Plastid redox state and sugars as interactive regulators of nuclear photosynthetic gene expression

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ABSTRACT

Feedback control of photosynthesis by carbon metabolites has long been recognised but the underlying cellular mechanisms that regulate this process remain unclear. As part of this thesis project it was demonstrated that in an Arabidopsis cell culture a block in photosynthetic electron flux prevents the increase in transcript levels of chlorophyll *a/b* binding protein (*CAB*) and the small subunit of Rubisco (*RBCS*) that typically occurs when intracellular sugar levels are depleted. In contrast, the expression of the nitrate reductase (*NR*) gene, which is induced by sugars, was not changed. These findings were confirmed *in planta* using Arabidopsis carrying the firefly luciferase reporter gene (*LUC*) fused to the plastocyanin (*PC*) and *CAB2* photosynthetic gene promoters. Transcription from both promoters increased upon carbohydrate deprivation. Blocking photosynthetic electron transport with 3-(3', 4'-dichlorophenyl)-1,1'-dimethylurea (DCMU) prevented this increase in transcription. This work led to the conclusion that plastid derived redox signalling can over-ride the sugar regulated expression of nuclear encoded photosynthetic genes. In the sugar response mutant *sun6* (sucrose uncoupled) *PC-LUC* transcription actually increased in response to exogenous sucrose rather than decreasing as in the wild type. Interestingly, plastid derived redox signals do not influence this defective pattern of sugar regulated gene expression in the *sun6* mutant. A model, which invokes a positive inducer originating from the photosynthetic electron transport chain, was proposed to explain the nature of the plastid-derived signal (Oswald, O., Martin, T., Dominy, P. J. and Graham, I. A., 2001: Plastid redox state and sugars: Interactive regulators of nuclear gene expression. *Proc.Natl.Acad.Sci.USA* **98:**2047-2052).

Previous studies in algal systems have shown that light and temperature are critical regulators of plastid redox state. When the redox state of the photosynthetic electron transport chain in the Arabidopsis culture was altered by changing temperature and light conditions differential effects were observed in carbon starved and carbon supplied cells. These results confirm that various environmental parameters can affect the plastid redox state and consequently nuclear gene expression in higher plants.

Over the last few years a growing number of plant 14-3-3 binding proteins, including DNA binding proteins and key proteins of carbon metabolism, have been identified. The binding of these proteins to 14-3-3 proteins affects their phosphorylation state and

consequently cellular activity. Sugar deprivation has recently been shown to have a dramatic effect on 14-3-3 binding protein abundance in the same cell culture that was used in this thesis work. However, the plastid redox signal as identified in this thesis work did not result in an altered regulation of 14-3-3 binding proteins by sugars, providing evidence that at least this proposed part of the sugar signalling pathway is not regulated by the redox state of the plastid.

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Abbreviations

AICAR	5-aminoimidazole-4-carboxamide riboside
AMP	adenosine monophosphate
AMPK	mammalian AMP activated kinase
ARF	gene for the member of the ARF subfamily of GTP-binding proteins
ATP	adenosine triphosphate
CAB	chlorophyll *a/b* binding protein
CAB	chlorophyll *a/b* binding protein gene
cai	carbohydrate insensitive mutant
CaMV35S	cauliflower mosaic virus 35S promoter
cen	*cai* EN mutant
CHS	chalcone synthase
CHS	chalcone synthase gene
cue	*CAB* underexpressed mutant
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone
DCMU	3-(3', 4'-dichlorophenyl)-1,1'-dimethylurea
DEPC	diethylpyrocarbonate
DIG	Digoxigenin
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid (disodium salt)
EF-1α	elongation factor 1 alpha gene
F_0	fluorescence of fully oxidised Q_A
F_{max}	maximal chlorophyll *a* fluorescence
F_S	steady state chlorophyll *a* fluorescence
GSH	reduced glutathione
gun	genome uncoupled mutant
HPLC	high performance liquid chromatography
kDa	kiloDalton
MOPS	3-(N-morpholino)propanesulphonic acid
mRNA	messenger ribonucleic acid
MSMO	Murashigge-Skoog medium with minimal organics
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NR	nitrate reductase
NR	nitrate reductase gene
PAR	photosynthetically active radiation
PC	plastocyanin
PC	plastocyanin gene
PCI	phenol: chloroform: isoamylalcohol 25:24:1
PCR	polymerase chain reaction
PET	photosynthetic electron transport
PRL1	pleiotrophic response regulator 1
psaAB	gene coding for the core protein of PSI
psbA	gene coding for the core protein of PSII

PSI	photosystem one
PSII	photosystem two
SDS	sodium dodecyl sulphate
SNF1	sucrose non fermenting 1
SnRK1	SNF1 related kinase of plants
SPS	sucrose phosphate synthase
sun	sucrose uncoupled mutant
TPS	trehalose-6-phosphate synthase
Tris	tris (hydroxymethyl)methylglycine
TTP	thymidine triphosphate
UDP	uridine diphosphate
UTP	uridine triphosphate
w/v	weight per volume (expressed as percentage)

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Declaration:

I declare that this thesis for the degree of doctor of philosophy has been composed entirely by myself and the work presented herein was performed by myself unless stated otherwise.

Chapter 1: Introduction

1.1 *Context*

All organisms must adjust their development to a variety of environmental signals. Due to their sessile nature this is especially true for plants. Environmental parameters, such as light, temperature and nutrient availability impact directly on the nutritional status of plant cells. Changes in the nutritional status therefore could acts as signals that reflect current environmental conditions. In this context sugars in particular have been a focus of plant research over the last decade (Sheen, 1990; Stitt et al., 1995; Graham and Martin, 2000; Smeekens, 2000). Sugars are involved in regulating a variety of processes from flowering, seed development and seedling establishment to carbon allocation and partitioning. A major consequence of intracellular signals generated by sugars is the alteration of nuclear gene expression (see Fig. 1.1).

However, environmental parameters may also have a much more direct effect on nuclear gene expression than that which occurs through the indirect route of altering the nutritional status. For example, there have been a limited number of reports in recent years (Maxwell et al., 1995; Escoubas et al., 1995; Karpinski et al., 1997; Petracek et al., 1998) which implicate the redox state of the chloroplast, and in particular the redox state of the photosynthetic electron transport (PET) chain, as a regulator of nuclear gene expression (Fig. 1.1).

Whilst there are a number of reports of the regulation of photosynthetic genes by the plastid redox state in algae (Maxwell et al., 1995; Escoubas et al., 1995), there have been no convincing reports of this phenomenon operating in higher plants. However, there are numerous reports of sugars regulating these same genes in higher plants (Koch, 1996).

The major objective of this thesis was to investigate the relative contribution that carbon nutritional status in the form of sugars and plastid redox signalling make to the control of photosynthetic gene expression in the model plant *Arabidopsis thaliana.*

The specific aims of this PhD project were to:

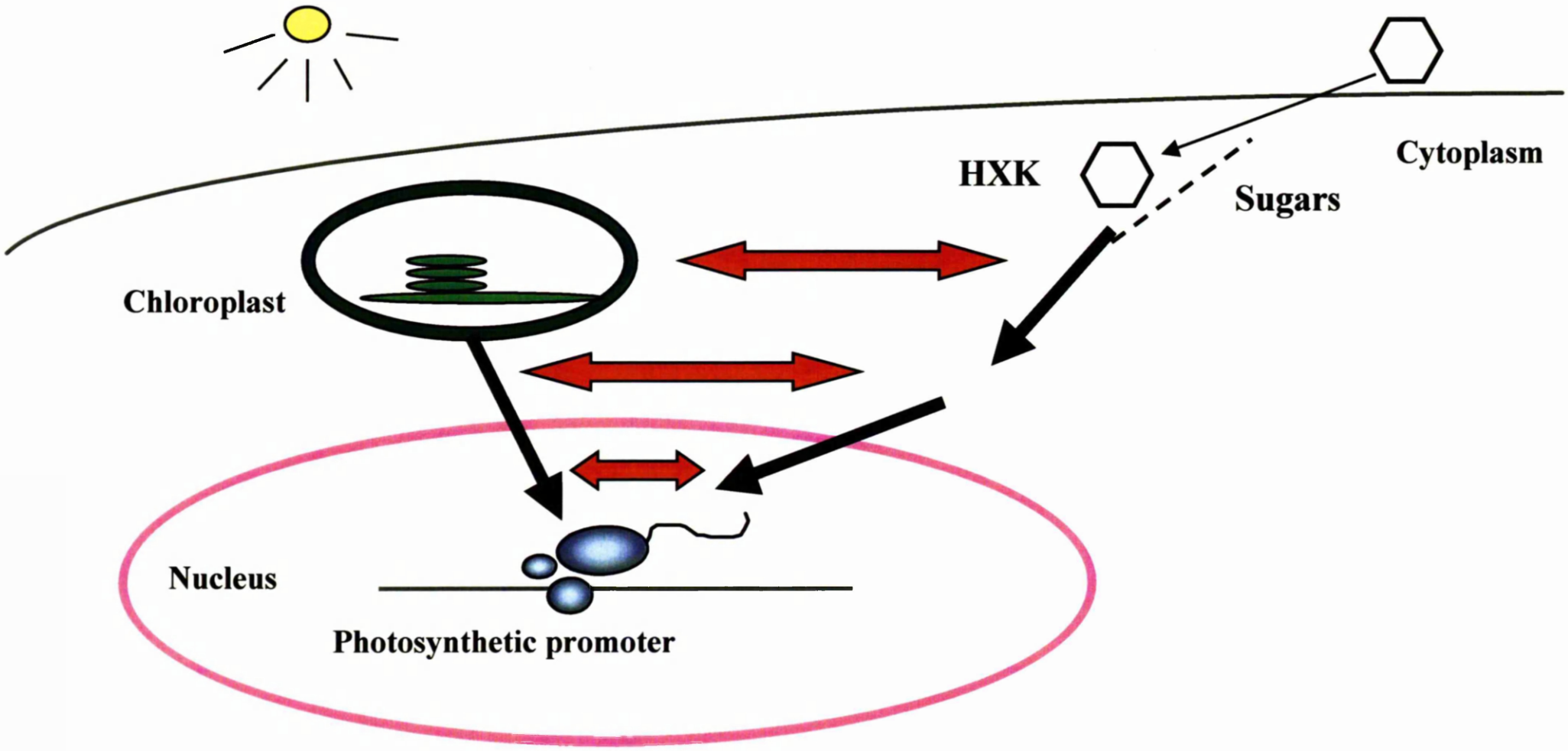
1. Establish if both sugars and plastid redox state regulate the expression of nuclear encoded photosynthetic genes in higher plants.
2. Identify if these two signals interact in their regulation of nuclear gene expression, and if so to identify at what level the interaction might occur.

Figure 1.1: Model depicting the hypothesis that sugars and chloroplast signals interact on nuclear photosynthetic gene expression

Both sugars and plastid redox state can initiate a signal regulating the expression of photosynthesis genes in the nucleus. These two signal transduction pathways might interact at different levels.

'HXK' Hexokinase

Sugars are depicted by a hexagon.


Cytoplasm
HXK
Sugars
Chloroplast
Nucleus
Photosynthetic promoter

1.2 Sugars regulate nuclear gene expression

1.2.1 A variety of plant genes are regulated by sugars

Metabolic regulation of nuclear encoded genes by sugars and acetate was first reported by Sheen using photosynthetic promoters fused to a CAT reporter gene in transiently transfected protoplasts (Sheen, 1990). It soon became apparent that not only a range of photosynthetic genes (Krapp et al., 1993) are regulated by sugars, but also genes in carbon metabolism such as malate synthase and isocitrate lyase (Graham et al., 1994a). A large number of nuclear genes involved in different aspects of carbon metabolism as well as photosynthetic genes and defence genes are now known to be sugar-regulated (reviewed in Sheen (1994); Stitt et al. (1995); Koch (1996); Graham (1996); Graham and Martin (2000)). However, while there is a comprehensive knowledge of patterns of gene regulation in response to sugars the mechanism(s) of sugar sensing in plants are still poorly understood.

1.2.2 Sugar sensing in yeast and mammalian cells

In micro-organisms such as yeast (*Saccharomyces cerevisiae*) glucose represses genes involved in the metabolism of other carbon sources through a process known as carbon catabolite repression (Gancedo, 1998).

Although the understanding of sugar sensing in yeast is still limited, it appears that there are at least two sensing systems operating for glucose. The first involves a signal generated by glucose binding to glucose transporters or transporter-like proteins. Two glucose sensors with homology to glucose membrane transporters have been identified (Özcan et al., 1996; Özcan et al., 1998). One, SNF3, senses low glucose availability while the other one, RGT2, senses high levels of glucose. In addition at least one G-protein coupled glucose receptor, GPR1, located in the membrane has been identified (Kraakman et al., 1999). GPR1 specifically activates signalling via cAMP while SNF3 and RGT2 act via a different mechanism (see Carlson (1998) and Schmidt et al. (1999)). The other glucose sensing mechanism operating in yeast requires the presence of active hexokinase (Thevelein and Hohmann, 1995; Gancedo, 1998; Carlson, 1998). It is unclear how hexokinase initiates a sugar signal. The downstream signalling components appear to involve the *sucrose non fermenting 1* SNF1 complex (Thevelein and

Hohmann, 1995; Gancedo, 1998; Carlson, 1998). Downstream of the SNF1 complex several DNA binding proteins have been identified in the signal transduction pathway (see Carlson (1998) for details).

In mammalian cells glucokinase / hexokinase has also been suggested as the sugar sensor (Epstein et al., 1992; Matschinsky et al., 1993; Grupe et al., 1995). Interestingly, a protein competing with glucose for binding to glucokinase and regulating the glucokinase activity has recently been identified in mice (Grimsby et al., 2000).
The mammalian homologue of the SNF1 complex is the AMP-activated kinase (AMPK) complex (Hardie et al., 1998). An increase in the AMP to ATP ratio activates AMPK in several ways (summarised in Halford et al. (1999)). In yeast an increase of the AMP / ATP ratio also coincided with SNF1 action (Wilson et al., 1996). Since the hexose phosphorylation by hexokinase will result in an increase of AMP and a decrease of ATP, it has been argued that the effect of hexokinase on sugar signal generation could be indirect and that the sugar signal could act through an AMPK like mechanism (Halford et al., 1999). This has led to the role of hexokinase in sugar sensing being questioned (Halford et al., 1999).

1.2.3 Sugar sensing in plants

Sugar sensing and signalling has been a major focus of plant research and has been frequently reviewed (Sheen, 1994; Stitt et al., 1995; Graham, 1996; Roitsch, 1999; Sheen et al., 1999; Graham and Martin, 2000; Smeekens, 2000; Pego et al., 2000).
In particular mutants of Arabidopsis have proven a promising approach, and yield first information about sugar sensing / signalling and the interaction with other signal transduction pathways (see below and Mita et al. (1997); Martin et al. (1997); Dijkwel et al. (1997); Zhou et al. (1998); Nemeth et al. (1998); Boxall (1999); Huijser et al. (2000); Pego et al. (2000); Laby et al. (2000); Arenas-Huertero et al. (2000)).

1.2.3.1 Hexose sensing in plants

1.2.3.1.1 Hexose transport

In yeast the binding of glucose to glucose transporter-like proteins is able to initiate a glucose signal although no actual transport is required as determined by the study of various truncated versions of these proteins and construction of glucose transporters – signalling domain constructs (Özcan et al., 1998). In plants hexose transport has also been suggested to result in a sugar signal. Hexose analogues that are transported into the cell, but can not be phosphorylated by hexokinase such as 3-O-methyl-glucose or 6-deoxy-glucose have been employed (Roitsch et al., 1995; Martin et al., 1997), sometimes in combination with direct studies of hexose transporters (Hilgarth et al., 1991; Sherson et al., 2000).

1.2.3.1.2 Hexokinase

The first clear demonstration that sugars regulate plant gene expression at the level of transcription was reported from work using a maize protoplast transient expression system (Sheen, 1990). The first evidence for the involvement of hexokinase in this process was provided by studies measuring the regulation of malate synthase and isocitrate lyase by sugars (Graham et al., 1994a; Graham et al., 1994b). The gene expression of these two enzymes of the glyoxylate cycle was induced in cucumber cell cultures and protoplasts when intracellular levels of sucrose, glucose and fructose fell below 3mM (Graham et al., 1994a). Sugars repressed the expression of these two genes, but the glucose analogue 3-O-methylglucose, which is imported into the cytoplasm but can not be phosphorylated by hexokinase, had no effect (Graham et al., 1994a; Graham et al., 1994b). Transcription from photosynthetic promoters were also repressed by hexoses and glucose analogues that can be phosphorylated by hexokinase but not efficiently metabolised further (Jang and Sheen, 1994). An inhibitor of hexokinase, mannoheptulose, could prevent the effect of sugars, and glycolytic intermediates had no effect on photosynthetic promoters (Jang and Sheen, 1994). Other studies have used analogues of glucose that can or can not be phosphorylated by hexokinase to elucidate the role of this enzyme in the generation of sugar signals in plants (Krapp et al., 1993; Pego et al., 1999). In particular the hexose sugar mannose has been employed, since it is

phosphorylated by hexokinase, but supposedly not metabolised at significant rates (Pego et al., 1999).

Using the inhibitors mannoheptulose and glucosamine Guglielminetti and co-workers concluded that hexokinases and glucokinase, but not fructokinase appear to be involved in sugar sensing in rice embryos (Guglielminetti et al., 2000).

Overexpression of Arabidopsis hexokinases in Arabidopsis resulted in hypersensitivity of seedlings to 6% (w/v) glucose while reducing their levels by antisense technology reduced the sensitivity to glucose (Jang et al., 1997). Overexpression of the yeast hexokinase PII in Arabidopsis increased total hexokinase activity, but resulted in reduced sensitivity to glucose (Jang et al., 1997). These results were taken as evidence for the role of hexokinase in sugar sensing. However, when Boxall overexpressed the same construct for Arabidopsis hexokinase 1 in the sugar sensing mutant *carbohydrate insensitive 10* (*cai10*) and wt Arabidopsis the results did not agree with those of Jang and colleagues, since no increase in sensitivity to glucose was present in seedlings overexpressing hexokinase (Boxall, 1999). Overexpression of the endogenous hexokinase in potato resulted in no significant changes, while reduction by antisense technology lead to an increase in transient starch in leaves, but no change of tuber metabolism (Veramendi et al., 1999). In contrast overexpression of Arabidopsis hexokinase 1 in tomato plants resulted in decreased photosynthesis, early senescence and altered fruit development (Dai et al., 1999). Grafting experiments showed that overexpression of hexokinase in photosynthetic tissue was responsible for the severe phenotype (Dai et al., 1999).

The importance of hexokinase in plant sugar sensing has recently been challenged on the basis that the majority of the experiments implicating hexokinase also impact on the AMP to ATP ratio (Halford et al., 1999). This factor could, along with the AMPK homologue (SnRK1, see below) be responsible for sugar sensing rather than hexokinase. However, the main hypothesis for sugar sensing in plants still assigns a central role to hexokinase (Smeekens, 2000). Further clarification of the role of hexokinase in plants is therefore required in future.

1.2.3.2 Sucrose sensing

Sucrose is readily hydrolysed to glucose and fructose, yet recent experimental evidence demonstrates that sucrose itself appears to induce a separate sugar signal. Gene expression controlled by the patatin (Wenzler et al., 1989; Jefferson et al., 1990) and rolC (Yokoyama et al., 1994) promoters is induced in response to sucrose, but the combination of fructose and glucose was less effective. The mRNA abundance of the *Beta vulgaris BvSUT1* sucrose transporter is repressed by sucrose (Chiou and Bush, 1998), but not by glucose. Mannoheptulose did not prevent sucrose repression. Other sucrose-specific effects, such as translational control of the Arabidopsis ATB2 have been reported (Rook et al., 1998). Sucrose transporters and their role in sugar sensing have recently been reviewed (Lalonde et al., 1999). From the evidence presented it appears that sucrose uptake, but not its breakdown products, is in some circumstances responsible for the generation of a sugar signal.

1.2.3.3 Trehalose as modulator of sugar sensing?

Trehalose is a disaccharide consisting of two linked glucose moieties. Its synthesis occurs in a two-step process: UDP-glucose and glucose-6-phosphate are linked to trehalose-6-phosphate by the enzyme trehalose phosphate synthase. Trehalose phosphate phosphatase then forms trehalose. Although known from micro-organisms and animal systems, trehalose synthesis proved elusive in plants until recently (reviewed in Goddijn and Smeekens (1998)). Trehalose was known as a stress protectant in micro-organisms and desiccation plants, but more importantly with respect to carbon metabolism it appears that the precursor, trehalose-6-phosphate, is an inhibitor of hexokinase and might act as a regulator of glycolysis in yeast (Thevelein and Hohmann, 1995). If trehalose-6-phosphate plays a similar role in plants is unclear. However, the recent analysis of an Arabidopsis trehalose phosphate synthase knockout mutant demonstrates a crucial role for trehalose-6-phosphate synthesis in embryo development (Eastmond et al., 2001). In the mutant homozygous embryo maturation is arrested at the stage when storage product accumulation normally occurs. A partial rescue of the embryo is possible if the extracellular sugar levels are decreased, indicating a regulatory role of trehalose synthesis on carbon metabolism and storage allocation (Eastmond et al., 2001). However, inhibition of Arabidopsis hexokinases by trehalose-6-phosphate appears to be much weaker than the one observed for yeast

hexokinase (Eastmond et al., 2001). Our understanding of the role of trehalose and trehalose-6-phosphate in plants has to await further progress in this field.

1.2.4 Suggested sugar signal transduction mechanisms

Little is known about the transduction of sugar signals in plants. The following components of signal transduction mechanisms have been implicated:

- Calcium and calmodulin. These ubiquitous second messengers have been reported to play a role in sugar induction of a β-amylase promoter - GUS reporter gene construct in tobacco (Ohto and Nakamura, 1995) and sugar-induced anthocyanin accumulation in *Vitis vinifera* (Vitrac et al., 2000).
- Protein kinases: inhibitors of protein kinases, in particular inhibitors of Ser/Thr protein kinases, proved successful in impairing sugar signal transduction in various systems (Ohto and Nakamura, 1995; Fujiki et al., 2000; Vitrac et al., 2000).
- Protein phosphatases: inhibitors of protein phosphatase action such as okadaic acid and microcystin-LR interfered with sugar signalling in sweet potato and tobacco (Takeda et al., 1994), *Chenopodium rubrum* cultures (Ehness et al., 1997), Arabidopsis suspension cultures (Fujiki et al., 2000) and *Vitis vinifera* cultures (Vitrac et al., 2000), as determined by the use of protein phosphatase inhibitors.
- Combinations of these appear to operate *in vivo* (as suggested by several reports cited above). Ohto and colleagues reported on a sugar-inducible calcium-dependent Ser/Thr protein kinase associated with the plasma membrane in tobacco leaf tissue (Ohto and Nakamura, 1995). Interestingly, Ehness and co-workers (1997) demonstrated that fungal stress elicitors could mimic glucose effects in a *Chenopodium rubrum* cultures with respect to the expression of *RBCS*, a gene for cell wall invertase and a gene for phenylalanine ammonium lyase. However, when protein kinase inhibitors were used the response to fungal stress elicitors proved to be staurosporine sensitive, i.e. kinase dependent, but not the glucose response. Yet protein phosphatase inhibitors could mimic the glucose effects.

Whilst all the evidence indicates a complex web of signal transduction mechanisms is operating *in vivo*, the SNF1 kinase complex has been assigned a central role as discussed below.

1.2.5 SNF1 as a possible key component of sugar signal transduction

The *sucrose non-fermenting 1* (*SNF1*) mutant of *Saccharomyces cerevisae* led to the identification of the SNF1 Ser/Thr protein kinase. Together with 2 other subunits it forms the SNF1 complex of yeast, which appears to play an important role in sugar signalling in yeast (reviewed in Carlson (1998)). Homologues of SNF1 have been found in mammalian and plant cells and led to the suggestion of the SNF1 complex as a common eukaryotic component of carbohydrate sensing (Hardie et al., 1998).

SNF1 homologues in plants were named SNF1-related Kinases (SnRK1). SnRK1 like kinases have been found in Arabidopsis and a number of other species (see Halford and Hardie (1998)) together with homologues of the other subunits of the SNF1 complex (Bouly et al., 1999). It was suggested that the SnRK1 complex acts as global regulators of carbon metabolism (Halford and Hardie, 1998). The SnRK1 complex is required for the induction of sucrose synthase by sucrose in potato (Purcell et al., 1998), implying that SnRK 1 contributes to the transduction of a sugar signal. Activation of SnRK1 like protein kinases by sugars has been demonstrated in Arabidopsis (Bhalerao et al., 1999). SnRK1 activity was shown to be regulated by kinases and phosphatases, as well as AMP (Sugden et al., 1999a).

Although the SnRK1 complex was given a central role in relaying sugar signals (Halford et al., 1999) initiated by sucrose sensing, hexokinase-mediated hexose sensing or hexokinase-independent hexose sensing (see above) the evidence supporting this model is still largely circumstantial rather than direct. It therefore is still possible that other signal transduction pathways that do not involve the SnRK1 complex do exist.

1.2.6 Targets of SNF1 action

Under low glucose conditions the SNF1 complex in yeast phosphorylates MIG1 a mediator of glucose repression. When phosphorylated MIG1 can no longer interacts with the repressor complex SSN6/TUP1. The repressor complex dissociates from DNA and chromatin re-modelling allows expression of the previously repressed genes for the utilisation of alternative carbon sources. This represents the end of glucose repression (see Carlson (1998) for details).

In mammalian and plant cells the target proteins of the SNF1 homologue appear to include a group of proteins called 14-3-3 binding proteins. After phosphorylation these proteins associate with proteins of the 14-3-3 type. Proteins of this type belong to a highly conserved family of proteins named after their particular migration pattern on 2D DEAE-cellulose chromatography and starch gel electrophoresis (Aitken, 1996). These 14-3-3 proteins form hetero- and homodimers, but also bind at least one sequence motif containing a phosphorylated serine residue on other proteins, hence grouped as 14-3-3 binding proteins.

In plants 14-3-3 binding proteins include G-box binding transcription factors (Lu et al., 1992; de Vetten et al., 1992; Schultz et al., 1998), regulatory proteins such as a calcium dependent kinase regulating NR activity (Toroser et al., 1998; Moorhead et al., 1999) and key metabolic proteins such as NR (Moorhead et al., 1996; Bachmann et al., 1996; Sugden et al., 1999b), sucrose-phosphate synthase (SPS), trehalose-6-phosphate synthase (TPS), glutamine synthases (GS) (Moorhead et al., 1999), sucrose synthase and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Sugden et al., 1999b), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cotelle et al., 2000) and glutamyl-tRNA synthetase (GluRS) (Cotelle et al., 2000). Phosphorylation of enzymes that are 14-3-3 binding proteins and subsequent binding of 14-3-3 proteins is thought to modulate enzymatic activity, as was reported for the phosphorylation and subsequent inactivation of nitrate reductase (NR) (Moorhead et al., 1996; Bachmann et al., 1996; Sugden et al., 1999b). In humans and mammals 14-3-3 binding proteins are also implicated in a multitude of processes and include amongst others the signal transduction molecule Raf or the cell cycle control enzyme Cdc25 phosphatase (for a more detailed listing see (Aitken, 1996)).

From this array of targets it appears that a signal transduced via SnRK1 could affect transcription of genes (cf. SNF1 complex in yeast) and alter the activity of key components of carbon metabolism.

1.2.7 Interaction with other signalling pathways

A key nutrient-signalling pathway such as the one for sugars is expected to be tightly interwoven with other signalling pathways involved in the cellular response to environmental factors. Indeed evidence is now emerging about links between sugar signalling and other signal transduction pathway (reviewed in Smeekens (2000)). Such

pathways include phytochrome signalling (Cheng et al., 1992; Dijkwel et al., 1996), and hormone signal transduction pathways for ethylene (Zhou et al., 1998) and abscisic acid (Huijser et al., 2000; Laby et al., 2000; Arenas-Huertero et al., 2000). The *pleiotrophic response regulator 1* (*PRL1*) identified in Arabidopsis (Nemeth et al., 1998) binds to two SNF1 like protein kinases of Arabidopsis and acts as a negative regulator (Bhalerao et al., 1999). If the *PRL1* gene is disrupted by mutation the result is hypersensitivity to glucose and sucrose as well as for the hormones cytokinin, ethylene, abscisic acid and auxin (Nemeth et al., 1998). Future genetic approaches should allow the isolation of more and more shared components of the signal transduction pathways. Considering its importance for photosynthetic cells one such pathway expected to interact with sugar signalling should be the plastid-to-nucleus signal that has been proposed by a number of researchers (Allen et al., 1995).

1.3 Plastid redox state as a signal

The redox state of a cell and its compartments such as plastids is given by the total of all molecules existing in a reduced and an oxidised form, and the balance between the two forms of each molecule. This balance will be altered by oxidative processes of benign nature (for example in assimilatory processes) or by potentially harmful substances such as active oxygen species. Production of reduction equivalents and antioxidants counteracts the oxidative processes and keeps the redox balance checked (reviewed in Noctor and Foyer (1998); Noctor et al. (2000); Foyer and Noctor (2000); Smirnoff (2000); Mullineaux et al. (2000)).

In the light PET generates reduction equivalents that can be used to produce NADPH or the reduced forms of thioredoxin and the antioxidants glutathione and ascorbate. Reduced glutathione, ascorbate and thioredoxin were found to be important in regulation of plastid enzyme activities and protection from active oxygen species. In addition, the redox state of the plastid appears to regulate processes in the cytoplasm and gene expression in the nucleus (reviewed in Noctor and Foyer (1998); Noctor et al. (2000); Foyer and Noctor (2000); Mullineaux et al. (2000)). The origin of a suggested signal conveying the plastid redox state to the cytoplasm and nucleus is unknown. In addition to the redox state of the ferredoxin-thioredoxin couple, several components of the PET chain have been implicated as the possible redox sensor, including the Q_A site of PSII, the PQ pool or the cytochrome b_6/f complex (reviewed in Huner et al. (1996);

see also Fig. 1.2). Antioxidants or hydrogen peroxide may play a contributory role to the signalling process, and in the case of hydrogen peroxide even act in systemic signalling (Karpinski et al., 1999). However, in cellular signalling they are of secondary importance to the redox state of a PET chain member, potentially PQ (Karpinski et al., 1997; Karpinski et al., 1999).

1.3.1 Active oxygen species and antioxidants

Active oxygen species such as hydroxyl radicals, singlet oxygen, superoxide and hydrogen peroxide, will primarily occur in peroxisomes, mitochondria and chloroplasts. In the latter, the Mehler reaction and other antioxidant processes will operate to nullify active oxygen species produced by PET (see Fig. 1.3). In the Mehler reaction, superoxide produced by direct electron transfer from PSI to oxygen will be converted to hydrogen peroxide and then reduced to water by a series of steps involving ascorbate as electron donor. Glutathione is used in regeneration of ascorbate and glutathione itself is regenerated with the help of NADPH (see Fig. 1.3).

Active oxygen species, and in particular hydrogen peroxide have been implicated in a number of signalling events including wounding and pathogen attack, and recently systemic signalling of excess excitation energy (reviewed in Mullineaux et al. (2000). However, their potentially deleterious effects mean that they have to be tightly controlled. Antioxidant systems scavenging active oxygen species appear to normally operate with redundancies: When catalase activity (processing hydrogen peroxide without the use of ascorbate) is reduced by several means this does not result in increased foliar hydrogen peroxide; chloroplasts are capable of rapidly detoxifying hydrogen peroxide of external origin (see Foyer and Noctor (2000) and therein for details).

The importance of antioxidants is shown by their abundance: ascorbate and glutathione are found in mM concentrations in foliar tissue, with ascorbate concentrations being about 10-fold higher, making ascorbate one of the most abundant metabolites representing up to 10% of the carbon pool (Smirnoff, 2000). Antioxidant systems are found in the cytosol as well as in cellular compartments and the apoplast. Some systems are limited to certain compartments, for examples is catalase absent from chloroplasts (Foyer and Noctor, 2000). Others operate in different compartments as different isoforms, e.g. ascorbate peroxidase which has five isoforms in Arabidopsis (Mullineaux

et al., 2000). Ascorbate peroxidase in its different isoforms and catalase appear to act co-operatively to remove hydrogen peroxide in leaves ensuring maximal hydrogen peroxide destruction at a minimal cost in terms of reducing power (Foyer and Noctor, 2000).

1.3.2 Plastid redox state regulates plastid enzyme activities

Already in 1981 Buchanan and colleagues (Nishizawa and Buchanan, 1981) demonstrated that ferredoxin reduces thioredoxin, which in turn regulates the activity of enzymes in the stroma by breaking disulphide bridges (summarised in Buchanan (1984)). Our knowledge about plastid enzyme regulation via the redox state of thioredoxin has improved considerably since then (see figure 1.4 and Foyer and Noctor (2000), although the mechanism by which this transfer of electrons from ferredoxin to thioredoxin is accomplished has only recently been identified (Dai et al., 2000). Enzymes, whose activity is regulated by redox state, for example by the breaking of disulphide bridges by reduced thioredoxin, include the Calvin cycle enzymes seduheptulose-1,7-bisphosphatase and glyceraldehyde-3-phosphate dehydrogenase. When the level of thioredoxin regulated enzymes such as seduheptulose-1,7-bisphosphatase or glyceraldehyde-3-phosphate dehydrogenase was decreased by antisense technology (Raines et al. (1999) and Ruuska et al. (2000), respectively), the remaining low level of enzyme could often compensate for the loss due to the highly regulated nature of these enzymes. This demonstrates the tight coupling of the activity of Calvin cycle enzymes with light absorption and the redox state of the plastid.

1.3.3 Translation of plastid-encoded transcripts is modulated by the redox state

The translation of mRNA in the chloroplast was shown to be modulated by the plastid redox state in work reported by Mayfield and co-workers (Danon and Mayfield, 1994). The group went on to identify redox control by a protein disulphide isomerase acting on the chloroplast polyadenylate-binding protein (cPABP) essential for mRNA translation (Kim and Mayfield, 1997). The importance of the plastid redox conditions for the translation as well as the assembly of the D1 protein of PSII was demonstrated using

various thiol reagents (Zhang et al., 2000). Progress in the understanding of the regulation of plastid translation was reviewed recently (Bruick and Mayfield, 1999).

1.3.4 In cyanobacteria the redox state controls gene transcription

In cyanobacteria it appears that the expression of the *psbA* gene coding for the D1 core protein of PSII is modulated by redox factors which themselves depend on PET (Alfonso et al., 2000; Sippola and Aro, 2000). However, which factor is the determining redox factor in this regulation is still unclear (Alfonso et al., 2000; Sippola and Aro, 2000).

1.3.5 Plastid encoded photosynthetic gene expression in plants is controlled by redox state

In higher plants, redox control of photosynthetic genes encoded in the plastid, such as *psbA* has been suggested (Pfannschmidt et al., 1999). Models for such a regulation by the redox state of PET chain components or the plastid redox state in general have been put forward (Link, 1996). Plastid-encoded reaction centre proteins for both photosystems have recently been reported to be regulated at the transcriptional level by the redox state of the PQ pool (Pfannschmidt et al., 1999). The regulation of a kinase determining the transcription rate of plastid encoded genes such as *psbA* was also recently suggested to be controlled by the redox state of glutathione (Baginsky et al., 1999).

Taken together the reports indicate the importance of the plastid redox state in the regulation of normal chloroplast function at many levels.

Figure 1.2: A cartoon depicting the photosynthetic electron transport chain and the sites of inhibitor action

The two photosystems are linked by the plastoquinone (PQ) pool, the cytochrome b_6/f complex (Cyt. b_6/f) and plastocyanin (PC). PSII, the PQ pool, the cytochrome b_6/f complex and the ferredoxin (Fd) –thioredoxin (TRX) system at the downstream end of PSI have all been implicated as the source of the plastid redox signal (black arrows). The inhibitor DCMU blocks the electron transport from Q_A to Q_B, while DBMIB blocks the flow of electrons from the PQ pool to the cytochrome b_6/f complex (red arrows). The sugar supply status of the cell could alter the Calvin cycle activity and this in turn could also result in n alteration of the redox state of PET as indicated by the yellow arrow.

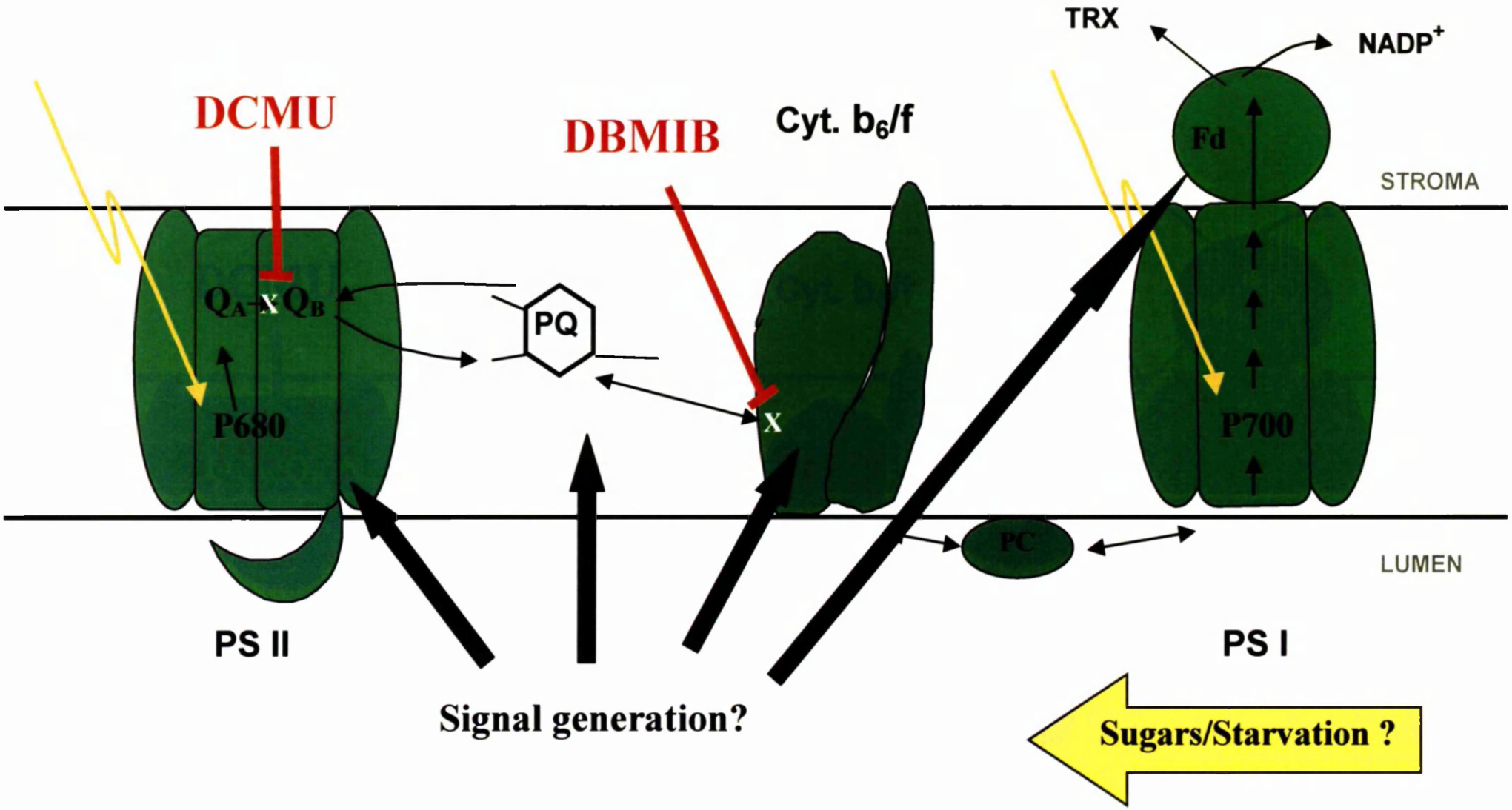

TRX
NADP+
DCMU
DBMIB
Cyt. b6/f
Fd
STROMA
QA
X
QB
PQ
P680
X
P700
PC
LUMEN
PS II
PS I
Signal generation?
Sugars/Starvation ?

Figure 1.3: The Mehler reaction scavenges active oxygen species produced at PSI

The water-water cycle in chloroplasts, showing photoreduction of dioxygen to generate superoxide, disproportionation of superoxide to hydrogen peroxide, reduction of hydrogen peroxide to water and the associated reduction of oxidised ascorbates to ascorbate, and microcompartmentation of the participating enzymes. APX, ascorbate peroxidase; Asa, ascorbate; Cu, Zn-SOD, copper-zinc superoxide dismutase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; Fd, ferredoxin; FR, Fd-$NADP^+$ reductase; GR, glutathione reductase; MDA, monodehydroascorbate radical; MDAR, MDA reductase; sAPX, stromal APX; SF, stromal factor for enhanced photoreduction of dioxygen; tAPX, thylakoid-bound APX; VDE, violaxanthin de-epoxidase; O_2^- = superoxide

The graphic is taken from Asada 2000.

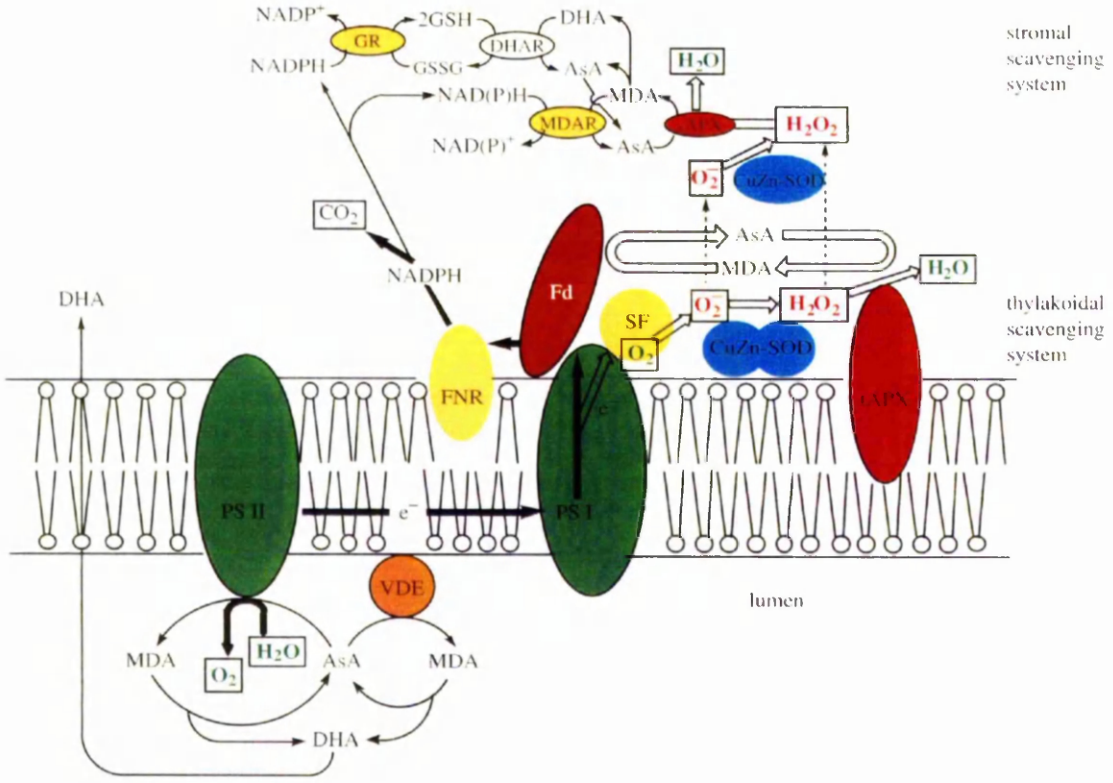
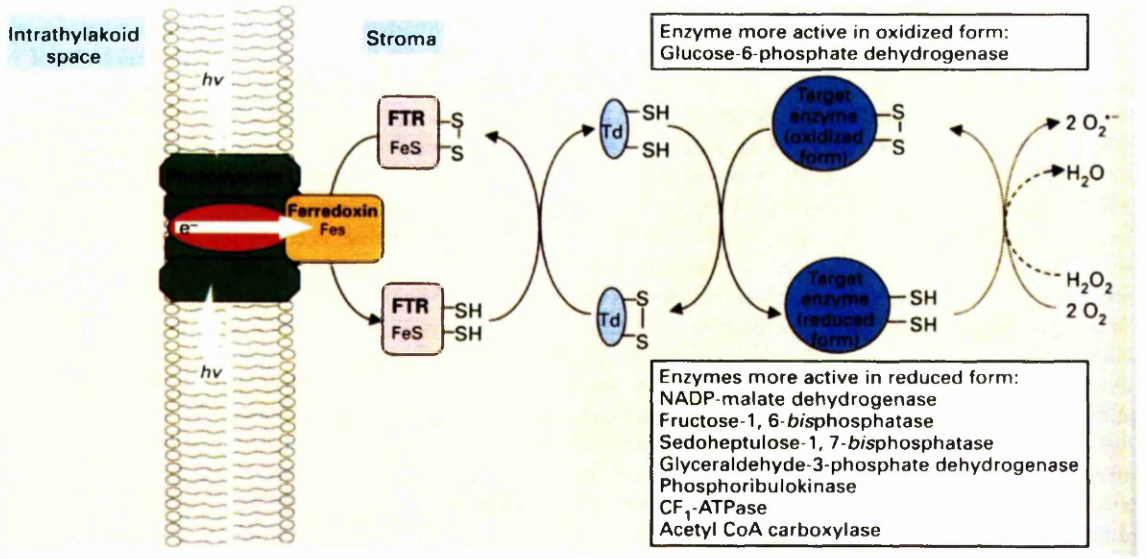

NADP+
GR
2GSH
DHAR
DHA
NADPH
GSSG
AsA
NAD(P)H
MDAR
MDA
NAD(P)+
AsA
H2O
APX
H2O2
O2-
CuZn-SOD
stromal scavenging system
CO2
NADPH
Fd
AsA
MDA
H2O
DHA
SF
O2-
O2
H2O2
CuZn-SOD
thylakoidal scavenging system
FNR
cytb6f
tAPX
PS II
e−
PS I
VDE
lumen
MDA
H2O
O2
AsA
MDA
DHA

Figure 1.4: The ferredoxin-thioredoxin system

When PET is active ferredoxin receives electrons from PSI, which it transfers to one of its targets. One of these is thioredoxin, and the enzyme involved is called ferredoxin:thioredoxin reductase (FTR). The reduced thioredoxin in turn can reduce the disulphide bridges on plastid enzymes, altering their activity. Oxygen or hydrogen peroxide can oxidise the reduced target enzymes and reverse the action of thioredoxin. However, the reduced thioredoxin pool is continuously replenished in the light.
The list of target enzymes is not intended to be exhaustive. $O_2^{\bullet-}$ = superoxide
The graphic is taken from Foyer and Noctor 2000.


