Investigation of the Regulation of carbohydrate metabolism in *Arabidopsis thaliana* using a genetic approach

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Abbreviations

ADP	adenosine diphosphate
AK/HSD	aspartate kinase/homoserine dehydrogenase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
cab	chlorophyll a/b binding protein
cAMP	cyclic 3', 5'-cyclic monophosphate
cDNA	complementary DNA
CHS	chalcone synthase enzyme
chs	chalcone synthase gene
CoA	coenzyme A
CTAB	hexadecyltrimethyl ammonium bromide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid (disodium salt)
ER	endoplasmic reticulum
F6P	fructose-6-phosphate
G6P	glucose-6-phosphate
GUS	β-glucuronidase
HAI	hours after imbibition
ICL	isocitrate lyase enzyme
icl	isocitrate lyase gene
kDa	kilodalton
M1P	mannose-1-phosphate
M6P	mannose-6-phosphate
M&S	Murashige and Skoog
MES	4-morpholineethane-sulphonic acid
MODY	maturity onset diabetes of the young
mRNA	messenger RNA
MS	malate synthase enzyme
ms	malate synthase gene
MOPS	3-(N-morpholino)propanesulphonic acid
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NADP	nicotinamide adenine dinucleotide phosphate (oxidised form)
NAPH	nicotinamide adenine dinucleotide phosphate (reduced form)

NPTII	neomycin phosphotransferase, type II
nr	nitrate reductase gene
PEP	phosphoenolpyruvate
PEPCK	phosphoenolpyruvate carboxykinase
PFK	phosphofructokinase
Pi	inorganic phosphate
PMI	phosphomannose isomerase
rbcS	ribulose-1,5- bisphosphate carboxylase small subunit gene
RNA	ribonucleic acid
Rubisco	ribulose-1,5- bisphosphate carboxylase/oxygenase
SDS	sodium dodecylsulphate
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
SNF-1	sucrose non-fermenting protein kinase 1
SPS	sucrose phophate synthase
SUSY	sucrose synthase
TCA	tricarboxylic acid
T-DNA	transfer-DNA
TE	tris-EDTA buffer
Tris	tris (hydroxymethyl) methylglycine
v/v	volume to volume (expressed as a percentage)
w/v	weight to volume (expressed as a percentage)

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Abstract

The regulation of carbohydrate metabolism in *Arabidopsis thaliana* was investigated using a genetic approach. A new class of carbohydrate insensitive mutant (*cai*) was characterised in order to gain insight into the control of carbohydrate metabolism. Wild type seedlings germinated on media containing 100 mM sucrose and 0.1 mM nitrogen but their cotyledons did not expand and accumulated anthocyanins. After 1 week growth was arrested. The internal carbohydrate content increased accompanied by repression of photosynthetic genes and induction of *chs* gene expression. *cai* mutants germinated on agar media containing 100 mM sucrose and 0.1 mM nitrogen but their cotyledons expanded and greened. After initial characterisation of a number of the mutants, two were selected for further analysis. When germinated on a range of different carbon; nitrogen ratios *cai* 10 and *cai* 28 displayed a reduced sensitivity to the high carbohydrate and low nitrogen conditions. *cai* 10 also displayed a mannose insensitive *(mig)* phenotype compared to the post-germinative growth of wild type which was arrested by mannose. This growth arrest in the wild type on mannose correlates with phosphate sequestration. *cai* 10 metabolises mannose at a different rate and accumulates less hexose phosphate than the wild type when germinated on mannose, thus indicating that the mannose insensitive phenotype may be a consequence of a disruption in metabolism. Overexpression of *Arabidopsis* hexokinase 1 in *cai* 10 did not complement the *cai* 10 phenotype. In contrast to previous results by Jang *et al* ., (1997), who found that plants overexpressing hexokinase were hypersensitive to sugars, our results indicate that they are less sensitive than wild type. This is not in agreement with the proposed model of hexokinase as a sugar sensor (Jang *et al.*, 1997).

Seeds of the hexokinase overexpressors germinated rapidly (within 18-20 h). The seeds also contained elevated levels of some amino acids, smaller lipid bodies and less lipid than the wild type. It is proposed that hexokinase overexpression increases glucose-6-phosphate concentration which activates phosphoenolpyruvate carboxylase (PEPCase) and in so doing diverts carbon from lipid biosynthesis to amino acid synthesis.

Chapter 1

Introduction

1.1.1 Source-sink relations in plants

In order to survive, all organisms need to be able to modify their cellular metabolism and growth in response to environmental and developmental cues, and in particular the availability of nutrients. Sugars not only serve a major function as the substrates for growth and development, but are also capable of signalling the metabolic status of cells which affects sugar sensing systems and initiates changes in gene expression. Plants must be able to adapt their metabolism to perceived changes in nutrient status and stresses because they are, on the whole, unable to move to more favourable conditions.

Plants are autotrophic and must coordinate photosynthetic production of carbohydrate with its mobilisation, allocation and utilization. The ability to sense and respond to the sugar status in different organs is vital to the efficient metabolism of the plant. Sugar-regulated genes not only provide a means of balancing carbon metabolism with the requirements of individual cells but also initiate carbon transport from source cells (such as in photosynthetic leaves) to sink cells (such as developing seeds or roots). Thus, sugar sensing must be integrated at the level of cell, tissue, organ and whole plant and hence, there must be cross-talk between sugar- and other signalling pathways such as those that signal developmental stage. Much work needs to be done to establish the mechanisms used by plants to sense sugars. However, there is evidence to show that plant sugar signalling has parallels with the situation in yeast and bacteria, mutants of which have been extensively studied (Trumbly, 1992, Saier, *et al.*, 1995).

Sucrose produced in source leaves is the predominant source of carbon for developing sink tissues in most higher plants. The flux of sugars into the sinks is thought to be a major determinant of plant growth and crop yield. Sucrose is the major form of transportable carbohydrate in most plants and much work has gone into studying the regulation of sucrose biosynthesis with the aim of altering crop yield (Stitt and Quick, 1989). In recent years, the manipulation of specific enzyme activities in transgenic plants

has been used to probe the control of assimilate flow. Several groups have tried to alter the partitioning of carbon using transgenic plants.

1.1.2 Manipulation of sucrose synthesis in source tissues

The major controlling steps in the sucrose biosynthetic pathway are thought to be the interconversion of fructose-1,6-bisphosphate (F16BP) to fructose-6-phosphate (F6P), the formation of sucrose-6-phosphate (S6P) from UDP-glucose (UDPG) and F6P (Stitt and Quick, 1989) and the dephosphorylation of S6P to yield sucrose. F16BP is converted to F6P by F-1,6-bisphosphatase (F16BPase) and the reverse reaction is catalysed by phosphofructokinase (PFK). A third enzyme, pyrophosphate dependent: phosphofructokinase (PFP) is able to catalyse the reaction in both directions. In contrast, only one enzyme (sucrose phosphate synthase) is responsible for the formation of S6P and one enzyme (sucrose phosphate phosphatase) dephosphorylates S6P to sucrose.

The activities of FBPase and PFP are subject to allosteric control by the signal metabolite fructose-2,6-bisphosphate (F-2,6-BP) (Stitt, 1990). Cytosolic FBPase is also regulated at the transcriptional and post-translational level (Harn and Daie, 1992, Khayat, *et al.*, 1993).

The importance of the interconversion of F-1,6-BP and F6P *in vivo* was studied in transgenic potato and tobacco by expressing a pyrophosphatase from *E. coli* (Jelitto, *et al.*, 1992), antisense inhibition of FBPase (Zrenner, *et al.*, 1996) or antisense inhibition of PFP (Hajirezaei, *et al.*, 1994, Paul, *et al.*, 1995). Overexpression of *E. coli* pyrophosphatase decreased the PPi pool and effectively made the reaction catalysed by PFP irreversible, thus increasing the flux of carbon towards sucrose. However, the growth of the plants was stunted and carbon was allocated to sucrose in preference to starch (Jelitto, *et al.*, 1992). Reduction of the cytosolic FBPase in potato limited photosynthetic sucrose biosynthesis but did not alter plant growth and tuber yield (Zrenner, *et al.*, 1996). Reduction of PFP in tobacco plants also had no affect on the phenotype, and the partitioning of sucrose and starch in source leaves was identical to wild type (Paul, *et al.*, 1995). Transgenic potato plants with strongly decreased expression of PFP also showed no visible phenotype

(Hajirezaei, et al., 1994). It was suggested that PFP does not play a significant role in photosynthate partitioning. Numerous roles for PFP have been proposed including regulation of PPi concentration and general adaptability to stress (Black, et al.,1987) and gluconeogenesis (Botha and Botha, 1993) although the precise function of PFP remains an enigma.

The relative importance of other Calvin cycle enzymes in regulating carbon flow and partitioning has also been investigated using transgenic plants. Photosynthetic carbon assimilation is thought to be limited by Rubisco in high light intensities and temperature or low CO_2 concentration (Stitt and Schulze, 1994), whereas at low light intensities the regeneration of ribulose-1,5-bisphosphate (RuBP) is thought to limit carbon assimilation (Gray *et al.*, 1995). Photosynthesis was not inhibited by Rubisco until 40-50 % of Rubisco protein was removed (Stitt and Schulze, 1994) when grown under constant low light conditions but when transferred to high light conditions the control coefficient of Rubisco increased. Plastid FBPase did not affect photosynthesis until 60 % of wild type activity was removed (Kossmann, *et al.*, 1995). Plants with less than 15 % of wild type sedoheptulose-1,7-bisphosphatase (SBPase) activity were found to have reduced carbon assimilation rates and contain less than 5 % of wild type starch levels (Harrison, *et al.*, 1998). Phosphoribulokinase (PRK) did not affect carbon assimilation rate until less than 15 % of wild type activity had been remained (Gray, *et al.*, 1995). When PRK activity was 5-15 % of wild type the transformants contained 20 % more starch and 20 % less soluble sugars. Photosynthesis was inhibited when NADP-glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPDH) activity was reduced to 65 % of wild type activity (Price, *et al.*, 1995). In none of the above studies has the decrease in enzyme activity been found to correlate with a proportionate decrease in photosynthesis. However, small changes in the activity of plastid aldolase, a Calvin cycle enzyme which has no known regulatory properties and was thought to be irrelevant in control of metabolism and growth (Haake, *et al.*, 1998), were found to have marked consequences for photosynthesis, carbon partitioning and growth (Haake, *et al.*, 1998).

Modulation of SPS by protein phosphorylation has been well documented, with changes in the phosphorylation state of the protein occurring in response to light-dark transitions (Huber, *et al.*, 1989), G6P, Pi, pH (Doehlert and Huber, 1983), and changes in the total amount of protein (Walker and Huber, 1989). Maize SPS was overexpressed in tomato (Gaultier, *et al.*, 1993) but analysis of the plants revealed only a very small stimulation in sucrose synthesis. Another study showed that most of the excess SPS protein was deactivated, probably due to post-translational modification (Krause, 1994). Overexpressing SPS in *Arabidopsis* led to overall lower carbohydrate levels in leaves possibly due to increased export of sucrose. Unlike the SPS in many species, *Arabidopsis* SPS is not regulated by light-dark induced phosphorylation and so the expression of the gene is reflected in the enzyme activity (Signora, *et al.*, 1998).

The major conclusion of these experiments was that a single gene does not control carbon flux and sucrose biosynthesis. To increase the flux through a pathway it may not be enough to increase the expression of 'key' regulatory enzymes. Moreover, there is no evidence that the importance of a particular enzyme can be gauged by its regulatory properties or its ability to catalyse irreversible reactions (Haake, *et al.*, 1998).

1.1.3 Allocation of carbon in sink tissues

Assimilates that are produced in the chloroplasts of mature leaves are exported to the cytosol. The surplus that is not needed for leaf metabolism is converted to sucrose or amino acids and is transported through the vascular system to the sink tissues. Seeds, roots, fruits, tubers and young leaves can all be classed as sink tissues during their development. In seeds, sucrose is transported to the developing embryo via the phloem. In many species the embryo is symplastically separated from the maternal seed coat (Thorne, 1985). Assimilates, therefore, have to pass at least 2 membranes and possibly 2 different transport systems (Frommer and Sonnewald, 1995, McDonald, *et al.*, 1996, Weber, *et al.*, 1996).

There is continuing discussion over whether the rate of import into growing sinks is source- or sink-limited (Farrar, 1996). There is evidence for both source- and sink-limitation

of carbon metabolism depending on the growth conditions (Wardlaw, 1990, Zrenner, *et al.*, 1995, Weber, *et al.*, 1996, Sweetlove, *et al.*, 1998).

Imported sucrose must be cleaved prior to use in metabolism or storage product synthesis. Sucrose synthase and invertase are involved in catalysing this process and it has been proposed that each enzyme operates in a specific metabolic pathway (Weber, *et al.*, 1997, Weber, *et al.*, 1998a). In general the invertase pathway is directed towards growth and cell expansion, whereas seed tissues actively synthesising starch often depend on a sucrose synthase (Edwards and apRees, 1986, Weber, *et al.*, 1995). The latter mechanism, which can be classed as sink regulation of metabolism, lowers the sucrose concentration in the embryo and creates a sucrose gradient which drives import (Weber, *et al.*, 1997).

Several groups have overexpressed invertase in sink tissues in an attempt to increase the strength of storage sinks. In both potato tubers and *Vicia narbonensis* cotyledons overexpression of invertase lead to a decrease in sucrose and an increase in hexoses which resulted in less carbon being partitioned into starch (Sonnewald, *et al.*, 1997, Weber, *et al.*, 1998b). It has been suggested that the sink does not have the capacity to metabolise glucose fast enough to keep up with its production (Trethewey, *et al.*, 1998). Glucokinase was introduced into invertase overexpressing potato tubers in an attempt too increase the rate of glucose metabolism and increase starch accumulation but starch synthesis was again drastically reduced (Trethewey, *et al.*, 1998). However, glycolysis was stimulated and there were large increases in glycolytic intermediates, organic acids, amino acids and 3-5 fold increase in CO_2 production. Flux through glycolysis was increased at the expense of starch production (Trethewey, *et al.*, 1998). Hexokinase activity was increased both in tubers overexpressing invertase (Trethewey, *et al.*, 1998) and in tubers where the activity of SUSY was inhibited by antisense repression (Zrenner, *et al.*, 1995). Recent work, varying the flux to growing potato tubers by changing the light intensity or using transgenic manipulations that specifically affect the source or the sink, found that the flux control coefficient of the source was 0.8 and the sink was 0.2 (Sweetlove, *et al.*, 1998). However, this work is restricted to the short-term control of flux and does not take into account long-term regulation of enzyme activity through changes in gene expression. The authors

suggested that the best way of manipulating tuber yield will involve photosynthetic capacity rather than sink metabolism (Sweetlove, *et al.*, 1998). This is in agreement with the work of Weber *et al*, (1998) who found that starch accumulation in *Vicia narbonensis* cotyledons is a function of sucrose concentration. In contrast, inhibition of SUSY in potato tubers also lead to a decrease in starch accumulation and supports the hypothesis that SUSY is the major determinant of sink strength (Zrenner, *et al.*, 1995). The debate continues.

Active storage organs like seeds often contain high levels of sugars. In *Vicia faba*, high concentrations of hexoses are found in non-differentiated premature regions of the cotyledon whereas mature starch-accumulating regions contain low glucose concentrations (Borisjuk, *et al.*, 1998). The glucose distribution is related to the developmental gradient and high hexose state is correlated with growth and mitotic activity (Weber, *et al.*, 1997, Borisjuk, *et al.*, 1998).

In mature *Brassica napus* seeds the main storage products are oil and protein (Murphy and Cummins, 1989) but starch accumulates transiently during the early phase of oil deposition (Kang and Rawsthorne, 1994). Of a number of carbon substrates tested, G6P was most effective at synthesising starch and pyruvate was best substrate for fatty acid synthesis (Kang and Rawsthorne, 1994). There is little competition between the 2 substrates for fatty acid and starch synthesis. Fatty acid synthesis requires both NADPH and NADH due to the specificities of the enzymes in the pathway to C-18 fatty acids (Harwood, 1988). Metabolism of G6P via the plastidial OPPP can potentially contribute to the NADPH requirement for fatty acid synthesis (Kang and Rawsthorne, 1996).

1.1.4 Interactions between carbon and nitrogen assimilation: Regulation of carbon flow into amino acids

Carbohydrate and nitrogen metabolism are interconnected. The flow of carbon into sucrose, starch and amino acids is subject to regulation by light, photosynthesis related metabolites and nitrogenous compounds (Champigny and Foyer, 1992, Lam, *et al.*, 1995); Champigny, 1992). In conditions of high sugar but limiting nitrogen, carbohydrates accumulate in preference to amino acids whereas in conditions of high sugars and plentiful

nitrogen a larger proportion of the carbon is partitioned into amino acids (Champigny and Foyer, 1992, Sadka, *et al.*, 1994).

In tobacco, nitrate can act as a signal to induce organic acid metabolism and repress starch metabolism (Scheible, *et al.*, 1997). Sucrose feeding stimulates amino acid assimilation and increased rates of α-ketoglutarate synthesis in leaves (Moracuende, *et al.*, 1998).

The main routes of entry of carbon into the citric acid cycle are via phosphoenolpyruvate carboxylase (PEPCase) and pyruvate kinase (PK). There is substantial evidence that they regulate the increase in anapleurotic carbon flow during nitrogen assimilation (Turpin, *et al.*, 1990). PEPCase is a cytosolic enzyme that converts PEP from glycolysis to oxaloacetate (OAA). The OAA produced is rapidly converted to malate and is imported into the mitochondrion where it enters the TCA cycle to replace ketoacids which have been siphoned off for amino acid synthesis (Turpin, *et al.*, 1997). The addition of nitrogenous compounds induces PEPCase expression in maize leaves (Sugiharto, *et al.*, 1992) and activates PEPCase enzyme (Mahn, *et al.*, 1993, Chollet, *et al.*, 1996). PEPCase enzyme is also activated by phosphorylation by PEPC kinase and allosterically by G6P (Chollet, *et al.*, 1996). Pyruvate kinase, the other route of carbon into the citric acid cycle, is inhibited by amino acids such as glutamate and glutamine (Podesta and Plaxton, 1994). In nitrogen-rich conditions, there is also an increase in cellular respiration, because of the demand placed on respiratory pathways to supply carbon skeletons needed for the incorporation of nitrogen into organic form. This regulation of respiration, and PEPCase and PK activities coordinates the supply of carbon in glycolysis and the need for carbon skeletons for amino acid synthesis.

The primary route of amino acid biosynthesis is the incorporation of ammonium and glutamate into glutamine by glutamine synthetase (GS). The glutamate synthase enzyme (GOGAT; L-glutamate : ferredoxin oxidoreductase[*trans*-aminating]) transfers the glutamine amide group to α-ketoglutarate to yield 2 molecules of glutamate. Glutamate is the net product of the GS/GOGAT cycle. Glutamate is then transaminated into aspartate, which is then either converted into asparagine by asparagine synthetase, or converted to

other amino acids in the aspartate family (lysine, threonine, methionine or isoleucine) involving aspartate kinase/homoserine dehydrogenase (AK/HSD) (Zhu-Shimoni and Galili, 1998).

The synthesis of glutamine, glutamate, aspartate and asparagine is subject to coordinate metabolic regulation. The expression of *Arabidopsis* and pea glutamine synthetase is stimulated by light (Lam, *et al.*, 1994, Lam, *et al.*, 1995) and the expression of AK/HSD is metabolically induced by sucrose and repressed by phosphate but is not regulated by nitrogenous compounds (Zhu-Shimoni and Galili, 1998). The expression of asparagine synthetase is repressed by light and sucrose and stimulated by dark and nitrogen (Tsai and Coruzzi, 1990, Tsai and Coruzzi, 1991, Lam, *et al.*, 1994, Lam, *et al.*, 1995, Chevalier, *et al.*, 1996). The sucrose repression affect on asparagine synthetase can be partially rescued by the addition of exogenous amino acids (Lam, *et al.*, 1994). Thus asparagine which has a high nitrogen: carbon ratio is synthesized when nitrogen is abundant and sugars levels are low. This suggests that the nitrogen : carbon ratio rather than carbon alone is responsible for the regulation of the asparagine synthetase gene (Lam, *et al.*, 1994). It has been suggested that during the day, when glutamine synthetase and AK/HSD are expressed, plants accumulate glutamine, glutamate and aspartate, which are used for the synthesis of other amino acids and at night, when asparagine synthetase is expressed, aspartate is converted to asparagine for storage (Lam, *et al.*, 1995, Zhu-Shimoni and Galili, 1998).

1.2 *Arabidopsis* Seeds

1.2.1 Deposition of reserves within seeds

Under optimal conditions, a single *Arabidopsis thaliana* plant can produce 20, 000 seeds in fruits known as siliques. The seeds are very small, with the wild type being only 0.5 mm long and 18-30 μg in weight at maturity (Meinke, 1994). Within a single silique, the development of seeds is generally synchronous and the seeds are usually the same size.

The accumulation of seed reserve materials in developing *Arabidopsis* has been studied in some detail, storage protein and lipid synthesis being most extensively examined.

The reserves accumulate during a well-defined and relatively short period during late embryogenesis 144-216 hours after fertilisation (HAF)(Mansfield and Briarty, 1992).

1.2.2 Amino acid transport to seeds

Amino acid import is essential for seed development since the accumulation of storage compounds must be preceded by import and since metabolites are involved in storage protein accumulation (Martin, *et al.*, 1997). Amino acids produced in the roots or shoots are transported to sink organs via the phloem and xylem. In *Arabidopsis*, asparagine, aspartate, glutamine and glutamate are the major transport forms of amino acids (Lam, *et al.*, 1995). Amino acids arriving at the seed via the phloem are taken up symplastically. They have to cross a plasma membrane and so two transport steps are necessary (Thorne, 1985) and several types of amino acid transporters have been isolated (Frommer, *et al.*, 1995; Hirner, *et al.*, 1998).

1.2.3 Storage proteins

Seed storage proteins accumulate in membrane-bound protein bodies present in the hypocotyl and cotyledons of developing embryos (Patton and Meinke, 1990, Mansfield and Briarty, 1992). Microscopy studies of developing legume seeds revealed that protein bodies are of vacuolar origin, and the presence of vacuolar enzymes within the protein bodies supports this theory (Casey, *et al.*, 1997). At the end of the cell expansion phase the cotyledon cells usually contain 1 or 2 vacuoles. At the onset of storage protein deposition, the protein accumulates on the luminal side of the vacuolar membrane. The membrane then surrounds these deposits and small, virtually mature protein bodies are pinched off. This is repeated throughout the protein accumulation phase during which the central vacuole divides and is replaced by many small vacuoles and protein bodies (Casey, *et al.*, 1997).

Cereal seed proteins have been classified into 4 different groups, albumins, globulins, glutelins and prolamins depending on their solubility. The nutritional value of these proteins is determined by their content of amino acids. Albumins and globulins are not seriously deficient in any amino acids. Prolamins, which predominate in maize, are

seriously deficient in lysine. Attempts have been made to increase the content of lysine in maize. The maize *opaque-2* mutant is high in lysine and contains less prolamin storage protein and more lysine rich glutelin resulting in an altered total amino acid composition (Mertz, *et al.*, 1964).

Most storage proteins should not be thought of as a single protein but rather as a complex of individual proteins bound together by a combination of intermolecular disulphide groups, hydrogen bonding, ionic bonding and hydrophobic bonding (Bewley and Black, 1994). Characteristically, storage proteins are made up of 2 or more subunits which may be made up of several slightly varying polypeptides that differ in amino acid composition. For instance, *Arabidopsis* contains abundant 12 S (cruciferin) and 2 S (arabin) proteins. Separation of the subunits by SDS-PAGE reveals that in total the subunits are composed of at least 9 different polypeptides (Heath, *et al.*, 1986).

1.2.4 Protein body inclusions

The protein bodies of *Arabidopsis* and many other species contain inclusions called globoids which are non-crystalline, globular structures (Mansfield and Briarty, 1992). Globoids appear in the developing protein bodies before storage protein accumulates and act as nuclei for the deposition of material (Mansfield and Briarty, 1992). Globoids are the sites of phytin deposition. Phytin is the collective name for the insoluble potassium, magnesium and calcium salts of phytic acid (myo-inositol hexaphosphoric acid). Phytin is present in relatively minor quantities but it is an important source of phosphate and mineral elements (Bewley and Black, 1994). Although the biosynthetic pathway of phytin is unclear, it is likely that myoinositol-1-P is synthesized from G6P and then a further 5 phosphates are added, with ATP as the donor, to form myoinositol hexaphosphoric acid (phytic acid). The association of ions with phytic acid is believed to occur randomly by attraction of the metallic cations to the strong negative charges on the exposed phosphate groups (Bewley and Black, 1994). Phytase hydrolyses phytin during germination to release phosphate, its associated cations and myoinositol. The released myoinositol phosphate may be used by the growing seedling for cell wall synthesis since this sugar is a precursor of pentosyl and

uronosyl sugar units normally associated with pectin and other cell wall polysaccharides (Bewley and Black, 1994).

1.2.5.1 Lipid reserves

Lipids in the form of energy rich triacyl glycerols (TAG) are a major form of storage carbon in many seeds. The seed lipids of *Arabidopsis* contain substantial proportions of both unsaturated 18-carbon fatty acids (30 % 18:2, 20 % 18:3) and long chain fatty acids (22 % 20:1) derived from 18 :1 (Browse and Somerville, 1994). The *de novo* synthesis of 16-carbon and 18-carbon fatty acids takes place almost exclusively in the chloroplasts (Ohlrogge, *et al.*, 1991, Ohlrogge, *et al.*, 1993) whereas this process occurs in the cytoplasm of animals and yeast (Schmid, *et al.*, 1997). Subsequent elongation steps that provide long-chain fatty acids for seed triacyl glycerols are believed to occur in the endoplasmic reticulum.

The fatty acid synthesis pathway uses acetyl-CoA as the building block for assembly of carbon chains (C16 and C18). Pyruvate, G6P, DHAP, acetate and malate can all serve as substrates for acetyl CoA synthesis in isolated oil seed rape plastids (Kang and Rawsthorne, 1994). Of these, pyruvate and G6P support the highest rate of fatty acid synthesis although G6P was utilised at 70% of the rate at which pyruvate was used (Kang and Rawsthorne, 1994). Acetyl CoA is probably primarily supplied to the pathway by the action of the pyruvate dehydrogenase complex (PDC) which catalyses the decarboxylation of pyruvate to acetyl CoA (Schmid, *et al.*, 1997). In chloroplasts, pyruvate can also be derived from the 3-phosphoglycerate produced in the Calvin cycle. In addition, plastids contain very active acetyl CoA synthetase, and *in vitro*, incorporate acetate into acetyl CoA. Therefore, it appears that acetyl CoA for fatty acid biosynthesis can be derived from the PDC reaction inside the plastid or from extraplastidial production of acetate followed by its activation inside the plastid by acetyl CoA synthetase (Schmid, *et al.*, 1997).

Nine 2 carbon units derived from acetyl CoA are condensed in the synthesis of a C18 fatty acid. The first committed step of fatty acid synthesis is the activation of acetyl CoA which is carboxylated to malonyl-CoA by acetyl CoA carboxylase (ACCase)

(Harwood, 1988). After formation of malonyl CoA all further steps of plant fatty acid synthesis require the acyl carrier protein (ACP). A repeated series of condensation, reduction, dehydration and saturation leads to the production of a fatty acid which is 16 or 18 carbons long (Schmid, *et al.*, 1997). In both seeds and leaves, 16:0-ACP is elongated to 18:0-ACP and then desaturated to 18:1-ACP by soluble desaturase (Shanklin and Somerville, 1990). The fatty acids are hydrolysed from ACP by a thioesterase and the free fatty acids are thought to move through the plastid envelope and are converted to acyl-CoA thioesters on the outer envelope, from where they are transported to the endoplasmic reticulum. The 18:1 free fatty acid can then undergo a series of modification reactions to produce TAG, polyunsaturates, epoxides, wax esters, hydroxides, conjugates or very long chain fatty acids (Murphy, 1993).

1.2.5.2 Lipid bodies

The TAG reserves in seeds are laid down in discrete subcellular organelles generally known as lipid or oil bodies. In *Arabidopsis* seeds, the oil bodies occupy a substantial volume of the cell. The oil bodies are surrounded by what appears to be half of a normal bilayer membrane (Yatsu and Jacks, 1972). The polar head groups of the membrane face the cytoplasm and the hydrophilic acyl groups face the centre of the oil body. The unit membrane is embedded with abundant structural proteins termed oleosins. Oleosins are alkaline proteins with a molecular mass of 15-24 kDa depending on species (Napier, *et al.*, 1996). They completely cover the surface of the oil body and can account for as much as 8-15 % of total seed protein (Murphy, 1993, Huang, 1996). Oil bodies remain as individual small organelles even after prolonged storage in plant seeds (Slack, *et al.*, 1980). Their stability is a consequence of steric hindrance and electronegative repulsion provided by proteins on the surface of the oil bodies (Tzen, *et al.*, 1992)

The mechanism of oil body biogenesis in plants is the subject of controversy and there are several hypotheses to explain their ontogeny. Oil bodies are first observed in oilseeds at an early to mid stage of seed development, and appear to lack any clear association with the protein synthesis machinery. Thus it has been postulated that they

originate by a budding off from the ER (Frey-Wyssling, *et al.*, 1963). It has been proposed that TAG is deposited between the leaflets of the phospholipid bilayer in the ER. Its accumulation causes a localised swelling of the ER, which buds off to form a small oil body enclosed by phospholipid monolayer (Wanner, *et al.*, 1981). Several observations support a second theory which proposes that oil bodies originate as naked oil droplets in the cytoplasm. Firstly, there is, as yet, no convincing electron microscopic evidence of budding off from the ER membrane (Bergfield, *et al.*, 1978). There is also evidence that isolated ER membranes are capable of synthesising TAG *in vitro*, forming oil droplets which do not coalesce and do not appear to have a phospholipid boundary (Stobart et al., 1986). From these results, TAG synthesis was postulated to occur on the outer surface of the ER (Stobart et al., 1986).

In addition to the continuing debate on the progenitor of oil bodies, there is also a great deal of uncertainty as to when the oleosins are incorporated. There is evidence that TAG and oleosins are synthesized concomitantly in the ER, from which a nascent mature lipid body is formed (Loer and Herman, 1993; Tzen *et al.*, 1993). Other investigations show that oleosin accumulation lags temporally behind that of TAG, and that oleosin is subsequently inserted into the oil bodies in the cytoplasm (Murphy and Cummins, 1989; Cummins and Murphy 1990). A third hypothesis suggests that during early seed development, oil bodies have very little oleosin on the surface. This correlates with low levels of oleosin transcript present during the early stages of TAG biosynthesis. These oil bodies are able to coalesce and the increasing surface density of the oleosins following each fusion event would limit their final size. During the mid late stages of seed development, when the rate of oleosin synthesis is higher, new oil bodies contain a relatively dense coating of oleosin. The oil bodies produced during late seed development would undergo fewer fusion events and the final size would be smaller than oil bodies made during an earlier stage, as has been observed experimentally (Rest and Vaughan, 1972; Cummins and Murphy, 1990).

1.2.6 Seeds with altered storage products

A number of mutants of *Arabidopsis*, including *wri1*, *fus3*, *lec1* and *tag* have been isolated which do not accumulate TAG in their seeds to the same extent as the wild type (Bäumlein, *et al.*, 1994, Meinke, *et al.*, 1994, Katavic, *et al.*, 1995, Focks and Benning, 1998).

A wrinkled seed mutant (*wri*1) has been isolated which has a low lipid content and a low seed weight (Focks and Benning, 1998). The *wri*1 mutation maps to the bottom of chromosome 3 and causes an 80 % reduction in seed oil content. The mature plants of *wri*1 and wild type are indistinguishable. However, developing seeds of *wri*1 mutants are impaired in the incorporation of sucrose and glucose into TAG, although pyruvate and acetate are incorporated at an increased rate. The activities of several glycolytic enzymes, including hexokinase and pyrophosphate dependent-phosphofructokinase are reduced in the developing seeds of homozygous *wri*1 mutants. The authors suggest that WRI1 is involved in developmental regulation of carbohydrate metabolism during seed filling. They suggested that WRI1 is either a regulatory protein governing carbohydrate metabolism during seed development or a novel hexokinase that may act as a sugar sensor in developing seeds, controlling the activity or expression of other glycolytic enzymes (Focks and Benning, 1998).

In the *tag* mutant, AS11, a decreased diacylglycerol acyltransferase (DGAT) activity is correlated with delayed seed development, a reduced TAG content and a repression of very long chain fatty acid synthesis (Katavic, *et al.*, 1995). The *tag* mutants have low 20:1 and 18:1, and high 18:3 fatty acids. Low diacylglycerol acyltransferase activity leads to a reduction of 20: 1 biosynthesis during seed development, leaving more 18:1 available for desaturation to an 18:3 chain (Katavic, *et al.*, 1995). The authors suggested that overexpression of DGAT earlier in development might provide a means of channelling more carbon into very long chain fatty acids and, ultimately, into TAG.

Leafy cotyledon mutants of *Arabidopsis* (*lec1*, *lec2* and *fus3*) also have altered storage product accumulation during embryo development (Meinke, *et al.*, 1994). Wild type embryos are normally filled with protein and lipid bodies but contain relatively small

amounts of starch at maturity and the hypocotyl and cotyledons are indistinguishable with respect to major storage products (Mansfield and Briarty, 1992). The cotyledons of *lec1* and *fus3* (allelic mutants) primarily contained starch. In *fus3*, two major classes of storage proteins, the 12S cruciferins and the 2S arabins are nearly absent, storage lipids are reduced and their composition is changed (Bäumlein, *et al.*, 1994). *lec2* have a gradient of starch and protein bodies in the cotyledons (with more starch at the tip and more protein bodies at the base of the cotyledon) but contained abundant lipid bodies in the hypocotyl (Meinke, *et al.*, 1994).

The *fus3* mutants seeds are desiccation intolerant although the immature seeds germinate precociously if transferred from the immature silique to humid conditions. Moreover, mutant alleles of the FUS3 gene are specifically defective in the gene expression programme responsible for seed maturation (Luerßen, *et al.*, 1998). Transcripts of abnormal sizes were found in the *fus3* mutants due to aberrant splicing caused by point mutations at intron termini (Luerßen, *et al.*, 1998). It has also been shown that the FUS3 gene product strongly induces the activity of several seed-specific gene promoters (Bäumlein, *et al.*, 1994).

Seed specific immunomodulation of abscisic acid was achieved in *Arabidopsis* by transforming plants with the gene for an antibody to abscisic acid under the control of the seed specific USP promoter (Phillips, *et al.*, 1997). In this way plants were created which had low or no abscisic acid in the seeds. The embryos contained fewer protein and oil bodies. Like the *fus3* mutants, seeds of plants with seed-specific immunomodulation of abscisic acid were intolerant of desiccation and germinated precociously if removed from the siliques during development.

1.2.7 Pattern of reserve mobilisation

Arabidopsis seed is said to have germinated when the radicle has protruded from the seed coat. The stimulation of embryo growth is the consequence of a signal cascade which emanates from the required environmental signal. In *Arabidopsis*, light is required for

germination and the active form of phytochrome (Pfr) is thought to be the initial trigger of this pathway (Shropshire, *et al.*, 1961).

The transition in metabolism between the phases of seed development and germination is remarkable. During seed development, metabolism is anabolic, manufacturing large amounts of lipid, proteins and carbohydrates. Germination and post-germinative growth is dominated by catabolic metabolism, when the reserves are broken down for seedling growth. These 2 opposite processes are separated by seed maturation and desiccation. During this period storage protein synthesis slows down and the storage protein mRNAs are degraded (Casey, *et al.*, 1997). Upon germination a different set of mRNAs are expressed in order to perform the catabolic function of storage reserve mobilisation.

Imbibition and germination in a small seeded weed-species such as *Arabidopsis thaliana* is a rapid process. The transition from the resting state to a phase of rapid reserve mobilisation and organelle differentiation occurs extremely quickly in the cotyledons, with major developmental changes occurring 24-36 h after imbibition (HAI) (Mansfield and Briarty, 1996). Structurally and biochemically, the transition from storage to photosynthetic tissues occurs within 48-60 hours after imbibition. After this point the tissue is no longer dependent on diminishing storage reserves. There are 3 phases in the development of the seedling: imbibition 0-24 HAI, seedling emergence 24-48 HAI, and the seedling development after emergence 48+ HAI (Mansfield and Briarty, 1996). In *Arabidopsis*, the hydrated cells become activated in an ordered sequence. Changes begin near the radicle apex and proceed acropetally with respect to the cotyledons. Reserves are mobilised first in all the epidermal cells. Reserve mobilisation in the radicle begins at the tip and progresses towards the hypocotyl (Mansfield and Briarty, 1996).

During protein hydrolysis, the protein bodies undergo significant changes in volume (V), surface area (SA) and V: SA ratio, as they are converted from a protein store to a sap-filled vacuole. In all oil seed species studied so far, including *A. thaliana*, the protein bodies swell initially and then fuse during hydrolysis, forming a large central vacuole (Mansfield and Briarty, 1996).

Hydrolysis of storage proteins to their constituent amino acids requires the presence of proteinases. The amino acids liberated from the storage proteins may be reutilized for protein synthesis or deaminated to provide carbon skeletons for respiration (Casey, *et al.*, 1997). Amides, such as asparagine and glutamine, are the major transportable form of amino acid and are carried by the vascular system from the storage organs to the growing seedling. Some amino acids, e.g., aspartate, glutamate, alanine, glycine and serine can be converted to sucrose and transported as sugar to the growing seedling (Bewley and Black, 1994).

In *Arabidopsis* extremely few microbodies are observed during seed development (Mansfield and Briarty, 1992) or maturation and the vast majority of microbodies are probably synthesized during germination. The increase in single microbody volume during early germination (12-36 hours) support the theory that enzymes are added to the microbodies present in early imbibed tissue (Kunce, *et al.*, 1984). Following germination all the gloxysomal enzymes increase during the first 2-3 days of growth (if grown at a constant 25-30 °C) and then decline.

When seeds germinate the TAG reserves are mobilised. The process commences with lipases which catalyse the hydrolysis of fatty acid from the glycerol backbone (Huang, 1987). The fatty acids are converted to oxaloacetate by the enzymes of ß-oxidation (Gerhardt, 1992, Gerhardt, 1993) and the glyoxylate cycle (Weir, *et al.*, 1980). These reactions are confined to the glyoxysome. In ß-oxidation, 2 carbon units are cleaved from a fatty acyl-CoA beginning at its carboxyl end. Each 2-carbon is oxidised by a series of reactions culminating in the release of acetyl CoA. The oxidation of acyl CoA to enoyl CoA releases hydrogen peroxide which is detoxified by catalase, a key enzyme in the glyoxysome (Gerhardt, 1992). In the process of gluconeogenesis, the glyoxylate cycle converts acetyl CoA to 4-carbon compounds. The acetyl CoA condenses with oxaloacetate to form citrate which is then isomerised to isocitrate. Isocitrate lyase, an enzyme unique to the glyoxylate cycle, converts isocitrate to succinate and glyoxylate. Malate synthase, also a unique glyoxylate cycle enzyme, combines the acetyl CoA with glyoxylate to form malate (Graham, *et al.*, 1989). Oxaloacetate is regenerated from malate to complete the cycle. The

net gain of the glyoxylate cycle is succinate. Succinate is metabolised in the mitochondria by citric acid cycle enzymes to form oxaloacetate, from where it migrates to the cytosol. There it is converted to PEP, and sucrose is produced by gluconeogenesis (ap Rees, *et al.*, 1974).

1.3 Carbohydrate control of gene expression in higher plants

1.3.1 Introduction

Sugars control the expression of many plant genes and thereby many metabolic and developmental processes (Koch, 1996). Genes involved in photosynthesis, reserve mobilisation and reallocation are down regulated by high sugar levels. In plants, the regulation of gene expression by sugars may function to control carbohydrate metabolism among tissues and organs (Koch, 1996). The regulation of photosynthetic genes by sugars guarantees the efficient use of carbon, ensuring that excessive carbohydrate is not manufactured in source leaves and that sink tissues receive enough sugars for metabolism and storage. Genes involved in carbohydrate allocation, and accumulation of storage reserves are induced when sugar levels are high. For instance, in developing seeds, where sugar concentrations are high, the genes for its utilization in storage products are induced. Similarly, when cellular carbohydrate levels are low the expression of genes required for sucrose production by photosynthesis and storage remobilization is induced.

1.3.2 Sugar repression of gene expression

1.3.2.1 Photosynthesis

Repression of transcription of many photosynthetic genes is an important mechanism for the sustained feedback inhibition of photosynthesis and ensures that levels of carbohydrates in the source tissues are maintained (Krapp, *et al.*, 1993, Krapp and Stitt, 1995). The photosynthetic genes down-regulated by sugar accumulation include those enzymes involved in the primary fixation of CO_2 of both C3 and C4 plants and other genes crucial to photosynthesis (Sheen, 1990, Cheng, *et al.*, 1992, Sheen, 1994).

When sucrose levels are elevated above a particular threshold, i.e. by high photosynthetic activity, the expression of genes involved in photosynthetic sucrose biosynthesis are repressed. Sugar repression of photosynthetic genes has been studied in several situations where the condition of high photosynthetic activity has been intentionally mimicked. These include sugar feeding of cell cultures, detached leaves and leaf disks; cold-girdling and transfer of ambient grown plants to 5°C, and overexpression of invertase in the apoplast, all of which result in elevated levels of intracellular sugars and repression of photosynthesis.

Initial work using a maize protoplast transient expression system, showed that seven maize photosynthetic promoters are repressed by the photosynthetic end-products, sucrose, glucose and fructose and by exogenous acetate (Sheen, 1990). Krapp *et al* . (1993) found that high levels of sugars lead to a rapid and reversible decrease in transcription rates and the steady state level of *rbcS*, *cab*, and *atpD* in autotrophic *Chenopodium* cell-suspension cultures. Cold-girdling of the petiole of tobacco and potato leaves showed a similar rapid decrease of transcripts (Krapp, *et al.*, 1993). Similarly, in plants that were grown at 23°C and then transferred to 5°C, there was severe repression of photosynthesis which corresponded with a decrease in transcripts for *rbcS* and *cab* (Strand, *et al.*, 1997). The transient activation of the plastocyanin gene of *Arabidopsis* is repressed by sucrose (Dijkwel, *et al.*, 1996). Glucose or sucrose feeding also led to repression of a number of genes encoding other Calvin cycle enzymes (Fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase, phosphoglycerate kinase) in wheat or sugar beet (Jones, *et al.*, 1996, Lee and Daie, 1997).

Sugar repression of photosynthetic genes overrides other regulation such as light, tissue type and developmental stage (Sheen, 1990). This is also seen in rape seed culture where 2 % sucrose inhibits the light-dependent accumulation of chlorophyll a/b binding protein (Harter, *et al.*, 1993), and *Arabidopsis*, where 2 % sucrose or glucose represses the light induction of *rbcS* in dark-adapted seedlings (Cheng, *et al.*, 1992) and sucrose represses the developmentally controlled transient expression of the plastocyanin gene (Dijkwel, *et al.*, 1996). Photosynthetic genes are often repressed more strongly by hexoses than sucrose

(Sheen, 1990, Jang and Sheen, 1994, Sheen, 1994). However, acetate also represses photosynthetic genes and often has a stronger affect than sugars (Sheen, 1990). Repression by sugars and by acetate appear to be mediated by different mechanisms (Sheen, 1990) and interact with a number of other regulatory signalling pathways.

1.3.2.2 Repression of photosynthetic gene expression by elevated CO_2

Photosynthesis is inhibited when production of photosynthate exceeds the rate of utilization, and carbohydrate accumulates in the leaves (Krapp, *et al.*, 1991, Krapp, *et al.*, 1993, Sheen, 1994, Krapp and Stitt, 1995). High CO_2 treatment of plants leads to an initial increase in photosynthesis followed by a long term decrease (Van Oosten, *et al.*, 1994). The negative acclimation of photosynthesis observed in plants subjected to CO_2 enrichment has been explained in part by repression of photosynthetic genes (Sheen, 1994, Van Oosten, *et al.*, 1994, Van Oosten and Besford, 1994, Krapp and Stitt, 1995). Nuclear genes encoding enzymes functioning in the chloroplast are repressed to a greater extent than chloroplastic genes when plants are exposed to high CO_2 or supplied with sugars (Van Oosten, *et al.*, 1994, Van Oosten and Besford, 1994). Increasing SPS may be valuable in preventing the high CO_2 induced acclimation of photosynthesis (Signora, *et al.*, 1998). Evidence for this comes from *Arabidopsis* plants overexpressing SPS. When grown in high CO_2, overexpression of SPS led to a decrease in the foliar carbohydrate content whilst the sugar: starch ratio and photosynthetic capacity were increased.

Contrasting studies indicated that there was little correlation between increased soluble carbohydrate, decreased levels of nuclear transcripts and acclimation of photosynthesis in high CO_2 (Nie, *et al.*, 1995, Moore, *et al.*, 1998). However, in high CO_2 there was a relationship between low Rubisco, acid invertase activity, and the leaf hexose: sucrose ratio. The data indicated that the carbohydrate repression of photosynthetic gene expression at elevated CO_2 may involve leaf sucrose cycling through acid invertase and hexokinase (Moore, *et al.*, 1998).

Arabidopsis mutants which are impaired in sucrose repression of genes have been isolated by several groups. These will be useful in the dissection of the mechanism of sugar

repression in higher plants and should lead to the identification of components of the signal transduction pathways. Sucrose uncoupled (*sun*) mutants have been isolated which showed reduced repression of the plastocyanin promoter-luciferase fusion gene (Dijkwel, *et al.*, 1997). Plastocyanin gene (*pc*) is activated independently of light during early seedling development (Dijkwel, *et al.*, 1996). In etiolated seedlings, *pc* mRNA levels peak transiently after 2 days growth in darkness. The transient increase in mRNA can be repressed by sugars which are phosphorylatable by hexokinase, suggesting that hexokinase induces the signal (Dijkwel, *et al.*, 1996, Van Oosten, *et al.*, 1997). As well as being repressed by sugars, the plastocyanin gene is also developmentally- and light-regulated. The affect of sucrose on far-red high irradiance responses was studied in wild type and several *sun* mutants. In wild type seedlings, sucrose repressed the far red light-induced opening of cotyledons and inhibition of hypocotyl elongation. *sun7* showed a reduced repression of these responses. The results provide evidence for a close interaction between sucrose and light signalling pathways (Dijkwel, *et al.*, 1997).

The development of *Arabidopsis* wild type seedlings is arrested on 6 % glucose. A number of glucose insensitive (*gin*) mutants have been isolated which are less sensitive to the presence of 6 % glucose in the growth medium (Zhou, *et al.*, 1996).

1.3.3 Induction of genes under low carbohydrate conditions

Genes for the synthesis and remobilization of sugars and other small molecules from storage products such as lipids, starch and proteins are induced by starvation conditions and repressed by sugars.

Malate synthase (MS) and isocitrate lyase (ICL) are key components of the glyoxylate cycle and their gene expression has been shown to be repressed by sugars (Graham, *et al.*, 1992, Graham, *et al.*, 1994a, Graham, *et al.*, 1994b, McLaughlin and Smith, 1994, Sarah, *et al.*, 1996). *ms* and *icl* are expressed during early post-germinative growth of lipid storing seedlings, when the glyoxylate cycle converts two molecules of acetyl CoA, derived from fatty acids, to succinate which is then converted to sucrose via gluconeogenesis. *ms* and *icl* are expressed again at the onset of cotyledon senescence, or

during sugar starvation conditions (Graham, *et al.*, 1992, Graham, *et al.*, 1994b). It has been proposed that expression of *ms* and *icl* can be repressed by sugars during senescence or starvation conditions but not during germination (Kim and Smith, 1994b, Kim and Smith, 1994a, Reynolds and Smith, 1995). Evidence to support this comes from analysis of the cucumber *ms* promoter which was found to contain distinct regions required for carbohydrate control and for regulation of gene expression during germination (Sarah, *et al.*, 1996).

In germinating monocot seeds, endosperm starch is hydrolysed by enzymes such as α-amylase to provide sugars for export to the developing seedling. In rice, at least 10 genes encode α-amylase isoforms but only two of them, RAmy3D and RAmy3E, are strongly under the control of sugars and the expression is inversely related to the sugar concentration in the culture medium (Huang, *et al.*, 1993, Yu, *et al.*, 1996). Expression of α-amylase genes is induced in the aleurone tissue by gibberellin (Fincher, 1989) and repressed in the embryo by sugars (Yu, *et al.*, 1991, Huang, *et al.*, 1993, Sheu, *et al.*, 1994). Hexokinase has been implicated as a possible sugar sensor for sugar repression of α-amylase gene expression (Perata, *et al.*, 1997, Umemura, *et al.*, 1998). Studies with okadaic acid, a potent and specific inhibitor of protein serine/threonine phosphatases 1 and 2A, have shown that protein phosphorylation is required for the induction of αAmy3 whereas inhibitors of protein kinase c, calcium, calmodulin and calcium dependent protein kinases had no affect (Lue and Lee, 1994). Three *cis*-elements are required for the high level gene expression of rice α–amylase Amy3D and αAmy3 (Hwang, *et al.*, 1998, Lu, *et al.*, 1998). Nuclear proteins which bind to the TATCCA element in a sequence specific and sugar dependent manner have also been identified (Lu, *et al.*, 1998). The TATCCA element is also an important component of the gibberellin response complex of the α-amylase genes in germinating cereal grains, suggesting that the regulation of α-amylase gene expression by sugar and hormonal signals may share common regulators (Hwang, *et al.*, 1998, Lu, *et al.*, 1998). An interaction between hormonal and metabolic regulation of α-amylase genes has also been suggested in barley grains (Perata, *et al.*, 1997).

The extent of protein remobilization (and associated gene expression) varies with the degree of carbohydrate depletion (Elamrani, *et al.*, 1994, James, *et al.*, 1994). Starvation conditions induce a protease in maize root tips and breakdown of leaf storage proteins in non-photosynthetic cells (James, *et al.*, 1994, Stepien, *et al.*, 1994).

Mannitol, the key source of carbon in celery, is broken down by mannitol dehydrogenase to mannose (Stoop and Pharr, 1993). In cultured celery cells, mannitol dehydrogenase is induced by sugar depletion and repressed by sugars (Prata, *et al.*, 1997). However, Pi is required for mannitol dehydrogenase derepression upon sugar depletion. The *rbcS* gene has also been reported as being insensitive to carbohydrate levels in Pi-limited seedlings (Stitt, *et al.*, 1995). Thus, it has been suggested that the dramatic affect of Pi may be due to the essential nature of Pi in the central metabolism rather than to a role as a signal molecule (Prata, *et al.*, 1997).

Both light and sucrose repress the gene expression of asparagine synthetase in *Arabidopsis* (Lam, *et al.*, 1994). The sucrose repression affect can be partially rescued by the addition of exogenous amino acids, which suggests that the nitrogen : carbon ratio rather than carbon alone is responsible for the regulation of the asparagine synthetase gene (Lam, *et al.*, 1994).

1.3.4 Induction of Gene Expression under High Carbohydrate Conditions

Conditions of high cellular carbohydrates inhibit photosynthetic production of sucrose and permit the reallocation of carbon and nitrogen to other processes such as storage product assimilation. Abundant carbohydrate induces the expression of genes encoding starch biosynthetic enzymes, storage proteins, defence related proteins and secondary metabolites. Sugar also up-regulates genes for nitrate reductase so that plants will reduce nitrate only when there is sufficient carbohydrate in the cells for incorporation into amino acids (Cheng, *et al.*, 1992). This may prevent plants from over taxing the carbohydrate reserves by excess nitrate reduction (Cheng, *et al.*, 1992).

