r) (Thornley, 1972a & b; Reynolds and Thornley, 1982). The property of a sink which determines the rate at which assimilate is imported into that sink is termed "sink strength" (Farrar, 1993a; Farrar, 1996). There is consensus that sinks compete with one another, but the manner of competition and the means by which it may be characterised remain the subject of great debate, focused in particular on concepts of "sink strength" (Farrar, 1993a, and following articles). Many observers have noted an apparent hierarchy of sinks in a growing plant (Brouwer, 1983; Ho, 1988; Wardlaw, 1990 for reviews), summarised by the order of priority: Seeds > fleshy fruit parts = shoot apices and leaves > cambium > roots > storage (Minchin and Thorpe, 1996).

Growth of fruits and seeds dominates vegetative growth, with the coda that flowers are less successful than established fruits in competition for assimilates when availability of assimilates is short (Ho, 1984; Ho, 1988; Wardlaw, 1990; Minchin and Thorpe, 1996).

The hypothesis that resource allocation is due to competition between sinks for resources can be tested by growing plants under limiting conditions and determining differences in allocation due to reduced availability of resources. Wild-type and *abi3-1* plants were grown under conditions of reduced carbon and nitrogen availability.

The fundamental nutrients required for plant growth are carbon and nitrogen. Carbon is fixed by photosynthesis and partitioned within photosynthetic tissues into either a transient starch pool, or, ultimately sucrose for export to growing tissues (see Chapter 1, Introduction, Section 1.2). Nitrogen is taken up by the roots, predominately in the form of nitrate from the soil, and is fixed either in the roots, or transported to leaf tissue, where it is fixed into organic amino acids. Resultant amino acids may thereafter be metabolised, or incorporated into Rubisco, which may function as a storage protein under non-limiting conditions of nitrate availability (Chapin *et al.*, 1990).

Within this system of interaction between carbon and nitrogen metabolism in plants, previous experiments have intended to perturb source-sink interactions to examine any competition for

resources within plants. Data from *Pisum* in which pods have been removed from the plant has been interpreted as evidence for long-distance signals that coordinate reproductive development and growth (Lockhart and Gottschall, 1961). In the G2 variety of pea, pod removal increases the initiation of new flowers (Reid, 1980). This behaviour occurs only under long-day conditions, even though the timing of flowering is unaffected by photoperiod (Reid, 1980), and this has been correlated with differences in the rate of fruit growth (Gianfanga and Davies, 1981). Initiation of new flowers may therefore be limited by demand for nutrients in already developing seeds; i.e. there is competition for allocation of resources in the plant. Surgical intervention as a mode of experimental method is not physiologically ideal, and the phenotype described in Chapters 3 and 4 of this thesis allow an opportunity to examine resource competition as a model of regulation of allocation of resources without the disadvantages of physical intervention.

In order to test the hypothesis that the phenotype of *abi3-1* plants is due to competition for resources in *Arabidopsis*, plants were grown under standard lighting conditions (250 $\mu$mol $m^{-2}$ $s^{-1}$ ) or shaded with Muslin cloth (90 $\mu$mol $m^{-2}$ $s^{-1}$), and watered to field capacity with either full strength Johnson's Medium, or with Johnson's Medium in which available nitrate had been reduced to either $^{1}/_{10}^{th}$ or $^{1}/_{5}^{th}$ of the standard solution. Control plants were therefore raised in identical conditions to those in Chapter 3. Plants were harvested at an early to mid-stage of seed development, or at a late stage of seed development; i.e. before and during expression of *ABI3* in the wild-type in accord with the results in Chapter 3. The aim of this experimental design was

firstly to determine any differences in growth responses and resource allocation in plants grown under reduced resource availability, and secondly, to dissect competition for carbon from competition for nitrogen within the plant. Thirdly, it was intended to discern any effects had by the *abi3-1* mutation on competition for resources with the plant, and, in case of effects, to further hypothesise on the mechanism and phenotype of the *abi3-1* mutation.

The phenotype of *abi3-1* plants described in Chapters 3 and 4 suggest that the sink strength of *abi3-1* seeds is less than that of wild-type seeds as the rate floral induction is slower in *abi3-1* plants compared with wild-type. However, the increased weight and number of siliques in the mutant suggest a greater sink strength in terms of the whole plant lifecycle. This is a result of prolonged floral initiation, resulting from the mutation, and, as has been demonstrated in both the short-terms and the long-term, leads to concomitant delay in senescence of cauline leaves. In this context it is suggested that the sink strength of the developing mutant seeds is weaker compared with the wild-type, and that in the context of the whole plant lifecycle one would expect a reduction in the mutant phenotype, relative to the wild-type, when resources (i.e. carbon and nitrate) are limiting.

The essential reasoning behind this approach was to use the established differences in resource allocation between wild-type and mutant plants (from Chapters 3 and 4) to examine the effects of reduction of the availability of the fundamental nutrients carbon and nitrogen. The effect of

shading was intended to reduce carbon fixation by reduction of photosynthesis. Nitrogen availability was reduced by reduction of available nitrate in the otherwise standard nutrient medium.

## 5.2 Results

Control conditions of full strength Johnson's Medium and saturating light (250 $\mu$mol $m^{-2}$ $s^{-1}$) are directly comparable to the conditions of the detailed growth analysis presented in Chapter 3. These conditions are shown in charts 'a' in Figures 5.1 – 5.6. Comparisons between charts 'a' and 'b' in these figures concern the effects of reduced availability of soil nitrogen, comparisons between charts 'a' and 'c' concern the effects of shading, and comparisons between figures 'a' and 'd' concern the combined effects of reduced availability of soil nitrogen and shading. In all treatments, early vegetative development was observed to be very similar in the two genotypes and no difference in either rate or frequency of germination was detected (data not shown).

Figure 5.1 a-d shows tissue dry weights in wild-type and *abi3-1* plants at c. 35 d after germination and the effects of reduced light concentration and 90% reduction of nitrate

**Figure 5.1** Tissue dry weights in wild-type and *abi3-1* plants at 35 d after germination grown under altered concentrations of light and nitrogen

Plants were grown at either 250 or 90 $\mu$mol $m^{-2}$ $s^{-1}$, and at either full, or $^{1}/_{10}^{th}$ strength nitrate. Wild-type (open bars) and *abi3-1*(stippled bars) were harvested at 35 d after germination, and dissected into roots, rosettes, racemes, cauline leaves, and siliques. Dry weights were determined after incubation at 70° C until constant weights were achieved. Values are the mean ± SE ($n$ = 8).

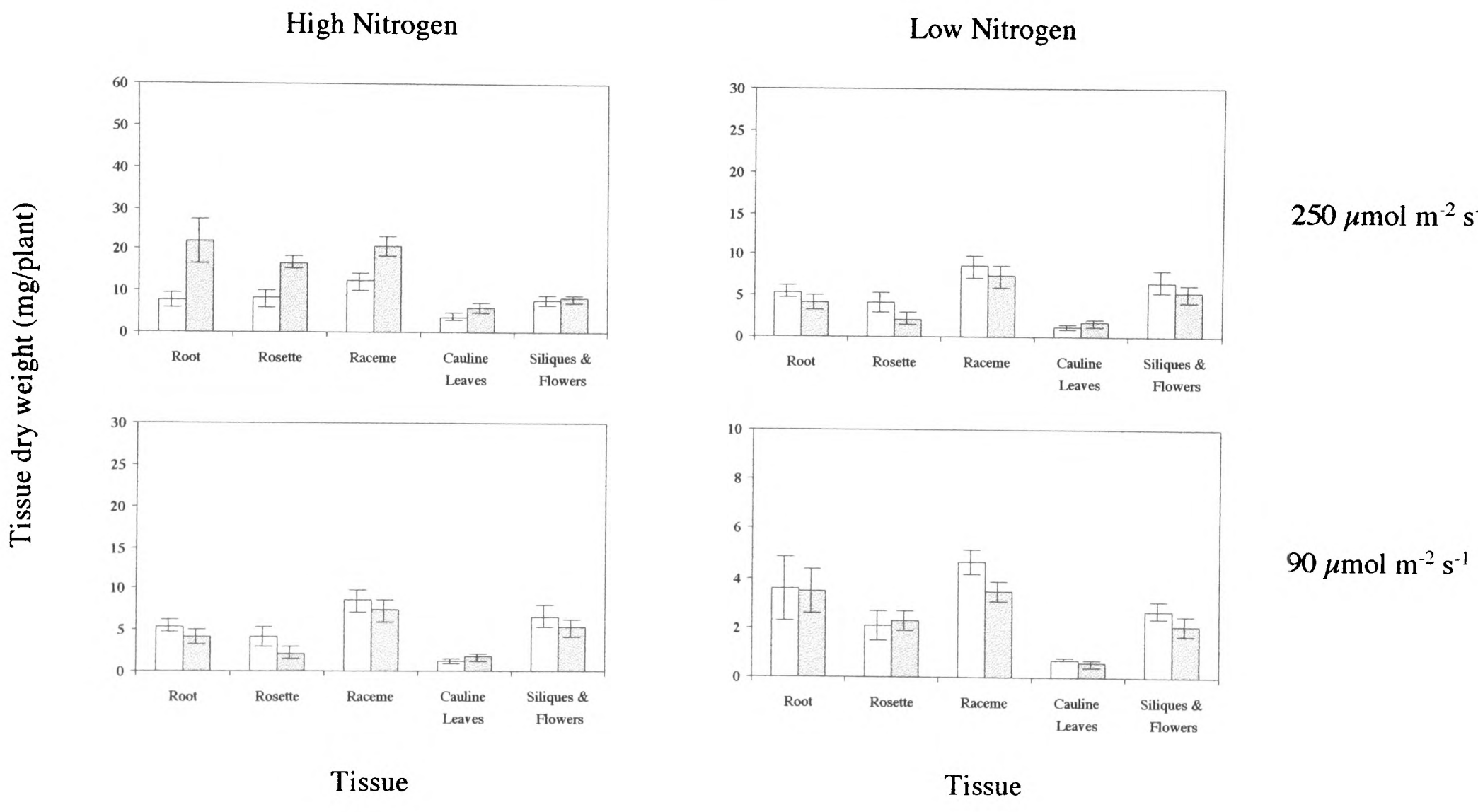


**Figure 5.2** Rosette and cauline leaf areas in wild-type and *abi3-1* plants grown under altered concentrations of light and nitrogen

Plants were grown at either 250 (a, b) or 90 (c, d) $\mu$mol $m^{-2}$ $s^{-1}$, and at either full (a, c), or $^{1}/_{10}^{th}$ (b, d) strength nitrate. Wild-type (open bars) and *abi3-1*(stippled bars) were harvested at 35 d after germination, and photocopied. Opaque leaf shapes were cut out and weighed and areas were calculated by relation to weight of a known area of paper. Values are the mean ± SE ($n$ = 8).

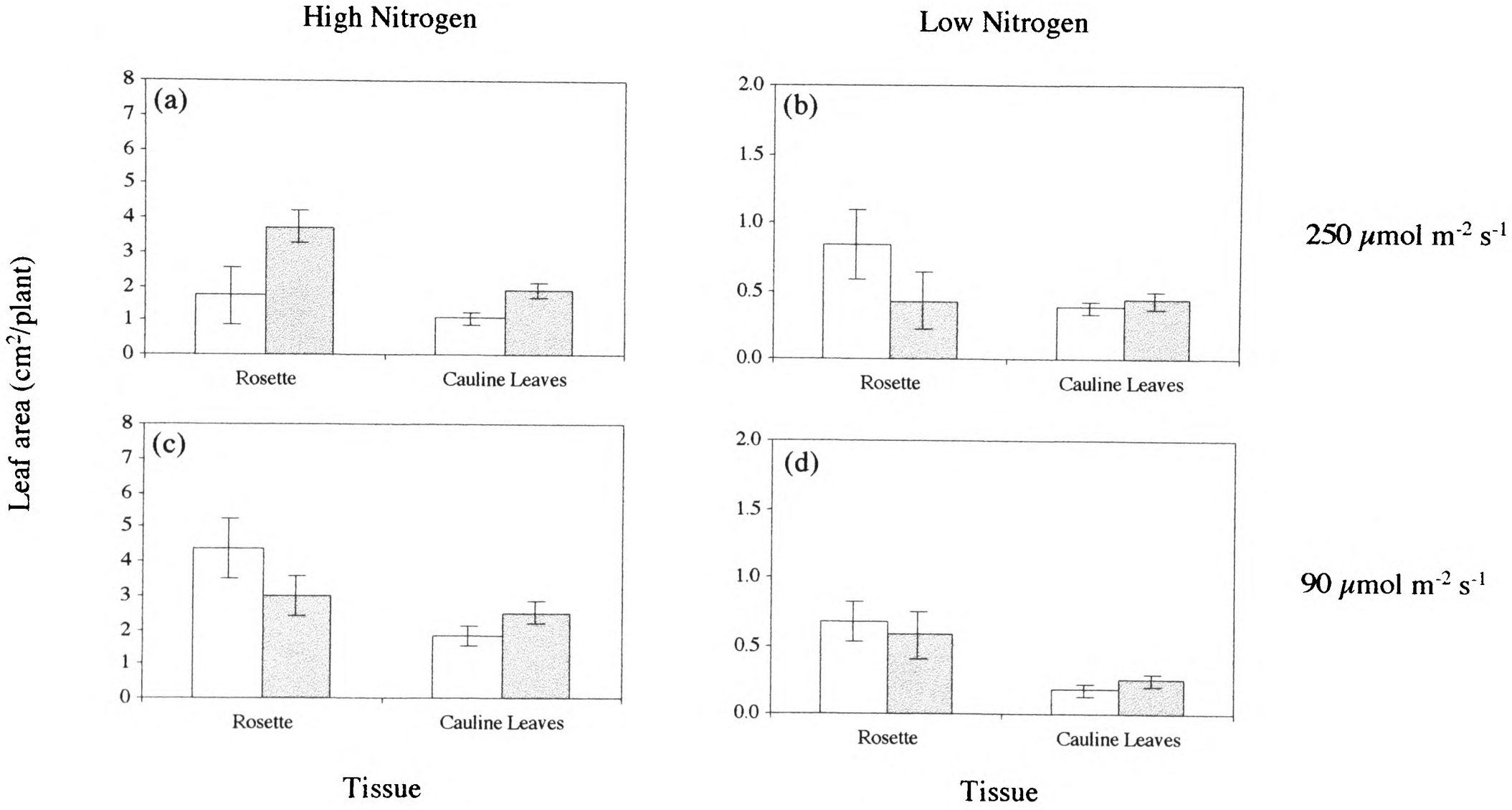


**Figure 5.3** Tissue dry weights in wild-type and *abi3-1* plants at 40 d after germination grown under altered concentrations of light and nitrogen

Plants were grown at either 250 (a, b) or 90 (c, d) $\mu$mol $m^{-2}$ $s^{-1}$, and at either full (a, c), or $^{1}/_{10}^{th}$ (b, d) strength nitrate. Wild-type (open bars) and *abi3-1*(stippled bars) were harvested at 40 d after germination, and dissected into roots, rosettes, racemes, cauline leaves, and siliques. Dry weights were determined after incubation at 70° C until constant weights were achieved. Values are the mean ± SE ($n$ = 8).

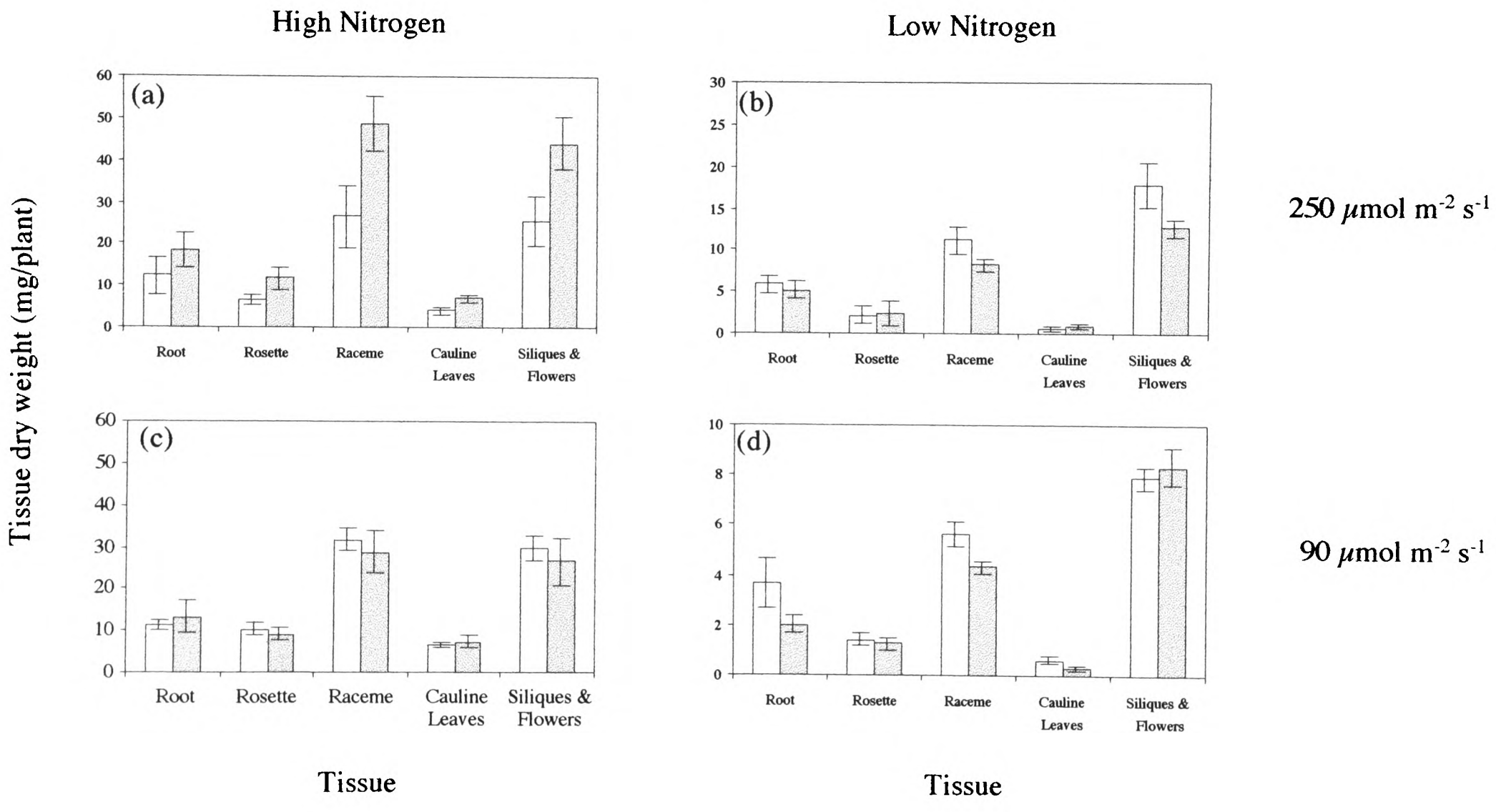


**Figure 5.4** Rosette and cauline leaf areas in wild-type and *abi3-1* plants grown under altered concentrations of light and nitrogen

Plants were grown at either 250 (a, b) or 90 (c, d) $\mu$mol $m^{-2}$ $s^{-1}$, and at either full (a, c), or $^{1}/_{10}^{th}$ (b, d) strength nitrate. Wild-type (open bars) and *abi3-1*(stippled bars) were harvested at 40 d after germination, and photocopied. Opaque leaf shapes were cut out and weighed and areas were calculated by relation to weight of a known area of paper. Values are the mean ± SE ($n$ = 8).

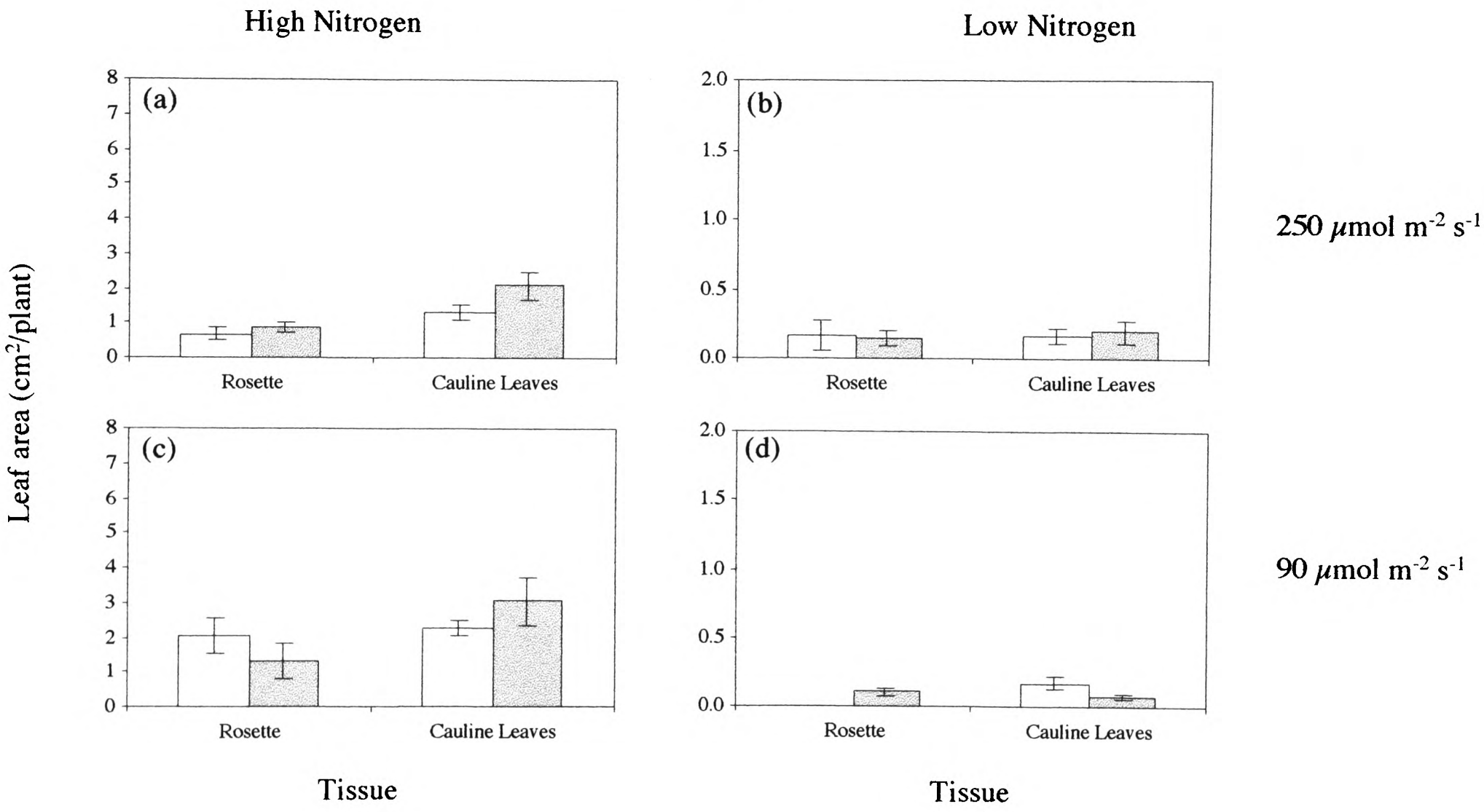


**Figure 5.5** Tissue dry weights in wild-type and *abi3-1*plants, 40 d after germination grown under altered concentrations of light and nitrogen

Plants were grown at either 250 (a, b) or 90 (c, d) $\mu$mol $m^{-2}$ $s^{-1}$, and at either full (a, c), or $^1/_5{}^{th}$ (b, d) strength nitrate. Wild-type (open bars) and *abi3-1*(stippled bars) were harvested 40 d after germination, and dissected into roots, rosettes, racemes, cauline leaves, and siliques. Dry weights were determined after incubation at 70° C until constant weights were achieved. Values are the mean ± SE ($n = 8$).