Intrathylakoid space
Stroma
hv
hv
FTR
FeS
Ferredoxin
Fes
e⁻
FTR
FeS
SH
SH
Td
SH
SH
Td
S
S
Target enzyme (oxidized form)
Target enzyme (reduced form)
SH
SH
2 O2•⁻
H2O
H2O2
2 O2
Enzyme more active in oxidized form:
Glucose-6-phosphate dehydrogenase
Enzymes more active in reduced form:
NADP-malate dehydrogenase
Fructose-1, 6-bisphosphatase
Sedoheptulose-1, 7-bisphosphatase
Glyceraldehyde-3-phosphate dehydrogenase
Phosphoribulokinase
CF1-ATPase
Acetyl CoA carboxylase

1.3.6 Redox signals appear to affect nuclear gene expression in algae and higher plants

The existence of a so-called 'plastid signal' from the functional chloroplasts to the nucleus has been claimed for some time now (Burgess and Taylor, 1988; Taylor, 1989; Allen et al., 1995; Durnford and Falkowski, 1997; Durnford et al., 1998; Somanchi and Mayfield, 1999). Some evidence for such a signal has come from the disruption of caretonoid biosynthesis, either by mutation or inhibitors such as Norflurazon (Batschauer et al., 1986; Susek et al., 1993). Most evidence for such a signal however was discovered by studying photoacclimation, predominantly in algae (Maxwell et al., 1995; Escoubas et al., 1995; Huner et al., 1996; Savitch et al., 1996).

The plastid redox state has been suggested to affect mRNA stability of nuclear-encoded genes (Maxwell et al., 1995; Escoubas et al., 1995; Huner et al., 1996; Savitch et al., 1996; Petracek et al., 1998; Kropat et al., 2000). In higher plants nuclear genes reported to be regulated by the plastid redox state include cytosolic ascorbate peroxidase (Karpinski et al., 1997), early light induced proteins (Montane et al., 1998) and heat shock protein 70 (Kropat et al., 2000). However, *CAB* expression in barley leaves was suggested not to be regulated by the redox state of PSII or intersystem electron transport components (Montane et al., 1998). The regulation of transcription of nuclear-encoded photosynthetic genes such as *CAB* and *RBCS* in higher plants by the redox state of PET had not been proven prior to this PhD project. However studies of algae provided evidence for such a regulation by the redox state of PET at least in these organisms (Maxwell et al., 1995; Escoubas et al., 1995; Huner et al., 1996; Savitch et al., 1996).

1.3.7 Organelle-nuclear signalling

Co-ordinating the regulation of biogenesis and operation of organelles such as plastids and mitochondria is likely to involve a substantial amount of crosstalk between the organelle and nucleus. An example for this kind of crosstalk is the redox regulation of the mammalian transcription factor GABP regulating the expression of cytochrome c oxidase subunits IV and Vb, as well as the expression of mitochondrial transcription factor 1 (Martin et al., 1996). The biogenesis of plastids requires signalling from the nucleus to the plastid, although in turn some feedback from the plastid to the nucleus appears to be also required (summarised in Somanchi and Mayfield (1999)).

Inhibitor studies have indicated that protein phosphatase activity of the PP1 type might be involved in plastid to nucleus signalling (Sheen, 1993; Fujiki et al., 2000). Other groups recently suggested chlorophyll precursors or enzymes involved in their production could act in plastid-to-nucleus signal transduction (Kropat et al., 2000; Vinti et al., 2000; Mochizuki et al., 2001). With respect to signalling of the PET redox state, Karpinski and co-workers suggested a role for reduced glutathione in the signal transduction process (Karpinski et al., 1999). However, it is fair to say that the nature of the signal or signals linking the plastid to nuclear functions is still poorly understood.

1.3.8 Interaction with other signalling pathways

Not surprisingly, the plastid-to-nucleus signalling has been suggested to interact with other signalling processes in plants, although the evidence for a complex web of interactions similar to that for sugar signalling has not been demonstrated. Such evidence has mainly been achieved with respect to interactions with phytochrome signalling. Vinti and co-workers studied the *gun* and *hy1* mutants and suggested a signal pathway from the plastid to the nucleus shared with the phytochrome signal pathway, but also an additional pathway not shared with the phytochrome pathway (Vinti et al., 2000). Weston and colleagues found little influence of blue light receptors on light acclimation in Arabidopsis (Weston et al., 2000). Studying the *CAB underexpressed* (*cue*) mutants with respect to de-repression of *CAB* by phytochrome activation, a group around J.Chory showed that most mutants were compromised in this response (Lopez-Juez et al., 1998).

1.4 Conclusions

1.4.1 Summary

Metabolic regulation of nuclear gene expression by sugars has been recognised as an important means for most organisms to adjust to their carbon supply status. In plant cells, sugars regulate a large number of genes, including photosynthetic genes encoded in the nucleus. These are repressed in the presence of sugars and induced in their absence.

Over the last few years, evidence has been accumulated for a signal connecting the redox state of the plastid with the nucleus. In unicellular algae, a signal originating from the redox state of the PET chain appears to act on nuclear-encoded genes for photosynthesis such as *CAB,* which in higher plants are known to be regulated by sugars. PET provides the Calvin cycle with NADPH and ATP required for carbon fixation, which results in triosephosphate export to the cytosol and subsequent formation of sugars from triosephosphates (see figure 1.5). A possible link between sugar signalling and plastid redox signalling could exist in some form of feedback mechanism from sugars to the Calvin cycle and in turn to the redox state of PET (Fig. 1.5). In support of this hypothesis the triosephosphate-phosphate-transporter of the chloroplast membrane has been shown to limit maximum rates of photosynthesis and sucrose formation (Hausler et al., 2000). However, even if a plastid redox signal that interacts with the sugar signalling pathway exists in higher plants this does not necessarily mean that the interaction is via a simple feedback mechanism. Possible crosstalk between the different pathways could occur at any one of a number of levels of control, from metabolic effects in the plastid or cytosol to effects on transcription factors in the nucleus.

1.4.2 Project aims

The objectives of this PhD project were:

1. To establish a model system suitable to study sugar signalling and plastid redox signalling in plants.
2. To elucidate whether a signal generated by the redox state of PET components exists in higher plants which modulates a sugar-related signal, or vice versa, with respect to nuclear-encoded photosynthetic gene expression.
3. If such a mechanism was shown to exist the further aim was to obtain evidence about the origin of the plastid redox signal and the mode of interaction with the sugar signal.

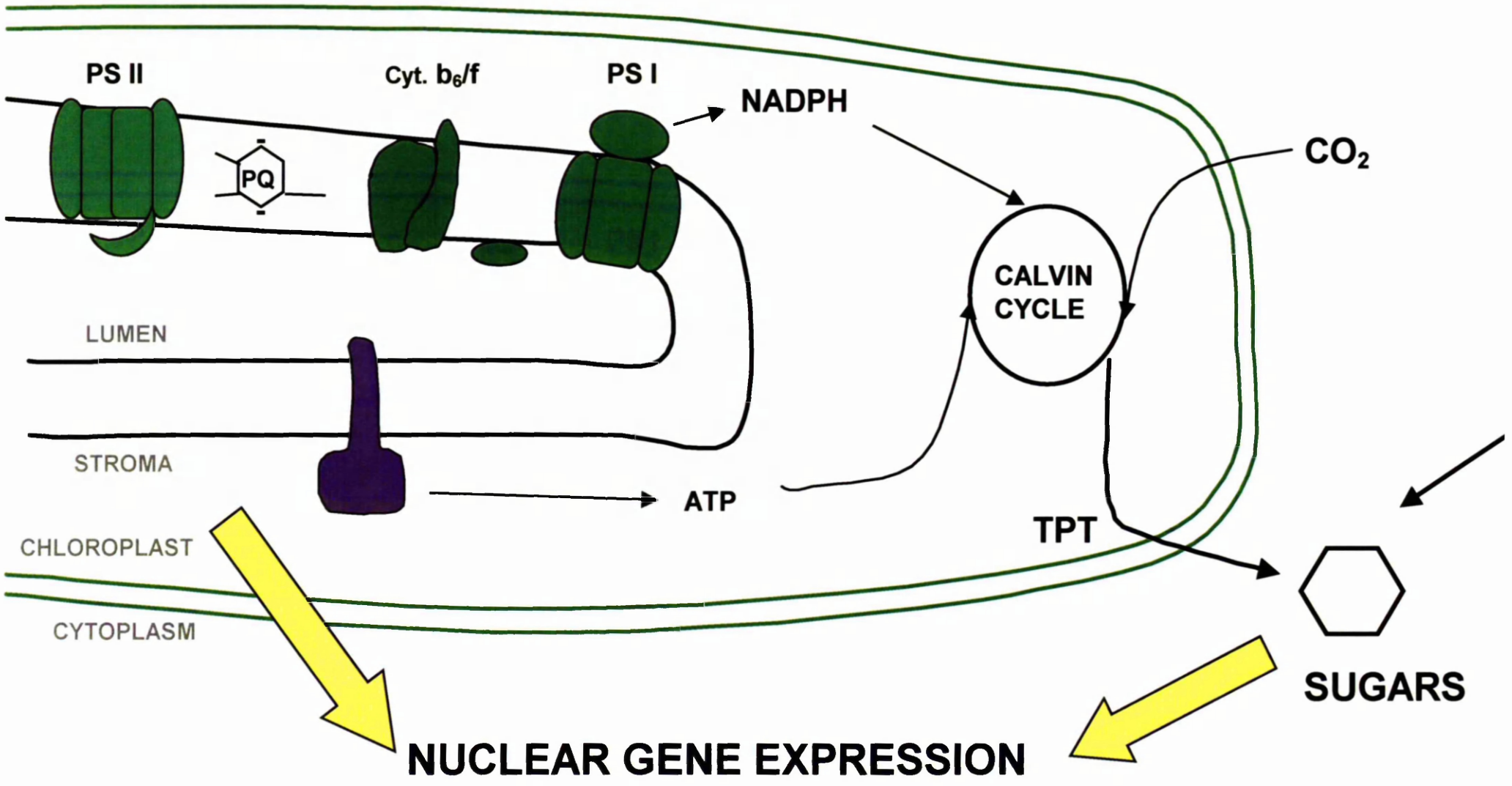
An Arabidopsis cell suspension culture system (May and Leaver, 1993) was characterised with respect to sugar and PET redox signalling and found to be a suitable model system. Using this experimental system a signal generated by the plastid redox state was found to interact with sugar signalling modulating nuclear encoded

photosynthetic gene expression. These findings were subsequently confirmed *in planta*. The results of this research have been published recently (Oswald, O., Martin, T., Dominy, P. J. and Graham, I. A. 2001: Plastid redox state and sugars: Interactive regulators of nuclear gene expression. *Proc.Natl.Acad.Sci.USA* **98:** 2047-2052). Finally, attempts were made to clarify at what level the interaction between the two signals occurs.

Figure 1.5: Model showing a possible link between sugar and plastid redox signals

PET provides NADPH and ATP for the Calvin Cycle. Carbon dioxide is fixed in the Calvin Cycle and results in the export of triosephosphates. Triosephosphates are combined to produce sugars in the cytosol.
Both the redox state of the PET chain and cytosolic sugars generate a signal to the nucleus. The Calvin cycle could provide a link between the two sources of signals.

PS	Photosytem
Cyt. b_6/f	cytochrome b_6/f complex
PQ	plastoquinone pool
TPT	Triosephosphate-phosphate-translocator


PS II
Cyt. b6/f
PS I
NADPH
CO2
PQ
CALVIN CYCLE
LUMEN
STROMA
ATP
TPT
CHLOROPLAST
CYTOPLASM
SUGARS
NUCLEAR GENE EXPRESSION

Chapter 2: Materials and Methods

2.1 Biological material

2.1.1 Plant material

Arabidopsis thaliana (L) Heynh. var. Landsberg erecta photomixotrophic cell suspension culture growing with 3% (w/v) sucrose were obtained from May and Leaver (University of Oxford, Oxford, United Kingdom) (May and Leaver, 1993). Over the period of 12 months the culture was used to acclimatise cultures to growth on 1%, 0.8%, 0.6% and 0.4% (w/v) sucrose.

Arabidopsis thaliana (L) Heynh var. Columbia seeds carrying the *CAB2* promoter – luciferase reporter gene and CaMV35S promoter – luciferase reporter gene constructs (*CAB2-LUC* and *35S-LUC* lines, respectively) were donated by Dr. Andrew Millar (University of Warwick, Warwick, United Kingdom). The *Arabidopsis thaliana* (L) Heynh var. C24 seeds carrying the plastocyanin promoter – luciferase reporter gene (*PC-LUC*) and seeds of the *sucrose uncoupled* 6 (*sun*6) were obtained from Dr. Sjef Smeekens (University of Utrecht, Utrecht, The Netherlands). Dr. Hoai-Nam Truong kindly provided seeds of the *Arabidopsis thaliana* (L) Heynh var. Columbia carrying the tobacco nitrite reductase promoter – luciferase reporter gene construct (*Nii-LUC* line).

2.1.2 cDNA clones

2.1.2.1 Expressed Sequence Tags (ESTs)

Nitrate reductase Clone 160F3T7

RuBisCo Clone ID 33G4T7

These EST were obtained from the Arabidopsis Biological Resource Centre (Ohio State University).

The identity of all ESTs was verified by sequencing.

2.1.2.2 Other cDNAs

A genomic clone for the Arabidopsis chlorophyll *a/b* binding protein (Lhcb1*1) in plasmid pAB165 propagated in *E. coli* JM103 (Leutwiler et al., 1986)

A cDNA for chalcone synthase (pCHS) in *E.coli* DH5-α (Trezzini et al., 1993)

ARF cDNA fragment cloned into pBS SK^- in XL1-Blue (Regad et al., 1993)

EF-1α cDNA fragment cloned into pBS SK^- in TG1 (Axelos et al., 1989)

PsbA in TOP10 (see section 2.2.7)

The identity of all cDNA was verified by sequencing.

2.1.3 Antibodies

Anti-Digoxigenin AP Fab fragment (Boehringer Mannheim)

Anti-Digoxigenin-POD Fab fragment (Boehringer Mannheim)

Anti-14-3-3-antibody, polyclonal; produced in sheep against α 14-3-3 of spinach, third bleed (kindly provided by Dr. C. MacKintosh; as in Moorhead et al. (1996))

Anti-sheepAb-antibody linked to peroxidase from goat (Boehringer Mannheim)

2.1.4 Chemicals

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

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

BDH Chemicals, Poole, Dorset, U.K.

Enzymes and Digoxigenin detection reagents and antibodies 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 and techniques of the cell culture work

2.2.1 Cell culture growth conditions

Cell suspension cultures were initiated by May and Leaver (May and Leaver, 1993). Cultures were grown as described in (Christie and Jenkins, 1996), except that Murashige-Skoog medium with minimal organics was used. The growth conditions were:

	Condition	Supplier/Equipment
Culture medium	Murashige-Skoog medium with minimal organics (Linsmaier and Skoog, 1965), pH 5.8	Sigma
	Plus 30g/l sucrose (3% w/v)	Sigma
Hormones	0.5 mg/l α-Naphtalene acetic acid (NAA)	Sigma
	0.05 mg/l Kinetin	Sigma
Culture vessel	200 ml culture in 500 ml conical glass flask, 100 ml culture in 250 ml conical glass flask	Fisher
	Sealed with cotton buns and aluminium foil	
Shaking	130 rpm on an orbital shaker	Orbital Platform Shaker Model Labotron TR-112 (Fisher)
Light	Continuos white light of 20μmol photons m^{-2} s^{-1} Photosynthetically Active Radiation (PAR)	Warm white fluorescent tubes (Osram, München, Germany)
Temperature	22°C	Temperature controlled room, Sanyo Gallenkamp , Osaka, Japan
Subculturing	Every 7 days 1/10 of establish culture added to 9/10 fresh medium	

Back-up callus cultures were grown in culture media supplemented with 0.8 % (w/v) agar (Sigma) and subcultured to fresh growth media every 3-4 weeks.

2.2.2 Fresh weight measurements

Either of the two methods was used:

1. Larger volumes of culture were harvested using a scintered glass plate and Buchner flask connected to a vacuum pump (KNF Neuberger GmbH, Freiburg Germany). Cells were washed once with an equal volume of de-ionised water, dried under vacuum suction for 30 s and transferred onto pre-weighed pieces of liquid nitrogen cooled aluminium foil. After wrapping the sample was frozen in liquid N_2 and the weight determined while the sample was kept frozen. Samples were stored at –70°C until further use. This method took approximately 3 minutes from taking the culture of the shaker to the frozen sample.
2. 1 ml aliquots of cell culture were transferred into pre-weighed 1.5 ml Eppendorf tubes. To avoid any blocking during the aspiration, the pipette tips used were cut to give a tip opening of at least 5 mm diameter. The cells were allowed to settle in the Eppendorf tube. The liquid was carefully removed with a Pasteur pipette. If sugar assays were to be performed cells were washed twice with 4°C cold de-ionised water, and cells allowed to settle between washes. When the supernatant had been removed, the remaining liquid in the cell pellet was removed by carefully inserting the tip of the Pasteur pipette to the bottom of the tube and thoroughly aspirating the pellet which resulted in a change in colour to light green.
   Samples were frozen and stored at –20°C or –70°C. The weight of the Eppendorf tube containing the sample was taken before or after freezing and the fresh weight determined. This method took between 5 and10 minutes from taking the sample until they were placed in the freezer (depending on the number of samples taken in parallel).

Method 1 was employed at first. However, it required larger sample volumes. Method 2 was devised and tested. The impact of the harvesting procedure on levels of sugars and chlorophyll is negligible.

2.2.3 Isolation of total RNA

The method described by Kay and colleagues (Kay et al., 1987) was used. All solutions, except Tris, were treated with 0.1 % diethylpyrocarbonate (DEPC) to destroy RNase activity and 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, ca. 1M β–mercaptoethanol, pH 8.0) was added to the frozen powder in the mortar and was ground into the plant material. 900 µl of PCI (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 PCI was added. After vortexing the tube 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 400 µl of DEPC-treated H_2O. 0.1 volumes of 3M sodium acetate (pH 5.5) and 2.5 volumes of ethanol were added to precipitate the RNA. The sample 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 re-dissolved in a minimal volume of DEPC-treated H_2O and allowed to re-hydrate on ice for 3-4 h.

Alternatively, total RNA was isolated using the Qiagen RNAeasy Plant Kit (Qiagen, Crawley, West Sussex, UK) following the manufacturer's instructions.

The quantity and purity of the RNA was determined spectrophotometrically by measuring 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 absorbance at 260 nm to 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.

2.2.4 Denaturing agarose gel electrophoresis of RNA

Samples of 5 or 7 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 10 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 (Sambrook et al., 1989).

2.2.5 Plasmid DNA isolation

A sterile yellow pipette tip was used to remove a small sample of the frozen glycerol stock and to spread it on LB agar plates (1% (w/v) Bacto Tryptone, 0.5% (w/v) Bacto yeast extract, 1 % (w/v) NaCl, pH 7.5 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.
A single colony was picked from the selection plate (see above and section 2.2.7) 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 (Sigma). 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.

2.2.6 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. 1 % (w/v) agarose gels were prepared and run in 1 x TBE (89 mM Tris-borate, 89 mM Boric acid, 2 mM EDTA, pH 8.3) containing 0.25 μg/ml ethidium bromide. Samples of DNA or RNA were mixed with 6 X loading buffer (0.025 % (w/v) bromophenol blue and 40 % (v/v) sucrose in water (Sambrook et al., 1989)) 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.

2.2.7 Cloning of Arabidopsis *PsbA*

Bacteriophage P1 (Liu et al., 1995) clones MAB17, MCI3 and MAH2 of *Arabidopsis thaliana* plastid genomic DNA were obtained from Dr. Satoshi Tabata of the Kazusa DNA Research Institute, Yana, Kisarazu, Japan. Primers specific for the *PsbA* gene were designed using the 'Primer3' software (Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, USA). The nucleotide sequences of the primers were:

Primer PSBA-14: 5' TAG ATG GAG CCT CAA CAG CA 3'

Primer PSBA –910: 5' TCA TTG CTG CTC CTC CAG TA 3'

Note: The number indicates the base number of the *PsbA* sequence at which the primer starts.

Primers were synthesised by MWG Biotech (Ebersberg, Germany). *PsbA* was amplified by the polymerase chain reaction (PCR) (Mullis and Faloona, 1987) from the clone MCI3 using the following procedure:

The 50 µl reaction mix was comprised of 5 µl 10 X PCR buffer (500 mM KCl, 100 mM Tris-Cl, pH 8.4, 15 mM $MgCl_2$, 200 µg/ml gelatine), 5 µl $MgCl_2$ 25mM, 1 µl PSBA-14 primer (20pmol/µl), 1 µl PSBA--910 primer (20pmol/µl), 5 µl 2 mM dNTP, 0.3 µl Taq polymerase (Promega), 32.7 µl water. A sterile yellow tip was dipped into the chosen bacterial colony on the selection plate and used to stir the reaction mix strongly. The reaction mix was covered with a layer of Chill-Out 14 liquid wax (MJ Research Inc., Waltham MA, USA) (25 µl) to reduce evaporation. The reaction was performed on a thermocycler (Perkin Elmer Gene Amp PCR System 2400). The reaction conditions were:

	94°C	2 minutes
followed by 30 cycles of:	94°C	30 seconds
	55°C	30 seconds
	72°C	60 seconds
followed by	72°C	10 minutes

The success of the PCR reaction was checked by electrophoresis on a 1.0 % agarose gel buffered by TBE (see section 2.2.6).

PsbA was subcloned into the pCR2.1 TOPO vector and was transformed into *E.coli* TOP 10 using the TOPO TA cloning Kit (Invitrogen, Groningen, the Netherlands) according to the manufacturer's instructions. Successful clones were isolated on LB agarose plates (see 2.2.5) with 100 µg/ml ampicillin and 50 µg/ml X-Gal (Sigma) and confirmed by PCR and sequencing of the insert in plasmids isolated as described in section 2.2.5. Glycerol stocks were initiated following the instruction in (Sambrook et al., 1989).

2.2.8 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 or 1 % (w/v) agarose gel buffered with 1 x TBE TBE (89 mM Tris-borate, 89 mM Boric acid, 2 mM EDTA, pH 8.3). 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^{TM} 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 synthesised, the probe was denatured by heating to 95-100°C for 5 minutes followed by chilling on ice before adding to the hybridisation solution.

2.2.9 Preparation of DIG labelled probes

The polymerase chain reaction (Mullis and Faloona, 1987) was used to specifically amplify fragments of cDNA or genomic clones for use as probes for hybridisation using the Digoxigenin (DIG) labelled nucleotides system. The 50 µl reaction mix was comprised of 5 µl 10 X PCR buffer (500 mM KCl, 100 mM Tris-Cl, pH 8.4, 15 mM $MgCl_2$, 200 µg/ml gelatine), 5 µl $MgCl_2$ 25mM, 1 µl T7 primer (20pmol/µl), 1 µl T3 primer (20pmol/µl), 5 µl 2 mM dNTP, 2U Taq, 32.7 µl water plus 25 ng plasmid DNA (see section 2.2.5). The reaction mix was covered with a layer of Chill-Out 14 liquid wax (MJ Research Inc., Waltham MA, USA) (25 µl) to reduce evaporation. The

reaction was performed on a thermocycler (MJ Research Inc., Waltham MA, USA). The reaction conditions were:

	94°C	2 minutes
followed by 30 cycles of:	94°C	30 seconds
	50°C	60 seconds
	72°C	120 seconds
followed by	72°C	10 minutes

The success of the PCR reaction was checked by electrophoresis on a 1.0 % agarose gel buffered by TBE. The concentration of amplified fragment was determined spectrometrically by measuring the absorbance at 260 nm and 280 nm. An $A_{260\ nm}$ of 1 is equivalent to an DNA concentration of 50 µg/ml.
The fragment obtained this way was then labelled with DIG by random priming using the DIG DNA Labelling Kit ('DIG-High Prime', Boehringer) according to the manufacturer's instructions.

In some cases the PCR reaction itself was used to introduce DIG label. For this purpose the PCR DIG Probe synthesis kit (Boehringer Mannheim) was used. The solutions listed above were substituted with the solutions provided by the kit's manufacturer according to the manufacturer's protocol. The same primers (T7 and T3) were used, and the same reaction conditions as listed above. The success of the PCR reaction was checked by electrophoresis on a 1.0 % agarose gel buffered by TBE (see section 2.2.5).

For amplification of the *CAB2* DNA the DIG labelling kit was used, but with the following modifications:
Due to the long fragment of genomic CAB2 DNA (>1 kb) inserted into the plasmid, the PCR reaction mix was altered as recommended by the kit's manufacturer. The concentration of DIG labelled UTP was reduced by replacing some of the nucleotide mix with a mix of unlabelled nucleotides containing TTP instead of DIG-UTP. 2.5 µl M13-20 primer (10pmol/µl) and 2.5 µl M13 reverse primer (10pmol/µl) were used instead of T7 and T3 primers. The volume of water was adjusted accordingly.

The PCR reaction conditions were as follows:

	94°C	2 minutes
followed by 30 cycles of:	94°C	45 seconds
	41°C	45 seconds
	72°C	180 seconds
followed by	72°C	10 minutes

The success of the PCR reaction was checked by electrophoresis on a 1.0 % agarose gel buffered by TBE (see section 2.2.5).
The effectiveness of the DIG labelling process was assessed using the DIG teststrips (Boehringer) following the manufacturer's instructions.

2.2.10 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 and Konica UK, Feltham, UK) 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).

2.2.11 Hybridisation using the DIG detection method

Hybridisation of the mRNA blots with DIG labelled probes and development of chemiluminescent images were performed according to the manufacturer's instructions. DIG EasyHyb , anti-DIG AP Fab fragment and the chemiluminescent substrate CSPD were used (all from Boehringer). DIG labelled probes were transferred into 50ml Falcon tubes and stored at –20°C for further use. Konica x-ray film (Konica UK, Feltham, UK) was used to record the chemiluminescence. Typical exposure times were between 45 and 120 minutes at 37°C. Film images were scanned using an Epson Perfection 1200S

scanner (EPSON (UK) Ltd., Hemel Hempstead, UK). For repeated hybridisation the mRNA blots were stripped of the previous probe using the SDS method described by the kit's manufacturer before the hybridisation procedure was repeated.

2.2.12 Sucrose, glucose and fructose measurements

Samples for sugar assays were harvested and their fresh weight determined as described in paragraph 2.2.2. The aliquots in Eppendorf tubes were 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 re-dissolved in 50 µl H_2O. Metabolites were measured using enzyme-linked assays according to (Stitt et al., 1989).

2.2.13 Induction of cell culture starvation or feeding different sugars to 3 d cultures

Suspension cultures in the early exponential phase (3-days-old) were washed three times with equal volumes of medium without sucrose using a 100 µm sieve. They were re-suspended to the original cell density in medium without sugar or washed once with medium with 3% (w/v) sucrose and re-suspended in medium with 3% (w/v) sucrose. Aliquots of 8ml were transferred into 25 cm^3 tissue culture flasks and put back into the growth room but shaken at 80 rpm. Samples were taken both before and after the wash, and at the times indicated. Samples were harvested as described in section 2.2.2.

2.2.14 Chlorophyll measurements

The method described by (Wintermans and Mots, 1965; Leutwiler et al., 1986) was adapted as follows: 95% ethanol was added to samples depending on the amount of sample to be extracted. Approximately 1 µl ethanol was added to the Eppendorf tube per mg sample present. The samples were vortexed very well and stored at 4°C in the dark for at least 3 to 4 hours, more typically overnight. Cells were centrifuged (Microcentaur, 13,000 rpm, 4°C) and all or some of the supernatant transferred into a new Eppendorf tube avoiding the transfer of any particles. The extracts were centrifuged again to remove any remaining particles. To determine the quantity and

nature of chlorophylls 60 or 100 µl of the extracts were measured spectrometrically. The absorbance at 649 nm, 654 nm and 665 nm was taken. From these readings total chlorophyll, chlorophyll *a* and chlorophyll b content was calculated as follows:

Chl. a = 13.7 x A_{665} – 5.76 x A_{649} (µg/ml extract)

Chl. b = 25.8 x A_{649} – 7.6 x A_{665} (µg/ml extract)

Total chlorophyll = 20.4 x A_{649} + 6.1 x A_{665} (µg/ml extract)

As a control, chlorophyll *a* plus b can be calculated as 25.1 x A_{654} (µg/ml extract)

2.2.15 Chlorophyll *a* fluorescence

When photons are absorbed by PSII, the energy can take one of several paths. It can be used in the reaction centre to dissociate an electron and result in reduced Q_A. The reaction centre quickly regains an electron from the water splitting complex. If this process cannot happen (for example, because Q_A is already reduced), or not quickly enough, the energy is either dissipated as heat (non-photochemical quenching) or re-emitted as photons of a longer wavelength (in the case of PSII around 680 nm). Either of the two mechanisms serves to minimise other, less favourable reactions leading to reactive molecules such as reactive oxygen species. The re-emission of photons by the chlorophyll *a* of the PSII reaction centre within a fraction of a second is termed chlorophyll *a* fluorescence. It can be taken as an indicator of the reduction state of Q_A: the more Q_A are reduced, the more PSII reaction centres will re-emit absorbed energy as chlorophyll *a* fluorescence (Papegorgiou, 1975; Bradbury and Baker, 1981; Duysens et al., 1995). Measuring the chlorophyll a fluorescence associated with a certain absorbed light quality and quantity will reflect the reduction state of PSII under the given conditions (Bradbury and Baker, 1981). Since unloading of electrons from PQ is considered the rate-limiting step in PET, the reductions state of Q_A as measured by chlorophyll fluorescence can be taken as a measure of the reduction state of the PQ as well.

Since the inhibitor of PET DCMU blocks transfer of electrons from Q_A to Q_B, it will result in fully reduced Q_A, and hence maximal chlorophyll *a* fluorescence.

Fig. 2.1 explains the experimental procedure in detail. 1 or 3 ml of culture were transferred into a quartz cuvette of the appropriate size containing a small stirring bar.

The cuvette was placed in the thermostatted (22°C) holder of the instrument (Perkin Elmer LS-50 luminometer operating in the prompt fluorescence time base mode), where the cells were stirred in darkness for at least 150 s (darkness is indicated by a black bar in Fig. 2.1). Once the cells had been in the dark for the given period, the weak excitation beam (440nm) was turned on. This will lead to enough chlorophyll *a* fluorescence from PSII to be recorded yet is sufficiently weak not to reduce Q_A significantly.

The instrument's settings were as in (Rouag and Dominy, 1994) with the following modifications: excitation 440nm, 2.5nm slit width delivering 0.5 μmol m^{-2} s^{-1} onto the cuvette face; emission recorded at 685nm.

The cells were kept in complete darkness bar the excitation beam for another 300 s. The blue line in Fig. 2.1 represents the emitted chlorophyll *a* fluorescence measured every second or half of a second. Once the fluorescence had settled after the excitation beam had been switched on, the average of the values measured was taken as the F_0 value as indicated in Fig.2.1. When the 300 s had passed, the cells were then exposed to 20 μmol m^{-2} s^{-1} PAR (unless indicated otherwise) via a fibre optic (yellow bar in Fig. 2.1). Actinic light intensities were attenuated with Balzers' neutral density filters (Lichtenstein) and measured using a Li-Cor LI-190SB (Lincoln, NE, USA) quantum sensor. This resulted in an increase in fluorescence to a new level indicating the percentage of Q_A now reduced by the light. When the fluorescence had reached a steady level the average of the measured values would be taken as the F_S value (Fig. 2.1).

At least 300 seconds after the light had been turned on DCMU was added directly into the cuvette while the measurement continued. The final concentration of DCMU was 10μM, the volume added was 1/1000. Once the maximal fluorescence was reached, the average of the values measured was taken as the F_{max} value. 300 s after the addition of DCMU the experiment was stopped.

From the chlorophyll *a* fluorescence measurements the redox state of Q_A was calculated as:

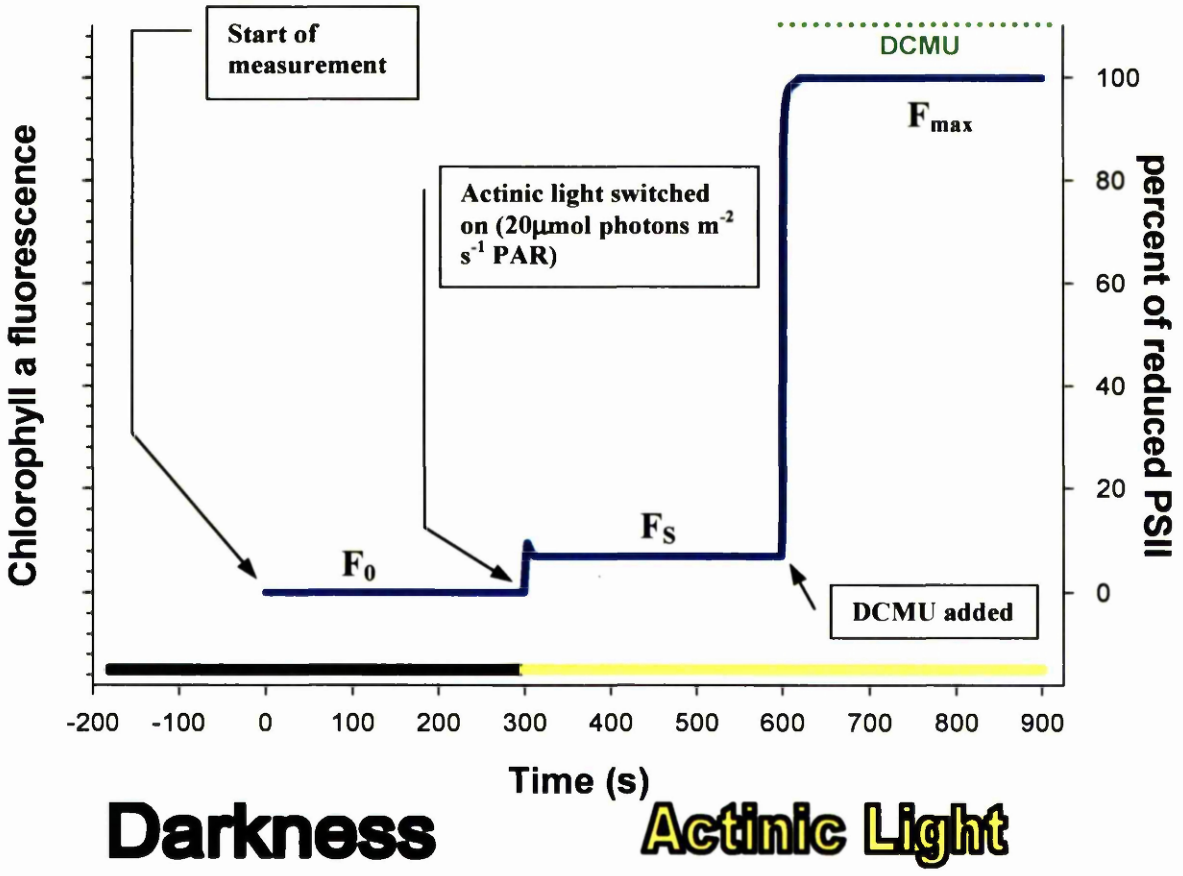
$$(F_S - F_0) / (F_{max} - F_0)$$

(Bradbury and Baker, 1981)

For measurements of cells incubated at 4°C the cuvette was cooled to 4°C before and during the measurements.

Figure 2.1: Schematic of a chlorophyll a fluorescence measurement

The blue line depicts the typical chlorophyll a fluorescence during the measurements. The black bar indicates dark treatment, the yellow bar indicates the period of actinic light treatment. The green dotted line indicates the presence of DCMU which fully reduces PSII (Q_A). See the text for a detailed description.


Start of measurement
Actinic light switched on (20µmol photons m-2 s-1 PAR)
DCMU
DCMU added
F0
FS
Fmax
Chlorophyll a fluorescence
percent of reduced PSII
Time (s)
-200
-100
0
100
200
300
400
500
600
700
800
900
100
80
60
40
20
0
Darkness
Actinic Light

2.2.16 Respiration and Photosynthesis measurements

After 24 h incubation samples were diluted to a cell density of 5 ± 0.5 mg fresh weight per ml with cell culture medium with or without 3% (w/v) sucrose. Sodium bicarbonate, freshly buffered to pH 5.8 was added to a concentration of 3mM to avoid limitation of photosynthesis by CO_2 availability. Respiration and photosynthesis rates were measured using a Hansatech (King's Lynn, UK) oxygen electrode thermostatted at 22 ± 0.05°C. Where appropriate, samples were irradiated with a 50 Watt quartz-halogen dichroic lamp (Osram) attenuated with Balzers' neutral density filters (Lichtenstein) to provide an intensity of 20μmol photons m^{-2} s^{-1} PAR. Where indicated 5mM sodium azide was added to the sample to block the mitochondrial cytochrome c oxidase activity and allow the measurement of the alternative oxidase activity.

2.2.17 Measurements of lipids and CoA

2.2.17.1 Fatty acid analysis

Total fatty acids were extracted and measured with a gas chromatograph based on the method described in Browse et al. (1986) and Larson and Graham (2001). Fatty acid profiling of plant material was done by direct transmethylation of tissue lyophilized in 2 ml screw-top vials containing 10 μg heptadecanoic acid (Sigma) as an internal standard. To each vial was added 500 μl 1 N HCl in methanol (Supelco, Poole,Dorset, UK) and 200 μl hexane (Rathburn). The vials were heated at 85°C for 2 h, cooled at room temperature, and the hexane phase containing the fatty acid methyl esters partitioned from the aqueous phase by the addition of 250 μl 0.9% KCl. The hexane phase was transferred to Teflon-capped vials, and 1 μl aliquots analysed by gas chromatography with flame ionisation detection (GC8000 Top, Thermoquest Separation Products, Manchester, UK). Injections were made into a 30 m long 0.25 mm ID SGE BPX70 column (SGE, Milton Keynes, UK) using He as a carrier gas at 1 ml min^{-1} with a 30 : 1 split ratio. The oven was run isothermally at 110°C for 1 min, then ramped to 180°C at 20°C min^{-1} then to 221°C at 2.5°C min^{-1}.

2.2.17.2 Fatty acyl CoA measurements

A novel method was used to determine fatty acyl CoA levels (Larson and Graham, 2001). This method is based on the derivatisation of acyl CoAs to their corresponding acyl etheno compounds that can be readily detected by fluorescence.
Between 5 and 30 mg frozen plant material was extracted, derivatised and measured exactly as described in Larson and Graham (2001).

2.2.18 DCMU treatment of cell cultures

Three-day-old cultures were washed, as described in section 2.2.15. Controls were re-suspended in medium with 3% (w/v) sucrose. Where appropriate DCMU [3-(3', 4'-dichlorophenyl)-1,1'-dimethylurea] in ethanol was added at time 0 to a final concentration of 10μM, controls were treated with ethanol (0.1%). Eight-ml aliquots were incubated for 24 h under the normal growth conditions given in section 2.2.1.

2.2.19 Quantification of Northern data

Autoradiographs or phospho-image plates exposed as described in 2.2.10 and 2.2.11 were scanned using either an Epson Perfection 1200S scanner (EPSON (UK) Ltd., Hemel Hempstead, UK) or the Fuji Bio-Imaging Analyser (Fuji Photo Film Co. Ltd., Japan). The intensity of bands on the acquired images was quantified using either the IFS32 software (4C, Maidenhead, UK & Photek, St.Leonards on Sea, UK) (for scanned autoradiographs) or the Mac-Bas software (Fuji Photo Film Co. Ltd., Japan) (for phospho-image data). For each exposed blot, the 3% sucrose fed samples were used to standardise the other values. All values were also standardised on the quantified levels of the constitutively expressed ARF gene.

2.2.20 Statistical analysis of Northern Blot data

The double standardised data obtained by the procedure described above was subjected to analysis of variance (ANOVA) using the MiniTab software (MiniTab Inc., State University of Pennsylvania, USA). The 'Tukey' setting was used, and the confidence interval set to 1%. The results of this analysis are shown in Apendix A in detail.

2.2.21 Dark and DCMU treatment of cell cultures

Three-day-old cell culture was washed and resuspended in medium with or without 3% sucrose as described in sections 2.2.13 and 2.2.18, except that the samples were wrapped in aluminium foil. One flask of starved cells was unwrapped as a control. The tissue culture flasks were incubated under the normal growth conditions for 24h and harvested as described in 2.2.2.

2.2.22 Repression experiments with the cell culture using DCMU

Cultures were treated as described in section 2.2.13. When the cells had been starved for 24h, DCMU was added to a final concentration of 10μM to half the starved and sucrose fed cells. Controls were treated with ethanol (0.1%). All samples were then left under the growth conditions for 165 minutes or longer and harvested as described in 2.2.2.

2.2.23 Repression experiments with the cell culture using DBMIB

Cultures were treated as described in section 2.2.13. When the cells had been starved for 24h, DBMIB was added to a final concentration of 5 and 10μM to starved and sucrose fed cells. Sucrose fed samples were diluted before the first addition to approximately the same cell density as starved samples to ensure the ratio of DBMIB to PSII cytochrome b_6/f was similar. Controls were treated with ethanol (0.1%). All samples were left under the usual growth conditions. Every 25 minutes the additions were repeated. Small aliquots were taken to monitor chlorophyll *a* fluorescence as described in section 2.2.15. Respiration was also monitored using a procedure similar to that described in section 2.2.16. After a total of 150 minutes the samples were harvested as described in 2.2.2.

2.2.24 Low temperature treatment of cell culture

Three-day-old cell suspension cultures were treated as described in section 2.2.13, except that half the samples were kept at 4°C instead of 22°C for 24h.

2.2.25 High light and low temperature treatment of cell culture

Three-day-old cell suspension cultures were treated as described in section 2.2.13, except that one half the samples were kept at 20µmol photons m^{-2} s^{-1} PAR at 22°C and 4°C. The other half was kept under 80µmol photons m^{-2} s^{-1} PAR at 22°C and 4°C. After 24h all samples were harvested.

2.2.26 Acclimation of cell suspension cultures to lower levels of sucrose in the growth medium

Seven-day-old cell suspension culture grown in medium with 3% (w/v/) sucrose were subcultured into new medium containing only 1% (w/v) sucrose and grown under the usual growth conditions. Every 7 days the new 1% culture was subcultured into fresh medium with 1% (w/v) sucrose. This initial acclimation was done in collaboration with Dr. Katrina Patterson.

After 2 months, a seven day old 1% sucrose culture was subcultured into medium containing 0.8% (w/v) sucrose. Every 7 days the new 0.8% culture was subcultured into fresh medium with 0.8% (w/v) sucrose. Subsequently, a 0.6% (w/v) sucrose culture and a 0.4% (w/v) sucrose were obtained in a similar procedure, always using the culture already acclimatised to the lowest concentration of sucrose. A minimum of 6 weeks under new culture conditions was necessary before experiments or the acclimation to a lower sucrose supply. This period ensured complete acclimation of the culture to the new sugar level.