Much of the research into induction of storage products has been carried out in potato (*Solanum tuberosum*), sweet potato (*Ipomoea batatas*) and maize (*Zea mays*) all of which store large amounts of starch and protein in their respective tubers or kernels.

Sugar inducible genes involved in carbohydrate metabolism in potato include ADP-glucose pyrophosphorylase (AGPase) (Müller-Röber, *et al.*, 1990), plastidic phosphorylase (St-Pierre and Brisson, 1995, Duwenig, *et al.*, 1997), starch-branching enzyme (Kossmann, *et al.*, 1991), starch synthases (Visser, *et al.*, 1991, Abel, *et al.*, 1996) and sucrose synthase (Salanoubat and Belliard, 1989).

Sugar also modulates two enzymes, invertase and sucrose synthase which catalyse the cleavage of sucrose during its import into sink cells. It has been proposed that each operates in a pathway of specific significance. Invertase converts sucrose to fructose and glucose favouring growth and cell expansion (Weber, *et al.*, 1997) and SUSY converts sucrose to UDP-glucose and D-fructose favouring starch synthesis (Weber, *et al.*, 1997). Both enzyme activities are comprised of 2 isozymes which are differentially expressed by sugars (Maas, *et al.*, 1990, Koch, *et al.*, 1992, Xu, *et al.*, 1996). The maize sucrose synthase gene, *Sus1*, is upregulated by high carbohydrate conditions whereas the *Sh1* is expressed even under carbon starvation conditions. Similarly one of the genes coding for invertase is expressed in high carbohydrate conditions (*Ivr2*) and the other is expressed in carbon starvation conditions (*Ivr1*). The two classes display differential expression throughout development with sugar enhanced genes (*Sus1* and *Ivr2*) expressed in many importing organs. The sugar repressed, starvation tolerant *Sh1* and *Ivr1* are expressed primarily during reproductive development (Xu, *et al.*, 1996). Sucrose modulation and developmental expression regulates both pathways of sucrose metabolism and as such can have a profound affect on the allocation of carbon among plant parts (Xu, *et al.*, 1996). Induction of the sugar inducible sucrose synthase in *Chenopodium rubrum* by 6-deoxyglucose suggests that the signal for sugar induction is non-phosphorylated-glucose (Godt, *et al.*, 1995).

Vegetative storage proteins accumulate in the somatic tissues of organs where dehydration is not normally required (Berger, *et al.*, 1995). Examples include patatin in potato (Rocha-Sosa, *et al.*, 1989, Wenzler, *et al.*, 1989b), sporamin in sweet potato (Hattori

and Nakamura, 1988, Hattori, *et al.*, 1991), and VSP A/B (DeWald, *et al.*, 1994) and lipoxygenase (Tranbarger, *et al.*, 1991) in soybean.

The expression of genes encoding patatin, which accounts for 30-40 % of the total soluble protein in potato tubers, is not specific to tubers as it can be induced by sugars in stems and leaves (Wenzler, *et al.*, 1989b). In sweet potato (*Ipomoea batatas*), the genes encoding sporamin and ß-amylase are induced by sugars with the concomitant accumulation of starch (Nakamura, *et al.*, 1991). Like patatin, sporamin and ß-amylase can be induced in leaf-petiole cuttings which have been supplied with a sugar solution (Nakamura, *et al.*, 1991, Mita, *et al.*, 1995). The expression of the gene ß-amylase is also induced in starchless mutants of *Arabidopsis* which accumulate high levels of soluble sugars during the photoperiod (Caspar, *et al.*, 1989). The evidence that patatin, sporamin and ß-amylase can be induced by sugars in tissues where they are not normally expressed implies that the expression may be regulated by the carbon partitioning and source-sink relations of the whole plant (Mita, *et al.*, 1995) and that sugar signals may be dominant over other signals (Graham, 1996).

Many of the vegetative storage proteins have enzymatic activity and these have been implicated in the defence response. Patatin is a lipophilic acyl hydrolase and may cleave fatty acids from membrane lipids as part of a wounding (Andrews, *et al.*, 1988) and sugar response (Wenzler, *et al.*, 1989a, Wenzler, *et al.*, 1989b). Proteinase inhibitor II, another abundant protein in potato tubers is also induced by sugars (Johnson and Ryan, 1990, Kim, *et al.*, 1991) and wounding (Ryan and An, 1988). The inhibitor is thought to protect the plant against herbivores by decreasing the digestibility and nutritional quality of the leaf proteins (Ryan, 1989). The soybean *VspA* and *VspB* encode proteins with phosphatase activity (Mason, *et al.*, 1992). Another soybean vegetative storage protein has lipoxygenase activity (Grimes, *et al.*, 1993). Studies of sugar responsiveness in these genes have also revealed important interactions between carbohydrate status and other signals. The sugar inducible genes encoding the vegetative storage proteins are activated by jasmonate, wounding, sugars and light and down-regulated by phosphate and auxin (Bell and Mullet, 1991, Mason, *et al.*, 1992, DeWald, *et al.*, 1994, Sadka, *et al.*, 1994).

Chalcone synthase is a key enzyme in the synthesis of anthocyanins. Anthocyanins are secondary metabolites which pigment flower petals and autumn leaves and are involved in plant defence. Sugars induce the expression of the gene encoding chalcone synthase (*chs*). CHS is also inducible by stresses such as high light, particularly UV, and nitrogen starvation (Tsukaya, *et al.*, 1991).

Extracellular invertases are induced by wounding or bacterial infection (Sturm and Chripeels, 1990). The hydrolysis of apoplastic sucrose by the extracellular invertase may lead to a higher influx of glucose and fructose which in turn triggers the repression of photosynthetic genes (Jang and Sheen, 1994). Increased soluble sugar concentrations may also stimulate the defence related proteins such as lipoxygenase (Bell and Mullet, 1991, Mason, *et al.*, 1992), proteinase inhibitor II (Johnson and Ryan, 1990), patatin (Andrews, *et al.*, 1988, Wenzler, *et al.*, 1989a), pathogenesis related proteins (Herbers, *et al.*, 1995, Herbers, *et al.*, 1996b) and chalcone synthase (Tsukaya, *et al.*, 1991).

It is known that abundant carbohydrates promote an increase in respiration in leaves (Azcón-Bieto and Osmond, 1983) and potato tubers (Trethewey, *et al.*, 1998). Some genes encoding enzymes involved in the cytoplasmic portion of respiration are induced by sugars (Koch, 1996). Recently, sugars have also been shown to induce the expression of cytochrome c in sunflower mitochondria (Feletti and Gonzalez, 1998). The affect could be mimicked by sugars, such as mannose and 2-deoxyglucose, that are phosphorylated by hexokinase but not further metabolised suggesting that the hexokinase reaction was involved in the induction of cytochrome c by sugars (Feletti and Gonzalez, 1998). It has been suggested that carbohydrates provide the link for the coordinated expression of genes involved in photosynthesis and respiration (Koch, 1996).

1.3.5 Promoter analysis and signal transduction of sugar inducible genes

Little is known about the signalling pathways for sugar induction. However, some progress has recently been made in determining the factors involved in the sugar induction of patatin and ß-amylase. Carbohydrates, nitrogen supply and amino acids appear to be involved in the induction of class I patatin genes (Peña-Cortés, *et al.*, 1992, Martin, *et al.*,

1997). Dissection of the class I patatin promoter has revealed that separate *cis* sequences and *trans* factors regulate the metabolic and developmental expression of patatin (Grierson, *et al.*, 1994). The region found to confer sugar inducibility of patatin has homology with other sugar enhanced genes (Grierson, *et al.*, 1994). The patatin promoter contained sequences called SUREs (Sucrose Response Elements) that are similar to a motif called SP8 found in sweet potato (Ishiguro and Nakamura, 1992), other motifs have been found in the promoters of sugar inducible ß-amylase, and sporamin (Ohta, *et al.*, 1991) and proteinase inhibitor II (*pin2*) (Kim, *et al.*, 1991). The evidence indicates that sequences for sugar inducible expression are conserved among several genes (Grierson, *et al.*, 1994).

Analysis of the patatin class I promoter in *Arabidopsis* roots revealed that expression requires transport of sugar into the cells but not hexokinase activity (Martin, *et al.*, 1997). Mutants showing reduced sugar response (*rsr*) and modified expression patterns (*mep*) have been isolated (Martin, *et al.*, 1997). Further analysis and cloning of the mutations will contribute to the understanding of sugar sensing (Martin, *et al.*, 1997).

Mutants with reduced expression of ß-amylase, designated *low-level beta-amylase* (*lba1* and *lba2*) mutants have been identified in *Arabidopsis* which show reduced responsiveness to sugars (Mita, *et al.*, 1997b). Landsberg erecta ecotype naturally has the *lba2* mutation and has a reduced level of sugar inducible ß-amylase compared to other ecotypes. This may partly explain why different ecotypes respond differently to different sugars (personal observation). Although the mutations *lba1* and *lba2* did not affect sugar inducible expression in general, the lesions caused a decrease in anthocyanin accumulation in the plants in response to sucrose, suggesting that there is a link between the expression of ß-amylase and anthocyanin accumulation (Mita, *et al.*, 1997b). The opposite type of mutant, the *high-level beta-amylase* (*hba*) mutants have also been found (Mita, *et al.*, 1997a). These have a hypersensitive response to sugar which stimulates ß-amylase gene expression even at low concentrations (Mita, *et al.*, 1997a). Like the *lba* mutants, the *hba* mutants do not have altered general carbohydrate metabolism but do accumulate high levels of anthocyanins (Mita, *et al.*, 1997a). The results suggest that there is a linkage between the signal cascade

of ß-amylase and anthocyanin production and that the expression of sugar induced genes may be regulated by several signal transduction pathways (Mita, *et al.*, 1997b).

Okadaic acid, a potent and specific inhibitor of protein phosphatases strongly inhibites the sucrose-inducible accumulation of mRNA for sporamin, ß-amylase and the small subunit of ADP-glucose pyrophosphorylase in sweet potato (Takeda, *et al.*, 1994). Thus, the authors suggest that the continuous dephosphorylation of proteins by protein phosphatases is required for expression of at least some sugar inducible genes (Takeda, *et al.*, 1994). There is also evidence that calcium signalling and plasma-membrane associated calcium dependent protein kinases are involved in the sugar inducible expression of genes encoding sporamin and ß-amylase (Ohto, *et al.*, 1995, Ohto and Nakamura, 1995). It has been proposed that the calcium dependent protein kinases are involved in the regulation of sugar transport because they are associated with the plasma membrane (Ohto and Nakamura, 1995).

There is now a large body of evidence which supports the idea that there is cross talk between signals for carbohydrate status and signals for many other factors in plants. We now know that sugar responsive expression of many genes occurs in a tissue specific manner under the influence of other factors such as phytohormones (Hattori, *et al.*, 1991, Mason, *et al.*, 1992, Ohto and Nakamura-Kito, 1992, DeWald, *et al.*, 1994), light (Peña-Cortés, *et al.*, 1992, Mita, *et al.*, 1995), and wounding stress (Johnson and Ryan, 1990). This suggests that sugar-responsive gene expression in plants might occur via a complex regulatory network of signal transduction cascades (Mita, *et al.*, 1997b).

1.6 Sugar sensing

1.6.1 Sugar sensing in Yeast

Glucose repression is a widespread phenomenon in yeast (*Saccharomyces cerevisiae*) and bacteria whereby cells grown on glucose repress a large number of genes that are required for the metabolism of alternative carbon sources such as sucrose, maltose and galactose (Gancedo, 1992, Trumbly, 1992, Ronne, 1995, Saier, *et al.*, 1995, Thevelein

and Hohmann, 1995). Sugar sensing and glucose repression have been extensively investigated in yeast and as such yeast serves as a eukaryotic model for plants.

The mechanism for glucose repression is not fully understood but hexokinase seems to play an important role. Yeast contains three glucose phosphorylating enzymes, hexokinase PI (*HXK1*), hexokinase PII (*HXK2*)and glucokinase (GLK1). The hexokinase PII appears to play an important role in the glucose mediated response (Entian, 1980, Ma, *et al.*, 1989, Rose, *et al.*, 1991). Mutations in hexokinase PII (HXK2) abolish glucose repression (Entian, 1980, Özcan and Johnston, 1995). This led to the speculation that hexokinase PII could have a regulatory role that was distinct from its catalytic function. However, if overexpressed, the hexokinase PI can also mediate glucose repression, and the catalytic and regulatory domains have so far been inseparable (Ma, *et al.*, 1989, Rose, *et al.*, 1991).

A feature of the hexokinase PII is that it is a phosphoprotein. This is important because protein phosphorylation is essential in most metabolic signal transduction pathways in eukaryotes (Randez-Gil, *et al.*, 1998). The hexokinase PII enzyme exists *in vivo* in a monomeric-dimeric equilibrium which is affected by phosphorylation. Only the monomeric form is phosphorylated. The reversible phosphorylation of the hexokinase PII protein is dependent on the carbon source, the protein being more highly phosphorylated on poor carbon sources such as galactose, raffinose and ethanol. Addition of glucose promotes the dephosphorylation of hexokinase PII. This affect is not present in glucose repression mutants *cat80/grr1*, *hex2/reg1* and *cid1/glc7* (Randez-Gil, *et al.*, 1998). The authors suggest that CID1/GLC7 phosphatase together with its regulatory HEX1/REG1 subunit are involved in the dephosphorylation of the hexokinase PII monomer. A mutant hexokinase PII protein which is unable to be phosphorylated could not cause glucose repression of invertase. Other recent work has revealed that the hexokinase PII protein is located in both the nucleus and the cytoplasm of *S. cerevisiae* cells and nuclear localisation of the hexokinase (Herrero, *et al.*, 1998) PII protein is necessary for glucose repression signalling of the SUC2 gene. Furthermore, the hexokinase PII protein participates in regulatory DNA-protein complexes which are necessary for the glucose repression of the SUC2 gene (Herrero, *et al.*, 1998).

Hexokinase PII is also required for the induction of the *HXT* gene encoding a hexose transporter (Özcan and Johnston, 1995). The glucose induction of *HXT* gene expression is also affected in cells expressing the mutated hexokinase PII. The results suggest that the phosphorylation of hexokinase is essential for glucose signal transduction *in vivo* (Randez-Gil, *et al.*, 1998).

Initially, it was thought that steps in glycolysis after hexokinase were not involved in glucose repression. However, mutants lacking glucose-6-phosphate isomerase do not initiate glucose repression when given a pulse of glucose (Sierkstra, *et al.*, 1993). In opposition to this, fructose which enters glycolysis beyond glucose-6-phosphate isomerase does trigger repression. However, fructose repression is only seen after 15-30 minutes, which coincides with the time that glycolysis is resumed after a transient inhibition. This was used as evidence that repression is triggered by glycolytic flux and not by an early intermediate of glycolysis (Sierkstra, *et al.*, 1993).

Hexokinase PII initiates a signal transduction pathway that is perceived by the GLC7 complex (Tu and Carlson, 1995). This activates the SSN6/TUP1 complex which, by binding to the transcription factor MIG1, modulates chromatin structure. Exactly how the HXK2, GLC7, and SSN6/TUP/MIG1 complexes are connected is unknown.

In the absence of glucose the glucose-repressed genes are derepressed by a different set of gene products. The SNF1 kinase (sucrose non-fermenting) forms a complex with other proteins, including the activating subunit SNF4 and other adaptor proteins. The interaction between SNF1 and SNF4 is strongly regulated by glucose and is affected by the components upstream in the glucose signalling pathway, such as HXK2, GLC7 and REG1. The SNF2-containing chromatin modulation complex is also involved in the derepression process. It consists of approximately 10 proteins and reverses the glucose induced closed chromatin conformation into an open conformation that can be transcribed. Like the SSN6/TUP1 complex, the SNF2 complex is a general chromatin modulator and is directed to a specific chromosomal target by transcription factors. The glucose repressing and derepressing pathways interact with the GLC7 phosphatase complex, antagonising the

function of the SNF1 protein kinase. The mechanism by which cellular glucose levels are perceived and a signal is created is still not understood (Wilson, *et al.*, 1996).

Hexokinase PI and hexokinase PII activities are inhibited by physiological concentrations of trehalose-6-phosphate (T6P) (Blázquez, *et al.*, 1993, Thevelein and Hohmann, 1995). Yeast strains with mutations in T6P synthase or with a very low content of this enzyme cannot properly control sugar influx into glycolysis (Thevelein and Hohmann, 1995). This is indicative that T6P synthase controls hexokinase activity. By controlling hexokinase activity, trehalose-6-phosphate may be a regulator of glucose induced signalling. Whether this control requires direct interaction of the hexokinases with subunits of the trehalose-6-phosphate synthase /phosphatase complex is not known.

Hexokinase is not the only sugar sensor in yeast. Two glucose transporters are also involved in sugar sensing and generate an intracellular glucose signal (Özcan, *et al.*, 1996). The snf3p high affinity glucose transporter appears to function as a low glucose sensor since it is required for the induction of several hexose transporter genes by low levels of glucose (Özcan, *et al.*, 1996). Another glucose transporter Rgt2p (similar to snf3p) is required for maximal induction of gene expression in response to high levels of glucose. Dominant mutations in RGT2 or SNF3 caused constitutive expression of several hexose transporter genes. Thus the glucose transporters appear to act as glucose receptors that generate an intracellular glucose signal, suggesting that glucose signalling in yeast is a receptor mediated process (Özcan, *et al.*, 1996) and does not require metabolism of glucose.

1.6.2 Sugar sensing in animals

The hexokinase pathway seems to be at least partially conserved in eukaryotes as hexokinase has been implicated in sugar-mediated gene regulation in animals. In mammals glucose sensing is mediated through an increase in the rate of intracellular catabolism of glucose. Glucokinase (HXKIV) has been proposed as being the glucose sensor (Matchinsky, *et al.*, 1993) but no direct evidence for its function as a sensor has been found. It is possible that the catalytic activity of glucokinase alters the flux of carbon, perturbing metabolite concentrations. Either carbon flux or other metabolites may be sensed. Functional

glucokinase is mainly expressed in the pancreatic ß-cells and in liver with the primary role of glucose sensing and control of blood glucose homeostasis. Mammalian glucokinase is very similar to yeast hexokinase. In liver cells glucokinase is essential for the disposal of excess glucose through glycogen accumulation, whereas in the pancreas it is required for glucose stimulated insulin release from ß-cells. Mutations in the glucokinase gene affecting Vmax and/or Km cause MODY2 (maturity onset diabetes of the young) in humans (Hattersley, *et al.*, 1992, Froguel, *et al.*, 1993). Targeted disruption of the glucokinase gene in mice causes a severe diabetic phenotype in homozygous knockout animals and a weak MODY like diabetes in heterozygous animals (Grupe, *et al.*, 1995).

1.6.3 Sugar sensing in plants

1.6.3.1 Evidence for hexokinase mediated sugar signalling

Evidence for hexokinase mediated sugar signalling in plants has come from several different sources. Initial work using a maize protoplast transient expression system found that phosphorylation of hexoses by hexokinase was critical for signalling because only hexoses and glucose analogues that can be phosphorylated by hexokinase were effective (Jang and Sheen, 1994, Jang, *et al.*, 1997, Jang and Sheen, 1997). The affect was reversed by adding mannoheptulose, a competitive inhibitor of hexokinase. In addition, when delivered directly into the maize leaf cells by electroporation, glucose, but not G6P or other downstream metabolites in the glycolytic pathway, triggered the repression. It was concluded that the hexokinase reaction caused the signal. Hexokinase was also thought to be involved in the sugar repression of *rbcS* in *Chenopodium* cell culture (Krapp, *et al.*, 1993).

The hexokinase reaction has also been implicated in the initial sugar sensing step resulting in repression of *ms* and *icl* (Graham, *et al.*, 1994a, McLaughlin and Smith, 1994, Sarah, *et al.*, 1996). In cucumber cell cultures, *ms* and *icl* were derepressed when the intracellular levels of sucrose, glucose and fructose fell below a threshold of approximately 3 mM (Graham, *et al.*, 1994b). Maintenance of cells in glucose, fructose or sucrose containing media resulted in the repression of *ms* and *icl* expression, whereas culture in 3-

O-methylglucose does not result in repression. Similar results were obtained in a cucumber protoplast transient expression system (Graham, *et al.*, 1994a). Hexokinase or the events associated with the hexokinase reaction are implicated in the initial sugar-sensing step.

There is also evidence that hexokinase is involved in the repression of mannitol dehydrogenase (Prata, *et al.*, 1997) and α-amylase (Perata, *et al.*, 1997, Umemura, *et al.*, 1998), and in the induction of cytochrome c (Feletti and Gonzalez, 1998).

Two *Arabidopsis* hexokinase genes have been cloned. *AtHXK1* is located on chromosome 4, and *AtHXK2* is located on chromosome 2 (Jang, *et al.*, 1997). The hexokinase clones were used in overexpression and antisense experiments to investigate the *in vivo* function of hexokinase sugar sensing (Jang, *et al.*, 1997). Although wild type plants germinated on 6 % glucose, the greening and expansion of the cotyledons, the initiation of true leaves, and the elongation of the hypocotyl and root were suppressed. In addition greening of cotyledons was suppressed in wild type germinated on 0.2 mM 2-deoxyglucose. Plants carrying the sense hexokinase transgene showed hypersensitivity to 6 % glucose as indicated by extremely stunted cotyledons, hypocotyls and roots. Plants carrying the antisense hexokinase greened, expanded and elongated normally on 6% glucose plates, suggesting that they were relatively hyposensitive to glucose (Jang, *et al.*, 1997). This altered sensitivity was also observed for the regulation of expression of *cab* and *rbcS* genes. Furthermore, overexpressing the yeast sugar sensor YHXK2 caused a dominant negative affect by elevating the hexokinase catalytic activity but did not provide the regulatory function for signalling in the transgenic plants. In fact, the transgenic plants overexpressing the yeast YHXK2 gene were less sensitive to glucose. They also found that it was possible to restore the catalytic activity but not the regulatory function in the yeast hxk1 hxk2 double mutant using *At*HXKI. The authors suggest that the transgene exerts a dominant negative affect, presumably by competing with the endogenous hexokinase for substrate. They also suggest that hexokinase in yeast and plants is not interchangeable with respect to the regulatory function required for control of gene expression (Jang, *et al.*, 1997). This is supported by the evidence that even the closely related yeast, *Kluveromyces lactis*, can only

complement the catalytic function, and not the regulatory function, in *S. cerevisiae* (Prior, *et al.*, 1993).

In plants sugar induction of apoplastic invertase and sucrose synthase in *Chenopodium* cell cultures does not involve the hexokinase reaction (Godt, *et al.*, 1995, Roitsch, *et al.*, 1995). The affect of induction of extracellular apoplastic invertase and sucrose synthase by glucose could be mimicked by the non-metabolisable glucose analogue 6-deoxyglucose (Godt, *et al.*, 1995, Roitsch, *et al.*, 1995). This suggests that hexose sugars themselves act as the primary signal for sugar induction of these genes. However, the sugar inducible promoter of the potato proteinase inhibitor II gene is not induced by 6-deoxyglucose (Kim, *et al.*, 1991). This indicates that there are at least two mechanisms for transmitting the signal for sugar induction of genes. This type of sugar sensing seems to be evolutionarily conserved, since it is also reported for the unicellular algae *Chlorella kessleri*. This alga is able to grow both autotrophically, and heterotrophically on glucose in the dark. Several genes, including a glucose transporter (HUP1) are induced when glucose is added to *C. kessleri*. The glucose affect may be mimicked by adding 6-deoxyglucose, and it was suggested that a glucose transporter functions as a sensor (Hilgarth, *et al.*, 1991).

The promoter of the sugar- and amino acid- induced class I patatin is induced by 6-deoxyglucose and 3-O-methylglucose, glucose analogues which are transported but not phosphorylated by hexokinase. This is evidence that in intact plants hexose transport but not hexose metabolism is required for sugar induction of genes (Martin, *et al.*, 1997). In yeast specialised hexose transporters can function as sensors (Özcan, *et al.*, 1996), but there is no evidence for sugar transporters having the ability to transduce sugar signals. The above results can be explained by assuming the presence of sugar binding proteins with a signalling function (Smeekens and Rook, 1997).

Expression of yeast invertase in either the cytosol, apoplast or vacuole of transgenic tobacco plants leads to sucrose hydrolysis and an increase in glucose and fructose levels which are stored in the vacuole (Heineke, *et al.*, 1994). The excess glucose and fructose were only sensed in the vacuole and apoplasm resulting in bleaching of the plants, reduced photosynthesis-related gene transcripts and an increase in the transcripts of a

pathogenesis related protein (Herbers, *et al.*, 1996a). The plants which expressed invertase in the cytosol did not sense the hexoses because they did not show these affects. Hexokinase is a cytosolic enzyme so if it is involved in sugar sensing the plants with invertase expressed in the cytoplasm would have shown signs of bleaching. The authors proposed that sugar signalling for the activation of defence related genes and repression of photosynthetic genes is associated with sensing mechanisms located at the secretory membrane system, possibly at the endoplasmic reticulum or Golgi apparatus (Herbers, *et al.*, 1996a). The apoplastic and vacuolar invertases both traverse the endomembrane system and are enzymatically active in this compartment. In addition it is known that sucrose is present in the endomembrane system because plants expressing a fructosyl transferase in this compartment accumulate fructans (Turk, *et al.*, 1996). The monosaccharides generated in the endomembrane system by this fructosyl transferase must be sensed because the plants have a severe chlorotic phenotype (Turk, *et al.*, 1996).

As mentioned previously, yeast hexokinase PI and hexokinase PII activities are inhibited by physiological concentrations of trehalose-6-phosphate. Trehalose is synthesized from G6P and UDP-glucose in a two-step process, analogous to sucrose synthesis, by trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase (Cabib and Leloir, 1958). However, until recently trehalose was thought to be absent from higher plants. In yeast and other micro-organisms it has an established role in the protection from various stress factors such as heat and desiccation (Vogel, *et al.*, 1998). Several groups have transformed tobacco with microbial genes for trehalose synthesis, in the hope of increasing the plants stress tolerance (Gaff, 1996, Holmström, *et al.*, 1996, Romero, *et al.*, 1997). Some transformed plants exhibited slight drought tolerance but the expression of microbial genes for trehalose synthesis always gave rise to severe growth defects, such as dwarfism, although only traces of trehalose were found. This gave hints that trehalose or related metabolites might have a function as regulators of plant growth and development. Furthermore, in yeast, trehalose seems not only to be involved in stress protection, but its precursor, possibly T6P, appears to be an important regulator of glucose influx and metabolism (Thevelein and Hohmann, 1995). Moreover, results indicate that trehalose may

interfere with sugar-regulated gene expression as application of trehalose to roots of soy bean induces sucrose synthase expression (Müller, *et al.*, 1998).

1.6.3.2 Do plants sense specific sugars?

Sucrose is readily hydrolysed to glucose and fructose so it is difficult to establish the direct function for particular sugar molecules. However, there is increasing evidence that there are specific sugar sensing and signalling systems in plants. Sucrose-specific induction of gene expression has been reported for the patatin promoter (Wenzler, *et al.*, 1989a, Jefferson, *et al.*, 1990) and for the phloem specific *rolC* promoter (Yokoyama, *et al.*, 1994). In these cases a combination of glucose and fructose was less effective. In addition, fructose was consistently more effective than sucrose or glucose at inducing the gene for cytochrome c in sunflower (Feletti and Gonzalez, 1998). The *Arabidopsis* bZIP transcription factor gene ATB2 is specifically repressed post-transcriptionally by sucrose. Other sugars alone or in combination were ineffective (Rook, *et al.*, 1998).

In potato, sucrose and glucose exert different affects on the metabolism of growing tuber parenchyma. In particular, sucrose selectively stimulated starch synthesis whereas glucose stimulated respiration (Geiger, *et al.*, 1998). The work concentrated on the rapid changes induced by sugars, presumed to be due to regulation by effectors or post-translational mechanisms. It is not yet known whether sucrose and glucose have different affects on the expression of the genes involved (Geiger, *et al.*, 1998).

1.6.3.3 How is the signal transduced?

It has been postulated that sugar sensors activate a signal transduction cascade that initiates gene repression or induction. However, the evidence for this is fragmentary. In yeast, SNF1 protein ser/thr kinases play a role in the derepression of several glucose repressed genes. SNF1 homologues have been identified in plants and animals (Hardie, *et al.*, 1994). The mammalian member of the family is adenosine monophosphate-activated protein kinase (AMPK) and a cDNA encoding its catalytic subunit has been cloned from rat (Carling, *et al.*, 1994). Plant members of the SNF1 family have been found in rye (RKIN1)

(Alderson, *et al.*, 1991), barley (BKIN1 and BKIN12) (Halford, *et al.*, 1992, Hannappel, *et al.*, 1995), *Arabidopsis* (AKIN10) (Le Guen, *et al.*, 1992), tobacco (NPK5) (Muranaka, *et al.*, 1994) and potato (PKIN1) (Man, *et al.*, 1997).

Antisense expression of a SNF1-homologue in potato resulted in a decrease in the expression of sucrose synthase and loss of sucrose inducibility of sucrose synthase transcripts in leaves. Activities of other enzymes which catalyse the conversion of sucrose to glucose and hexose phosphate, i.e. invertases, glucokinase and fructokinase were unaffected (Purcell, *et al.*, 1998). PKIN1 may be involved in a signal transduction pathway that mediates the response of changing sugar levels on the expression of sucrose synthase, and possibly other enzymes of carbohydrate metabolism. However, as yet, plant SNF1-related protein kinase activity has not been shown to be regulated by glucose or other hexose or hexose phosphate levels (Purcell, *et al.*, 1998) .

As has been mentioned previously, a number of mutants in different sugar sensing pathways have been isolated in *Arabidopsis*. Sucrose uncoupled (*sun*) (Dijkwel, *et al.*, 1997) and glucose insensitive (*gin*) (Zhou, *et al.*, 1996) mutants are altered in sugar repression signalling. Reduced sucrose response (*rsr*) mutants are defective in the sugar induced expression of the class I patatin promoter (Martin, *et al.*, 1997). Mutants showing reduced sugar-induced ß-amylase gene expression (*low-level beta-amylase;lba*) (Mita, *et al.*, 1997b) and *high-level beta-amylase* gene expression (*hba*) (Mita, *et al.*, 1997a) have also been isolated. Further characterisation of the mutants and cloning of the mutant genes will be useful in the dissection of the signal transduction pathways. If hexokinase does play a role in signal transduction of carbohydrate status, mutants in hexokinase regulatory and / or catalytic activities might be expected. Similarly sugar transport genes might also be isolated in the *rsr* mutants. In addition, it is possible that components which are involved in the intersection of several signal transduction pathways could be isolated.

The aim of my project was to characterise a new class of *Arabidopsis* mutant which are altered in the response to high sucrose concentration and low nitrogen concentration in the growth medium. They have been classed as carbohydrate insensitive (*cai*) mutants. It is expected that this class of mutant will contain mutants in sugar sensing and signalling,

nitrogen sensing and signalling and even carbon: nitrogen ratio sensing and signalling. In addition, it is likely that the *cai* class also contains mutants which are altered in carbon metabolism.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Plant material

Arabidopsis thaliana ecotypes columbia-2 and Wassilewskija were propagated from seeds obtained from Prof. C. Somerville's laboratory, Carnegie Institute, Wahington, 290 Panama St., Stanford, CA94305, USA.

2.1.2 Bacterial strains

Escherichia coli

XL1 Blue *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F'*proABlac*^{q} *ZΔM15* Tn*10* (Tet^{r})]

DH5-α φ80d*lacZΔM15 recA1 endA1 gyrA96 thi-1 hsdR17*(r_k^-,m_k^+) *supE44 relA1 deoR* *Δ(lacZYA-argF)U169*

JM103 *endA1 hsdR supE sbcB15 thi-1 strA Δ(lac-proAB)* [F' *traD36 proAB lacI*^{q}*ZΔm15*]

Agrobacterium tumifaciens strain GV3101(pMP90) (Koncz and Schell, 1986)

2.1.3 cDNA clones

2.1.3.1 Expressed Sequence Tags (ESTs)

ADP glucose pyrophosphorylase clone ID 135L24T7

Hexokinase Clone ID 84G1T7

Isocitrate lyase Clone ID VBV01-30492

Malate synthase 1 clone ID 34F10T7

Nitrate reductase Clone 160F3T7

Rubisco Clone ID 33G4T7

All ESTs verified by sequencing.

2.1.3.2 Other cDNAs

chlorophyll ab binding protein pAB165 in JM103 (Leutwiler, *et al.*, 1986)

chalcone synthase (pCHS) in DH5-α (Trezzini, *et al.*, 1993)

plastocyanin pPC8.130 (Vorst, *et al.*, 1988)

AtHXK 1 and 2 cDNAs cloned into a pBIN19 35S GUS derived vector (Jang, *et al.*, 1997)

H1 plasmid pH1 is in PAT153 (Lawton and Lamb, 1987)

2.1.4 Antibodies

anti-AtHXK1 polyclonal antibody (production bleed) (Jang, *et al.*, 1997)

2.1.5 Chemicals

Unless stated, all chemicals were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. or

Fisher Scientific (U.K.) Ltd., Loughborough, U.K., or

BDH Chemicals, Poole, Dorset, U.K.

Enzymes were from Boehringer Mannheim Corp. (London) Ltd., Lewes, Sussex, U.K.

Restriction enzymes were from Promega (U.K.) Ltd., Southampton, U.K.

Radioisotopes and Hybond-N were from Amersham International, Bucks, U.K.

2.2 Methods

2.2.1 Plant growth conditions

2.2.1.1 Seed sterilisation

Surface sterilising solution was freshly prepared each time it was required by dissolving 1 Covclor tablet (Coventry Chemicals Ltd., Coventry) in 35 ml H2O, 1% Tween-20. The working solution was made from this solution by adding 5 ml to 45 ml of 95% ethanol. Seeds were soaked in the working solution for 10 minutes and were rinsed twice in 95% ethanol. The sterile seeds were allowed to dry in a sterile flow hood.

2.2.1.2 *cai* screen

A modified Murashige and Skoog medium (M&S) (Murashige and Skoog, 1962) was prepared containing with 0.1 mM nitrogen (ammonium nitrate and potassium nitrate) plus 100 mM sucrose, pH 5.6-5.8 solidified with 0.8% agar. Table 2.1 shows the components of

M&S solutions with reduced nitrogen concentrations. The loss of potassium ions in the M&S solution, caused by the reduction in potassium nitrate, was replaced by the addition of potassium chloride.

The following stock solutions were made:

(1) 100 X stock macronutrient solution (165 g/l ammonium nitrate and 190 g/l potassium nitrate).

(2) 100 X stock potassium chloride solution (130 g/l potassium chloride).

(3) 100 X micronutrient solution (0.62 g/l boric acid, 2.5 mg/l cobalt chloride, 2.5 mg/l cupric sulphate, 1.69 g/l manganese sulphate, 25 mg/l molybdic acid, 83 mg/l potassium iodide, 0.86 g/l zinc sulphate).

(4) 200 X stock solution containing 5.56 g/l ferrous sulphate and 7.45 g/l EDTA (disodium salt; 40 ml of a 0.5 M EDTA, pH8.0). The solution was boiled for 10 minutes until the colour changed from green to brown.

A 1l solution of 0.1 mM nitrogen M&S solution was made up by adding 0.016 ml of 100 X macronutrient solution, 9.984 ml of 100 X potassium chloride solution, 10 ml of 100 X micronutrient solution and 5 ml of 200 X ferrosulphate/EDTA solution. The calcium chloride, magnesium sulphate and potassium phosphate were added individually. The pH of M&S solution was increased to pH 5.6-5.8 by adding 0.1 M KOH. Sucrose (34.23 g/l) and agar (8 g/l) were added to the solution before autoclaving, and pouring plates in a sterile flow hood.

Sterile seeds were sown on the agar plates and were kept in darkness at 4 °C for 4 days before being transferred to continuous white light (120 µmol $m^{-2}s^{-1}$) at 22° C for 7 days.

2.2.1.3 *gin* screen

Sterile seed were sown on 1/2 strength M&S medium containing 333 mM glucose and 0.8% agar and grown as described in 2.2.1.2

2.2.1.4 *mig* screen

Sterile seed were sown on 1/2 strength M&S medium containing 5 mM mannose and 0.8% agar and grown as described in 2.2.1.2.

Murashige and Skoog Medium with varying Nitrogen concentrations

	Final nitrogen concentration (mM)				
M&S components	**60**	**6**	**0.6**	**0.1**	**0**
Macronutrients (mg / l)					
ammonium nitrate	1650	165	16.5	1.65	0
potassium nitrate	1900	190	19	1.9	0
potassium chloride	0	1170	1287	1298	1300
calcium chloride (anh)	332.2	332.2	332.2	332.2	332.2
potassium phosphate (monobasic salt)	170	170	170	170	170
magnesium sulphate	180.7	180.7	180.7	180.7	180.7
Micronutrients (mg / l)					
boric acid	6.2	6.2	6.2	6.2	6.2
cobalt chloride $6H_20$	0.025	0.025	0.025	0.025	0.025
cupric sulphate $5H_20$	0.025	0.025	0.025	0.025	0.025
manganese sulphate H_20	16.9	16.9	16.9	16.9	16.9
molybdicacid($2H_20$; Na salt)	0.25	0.25	0.25	0.25	0.25
potassium iodide	0.83	0.83	0.83	0.83	0.83
zinc sulphate	8.6	8.6	8.6	8.6	8.6
Iron/EDTA (mg/l)					
ferrous sulphate.$7H_20$	27.8	27.8	27.8	27.8	27.8
Na_2EDTA	37.26	37.26	37.26	37.26	37.26

2.2.1.5 *sig* screen

Sterile seed were sown on 1/2 Murashige and Skoog medium containing 350 mM sucrose and 0.8% agar. The plates were kept in darkness at 4 °C for 4 days before being transferred to continuous white light (120 μmol $m^{-2}s^{-1}$) at 22° C for 2 weeks.

2.2.1.6 Soil grown plants

Seedlings selected by the above screens were pricked out into 1:1 mixture of potting and bedding compost (William Sinclair Horticulture Ltd., Lincoln, U.K.), and sharp sand and grown in 16h light (120 $\mu mol^{-2}s^{-1}$), 8h dark at 22°C.

2.3 Genetic analysis

2.3.1 Crosses

The maternal flower was prepared for crossing when the bud appeared to reach its maximal size but before there was any protrusion of the petals. The sepals and petals were removed from the bud using watchmakers forceps. The bud was then emasculated by removing all 6 anthers leaving an exposed stigma. Ripe anthers were separated from the pollen donor and were brushed over the stigma to fertilise the maternal plant. The fertilised bud was wrapped in a small piece of plastic wrap for 24h to prevent dehydration during pollen tube growth. If the cross was successful a silique extended from the ovary over the next few days.

2.3.2 Parental crossing

Reciprocal crosses were performed between the *cai* mutants and their parental ecotypes to eliminate unwanted additional mutations. The F1 seed were grown on selection media to look for dominant mutations. The F2 were grown on selection media to look for recessive mutations which would have an expected ratio of 3:1 wild type:mutant.

2.3.3 Complementation analysis

The *cai* mutants were crossed reciprocally between themselves to determine whether the mutations were allelic. Recessive non-allelic mutations should complement each other and the F1 will have the wild type phenotype on the selection media. If the mutation of one parent is dominant the F1 phenotype will be mutant whether the mutations are allelic or not.

Complementation of a mutation by *Arabidopsis* transformation (see section 2.4.12)

2.4 Molecular techniques

2.4.1 Production of competent cells of *Escherichia coli*

Competent cells of *E. coli* were produced by the calcium chloride method. A 5 ml aliquot of LB broth (1% (w/v) Bacto Tryptone, 0.5% (w/v) Bacto yeast extract, 1 % (w/v) NaCl, pH 7.5) was inoculated with a single colony of XL1 Blue MRF' *E. coli* and incubated overnight at 37°C with shaking (approximately 225 rpm). This culture was used to inoculate 500 ml fresh LB broth and grown at 37°C with shaking for 3-4 h until an OD_{600} of between 0.4 and 0.6 was reached. The cells were centrifuged at 3000 rpm using a 6 X 250 ml angled-rotor (JS-14) in a Beckman centrifuge (model J2-21) for 10 minutes at 4°C. The pellet was gently resuspended in 250 ml ice-cold $CaCl_2$ and left on ice for 1 h. The cells were centrifuged again (3000 rpm, 4°C, 10 minutes) and the pellet was resuspended in 50 ml 50 mM of ice-cold $CaCl_2$/20 % glycerol. The cells were then aliquoted into 1.5 ml Eppendorf tubes, frozen in liquid nitrogen and stored at -70°C.

2.4.2 Transformation of *E. coli* with plasmid DNA

A 100 µl aliquot of competent cells was thawed on ice and 10-100 ng plasmid DNA was added. The solutions were mixed by gently flicking the tube. The tube was incubated on ice for 20 minutes followed by 42°C for 2 minutes. After cooling on ice, 1 ml of LB was added and the tube was incubated at 37°C for 1 h. 100 µl of the transformed cells were spread on LB agar plates (as for LB broth plus 1.5 % 9 (w/v) Bacto agar) containing 100

μg/ml ampicillin (added before pouring the plates when the temperature of the LB-agar is approximately 45°C). The plates were incubated overnight at 37°C.

2.4.3 Plasmid DNA isolation

A single colony was picked from the selection plate (see above section) using a sterile yellow tip. The whole tip was ejected into a sterile universal bottle containing 5 ml of LB broth plus 100 μg/ml ampicillin. The culture was grown overnight at 37°C with shaking. The plasmid DNA was isolated using the QIA-prep spin plasmid miniprep kit (Qiagen, U.K.) according to the manufacturer's instructions.

2.4.4 Electrophoresis of DNA and RNA in non-denaturing conditions

Samples of purified DNA and RNA were checked for integrity and molecular weight distribution using agarose gel electrophoresis. 0.8 % (w/v) agarose gels were prepared and run in 1 X TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) containing 0.25 μg/ml ethidium bromide. Samples of DNA or RNA were mixed with 4 X loading buffer (0.25 % (w/v) bromophenol blue, 0.25 5 (w/v) xylene cyanol and 30 % (v/v) glycerol in water) and loaded alongside 1 Kb DNA ladder (Gibco, BRL, U.K.). The gel was electrophoresed at 5 V/cm for 1-2 hours depending on the dimensions of the gel.

2.4.5 Isolation of total RNA

All solutions, except for Tris, were treated with 0.1 % diethylpyrocarbonate (DEPC) to destroy RNase activity and were subsequently autoclaved to destroy the DEPC (forms ethanol and CO_2 upon autoclaving). Total RNA was isolated from approximately 500 mg fresh weight plant material by grinding it to a fine powder in liquid nitrogen using a pestle and mortar. 900 μl of extraction buffer (25 mM Tris-Cl, 25 mM EDTA, 75 mM Na Cl, 1 % SDS, 7.8% β–mercaptoethanol, pH 8.0) was added to the frozen powder in the mortar and was ground into the plant material. 900 μl of PIC (phenol: chloroform: isoamylalcohol 25:24:1) was added to the mortar and the mixture was ground until it had melted. The mixture was transferred to an Eppendorf tube and centrifuged (Microcentaur, 13,000 rpm,

4°C) for 10 minutes. The aqueous layer was transferred to a fresh Eppendorf tube and 1 volume of PIC was added. After vortexing the tube it was centrifuged again and the aqueous layer was transferred to a clean Eppendorf tube. 10 M LiCl was added to the aqueous extract to a final concentration of 2M (to preferentially precipitate RNA) and the tube was vortexed immediately. The tube was incubated at 4°C overnight before being centrifuged (Microcentaur, 13,000 rpm, 4°C) for 10 minutes. The supernatant was gently poured off and the pellet was washed with ice-cold 2 M LiCl by shaking. The tube was centrifuged again to consolidate the pellet and all the supernatant was removed. The pellet was resuspended in 500 µl of DEPC-treated H_2O and then 0.1 volumes of 3M sodium acetate (pH 5.5) and 2.5 volumes of ethanol were added to precipitate the RNA. It was then incubated on ice for 20 minutes. The tube was centrifuged (Microcentaur, 13,000 rpm, 4°C) for 15 minutes and the supernatant was discarded. The pellet was washed with 70 % ethanol and then the pellet was dried for 5 minutes in a Speed Vac Plus (Savant, model SC110A) connected to a vacuum pump (Vacuubrand Gmbh and Co., Wertheim, Germany). The pellet was finally redissolved in a minimal volume of DEPC-treated H_2O and allowed to rehydrate on ice for 3-4 h .

The quantity and purity of the RNA was determined spectrophotometrically by measuring its absorbance at 260 nm and 280 nm. An $A_{260\ nm}$ of 1 is equivalent to an RNA concentration of 40 µg/ml. The purity of RNA is determined by the ratio of its absorbance at 260 nm to its absorbance at 280 nm. An $A_{260/280}$ ratio of between 1.8 and 2.0 indicates that the RNA is free from protein contamination. The quality of the RNA was determined by agarose gel electrophoresis and the ribosomal RNA bands were checked for signs of degradation.

2.4.6 Denaturing agarose gel electrophoresis of RNA

Samples of 5 µg RNA were separated by electrophoresis through a 1.3 % (w/v) agarose gel containing 10 % formaldehyde and 1 x MOPS buffer, pH 7.0 (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA). The RNA samples were mixed with 1 % (v/v) formaldehyde, 30 % (v/v) formamide, and 1x MOPS, pH 8.0. The samples were heated at

65°C for 2.5 minutes, snap cooled on ice and loaded on to the gel. The gel was run for 2 h at 100V in 1 x MOPS, pH 7.0.

2.4.7 Northern hybridisation using Hybond N

RNA was transferred onto Hybond N nitrocellulose membrane using 20 X SSC (3 M NaCl and 0.3 M sodium citrate, pH 7.0) as described in the manufacturers protocol (Amersham International plc). Prehybridisation and hybridisation were carried out in 0.5M Na_2HPO_4, 7 % SDS and 10 mg/ml BSA as described by Church and Gilbert (1984). Northern blots were exposed onto X-ray film (Fuji RX) using an intensifying screen at -70°C for 1-7 days. Radioactive blots were also phospho-imaged using a Fuji Bio-Imaging Analyser (Fuji Photo Film Co. Ltd., Japan) by exposing the blot on to a pre-blanked imaging plate for 1 - 24 h in a cassette at room temperature. Exposed plates were developed by the Fuji Bio-Imaging Analyser and the images were captured using Mac-Bas software (Fuji Photo Film Co. Ltd., Japan).

2.4.8 Preparation of radiolabelled DNA probes

Plasmid DNA was digested with restriction endonucleases to release the insert. The digestion products were separated by electrophoresis through a 0.8 % (w/v) agarose gel buffered with 1 x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The required fragment was excised and extracted from the agarose using the QIA-quick gel extraction kit according to the manufacturer's instructions. Radiolabelled DNA probes were prepared for use as probes on Northern blots by oligonucleotide random priming using the Decaprime™ DNA labelling kit (Ambion Inc., Austin, U.S.A.) according to the manufacturer's protocol. G-50 spin columns were used to separate the unincorporated nucleotides from the radiolabelled DNA probe. Once synthesized, the probe was denatured by heating to 95-100°C for 5 minutes followed by chilling on ice before adding to the hybridization solution.

2.4.9 Isolation of *Arabidopsis* genomic DNA

3-4 mature rosette leaves were ground to a fine powder in liquid nitrogen with a pestle and mortar. The powder was added to a 30 ml plastic tube (Sarstedt, U.K.) containing 15 ml extraction buffer (2 % (w/v) hexadecyltrimethyl ammonium bromide (CTAB), 1.34 M NaCl, 20 mM EDTA, 0.002 % (v/v) β-mercaptoethanol, 100 mM Tris-Cl, pH 8.0) at 65 °C. The tube was mixed by inversion and incubated at 65 °C for 30 minutes. An equal volume of chloroform: isoamylalcohol (24: 1) was added to the tube which was shaken for 15 minutes. The tube was centrifuged at 4°C for 10 minutes at 4000 rpm in a 8 X 50 ml angled rotor (JA-20) in a Beckman centrifuge (model J2-21). The aqueous layer was transferred to a new tube containing 0.67 volumes of isopropanol and was shaken for 20 minutes at room temperature. The tube was centrifuged, as before, at 4000 rpm for 10 minutes to pellet the nucleic acids and the supernatant was discarded. The pellet was resuspended in 500 µl TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) and transferred to an Eppendorf tube. The nucleic acid solution was treated with RNase (10 µl of 5 mg/ml stock) and incubated for 30 minutes at 37 °C. 1 volume of PIC (phenol: chloroform: isoamylalcohol; 50: 49: 1) was added and the mixture was vortexed. The aqueous layer was removed to a new tube and 0.1 volumes 3 M sodium acetate and 2 volumes of 95 % (v/v) ethanol were added to precipitate the DNA. The tube was incubated at -20 °C for 1 hour. The DNA was centrifuged (MicroCentaur, 13000 rpm, 4°C) for 10 minutes, the pellet was washed with 70 % (v/v) ethanol and desiccated. The pellet was resuspended in 50 µl T E. The quantity and purity of the DNA was determined spectrophotometrically by measuring its absorbance at 260 nm and 280 nm. An $A_{260\,nm}$ of 1 is equivalent to a concentration of 50 µg/ml. The purity of DNA is determined by it $A_{260/280}$ ratio. An $A_{260/280}$ ratio of between 1.8 and 2.0 indicates that the DNA is free from protein contamination.

2.4.10 Primer design

Primers were designed which were specific to the Arabidopsis *thaliana* hexokinase 1 and hexokinase 2 pBIN19 constructs (the full length coding sequences of At HXK 1 and 2 cloned into a pBIN19 35S GUS derived vector; a gift from Dr J-C Jang). The forward

primer was designed in a region of the 35S promoter and the reverse primers were designed in regions of the hexokinase genes which were non-homologous (Figure 2.2).

The primer sequences were:

hexokinase 1 reverse (athHXK1s-R) 5' -gAg gAT AgC CAA AAC ACg - 3'

hexokinase 2 reverse (athHXK2s-R) 5'-CAA TAT CTC TAT CAC TCT - 3'

35 S forward (camV 35S-F) 5'.-ggA TTg ATg TgA TAT CTC - 3'

2.4.11 The polymerase chain reaction (PCR)(Mullis and Faloona, 1987)

The polymerase chain reaction was used to specifically amplify fragments of AtHXK 1 or 2 flanked by T-DNA in order to verify whether plants were transformed with hexokinase 1 or hexokinase 2. The 20 µl reaction mix was comprised of 2 µl 10 X PCR buffer (500 mM KCl, 100 mM Tris-Cl, pH 8.4, 15 mM $MgCl_2$, 200 µg/ml gelatine), 2 µl $MgCl_2$, 0.5 µl 35 S forward primer, 0.5 µl of a specific hexokinase reverse primer, 0.18 µl 25 mM dNTP, 1U Taq, 14.62 µl water plus 10-100 ng genomic DNA. The reaction was performed on a thermocycler (Perkin Elmer Gene Amp PCR Syetem 2400). The reaction conditions were :

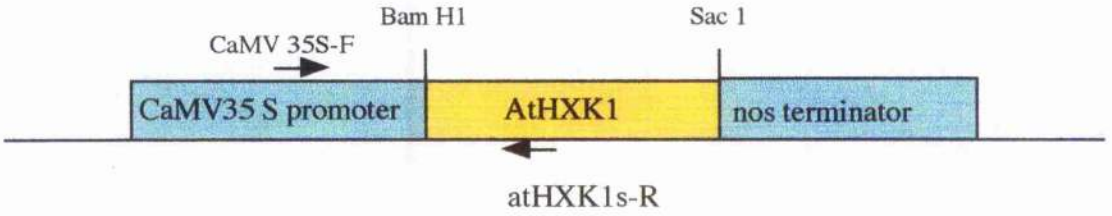
conditions were :	94 ° C	3 minutes
followed by 40 cycles of:	94 ° C	15 seconds
	55 ° C	15 seconds
	72 ° C	30 seconds
followed by	72 ° C	5 minutes

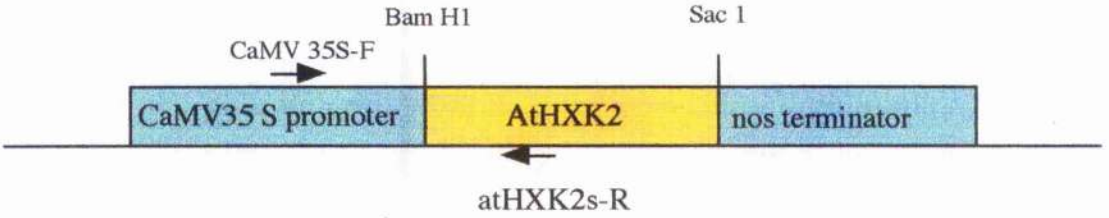
The PCR products were separated by electrophoresis through a 0.8 % agarose gel buffered by TAE.