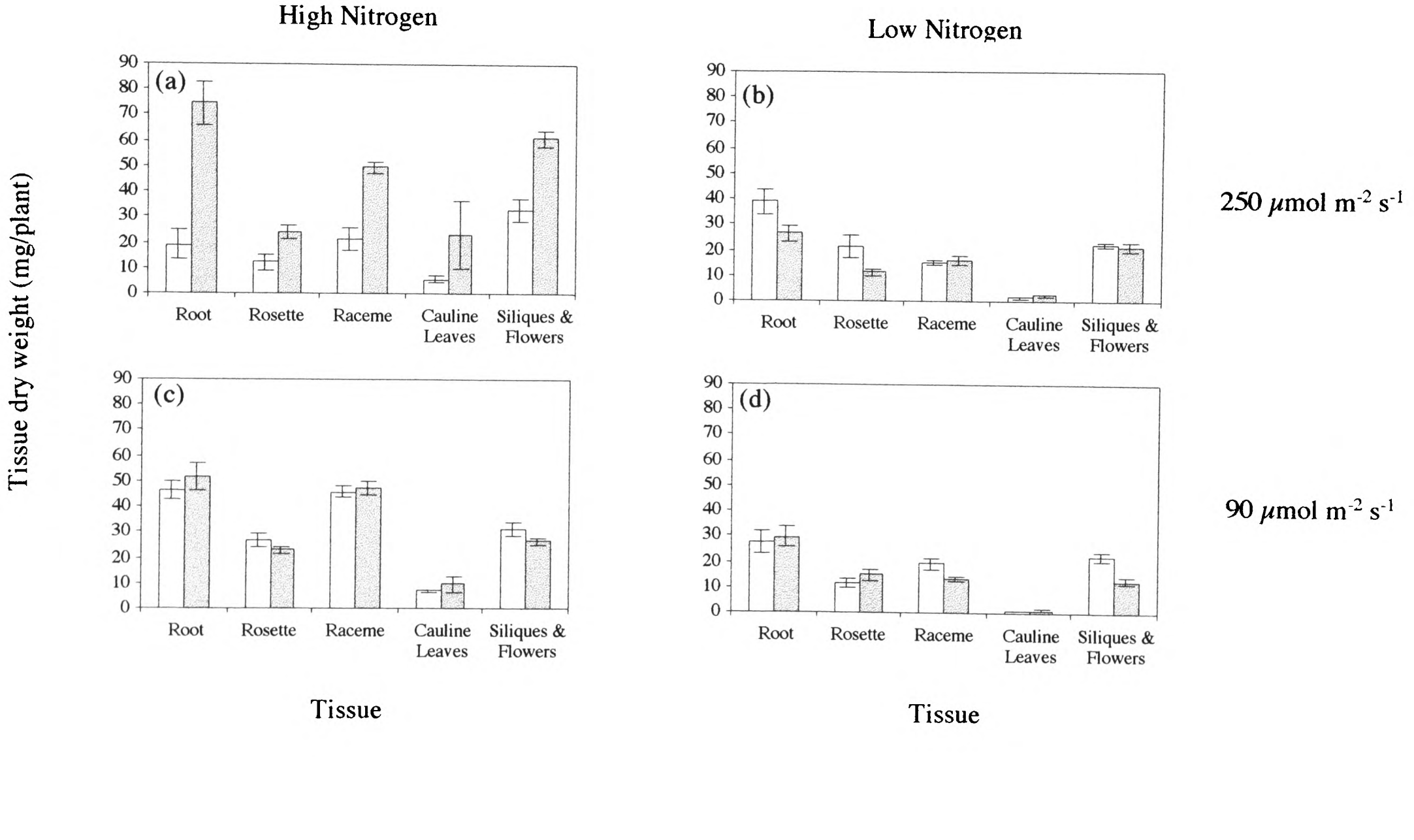


**Figure 5.6** Rosette and cauline leaf areas in wild-type and *abi3-1* plants grown under altered concentrations of light and nitrogen

Plants were grown at either 250 (a, b) or 90 (c, d) $\mu$mol $m^{-2}$ $s^{-1}$, and at either full (a, c), or $^1/_5{}^{th}$ (b, d) strength nitrate. Wild-type (open bars) and *abi3-1*(stippled bars) were harvested at c. 40 d after germination, and photocopied. Opaque leaf shapes were cut out and weighed and areas were calculated by relation to weight of a known area of paper. Values are the mean ± SE ($n = 8$).

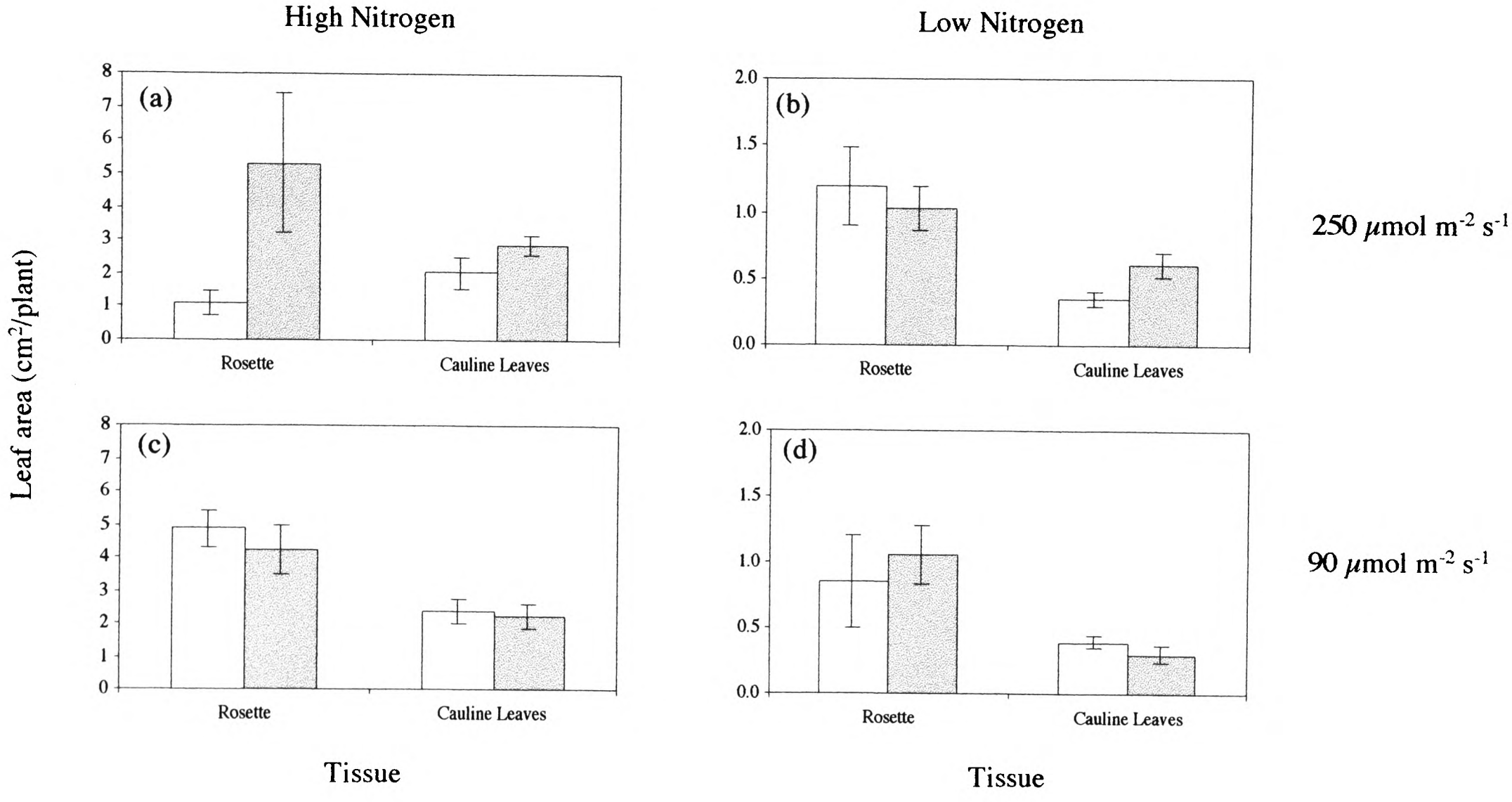
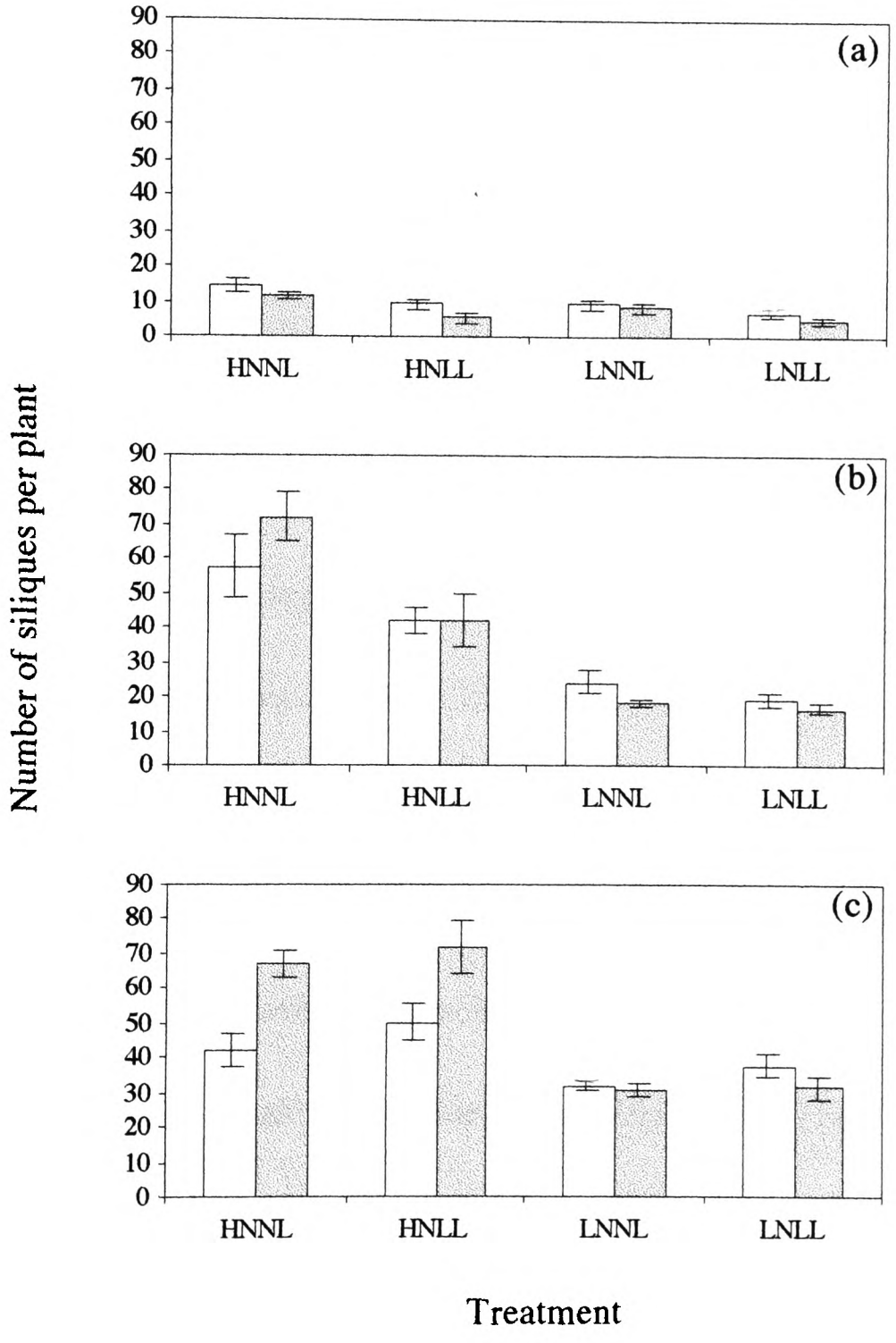


**Figure 5.7** Number of siliques in wild-type and *abi3-1* plants grown under altered concentrations of light and nitrogen

Plants were grown at either 250 or 90 $\mu E.m^{-2}.s^{-1}$, and at either full, $^1/_{10}{}^{th}$, or $^1/_5{}^{th}$ strength nitrate. Wild-type (open bars) and *abi3-1*(stippled bars) were harvested at 35 or 40 d after germination, and number of siliques were counted. HNNL = high nitrogen, normal light; HNLL = high nitrogen, low light; LNNL = low nitrogen, normal light; LNLL = low nitrogen, low light. Values are the mean ± SE ($n$ = 8).




$^1/_{10}{}^{th}$ strength nitrate, 35 d after germination

$^1/_{10}{}^{th}$ strength nitrate, 40 d after germination

$^1/_5{}^{th}$ strength nitrate, c. 40 d after germination

availability. There is significantly greater growth in the roots, rosette leaves and racemes of *abi3-1* plants compared with wild-type plants. Reference to Figure 3.2 (Chapter 3) shows that this determination of growth is in accord with the earlier growth analysis. The lack of significant differences in dry weights of cauline leaves, and siliques and flowers in each genotype demonstrates that the manifestation of the gross phenotype in *abi3-1* has yet to appear at 35 d after germination. The tissue dry weights of both genotypes under control conditions usefully illustrate resource allocation at a stage of development when wild-type cauline leaves are beginning to senesce relative to *abi3-1* cauline leaves. This is demonstrative of the very earliest manifestation of the previously characterised mutant phenotype. Figure 5.1 can therefore be used as a control in the development of the *abi3-1* phenotype at the earlier stages of seed development.

Figure 5.1a & b show the effect of reduced availability of nitrate at this control stage of development. Phenotypic differences between genotypes under control conditions are abolished under 90% reduction of nitrate availability. In relative terms, there is significant increase in the dry weight of wild-type rosette leaves under 90% reduction in available nitrate. This is reflected in increased dry weight of wild-type roots. In the mutant there is no significant difference in rosette dry weight at the same nitrate availability, relative to control conditions, but there is significant decrease in dry weight of all other tissues. The relatively high standard errors of means of root dry weight may be associated with persistent adhesion of the sand rooting medium

after harvesting. In general, wild-type tissue dry weights marginally increase whereas *abi3-1* tissue dry weights are reduced.

Figure 5.1a & c show the effect of shading at this control stage of development. The dry weights of each tissue and therefore of the whole plant, are reduced in both genotypes to approximately half those of controls as a result of shading, and there are no significant differences in tissue dry weights between genotypes. Root and leaf tissue dry weights are reduced in proportion to dry weights of definitively reproductive tissue (i.e. racemes, and siliques and flowers) in both wild-type and *abi3-1* plants, compared to those treated with reduced nitrate.

Figures 5.1 a & d show the effect of both shading and reduction of nitrate availability. There are no significant differences between genotypes under this treatment, and tissue dry weights, and therefore whole plant dry weights, are reduced to approximately one fifth of control plants grown in control conditions. Unlike shade treatment alone, there is no apparent stimulation of reproductive growth over vegetative growth. The combined limitations of reduced light and nitrate concentrations have a sum effect of reducing overall plant size to a large extent, cancel any phenotypic differences between genotypes.

Figure 5.2a-d shows leaf areas of plants from Figure 5.1. Under control conditions (Figure 5.1a), both rosette and cauline leaf areas are greater in *abi3-1* plants compared with wild-type. In *abi3-1* there is greater significant difference in control cauline leaf area than in control cauline leaf

weight, indicating relatively greater cauline leaf expansion has occurred by this stage in the mutant. This is not apparent in the wild-type. Differences in rosette leaf area and rosette leaf weights in and between both genotypes are not significantly different, indicating maturity of these leaves in both genotypes.

Figure 5.2b, nitrate limitation effectively reduces leaf areas in both genotypes by over a factor of two. There are no significant differences between genotypes in either leaf type. As tissue dry weights are comparable between genotypes at this treatment and under control conditions, the effect of lowered availability of nitrate is to reduce leaf expansion. In all treatments in this experiment, plants were watered to field capacity with appropriate nutrient medium as per treatment, and so this difference in relative leaf size (i.e. $cm^2.g^{-1}$) is not due to any difference in water availability.

By contrast to the effect of reduced availability of nitrate on leaf areas, Figure 5.2c shows an increase in leaf area in wild-type plants grown in reduced light concentration, and no effect of reduced light in *abi3-1* plants. In conjunction with the lower dry weights of these tissues, the effect of shading is increased expansion of leaf tissues in both genotypes. In parallel, these observations demonstrate that this effect is heightened in wild-type plants compared with mutants. The situation is therefore the obverse of that when nitrate is limiting. The two-fold

reduction in whole plant dry weight in both genotypes indicates that net carbon assimilation is reduced in shaded conditions.

Figure 5.2d shows no significant differences between leaf areas in either genotype under treatment with limiting light and nitrate. The approximate five-fold reduction of tissue, and hence whole plant, dry weights is mirrored by broadly comparable reduction in both rosette and cauline leaf areas. Closer comparison of Figure 5.2d to Figures 5.2b & c indicates greater relative leaf expansion under limiting light and nitrate than under single limitation of either resource. Combination of light and nitrate limitation combines to greatly reduce tissue weights and leaf areas, but the effect of shading predominates in causing relative leaf expansion.

Figure 5.3a-d shows the effects of reduced concentrations of light and nitrate on tissue dry weights of wild-type and *abi3-1* plants at c. 40 d after germination. Reference to Figure 3.2 shows this determination of growth is in accord with the earlier analysis of growth. There is little difference in dry weights of roots in either genotype and is also in accord with data shown in Figure 3.2. Dry weight of racemes are significantly greater in *abi3-1* compared with wild-type plants, and again these data accord with those in Figure 3.2. There is significant difference between wild-type and *abi3-1* silique and flower dry weights, which is expected as plants move from vegetative to reproductive growth, and is in accord with the previously characterised phenotype. This confirms expression of the previously characterised difference in allocation

between wild-type and *abi3-1* plants to siliques. Although these data are significantly greater in this experiment compared with the original growth analysis presented in Chapter 3, the pattern of tissue dry weights is retained allowing comparability with the control presented in Figure 5.3a and continuity of controls between Chapters 3 and 5.

Dry weight of rosette leaves in Figure 5.3a are consistent with data shown in Figure 5.1a with evidence of a marginal delay in senescence of rosette leaves in the mutant compared with the wild-type. However, although there is a significant difference between the cauline leaf dry weight between the genotypes, the greater cauline leaf dry weight in the mutant is not in the same order of significance suggested in the number of cauline leaves in Figure 3.4. Analysis of rosette and cauline leaf areas in both genotypes is presented in Figure 5.4a-d. Total leaf areas for each genotype derived from figure 5.4a when compared to total leaf areas from the earlier growth analysis shown in Figure 3.4a confirm the phenotype. Figure 5.4a shows no significant difference between genotypes in rosette leaf areas, but significant difference in cauline leaf areas in concurrence with the previously described phenotype. That the previous growth analysis accorded a greater difference between the genotypes in determination of cauline leaf number can therefore be seen as significant and indicative of increased inception of cauline leaf meristems in the mutant compared to the wild-type, and that this can be appended to the phenotype described in Chapters 3 and 4. To conclude, the control presented in Figure 5.4a is wholly satisfactory for use in the experiment outlined in the introduction to this chapter.

Figure 5.3b shows that under limiting conditions of nitrogen overall plant size is reduced by approximately half, and that differences between genotypes are lost or reversed. Figures 5.3b and 5.4b show massively reduced dry weights and areas of both rosette and cauline leaves and there are no differences between wild-type and *abi3-1* plants in either trait.

Figures 5.3a & c shows that under limiting concentration of light, the mutant phenotype is abolished. Root dry weights are similar between genotypes and between this treatment and the control. Differences between rosette and cauline leaf areas exist between treatment and control plants indicating delayed senescence of wild-type leaves relative to Figure 5.4a and in accord with Figure 3.4. Although leaf dry weights do not differ from controls, Figure 5.5c shows significantly greater leaf areas in shade treated plants, with the marginal exception of *abi3-1* rosette leaf area, indicating more expanded leaves. This again indicates leaf expansion to compensate for lower light concentration. Shade grown plants of both genotypes have greatly compensated the earlier low growth rate at c. 35 d after germination to achieve parity with control plants in all but leaf area.

Figure 5.3d shows the effects of reduced concentrations of light and nitrate on reproductively growing plants of both genotypes. There is little difference in tissue dry weights between genotypes, and the approximate ratios of tissue dry weights are preserved within plants. Limiting

concentration of light has reduced overall plant size by at least a factor of three. Limiting nitrate availability has enormously reduced both rosette and cauline leaf growth in terms of dry weight, and area (Figure 5.4d). There is a trend to lack or reversal of this difference in wild-type leaf dry weights.

Figures 5.5a-d and 5.6a-d repeat the experiment shown in Figures 5.2 and 5.3 except nitrate limitation is reduced to 80%. Increased problems with adhesion of sand from the growing medium to harvested roots caused root dry weight data to be significantly erroneous and incompatible in terms of credible root: shoot ratios and so will be ignored. The basic pattern of the control shown in Figures 5.5a is equivalent to that shown in Figure 5.3a, and the patterns in treatments is also similar to those shown in Figure 5.3.

Figures 5.5a & c and 5.6a & c shows dry weights of tissues and leaf areas of wild-type and *abi3-1* plants grown under control and shade conditions. Ignoring erroneous data for the roots, Figures 5.5a & c show the same pattern and order of tissue dry weights as in Figures 5.3a & c. Similarly, Figures 5.6a & c show the same pattern of distribution of leaf areas as Figures 5.4a & c. The very large standard error of the mean of the rosette leaf area in *abi3-1* plants in the control is erroneous and traceable to an outlying datum (= 18.74, recalculated mean = 3.4 ±0.48).

Figures 5.5c & d show the effects of 80% nitrate limitation as opposed to 90% nitrate limitation, relative to controls. Under 80% nitrate limitation wild-type tissue cauline leaf and silique dry weights are only slightly limited relative to controls. By contrast, the mutant phenotype is again abolished. In terms of dry matter accumulation in the whole plant, this pattern is the same as in Figure 5.3b. Differences in dry weights occur in the leaf tissues. Wild-type rosette leaf dry weight is increased by almost two-fold under 80% nitrate limitation, and reduced by the same factor in *abi3-1* plants. Rosette leaf areas for the two genotypes, shown in Figure 5.6b, taking the *abi3-1* rosette leaf control area to be 3.4 ±0.5 $cm^2$ per plant, shows no significant increase in wild-type rosette leaf area, and an approximately 3-fold decrease in *abi3-1* rosette leaf area relative to controls. Rosette leaf area in both genotypes in increased 3-fold by the 100% increase in available nitrate.