2.3 Methods and techniques used for in planta *reporter-gene studies*

2.3.1 Plant growth conditions

Plants were grown in soil at 22°C in a greenhouse with a 16 h light 8 h dark period. Supplementary metal halide lights (HOLOPHANE Prismalume, fitted with OSRAM Powerstar HQI-E 400w/d daylight bulbs) were used as necessary.

2.3.2 Experimental set-up for the luciferase experiments

Rosette stage plants were placed in the dark for 24 h to allow leaf starch breakdown. Leaves were then detached from submerged plants or 4mm diameter leaf discs removed. Leaf tissue was then floated on half strength Murashige and Skoog medium (Murashige and Skoog, 1962) with either 3% (w/v) sucrose or 1.6 % (w/v) mannitol as an osmotic control. Where appropriate, 10 μM DCMU was added to the incubation media.

2.3.3 Imaging of leaves and leaf disks

Samples were subjected to a pre-experimental spray of 5mM luciferin in 0.01% Triton X-100 for a minimum of 2 hours before the measurement as described by Millar (Millar et al., 1992), except that a cosmetic pump spraying device was used ('No more Tangles', Johnson & Johnson, 'Superdrug' store, York, United Kingdom). For the actual measurement leaf tissue was sprayed again with luciferin and kept in the dark for 20 minutes. Then images were acquired using a photon counting camera (Photek, St.Leonards on Sea, UK) and IFS32 software (4C, Maidenhead, UK & Photek, St.Leonards on Sea, UK) according to the following guidelines:

CAB2-LUC line	5 to 10 minutes
PC-LUC line	3 to 5 minutes
Sun6	3 to 5 minutes
35S-LUC line	3 to 5 minutes
Nii-LUC line	10 minutes or more

2.3.4 Quantification of leaf disk luminescence

The data obtained from leaf disks was quantified using the IFS32 software (4C, Maidenhead, UK & Photek, St.Leonards on Sea, UK).

2.3.5 Repression experiments with the leaf disks using DCMU

Leaf discs were prepared and treated as described in section 2.3.2, except that DCMU and ethanol were only added after 24h starvation. The leaf discs had been sprayed with 5mM luciferin 2 hours before the addition of DCMU, at the time of the DCMU addition and 2 and 4 hours after the addition of DCMU as described in section 2.3.3.

Luminescence of the leaf discs was measured at the time of DCMU addition and 2 , 4 and 6h after DCMU additions.

2.4 Methods involved in assessing the binding of 14-3-3 proteins

2.4.1 AICAR and DCMU treatments of low sucrose cultures

Three-day-old cell suspension cultures of all 5 culture types (see 2.2.26) were divided in two equal aliquots. One aliquot of each culture type was washed and re-suspended as described for the type 3 culture in section 2.2.13. The other aliquot of each culture type was split into 8ml samples in 25 cm^3 tissue culture flasks keeping them in their sucrose containing media. These were the '+ sucrose' samples. One third of all aliquots were treated with 10 μM DCMU, one third with ethanol as a control (0.1%) and the remaining third with 10mM AICAR dissolved in water (200mM stock). All samples were incubated under the usual growth conditions for 24h. Samples for sugar analysis, chlorophyll analysis, RNA analysis and protein extracts were processed as detailed in section 2.2.2. The samples for RNA and protein analysis were stored at –70°C, those for sugar or chlorophyll analysis at –20°C.

2.4.2 Protein extraction, protein gel and blotting

100-400 mg of frozen tissue were used for protein extractions. The frozen cells were disrupted by sonication in the presence of 50mM HEPES pH 7.5, 1mM Dithiothreitol, 10mM $MgCl_2$ (all from Sigma) and 1μM Microcystin-LR (a Ser/Thre phosphatase inhibitor; from Gibco BRL, UK). All samples were centrifuged for 10 minutes (Microcentaur, 13,000 rpm, 4°C) to remove cell debris and the supernatant frozen in aliquots. The protein concentration was determined by Bradford assay (Bradford, 1976) (Fisher). 45μg of total protein per lane were loaded onto a NuPAGE (Novex, San Diego CA, USA) Bis-Tris Acrylamide gradient gel (4-12%) with MOPS SDS DTT running buffer. The electrophoresis was performed using a NuPage Electrophoresis system (Novex) according to the manufacturer's instructions. Protein transfer onto nylon membrane pieces (Hybond ECL, Amersham) was performed as described by the electrophoresis system's manufacturer (Novex). The blots were stained with Ponceau stain, scanned and destained.

2.4.3 14-3-3 overlay assay

The blots were washed in TBS (25 mM Tris pH 7.5, 500mM NaCl; from Sigma) with 5% (w/v) skimmed milk powder (buffer A) for 16h. The membrane was then incubated for 2h at ambient temperature in buffer A containing 1mg ml^{-1} bovine serum albumin and 1μg ml^{-1} DIG-BMH1 probe (see below). Unbound probe was removed by repeatedly washing the membrane in buffer A over a 2h period. Blots were incubated for 45 minutes in buffer A containing 1mg ml^{-1} bovine serum albumin and 1/5000 anti-DIG antibody (Boehringer Mannheim), conjugated with horseradish peroxidase (0.03 U ml^{-1}). After washing the membrane in buffer A for 2 hours, the bands were visualised with the enhanced chemiluminescence system (Amersham) for detection on X ray film (Konica).

The DIG BMH1 probe used in these experiments was generated by Dr. Jean Harthill by overexpression of the yeast 14-3-3 protein BMH1 in *E.coli*, extraction and labelling with DIG (Boehringer Mannheim) as follows:

A 2 L flask containing 50 ml of LB-Amp medium (LB broth plus 100 mg/ml ampicillin) were inoculated with *Escherichia coli* DH5a cells expressing the BMH1 14-3-3 isoform from *Saccharomyces cerevisiae* with an N-terminal 6-His-tag (a gift from Dr. Marie Scarabel and Prof. Alastair Aitken, see Moorhead et al. (1996, 1999)). This starter culture was grown overnight at 37°C, shaking at 250 rpm, then used to inoculate 25 L of LB-Amp in a 25 L fermenter with 1 ml antifoam. The culture was grown aerobically, with stirring, at 37°C until mid-log phase (A600 0.6 - 0.7). Expression of the 14-3-3 construct was induced by adding 0.5 mM IPTG for 3 h. Bacteria were then harvested by centrifugation for 20 min at 3600 x g. The bacterial pellets were snap-frozen and stored at –80°C until use. Pellets were thawed in PBS containing 1 mM 2-mercaptoethanol, 1mM EDTA, 5 % glycerol, 1 mM PMSF, and cells were lysed using a French Press. The lysate was clarified by centrifugation at 70,000 x g for 30 min and added to Ni-NTA agarose that had been washed with 4-5 vol 50 mM $NiSO_4$ in 50 mM Tris pH 7.5, 0.5 M NaCl followed by 10-20 vol of 50 mM Tris pH 7.5, 0.5 M NaCl. Lysate and beads were mixed by rotating end-over-end for 30 min at 4°C. Beads were transferred to a 25 ml Econopac column holder and washed 3-4 times (10 column vol each time) with of 50 mM Tris pH 7.5, 0.5 M NaCl, 10 mM imidazole, then twice with 50 mM Tris pH 7.5, 0.15 M NaCl, 10 mM imidazole. His-tagged 14-3-3 proteins were then eluted from

the beads with 300 mM imidazole in 50 mM Tris pH 7.5, 150 mM NaCl. The protein peak was pooled and dialysed overnight into 25 mM Hepes-NaOH pH 7.5, 1 mM DTT, 50 % glycerol before storage at –20°C.

14-3-3 protein (100 mg of the 6-His-tagged BMH1) was incubated for 2 h at room temperature with 7.5 mg digoxygenin-O-methylcarbonyl-e-aminocaproic acid-N-hydroxysuccinimide (DIG) ester, added from a 2 mg/ml stock solution in dimethyl sulphoxide, and 400 ml PBS, pH 8.5, with occasional mixing. The mixture was dialysed extensively in PBS pH 7.2 at 4°C to remove unreacted DIG ester, and diluted to a final protein concentration of 1 mg/ml in 25mM Tris pH 7.5, 500mM NaCl, 2mg/ml BSA. The resulting DIG-14-3-3 probe was stored at 4°C with 0.05 % sodium azide. It was found that the probe could be reused many times. Initial high backgrounds seen with some batches of DIG-14-3-3 could often be reduced by incubating the probe with a "blank" blocked nitrocellulose membrane prior to use.

This probe will bind to phosphorylated 14-3-3 binding proteins as well as 14-3-3 proteins themselves.

2.4.4 Anti-14-3-3 Western Blotting

Protein blots used for the 14-3-3 overlays were stripped in 62.5 mM Tris pH 6.7, 2% SDS and 3 ml/l β-mercaptoethanol (all Sigma) at 50°C for 1h. The blots were washed in TBS until all SDS was removed. After blocking (TBS with 5% (w/v) skimmed milk powder, 3 x 10 minutes shaking) the blots were incubated for 1h in fresh aliquot of the same solution but with a sheep anti-14-3-3-antibody (1/5000 dilution) (Moorhead et al., 1999) diluted 1/5000 in it. After washes (TBS with 5% (w/v) skimmed milk powder, 3 x 10 minutes shaking), blots were incubated with an anti-sheep-Ab- antibody linked to peroxidase (in TBS with 5% (w/v) skimmed milk powder) for 1h. After washes (TBS with 5% (w/v) skimmed milk powder, 3 x 10 minutes shaking) the enhanced chemiluminescence system (Amersham) was used for detection (following the manufacturer's instructions).

Chapter 3: Characterisation of the Arabidopsis cell suspension culture

3.1 Introduction

An *Arabidopsis thaliana* v. Landsberg erecta cell suspension culture (May and Leaver, 1993) was used as a model system to study possible interactions between sugar and plastid redox signalling. This model system was considered to be suitable for this purpose for the following reasons:

- The culture is photomixotrophic, i.e. it is grown in the presence of 3% (w/v) sucrose, but it is green and photosynthetically competent.
- The culture has a short growth period of seven days during which the cell mass increases 10 fold thus providing ample amounts of tissue.
- With a cell suspension culture pharmacological and nutrient studies can easily be performed by additions to the medium or washing of the cells and resuspension in new medium of the desired composition, e.g. medium with no or an alternative carbon supply.
- Arabidopsis has been a major focus of molecular genetics studies to identify sugar sensing mutants and of genomic sequencing. An Arabidopsis system was therefore desirable to use for this study as it provides the option to eventually adopt a genetic approach for future work.

In this chapter a series of experiments involving the basic characterisation of the cell culture is presented. The culture's suitability for sugar and plastid redox studies is demonstrated.

3.2 Characterisation of the growth period of the cell suspension culture

An initial characterisation of the cell culture involved monitoring the fresh weight over a ten-day period after subculturing (Fig. 3.1). The culture displays an initial lag phase of 2 days, has entered exponential phase of growth by day 3 and reaches stationary phase by day 7 to 8.

The initiation of growth could readily be detected by Northern analysis of the transcript levels of EF-1α, a gene that encodes a translation elongation factor (Axelos et al.,

1989). Interestingly, the expression of EF-1α is quickly (5h) and strongly induced by subculturing (Fig. 3.2), lasting until the exponential phase of growth has been reached. However an increase in fresh weight was only detectable after 3 days, when the mRNA levels of EF-1α are already returning to lower levels (Fig. 3.1 and 3.2). In contrast, the expression of ARF, encoding an Arabidopsis member of the ARF subfamily of GTP-binding proteins involved in vesicle transport, was found to be constitutive over the growth period (Fig. 3.2), as previously reported (Regad et al., 1993).

Over the growth period, the sucrose levels in the culture medium start to decline after the initial 2 days and are depleted quickly when the cells reach stationary phase (Fig 3.3 A). Fructose and glucose levels in the medium are considerable but constant over the first 24 hours. By day 3 glucose is depleted while fructose reaches a peak value. However, by day 4 fructose has also become depleted. The presence of hexoses in the culture medium demonstrates that extracellular invertase must be present, leading to hexose levels in the medium within the range of values found inside the cells at some growth stages (cf. Fig 3.3 B). The more rapid depletion of glucose suggests it is taken up by the cells in preference to fructose. However, fructose is rapidly removed from the medium after the removal of glucose. The uptake of fructose by these cells could either be the result of a fructose specific transporter, expressed only after day 3, or a change in substrate transported by a non-specific hexose transporter. It is interesting to note that a fructose specific transporter of the plasmalemma has not yet been identified. The number of monosaccharide transporters in Arabidopsis is currently unclear, with 21 to 26 potential carbohydrate transporters identified in the Arabidopsis genome sequence (Lalonde et al., 1999; Munich information centre for protein sequences, 2001), and a range of substrates found for the few that have been characterised (Sauer et al., 1990; Boorer et al., 1994; Truernit et al., 1996; Truernit et al., 1999; Büttner et al., 2000; Sherson et al., 2000). It is therefore not possible to predict what transport mechanism is operating in these cells. Based on the changes that occur in the external medium in the early phases of growth sugar uptake appears to be predominantly as hexoses rather than sucrose. Only in the late exponential (day 5 and 6) and early stationary phase (day 7 to 8) does sucrose uptake across the plasma membrane appear to occur at any significant rate. However, it is possible that the observed rapid depletion of sucrose is entirely due to hexose rather than sucrose uptake.

Figure 3.1 Fresh weight of cell suspension culture over the growth period

The cell suspension culture was sampled daily for 10 days after subculturing at the times indicated. The fresh weight (FW) in g per ml culture over the growth period is shown. n= 3 independent samples, standard error are shown.
The fresh weight of the 7 d old cultures used for subculturing is shown ('starting culture').

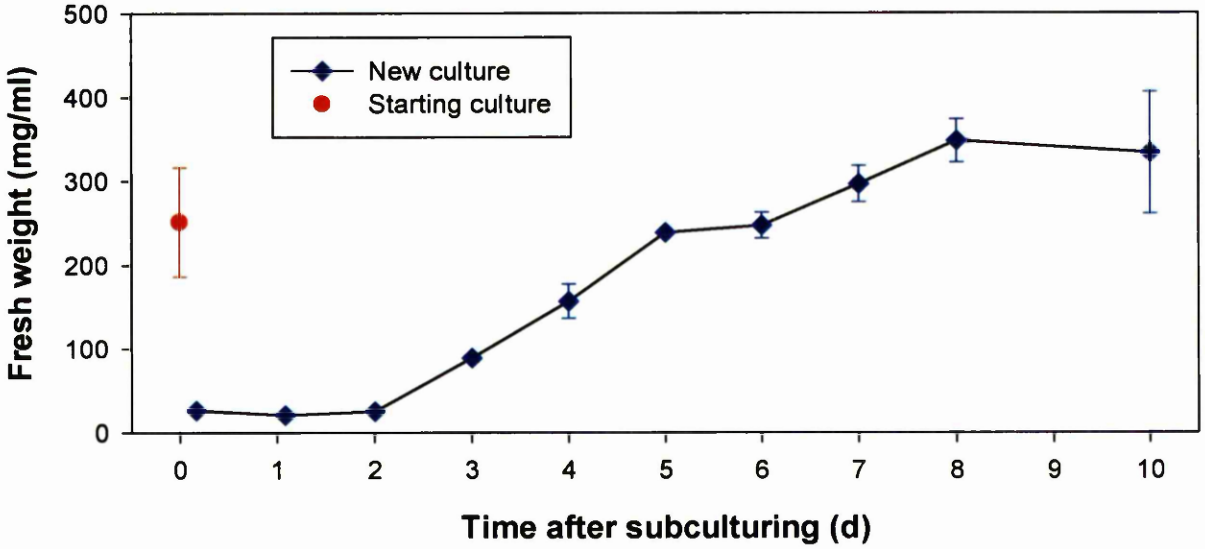

500
400
300
200
100
0
Fresh weight (mg/ml)
New culture
Starting culture
0
1
2
3
4
5
6
7
8
9
10
Time after subculturing (d)

Figure 3.2: Transcript abundance of EF-1α and ARF in cell suspension culture over the growth period

The cell suspension culture was sampled over the course of 8 days after subculturing at the times indicated. Total RNA was isolated from the samples and 7 μg total RNA analysed by Northern Blot analysis. The transcript abundance of EF-1α and of ARF were assessed. An image of the Ethidium Bromide stained rRNA on the gel is included for comparison of loading. P = pre-wash sample.

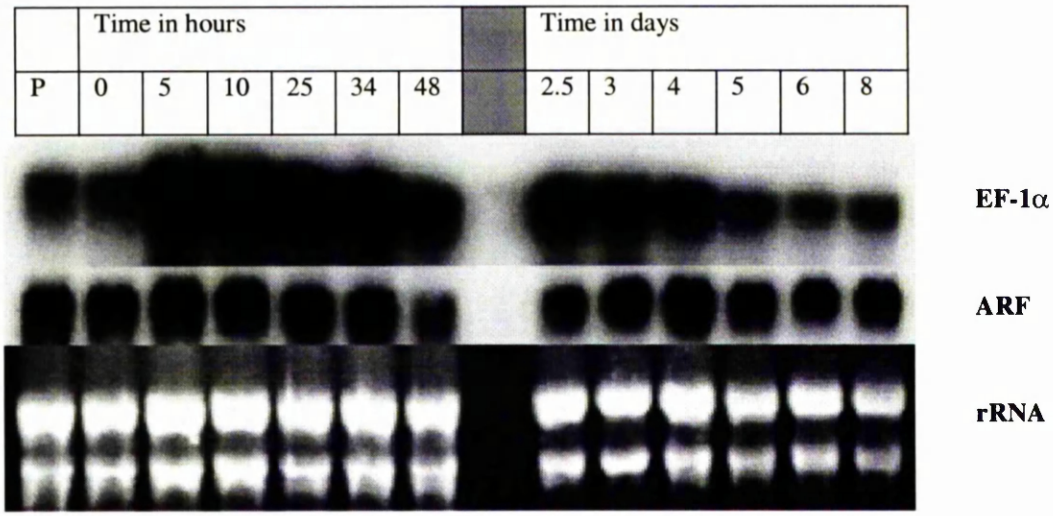
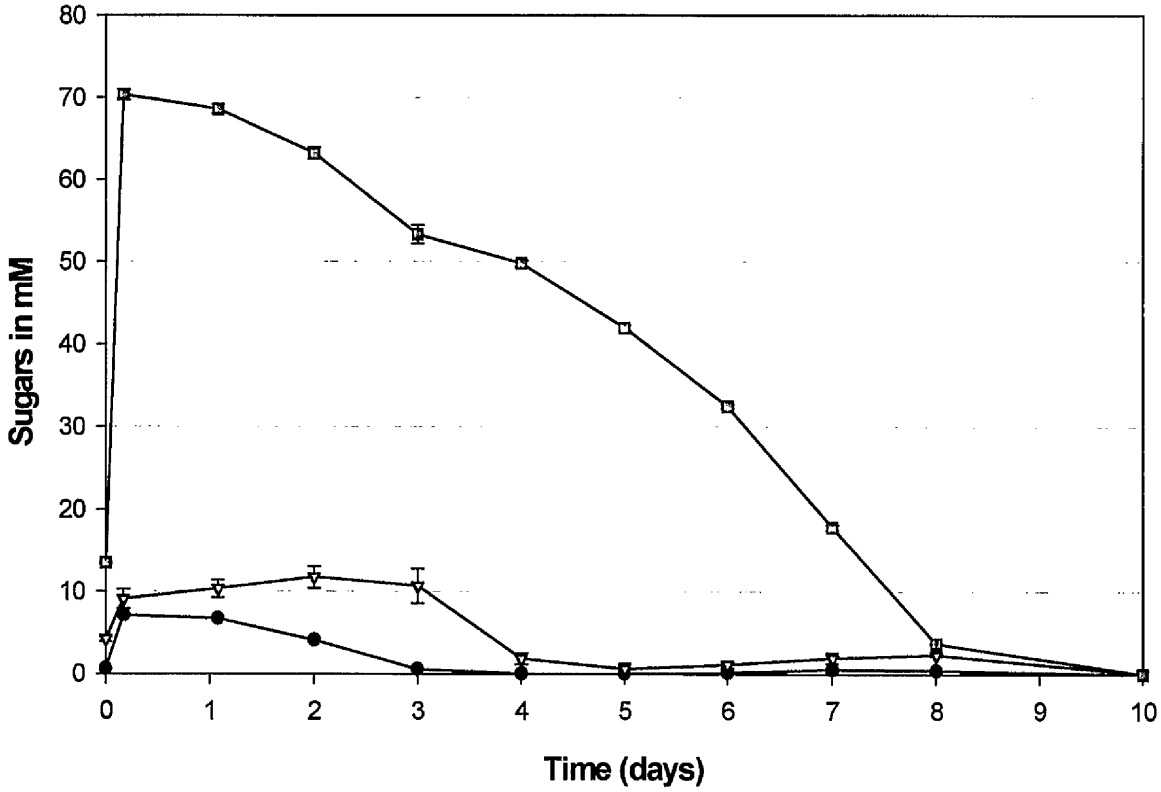

Time in hours
Time in days
P
0
5
10
25
34
48
2.5
3
4
5
6
8
EF-1α
ARF
rRNA

Figure 3.3: Sugar levels in culture medium and in suspension culture cells over the growth period

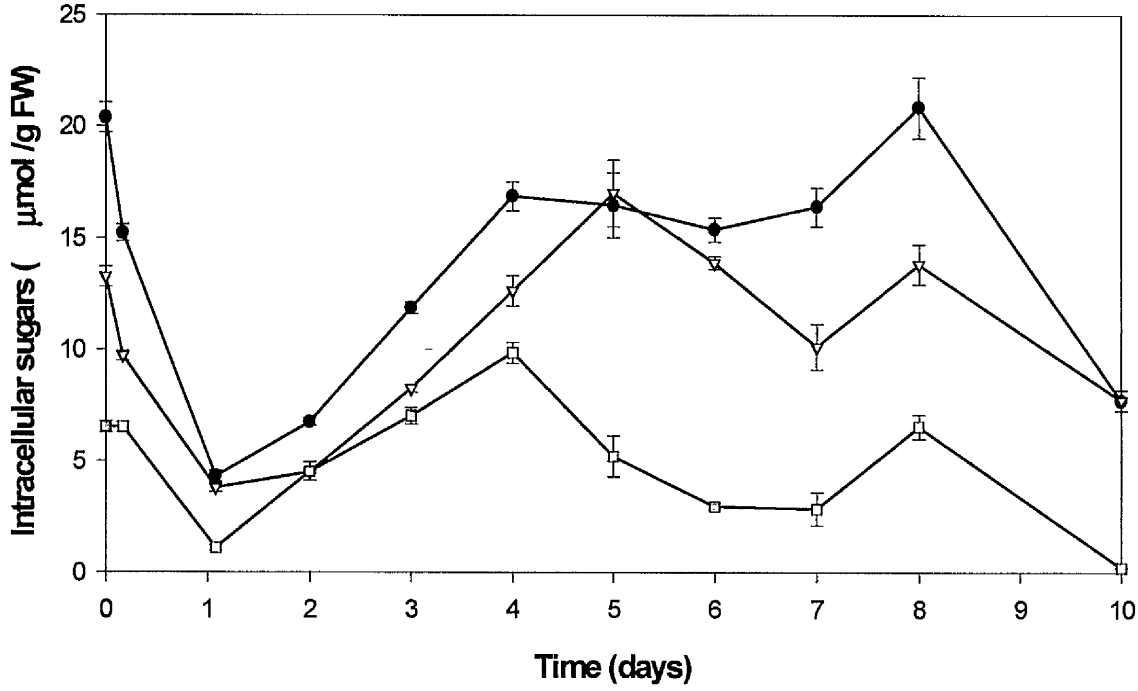
The cell suspension culture was sampled after subculturing daily until 10 days after subculturing. Samples were separated into cells and growth medium and the soluble sugar levels were determined. All values are the average and standard errors of the given number of replicates.

(*A*) Glucose (—●—), fructose (—▽—) and sucrose (—▣—) levels in the medium over the growth period (mM), n=3 independent samples; (*B*) intracellular glucose (—●—), fructose (—▽—) and sucrose (—▣—) levels over the growth cycle (in approximately mM), n=3 independent samples;

A


Sugars in mM
Time (days)
0
10
20
30
40
50
60
70
80
0
1
2
3
4
5
6
7
8
9
10

B


Intracellular sugars (µmol /g FW)
Time (days)
0
5
10
15
20
25
0
1
2
3
4
5
6
7
8
9
10

Intracellular sugar levels decrease in the 24h period after subculturing and recover from the initial low during day 2 and 3 (Fig 3.3 B). In the mid exponential phase sucrose (day 4) and fructose (day5) levels peak, while glucose reaches a new high plateau from day 4 to 7. Interestingly, all sugars show a second peak on day 8 when the cells have entered stationary phase, before the decline towards day 10. It is noteworthy that the entry into stationary phase coincides with sucrose in the culture medium being most rapidly taken up and becoming completely depleted on day 8 (Fig.3.3 A). The depletion of the external carbon source as well as other nutrients such as nitrogen and micronutrients from the medium together with an increase in waste products in the medium could initiate the transition to stationary phase. The transient peak in intracellular sugar levels at day 8 could be explained by the change from a metabolic state that involves high sugar usage in exponential phase to a state that uses much less sugar in the stationary phase.

The cell's preference for glucose in the early phase of growth is apparent when suspension cultures are subcultured and grown on medium with 3% (w/v) sucrose, 20 mM glucose or no external carbon source for 4 days (Fig 3.4). The cells grow as well on only 20 mM glucose as they do on 3% (w/v) sucrose (i.e. 88 mM sucrose) over the 4 day period. Cells with no external carbon supply also show some growth, more than doubling their fresh weight (Fig. 3.4 A).

3.3 The response of the cell culture to starvation

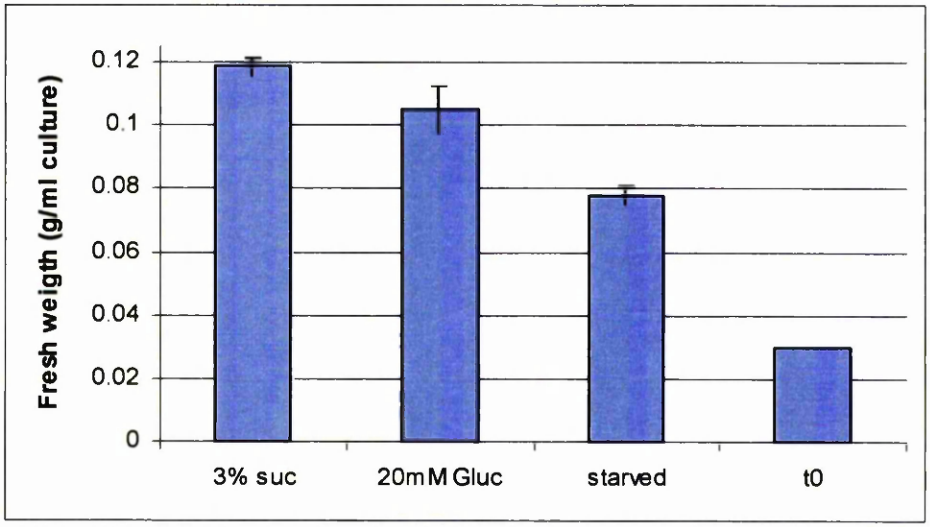
From the initial work presented above it is apparent that between day 3 and day 4 of the growth period the presence or absence of sugars would affect the suspension culture growth rate most strongly. The cells are in early exponential phase and their rapid growth leads to a great demand for carbon (Fig. 3.1). Hexose levels in the medium are being depleted and sucrose breakdown begins to accelerate (Fig. 3.3 A). Intracellular sugar levels increase and peak by day 4 (Fig. 3.3 B).

Based on these observations 3-day-old suspension culture was chosen to be subjected to a starvation regime. Cells were washed and resuspended in medium without any sugar. Samples were taken at the times indicated (Fig 3.5). Intracellular levels of glucose, fructose and sucrose began to decline after two hours of starvation, and reached a basal minimal level by 24 hours (Fig. 3.5 *A*). Starch levels remained very low at all times (data not shown). If sucrose was added back into the medium to a final concentration of

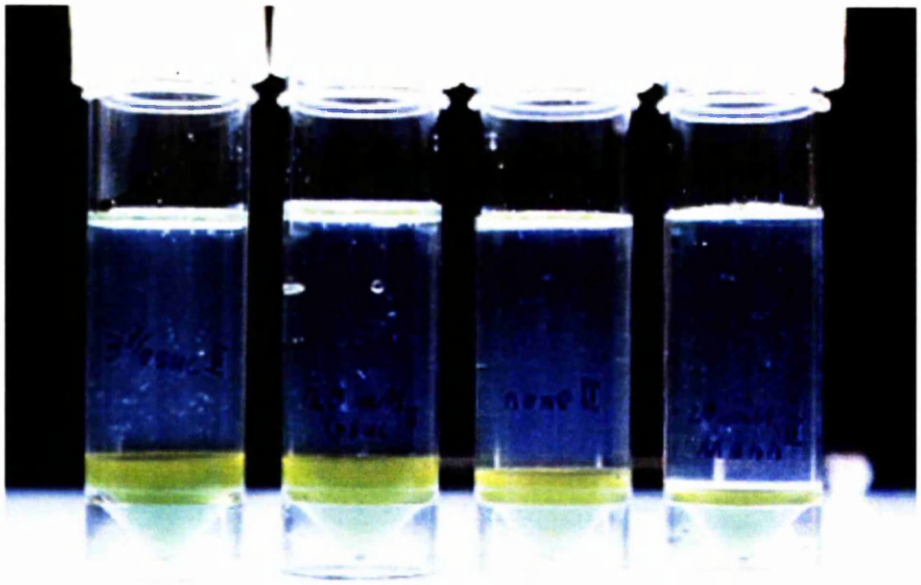
Figure 3.4: Growth of cell culture over 4 days with different carbon sources

Cell cultures were subcultured into fresh medium containing 3% (w/v) (88 mM) sucrose, 20 mM glucose, or no carbohydrates and cultured for 4 days. (*A*) Growth was assessed by comparison of fresh weight (n=2), and visually (*B*).

A



B



3% sucrose	20 mM glucose	starved	20 mM mannose

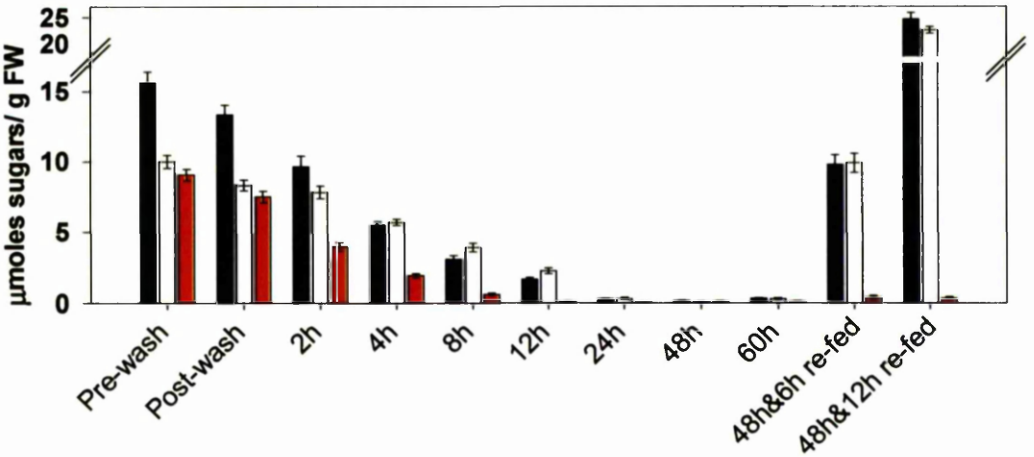
Figure 3.5: Starvation and re-feeding of three-day-old cell suspension cultures

Three-day-old Arabidopsis suspension culture was washed and re-suspended in medium with or without 3% (w/v) sucrose as described in section 2.2.13. Samples were taken before the wash ('Pre-wash'), at time 0 and at various intervals indicated

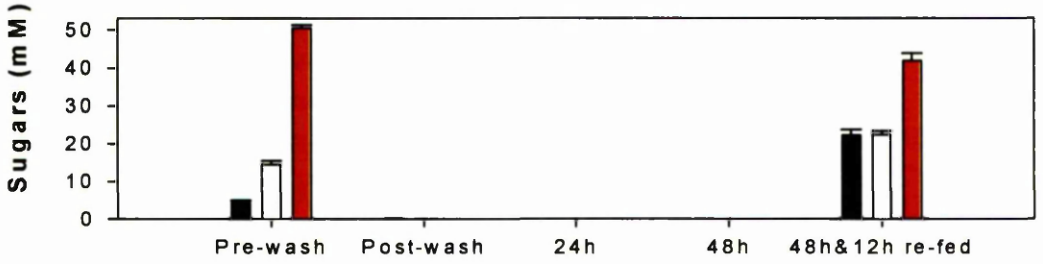
After starvation for 48 hours, where indicated, sucrose was added to a final concentration of 3% (w/v). All values are the average and standard errors of the given number of replicates. Codes: all numbers represent hours of starvation; where indicated cells were starved for 48 hours and then re-fed sucrose for 6 or 12 hours, respectively, before harvesting.

(*A*) Intracellular glucose (■), fructose (□) and sucrose (□) levels during starvation (in approximately mM), n=3 independent samples; (*B*) glucose (■), fructose (□) and sucrose (□) levels in the medium during starvation (mM), n=3 independent samples; (*C*), total chlorophyll (▩) and Chl. a / Chl. b ratios (●) n = 3 independent samples; (*D*), fresh weight (FW) in g per ml culture over the starvation period (—▲—), n= 6 independent samples; (*E*), redox state of Q_A of PSII under high light (300 µmol photons m^{-2} s^{-1} PAR) as assessed by chlorophyll a fluorescence (see section 2.2.15) of starved, re-fed and control cell cultures at the indicated time points during a pilot experiment (single measurements).

A

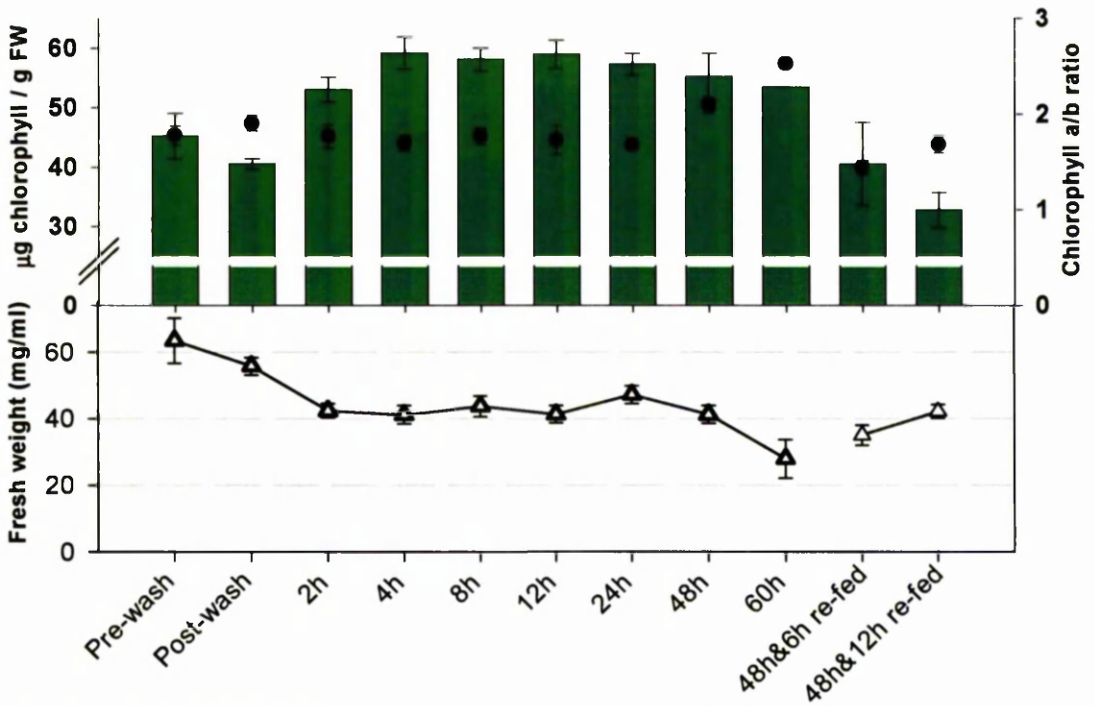

µmoles sugars/ g FW
25
20
15
10
5
0
Pre-wash
Post-wash
2h
4h
8h
12h
24h
48h
60h
48h&6h re-fed
48h&12h re-fed

B


Sugars (mM)
50
40
30
20
10
0
Pre-wash
Post-wash
24h
48h
48h&12h re-fed

C

D


µg chlorophyll / g FW
60
50
40
30
0
Chlorophyll a/b ratio
3
2
1
0
Fresh weight (mg/ml)
60
40
20
0
Pre-wash
Post-wash
2h
4h
8h
12h
24h
48h
60h
48h&6h re-fed
48h&12h re-fed

E

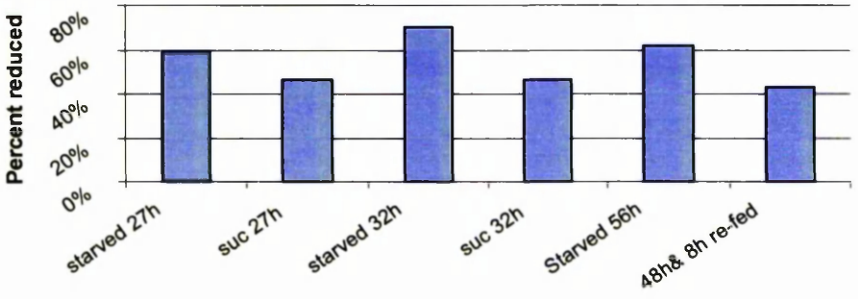
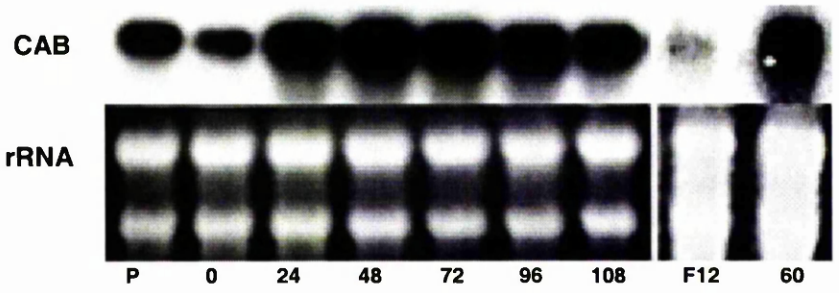

Percent reduced
80%
60%
40%
20%
0%
starved 27h
suc 27h
starved 32h
suc 32h
Starved 56h
48h& 8h re-fed

Figure 3.6: *CAB* mRNA levels in 3-day old culture are elevated in response to starvation and reduced after re-feeding sucrose

Three-day-old Arabidopsis suspension culture was washed and re-suspended in medium with or without 3% (w/v) sucrose. 'Pre-wash' and time 0 samples were taken, in addition to those at the indicated times. After starvation, where indicated, sucrose was added to a final concentration of 3% (w/v). RNA blot analysis on 7 μg total mRNA was performed using the *CAB2* gene as probe. Codes; P= pre-wash; 0= post-wash; all numbers represent hours of starvation; F12 cells were starved for 48 hours and then re-fed sucrose for 12 hours, , before harvesting. An image of the Ethidium Bromide stained rRNA on the gel is included for comparison.

The experiment was undertaken in collaboration with Dr. Thomas Martin. Except for the re-fed and 60 h samples, Dr. Thomas Martin performed the analysis.


CAB
rRNA
P
0
24
48
72
96
108
F12
60

c. 3% (w/v), the intracellular hexose values increased dramatically, while sucrose increased only minimally (Fig. 3.5 A). Sugar was absent from the medium until the re-admission of sucrose (Fig. 3.5 B). Significant hexose levels accumulate in the medium 12 hours after re-feeding sucrose, stressing the role of extracellular invertase in this culture.

Four hours after removal of sucrose a significant increase ($p < 0.05$) in total chlorophyll levels was observed suggesting the induction of genes associated with the photosynthetic apparatus (Fig. 3.5 C). No concomitant increase in the chlorophyll *a/b* ratio was detected indicating the synthesis of both the chlorophyll *a*-containing core complexes and of the peripheral light harvesting chlorophyll *a/b* complexes (*CAB*) (Fig. 3.5 C). Only after prolonged starvation (48h and more) did the chlorophyll *a/b* ratio increase, but dropped quickly after re-feeding sucrose (Fig 3.5 C). When measured under high illumination (300µmol photons m^{-2} s^{-1} PAR) the redox state of Q_A was more reduced in starved cells compared with cells not starved or re-fed with sucrose (Fig 3.5 D). This great difference in the Q_A redox state between starved and sucrose fed cells under elevated to high light conditions was very reproducible (cf. Fig. 3.7 D).

The fresh weight showed a decrease in the first 2 hours of starvation, but there was no further significant change until 48h of starvation. Longer starvation resulted in a further decrease in fresh weight (Fig.3.5 E). This loss of fresh weight could be prevented by re-feeding sucrose (Fig 3.5 E). RNA blot analysis on samples starved for different periods of time demonstrates that *CAB* transcript levels are inversely correlated with intracellular sugar concentrations, being suppressed when sugar levels are high (pre- and post-wash), and increasing only when sugar levels began to decline (>24 hours). Further, re-feeding cells with sucrose after 48 hours starvation resulted in a strong decrease in *CAB* transcript (Fig. 3.6).

These results are consistent with the findings from other plant systems which show that expression of nuclear encoded photosynthetic genes is inversely correlated with intracellular soluble sugar levels (Sheen, 1994; Stitt et al., 1995; Koch, 1996).

When fresh weight of 24 h starved cells was compared with cells supplied with 3% (w/v) sucrose for the same period, the sucrose fed cells had nearly double the fresh weight per ml culture. This is consistent with the rapid growth during the exponential phase when sugars are supplied, but only little growth when the cells are being starved (Fig 3.7 A). Both starved and sucrose replete cells showed a significant capacity for photosynthesis and respiration indicating functional electron transport chains (Fig. 3.7

B). However, cells starved for 24h show reduced rates of both respiration and photosynthesis compared with cells supplied with 3% sucrose over the same period.

In contrast, total chlorophyll was increased in starved cells, mostly due to an increase in chlorophyll *a* (Fig 3.7 C). The chlorophyll *a/b* ratio was also higher. The increase in both chlorophyll *a* and the chlorophyll *a* to b ratio indicate that not only the proteins of the antenna complex (encoded by the *CAB* gene family, and containing chlorophyll *a* and b) are increased, but also and more strongly the reaction centre proteins (encoded by *psbA* for example, and containing chlorophyll *a* only).

To assess the plastid redox state in cells starved for 24h the Q_A redox state was monitored using chlorophyll fluorescence (see section 3.5 and Fig. 3.9 for details). With both starved and sucrose-fed cells, increasing light intensity resulted in a progressive reduction of the steady state Q_A, and hence PQ pool, redox potential (Fig. 3.7 D, see also Fig. 3.5D). However, at the experimental growth conditions of 20μmol photons $m^{-2} s^{-1}$ PAR, the Q_A and PQ pool redox state of both starved and sucrose fed cells were not significantly different, and remained almost fully oxidised (Fig. 3.7 D).

3.4 Does autophagy of cellular components occur?

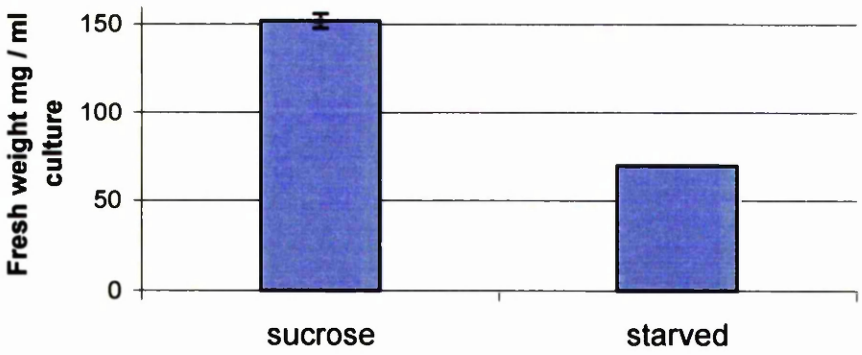
In cells starved for 24h, respiration and photosynthesis were decreased (Fig. 3.7 B). Douce and co-workers have reported autophagy of cytoplasmic components, including mitochondria and plastids, in starving sycamore cells after carbohydrate reserves have become depleted and respiration rates decline (Aubert et al. (1996) and therein). In their studies, Douce and colleagues found the breakdown of fatty acids from membrane lipids and the release of the membrane lipids' polar head groups as a good indicator of autophagy of cellular components. Plasma membrane located sterols did not decrease, but phospholipids and organelle specific galactolipids decreased and analysis of the cellular ultrastructure showed a simultaneous loss of organelles (Aubert et al., 1996).