2.4.12 Complementation of a mutation by *Arabidopsis* transformation (vacuum infiltration method)(Bechtold, *et al.*, 1993)

A mutation in a specific gene can be complemented by transforming the mutant with the correct gene in the sense orientation under the control of either its own promoter or a constitutively expressed promoter. Plants of the appropriate ecotype were grown to a stage at which the bolts were just emerging. These bolts were clipped to promote growth of

Figure 2.1 Vector derived from pBIN19 containing the 35S:AtHXK1 or 35S:AtHXK2 construct.


Bam H1
Sac 1
CaMV 35S-F
CaMV35 S promoter
AtHXK1
nos terminator
atHXK1s-R


Bam H1
Sac 1
CaMV 35S-F
CaMV35 S promoter
AtHXK2
nos terminator
atHXK2s-R

multiple secondary bolts and infiltration was done 4-8 days after clipping. A 400 ml culture of *Agrobacterium* (GV3101) containing the AtHXK 1 construct was prepared in LB plus 25μg/ml gentamycin and 50 μg/ml kanamycin and grown to an OD_{600} >2.0. The cells were harvested by centrifugation (5K, 10 minutes in a GSA rotor at RT) and resuspended in a volume of infiltration medium (1/2 MS salts, 1 X B5 vitamins, 5 % sucrose, 0.044μM benzylaminopurine) calculated to give an OD_{600} of approximately 0.8. The bolts and rosette leaves of the *Arabidopsis* were submerged in the *Agrobacterium* containing infiltration medium and placed under a 1.7 m^3/h vacuum (Vacuubrand MZ2C pump, Gmbh and Co. Wertheim, Germany) for 3 minutes. The vacuum was briefly released to dislodge trapped air bubbles before a final 30 second vacuum period. After this treatment the rosette leaves had lost turgidity and appeared dark green. The plants were removed from the solution and allowed to drain before being put back in the growth room. The plants were allowed to bolt and the siliques were collected when they were mature and dry. Putative transformant seeds were sterilised, resuspended in 0.1 % agar and spread on selection plates (2000 seed per 150 x 15 mm plate: 1/2 MS salts, 0.8% agar, 1 x B5 vitamins, 50μg/ml kanamycin, 25 mg/l nystatin). After 2 days in the cold room the plates were moved to the growth room . After about 7 days in the growth room transformants were identified as dark green seedlings with secondary leaves and roots that extend into the selective medium. Transformants were transferred to soil and allowed to set seed.

2.5 Protein Analysis

2.5.1 Soluble protein extraction for western blotting and hexokinase assays

Plant tissue was ground up using a pestle and mortar in 50 mM Tris-Cl, pH 7.3, 1 mM DTT, 15 % (v/v) glycerol, 5 mg/ml BSA (1g fw/ml buffer), and 120 mg/g fw polyvinylpolypyrollidone at 4°C and transferred to an Eppendorf tube. The sample was centrifuged (Microcentaur, 13000 rpm) for 5 minutes at 4°C. The extracts were rapidly desalted by a centrifugation (Microcentaur, 400 rpm, 2 minutes) through a 1 ml Sephadex G50 mini-column pre-equilibrated with the extraction buffer and the eluate was used in enzyme assays and westerns.

2.5.2 Storage protein Extraction

Seeds were ground up in 50 mM Tris-Cl, pH 7.3, 1 mM EDTA, 1 mM DTT, 120 mg/g fw PVPP, 1 % SDS and were boiled for 5 minutes to solubilise and denature the proteins. The sample was centrifuged (Microcentaur, 13000 rpm) for 5 minutes at 4°C.

2.5.3 Quantitation of total protein

The protein concentration of the extracts was measured by 2 methods. Soluble protein extract concentrations were determined using Biorad Bradford's reagent (Bradford, 1976). The Bradford method depends on the binding of Coomasie Brilliant Blue to an unknown protein and comparing this binding to that of different amounts of a known protein, IgG. The Bradford method cannot be used to measure protein concentration in samples containing SDS. Storage protein concentration was measured using the method of Lowry (Lowry, *et al.*, 1951, Peterson, 1977) which is not perturbed by the presence of SDS in the extract. The Lowry method depends on quantitating the colour obtained from the reaction of Folin-Ciocalteu phenol reagent with the tyrosyl residues of an unknown protein and comparing this colour value to the colour values derived from a standard curve. 1-10 µl of the unknown protein extract was placed in an Eppendorf and the volume was made up to 200 µl with water. 20 µl of 0.15% (w/v) sodium deoxycholate was added, the tube was vortexed and allowed to stand for 10 minutes at room temperature. 20 µl of 72 % (w/v) trichloroacetic acid was added and the tube was vortexed. The tube was centrifuged at 3000 x g and the pellet was redissolved in 200 µl water. 200 ul of copper tartrate/carbonate solution [0.1 % (w/v) $CuSO_4.5H_2O$ and 0.1 % (w/v) potassium tartrate, 10 % (w/v) sodium carbonate] was added, the tube was vortexed and left to stand for 10 minutes at room temperature. 100 µl of 20 % Folin-Ciocalteu was added, the tube was vortexed and left to stand for 30 minutes at room temperature. The A_{750} was determined and was compared to a standard curve of between 1 and 20 µg of protein.

2.5.4 Electrophoretic separation of proteins (SDS PAGE)

Proteins were separated by discontinuous SDS-PAGE (Laemmli, 1970). Storage protein gels were made with a 12.5 % polyacrylamide separating gel and all other gels were made with an 8 % polyacrylamide separating gel. The stacking gel was 3 % polyacrylamide. Protein samples were denatured by boiling for 4 minutes in 25 mM Tris-HCl, pH 6.8, 0.5 % (w/v) SDS, 5 % (w/v) glycerol, 0.01 % (w/v) bromophenol blue and 0.5 % (w/v) 2-mercaptoethanol. The samples were then loaded onto the gel and electrophoresed at 60 mA for 2-2.5 hours.

2.5.5 Staining SDS PAGE gels

The stacking gel was removed and the separating gel was stained for 30 minutes at 37 °C in 0.1 % Coomassie Brilliant Blue G250, 50 % (v/v) methanol, 10 % (v/v) glacial acetic acid, and destained in several changes of 10 % (v/v) methanol, 10 % (v/v) glacial acetic acid at 37 °C.

2.5.6 Western blot analysis

After electrophoresing protein extracts against prestained markers (Biorad, UK.) by SDS PAGE, the protein was transferred to nitrocellulose using a miniblot system (Biorad, U.K.) using the manufacturers protocol. The nitrocellulose was stained with Ponceau S solution (Salinovich and Montelaro, 1986) to stain the proteins and to check that loading of the wells was equal. The filter was then completely destained in water. The filter was placed overnight in a plastic box containing the blocking solution (25 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05 % Tween-20, 5% Marvel). The blocking solution was poured off and the blot was incubated with the *At*HXK antibody (1 in 1000 dilution; a gift from J-C. Jang) in TBS-Tween-milk for 4 h. The blot was washed 3 times in TBS-Tween and then incubated for 2 hours with the secondary anti-rabbit antibody linked to alkaline phosphatase diluted 1 to 20000 in TBS-Tween. The blot was washed 3 times in TBS-Tween. The blot was developed with an alkaline phosphatase/ nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) system for immunodetection. The blot was incubated in 20 ml of

developer buffer (0.1 M Tris-Cl, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$, 4.4 mg NBT and 3.3 mg BCIP) at 37°C in the dark. The reaction was stopped by washing in distilled water.

2.5.7 Hexokinase assays

Hexokinase activity in the protein extract was assayed using a modified version of that described by Bouny and Saglio (1996). The rate of NADP reduction was recorded (ATi Unicam UV/Vis Spectrometer-UV4) at 340nm (25°C or 37°C) in a cuvette containing the protein extract plus 50 mM Tris-Cl, pH 7.3, 1 mM glucose, 1mM $MgCl_2$, 1 mM NADP, 1 mM ATP, 0.1 mM EDTA, 5 units G6PDH.

2.6 Metabolite Measurements

2.6.1 Radioactive ^{14}C -mannose and ^{14}C -glucose feeding experiments

10 mg dry weight of sterile seeds was sown in a 24 well culture plate on 3 discs of 3M filter paper soaked in 150 µl 1/2 MS salts containing 1) 5 mM mannose plus 1 µCi D-[U-^{14}C]mannose; 2) 5 mM glucose plus 1 µCi D-[U-^{14}C]glucose or 3) 5 mM mannose plus 1 µCi D-[U-^{14}C]mannose plus 50 mM glucose. The plate was sealed with Nescofilm and the seeds were imbibed at 4°C in the dark for 4 days. Transfer to the growth room (22 °C, 24 h continuous white light, 120 $\mu Em^{-2}\ s^{-1}$) represents time zero. Samples were taken at time intervals thereafter. The germinating seeds were washed in ice cold water to remove any exogenous sugars and MS salts. The seedlings were then finely ground in a glass homogeniser in an excess of 80 % ethanol and left to extract for 1 hour at room temperature. The solution was centrifuged, the supernatant was lyophilised in a Speed Vac Plus (Savant, model SC110A) connected to a vacuum pump (Vacuubrand Gmbh and Co., Wertheim, Germany) and the resulting pellet was resuspended in 40 µl H_2O. The samples were then loaded onto a paper chromatogram (PC) and developed for 18 h in ethyl acetate: pyridine: water (8:2:1) as described by Fry (1988). The PC was dried and imaged using a Packard Instant Imager (Packard Instrument Company, USA) which allows visualisation and accurate quantitation of the ^{14}C metabolites, amino acids and deacetylated amino sugars.

2.6.2 Alkaline phosphatase treatment of metabolites

Metabolites extracted from germinating seeds fed with ^{14}C labelled compounds were treated with alkaline phosphatase to determine the amount of hexose phosphates that were present. 25 µl of extract was incubated for 3 hours at 25°C in a 100 µl reaction mixture containing 50 mM NH_4HCO_3 (pH 8.0), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 5 units alkaline phosphatase. The digested extract was then separated by paper chromatography and compared with the undigested extract.

2.6.3 Invertase treatment of metabolites

Metabolites extracted from germinating seeds fed with ^{14}C labelled compounds were treated with invertase to identify a radioactive compound which had the same Rf value as sucrose. 25 µl of extract was incubated for 1 h at 37°C in a 100 µl reaction mixture containing 100 mM imidazole (pH 6.9), 5 mM $MgCl_2$ and 5 units invertase. The digested extract was then separated by paper chromatography.

2.6.4 Silver nitrate staining of sugars and alditols

The PCs were stained with silver nitrate to detect sugars and alditols (Fry, 1988). After drying, the PC was briefly dipped in solution 1 (0.5 ml saturated aqueous silver nitrate added to 100 ml acetone). The PC was dried again and was dipped in solution 2 (1.25 ml 10N NaOH added to 100 ml ethanol) which stains the sugars brown. After drying again, the PC was dipped in 10 % (w/v) sodium thiosulphate to stabilise the colour reaction and prevent fading.

2.6.5 Extraction and paper chromatographic analysis of amino acids and de-acetylated amino sugars (Fry, 1988)

50 seeds were ground in a glass homogeniser in 0.3 ml 80 % (v/v) ethanol. The extracts were centrifuged (Microcentaur, 13000 rpm) for 10 minutes at RT and the pellet was re-extracted in 0.3 ml 80 % ethanol. After centrifugation (Microcentaur, 13000 rpm, 10 minutes), the pellet was re-extracted with 20 % (v/v) ethanol. The suspension was

centrifuged as before and all 3 supernatants were combined and lyophilised in a Speed Vac Plus (Savant, model SC110A) connected to a vacuum pump (Vacuubrand Gmbh and Co., Wertheim, Germany) and redissolved in 50 µl H_2O. The amino acids were separated by paper chromatography in butan-1-ol: acetic acid: water (120: 30: 50) for 18 hours. The paper chromatogram was dried and briefly dipped in a solution of 0.5 % (w/v) ninhydrin in acetone. The paper was dried and then heated to 105 °C to allow the colours to develop. Most amino compounds are violet and proline and hydroxyproline are yellow.

2.6.6 Sucrose, glucose and fructose measurements

Frozen plant tissue (200-300 mg) was heated for 1 hour at 70°C in 3 X 500 µl 80 % ethanol. The extracts were combined, lyophilised in a Speed Vac Plus (Savant, model SC110A) connected to a vacuum pump (Vacuubrand Gmbh and Co., Wertheim, Germany) and redissolved in 50 µl H_2O. Metabolites were measured using enzyme-linked assays according to Stitt *et al* (1989).

2.6.7 Chlorophyll measurements

Chlorophyll was extracted from whole seedlings using a modification of the method described by Arnon (1949). 50 mg plant material was ground up in 200 µl 80 % acetone in an Eppendorf tube using a plastic homogeniser. The sample was centrifuged (Microcentaur, 13000 rpm, 2 minutes) and the supernatant was removed to a new tube. The pellet was re-extracted with 2 X 200 µl 80 % acetone until it was colourless and the supernatants were combined. The absorption of the supernatant was measured at 663 and 645 nm. The equations for determining chlorophyll concentration is set out below.

Chlorophyll a = 12.7 X A_{663} - 2.69 X A_{645} (mg/l)

Chlorophyll b = 22.9 X A_{645} - 4.68 X A_{663} (mg/l)

Total chlorophyll = 20.2 X A_{645} + 8.02 X A_{663} (mg/l)

2.6.8 Lipid extraction

50 seeds were ground on ice in a glass homogeniser in 800 µl chloroform :methanol :formic acid (10: 10 :1(v/v)). The mixture was transferred to an Eppendorf tube, centrifuged (Microcentaur, 13000 rpm, 2 minutes) and the supernatant was kept. The pellet was re-extracted in 300 µl chloroform :methanol :water (5: 5 :1(v/v)), centrifuged again and the supernatant was combined with the first. The supernatant was washed with 400 µl 0.2 M H_3PO_4, 1 M KCl and the lipid was recovered in the chloroform phase. The chloroform phase was dried in a Speed Vac Plus (Savant, model SC110A) connected to a vacuum pump (Vacuubrand Gmbh and Co., Wertheim, Germany) and the lipids were redissolved in 50 µl of chloroform. The sample was then separated by thin layer chromatography (TLC).

2.6.9 Thin layer chromatography (TLC) of lipids

Lipid samples were applied to a silica plate (General Purpose, silica on glass, Sigma) 150 mm apart and 150 mm from the bottom of the plate. The TLC was developed immediately in a filter paper lined tank containing the solvent (hexane: ether: acetic acid (70: 30: 1)) until the solvent front was 200 mm from the top of the plate. The TLC was sprayed with a fine mist of 50 % (v/v) sulphuric acid and was baked for 3-5 minutes at 160 °C to char the lipids. Alternatively, the lipids can be visualised by putting the TLC in a tank saturated with iodine gas.

2.6.10 Amino acid extraction for HPLC analysis

Fifty seeds were ground up in a glass homogeniser in 300 µl 80 % ethanol. The extract was transfered to an Eppendorf tube and a further 300 µl of 80 % ethanol was used to wash out the homogeniser. The extracts were combined and centrifuged (Microcentaur, 13000 rpm, 5 minutes). The supernatant was transfered to another tube and the pellet was reextracted in 300 µl 20 % ethanol. The tube was centrifuged and the supernatant was added to the 80 % extracts. The extract was lyophilized in a Speed Vac Plus (Savant, model SC110A) connected to a vacuum pump (Vacuubrand Gmbh and Co., Wertheim, Germany) and was resuspended in 50 µl H_2O.

2.6.11 Amino acid HPLC analysis

Amino acid samples analysed by HPLC (Spheri-5PTC column 5um, 0.2 X 22 cm; solvent A: 50 mM sodium acetate, pH 5.4; solvent B: 70 % acetonitrole, 32 mM sodium acetate pH 6.1; flow: 300ul/minute; target pressure 2000 Kpa, target time 0.1 minutes). The samples were sent to Dr M. Cusack, who provides an analytical HPLC service at the University of Glasgow, Molecular Palaeontology Department.

2.6.12 Fatty acid measurements using GC (performed by Dr T. Larson)

Fifty seeds were placed in a 16 x 100 mm screw-top glass tube. 100 µl hexane and 10 µl of a 5mg/ml heptadecanoic acid dissolved in chloroform (internal standard) was added. 1 ml 1N HCl in methanol was added to the tube which was then sealed and incubated at 85 ° C for 1 h. The solution was allowed to cool for 10 minutes and then 0.5 ml 0.9 % (v/v) KCl (aq) was added. The tube was vortexed. The upper hexane layer containing the fatty acid methyl ester derivatives was aspirated into an Eppendorf tube. 1 µl of the fatty acid methyl ester derivatives was injected into the GC (GC8000, CE Instruments) equipped with a 30 m long 0.32 mm I. D. Hewlett Packard Innowax crosslinked polyethylene glycol column (HP part number 19091N-213). The carrier gas was Helium with a 1 ml/minute flow rate, split ratio 30:1. Peaks were eluted at 50° C for 5 minutes then ramped at 10 ° C /minute to 250 °C and held at this temperature for 5 minutes. The peaks were detected with a flame ionisation detector. The peaks were analysed using Chromcard software.

2.7 Treatment of data

All experiments were carried out twice unless otherwise stated and the average value was presented. The error bars represent the standard deviation P of the data. Standard deviation P is the calculation of the standard deviation based on the entire population given as arguments. The standard deviation is a measure of how widely values are dispersed from the average value. The standard deviation P was calculated using the formula described in Microsoft Excel Help.

Chapter 3

Characterisation of carbohydrate insensitive (*cai*) mutants

3.1 Introduction

Tobacco seedlings which have been grown on a medium containing high levels of carbon (100 mM sucrose) and low levels of nitrogen (0.48 mM) accumulate sugars ((Paul and Stitt, 1993). This leads to a decrease in the level of protein in the shoots and, particularly, Rubisco and chlorophyll. A possible explanation for this carbohydrate accumulation is that there is insufficient nitrogen to support the flux of carbon into amino acids. This would lead to a decrease in flux through glycolysis, and hence, a build up of hexoses. A further consequence of growing the tobacco seedlings on high carbon/ low nitrogen is that the root : shoot ratio increases.

The observations of Paul and Stitt (1993) formed the basis of a screen which was developed by Dr I. A. Graham to select for carbohydrate insensitive (*cai*) mutants of *Arabidopsis thaliana.* In a prototype experiment Dr Graham grew wild type *A. thaliana* were grown on an agar medium containing Murashige and Skoog salts (M&S) with 0.48 mM nitrogen and 100 mM sucrose and were characterised with regard to the root: shoot ratio. In addition to an increase in root : shoot ratio, the seedlings also had purple cotyledons suggesting the accumulation of elevated levels of anthocyanins. Anthocyanin production is a typical stress response and in this case it was probably triggered by the high concentration of soluble sugars. Such conditions have previously been found to induce chalcone synthase expression in Petunia (Tsukaya, *et al.*, 1991). CHS is a key enzyme in anthocyanin biosynthesis. It is also possible that the *Arabidopsis* seedlings were stressed due to limiting nitrogen. Dr. Graham used these conditions (100 mM sucrose, 0.48 mM nitrogen) to screen a population of ethyl methanesulphonate (EMS) mutagenised *Arabidopsis* seeds (ecotypes Columbia-2 and Wassilewskija) for seedlings which developed healthy green cotyledons rather than the typical purple cotyledons produced by the wild type. Approximately 70,000 seeds were screened and around 70 putative mutants (putants) were selected by virtue of their green cotyledons, of which 30 survived. It was envisaged that these putants could represent plants which were insensitive to growth on high sugar/ low nitrogen

concentrations due to a mutation in either the sugar sensing mechanism, carbon metabolism or the uptake of sucrose and/or nitrogen.

The objectives of the work described in this chapter were to:

(1) characterise the wild type under the *cai* screen,

(2) identify which of the M2 putants were real mutants in carbohydrate signalling and

(3) describe the *cai* phenotype.

3.2 Results

3.2.1 Growth of wild type *Arabidopsis* seeds on *cai* selection conditions

Seeds of wild type *Arabidopsis* were germinated in high carbon/ low nitrogen conditions in order to determine whether they responded like tobacco seedlings as described in Paul and Stitt (1993). Figure 3.1a shows wild type (col-2 ecotype) which were grown on M&S salts with decreasing concentrations of nitrogen plus 100 mM sucrose for 11 days. The root : shoot ratio increased as the nitrogen concentration decreased. In addition, the cotyledons of the seedlings became increasingly purple as the nitrogen concentration decreased. In Figure 3.1b sucrose was replaced by the non-metabolizable alditol, mannitol. This simulates the osmotic affect of sucrose but avoids the accumulation of soluble sugars associated with exogenous sucrose. The cotyledons do not have purple cotyledons and develop primary leaves as normal (Figure 3.1b). Figure 3.2a shows that as the nitrogen concentration decreases *rbcS*, *cab* and *pc* expression is repressed and *chs* expression is induced. The fall in *rbcS*, *cab* and *pc* expression correlates with an increase in seedling soluble sugars (Figure 3.2b) and a decrease in chlorophyll (Figure 3.2c). As the nitrogen becomes limiting the root : shoot ratio increases (Figure 3.2d) and fresh weight decreases (Figure 3.2e). The actual root length increases as nitrogen concentration decreases from 60 mM to 6 mM and may be due to the seedlings having to scavenge for nitrogen (Figure 3.2 f). Figure 3.3 shows seedlings of col-2 grown on decreasing nitrogen concentration plus 100 mM sucrose and complements the data illustrated in Figure 3.2 A-F. Figure 3.3 also shows that the phenotypes of the population vary as some seedlings have green cotyledons and others have purple cotyledons. This may be a result of varying quantities of seed reserve.

Figure 3.1 The affect of carbon-nitrogen ratio on seedling phenotype.

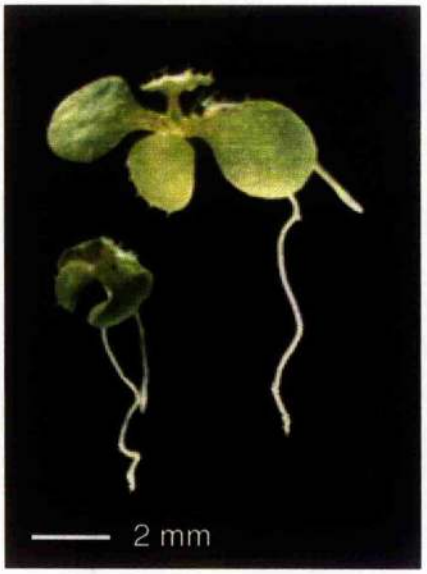
a: Wild type *A. thaliana* (col-2) were grown on M&S agar media containing 100 mM sucrose and varying concentrations of nitrogen for 7 days at 22 °C in continuous white light (120 $\mu Em^{-2}s^{-1}$).

b: Wild type *A. thaliana* (col-2) were grown on M&S agar media containing 100 mM mannitol and varying concentrations of nitrogen for 7 days at 22 °C in continuous white light (120 $\mu Em^{-2}s^{-1}$).

a

100 mM sucrose plus	60 mM nitrogen	2.4 mM nitrogen	0.5 mM nitrogen	0.1 mM nitrogen

b



100 mM mannitol plus	60 mM nitrogen	0.5 mM nitrogen

Figure 3.2 Changing carbon nitrogen ratios affects a range of growth parameters in 7 day old *A. thaliana* seedlings.

a: Northern blot analysis of the expression of *rbcS, cab, pc* and *chs*.

b: Seedling soluble sugars increase as the carbon-nitrogen ratio of the media increases.

c: Chlorophyll concentration decreases as carbon nitrogen ratio increases.

d: Root: shoot ratio

e: Fresh weight

f: Root length

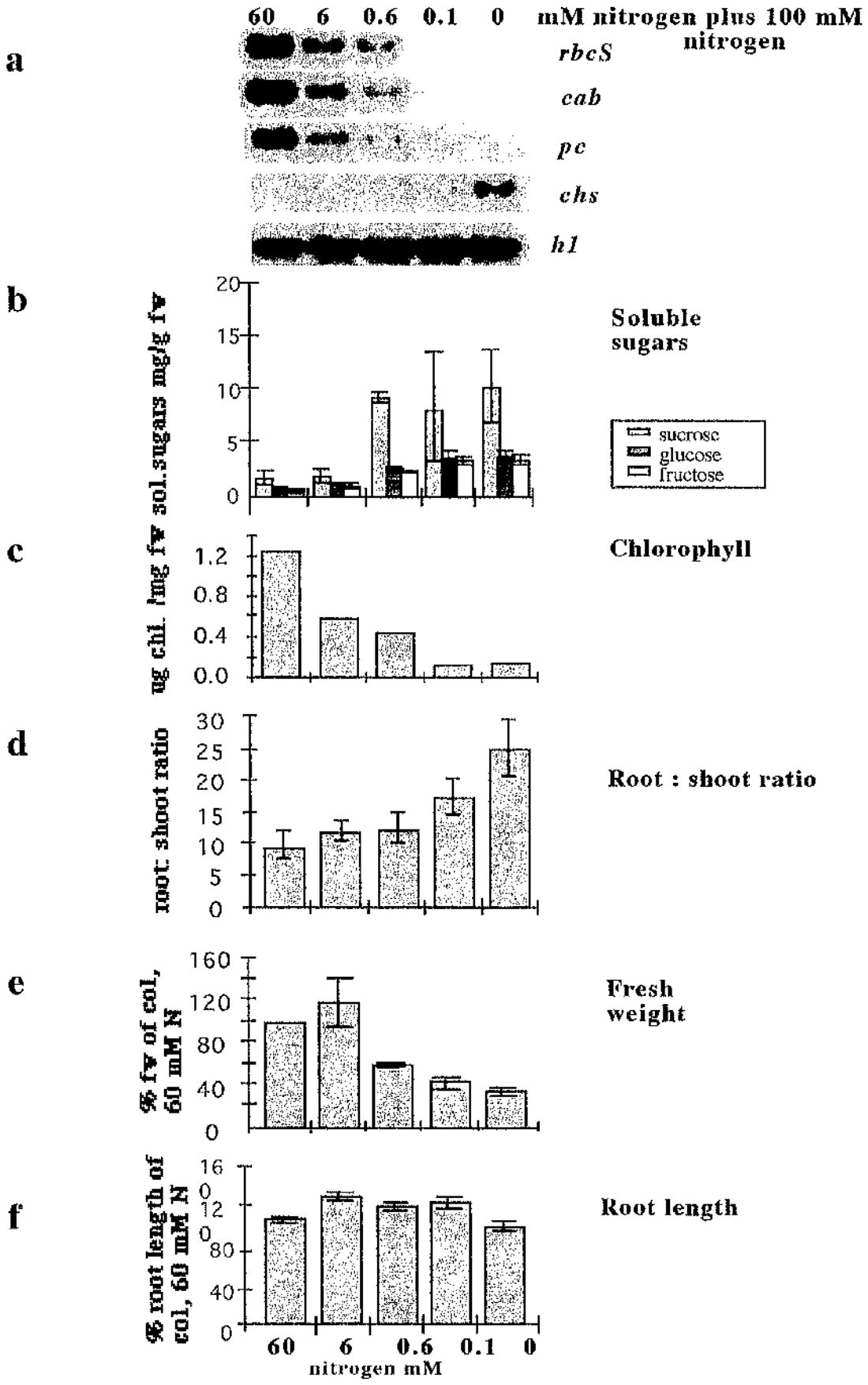
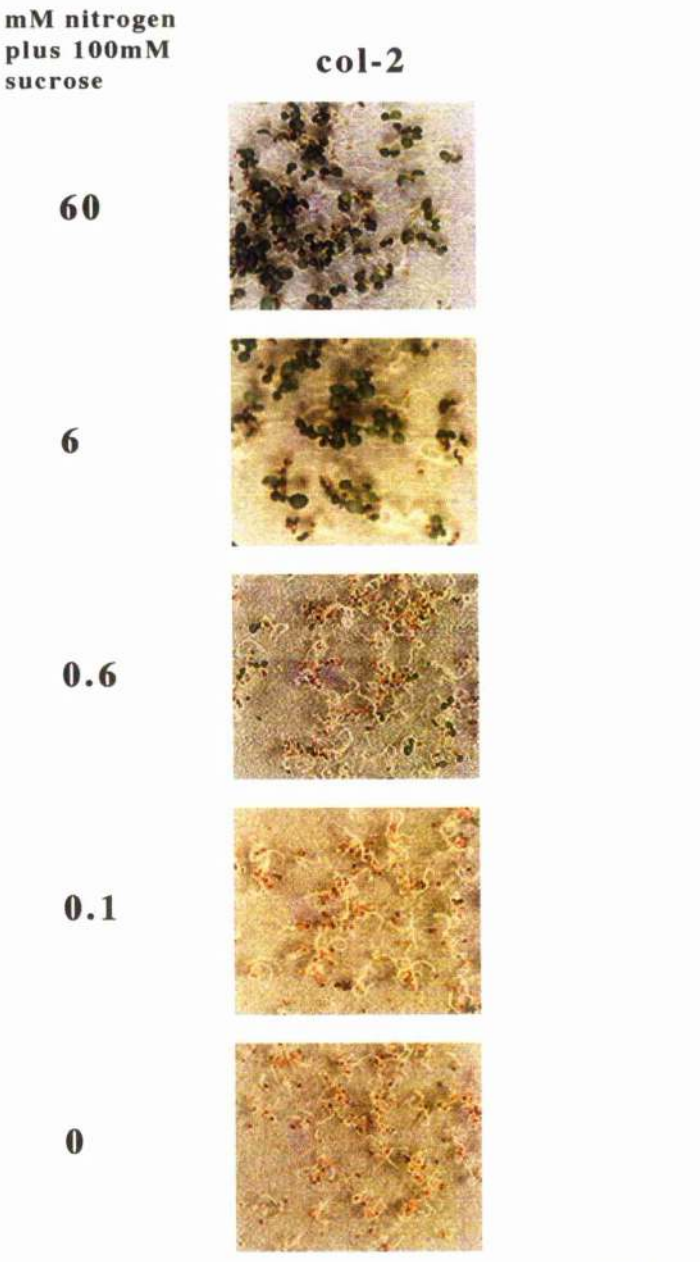

a
60 6 0.6 0.1 0 mM nitrogen plus 100 mM nitrogen
rbcS
cab
pc
chs
h1
b
Soluble sugars
sol.sugars mg/g fw
sucrose
glucose
fructose
c
Chlorophyll
ug chl. /mg fw
d
Root : shoot ratio
root: shoot ratio
e
Fresh weight
% fw of col, 60 mM N
f
Root length
% root length of col, 60 mM N
60 6 0.6 0.1 0
nitrogen mM

Figure 3.3 The variable phenotypes within a population of *A. thaliana* grown on different carbon-nitrogen ratios.

Wild type *A. thaliana* (col-2) were grown on M&S agar media containing 100 mM sucrose and varying concentrations of nitrogen for 7 days at 22 °C in continuous white light (120 $\mu Em^{-2}s^{-1}$).


mM nitrogen plus 100mM sucrose
col-2
60
6
0.6
0.1
0
1.5 cm

3.2.2 Optimisation of the *cai* screen

A problem encountered with the screening conditions of high carbon / low nitrogen was background levels of green seedlings in the wild type. The frequency of purple versus green seedlings varied in response to slight changes in temperature and light conditions. This made genetic analysis very difficult as one could not be certain (without further genetic analysis in subsequent generations) that a seedling with green cotyledons actually was a *cai* mutant. The growth parameters (imbibition time, light regime, light intensity and temperature) were changed systematically to optimise the *cai* screen. The results of a substantial amount of optimisation work are as follows: sterilised seeds were sown on to agar plates containing M&S salts with 0.1 mM nitrogen and 100 mM sucrose. The plates were given a 4 day cold treatment (4°C) in the dark and were transferred to a growth room with continuous fluorescent white light (120 μmol m^2 s^{-1}) at 22°C for 1-2 weeks. These parameters gave the best reproducibility of results although wild type seedlings still had a background of 5 - 10% green cotyledons.

3.2.3 Penetrance of the *cai* phenotype

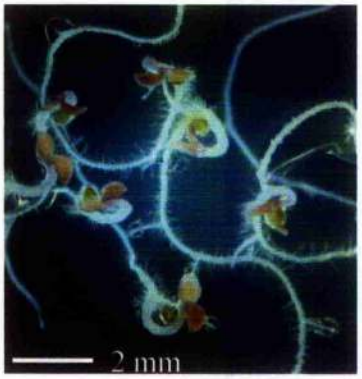
Initial work included rescreening the 30 putative mutants (M2 generation) on M&S salts with 0.1 mM nitrogen concentration and 100 mM sucrose. Figure 3.4 shows col-2 and *cai* 28 grown for 7 days on *cai* selection conditions. Six mutants, from a different sibling pools, were selected for further experimentation. Figure 3.5 shows five of these mutants grown on M&S salts with 0.1 mM nitrogen plus 100 mM sucrose. As can be seen all five plants are greener and have a larger root system than the wild type plant in figure 3.1a. Figure 3.6 shows the *cai* 10 and *cai* 28 in soil for 25 days. *cai* 10 has a phenotype similar to ws. *cai* 28 is slower to grow, is smaller and has paler green leaves than ws.

Whilst rescreening of the M2 and subsequent generations of the *cai* mutants it was repeatedly found that only a percentage of the seedlings had green cotyledons (typically 20-30 % penetrance for *cai* 10 and 28) even though the seeds theoretically had identical genotypes (Table 3.1). The results also illustrate the variation in the penetrance of the *cai* phenotype between experiments. To determine whether this was due to incomplete

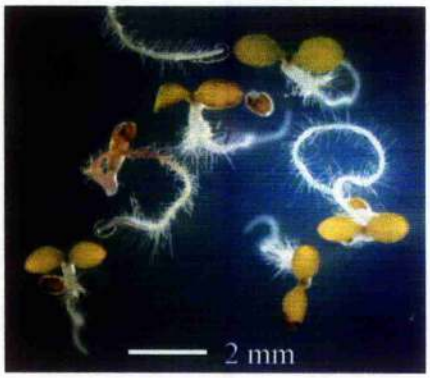
Figure 3.4 An example of the *cai* phenotype on *cai* selection conditions.

a: Wild type seedlings were grown on M&S agar media containing 100 mM sucrose and 0.1 mM nitrogen for 7 days at 22 °C in continuous white light (120 $\mu Em^{-2}s^{-1}$).

b: *cai* 28 seedlings were grown on M&S agar media containing 100 mM sucrose and 0.1 mM nitrogen for 7 days at 22 °C in continuous white light (120 $\mu Em^{-2}s^{-1}$).



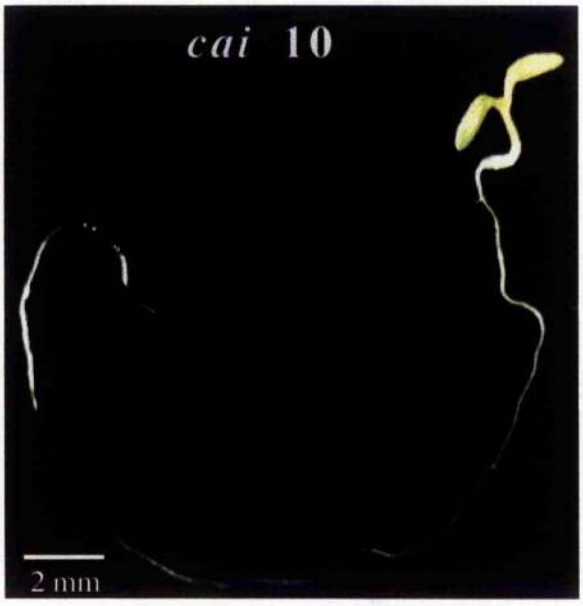
wild type (ws)



***cai* 28**

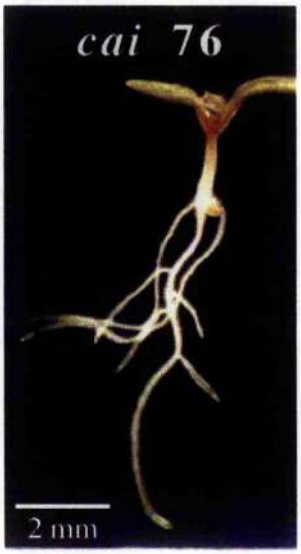
Figure 3.5 *Cai* mutants grown on M&S agar media containing 100 mM sucrose and 0.1 mM nitrogen.

cai mutant seedlings were grown on M&S agar media containing 100 mM sucrose and 0.1 mM nitrogen for 7 days at 22 °C in continuous white light (120 $\mu Em^{-2}s^{-1}$).


cai 10
2 mm


cai 28
2 mm


cai 31
2 mm


cai 76
2 mm


2 mm
cai 172

Figure 3.6 The phenotype of adult *cai* mutants grown on soil.

The *cai* mutants were grown for 25 days in soil at 22 °C in 120 $\mu Em^{-2}s^{-1}$ white light (16h light, 8 h dark regime).


10 (M7)
25 day old
28 M9
25 day old
ws
25 day old
2 cm

Table 3.1 The percentage seedlings with a *cai* phenotype is highly variable between experiments and with different nitrogen concentrations.

cai mutant seeds were sown on M&S agar media containing 100 mM sucrose and varying concentrations of nitrogen. The plates were imbibed for 4 days at 4 °C and were transferred to a growth room at 22 °C in continuous white light (120 $\mu Em^{-2}s^{-1}$) for 7 days. The percentage of seedlings which had a *cai* phenotype (green cotyledons) was calculated.

% penetrance of *cai* phenotype on different nitrogen concentrations (mM) plus 100 mM sucrose						
***cai* mutant**	0	0	0.1	0.1	0.1	0.48
***cai*10**	15	58		13	6.3	25
***cai* 28**	16		33.3	14	25	79
***cai* 31**	0			0	28	77
***cai* 76**	2	15		1	24	48
***cai* 112**	6		29	5		49
***cai* 172**	0		16	1		29
col-2	0	7	2	3.8	5	13
ws	1	10	3.6	2.7	9	20

penetrance of the phenotype, seedlings with either purple or green cotyledons were rescued from the selection media and grown up for seed. Their progeny were then also grown on *cai* selection media. The progeny of both purple and green parents had a mixture of seedlings with purple and green cotyledons (Table 3.2). However, the progeny of parents with a 'very pale with purple halo' phenotype do have a lower penetrance than progeny of green parents. This indicates that there may be some segregation of the *cai* mutation in the M5 generation. This implies that both segregation and penetrance influence the phenotype of *cai* 28. Reasons for incomplete penetrance of a gene are not well understood but it is known that they arise when the trait is modified strongly by environmental factors; e.g., light intensity or light quality during seed development or post-germinative growth. In *Arabidopsis* this is the case with genes affecting flowering time, seed dormancy, photomorphogenesis and anthocyanin formation (Koornecf, 1994). Communication with Dr S. Smeekens and Dr J-C Jang revealed that their sugar insensitive mutants (*sun*, *mig* and *gin* ; see section 3.2.7.2) also display a low penetrance so it appears that several classes of sugar sensing mutants have this phenomenon.

3.2.4 Ecotypes of the *cai* mutants

The original pool of EMS mutagenised seed that had been screened for *cai* mutants were reportedly col-2 and so for 3 years col-2 was used as the wild type control for *cai* 10 and *cai* 28. However, while mapping *cai* 10 Dr F. Regad, a postdoc in Dr Graham's lab, discovered that the *cai* 10 and *cai* 28 and a number of the other *cai* mutants were actually in the Wassilewskija (ws) background. The *cai* mutants were crossed into Landsberg errecta (Ler) wild type. Simple sequence length polymorphism (SSLP) markers were chosen which had a known banding pattern in ecotypes col-2 and Ler. However, when used with the *cai* 10/Ler F2 crosses, these markers did not give the bands expected for col-2 and Ler. Use of other SSLP markers revealed that the *cai* 10/Ler F2 crosses matched the banding patterns expected from ws /Ler F2 crosses. We now believe that the initial EMS mutagenised seed pool that was screened for these mutants was a mixture of col-2 and ws.

Table 3.2 Penetrance of the *cai* phenotype in *cai* 28.

cai 28 seeds were sown on M&S agar media containing 100 mM sucrose and 0.1 mM nitrogen and were grown for 7 days at 22 °C in continuous white light. Seedlings which had a range of phenotypes from dark green to very pale green with a purple halo were selected, transferred to soil and allowed to set seed. The progeny were sown on M&S agar media containing 100 mM sucrose and 0.1 mM nitrogen and their phenotypes were assessed after 7 days.

	Phenotype of progeny on 0.1 mM nitrogen plus 100 mM sucrose (*cai* screen)		
Parental phenotype	**No. of green seedlings**	**No. of purple seedlings**	**% penetrance of *cai* phenetype**
dark green M5 28	58	58	50
mid green M5 28	46	110	29
pale green M5 28	50	78	39
v.pale with purple halo M5 28	11	151	7
col-0	5	131	3.6
ws	6	110	5.1

3.2.5 Characterisation of the growth of wild type and *cai* mutants on media containing varying carbon : nitrogen ratios

*cai*10 and *cai* 28 were chosen for further analysis because they had the highest penetrance and had *cai* phenotypes that were most reproducible. *cai* 10, *cai* 28, col-2 and ws were grown on various carbon : nitrogen ratios in order to characterise their response. Seeds were sown on M&S agar plates containing 100 mM sucrose and either 60 mM, 6 mM, 0.6 mM, 0.1 mM or 0 mM total nitrogen. The plates were incubated at 4°C for 4 days in the dark and then transferred to the growth room for 7 days. The whole population of each plate was used for all parameters measured. Seeds to be analysed for root length were grown on vertical plates and seeds to be analysed for other parameters were held horizontally. The root: shoot ratio of *cai* 10 increased as nitrogen concentration decreased but there was only a slight increase in root :shoot ratio for *cai* 28 (Figure 3.7a). The root : shoot ratio of col and ws also increased as nitrogen decreased. The root length of the *cai* mutants was measured (Figure 3.7b). The root length of *cai* 10 increased as nitrogen decreased from 60 to 0.6 mM. Below 0.6 mM nitrogen the root length decreased probably because the seedling growth was restricted by lack of nitrogen for amino acid production. At lower concentrations of nitrogen the root length of *cai* 10 was higher than the wild type. In contrast, nitrogen concentration had very little affect on root length of *cai* 28. It appears that *cai* 28 is either less sensitive to carbon-nitrogen ratio or nitrogen concentration than the wild type. An alternative explanation could be that *cai* 28 has greater nitrogen reserves in the seed or metabolises its nitrogen reserves more slowly than the wild type and consequently nitrogen does not limit carbon incorporation into amino acids. The fresh weights of the seedlings were also measured under these conditions (Figure 3.7c). In all cases seedlings on 6 mM nitrogen had the highest fresh weight. 60 mM nitrogen appears to have been inhibitory to biomass production. As is expected fresh weight decreases as nitrogen is restricted. However, *cai*10 has a greater fresh weight than *cai* 28 and both are greater than ws at 0 mM nitrogen. Chlorophyll was extracted from the seedlings (Figure 3.7d). It can be seen that while chlorophyll decreased in *cai* 10 and *cai* 28 this does not occur to the extent as observed for the wild types. This correlates with the colour of the cotyledons under these

Figure 3.7 The phenotype of the *cai* mutants grown for 7 days on varying carbon-nitrogen ratios.

Seeds were grown on M&S agar media containing 100 mM sucrose and varying concentrations of nitrogen for 7 days at 22 °C in continuous white light (120 $\mu Em^{-2}s^{-1}$).

col-2 ws *cai* 10 *cai* 28

a: Root: shoot ratio

b: Root length

c: Fresh weight

d: Chlorophyll

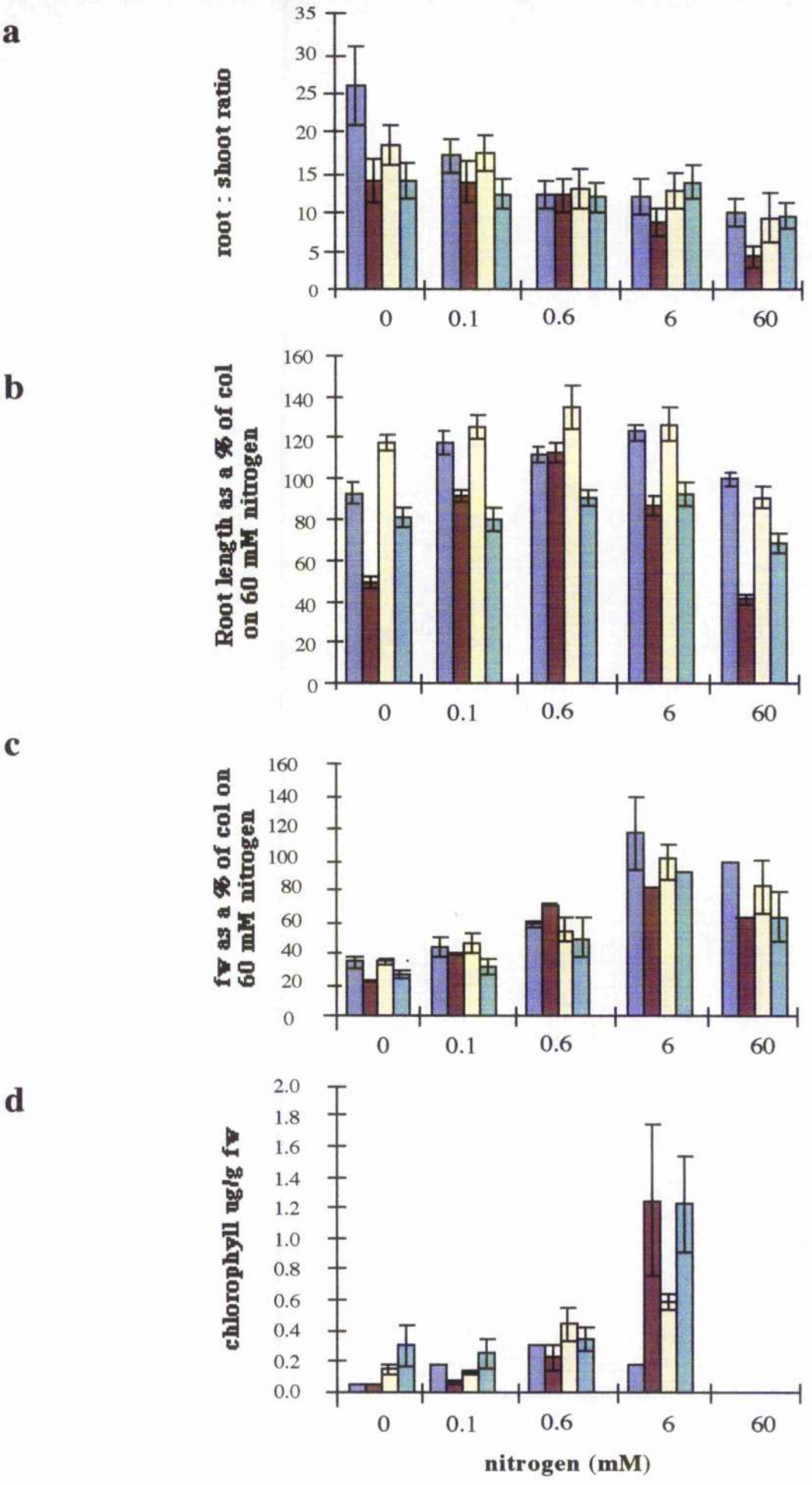
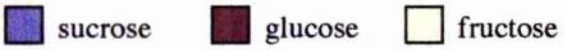
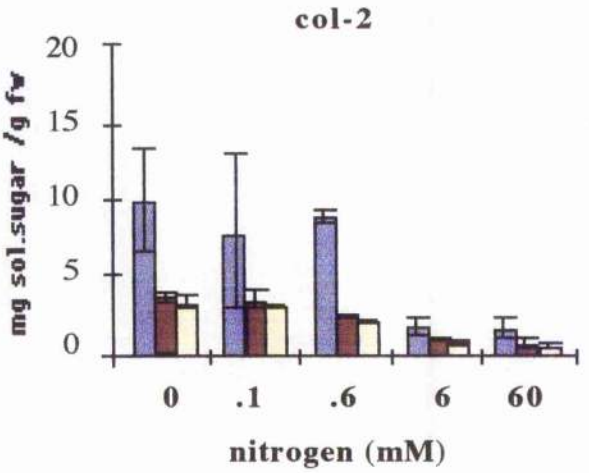
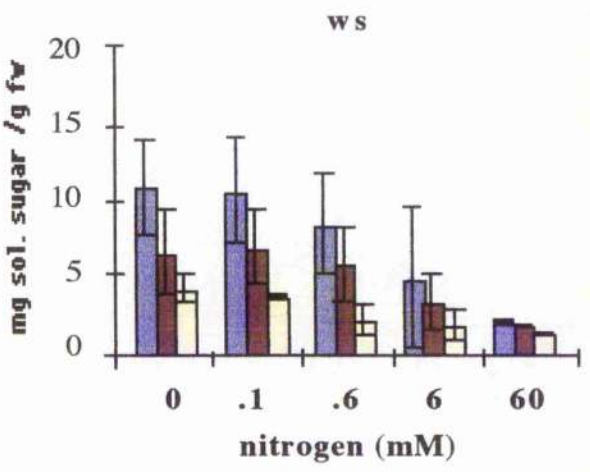

a
root : shoot ratio
35
30
25
20
15
10
5
0
0
0.1
0.6
6
60
b
Root length as a % of col on 60 mM nitrogen
160
140
120
100
80
60
40
20
0
0
0.1
0.6
6
60
c
fw as a % of col on 60 mM nitrogen
160
140
120
100
80
60
40
20
0
0
0.1
0.6
6
60
d
chlorophyll ug/g fw
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
0.1
0.6
6
60
nitrogen (mM)

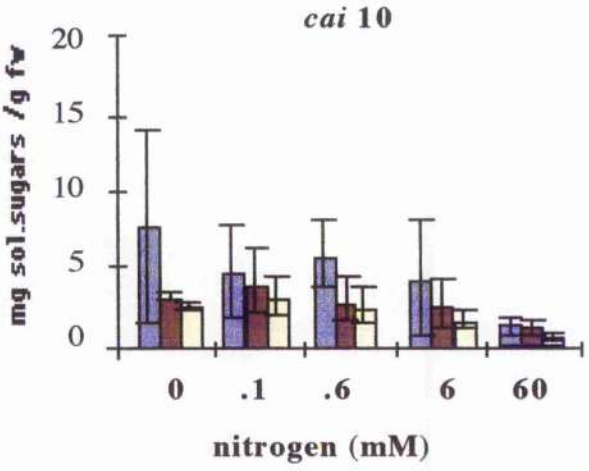
Figure 3.7e Seedling cellular soluble sugars

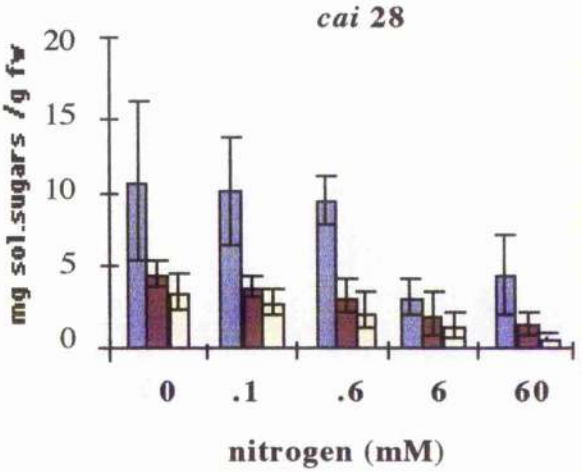
Seedlings were washed in ice-cold water and the soluble sugars were extracted. The concentration of soluble sugars was measured using enzyme linked assays.




col-2
mg sol.sugar /g fw
20
15
10
5
0
0
.1
.6
6
60
nitrogen (mM)


ws
mg sol. sugar /g fw
20
15
10
5
0
0
.1
.6
6
60
nitrogen (mM)


cai 10
mg sol.sugars /g fw
20
15
10
5
0
0
.1
.6
6
60
nitrogen (mM)


cai 28
mg sol.sugars /g fw
20
15
10
5
0
0
.1
.6
6
60
nitrogen (mM)

conditions and is further evidence for carbohydrate repression of photosynthesis in the wild types. The concentration of soluble sugars was measured in the seedlings grown under these conditions (Figure 3.7e). In all cases the concentration of seedling cellular soluble sugars increases as nitrogen concentration decreases corroborating the evidence that was found in tobacco seedlings (Paul and Stitt, 1993). There are no significant differences between the wild type and the *cai* mutants. However, the standard error is quite large. The experiment needs to be repeated several times in order to ascertain whether there are significant differences in the soluble sugar content of the *cai* mutants.