Cauline leaf areas shown in Figure 5.6b show that the *abi3-1* cauline leaf phenotype is maintained under 80% nitrate limitation. Both wild-type and *abi3-1* cauline leaves are smaller in area than in controls. This confirms the effect of 90% nitrate limitation, but the phenotype of delayed cauline leaf senescence in the mutant is apparent, suggesting that this effect is independent of the yield phenotype.

Dry weights of cauline leaves are greater in both genotypes grown at 80% nitrate limitation relative to plants grown under 90% limitation of nitrate, and are not significantly different from

one another. Cauline leaf areas for both genotypes are approximately 2-fold greater under 80% limitation of nitrate, but still 3-fold less than plants grown in control conditions.

Under conditions of 80% limiting nitrate and shade, the *abi3-1* phenotype is lost. Plants of both genotypes are marginally smaller than when unshaded, but have 2-3-fold greater dry weight than plants grown under 90% nitrate limitation and shade. The pattern of distribution of tissue dry weights is otherwise unchanged from unshaded plants and controls. There are no differences in rosette or cauline leaf areas between genotypes, though both are approximately one third less than non-shaded plants.

Figure 5.7a shows no significant differences between genotypes in number of siliques per plant at 35 d after germination. There is barely significant reduction in silique initiation under conditions of low light and 90% reduction in available nitrate. Figure 5.7 b shows greater silique initiation in mutant plants under control conditions compared with wild-type, and though this is not a significant difference it is in accord with the previously described phenotype. Shading reduces overall silique initiation and abolishes any differences between genotypes. Nitrate limitation reduces silique initiation still further and reverses the phenotype, indicating wild-type siliques compete more effectively for amino acids compared to *abi3-1* plants. This effect is abolished when plants are limited by shading as well as reduced availability of nitrate.

Allowing for the unavoidable alterations to experimental conditions accounted above, there is no significant difference between controls between Figures 5.7b and 5.7c. The control phenotype is more pronounced in the latter and is so confirmed. In this experiment, no effect is seen by shade treatment. This is contradictory to the results show in Figure 5.7b. Reference to Figures 5.3c and 5.5c suggest that this result may be anomalous as there is no difference in silique dry weights in shade grown plants in either experiment. In literal interpretation however, the maintenance of the control phenotype in shade treated plants shown in Figure 5.7c shows reduced silique weight in these plants if dry weight of this tissue is not increased (Figure 5.5c).

Figures 5.7b & c show no differences between wild-type and *abi3-1* plants in initiation of siliques. Reduction of nitrate limitation to 80% marginally relieves limitation of initiation compared with 90% nitrate limitation. This effect is maintained in shade grown plants treated with 80% limitation of nitrate availability, and shade effects are again shown to not limit silique initiation when nitrate is limiting (Figures 5.7c cf. 5.7b).

Leaf area ratios (F) and number of raceme branches decreased and increased respectively from mid- to later stages of seed development, but no differences were found between genotypes under all treatments (data not shown).

## 5.3 Discussion

The aim of the work described in this chapter was to investigate the effects of limitation of carbon and nitrate assimilation on resource allocation in plants during vegetative and reproductive growth. Wild-type and *abi3-1* plants were grown under limiting conditions of light and nitrate availability to test the hypothesis that resource allocation is regulated by competition for resources in growing sink tissues. Plants were grown under regimes of normal or shaded light conditions and of 90% and 80% reduction in available nitrate. Plants were harvested before and during expression of the ABI3 gene product (i.e. within and after 12 d after anthesis, Parcy *et al.*, 1994) and growth analyses performed to determine any differences in resource allocation in and between both genotypes.

Differences in resource allocation and competition for carbon and nitrogen between wild-type and *abi3-1* mutant plants were investigated in conditions of reduced resource availability.

Under all treatments there is no significant differences between genotypes and treatments in leaf area ratio and number of branches in racemes. This shows reduction of available concentrations of light and nitrate therefore has no effect on these traits of plant architecture.

At 35 d after germination, shading appears to promote reproductive over vegetative growth. Transition to reproductive growth is a possible plant response to pervasive environmental stress, effectively enabling movement by means of seed dispersal. Shading to 90 $\mu$mol $m^{-2}$ $s^{-1}$ light availability may therefore be stressful. The reduction in root and leaf tissue dry weights in proportion to dry weights of definitively reproductive tissue (i.e. racemes, and siliques and flowers) in both wild-type and *abi3-1* plants, compared to those treated with reduced nitrate, indicate shading as being more stressful than nitrate limitation to plants, with resulting stimulation of reproduction. The combined limitations of reduced light and nitrate concentrations have a sum effect of reducing overall plant size to a large extent, and cancelling any phenotypic differences between genotypes.

Control rosette and cauline leaf areas at 35 d after germination concur with the earlier control in Chapter 3. Ten-fold reduction in available nitrate causes significant reduction in leaf areas relative to controls. The size of these plants compared to controls indicates little difference in total carbon accretion, and so it can be inferred that net rates of photosynthetic carbon fixation are uncompromised by reduced availability of nitrate.

Traditional mechanical models of photosynthesis have ascribed Rubisco as being the 'rate-limiting step' in the acquisition of $CO_2$ according to prevalent conditions of saturating light, and limiting low concentrations of $CO_2$ (Farquhar and von Caemmerer, 1982; Sharkey,

1989; Sage, 1990). Rubisco has also been shown to act as storage protein when nitrate is non-limiting (Chapin *et al.*, 1990). It is therefore suggested that 90% reduction in nitrate availability may have a direct effect on Rubisco concentrations in leaves. Nitrate limitation causes increased efficiency of Rubisco (Quick *et al.*, 1992; Stitt and Schulze, 1994). The reduction in leaf area is therefore likely to pertain to reduced concentration of Rubisco in these leaves, but increased efficiency of that enzyme in its role in photosynthetic carbon fixation.

In contrast, the wild-type response to shading is opposite to that of reduced availability of nitrate. Increased leaf size indicates wild-type plants respond to shade by increasing leaf area, and so light interception. As nitrate is not limiting, it is assumed that the concentration of Rubisco is not affected by shading. The reduction in carbon fixation, inferred from reduced plant dry weights, is indication that the efficiency of photosynthesis does not increase under this treatment to compensate for this treatment. That *abi3-1* plants do not respond to the same degree as wild-type plants may indicate an early manifestation of the *abi3-1* phenotype at this stage when there are otherwise indistinguishable differences between the two genotypes. Although this time point reflects an early stage of reproductive growth in both genotypes, ABI3 is beginning to be expressed at this time (> 12 d) after anthesis (Parcy *et al.*, 1994) in the wild-type. Regulation of seed development in the wild-type, by ABI3, affects plant response to shade.

At 40 d after germination the lack of difference between genotypes in root dry weights, relative to at 35 d after germination, is expected as plants move from vegetative to reproductive growth and root: shoot decreases (Brouwer, 1983). This is also in accord with the data shown in Figure 3.2. Dry weights of racemes and siliques are similarly in accord with Figure 3.2 and the previously characterised phenotype.

Under 90 % nitrate limitation the trend towards greater dry weight of racemes and siliques and flowers in wild-type is possibly due to a combination of the significant reduction in leaf size under nitrate limitation, with increased photosynthesis occurring in racemes and siliques (and flowers), (cf. Figure 4.4a). This may reflect a greater efficiency of nitrate use in the wild-type compared *abi3-1* plants. This latter point may be regarded as evidence of more effective competition for resources by reproductive sinks in the wild-type, or reduced competitive ability of mutant reproductive sinks for resources.

At 40 d after germination the data shows that plants have increased specific leaf area ($cm^2 . g^{-1}$) to compensate for the lower light concentration. Shade grown plants have greatly compensated for the early low growth rate up to 35 d after germination, and achieve parity with control plants in all but leaf area.

The earlier initiation and favouring of reproductive growth as a stress response may have considerably helped in the whole plant's adaptive reproductive response to limiting light concentration. This is at the expense of increased silique yield in the mutant, which indicates reduced ability of reproductive sinks to compete for resources in *abi3-1* plants.

The combination of shading and 90 % reduction in nitrate availability caused at least a 3-fold reduction in overall plants size in both genotypes. Leaf dry weights and areas are greatly reduced. The abolishment of the phenotypic difference between *abi3-1* and wild-type plants indicates a reduced ability in the reproductive sinks of *abi3-1* to compete for resources compared with the wild-type.

Wild-type plant dry weights are only slightly limited by 80 %, as opposed to 90 % nitrate limitation. The *abi3-1* dry weights show the mutant phenotype is abolished under 80 % nitrate limitation, as at 90 % nitrate limitation. Leaf areas are more greatly affected by the slight increase in available nitrate. Both genotypes demonstrate a 3 fold increase in leaf area in response to the reduction in nitrate limitation. Wild-type rosette leaf area is not affected by 80 % reduction of nitrate availability relative to controls. However *abi3-1* rosette leaves are approximately 3 fold smaller in area than controls. And so with no difference between genotypes in rosette leaf dry weight and no change in wild-type rosette leaf area from control, wild-type plants are not affected by 80% reduction in nitrate availability in vegetative leaf growth, but *abi3-1* plants remain so.

The effect of 90 % nitrate limitation on cauline leaf areas is confirmed in both genotypes when grown at 80 % nitrate limitation. However, the maintenance of the *abi3-1* cauline leaf delayed senescence phenotype is seen to be independent of the silique yield phenotype, which is not changed from the more severe 90 % nitrate limitation.

This effect is apparently inconsistent with expression of ABI3 specific to seeds (Parcy *et al.*, 1994). However, analysis of the number of siliques demonstrates maintenance of the silique phenotype under 80 % nitrate limitation in this trait, even though silique dry weights are no different form the more severe 90% nitrate limitation. With unchanged initiation of silique but reduced silique dry weights, it appears that the effect of the slight reduction in nitrate limitation is that developing seeds fill less than wild-type. This indicates that *abi3-1* siliques cannot compete for resources within the plant as well as wild-type siliques can within wild-type plants when nitrate is restricted by 80 % of non-limiting availability. Competition for carbon resources may be reduced as a result of nitrate limitation, or may result in competition for amino acids.

Combination of shade treatment with 80 % reduction in available nitrate again causes abolition of the mutant phenotype. Dry weights of tissues of both genotypes are 2-3 fold greater than when nitrate availability was reduced by 90 %, and are only slightly lower than controls. This marked difference indicates that nitrate is the major limiting nutrient causing abolition of the *abi3-1*

phenotype. This is supported by the lack of differences in rosette and cauline leaf areas between genotypes, though these are reduced to two-thirds those of control plants.

In summary, when carbon assimilation is limited by shading the silique initiation phenotype occurs, but seeds do not fill as much as under control conditions. Competition for carbon resources in *abi3-1* plants is therefore reduced in shade conditions compared to wild-type. Both genotypes are severely limited by 90 % reduction in available nitrate. Reducing this limitation to 80 % reduction in available nitrate relieves wild-type plants to a greater extent than *abi3-1* plants indicating the latter as being more sensitive to nitrate limitation. In conclusion, the mutant phenotype described in Chapters 3 and 4 depends on there being a non-limiting supply of nutrients. This concurs with the hypothesis that *abi3-1* seeds compete less well than wild-type seeds for resources.

Differences between seeds of *abi3-1* and wild-type seeds in competition for resources having been identified in the above experiments, the remaining hypothesis, from Chapter 4, has not been discounted. Differences in resource allocation between wild-type and mutant plants may be due to differences in signalling between sources and sinks in each genotype, the likely candidate signal being sucrose. Further evidence of differences in competition between wild-type and *abi3-1* seeds is sought in the short term, and evidence of sucrose as signalling, as well as transport molecule, is sought in Chapter 6.

# Chapter 6

## Differences in short-term resource allocation between wild-type and *abi3-1* plants at different concentrations of $CO_2$

## 6.0 Differences in short-term resource allocation between wild-type and *abi3-1* plants at different concentrations of $CO_2$

The aim of the work described in this chapter was to further investigate the consequences of altered resource supply on allocation in wild-type and *abi3-1* plants by varying short-term $CO_2$ concentrations.

## 6.1 Introduction

This chapter presents an investigation of the fate of $^{14}C$ supplied at different concentrations of $CO_2$ to previously unadapted, photosynthesising wild-type and *abi3-1* plants, to further test the hypothesis, arising from Chapter 4, that continued floral initiation in the mutant is due to differences in competition for resources between wild-type and mutant seeds. The investigation presented in this chapter focuses on carbon assimilation and allocation, specifically plant responses to exposure to 60% and 230% ambient $CO_2$ concentration.

The results presented in Chapter 5 show that wild-type *Arabidopsis* plants respond to long-term shade conditions by increasing relative leaf area in comparison to controls. In contrast, *abi3-1* plants do not show significant response to shade treatment. Leaf dry-weights of shade grown

plants of both genotypes are significantly less than controls indicating the response to shade is specifically leaf expansion. This experiment also showed that rosette leaf senescence is delayed by shade treatment, that this response becomes less marked in rosette leaves in later seed development, that there is no significant difference between control and shaded cauline leaves during later seed development, and that the mutant cauline leaf phenotype is abolished (see Chapter 5, Results).

These responses are the result of growing plants under shaded conditions in the long–term and, as with Chapter 3, although mechanisms underlying these responses can be proposed from these data, evidence of these mechanisms is desirable from experiment on plant responses in the short-term, as with Chapter 4.

The short term pattern of carbon assimilation and allocation was investigated using the same apparatus as in Chapter 4. As a control, $^{14}CO_2$ was supplied to plants in the sealed chamber for 1 h (pulse) followed by continued photosynthesis for 3 h in the presence of $^{12}CO_2$ (chase). Plants were harvested at the end of the pulse (1 h time point) and then 3 h into the chase (4 h time point) and the amount of $^{14}C$ recovered in water-soluble, chloroform-soluble (lipids and pigments) and insoluble components of siliques and flowers, cauline leaves, racemes, and rosette leaves was

determined. Net source tissue is expected to assimilate a large proportion of the label during the pulse and to export label during the chase. A sink tissue is expected to incorporate label throughout the experiment. The total incorporation of label into water-soluble, chloroform-soluble and insoluble components gives a measure of the total rate of assimilation. The experiment was carried out on plants at around 35 d after germination, when the phenotype, described in Chapters 3 and 5, is beginning to be expressed.

Treatments were as follows: In the first instance the long-term responses to shade treatment were investigated in the short term by determination of the fate of $^{14}C$ supplied at 60% of ambient concentration of $CO_2$ to wild-type and *abi3-1* plants. Secondly, the fate of $^{14}C$ supplied at 230% of ambient concentration of $CO_2$ to wild-type and *abi3-1* plants was determined.

## 6.2 Results

Figure 6.1 shows mean tissue fresh weights of wild-type and *abi3-1* plants harvested for pulse-chase feeding experiments under different concentrations of $CO_2$. There is no significant difference in fresh weights of racemes or siliques and flowers, in accord with development at 35 d after germination (see Chapter 3). The difference in fresh weight of rosette leaves shown is also in accord with observations made in Chapter 3 (see Figure 3.1b). The fresh weight of mutant

**Figure 6.1** Pooled tissue fresh weights of wild-type and *abi3-1* plants from pulse-chase feeding experiment under different concentrations of $CO_2$.

Fresh weights were determined for each tissue of wild-type (open bars) and *abi3-1* (stippled bars) harvested at both 1 h pulse and 3 h chase of $\Delta[^{14}CO_2]$ pulse-chase feeding experiment. Pooled values are means ± SE ($n$ = 18).

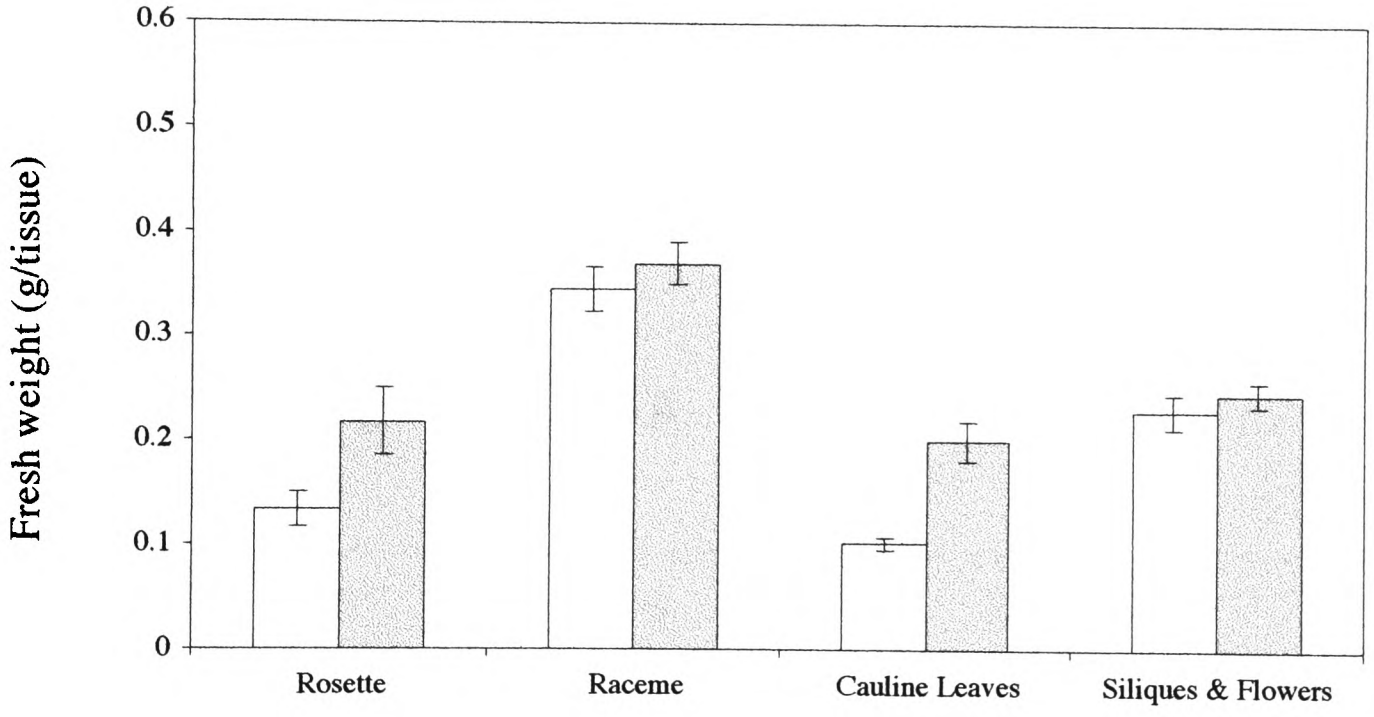


**Figure 6.2** Incorporation of $^{14}C$ from $^{14}CO_2$ by wild-type and *abi3-1* plants at different concentrations of $CO_2$. Intact plants (c. 35 d old) were exposed to $^{14}CO_2$ and allowed to photosynthesise for a 1 h pulse. A subset of plants were harvested and the remainder incubated for a further 3 h (4 h time point). At each point the total $^{14}C$ incorporated per plant was determined. Values are mean ± SE ($n = 3$). (a) wild-type, 200 ppm $CO_2$, (b) *abi3-1*, 200 ppm $CO_2$, (c) wild-type, 400 ppm $CO_2$, (d) *abi3-1*, 400 ppm $CO_2$, (e) wild-type, 800 ppm $CO_2$, (f) *abi3-1*, 800 ppm $CO_2$.

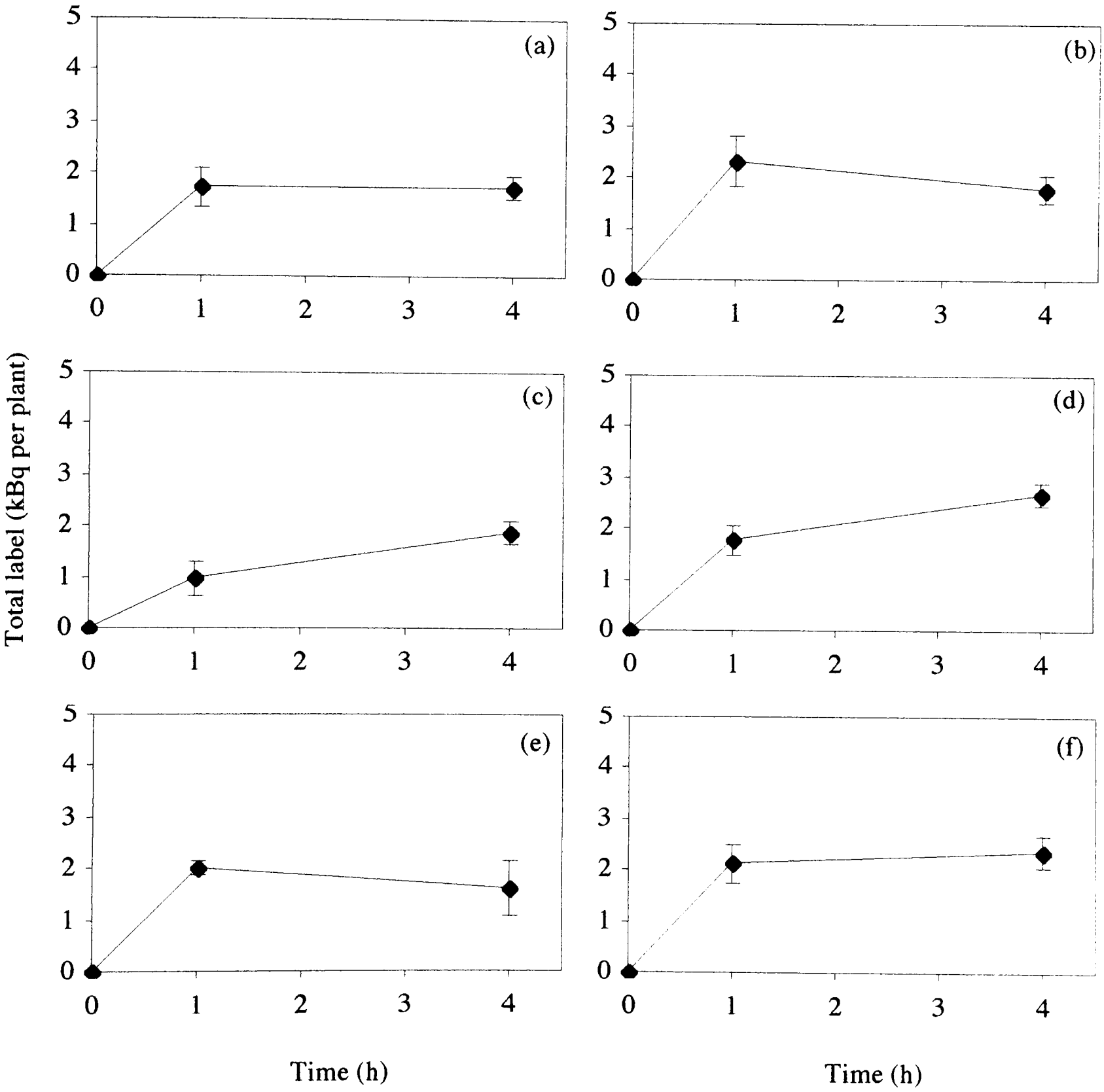


**Figure 6.3** Metabolism of $^{14}CO_2$ by wild-type and *abi3-1* plants, c. 35 d after germination under ambient concentration of $^{12}CO_2$.