To investigate whether this process might occur in these Arabidopsis cells after 24h starvation, fatty acid and fatty acyl-CoA levels in starved and sucrose fed cells were analysed by gas chromatography and high performance liquid chromatography (HPLC) (Fig 3.8). Sucrose fed cells showed a marked increase in fatty acids and fatty acyl-CoAs over the 24h period when compared with the pre-wash samples. In contrast, the levels of the most abundant fatty acids in starved samples stayed similar to those in the pre-wash samples (Fig. 3.8 A). However, the fatty acids incorporated in plastid membrane

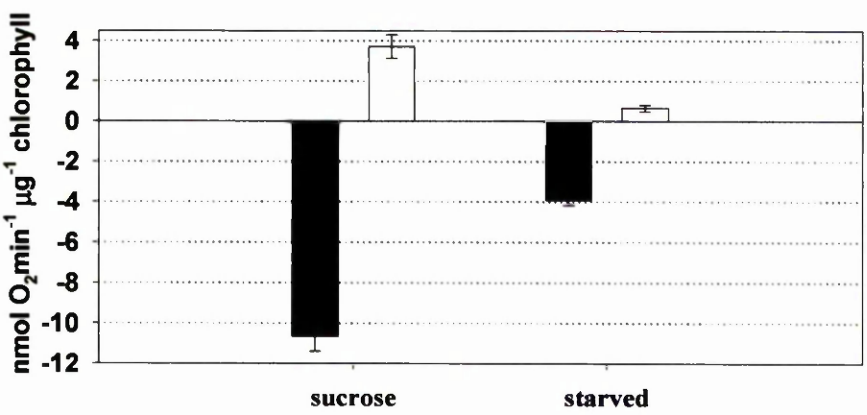
Figure 3.7: Fresh weight, respiration, chlorophyll and PQ redox state of starved and sucrose fed cells

Three-day-old Arabidopsis suspension culture was washed and re-suspended in medium with or without 3% (w/v) sucrose. Samples were taken after 24 hours. (*A*), fresh weight of cell culture (mg/ml), n=3; (*B*), respiration (black bars) and photosynthesis (white bars) rates after 24 hours of starvation, n=3; (*C*), chlorophyll levels and chlorophyll a-to-chlorophyll b ratio in sucrose treated (darker bars) and starved cells (lighter bars), n = 3; (*D*), The effect of light on PQ redox state in starved and sucrose-replete cells. Chlorophyll fluorescence emanating from Q_A was measured over the range of indicated light intensities (5 - 155 μmol photons m^{-2} s^{-1}PAR) and the redox state of plastoquinone (PQ) was calculated as $[F_S - F_0] / [F_{max} - F_0]$ (see chapter 2 section 2.2.12 for details). Each value represents the average and standard error of n=3 (starved) or n=2 (sucrose-fed) independent samples. Open symbols, 3% (w/v) sucrose; closed symbols, starved for 24 hours.

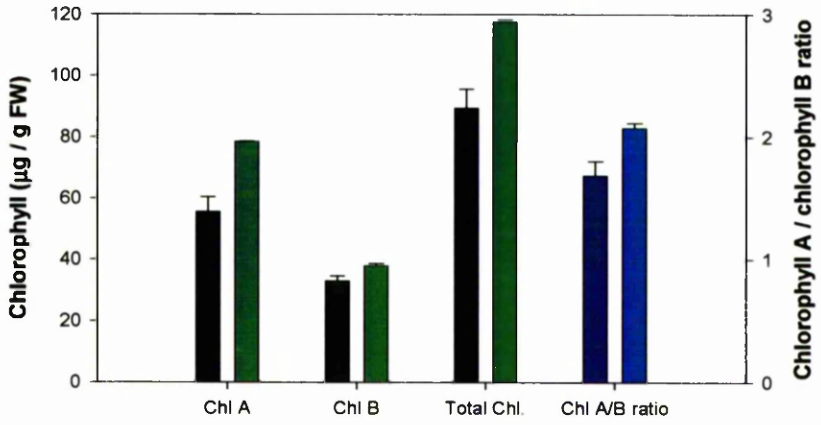
A


Fresh weight mg / ml culture
150
100
50
0
sucrose
starved

B


nmol O_2min^{-1} µg^{-1} chlorophyll
4
2
0
-2
-4
-6
-8
-10
-12
sucrose
starved

C


Chlorophyll (µg / g FW)
120
100
80
60
40
20
0
3
2
1
0
Chlorophyll A / chlorophyll B ratio
Chl A
Chl B
Total Chl.
Chl A/B ratio

D

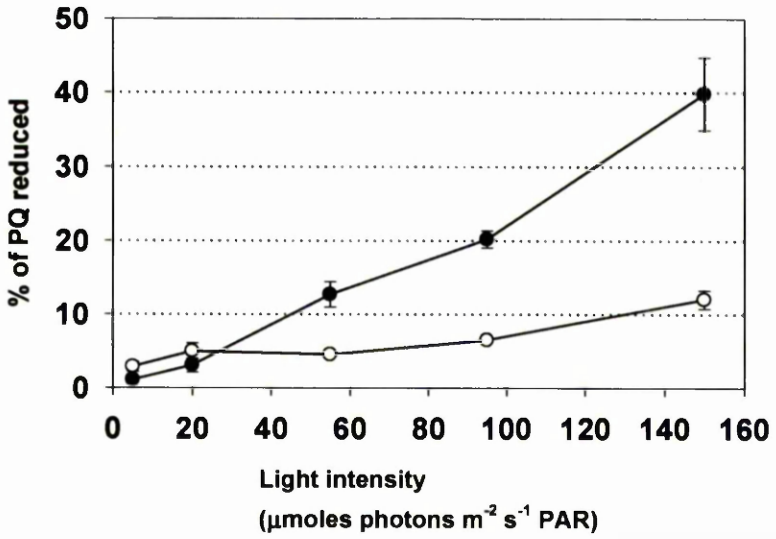

% of PQ reduced
50
40
30
20
10
0
0
20
40
60
80
100
120
140
160
Light intensity
(µmoles photons m^{-2} s^{-1} PAR)

Figure 3.8: Total fatty acid and fatty acyl-CoA levels in response to starvation of cell culture

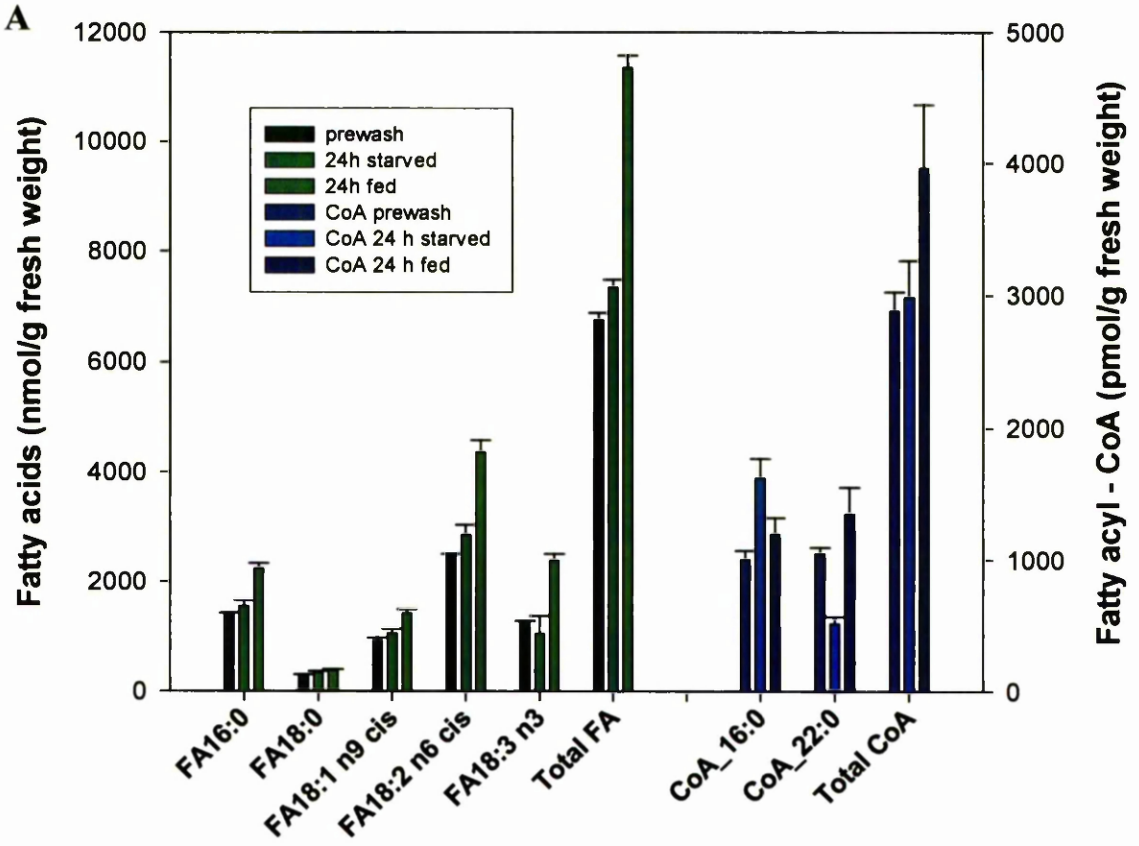
Three-day-old cell suspension culture was washed and subjected to starvation as described in section 2.2.13. Samples were taken before the wash and after 24 hours of starvation or sucrose feeding as control. Samples were harvested and stored at –80°C until they were extracted and analysed by gas chromatography and high performance liquid chromatography as described in chapter 2 section 2.2.17. The averages and standard errors are shown; n = 3 for pre-wash samples, n = 6 for starved samples and sucrose controls.

FA	fatty acids	green bars
CoA	fatty acyl CoenzymeA ester	blue bars

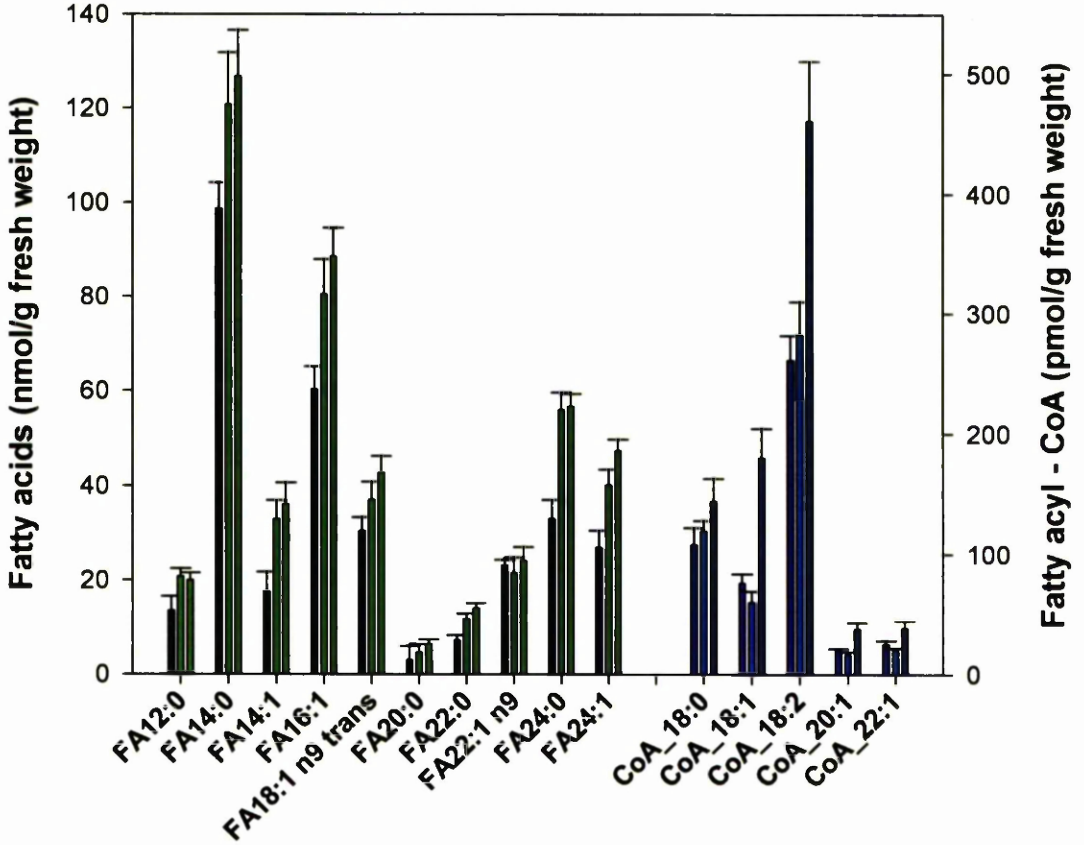
The numbers indicate the chain length and double bond number and position of the fatty acyl moiety.

This analysis was performed in collaboration with Dr. Tony R. Larson and Dr. Teresa Egdell.

A


Fatty acids (nmol/g fresh weight)
Fatty acyl - CoA (pmol/g fresh weight)
prewash
24h starved
24h fed
CoA prewash
CoA 24 h starved
CoA 24 h fed
FA16:0
FA18:0
FA18:1 n9 cis
FA18:2 n6 cis
FA18:3 n3
Total FA
CoA_16:0
CoA_22:0
Total CoA

B


Fatty acids (nmol/g fresh weight)
Fatty acyl - CoA (pmol/g fresh weight)
FA12:0
FA14:0
FA14:1
FA16:1
FA18:1 n9 trans
FA20:0
FA22:0
FA22:1 n9
FA24:0
FA24:1
CoA_18:0
CoA_18:1
CoA_18:2
CoA_20:1
CoA_22:1

lipids (such as 16:0, 18:2, 18:3) did not decrease significantly in starved samples compared to the pre-wash samples (Fig. 3.8 A). This indicates that autophagy of the plastids is not being induced after 24 h starvation. The levels of the less abundant fatty acids in starved samples increased compared to the pre-wash samples and were similar to those detected in sucrose supplied cells (Fig. 3.8 D). The levels of fatty acyl-CoAs in starved cells remained at the level of the pre-wash samples, except for CoA_20:0 and CoA_22:0 which decreased, and CoA_16:0, which increased even more in starved than it did in sucrose fed cells (Fig. 3.8 A and B). The reason for the changes in only these three fatty acyl-CoAs is unclear. The total amount of fatty acyl-CoA was of the same magnitude as previously reported for 2-day-old Arabidopsis seedlings (Larson and Graham, 2001).

Based on the fact that there is no dramatic change in fatty acid levels, including those associated with plastid membrane lipids, and only a slight depletion in fatty acyl-CoAs it can be concluded that over the 24h starvation period there is no significant induction of autophagy in the Arabidopsis cell suspension.

The sycamore cells started to display a loss of lipids indicating autophagy after 35h of starvation. The Douce group has shown that glycerol or pyruvate feeding of sucrose-deprived sycamore cells could prevent autophagy by maintaining the normal respiration rates while carbohydrate levels were still depleted (Aubert et al., 1996). In the sycamore cells, which appear not to be photosynthetically competent, the onset of autophagy is determined by the supply of respiratory substrates from sucrose hydrolysis and starch breakdown in the amyloplasts. The Arabidopsis cells have negligible starch levels yet maintain a reduced, but considerable respiration rate despite the depletion of sugars after 24h of starvation (Fig. 3.5 A and 3.7 B). Photosynthesis in these cells could have a similar role to starch breakdown in the sycamore cells by supplying respiratory substrates and hence preventing autophagy despite only minimal levels of soluble sugars. Alternatively photosynthesis could evoke a signal that suppresses autophagy.

3.5 Chlorophyll a *fluorescence as a measure of the redox state of the Q_A site of PSII*

When PSII or its antenna absorbs a photon its energy can suffer one of several fates. If the reaction centre is open (i.e. oxidised) and the water splitting complex is active, the

energy can be used to transport an electron from the water splitting complex to the Q_A and consecutively to the other components of the PET chain (see Fig. 1.2). However, if the energy absorbed in PSII can not be used within a very short time period, it will be re-emitted as light of a longer wavelength (around 680nm). The energy difference will be dissipated as thermal energy.

At any given time, some of the absorbed light will be re-emitted as fluorescence by PSII within a fraction of a second. However, the more reaction centres that are already reduced, the higher the chlorophyll *a* fluorescence. The absolute level of fluorescence depends in each case on the number of PSII reaction centres and hence on cell, plastid and PSII copy number. Any increase in fluorescence above the level associated with a fully oxidised Q_A reflects an increase in PSII reaction centre reduction state. If electron transport is blocked by DCMU, Q_A and hence PSII reaction centres will be fully reduced and the fluorescence will be maximal. However, it is important to remember that the downstream members of the PET chain will be fully oxidised when DCMU is present, although this is not relevant for these measurements (in contrast to the interpretation of the results presented in chapter 4).

By measuring the chlorophyll *a* fluorescence of cells in the dark (Q_A and PQ fully oxidised), under a given illumination (Q_A and PQ partially oxidised, partially reduced) and after the addition of DCMU (Q_A fully reduced, since transfer to PQ blocked) the redox state of Q_A (representative of PSII redox state) under that light intensity can be measured. In addition, this should also reflect the redox state of the PQ pool (yet PQ pool size will be larger than Q_A pool size).

Fig. 3.9 explains the experimental procedure and fluorescence data interpretation. An aliquot of culture was transferred into a quartz cuvette containing a small stirring bar. The cuvette was placed in the thermostatted holder of the instrument, where the cells were stirred in darkness for at least 150 s (darkness is indicated by a black bar in Figs. 3.9 and 3.11). In pre-experimental tests this period of darkness proved more than sufficient to result in fully oxidised Q_A in both starved and sucrose fed cells (data not shown). This could be assured once the excitation beam was turned on by comparison of the fluorescence level after longer periods of darkness (5 to 10 min) and by exposure to a weak light of 718 nm. This wavelength will drive PSI but not PSII, leading to oxidation of Q_A if possible. However, light of 718 nm was unable to reduce the chlorophyll *a* fluorescence from PSII after this dark treatment, since Q_A was already fully oxidised (data not presented).

Once the cells had been in the dark for the given period, the excitation beam was turned on. The excitation beam was a weak beam of 440nm wavelength. This will lead to enough chlorophyll *a* fluorescence from PSII to be recorded yet is sufficiently weak not to reduce Q_A significantly. Simultaneous illuminations with the 718nm light and interrupting periods of full darkness assured this was the case in pre-experimental tests (data not shown).

The cells were kept in complete darkness bar the excitation beam for another 300 s. The blue line in Fig. 3.9 represents the emitted chlorophyll *a* fluorescence measured every second or half of a second. Once the fluorescence had settled after the excitation beam had been switched on, the average of the values measured was taken as the F_0 value as indicated in Fig.3.9. When the 300 s had passed, light (represented by the yellow bar in Figs. 3.9 and 3.11) was shone upon the cuvette via a fibre optic. This resulted in an increase in fluorescence to a new level indicating the percentage of Q_A now reduced by the light. In most cases the light intensity was 20μmol photons m^{-2} s^{-1} PAR, though other light intensities between 2 and 300 μmol photons m^{-2} s^{-1} PAR were possible (cf. Figs. 3.5 D and 3.7 D). When the fluorescence had reached a steady level the average of the measured values would be taken as the F_S value (Fig. 3.9).

At least 300 seconds after the light had been turned on DCMU was added directly into the cuvette while the measurement continued. The final concentration of DCMU was 10μM, the volume added was 1/1000. More or less immediately after the injection of DCMU into the cuvette the fluorescence would shoot up and approach its maximum value (Fig. 3.9). Once the maximal fluorescence was reached, the average of the values measured was taken as the F_{max} value. 300 s after the addition of DCMU the experiment was stopped.

In pre-experimental test 10μM DCMU had proven to be fully sufficient to completely block electron transport (data not shown). In no case did further additions of DCMU lead to any further reduction of Q_A. If the light were turned off after the addition of DCMU, the chlorophyll fluorescence would slowly return to lower values, indicating re-oxidation of PSII by quenching processes (data not shown).

Because the oxidation / reduction rates of the downstream PQ pool are several orders of magnitude slower than those of PSII, the redox level of Q_A can also be taken to reflect the redox level of the PQ pool. From the chlorophyll *a* fluorescence measurements the redox state of Q_A and PQ was calculated as:

$$(F_S - F_0) / (F_{max} - F_0)$$

The primary data from chlorophyll *a* measurements in starved, sucrose, glucose or mannose treated cells are discussed in the following section and shown in figure 3.11.

3.6 The effect of the glucose analogue mannose on the cell culture

Mannose has been used as an analogue to glucose in studies investigating metabolic regulation by sugars in plants (Jang and Sheen, 1994; Graham et al., 1994a; Pego et al., 1999). Like glucose it is phosphorylated by hexokinase, but is not metabolised to the same extent as the latter. Its sugar-like effect on gene expression and germination is attributed to its ability to generate a sugar signal at the hexokinase step (Pego et al., 1999). To evaluate its usefulness in studies of sugar and plastid redox signalling, the Arabidopsis cell suspension culture was subcultured into medium containing 20 mM mannose as the sole carbon source. After 4 days the growth of the cell culture was assessed (Fig. 3.10 A). Mannose completely inhibited cell growth in the culture. It could be argued that mannose might not be metabolised at sufficient rates to allow growth, leading to starvation. However, this is not the case, since cell culture starved of all carbohydrates shows significant growth. When equimolar amounts of glucose were present at the same time as mannose cell growth was still absent, providing further evidence for a specific effect of mannose. As shown in figure 3.4 B mannose *per se* was not toxic to the cells: after 4 days with 20mM mannose the cells look healthy, though they had not grown. When *CAB* transcript levels were compared from cells maintained for 4 days on 3% sucrose, 20mM glucose or 20mM mannose, mannose treated cells showed strong repression, just like sucrose fed cells (Fig.3.10 B). Cells kept with 20 mM glucose showed higher *CAB* mRNA levels, probably because 20 mM glucose had already been depleted from the medium at this stage and the sugar repression of *CAB* had ended. When soluble sugar levels in these cells were analysed, it was found that glucose fed cells indeed had much lower sugar values than sucrose fed cells (Fig. 3.10 C) although the intracellular hexose levels were still considerable. Starved cells showed

only minimal levels of the sugars. Surprisingly mannose fed cells showed significant levels of glucose and fructose. Mannose treatment might either result in glucose and fructose being depleted more slowly than in starved cells, or mannose itself could potentially be metabolised (see also Boxall (1999).

Although hexose levels were significant in mannose fed cells they were lower than those in glucose fed cells, yet *CAB* was strongly repressed (Fig. 3.10 B and C). This provides evidence that the intracellular hexose levels *per se* are not responsible for the regulation of photosynthetic gene expression. However, mannose could still act as a potent signal for the putative hexokinase sensing system. Different hexoses might evoke signals of different intensity and the flux rather than absolute levels of hexoses may be important (see also Stitt et al. (1995))

To study the effect of mannose on plastid redox state, 3 day old suspension culture was washed and resuspended in medium with either 3% (w/v) sucrose, 20mM glucose, 20mM mannose or no carbohydrates. Since it has been suggested that mannose will lead to sequestering of phosphate due to the build-up of mannose-6-P, some cells were resuspended in medium containing 20mM mannose and 20mM phosphate in addition to the usual amount of phosphate present in the medium. All treatments were incubated for 24h under the given growth conditions.

The redox state of PSII reaction centres and the plastoquinone pool was estimated by measuring chlorophyll *a* fluorescence (Figs. 3.11). When after 24 h treatments starved, glucose and sucrose fed cells were illuminated with an actinic beam of 20μmol photons m^{-2} s^{-1} PAR, chlorophyll *a* fluorescence increased slightly, indicating that a small percentage of Q_A and PQ was now reduced (Fig. 3.11 A). When the electron flow was blocked by the addition of 10 μM DCMU, Q_A became fully reduced (maximal fluorescence) (Fig. 3.11A).

Pre-treatment with mannose for 24 hours had a major effect on the fluorescence (Fig. 3.11 B). For example, when compared with starved cells, the initial F_0 level of fluorescence was significantly higher and the final F_{max} level significantly lower. Furthermore, upon exposure to actinic light (20μmol photons m^{-2} s^{-1} PAR), there was no evidence for any reduction of the Q_A pool (c.f. small increase from F_0 to F_S in starved cells, Fig. 3.11 B). If we assume that the addition of DCMU results in 100% reduced Q_A then we conclude that in the mannose treatment the initial state of Q_A is significantly more reduced than in the glucose, sucrose or starvation treated cells.

Figure 3.9: Schematic of a chlorophyll a fluorescence measurement (copy)

A copy of figure 2.1, provided here for ease of comparison with figure 3.11

The blue line depicts the typical chlorophyll a fluorescence during the measurements. The black bar indicates dark treatment, the yellow bar indicates the period of actinic light treatment. The green dotted line indicates the presence of DCMU which fully reduces PSII (Q_A). See the text for a detailed description.

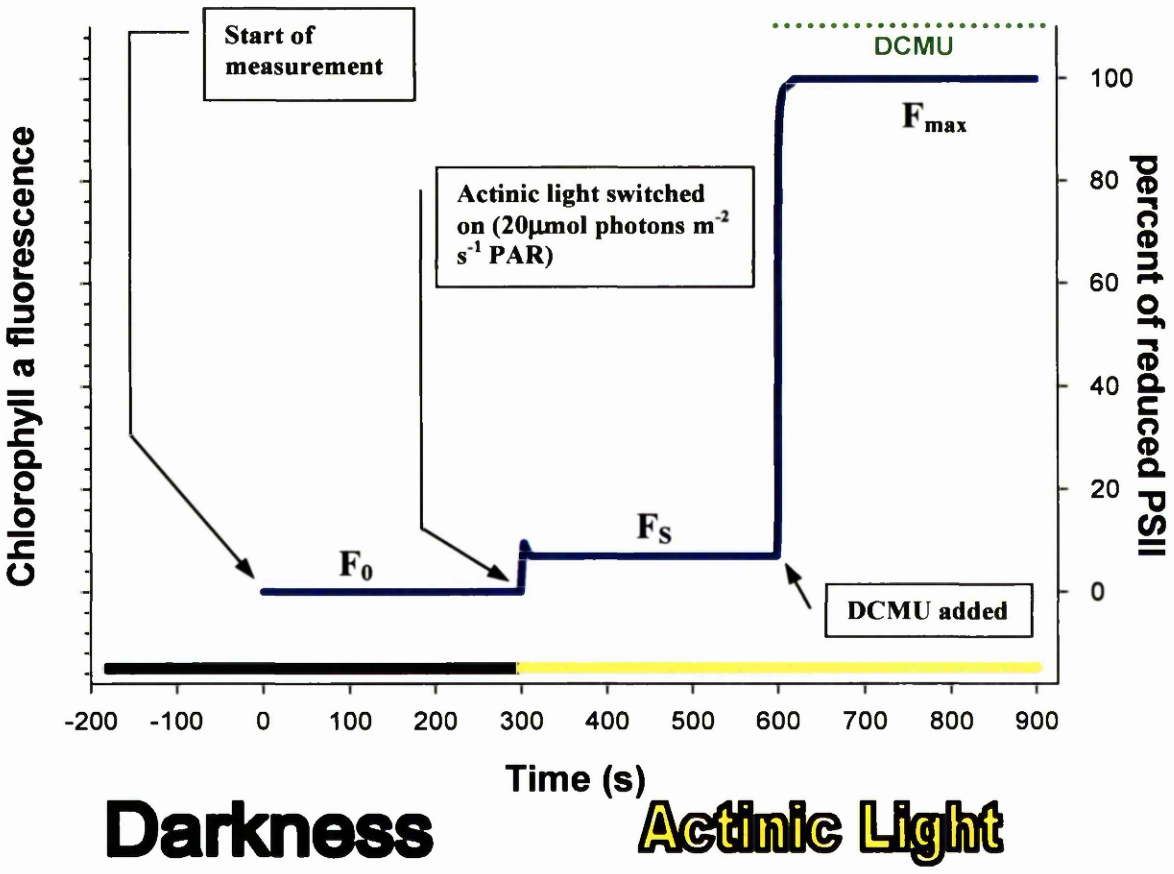
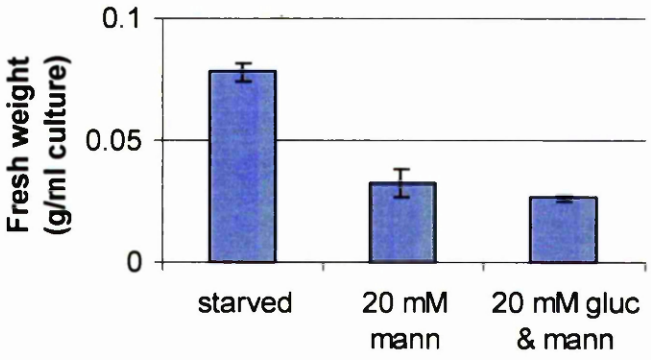

Start of measurement
Actinic light switched on (20µmol photons m-2 s-1 PAR)
DCMU
DCMU added
F0
FS
Fmax
Chlorophyll a fluorescence
percent of reduced PSII
0
20
40
60
80
100
-200
-100
0
100
200
300
400
500
600
700
800
900
Time (s)
Darkness
Actinic Light

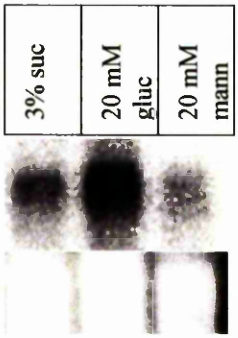
Figure 3.10: Growth of cell culture in the presence of 20mM mannose

Cell cultures were subcultured into fresh medium containing 3% (w/v) sucrose (i.e. 88 mM) sucrose (suc), 20 mM glucose (gluc), no carbohydrates (starved), 20 mM mannose (mann) or 20 mM mannose plus 20mM glucose and grown for 4 days.
(*A*) Comparison of the fresh weight of starved, mannose or mannose plus glucose treated cells (averages and standard deviation of 2 replicates). (*B*) *CAB* mRNA abundance in sucrose, glucose and mannose treated cells (7 μg total mRNA , Ethidium Bromide stained rRNA on the gel shown is shown for comparison) (*C*) Intracellular glucose (■), fructose (□) and sucrose (□) levels in sucrose-, glucose-, mannose-treated and starved cells (measured in triplicate, standard errors are shown).

A


Fresh weight (g/ml culture)
0.1
0.05
0
starved
20 mM mann
20 mM gluc & mann

B


3% suc
20 mM gluc
20 mM mann

C

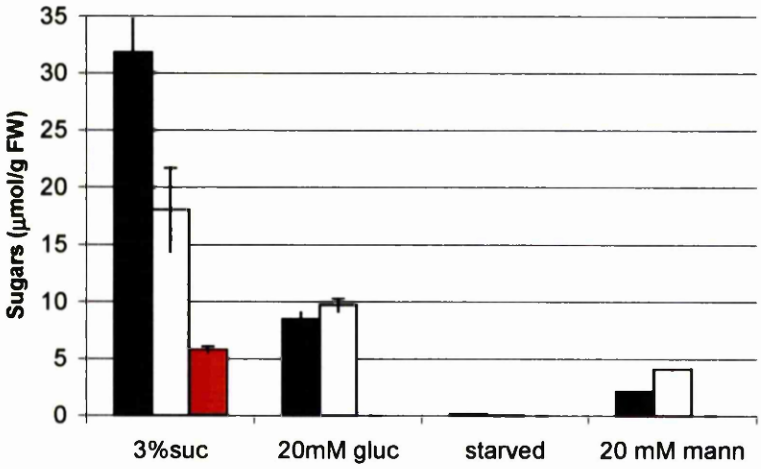
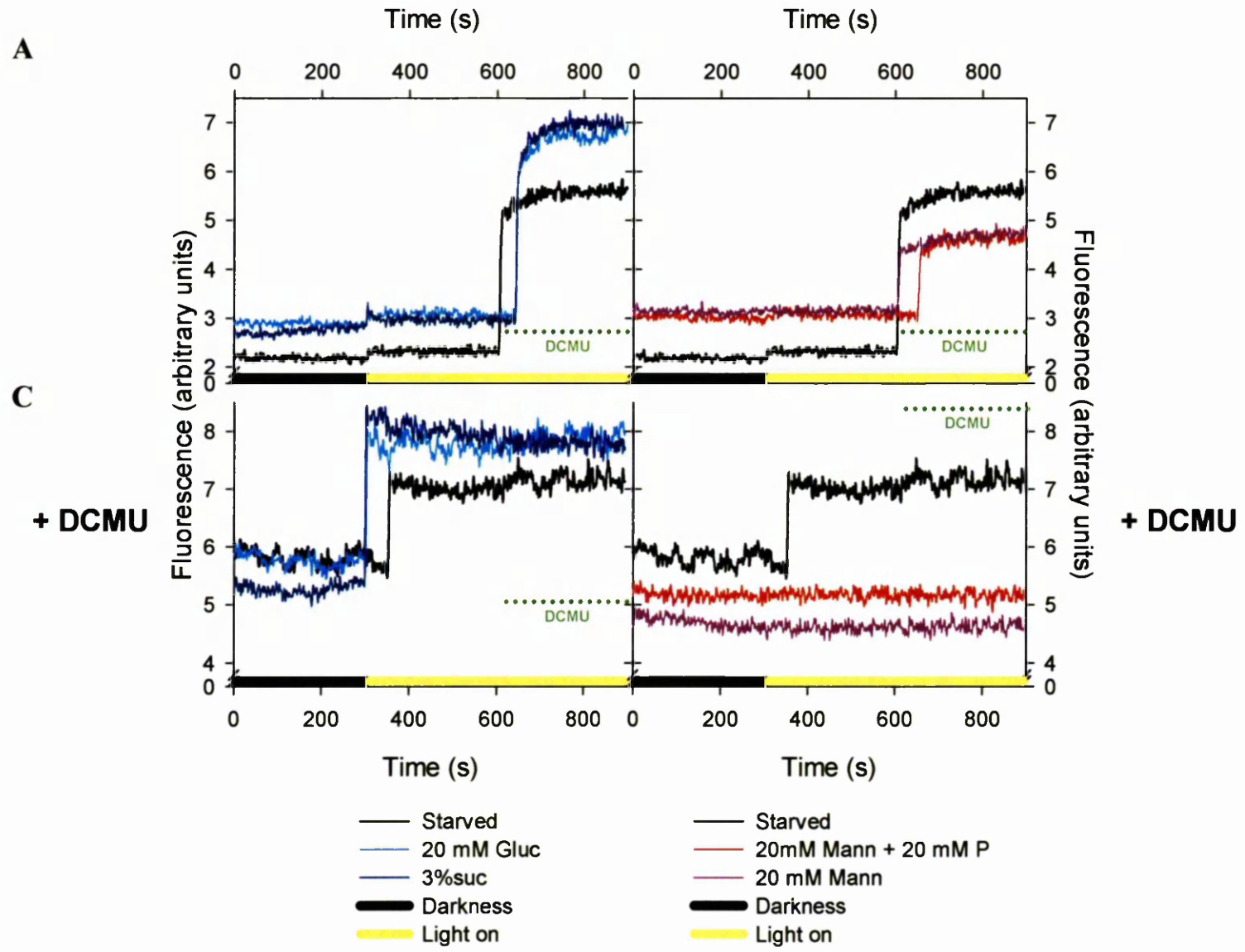

Sugars (µmol/g FW)
35
30
25
20
15
10
5
0
3%suc
20mM gluc
starved
20 mM mann

Figure 3.11: Chlorophyll a fluorescence at 685 nm in response to starvation, glucose or mannose feeding

Three-day-old *Arabidopsis thaliana* suspension culture was washed and re-suspended in medium with 3% (w/v) sucrose (suc), 20mM glucose (gluc), 20 mM mannose (mann), 20 mM mannose plus 20 mM phosphate (P) or no carbohydrate (starved) as described in chapter 2 section 2.2.13. After 24 hours under the normal growth conditions, samples were kept in the dark for at least 2 minutes and then chlorophyll a fluorescence at 685 nm was measured as described in chapter 2 section 2.2.15 (*A*, *B*). In parallel experiments, samples were incubated under the same conditions except that DCMU was added to a final concentration of 10 μM at the start of the incubation. Chlorophyll a fluorescence was measured as before (*C*, *D*).

The intensity of the actinic light beam was 20 μmol photons m^{-2} s^{-1} PAR.

The black and yellow bars at the bottom of each graph represent dark and actinic light treatments, respectively.


A
B
C
D
+ DCMU
+ DCMU
Time (s)
Time (s)
Time (s)
Time (s)
Fluorescence (arbitrary units)
Fluorescence (arbitrary units)
DCMU
DCMU
DCMU
DCMU
Starved
20 mM Gluc
3%suc
Darkness
Light on
Starved
20mM Mann + 20 mM P
20 mM Mann
Darkness
Light on

A parallel set of experiments were performed and another set of aliquots were treated in exactly the same way with the exception that DCMU (final concentration 10 μM) was added at the start of the 24h incubation period. When starved cells or cells supplied with sucrose or glucose were analysed after 24 h for chlorophyll *a* fluorescence, they showed the predicted pattern: since the electron transport was blocked, chlorophyll *a* fluorescence increased to the maximal value as soon as the samples were illuminated (Fig. 3.11 C). Additions of DCMU did not increase the fluorescence any further, since Q_A was already fully reduced. However, mannose supplied cells showed no response to light or further additions of DCMU. The presence of phosphate did not alter this response in mannose treated cells (Fig. 3.11 D).

It appears that mannose treatment results in a decrease in the maximal level of fluorescence (F_{max}; Figs. 3.11 B and D), possibly due to the loss of chlorophyll. However, in addition, mannose seems also to effect an increase in the F_0 level of fluorescence (Fig. 3.11 B) resulting in an apparent decrease in photosynthetic capacity (F_{max}-F_0 transition). When samples are incubated for 24h with mannose and DCMU the F_0 level of fluorescence appears to be completely abolished (Fig. 3.11 D).

These anomalic effect on chlorophyll fluorescence can be explained by suggesting that with mannose either Q_A (and the PQ pool) is not fully oxidised in the dark (F_0 is proportionally higher), or that a significant fraction of the cells become photosynthetically inactive thereby contributing a fixed level of fluorescence to the signal in the dark, as in the light and in the presence of DCMU. Additional phosphate could not prevent this effect of mannose consistent with the inability of phosphate to prevent other mannose effects, despite the rapid uptake of phosphate (Pego et al., 1999).

To investigate the effects of mannose in more detail, 3-day-old cell culture was washed and resuspended in medium containing 20 mM mannose. Chlorophyll levels, chlorophyll *a* fluorescence, respiration and photosynthetic oxygen evolution were monitored up to 27h after the start of the incubation (Fig. 3.12).

Mannose fed cells showed a transient increase in chlorophyll levels that was followed by a dramatic decrease in chlorophyll levels to less than 2/3 of the starting level after 9 hours of mannose treatment (Fig. 3.12 A). The chlorophyll levels remained at this reduced level for the remainder of the treatment. After 6 hours of mannose treatment the chlorophyll *a/b* ratio also decreased, yet not quite as dramatically as the total

chlorophyll levels (Fig. 3.12 A). The chlorophyll data seems to indicate that after a transient increase in light harvesting complex abundance mannose leads to the loss of light harvesting complex and the loss of reaction centres commencing little more than 3 hours after the exposure to mannose.

Chlorophyll *a* fluorescence was measured at the times indicated. When the percentage of reduced Q_A in response to light (20µmol photons m^{-2} s^{-1} PAR) was determined as shown above (cf. Fig.3.9), the redox state of the mannose-treated cells was shown to increase over the first 9 hours from 5 % to 9%. However a more detailed analysis proved that all three fluorescence parameters (F_0, F_S and F_{max}) were decreasing in mannose treated cells (Fig.3.12 B), making the calculations of the Q_A redox state as described above void. However, two photosynthetic parameters could be assessed as follows:

$$F_V / F_{max} = (F_{max} - F_0) / F_{max}$$

This is called the quantum yield and describes the fraction of absorbed energy diverted to PET

and

$$F_{V'} / F_{max} = (F_{max} - F_S) / F_{max}$$

This value describes the percentage of closed photosynthetic reaction centres.

Both parameters can be taken as an estimate of the efficiency of photosynthesis at PSII. When these values were calculated and plotted, a strong decrease of both parameters in cells treated with mannose for more than 6 hours was observed, while sucrose-fed cells and starved cells did not decrease (Fig. 3.12 C).

Taken together the chlorophyll and fluorescence data indicate a loss of photosynthetic reaction centres (decrease in chlorophyll *a/b* ratio and loss of light inducibility of fluorescence) and antenna complexes (decrease in total chlorophyll) in response to mannose treatment.

Respiration and photosynthesis decrease quickly after the exposure to mannose (Fig. 3.12 D). Within 3 h the respiration rate has fallen to only half the respiration rate at t0, and after 24 h the respiration in mannose-treated cells is half the respiration rate for cells starved for the same period (Fig. 3.12 D). Levels of glucose and fructose are higher than in starved cells at that point (Fig. 3.10 C). Interestingly, the azide resistant

Figure 3.12: Monitoring of the effects of mannose on cell culture over a 27h period

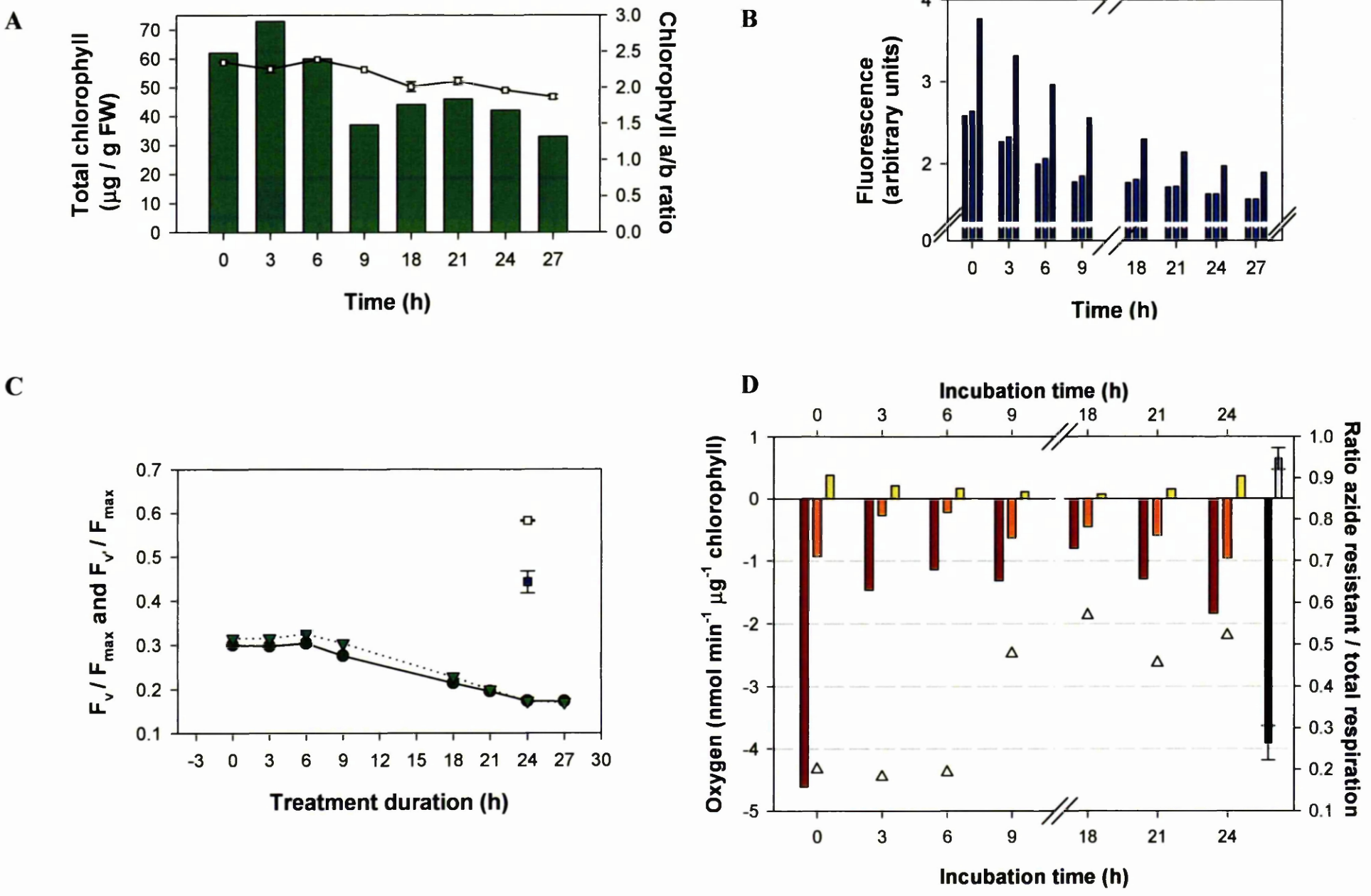
Three-day-old Arabidopsis suspension culture was washed and re-suspended in medium with 20mM Mannose. Samples were taken at the times indicated over a total of 27 hours. (*A*) Chlorophyll levels (bars) and chlorophyll a to chlorophyll b ratio (—□—) in mannose treated cells.