As previously mentioned, the whole population of each plate was used for measurement of growth parameters although only a percentage of the plate showed the *cai* phenotype. Thus, any differences between the *cai* mutants and wild types are caused by a minority of the seedling population. In growth conditions where the penetrance of the phenotype was 100 %, the differences between *cai* mutant and would be expected to be much greater.

RNA was isolated from seedlings grown on varying carbon/ nitrogen conditions and transcript hybridisation analysis was performed using a range of genes (Figure 3.8). Two sets of hybridisation data have been presented because the data varied slightly between experiments. In both experiments the *rbcS*, *cab* and *pc* transcripts decrease as nitrogen concentration decreases and soluble sugar concentrations increase (Figure 3.8 a and b). In figure 3.8a the repression of *rbcS*, *cab* and *pc* is less severe in the *cai* mutants than in col-2. However, in figure 3.8b gene expression of *rbcS*, *cab* and *pc* in the *cai* mutants is higher than ws but similar to col-2. This is typical of the small variations found between experiments and is probably due to slight fluctuations in growth conditions. In both wild type and *cai* mutants, *chs* and *nr* were induced by the low nitrogen, high sucrose conditions. *agpase* was approximately constitutive under these conditions.

During post-germinative growth of oil seed plants, the glyoxylate cycle functions together with ß-oxidation to mobilise stored fat for conversion into sugars. The sugars are then transported to the growing regions of the seedlings and are used as a carbon source in the period before the seedling becomes fully photosynthetic. The synthesis of the glyoxylate

Figure 3.8 The steady state gene expression of sugar modulated genes in the *cai* mutants varies with carbon nitrogen ratio and between experiments.

RNA was extracted from seedlings which had been grown on M&S agar media containing 100 mM sucrose and varying concentrations of nitrogen for 7 days at 22 °C in continuous white light (120 $\mu Em^{-2}s^{-1}$). The RNA was separated by denaturing agarose gel electrophoresis and the RNA was transferred to HybondN membrane. The RNA was hybridised with various radioactive probes and visualised using a phosphoimager.

a: experiment 1.

b: experiment 2

rbcS - small subunit of Rubisco

cab - chlorophyll a/b binding protein

pc- plastocyanin

chs- chalcone synthase

nr - nitrate reductase

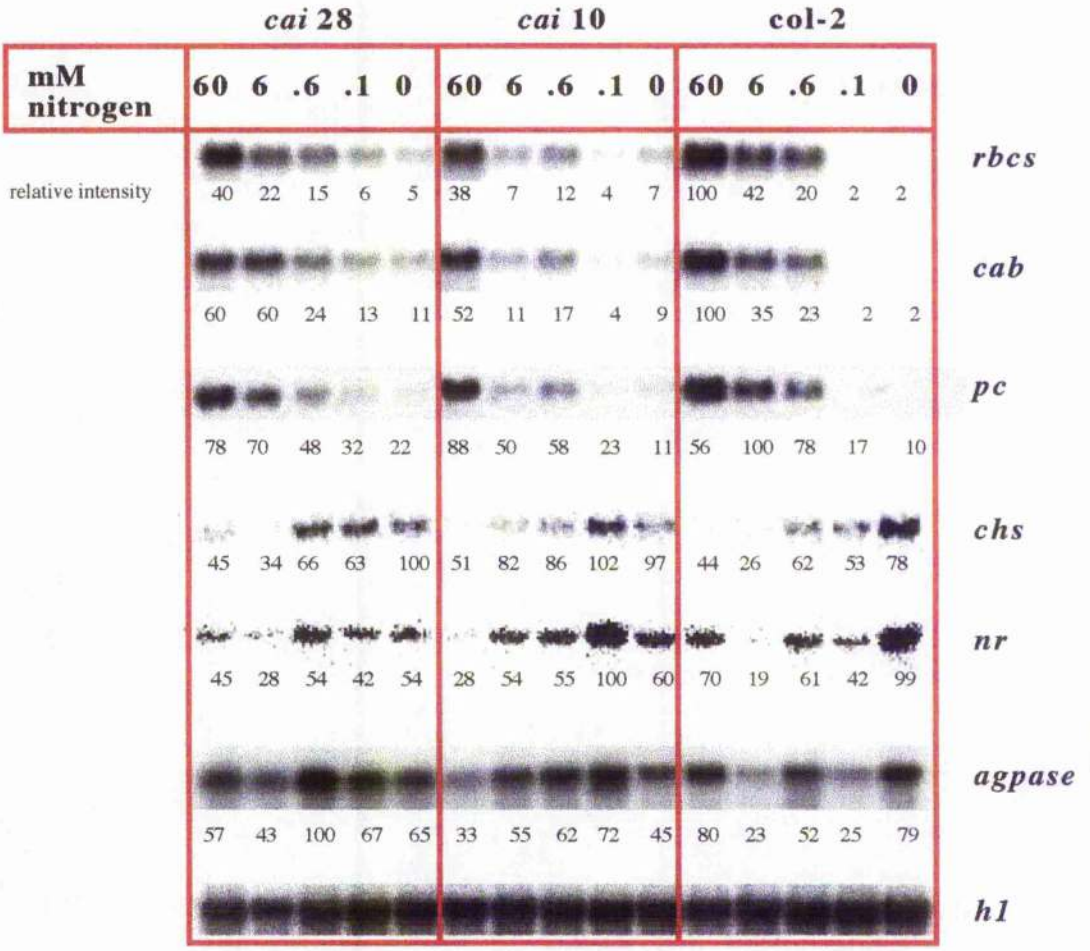
agpase- ADP-glucosepyrophosphatase

ms - malate synthase

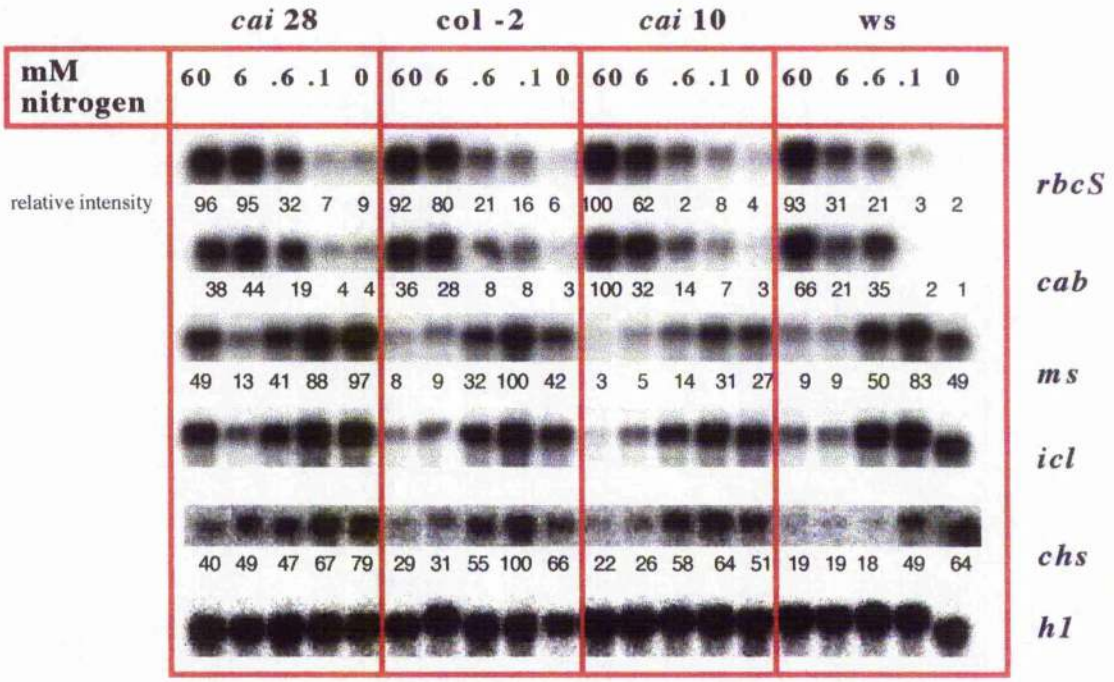
icl - isocitrate lyase

h1 - constitutive probe encoding a gene product of unknown function.

a


cai 28
cai 10
col-2
mM nitrogen
60 6 .6 .1 0 60 6 .6 .1 0 60 6 .6 .1 0
relative intensity
rbcs
40 22 15 6 5 38 7 12 4 7 100 42 20 2 2
cab
60 60 24 13 11 52 11 17 4 9 100 35 23 2 2
pc
78 70 48 32 22 88 50 58 23 11 56 100 78 17 10
chs
45 34 66 63 100 51 82 86 102 97 44 26 62 53 78
nr
45 28 54 42 54 28 54 55 100 60 70 19 61 42 99
agpase
57 43 100 67 65 33 55 62 72 45 80 23 52 25 79
h1

b


cai 28
col -2
cai 10
ws
mM nitrogen
60 6 .6 .1 0 60 6 .6 .1 0 60 6 .6 .1 0 60 6 .6 .1 0
relative intensity
rbcS
96 95 32 7 9 92 80 21 16 6 100 62 2 8 4 93 31 21 3 2
cab
38 44 19 4 4 36 28 8 8 3 100 32 14 7 3 66 21 35 2 1
ms
49 13 41 88 97 8 9 32 100 42 3 5 14 31 27 9 9 50 83 49
icl
chs
40 49 47 67 79 29 31 55 100 66 22 26 58 64 51 19 19 18 49 64
h1

cycle enzymes, malate synthase (MS) and isocitrate lyase (ICL) are coordinately and developmentally controlled during germination (Weir, *et al.*, 1980) and senescence (De Bellis, *et al.*, 1990). In cucumber seedlings *ms* and *icl* expression rise to a peak 3 days after seed imbibition and then again during senescence or starvation (Graham, *et al.*, 1992). The dark induced senescence of *ms* can be repressed by incubation in the dark with sugars (Sarah, *et al.*, 1996). The northern data shows that, as expected, both wild types expressed very little *ms* and *icl* on 60 mM nitrogen plus 100 mM sucrose after 7 days. Interestingly, *ms* and *icl* were induced even after 7 days post-germinative growth when the nitrogen concentration was decreased and seedling soluble sugar concentrations increased. This is unexpected because *ms* and *icl* are normally not expressed after 3 days of post-germinative growth and because *ms* and *icl* are also known to be repressed by sucrose. However, the *ms* germination response is thought to be dominant over the *ms* sugar response (I. Graham, pers. comm.). These data may imply that seedlings grown on low nitrogen and 100 mM sucrose are developmentally retarded because the whole of metabolism is slowed down and could mean that there is still lipid in the cotyledons after 7 days in these conditions. The low nitrogen conditions may trigger a general/carbon starvation response which would induce *ms* and *icl* expression. In this case it appears that the cause of *ms* and *icl* induction is dominant over the sugar repression response. At 60 mM nitrogen, *cai* 10 behaves like the wild types with respect to *ms* and *icl* expression but *cai* 28 is significantly different. *ms* expression is 5-fold higher in *cai* 28 than ws. It is difficult to speculate why this occurs without further experimentation. However, *cai* 28 does germinate more slowly than ws and is less developed than ws after 7 days on high nitrogen concentration plus sucrose.

3.2.6 Analysis of *cai* mutant seed reserves

One theory to explain why *cai* mutants maintain green cotyledons on high carbon low nitrogen conditions is that their seeds contain a large reserve of nitrogen which can be utilised during early seedling growth. The seed storage reserves were analysed to investigate this. Variations in penetrance of the *cai* phenotype on high carbon, low nitrogen conditions

could also be caused by differences in the seed storage reserves as some seeds may have more reserves than others.

Storage proteins were extracted from wild type and mutant seeds and seedlings during post-germinative growth to examine whether there were any differences between the *cai* mutants and the wild types. The protein concentration was measured using the Lowry method (Lowry, *et al.*, 1951). 20 ug of each sample was loaded onto a 12.5 % acrylamide gel and the proteins were separated by SDS-PAGE (Figure 3.9). The storage proteins were broken down during days 1 and 2 and are used as a source of carbon and nitrogen during early seedling growth. The results show that the composition of seed storage proteins appears to be similar in both the wild type and the mutants. The storage proteins in *cai* 28 appear to be broken down more slowly than in the wild type (see day 2 low molecular weight proteins). The observation correlates well with the observation that *cai* 28 is slower to germinate than wild type. It does, however, lead to difficulties in interpreting the results as it is not easy comparing the proteins in seedlings that are at different developmental stages.

The amino acid contents of the wild types and *cai* 10 were analysed by paper chromatography. The paper chromatogram gave an indication that ws had a higher concentration of amino acids than col-2. The data also suggested that the *cai* mutants had a higher concentration of amino acids than ws (data not shown). The amino acid contents of col-2, ws and *cai* 10 were further analysed by HPLC (Figure 3.10). Preliminary results indicate that *cai* 10 has an elevated amount of glutamic acid and histidine compared to the wild types. The soluble sugar content of *cai* mutant seeds was analysed (Figure 3.11). The extracts were separated by paper chromatography and was stained with silver nitrate to visualise the sugars. ws contains a sugar which runs at the same Rf as myoinositol which is present in only a low concentration in col-2. *cai* 10 and *cai* 28 (both ws background) also possess a large amount of the sugar that runs at the same Rf as myoinositol. *cai* 31 (col-2 background) contains only a small amount of this sugar. These results indicate ws and col-2 have different soluble sugar contents and that the *cai* mutants may be segregated into col-2 and ws genetic backgrounds by analysis of their sugars. The differences between the wild

Figure 3.9 The breakdown of seed storage proteins in germinating seeds is retarded in *cai* 28.

Seedlings were grown on 1/2 strength M&S agar media containing 20 mM sucrose for 0-3 days at 22 °C in continuous white light (120 $\mu Em^{-2}s^{-1}$). Total proteins were extracted from seedlings and 20 ug of each samples was separated by SDS-PAGE on a 12.5 % acrylamide gel. The proteins were stained with Coomassie brilliant blue.

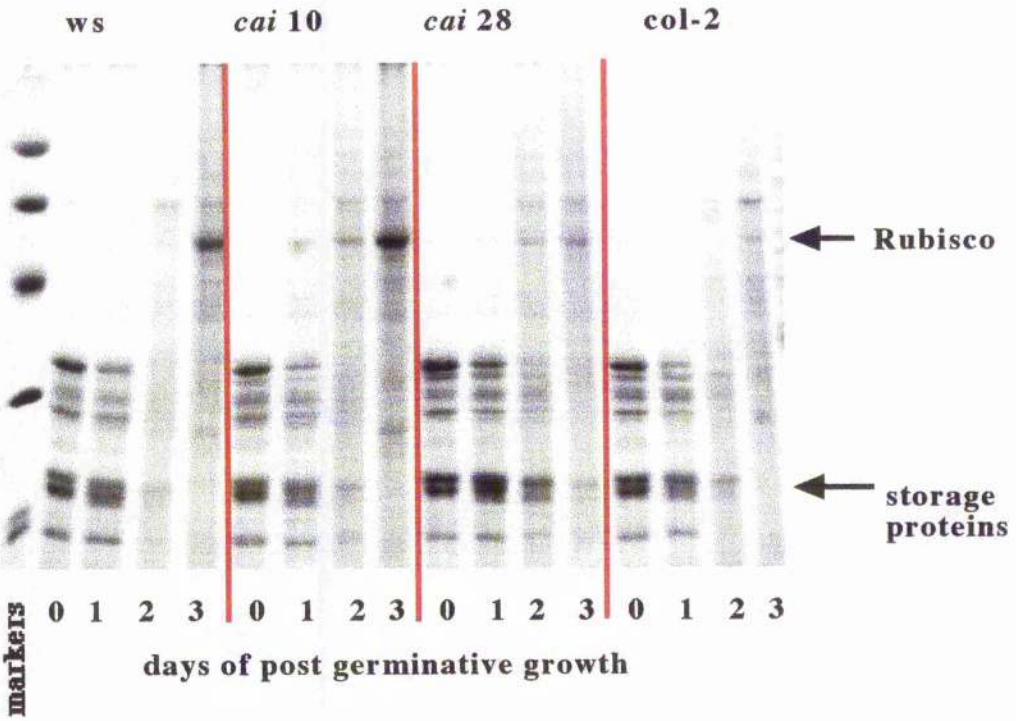

ws
cai 10
cai 28
col-2
Rubisco
storage proteins
markers
0 1 2 3 0 1 2 3 0 1 2 3 0 1 2 3
days of post germinative growth

Figure 3.10 Seeds of *cai* 10 have a higher concentration of glutamic acid and histidine than wild type.

Amino acids were extracted from 50 seeds and the concentrations of individual amino acids was determined by HPLC.

col-2 ws *cai* 10

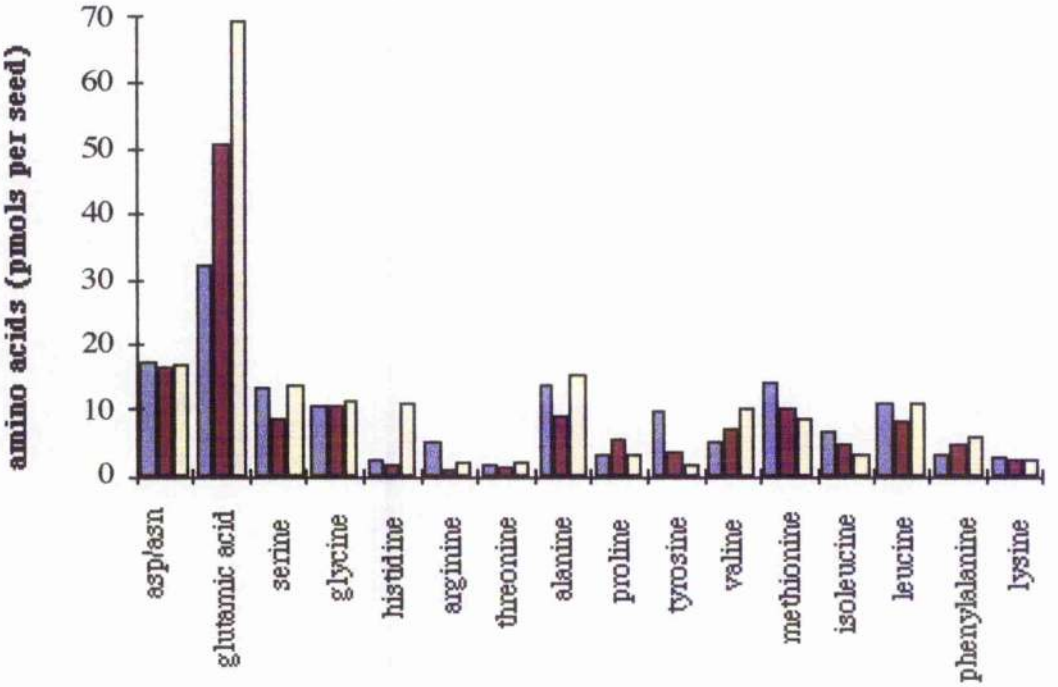

amino acids (pmols per seed)
70
60
50
40
30
20
10
0
asp/asn
glutamic acid
serine
glycine
histidine
arginine
threonine
alanine
proline
tyrosine
valine
methionine
isoleucine
leucine
phenylalanine
lysine

Figure 3.11 Variation in the soluble sugar content of *cai* mutant seeds.

Soluble sugars were extracted from 50 seeds. The soluble sugars were separated by paper chromatography for 18 hours (ethyl acetate: pyridine :water; 8 :2 :1) and were stained with silver nitrate.

1 = fructose
2 =xylose
3 = glucose
4 = fucose
5 = arabinose
6 = galactose
7 = mannose
8 = trehalose
9 = myoinositol
10 = xylitol
11 = galactitol
12 = sucrose

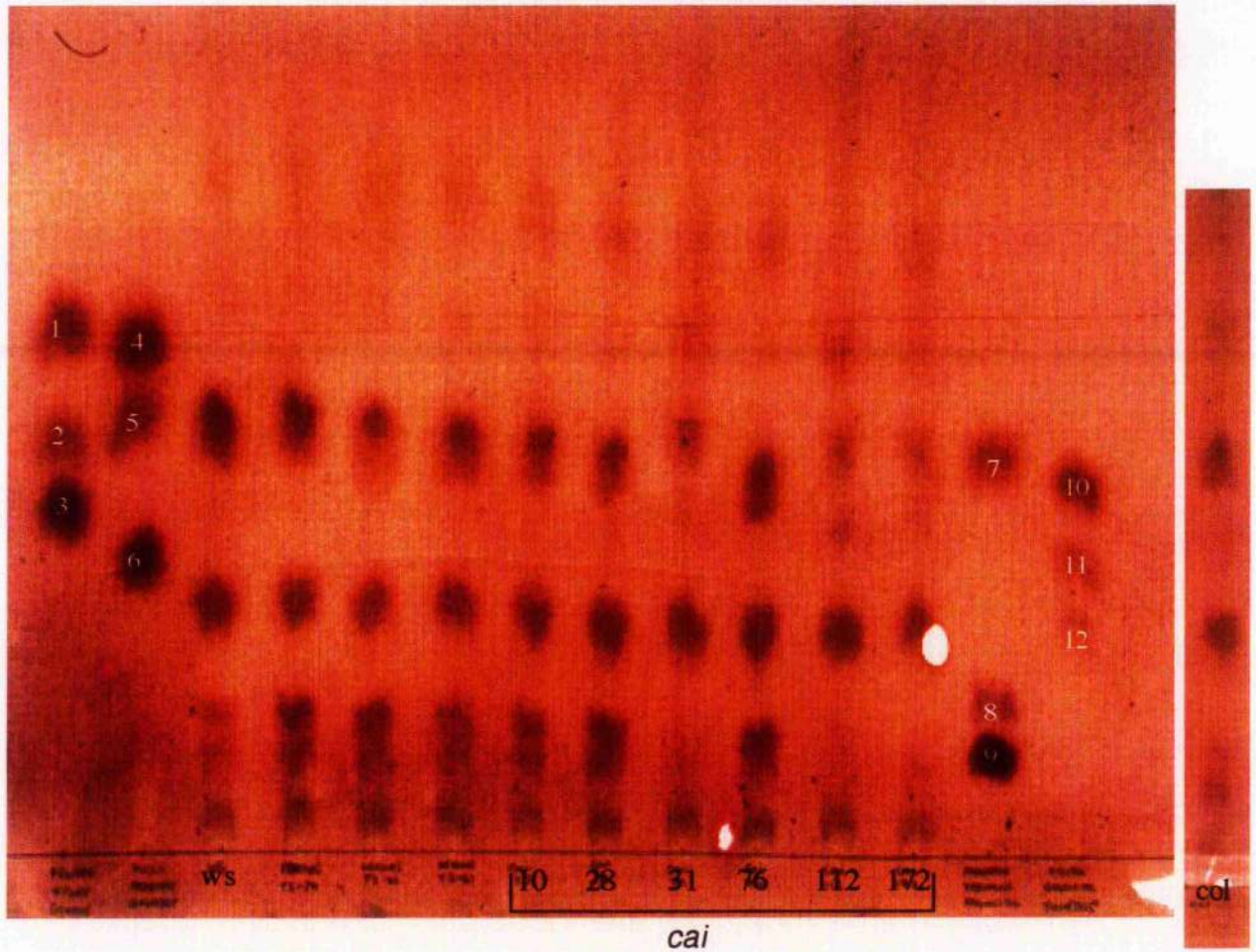

1
2
3
4
5
6
7
8
9
10
11
12
Ws
10
28
31
76
112
172
col
cai

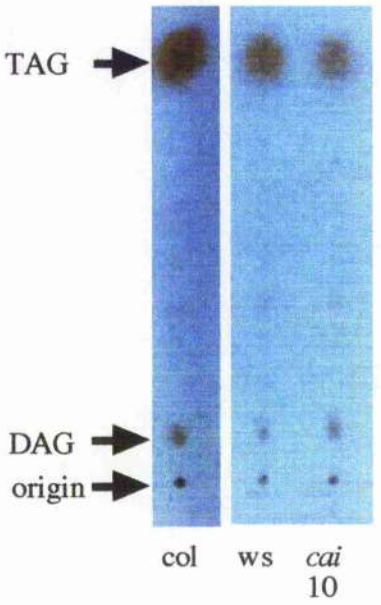
Figure 3.12 *cai* 10 seeds contain less triacyl glycerol than wild type.

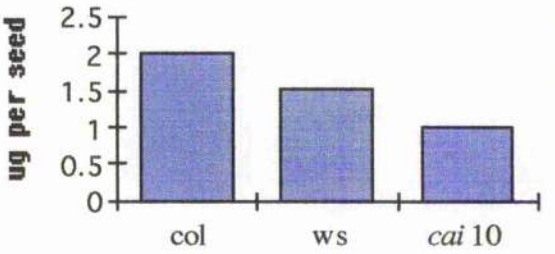
Triacylglycerol (TAG) was extracted from 10 seeds and separated by thin layer chromatography (TLC)(a). The TAG was visualised by spraying the TLC with 50 % (w/w) sulphuric acid and charring in an oven. The TAG was quantified using a standard curve and plotted on a histogram (b).

a

b

TLC


TAG
DAG
origin
col
ws
cai
10


ug per seed
2.5
2
1.5
1
0.5
0
col
ws
cai 10

type sugar contents may also help to explain why col-2 and ws behave differently on the *cai* screen.

Lipids were extracted from the seeds of the *cai* 10 and the wild types. Triacyl glycerols (TAG) were analysed by TLC (Figure 3.12 a and b) and quantified using a standard curve. *cai* 10 has less TAG than col-2 and ws. The total lipid content and the fatty acid profile of *cai* 10, ws and col were analysed by gas chromatography (collaboration with Dr. T. Larson; Figure 3.13) which is a more accurate method than TLC. col-2 has slightly more total fatty acids than ws and ws has slightly more total fatty acid than *cai* 10. The fatty acid profile is similar in all cases.

These preliminary results indicate that *cai* 10 may have an altered flux of carbon into lipids and amino acids. Further experimentation is required to establish if this is the case.

3.2.7 Characterisation of the *cai* mutants on different growth conditions

3.2.7.1 Growth of *cai* mutants in the dark on 0 % and 3 % sucrose

A second approach was employed in an attempt to find a clear difference between the wild type and the *cai* mutants at the level of transcript abundance. The experiment was based on evidence that the plastocyanin gene (*pc*) is activated independently of light during early seedling development (Dijkwel, *et al.*, 1996). In etiolated seedlings, *pc* mRNA levels increase transiently and the maximum dark level is reached after 2 days growth in darkness. The transient increase in mRNA can be repressed by sucrose and it was hypothesised that the high concentration of sugars found in seedlings grown on 3 % sucrose induce the signal for *pc* repression. Only sugars which are phosphorylatable by hexokinase cause the repression of gene expression and the authors suggested that hexokinase induces the signal (Dijkwel, *et al.*, 1996). *pc* is not the only gene to display a transient increase in expression. A similar increase of nuclear encoded photosynthetic genes has been found in other species (Walden and Leaver, 1981, Fiebig, *et al.*, 1990) and in *Arabidopsis* chlorophyll a/b-binding protein (CAB) and the small subunit of RUBISCO were transiently expressed during etiolated seedling development (Brusslan and Tobin, 1992). The transient expression of

Figure 3.13 *cai* 10 seeds contain less total fatty acids than wild type although the fatty acid profile remains the same.

Fatty acids were extracted from 10 seeds and analysed by gas chromatography (GC).

 individual fatty acids  total fatty acid

col-2

ws

***cai* 10**

rbcS is also repressed by 3 % sucrose (Dr.S. Smeekens; pers. comm.). Sucrose uncoupled (*sun*) mutants had been isolated which showed reduced repression of the plastocyanin promoter-luciferase fusion gene (Dijkwel, *et al.*, 1997). The aim of this experiment was to establish if the *cai* mutants were also insensitive to sucrose repression of the transient expression of sugar modulated genes. Seeds were sown on 1/2 strength M&S agar medium containing either 0% or 3% sucrose and were kept at 4 °C for 4 days. The seeds were then given a 45 minute red light treatment to promote germination (Dijkwel, *et al.*, 1996) before being incubated at 23 °C in the dark for 1-3 days. The etiolated seedlings were harvested and samples were taken for RNA expression analysis and soluble metabolite measurements. The expression of several photosynthetic and sugar modulated genes was analysed in etiolated seedlings of the *cai* mutants grown on 0 or 3 % sucrose (Figure 3.14). The wild types ws and col expressed *rbcS* after 2 days growth in the dark on 0 % sucrose, whereas 3 % sucrose repressed this expression. On day 3 *rbcS* expression on 0 % sucrose increased and was only partly repressed by 3 % sucrose. *cab* and *pc* were expressed on 0% sucrose and repressed by 3 % sucrose on day 3. *chs* was highly expressed in seedlings grown on 3 % sucrose. The *cai* mutants were sampled after 2 days growth in the dark. Hybridisation analysis shows that *cai* 10, *cai* 28 and *cai* 172 show the same repression of photosynthetic genes as the wild types. In contrast, *cai* 31 and *cai* 112 express *rbcs, cab* and *pc* on day 2 although the wild types do not express *cab* and *pc* until day 3. This may be significant but is more likely to be due to a difference in developmental stage as *cai* 112 is known to germinate rapidly in the dark (personal observation). Rapid germination could account for the precocious peak of *cab* and *pc* expression.

Soluble hexoses were extracted from the seedlings grown on 0 % and 3 % sucrose (Figure 3.15). The results show that during the first 3 days of post-germinative growth wild type cellular hexoses increased. Seedlings grown on 3% sucrose had a higher internal soluble hexose concentration than seedlings grown on 0% sucrose (Figure 3.15) indicating that they do take up the exogenous sucrose. The concentration of hexoses in the wild types rose dramatically after 2 days growth on 3% sucrose. The concentration of hexoses was measured in *cai* 10, *cai* 28 and *cai* 31 after 2 days. In each mutant the concentration of

Figure 3.14 Sucrose represses the developmentally-induced expression of sugar -modulated genes.

Seeds were grown for 1-3 days in darkness on M&S agar media containing 0% or 3% sucrose. The seedlings were harvested and their RNA was extracted. The RNA was separated by denaturing agarose gel electrophoresis and transferred to HybondN membrane. The RNA was hybridised with radioactive probes of several sugar-modulated genes and was visualised using a phosphoimager.

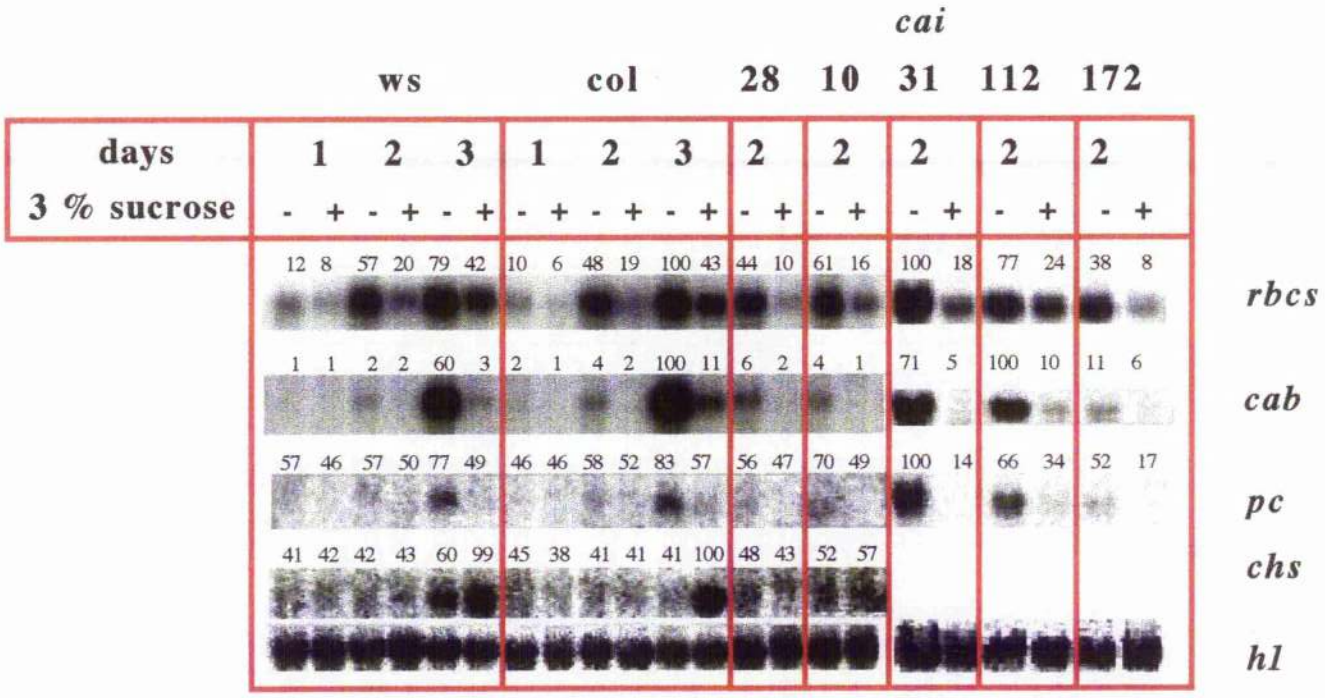
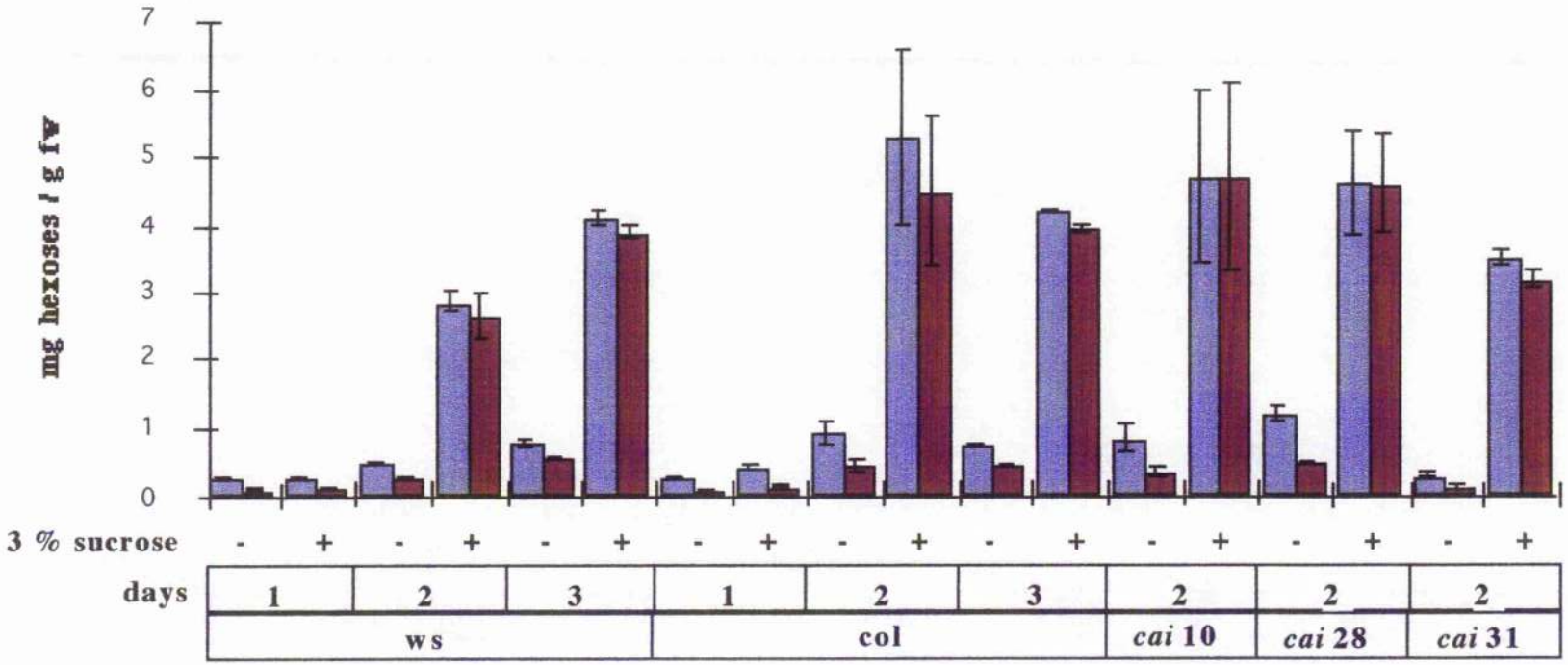

cai
ws
col
28
10
31
112
172
days
3 % sucrose
1 2 3 1 2 3 2 2 2 2 2
- + - + - + - + - + - + - + - + - + - + - +
12 8 57 20 79 42 10 6 48 19 100 43 44 10 61 16 100 18 77 24 38 8
rbcs
1 1 2 2 60 3 2 1 4 2 100 11 6 2 4 1 71 5 100 10 11 6
cab
57 46 57 50 77 49 46 46 58 52 83 57 56 47 70 49 100 14 66 34 52 17
pc
41 42 42 43 60 99 45 38 41 41 41 100 48 43 52 57
chs
hl

Figure 3.15 The soluble hexose content of seedlings grown in the dark on 0 % or 3% sucrose.

Seeds were grown for 1-3 days in darkness on M&S agar media containing 0% or 3% sucrose. The seedlings were harvested, the soluble hexoses were extracted and analysed using enzyme linked assays.


mg hexoses / g fw
7
6
5
4
3
2
1
0
3 % sucrose
- + - + - + - + - + - + - + - + - + - +
days
1 2 3 1 2 3 2 2 2
ws
col
cai 10
cai 28
cai 31

glucose and fructose was higher in seedlings grown on 3 % sucrose than 0% sucrose. The elevated cellular sugars are likely to have caused the repression of photosynthetic genes during dark germination on 3 % sucrose. The concentration of soluble hexose is similar in the wild types and the *cai* mutants. Thus the *cai* mutants behaved like the wild types throughout this experiment.

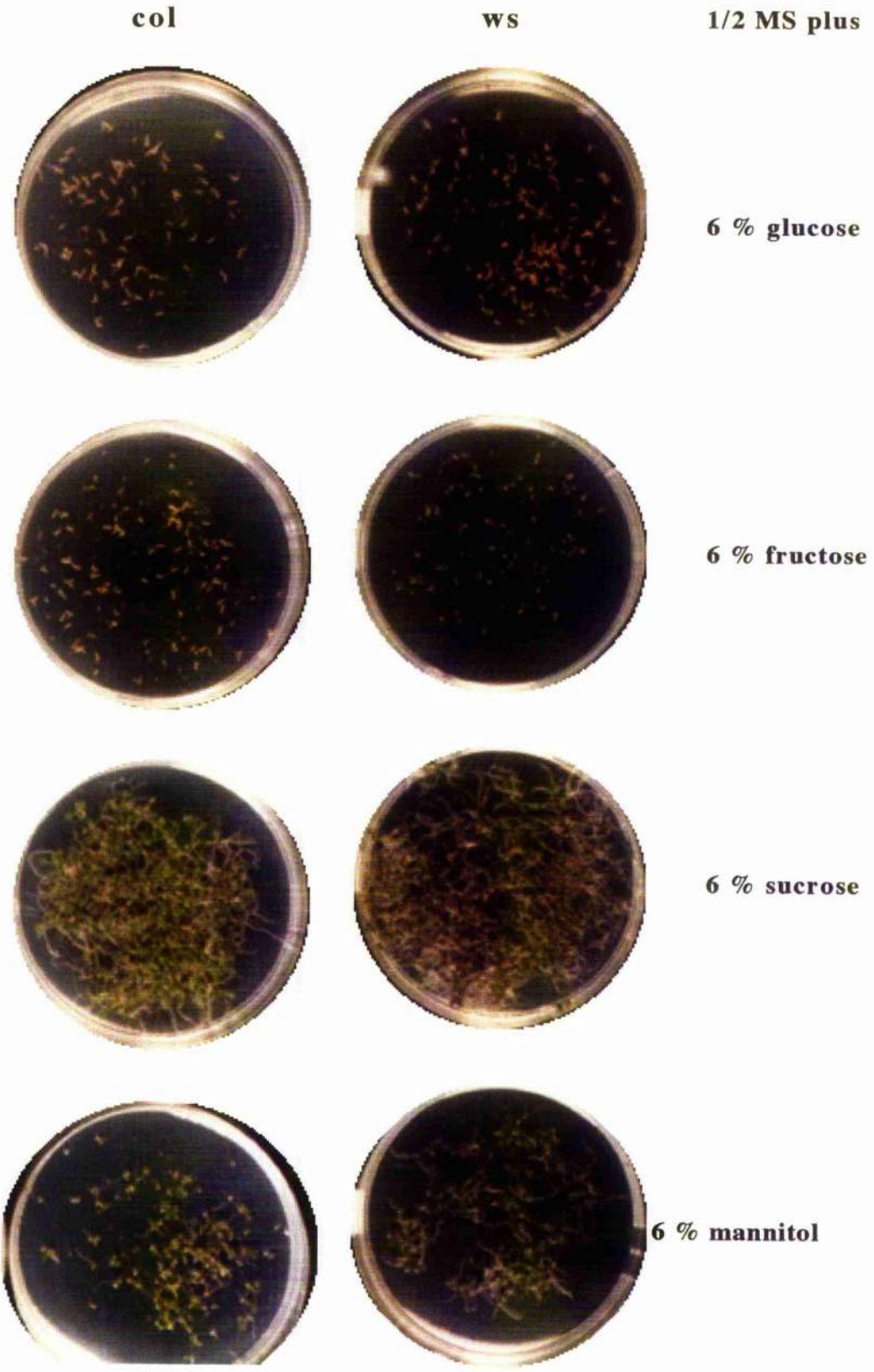
3.2.7.2 Growth of the *cai* mutants on other sugars

At the same time as the *cai* mutants were being selected, workers in other laboratories in Europe and USA were isolating other sugar insensitive *Arabidopsis* mutants using different screens. Dr J-C. Jang designed a screen for glucose insensitive (*gin*) mutants based on work with hexokinase antisense plants which were found to be insensensitive to 6 % glucose (Jang, *et al.*, 1997). Growth of wild type seedlings was arrested at the cotyledon stage on 6 % glucose. *gin* mutants were isolated by virtue of their continued development on 6 % glucose. Dr S. Smeekens isolated sucrose insensitive growth (*sig)* mutants. Wild type *Arabidopsis* accumulated anthocyanins and growth was arrested at the cotyledon stage on 12 % sucrose but the *sig* mutants continued to develop. J. Pego and Dr. S. Smeekens also isolated mannose insensitive growth (*mig*) mutants (Pego, *et al.*, 1999). Wild type *Arabidopsis* germinated on 5 mM mannose and produced a radicle of 1-3 mm but no cotyledons emerged. The *mig* mutants developed green cotyledons on 5 mM mannose. The *cai* mutants were germinated on the screening conditions for *gins, sins* and *migs* with the aim of establishing whether any of the *cai* mutants were also insensitive to other sugars.

col-2 and ws were grown on 1/2 strength M&S agar medium containing 6 % (W/V) glucose, fructose, sucrose or mannitol for 8 days (Figure 3.16). The seedlings developed cotyledons but did not produce primary leaves when germinated on 6 % glucose or 6 % fructose although growth on 6 % mannitol was normal indicating that it is hexose sugars *per se* and not the osmotic effect that is affecting seedling growth. Seedling growth was enhanced at 6 % sucrose compared to 6 % mannitol. However, if seedlings are grown on 12 % sucrose, vigour is poor and the cotyledons contain a lot of anthocyanins. 12 % sucrose (350 mM) is approximately equivalent to 6 % glucose or fructose (333 mM). However, 12 % mannitol also arrests seedling development (data not shown) and this may indicate that

Figure 3.16 The affect of different sugars on wild type seedling growth.

Wild type *A. thaliana* (ecotypes col-2 and ws) were grown for 7 days on 6 % glucose, 6 % fructose, or 6 % sucrose. 6 % mannitol was used as an osmotic control.


col
ws
1/2 MS plus
6 % glucose
6 % fructose
6 % sucrose
6 % mannitol
2 cm

the affect of 12 % sucrose is mostly osmotic. The germination frequency of the *cai* mutants sown on the above sugars was calculated. The differences between wild type and mutant were not great enough for use as a screen in genetic analysis.

ws and col-2 were also sown on 1/2 strength M&S agar medium containing 5 mM mannose, a hexose which is phosphorylated by hexokinase but further metabolised less readily than other hexose sugars. The seedlings germinated but post-germinative growth was arrested when 1-3 mm of radicle had emerged. The *cai* mutants were also sown on 5 mM mannose (Figure 3.17) and 4 of the 30 putants displayed mannose tolerance. *cai*10 had almost 100 % germination on 5 mM mannose and will be discussed in detail in chapter 4.

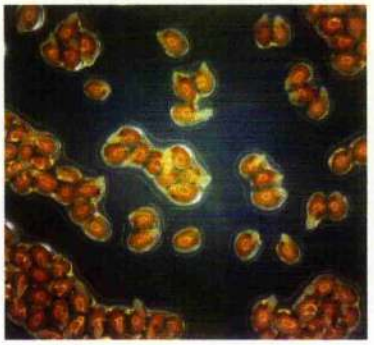
3.2.8 Genetic analysis of *cai* mutants

3.2.8.1 Parental crosses

Reciprocal parental crosses were carried out extensively between the *cai* mutants and col gl1 -1 (joint work in collaboration with Drs T. Martin and F. Regad) in order to remove any secondary mutations from the genotype and to establish whether the *cai* mutations were dominant or recessive. EMS mutation usually gives rise to recessive mutant phenotypes because the nature of mutagenesis is to disrupt genes, thereby destroying or diminishing the activity of the proteins they encode. For a recessive mutation, the F1 of a parental backcross has the wild type phenotype and for a dominant mutation the F1 will have the mutant phenotype. However, very few seeds are produced with each cross so the F2 were analysed. A recessive mutation gives rise to an F2 with a 3:1 ratio of wild type to mutant phenotype whereas a dominant mutation gives rise to an F2 with a 9: 7 ratio of wild type to mutant phenotype. The segregation ratios of the F2 were analysed on *cai* selection media (M&S agar containing 0 mM nitrogen, 100 mM sucrose). However, there are 2 reasons why the data has not been included. (1) The *cai* mutants were crossed with col gl1-1 which was at a later date discovered not to be the parent ecotype as *cai* 10 and 28 were from the ws background. (2) Analysis of the F2 progeny proved to be very difficult because the segregation ratios were distorted by low penetrance. However, these crosses did prove to be useful in mapping *cai* 10 (carried out by Dr. F. Regad) since *cai* 10 is 100 % resistant to

Figure 3.17 Cotyledon emergence is inhibited by mannose in wild type seedlings but not in *cai* 10.

Seeds of wild type and several *cai* mutants were sown on 1/2 strength M&S agar medium containing 5 mM mannose. The plates were imbibed at 4 °C for 4 days and were then transferred to a growth room (22 °C, 120 $\mu Em^{-2}s^{-1}$ continuous white light) for 5 days.

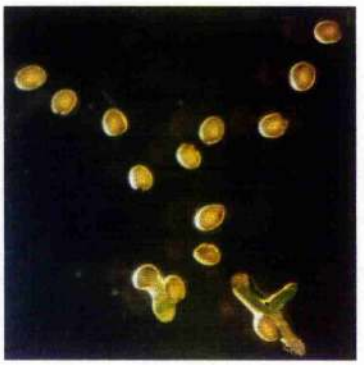


w s

cai 10

cai 24

cai 27



cai 171

2 mm

Table 3.3 F1 analysis of the *cai* mutant crosses indicates that *cai* 10 has a dominant mutation.

The cai mutants were crossed and the F1 seeds were collected. The F1 seeds were sown on 1/2 strength M&S agar media containing 5 mM mannose. The plates were imbibed for 4 days at 4 °C and were transferred to a growth room at 22 °C in continuous white light (120 $\mu Em^{-2}s^{-1}$) for 5 days. The percentage of seedlings which had a *mig* phenotype was calculated. For each cross the female parent is written first.

Crosses on mannose screen	
F1	% *mig*
cai 10 X colgl1-1	80
colgl1-1 X cai 10	22
colgl1-1 X *cai* 31	4
colgl1-1 X *cai* 31	8
cai 31 X colgl1-1	14
cai 10 X *cai* 27	65
cai 10 X *cai* 28	85
cai 10 X *cai* 31	84
cai 10 X *cai* 31	96
cai 10 X *cai* 76	75
cai 31 X *cai* 28	51
F0	
col	0
col gl1-1	12
cai 10	80
cai 27	1.
cai 28	10
cai 31	0
cai 76	22

mannose. Mannose was used to select the F2 progeny of the crosses which had a *mig* phenotype (Table 3.3).

3.2.8.2 Complementation crosses

It is possible to determine whether 2 mutants are allelic by crossing them and analysing their progeny. If two allelic mutants are crossed the F1 progeny have the mutant phenotype. If the mutations are non-allelic and recessive the progeny will have the wild type phenotype but if one or both of the mutations is dominant the F1 will have a mutant phenotype. The *cai* mutants were divided into groups and crossed in an attempt to organise them into complementation groups (collaborative work with Drs T. Martin and F. Regad). The F1 were germinated on *cai* selection media and the phenotypes were recorded. A percentage of each of the F1s had the *cai* phenotype which could be interpreted as allelism between all the *cai* mutants. However, the low penetrance of the phenotype made it difficult to be confident of the data and the work was not continued.