Intact plants were exposed to $^{14}CO_2$ and allowed to photosynthesis for a 1 h pulse in ambient concentration of $^{12}CO_2$. A subset of plants were harvested and the remainder incubated for a further 3 h chase in ambient concentration of $^{12}CO_2$ (4 h time point). At each point the plants were dissected into siliques, cauline leaves, racemes, and rosette leaves. Each tissue was extracted in methanol : chloroform : $(NH_4)_2CO_3$. Extracts were separated into three fractions: chloroform soluble (solid bars); water soluble (stippled bars); and insoluble (open bars). The $^{14}C$ content of each fraction was determined and summed to give the total $^{14}C$ per tissue. Data is expressed as a percentage of total $^{14}C$ incorporated (see Figure 4.2) and values are the mean ± SE ($n$ = 3). The error bars refer to the error on the total $^{14}C$ per tissue, not the individual fractions. (a) = wild-type siliques, (b) = *abi3-1* siliques, (c) = wild-type cauline leaves, (d) = *abi3-1* cauline leaves, (e) = wild-type racemes, (f) = *abi3-1* racemes, (g) = wild-type rosette leaves, (h) = *abi3-1* rosette leaves.

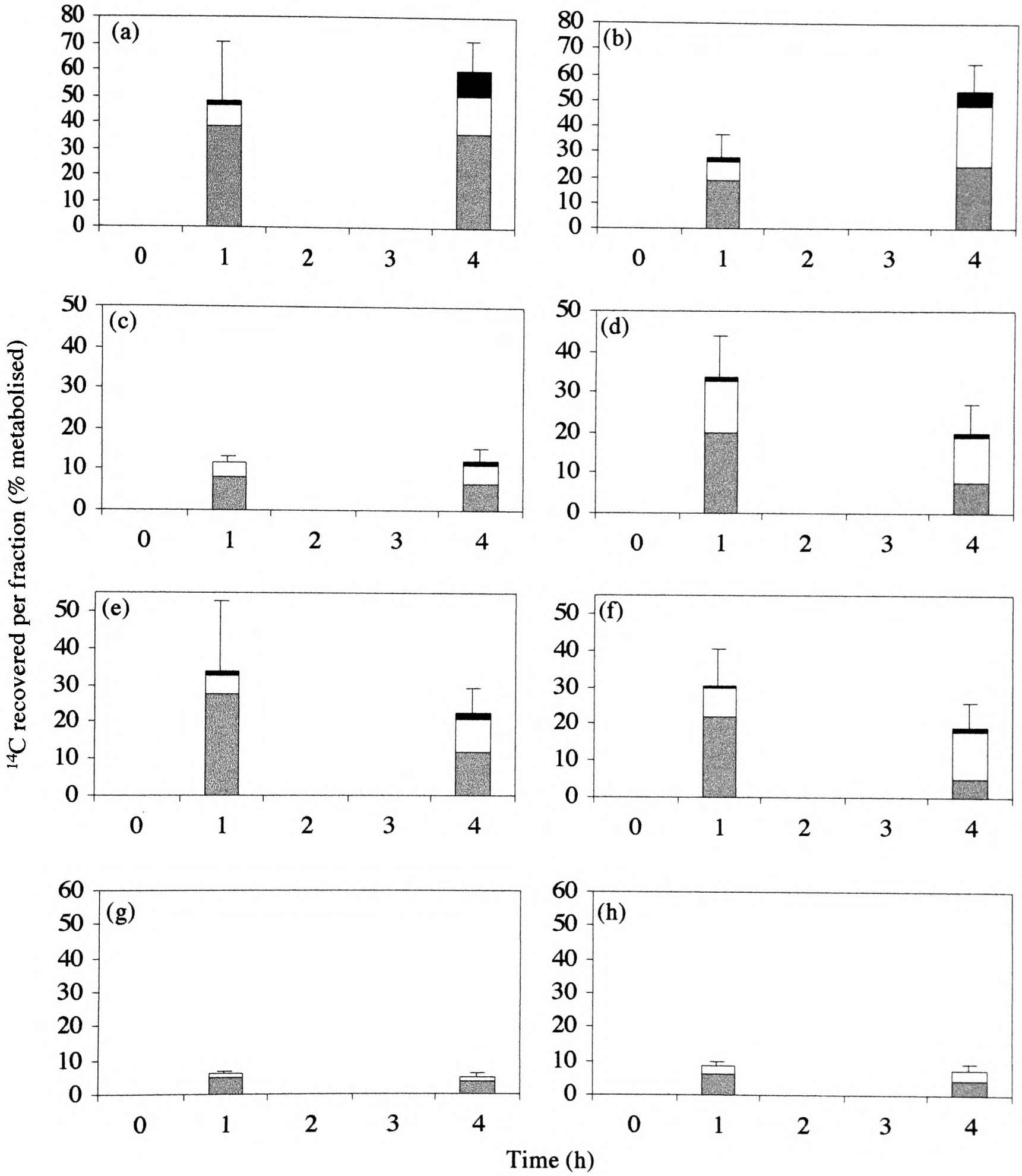

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
$^{14}$C recovered per fraction (% metabolised)
Time (h)

**Figure 6.4** Metabolism of $^{14}CO_2$ by wild-type and *abi3-1* plants, c. 35 d after germination under 200 ppm concentration of $^{12}CO_2$.

Intact plants were exposed to $^{14}CO_2$ and allowed to photosynthesis for a 1 h pulse in a 200 ppm concentration of $^{12}CO_2$. A subset of plants were harvested and the remainder incubated for a further 3 h chase in 200 ppm concentration of $^{12}CO_2$ (4 h time point). At each point the plants were dissected into siliques, cauline leaves, racemes, and rosette leaves. Each tissue was extracted in methanol : chloroform : $(NH_4)_2CO_3$. Extracts were separated into three fractions: chloroform soluble (solid bars); water soluble (stippled bars); and insoluble (open bars). The $^{14}C$ content of each fraction was determined and summed to give the total $^{14}C$ per tissue. Data is expressed as a percentage of total $^{14}C$ incorporated (see Figure 4.2) and values are the mean ± SE ($n = 3$). The error bars refer to the error on the total $^{14}C$ per tissue, not the individual fractions. (a) = wild-type siliques, (b) = *abi3-1* siliques, (c) = wild-type cauline leaves, (d) = *abi3-1* cauline leaves, (e) = wild-type racemes, (f) = *abi3-1* racemes, (g) = wild-type rosette leaves, (h) = *abi3-1* rosette leaves.

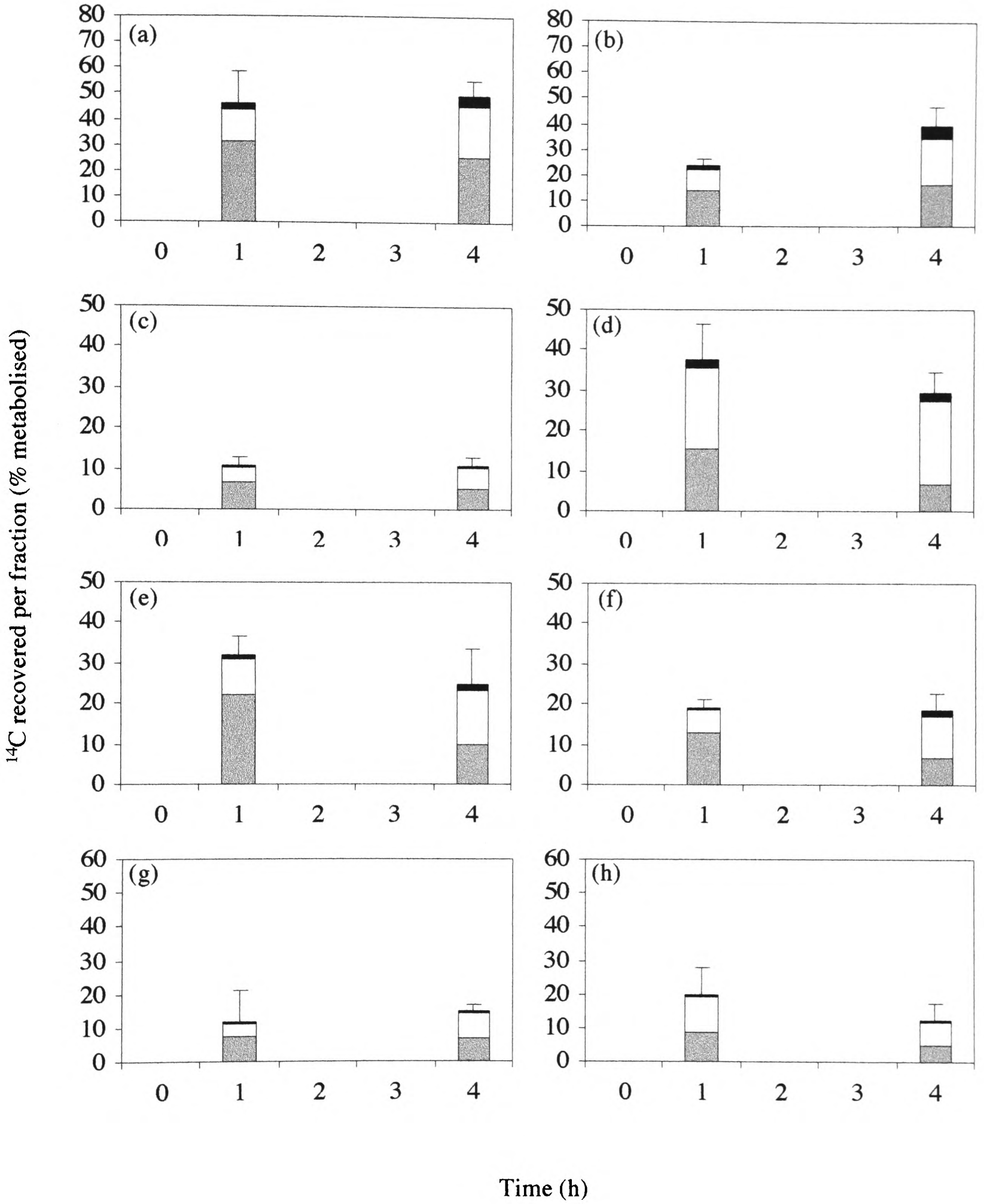
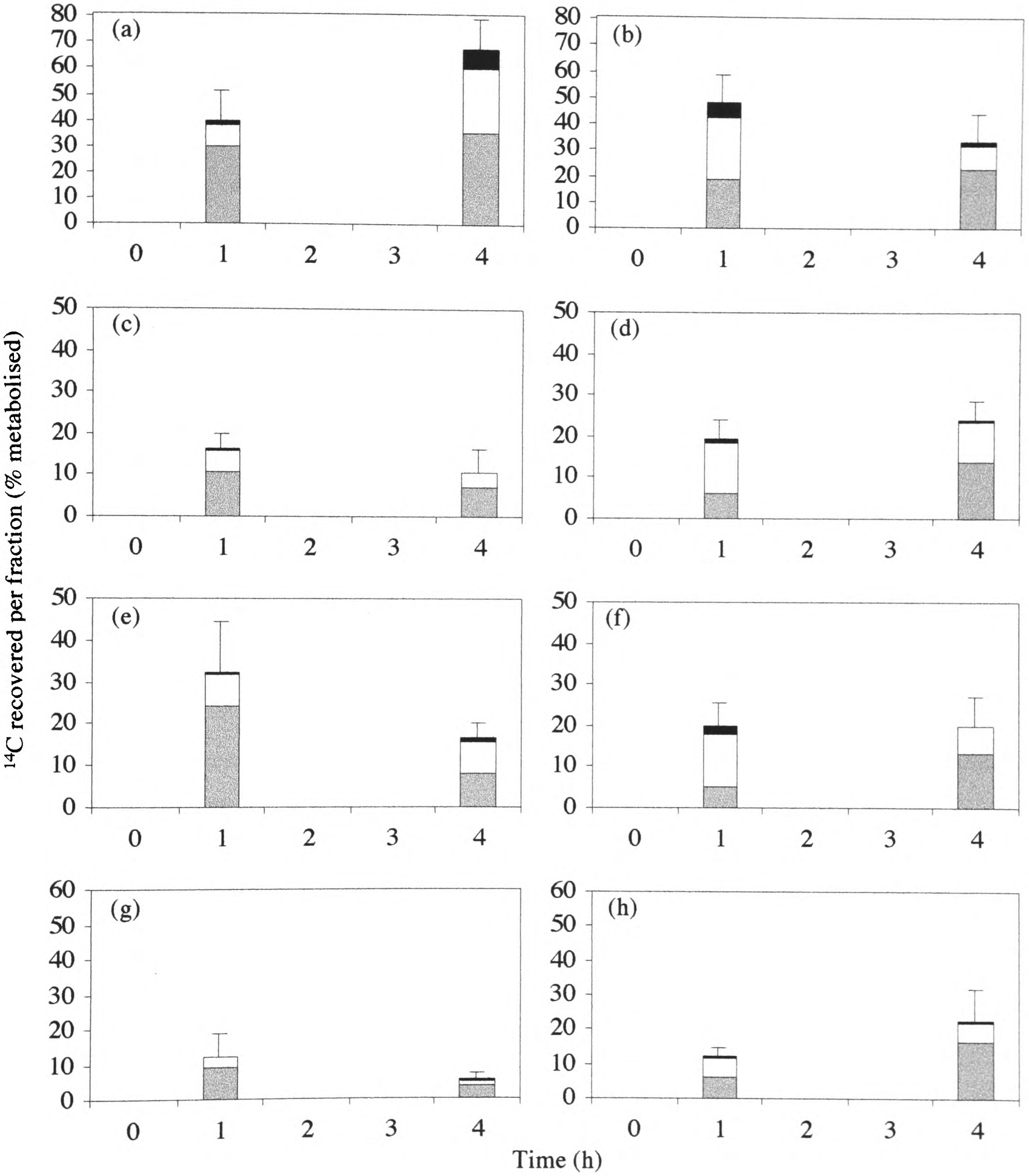

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
$^{14}$C recovered per fraction (% metabolised)
Time (h)

**Figure 6.5** Metabolism of $^{14}CO_2$ by wild-type and *abi3-1* plants, c. 35 d after germination, under 800 ppm concentration of $^{12}CO_2$.

Intact plants were exposed to $^{14}CO_2$ and allowed to photosynthesis for a 1 h pulse in a 800 ppm concentration of $^{12}CO_2$. A subset of plants were harvested and the remainder incubated for a further 3 h chase in 800 ppm concentration of $^{12}CO_2$ (4 h time point). At each point the plants were dissected into siliques, cauline leaves, racemes, and rosette leaves. Each tissue was extracted in methanol : chloroform : $(NH_4)_2CO_3$. Extracts were separated into three fractions: chloroform soluble (solid bars); water soluble (stippled bars); and insoluble (open bars). The $^{14}C$ content of each fraction was determined and summed to give the total $^{14}C$ per tissue. Data is expressed as a percentage of total $^{14}C$ incorporated (see Figure 4.2) and values are the mean ± SE ($n = 3$). The error bars refer to the error on the total $^{14}C$ per tissue, not the individual fractions. (a) = wild-type siliques, (b) = *abi3-1* siliques, (c) = wild-type cauline leaves, (d) = *abi3-1* cauline leaves, (e) = wild-type racemes, (f) = *abi3-1* racemes, (g) = wild-type rosette leaves, (h) = *abi3-1* rosette leaves.


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
0
1
2
3
4
Time (h)
$^{14}$C recovered per fraction (% metabolised)

cauline leaves is significantly greater than wild-type, indicating early manifestation of the mutant phenotype and in agreement with the pattern of dry weights shown in Chapter 5, Figure 5.1a. The plants used in the pulse-chase feeding experiment presented in this chapter can therefore be considered as being at the same stage of reproductive growth as those in Chapter 5, Figure 5.1a. The total incorporation of $^{14}C$ into water-soluble, chloroform-soluble and, insoluble components is shown in Figure 6.2 and gives a measure of the total rate of assimilation. At 35 d after germination there is significant fixation of $^{14}C$ by both wild-type and *abi3-1* plants (Figure 6.2a-f).

The amount of label incorporated into plants at ambient concentration of $CO_2$ (Figure 6.2c & d) apparently increases during the pulse in both genotypes, although the effect is more pronounced in the wild-type. As label was supplied to plants only during the pulse, this is impossible. This anomaly can be traced to low recovery of label from the cauline leaves in the wild-type, relative to equivalent recovery from tissue in Chapter 4, in two out of the three replicates. The recovery of label from the third replicate at the 1 h time point is not low compared to equivalent recovery from cauline leaf tissue in Chapter 4.

Other than the above anomaly, there is no change in the amount of label between the pulse and the chase at any stage (Figure 6.2a & b, e & f). The total rate of assimilation from the labelling is

approximately 2-3 $\mu$mol $CO_2$ $h^{-1}$ per plant. The rate of assimilation in wild-type plants at ambient $CO_2$ is less at 1-2 $\mu$mol $CO_2$ $h^{-1}$ per plant (Figure 6.2c) and this is consistent with the erroneously low recovery in two out of three wild-type cauline leaf tissue samples. In summary, Figure 6.2 shows that changing concentration of $CO_2$ does not affect the rate of photosynthesis.

The pattern of label distribution through wild-type and mutant plants at 35 d after germination under ambient concentrations of $CO_2$ is shown in Figures 6.3a-h. Recovery of label from roots is not shown as roots were previously found to accumulate less than 2% of total label in each genotype (see Chapter 4, Figure 4.3i & j). In both genotypes the rosettes show little assimilation of carbon during the pulse, and no export of label during the chase (Figure 6.3g & h). The marginally greater fresh weight of mutant rosette leaves (Figure 6.1) is reflected by marginally greater accumulation of label in this tissue compared to the wild-type rosette (Figures 6.3h cf. 6.3 g).

There are no significant changes in the proportion of label incorporated by the racemes in either genotype, and the proportion does not change through the pulse (Figures 6.3e & f). This is concurs with the pattern of incorporation in Figures 4.3e & f, and is compatible with this tissues role in transport. In both genotypes there is an increase in the proportion of water-soluble components which is consistent with an increasing proportion of label in transit through the

phloem, most likely in the form of sucrose. The increase in chloroform-soluble and insoluble components may indicate the presence of developing apices in this tissue in both genotypes (Figures 6.3e & f).

Incorporation of label into wild-type cauline leaves during the pulse is less than at 30 d after germination (Figure 6.3c cf. Figure 4.3c), indicating that the wild-type cauline leaves are senescent at 35 d after germination. Similarly, there is no evidence of export from wild-type cauline leaves at 35 d after germination. By contrast, significantly more label is incorporated into the cauline leaves of *abi3-1* plants during the 1 h pulse (Figures 4.3c & d) compared with wild-type cauline leaves, particularly in the water-soluble fraction. The proportion of label in water-soluble fraction decreases greatly during the chase, likely reflecting incorporation of label into, and export as, sucrose.

At 35 d after germination considerably more label is incorporated into wild-type siliques and flowers compared with incorporation into the same tissues at 30 d after germination (Figures 6.3a cf. 4.3a). The proportion of label in the water soluble fraction is particularly high, and does not diminish during the chase. The proportion of both the insoluble and chloroform soluble fractions increases during the chase. As no tissue shows export of label in the wild-type, the overall increase in total label in the siliques and flowers suggests that most of the assimilation of label

occurs in the siliques. This is supported by the pattern of partitioning between the water-soluble, chloroform-soluble and insoluble fractions, with the high proportion of label in the water soluble fraction being indicative of photosynthesis occurring in the silique pod wall, and assimilated label being incorporated into maturing seeds, most notably as chloroform-soluble oils in the seed.

By contrast, the increase in label in *abi3-1* siliques and flowers is reflected by the export of label from the cauline leaves (Figures 6.3b & c). The percentage of label in the siliques and flowers in the mutant at 35 d after germination is increased over that at 30 d after germination (Figure 6.3b cf. Figure 4.3b) and the pattern of partitioning of label within this tissue shows an increased proportion of the water-soluble fraction compared with at 30 d after germination (Figure 6.3b cf. Figure 4.3b). There is no decrease in this fraction during the chase, but there is marked increase in the insoluble and chloroform-soluble fractions. The increase in the latter fraction likely reflects greater partitioning into oil in developing seeds, but the greatly increased proportion of the insoluble fraction is likely a reflection of increased silique formation.

The pattern of label distribution through wild-type and mutant plants at 35 d after germination under 200 ppm concentration of $CO_2$ is shown in Figures 6.4a-h. Recovery of label from roots is again not shown. In both genotypes the rosettes show greater assimilation of carbon during the

pulse compared with plants at ambient concentration of $CO_2$, but no export of label during the chase (Figure 6.4g & h).

There are no significant changes in the proportion of label incorporated by the racemes in either genotype, and the proportion does not change through the pulse (Figures 6.4e & f). This concurs with the pattern of incorporation in Figures 6.3e &f and 4.3e & f.

The pattern of assimilation and partitioning of label shown in Figures 6.4c & d is not significantly different to that shown in Figures 6.3c & d. Similar inferences are made.

The pattern of assimilation and partitioning of label shown in Figures 6.4a & b is not significantly different to that shown in Figures 6.3a & b. Similar inferences are made.

The pattern of label distribution through wild-type and mutant plants at 35 d after germination under 800 ppm concentration of $CO_2$ is shown in Figures 6.5a-h. Recovery of label from roots is again not shown. Both genotypes incorporate more label in 800 ppm $CO_2$ than the control at ambient $CO_2$ concentration (Figures 6.5g & h) with wild-type rosette leaves incorporating a higher proportion of label into the water-soluble fraction compared with the mutant, and a relatively lower proportion into the insoluble fraction. The proportion of the label in the water-

soluble fraction decreases in the course of the chase and there is overall export from the wild-type rosette. In contrast, the water soluble fraction in *abi3-1* rosette leaves increases significantly, although the apparent net import of total label into *abi3-1* rosette leaves during the chase is not significant.

Incorporation of label into wild-type raceme tissue under 800 ppm $CO_2$ is not significantly different in amount or proportion in each fraction to the control or 200 ppm treatment (Figure 6.5e cf. Figures 6.4e and 6.3e). During the chase, however, the proportion of label in the water-soluble fraction decreases significantly and there is overall net export from the raceme tissue in the wild-type as a result of this decrease in the water-soluble fraction. In the mutant, as with wild-type raceme tissue, there is no significant difference in total incorporation of label in the pulse compared with the control or 200 ppm treatment (Figures 6.5f cf. Figures 6.4f and 6.3f) . However, the proportion of label incorporated into the insoluble tissue fraction is markedly greater than at the other $CO_2$ concentrations. There is no change in the amount of label in *abi3-1* racemes during over the course of the chase, but the proportion of label in the water-soluble fraction increases significantly in the chase, with a concomitant decrease in the proportion of label in the insoluble fraction.