(*B*) Chlorophyll fluorescence emanating from PS II was measured at 20 µmol photons m^{-2} s^{-1} PAR. F_0 (▬), F_S (▬) and F_{max} (▬) are shown.

(*C*) Decline of the photosynthetic parameters F_v/F_{max} (the quantum yield, calculated as $(F_{max} - F_0)/F_{max}$) (··▽··) and $(F_{max} - F_S)/F_{max}$ (—●—) in mannose treated cells. For comparison, the quantum yield F_v/F_{max} of cells starved for 24 hours (■; n=3) or sucrose-fed controls (□; n=2) are shown.

Note: Since no true time zero value was available for the starved and sucrose-fed control samples, it is not clear if the higher values observed for these samples compared to the t0 value shown here are true increases from t0 or represent a difference between batches. However, given the data and other evidence it appears unlikely that the values in these two treatments show any decrease like the mannose treated samples do.

(*D*) Respiration (▬), azide resistant respiration via the alternative oxidase (▬) and photosynthesis (▬). Respiration (▬) and photosynthesis (▬) of cell starved for 24 h are shown for comparison. The contribution of azide resistant respiration to the total respiration is shown as azide resistant respiration / total respiration (triangles)


A
Total chlorophyll (µg / g FW)
Chlorophyll a/b ratio
Time (h)
B
Fluorescence (arbitrary units)
Time (h)
C
F_v / F_{max} and $F_{v'} / F_{max}$
Treatment duration (h)
D
Incubation time (h)
Oxygen (nmol min^{-1} μg^{-1} chlorophyll)
Ratio azide resistant / total respiration
Incubation time (h)

respiration via the alternative oxidase is reduced initially similar to the total respiration, but recovers after 9 hours and is at approximately pre-treatment level after 24 h (Fig. 3.12 D). Its contribution to the cellular respiration increases from less than 20% to about 50% (triangles in figure 3.12 D).

Photosynthesis decreases after the addition of mannose though there appears to potentially be a transient peak or recovery after 24h of mannose exposure. Interestingly cells starved for 24h showed an increase in photosynthesis, and similar respiration rates, compared to cells before the start of the experiment, yet their soluble sugar levels were minimal (Fig. 3.12 D and 3.5 A).

Two explanations can account for these observations:

- Mannose treatment reduces the rate at which glucose and fructose are depleted. The reduced total respiration found after mannose treatment could indicate an inhibition of respiration via complex IV by mannose. After the initial inhibition the contribution of respiration via the alternative oxidase increases to partially compensate for the inhibition of complex IV respiration. Although the flux of sugars through glycolysis should be lower in this scenario, *CAB* mRNA abundance is repressed by mannose (Fig. 3.10 B) possibly via a hexokinase signal. Adding equimolar amounts of phosphate to the medium could not prevent the effects of mannose on PET (Fig. 3.11). Phosphate depletion was reported not to be responsible for the inhibition of germination by mannose (Pego et al., 1999). However, a sequestration of phosphate by mannose could contribute to the observed effects in this scenario.
- Mannose itself is metabolised allowing the cells to partly maintain glucose and fructose levels despite the absence of sucrose, glucose or fructose from the medium. The metabolism of mannose has a strong repressive effect on *CAB* transcript levels. However, respiration, in particular via complex IV, is inhibited. Evidence for an efficient metabolism of mannose in Arabidopsis seedlings was provided by Boxall (1999)

If mannose inhibits PET in some way, this would first lead to a higher responsiveness to low light followed by a generally more reduced PET chain. In the light photodamage of the photosynthetic reaction centres and antenna complexes would occur at an increased rate. Furthermore, chlorophyll *a* fluorescence and oxygen evolution would be expected

to decrease. A decrease in chlorophyll *a* fluorescence and photosynthesis was in fact observed (Fig. 3.12 B - D) and PSII was more reduced (Fig. 3.11 B). Light harvesting complex abundance and reaction centre abundance also decreased (Fig. 3.12 A & B).

To summarise: mannose results in a strong repression of *CAB* gene expression, stops cell growth, results in reduced respiration via complex IV and has a remarkable effects on photosynthetic and respiratory electron transport chains. The mechanism by which mannose causes these effects is unclear but is appears to be much more complex than simply acting as an analogue of glucose to generate a hexokinase mediated signal as previously proposed (Graham et al., 1994a; Pego et al., 1999).

Chapter 4: Blocking photosynthetic electron transport

4.1 Introduction

The results presented in chapter 3 demonstrate that the Arabidopsis cell suspension culture is a suitable model system for the study of possible interactions between sugar regulated gene expression and redox signalling. When the impact of starvation on photosynthesis in the cell culture was assessed, it was discovered that photosynthetic oxygen evolution is decreased (Fig. 3.6 B) and nuclear encoded transcript levels of *CAB* are increased in starved cells (Fig 3.5 F). While a difference between starved and sucrose fed cells in the capacity to transport electrons along the PET chain was found at elevated light intensities, the redox state of the PET chain at the normal growth and experimental condition of 20μmol photons m^{-2} s^{-1} was similar (Fig. 3.6 D). At this light intensity there was no direct effect of starvation or sucrose feeding on the redox state of the PET chain. To establish if an alteration in the redox state of the PET chain affected the regulation of nuclear photosynthetic genes by the sugar status, a series of experiments using the inhibitor DCMU were performed. This compound inhibits electron transfer between PSII and the PQ pool, and was chosen for its selectivity for the PET chain only, its stability, ease of use and widespread application in studies of PET redox signalling (Pearson et al., 1993; Escoubas et al., 1995; Karpinski et al., 1997; Alfonso et al., 2000; Sippola and Aro, 2000). DCMU prevents the flow of electrons from the Q_A site of PSII to the Q_B site and the PQ pool (see graphic in Figure 1.2), leading to a fully reduced PSII (as can be observed by chlorophyll *a* fluorescence cf. Fig 3.11) but a mostly oxidised PET chain from the PQ pool downwards. A final concentration of 10μM of the inhibitor was found to achieve complete inhibition of electron transport within seconds and no decrease of inhibition was observed after 24 hours (Fig.3.9).

4.2 Plastid redox and sugar signals do interact

3-day-old suspension culture cells were washed and resuspended in medium with or without 3% (w/v) sucrose as described in chapter 2 section 2.2.13 of this thesis. DCMU

dissolved in ethanol was added to half of the starved and half of the sucrose fed samples (final concentration 10μM). Controls were treated with ethanol. The cells were incubated under the usual growth conditions for 24h and then harvested and stored at –70°C. Total RNA was extracted and analysed by RNA blotting.

The results obtained suggest that the response to starvation reported above is specific for the transcript levels of photosynthetic genes (Fig. 4.1). After 24 hours starvation *CAB* (Fig. 4.1*A*) and *RBCS* (Fig. 4.1*B*) transcript levels increased significantly ($p < 0.01$) over those of sucrose-fed cells. In contrast, the corresponding levels for *NR* showed a positive correlation with intracellular sugars (Fig. 4.1*C*), whilst those of chalcone synthase were insensitive to sugar status (Fig. 4.1*D*).

The addition of DCMU to samples abolished the starvation-induced increase in both *CAB* and *RBCS* transcript levels (Fig. 4.1*A* & 4.1*B*), but had no effect on *NR* transcript abundance (Fig. 4.1*C*). Autoradiographs of *CAB*, *NR* and *ARF* (constitutive control) probed Northern blots are shown. (Fig 4.1 E).

These results suggest that the normal starvation-induced increase in the transcript levels of nuclear-encoded photosynthetic genes requires PET and is modulated through a plastid-derived signal.

Surprisingly, when transcript level of the plastid encoded, photosynthetic gene *psbA* were analysed, these were induced in starved cells but in contrast to nuclear photosynthetic genes *psbA* transcript abundance was not affected by DCMU (Fig 4.1 F). Yet *psbA* mRNA levels - just like nuclear photosynthetic gene transcripts - were lower in the presence of sugar. It is noteworthy that the chlorophyll measurements of starved and sucrose fed cells implied a similar situation for the abundance of the chlorophyll *a* containing reaction centre proteins (Fig. 3.7 C). To my knowledge this is the first report of a sugar regulation of *psbA* transcript abundance. Previously mRNA levels for other plastid genes such as *rbcL* were reported not to change in response to sugars (Vanoosten and Besford, 1994), or only after prolonged treatment compared to nuclear encoded *RBCS* transcript levels and Rubisco protein levels (Criqui et al., 1992).
Pfannschmidt and co-workers reported that the expression of the two plastid genes *psaAB* and *psbA* were regulated by the redox state of PQ in *Sinapis alba* seedlings

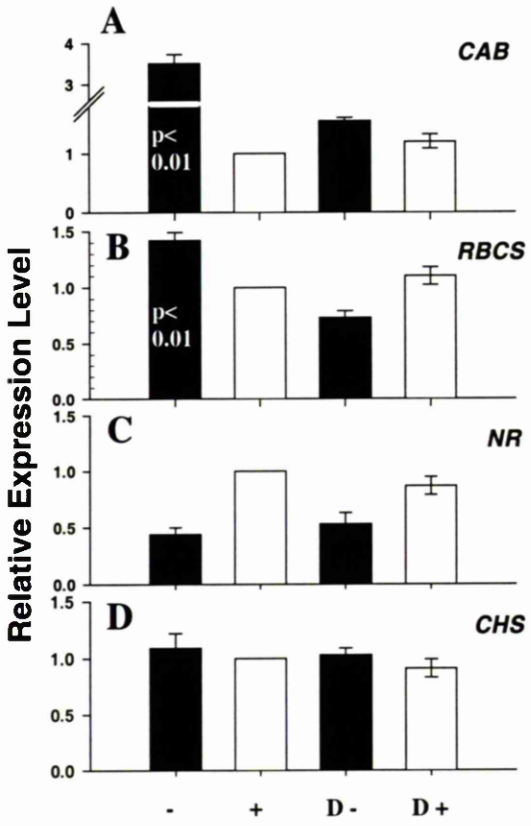
Figure 4.1: DCMU prevents the starvation-induced increase in transcript levels of nuclear encoded photosynthetic genes

Three-day-old cell cultures were treated as described for figure 3.5. DCMU in ethanol was added at time 0 to a final concentration of 10μM, controls were treated with ethanol (0.1%). Samples were harvested after 24h. Total RNA was isolated from the samples and 7 μg total mRNA analysed by Northern Blot analysis. Autoradiographs were scanned and signal intensity quantified; the values presented are the averages and standard errors from 6 independent experiments; the values have been normalised on the constitutively expressed gene (*ARF*), and are presented relative to the expression level in sucrose treated cells. An analysis of variance test was performed on the data and significant probability levels given on the graph. (*A*) Chlorophyll a/b binding protein - *CAB*; (*B*) small subunit Rubisco - *RBCS*; (*C*) nitrate reductase - *NR*; (*D*) chalcone synthase – *CHS*. Filled bars represent starved samples, white bars sucrose treated samples.

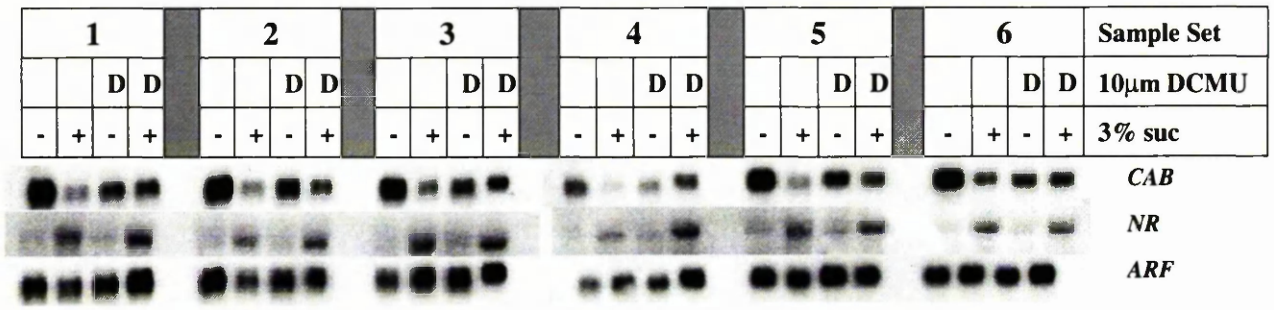
(*E*) Autoradiographs showing the abundance of *CAB*, *NR* and *ARF* gene transcripts in the 6 sets of samples.

(*F*) Autoradiographs showing the abundance of the plastid encoded *psbA* gene transcript in the same samples. An image of the Ethidium Bromide stained rRNA on the gel is included for comparison..

Codes: (-) and (+), absence and presence of 3 % (w/v) sucrose; D, presence of 10μM DCMU.


A
CAB
4
3
1
0
p< 0.01
B
RBCS
1.5
1.0
0.5
0.0
p< 0.01
C
NR
1.5
1.0
0.5
0.0
D
CHS
1.5
1.0
0.5
0.0
Relative Expression Level
-
+
D -
D +

E


1
2
3
4
5
6
Sample Set
D D
10μm DCMU
- + - +
3% suc
CAB
NR
ARF

F

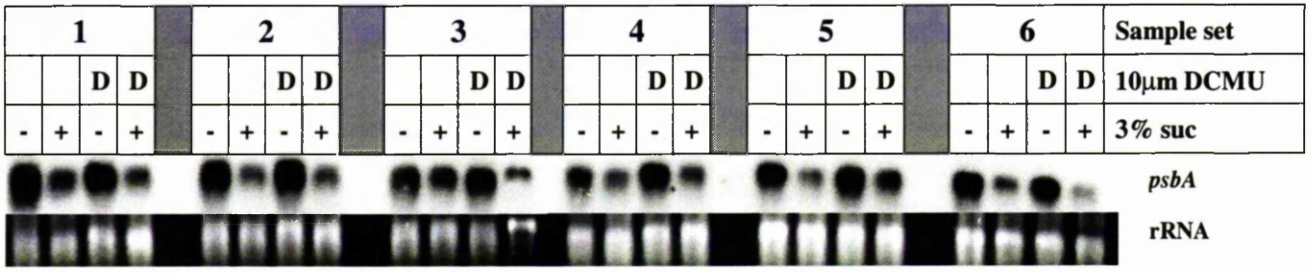

1
2
3
4
5
6
Sample set
D D
10μm DCMU
- + - +
3% suc
psbA
rRNA

Figure 4.2: Sugar levels in DCMU treated cells

Three-day-old cultures were treated as described for Fig. 4.1. Samples were taken after 8 and 24 hours and the intracellular sugar content was analysed in triplicate as described in chapter 2 paragraph 2.2.12. Sugar levels in 3-day old and 4-day old controls are shown for comparison.

Codes: (+) presence of 3% (w/v) sucrose; (-) starved ; 'D' indicates the presence of 10 µM DCMU, controls were treated with ethanol; (■) glucose, (□) fructose and (□) sucrose in µmol / g FW (approx. mM).

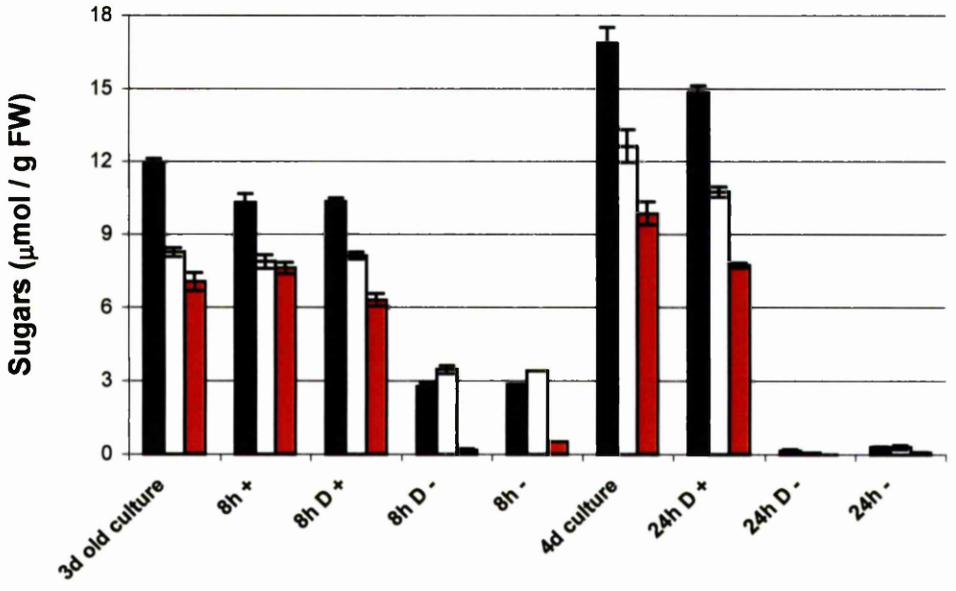
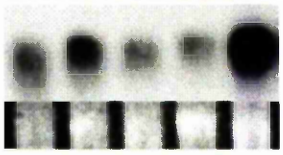

Sugars (µmol / g FW)
18
15
12
9
6
3
0
3d old culture
8h +
8h D +
8h D -
8h -
4d culture
24h D +
24h D -
24h -

Figure 4.3: *RBCS* transcript levels in dark treated cell culture are at basal levels after 24 h independent of sugar status or the presence of DCMU

Three-day-old Arabidopsis suspension culture was washed and re-suspended in medium with or without 3% (w/v) sucrose. The cultures were treated with 10μM DCMU or ethanol and kept in darkness for 24 h. Total RNA was isolated. Total RNA was isolated from the samples and 7 μg total mRNA analysed for *RBCS* transcript levels by Northern Blot analysis. An image of the Ethidium Bromide stained rRNA on the gel is included for comparison.

The experiment was undertaken in collaboration with Dr. Thomas Martin, who performed the hybridisation and exposure of the autoradiographs.

	D		D		DCMU
+	+	-	-	-	3% sucrose
d	d	d	d	l	dark / light



(Pfannschmidt et al., 1999). While *psbA* codes for the D1 core protein of PSII, *psaAB* codes for the core protein of PSI. Expression of either gene could be induced by altering the redox state of PQ with light regimes favouring PSI or PSII as measured by run-on assays of isolated chloroplasts (Pfannschmidt et al., 1999). However, in contrast to *psaAB*, *psbA* showed no or very little decrease in transcription rate or mRNA abundance *in vivo*, and was unresponsive *in vitro* to attempts to alter PQ redox state by light or the use of inhibitors such as DCMU and DBMIB (Pfannschmidt et al., 1999). While *psaAB* expression showed a repression or induction correlating with the redox state of PQ *in vivo* and *in vitro* (Pfannschmidt et al., 1999) the regulation of *psbA* transcript abundance could be influenced by other factors. In the algae *Chlamydomonas reinhardtii* the translation of *psbA* mRNA has previously reported to be regulated by the plastid redox state (Danon and Mayfield, 1994). However, the authors used thiol redox reagents such as DTT and thioredoxin to demonstrate the translational control by the redox state, but not inhibitors altering the redox state of PQ. It is entirely possible that not only *psbA* translation (Danon and Mayfield, 1994) but also transcription is regulated by the redox state of thioredoxin, but not by the PQ redox state, in contrast to *psaAB*. Indeed the activity of one component of the plastid major RNA polymerase complex in mustard was shown to be regulated by reduced glutathione (Baginsky et al., 1999). However the role of thioredoxin in this regulation was not explored in that study.

In the experiment described here (Fig.4.1 F) only the transcript abundance of *psbA* could be assessed. However, transcript levels depend on both transcription and mRNA stability. It has been suggested, that plastid encoded photosynthetic genes are mostly regulated on the level of mRNA stability and translation, while nuclear encoded photosynthetic genes are mostly regulated at the transcriptional level in response to changes in illumination (Bruick and Mayfield, 1999). A model for the regulation of *psbA* translation in *Chlamydomonas reinhardtii* by the plastid redox state has been presented, yet the situation in higher plants remains unclear (Bruick and Mayfield, 1999). The results described in figure 4.1 are entirely consistent with the suggested regulation of nuclear photosynthetic genes on the level of expression (Fig. 4.1 A, B and E) and a regulation of plastid encoded *psbA* on the level of translation, but not mRNA abundance (Fig. 4.1 F).

DCMU addition had no effect on the rates at which sugars were depleted upon starvation (Fig. 4.2) thus indicating that DCMU has no major impact on glycolysis and respiration in these cultures. When cell cultures were treated with DCMU as described

before, but kept in the dark over the starvation period, *RBCS* transcript levels were very low even in the starved samples. No effect of DCMU could be observed, and only a very basal level of *RBCS* mRNA could be found in starved or sucrose treated cells submitted to darkness (Fig 4.3). This shows the necessity of light perception for the basal expression of nuclear encoded photosynthetic genes.

The evidence presented above demonstrates that high intracellular sugars repress the abundance of nuclear-encoded photosynthetic gene transcripts through the action of an uncharacterised cytosolic signalling mechanism that may involve hexokinase. However, the removal of the sugar-repression signal alone does not result in increased transcript levels for these genes; the experiments with DCMU clearly indicate that an additional signal is required which is dependent upon PET.

4.3 In planta *analysis of the influence of PET on sugar regulated nuclear gene expression*

Having established the effect of DCMU on nuclear encoded photosynthetic transcript levels in starved cell suspension culture, two further questions had to be addressed:

- Does the phenomenon of plastid redox and sugar signal interaction observed in the cell culture also occur in whole plant tissue?
- At what level is the control of nuclear encoded photosynthetic genes excerted, i.e. is it transcriptional or post-transcriptional?

To address both of these questions, Arabidopsis plants carrying the firefly luciferase reporter gene under the control of nuclear photosynthetic gene promoters were chosen for further studies. Firefly luciferase has been used as a reporter gene in animals and plants due to its ease and speed of detection and shorter half-life compared to green fluorescent protein. To evaluate the reporter gene expression the plant material has to be sprayed simply with the substrate luciferin in a weak detergent solution. After a short time the luminescence can be measured using photon-counting equipment. Decreases in reporter gene expression can be monitored after pre-treatment of the material since the firefly luciferase has a lifetime of 2 h after substrate application (Millar et al., 1992). Moreover, constructs of nuclear photosynthetic promoters and luciferase reporter genes have been used before to investigate circadian rhythms (Millar et al., 1992) and even to

isolate sugar sensing mutants (VanOosten et al., 1997). These two transgenic *Arabidopsis* lines (*CAB2-LUC* (Millar et al., 1992) and *PC-LUC* (Dijkwel et al., 1996)) carrying constructs of the luciferase reporter gene (*LUC*) fused behind the endogenous *CAB2* and the plastocyanin gene (*PC*) promoters were used to test this DCMU effect *in planta*. For this purpose detached rosette leaves or leaf discs from rosette leaves were floated on solutions containing 3% (w/v) sucrose or 1.6% (w/v) mannitol as an osmotic control. Similar systems have been used for sugar feeding experiments before (Krapp et al., 1991) and these have proven to be an effective method for imposing different treatments on the experimental tissue. Mannitol has been widely used as an osmoticum in experiments with Arabidopsis and other plant species (Curti et al., 1993; Saleki et al., 1993; Thomas et al., 1995; Wu et al., 2000; Kiegle et al., 2000). Although it can not currently be ruled out that mannitol might be metabolised to a small degree (Steinitz, 1999), it does not invoke a sugar signal and can be used as an osmotic control for sucrose, glucose or fructose feeding (Mita et al., 1995; Dejardin et al., 1999).
Figure 4.4 *A* presents the results from a typical experiment on detached leaves from the *PC-LUC* line. Luminescence was low in the presence of sugar (+) and strongly increased in starved samples (-). However, luminescence remained low when leaves were starved for 24 hours in the presence of DCMU (D-). Similar results were obtained with detached leaves from *CAB2-LUC* lines (Fig. 4.4B). In order to quantify the luminescence experiments were performed on leaf discs from the *CAB2-LUC* (diagonal shading) and *PC-LUC* (solid shading) lines (Fig. 4.4C). The results from the detached leaves and leaf discs are entirely consistent with those obtained from the cell suspension cultures (Figs. 4.1).

Figure 4.4D presents detached leaf images from a similar experiment using the *Arabidopsis* mutant *sun6* (*sucrose-uncoupled*), also carrying the *PC-LUC* reporter gene construct. Quantified data from leaf discs of the *sun6* mutant is presented in Figure 4.4C (hatched shading). *Sun6* was isolated as a line in which sugar failed to repress *PC* expression (VanOosten et al., 1997) and it has been shown recently that *sun6* is allelic to *abi4* (*abscisic acid insensitive*) due to a disruption of an *APETELLA* 2-like transcription factor (Huijser et al., 2000). In sharp contrast to the wild type lines, sugar produced a 3-fold increase in luciferase activity over the levels in starved tissues. Our results suggest that, rather than being repressed by sugars, PC transcription is actually increased in the rosette leaves of the *sun6* mutant. Furthermore, blocking PET with

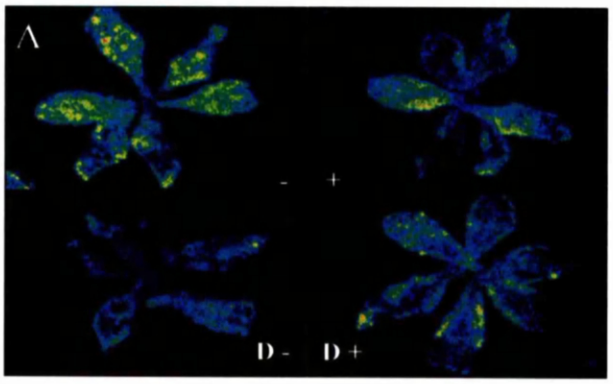
Figure 4.4: DCMU prevents induction of photosynthetic genes in transgenic plants, but not in the sugar response mutant *sun6*

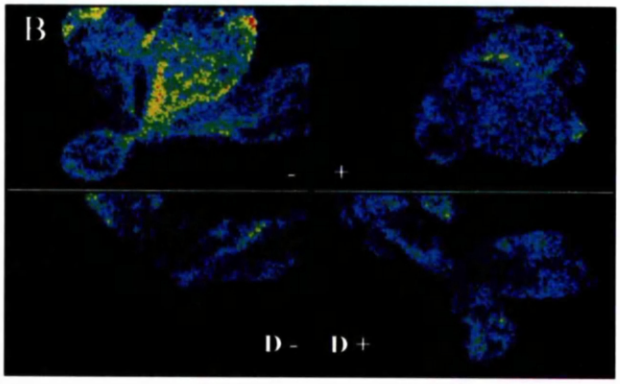
Luminescence from detached *Arabidopsis thaliana* leaves carrying the *PC-LUC* (*A*), *CAB2-LUC* (*B*), *PC-LUC* in *sun-6* background (*D*) and *CaMV35S-LUC* (*E*) constructs. Detached leaves were harvested and treated as described in chapter 2 paragraphs 2.3.2 to 2.3.4. Pseudo-colours represent luminescence intensity, low (dark blue), very high (red).

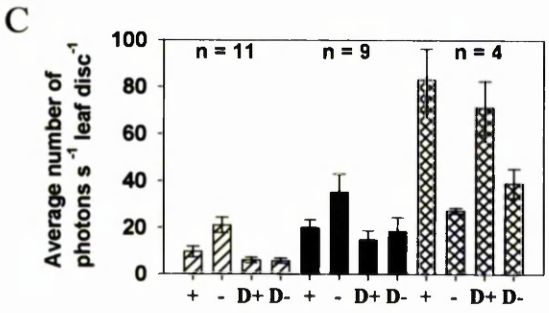
(*C*) Quantified luminescence of 4 mm diameter leaf disks from *CAB2-LUC* (diagonal shading), *PC-LUC* wt (solid shading) and *sun6* (hatched shading) plants. Values represent the averages and standard errors of number of photons emitted per leaf disk per s.

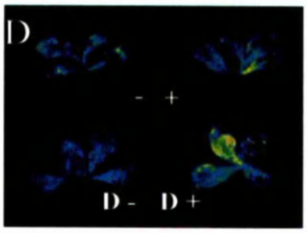
(*F*) Luminescence from detached leaves of the nitrite reductase promoter - luciferase reporter gene plants (*Nii-LUC*). These show slightly increased luminescence in response to sucrose.

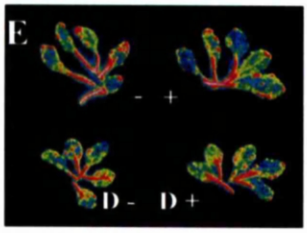
Treatment codes: (-) and (+), absence and presence of 3 % (w/v) sucrose for 24 hours; D, presence of 10μM DCMU for 24 hours prior to measurement.

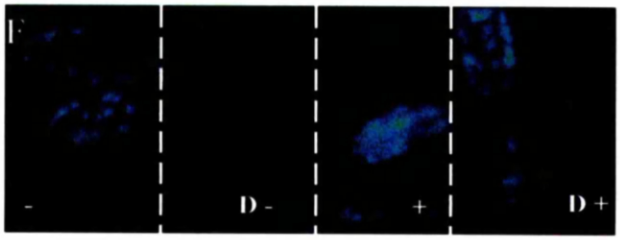

A
- +
D - D +


B
- +
D - D +


C
Average number of photons s-1 leaf disc-1
100
80
60
40
20
0
n = 11
n = 9
n = 4
+ - D+ D- + - D+ D- + - D+ D-


D
- +
D - D +


E
- +
D - D +


F
-
D -
+
D +

DCMU does not affect the sucrose-dependent increase of PC transcription in the *sun6* mutant. Therefore, it can be concluded that a lesion in the *sun6 / abi4* gene, which disrupts the sugar repression response in young seedlings, causes sugar hypersensitivity in fully expanded leaves. This hypersensitive response is not influenced by the plastid-derived signal.

In vivo luciferase activity is dependent on endogenous ATP. To ensure that the effects of sugar and DCMU seen in the *CAB2-LUC* and a *PC-LUC* lines did not arise as a consequence of altered ATP levels, experiments were carried out on a transgenic line carrying the constitutively expressed *CaMV35S-LUC* construct. Neither sugar nor DCMU affect luciferase activity in this line (Fig. 4.4E).

The expression of the non-photosynthetic, nuclear gene nitrite reductase was not altered by DCMU, as can be seen by the luminescence of leaves carrying a construct of the tobacco nitrite reductase promoter and firefly luciferase (Dorbe et al., 1998). The luminescence was higher in sugar fed leaves and not affected by DCMU (Fig. 4.4F), consistent with the results obtained for NR transcript levels in the cell culture (Fig.4.1D)

4.4 Repression of photosynthetic genes by PET does not occur

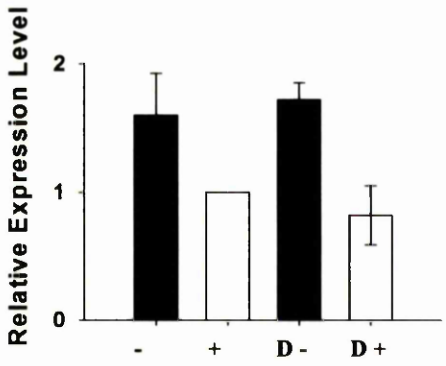
The requirement for PET for the induction of nuclear-encoded photosynthetic gene transcription was uncovered by DCMU addition at the time of sugar removal from transcriptionally repressed samples (see above). However it had to be established whether the blocking of PET could reverse the induction of photosynthetic genes when these were already induced due to starvation. DCMU was added to cell culture (Fig 4.5*A* and *B*) and *PC-LUC* leaf discs both already starved for 24 hours (Fig. 4.5*C*). These results clearly show that DCMU does not repress photosynthetic genes of the nucleus once they have been de-repressed due to sugar removal.

To summarise, the experiments on the effects of sugar and DCMU on *CAB2* and *PC* expression *in planta* support the conclusion drawn from RNA analysis on cell suspension cultures, that a chloroplast-derived signal modulates the de-repression of nuclear-encoded photosynthetic genes. However, this same signal does not have an effect on *CAB* and *PC* genes once they are de-repressed.

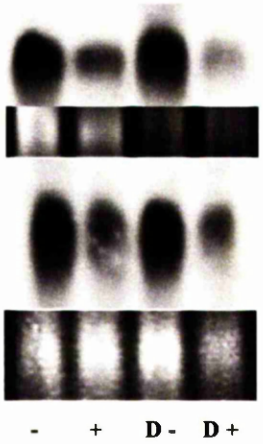
Figure 4.5: DCMU is unable to repress photosynthetic genes in starving cells

Cell cultures and leaf disks from the *PC-LUC* lines were prepared, as described in Figs 4.1 and 4.4, except that where indicated, DCMU was added only after the samples had been starved for 24 hours. (*A*) *CAB* transcript levels were determined 3 hours after DCMU addition by RNA blot analysis (7 μg total mRNA) as described in Fig. 4.1. The averages and standard errors are shown (n=3); black bars represent starved samples, white bars the sucrose-fed controls (*B*) Autoradiographs showing *CAB* and *psbA* mRNA levels in one of the three sets. An image of the Ethidium Bromide stained rRNA on the gel is included for comparison. Codes: (-) and (+), absence and presence of 3 % (w/v) sucrose; D, presence of 10μM DCMU.
(*C*) Pseudo-colour luminescence images of *PC-LUC* leaf disks 4 hours after DCMU addition (blue = low, red = high luminescence).

A



B



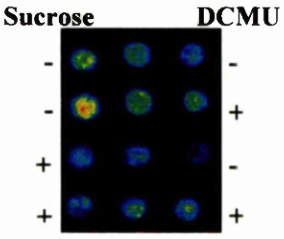
CAB

rRNA

psbA

rRNA

C



4.5 The use of the inhibitor DBMIB to elucidate the role of PQ redox state

In addition to DCMU, DBMIB has often been used to block photosynthetic electron transport (Escoubas et al., 1995; Karpinski et al., 1997; Pfannschmidt et al., 1999; Alfonso et al., 2000; Sippola and Aro, 2000). In contrast to DCMU DBMIB blocks electron transfer from the PQ pool to the cytochrome b_6/f complex (Durnford et al., 1998). Whereas DCMU blocks between Q_A and Q_B and therefore leads to a fully oxidised PQ pool, DBMIB leads to a fully reduced PQ pool, as well as fully reduced PSII. Since the Q_A site of PSII, the PQ pool itself or the cytochrome b_6/f complex have been implicated (Pearson et al., 1993; Escoubas et al., 1995; Alfonso et al., 2000) as the origin of a PET redox signal (see Fig. 1.2) the two inhibitors have been used to help establish which is the signal generation site. However, in the past results from experiments with DCMU and DBMIB have not been without contradiction (Pearson et al., 1993; Escoubas et al., 1995; Alfonso et al., 2000; Sippola and Aro, 2000). DCMU is highly specific for the photosynthetic electron transport chain, DBMIB is not as specific and can result in an inhibition of the respiratory electron transport chain as well. In addition DBMIB is unstable at higher irradiances (Trebst A, 1980; Rich et al., 1991).

DBMIB treatments of the cell culture showed that it could block electron transport in the suspension cells within 20 seconds of the addition at concentrations of 1 to 3 μM (data not shown). However the effect of DBMIB on chlorophyll fluorescence and hence electron transport disappeared within 30 minutes of application. This was not due to light induced breakdown or a chemical reagent in the culture medium, since the same loss of inhibition was observed in darkness and required the presence of cells (data not presented). This inactivation, excretion or breakdown of DBMIB meant that 24h treatments were not possible to perform.

When 3-day-old cells were starved for 24 h and then treated with 5 and 10 μM DBMIB every 25 minutes over a period of 150 minutes, PSII could be maintained in a greatly reduced state as determined by monitoring chlorophyll fluorescence (data not shown). When *CAB* transcript levels were analysed in the samples of this treatment, 5 and 10 μM DBMIB seemed to cause lower *CAB* transcript levels in starved cells, compared to the ethanol treated controls (Fig 4.6). This is in contrast to the results obtained with

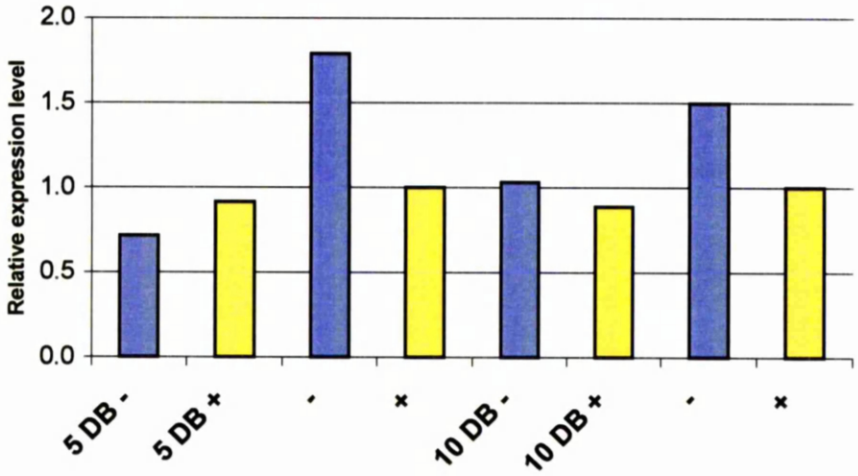
DCMU (Fig. 4.5). In cells starved for 24 h before the application of DCMU, DCMU had no effect when cells were incubated for nearly 3 hours.
Since DBMIB can inhibit respiratory electron transport, respiration of the samples was monitored as well. It was found that 10 μM DBMIB did indeed inhibit the respiration of the cells between 10 to 25 percent, though no significant effect on respiration was found for 5 μM (data not shown).

Based on the fact that DBMIB was rapidly inactivated along with the non-specific nature of the inhibitor it was decided to discontinue these experiments. The role of the redox state of the PQ pool in the observed phenomenon could not be clarified. In future experiments other, more specific inhibitors, light qualities specific for either PSI or PSII and antisense plants with reduced amounts of components of the PET chain will have to be employed to address this question.

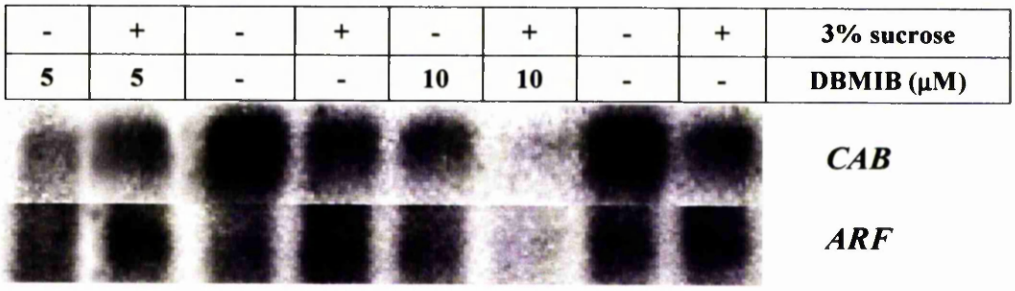
Figure 4.6: Repeated additions of DBMIB appear to repress *CAB* expression in starving cells

Cell cultures were prepared as described in chapter 2 paragraph 2.2.13 except that where indicated, DBMIB was added after samples had been starved for 24 hours. The final concentration of DBMIB was 5 and 10 μM. Since DBMIB was inactivated quickly, the additions were repeated every 25 minutes over 150 minutes. Controls were treated with ethanol. Total RNA was extracted and 7 μg used for RNA blot analysis.
(*A*) *CAB* transcript levels were determined as described in Fig. 4.1. Codes: (-) and (+), absence and presence of 3 % (w/v) sucrose; 5 DB, presence of 5μM DBMIB; 10 DB, presence of 10μM DBMIB.
(*B*) Autoradiographs. Levels of the constitutive ARF transcript are shown for comparison.

A


2.0
1.5
1.0
0.5
0.0
Relative expression level
5 DB -
5 DB +
-
+
10 DB -
10 DB +
-
+

B


- + - + - + - + 3% sucrose
5 5 - - 10 10 - - DBMIB (μM)
CAB
ARF

Chapter 5: Altering the redox balance by low temperature and increased light intensities

5.1 Introduction

In the work described in chapter 4, photosynthetic inhibitors were used to alter the redox state of the PET chain. However, the dark reactions of photosynthesis are temperature dependent biochemical processes, while the light reactions of photosynthesis are largely temperature independent, light driven processes (Fig. 5.1). Elevated illumination leads to a reduction in the redox state of the PET chain and has been used in combination with photosynthetic inhibitors (Karpinski et al., 1997) or low O_2 and CO_2 partial pressures (Montane et al., 1998). On the other hand a lower temperature which decreases the flux of carbon through the Calvin cycle also leads to a reduced redox state of the electron transport components (Maxwell et al., 1995; Huner et al., 1996; Savitch et al., 1996). Both light and cold have been shown to alter the redox state of the PET chain without the use of inhibitors (reviewed in Huner et al. (1998)). Huner and co-workers used a combination of these environmental parameters to create low and high redox states in the algae *Dunaliella salina* at different temperatures and illuminations (Maxwell et al., 1995). The results from this work imply that the redox state of PET gives rise to a signal that regulates *CAB* expression in the nucleus. However, in contrast to these findings, work carried out in barley plants by Montané and co-workers using a combination of low O_2 and CO_2 partial pressures and elevated light intensities found *CAB* expression and light harvesting complex II (LHCII) antenna size were unlikely to be controlled by the redox state of the PET chain (Montane et al., 1998). The authors concluded that photoacclimation of photosynthesis seems to differ between higher plants and algae.

Huner and co-workers subjected cultures of the algae *Chlorella vulgaris* to various light and temperature combinations that resulted in similar redox states of PSII (Savitch et al., 1996). For example, cells grown at 5°C and 20 μmol photons m^{-2} s^{-1} PAR and those grown at 27°C and 150 μmol photons m^{-2} s^{-1} PAR had comparable redox states of PSII.

Figure 5.1: Influence of temperature and light on the light and dark reactions of photosynthesis

The biophysical processes (light reactions) of the PET chain are largely dependent on the illumination but are not influenced strongly by temperature changes. In contrast, the biochemical processes of the Calvin cycle (dark reactions) and other metabolic processes are limited by temperature, but are not directly influenced by light. Consequently an increase in illumination or a reduction in temperature can result in a more reduced electron transport chain.