3.2.8.3 Crosses on the mannose screen

cai 10 displayed 90-100 % mannose insensitive growth (*mig*) on 5 mM mannose and ws and col-2 were 0 % mannose resistant. Therefore, 5 mM mannose was chosen as a selection for genetic analysis of *cai* 10. If the mutation is recessive none of the F1 would be expected to have a *mig* phenotype because the mutation is complemented by the wild type allele. However, the results show that the F1 has 22 % *mig* phenotype (Table 3.3). This suggests that the *cai* 10 mutation is dominant. Further work by Dr F. Regad corroborated this data. As stated in the previous section, EMS mutation rarely cause a dominant mutation because the nature of mutagenesis is to knock-out genes, destroying or diminishing the activity of the proteins they encode. However, there are circumstances in which a dominant mutation is possible. For example, a dominant mutation could cause the binding site for a negative regulator of a gene to be disrupted. This would lead to enhanced expression of the gene which would not be complemented by the wild type allele. A dominant mutation could also cause an alteration of the regulatory or activity domain of an enzyme which could

result in increased enzyme activity. The crossing of *cai* 10 with other *cai* mutants (which do not have a *mig* phenotype) also results in F1 which have *mig* phenotype (Table 3.3) and further corroborates the evidence that *cai* 10 is a dominant mutation.

3.3 Discussion

Wild type seedlings accumulate cellular soluble sugars under conditions of limiting nitrogen and 100 mM exogenous sucrose causing repression of photosynthesis. The lack of nitrogen for incorporation of carbon skeletons into amino acids is thought to be responsible for the build up of cellular soluble sugars which reach toxic concentrations and repress photosynthesis. As the carbon : nitrogen ratio of the media increases the root: shoot ratio increases, photosynthetic gene expression is repressed and chlorophyll is depleted. The *cai* mutants are less sensitive to high carbon : nitrogen ratios than the wild types and maintain green cotyledons. The repression of *rbcS*, *cab* and *pc* is reduced in *cai* 10 and *cai* 28.

cai 10 accumulates less soluble sugars than wild type on high carbon/low nitrogen conditions. This indicates that *cai* 10 may be a sucrose uptake mutant or it may be able to metabolise sugars rapidly into other compounds. *cai* 10 does not have a *gin, sun* or *sin* phenotype but it does show mannose insensitive growth (*mig*). On soil, *cai* 10 has a phenotype identical to wild type. Preliminary results indicate that *cai* 10 seeds have a higher amino acid content than wild type and a lower lipid content. This may indicate that *cai* 10 diverts carbon from lipid synthesis into amino acids.

cai 28 germinates slowly and has paler green leaves than wild type when grown on soil. The storage proteins are also broken down more slowly than wild type during germination but are identical in composition. *cai* 28 also differs from wild type when germinated on different carbon: nitrogen ratios. It does accumulate soluble sugars at similar concentrations to wild type which indicates that it is not a sucrose uptake mutant. It may thus respond differently to wild type on high carbon/ low nitrogen conditions because it is a mutant in sugar sensing, carbon: nitrogen sensing or nitrogen sensing. In addition it may be able to compartmentalise the soluble sugars such that critical sugar sensitive processes are unaffected. The root length of *cai* 28 is shorter than the wild type and does not respond to

the decrease in nitrogen. The response of*cai* 28 to 60 mM nitrogen plus 100 mM sucrose is also different from the wild type. After 7 days growth on 60 mM nitrogen plus 100 mM sucrose *ms* and *icl* are expressed in *cai* 28 although they are not present in the wild type. *ms* and *icl* are not expressed at 6mM nitrogen which may indicate that *cai* 28 is altered in nitrogen sensing. This phenomenon needs to be further investigated. Preliminary data indicate that *cai* 28 has a higher concentration of amino acids than wild type and this may be one reason why the seedlings are are tolerant to low nitrogen conditions. The *cai* 28 seedlings may be able to survive for longer in high carbon: nitrogen conditions as nitrogen does not limit as soon as occurs in the wild type.

The penetrance of the *cai* phenotype is low. Communication with Dr S. Smeekens and Dr J-C. Jang revealed that their *sun*, *mig* and *gin* mutants also display a low penetrance so it appears that several classes of sugar sensing mutants manifest this phenomenon. The growing conditions of all classes of sugar sensing mutant has to be very precise during selection as any fluctuation in temperature or light levels results in the wild type behaving like the mutants. The conditions during seed development are also likely to be a factor in reduced penetrance as each seed may receive a slightly different amount of sucrose, nitrogen or light resulting in only a certain proportion of the seedlings being subjected to nitrogen limiting/ sucrose accumulating threshold which causes the *cai* phenotype. Genetic analysis of the *cai* mutants proved very difficult due to the low penetrance of the phenotype.

The *cai* phenotype is a distinct class of sugar sensing mutants as many of the *cai* mutants do not fall into the *sun*, *mig*, *gin* or *sig* categories. *cai* 10 however, is also a *mig* and displays 100 % penetrance. This enabled it to be genetically analysed and results of segregation of the parental crosses and complementation crosses (F1, F2 and F3) on 5 mM mannose indicated that the *cai* 10 mutation was dominant. This is a very unusual occurrence in EMS mutagenised plants as the process knocks out genes. However, there are several circumstances when a EMS mutation could cause a dominant mutation: (1) the mutation is in a negative regulator of a gene and so expression of the gene is enhanced rather than reduced, (2) the mutation is in a regulatory domain of a gene which makes it more sensitive

to a regulory compound or (3) the mutation is in a regulatory domain of an enzyme which makes it more active.

In theory, the *cai* screen can select mutants in sugar, nitrogen or carbon: nitrogen ratio signalling but it could also select mutants in metabolism and sugar uptake. The metabolic mutants could be altered in nitrogen storage reserves which could decrease the nitrogen limitation affect of the *cai* screen and maintain chlorophyll concentration in the cotyledons. Other metabolic mutants which could avoid the build up of soluble sugars would also be selected on the *cai* screen. These include seedlings that can metabolise sugars so rapidly that their concentration never reaches a toxic threshold or reaches it later than in wild type. Theoretical candidates for the *cai* mutants might be plants altered in glycolysis or biosynthesis of sink products such as starch, lipids, proteins or ascorbic acid, all of which are high in carbon and can be present in the cells at a high concentration. Several groups have designed screens for sugar sensing mutants but it is likely that a large proportion of the plants will be metabolic mutants.

Chapter 4

Detailed characterisation of *cai* 10

4.1 Introduction

A. thaliana plants will flourish on growth media containing relatively high concentrations (i.e. 100 mM) of the metabolisable hexoses, such as glucose and fructose if there are sufficient macronutrients for their incorporation into amino acids and other carbon-rich molecules. However, mannose, another hexose, is toxic to many higher plants even at low concentrations (i.e. 5 mM). The entry of glucose, fructose and mannose into metabolism is catalysed by hexokinase which phosphorylates the carbon chain at position 6 resulting in hexose-6-phosphate. Glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) are inter-convertible by the action of phosphoglucose isomerase (PGI) and can be diverted directly into other pathways for energy production or biosynthesis (e.g. glycolysis or sucrose synthesis). *In vitro*, PGI from yeast does not act on mannose-6-phosphate (M6P)(personal observation). Plants require phosphomannose isomerase (PMI) to utilise M6P for entry into glycolysis. PMI converts M6P to F6P and only certain higher plants possess this enzyme in a high enough concentration to allow them to utilise exogenously supplied mannose. Celery cell cultures have very high PMI activity (Stoop and Pharr, 1993) and are able to survive on mannitol which is oxidised to mannose and phosphorylated by hexokinase to M6P before being isomerised to F6P by PMI. Mannose has an herbicidal affect on plants that do not contain PMI or which have low levels of enzyme. In mannose sensitive plants, it is thought that mannose exerts its toxic affect by sequestering inorganic phosphate (Pi) as M6P (Herold and Lewis, 1977) Sequestration of Pi may cause a decrease in phosphorylated metabolites and ATP formation, and inhibit photosynthesis by perturbing Pi : DHAP ratios.

During germination and post-germinative growth the glyoxylate cycle is required for gluconeogenesis, a process which generates sucrose from the breakdown products of seed storage triacyl glycerols. The sucrose is used as a carbon source by the developing seedling before it becomes fully photosynthetic. Malate synthase (MS) and isocitrate lyase (ICL) are 2 key components of the glyoxylate cycle and their gene expression has been

shown to be repressed by sugars (Graham *et al*, 1994 a and b). The hexokinase reaction has been implicated in the initial sugar sensing step resulting in repression of *ms* and *icl*. Thus, when mannose is supplied to a germinating seedling, the phosphorylation of mannose or the build up of M6P may instigate the signal for catabolite repression of glyoxylate cycle genes.

Wild type *Arabidopsis* seeds germinate on 5 mM mannose, producing a 1-3 mm radicle but do not develop green cotyledons (Pego, *et al.*, 1999). *Arabidopsis* is likely, therefore, to have little or no PMI. Pego and Smeekens (J. Pego; pers. comm.) used transgenic *Arabidopsis* containing the *ms* promoter linked to the GUS coding region to study the affect of mannose during germination. Their results showed that GUS activity was repressed by 5 mM mannose. They hypothesised that mannose is toxic during germination because it represses glyoxylate cycle gene expression and thus gluconeogenesis. This was the first report of sugar repression causing developmental arrest during post-germinative growth. However, this theory does not exclude the possibility that sequestration of Pi is the cause of seedling arrest during post-germinative growth. A series of experiments were designed to address this, the results of which form a large part of this chapter.

Previously, Pego *et al.* (1999) had selected *Arabidopsis* mutants which were insensitive to mannose. These mutants were defined as having a *mig* (mannose insensitive growth) phenotype. Several different types of mutant might be insensitive to mannose. Mutants in sugar sensing might be insensitive to mannose because the signal for sugar repression is not transduced. More specifically, mutants in hexokinase may be insensitive to mannose either because Pi is not sequestered or because the signal for sugar repression is not transduced. Mutants overexpressing PMI may also be insensitive to mannose because mannose phosphate is converted to fructose phosphate which can then be quickly metabolised releasing the Pi. With these possible mechanisms for mannose resistance in mind, the *cai* mutants were rescreened on mannose to see if any of them have a *mig* phenotype. *cai* 10 was found to have a *mig* phenotype with a penetrance of 100 %. This made *cai* 10 suitable for biochemical characterisation and genetic analysis. The experiments in this chapter investigate mannose metabolism, the mechanism of mannose toxicity and attempt to ascertain why *cai* 10 is mannose insensitive.

4.2 Results

4.2.1 Screening the *cai* mutants for mannose insensitive growth (*mig*) phenotype

All the *cai* mutants described in chapter 3 were screened for mannose insensitive growth on 1/2 M&S agar containing 5 mM mannose. Four of the *cai* mutants (vai 10, 24, 27 and 171) had mig phenotypes. *cai* 24, *cai* 27 and *cai* 171 had approximately 5 % penetrance of the *mig* phenotype (Figure 3.17, chapter 3) and were not further characterised. *cai* 10 was found to exhibit 100 % mannose insensitive growth (*mig*) phenotype during germination whereas ws displayed 0% *mig* phenotype (Figure 4.1). ws extends a radicle of 1-3 mm in length before growth is arrested whereas *cai* 10 develops green cotyledons and a short root.

4.2.2 Glucose alleviates the mannose affect

The affect of mannose on wild type *Arabidopsis* seeds can be relieved by adding 50 mM glucose or mannoheptulose (a specific hexokinase inhibitor) (Coore and Randle, 1964) to the growth medium (Pego, *et al.* 1999). This affect was repeatable with ws and col-2 (Figure. 4.2). To determine the concentration at which glucose can relieve the affect of mannose, wild type seeds (col-2) were allowed to germinate on 1/2 M&S agar plates containing 5 mM mannose and 0 - 50 mM glucose for 5 days. Figure 4.2 shows that at 5 mM mannose and 0 mM glucose none of the seeds developed green cotyledons. At 30 mM glucose 95 % of the seeds had developed green cotyledons. The concentration at which there was 50 % greening of cotyledons was about 7.5 mM glucose plus 5 mM mannose. This suggests that glucose competes directly with the mechanism causing mannose toxicity and that mannose is taken up and /or phosphorylated in slight preference to glucose.

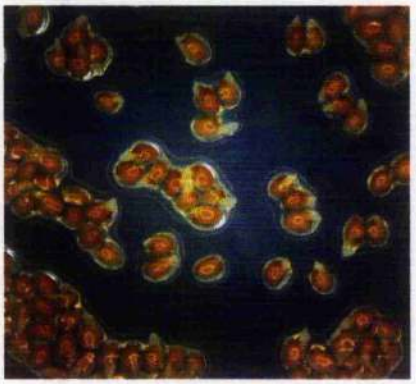
4.2.3 Investigating the route of mannose metabolism using ^{14}C-mannose and tritiated mannose feeding experiments

Figure 4.3 shows two possible fates of mannose metabolism. D-mannose is phosphorylated to M6P which can either be converted to F6P by PMI (reaction 1) or to mannose-1-phosphate (M1P) by phosphomannomutase (reaction 2). F6P is diverted into glycolysis, storage compounds and carbon skeletons (Pharr *et al.*, 1995) Sucrose is

Figure 4.1 The affect of 5 mM mannose on the post-germinative growth of ws and *cai* 10.

Seeds were sown on 1/2 strength M&S agar media containing 5 mM mannose. The seeds were imbibed for 4 days at 4 °C and were then transferred to a growth room (22 °C, 120 $\mu Em^{-2}s^{-1}$ continuous white light) for 5 days.

***cai* 10**



2 mm

w s

Figure 4.2 Glucose relieves the affect of mannose on seedling post-germinative growth.

Wild type seeds were sown on M&S agar media containing 5 mM mannose and increasing concentrations of glucose. The plates were imbibed for 4 days at 4 °C and were transferred to a growth room (22 °C, 120 $\mu Em^{-2}s^{-1}$ continuous white light) for 5 days when the phenotype was assessed. The arrow indicates the concentration of glucose added to the medium at which 50 % of the seedlings develop green cotyledons.

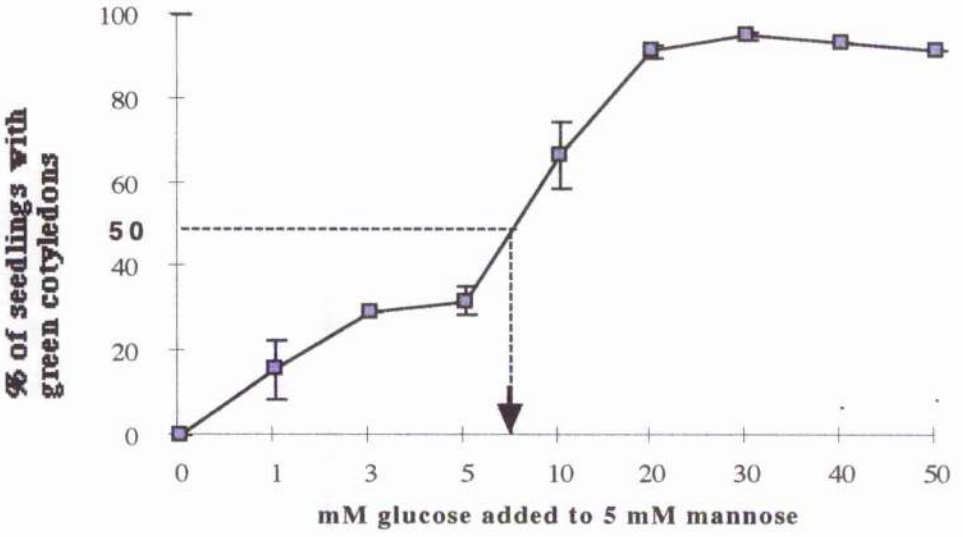

% of seedlings with green cotyledons
100
80
60
50
40
20
0
0
1
3
5
10
20
30
40
50
mM glucose added to 5 mM mannose

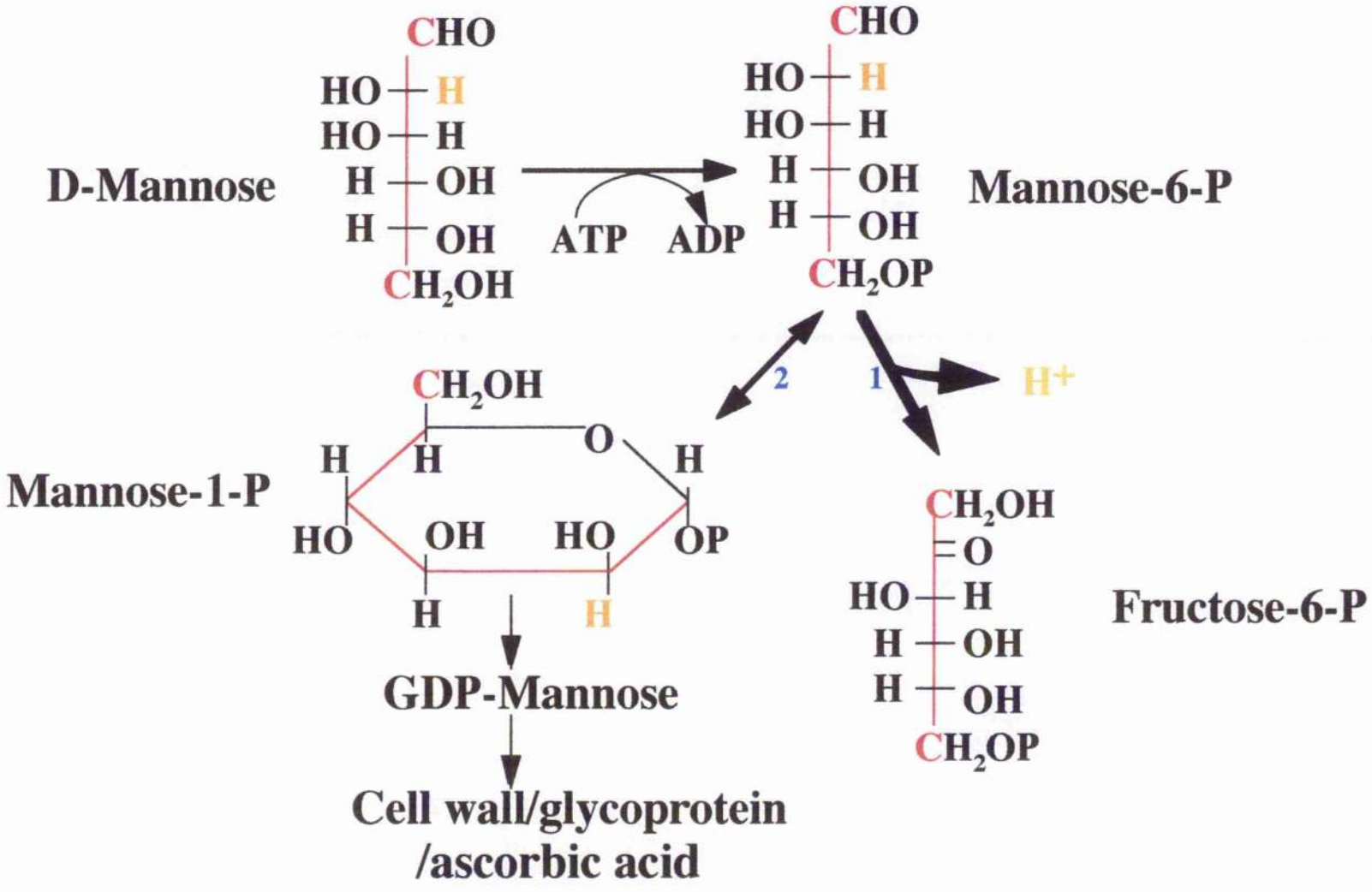
Figure 4.3 The fate of the tritiated and ^{14}C moieties of mannose during metabolism.

C_____C = ^{14}C labelled carbon

H = tritium

1 = phosphomannose isomerase

2 = phosphomannomutase


D-Mannose
ATP
ADP
Mannose-6-P
Mannose-1-P
2
1
H+
GDP-Mannose
Fructose-6-P
Cell wall/glycoprotein
/ascorbic acid

synthesised from F6P by the enzymes G6P isomerase, phosphoglucomutase, UDP-glucose pyrophosphorylase, sucrose phosphate synthase and sucrose phosphatase. M1P is converted by GTP-mannose-1-phosphate guanyltransferase to GDP-mannose (De Asua *et al.*, 1966) the building blocks of cell wall polymers, glycoproteins and also a precursor of L-ascorbic acid (Wheeler *et al.*, 1998).

4.2.3.2 D-[U-^{14}C] mannose feeding experiments

D-[U-^{14}C] mannose was supplied to *cai* 10 and ws to investigate the metabolism of mannose in mannose sensitive and mannose insensitive plants. The aims of the experiment were to (1) determine whether mannose is metabolised in ws and *cai* 10 and if so, into which compounds and (2) to establish if there are differences in mannose metabolism in *cai* 10 and ws.

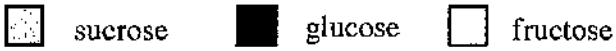
10 mg aliquots of dry seed were imbibed for 4d at 4 °C on filter paper soaked in 5 mM mannose (1/2 M&S salts) spiked with D-[U-^{14}C]mannose. The seeds were then put into continuous white light (120 umols /m^2/s) at 21 °C to germinate for 36 hours. After this period of germination both ws and *cai* 10 possess a 1-3 mm radicle emerging from the seed and appear developmentally identical. The seedlings were rinsed off extensively in cold water and ground up in 80 % ethanol to extract the metabolites. The extracts were loaded on to paper chromatograms and developed in EPW (100:35:25). The radioactive metabolites of D-[U-^{14}C]mannose were measured using a ^{14}C image analyser which was used to quantitate the amount of radioactivity present in the resolved compounds.

4.2.3.3 Invertase treatment of the D-[U-^{14}C]mannose metabolites

Figure 4.4 shows that D-[U-^{14}C] mannose is metabolised in ws and *cai* 10 as the D-[U-^{14}C] mannose has been converted into other compounds visible as 'hot' spots on the paper chromatogram. The 'hot' spot at the origin is made up of polar compounds (i.e. sugar phosphates) which do not migrate with the solvents used. In order to identify whether the spots which run at an Rf similar to sucrose actually were sucrose, a portion of the sample was pre-treated with invertase before loading onto the chromatogram. Invertase cleaves any terminal fructosyl moieties from polysaccharides and sucrose. The digestion of the

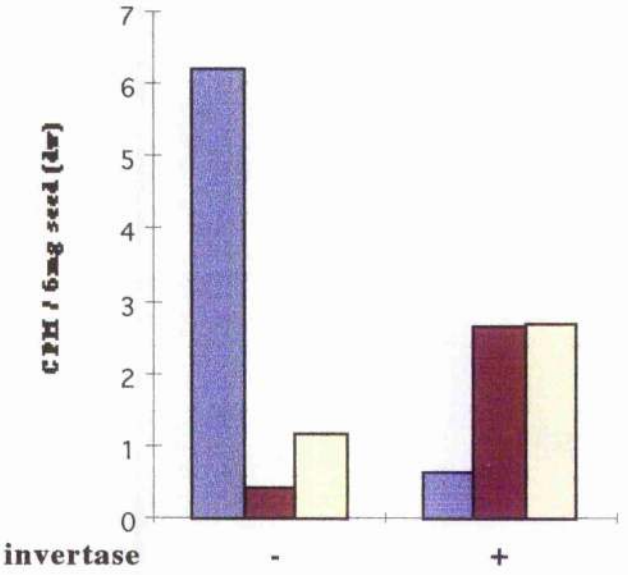
Figure 4.4 Invertase treatment identifies sucrose as a metabolite of mannose.

Seeds were sown on 1/2 strength M&S medium containing 5 mM mannose spiked with ^{14}C mannose. The seeds were imbibed at 4 °C for 4 days and transferred to a growth room (22 °C, 120 $\mu Em^{-2}s^{-1}$ continuous white light) for 36 hours. The soluble sugars were extracted from the seedlings. One aliquot of the extract was treated with invertase. An invertase treated sample and an untreated control were separated by paper chromatography. (a) shows the quantification of radioactivity in the metabolites of mannose before and after invertase treatment.

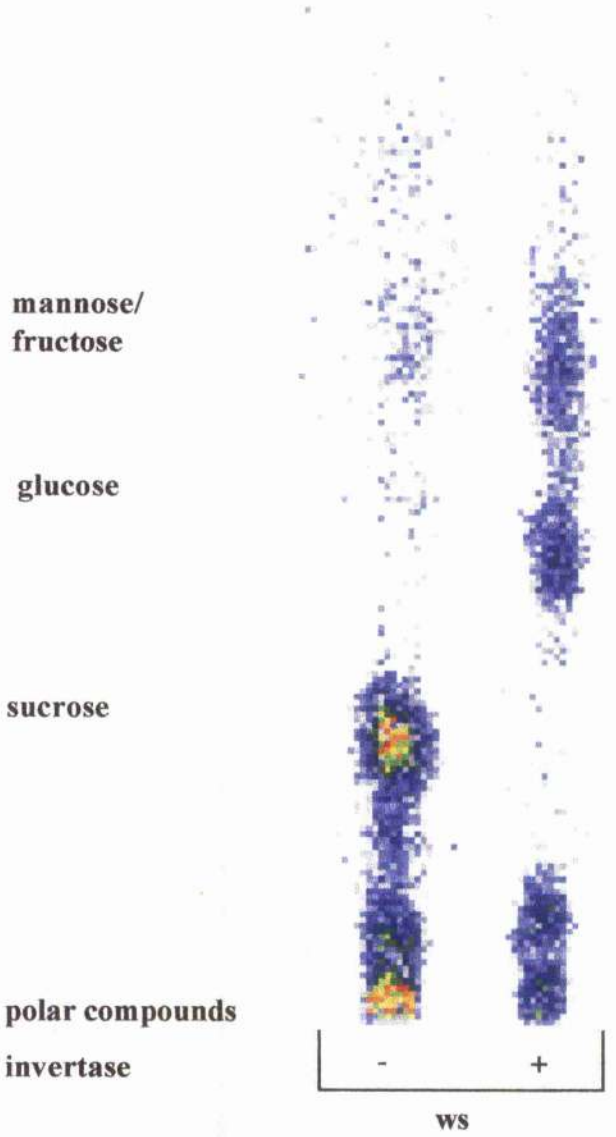


(b) shows the radioactive metabolites of mannose separated by paper chromatography. The relative intensity of radioactivity in each compound corresponds to the colour of the image. The intensity of radioactivity is illustrated by a colour spectrum. Red is the most intense radioactivity and violet is the least intense radioactivity.

a


CPM / 6mg seed (dw)
7
6
5
4
3
2
1
0
invertase
-
+

b


mannose/
fructose
glucose
sucrose
polar compounds
invertase
-
+
ws

"sucrose" spots by invertase into spots which migrate at the same Rf s as glucose and fructose confirms that a large proportion of mannose fed to both ws and *cai* 10 seedlings is metabolised to sucrose.

4.2.3.4 Alkaline phosphatase treatment of the D-[U-^{14}C]mannose metabolites

To test the hypothesis that mannose-fed seedlings accumulate high concentrations of mannose phosphates, seedling extracts were treated with alkaline phosphatase prior to loading on to a paper chromatogram. Before alkaline phosphatase treatment a large proportion of D-[U-^{14}C] radioactivity is visible as polar compounds at the origin of the paper chromatogram (Figure 4.5). Theoretically, the polar compounds will include M6P and F6P (precursors of sucrose) and also M1P which is metabolised via a different pathway to cell wall polymers. The samples that were digested with alkaline phosphatase show a dramatic decrease in the polar compounds and a concurrent increase in mannose and/or fructose (fructose and mannose have the same Rf using this solvent system). This indicates that the polar compounds were mannose phosphate and/or fructose phosphate but it is not possible to determine whether the mixture contained M6P, M1P or both.

4.2.3.5 Alkaline phosphatase treatment of seedlings fed D-[U-^{14}C]mannose plus 50 mM glucose

As shown in figure 4.2 glucose relieves the affect of mannose on seedling development resulting in ws seedlings developing normally at 50 mM glucose plus 5 mM mannose. In figure 4.6 extracts of seedlings germinated for 36 h on 5 mM mannose spiked with D-[U-^{14}C]mannose plus 50 mM glucose were digested with alkaline phosphatase prior to loading on a paper chromatogram. The figure shows that the concentration of radioactive sugar phosphates in seedlings fed D-[U-^{14}C]mannose plus glucose is significantly less than seedlings germinated on 5 mM mannose alone. This data supports the hypothesis that germination is stopped because of sequestration of Pi.

Figure 4.5 Identification of hexose phosphates metabolites of mannose by alkaline phosphatase treatment.

Seeds were sown on 1/2 strength M&S medium containing 5 mM mannose spiked with ^{14}C mannose. The seeds were imbibed at 4 °C for 4 days and transferred to a growth room (22 °C, 120 $\mu Em^{-2}s^{-1}$ continuous white light) for 36 hours. The soluble sugars were extracted from the seedlings. An aliquot was treated with alkaline phosphatase. An alkaline phosphatase-treated sample and an untreated control were separated by paper chromatography.

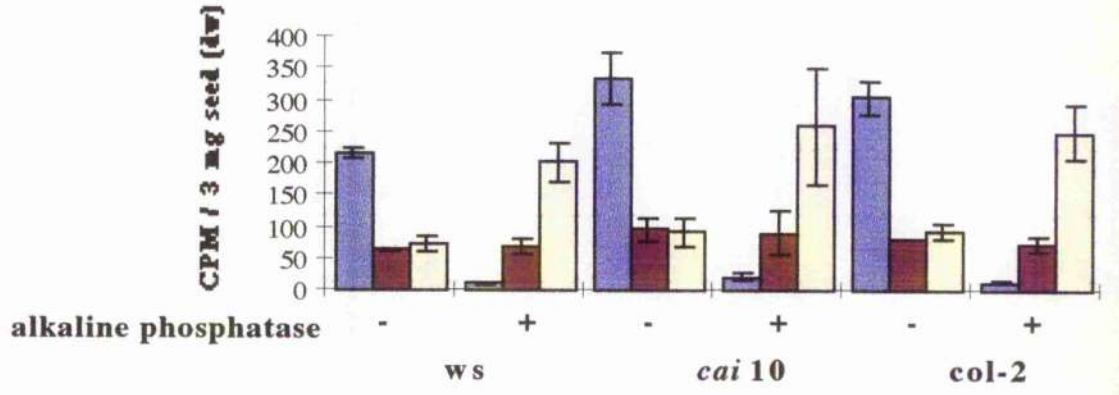
(a) shows the quantification of radioactivity in the metabolites of mannose before and after alkaline phosphatase treatment.

▨ phosphates ■ sucrose □ mannose/ fructose

(b) shows the radioactive metabolites of mannose separated by paper chromatography.

The relative intensity of radioactivity in each compound corresponds to the colour of the image. The intensity of radioactivity is illustrated by a colour spectrum. Red is the most intense radioactivity and violet is the least intense radioactivity.

a


CPM / 3 mg seed (dw)
400
350
300
250
200
150
100
50
0
alkaline phosphatase
-
+
-
+
-
+
ws
cai 10
col-2

b

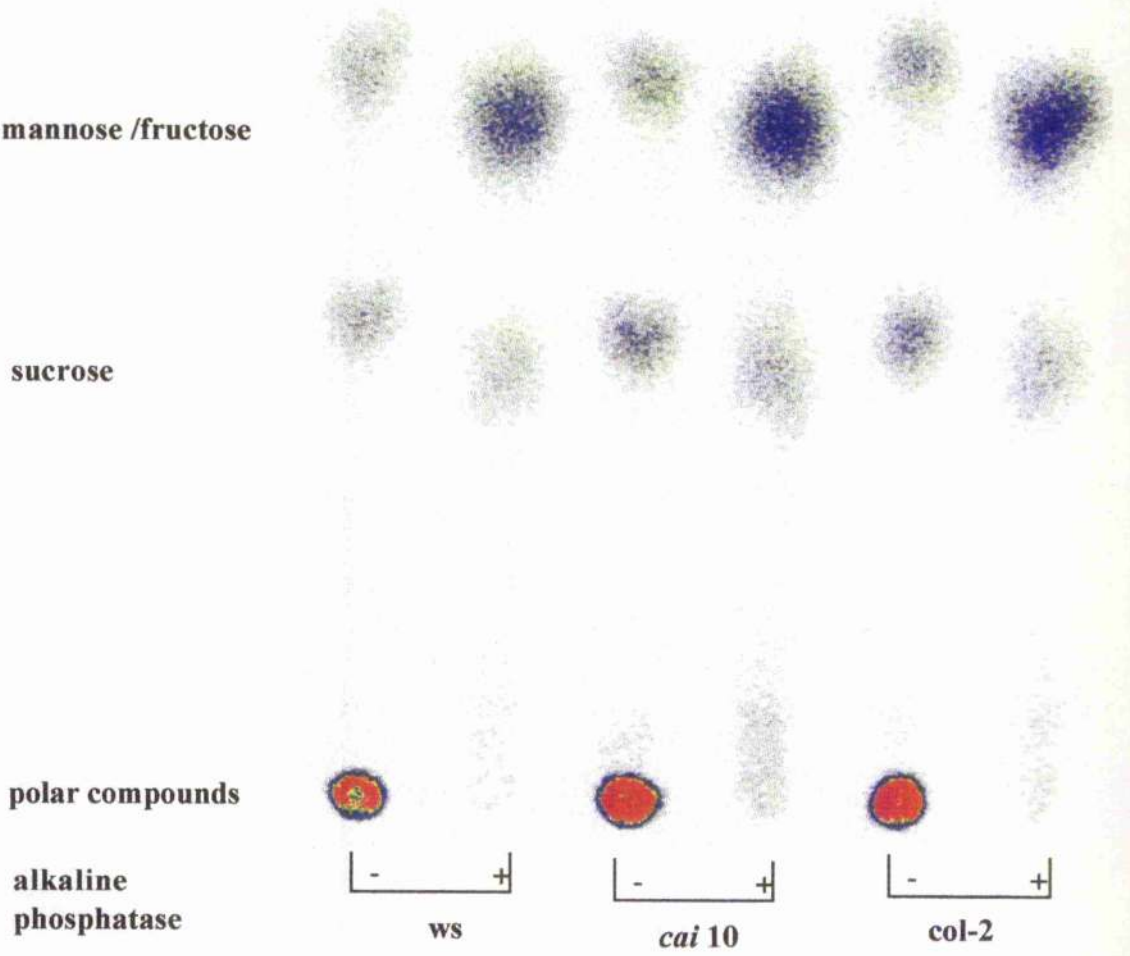

mannose /fructose
sucrose
polar compounds
alkaline phosphatase
-
+
-
+
-
+
ws
cai 10
col-2

Figure 4.6 Identification of hexose phosphates metabolites of ^{14}C mannose by alkaline phosphatase treatment.

Seeds were sown on 1/2 strength M&S medium containing 5 mM mannose spiked with ^{14}C mannose plus 50 mM glucose. The seeds were imbibed at 4 °C for 4 days and transferred to a growth room (22 °C, 120 $\mu Em^{-2}s^{-1}$ continuous white light) for 36 hours. The soluble sugars were extracted from the seedlings. An aliquot was treated with alkaline phosphatase. An alkaline phosphatase-treated sample and an untreated control were separated by paper chromatography.

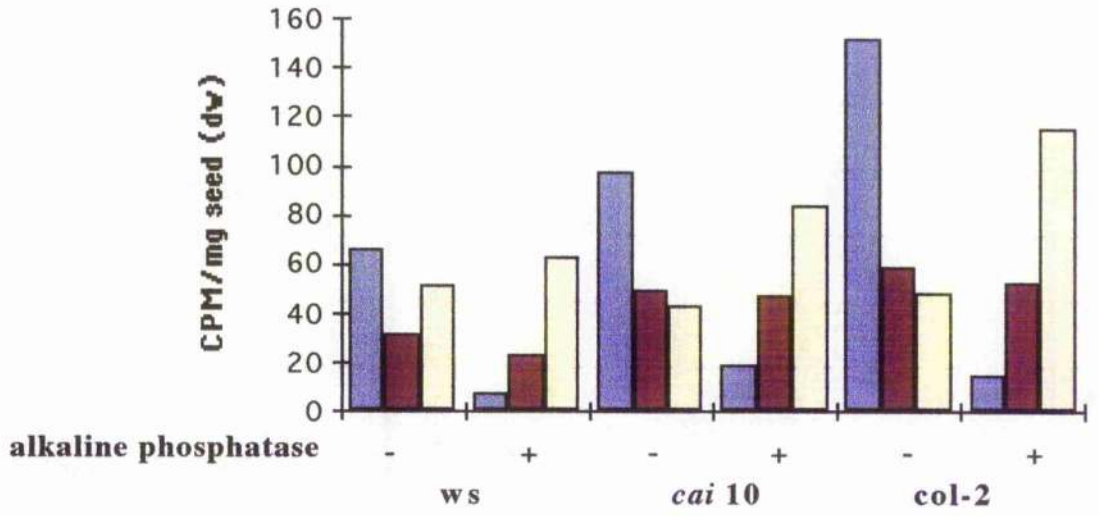
(a) shows the quantification of radioactivity in the metabolites of mannose before and after alkaline phosphatase treatment.

▒ phosphates ■ sucrose □ mannose/ fructose

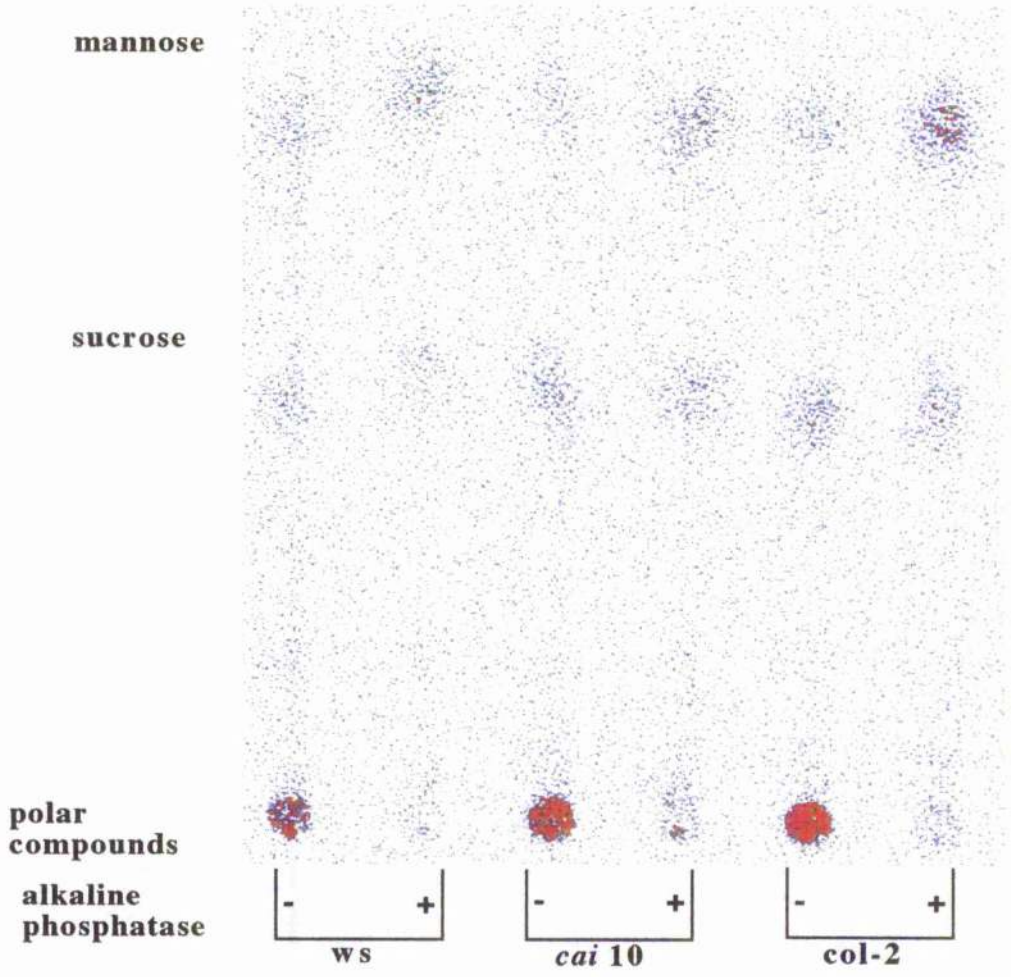
(b) shows the radioactive metabolites of mannose separated by paper chromatography.

The relative intensity of radioactivity in each compound corresponds to the colour of the image. The intensity of radioactivity is illustrated by a colour spectrum. Red is the most intense radioactivity and violet is the least intense radioactivity.

a


160
140
120
100
80
60
40
20
0
CPM/mg seed (dw)
alkaline phosphatase
-
+
-
+
-
+
ws
cai 10
col-2

b


mannose
sucrose
polar compounds
alkaline phosphatase
-
+
-
+
-
+
ws
cai 10
col-2

4.2.3.6 Determining the proportions of mannose diverted into sucrose metabolism and cell wall polymers

It was not possible to distinguish between mannose and fructose phosphates in the D-[U-^{14}C]mannose feeding experiments; nor was the route of mannose metabolism to sucrose determinable by this method. In this experiment tritiated mannose was used to distinguish between M6P and F6P and to establish the route of sucrose biosynthesis. The proportion of mannose that is either recruited by glycolysis or directed to structural polymers or ascorbate can be determined by feeding seeds with D-[2-^{3}H]mannose. The tritium on C-2 of M6P is lost as tritiated water when M6P is isomerised to F6P but the tritium is retained when M6P is converted to M1P. On drying, tritiated water is lost from the extract leaving a residue of tritiated M1P and tritiated mannose. The loss of radioactivity is equivalent to the amount of mannose that is converted to F6P.

cai 10, ws and col-2 seeds were germinated for 36h on 1/2 strength M&S solution containing 5 mM mannose spiked with [2-^{3}H]mannose. The seedlings were rinsed with cold water and ground up in 80 % ethanol to extract the metabolites. The sample was divided into two equal aliquots. One aliquot was dried and the other was not. The amount of tritium was measured in each sample using a liquid scintillation counter (Figure 4.7). The fact that 80 % of the radioactivity is lost though evaporation indicates that this is the amount of tritiated mannose converted from M6P to F6P.

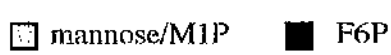
In *cai* 10, ws and col-2 approximately 80 % of the tritiated mannose is metabolised to F6P and 20% remains as unmetabolised mannose or has been converted to M1P. Thus a considerable amount of the exogenous mannose is metabolised via F6P to sucrose. This correlates with the large sucrose spot on the paper chromatogram (Figure 4.4), which is visualised as a consequence of D-[U-^{14}C]mannose labelling, as this label is not lost.

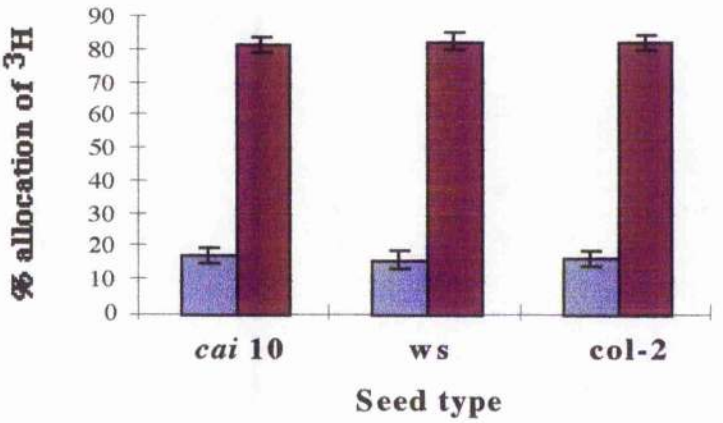
4.2.4.1 Measuring the flux of mannose and glucose into metabolism in *cai* 10 and ws

The metabolites of D-[U-^{14}C]mannose and D-[U-^{14}C]glucose in ws and *cai* 10 were measured over the 36 h germination period. The aims of this experiment were to (1)

Figure 4.7 The percentage of mannose metabolised into mannose/mannose-1-phosphate and fructose -6-phosphate.

Seeds were sown on 1/2 strength M&S medium containing 5 mM mannose spiked with ^{3}H-mannose. The seeds were imbibed at 4 °C for 4 days and transferred to a growth room (22 °C, 120 $\mu Em^{-2}s^{-1}$ continuous white light) for 36 hours. The soluble sugars were extracted from the seedlings and were divided into equal aliquots. One aliquot was air-dried and then the radioactivity in each aliquot was measured by a scintillation counter. The loss of radioactivity from the air dried sample is equivalent to the percentage of mannose which is metabolised to fructose-6-phosphate.




% allocation of ^{3}H
90
80
70
60
50
40
30
20
10
0
cai 10
ws
col-2
Seed type

establish if ws and *cai* 10 metabolise mannose at the same rate and (2) determine whether mannose and glucose were metabolised at the same rate.

4.2.4.2 Mannose metabolism

Figure 4.8 shows the metabolism of D-[U-^{14}C]mannose to hexose phosphates and sucrose. In both ws and *cai* 10 metabolism of D-[U-^{14}C]mannose began 4-8 hours after transferral to the light at 22 °C. By 12 to 18 hours ws was accumulating more hexose phosphates and sucrose than *cai* 10. By 36 hours ws had accumulated 5-fold more hexose phosphates and 3-fold more sucrose derived from D-[U-^{14}C]mannose than *cai* 10. The data indicate that *cai* 10 is metabolising mannose at a different rate to ws but it is not possible to say whether it is metabolising it faster or slower than ws. The reason for this is that although sucrose is not accumulating as much in *cai* 10 as ws, it may be respiring or converting the D-[U-^{14}C]sucrose to other compounds (such as proteins and fatty acids) faster than ws and this would not be detected by this method. However, whichever strategy *cai* 10 employs, hexose phosphates do not accumulate and Pi is, therefore, presumably not sequestered to the same extent.

4.2.4.3 Glucose metabolism

Figure 4.9 shows the accumulation of metabolites of D-[U-^{14}C]glucose by ws and *cai* 10. As for D-[U-^{14}C]mannose, ws and *cai* 10 started to metabolise D-[U-^{14}C]glucose after about 4 h in the light. Between 12 and 18h ws accumulated more hexose phosphates and sucrose than *cai* 10. Again this can be interpreted as *cai* 10 metabolising glucose more slowly than ws or respiring the end product sucrose more rapidly than ws. A comparison of mannose and glucose metabolism shows that ws and *cai* 10 both accumulate more than twice the amount of sucrose when metabolising glucose rather than mannose. The critical difference between glucose and mannose metabolism in ws is that hexose phosphates do not accumulate to the same extent when glucose is fed as substrate compared to when mannose is fed as a substrate. Therefore, when ws is germinated on glucose, Pi is not sequestered. Like ws, when *cai* 10 is germinated on glucose more sucrose but less hexose phosphate

Figure 4.8 The uptake timecourse of ^{14}C mannose by *cai* 10 and ws during germination.

Seeds were sown on 1/2 strength M&S medium containing 5 mM mannose spiked with ^{14}C mannose. The seeds were imbibed at 4 °C for 4 days and transferred to a growth room (22 °C, 120 $\mu Em^{-2}s^{-1}$ continuous white light). Transfer to the growth room is the 0 hour time point. Seedlings were taken at time intervals for 36 hours. The soluble sugars were extracted from the seedlings. The soluble sugars were separated by paper chromatography and the radioactive metabolites of ^{14}C mannose were quantified.

—□— hexose phosphates in *cai* 10 —■— sucrose in *cai* 10

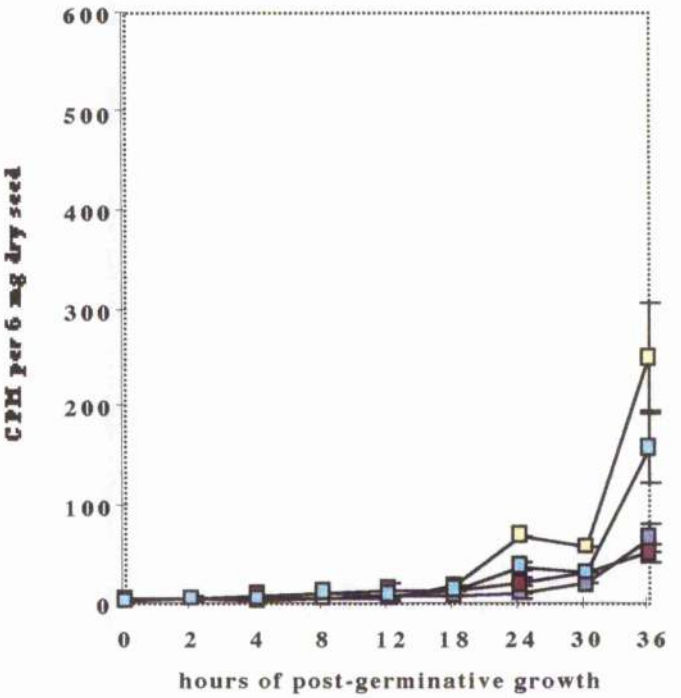
—□— hexose phosphates in ws —□— sucrose in ws

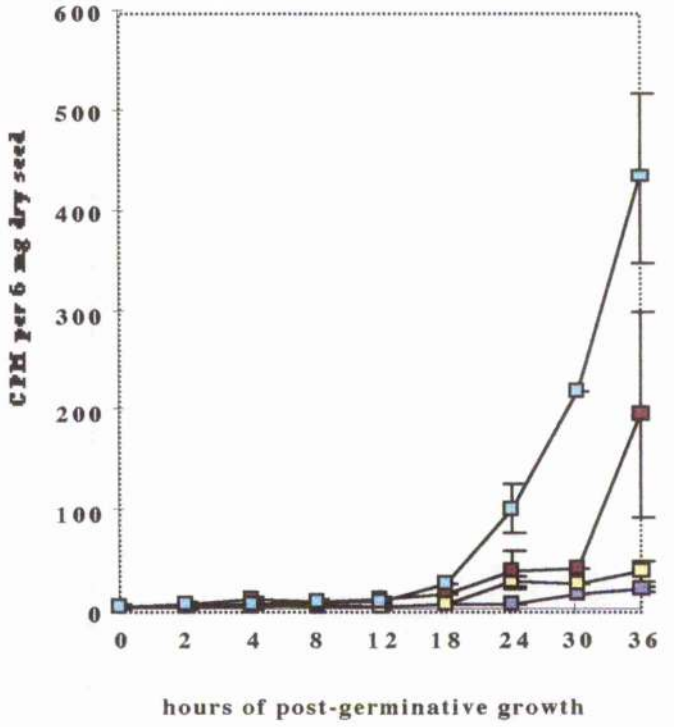
Figure 4.9 The uptake timecourse of ^{14}C glucose by *cai* 10 and ws during germination.

Seeds were sown on 1/2 strength M&S medium containing 5 mM glucose spiked with ^{14}C glucose. The seeds were imbibed at 4 °C for 4 days and transferred to a growth room (22 °C, 120 $\mu Em^{-2}s^{-1}$ continuous white light). Transfer to the growth room is the 0 hour time point. Seedlings were taken at time intervals for 36 hours. The soluble sugars were extracted from the seedlings. The soluble sugars were separated by paper chromatography and the radioactive metabolites of ^{14}C mannose were quantified.

—□— hexose phosphates in *cai* 10 —■— sucrose in *cai* 10

—□— hexose phosphates in ws —□— sucrose in ws


600
500
400
300
200
100
0
CPM per 6 mg dry seed
0 2 4 8 12 18 24 30 36
hours of post-germinative growth


600
500
400
300
200
100
0
CPM per 6 mg dry seed
0 2 4 8 12 18 24 30 36
hours of post-germinative growth

accumulates than when germinated on mannose. These results provide an explanation for the *cai* 10 phenotype on mannose. An interesting feature of the D-[U-^{14}C]mannose uptake time course was that both ws and *cai* 10 metabolised a small amount of mannose but not glucose into arabinose and xylose within 2 h (data not shown). This is evidence that mannose is metabolised into cell wall precursors very soon after imbibition of the seed.