The pattern of assimilation and partitioning of label in wild-type cauline leaves, shown in Figures 6.5c, is not significantly different to that shown in the control or 200 ppm $CO_2$ treatment (Figures 6.3c and 6.4c). Assimilation of label into mutant cauline leaves is, however, markedly decreased compared with the control and 200 ppm $CO_2$ treatment (Figure 6.5d, cf. Figures 6.3d and 6.4d), though the proportions of label in each fraction are comparable. There is no significant increase in the total amount of label in the cauline leaves of the mutant under 800 ppm $CO_2$ concentration, but there is a significant increase in the amount of label in the water-soluble fraction, with a concomitant decrease in the amount of label in the insoluble and chloroform-soluble fractions (Figure 6.5d).

Incorporation and partitioning of label into wild-type siliques and flowers is not significantly different compared with the control $CO_2$ concentration and 200 ppm treatment (Figure 6.5a cf. Figures 6.3a and 6.4a). There is overall net import of label into wild-type siliques in plants treated with 800 ppm $CO_2$ with significant increase in the proportion of label incorporated into both the insoluble and chloroform-soluble fractions (Figure 6.5b), and the proportion of label in the water-soluble fraction of wild-type siliques and flowers remains unchanged. In *abi3-1* siliques and flowers, significantly greater label is incorporated during the pulse than under control $CO_2$ concentration, or 200 ppm $CO_2$ treatment (Figure 6.5b cf. Figures 6.3b and 6.4b), due to relatively greater proportions of label being incorporated into the insoluble and chloroform-

soluble fractions. The total amount of label in the mutant siliques and flowers does not decrease significantly during the chase, but proportion of label in the insoluble and chloroform-soluble fractions decrease markedly, with a concomitant slight increase in the amount of label in the water-soluble fraction (Figure 6.5b).

## 6.3 Discussion

The aim of the experiments presented in this chapter was to analyse short-term responses of both wild-type and *abi3-1* plants to raised and lowered concentrations of $CO_2$ to investigate sink-competition for carbon in these conditions. This was investigated by conducting $^{14}CO_2$ pulse-chase feeding experiments on plants at 35 d after germination, that is at the stage, demonstrated in Chapter 5, when the earliest manifestation of the *abi3-1* phenotype is apparent as delayed in senescence of cauline leaves. Control was established by analysis of assimilation and allocation of $^{14}C$ radiolabel in a pulse-chase feeding experiment performed on both genotypes at ambient concentration of $CO_2$. The effect of shading plants to reduce carbon assimilation by photolimitation of photosynthesis, demonstrated in Chapter 5, was investigated by conducting a pulse-chase feeding experiment on both genotypes at 60% of ambient $CO_2$ concentration (i.e. 200 ppm [$CO_2$]). The effect of increased concentration of $CO_2$ was investigated by performing a pulse-chase feeding experiment on both wild-type and *abi3-1* plants at 230% ambient $CO_2$

concentration (i.e. 800 ppm [$CO_2$]). In all experiments, plants used were not previously acclimated to the experimental concentrations of $CO_2$.

Short-term competition by reproductive sinks in whole plants was successfully investigated in each of the pulse-chase feeding experiments outlined above.

The analysis of the short-term responses of plants at 35 d after germination under control conditions successfully establishes a control in the series of experiments presented in this chapter. The control experiment presented in Chapter 5 (see Figures 5.1a and 5.2 a) demonstrated the earliest quantifiable trait of the mutant phenotype to be delayed senescence in cauline leaves. The phenotype of increased initiation of flowers, and so yield of siliques, does not become clearly manifest until later in the life-cycle. However, previous analysis of expression of the ABI3 gene product has shown that it is expressed from 12 d after anthesis in wild-type plants (Parcy *et al.*, 1992). The phenotype of delayed senescence of cauline leaves in mutant plants can therefore be expected to be manifested 12 d after anthesis which occurs at 17 d after germination.

The earlier pulse-chase feeding experiment described in Chapter 4 demonstrated a shift in source tissues in the wild-type from rosette leaves at 30 d after germination, to silique pod-walls at 38 d after germination. In the mutant, there was a shift in source tissues from rosette leaves at 30 d after germination, to cauline leaves at 38 d after germination. This is interpreted as demonstration

that the effect of the expression of the mutant in the seed causes delayed senescence of cauline leaves which, in turn, support the prolonged floral initiation that gives rise to the increase in yield of siliques per plant in *abi3-1* plants. What is left unclear is whether there is an equivalent stage of cauline leaves acting as a predominant source tissue in the wild-type. The control experiment presented in this chapter demonstrates that is not the case (Figure 6.3 cf. Figure 4.3). However, it is demonstrated that wild-type cauline leaves are photosynthetically contributing the carbon assimilation throughout reproductive growth, but no tissue predominates as a source.

The metabolism of $^{14}CO_2$ by wild-type and *abi3-1* plants at 35 d after germination under 200 ppm $CO_2$ concentration (Figure 6.4) shows no significant differences from the control (Figure 6.3). This is in marked contrast to the effect of growing plants under shade (see Figures 5.1c and 5.2c). However, as plants used in the pulse-chase feeding experiment, shown in Figure 6.4, were not previously acclimated to lowered concentration of $CO_2$ it is demonstrated that plants of both genotypes are exhibiting resilience to any effects of reduced concentration of $CO_2$ in the short-term. The effect on dry weights, and so carbon assimilation and allocation, of plants grown on limiting availability of nitrate is negligible at 35 d after germination (Figures 5.1b cf. 5.1a), and this demonstrates that photosynthetic capacity of such plants is unimpaired. As was discussed in Chapter 5, this is likely due to modulation of the efficiency of Rubisco, as quantities of this key photosynthetic enzyme are likely reduced under nitrate-limiting conditions. That the plants used

in the pulse-chase feeding experiment at 200 ppm $CO_2$ concentration were raised without nitrate limitation is it suggested that there is spare capacity of Rubisco in these plants as they are readily able to cope with short-term reduction in availability of carbon.

The responses of each genotype to treatment with 800 ppm $CO_2$ are remarkably different. In the wild-type there is apparent export of label from the rosettes and the racemes, with the pattern of assimilation and allocation of carbon in the cauline leaves being notably unaffected by the increased $CO_2$ concentration. Export from the rosette is barely significant, but notably emanates from the water-soluble fraction suggesting export in the form of sucrose. Similarly, export from the raceme emanates from the water-soluble fraction and is also likely to be in the form of sucrose. This suggests that under high $CO_2$ concentration, the wild-type raceme becomes net photosynthetic. Export from these tissues is reflected in the net import of label displayed by the siliques and flowers. The increased proportion of insoluble and chloroform soluble fractions reflects partitioning of imported assimilates into the developing seeds, in the case of the chloroform-soluble constituents, notably into seed lipids. The maintenance of the amount of label in the water-soluble fractions at each time point in the wild-type siliques is evidence that the silique walls are also photosynthesising.

In marked contrast, the responses in each tissue of the *abi3-1* plants treated with 800 ppm $CO_2$ show transport within the plant is greatly affected. There is a notable pattern in all tissues in which the proportion of label incorporated into the insoluble fractions during the pulse diminishes during the chase, with a concomitant increase in the proportion of label incorporated into the water soluble fractions in the course of the chase. That the species into which the label is incorporated in the insoluble fractions can be mobilised strongly suggests that a great deal of photosynthate is metabolised into starch during the pulse, and this forms the basis of the high proportion of label found in the insoluble fractions.

Elevated concentrations of $CO_2$ increases starch accumulation in many species, and concomitantly decreases photorespiratory carbon metabolism (Farrar and Williams, 1991, and references therein). Tissue concentrations of hexoses and sucrose have also been reported to increase in both the short-term (less than one photoperiod) (Geiger *et al.*, 1983) and the long-term (days) (Farrar and Williams, 1991, and references therein). The results presented here are observations of metabolism at a resolution of 1 h or less, and sequentially concur with the above reports. The sum of the above observations (Geiger *et al.*, 1983) may contain such sequential metabolic events as reported here.

From initial starch accumulation during the pulse, the following shift in the partitioning of label from the insoluble fractions to the water soluble fractions suggests that a significant proportion of this starch is mobilised into sucrose during the chase. There is the suggestion in the data that export actually occurs from the siliques and flowers in the course of the chase (Figure 6.5b), and this is reflected by apparent import into the rosettes (Figure 6.5h). Further analysis of the raw data, in kBq per tissue confirms this behaviour (data not shown). This represents a complete inversion of normal source-sink relations during reproductive growth, which may result from accumulation of sucrose in all tissues cancelling, or inverting, the sucrose concentration gradient driving Münchian mass-flow in the phloem.

As the plants used in these experiments were not previously acclimated to the experimental conditions, and, in particular, were grown under non-limiting availability of nitrate, these experiments are evidence that photosynthetic responses enabled adaptation to reduced carbon availability within the 1 h pulse in both genotypes so that phenotypic differences were maintained. Neither genotype is affected by reduced concentration of $CO_2$ in the short-term. However, the disparate responses of each genotype to increased $CO_2$ concentration show that the wild-type plant is able to maintain seed development, albeit with alteration as to source tissue.

A significant difference between genotypes at 1 h in 800 ppm $CO_2$ is the starch status in source tissues. In the wild-type there is no change in the relative amount of label incorporated into the insoluble tissue fractions of source tissues. In contrast there is a significant increase in the amount of label incorporated into these tissue fractions in *abi3-1* plants, and it is mobilisation from these fractions that give rise to the relatively increased amounts of label in the water-soluble fractions during the chase. High concentrations of $CO_2$ therefore have a significant effect on the carbon partitioning within photosynthetic tissues of the mutant and, subsequently, a gross effect on carbon allocation within the plant.

Results presented in Chapter 5 demonstrate *abi3-1* plants to be more nutient sensitive than wild-types. It has been found that responses to $CO_2$ enrichment differ in two mutants differing in their sensitivity to nutrient deprivation (Zhang and Lechowicz, 1995). The more nutrient-sensitive mutant was found to be more responsive to $CO_2$ enrichment (Zhang and Lechowicz, 1995). This concurs with the observations from Chapter 5 that mutant plants are more nutrient sensitive, in particular that *abi3-1* plants respond less well to reduced nitrate limitation (from 90 % to 80 % limitation) than wild-type plants, and are concomitantly more sensitive to $CO_2$ enrichment.

In conclusion, the wild-type reproductive sink out-competes other tissues for resources and so maintains allocation to the seed. In contrast, the *abi3-1* reproductive sink strength is less than

wild-type, and so mutant sinks are less able to compete for resources with other tissues. Consequently the allocation of carbon breaks down. Sucrose status in each tissue is strongly implicated in this breakdown in functional allocation.

Further to the hypotheses put forward in Chapter 4, that resource allocation is regulated by either competition for resources within the plant, or, by the production of a factor by growing sinks that acts as a long-distance signal between tissues, most likely sucrose, it is proposed that resource allocation is regulated by competition for resources within the plant, and the mechanism underlying such competition is signalling between tissues by means of sucrose as the molecule of transport.

# Chapter 7

Investigation of the effects of the *abi3-1* mutation on sugar status in the source, transport and sink tissues of *Arabidopsis* plants

## 7.0 Investigation of the effects of the *abi3-1* mutation on sugar status in the source, transport and sink tissues of *Arabidopsis* plants

The aim of the work described in this chapter was to investigate any effects that the *abi3-1* mutation might have on sugar status within the leaves, racemes and siliques of plants to test the hypothesis that sucrose as a molecule of communication is the mechanism underlying the phenotype of *abi3-1* plants described in chapters 3 and 4.

## 7.1 Introduction

This chapter describes experimental attempts to demonstrate that sucrose, as the major molecule of carbon transport in plants, is central to the mechanism underlying the previously described phenotype resulting from the *abi3-1* mutation. The first approach was to enzymologically measure sucrose concentrations in each tissue of the whole plant to determine any significant differences between wild-type and mutant plants. The second approach was to experimentally test rate of import from the phloem into siliques.

The major form of carbon exported from photosynthesising leaves is sucrose. Sucrose is loaded into the phloem for transport to growing sinks. The best supported model of phloem function is the Mass Flow Hypothesis (Münch, 1930). Chapters 3 and 4 provide good evidence that changes

in long distance signalling in *abi3-1* plants lead to changes in the allocation of resources and growth. The altered metabolism in the mutant seed may affect the strength of the seeds as sinks. In particular, greater accumulation of sucrose in the seed (Ooms *et al.*, 1993) might be expected to affect the concentration of sucrose in the phloem at the point of unloading. Sucrose breakdown and phloem unloading have been identified as key determinants of sink growth in maize (Miller and Chourey, 1992) and bean (Weber *et al.*, 1996) and so it is hypothesised that the changes in resource allocation in *abi3-1* so far identified are direct result of altered sucrose status in the whole plant.

In order to test this hypothesis it was decided to determine sucrose concentration in sources, sinks and transport tissue in the whole plant. Plants were harvested at a mid point of seed development and at a later stage of seed development. At each point, shoots of harvested plants were dissected into rosette leaves, racemes, cauline leaves and siliques and fresh weights were determined. Each tissue was killed and extracted in boiling 80% (v/v) ethanol and ethanol soluble fractions were evaporated to dryness *in vacuo* before resuspension in de-ionised water to a known volume. Prepared samples were then enzymoligically assayed for sucrose content and these data expressed per fresh weight of each tissue.

A second approach to testing the hypothesis, that changes in resource allocation in *abi3-1* result from the effects of the mutation on seed metabolism and sucrose concentration in the phloem,

was to investigate distribution of a radiolabelled carbon species supplied to detached, silique-bearing racemes. Silique-bearing racemes were harvested from plants and incubated in [U-$^{14}$C]glucose. Glucose was chosen as the tracer species to avoid experimentally altering the sucrose concentration in the phloem. To assess whether label was transported via the phloem two treatments were used that were expected to inhibit phloem transport. Control samples were trimmed in 50 mM EGTA to prevent wound responses occurring at cut points. Treatments were to trim tissue without EGTA, and to incubate tissue in the presence of PCMBS (*para*-chloromecuribenzene sulphonic acid), an inhibitor of membrane transport proteins. The aim of the treatment without EGTA was to allow wound responses to occur in expectation that those samples treated in this manner would show reduced uptake of label from the incubating medium. The aim of incubation with PCMBS was to inhibit phloem unloading in the expectation that label would be taken up into the phloem but would not be unloaded into the sinks.

## 7.2 Results

### 7.2.1 Assay of sucrose concentration in wild-type and *abi3-1* tissues

The first approach to test the hypothesis that sucrose, as a molecule of communication, is the mechanism underlying the phenotype of *abi3-1* plants previously described, was to enzymologically measure sucrose concentrations in each tissue of the whole plant to determine any differences between wild-type and mutant plants.

Figure 7.1 shows change in tissue fresh weights in wild-type and mutant plants at c. 32 and c. 39 d after germination. Figure 7.1a shows no significant difference between wild-type and *abi3-1* tissue fresh weights 32 d after germination. Figure 7.1b shows significantly greater fresh weights of racemes, cauline leaves and siliques in *abi3-1* plants compared with wild-type plants 39 d after germination. The lack of significant differences between tissue fresh weights in Figure 7.1a is in accord with the growth analysis presented in Chapter 3, and the significant differences in Figure 7.1b are in accord with the phenotype described in the growth analysis in Chapter 3.

Figure 7.2a shows the concentration of sucrose in wild-type and *abi3-1* tissues at 32 d after germination and shows that there is no significant difference in sucrose concentration in any of the tissues. Figure 7.2b shows the concentration of sucrose in wild-type and *abi3-1* tissues at 39 d

**Figure 7.1** Tissue fresh weight change in wild-type and *abi3-1* plants between 32 and 39 d after germination

Wild-type (open bars) and *abi3-1* (stippled bars) plants were harvested at 32 (a) and 39 (b) d after germination and dissected into rosette leaves, racemes, cauline leaves and siliques. Values are the mean ± SE ($n$ = 10).

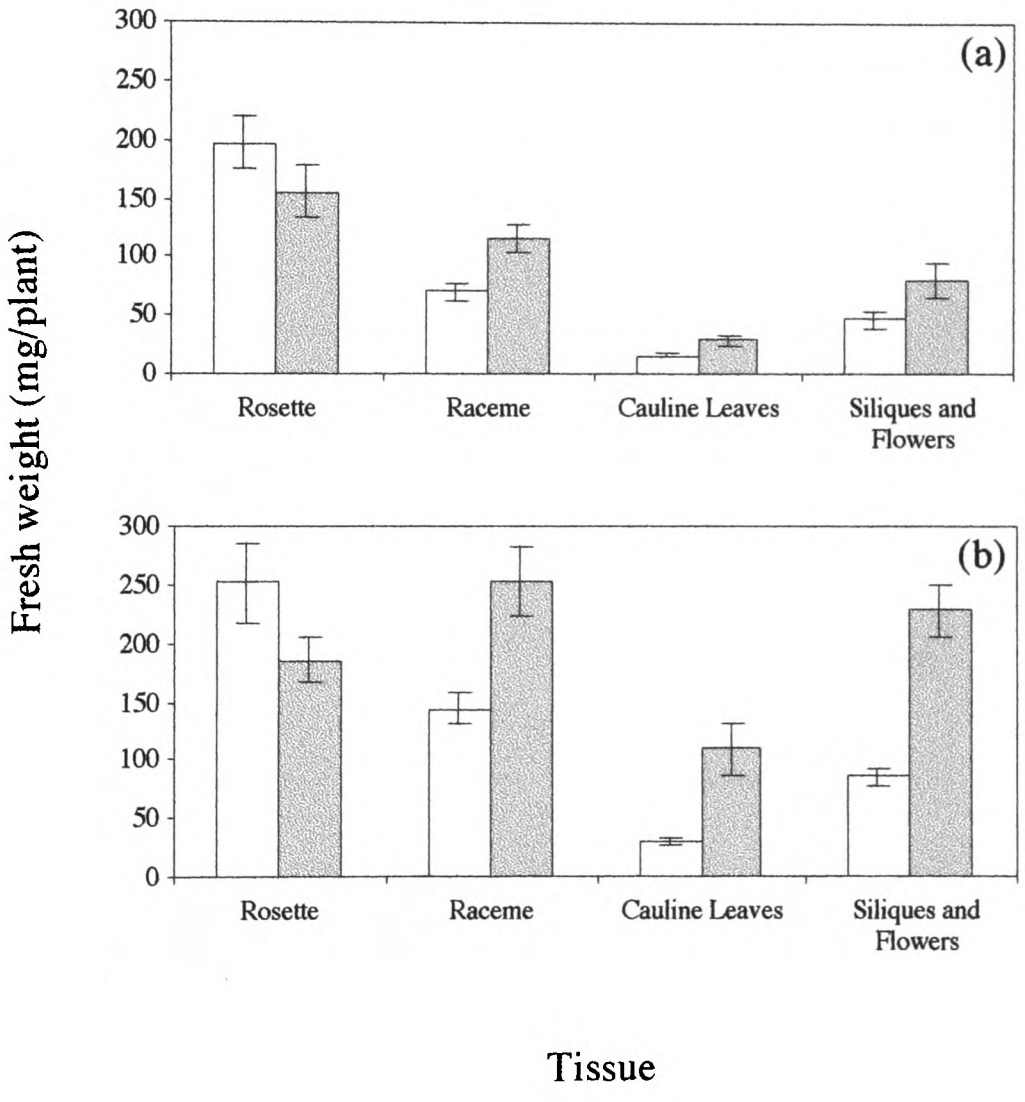


**Figure 7.2** Concentration of sucrose in wild-type and *abi3-1* tissues at 32 and 39 d after germination Wild-type (open bars) and *abi3-1* (stippled bars) plants were harvested at 32 (a) and 39 (b) d after germination and dissected into rosette leaves, racemes, cauline leaves and siliques. Fresh weights were determined (see Figure 7.1) and concentration of sucrose in each tissue was determined enzymologically. Values are the mean ± SE ($n$ = 10).

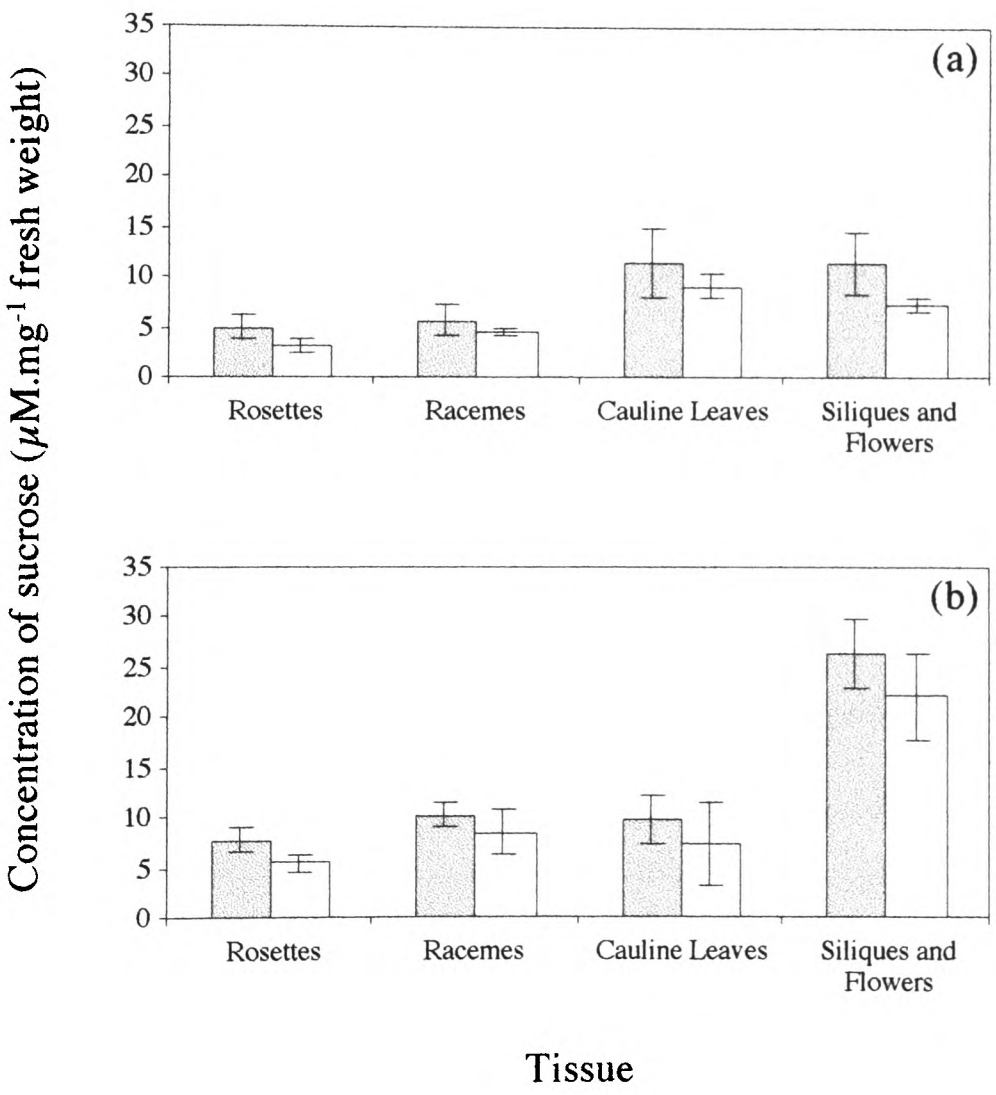


**Table 7.1:** Percentage distribution of [U-$^{14}$C]glucose through silique bearing racemes.

Silique-bearing racemes were detached from palnts and trimmed in 50 mM EGTA (control), without EGTA (-EGTA), or incubated in 5 mM PCMBS. The treatments were chosen as likely inhibitors of phloem transport. Racemes were uncubated in [U-$^{14}$C glucose] for 5 h. Distribution of label was determined following killing and extraction of tissue. Values are the mean ± SE ($n = 3$).

| | % of $^{14}$C recovered in | | | | | |
|---|---|---|---|---|---|---|
| | **Wild Type** | | | ***abi3-1*** | | |
| | **control** | **-EGTA** | **+PCMBS** | **control** | **-EGTA** | **+PCMBS** |
| **Total DPM** | | | | | | |
| **$CO_2$** | 10.0 ±1.3 | 12.4 ±1.1 | 9.8 ±0.8 | 9.9 ±1.4 | 10.4 ±1.1 | 10.2 ±2.2 |
| **Siliquæ** | 3.0 ±0.4 | 4.3 ±0.6 | 3.9 ±0.5 | 2.5 ±0.4 | 2.5 ±0.4 | 2.7 ±0.5 |
| **Racemes** | 86.9 ±6.5 | 83.3 ±8.0 | 86.3 ±10.2 | 87.6 ±8.4 | 87.0 ±9.0 | 87.1 ±10.8 |

**Table 7.2:** Mean percentage distribution of [U-$^{14}$C]glucose in wild type and *abi3-1* siliques.

Values ± SE calculated from means of means in above table ($n = 9$).

| | **Wild Type** | ***abi3-1*** |
|---|---|---|
| **$CO_2$** | 10.7 ±1.1 | 10.1 ±1.6 |
| **Siliquæ** | 3.7 ±0.4 | 2.6 ±0.5 |
| **Racemes** | 85.5 ±8.3 | 87.2 ±9.4 |

after germination, and again shows no significant differences in sucrose concentration between tissues.