PS	Photosytem
Cyt. b_6/f	cytochrome b_6/f complex
PQ	plastoquinone pool
TPT	Triosephosphate-phosphate-translocator

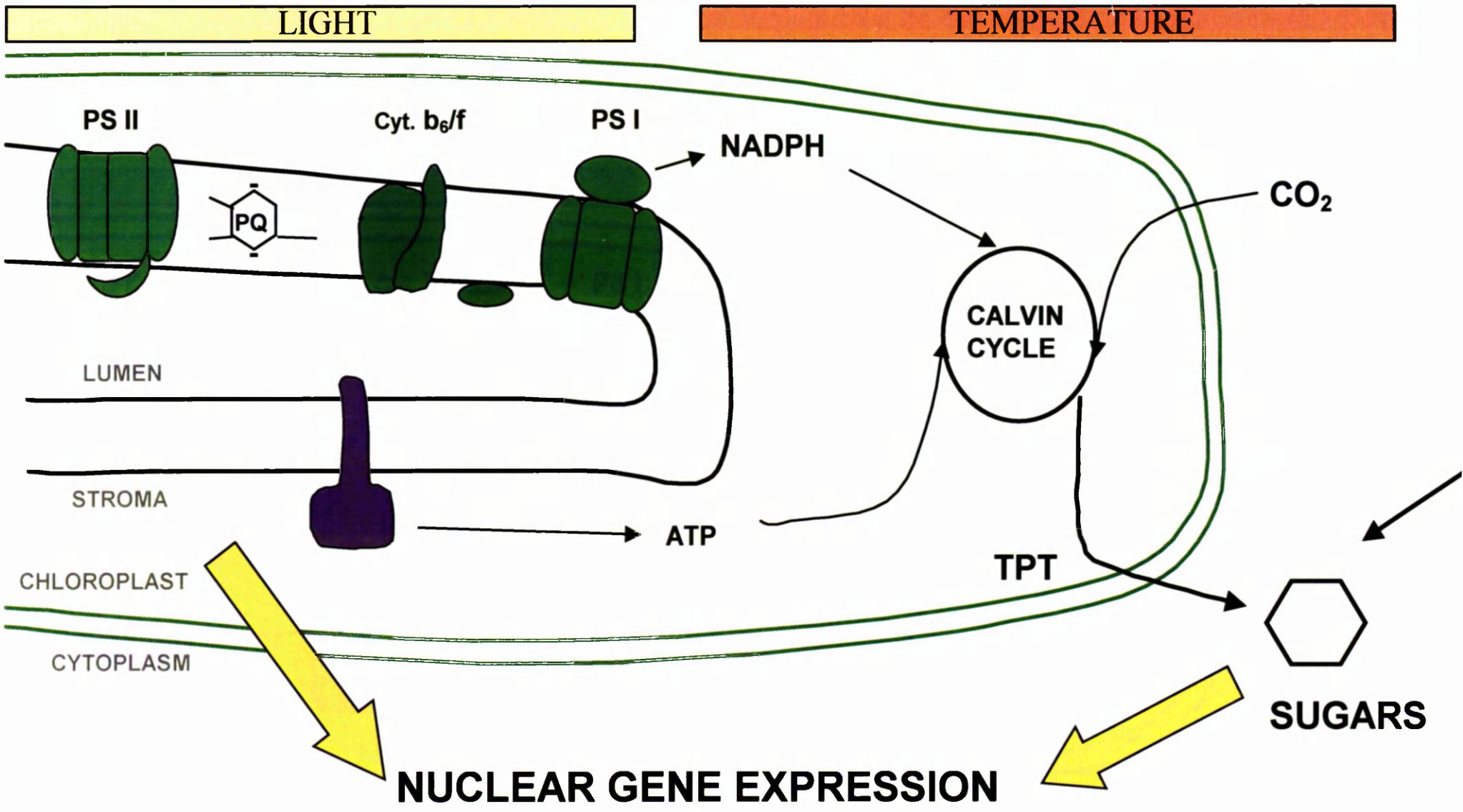

LIGHT
TEMPERATURE
PS II
Cyt. b6/f
PS I
NADPH
CO2
PQ
CALVIN CYCLE
LUMEN
STROMA
ATP
TPT
CHLOROPLAST
CYTOPLASM
SUGARS
NUCLEAR GENE EXPRESSION

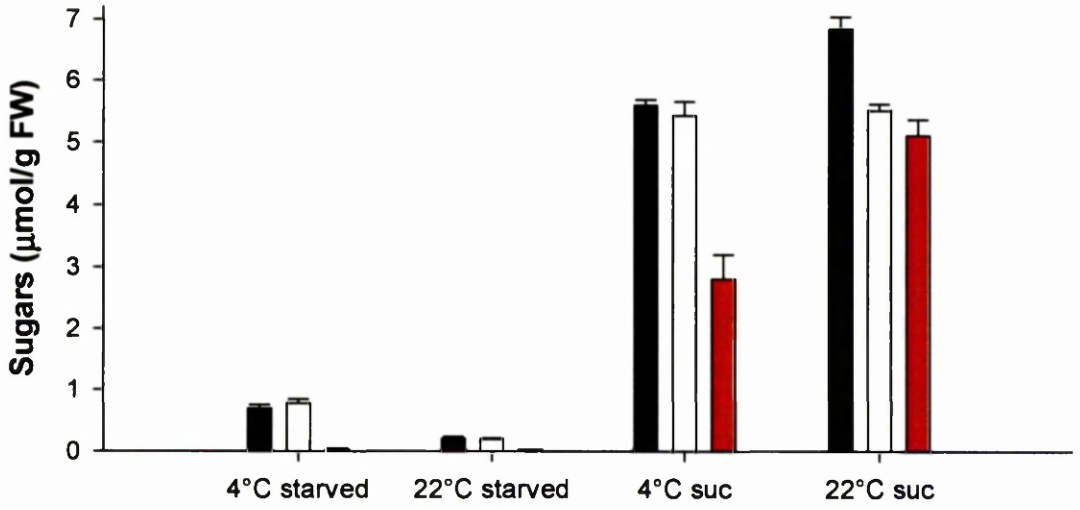
Figure 5.2: Intracellular sugar levels, chlorophyll and fresh weight of cell suspension culture starved at 4°C

3-day old cultures were submitted to starvation. Half the aliquots were kept under the normal growth conditions, the other half was cultured at 4°C rather than 22°C. Sucrose fed controls were treated accordingly.

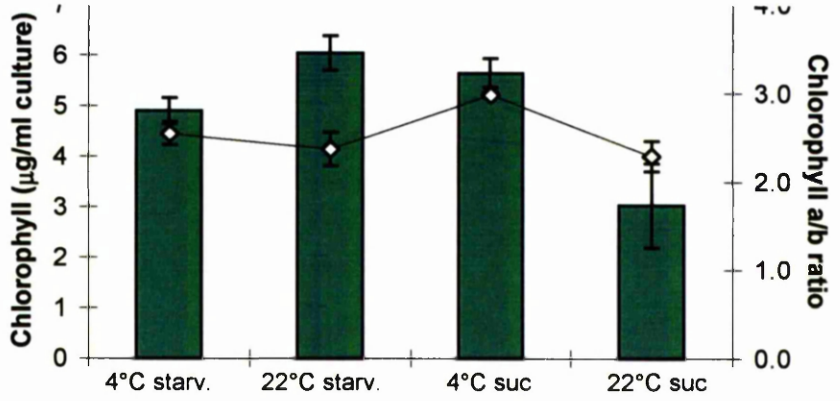
(*A*) Intracellular glucose (■), fructose (□) and sucrose (□) levels after starvation at 4°C and 22°C for 24 h (in approximately mM). (*B*) Total chlorophyll (▭) and chlorophyll a to chlorophyll b ratio (—◇—). (*C*) Fresh weight of cells after 24h (in mg/ml). (*D*) Compact cell volume after 4 days at 4°C or 22°C (i.e. 7 days after subculturing).

n=2 independent samples

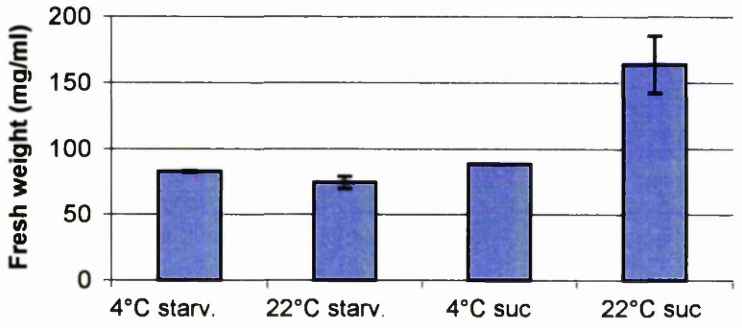
A


Sugars (µmol/g FW)
0
1
2
3
4
5
6
7
4°C starved
22°C starved
4°C suc
22°C suc

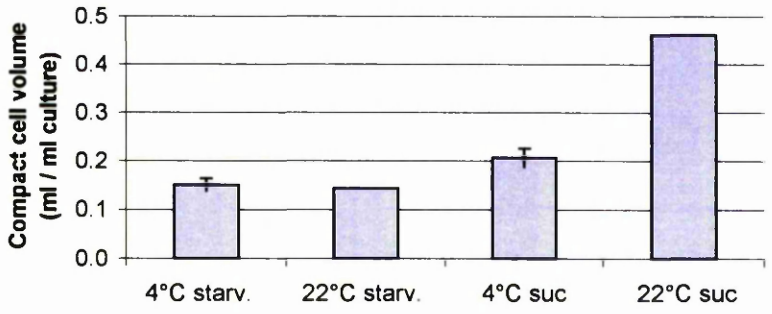
B


Chlorophyll (µg/ml culture)
0
1
2
3
4
5
6
Chlorophyll a/b ratio
0.0
1.0
2.0
3.0
4°C starv.
22°C starv.
4°C suc
22°C suc

C


Fresh weight (mg/ml)
0
50
100
150
200
4°C starv.
22°C starv.
4°C suc
22°C suc

D


Compact cell volume (ml / ml culture)
0.0
0.1
0.2
0.3
0.4
0.5
4°C starv.
22°C starv.
4°C suc
22°C suc

Similarly, exposure of *C.vulgaris* to 27°C and 2200 μmol photons m^{-2} s^{-1} PAR resulted in a similar reduction of PSII as 150 μmol photons m^{-2} s^{-1} PAR at 5°C (Savitch et al., 1996). These studies showed, that pigment content and composition, LHCII abundance, cell ultrastructure and *CAB* mRNA levels correlated better with PQ redox state than with the environmental conditions, and were poorly correlated with cellular levels of carbohydrates and related metabolites, which have both been widely implicated in the control of gene expression state (Savitch et al., 1996). Based on these results the authors ruled out any role for sugar sensing in photoacclimation of photosynthesis and concluded that only the redox state of the PET chain was the defining signal.

Soluble sugar levels decreased in *Chlorella* grown at 5°C, but the opposite was found when *Arabidopsis* leaves were shifted to or grown at 5°C (Strand et al., 1997). Leaves shifted to or grown at low temperature showed increased levels of sugars and associated metabolites, but only leaves shifted to the low temperature had decreased levels of *CAB* and *RBCS* transcripts. In plants transferred to 5°C the *RBCS* mRNA levels seemed to recover after a longer period (10 to 21 days). *CAB* and *RBCS* mRNA levels in cold developed leaves were comparable to those in leaves grown at 23°C. When shifted to 5°C and subsequently measured at the same temperature, Q_A was more reduced than in control leaves. The degree of reduction increased with the duration of the cold incubation and cold developed leaves had a more reduced PSII at all temperatures (Strand et al., 1997). One interpretation of these results is that a plastid redox signal can over-ride the repression of nuclear encoded photosynthetic genes by elevated sugar levels at low temperatures.

5.2 The effects of cold and light treatment on the cell suspension culture

However, what is not clear from the published results is exactly how PET redox state and sugars may interact to control nuclear photosynthetic gene expression in response to temperature changes. A number of experiments were designed to investigate this phenomenon further.

Three-day-old cell suspension culture was submitted to starvation as described before, except that after the washing step, cultures were maintained at 4°C for 24h. Intracellular sugar levels, chlorophyll and fresh weight were analysed (Fig 5.2).

In contrast to the work reported by Strand et al. (1997) sugar levels did not increase in sucrose fed cell cultures when they were incubated at 4°C rather than 22°C for 24h (Fig. 5.2 A). Rather the opposite seemed to be the case, cells at 4°C had glucose levels approximately 80% and sucrose values approximately 50% of cells at 22°C. In cells starved at 4°C hexose levels were approximately double the levels of hexoses of cells starved at 22°C, but the levels of sugars were still low. The fact that cells treated at 4°C had more hexoses could be due to a slower metabolism at 4°C.

When total chlorophyll content and the chlorophyll *a/b* ratio of the cells were analysed, cells maintained at 4°C with sucrose had a higher chlorophyll content than cells maintained at 22°C with sucrose (Fig. 5.2 B). In fact the chlorophyll content in cells supplied with sucrose at 4°C was similar to the chlorophyll content in cells starved at 4°C or 22°C. The chlorophyll *a/b* ratio appeared to be higher as well in cells supplied with sucrose at 4°C than at 22°C.

It was previously shown that there is an inverse correlation between sugar levels and the chlorophyll content at 22°C (cf. Fig. 3.5 A and C) and this is also apparent from Figure 5.2 A and B. However, there is no such correlation when sugar and chlorophyll levels are compared for 4°C treated cells (Fig. 5.2 A and B). Therefore another parameter other than the intracellular sugar levels is likely to be involved in the regulation of chlorophyll levels.

Cell growth, measured as fresh weight per ml culture, was reduced in cells fed sucrose at 4°C compared to 22°C (Fig. 5.2 C). Starved cells at either temperature showed reduced growth. Continued growth of the cells under these four conditions was monitored for a further 3 days (i.e. to day 7 after subculturing). Compact cell volume measurements reflected the growth pattern of cells treated for 24h (Fig. 5.2 C), with the exception that sucrose fed cells maintained at 4°C performed slightly better than the cells starved at either temperature (Fig 5.2 D).

When Chlorophyll *a* fluorescence was measured, it was found that the redox state of Q_A appeared to be more reduced at low temperatures (Table 5.1).

Table 5.1: The redox state of Q_A in 4°C and 22°C treated cells as assessed by chlorophyll *a* fluorescence

	Q_A reduced (%)	stdev
4°C starved	35%	14%
22°C starved	19%	9%
4°C sucrose	25%	9%
22°C sucrose	13%	1%

Fluorescence measurements were made according to the procedure described in section 2.2.15. The averages of 2 replicates are shown. stdev = standard deviation

Previous studies on algae and Arabidopsis leaves had also shown a more reduced Q_A (as discussed above), although the difference was less clear in this study.

To asses the impact of the altered redox state on photosynthetic mRNA levels, three-day-old culture was washed and resuspended in medium with or without sucrose as described above. One half of the samples were incubated for 24h at 4°C and 20 µmol photons m^{-2} s^{-1}, the other for 24h at 22°C and 20 µmol photons m^{-2} s^{-1}. The samples were then harvested, total mRNA extracted and RNA blot analysis for *CAB* transcript levels performed (Fig 5.3, columns 1-4).

CAB transcript levels in cold treated cells appeared to be similar under sucrose feeding and starvation conditions (columns 1 and 3) and low when compared with cells starved at 22°C (columns 2). *CAB* mRNA levels in sucrose fed cells at 22°C were very low (column 4).

In a separate experiment 3 day old cultures were submitted to starvation at 22°C and 4°C, but in addition to the previous experiment samples at both temperatures were kept at either the normal low light intensity (20 µmol photons m^{-2} s^{-1}) or exposed to an elevated light intensity (80 µmol photons m^{-2} s^{-1}). Again, *CAB* transcript levels were measured (Fig. 5.3, columns 5-10).

As seen in the previous experiment, *CAB* transcript levels were low in starved cells when they were kept under cold conditions and 20 µmol photons m^{-2} s^{-1} (Fig.5.3, columns 5 vs. 6). However, when the illumination was increased to 80 µmol photons m^{-}

^{2} s^{-1} during the starvation period, the reverse results were obtained: cells starved at 22°C showed low *CAB* transcript abundance. In contrast, cells starved at 4°C and 80 µmol photons m^{-2} s^{-1} showed high levels of *CAB* transcript (columns 7 vs. 8)! Sucrose fed cells showed minimal levels of *CAB* transcript when exposed to elevated light, irrespective of the temperature (columns 9 & 10).

Cold treatment (Fig. 5.3 columns 1 and 5) or increased illumination (column 8) of starved cell culture evoked the expected photoacclimation response: *CAB* mRNA abundance was low. However, when both these environmental parameters act in combination on starved cells, they result in high levels of CAB transcript (Fig.5.3 column 7). The mechanism responsible for this effect is unknown.

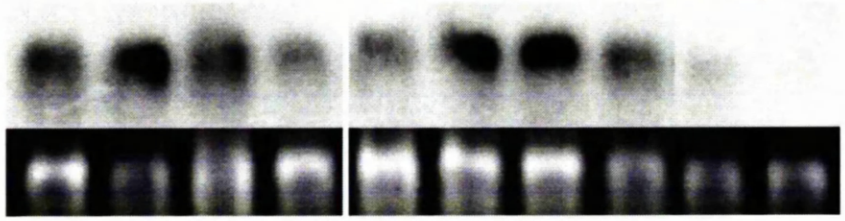
Cold treatment of cell culture did not result in elevated sugar levels as reported for *Arabidopsis* leaves (Strand et al., 1997). Under these conditions it is important to note that although the cell culture is photosynthetically active, it is still a net consumer of carbon and thus effectively a 'sink' tissue. The rosette leaves used by Strand and co-workers were 'source' tissues and export of carbohydrates from source tissue might be particularly affected by cold. The chlorophyll content of cells fed sucrose at 4°C was similar to cells starved at either temperature. Growth was reduced to a similar extent in sucrose fed cells when maintained at 4°C and in starved cells at 4°C or 22°C. Cells of both sugar treatments showed Q_A to be more reduced when exposed to cold and *CAB* transcript levels were also similar (Fig. 5.3 columns 1, 3 and 5). It is possible that PET is slower at 4°C than at 22°C due to feedback from the Calvin cycle, and this could lead to a more reduced PSII under the same illumination. If sugars do not repress photosynthetic gene expression at 4°C and PET is decreased, this could explain the intermediate level of *CAB* in both sucrose fed and starved cultures. However, when light intensity is increased under cold conditions, and hence PSII reduced even further, the cells seem to respond differently, depending on their sugar status; sucrose fed cells show reduced transcript levels (Fig. 5.3 A column 9), displaying a photoacclimation response similar to sucrose fed and starved cells at 22°C. Conversely, cells starved at 4°C show increased *CAB* levels when illuminated with elevated light intensities (Fig. 5.3 A column 7). A more detailed study would be required for a full investigation of the observed effects of cold and light treatments on *CAB* mRNA abundance in the cell culture.

Figure 5.3: *CAB* mRNA levels are modulated differently by carbohydrate status and light intensity at 22°C and 4°C

Three-day-old cultures were starved at 22°C and 4°C. The samples were harvested after 24 hours. (Columns 1 – 4)
In a separate experiment, cultures were treated the same way, except that one half of the samples was illuminated with 80 µmol photons m^{-2} s^{-1} at 4°C or 22°C. (Columns 5-10)

Total RNA was isolated from the samples and 7 µg total mRNA analysed for *CAB* transcript abundance by Northern Blot analysis. An image of the Ethidium Bromide stained rRNA on the gel is included for comparison.
Codes: Presence (+) or absence (-) of 3 % sucrose. All light intensities are given as µmol photons m^{-2} s^{-1}.

1	2	3	4	5	6	7	8	9	10	Column #
-	-	+	+	-	-	-	-	+	+	Sucrose
20	**20**	**20**	**20**	**20**	**20**	**80**	**80**	**80**	**80**	Light intensity
4	**22**	**4**	**22**	**4**	**22**	**4**	**22**	**4**	**22**	Temp. (°C)



CAB mRNA

Gel loading (rRNA)

Chapter 6: Investigation of the phosphorylation state of 14-3-3 binding proteins in response to DCMU and the AMP analogue AICAR using a range of cell cultures

6.1 Introduction

In chapter 4 it was shown that DCMU could prevent the induction of nuclear encoded photosynthetic genes under starvation conditions (Figs. 4.1 and 4.4). Given that the PET derived signal was interacting with sugar regulated photosynthetic gene expression the question then arises as to where this interaction occurs. For example, a plastid derived signal could interact with a cytosolic component of a sugar signalling mechanism or the interaction could occur in the nucleus, possibly involving independent transcription factors on the same gene promoter. The aim of the work presented in this chapter was to establish if DCMU, and thus plastid-derived signals, could modulate the carbohydrate signal upstream of the promoter level.

In sugar signalling in yeast, mammalian cells and plants the SNF1 complex is thought to play a central role (reviewed in Smeekens and Rook (1997); Halford et al. (1999); Smeekens (2000)). The SNF 1 complex in *Saccharomyces cerevisae* consists of a Serine/Threonine protein kinase called sucrose non-fermenting 1 (SNF1), an activator subunit SNF4 and one of three subunits connecting the first 2 subunits (Jiang and Carlson, 1996). The mammalian homologue of the SNF1 complex is the AMP activated kinase (AMPK) which also consists of three subunits (α (=SNF1),γ (=SNF4) and one β). In plants the SNF1 homologue is called SNF1-related Kinase (SnRK1) and SnRK1 like kinases have been found in Arabidopsis and a number of other species (see Halford and Hardie (1998)) together with homologues of the other subunits of the SNF1 complex (Bouly et al., 1999). Plant homologues of yeast SNF1 subunits can complement mutations in the yeast SNF1 complex (Halford and Hardie, 1998; Hardie et al., 1998; Bouly et al., 1999; Lakatos et al., 1999) and a functioning SNRK1 complex is required for sugar inducibility of sucrose synthase in potato (Purcell et al., 1998), indicating a role for SnRK 1 action in relaying a sugar signal. Activation of SnRK1 like protein kinases by sugars has been demonstrated in Arabidopsis (Bhalerao et al., 1999).

Amongst the target proteins for SnRK1 are a group of proteins called 14-3-3 binding proteins so named because of their ability – after phosphorylation at a specific sequence

- to bind proteins of the 14-3-3 type (Aitken, 1996). Phosphorylation of 14-3-3 binding proteins and subsequent binding of 14-3-3 proteins is thought to modulate enzymatic activity, as was reported for the phosphorylation and inactivation of nitrate reductase (NR), a 14-3-3 binding protein (Moorhead et al., 1996; Bachmann et al., 1996).

In addition to nitrate reductase other key proteins of metabolism and cellular function have recently been identified amongst the approximately thirty Arabidopsis proteins found to bind 14-3-3s in the same Arabidopsis cell suspension culture used for the experiments described in chapters 3 to 5, (Moorhead et al., 1999; Cotelle et al., 2000). These include a calcium dependent protein kinase (CDPK) able to phosphorylate NR, sucrose-phosphate synthase (SPS), glutamyl-tRNA synthase (GluRS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cotelle et al., 2000). When sucrose was present in the culture medium, 14-3-3 binding proteins were phosphorylated, possibly by the SnRK1 complex and maybe other protein kinases, and able to bind the 14-3-3 motif (Cotelle et al., 2000). However, when 3 day old cell culture was subjected to starvation in the same way as it was done in the experiments described in chapters 3 to 5 of this thesis, Cotelle and co-workers reported the complete loss of any 14-3-3 binding. This was found to be the result of a starvation-induced protease that lead to cleavage of the 14-3-3 binding proteins (Cotelle et al., 2000). *In vitro* assays showed that 14-3-3 proteins could protect their binding partners from proteolysis (Cotelle et al., 2000). Since de-phosphorylated 14-3-3 binding proteins are unable to bind 14-3-3 proteins (Moorhead et al., 1999) one hypothesis is that starvation leads to de-phosphorylation of the 14-3-3 binding proteins, disrupting their association with the 14-3-3s and allowing the proteolytic attack of the simultaneously induced protease (Cotelle et al., 2000). Re-feeding sugars resulted in 14-3-3 binding, probably due to re-phosphorylation of 14-3-3 binding proteins in response to sugars (Cotelle et al., 2000).

The hypothesis to be tested was that there might exist an interaction between the plastid redox state and the sugar signalling pathway upstream of the de-phosphorylation and proteolysis of the 14-3-3 binding proteins. If so, application of DCMU might somehow result in a sugar-like signal upstream of the SnRK1 complex without altering the sugar levels. Such a signal would potentially not only result in the difference in photosynthetic gene expression observed (see chapter 4), but as well in the protection of 14-3-3 binding proteins from degradation similar to the reported effect of AICAR (5-aminoimidazole-4-carboxamide riboside) (Cotelle et al., 2000). This compound is

converted intracellularly into the AMP analogue ZMP. AMP inhibits the dephosphorylation of SnRK1 and hence activates it (Sugden et al., 1999a). As previously reported for the same cell culture AICAR is able to maintain the 14-3-3 binding of SnRK1 target proteins and to protect them from proteolysis under starvation conditions (Cotelle et al., 2000).

As can be seen in the results presented in chapter 3 and 4 the presence of 3 % (w/v) sucrose in the culture medium causes a significant repression of photosynthetic gene expression and chlorophyll accumulation. At an early stage in the current thesis project it was decided to attempt to acclimatise the cell cultures to growth on lower concentrations of carbohydrates as this will increase photosynthesis and reduce the importance of externally supplied carbohydrates. 3% (w/v) sucrose grown cell suspension cultures were acclimatised in steps over a period of a year to grow on reduced amounts of sucrose. In addition to the usual 3% (w/v) sucrose cultures growing with only 1%, 0.8%, 0.6% and 0.4% sucrose were obtained (Fig 6.1). The different cultures will be referred to as culture type 3, 1, 0.8, 0.6 and 0.4 with the number denoting the percentage (w/v) of sucrose in the growth medium.

6.2 Investigation of the 14-3-3 binding proteins in a range of cell cultures and treatments

An experiment using the range of cell suspension cultures acclimatised to the different sucrose levels was performed in collaboration with Carol MacKintosh's group to address the following questions:

1. Since DCMU maintains photosynthetic transcript levels on a sugar repressed level, will it influence the pattern of phosphorylated 14-3-3 binding proteins under starvation conditions, i.e. is there an interaction between the plastid redox state and the sugar signalling pathway upstream of the de-phosphorylation and degradation of the 14-3-3 binding proteins?
2. Will the low sucrose cultures respond differently to starvation, DCMU or AICAR since they are acclimatised to a reduced carbon presence?

3-day-old cultures of each of the five available types (3%, 1%, 0.8%, 0.6% and 0.4% (w/v) sucrose) were washed and resuspended in medium without sugars as described earlier (-suc samples). The sucrose fed controls for each culture type were kept in their original media without any washing step (+suc samples).

Please note that this was in contrast to the previous experimental procedure, where all samples were washed and re-suspended. However in this case this would have extended the total washing time to over 2 hours, which by itself might cause secondary effects. Since the sugar content in the medium of type 3 culture was still high after the first 3 days (see Fig. 3.3A), it was decided to adopt this new experimental procedure. Any potential effect should be visible by comparison of the type 3 culture controls with previous results.

Minus sucrose and plus sucrose samples for each culture type were treated with10 μM DCMU, ethanol as a control or 10 mM AICAR. The samples were left for 24h under their usual growth conditions, quickly harvested and stored at -80°C until 14-3-3 overlay assays could be performed.

Starvation of type 3 culture leads to loss of all bands in the 14-3-3 over lay assay (Fig. 6.2 A column 1 and 2), proving the degradation of 14-3-3 binding proteins reported before. The presence of DCMU does not prevent the degradation of these proteins and also has no effect on the 14-3-3 binding proteins in sucrose fed cells (Fig. 6.2 A columns 3 and 4). Similar results were obtained for the type 0.4 culture: DCMU can not protect the 14-3-3 binding proteins from degradation in response to starvation (Fig. 6.3 A columns 1 to 4). These findings were confirmed using samples of a repeat experiment conducted in parallel (Fig. 6.4 A columns 1 to 4).

In DCMU treated cell culture the 14-3-3 binding proteins are subject to the usual starvation response and sugar levels are minimal (Fig. 4.2), yet DCMU can counteract this sugar depletion signal with respect to photosynthetic genes of the nucleus. These results confirm again that DCMU does prevent the induction of nuclear encoded photosynthetic genes by a specific mechanism of plastid origin and not by a more general, unspecific effect.

In contrast to DCMU, AICAR alters the response to starvation on the level of the 14-3-3 binding proteins: Cells of the type 3 culture starved in the presence of 10mM AICAR

show a weak preventative effect of AICAR. In accordance to other studies, this concentration of AICAR results in the retention of small amounts of the 14-3-3 binding proteins, as visible by the faint bands (Fig. 6.2 A column 6). MacKintosh and co-workers have shown that with larger concentrations of AICAR this preventative effect becomes stronger in the type 3 culture submitted to starvation (Cotelle et al., 2000).
Interestingly, 10mM AICAR has a much more pronounced effect in the type 0.4 culture (Fig. 6.2 A column 10 and Fig. 6.3 A column 6). Most bands are strongly retained in these cells when AICAR is present during the starvation treatment. The exception are three bands (see arrows in Fig. 6.3 A) that appear much weaker than the other bands. AICAR seems unable to prevent their degradation or loss of 14-3-3 binding. However the identity of 14-3-3 binding proteins of these sizes is not known. AICAR has the strongest effect in the cultures acclimatised to the lowest supply of sucrose and was less preventive if the cultures are conditioned to growth on medium to high sucrose (type 0.8 to type 3) (Fig. 6.4 A columns 5 to 10 as well as columns 6 and 10 in Figs. 6.2A and 6.3A).
As found in other studies AICAR can maintain 14-3-3 binding and prevent 14-3-3 binding proteins to some extent from degradation under starvation conditions. If the cells have been acclimatised to a lower carbon supply, AICAR excerts a greater effect. However, AICAR does not appear to protect all 14-3-3 binding proteins in the same way in the cells acclimatised to low sucrose. At least three proteins seem to still be degraded.

Three additional observations were made with respect to differences between the different cultures:

- Cultures acclimatised to low or medium sucrose levels show a stronger binding of 14-3-3 peptides under sucrose conditions than the type 3 culture (compare columns 1,3 and 5 with columns 7 to 9 in Fig.6.2 A; see Fig.6.4 B columns 1-5). In particular, three proteins of approximately 97 kDa, 66 kDa and 55 kDa size result in much stronger bands in low sucrose cultures. In contrast, one protein band of a large molecular mass appears to bind 14-3-3 peptides more strongly in the type 3 culture when sucrose is supplied (arrow in Fig. 6.2 A). Interestingly sucrose phosphate synthase, a known 14-3-3 binding protein, was shown to be of an approximately similar size and is detected well in the 14-3-3 overlay assay (Moorhead et al., 1999; Cotelle et al., 2000).

Figure 6.1: A range of cell suspension cultures acclimatised to growth on lower sucrose concentrations.

Cultures growing with 3% (w/v/) sucrose were slowly adapted to growth on lower levels of carbohydrates. As a result, a range of culture types acclimatised to growth in media containing, 3%(w/v) (ca. 88 mM), 1% (ca. 29 mM), 0.8% (ca. 23 mM), 0.6% (ca. 18 mM) and 0.4% (ca. 12 mM) sucrose were obtained.

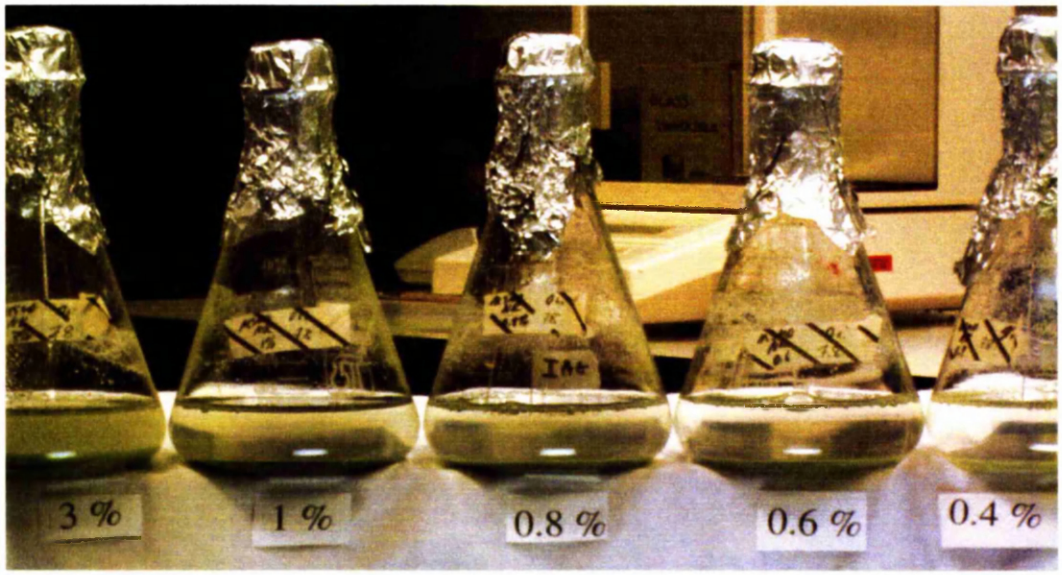

3 %
1 %
0.8 %
0.6 %
0.4 %

Figure 6.2: AICAR but not DCMU can prevent 14-3-3 binding proteins from starvation induced degradation.

Three-day old 3% (w/v) sucrose culture was washed and resuspended as described in section 2.2.13. After resuspension, DCMU was added to a third of the aliquots to a final concentration of 10 μM. one third of the samples was treated with ethanol (0.1%) and the remaining third was treated with AICAR (final concentration 10 mM). All samples were incubated under the normal growth conditions for 24h. Samples were harvested and stored at –70°C. Total protein extracts, protein blots, 14-3-3 overlay assays and anti-14-3-3 Western Blot analysis were performed as in section 2.4. Some samples of the type 0.4 culture, treated in the same way, are shown for comparison (columns 7 to 10).

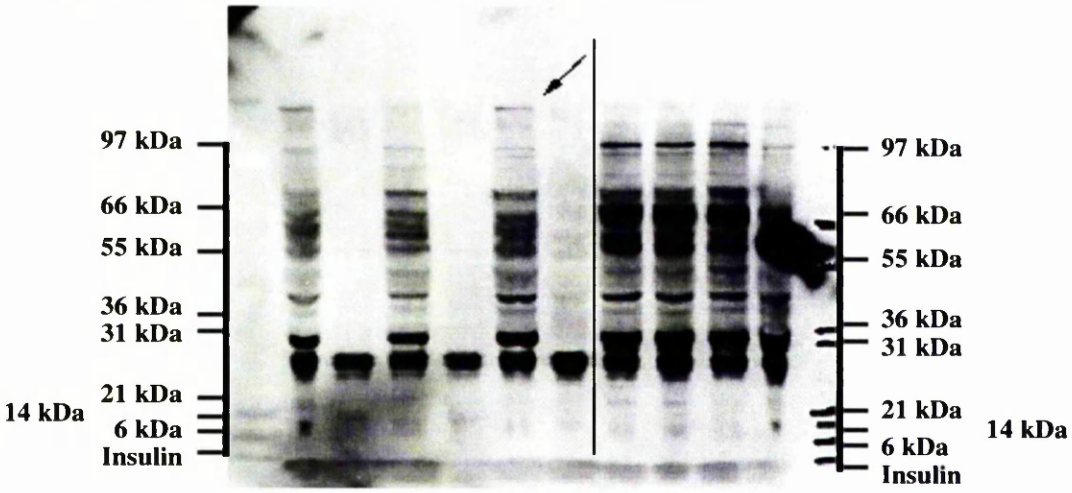
(*A*) 14-3-3 overlay assay. DCMU has no effect on 14-3-3 binding. The arrow points out a protein band showing different intensity in type 3 culture compared to the type 0.4 culture (columns 7-10). (*B*) and (*C*) show different exposures of the results of an anti 14-3-3 Western Blot analysis of the same blot. The arrow in (*B*) indicates the near absence of degradation products when AICAR is present. The inability of AICAR to maintain one particular protein band, in contrast to the other bands detected, is pointed out by the arrow in (*C*). (*D*) shows an image of the Ponceau stained blot after protein transfer. 45 μg total protein were loaded in each lane. The degradation of large molecular weight proteins in the presence or absence of DCMU is visible. AICAR prevents this degradation.

Codes: Treatment codes are 'C' for ethanol treated controls, 'D' for 10 μM DCMU treatment and 'A' for 10 mM AICAR treatment. The presence or absence of sucrose in the different culture types is indicated by '+' and '-' respectively. The markers on each side (in kDa) indicate the approximate size of the protein bands.

This analysis was performed in collaboration with Dr. Carol MacKintosh, Dr. Sarah E.M. Meek and Dr. Valérie Cotelle.

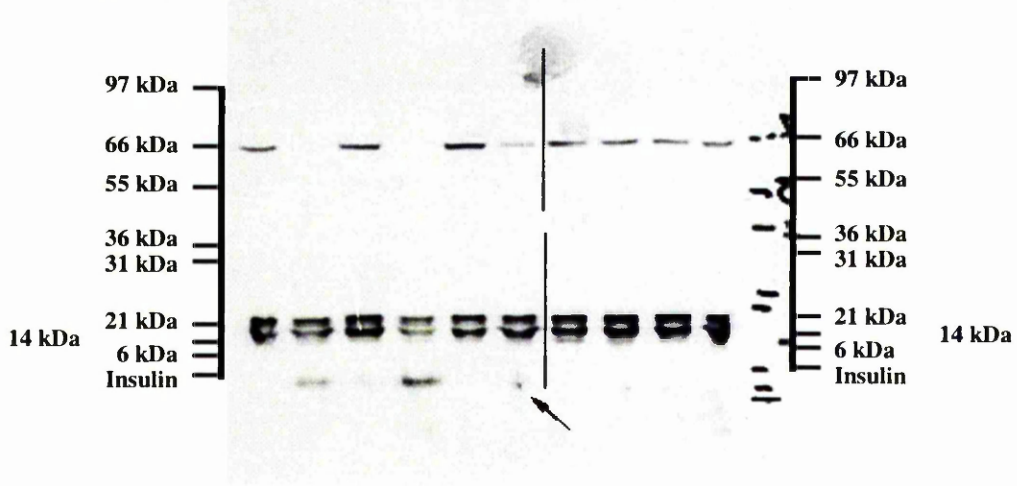
A

Column	0	1	2	3	4	5	6	7	8	9	10
Culture type	M	3						0.4			
Treatment		C	C	D	D	A	A	C	D	A	A
Sugar		+	-	+	-	+	-	+	+	+	-



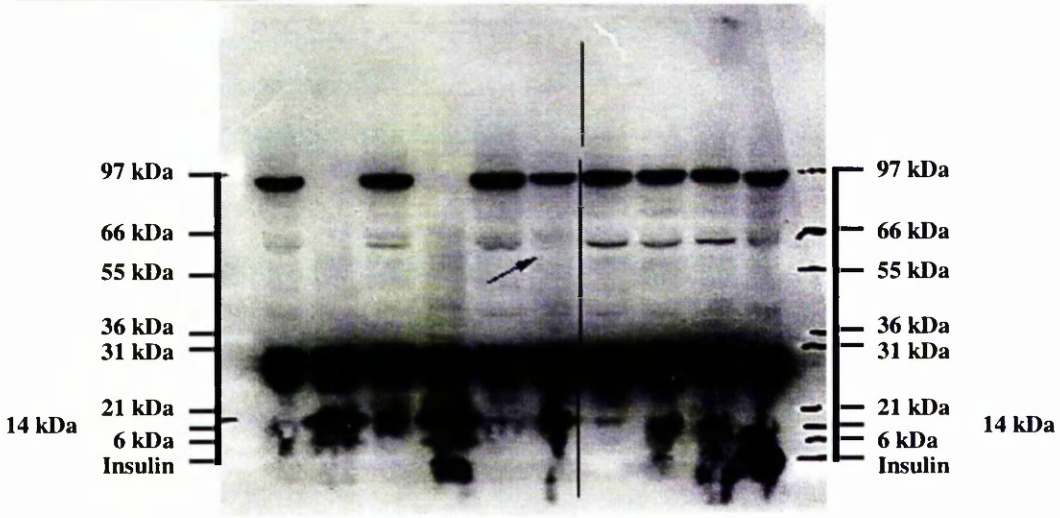
B

Column	1	2	3	4	5	6	7	8	9	10
Culture type	3						0.4			
Treatment	C	C	D	D	A	A	C	D	A	A
Sugar	+	-	+	-	+	-	+	+	+	-



C

Column	0	1	2	3	4	5	6	7	8	9	10
Culture type	M	3						0.4			
Treatment		C	C	D	D	A	A	C	D	A	A
Sugar		+	-	+	-	+	-	+	+	+	-



D

Column	0	1	2	3	4	5	6	7	8	9	10
Culture type	M	3						0.4			
Treatment		C	C	D	D	A	A	C	D	A	A
Sugar		+	-	+	-	+	-	+	+	+	-

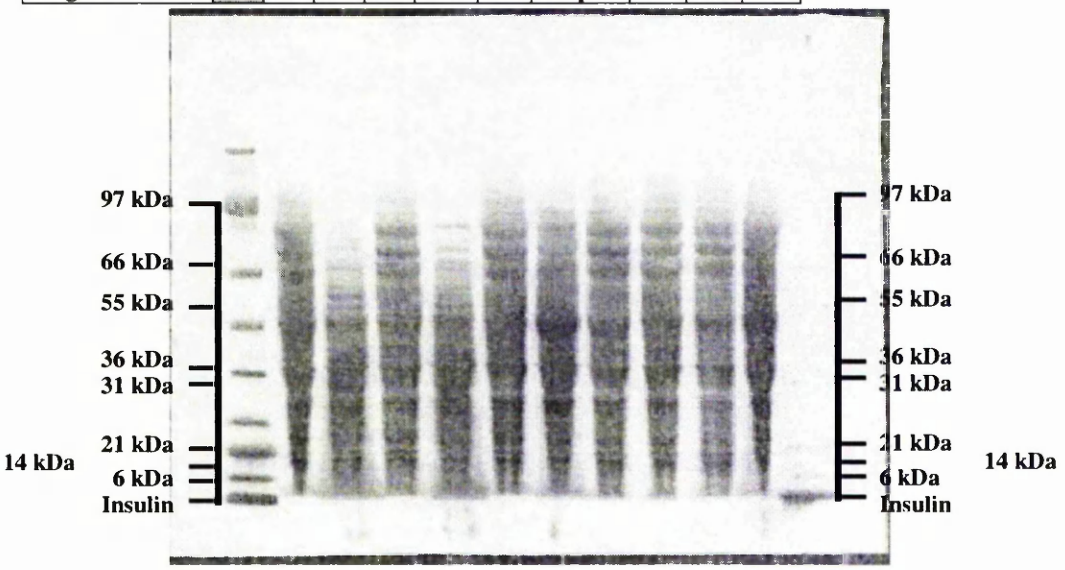


Figure 6.3: 14-3-3 overlay assays on 0.4% sucrose cultures treated with DCMU and AICAR during starvation

Three-day old 0.4% (w/v) sucrose culture was treated as described in Figure 6.2 for the 3% sucrose culture. Some samples of the 3% sucrose culture, treated in the same way, are shown for comparison (columns 8 to 10).

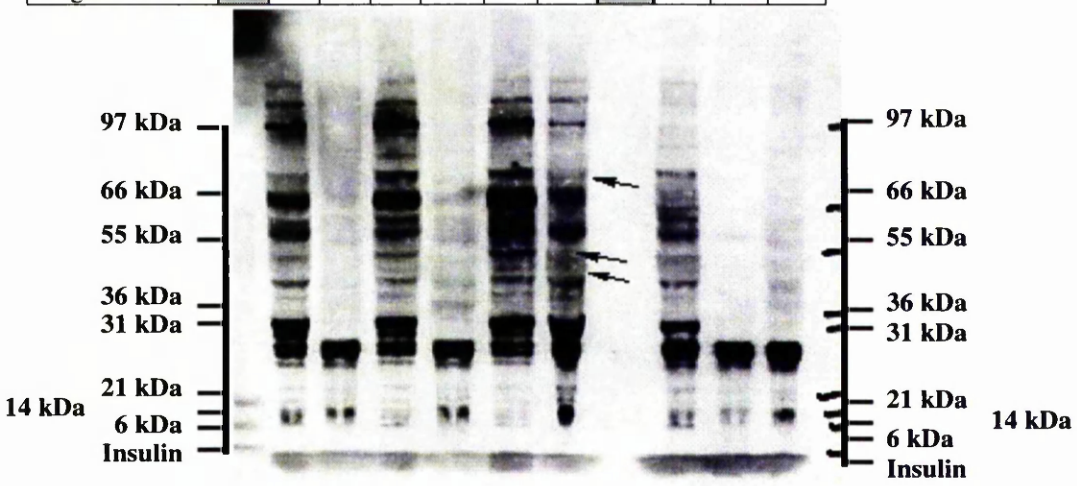
(*A*) 14-3-3 overlay assay. Arrows point out protein bands that are not protected in the AICAR treatment. (*B*) and (*C*) show different exposures of the results of an anti 14-3-3 Western Blot analysis of the same blot. The arrow in (*B*) marks a band of greater intensity in the low sucrose culture types when compared to the type 3 samples (columns 8 to 10). In (*C*) the arrows pointing upwards point out two minor bands that are protected by AICAR. The downward arrows indicate a band that is unaffected by starvation in the type 0.4 cultures, yet completely absent in starved type 3 cultures. (*D*) shows an image of the Ponceau stained blot after protein transfer. 45 μg total protein were loaded in each lane. The degradation of large molecular weight proteins in the absence of AICAR is not as prevalent in starved type 0.4 cultures as it is in type 3 cultures (column 9 and Fig. 6.2).

Codes: Treatment codes are 'C' for ethanol treated controls, 'D' for 10 μM DCMU treatment and 'A' for 10 mM AICAR treatment. The presence or absence of sucrose in the different culture types is indicated by '+' and '-' respectively. The markers on each side (in kDa) indicate the approximate size of the protein bands.

This analysis was performed in collaboration with Dr. Carol MacKintosh, Dr. Sarah E.M. Meek and Dr. Valérie Cotelle.