4.2.5 Does mannose alter the conversion of lipid into sugars during germination?

4.2.5.1 Analysis of soluble sugars in seedlings germinated on mannose

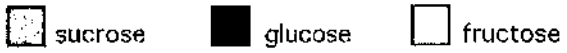
Figure 4.10a-d shows the differences in metabolites extracted from seedlings germinated for 36 hours on 5 mM mannose, 5 mM mannose plus 50 mM glucose, 50 mM glucose and zero carbon source. A striking feature of figure 4.10a is that *cai* 10 seedlings possess approximately 30 % of the amount of sucrose, glucose and fructose present in ws and col-2 when germinated on 5 mM mannose. This agrees with the radiolabelling data in figure 4.8. In addition seedlings germinated on mannose have higher cellular soluble sugars than seedlings germinated on no exogenous carbon source (Figure 4.10 d). This agrees with the evidence that mannose is being used as carbon source and corroborates the radioactive labelling data (Figure 4.8). In figure 4.10b the seedlings all have the potential to develop to the green cotyledon stage because glucose is relieving the affect of mannose. The metabolite concentrations in all 3 seed types are similar but are much higher than in the seedlings which were germinated on 5 mM mannose. When germinated on 50 mM glucose only (Figure 4.10c) the metabolite concentrations are similar between each seed type and are also similar to those of seedlings grown on 5 mM mannose plus 50 mM glucose (Figure 4.10b). Not surprisingly, seedlings germinated on media containing no exogenous sugar the metabolite concentrations are very low (Figure 4.10d).

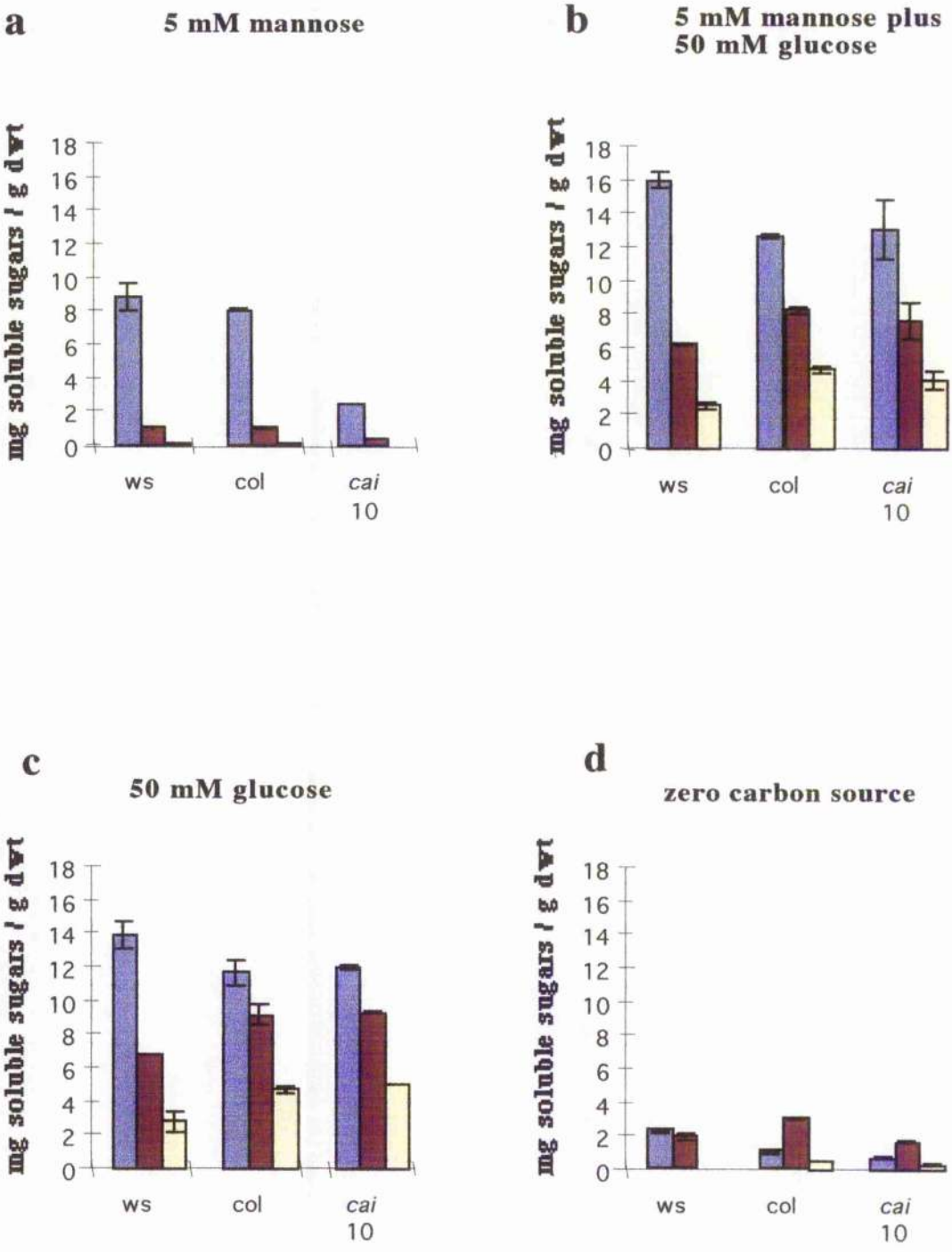
4.2.5.2 Analysis of steady state transcript levels in seedlings germinated on 5 mM mannose

To investigate the hypothesis that mannose arrests seedling development by repressing glyoxylate cycle gene expression and in so doing prevents the conversion of

Figure 4.10 Mannose is used as a carbon source during germination.

Seeds were sown on 1/2 strength M&S agar medium containing (a) 5 mM mannose, (b) 5 mM mannose plus 50 mM glucose, (c) 50 mM glucose or (d) zero carbon source. The plates were given a 4 day cold treatment at 4 °C and were transferred to a growth room at 22 °C in continuous white light (120 $\mu Em^{-2}s^{-1}$) for 36 hours. The seedlings were rinsed in ice-cold water and the soluble sugars were extracted and analysed using enzyme linked assays.

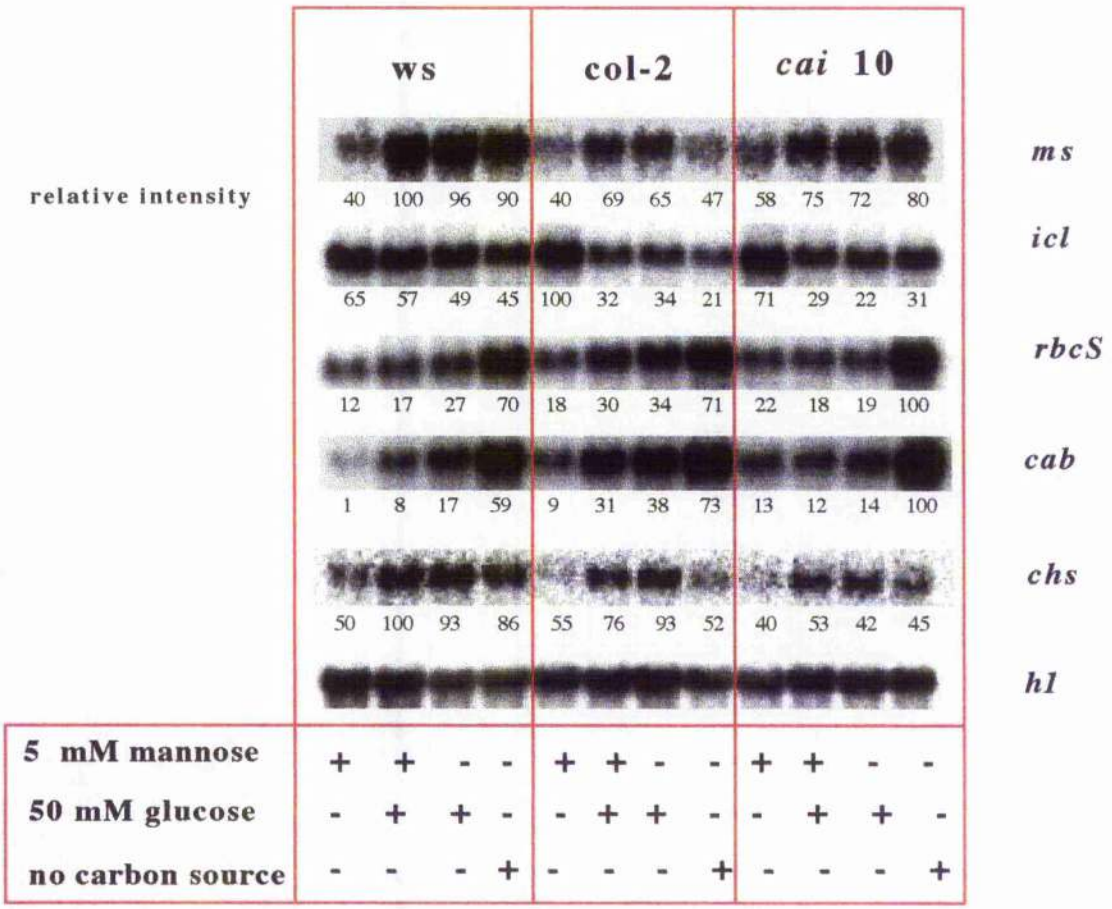



a
5 mM mannose
b
5 mM mannose plus
50 mM glucose
c
50 mM glucose
d
zero carbon source
mg soluble sugars / g d wt
0
2
4
6
8
10
12
14
16
18
ws
col
cai
10

lipids into sugars ws, col-2 and *cai* 10 were germinated for 36 h on 1/2 M&S agar plates containing 5 mM mannose, 5 mM mannose plus 50 mM glucose, 50 mm glucose or no carbon source. RNA was isolated from the seedlings and 5 ug of each sample was separated on a formaldehyde gel. The RNA gel blot analysis (Figure. 4.11) shows the steady state transcript levels of several genes which are known to be regulated by sugars. The glyoxylate cycle genes *ms* and *icl* are known to be co-ordinately repressed by sucrose in germinating cucumber cell cultures and protoplasts (Graham et al., 1994 a and b). In contrast to all published sucrose repression data the results presented in figure 4.11 show that *ms* is repressed but *icl* is induced by mannose. This is the first published report of conditions which cause uncoordinated expression of *ms* and *icl*. However, although *icl* appears to be induced by 5 mM mannose it is not possible to discern from this whether mannose has an affect on transcription of the ICL gene or whether *icl* RNA stability has been affected. 5mM mannose decreases the expression of 2 other genes (*rbcS, cab*) known to be repressed by sucrose. The addition of glucose to the medium containing mannose partially relieves the repression of *cab*, *rbcS* and *ms* in ws and col-2. In *cai* 10, 5 mM mannose has the same repression affect on *rbcS* and *cab* as 50 mM glucose but 5 mM mannose plus 50 mM glucose does not reduce the repression in *cai* 10 as it does in ws and col-2. In both wild types 5 mM mannose is much more effective than 50 mM glucose at repressing *rbcS*. In *cai* 10, 50 mM glucose is as effective as 5 mM mannose at repressing *rbcS*. In ws and col-2, *chs* is induced by glucose and repressed by mannose. Glucose relieves the affect of mannose repression. Mannose and glucose have little affect on *chs* expression in *cai* 10, the transcript abundance being little different to seedlings germinating on no carbon source. It is interesting to note that in all seed types *chs* expression is repressed by mannose even though glucose induces its expression. One explanation for this is that mannose cannot mimic glucose in the induction of *chs*. Alternatively, 5 mM mannose (by sequestering Pi) may be depleting the seedling's pool of carbon skeletons which would induce a carbohydrate starvation signal. The carbon starvation signal could prevent carbon skeletons from being diverted into non-essential pathways such as anthocyanin biosynthesis by repressing *chs*.

Figure 4.11 Mannose represses *ms, rbcS* and *cab* but induces *icl* gene expression after 36 hours of germination.

Seeds were sown on 1/2 strength M&S agar medium containing 5 mM mannose, 5 mM mannose plus 50 mM glucose, 50 mM glucose or zero carbon source. The plates were given a 4 day cold treatment at 4 °C and were transferred to a growth room at 22 °C in continuous white light (120 $\mu Em^{-2}s^{-1}$) for 36 hours. RNA was extracted from the seedlings, separated by denaturing agarose gel electrophoresis and transferred to HybondN membrane. The RNA was hybridised with radioactive probes of several sugar-modulated genes and was visualised using a phosphoimager.


ws
col-2
cai 10
relative intensity
ms
icl
rbcS
cab
chs
hl
40 100 96 90 40 69 65 47 58 75 72 80
65 57 49 45 100 32 34 21 71 29 22 31
12 17 27 70 18 30 34 71 22 18 19 100
1 8 17 59 9 31 38 73 13 12 14 100
50 100 93 86 55 76 93 52 40 53 42 45
5 mM mannose + + - - + + - - + + - -
50 mM glucose - + + - - + + - - + + -
no carbon source - - - + - - - + - - - +

4.2.6 *cai* 10 is only mannose insensitive during post-germinative growth

So far the experiments in this chapter have all been aimed at establishing why *cai* 10 is insensitive to mannose during post-germinative growth. An experiment was designed to investigate whether older *cai* 10 seedlings were also insensitive to mannose. *cai* 10 seeds were germinated on vertical agar plates containing 1/2 M&S plus 50 mM glucose for 7 days. The seedlings were then transferred to vertical agar plates containing 5 mM mannose and the plates were turned through 90 degrees. After a further 7 days growth the seedlings were assessed. Figure 4.12 shows that the roots of *cai* 10 do not extend after the seedlings have been transferred to mannose. The slightly bulbous root tip is a typical response to toxicity. ws responds in the same way as *cai* 10. Thus the mutation that makes *cai* 10 mannose insensitive has a germination specific effect. The roots of control seedlings which were transferred to identical 50 mM glucose agar plates after 7 days continued to grow and after 14 days were approximately 42 mm long (data not shown).

4.8 Discussion

Mannose is toxic to wild type *Arabidopsis* and arrests seedling growth after radicle emergence. *cai* 10 is mannose insensitive at the germination stage and develops cotyledons on growth media containing 5 mM mannose.

Experiments feeding D-[U-^{14}C]mannose to germinating wild type (ws) *Arabidopsis* show that although growth is arrested by mannose, the mannose itself is metabolised. Treatment of extracts with invertase and alkaline phosphatase allowed identification of sucrose and hexose phosphate spots on paper chromatograms. It was found that the wild type metabolises mannose to hexose phosphates and sucrose. Feeding germinating seedlings with mannose tritiated at carbon-2 demonstrated that the route of mannose metabolism to sucrose was via F6P and that 80 % of supplied mannose was metabolised to F6P. The remaining mannose was split between unmetabolised mannose and M1P, a precursor for the biosynthesis of cell wall polymers and ascorbate. Indeed, within 2 hours of transferring germinating seedlings to light some mannose had been converted into compounds such as arabinose and xylose (cell wall precursors).

Figure 4.12 *cai* 10 is not insensitive to mannose after 7 days post-germinative growth. Seeds of *cai* 10, ws and col-2 were sown on 1/2 strength M&S agar medium containing 50 mM glucose. The plates were given a 4 day cold treatment at 4 °C and were transferred to a growth room at 22 °C in continuous white light (120 $\mu Em^{-2}s^{-1}$) where the plates were held vertically for 7 days. The seedlings were gently transferred to plates containing 1/2 strength M&S agar medium plus 5 mM mannose. The plates were turned 90 ° and were held vertically for 7 days in the same growth conditions. Root growth on mannose was noted.

ws

***cai* 10**

col-2

Wild type and *cai* 10 were fed D-[U-^{14}C]mannose and D-[U-^{14}C]glucose and samples were taken during the first 36 hours of germination in order to investigate the differences in mannose and glucose metabolism (Figures 4.8 and 4.9). When D-[U-^{14}C]mannose was fed to wild type a large amount of hexose phosphates and sucrose accumulated. However, when wild type was supplied with D-[U-^{14}C]glucose, little hexose phosphate accumulated although a large amount of sucrose was synthesized. In contrast, when *cai* 10 was fed D-[U-^{14}C]mannose or D-[U-^{14}C]glucose very little hexose phosphate accumulated and 2-fold less sucrose accumulated than in wild type. However, there is a discrepancy between the amounts of hexose phosphates which accumulate in figures 4.5a and 4.8. In figure 4.5a ws and *cai* 10 accummulate similar amounts of hexose phosphates after 36 h, whereas in figure 4.8 *cai* 10 accumulates 3-fold less hexose phosphates than ws. However, figure 4.8 shows a more complete representation of mannose metabolism during germination than figure 4.5a as metabolite measurements have been taken at several time points indicating that *cai* 10 and ws do metabolise mannose at different rates. Another possible explanation for the difference between figure 4.5a and 4.8 is that the rate of germination varied between experiments due to subtle changes in environmental conditions. The critical difference in mannose metabolism between wild type (ws) and *cai* 10 seen in figure 4.8 is that ws accumulates hexose phosphates and *cai* 10 does not. This implies that sequestration of Pi may have a role in mannose toxicity in ws plants, a fact which is supported by previous work on other species (Herold and Lewis, 1977).

Another important point is that *cai* 10 accumulates less sucrose and hexose phosphate than wild type when it is supplied with D-[U-^{14}C]mannose and D-[U-^{14}C]glucose. This suggests that *cai* 10 metabolises mannose and glucose at a different rate to wild type but it is not possible to say whether it metabolises them faster or slower than wild type because although it does not accumulate as much sucrose as wild type, *cai* 10 may simply respire the D-[U-^{14}C]sucrose faster than wild type. This would not be detected with the method used here. The respiration rate of D-[U-^{14}C]mannose and D-[U-^{14}C]glucose needs to be measured in wild type and *cai* 10 to clarify this. This could be done by capturing the respired $^{14}CO_2$ with KOH and measuring the radioactivity in a scintillation counter.

When wild type seed was germinated on 5 mM mannose spiked with D-[U-^{14}C]mannose plus 50 mM glucose the concentration of D-[U-^{14}C] hexose phosphates in seedlings was significantly lower than in seedlings germinated on 5 mM mannose spiked with D-[U-^{14}C]mannose alone. This suggests that glucose relieves the toxic effect of mannose by reducing the amount of Pi sequestration and maintaining a pool of carbon skeletons for biosynthesis into other compounds.

Although sequestration of Pi appears to have a role in mannose toxicity it does not exclude the possibility that mannose could also repress expression of genes that are vital to post-germinative growth. Since the phenotypes of *cai* 10 and wild type are so different on 5 mM mannose it seemed appropriate to investigate the steady state transcript level of a range of genes during germination. To avoid comparing transcript abundance in an arrested seed and a green seedling, the time point for RNA isolation was chosen when all the seedlings looked similar, i.e. radicle emergence. From this experiment it is possible to say that mannose may arrest seedling development in the wild types by repressing *ms* transcript abundance or increasing the turnover of *ms* transcript, especially as in *cai* 10 the *ms* transcript level was decreased to a lesser extent. Unexpectedly, the transcript abundance of *icl* was induced by mannose in all cases. This result is contrary to all previously published data on *ms* and *icl* and is the first report of non-coordinate regulation of these two genes. However, it is not possible to discern from hybridisation analysis whether *icl* RNA transcription stability was affected by growth in 5 mM mannose. Promoter-reporter gene studies in transgenics could be used to investigate this.

The stabilities of the MS and ICL enzymes may also differ in *cai* 10 during germination which might result in the presence of a complete glyoxylate cycle and thus continued gluconeogenesis even though the *ms* gene is not being expressed. It would be necessary to assay MS and ICL enzyme activities to determine if the glyoxylate cycle is in operation. In addition malate synthase from germinating castor beans has been found to undergo *in vitro* phosphorylation on a serine residue, although the affect of phosphorylation on the *in vivo* activity of the enzyme was not determined (Yang *et al.*, 1988). If phosphorylation is vital for MS activity, mannose may affect MS activity by sequestering

Pi. This could prevent post-germinative growth in the wild type and cause negative feedback regulation of *ms* gene expression.

Soluble sugar measurements of seedlings germinated on 5 mM mannose for 36 hours revealed that *cai* 10 seedlings have approximately 70 % less sucrose, glucose and fructose than the wild types. This data correlates with the uptake time course and is further evidence that *cai* 10 metabolises mannose at a different rate to the wild type. *cai* 10 has a lower concentration of soluble sugars when grown on 5 mM mannose than ws and col-2. This fact might also explain the decreased repression of *ms* by mannose in *cai* 10. It is possible that the wild types fail to develop beyond the 1-3 mm radicle stage because MS activity is too low to support gluconeogenesis.

In conclusion, the results suggest that *cai* 10 resists the toxicity of mannose during germination by avoiding the sequestration of Pi into hexose phosphate pools, and its low soluble sugar concentrations may lessen the affect of mannose repression of gene expression. The low soluble sugar concentration and altered hexose metabolism in *cai* 10 indicate that it is more likely to be a metabolic/uptake mutant than a sugar signalling mutant. *cai* 10 is only insensitive to mannose during post-germinative growth. Seven day old seedlings are no longer insensitive to mannose. This may imply that the sugar repression of *ms* is important in mannose metabolism. It would be interesting to compare the mannose metabolism in *cai* 10 during post-germinative growth and in 7 day old seedlings.

Chapter 5

Characterisation of *cai* 10 overexpressing hexokinase 1 (*At*HXK1)

5.1 Introduction

Hexokinase has been proposed as a key component of the signal transduction cascade which leads to carbohydrate repression of a range of metabolic genes (Graham, *et al.*, 1994b, Jang and Sheen, 1994, Jang and Sheen, 1997). Studies involving feeding sugars to cucumber cell cultures found that 2-deoxyglucose and mannose which are phosphorylated by hexokinase can initiate the signal for repression of *ms* and *icl* in a manner similar to that of metabolisable sugars (Graham, *et al.*, 1994b). However, 3-methyl glucose, which was taken up by the cell culture but is not known to be phosphorylatable by hexokinase, did not cause repression of the expression of *ms* and *icl* transcripts. From this evidence it was suggested that hexose sugars are important in the sugar response. Hexose sugars themselves, were excluded as the initiators of the signal because 3-methyl glucose, which is not phosphorylated, did not induce the sugar response. Furthermore, a specific hexokinase inhibitor, mannoheptulose (Coore and Randle, 1964), can block glucose repression of photosynthetic gene expression in maize protoplasts (Jang, *et al.*, 1997). The evidence showing that mannose and 2-deoxyglucose, which are phosphorylated by hexokinase, caused sugar repression, and that mannoheptulose can block sugar repression, suggests that the hexokinase reaction transduces the signal reporting the metabolic status of the cell. The signal from the hexokinase reaction could be initiated by (1) the ATP substrate, (2) the phosphorylated products, (3) the flux through the hexokinase reaction, (4) or a combination of the above. Hexokinase has also been implicated in initial transduction of the signal leading to carbohydrate repression in *S. cerevisiae* as mutation of the hexokinase PII (HXK2) results in constitutive expression of glucose repressible genes (Entian, *et al.*, 1985).

A mutant which does not express active hexokinase may be unable to sense sugars (Jang and Sheen, 1997) including mannose or its product M6P and so may not activate the signal transduction cascade which leads to the carbohydrate repression of *ms* and *icl* . Seeds unable to break down their lipid storage reserves are unable to grow without an external carbohydrate source. This may be one reason why glucose can relieve the affect of mannose

on seedling post-germinative growth. If the hexokinase reaction initiates the signal transduction pathway for sugar repression and communicates the metabolic status of the cell, a plant with mutant hexokinase may not be able to sense the presence of hexoses. Previous experiments showed that wild type seedlings do not develop cotyledons when germinated on a media containing mannose, a hexose which is phosphorylated by hexokinase. The fact that *cai*10 germinates and develops green cotyledons on mannose implied it may not sense mannose and thus, might be a hexokinase mutant. An additional hypothesis to explain the *cai* 10 phenotype on mannose is that *cai* 10 is mutated in the signal transduction pathway downstream from hexokinase. This could lead to reduction or abolition of the sugar repression signal.

Genetics can prove a phenotype is due to a mutation in a single locus. Having cloned a candidate gene for a mutant locus, the definitive proof that a mutation in that gene caused the mutant phenotype is complementation. This involves transforming the mutant plant with the wild type gene. To prove that the mutant gene causes a phenotype the corresponding wild type gene must restore the plant to the wild type phenotype.

The experiments in this chapter were initiated before all the data presented in the previous chapters had been obtained. For instance, it is now known that *cai* 10 is a dominant mutation. However, when the work was started the aim was to determine whether *cai* 10 was a hexokinase mutant using complementation with the wild type hexokinase gene.

The rationale of the work was as follows: if *cai* 10 had a recessive mutation in hexokinase or an upstream signalling component, overexpression of hexokinase 1 in *cai* 10 could restore the wild type phenotype on mannose by (1) complementing a mutation in hexokinase or (2) by increasing the amplitude of the hexokinase signal thereby overriding a weak signal caused by a mutation in the signal cascade upstream of hexokinase. The aim of this chapter was to assess whether the *cai* 10 lesion could be complemented by transformation by overexpression of hexokinase 1 and to characterise the affect of hexokinase 1 overexpression on wild type.

5.2 Results

5.2.1 Immunoblot analysis of hexokinase in *cai* 10

Protein extracts were made from *cai* 10 seeds and seedlings during post-germinative growth. The protein extracts were rapidly desalted, separated by SDS-PAGE and blotted onto nitrocellulose. The hexokinase isozymes were visualised by immunodetection with hexokinase polyclonal antibodies (a gift from Dr J-C. Jang; Figure 5.1). The western blot reveals that *cai* 10 manufactures two proteins that are detected by the hexokinase antibody, one of approximately 25 kDa in seeds and another of 56 kDa in germinating seedlings. This corroborates the work of Jang *et al.*, (1997) who, using the same antibody, detected a 56 kDa protein in 6 day old seedlings. The hexokinase antibody also shows that the pattern of hexokinase expression is similar in both wild type and *cai* 10. However, it is not possible to ascertain whether the hexokinase proteins are active in the *cai* 10 mutant, or whether a depletion of 1 isozyme is masked by another isozyme of the same size.

5.2.2.1 Transformation of *cai* 10 with sense-orientated hexokinase 1 (35S:*At*HXK1)

col-2, ws and *cai* 10 were transformed with *A. thaliana* hexokinase 1 under the control of the CaMV 35S promoter in the sense orientation (35S:*At*HXK1; a gift from Dr. J-C. Jang; Jang, *et al.*, 1997) using an *A. tumifaciens* vacuum infiltration method (Bechtold, *et al.*, 1993). Transformation with sense-orientation hexokinase resulted in 2 lines of *cai* 10, 9 lines of ws and 14 lines of col-2 overexpressing hexokinase 1.

5.2.2.2 Analysis of the hexokinase 1 transformants on different sugar selection media

The T2 lines were selected on kanamycin and the T3 generation were germinated on either 50 μg/ml kanamycin; 5 mM mannose; 0 mM nitrogen and 100 mM sucrose; or 6 % glucose (Figure 5.2). Some of the lines were still segregating as revealed by a less than 100 % resistance to kanamycin. As was expected, there were large phenotypic variations between the different lines probably due to position affect of the transgene. The results show that in the 2 lines obtained, hexokinase 1 overexpression did not restore a wild type

Figure 5.1 Western blot analysis reveals that *cai* 10 contains hexokinase protein

Seeds of *cai* 10 and ws were sown on 1/2 strength M&S agar medium containing 20 mM sucrose. The plates were given a 4 day cold treatment at 4 °C and were transferred to a growth room at 22 °C in continuous white light (120 $\mu Em^{-2}s^{-1}$) for 3 days. Transfer to the growth room is the day 0 time point. Seedlings were harvested after 0-3 days. Total protein was extracted. 50 ug of each protein sample was separated by SDS-PAGE. The proteins were transferred to nitrocellulose and were cross-reacted with polyclonal hexokinase antibodies.

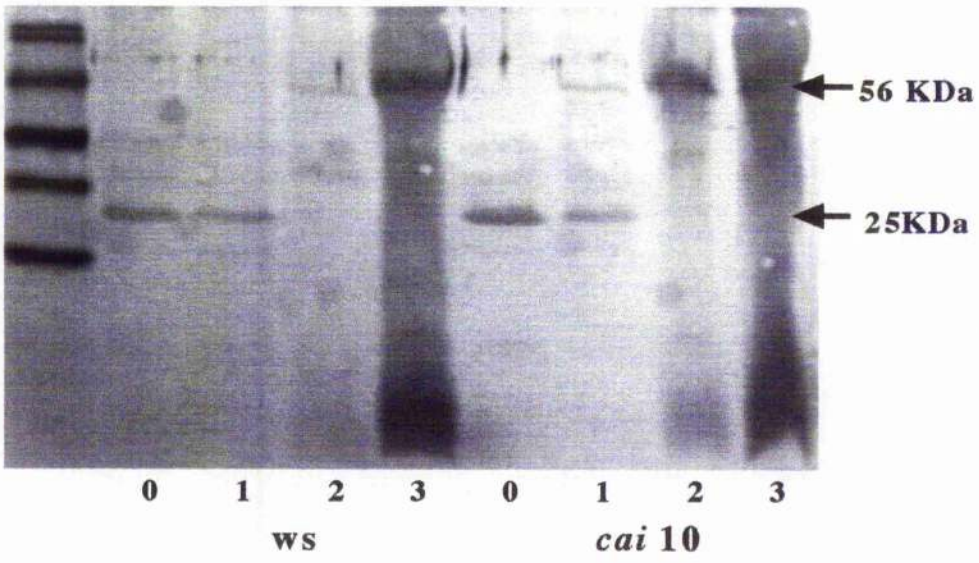

56 KDa
25KDa
0 1 2 3 0 1 2 3
ws
cai 10

Figure 5.2 Growth of T3 transformants overexpressing hexokinase on different selection media.

The T3 transformant lines were sown on 1/2 strength M&S agar media containing 50 μg/ml kanamycin, 5 mM mannose, 100 mM sucrose minus nitrogen, or 6 % glucose and were imbibed at 4 °C for 4 days. The plates were transferred to growth room conditions (22 °C, 120 $\mu Em^{-2}s^{-1}$) for 7 days and the seedling phenotypes were assessed. *mig* = mannose insensensitive growth phenotype. *cai* = carbohydrate insensitive growth phenotype. *gin* = glucose insensitive growth phenotype. MSCD= kanamycin resistant control.

☐ % kanamycin resistance ■ % *mig* ☐ % *cai* ▨ % *gin*

Percentage
0
10
20
30
40
50
60
70
80
90
100
10xhxkl-23
10xhxkl-24
c#10
ws#xhxkl-40
ws#xhxkl-61
ws
col x hxk 1-1
col x hxk 1-2
col x hxk 1-3
col x hxk 1-4
col x hxk 1-5
col x hxk 1-6
col x hxk 1-7
col x hxk 1-8
col x hxk 1-9
col x hxk 1-10
col x hxk 1-11
col x hxk 1-12
col x hxk 1-13
col x hxk 1-14
col-2
MSCD

phenotype in *cai* 10 when germinated on mannose (Figure 5.2). The observation that hexokinase overexpression does not complement the *cai* 10 lesion suggests that *cai* 10 is not a hexokinase mutant. It does not exclude the possibility that the *cai* 10 lesion completely blocks a possible sugar repression signal downstream of hexokinase. *cai* 10 hexokinase 1 overexpressors also exhibit similar percentages of *mig*, *cai* and *gin* phenotypes as *cai* 10 which have not been transformed (Figure 5.2). Interestingly, lines of col-2 overexpressing hexokinase 1 displayed a good *mig* phenotype and / or *cai* phenotype and some also had a *gin* phenotype.

Kanamycin resistant T3 seedlings were grown up for seed (T4). Seed from the T4 generation of each transformed line was also grown on a range of selective media (Figure 5.3). The percentage of kanamycin resistance in the T4 generation was higher than in the T3 generation. The col-2 hexokinase overexpressors all displayed some degree of mannose resistance and *cai* phenotype but no *gin* or *sig* phenotype. This apparent loss of *gin* phenotype from T3 to T4 could have been due to slight environmental fluctuations which can affect selection on the *gin* screen. The fructokinase mutant (*mig 7*; a gift from Dr S. Smeekens) which has a ws genetic background was used as a control. It is interesting to note that both seedlings overexpressing hexokinase 1 and a fructokinase deletion mutant are mannose resistant. MSCD seedlings were also used as a control as they contain the kanamycin resistance gene. Since MSCD seedlings do not have *mig cai* or *gin* phenotypes it is assumed that transformation with the kanamycin gene does not confer the sugar insensitive phenotypes observed in the hexokinase overexpressors.

5.2.2.3 Assessing the transformants for T-DNA insertion

DNA was extracted from the kanamycin resistant transformants. PCR primers were designed to the hexokinase 1 construct (collaboration with Dr F. Regad) in order to check the transformants for the presence of the hexokinase 1 transgene. These were used in a PCR reaction to verify that the transformants actually contained the hexokinase 1 transgene (Figure 5.4). There was no band in col-2, *cai* 10 or the water control. Furthermore, the primers do not detect hexokinase 2 as can be seen in the lane containing the vector

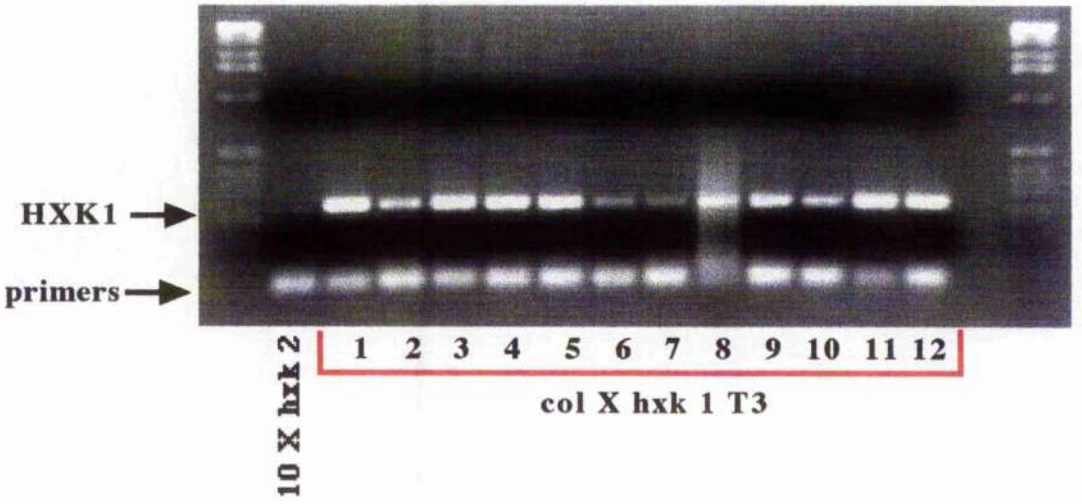
Figure 5.3 Growth of T4 transformants overexpressing hexokinase on different selection media.

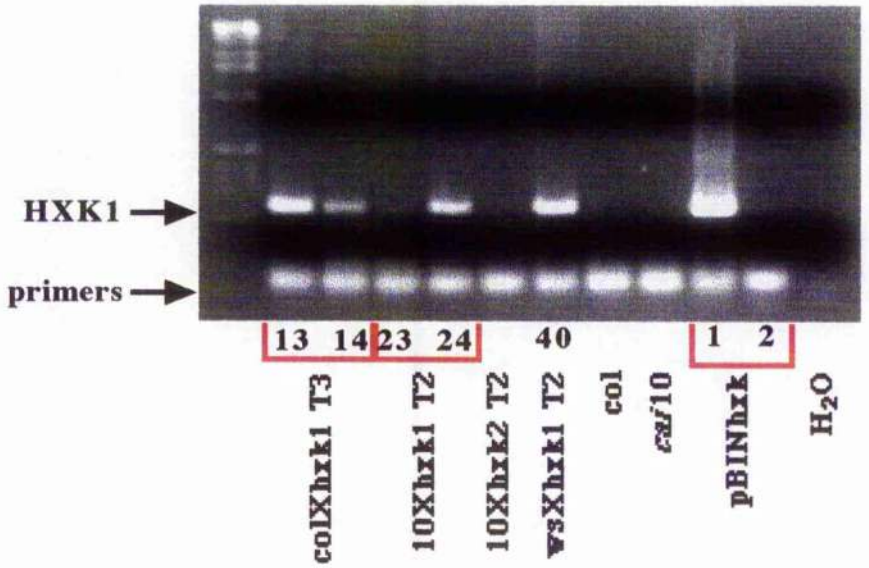
The T4 transformant lines were sown on 1/2 strength M&S agar media containing 50 μg/ml kanamycin, 5 mM mannose, 100 mM sucrose minus nitrogen, 6 % glucose or 12 % sucrose and were imbibed at 4 °C for 4 days. The plates were transferred to growth room conditions (22 °C, 120 $\mu Em^{-2}s^{-1}$) for 7 days and the seedling phenotypes were assessed.

Percentage
100
90
80
70
60
50
40
30
20
10
0
colgl1-1
WS
mig 7
mig 3
mig 2
cal 10
col
colxhxkl-14
colxhxkl-13
colxhxkl-12
colxhxkl-11
colxhxkl-8
colxhxkl-7
colxhxkl-6
colxhxkl-5
colxhxkl-4
colxhxkl-3

Figure 5.4 Presence of the 35S:AtHXK1 construct in the transgenic plants was assessed using PCR.

DNA was extracted from the leaves of plants transformed with the 35S:AtHXK1 construct. The transgenic DNA was amplified using PCR, the products were separated by agarose gel electrophoresis. pBINhxk1 and pBINhxk2 were used as controls. pBINHXK1 contains the 35S:AtHXK1 construct. pBINhxk2 contains the 35S:AtHXK2 construct and is not amplified by the primers which are specific for the 35S:AtHXK1 construct.


HXK1
primers
10 X hxk 2
1 2 3 4 5 6 7 8 9 10 11 12
col X hxk 1 T3


HXK1
primers
13 14 23 24 40 1 2
colXhxk1 T3
10Xhxk1 T2
10Xhxk2 T2
wsXhxk1 T2
col
cat10
pBINhxk
H2O

pBINhxk2. All the putative col-2 transformants contain the hexokinase 1 construct. Line 24 of the *cai* 10 transformants and line ws hxk-40 also contain the hexokinase 1 construct. Line 23 of the *cai* 10 transformants was a false positive.

5.2.2.4 Analysis of hexokinase transcript expression in the transformants

RNA was extracted from 3 individual kanamycin resistant plants per line. It was separated by agarose gel electrophoresis, blotted onto Hybond N and probed with a ^{32}P radiolabelled hexokinase probe which hybridises to hexokinase 1 and 2 (Figure 5.5). col-2 hxk lines 3, 4, 6, 7, 8, 10, 12, 13 and 14 all have increased steady state levels of hexokinase mRNA. Lines 5, 9 and 11 are low hexokinase expressors even though they do contain the T-DNA (Figure 5.4). The constitutive probe *h1* indicates that the loading and quality of the RNA is approximately equal in all lanes and thus low hexokinase expression is not due to variations in the RNA loaded. However, although lines 5, 9 and 11 show low expression of hexokinase RNA in leaf tissue, they all display some degree of mannose resistance in both the T3 and T4 generations (Figures 5.2 and 5.3) indicating that hexokinase 1 RNA expression in leaves does not necessarily correlate with a *mig* phenotype in seedlings. Line 12 is segregating between high and low expressors. Of the 2 *cai* 10 transformants only line 24 shows high expression of hexokinase which agrees with PCR data (Figure 5.4) and of the 9 ws transformants only lines 43, 44 and 49 are high expressors.

5.2.3 Analysis of the hexokinase protein in the transformants

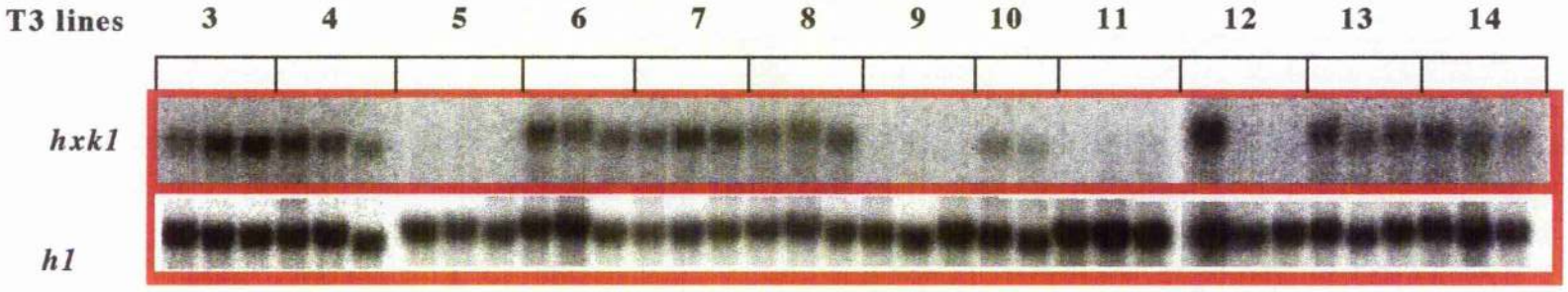
5.2.3.1 Immunodetection of hexokinase in the transformants

Protein was extracted from rosette leaves of the T3 transformants and 20 µg of each sample was separated by SDS-PAGE, blotted onto nitrocellulose and cross-reacted with hexokinase antibodies (a gift from Dr J-C. Jang;(Jang, *et al.*, 1997)). Ponceau S staining of the nitrocellulose blots showed that the amount loaded was even (Figure 5.6 b). It also shows that there is a high concentration of BSA which was added to the extract to stabilise the proteins. Figure 5.6a reveals that the hexokinase 1 overexpressors (Figure 5.5) do manufacture more hexokinase protein in leaves than the low hexokinase expressors, e.g. compare line 9 and 3 in figures 5.5 and 5.6. The hexokinase protein has a size of 49.5 KDa.

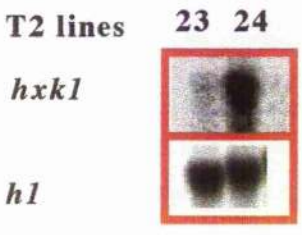
Figure 5.5 Hexokinase is overexpressed in several transformant lines containing the 35S:AtHXK1 construct.

RNA was extracted from leaves of 3 plants from each T3 col-2 line transformed with the 35S:AtHXK1 construct. RNA was also extracted from the T2 lines of *cai* 10 and ws transformed with the 35S:AtHXK1 construct. The RNA was separated by agarose gel electrophoresis and was blotted onto Hybond N membrane. The RNA was hybridised with a hexokinase probe and was analysed using a phosphoimager. hxk1 = hexokinase probe and *hI* = constitutive probe.

Col-2 transformed with 35S:*At*HXK1



cai 10 transformed with 35S:*At*HXK1



ws transformed with 35S:*At*HXK1

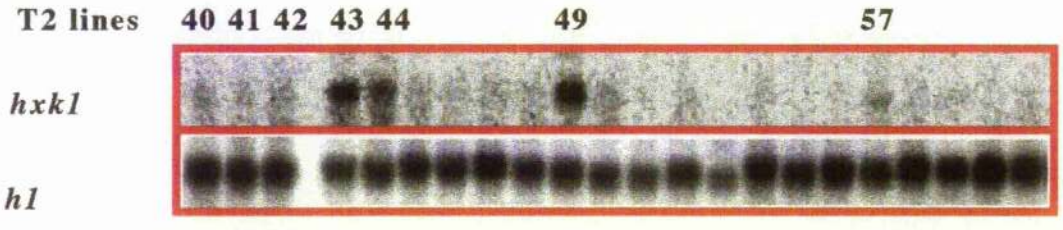


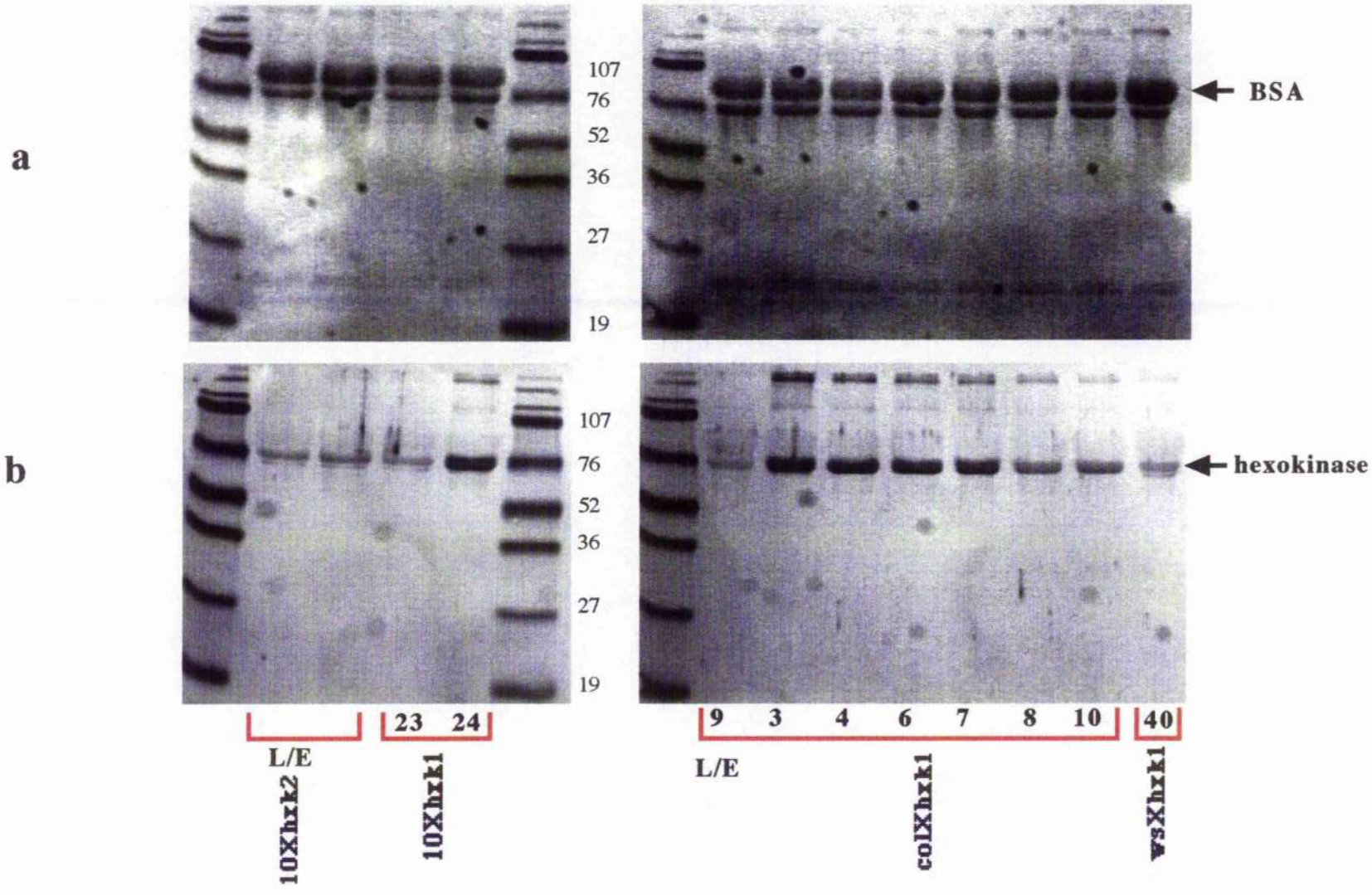
Figure 5.6 Immunodetection with hexokinase polyclonal antibodies detects the overexpression of the hexokinase protein in the hexokinase 1 overexpressors.

L/E = low expressor control

10 X hxk2 = *cai* 10 transformed with hexokinase 2

(a) The blots were stained with Ponceau S to assess accuracy of equal loading.

(b) Immunodetection with hexokinase polyclonal antibodies.


a
b
107
76
52
36
27
19
BSA
hexokinase
9 3 4 6 7 8 10
40
23 24
L/E
L/E
10Xhxk2
10Xhxk1
colXhxk1
vsXhxk1

This is smaller than the size reported for hexokinase in figure 5.1 and may be a result of distortion of the protein migration due to the excess of BSA.

5.2.3.2 Analysis of hexokinase activity in transformant leaves and whole seedlings

Hexokinase assays were performed on rapidly desalted protein extracts from both leaf and seedlings (Figures 5.7 a and b). For the leaf assays (Figure. 5.7a) line 9 was used as a control for base rate hexokinase activity for the col-2 transformants because it contains the sense hexokinase 1 construct but RNA is not overexpressed. Overexpressors exhibited 1.8-fold to over 5-fold increases in hexokinase activity compared to the control. *cai* 10 hexokinase 1 overexpressor line 24 also displayed a 2-fold increase in hexokinase activity compared to its control line 23. Hexokinase activity in 7 day old seedlings was determined (Figure 5.7b). In this case line 11 was used as the low expressor control. Line 14 which had the second highest hexokinase activity in leaf had very low activity in seedlings. The only lines which had increased seedling activities were line 3 and 6 and overall the hexokinase activity in seedlings was much less than in rosette leaves. This could be a feature of the position of transgene insertion, or the 35S promoter being more highly expressed in leaves than in 7 day old seedlings.

5.2.3.3 Mannose metabolism in transformants overexpressing hexokinase 1

Since the transformants overexpressing hexokinase 1 germinated rapidly and showed mannose insensitive growth (*mig*), the rate of D-[U-^{14}C]mannose metabolism was investigated as in chapter 4, section 4.2.4. The labelled products of D-[U-^{14}C]mannose metabolism, ^{14}C hexose phosphate and ^{14}C sucrose, were measured after 8 h growth. Figure 5.8 shows that several of the lines of transformants overexpressing hexokinase 1 accumulated a much higher concentration of labelled hexose phosphates and sucrose than the wild type. The low expressor line 5 from leaf analysis accumulates wild type amounts of hexose phosphates and sucrose but the low expressor lines 9 and 11 accumulate elevated levels of these compounds. In addition, the T4 generation of line 5 shows a 10 % *mig* phenotype whereas line 9 and 11 both show approximately 50 % *mig* phenotypes. There is,

Figure 5.7 Hexokinase activity in seedlings and leaves of the transformants overexpressing hexokinase 1.

Seeds were sown on 1/2 strength M&S agar medium containing 20 mM sucrose. The plates were imbibed for 4 days at 4 °C. The plates were transferred to growth room conditions (22 °C, 120 $\mu Em^{-2}s^{-1}$) for 7 days. Soluble proteins were extracted from the seedlings, the extracts were rapidly desalted and the hexokinase activity was measured spectrophotometrically. The hexokinase activity from 2 extracts of col-2 from leaves and seedlings was measured 3 times. The range of hexokinase between the 2 leaf extracts was negligable. The range of hexokinase between the 2 seedling extracts was also negligable. Hexokinase activity in seedlings (a) and in rosette leaves (b) of transformants. 10 X hxk2 is *cai* 10 transformed with a hexokinase 2 (low expressor control).

a

nmols/min/mg protein
25
20
15
10
5
0
10 x hxk2
col x hxk-3
col x hxk-4
col x hxk-6
col x hxk-7
col x hxk-8
col x hxk-9
col x hxk-10
col x hxk-12
col x hxk-13
col x hxk-14
10 x hxk-23
10 x hxk-24
ws x hxk-30
ws x hxk-40
cw10
col-2

b

nmols/min/mg protein
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
col x hxk-3
col x hxk-6
col x hxk-8
col x hxk-11
col x hxk-14
cw10
cw31
cw76
cw112
cw172
mg2
mg3
mg7
ws
col-2

Figure 5.8 Mannose is metabolised by the hexokinase overexpressors.