### 7.2.2 Metabolism of [U-$^{14}$C]glucose in detached, silique-bearing racemes

The second approach to test the hypothesis that sucrose, as a molecule of communication, is the mechanism underlying the phenotype of *abi3-1* plants previously described, was to experimentally test the rate of import from the phloem into developing wild-type and *abi3-1* siliques using [U-$^{14}$C]glucose radiolabelling.

Table 7.1 shows no significant difference between treatments. The data do, however, suggest a consistently greater amount of label in wild-type siliques compared with *abi3-1* siliques.

The lack of significant difference between the control and the treatment without EGTA shows that there is no difference in uptake from the feeding medium regardless of application of EGTA. Similarly, there is no significant difference between these data and those from treatment with PCMBS. From this it can be concluded that PCMBS does not have the hypothesised effect of inhibiting phloem function in this experiment. The lack of significant difference between the treatments and the control mean that the data can be pooled.

Table 7.2 shows no difference in the amount of label in the $CO_2$ or raceme fractions when data from Table 7.1 are pooled together. A greater amount of label is incorporated into wild-type siliques compared with *abi3-1* siliques, however this difference is barely significant (t-test, p = 0.05).

## 7.3 Discussion

The aims of the experiments described in this chapter were to firstly; investigate whether there were any differences in the concentration of sucrose in the whole plant that might explain the phenotype described in Chapter 3, and secondly; to investigate phloem unloading into developing siliques to determine any differences in rate of unloading between wild-type and *abi3-1* plants that could reveal the mechanism of long distance signalling from sink to source as being sucrose.

Assays of sucrose concentration in tissues of wild-type and *abi3-1* plants reveal no significant differences in sucrose concentration at either 32 d after germination, when the phenotype of the plants is similar, or at 39 d after germination when the phenotype of the *abi3-1* mutant is apparent.

That this experiment did not reveal any differences in concentration of sucrose in tissues does not confirm that such differences do not occur. Harvesting of plants some 4 h into the light period was carried out to investigate if any differences in tissue sucrose concentrations might be apparent when plants were in stable, net photosynthesis, as demonstrated in Chapter 4, Figure 4.1.

The data from the [U-$^{14}$C]glucose feeding experiments show barely significant differences in the metabolism of the siliques between wild type and mutant plants. However a low percentage of the counts (< 5 %) reached the siliques in either case. The use of detached silique-bearing racemes over the course of a 5 h feeding experiment is not a physiologically ideal system. The efficacy of the treatments is also open to criticism.

The absence of EGTA in the bath in which racemes were cut does not mean that wound response occurred. As there was no significant reduction of phloem transport this possibility may have occurred differentially in the course of the experiments. This may explain the lack of statistically significant differences between this treatment and the control and between mutant and wild type.

The treatment using PCMBS was an attempt to actively inhibit phloem function. However, the specificity of its action in these experiments is unknown. It may be that the PCMBS applied to the cut racemes did not reach the distal end of the racemes and so did not affect phloem

unloading. This may have been due to the action of PCMBS on the tissue at the proximal end of the racemes. Also, the action of PCMBS is not as specific as has been implied, e.g.; Giaquinta, 1976. It has been shown to block the function of Aquaporin, a water-channel membrane protein (Prof. A. Cossins, University of Liverpool, UK, personal communication) and so is not specific to solute transport. The lack of statistical significance in the results of the PCMBS treatment is possibly due to; a) insufficiently specific application of the treatment and, b) insufficient specificity of treatment effect.

The observation taken from the [U-$^{14}$C]glucose feeding experiment that over the 5 h period of the experiment the wild type siliques took up more label than the *abi3-1* mutant siliques (Table 7.2) indicates slower seed fill in the *abi3-1* seeds compared with wild-types. This is in accord with previously characterised perturbations to seed metabolism in *abi3* plants: 12S (cruciferin) and 2S (arabin) proteins are absent in the severe alleles *abi3-4* and *abi3-5* and this correlates with levels of dormancy in a range of *aba* and/or *abi* genotypes (Koornneef *et al.*, 1989; Nambara *et al.*, 1992). In *abi3* seed the major storage form of fatty acid with more than 20 carbon units, eicosenoic acid (20:1), is one third the level found in wild-type seeds. However, precursor forms, 18:1 and 18:2, are correspondingly higher (Finkelstein and Somerville, 1990). The *abi3-1* homozygote has a mono:oligosaccharide ratio of 1.28, compared to 1.02 in the wild-type (Ooms *et al.*, 1993). The total sugar content of the *abi3-1* seeds was found to be 2.5x higher than in wild-

type, percentage sucrose content in *abi3-1* measured at 88.8% compared to 77.9% in the wild-type (Ooms *et al.*, 1993).

This list of differences in storage metabolism in developing seeds, although no wholly specific to *abi3-1* seeds, portrays less efficient storage in *abi3* seeds compared to wild-types in terms of build up of precursor fatty acids to the normal storage form, and absence of specific proteins in seeds of severe *abi3* alleles which may underlie their reduced dormancy/ vivipary. However, the specific changes to *abi3-1* seed metabolism relate most notably to sugar status, affecting not only the mono: oligosaccharide ratio, thereby reducing dessication tolerance in mutant seed, but also the percentage sucrose content (Ooms *et al.*, 1993). That the mutant has 2.5x greater sugar content than the wild-type, and specifically sucrose content at 89 % compared to 78 % in the wild-type seeds, explains how unloading into mutant seeds may be slowed by the reduced concentration gradient between phloem and seed in the mutant. Rather than being less competitive for resources than wild-type seeds, *abi3-1* seed may have reduced 'sink strength' in terms of their "capacity to grow" (Farrar and Williams, 1991). It is envisaged that this increased sucrose concentration in mutant seed may therefore give rise to increased concentration of sucrose feeding back into the whole plant from the seeds via the phloem, and this may be the root cause of the phenotype previously described in Chapters 3 and 4.

# Chapter 8

## General Discussion

## 8.0 General Discussion

The aim of the work described in this thesis was to address the effects of altered seed metabolism on resource allocation during reproductive growth in the model plant *Arabidopsis thaliana* L. Heynh, and to investigate how resource allocation may be regulated.

Selection of the *abi3-1* mutant from the extensive range of PGS mutants available in *Arabidopsis* was useful to investigation of the above aim because *abi3-1* causes mild perturbation of seed metabolism relative to wild-type seeds. More severe alleles variously exhibit reduction in seed dormancy and viviparous germination, and gross changes to seed metabolism (Koornneef *et al.*, 1989; Finkelstein and Somerville, 1990; Nambara *et al.*, 1992; Ooms *et al.*, 1993; Parcy *et al.*, 1994). Rates of germination in *abi3-1* seeds are not significantly different to wild-type and in comparison to alternative alleles, changes to metabolism in the seed are quite moderate.

*abi3-1* was selected because of its supposed seed-specific expression (Parcy *et al.*, 1994). Seeds have the highest priority of sinks on a developing plant (Minchin and Thorpe, 1996). Use of a mutant with specific alterations to metabolism within the seed offers a discrete and distinct treatment for use in experiments investigating regulation of allocation between sources and sinks.

Since the research presented in this thesis was started, expression of ABI3 has been reported in vegetative tissues under specific conditions, e.g. dark grown seedlings (Rohde *et al.*, 1999; 2000). Although such vegetative expression of ABI3 has been linked with other regulators of maturation processes, e.g. deetiolated (DET1) (Rohde *et al.*, 2000), and these correlations are of potentially great import in dissection of regulation of maturation at the molecular level (Kurup *et al.*, 2000), the experimental conditions and severe alleles used in such experiments, e.g. *abi3-4* (Parcy *et al.*, 1994) differ considerably from the normal plant phenotype and growth conditions. It remains the case that the most significant expression of *abi3-1* is in developing seeds, and that *abi3-1* has primary, well characterised effects on seed metabolism (Ooms *et al.*, 1993).

PGSs have been implicated in many processes of plant growth and development. ABA in particular has been implicated in many processes in the biology of higher plant seeds. Yet, the mechanisms by which PGSs function remain largely unresolved. Use of a PGS mutant therefore allows some degree of dissection of the extent of PGS contribution to whole plant resource allocation. There are several difficulties and weaknesses of interpretation of experiments using exogenous application of PGSs, or *in vivo* alterations of PGS biosynthesis. There continues to be much debate on the question of tissue and organ sensitivity to PGSs (Trewavas, 1991). Use of a mutant specifically insensitive to ABA avoids such difficulties. Changes in dose are essentially active experimental treatments, whereas use of a mutant with PGS-specific insensitivity is experimentally passive and consistent.

In summary, *abi3-1* offers a useful genotype to investigate regulation of resource allocation during reproductive growth. *abi3-1* is, for the intents and purposes of this thesis, seed specific in altering metabolism relative to wild-type, and so is a discrete and distinct treatment for use in experimental investigation of regulation of resource allocation between sources and sinks. Secondarily, *abi3-1* offers an effective experimental treatment to address broad questions regarding PGS contribution to regulation of resource allocation, while avoiding difficulties of interpreting experiments altering dose of PGSs or alterations to biosynthesis of PGSs.

The first experiment, described in Chapter 3, was to determine any differences between *abi3-1* and wild-type plants, by means of a detailed analysis of plant growth, to test the hypothesis that perturbation of seed metabolism and maturation in *abi3-1* plants would alter resource allocation at the whole plant level.

It was found that *abi3-1* plants continue to initiate new flowers, and hence siliques, for longer than wild-type plants. This was found to be coincident with increased initiation and delayed senescence of cauline leaves in the mutant. The increased harvest index coupled with delayed senescence of cauline leaves in the mutant, compared to the wild-type, implied increased carbon assimilation in the mutant was achieved via the cauline leaves.

The experiment described in Chapter 4 used $^{14}CO_2$ pulse-chase feeding of plants at early and late stages of wild-type seed development to investigate the hypothesis, arising from Chapter 3, that, in order to produce a greater number of siliques, mutant plants must assimilate more carbon, and that this increase in carbon assimilation takes place in the cauline leaves and is achieved by the delayed senescence of the cauline leaves on mutant plants. A secondary aim of this work was to try and establish that differences in growth between wild-type and mutant plants in the long-term are reflected in short-term metabolism.

Estimated total carbon assimilation was the same in both genotypes during early reproductive growth, i.e. 30 d after germination. This rate of no significantly different in the mutant at 38 d after germination, but had fallen by 30-50 % in wild-type plants at the same time. This was consistent with the pattern of leaf senescence in the wild-type. It was shown that there was a shift in the source tissue in the wild-type from the rosette leaves to the silique walls between 30 and 38 d after germination. In *abi3-1* plants the cauline leaves were shown to be acting as the significant source of fixed carbon during the prolonged period of silique initiation after rosette senescence. These observation support the hypothesis that increased carbon assimilation required to support prolonged initiation of siliques in *abi3-1* plants takes place via the cauline leaves. That the ABI3 gene product is not normally expressed outside of seed tissue leads to the conclusion that the cauline leaf phenotype in *abi3-1* plants results from the production of a long-distance signal, or

signals, by the seeds, resulting from abscisic acid secondary signalling effects within maturing seeds, that alters cauline leaf initiation and senescence, and causes continued floral initiation.

A mechanistic explanation of continued floral initiation in *abi3-1* plants is that there is some difference in competition for resources by wild-type and *abi3-1* seeds within each plant. This hypothesis was investigated in Chapter 5 by detailed comparative analysis of growth of wild-type and *abi3-1* plants raised in shade, so as to reduced photosynthesis and hence carbon fixation, and with reduced availability of nitrate.

It was found that the mutant phenotype described in Chapters 3 and 4 depends on non-limiting availability of light and nitrate. Restriction of carbon and or nitrogen fixation abolishes the mutant phenotype. It was found that shading alone favours transition from vegetative to reproductive growth. Shading also caused increase in leaf area relative to leaf weight. In contrast, leaf area was reduced relative to leaf weight in both genotypes when nitrate was limiting. Reduction in nitrate limitation from 90 to 80 % was considerably less limiting for growth of wild-type plants, but remained grossly limiting for plants carrying the *abi3-1* mutation.

That *abi3-1* seeds fill less (i.e. silique weights are reduced) when plants are shade-grown, but the silique initiation phenotype is unaffected, shows that mutant seeds compete less well for available carbon resources compared with wild-type seeds. It was therefore decided to examine the effects

of varying availability of carbon for assimilation more closely and in the short term. This was undertaken by conducting pulse-chase feeding experiments along the same lines as those described in Chapter 4, but with treatments of 200 and 800 ppm concentration of carbon dioxide, to test the hypothesis that the mutant phenotype is dependent on carbon resources, in particular, being non-limiting, and that great excess of $CO_2$ will enhance the mutant phenotype.

Using previously non-acclimatised plants, no difference in the assimilation and allocation of the $^{14}C$ radiolabel was found in either genotype in the short-term relative to controls. At 800 ppm the wild-type was similarly unaffected by the increased concentration of $CO_2$. However, in *abi3-1* plants , the pattern of carbon allocation in the short-term was grossly affected. A significantly large amount of radiolabel was incorporated into the insoluble fraction of all tissues during the pulse and this proportion shifted during the chase into the water soluble fraction in each tissue. Because of the mobility of the large portion label in the insoluble fraction after the pulse is was concluded that this label was temporarily incorporated into starch. The large proportion of label in this starch pool suggests significant increase in the size of this pool under 800 ppm compared to controls. As the breakdown of starch yields sucrose, the increase in label in the water soluble fraction at the end of the chase is suggested to be incorporated in sucrose.

In the whole plant there is not a normal pattern of export and import from "source" to "sink" tissues. Indeed there is apparent export from the silique walls to rosette leaves. It is iterated, and

so stressed, that the plants used in these experiments were not previously acclimated to the treatments conditions. However, the total abolition of the mutant phenotype and apparent reversal of normal source-sink relations in the short-term is apparently due to perturbation of partitioning between starch and sucrose caused by 800 ppm $CO_2$. The large amount of label ending up in the water-soluble fractions of mutant tissues at the end of the chase, if as sucrose, supports abolition of Münchian Mass Flow by cancellation of gradients of sucrose concentration within the phloem.

It was therefore proposed that resource allocation is regulated by competition for resources between sinks maintaining sucrose concentration gradients in the phloem, and that sucrose is both the transport and signalling molecule in the mechanism described.

In order to test that sucrose is the signalling molecule in regulation of resource allocation during reproductive growth, two experimental approaches were undertaken, described in Chapter 7. The first was to assay concentration of sucrose in each tissue of both wild-type and *abi3-1* plants at 4 h into the light period, that is, when plants would be in steady net photosynthesis. No difference between genotypes was found. In the second experiment, rate of import of [U-$^{14}$C]glucose into siliques on detached racemes was investigated. It was found that mutant siliques import marginally, but significantly, more slowly from the phloem than wild-type siliques. Rather than being less competitive, *abi3-1* seeds may be seen as having reduced capacity for growth.

This thesis was born of the continuing debate surrounding how plants regulate their growth. That growth is regulated is known from the allometric ratio of root: shoot growth, the ratio *k* being heritable. PGSs have historically been the explanation of how plant growth is regulated, and indeed how developmental transitions are mediated. However, PGS centred theories have been undermined by questions raised as to exactly how PGSs function (Trewavas, 1991) and by competing theories, such as Functional Equilibrium (Brouwer, 1983) and sucrose as both transport and signalling molecule (von Schaewen *et al.*, 1990; Sonnewald *et al.*, 1991; Dickinson *et al.*, 1991; Sonnewald and Willmitzer, 1992) These latter two theories have been successfully mathematically modelled (Thornley and Johnson 1990; Minchin *et al.*, 1993; Minchin and Thorpe, 1996) and there is considerable experimental evidence supporting mechanisms suggested by these models. Indeed the action of nitrate within functional equilibrium theory has been dissected away by work suggesting the $NO_3^-$ may act as a PGS in itself (Scheible *et al.*, 1994; Scheible *et al.*, 1997) The aim of the work described in this thesis has been to investigate these allegedly competing theories using a seed-specific abscisic acid-insensitive mutant causing moderate alterations to metabolism in developing seeds in the model plant *Arabidopsis thaliana* (Parcy *et al.*, 1994).

This thesis has achieved *firstly*, a fundamental characterisation of the *abi3-1* phenotype at the whole plant level. This phenotype shows significantly greater yield of seeds and concomitant delay in senescence of cauline leaves when compared to wild-type controls.

This thesis has *secondly* achieved the first demonstration that changes in long-term growth are based on changes in short-term metabolism.

*Thirdly*, this thesis has shown that the effects of nitrate limitation are separable from limitation of photosynthesis by shading. It has shown that perturbation of carbon assimilation and allocation has a more fundamental effect on competition for resources between sinks on a plant than does effects of reduced availability of nitrate.

*Fourthly*, this thesis has shown that the effects of carbon limitation are below the resolution of a single photoperiod and so may be cumulative. The effects of excess carbon dioxide on *abi3-1* plants are dramatic and show abolition of normal carbon partitioning behaviour in all photosynthetic tissues and the suggestion that sucrose is central to the mechanism of regulation of resource allocation during reproductive growth and is the mechanism by which the *abi3-1* whole plant phenotype is wrought.

*Fifthly* the work described in this thesis has shown that loading from the phloem into developing *abi3-1* seeds is significantly slower than loading into wild-type seeds.

ABA is a PGS associated with many aspects of seed biology (Black, 1991). ABI3 confers sensitivity to ABA. That *abi3-1* plants show altered resource allocation during reproductive

growth demonstrates that PGSs, in this case ABA, may have a role in whole plant resource allocation. The effect of ABI3 is to regulate gross metabolism within developing seeds. *abi3* mutants show altered fatty acid and protein storage behaviour and there is perturbation of the mono-oligo saccharide ratios in *abi3* mutant seeds. Specifically, *abi3-1* seeds have sucrose concentration of 89 % compared with 78 % in wild-types (Ooms *et al.*, 1993).

The gross phenotype of *abi3-1* whole plants is prolonged floral initiation with coincident delay in senescence of cauline leaves. Study of short-term resource allocation and metabolism using $^{14}CO_2$ pulse-chase radiolabelling confirms the phenotype from the long-term growth analysis. Further growth analysis of plants grown with reduced availability of nitrate and in shade reveal that nitrate limitation causes significant changes in leaf development but has a lesser impact on resource allocation than the effect of shading intended to reduce photosynthesis in wild-type plants. *abi3-1* plants show greater sensitivity to nitrate limitation than wild-types. Shading significantly reduces seed filling in mutant, compared with wild-type plants. The mutant phenotype is dependent on non-limiting availability of essential nutrients, but is shown that *abi3-1* seeds compete for resources less well *in planta* than wild-type seeds. With no difference between genotypes in short-term response to reduced $CO_2$ concentration, increased $CO_2$ concentration had significant effect on initial partitioning of assimilated carbon in the photosynthetic tissues of the mutant with greatly increased initial assimilation into starch followed by a subsequent great increase in sucrose concentration in all photosynthetic tissues.

This causes total breakdown of export from normal source tissues to normal sink tissues and apparent inversion of the source sink relationship.

The evidence of reduced sink strength in *abi3-1* siliques (i.e. their ability or capacity to compete for resources) and greatly perturbed photosynthetic carbon partitioning, direct evidence of altered sucrose status in tissues was sought, but not found. In a second approach it was shown that import of C-species from the phloem into mutant siliques is significantly slower in the mutant compared with the wild-type.

These results in the context of the phenotype described in Chapter 3 and confirmed in the short-term in Chapter 4, lead to the proposal of the following model:

Altered seed metabolism in *abi3-1* plants causes slower seed loading from the phloem compared to wild-type. This causes reduction in the concentration gradient of sucrose in the phloem between source and sink tissues by relative raising of sucrose concentration at the sink end of the transport system distal to the source tissue. That cauline leaf senescence is delayed in *abi3-1* plants suggests that this increase in sucrose concentration does not feed back through the phloem to the source tissue, as rising sucrose concentrations normally induce leaf senescence. As the final yield of siliques in *abi3-1* plants is very much greater than in the wild-type it is proposed that the feedback of increased sucrose concentration in the phloem stimulates continued floral

initiation. Flowering has been shown to be response to sucrose availability in dark-grown plants (Roldán *et al.*, 1999). The increase demand for photosynthate due to the increase in floral initiation in the mutant thereby maintains the overall sucrose concentration gradient between sources and sinks in the whole plant, and so continues to demand photoassimilates from the source tissues, so delaying their senescence. As ABI3 is expressed in seeds only after 12 d after anthesis, this phenotype is peculiar to reproductive growth. *Arabidopsis* exhibits classical monocarpic senescence and so senescence of rosette leaves is unaffected by the mechanism described as rosette leaves are essentially vegetative organs.

This model requires testing. Recent work has shown interactions between *ABI3* and gene loci that play major roles in regulating embryo development and maturation (Kurup *et al.*, 2000). Moreover, interaction was demonstrated between *ABI3* and the plant transcription factor gene *CONSTANS* which has been shown to promote flowering (Putterill *et al.*, 1995; Kurup *et al.*, 2000). In light of these data the expression patterns of *ABI3* reported by Rohde *et al* (1999 & 2000) present a new and exciting area of potential direct genetic regulation underlying the phenotype of *ABI3* plants. In the context of this thesis however, considerable development needs to be undertaken before a molecular model can be proposed. Further, the physiological model proposed above need not be superseded by development of a molecular model, but may be shown to be resultant from activity of interacting transcription factors.