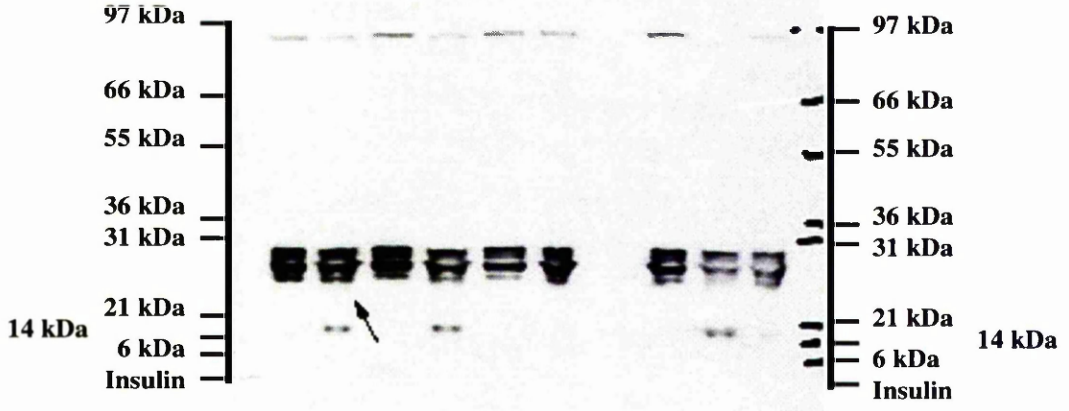
A

Column	0	1	2	3	4	5	6	7	8	9	10
Culture type	M	0.4							3		
Treatment		C	C	D	D	A	A		C	C	A
Sugar		+	-	+	-	+	-		+	-	-



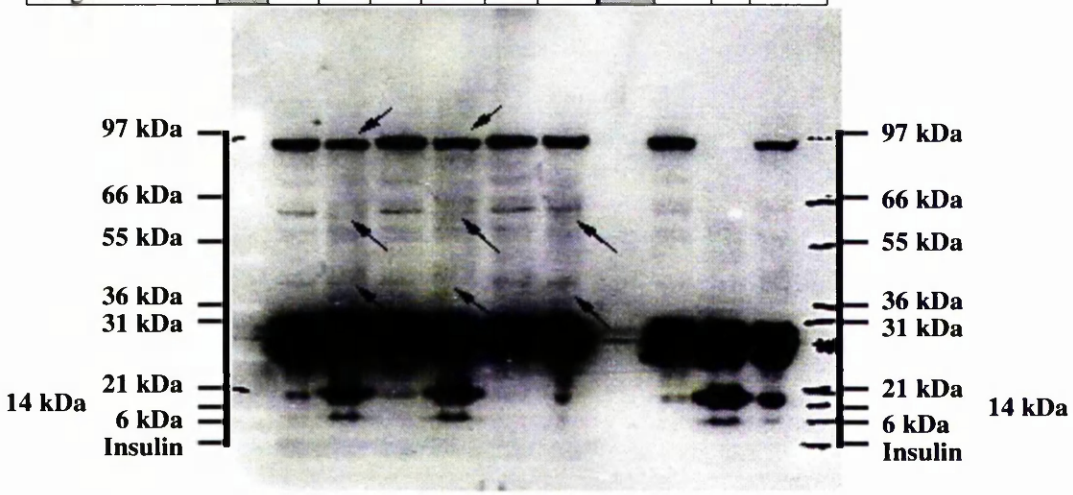
B

Column	0	1	2	3	4	5	6	7	8	9	10
Culture type	M	0.4							3		
Treatment		C	C	D	D	A	A		C	C	A
Sugar		+	-	+	-	+	-		+	-	-



C

Column	0	1	2	3	4	5	6	7	8	9	10
Culture type	M	0.4							3		
Treatment		C	C	D	D	A	A		C	C	A
Sugar		+	-	+	-	+	-		+	-	-



D

Column	0	1	2	3	4	5	6	7	8	9	10
Culture type	M	0.4							3		
Treatment		C	C	D	D	A	A		C	C	A
Sugar		+	-	+	-	+	-		+	-	-

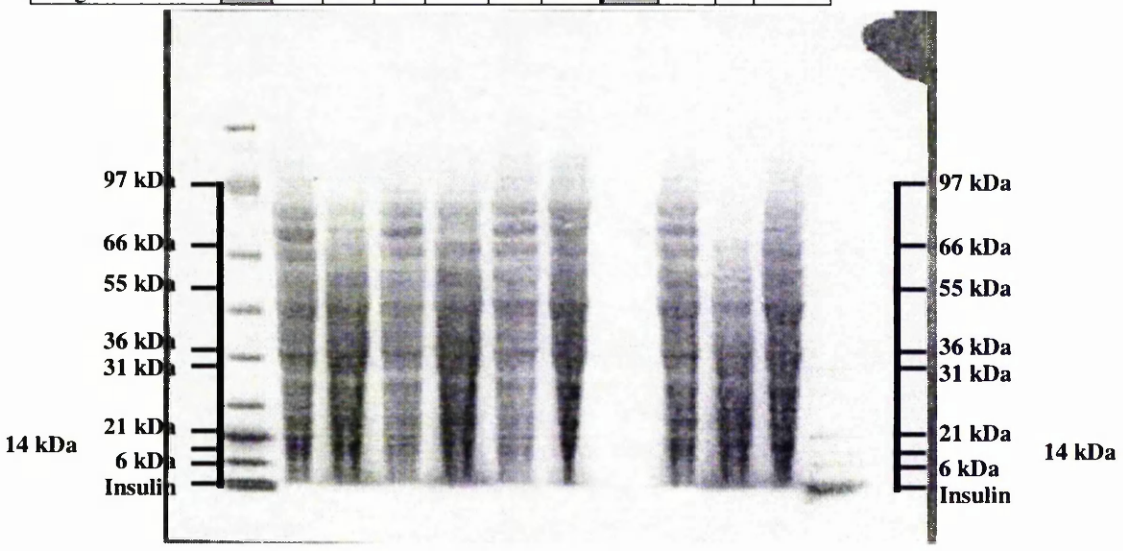


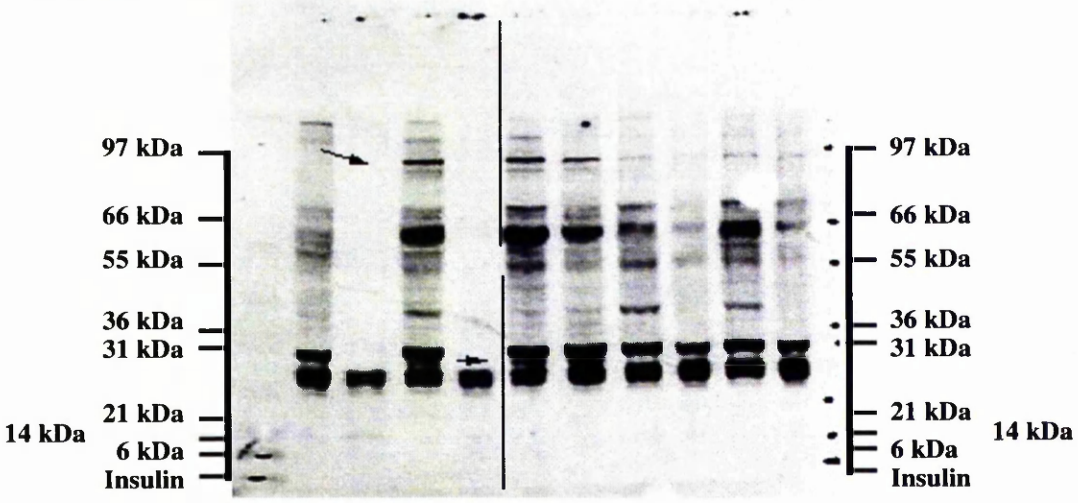
Figure 6.4: 14-3-3 overlay assays (continued)

Three-day old type 0.4, 0.6, 0.8, 1 and 3 culture was treated as described in Figure 6.2 for the type 3 culture.
(*A*) 14-3-3 overlay assay. 2 replicates of 3% and 0.4% sucrose cultures confirm that DCMU has no effect on 14-3-3 binding (columns 1 to 4). The effect of AICAR on the 0.6%, 0.8% and 1% sucrose cultures is shown by the samples in column 5 to 10. AICAR has a stronger protective effect in the cultures acclimatised to the lower amounts of sucrose, as seen in Figs. 6.2 and 6.3 as well. However, the arrows point out protein bands that are only present in low sucrose culture samples. (*B*) shows an image of the Ponceau stained blot after protein transfer. 45 μg total protein were loaded in each lane. (*C*) 14-3-3 overlay assay on starved and sucrose treated samples of the range of cell cultures. (*D*) shows an image of the Ponceau stained blot after protein transfer. 45 μg total protein were loaded in each lane. The degradation of 14-3-3 binding proteins is the strongest in the 3% sucrose culture as shown in (*C*) and (*D*).
Codes: Treatment codes are 'C' for ethanol treated controls, 'D' for 10 μM DCMU treatment and 'A' for 10 mM AICAR treatment. The presence or absence of sucrose in the different culture types is indicated by '+' and '-' respectively. The markers on each side (in kDa) indicate the approximate size of the protein bands.

This analysis was performed in collaboration with Dr. Carol MacKintosh, Dr. Sarah E.M. Meek and Dr. Valérie Cotelle.

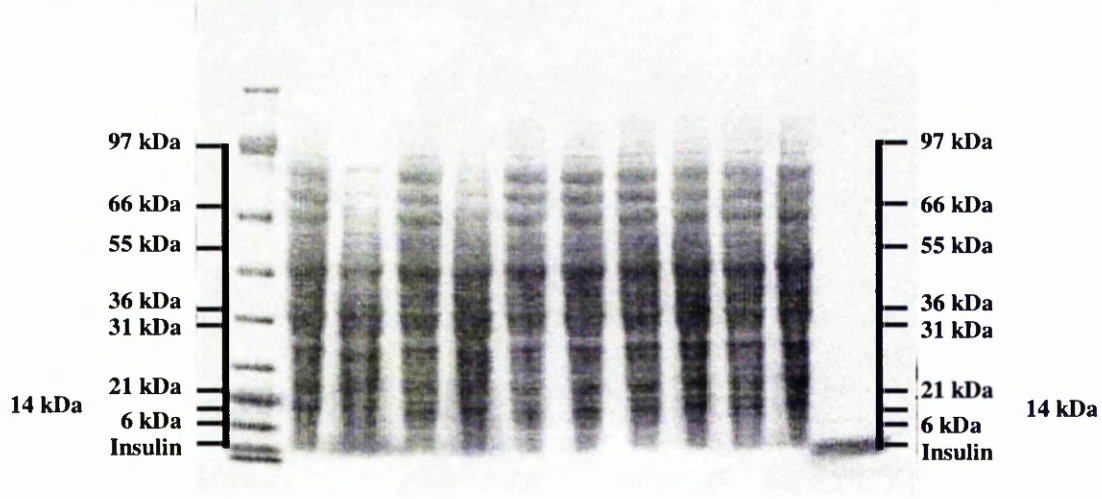
A

Column	0	1	2	3	4	5	6	7	8	9	10
Culture type	M	3		0.4		0.6		0.8		1	
Treatment		D	D	D	D	A	A	A	A	A	A
Sugar		+	-	+	-	+	-	+	-	+	-



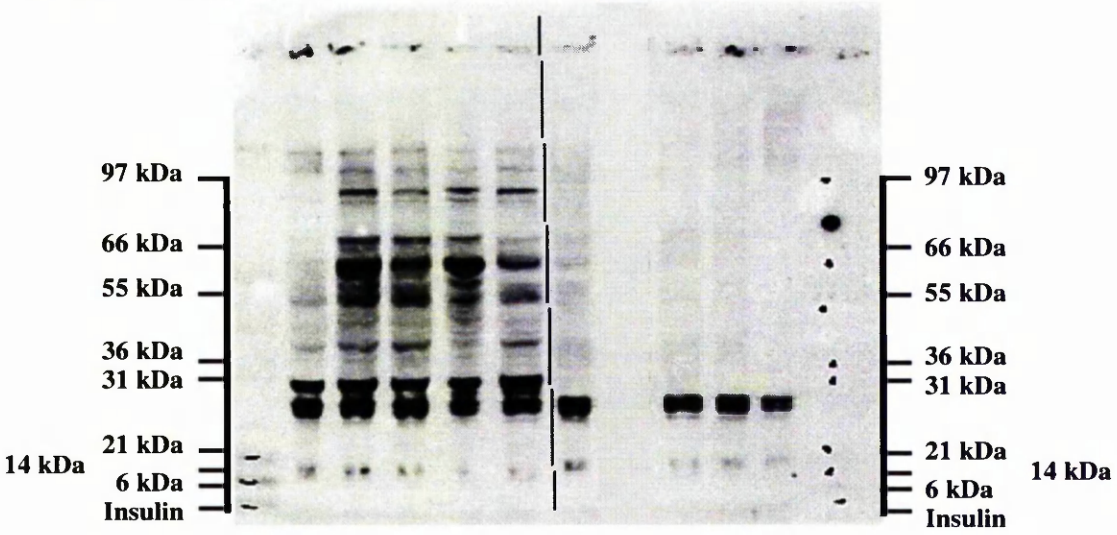
B

Column	0	1	2	3	4	5	6	7	8	9	10
Culture type	M	3		0.4		0.6		0.8		1	
Treatment		D	D	D	D	A	A	A	A	A	A
Sugar		+	-	+	-	+	-	+	-	+	-



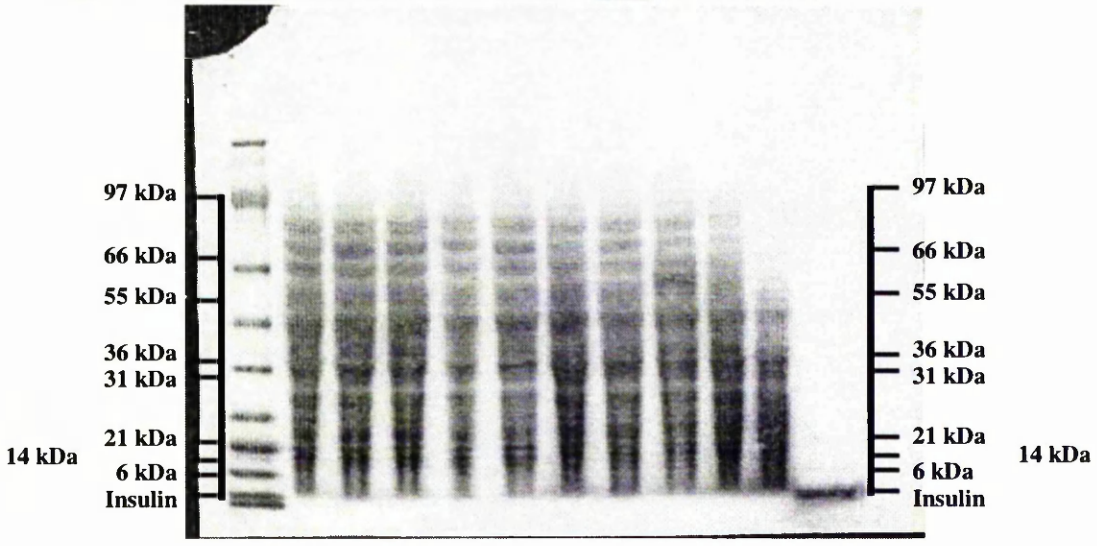
C

Column	0	1	2	3	4	5	6	7	8	9	10
Culture type	M	3	1	.8	.6	.4	.4		.8	1	3
Treatment		C	C	C	C	C	C		C	C	C
Sugar		+	+	+	+	+	-		-	-	-



D

Column	0	1	2	3	4	5	6	7	8	9	10
Culture type	M	3	1	.8	.6	.4	.4	.6	.8	1	3
Treatment		C	C	C	C	C	C		C	C	C
Sugar		+	+	+	+	+	-		-	-	-



- Parts B and C of figures 6.2 and 6.3 are different exposures of the results of anti-14-3-3 Western blots. The 14-3-3 peptide will not only detect 14-3-3 binding proteins in the overlay assay, but as well 14-3-3 proteins themselves, because of the ability of these to form homodimers. The 14-3-3 proteins are represented by the bands of about 30 kDa size. They show little change in response to starvation (Fig. 6.2 and 6.3 A to C). However, the anti 14-3-3 antibody detected a major band of slightly less than 97 kDa and 2 minor bands of less than 66 kDa and approximately 43 to 45 kDa respectively. In contrast to the 14-3-3 proteins, the major band of 97 kDa disappears when type 3 culture cells are starved, except if AICAR is present (Fig.6.2 C). However, the low sucrose culture retain this protein even under starvation conditions (downward arrows in Fig. 6.3 C). The two minor bands detected by this antibody still disappear in these cells when starved, but are retained when AICAR is present (upwards arrows in Fig. 6.3 C). Again the type 3 cultures show a different response, here the band of less than 66 kDa size is not retained when AICAR is present (arrow in Fig. 6.2 C)
- The loss of total protein, in particular of large molecular weight proteins is apparent in starved type 3 cultures but decreases with increasing acclimation to low sucrose levels (see columns 6 to 10 in Figure 6.4 D). Low sucrose cultures cope better with the total absence of carbon from the medium. It should be noted that this loss of total protein in response to carbon depletion is most likely caused by the increased use of proteins as a carbon and energy source. However, the loss of total protein is linked to the regulatory degradation of the 14-3-3 binding proteins only in so far, as both occur in response to the same environmental condition (i.e. carbon starvation). In contrast to the loss of 14-3-3 binding proteins, the total loss of protein is not always observed when cell culture is subjected to carbon depletion and might depend on slightly varying experimental conditions (cf. Cotelle et al. (2000))

Taken together the results demonstrate the effect of acclimation to a reduced carbon supply on the sugar signalling cascade involving 14-3-3- binding proteins. Further work characterising the low sucrose cultures and their response to AICAR had to be carried out before any attempt to explain the observed differences between low and high sucrose cultures could be made.

6.3 Characterisation of the range of cell cultures

The low sucrose cultures show a similar growth pattern to the type 3 culture. They enter exponential phase at about day three and reached the stationary phase at day 7. The lower the amount of sucrose in the medium the sooner the cultures start to decay once they have reached stationary phase as assessed by daily visual inspection. The fresh weight per ml culture at day 4 and 7 is correlated to the amount of sucrose present in the medium (Figs. 6.5). Total chlorophyll in 7-day-old cultures is increased in the cultures grown on 1% and 0.8% sucrose, but the chlorophyll *a/b* ratio dos not change compared to the type 3 culture (Fig. 6.6 A). In contrast the low sucrose cultures (type 0.6 and 0.4) have reduced amounts of total chlorophyll per g fresh weight compared to the medium sucrose cultures and only slightly higher levels than the type 3 culture. However, the chlorophyll *a/b* ratio is increased in these low sucrose cultures compared to the type 0.8 to 3 cultures (Fig. 6.6 A).

To characterise the effect of AICAR on the range of cultures further, Northern Blot analysis, sugar assays and chlorophyll measurements were performed on type 3, 0.8, 0.6 and 0.4 cultures. (Fig 6.7)

When treated with 10mM AICAR for 24 hours, AICAR does not have any effect on *CAB* mRNA levels in sucrose fed cells. However, AICAR treatment in starved type 3 culture results in a strong induction of *CAB* transcript (Fig.6.7 A). This is not the case for low sucrose culture cells starved in the presence of AICAR (Fig. 6.7 b to D). In the type 0.4-0.8 culture *CAB* mRNA levels are as low as in sucrose fed cells when AICAR was present during the starvation

Chlorophyll levels and chlorophyll *a/b* ratios do not change significantly in any of the samples treated with AICAR (Fig. 6.8 A). Interestingly, the low sucrose cultures (type 0.4 and 0.6) showed slightly higher chlorophyll levels when sucrose was supplied than under starvation conditions, an indication of their carbon limitation. (Fig 6.8 A)

Figure 6.5: The fresh weight of cell suspension cultures is correlated to the amount of sucrose present in the media

The different culture types were subcultured into fresh medium containing the appropriate concentration of sucrose as described in section 2.2.26.
Fresh weight was assessed in triplicate after 4 (■) and 7 (□) days.

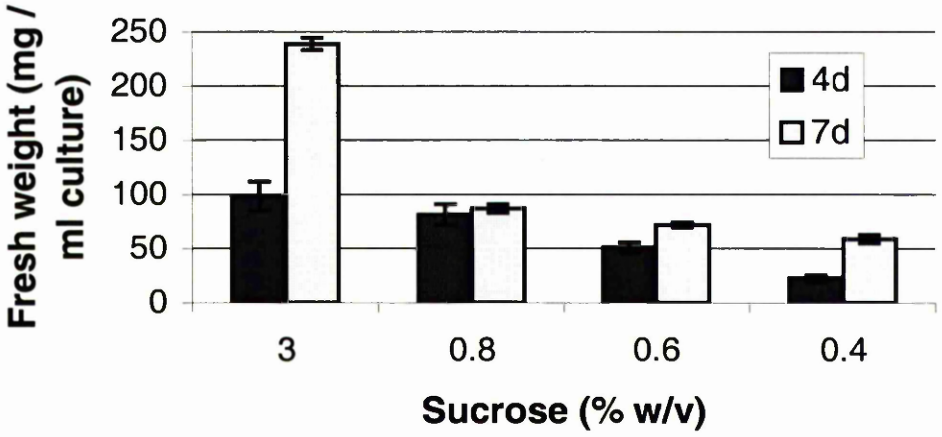
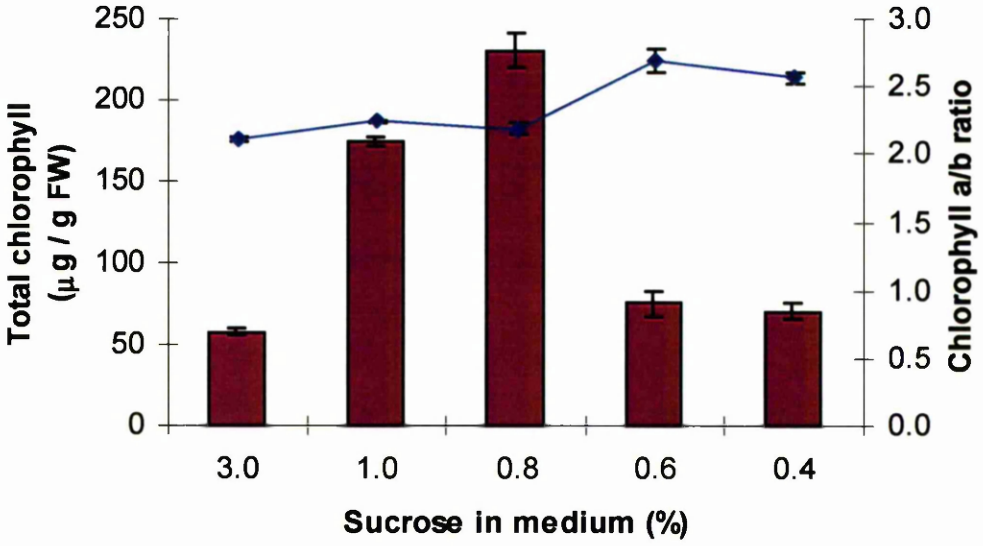

Fresh weight (mg / ml culture)
250
200
150
100
50
0
4d
7d
3
0.8
0.6
0.4
Sucrose (% w/v)

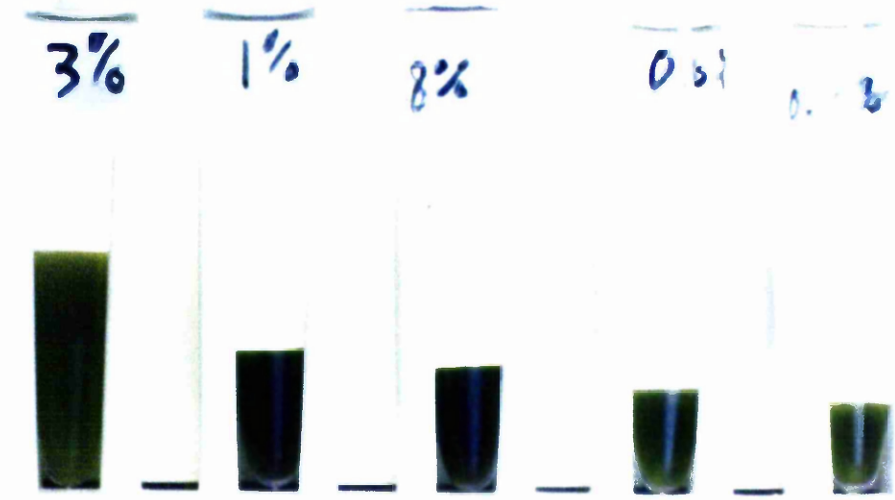
Figure 6.6: Acclimation to lower levels of sucrose in the growth medium results in changes to photosynthetic pigment abundance and composition

The range of cell cultures was grown under the normal growth conditions for 7 days.
(*A*) Total chlorophyll (▭) and chlorophyll a to chlorophyll b ratio (◆), n=3. Averages and standard errors are shown.
(*B*) Image of 8 ml aliquots of 7d old cultures after the cells have settled.
Code: The numbers indicate the percentage of sucrose (% suc) (w/v) present in the culture medium.

A



B



3	1	0.8	0.6	0.4	% suc

Sugar levels in AICAR treated, starved cell cultures decreased, but in comparison to the samples starved without AICAR they appeared to stay at a slightly higher level (Fig 6.8 B-E). This is most pronounced in the type 0.8 culture (Fig. 6.8 C). In this culture, sugar levels are the least affected by starvation in the absence of AICAR and even less in the presence of AICAR. When compared to the sucrose-fed controls without AICAR, type 0.8 cultures starved in the presence of AICAR retain nearly half their level of glucose, 2/3 of their level of fructose and similar levels of sucrose (Fig.6.8 C). Interestingly, this culture shows a tripling in sucrose levels when sucrose supplied cells are treated with AICAR (Fig. 6.8 C). Since sucrose phosphate synthase has been shown to be regulated by 14-3-3- binding (Toroser et al., 1998), this could be an indirect effect of AICAR activating SnRK 1. Alternatively, AICAR might affect other, AMP sensitive processes of carbohydrate metabolism in this culture.

When sucrose was supplied, hexose levels in type 3 and 0.8 cells were higher than sucrose levels. The reverse appeared to be the case in the type 0.6 and 0.4 cultures: Sucrose levels were higher than hexose levels in these cells. This indicates that different metabolic programmes might be operating in low sucrose and medium to high sucrose cultures. The latter have abundant carbon for most of their growth cycle, but might be limited by other nutrients such as nitrogen. The type 0.4 and 0.6 cultures appear to be carbon limited but have probably an abundant supply of nitrogen and other nutrients.

During the AICAR treatments, two further observations in the physical appearance of the culture were made: AICAR treated cells particularly when supplied with sucrose showed a slight shift in colour towards a brown-yellower colour. This was more visible in the low sucrose cultures. Secondly, starved cell cultures had a characteristic, slightly phenolic odour. This odour was the strongest in starved type 3 cultures and the weakest in starved low sucrose cultures. However, even when fed with their usual amount of sucrose the low sucrose cultures emitted this odour in a weak form. When AICAR was present, none of the samples produced any detectable odour. Due to the constraints of time, these observations could not be investigated in more detail.

Figure 6.7: *CAB* mRNA levels in AICAR treated culture types

Three-day old type 0.4, 0.6, 0.8 and 3 cultures were washed and resuspended as described in section 2.4.1. After resuspension one half of the samples was treated with ethanol (0.1%) and the remaining half was treated with AICAR (final concentration 10 mM). All samples were incubated under the normal growth conditions for 24h. RNA blot analysis on 7 μg total mRNA was performed using the *CAB2* gene as probe.
(*A*) type 3 culture; (*B*) type 0.8 culture; (*C*) type 0.6 culture; (*D*) type 0.4 culture.
Codes: Presence (+) and absence (-) of sucrose in different culture types; 'A' marks the presence of 10 mM AICAR
An image of the Ethidium Bromide stained rRNA on the gel is included for comparison.

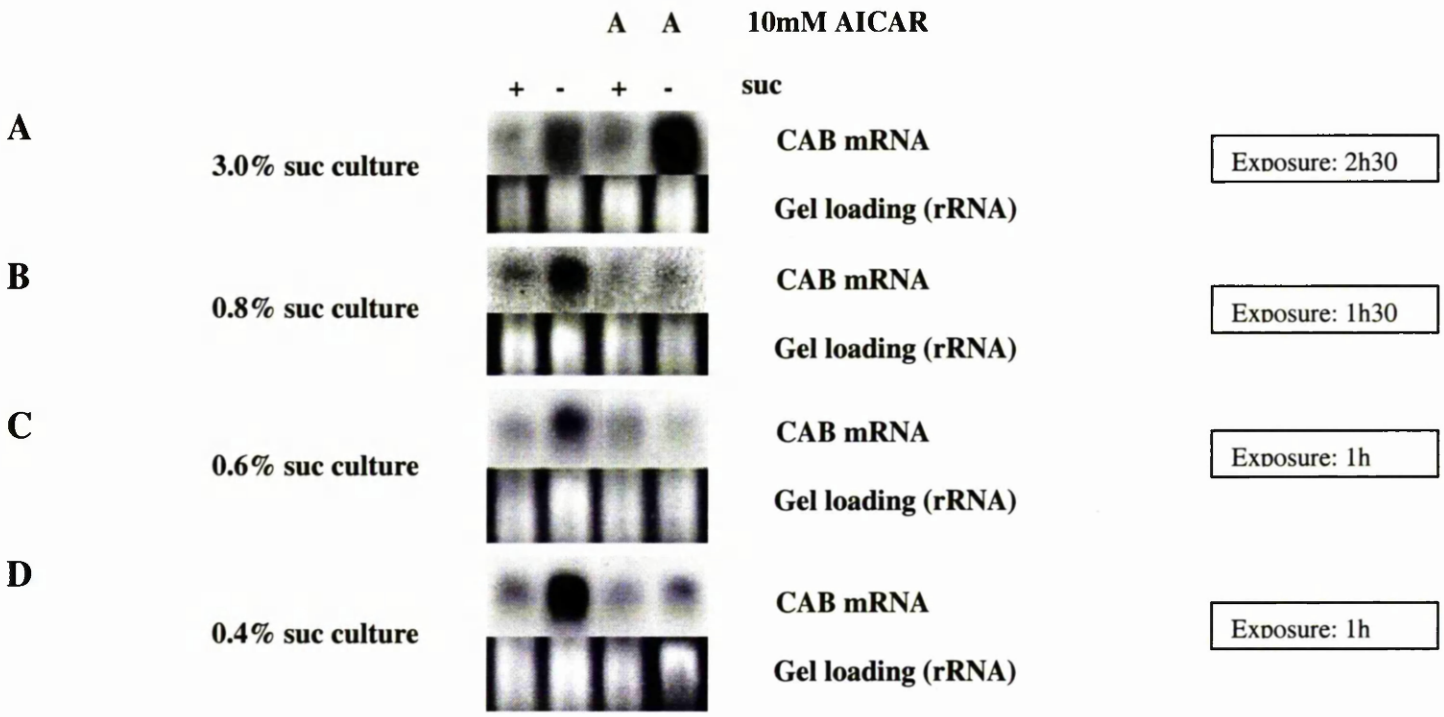
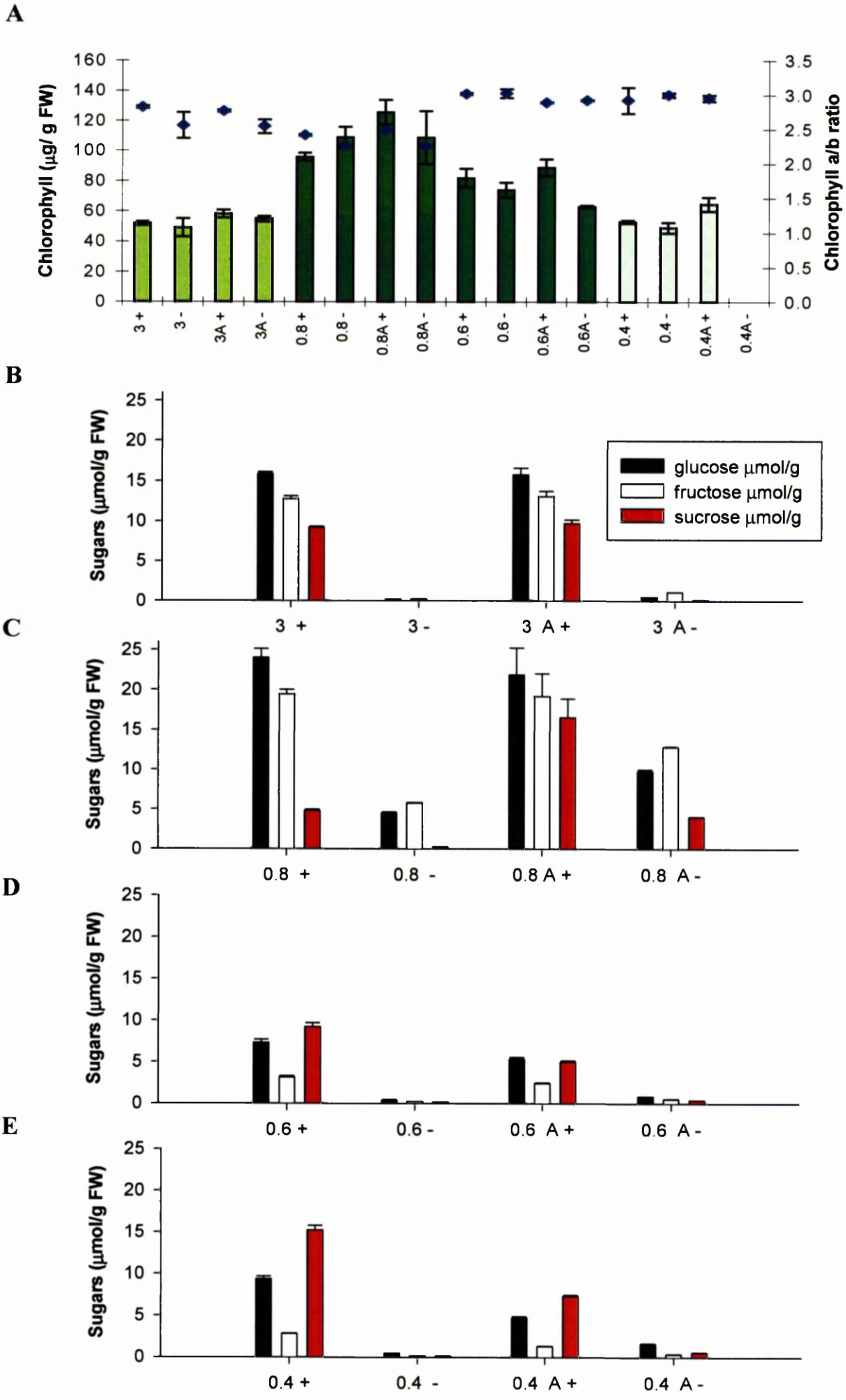

A A
10mM AICAR
+ - + -
suc
A
3.0% suc culture
CAB mRNA
Gel loading (rRNA)
Exposure: 2h30
B
0.8% suc culture
CAB mRNA
Gel loading (rRNA)
Exposure: 1h30
C
0.6% suc culture
CAB mRNA
Gel loading (rRNA)
Exposure: 1h
D
0.4% suc culture
CAB mRNA
Gel loading (rRNA)
Exposure: 1h

Figure 6.8: Chlorophyll and sugar levels in AICAR treated low sucrose cultures

Three-day old type 0.4, 0.6, 0.8 and 3 cultures were treated as described in figures 6.7. After 24h incubations samples were taken for chlorophyll and sugar analysis.
(*A*) Total chlorophyll levels in type 3 (▨), 0.8 (▩), 0.6 (▩) and 0.4 (□) cultures. Chlorophyll a to chlorophyll b ratios are given by blue diamonds (◆), n=2. Intracellular glucose (■), fructose (□) and sucrose (□) levels (μmol/g FW) after 24 h treatment of type 3 (*B*), 0.8 (*C*), 0.6 (*D*) and 0.4 (*E*) cultures measured in triplicate. Codes: The culture type is given by the number. The presence (+) or absence (-) of sucrose corresponding to the culture type is indicated. 'A' = 10 mM AICAR treatment


A
Chlorophyll (µg/ g FW)
Chlorophyll a/b ratio
160
140
120
100
80
60
40
20
0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
3 +
3 -
3A +
3A -
0.8 +
0.8 -
0.8A +
0.8A -
0.6 +
0.6 -
0.6A +
0.6A -
0.4 +
0.4 -
0.4A +
0.4A -
B
Sugars (µmol/g FW)
glucose µmol/g
fructose µmol/g
sucrose µmol/g
25
20
15
10
5
0
3 +
3 -
3 A +
3 A -
C
Sugars (µmol/g FW)
0.8 +
0.8 -
0.8 A +
0.8 A -
D
Sugars (µmol/g FW)
0.6 +
0.6 -
0.6 A +
0.6 A -
E
Sugars (µmol/g FW)
0.4 +
0.4 -
0.4 A +
0.4 A -

Chapter 7: General discussion

7.1 Introduction

Plastids are the site of photosynthesis and numerous other essential metabolic processes in higher plants. To perform these tasks both plastid-encoded and nuclear-encoded proteins are required, but to function optimally, plastid metabolism must be tightly integrated with that of the whole cell.

The aim of this PhD project was to establish whether any plastid-derived signals influence nuclear-encoded photosynthetic gene expression, if they interact with the sugar sensing/signalling mechanism and the level of signal generation or signal transduction at which the interaction might occur (see. Fig.1.1).

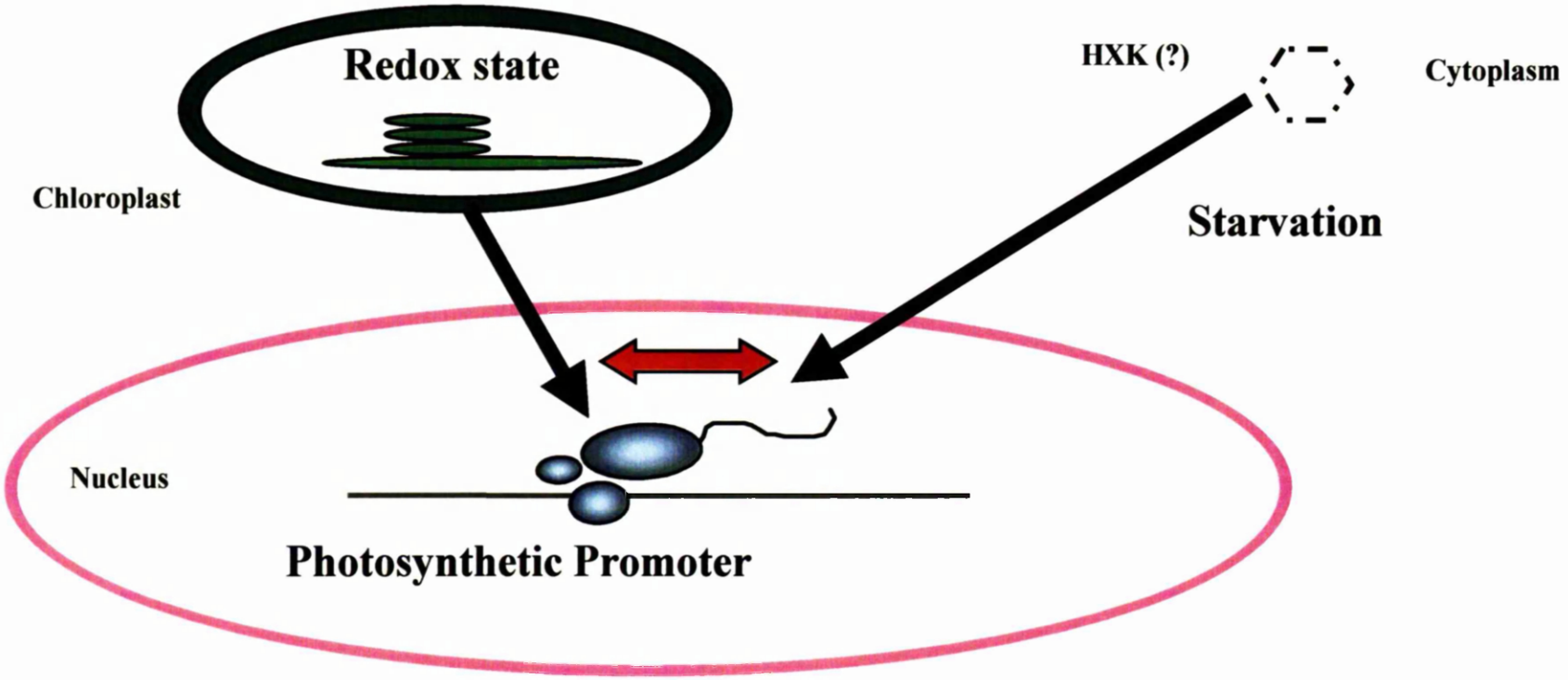
Within the plastid a complex array of mechanisms operating at the level of transcription and post-translation have been identified (Link, 1996; Somanchi and Mayfield, 1999) including redox regulation of both C-3 cycle enzymes (Raines et al., 1999; Ruuska et al., 2000)as well as transcript abundance (Pfannschmidt et al., 1999) and translation (Danon and Mayfield, 1994) of the plastid-encoded genes for the photosynthetic reaction centres. While the redox state of the photosynthetic electron transport chain has been shown to regulate the expression of some plastid-encoded photosynthetic genes (Pfannschmidt et al., 1999), the work described here is the first convincing demonstration of a plastid-derived redox signal operating to regulate nuclear-encoded photosynthetic genes in higher plants. In contrast, a large volume of literature has been accumulated in support of a sugar sensing mechanism. Here, high levels of intracellular sugars are thought to represses the expression of the nuclear-encoded photosynthetic genes *CAB, PC* and *RBCS*, and high rates of transcription occur only when sugar levels fall below some threshold level (Koch, 1996).

This study shows that the sugar-regulated mechanism interacts with the plastid redox signal with respect to the regulation of the expression of nuclear photosynthetic genes (Fig. 7.1).

Figure 7.1: Cartoon summarising the findings of this thesis

A signal originating from the plastid redox state and a sugar signal do interact with respect to the regulation of photosynthetic genes of the nucleus. The interaction occurs at the level of transcription. Upstream components of the sugar signal transduction pathway do not appear to be affected by the plastid redox signal.

HXK Hexokinase


Redox state
Chloroplast
HXK (?)
Cytoplasm
Starvation
Nucleus
Photosynthetic Promoter

7.2 The Arabidopsis cell suspension culture is a suitable model system for studies of sugar and plastid redox sensing

The first objective of this thesis project was to establish if Arabidopsis cell suspension cultures are a valuable model system to study sugar and plastid redox signalling. The cell suspension cultures are photomixotrophic, depending largely on extracellularly supplied carbon (Fig.3.3 and 3.4) yet having a fully functioning photosynthetic apparatus and considerable photosynthetic activity (Fig. 3.7). The cells' carbohydrate status can easily be manipulated (Fig. 3.4 to 3.7). Glucose can readily replace sucrose as the extracellular carbon source (Fig. 3.4). Since the cells take up considerable amounts of carbohydrates as hexoses, in particular glucose, rather then sucrose (Fig. 3.3) this is not surprising. When the cells were deprived of extracellular carbon in the exponential growth phase, there was a rapid decrease of intracellular sugar levels, an increase of chlorophyll levels and an increase of photosynthetic gene transcripts. When sucrose was returned to the growth medium, these changes were reversed quickly (Fig. 3.5 and 3.6). These results on sugar-regulated photosynthetic gene expression are consistent with the previous observation that the expression of nuclear encoded photosynthetic genes is inversely correlated with intracellular soluble sugar levels (Sheen, 1994; Stitt et al., 1995; Koch, 1996). Interestingly, in this thesis project it was also shown that the plastid encoded photosynthetic gene *psbA* is also regulated by the sugar status of the cells (Fig. 4.1 F).

While some starvation-related changes to the cell culture were observed after 24 hours, this period was not sufficient to trigger cellular autophagy (as observed by monitoring fatty acid levels and composition, see Fig. 3.8) or reduce respiration significantly when compared to the respiration rates before the onset of the starvation (Fig.3.12 D).

Another advantage of the cell suspension cultures is the ease with which the redox state of the PSII and indirectly of the PQ pool can be monitored (Fig. 3.5, 3.7 and 3.11) and manipulated using electron transport inhibitors (Fig.3.11) or light and temperature changes (Table 5.1).

Changes in sugar status alone did not alter the redox state of PET under the normal growth conditions (Fig. 3.7 D). At the beginning of this project direct feedback from the Calvin cycle to the PET chain depending on the sugar supply status of the cell was

considered plausible (Fig.1.5). The evidence gathered during this study suggest that this feedback does occur, but it does not seem to be a single major factor regulating the redox state of PSII and the PQ pool. Only if other parameters, such as light intensity (Fig. 3.5 E and 3.7 D) and temperature (Table 5.1) are changed, does the carbohydrate status modulate the redox state of the PET chain.