Seeds were sown on 1/2 strength M&S medium containing 5 mM mannose spiked with ^{14}C mannose. The seeds were imbibed at 4 °C for 4 days and transferred to a growth room (22 °C, 120 $\mu Em^{-2}s^{-1}$ continuous white light). Transfer to the growth room is the 0 hour time point. The seeds were allowed to germinate for 8 hours. The soluble sugars were extracted from the seedlings. The soluble sugars were separated by paper chromatography and the radioactive metabolites of ^{14}C mannose were quantified.

 hexose phosphates  sucrose

 *% mig* phenotype

CPM per mg seed (dw)
0
5
10
15
20
25
30
35
40
45
col x hxk-3
col x hxk-5
col x hxk-6
col x hxk-7
col x hxk-8
col x hxk-9
col x hxk-10
col x hxk-11
col x hxk-12
col x hxk-13
col x hxk-14
col
mig2
mig3
mig7
ws
ws x hxk-40
ws x hxk-61
10 x hxk-24
nd
col10
coll
% mig phenotype
0
20
40
60
80
100

in general, a much better correlation between the accumulation of elevated hexose phosphates and sucrose and the *mig* phenotype after 8 h than with RNA and enzyme activity in leaves and seedlings. This is not surprising as the *mig* phenotype is dependent on alterations in metabolites or signalling very early in post germinative growth as has been demonstrated for *cai* 10. Interestingly, *mig* 7 which is a fructokinase deletion mutant also accumulated elevated levels of hexose phosphates and sucrose after 8h. The other *migs*, *mig* 2 *mig* 3 and *cai* 10 accumulate similar amounts as their respective wild types. This is in agreement with figure 4.8 which shows that *cai* 10 only differs from the wild type after 18-24 h.

5.2.4 Sensitivity of hexokinase 1 overexpressors to mannose after the germination stage

The hexokinase 1 overexpressors were examined in order to establish whether they were insensitive to mannose after the germination stage as in chapter 4 section 4.2.6. Seeds were germinated for 7 days on vertical agar plates containing 1/2 strength M&S media and 50 mM glucose. The seedlings were then transferred to 1/2 strength M&S agar medium plates containing 5 mM mannose and the plates were turned through 90°. After 7 days the root growth of the seedlings was analysed. Figure 5.9 shows that the hexokinase 1 overexpressor (col X hxk-14) grows initially on 5 mM mannose and then root growth is terminated. *mig* 7 and *cai* 10, which also display mannose insensitive growth during germination display the same sensitivity to mannose after the cotyledon stage. The roots of control seedlings which were transferred to identical 50 mM glucose agar plates after 7 days continued to grow and after 14 days were approximately 40 mm long (data not shown).

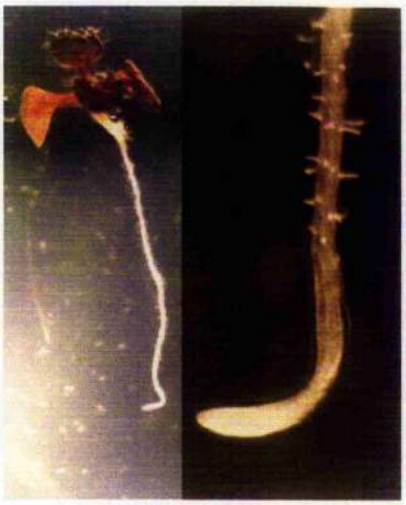
5.3 Discussion

The definitive way of testing whether a phenotype is caused by a recessive mutation in a specific gene is to transform the genome with the wild type gene. To link the mutant gene to a phenotype, the wild type gene must complement the mutant gene, restoring the wild type phenotype. At the time when this work was planned, *cai* 10 was assumed as being recessive because it was created using EMS and the majority of EMS induced

Figure 5.9 Root growth of 7 day old hexokinase overexpressor seedlings is arrested by mannose.

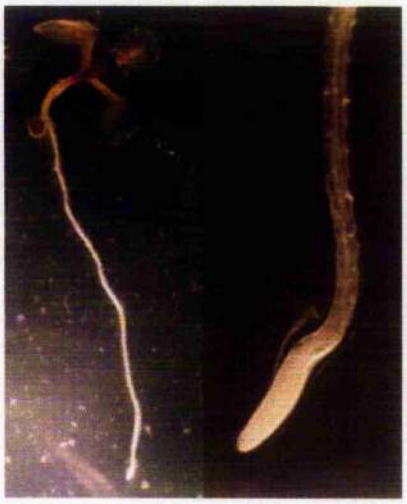
Seeds of the hexokinase overexpressors were sown on 1/2 strength M&S agar medium containing 50 mM glucose. The plates were given a 4 day cold treatment at 4 °C and were transferred to a growth room at 22 °C in continuous white light (120 $\mu Em^{-2}s^{-1}$) where the plates were held vertically for 7 days. The seedlings were gently transferred to plates containing 1/2 strength M&S agar medium plus 5 mM mannose. The plates were turned 90° and were held vertically for 7 days in the same growth conditions. Root growth on mannose was noted.

col X hxk 14



***mig* 7**

col-2



ws

mutants are recessive. When a transformant of *cai* 10 overexpressing hexokinase 1 was germinated on mannose the phenotype was identical to the *cai* 10 control.

The results presented here are consistent with more recent work which indicates that *cai* 10 contained a dominant mutation. A dominant mutation in hexokinase could result in several different phenotypes. A dominant mutation in the proposed regulatory domain of hexokinase could result in a constitutive sugar signal resulting in plants that perceive a high concentration of sugars even when none is present. A dominant lesion in the catalytic domain would result in high hexokinase activity. The hexokinase activity in *cai* 10 soluble protein extracts was undetectable indicating that *cai* 10 was not originally a dominant hexokinase mutant. This did not rule out the possibility that the *cai* 10 lesion specifically affects the signalling domain of hexokinase and does not affect hexokinase activity.

Previous studies using glucose analogues had found that hexokinase was a putative sugar sensor (Graham, *et al.*, 1994b, Jang and Sheen, 1994). The role of hexokinase *in vivo* was further investigated using overexpression and antisense technologies (Jang, *et al.*, 1997). Transformants overexpressing hexokinase were hypersensitive to sugars as had been predicted. The prediction was based on the hypothesis that an increased number of sugar sensors would transduce a larger sugar signal which would result in a more severe response. Plants expressing antisense hexokinase 1 were less sensitive to sugars and it was suggested that this was because there were fewer hexokinase enzymes to sense the sugars present (Jang, *et al.*, 1997).

cai 10 was transformed with sense *AtHXK1* in an attempt to reverse the sugar insensitive mutation. Overexpression of hexokinase in *cai* 10 did not make the plants more sensitive to sugars which implied that *cai* 10 does not have a lesion in the hexokinase signalling domain. Moreover, *cai* 10 has recently been mapped to a region at the top of chromosome 1 (Dr F. Regad; postdoc in Dr I. Graham's lab). This region of chromosome 1 has been sequenced and there are no hexokinase genes present on it. Since the two known hexokinase genes in *Arabidopsis* map to chromosome 2 (hexokinase 2) and chromosome 4 (hexokinase 1), it is highly unlikely that *cai* 10 was a hexokinase unless there are other as yet unmapped alleles of hexokinase in the genome.

In this experiment, the controls proved to be very informative. Some col-2 transformants that overexpressed hexokinase 1 displayed a range of sugar resistant phenotypes. All the lines displayed a degree of mannose insensitive growth (*mig*) and a *cai* phenotype. However, hexokinase gene expression and protein activity in the leaf did not correlate well with *mig*, *cai* and *gin* phenotypes in the seedlings. In contrast, the *mig* phenotype in the T4 did correlate with the accumulation of elevated levels of hexose phosphates and sucrose after 8 h and it is during early post germinative growth that the *mig* phenotype appears. Thus it appears that the time point at which hexokinase activity is analysed is crucial. Later in development hexokinase activity and gene expression do not correlate with the *mig* phenotype. However, it must be noted that the phenotypes that I have observed are not what is predicted from the conventional model of hexokinase, since this suggests that hexokinase overexpressors are hypersensitive to sugars.

Remarkably, a fructokinase knockout mutant, *mig* 7 also accumulated an increased amount of hexose phosphates and sucrose. It is interesting to note that both a hexokinase 1 overexpressor and a fructokinase knockout mutant are both mannose resistant and both accumulate metabolites of mannose at the same rate. A possible explanation for this is that the fructokinase mutant compensates for its lack of fructokinase by overexpressing hexokinase. This is in stark contrast to *cai* 10, also a *mig* but which accumulates mannose metabolites to a lesser degree.

Overexpression of hexokinase may cause a *mig* phenotype in several ways: (1) The activity of the hexokinase may be so high that mannose is rapidly phosphorylated to M6P and sucrose. This may enable the seedling to prevent phosphate sequestration because sucrose production releases Pi. (2) If mannose itself is toxic to the seedling, its affect would be alleviated by high hexokinase activity because mannose would be rapidly converted to M6P. (3) High activity of hexokinase in the leaves may have a maternal affect on the phenotype of the seeds by increasing the flux of carbon into sugars in the seeds. An increased sugar content in the seeds could compete with mannose for phosphorylation by hexokinase during germination and thereby prevent phosphate sequestration, a possible cause of mannose toxicity.

Overexpression of hexokinase could cause a *cai* phenotype by rapidly metabolising sugars which build up in seedlings grown in high carbon / low nitrogen conditions, thus preventing the internal sugar concentration from reaching a toxic threshold. Alternatively, high hexokinase activity in the rosette leaves of the overexpressors could lead to an increase of nitrogen storage products in the seeds which would prevent the seedling from becoming nitrogen limited during germination on *cai* conditions.

The final results chapter investigates the above hypotheses and analyses the seed storage contents of *Arabidopsis* overexpressing hexokinase.

Chapter 6

Analysis of the seed storage reserves of *Arabidopsis* transformants overexpressing hexokinase 1

6.1 Introduction

Apart from the constituents found in all plant tissues, seeds contain additional quantities of compounds for use as a source of nutrients to support germination and early seedling growth. These are principally lipids, proteins and carbohydrates but also include minor but important reserves (e.g. phytin, alkaloids, lectins, proteinase inhibitors, raffinose and oligosaccharides). The chemical composition of seeds is ultimately determined by genetic factors and thus varies from species to species. However, some modification of composition may result from growth conditions i.e. light, temperature and addition of fertiliser, but the changes are relatively minor (Bewley and Black, 1994).

A major constituent of *Arabidopsis* seed are the triacylglycerols (TAG) which are esters of glycerol and fatty acids. TAG reserves are laid down in discrete subcellular organelles called oil bodies and range in size from 0.006 to 25 μm (Murphy, *et al.*, 1997). In high TAG-containing seeds, such as *Arabidopsis*, the oil bodies occupy a substantial volume of the cell. The oil bodies are surrounded by oleosins, proteins of approximately 19 KDa in *Arabidopsis* (M. Hill, pers comm.). They are believed to preserve the individual lipid bodies as discrete entities (Huang, 1996).

Arabidopsis contains 2 major classes of seed storage protein, the 12 S (cruciferin) protein and the 2S (arabin) protein (Heath, *et al.*, 1986). Seed storage proteins are usually deposited within specialized organelles called protein bodies which are 0.1-25 μm in diameter and are contained within a single membrane. Inclusions frequently occur in the protein bodies, particularly crystalloids (insoluble proteinaceous inclusions), and globoids (non crystalline sites of deposition of phytin) and occasionally druse (calcium oxalate) (Bewley and Black, 1994).

Free sugars are rarely the main storage carbohydrate in seeds but disaccharides (sucrose) and oligosaccharides (raffinose and stachyose) are often found as minor reserves in the embryo and reserve tissue. There is increasing evidence that they are an important

source of sugars for respiration during germination and early seedling growth (Bewley and Black, 1994). Sucrose, raffinose and stachyose increase in association with the onset of desiccation tolerance. In the desiccation intolerant stage of seed development hexoses (glucose, fructose, mannose and galactose) predominate. ABA mutants (*abi*3-4, *abi*3-5) which have a low or no desiccation tolerance (*abi*3-1, *aba* 1) have relatively high amounts of total sugars (mono- and disaccharides) but have much lower oligosaccharides (raffinose and stachyose) (Bewley and Black, 1994).

In mature seed of oilseed rape, the main storage products are oil and protein (Murphy and Cummins, 1989), but starch accumulates transiently during the early phase of oil deposition (Kang and Rawsthorne, 1994, Kang and Rawsthorne, 1996). *Arabidopsis* is also an oilseed and also accumulates starch transiently during the early phase of oil deposition (Mansfield and Briarty, 1992).

Recently, a wrinkled seed mutant (*wri*1) has been isolated which has a low lipid content and a low seed weight (Focks and Benning, 1998). The *wri*1 mutation maps to the bottom of chromosome 3 and causes an 80 % reduction in seed oil content. The mature plants of *wri*1 and wild type are indistiguishable. However, developing seeds of *wri*1 mutants are impaired in the incorporation of sucrose and glucose into TAG, although pyruvate and acetate were incorporated at an increased rate. The activities of several glycolytic enzymes, including hexokinase and pyrophosphate dependent-phosphofructokinase are reduced in the developing seeds of homozygous *wri*1 mutants and the authors suggested that WRI1 is involved in developmental regulation of carbohydrate metabolism during seed filling. The authors suggested that WRI1 is either a regulatory protein governing carbohydrate metabolism during seed development or a novel hexokinase that may act as a sugar sensor in developing seeds, controlling the activity or expression of other glycolytic enzymes (Focks and Benning, 1998).

A number of other *Arabidopsis* mutants with low seed lipid have also been isolated. The seeds of *fus3*, *lec1* and *tag1* do not accumulate TAG to the same extent as the wild type (Bäumlein, *et al.*, 1994, Meinke, *et al.*, 1994, Katavic, *et al.*, 1995).

While the work in this chapter was being carried out, evidence was published which corroborated our work. The overexpression of glucokinase and invertase in potato tubers lead to stimulation of glycolysis and a dramatic reduction in starch biosynthesis (Trethewey, *et al.*, 1998). In addition, the tubers contained an increase in metabolic intermediates, organic acids and amino acids. The tubers also had a 2-3 fold increase in maximum catalytic activities of several key respiratory enzymes and a 3-5 fold increase in carbon dioxide production. Parallels will be drawn between the evidence presented in this chapter and this work in the discussion.

6.1.1 Rationale

One of the most exciting discoveries about the hexokinase overexpressing transformants was that they appear to germinate extremely quickly, the first cotyledons emerging after only 18h-20h in the growth room (Figure 6.1) compared to wild type which usually takes 2-3 days to germinate. This observation led to several experiments aimed at determining the factor that caused the rapid germination. There are several hypotheses that could explain the rapid germination observed in the hexokinase overexpressors: (1) Seed lipid reserves are mobilised more rapidly than wild type due to an increase in flux through ß-oxidation or gluconeogenesis which could be the result of high hexokinase activity causing a depletion of hexoses and induction of *ms* and *icl*; (2) the metabolic rate of the seed is increased due to increased glycolytic flux; (3) the seed storage reserves have a different composition, i.e. high elevated levels of compounds which can be mobilised more rapidly than the reserves in the wild type. Seed storage compounds could be increased or altered because high hexokinase activity in the rosette leaves of the parent could perturb the sink strength of the seed or modify carbon / nitrogen allocation during seed development.

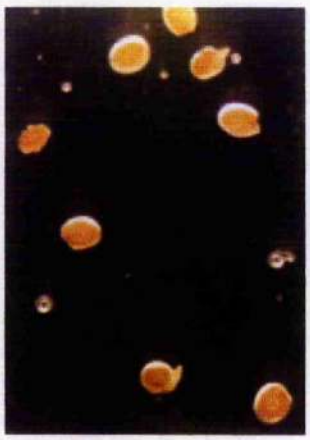
6.2 Results

6.2.1 Lipid Analysis

In order to assess whether the rate of TAG breakdown was greater in the hexokinase 1 overexpressors seeds were germinated on 1/2 strength M&S agar medium

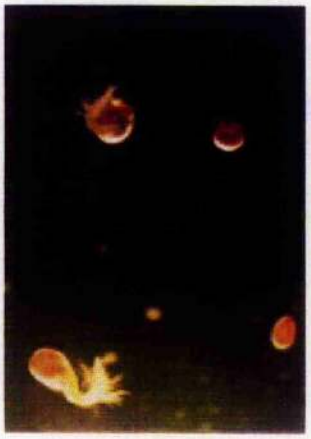
Figure 6.1 Rapid germination of hexokinase overexpressors.

Seeds of the hexokinase overexpressors were sown on 1/2 strength M&S agar medium containing no exogenous carbon source. The plates were imbibed for 4 days at 4 °C. The plates were transferred to growth room conditions (22 °C, 120 $\mu Em^{-2}s^{-1}$) for 20 hours after which time they were assessed.

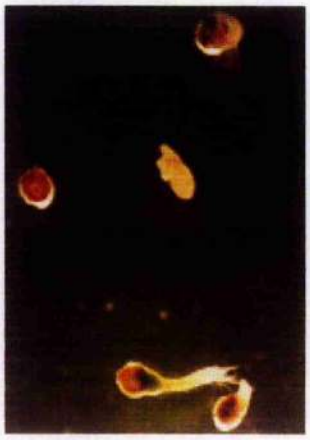


col X hxk-3

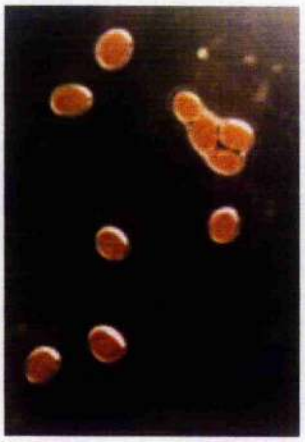
col X hxk-6



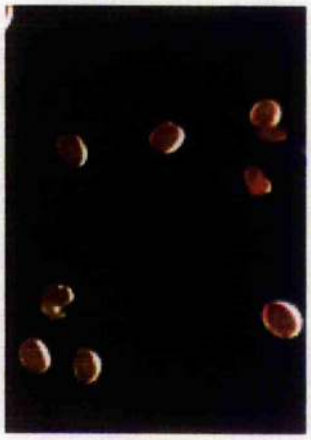
col X hxk-7



col X hxk-14



col-2



***cai* 10**

1 mm

containing no exogenous sugar for 1-4 days. Fifty germinating seedlings were harvested per sample and the lipid was extracted and separated by TLC (Figure 6.2). The TLC shows that the hexokinase overexpressors do not break down their storage triacyl glycerols (TAG) more rapidly than the wild type. The seeds transformed with sense hexokinase 1 contain decreased TAG content, the value ranging between 34 and 82 % of the amount in wild type seeds (Figure 6.3a and b). However, the seeds of lines 5, 9 and 11 which expressed low amounts of hexokinase RNA in leaves also showed a decrease in TAG. It is therefore possible kanamycin resistance causes a decrease in lipid. Further experimentation is required to ascertain the actual cause of low TAG in the seeds. The total lipid content of the seeds was analysed by GC (performed by Dr T. Larson, a postdoc in Dr I Graham's lab;Figure 6.4). The GC data corroborated the TLC evidence that lipid content is lower in the overexpressors. GC analysis was also used to establish whether a decrease in flux into seed storage lipids could alter the fatty acid composition as a consequence of enzymes in fatty acid biosynthesis having differential activity dependent on substrate concentration. A decreased flux of carbon into seed lipid storage could cause an alteration in the fatty acid composition. However, the fatty acid composition of the seed lipid is not significantly different from the wild type (Figure 6.4).

6.2.2 Seed dry weight

The dry weight of seeds from each line was measured using a fine balance to determine whether the decrease in lipid content was a consequence of lower seed mass. Figure 6.5 shows that, in general, the seed mass of the overexpressors is lower than the wild type. This could be significant as smaller seeds would have proportionally less lipid. However, the hexokinase overexpressors did germinate faster than the wild type so they had to have some rapidly mobilizable reserve.

6.2.3 Seed carbohydrate analysis

The seeds of the hexokinase 1 overexpressors appeared to be slightly wrinkled (Figure 6.6) which indicated that the seeds might contain a compound with high osmotic

Figure 6.2 Fast germination of the hexokinase overexpressors is not a result of rapid triacylglycerol (TAG) breakdown.

Seeds of the hexokinase overexpressors were sown on 1/2 strength M&S agar medium containing no exogenous carbon source. The plates were imbibed for 4 days at 4 °C. The plates were transferred to growth room conditions (22 °C, 120 $\mu Em^{-2}s^{-1}$) and germinating seeds were harvested at time intervals. Lipids were extracted from 25 seedlings of each line and the lipid and TAG content were analysed by TLC. C = TAG standard.

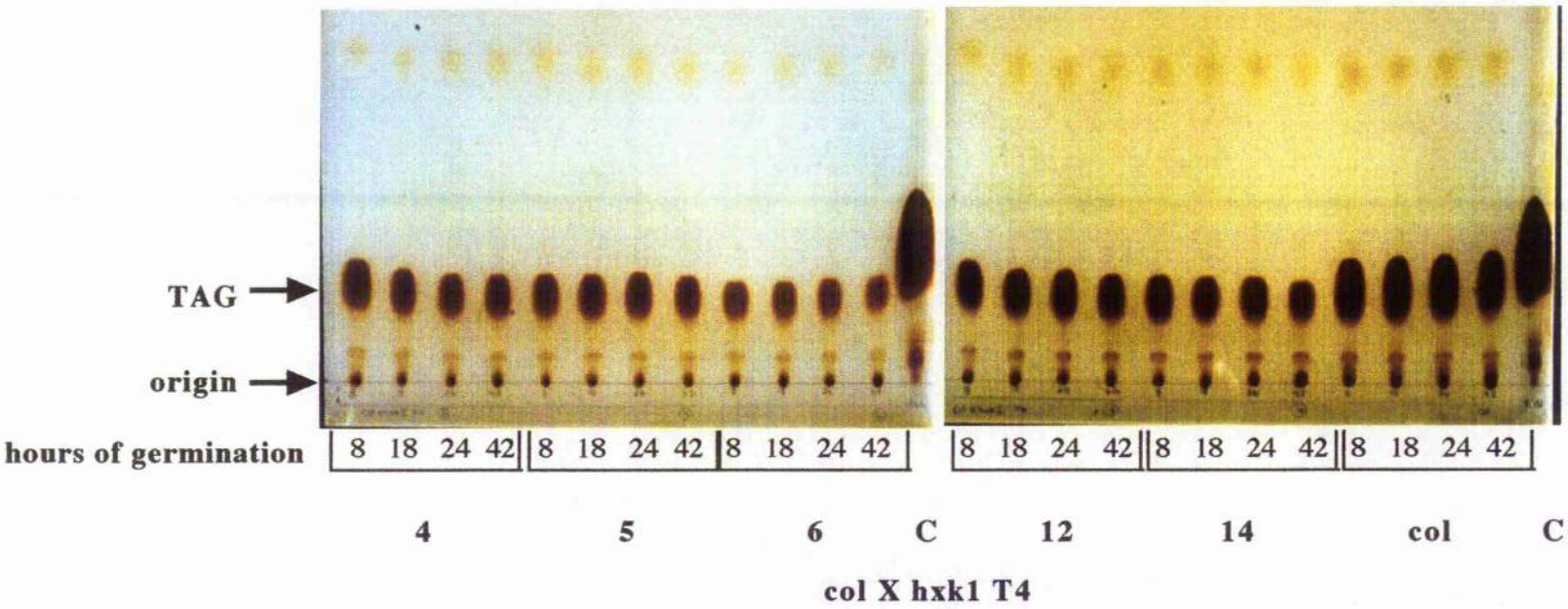
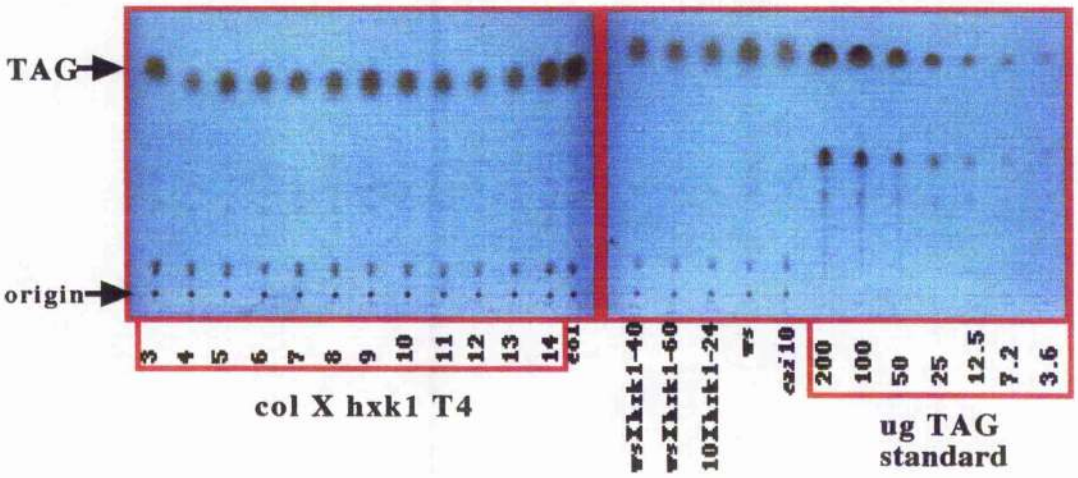

TAG
origin
hours of germination
8 18 24 42 8 18 24 42 8 18 24 42 8 18 24 42 8 18 24 42 8 18 24 42
4 5 6 C 12 14 col C
col X hxk1 T4

Figure 6.3 Hexokinase overexpressors contain a reduced amount of TAG in their seeds.

Lipids were extracted from 10 dry seeds of each line and the lipid and TAG content were analysed by TLC (a). The TAG content was quantified using standard curve (b).

a


TAG
origin
3
4
5
6
7
8
9
10
11
12
13
14
col
col X hxk1 T4
ws
ws X hxk1-40
ws X hxk1-60
10X hxk1-24
cai 10
200
100
50
25
12.5
7.2
3.6
ug TAG standard

b

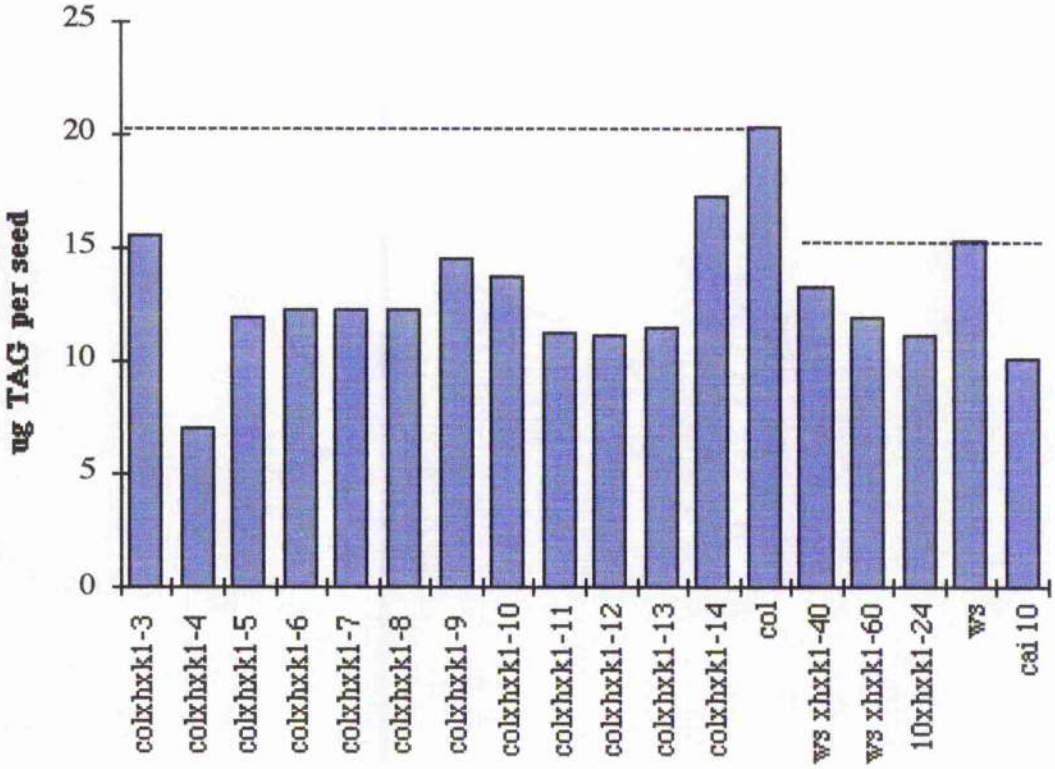

ug TAG per seed
25
20
15
10
5
0
colxhxk1-3
colxhxk1-4
colxhxk1-5
colxhxk1-6
colxhxk1-7
colxhxk1-8
colxhxk1-9
colxhxk1-10
colxhxk1-11
colxhxk1-12
colxhxk1-13
colxhxk1-14
col
ws xhxk1-40
ws xhxk1-60
10xhxk1-24
ws
cai 10

Figure 6.4 GC Analysis of the fatty acid content of the hexokinase overexpressors.

The fatty acids were extracted from dry seeds and were separated and quantified by GC. The purple bars represent the individual fatty acids and the pink bars represent the total fatty acid content. uncal equals the unidentified fatty acids in the seeds.

col-2

col X hxk-3

col X hxk-4

col X hxk-6

col X hxk-7

col X hxk-14

Figure 6.5 The dry weight of the seeds of the hexokinase overexpressors is generally less than the wild type.

Three batches of fifty seeds from a single T4 plant were counted and weighed. The seed were all from plants which had been grown at 120 μEm-2s-1 at 22°C in 16 h light and 8 h dark.

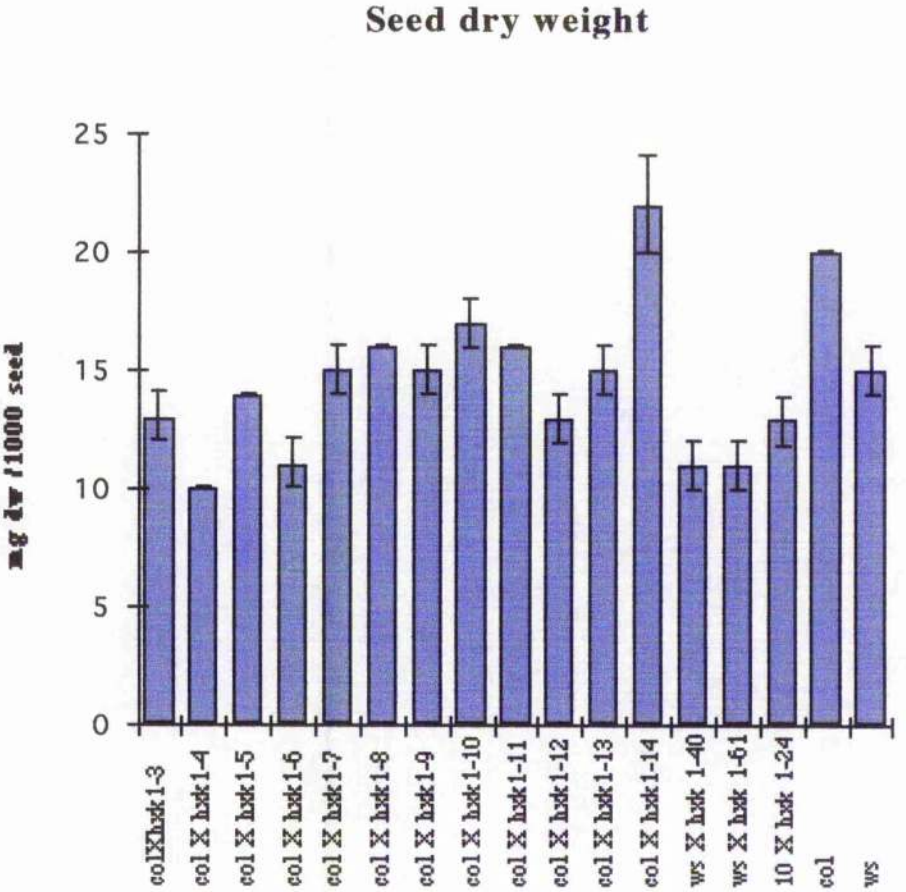

Seed dry weight
mg dw /1000 seed
25
20
15
10
5
0
col X hxk1-3
col X hxk1-4
col X hxk1-5
col X hxk1-6
col X hxk1-7
col X hxk1-8
col X hxk1-9
col X hxk1-10
col X hxk1-11
col X hxk1-12
col X hxk1-13
col X hxk1-14
ws X hxk 1-40
ws X hxk 1-61
10 X hxk 1-24
col
ws

Figure 6.6 Seed phenotype of the hexokinase overexpressors.

Seeds were observed with a binocular microscope using dark field illumination.

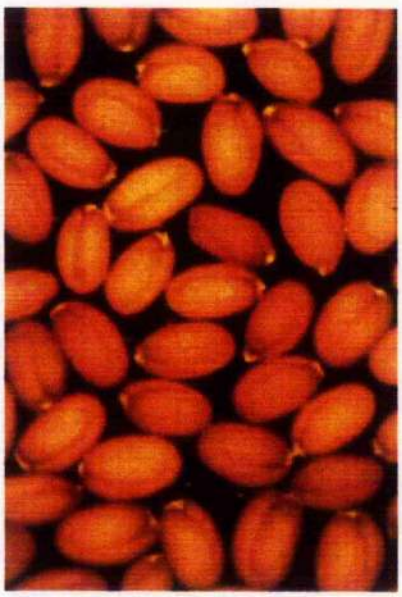
col X hxk 4

col X hxk 6

col X hxk 14

0.5 mm

col X hxk 9



col-2

strength. Seed extracts were assayed for sucrose, glucose and fructose content but levels of all 3 metabolites in the overexpressing lines were similar to the wild type on a per seed basis (Figure 6.7a). However, when measured on the basis of mg sugars per g dry weight, the hexokinase overexpressors contained more sucrose than the wild type (Figure 6.7b). This indicates that a larger proportion of carbon is allocated to sugars in the hexokinase overexpressors than in wild type and may account for the slightly wrinkled appearance of the seeds. Soluble sugars were separated by paper chromatography and stained with silver nitrate. Figure 6.8 shows that the profile of sugars in the wild type and the hexokinase overexpressors are slightly different. For instance, the hexokinase overexpressors have more of the compound that runs between *myo*inositol and trehalose. GC analysis of the sugars will be necessary to clarify the differences between the wild type and the hexokinase overexpressors.

Iodine staining of seeds established that both wild type and overexpressors contain undetectable amounts of starch.

6.2.4 Seed amino acid analysis

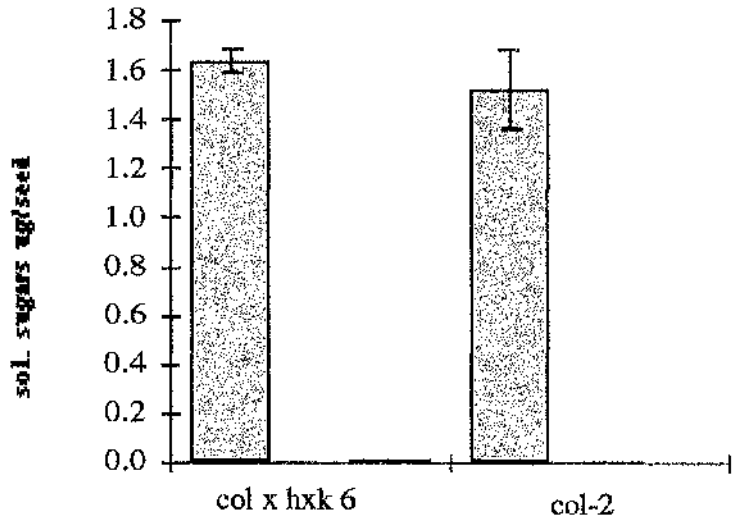
Ethanol extracts of seeds were separated by paper chromatography and were stained with ninhydrin. The results indicated that there was an increase in amino acids in seeds of plants overexpressing hexokinase 1 (Figure 6.9). The amino acids were analysed in detail by HPLC by Dr M. Cusack; Molecular Palaeontology, Glasgow University. The amino acid concentrations of aspartate, asparagine, glutamine, serine, histidine and arginine are much higher in the hexokinase overexpressors than in col-2 (Figure 6.10). In addition to this there is a huge peak which runs at a retention time slightly shorter than serine which appears in lines 4, 6 and 14 but which is not in col-2. It is not possible to discern whether this compound is serine or a different compound. The structure of this compound needs to be resolved by LC-MS.

Figure 6.7 The soluble sugar content of seeds of the hexokinase overexpressors.

(a) μg soluble sugars per seed

(b) mg soluble sugars per g dry weight of seed.

a


sol. sugars ug/seed
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
col x hxk 6
col-2

b

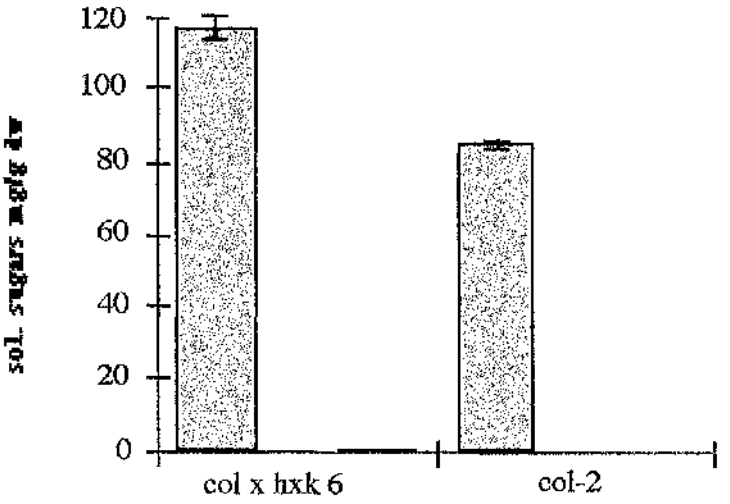

sol. sugars mg/g dw
120
100
80
60
40
20
0
col x hxk 6
col-2

Figure 6.8 Analysis of the seed sugar content of the hexokinase overexpressors using paper chromatography.

The samples were separated by paper chromatography in ethyl acetate :pyridine : water (8 :2 :1) for 18 hours. The sugars on the paper chromatogram were stained with silver nitrate.

1 = fructose
2 = xylose
3 = glucose
4 = fucose
5 = arabinose
6 = galactose
7 = mannose
8 = trehalose
9 = myoinositol
10 = xylitol
11 = galactitol
12 = sucrose

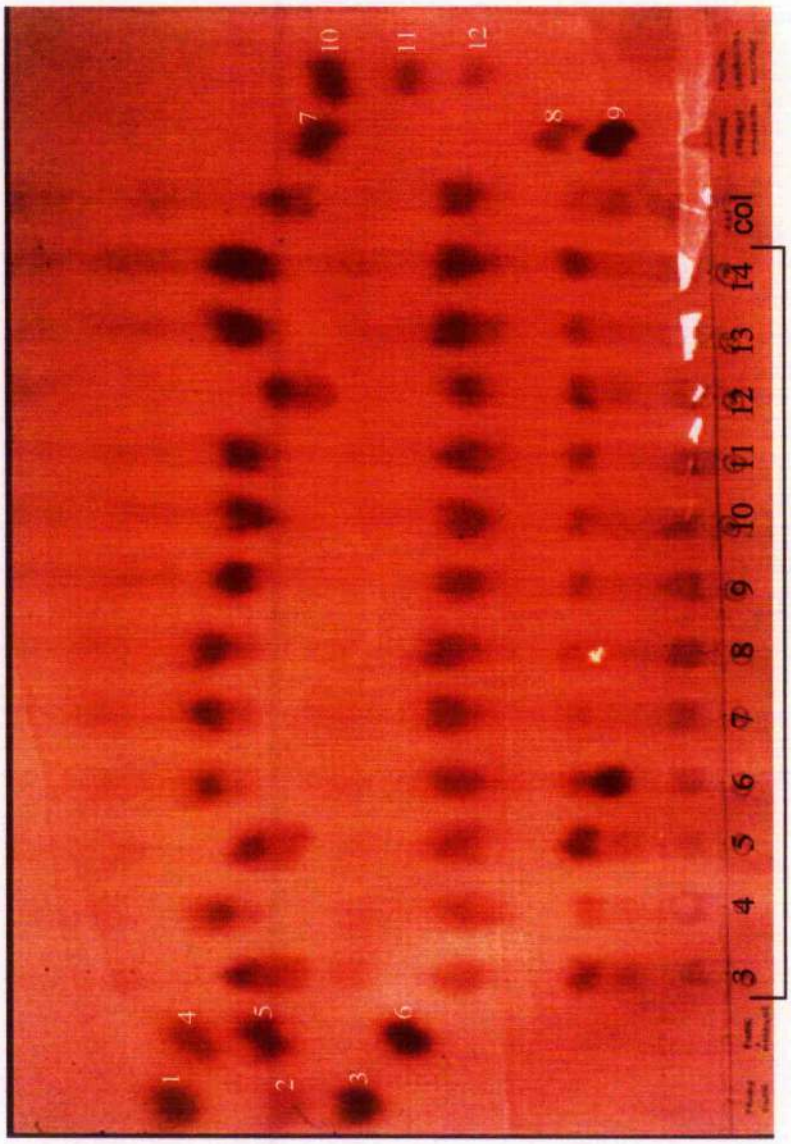

1
4
5
2
3
6
7
10
11
12
8
9
3
4
5
6
7
8
9
10
11
12
13
14
col
col x hxk1

Figure 6.9 Paper chromatographic analysis indicates that seeds of a hexokinase overexpressor contain an elevated level of amino acids.

Amino acids were extracted from seeds and were separated by paper chromatography (butan-1-ol :acetic acid: water ; 120:30:50) for 18 hours. The paper chromatogram was then stained with 0.5 % (w/v) ninhydrin solution.

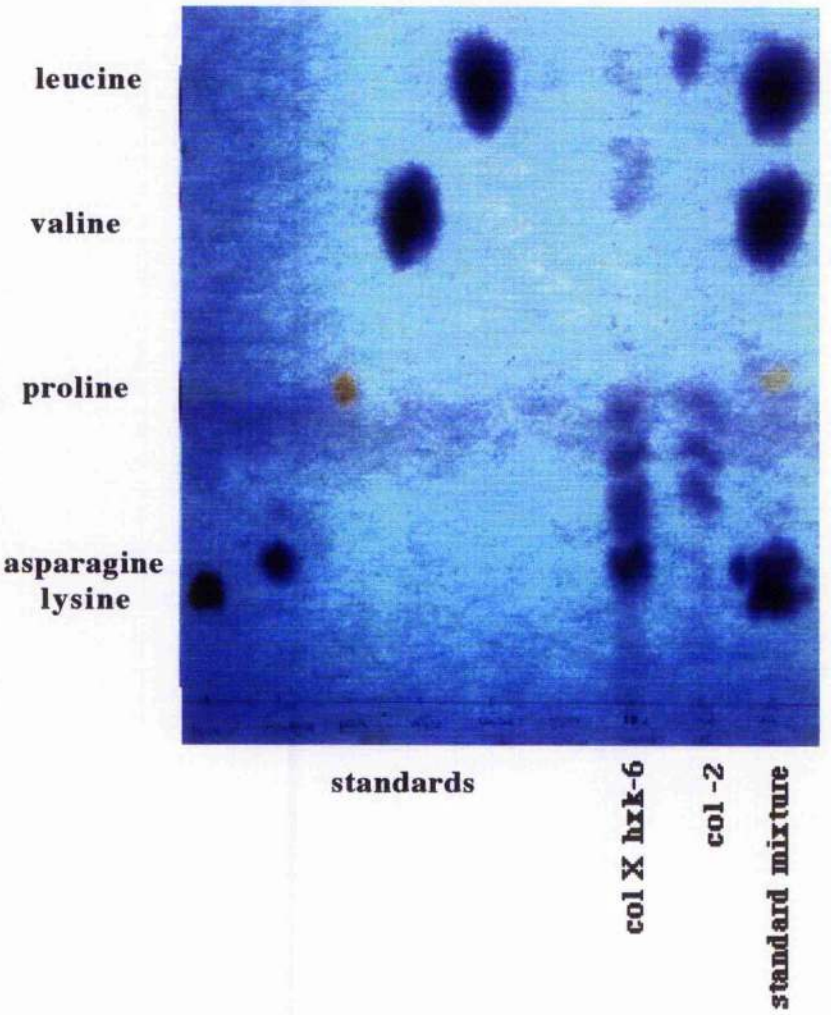
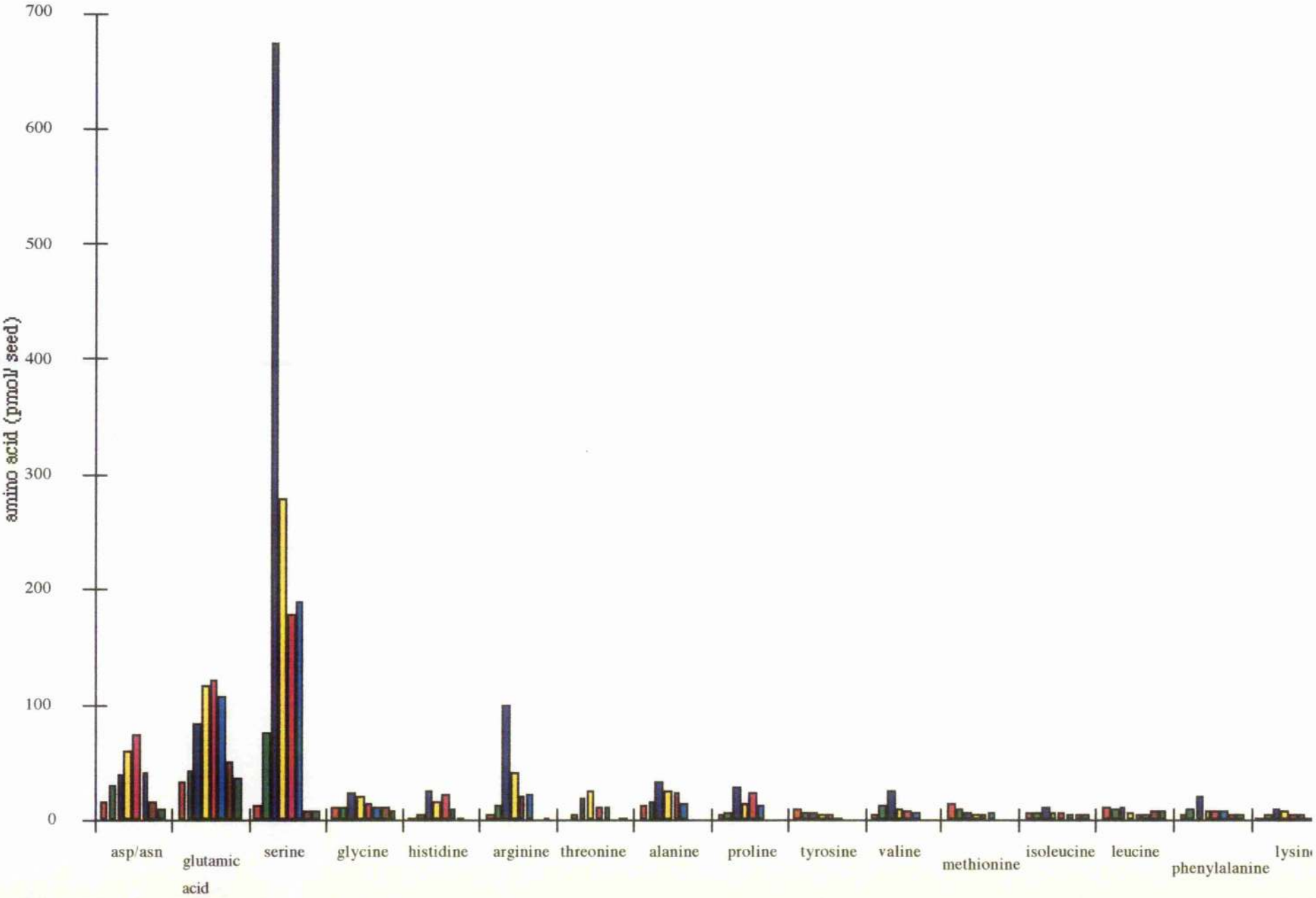

leucine
valine
proline
asparagine
lysine
standards
col X hzk-6
col -2
standard mixture

Figure 6.10 HPLC analysis indicates that seeds of the hexokinase overexpressors contain elevated levels of amino acids.

Amino acids were extracted from seeds and were analysed by HPLC.


amino acid (pmol/ seed)
700
600
500
400
300
200
100
0
asp/asn
glutamic acid
serine
glycine
histidine
arginine
threonine
alanine
proline
tyrosine
valine
methionine
isoleucine
leucine
phenylalanine
lysin

6.2.5 Storage protein analysis

Storage proteins were extracted from seeds of the overexpressors. The protein concentration was measured using the Lowry method and 20 µg of each sample was separated by SDS-PAGE on a 12.5 % acrylamide gel (Figure 6.11). Oleosin, which has a size of 19 KDa is present in approximately equal amounts in both col-2 and overexpressors. There are no striking differences in storage protein composition between the wild type and the transformants.

6.2.6 Electron microscopy of dry seeds

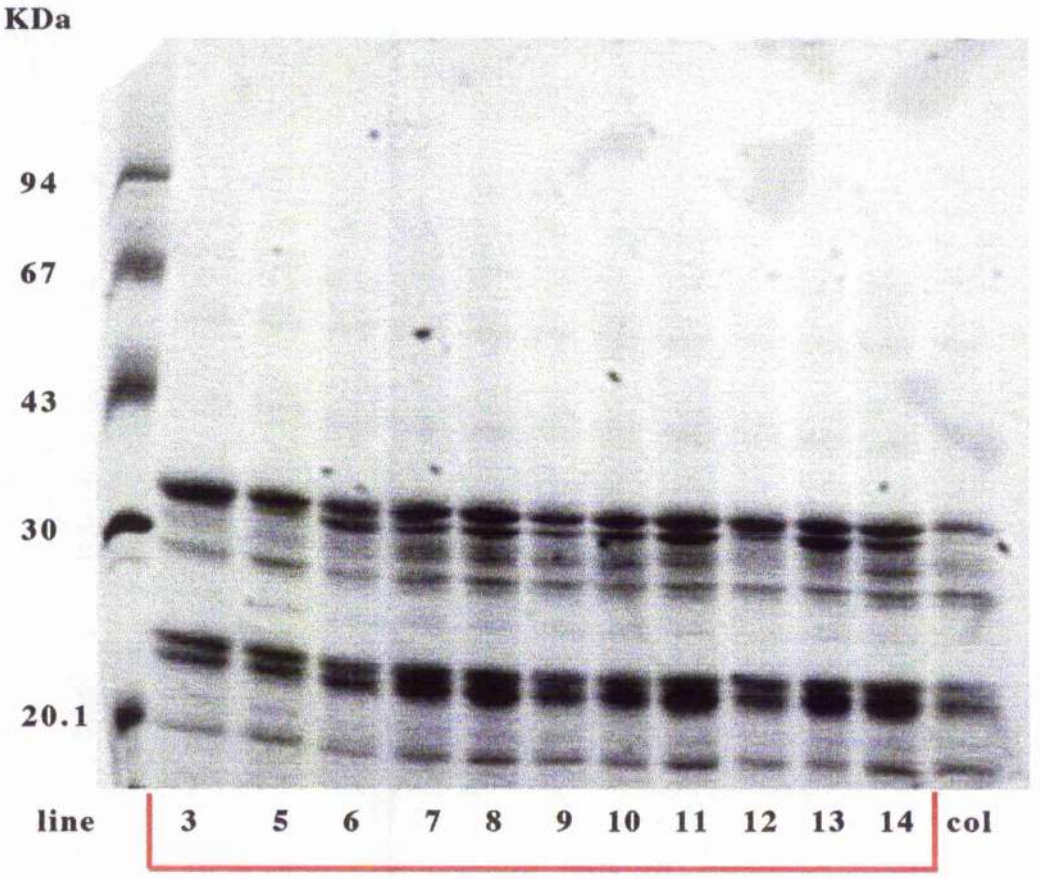
Electron microscopy of the dry seeds revealed that the histology of sense-hexokinase 1 transformants is very different to col-2 (Figure 6.12). The oil bodies in col-2 and ws are large and coalesced, filling the cells. Electron micrographs of the transformants show that the oil bodies are smaller than the wild type and there are about 4 times more of them. The overall size of the cells also appears to be smaller in the overexpressors than the wild type. It must be noted that line 9 which expressed low amounts of hexokinase 1 in leaves but which had high hexokinase activity in germinating seedlings also shows the small oil body phenotype. The lack of correlation between hexokinase expression in leaves and the phenotype of the transformant at other stages in development has been observed repeatedly. This may be a consequence of differential expression of the 35S promoter during different stages of development.