Continuation of the research described in this thesis should initially focus on the carbohydrate status of tissues throughout the diurnal and life cycles in both genotypes. Comprehensive determination of starch and sucrose pool sizes in source, transport and sink tissues in conjunction with assays expression and activity of key enzymes regulating carbon partitioning (i.e. sucrose synthase and ADP-glucose pyrophosphorylase) may contribute direct evidence of differences in carbohydrate status that may explain how metabolic differences in mutant seed may affect whole plant resource allocation. Another route of further research might then be to compare pathways of nitrate uptake and assimilation. In the context of a comprehensive audit of carbohydrate status in all tissues, a detailed molecular and biochemical picture of carbon and nitrogen interactions may be developed which might further explain the different mutant responses to alterations of carbon and nitrogen availability. A final suggestion for continuing investigation of the phenotype and mechanisms investigated in this thesis would be to seek direct evidence of continuing floral induction in *abi3-1* plants as a response to any differences in carbohydrate status. Identification of sucrose-sensitive genes in initiating floral meristems in the mutant after any such expression has ceased in wild-type plants would provide compelling evidence in support of the model proposed above.

# References

Albini S. M. (1994) A karotype of the *Arabidopsis thaliana* genome derived from synaptonemal complex analysis at prophase 1 of meiosis. *The Plant Journal* **5** (5) 665-672.

Allen S. & Smith J.A.C. (1986) Ammonium Nutrition in *Ricinis communis:* Its Effect on Plant Growth and the Chemical Composition of the Whole Plant, Xylem & Phloem Saps. *Journal of Experimental Botany* **37**, 1599-1610.

Andrews J.T. & Lorimer G.M. (1987) Rubisco: structure, mechanism and prospects for improvement. In: The biochemistry of plants, vol.10 (eds. Hatch M.D. & Boardman N.K.) Academic Press, New York, 132-219.

Audals C., Raquin C., Machon N., Godelle B., Mousseau M. (1998 ) Direct and Material effects of elevated CO2 on early root growth of germinating *Arabidopsis thaliana* seedlings. *Annals of Botany* **813**,405-411.

Bagnall D.J. (1992) Control of Flowering in *Arabidopsis thaliana* by Light, Vernalisation and Gibberellins. *Australian Journal of Plant Physiology* **19**, 401-9.

Beck E. (1994) The Morphogenic Response of plants to soil nitrogen: Adaptive Regulation of Biomass Distribution and nitrogen metabolism by Phytohormones. In: Flux control in biological systems. From enzymes to populations and ecosystems. (ed. Schulze E.-D.), Academic Press, Inc., San Diego, pp 119-151.

Bernier G., Havelarge A., Houson C., Petitgean A. & Lejeune P. (1993) Physiological Signals that Induce Flowering. *The Plant Cell* **5**, 1147-1155.

Black C.C. (1993) Sink Strength: Is it real, or measurable? *Plant, Cell & Environment* **16,** 1037-1038. Review, cf. Farrar, 1993a.

Black M. (1991) Involvement of ABA on the physiology of developing and mature seeds. In: Abscisic Acid physiology & biochemistry (eds. Davies, W.J. and Jones, H.G.). 8, 99-119. Bios.

Blocker A.B, & Patterson S. E. (1997) Last Exit: Senescense, Abscission, and Meristem Arrest in *Arabidopsis*. *The Plant Cell* **9**, 1169-1179.

Bodson M., King R,.W., Evans L.T. & Bernier G. (1977) The Role of Photosynthesis in Flowering of the Long-day Plant *Sinapsis alba*. *Australian Journal of Plant Physiology* **4**, 467-478.

Boergan W., Cerveron M.T., Delarue M., Breckman T., Dewitte W., Bellini C., Caboche M., Van Orckelen H., Van Monagu M., & Inzé. (1995) *Superroot*, a Recessive Mutation in *Arabidopsis* Confers Auxin Overproduction. *The Plant Cell* **7**, 1405-1419.

Brouwer R. (1983) Functional Equilibrium: sense or nonsense? *Netherlands Journal of Agricultural Science* **31** pp 335-348.

Brown G.C., Hafner R.P. & Brand M.D. (1990) A 'top-down' approach to the determination of control coefficients in metabolic control theory. *European Journal of Biochemistry* **188**, 321-325.

Carol P., Ping J., & Harberd N.P. (1995) Isolation and preliminary characterization of *gas 1-1*, a mutation causing partial suppression of a phenotype conferred by gibberellin-insensitive (*gai*) mutation in *Arabidopsis thaliana* (L.) Heynh. *Planta* **197,** 414-417.

Caspar T., Huber S.C., & Somerville C. (1985) Alterations in Growth, Photosynthesis and Respiration in a Starchless Mutant of *Arabidopsis thaliana* (L.) Deficit in Chloroplastic Phosphoglucomutase Activity. *Plant Physiology* **79**, 11-17.

Caspar T., Lin T.P., Kalsefrida G., Benbow L., Preiss J. & Somerville C. (1991) Mutants of *Arabidopsis* with Altered Regulation of Starch Degradation. *Plant Physiology* **95**, 1181-1188.

Chaudhury A.M. (1993) Nuclear genes controlling male fertility. *Plant Cell* **5**, 1277-1283.

Chaumont S., (1993) Sink Strength: the key for plant yield modelling. *Plant, Cell and Environment* ***16***, 1033-1034. Review cf. Farrar, 1993.

Chandler J.W. & Bartels D. (1997) Structure and function of the VP1 gene homologue from the resurrection plant *Craterostigma plantagineum* Hochst. *Molecular & General Genetics* **256** 539-546.

Chapin III F.S., Schultze E.D. & Mooney H.A. (1990) The Ecology & Economics of Storage in Plants. *Annual Review of Ecology & Systematics* **21**, 423-447.

Cheng C.L., Acedo G.N., Cristinsin M. & Conkling M.A. (1992) Sucrose mimics the light induction of *Arabidopsis* nitrate reductase gene-transcription. *Proceedings of the National Academy of Sciences USA* **89**, 1861-1864.

Cleland R.E. (1983) Is plant development regulated by changes in the concentration of growth substances or by changes in the sensitivity to growth substances? Changes in hormone concentration are important too. *Trends in Biochemical Science* **8**, 354-357.

Clouse S.D. (1996) Molecular genetic studies confirm the role of brassinosteriods in plant growth & development. *The Plant Journal* **10** (1), 1-8.

Corbinier L., Lejeune P. & Bernier G (1998) The role of carbohydrates in the induction of flowering in *Arabidopsis thaliana* comparison between wild-type and a starchless mutant. *Planta* **206**, 131-137.

Cullimore J.V. & Bennett M.J. (1992) Nitrogen assimilation in the legume root-nodule: current status of the molecular biology of the plant enzymes. *Canadian Journal of Microbiology* **38**, 461-466.

Dancer J., David M. & Stitt M. (1990) Water stress leads to a change of positioning in favour of sucrose in heterotrophic cell suspension cultures of *Chenopodium rubrum*. *Plant Cell & Environment* **13**, 957-963.

da Silva P.M.F.R., Eastwood P.J., Hill L.M., Smith A.M. & Rawsthorne S. (1997) Starch metabolism in developing embryos of oilseed rape. *Planta* (1997) **203**: 480-487.

de Bruign S.M. & Vreugdenhil D. (1992) Abscisic Acid and Assimilate Partitioning to Developing Seeds. I. Does Abscisic Acid Influence the Growth Rate of Pea Seeds? *Journal of Plant Physiology* **140**, 201-206.

de Bruijn S.M. & Vreugdenhil D. (1993) Abscisic acid and Assimilate partitioning to developing seeds. II. Does abscisic acid influence the sink strength of *Arabidopsis* seeds? *Physiologia Plantarum* **88**, 583-589.

Dickinson C.D., Altabella T. & Chrispeels M.J. (1991) Slow growth phenotype of transgenic tomato expressing apoplastic invertase. *Plant Physiology* **95**, 420-425.

Eimert K., Wang S.M., Luc W.-L. & Chen J. (1995) Monogenic Recessive Mutations Causing Both Late Floral Limitation and Excess Starch Accumulation in *Arabidopsis*. *The Plant Cell* **7**, 1703-1712.

Epstein E. (1972) Mineral nutrition in plants : Principles & perspectives. Wiley & Sons, New York, NY.

Evert R.F., Escrich W. & Heyser W. (1978) Leaf structure in relation to solute transport and phloem loading in *Zea mays* L.. *Planta* **138**, 279-294.

Faiss M., Struad M., Redig P., Dolezal K., Hanus J., Van Onckelen H, & Scharülling T. (1996) Chemically induced expression of the *rolC* - encoded *B* - glucosidase in transgenic tobacco plants and analysis of cytokinin metabolism: *rolC* does not hydrolyze endogenous cytokinin glucosides *in planta*. *The Plant Journal* **10** (1), 33-46.

Faiss M., Zalubilová J., Struad M., & Scharülling T. (1997) Conditional transgenic expression of the *ipt* gene indicates a function for cytokinins in paracrine signalling in whole tobacco plants. *The Plant Journal* **12** (2) 401-415.

Farquhar G.D. & von Caemmerer S. (1982) Modelling of photosynthetic response to environmental conditions. In: Encyclopedia of plant physiology, vol. 12b (eds. Lange O.L., Nobel P.S., Osmond C.B. & Ziegler M.) 549-587. Springer, Berlin, Heidelberg, New York.

Farrar J,F. & Minchin P.E.H. (1991) Carbon Partitioning in Split Root Systems of Barley: Relation to Metabolism. *Journal of Experimental Botany* **42** (243), 1261-1269.

Farrar J.F. and Williams M.L. (1991) The effects of increased carbon dioxide and temperature on carbon partitioning, source-sink relations and respiration. *Plant, Cell and Environment* **14**, 819-830.

Farrar J.F. (1992) The whole plant : carbon partitioning during development. In: Carbon Partitioning within and between organisms (eds. Pollock, C.J., Farrar, J.F. and Gordon, A.J.), 163-176. Bios.

Farrar J.F. (1993a) Sink Strength : What is it and how do we measure it? Introduction. *Plant Cell and Environment* **16**, pp. 1015-1016.

Farrar J.F. (1993b) Sink Strength : What is it and how do we measure it? A summary. *Plant Cell and Environment* **16**, 1045-1046.

Farrar J.F., Minchin P.E.H. & Thorpe M.R. (1994) Carbon import into barley roots : stimulation by galactose. *Journal of Experimental Botany* **45** (270), 17-22.

Farrar J.F. (1996) Sinks - integral parts of a whole plant *Journal of Experimental Botany*, **47** Special Issue, pp. 1273-1279.

Fell D.A. (1997) Understanding the Control of Metabolism. Portland Press, London.

Fellows R.J. & Geiger D.R. (1974) Structural and physiological changes in sugar beet leaves during sink to source conversion. *Plant Physiology* **54**, 877-885.

Finkelstein R.R. & Somerville C.R. (1990) Three classes of Abscisic Acid (ABA) - Insensitive mutation of *Arabidopsis* define genes that control overlapping subsets of ABA responses. *Plant Physiology* **94**, 1172-1179.

Finkelstein R.R. (1994) Mutations at 2 new *Arabidopsis* ABA response loci are similar to the ABI3d mutations. *Plant Journal* **5**, 765-771.

Fisher D.G. & Evert R.F. (1982) Studies on the leaf of *Amaranthus retroflexus* (Amaranthaceæ): ultrastructure, plasmodesmatal frequency, and solute concentration in relation to phloem loading. *Planta* **155**, 377-387.

Fitchner K., Quick W.P., Schultze E.-D., Mooney H.A. Rodermel S.R., Bogorad L., & Stitt M., (1993) Decreased ribulose - 1,5 - bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" *rbcS* V. Relationship between photosynthetic rate, storage, strategy, biomass allocation and vegetative plant growth at three different nitrogen supplies. *Planta* **190**, 1-9.

Fliege R., Flügge U.-I., Werdan K., & Heldt H.W. (1978) Specific transport of inorganic phosphate, 3-phosphoglycerate and triose phosphate across the inner membrane of the envelope in spinach chloroplasts. *Biochim. Biophys. Acta* **502** 232-247.

Flügge U.-I. & Heldt H.W. (1991) Metabolic translocator of the chloroplast envelope. *Annual Review of Plant Physiology and Plant Molecular Biology* **42**, 129-144.

Fondy B.R. & Geiger D.R. (1982) Diurnal patterns of translocation and carbohydrate metabolism in source leaves of *Beta vulgaris* L. *Plant Physiology* **70**, 671-676.

Foyer C.H. (1988) Feedback inhibition of photosynthesis through source-sink regulation in leaves. *Plant Physiology & Biochemistry* **26**, 483-492.

Gan S. & Amasino R.M. (1995) Inhibition of leaf senescence by autoregulated production of cytokinin. *Science* **270**, 1986-1988.

Geigenberger P., Langenberger S., Wilke I., Heineke D., Heldt H.W. & Stitt M. (1994) Sucrose is metabolised by sucrose synthase and glycolysis within the phloem complex of *Ricinis communis* L. seedlings. *Planta* **190**, 446-453.

Geigenberger P., Lerchl J., Stitt M. & Sonnewald U. (1996) Phloem-specific expression of pyrophosphatase inhibits long-distance transport of carbohydrates and amino acids in transgenic plants. *Plant, Cell & Environment*, **19**, 43-55.

Geiger D.R., Giaquinta R.T., Sovonik S.A. & Fellows R.J. (1973) Solute distribution in sugar beet leaves in relation to phloem loading and translocation. *Plant Physiology* **52**, 585-589.

Geiger D.R., Sovonik S.A., Schock T.L. & Fellows R.J. (1974) Role of free space in translocation of sugar beet. *Plant Physiology* **54**, 892-898.

Gianfanga T.J. & Davies P.J. (1981) The relationship between fruit growth and apical senesence in the G2 line of peas. *Planta* **152**, 356-364.

Giaquinta R (1976) Evidence for phloem loading from the apoplast. Chemical modification of membrane sulfhydryl groups. *Plant Physiology* **75**, 872-875.

Giaquinta R. (1977) Phloem loading of sucrose. pH dependence and selectivity. *Plant Physiology* **59**, 750-755.

Gifford R.M., Thorne J.H., Hitz W.D. & Giaquinta R.T. (1984) Crop productivity and photoassimilate partitioning. *Science* **225**, 801-808.

Gifford R.M. & Thorne J.H. (1986) Phloem unloading in soybean seed coats: dynamics and stability of efflux into attached "empty" ovules. *Plant Physiology* **80**, 464-469.

Giraudat J., Hauge B.M. Valon C., Smalle J., Parcy F. & Goodman H.M. (1992) Isolation of the *Arabidopsis ABI3* gene by positional cloning. *Plant Cell* **4**, 1251-1261.

Gordon A.J. (1986) Diurnal patterns of photosynthate allocation and partitioning among sinks. In: Phloem Transport (eds. Cronshaw J., Lucas W.J. and Giaquinta R.T.), Alan R. Liss, New York, 499-518.

Graham I.A., Denby K.J. & Leaver C.J. (1994) Carbon catabolite repression regulates glyoxylate cycle gene expression in cucumber. *Plant Cell* **6**, 761-772.

Gregerson R.G., Miller S.S., Twary S.N., Gantt J.S. & Vance C.P. (1993) Molecular characterisation of NADH-dependent glutamate synthase from alfalfa nodules. *Plant Cell* **5**, 215-226.

Gunning B.E.S., Pate J.S., Minchin F.R. & Marks I. (1974) Quantitative aspects of transfer cell structure in relation to vein loading in leaves and solute transport in legume nodules. *Symposium of the Society of Experimental Biology* **18**, 87-126.

Harter K., Talkemesserer C., Barz W. & Schafer E. (1993) Light-dependent and sucrose-dependent gene expression in photomixotrophic cell-suspension cultures and protoplasts of rape (*Brassica napus* L.). *The Plant Journal* **4**, 507-516.

Hattenbach A., Müller-Röber B., Nast G. & Heineke D. (1997) Antisense repression of both ADP-glucose pyrophosphorylase and triose-phosphate translocator modifies carbohydrate partitioning in potato leaves. *Plant Physiology* **115**, 471-475.

Heineke D., Sonnewald U., Büssis D., Günter G., Leidreiter K., Wilke I., Raschke K., Willmitzer L. & Heldt H.W. (1992) Expression of yeast-derived invertase in the apoplast of potato plants results in an inhibition of photosynthesis caused by an increase of the osmotic pressure in leaf cells due to the accumulation of hexoses and amino acids, and affects an increase in the protein to starch ratio in the tubers. *Plant Physiology* **100**, 301-308.

Heineke D., Kruse A., Flügge U.-I., Frommer W.B., Riesmeier J., Willmitzer L. & Heldt H.W. (1994) Effect of 'antisense' repression of the chloroplast triose-phosphate translocator on photosynthesis metabolism in trnasgenic potato plants. *Planta* **193**, 174-180.

Hendrix D.L. & Huber S.C. (1986) Diurnal fluctuations in cotton leaf carbon export, carbohydrate content and sucrose synthesising enzymes. *Plant physiology* **81**, 584-586.

Hensel L.L. & Bleecker A.B. (1992) *Arabidopsis* as a model system for analysis of leaf senescence and inflorescence-meristem longevity. In: Proceedings of the Twenty-First Steenbock Symposium (ed. Amasino R.M.), 123-130. Plenum Press, New York.

Hensel L.L., Grbi´c V., Baumgarten D.A. & Bleecker A.B. (1993) Developmental and age-related processes that influence the longevity and senescence of photosynthetic tissues in *Arabidopsis*. *Plant Cell* **5**, 553-564.

Hensel L.L., Nelson M.A., Richmond T.A. & Bleecker A.B. (1994) The fate of inflorescence meristems is controlled by developing fruits in *Arabidopsis*. *Plant Physiology* **106**, 863-876.

Hetherington A.M. & Quatrano R.S. (1991) Tansley Review No. 31: Mechanisms of action of abscisic acid at the cellular level. *New Phytologist* **119**, 9-32.

Hildebrand F. (1881) Die Lebensdauer und Vegetationsweise der Pflanzen, ihre Ursache und ihre Entwicklung. *Botansiche Jahrbooke* **2**, 51-135.

Ho L.C. & Shaw A.F. (1977) Carbon economy and translocation of $^{14}CO_2$ in leaflets of the seventh leaf of tomato during leaf expansion. *Annals of Botany* **41**, 833-848.

Ho L.C. (1984) Partitioning of assimilates in fruiting tomato plants. *Plant Growth Regulation* **2**, 277-285.

Ho L.C. (1988) Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. *Annual Review of Plant Physiology and Plant Molecular Biology* **39**, 355-378.

Huber S.C. (1989) Biochemical mechanism for regulation of sucrose accumulation in leaves during photosynthesis. *Plant Physiology* **91**, 656-662.

Huber S.C., Huber J.L.A., & McMichael Jr. R.W. (1992) The regulation of sucrose synthesis in leaves. In: Carbon Partitioning within and between organisms (eds. Pollock C.J., Farrar J.F. & Gordon A.J.), 1-26. ßios.

Hunt R. (1978) Plant Growth Analysis. *Studies in Biology* **96**, Edward Arnold, Ltd.

Jackson M.B. (1993) Are plant hormones involved in root to shoot communication? *Advances in Botanical Research* **19**, 104-168.

Jang J.-C. & Sheen J. (1994) Sugar sensing in higher plants. *Plant Cell* **6**, 1665-1679.

Jelitto T., Sonnewald U. & Willmitzer L. (1992) Inorganic pyrophosphate content and metabolites in potato and tobacco plants expressing *E. coli* pyrophosphatase in their cytosol. *Planta* **188**, 238-244.

Jenner C.F. & Hawker J.S. (1993) Sink strength: soluble starch synthase as a measure of sink strength in wheat endosperm. *Plant, Cell Environment* **16**, 1023-1024.

Kaarsen C.M. (1982) Indirect effect of abscisic acid on the induction of secondary dormancy in lettuce seeds. *Physiologia Plantarum* **54**, 258-266.

Kacser H. & Burns J.A. (1973) The control of flux. *Symposium of the Society of Experimental Biology* **27**, 65-107.

Kacser H. & Porteous J.W. (1987) Control of metabolism: what do we have to measure? *Trends in Biological Science* **12**, 5-14.

Kalt-Torres W., Kerr P.S. Usuda H. & Huber S.C. (1987) Diurnal changes in maize leaf photosynthesis. Carbon exchange rate, assimilate export rate, and enzyme activities. *Plant Physiology* **83**, 283-288.

King S.P., Lunn J.E. & Furbank R.T. (1997) Carbohydrate content and enzyme metabolism in developing canola siliques. *Plant Physiology* **114**, 153-160.

Koorneef M., Reuling G. & Karsen C.M. (1984) The isolation and characterisation of abscisic acid-insensitive mutants of *Arabidopsis thaliana. Physiologia Plantarum* **61**, 377-383.

Koorneef M., Hanhart C.J., Hilhorst H.W.M. & Karsen C.M. (1989) *In vivo* inhibition of seed development and reserve protein accumulation. *Plant Physiology* **90**, 463-469.

Kossman J., Sonnewald U. & Willmitzer L. (1994) Reduction of the chloroplastic fructose-1,6-bisphosphatase in transgenic potato plants impairs photosynthesis and plant growth. *Plant Journal* **6**, 637-650.

Krapp A., Hofmann B., Schafer C. & Stitt M. (1993) Regulation of the expression of *rbcS* and other photosynthetic genes by carbohydrates – a mechanism for the sink regulation of photosynthesis. *Plant Journal* **3**, 817-828.

Kruger N.J. (1997) Carbohydrate synthesis and degradation. In: Plant Metabolism (eds. Dennis D.T., Turpin D.H., Lefebvre D.D. and Layzell D.B.), Addison Wesley Longman Ltd., 83-105.

Kurup S., Jones H.D. & Holdsworth M.J. (2000) Interactions of the developmental regulator ABI3 with proteins identified from developing *Arabidopsis* seeds. *The Plant Journal* **21**, 143-155.

Lauerer M., Safic D., Quick W.P., Labate C., Fichtner K., Schulze E.-D., Rodermel S.R. Bogorad L. & Stitt M. (1993) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" *rbc*S VI. Effect on photosynthesis in plants grown at different irradiances. *Planta* **190**, 332-345.

Lerchl J., Geigenberger P., Stitt M. & Sonnewald U. (1995) Impaired photoassimilate partitioning caused by phloem-specific removal of pyrophosphate can be complemented by a phloem-specific cytosolic yeast-derived invertase in transgenic plants. *Plant Cell* **7**, 259-270.

Lockhart J.A. & Gottshcall V. (1961) Fruit-induced and apical senescence in *Pisum sativum* L.. *Plant Physiology* **36** 389-398.

Lucas W.J., Olesinski A., Hull R.J., Haudenshield J.S., Deom C.M., Beachy R. N. & Wolf S. (1993) Influence of the tobacco mosaic virus 30 kD movement protein on carbon metabolism and photosynthate partitioning in transgenic tobacco plants. *Planta* **190**, 88-96.

Marcelis L.F.M (1996) Sink strength as a determinant of dry matter partitioning in the whole plant. *Journal of Experimental Botany* **47**, 1281-1291.