6.3 Discussion

Seeds of transformants overexpressing hexokinase 1 germinated more rapidly than wild type seeds. Extraction of TAG over the germination period showed that TAG reserves in hexokinase overexpressors were not depleted more rapidly than wild type reserves. This implied that the rate of ß-oxidation and gluconeogenesis was the same in both wild type and transformants. The hexokinase overexpressors did, however, have a lower TAG and fatty acid content per seed. Although there was less lipid in the hexokinase overexpressors than the wild type, the proportion of each fatty acid remained the same except for line 4 and 14

Figure 6.11 The storage proteins in seeds of the hexokinase overexpressors are qualitatively similar to the wild type.

Total protein was extracted from the seeds of the hexokinase overexpressors. 20 μg of each sample was separated by SDS-PAGE on a 12.5 % acrylamide gel. The proteins were stained with Coomassie brilliant blue.



col X hxk1 T4

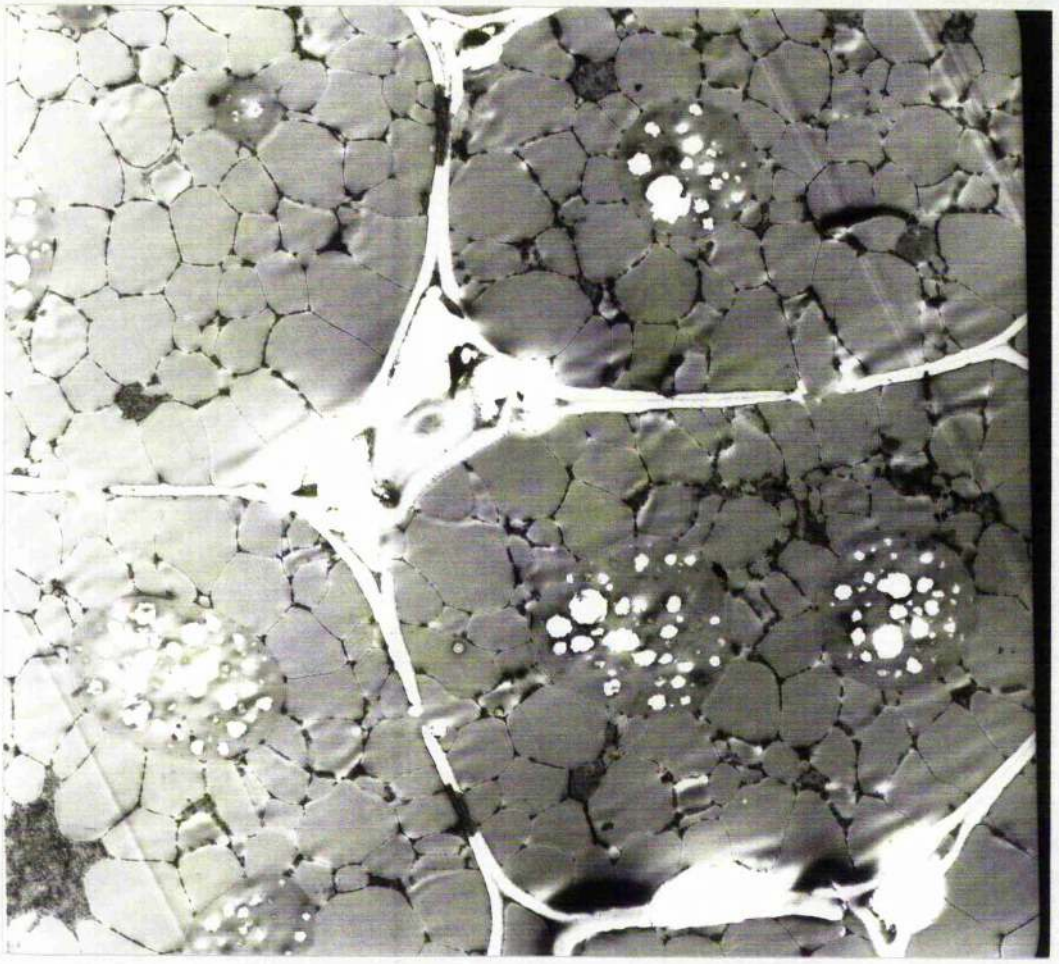
Figure 6.12 Electron micrographs of seeds of the hexokinase overexpressors reveal numerous small lipid bodies.

Magnification x 6000

(a) col-2

(b) col X hxk -4

a



b

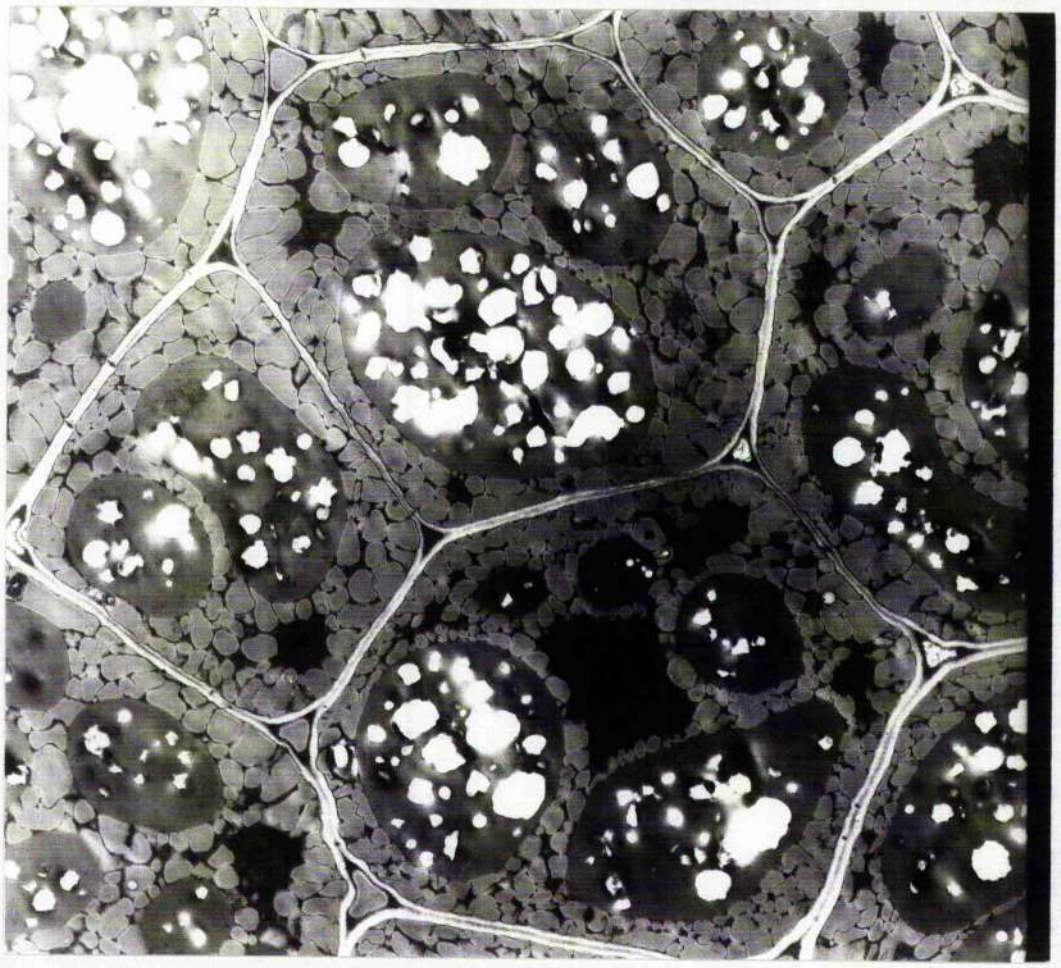


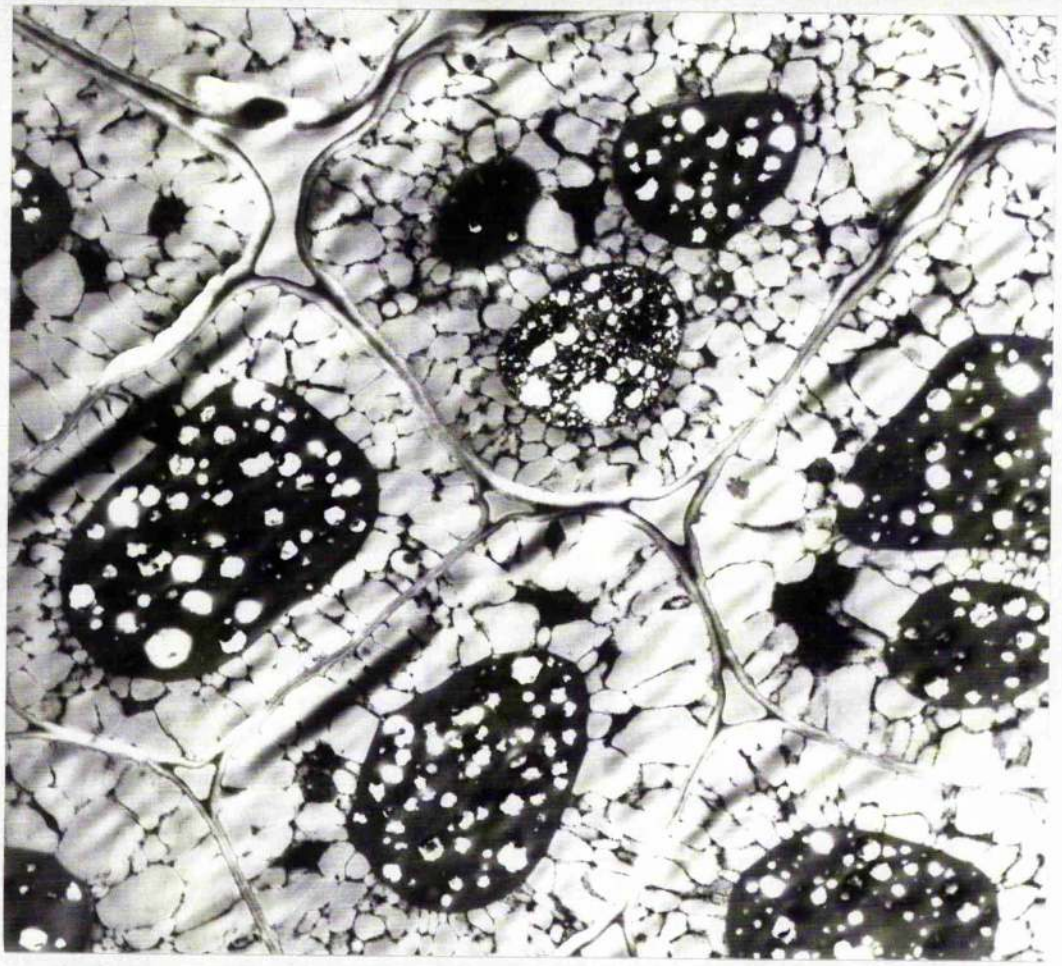
Figure 6.12 Electron micrographs of seeds of the hexokinase overexpressors reveal numerous small lipid bodies.

Magnification x 6000

(c) col X hxk -6

(d) col X hxk -14

c



d

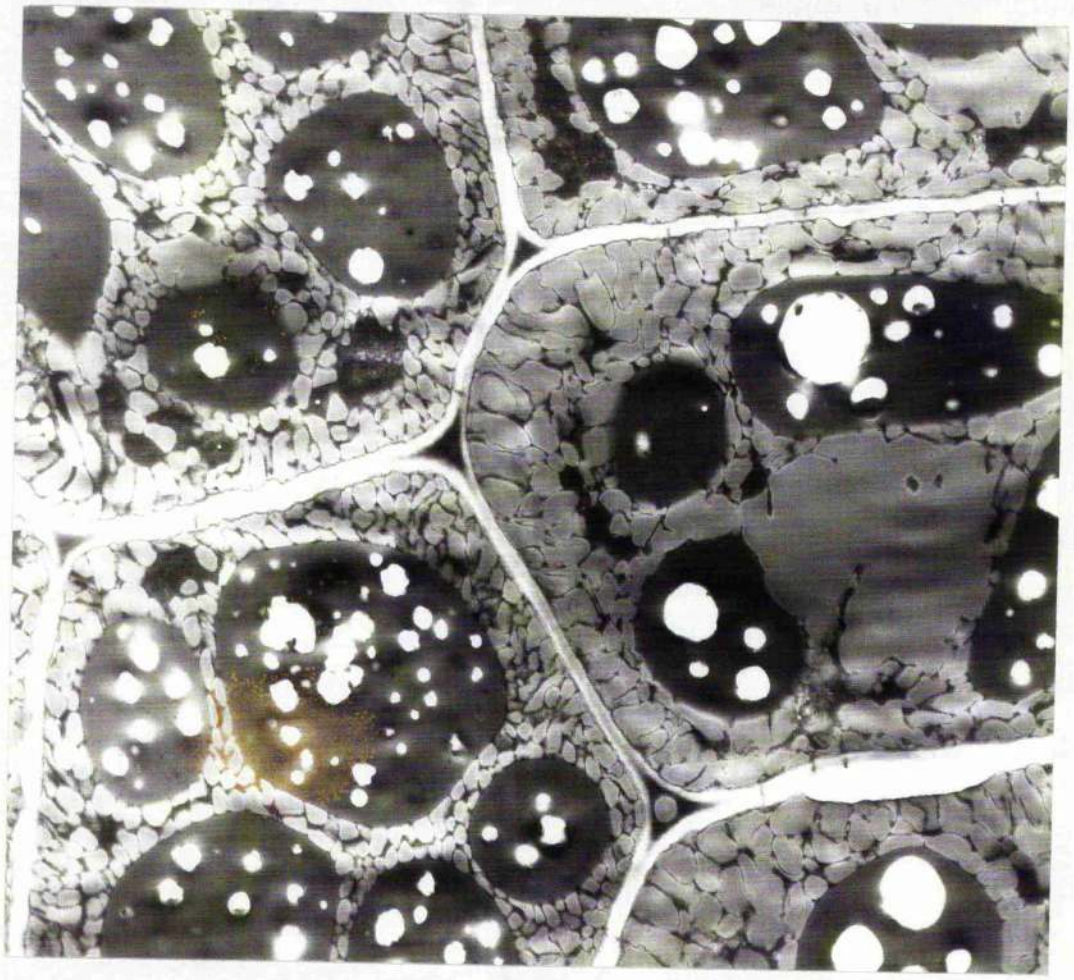


Figure 6.12 Electron micrographs of seeds of the hexokinase overexpressors reveal numerous small lipid bodies.

Magnification x 6000

(e) col X hxk -9 (low expressor control)

e

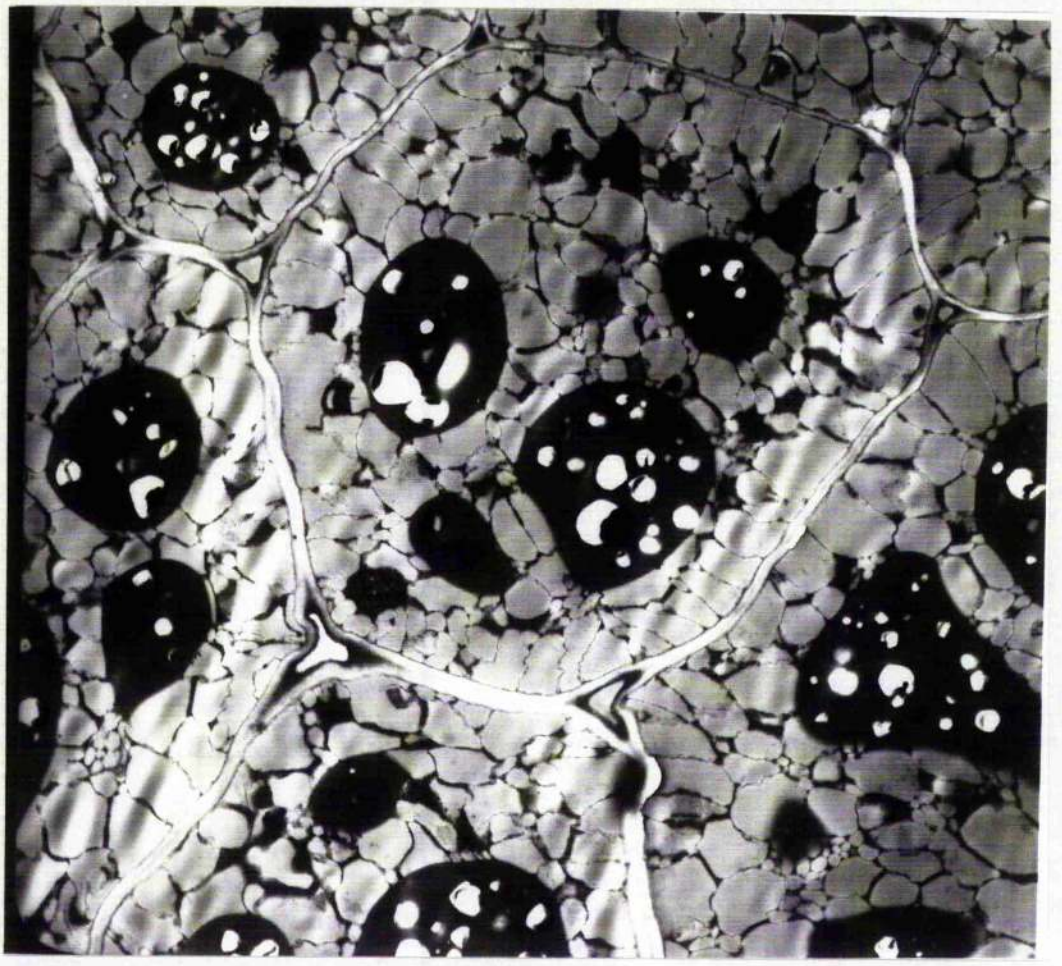


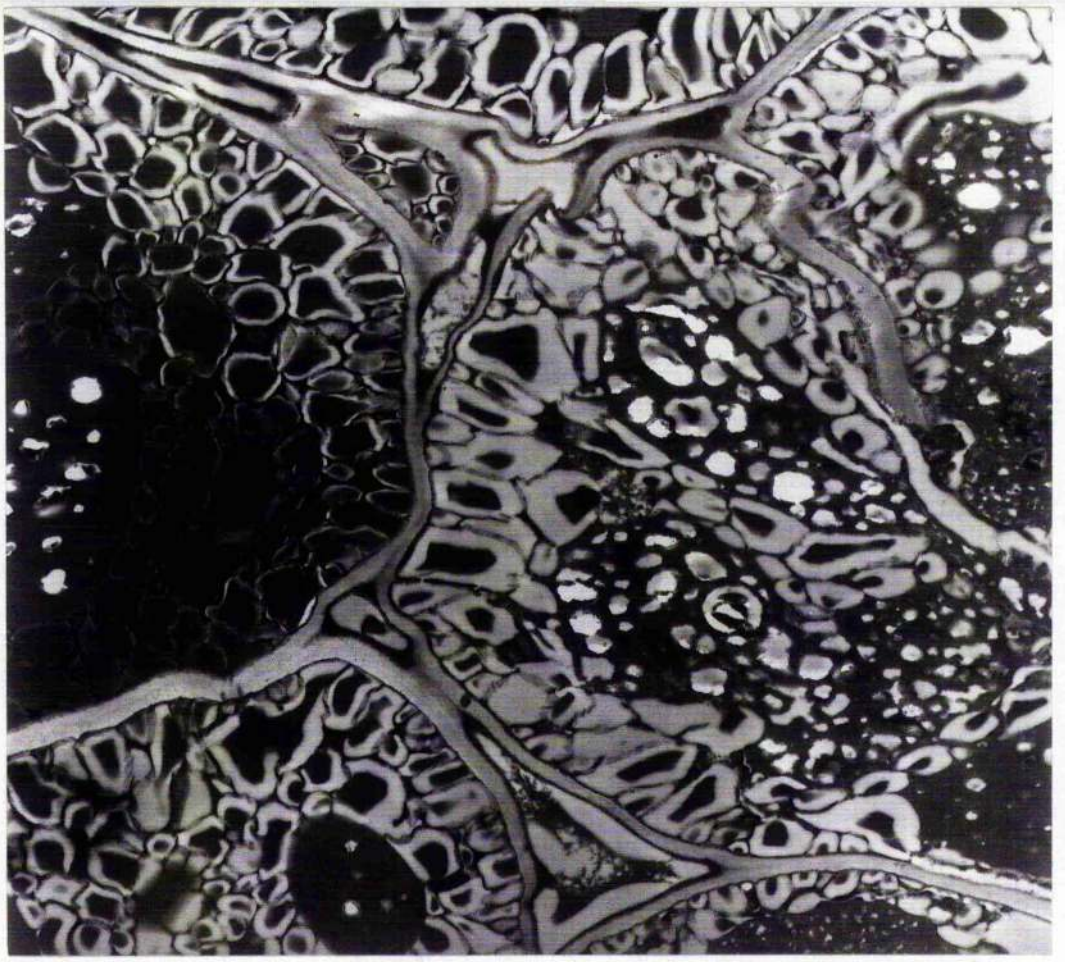
Figure 6.12 Electron micrographs of seeds of the hexokinase overexpressors reveal numerous small lipid bodies.

Magnification x 6000

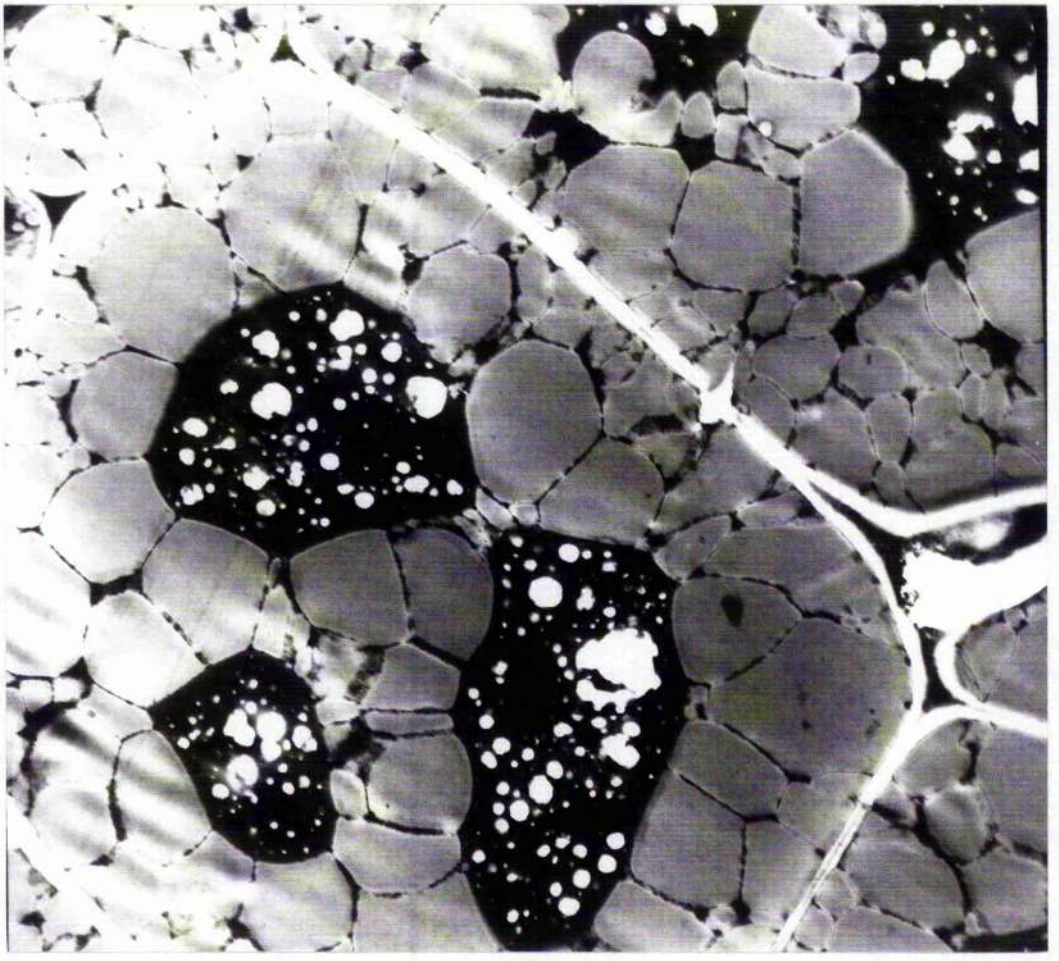
(f) *cai* 10

(g) ws

f



g



which had less 18:1 relative to other fatty acids. The dry weight of the transformant seeds was generally lower than the wild type, except for line 14, so it was not surprising that they contained less lipid. On a per seed basis there was a similar amount of sucrose, glucose and fructose in the wild type and transformants. Thus, the sucrose content of the hexokinase seeds is not the storage resource which causes rapid germination. Moreover, starch is not the storage compound enabling rapid germination because neither wild type or hexokinase overexpressors contain a detectable amount.

The total soluble sugars in seeds were qualitatively analysed by paper chromatography and there were some differences between wild type and hexokinase overexpressors. In particular, the hexokinase overexpressors appear to contain more of a compound which has a similar Rf value to trehalose.

Trehalose is synthesized from G6P and UDP-glucose in a two-step process, analogous to sucrose synthesis, by trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase(Cabib and Leloir, 1958) but until recently was thought to be absent from higher plants. In yeast and other micro-organisms it has an established role in the protection of various stress factors such as heat and desiccation (Vogel, *et al.*, 1998). Several groups have transformed tobacco with microbial genes for trehalose synthesis, in the hope of increasing the plants' stress tolerance (Gaff, 1996, Holmström, *et al.*, 1996, Romero, *et al.*, 1997). Some transformed plants exhibited slight drought tolerance but the expression of microbial genes for trehalose synthesis always gave rise to severe growth defects, such as dwarfism, although only traces of trehalose were found. This gave hints that trehalose or related metabolites might have a function as regulators of plant growth and development. Furthermore, in yeast, trehalose seems not only to be involved in stress protection, but its precursor, possibly T6P, appears to be an important regulator of glucose influx and metabolism (Thevelein and Hohmann, 1995). Moreover, results indicate that trehalose may interfere with sugar-regulated gene expression as application of trehalose to roots of soy bean induces sucrose synthase expression (Müller, *et al.*, 1998).

The hexokinase overexpressors could be expected to contain a steady supply of G6P which could be diverted into trehalose synthesis. If, by chance, the unknown

compound was trehalose, it could have some serious ramifications for the plants. Trehalose may be regulating glucose influx and metabolism, as in yeast, possibly resulting in the mannose tolerant phenotype. Opposing this speculation is the fact that the hexokinase overexpressors do not show any consistent growth defects, a trait seen in all trehalose overexpressing tobacco plants. The seed extracts need to be analysed by GC-MS to establish the identity of the sugars present in the hexokinase 1 overexpressors. It would also be interesting to study the expression of sugar regulated genes in the transformants and compare them to the wild type. If there is an abundance of trehalose, the transformants might be expected to express high levels of sucrose synthase.

Three metabolites of glycolysis and the citric acid cycle serve as the major withdrawal points for inorganic carbon in the syntheses of the amino acids; pyruvate, oxaloacetate and α-ketoglutarate. Since phosphoenolpyruvate (PEP) is dephosphorylated to pyruvate (by pyruvate kinase) and is carboxylated to form oxaloacetate (by PEP carboxylase) (Andreo, *et al.*, 1987), it is a very important compound for both lipid and amino acid biosynthesis. Oxaloacetate is a carbon donor for amino acid synthesis and pyruvate is a substrate for both amino acid and fatty acid biosynthesis (Kang and Rawsthorne, 1994). In the hexokinase overexpressors it would appear that the allocation of carbon has been diverted from lipid synthesis into amino acid production. The results suggest that more of the PEP is being carboxylated by PEP carboxylase and less is being dephosphorylated by pyruvate kinase to pyruvate.

The demand for carbon skeletons for amino acid synthesis varies according to many factors - age, time of day, stress, nutrient conditions. When nitrogen deficient plants are transferred to a nitrogen-rich environment the rate of amino acid synthesis increases. Concomitantly, there is an increase in cellular respiration because of the demand placed on respiratory pathways to supply carbon skeletons needed for the incorporation of nitrogen into organic form. The consumption of α-ketoglutarate by the nitrogen assimilating activities of GS/GOGAT cycle causes a depletion of citric acid cycle intermediates. The plant's response to this is to increase the activities of glycolysis, and PEP carboxylase which is thought to have the anapleurotic role of replenishing oxaloacetate to the citric acid cycle.

The increases in PEP carboxylase and glycolysis may be coordinated because G6P (an glycolytic intermediate), is known to activate PEP carboxylase.

The conditions for high amino acid biosynthesis may be being mimicked in the hexokinase overexpressors as follows : (1) the high hexokinase activity may stimulate glycolysis in the adult plant and provide a large pool of carbon skeletons which can be transported to the developing seeds (sink) and converted into the major reserves, lipids and amino acids. (2) As a result of high hexokinase activity and rapid phosphorylation of glucose, the plants have an elevated concentration of G6P. The G6P allosterically activates PEP carboxylase which can now effectively compete against pyruvate kinase for PEP. This would divert PEP into oxaloacetate synthesis in preference to pyruvate synthesis and could result in an increase in amino acid synthesis and a decrease in lipid biosynthesis. A future experiment would be to feed ^{14}C PEP to the developing embryos of the hexokinase overexpressors and monitor the proportion of label that went into oxaloacetate and pyruvate, and compare this with the wild type.

G6P is also a good substrate for lipid synthesis. In experiments supplying ^{14}C-labelled compounds to plastids of developing oilseed rape embryos, G6P, pyruvate, DHAP, malate and acetate were all found to be good substrates for fatty acid synthesis (Kang and Rawsthorne, 1994, Kang and Rawsthorne, 1996). However, amino acids were not measured in these experiments so it is not possible to ascertain whether the allocation of carbon to lipids and amino acids in the plastids was altered.

Aspartic acid/ asparagine, glutamic acid and a compound which may be serine were extremely elevated in the hexokinase overexpressing transformants. These 4 amino acids are all synthesized as primary products of ammonia assimilation, and then serve as precursors and amino donors in the synthesis of other amino acids (Ireland, 1997). Glutamate is the net product of the GS/GOGAT cycle and aspartate, asparagine, and serine are all products of its metabolism. Aspartate and asparagine are synthesized by the transamination of oxaloacetate by glutamate or alanine. Serine can be synthesized by 3 routes. It is a product of photorespiration : in this process, glycolate is oxidised to glyoxylate, which is transaminated to glycine. Then 2 molecules of glycine are converted to

a single molecule of serine. Serine can also be synthesized by 2 routes not involving glycine. Both of these pathways use phosphoglycerate, derived from the Calvin cycle or glycolysis, as a starting point. In the first, phosphoglycerate is converted to glycerate and then to hydroxypyruvate, which can be transaminated to serine. In the second, phosphoglycerate is converted to serine via phosphohydroxypyruvate and phosphoserine (Ireland, 1997). Excessive photorespiration could be the cause of the high 'serine' concentration but would not account for the elevated levels of asparagine, aspartate and glutamate because serine is not easily converted to these amino acids (deduction from amino acid synthesis pathways (Ireland, 1997). Furthermore, it is difficult to see how high hexokinase activity could lead to an increase in the oxygenase activity of RUBISCO. A more likely explanation, perhaps, is that high hexokinase activity increases the flux of carbon through glycolysis. Hexose phosphates are converted to F16BP. F16BP is converted to 3-phosphoglyceraldehyde, followed by 1, 3-bisphosphoglycerate and 3-phosphoglycerate. 3-phosphoglycerate is metabolised via 2-phosphoglycerate to PEP. Carbon can be diverted via phosphoglycerate to serine and also via PEP and oxaloacetate to aspartic acid, asparagine and glutamate.

In order to test this hypothesis the concentration of G6P and the activities of PEP carboxylase and other glycolytic enzymes should be measured. Hexokinase overexpressing transformants might also be expected to have an increase in sucrose phosphate synthase activity because this enzyme is activated by G6P and inhibited by Pi (Stitt, *et al.*, 1988), conditions which might prevail in these plants. This might also account for the slight increase of sucrose in the hexokinase overexpressors per mg of dry seed.

Interestingly, the amino acids which are elevated in the transgenic seeds are all gluconeogenic amino acids, and as such, can be converted to sucrose during germination (Bewley and Black, 1994). Therefore, the factor which enables the rapid germination of the hexokinase overexpressing seeds could be the pool of free amino acids. The rate of gluconeogenesis of these amino acids could be measured by supplying germinating seeds with ^{14}C-labelled serine, glutamine, aspartate and asparagine and monitoring the concentration of ^{14}C-labelled sucrose and its metabolites during germination.

Another possibility is that the compound which has been nominally called serine is actually a different compound which is masking the actual serine peak on the HPLC trace. Analysis of the compound by LC-MS will be necessary to confirm the identity of this compound.

Work by various groups have shown that oleosins are vital in determining oil body size. Oleosins are believed to stabilise oil body size and allow a large surface area to volume ratio (i.e. small oil bodies). A cell with a large amount of oleosin is hypothesised to have small oil bodies (Murphy, *et al.*, 1997). A large surface area: volume ratio may also be useful for rapid lipid breakdown as the lipid breakdown enzymes are in contact with a greater proportion of the total lipid at one time. In this case, however, rate of lipid breakdown appears to be equal in the transformants and the wild type.

The electron micrographs of the hexokinase overexpressors show that the seed cells contain numerous small oil bodies, whereas the wild type possess fewer, larger oil bodies. According to one hypothesis (Murphy, *et al.*, 1997), seeds with small lipid bodies are expected to have abundant oleosin synthesis. However, this was not apparent in the storage protein analysis of the hexokinase overexpressors.

The evidence for numerous, small lipid bodies correlates with the data indicating that the transgenic plants contain an altered lipid content, and rapid germination. Interestingly, other electron micrographs of identical seeds show lipid bodies with non-uniform contents although the shape of the organelles remain intact. An example of lipid bodies with non-uniform contents is illustrated by the electron micrograph of *cai* 10. This phenomenon may be an artefact of the fixation process because aqueous fixatives were used prior to embedding and sectioning. It is known that small seeds imbibe water very rapidly. When *Arabidopsis* seeds are put into aqueous fixatives the cells rapidly imbibe water, resulting in partial to complete hydration before adequate fixation occurs. This can lead to distortion of cellular contents, and the resultant picture is obviously not that of a dry cell. A better fixation, giving a truer picture of the inclusions within the dry cells, can be obtained by using non-aqueous fixatives or osmium vapour, or by freeze etching (Bewley and Black, 1994). In the electron micrographs cells in certain parts of the cotyledons showed non-

uniform lipid bodies whereas a few cells away, the lipid bodies appeared to be uniform. This again, may be due to the aqueous fixation process which allows partial imbibition before fixation occurs. The sequential hydration that appears to occur during fixation of dry seeds could be a consequence of the natural phenomenon of sequential hydration that occurs during seed imbibition (Mansfield and Briarty, 1996). However, this phenomenon does not invalidate the electron micrograph evidence for small lipid bodies.

The hexokinase activity of the transformants was higher than the wild type as was illustrated by germination of seeds supplied with ^{14}C-mannose (refer to chapter 5 section 5.2.3.3). After 8 hours germination, the hexokinase overexpressors had accumulated more hexose phosphates and sucrose than the wild type. This correlates with the evidence that hexokinase overexpressing transformants germinate fast because the seeds have a high metabolic rate. A further experiment would be to feed the specific hexokinase inhibitor, mannoheptulose to germinating hexokinase overexpressing seeds. If the rate of germination is a result of high hexokinase activity, then inhibition of hexokinase activity will slow germination. Of course, a problem with this experiment is that mannoheptulose may also inhibit other unknown processes which slow germination.

The *wri*1 mutants are similar to the hexokinase overexpressors with respect to low seed oil content (Focks and Benning, 1998). However, the *wri 1* seeds required sucrose for germination whereas the hexokinase overexpressors did not. In contrast to the *wri 1* mutants which displayed a reduction in glycolytic activities, the hexokinase activity was high in the hexokinase overexpressors. This, coupled with the evidence that both the hexokinase overexpressors and a fructokinase knockout mutant display increased glycolytic activity indicate that perturbations leading to increase or decrease of glycolytic activity cause altered allocation of carbon in the developing seed.

The evidence presented in this chapter parallels work which was done in potato tubers (Trethewey, *et al.*, 1998). In essence, overexpression of glucokinase and invertase stimulated glycolysis and diverted allocation of storage carbon from starch into amino acids and carbon dioxide. This is mirrored in the hexokinase overexpressors where an increase in a glycolytic enzyme lead to reallocation of carbon from lipid to amino acids. The group of

elevated amino acids was similar in both transgenic potato and the *Arabidopsis* hexokinase overexpressors described in this chapter, and could be attributed to increased flux of PEP into oxaloacetate catalysed by PEP carboxylase. The main difference was alanine, which accumulated highly in the potato plants but only slightly increased in hexokinase-overexpressing *Arabidopsis* plants. The *Arabidopsis* plants contained 5 - 47 fold of a compound designated as serine (based on its retention time on HPLC) than their control whereas the potato plants had 2.5-2.8 fold more serine than their controls.

In summary, the hexokinase overexpressing transformants may germinate faster than the wild type for several reasons. (1) they contain a large amount of gluconeogenic amino acids which may be rapidly mobilised or the 'serine' compound may fuel rapid germination; (2) the activity of hexokinase is higher than wild type 8 hours after being put in the growth room and this may lead to more rapid radicle emergence. Further experiments are necessary to determine whether one or both of these factors are involved in the rate of germination of the hexokinase overexpressors.

Chapter 7

Discussion

The work presented here investigates several mechanisms by which *Arabidopsis* seedlings avoid the affects of high cellular carbohydrate concentrations during post-germinative growth. Methods of escaping the affects of high cellular sugars include prevention of uptake of exogenous sugar, metabolism of the sugar such that its concentration does not reach a toxic threshold, and altered sugar sensing/signalling ability. Characterisation of several *cai* mutants has been used to investigate these methods and cloning of the *cai* mutants could reveal genes involved in each of these mechanisms. The role of hexokinase as a sugar sensor has been investigated using transgenic *Arabidopsis* plants which overexpress hexokinase. Analysis of mannose metabolism in sensitive and insensitive *Arabidopsis* seedlings has been useful tool for determining the mechanism of mannose toxicity and the way that mannose insensitive seedlings avoid its toxic affects.

Low concentrations of the glucose analogue, mannose, inhibit post-germinative growth of *Arabidopsis* seedlings. The results presented in this thesis serve as evidence that mannose is phosphorylated by hexokinase but only slowly further metabolised. The developmental arrest in wild type *Arabidopsis* coincides with an accumulation of hexose phosphates, indicating that sequestration of Pi may be a cause. The phenomenon mannose-induced seedling arrest has recently been studied by Pego *et al* (1999). The authors found that only phosphorylatable analogues of glucose, such as mannose and 2-deoxyglucose were capable of suppressing seedling development, whereas 6-deoxyglucose and 3-O-methylglucose (which are transported into cells but are not phosphorylated by hexokinase) were not. In addition, mannoheptulose, a specific inhibitor of hexokinase (Coore and Randle, 1964) relieved the affect of mannose. From this evidence it was proposed that the hexokinase reaction was involved in mannose-induced seedling arrest (Pego, *et al.*, 1999).

The results presented in this thesis imply that Pi sequestration is correlated to seedling arrest of wild type *Arabidopsis*. However, Pego et al (1999) reached a different conclusion. They found that it was not possible to relieve the affect of mannose by adding exogenous phosphate to the growth medium and proposed that the seed phosphate content is sufficient to

support germination even on mannose. There are several possible explanations for these different conclusions. One is that Pi levels are not, in fact, adequate for germination on mannose. It may be that it is the concentration of Pi in a particular compartment, or the ratio of Pi in one compartment relative to another compartment that is important for preventing sequestration of Pi. Phosphate feeding experiments may not be able to achieve the required concentrations or ratios. It is also possible that subtle differences in experimental conditions affect the extent to which phosphate availability controls germination. Another reason to explain why Pego *et al.*, (1999) could not relieve mannose germination arrest by adding phosphate whereas our results indicate that phosphate sequestration may involved, is that *cai* 10 and is a different type of *mig* from those selected by J. Pego.

It has also been proposed that seeds germinating on concentrations of mannose which repress seedling development contain sufficient ATP for germination (Pego, *et al.*, 1999). This was used as further evidence to support their hypothesis that mannose-induced seedling arrest is not a consequence of Pi sequestration. However, the experimental method which they employed to gain this information is open to question. ATP was measured per µg of soluble protein in seedlings allowed to germinate on agar plates containing 0 mM, 0.5 mM, 7.5 mM, 50 mM mannose. All the seeds germinated but the growth of seedlings on 7.5 mM and 50 mM mannose was arrested. Their results showed that the log moles ATP per ug soluble protein dropped by two orders of magnitude in the seedlings which were germinated on 0 mM or 0.5 mM mannose. In the seedlings which arrested (on 7.5 mM and 50 mM mannose), there was no drop in ATP concentration during 4 days of growth. However, *Arabidopsis* seeds contain a high proportion of insoluble storage protein which would not be measured by their method. It is known that the storage proteins are broken down during germination (see Figure 3.9), creating a large increase in soluble protein during early seedling development. The large drop in ATP per µg soluble protein seen in seedlings germinated on 0 mM or 0.5 mM mannose can be explained as followed: On day 0, there is relatively little soluble protein in the seeds so the concentration of ATP per ug soluble protein would be relatively high. After 2 days growth, when a significant proportion of the storage proteins would have broken down to soluble proteins, the relative proportion of ATP would be significantly lower. However,

when germinated on 7.5 mM mannose or above, the seedlings arrest so the storage proteins are unlikely to have broken down. Thus, the relative proportion of ATP to soluble protein would remain unchanged. Had the researchers measured total seedling protein and related this to the amount of ATP present the experiment might have been more informative. However, on days 0-1, before radicle emergence, the amount of ATP per ug protein was similar in both samples which had the potential to germinate and in samples which would have development arrested. This was used as evidence that there was sufficient ATP in seedlings germinated on 7.5 mM mannose and that ATP depletion was not a cause of seedling arrest. The results presented in figures 4.8 and 4.9 do not support this argument, since wild type accumulates more hexose phosphates when germinated for 24 h on mannose-containing medium than on glucose-containing medium.

Further evidence to support the role of Pi sequestration in mannose-induced seedling arrest is the observation that the mannose insensitive mutant, *cai* 10, sequesters less hexose phosphates than the mannose sensitive wild type (Figure 4.8). This could be a consequence of either a higher or lower rate of mannose metabolism such that only a small proportion of the total cellular Pi is sequestered at any one time. Since mannose also acts as a carbon source (Figure 4.10 a), it is possible that it induces respiration (Koch, 1996, Feletti and Gonzalez, 1998) which could either respire the mannose to CO_2 or convert it to lipid or protein. However, not all mannose insensitive seedlings accumulate less hexose phosphate. Most of the hexokinase overexpressors accumulate more hexose phosphates than their respective wild types (Figure 6.13). In contrast, *mig 7*, which is a fructokinase deletion mutant also accumulates more hexose phosphates than the wild type. This implies that there may be several mechanisms by which seedlings are able to be mannose insensitive. Plants with altered mannose metabolism may have a *mig* phenotype (1) by very slow metabolism of mannose such as may be the case for *cai* 10 and (2) by rapid phosphorylation of mannose by hexokinase and fast turnover of the hexose phosphates. Alternatively, a *mig* phenotype could be a consequence of the mutation of a sugar sensor (3). The fructokinase deletion mutant (*mig 7*) may have a mig phenotype because fructokinase is a sugar sensor and thus cannot sense the mannose and does not respond to the accumulation of hexose phosphates, or it may be that the

mig phenotype and the accompanying accumulation of hexose phosphates (Figure 6.13) is caused by the upregulation of another hexokinase gene in the germinating seedling to compensate for the lack of fructokinase.

Pego *et al* (1999) also proposed that hexokinase mediates the mannose repression of *Arabidopsis* post-germinative growth via energy depletion. They suggested that in *Arabidopsis* seeds, the phosphorylation of mannose by hexokinase triggers a signalling cascade leading to the repression of genes required for germination. It is known that the glyoxylate cycle genes *ms* and *icl* are repressed by sugars and there is evidence that hexokinase mediates the carbohydrate repression of these genes (Graham, *et al.*, 1994b). Detailed analysis of the *ms* and *icl* promoters has found that distinct regions are required for the response to sugar and for regulation of gene expression in during germination (Reynolds and Smith, 1995, Sarah, *et al.*, 1996). It has been shown that sugars can repress the expression of *ms* and *icl* during starvation and senescence (Graham, *et al.*, 1994b, Reynolds and Smith, 1995, Sarah, *et al.*, 1996). Pego *et al* (1999) suggest that genes required for germination are repressed by mannose. The results presented in Figure 4.11 show that mannose represses *ms* expression but induces *icl* expression in germinating wild type *Arabidopsis*. Transcript blot analysis simply measures the presence of RNA and it is not possible to determine whether transcription of the *icl* gene is actually occurring during germination on mannose. The enzyme activities of MS and ICL could be measured to give an indication of whether the glyoxylate cycle is active. Alternatively, transgenic plants carrying the MS or ICL promoter fused to GUS could be used to determine whether glyoxylate cycle genes are repressed in seeds germinating on mannose. In addition, the level of *ms* and *icl* transcription in seeds germinating on mannose could be measured by nuclear run-on assays.

The work of Jang et al (1997), showed that transgenic *Arabidopsis* plants overexpressing hexokinase were hypersensitive to 6 % glucose whereas in plants containing the antisense hexokinase construct were hyposensitive to 6 % glucose. This agreed with their model for hexokinase sugar sensing which had been devised on the basis of previous experiments with glucose analogues which found that sugar repression of photosynthetic gene expression required the hexokinase reaction. The hexokinase overexpressors described in this

thesis were transformed with the same construct (AtHXK1) as the plants described by Jang et al (1997), yet the opposite phenotype was reported. It must be noted, however, that the plants were all individual transformants and so could vary with the position of DNA insertion. A large proportion of the T3 generation of hexokinase overexpressors showed approximately 20 % penetrance of the *gin* phenotype. However, this was not reproducible in the T4 generation, possibly due to slight variations in growth conditions which are known to affect the phenotype. Lines in both the T3 and the T4 generation did have *cai* and *mig* phenotypes. This does not agree with the model of the hexokinase sugar sensor proposed by Jang et al (1997) which proposed that hexokinase overexpressors are more sensitive to sugars due to an increased number of sugar sensors. However, the authors did report that several lines of hexokinase 1 overexpressor had phenotypes similar to wild type on 6% glucose. They proposed that the sugar hyposensitivity in the transgenics carrying the 35S:AtHXK1 sense transgene resulted from cosuppression (Napoli, *et al.*, 1990). It is possible that these plants represent the true phenotype resulting from hexokinase 1 overexpression. The yeast hexokinase PII (YHXK2; the proposed sugar sensor in yeast) gene was also overexpressed in *Arabidopsis* (Jang, *et al.*, 1997). These plants were less sensitive to glucose although hexokinase catalytic activity was increased. The authors proposed that the regulatory domain of the yeast hexokinase PII is different to the hexokinase regulatory domain in plants and is thus not interchangeable. This is supported by the evidence that it was possible to restore catalytic but not the regulatory function in the yeast hxk1 hxk2 double mutant by complementation with AtHXK1 or AtHXK2 (Jang, *et al.*, 1997). They suggested that transgenic plants overexpressing YHXK2 were less sensitive to sugars because the transgene exerts a dominant negative affect, presumably competing with the endogenous hexokinase for substrate and thus reducing the sugar signal. The results with yeast hexokinase overexpression are similar to the results that we found with transgenic plants overexpressing AtHXK1. It would be interesting to see whether the plant overexpressing yeast hexokinase showed the same phenotype in our hands.

As has been described there are many unanswered questions relating to the mechanism by which mannose represses germination, and the role of hexokinase in sugar sensing in

plants. Our results indicate that sequestration of Pi, and possibly repression of *ms* are involved in mannose-induced seedling arrest. A future experiment would be to determine the relative importance of each of these factors in seedling arrest.

In addition it would be interesting to establish the underlying cause of the altered carbon partitioning in the seeds of the hexokinase overexpressors. It is not clear whether the increase in amino acids in the hexokinase overexpressors seeds is a maternal affect, whereby the high hexokinase activity in the leaves results in an upregulation of *nr*, leading to high leaf amino acid concentrations which are then transported to the developing seeds. To determine this, the amino acid content of leaves of the hexokinase overexpressors needs to be compared to wild types grown in the same conditions. Then, the amino acid content of their progenies should be analysed. If the concentration of amino acids is higher in the hexokinase expressors than the wild type then the composition of the progeny seed may be due to maternal affects.

Alternatively, the high hexokinase activity could result in a high concentration of G6P which could allosterically activate PEP carboxylase and increase the rate of amino acid synthesis relative to the rate of lipid synthesis. The activity of PEP carboxylase in leaves and developing embryos of the hexokinase overexpressors could be measured to answer this. In addition, it would be valuable to measure the activity in the hexokinase overexpressors of other key enzymes in central metabolism such as SPS, PFK, PFP, pyruvate kinase, acetyl CoA carboxylase, sucrose synthase and NR. This would give a clearer picture of how an increase in hexokinase activity can affect the flux of carbon in transgenic plants. Feeding ^{14}C PEP to developing embryos of the hexokinase overexpressors could be useful in determining the partitioning of PEP between lipids and amino acids.

The hexokinase plants germinate rapidly but then the plants grow more slowly and set seed later than the wild type. If high hexokinase activity is a cause of rapid germination it may be possible to engineer plants which germinate quickly but then set seed at the same rate as wild type by making transgenic plants which contain the hexokinase gene fused to the MS promoter so that hexokinase activity is only high during germination. In addition constructs could be made in which the napin seed storage promoter was fused to the hexokinase gene. This could result in transgenic plants which only overexpressed hexokinase during seed

development and may result in plants whose progeny contain altered carbon composition but develop in the same time as the wild type. Finally, plants containing both the ms promoter fused to the hexokinase and the napin promoter fused to the hexokinase may germinate rapidly, set seed in the same time as wild type, and produce seed with altered carbon composition.

Now that a large number of putative sugar sensing mutants have been isolated there is a lot of work to be done categorising them into allelic groups and cloning the mutant genes. Recent work on the hexokinase PII in yeast has revealed hexokinase PII participates in DNA-protein complexes, with *cis*-acting regulatory elements of the SUC2 gene promoter which is necessary for the glucose repression of the SUC2 gene in yeast (Herrero, *et al.*, 1998). Trying to identify homologous regions of glucose repressed genes in plants is one research area which needs to be expanded.

The observation that altering hexokinase activity in plants significantly alters carbon allocation indicates that it has an important role in the control of carbon metabolism. From the purely scientific viewpoint, this is interesting because much research has been done to determine which enzymes are important in metabolic flux control and many of these experiments have shown that altering the activity of enzymes in metabolism has a negligible affect. In addition, plants which overexpress hexokinase are potentially of economic value. Seeds which germinate rapidly have the potential to outcompete weeds. Thus, production of rapidly germinating transgenic plants could be of great commercial value. Furthermore, manipulation of the rate of carbon and the allocation of carbon into the different storage polymers is also highly desirable. For centuries, traditional plant breeders have been selecting plants which possess certain traits such as high protein content or high lipid content and the hexokinase overexpressors may provide an alternative method for manipulating carbon flow.

Chapter 8

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