Martin T., Frommer W.B., Salanabout M. & Willmitzer L. (1993) Expression of an *Arabidopsis* sucrose synthase gene indicates a role in metabolisation of sucrose both during phloem loading and in sink organs. *Plant Journal* **4**, 367-377.

McCarty D.R., Hattori T., Carson C.B. Vasil V., Lazar M. & Vasil I.K. (1991) The *Viviparous-1* developmental gene of maize encodes a novel transcriptional activator. *Cell* **66**, 895-905.

McGrath R.B. & Coruzzi G.M. (1991) A gene network controlling glutamine and asparagine biosynthesis in plants. *Plant Journal* **1**, 275-280.

Miller M.E. & Chourey P.S. (1992) The invertase deficient *miniature-1* seed mutation is associated with aberrant pedicel and endosperm development. *Plant Cell* **4**, 297-305.

Minchin P.E.H. & Thorpe M.R. (1987) Measurement of unloading and reloading of photoassimilate within the stem of bean. *Journal of Experimental Botany* **38**, (187) 211-220.

Minchin P.E.H. & Thorpe M.R. (1989) Carbon partitioning to whole versus surgically modified ovules of pea: an application of the *in vivo* measurement of carbon flows over many hours using the short-lived isotope carbon-11. *Journal of Experimental Botany* **38**, 211-220.

Minchin P.E.H., Thorpe M.R. & Farrar J.F. (1993) A simple mechanistic model of phloem transport which explains sink priority. *Journal of Experimental Botany* **44**, 947-955.

Minchin P.E.H & Thorpe M.R. (1996) What determines carbon partitioning between competing sinks? *Journal of Experimental Botany* **47**, 1293-1296.

Mita S., Suzuki-Fujii K. & Nakamura K. (1995) Sugar-inducible expression of a gene for ß-amylase in *Arabidopsis thaliana*. *Plant Physiology* **107**, 895-904.

Müller-Röber B., Sonnewald U. & Willmitzer L. (1992) Inhibition of the ADP-glucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage protein genes. *The EMBO Journal* **11**, 1129-1238.

Münch E. (1930) Die Stoffbewegungen in der Pflanze. Verlag von Gustav Fischer, Jena.

Nambara E., Naito S. & McCourt P. (1992) A mutant of *Arabidopsis* which is defective in seed development and storage protein accumulation is a new *abi3* allele. *The Plant Journal* **2**, 435-441.

Nambara E., Keith K., McCourt P. & Naito S. (1994) Isolation of an internal deletion mutant of the *Arabidopsis thaliana ABI3* gene. *Plant Cell Physiology* **35**, 509-513.

Nolte K.D. & Koch K.E. (1990) Companion cell specific localization of sucrose synthase in zones of phloem loading and unloading. *Plant Physiology* **101**, 899-905.

Noodén L.D., Singh S. & Letham D.S. (1990) Correlation of xylem sap cytokinin levels with monocarpic senescence in soybean. *Plant Physiology* **93**, 33-39.

Offler C.E. & Patrick J.W. (1984) Cellular structures, plasma membrane surface areas and plasmodesmatal frequencies of seed coats of *Phaseolus vulgaris* L. in relation to photosynthate transfer. *Australian Journal of Plant Physiology* **11**, 79-99.

Offler C.E. & Patrick J.W. (1986) Cellular pathway and hormonal control of short-distance transfer in sink regions. In: Phloem Transport (eds. Cronshaw J., Lucas W.J. and Giaquinta R.T.), Alan R. Liss, New York, 295-306.

Offler C.E., Nerlich S.M. & Patrick J.W. (1989) Pathway of photosynthate transfer in the developing seed of *Vicia faba* L.. Transfer in relation to seed anatomy. *Journal of Experimental Botany* **40**, 769-780.

Ooms J.J.J., Léon-Kloosterziel K.M., Bartels D., Koorneef M. & Karssen C.M. (1993) Acquisition of desiccation intolerance and longevity in seeds of *Arabidopsis thaliana*. A comparative study using abscisic acid-insensitive *abi3* mutants. *Plant Physiology* **102**, 1185-1191.

Parcy F., Valon C., Raynal M., Gaubier-Comella P., Delseny M. & Giraudat J. (1994) Regulation of gene expression programs during *Arabidopsis* seed development: roles of the *ABI3* locus and of endogenous abscisic acid. *Plant Cell* **6**, 1567-1582.

Parcy F. & Giraudat J. (1997) Interactions between the *ABI3* and the ectopically expressed *ABI3* genes in controlling abscisic acid responses in *Arabidopsis* vegetative tissues. *Plant Journal* **11**, 693-702.

Patrick J.W. (1983) Photosynthate unloading from seed coats of *Phaseolus vulgaris* L.. General characteristics and facilitated transfer. *Zeitschrift für Pflazenphysiologie* **111**, 9-18.

Patrick J.W. (1997) Phloem unloading: Sieve element unloading and post-sieve element transport. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**, 191-222.

Pearsall W.H. (1927) Growth studies. VI. On the relative sizes of growing plant organs. *Annals of Botany* **151**, 549-556.

Peoples M.B. & Gifford R.M. (1997) Regulation of the transport of nitrogen and carbon in higher plants. In: Plant Metabolism (eds. Dennis D.T., Turpin D.H., Lefebvre D.D. and Layzell D.B.), Addison Wesley Longman Ltd., 525-539.

Putterill J., Robson F., Lee K., Simon R. & Coupland G. (1995) The *CONSTANS* gene of *Arabidopsis* promotes flowering and encodes a protein showing similarities to zinc-finger transcription factors. *Cell* **80**, 847-857.

Quick W.P., Schurr U., Scheibe R., Schulze E.-D., Rodermel S.R., Bogorad L. & Stitt M. (1991a) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" *rbc*S I. Impact on photosynthesis in ambient growth conditions. *Planta* **183**, 542-554.

Quick W.P., Fichtner K., Shulze E.-D., Wendler R., Leegood R.C., Mooney H., Rodermel S.R. Bogorad L. & Stitt M. (1992) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" *rbc*S IV. Impact on photosynthesis in conditions of altered nitrogen supply. *Planta* **188**, 522-531.

Redinbaugh M.G. & Campbell W.H. (1993) Glutamine synthetase and ferredoxin-dependent glutamate synthase expression in the maize (*Zea mays*) root primary response to nitrate. *Plant Physiology* **101**, 1249-1255.

Reid J.B (1980) Apical senescence in *Pisum*: a direct or indirect role for the flowering genes. *Annals of Botany* **45**, 195-201.

Reynolds J.F. & Thornely J.H.M. (1982) A shoot: root partitioning model. *Annals of Botany* **49**, 585-597.

Riesmeier J.W., Flügge U.-I., Schulz B., Heineke D., Heldt H.W., Willmitzer L. & Frommer W. (1993) Antisense inhibition of the chloroplast triosephosphate translocator affects carbon partitioning in transgenic potato plants. *Proceedings of the National Academy of Sciences, USA* **90**, 6160-6164.

Riesmeier J.W., Willmitzer L. & Frommer W.B. (1994) Evidence for an essential role of the sucrose transporter in phloem loading and assimilate partitioning. *The EMBO Journal* **13**, 1-7.

Riens B., Lohaus G., Winter H. & Heldt H.W. (1994) Production and diurnal accumulation of assimilates in leaves of spinach (*Spinacia oleracea* L.) and barley (*Hordeum vulgare* L.). *Planta* **192**, 497-501.

Robichaud C. & Sussex I.M. (1986) The response of *viviparous-1* and wild-type embryos of *Zea mays* to culture in the presence of abscisic acid. *Journal of Plant Physiology* **126**, 235-242.

Rochasosa M., Sonnewald U., Frommer W., Stratmann M. & Schell J. (1989)Both developmental and metabolic signals activate the promoter of a Class-I patatin gene. *EMBO Journal* **8**, 23-29.

Rodermel S.R., Abbott M.S. & Bogorad L. (1988) Nuclear-organelle interactions: nuclear "antisense" gene inhibits ribulose bisphosphate carboxylase enzyme levels in transformed tobacco plants. *Cell* **55**, 673-681.

Rohde A., van Montague M. & Boerjan W. (1999) The *ABSCISIC ACID-INSENSTIVE 3 (ABI3)* gene is expressed during vegetative quiescence processes in *Arabidopsis*. *Plant, Cell & Environment* **22**, 261-270.

Rohde A., de Rycke R., Beekman T., Engler G., van Montagu M & Boerjan W. (2000) ABI3 effects on plastid differentiation in dark-grown *Arabidopsis* seedlings. *Plant Cell* **12**, 35-52.

Roldán M., Gómez-Mena C., Ruiz-Garcia L., Salinas J. & Martinez-Zapater J.M. (1999) Sucrose availability on the aerial part of the plant promotes morphogenesis and flowering of *Arabidopsis* in the dark. *The Plant Journal* **20**, 581-590.

Rufty Jr. TW., Huber S.C. & Volk R.J. (1988) Alterations in leaf carbohydrate metabolism in response to nitrogen stress. *Plant Physiology* **88**, 725-730.

Sage R.F. (1990) A model describing the regulation of ribulose-1,5-bisphosphate carboxylase-oxygenase, electron transport and triose phosphate use in response to light intensity and $CO_2$ in C3 plants. *Plant Physiology* **94**, 1728-1734.

Sakakibara H., Watanabe M., Hase T. & Sugiyama T. (1991) Molecular cloning and characterisation of complementary DNA encoding for ferredoxin-dependent glutamate synthase in maize leaf. *Journal of Biological Chemistry* **266**, 2028-2035.

Salisbury F.B. & Ross C.W. (1992) Plant Physiology (4th Edition). Wadsworth Publishing Company, Belmont, Calif., USA.

Sauer N., Baier K., Gahrtz M., Stadler R., Stoltz J. & Truernit E. (1994) Sugar transport across the plasma membranes of higher plants. *Plant Molecular Biology* **26**, 1671-1679.

Scheible W.-R., Lauerer M., Schulze E.-D., Caboche M. & Stitt M. (1997a) Accumulation of nitrate in the shoot acts as a signal to regulate shoot-root allocation in tobacco. *Plant Journal*, **11**, 671-691.

Scheible W.-R., González-Fontes A, Morceunde R., Lauerer M., Geiger M., Glaab J., Gojon A., Schulze E.-D. & Stitt M. (1997b)Tobacco mutants with a decreased number of functional nia genes compensate by modifying the diurnal regulation of trnascription, post-translational modification and turnover of nitrate reductase. *Planta* **203**, 304-319.

Scheible W.-R., González-Fontes A., Lauerer M., Müller-Röber B., Caboche M. & Stitt M. (1997c) Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. *Plant Cell* **9**, 783-798.

Schultz E.A. & Haughn G.W. (1991) *LEAFY*, a homeotic gene that regulates inflorescence development in *Arabidopsis*. *Plant Cell* **3**, 771-781.

Schulze W., Stitt M., Schulze E.-D., Neuhaus H.E. & Fichtner K. (1991) A quantification of the significance of assimilatory starch for growth of *Arabidopsis thaliana* L. Heynh.. *Plant Physiology* **95**, 890-895.

Schulze W., Schulze E.-D., Stadler J., Heilmeier H., Stitt M. & Mooney H.A. (1994) Growth and reproduction in *Arabidopsis thaliana* in relation to storage of starch and nitrate in the wild-type and in starch-deficient and nitrate-uptake-deficient mutants. *Plant, Cell & Environment* **17**, 795-809.

Scott R.J., Spielman M., Bailey J. & Dickinson H.G. (1998) Parent-of-origin effects on seed development in *Arabidopsis thaliana*. *Development* **125**, 3329-3341.

Sharkey T.D. (1989) Evaluating the role of Rubisco in photosynthesis of C3 plants. *Philosophical Transactions of the Royal Society, London, Biological Science* **323**, 435-448.

Sheen J. (1990) Metabolic repression of transcription in higher plants. *Plant Cell* **2**, 1027-1038.

Small J.R. & Fell D.A. (1990) Metabolic control analysis. Sensitivity of control coefficients to elasticities. *European Journal of Biochemistry* **191**, 413-420.

Somerville C.R. & Meyerowitz E.M. (1994) Introduction. In: *Arabidopsis* (eds. Meyerowitz E.M & Somerville C.R.) Coldspringharbor Laboratory Press.

Sonnewald U., Brauer M., von Schaewen A., Stitt M. & Willmitzer L. (1991) Transgenic tobacco plants expressing yeast-derived invertase in either the cytosol, vacuole or apoplast: a powerful tool for studying sucrose metabolism and sink/ source interactions. *The Plant Journal* **1**, 95-106.

Sonnewald U. (1992) Expression of *E.coli* pyrophosphatase in transgenic plants alters photoassimilate partitioning. *Plant Journal* **2**, 571-581.

Sonnewald U. & Willmitzer L. (1992) Molecular approaches to sink-source interactions. *Plant Physiology* **99**, 1267-1270.

Sonnewald U., Lerchl J., Zrenner R. & Frommer W. (1994) Manipulation of sink-source relations in transgenic plants. *Plant, Cell & Environment* **17**, 649-658.

Stitt M. (1993) Sink strength: integrated systems need integrated approaches. *Plant, Cell & Environment* **16**, 1041-1043.

Stitt M. (1996) Plasmodesmata play an essential role in sucrose export from leaves: a step toward an integration of metabolic biochemistry and cell biology. *Plant Cell* **8**, 565-571.

Stitt M. & Schulze D. (1994) Does Rubisco control the rate of photosynthesis and plant growth? An exercise in molecular ecophysiology. *Plant, Cell & Environment* **17**, 465-487.

Stitt M. & Schulze E.-D. (1994) Plant growth, storage and resource allocation: from flux control in the metabolic chain to the whole plant level. In: Flux control in biological systems. From enzymes to populations and ecosystems (ed. Schulze E.-D.) Academic Press, Inc. San Diego, 57-118.

Stitt M. (1997) The flux of carbon between the chloroplast and cytoplasm. In: Plant Metabolism (eds. Dennis D.T., Turpin D.H., Lefebvre D.D. and Layzell D.B.), Addison Wesley Longman Ltd., 382-401.

Strickland S. & Loeb J.N. (1981) Obligatory separation of hormone binding and biological response curves in systems dependent on secondary mediators of hormone action. *Proceedings of the National Academy of Sciences USA* **78** (3) 1366-1370.

Sung S.J.S., Xu D.P. & Black C.C. (1989) Identification of actively filling sucrose sinks. *Plant Physiology* **89**, 1117-1121.

Sweetlove L.J., Burrell M.M. & ap Rees T. (1996a) Characterization of transgenic potato (*Solanum tuberosum*) tubers with increased ADP-glucose phyrophosphorylase. *Biochemical Journal* **320**, 487-492.

Sweetlove L.J., Burrell M.M. & ap Rees T. (1996b) Starch metabolism in tubers of transgenic potato (*Solanum tuberosum*) with increased ADP-glucose phyrophosphorylase. *Biochemical Journal* **320**, 493-498.

Sweetlove L.J., Kossman J., Riesmeier J.W., Trethewey R.N. & Hill S.A. (1998) The control of source to sink carbon flux during tuber development in potato. *The Plant Journal* **15**, 697-706.

Taiz L. & Zeiger E. (1991) Plant Physiology. The Benjamin/Cummings Publishing Company, Inc., Redwood City, Calif., USA.

Takeda S., Mano S., Ohto M.-a. & Nakamura K. (1994) Inhibitors of protein phosphatases 1 & 2A block the sugar inducible gene expression in plants. *Plant Physiology* **106**, 567-574.

Telfer A. & Poethig R.S. (1998) *HASTY*: a gene that regulates the timing of shoot maturation in *Arabidopsis thaliana*. *Development* **125**, 1889-1898.

Thorne J.H. & Rainbird R.M. (1983) An *in vivo* technique for the study of phloem unloading in seed coats of developing soybean seeds. *Plant Physiology* **72**, 268-271.

Thornley J.H.M. (1972) A balanced quantitative model for root: shoot ratios in vegetative plants. *Annals of Botany* **36**, 431-441.

Thornley J.H.M & Johnson I.R. (1990) Plant and crop modelling: A mathematical approach to plant and crop physiology. Clarendon Press, Oxford.

Tingey S.V., Tsai F.Y., Edwards J.W., Walker E.L. & Coruzzi G.M. (1988) Chloroplast and cytosolic glutamine synthetase are encoded by homologous nuclear genes which are differentially expressed *in vivo*. *Journal of Biological Chemistry* **263**, 9651-9657.

Trewavas A.J. (1981) How do plant growth substances work? *Plant, Cell & Environment* **4**, 203-228.

Trewavas A.J. (1982) Growth substance sensitivity: the limiting factor in plant development. *Physiologia Plantarum* **55**, 60-72.

Trewavas A.J. (1983) Is plant development regulated by changes in the concentration of growth substances or by changes in the sensitivity to growth substances? Sensitivity is the regulating factor. *Trends in Biochemical Sciences* **8**, 354-357.

Trewavas A.J. (1991) How do plant growth substances work? II. *Plant, Cell & Environment* **14**, 1-12

Truernit & Sauer (1995) The promoter of the *Arabidopsis thaliana* SUC2 sucrose-$H^+$ symporter directs expression of GUS to the phloem: Evidence for phloem loading and unloading by SUC2. *Planta* **196**, 564-570.

Turgeon R. & Webb J.A. (1975) Leaf development and phloem transport in *Curcurbita pepo*: maturation of minor veins. *Planta* **121**, 265-269.

van Bel A.J.E. & Gamelai Y.V. (1991) Multiprogrammed phloem loading. In: Recent Advances in Phloem Transport and Assimilate Compartmentation (eds. Bonnemain J.-L., Delrot S., Lucas W.J. & Dainty J.),128-139. Ouest Editions, Nantes.

van Bel A.J.E. (1992) Pathways and mechanisms of phloem loading. In: Carbon Partitioning within and between organisms (eds. Pollock, C.J., Farrar, J.F. and Gordon, A.J.), 53-70. Bios.

von Schaewen A., Stitt M., Schmidt R., Sonnewald U. & Willmitzer L. (1990) Expression of a yeast-derived invertase in the cell wall of tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants. *The EMBO Journal* **9**, 3033-3044.

Vance C.P. (1997) The molecular biology of N metabolism. In: Plant Metabolism (eds. Dennis D.T., Turpin D.H., Lefebvre D.D. and Layzell D.B.), Addison Wesley Longman Ltd., 449-478.

Wardlaw I.F. (1990) Tansley Review No. 27: The control of carbon partitioning in plants. *New Phytologist* **116**, 341-381.

Weber H., Borisjuk L. & Wobus U. (1996) Controlling seed development and seed size in *Vicia faba*: a role for seed coat associated invertases and carbohydrate state. *Plant Journal* **10**, 823-834.

Weber H., Golombek S., Heim U., Borisjuk L., Panitz R., Manteuffel R. & Wobus U. (1998a) Integration of carbohydrate and nitrogen metabolism during legume seed development: implications for storage product synthesis. *Journal of Plant Physiology* **152**, 641-648.

Weber H., Heim U., Golombek S., Borisjuk L. & Wobus U. (1998b) Assimilate uptake and the regulation of seed development. *Seed Science Research* **8**, 331-345.

Weber H., Heim U., Golombek S., Borisjuk L., Manteuffel R. & Wobus U. (1998c) Expression of a yeast-derived invertase in developing cotyledons of *Vicia narbonensis* alters the carbohydrate state and affects storage functions. *Plant Journal* **16**, 163-172.

Williams J.H., Minchin P.E.H. & Farrar J.F. (1991) Carbon partitioning in split root systems of barley: the effect of osmotica. *Journal of Experimental Botany* **42**, 453-460.

Wolf S. & Lucas W.J. (1994) Virus movement proteins and other molecular probes of plasmodesmatal function. *Plant, Cell and Environment* **17**, 573-585.

Wolswinkel P. & Ammerlaan A. (1983) Phloem unloading in developing seeds of *Vicia faba* L.. The effect of several inhibitors on the release of sucrose and amino acids by the seed coat. *Planta* **158**, 205-215.

Woodrow I.E. & Berry J.A. (1988) Enzymatic regulation of photosynthetic $CO_2$ fixation in C3 plants. *Annual Review of Plant Physiology and Molecular Biology* **39**, 533-594.

Wray J.L. (1993) Molecular biology, genetics and regulation of nitrite reduction in higher plants. *Physiologia Plantarum* **89**, 607-612.

Yamaya T., Hayakawa T., Tanasawa K., Kamachi K., Mae T. & Ojima K. (1992) Tissue distribution of glutamate synthase and glutamine synthetase in rice leaves. *Plant Physiology* **100**, 1427-1432.

Zhang J. & Lechowitz M.J. (1995) Responses to $CO_2$ enrichment by two genotypes of *Arabidopsis thaliana* differing in their sensitivity to nutrient availability. *Annals of Botany* **75**, 491-499.

Zrenner R., Salanoubat M., Willmitzer L. & Sonnewald U. (1995) Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (*Solanum tuberosum* L. ). *Plant Journal* **7**, 97-107.

# Appendix 1

Formula of Johnson's Medium

## Johnson's Medium[1]

### Macronutrients

| Compound | MW | [stock]M | [stock]g.l$^{-1}$ | $^{\text{Stock volume}}/_{\text{Final volume}}$ ml | Element | Final [Element]$\mu$M |
|---|---|---|---|---|---|---|
| $KNO_3$ | 101.10 | 1.00 | 101.10 | 6.0 | N | 16000 |
| $Ca(NO_3)_2.4H_2O$ | 236.16 | 1.00 | 236.16 | 4.0 | K | 6000 |
| $NH_4H_2PO_4$ | 115.08 | 1.00 | 115.08 | 2.0 | Ca | 4000 |
| $MgSO_4.7H_2O$ | 246.49 | 1.00 | 246.49 | 1.0 | P | 2000 |
| | | | | | S | 1000 |
| | | | | | Mg | 1000 |

### Micronutrients[2]

| Compound | MW | [stock]mM | [stock]g.l$^{-1}$ | $^{\text{Stock volume}}/_{\text{Final volume}}$ ml | Element | Final [Element]$\mu$M |
|---|---|---|---|---|---|---|
| KCl | 74.55 | 50 | 3.728 | 1.0 | Cl | 50 |
| $H_3BO_3$ | 61.84 | 25 | 1.546 | 1.0 | B | 25 |
| $MnSO_4.H_2O$ | 169.01 | 2.0 | 0.338 | 1.0 | Mn | 2.0 |
| $ZnSO_4.7H_2O$ | 287.55 | 2.0 | 0.575 | 1.0 | Zn | 2.0 |
| $CuSO_4.5H_2O$ | 249.71 | 0.5 | 0.125 | 1.0 | Cu | 0.5 |
| $H_2MoO_4$ (85% $MoO_3$) | 161.97 | 0.5 | 0.081 | 1.0 | Mo | 0.5 |
| Fe-EDTA[3] | 346.08 | 20 | 6.922 | 1.0 | Fe | 20 |

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[1] After Johnson *et al.*, (1957), modified.

[2] A combined stock solution is made up combining all micronutrients except iron.

[3] Ferrous dihydrogen ethylenediaminetetraacetic acid.

# Appendix 2

Robinson C. K. & Hill S. A. (1999)

Altered resource allocation during seed development

in *Arabidopsis* caused by the *abi3* mutation.

*Plant, Cell & Environment* **22**, 117-123