7.3 Is mannose just an analogue of glucose?

The hexose sugar mannose has been used as an analogue for glucose in studies of sugar sensing (Graham et al., 1994a; Pego et al., 1999). However, when mannose was supplied to the cell cultures as the sole carbon source, it caused numerous effects: it stopped cell growth (Fig. 3.10 A), lead to a strong repression of *CAB* gene expression (Fig.3.10 B), affected glucose and fructose levels (Fig. 3.10 C) reduced total chlorophyll and respiration (Fig. 3.12 A and D respectively) and had a dramatic effect on the PET chain (Fig. 3.11 and 3.12 B & C). In contrast to total respiration, azide resistant respiration recovers after mannose treatments and contributes largely to the respiration in mannose supplied cells (Fig. 3.12 D). Overall mannose results in changed, but decreased respiration, altered sugar levels and strong repression of *CAB*. Photosynthetic reaction centres appear to be lost, in conjunction with antenna complexes. The data collected in this study seems to indicate that mannose leads to a generally more reduced PET chain. Increased rates of photodamage of existing photosynthetic reaction centres due to decreased electron flux together with a lower production of new reaction centres may account for the loss observed. However, until further information on the cellular effects of mannose is available this explanation has to remain hypothetical.

The mechanism(s) by which mannose causes any of these effects are unclear. However, it appears to be a much more complex situation than mannose simply acting as an analogue of glucose to generate a hexokinase-mediated signal as previously proposed (Graham et al., 1994a; Pego et al., 1999). The experimental evidence gathered in this work suggests that mannose should only be used with caution, since any observations could be attributed to any number of these effects.

7.4 Sugar signalling and plastid redox signalling interact in regulating nuclear encoded photosynthetic gene expression in planta

The main aim of this study was to identify any interactions between sugar signalling and signalling of the plastid redox state as determined by the redox state of the PET chain. For this purpose, the redox state of the PET chain was altered using the photosynthetic inhibitor DCMU. When cell suspension cultures were starved in the presence of DCMU, the previously observed increase in the transcript abundance of the nuclear encoded, photosynthetic genes *CAB* and *RBCS* was absent (Fig.4.1 A & B). This effect of DCMU did not affect the transcript levels of nuclear encoded, non-photosynthetic genes such as *NR* and *CHS* (Fig. 4.1 C & D). It is well established that DCMU is a specific inhibitor blocking electron flow beyond the Q_A binding site in PS II (see Figure 1.2). No other sites of DCMU action in plant cells are documented, and the results obtained confirm that DCMU does not affect the sugar status of cells during starvation (Fig.4.2) or respiration rates (data not shown).

The evidence gathered in this study demonstrates that high intracellular sugars repress the abundance of nuclear-encoded photosynthetic gene transcripts through the action of an uncharacterised cytosolic signalling mechanism that may involve hexokinase. However, the removal of the sugar-repression signal alone does not result in increased transcript levels for these genes. The experiments with DCMU clearly indicate that the normal starvation-induced increase in the transcript levels of nuclear-encoded photosynthetic genes requires an additional signal, which is dependent upon PET (Figs. 4.1 and 4.4). However, from the cell culture work alone it was not possible to deduce if this regulation was on the level of transcription itself or of a post-transcriptional nature (e.g. mRNA stability). However, the experiments using the Arabidopsis plants carrying nuclear photosynthetic gene promoters fused to the firefly luciferase gene (*CAB2-LUC* (Millar et al., 1992) and *PC-LUC* (Dijkwel et al., 1996)) did provide the answer to this question. The luminescence experiments with the *PC-LUC* and *CAB2-LUC Arabidopsis* lines gave results that are entirely consistent with those from the cell suspension cultures, and confirmed that the sugar sensing and chloroplast-derived signals modulate the rates of gene transcription rather than transcript stability. However, this same chloroplast-derived signal does not have an effect on *CAB* and *PC* genes once they are de-repressed (see Fig. 4.5).

In contrast to the nuclear encoded photosynthetic genes transcript, levels of the plastid encoded *psbA* (coding for the D1 protein of PSII) did not change in the cell suspension cultures in response to DCMU (Fig.4.1 F). *PsbA* transcript abundance was regulated only by the sugar status of the cells. The regulation of transcription of the plastid encoded *psaAB* (coding for the core protein of PSI) by the redox state of the PQ pool has previously been reported for *Sinapis alba* seedlings (Pfannschmidt et al., 1999). In contrast to *psaAB* the transcription of *psbA* could not be repressed by altering the redox state of PQ by light favouring PSI or PSII selectively (Pfannschmidt et al., 1999). Both the inhibitors DCMU and DBMIB had no effect on *psbA*. These findings are consistent with the results presented in this thesis which state that DCMU fails to prevent the induction of psbA in response to starvation.

At least in algae *psbA* and other plastid encoded photosynthetic genes appear to be regulated by the plastid redox state at the post-transcriptional level rather than at the transcriptional level (Danon and Mayfield, 1994; Bruick and Mayfield, 1999; Sippola and Aro, 2000). The situation in higher plants is still under investigation.

The findings of this study establish the hypothesis that nuclear encoded photosynthetic genes are regulated by the plastid redox state at the level of transcription, but the plastid encoded *psbA* is regulated post-transcriptionally (Bruick and Mayfield, 1999).

7.5 What redox component in the chloroplast evokes nuclear encoded gene transcription?

Several components of the PET chain have been implicated as the possible redox sensor generating a plastid redox signal (reviewed in Huner et al. (1996); see also Fig. 1.2). In particular the redox state of the PQ pool has been favoured (Escoubas et al., 1995; Allen et al., 1995; Petracek et al., 1998; Pfannschmidt et al., 1999). Alternatively the redox state of thioredoxin (Danon and Mayfield, 1994; Sippola and Aro, 2000) glutathione (Karpinski et al., 1997) or ascorbate (Noctor et al., 2000) have been suggested.

7.5.1 A case for active oxygen species?

When respiration and photosynthesis of starved and sucrose-fed cell culture were measured by monitoring oxygen evolution and consumption, starved cells showed low

oxygen evolution in the light (see Fig. 3.7 B). One interpretation is that of a carbon skeleton limitation of the Calvin cycle resulting in lower photosynthesis. However, two other explanations, both being associated with an increase in active oxygen species, can not be dismissed without thorough consideration:

I. Photorespiration

In this process Rubisco will use oxygen rather than carbon dioxide for its reaction. The processing of the resulting 2-phospho-glycollate involves cytosol, mitochondria and peroxisomes, where hydrogen peroxide is produced. Although converted to water by catalase, some hydrogen peroxide may leave the peroxisomes (see Foyer and Noctor (2000) for more information).

Under elevated light conditions and with limiting carbon dioxide, photorespiration can be a major source of hydrogen peroxide and could mask the true oxygen evolution at the water splitting complex.

However, the cells and plants in this study were not exposed to high light (with the exception of some experiments in chapter 5). In addition, the concentration of carbon dioxide in starved cells should still be high enough not to result in increased photorespiration, since the respiration in starved cells is still at normal levels (see Figs. 3.7 B and 3.12 D). Furthermore, photorespiration is a wasteful process with respect to energy and reduction equivalents. While this has been suggested to be of use in conditions of excess excitation energy (see Foyer and Noctor (2000) and therein), it does not seem favourable for carbon-deprived, starving cells to waste ATP and reduction equivalents in a process that in addition results in the loss of carbon as CO_2.

II. The Mehler reaction (see Fig. 1.3)

In this reaction, also described as part of the water-water cycle of photosynthesis, PSI transfers electrons, derived from PET and hence from the water splitting complex, to oxygen. The formed superoxide is converted to hydrogen peroxide by superoxide dismutase. The resulting hydrogen peroxide is reduced by ascorbate peroxidase to water. In the process ascorbate is oxidised and has to be regenerated using NADPH or glutathione (see (Asada (2000) for details).

Whilst oxygen evolution would be low, because of the water-water cycling, electron transport would still provide a thylakoid proton gradient and hence

ATP, but at the expense of reduction equivalents. Ascorbate and glutathione pools would become oxidised. In addition, some hydrogen peroxide might leave the plastid and act as signal.

It is plausible this process does occur in starved cells, but it might be disadvantageous for the cell to deplete its antioxidant stocks, and in particular ascorbate, with the result of less useful breakdown products, for the sole benefit of ATP production. If the Calvin cycle can not operate at maximum capacity, for example because carbon skeletons might be limiting in starved cells, ATP production could be achieved by cyclic electron transport, rather than by the Mehler reaction with the resulting loss of ascorbate, a major carbon metabolite in leaves.

The balance between the oxidised and reduced forms of ascorbate rather than the balance between the oxidised and reduced forms of glutathione have been suggested to be important in chloroplast for initiating an oxidative stress signal with respect to the induction of antioxidant systems (Noctor et al., 2000). The Mehler reaction in starved cells will not only result in hydrogen peroxide production (which might act as a signal per se), but consequently in consumption of reduced ascorbate. This will tip the balance towards oxidised ascorbate. Should a signal be initiated (by hydrogen peroxide or ascorbate) and should this signal be responsible for the observed induction of *CAB*, the resulting increase in antenna complexes would only increase photon flux through the PET chain, and lead to even more Mehler reactions.

Moreover, ascorbate is also involved in photoprotective mechanisms around PSII: it is a co-factor of violaxanthin de-epoxidase involved in zeaxanthin-dependent dissipation of excess excitation energy, regenerates α-tocopherol when this has been consumed to reduce lipid peroxyl radicals and can donate electrons to PSII (Smirnoff, 2000). These functions will be of greater importance when PET is blocked by DCMU and PSII has to dissipate rather than transduce the absorbed energy. In cells treated with DCMU this should lead to an increased consumption of reduced ascorbate. Should the altered balance between oxidised ascorbate and reduced ascorbate – either by these processes or the Mehler reaction - be the origin of the signal, then in starved or sucrose-supplied cells treated with DCMU the same signal should be initiated as in starved cells

with increased Mehler reactions. With respect to nuclear photosynthetic gene expression this is clearly not the case.

However, in their work the group around Karpinski and Mullineaux demonstrated a role for antioxidants and active oxygen species in regulating the induction of ascorbate peroxidase genes in response to elevated light conditions (Karpinski et al., 1997; Karpinski et al., 1999). Glutathione and ascorbate, supplied in their oxidised or reduced form before the shift to elevated light, could inhibit this induction (Karpinski et al., 1997). Pre-treatment of leaves with hydrogen peroxide also prevented this induction, while infiltration with catalase resulted in partial prevention (Karpinski et al., 1999). Even more, hydrogen peroxide was shown to be involved in systemic signalling of excess excitation energy (Karpinski et al., 1999).
However, for the signalling at the local level the group had already shown that a PET-derived signal precedes the hydrogen peroxide burst associated with exposure to excess excitation energy (Karpinski et al., 1997). Hydrogen peroxide could not overcome the block of induction when DCMU was present (Karpinski et al., 1999).
Chilling can also result in elevated hydrogen peroxide level, yet in this study chilling prevented higher *CAB* mRNA levels in starved cells (see Fig. 5.3) similar to the effects of DCMU at room temperature (discussed below in more detail).

It can therefore be concluded that a PET-derived factor is of greater significance for the signalling from plastid to nucleus than hydrogen peroxide, although the latter has a role in systemic signalling.

7.5.2 PET chain components

The redox state of the PQ pool has been suggested to be of prime importance for the signalling of excess excitation energy in algae and Arabidopsis (Karpinski et al., 1997; Durnford and Falkowski, 1997; Karpinski et al., 1999). However, other components of the PET chain have also been put forward as candidates for redox sensors (reviewed in Huner et al. (1996); see also Fig. 1.2).
The observation that in the experiments described in this study PQ remained almost fully oxidised in both starved (inducing) and sucrose replete (suppressing) conditions at 20 μmol photons s^{-1} m^{-2} (Fig. 3.7 D) suggests that PQ redox state is not involved.

Further, as the oxidation of PQ is the rate-limiting step in PET, it can be concluded that all components of the PET chain were oxidised, regardless of sugar status, and therefore it is unlikely that any of these give rise to the redox signal affecting nuclear gene transcription. The experiments using the inhibitor of PET, DCMU, support this view. With DCMU, all components downstream of its site of action (*i.e.* distal of the Q_A binding site), including PQ, would have been fully oxidised, whilst those upstream (proximal of Q_A) would have been fully reduced. It is difficult to reconcile the observed gene expression patterns in DCMU treated samples (always suppressed, reduced proximal / oxidised distal components) and untreated samples (induced by starvation, oxidised PET), with the concept that the redox status of a PET chain component gives rise to nuclear gene transcription. Anderson and co-workers (Anderson et al., 1997) studied tobacco plants with reduced levels of cytochrome b_6/f complex and also concluded that neither the redox state of Q_A nor of the PQ pool could be responsible singularly for redox control of gene expression.

From the experiments using the inhibitor DBMIB, which blocks downstream of PQ, it can not completely be ruled out that the PQ pool might play a role in the repression of *CAB* once the gene has already been induced (see Figs. 4.5 and 4.6). However, the labile nature of DBMIB under the given experimental conditions prevented the confirmation of this possibility.

Karpinski and co-workers employed DCMU and DBMIB to investigate the induction of ascorbate peroxidase genes in response to elevated light (Karpinski et al., 1997; Karpinski et al., 1999). DCMU did prevent the induction normally observed after transfer from low light to ten times higher light intensities. DBMIB in contrast induced the ascorbate peroxidase genes, even under low light conditions (Karpinski et al., 1997; Karpinski et al., 1999). The authors concluded that the redox state of PQ is the key factor of PET involved in this regulation. However, the concentrations of DBMIB used in their studies (12 to 14 μM) were above the maximal concentration (10μM) used in this study and observed to inhibit respiration (see section 4.5). Secondary effects of DBMIB with respect to the induction of ascorbate peroxidase genes can not be ruled out, since no respiration measurements were provided by Karpinski and colleagues (1997, 1999).

In this context it is noteworthy that the work by Sippola and Aro on *psbA* in cyanobacteria (Sippola and Aro, 2000) suggests that the PQ pool might only play a role in redox signalling under certain conditions, yet in their experiments thiol reducing

agents were effective under all conditions tested and could counteract any hypothetical effect from the redox state of the PQ pool.

When the redox state of the PET chain was altered by means of lower temperature (Table 5.1), it did affect *CAB* transcript abundance (Fig. 5.3). However, the lower temperature modulated the cellular responses to carbohydrate status as well as altering the plastid redox state (Fig. 5.2). Moreover, when lower temperatures were combined with elevated light intensities (also reducing the PET chain) the two treatments seemed to counteract each other in a manner depending on the carbohydrate status of the cells (Fig.5.3). These observations cannot be explained without further, detailed study of the effects of low temperature on plastid redox state and carbohydrate status. However, they don't appear to support a role of the PQ pool as the redox sensor – in the low temperature treatments the redox state of PQ was more reduced, in the treatments with DCMU it was mostly oxidised, yet the effects on CAB mRNA levels were similar (Fig. 4.1 A and Fig. 5.3).

Acclimation to elevated light (photoacclimation) and acclimation to cold share some common features (see Huner et al. (1998) for review). However, the results obtained during the course of this thesis project cannot clarify if the redox state of the PQ pool in higher plants is a determining factor in the response to photoacclimation as proposed for algae (Escoubas et al., 1995; Durnford and Falkowski, 1997). When the cell suspension culture was subjected to elevated illumination, starved cells showed a much stronger reduction of PS II and PQ than sucrose fed cells (Fig. 3.7 D). However, in both treatments *CAB* mRNA levels were decreased in response to exposure to 80 μmol photons s^{-1} m^{-2}, although more strongly in sucrose-fed cells (Fig. 5.3 columns 8 and 10). However, when the cells were starved under the elevated light conditions, but kept at 4°C during this treatment, *CAB* mRNA abundance was high (Fig. 5.3 column 7). In contrast, sugar-fed cells exposed to elevated light and cold still had low levels of *CAB* mRNA (Fig. 5.3 column 9). These results suggest that one treatment (elevated light) resulting in a more reduced PS II (Fig. 3.7 D) can be counteracted by another treatment (cold) which on its own also results in a more reduced PS II (Table 5.1) in a manner dependent on sugar status. The suggested role for the redox state of the PQ pool as the determining factor (Escoubas et al., 1995; Durnford and Falkowski, 1997) can not be reconciled with these findings without great difficulties. The situation under elevated

illumination is further complicated by the possibility that photoinhibition and photorespiration might occur at significant rates. A consequence of this might be an increase in active oxygen species and corresponding oxidation of antioxidants in the plastid stroma (e.g. glutathione, ascorbate and even thioredoxin) (Foyer and Noctor, 2000). The resulting changes in plastid redox state might mask any signal connected to the redox state of PQ.

Taken together, it can be concluded that PET is required to evoke the redox signal mechanism observed in this study, but that this mechanism probably operates through a stromal redox component. Possible candidates include thioredoxin (Danon and Mayfield (1994), Sippola and Aro (2000)) or glutathione (Karpinski et al., 1997). However, it is conceivable that subtle changes (within the 0 - 10% reduced range) in the redox state of one or several PET chain component are sufficient to trigger changes in nuclear gene transcription.

7.6 Is the plant homologue of the SNF1 complex involved in transmitting a sugar signal that has been modulated by the plastid redox signal?

Based on the fact that altering the intracellular sugar status did not alter the plastid redox state under the normal growth conditions (Fig. 3.7 D) and conversely altering the plastid redox state with DCMU did not change sugar status of the cells (Fig.4.2), it appears that these two different signals do not affect the generation of each other. The effect of the redox state on sugar regulated gene expression must therefore occur downstream of the metabolite levels, possibly in the sugar signalling pathway. The experiments assessing the binding of 14-3-3 proteins were designed to address this question. If the SnRK1 complex in plants plays a central role in transmitting sugar-mediated signals, as suggested by Halford and co-workers (Halford and Hardie, 1998; Halford et al., 1999) it could be expected that any factor modulating the sugar signal upstream or at the level of the SnRK1 complex could potentially affect the phosphorylation state of the 14-3-3 binding proteins as well.

In the Arabidopsis cell suspension culture grown for 3 days with 3% (w/v) sucrose, all 14-3-3 binding is lost in response to 24 hour starvation and 14-3-3 binding proteins are degraded by a starvation induced protease (Cotelle et al., 2000). Although DCMU

resulted in a sugar-like effect with respect to nuclear photosynthetic gene expression, it did not alter the dephosphorylation and degradation of 14-3-3 binding proteins in starved cell culture (Fig. 6.1 – 6.3). From these findings, two conclusions can be reached:

- DCMU does prevent the induction of nuclear encoded photosynthetic genes by a specific mechanism of plastid origin.
- Either the interaction between the plastid redox signal and the sugar signal does occur downstream of the SnRK1 complex, potentially even at the promoter level, or the SnRK1 complex does not transmit the modulated signal regulating photosynthetic gene expression. Although it currently appears unlikely, it can also not be ruled out that the sugar signal altered by the plastid redox state might potentially be transmitted by the SnRK1 complex, but by a branch of the signal transduction pathway not including action on 14-3-3 binding proteins. Only further detailed analysis of known and yet unknown signal transduction components will reveal the exact mode of interaction.

7.7 Summary

Summarising the results of the different experiments, it can now be concluded that (see Fig. 7.1):

1. A signal generated by the plastid redox state as determined by PET regulates the expression of nuclear-encoded photosynthetic genes.
2. The plastid-redox signal interacts with the sugar signalling mechanism with respect to nuclear photosynthetic genes. Non-photosynthetic genes of the nucleus appear to be unaffected by the plastid-redox signal.

7.8 Results obtained about the sugar signalling pathway

Although the experiments using the low sucrose acclimatised culture types and the compound AICAR did not directly address the interaction between plastid redox and sugar signalling, they contributed to the understanding of the transduction of the starvation signal with some interesting findings.

The presence of AICAR protected most, but not all 14-3-3 binding proteins from the starvation induced degradation (Fig.6.2 – 6.4). AICAR treatment of starved cells results in minimally higher sugar levels then in starved controls (Fig. 6.8), with the exception of the type 0.8 culture where AICAR had strong effects on sugar levels in sucrose fed and starved cells (Fig. 6.8 C). Although *CAB* mRNA levels in all but the type 3 culture were not increased (Fig. 6.7) under starvation conditions, AICAR did not affect total chlorophyll levels or chlorophyll *a/b* ratios significantly (Fig. 6.8 A). It should be remembered however, that these findings await confirmation by independent replication in the future

The effects of AICAR on sugar levels observed in this study could be caused by the activation of the SnRK1 complex by the AMP analogue, as reported for the activation of SnRK1 complex by AMP *in vitro* (Halford and Hardie, 1998; Sugden et al., 1999a). Subsequently the SnRK1 complex might act on 14-3-3 binding proteins involved in carbohydrate metabolism such as SPS, TPS and GAPDH (Moorhead et al., 1999; Cotelle et al., 2000) and alter sugar metabolism in this way. On the other hand it is possible that AMP (and its analogue ZMP) directly affect other proteins regulating carbohydrate metabolism.

In this context a striking difference with respect to the SNF1 complex between mammalian and yeast cells on one side, and plant cells on the other side is worth highlighting. AMP activates the plant SnRK1 by inhibiting its dephosphorylation and increasing its phosphorylation by the upstream kinase (Sugden et al., 1999a). The members of the SNF1 family in mammalian cells and yeast are also activated by AMP (Halford and Hardie (1998) and therein). High levels of AMP are generally associated with carbon deprivation, while high levels of ATP are associated with a generous 'fuel' supply (Wilson et al., 1996). In yeast and mammalian cells the SNF1 complex / AMPK is activated by sugar deprivation. When active it reduces ATP consumption and switches metabolism to alternative energy sources (Jiang and Carlson, 1996; Hardie et al., 1998). Although the regulation of the SnRK1 complex, its composition and its mode of action are similar to the situation in yeast and mammals, the plant SNF1 homologue is activated not by sugar deprivation, but by sugar feeding (Bhalerao et al., 1999)! When activated by the AMP analogue produced from AICAR (which doesn't result in increased ATP levels (Huber and Kaiser, 1996), it counteracts a sugar deprivation signal (chapter 6 and Cotelle et al. (2000)) although activation by AMP should be indicative of a low sugar supply. Instead of switching metabolism to alternative energy sources via

the action of the SNF1 / AMPK complex as it does in yeast and mammals, AMP appears to lead to a possible downregulation of photosynthesis, as the low levels of *CAB* mRNA in AICAR treated starved low sucrose cultures might indicate (Fig. 6.8). Should future research prove this speculation to be fact, it would indicate that plants employ a conserved signal transduction mechanism for the opposite function than their heterotrophic relatives.

The culture types acclimatised to different levels of sucrose showed remarkable differences: in low sucrose adapted cultures (type 0.4 and 0.6) starvation had a less dramatic effect with respect to the degradation of 14-3-3 binding proteins and AICAR conferred greater protection against proteolysis (Fig. 6.2-4). Differences in individual 14-3-3 binding proteins bands were visible between the low and high sucrose cultures, and type 0.4 cultures showed some 14-3-3 proteins that were absent in type 3 cultures when starved (Fig.6.2 & 6.3). Type 1 and 0.8 had very high chlorophyll levels (Fig. 6.6), while in type 0.4 and 0.6 cultures sucrose levels were higher than hexose levels in contrast to the type 0.8 and 3 cultures (Fig. 6.8). Although the growth period was the same, the fresh weight was correlated to the sucrose content of the medium (Fig. 6.5). These different culture types should be useful tools in further investigations of the role of sugar signalling and plastid-redox signalling under a range of physiological conditions, from carbon limited (type 0.4 and 0.6 cultures) over carbon challenged (type 0.8 and 1) to carbon abundant cultures (type 3).

7.9 A model for the regulation of nuclear photosynthetic gene expression by sugar and plastid redox signals

7.9.1 The model proposed

A model may be proposed to account for the observations on the dependence of nuclear-encoded photosynthetic gene expression on PET. This model relies on the generation of a positive regulator (inducer) by active PET and is summarised in Fig. 7.2. The positive regulator model predicts that under the experimental conditions used in these experiments (20μmol photons m^{-2} s^{-1} PAR) a moderately weak inductive signal arises from PET, but the strong antagonistic suppression arising from exogenous sugars

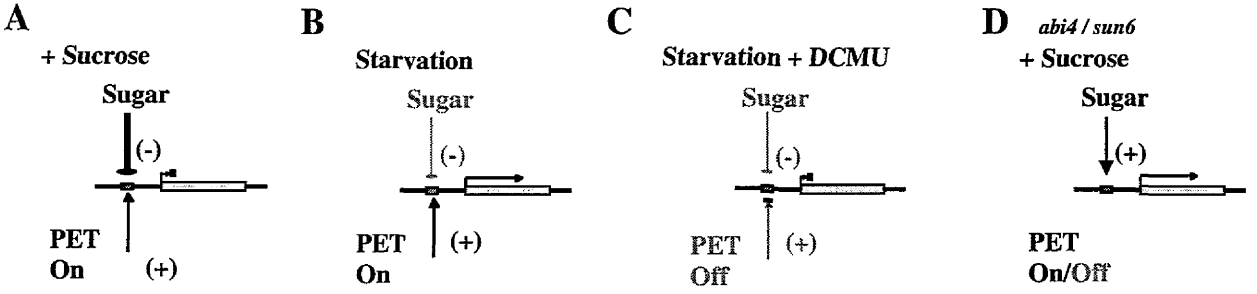
prevents transcription (Fig. 7.2 A). Starvation in the light results in the removal of the sugar-dependent suppression allowing the chloroplast-derived positive regulator to induce transcription (Fig. 7.2 B). However, transcription in starved cells is blocked if PET is inhibited (Fig. 7.2 C). From this model it follows that altering either the sugar status of the cell or the rate of PET can set the balance between sugar-dependent gene suppression and redox-dependent gene transcription. The experiments described in this study do not completely rule out the possibility that inactive PET could generate a negative signal regulating photosynthetic gene expression in the nucleus, rather than the positive regulator model as proposed. However, the findings of other groups support the positive regulator model:

- When carotenoid biosynthesis is completely disrupted, either by genetic (Wu et al., 1999) or chemical means (e.g. with Norflurazon), photooxidation will impair chloroplast development and result in the absence of photosynthetic structures in the plastid (LaRocca et al., 2000). Nuclear encoded, photosynthetic gene expression is inhibited, and the absence of this inhibition was used to study the *genome uncoupled* (*gun*) mutants (Susek et al., 1993). However, another, less stringent inhibitor of carotenoid biosynthesis, Amitrole, will allow for rudimentary photosynthetic membrane systems in barley (LaRocca et al., 2000). In their study La Rocca and co-workers found that leaves treated with Norflurazon had no detectable *CAB* mRNA levels, yet Amitrole treated leaves retained some *CAB* mRNA. The authors concluded, in accordance with work performed on carotenoid mutants, that only complete dismantling of thylakoids abolishes the plastid-derived positive regulator (LaRocca et al. (2000) and therein). Together with the results obtained in the experiments with DCMU in this study, it appears that active PET even at low levels can invoke the positive regulator signal.

- When chloroplast development is disrupted by the use of Norflurazon the Arabidopsis *gun* mutants exhibit nuclear encoded photosynthetic gene expression in the absence of functioning chloroplasts (Susek et al., 1993; Vinti et al., 2000; Mochizuki et al., 2001). These mutants maintain normal patterns of tissue specific, cell-type-specific, light-receptor dependent and dark-adapted *CAB* expression (Susek et al., 1993) indicating that not the basic machinery and regulation has been disrupted by the mutation.

Figure 7.2: Hypothesis for the control of nuclear encoded photosynthetic gene expression by sugar and plastid redox signals

Model showing the control of nuclear gene transcription by cytosolic sugar status and photosynthetic electron transport. PET gives rise to a positive signal that enhances nuclear gene transcription. However, the low light levels used in this study (20 μmol photons m^{-2} s^{-1} PAR) evoke only a moderately weak signal, and in the presence of the strong antagonistic repression arising from exogenous sugars, transcription is suppressed (*A*). Starvation in the light results in the removal of the sugar-dependent repression allowing the chloroplast-derived positive regulator to induce transcription (*B*). However, de-repression in starved cells is blocked if PET is inhibited (*C*). In the *sun6* mutant sugars induce photosynthetic gene expression irrespective of the status of PET (*D*).


A
+ Sucrose
Sugar
(-)
PET
On
(+)
B
Starvation
Sugar
(-)
PET
On
(+)
C
Starvation + DCMU
Sugar
(-)
PET
Off
(+)
D
abi4 / sun6
+ Sucrose
Sugar
(+)
PET
On/Off

However, in these mutants *CAB* expression does appear to be uncoupled from chloroplast function possibly due to a constitutive positive signal caused by the mutation.

- Fujiki and colleagues studied the transcript abundance of a group of dark induced (*din*) genes in another photomixotrophic Arabidopsis cell suspension culture (Fujiki et al., 2000). This group of *din* genes is repressed by sugars and expressed under starvation conditions similar to photosynthetic genes (Fujiki et al., 2000). The authors used a series of inhibitors, mainly protein kinase and phosphatase inhibitors, to investigate their effects on *din* and *CAB* mRNA levels in starved and sucrose fed cultures. While their findings showed some similarities in the transduction of a starvation signal with respect to *din* and the *CAB* mRNA accumulation, their work demonstrated that there are multiple signalling pathways differentially affecting not only mRNA levels of different members of the group of *din* genes, but also *CAB* transcript levels (Fujiki et al., 2000). Moreover, a starvation signal alone was not sufficient to result in an increase in *CAB* mRNA, but another signal, dependent on protein phosphatase action (most likely of the type 1 of protein phosphatases) was required (Fujiki et al., 2000). This is consistent with my findings that a starvation signal is necessary, but not sufficient, to induce nuclear photosynthetic genes, and that a signal connected to PET is also required. It is noteworthy that Fujiki and co-workers found that the transduction of the starvation signal did not require Tyrosine/Histidine kinase activity, but depended on Serine/Threonine kinases with respect to the expression of both *din* and *CAB* genes (Fujiki et al., 2000). The SNF1 complex and the kinase activating SnRK1 itself are Serine/Threonine kinases.

7.9.2 A possible involvement of the ABA signal transduction pathway

Several recent reports have demonstrated the involvement of the plant hormone abscisic acid in sugar mediated responses (Huijser et al., 2000; Laby et al., 2000; Arenas-Huertero et al., 2000). Using the *Arabidopsis sun6* mutant, which is allelic to the abscisic acid insensitive mutant *abi4*, it was demonstrated that *PC* transcription is induced rather than repressed by sugars and PET has no effect (Fig. 4.4 C & D and Fig. 7.2 D) It is interesting to note that in *sun6*, *PC* transcription is behaving in a similar fashion to that of *NR* in that it is induced by sugars (Fig 4.1 C and Fig 4.4 C). In the

case of *NR* PET also had no effect on transcript abundance. It is remarkable that the sugar repressible, PET sensitive, *PC* promoter is converted to being sugar inducible and PET insensitive by a mutation in the *ABI4* gene.

7.9.3 Implications

The model proposed (see Fig. 7.2) provides plants with a flexible mechanism for tailoring cell metabolic processes to the appropriate tasks determined by the whole plant in its unique environment. Clearly, the presence of abundant light should trigger the synthesis of new photosynthetic apparatus. However, this is an appropriate response only when there is a requirement for energy or carbon fixation to meet either the immediate demands of the photosynthetic cell, or the demands from sink tissues. When soluble sugar levels rise, for example due to an impairment of phloem loading by either genetic or environmentally determined growth cessation, the synthesis of new photosynthetic apparatus will be curtailed until the demand for carbon increases.

7.10 Future work

A major question that remains to be addressed is the source of the plastid redox signal. A two component response regulator similar to those present in bacteria has been suggested to measure the redox state of the PQ pool (Allen and Nilsson, 1997). Two component response regulators have indeed been found in Arabidopsis, but no involvement in redox signalling has so far been proven (Schaller, 2000). The PQ redox state has so far been linked only with the phosphorylation (and consecutive re-distribution between PSII and PSI) of the light harvesting complex (Allen and Nilsson, 1997) and the expression of plastid encoded photosynthetic genes (Pfannschmidt et al., 1999). However, it has recently been suggested that the redox state of the PQ pool alone is not the determining factor, but rather that in operates in conjunction with the redox state of the cytochrome b_6/f complex (Anderson et al., 1997; Alfonso et al., 2000). Other reports describe a role for the PQ pool in the phosphorylation of light harvesting complex only in conjunction with the redox state of the ferredoxin-thioredoxin system (Rintamaki et al., 2000) and suggest that reduced thiol group carriers play a major role in plastid gene expression (Sippola and Aro, 2000). There are indeed numerous reports linking thiol groups (in the form of thioredoxin or glutathione) to a multitude of plastid

processes such as phosphorylation of the light harvesting complex (Rintamaki et al., 2000), expression and translation of plastid encoded photosynthetic genes (Danon and Mayfield, 1994; Link, 1996; Kim and Mayfield, 1997; Baginsky et al., 1999; Alfonso et al., 2000; Sippola and Aro, 2000) and the regulation of the activity of key enzymes of the Calvin cycle (Raines et al., 1999; Ruuska et al., 2000).

Careful and detailed analysis of electron flux through the components of the photosynthetic electron transport chain, including cyclic electron transport, using a range of light intensities, light qualities specific for either PSI or PSII, antisense plants with reduced levels of PET chain components (e.g. Anderson et al. (1997)), mutants in PET chain components (e.g. Varotto et al. (2000)), thiol reducing and oxidising reagents (such as dithiothreitol and *N*-ethylmaleimide),more specific inhibitors and inhibitors blocking PET at different locations (e.g. Methylvialogen) should resolve this issue.

Little real evidence about the nature of the signal transduction pathway from the chloroplast to the nucleus has emerged so far. A good proportion of the plastid genome encodes for transdehydrogenases (18 open reading frames of a total of 123 genes in the tobacco plastid genome (Shinozaki et al., 1986)) and it is possible that they are involved in transferring a redox signal across the plastid membranes. Streatfield and co-workers studied the *CAB gene underexpressed 1* (*cue1*) mutant and located the mutation in the plastid inner envelope phosphoenolpyruvate/phosphate translocator (Streatfield et al., 1999). They interpreted this as an indication of the importance of metabolites dependent on a plastid supply of phosphoenolpyruvate in sensing and transducing plastid redox signals. Karpinski and colleagues proposed reduced glutathione to be involved in the signal transduction of a plastid redox signal from the chloroplast to the ascorbate peroxidase genes in the nucleus (Karpinski et al., 1997). Protein phosphatases have been implicated with the transmission of a plastid derived signal (Fujiki et al., 2000) although this might only affect transcript stability but not gene expression (Sheen, 1993; Kurotani et al., 1999). Chlorophyll precursors have also been implicated in the plastid-to-nucleus signal transduction (Kropat et al., 2000; Vinti et al., 2000; Mochizuki et al., 2001). However work with the *gun1hy1* double mutant suggests the existence of tetrapyrrole independent signalling from the plastid to the nucleus as well as a chlorophyll precursor dependent pathway (Vinti et al., 2000).

A genetic approach to identify components in the plastid redox signal pathway seems to be the most promising approach. Similar efforts are already underway to elucidate the sugar signalling network and have led to the isolation of a number of sugar sensing

mutants (reviewed in Smeekens (2000)). These are beginning to yield information about the crosstalk of sugar sensing and other signalling pathways. In particular, the crosstalk with signal transduction pathways for the plant hormones ethylene and abscisic acid (Laby et al., 2000; Arenas-Huertero et al., 2000) in sugar mediated responses has been demonstrated in several recent reports (Nemeth et al., 1998; Huijser et al., 2000; Laby et al., 2000; Arenas-Huertero et al., 2000).

Already the benefit of using sugar sensing mutants in the studies of the interaction between sugar and plastid redox signalling has been shown: Using the *Arabidopsis sun6* mutant, which is allelic to the abscisic acid insensitive mutant *abi4*, it was demonstrated that *PC* transcription is induced rather than repressed by sugars and PET has no effect (Fig. 4.4 C & D and Fig. 7.2 D)

Since the response of plant cell to carbohydrates is modulated by their nitrogen status (Boxall (1999) and Martin T., Oswald O. and Graham I.A., submitted for publication), high sucrose and low nitrogen conditions have previously been used to isolate *carbohydrate insensitive* (*cai*) mutants (Boxall, 1999). In parallel with the work described in this thesis I was involved, along with Dr. Farid Regad and Dr. Thomas Martin, in the isolation of transposon tagged *cai* mutants from a population of *Arabidopsis thaliana* var. Col0 plants (2200 lines), carrying the autonomous EN transposon of maize (Wisman et al., 1998), in two rounds of screening (data not shown). These mutants were labelled *cai* EN (*cen*) and two mutant lines were selected for further analysis (*cen* 2008 and *cen* 1427). After crossing with the wt to reduce the transposon number, the *cen* x Col0 were re-tested for their cai phenotype and the transposon number in plants with cai phenotype assessed by Southern Blotting (data not shown). Attempts to identify the mutations responsible for the *cai* phenotype and to assess the impact on the interaction between sugar and plastid redox signalling could not be completed during the period of this thesis project. However, significant results both for the research on sugar sensing and the interaction between plastid redox and sugar sensing can be expected from work with these mutants in the future.

Far less is known about signalling between organelles and the nucleus in plants compared to animals. For example, in animal cells heteromeric transcription factors regulating the expression of key components of mitochondrial electron transport in the nucleus are known to be subject to redox regulation by thiol groups (Martin et al., 1996). However, this example highlights an additional complexity in plant cells. In

response to extracellular sugars plant cells increase the flux through the mitochondrial electron transport, but decrease photosynthetic electron transport. They therefore have to co-ordinate nuclear encoded components for both types of organelle. Unicellular algae should provide some clues. However, since a plastid redox signal has been reported to evoke a systemic response in Arabidopsis (Karpinski et al., 1999) the situation in higher plants is expected to be more sophisticated than in unicellular algae. Taking the adaptivity and fluidity of plant metabolism into account a much more complex situation for signalling from the organelles to the nucleus can be expected in plants, with a network of signalling pathways involved in a complex web of crosstalk. This thesis project has provided a first glimpse of knowledge about these processes.

APENDIX A: Statistical analysis of standardised mRNA blot data

The output of the ANOVA analysis by the Minitab software is shown below:

Worksheet size: 100000 cells

MTB > Oneway 'cab' 'code';
SUBC> Tukey 5.

One-Way Analysis of Variance

Analysis of Variance on cab

Source	DF	SS	MS	F	p
code	3	21.6190	7.2063	81.98	0.000
Error	20	1.7582	0.0879		
Total	23	23.3772			

Individual 95% CIs For Mean
Based on Pooled StDev

Level	N	Mean	StDev	-+---------+---------+---------+-----
1	6	3.3450	0.4163	(--*--)
2	6	0.9767	0.2379	(--*--)
3	6	1.5033	0.3290	(--*--)
4	6	1.1167	0.1162	(--*--)

Pooled StDev = 0.2965 -+---------+---------+---------+----- 0.80 1.60 2.40 3.20

Tukey's pairwise comparisons

Family error rate = 0.0500
Individual error rate = 0.0111

Critical value = 3.96

Intervals for (column level mean) - (row level mean)

	1	2	3
2	1.8890 2.8477		
3	1.3623 2.3210	-1.0060 -0.0473	
4	1.7490 2.7077	-0.6193 0.3393	-0.0927 0.8660

```
MTB > Oneway 'cab' 'code';
SUBC>  Tukey 1.

One-Way Analysis of Variance

Analysis of Variance on cab
Source   DF      SS      MS      F      p
code      3  21.6190  7.2063  81.98  0.000
Error    20   1.7582  0.0879
Total    23  23.3772
                        Individual 95% CIs For Mean
                        Based on Pooled StDev
Level    N    Mean   StDev -+---------+---------+---------+-----
   1     6  3.3450  0.4163                      (--*--)
   2     6  0.9767  0.2379 (--*--)
   3     6  1.5033  0.3290       (--*--)
   4     6  1.1167  0.1162  (--*--)
                        -+---------+---------+---------+-----
Pooled StDev =  0.2965        0.80      1.60      2.40      3.20

Tukey's pairwise comparisons

   Family error rate = 0.0100
Individual error rate = 0.00202

Critical value = 5.02

Intervals for (column level mean) - (row level mean)

          1       2       3

   2   1.7607
       2.9760

   3   1.2340  -1.1343
       2.4493   0.0810

   4   1.6207  -0.7476  -0.2210
       2.8360   0.4676   0.9943


MTB > Oneway 'rbcS' 'code';
SUBC>  Tukey 5.

One-Way Analysis of Variance

Analysis of Variance on rbcS
Source   DF      SS      MS      F      p
code      3  232.49   77.50   22.96  0.000
```

```
Error     20    67.50     3.38
Total     23   300.00
                          Individual 95% CIs For Mean
                          Based on Pooled StDev
Level     N     Mean    StDev ---------+---------+---------+-------
   1      6   17.805    2.089                    (----*---)
   2      6   12.663    2.142          (---*----)
   3      6    9.067    0.965 (----*---)
   4      6   13.657    1.902            (---*---)
                          ---------+---------+---------+-------
Pooled StDev =    1.837              10.5      14.0      17.5
```

Tukey's pairwise comparisons

```
   Family error rate = 0.0500
Individual error rate = 0.0111
```

Critical value = 3.96

Intervals for (column level mean) - (row level mean)

```
          1       2       3

   2    2.172
        8.112

   3    5.768   0.627
       11.708   6.567

   4    1.178  -3.963  -7.560
        7.118   1.977  -1.620
```

```
MTB > Oneway 'rbcS' 'code';
SUBC>  Tukey 1.
```

One-Way Analysis of Variance

Analysis of Variance on rbcS

```
Source    DF      SS      MS      F      p
code       3  232.49   77.50   22.96  0.000
Error     20   67.50    3.38
Total     23  300.00
                          Individual 95% CIs For Mean
                          Based on Pooled StDev
Level     N     Mean    StDev ---------+---------+---------+-------
   1      6   17.805    2.089                    (----*---)
   2      6   12.663    2.142          (---*----)
   3      6    9.067    0.965 (----*---)
   4      6   13.657    1.902            (---*---)
```

```
                        ---------+---------+---------+-------
Pooled StDev =   1.837             10.5      14.0      17.5
```

Tukey's pairwise comparisons

```
   Family error rate = 0.0100
Individual error rate = 0.00202

Critical value = 5.02
```

Intervals for (column level mean) - (row level mean)

```
          1       2       3

   2   1.377
       8.907

   3   4.973  -0.168
      12.503   7.362

   4   0.383  -4.758  -8.355
       7.913   2.772  -0.825
```

```
MTB > Oneway 'chS' 'code';
SUBC>  Tukey 5.

One-Way Analysis of Variance

Analysis of Variance on chS
Source   DF      SS      MS      F      p
code      3  0.0272  0.0091   0.56  0.647
Error    20  0.3237  0.0162
Total    23  0.3509
                          Individual 95% CIs For Mean
                          Based on Pooled StDev
Level    N    Mean   StDev -+---------+---------+---------+-----
   1     6  0.5900  0.1321        (----------*----------)
   2     6  0.5683  0.1574        (----------*----------)
   3     6  0.5733  0.1253        (----------*----------)
   4     6  0.5017  0.0826  (----------*----------)
                          -+---------+---------+---------+-----
Pooled StDev =   0.1272         0.40      0.50      0.60      0.70
```

Tukey's pairwise comparisons

```
   Family error rate = 0.0500
Individual error rate = 0.0111
```

Critical value = 3.96

Intervals for (column level mean) - (row level mean)

	1	2	3
2	-0.1840		
	0.2273		
3	-0.1890	-0.2107	
	0.2223	0.2007	
4	-0.1173	-0.1390	-0.1340
	0.2940	0.2723	0.2773

```
MTB > Oneway 'nr' 'code';
SUBC>  Tukey 5.

One-Way Analysis of Variance

Analysis of Variance on nr
Source   DF      SS      MS      F      p
code      3  0.7846  0.2615  12.18  0.000
Error    20  0.4293  0.0215
Total    23  1.2139
                          Individual 95% CIs For Mean
                          Based on Pooled StDev
Level    N    Mean   StDev ---------+---------+---------+-------
   1     6  0.3500  0.1381  (-----*------)
   2     6  0.7917  0.1564                  (------*-----)
   3     6  0.4167  0.1726     (-----*-----)
   4     6  0.6717  0.1120               (------*-----)
                          ---------+---------+---------+-------
Pooled StDev =  0.1465             0.40      0.60      0.80

Tukey's pairwise comparisons

   Family error rate = 0.0500
Individual error rate = 0.0111

Critical value = 3.96

Intervals for (column level mean) - (row level mean)
```

	1	2	3
2	-0.6785		
	-0.2048		

```
   3  -0.3035   0.1381
      0.1702   0.6119

   4  -0.5585  -0.1169  -0.4919
     -0.0848   0.3569  -0.0181


MTB > Oneway 'nr' 'code';
SUBC>  Tukey 1.

One-Way Analysis of Variance

Analysis of Variance on nr
Source   DF      SS      MS      F      p
code      3  0.7846  0.2615  12.18  0.000
Error    20  0.4293  0.0215
Total    23  1.2139
                          Individual 95% CIs For Mean
                          Based on Pooled StDev
 Level    N    Mean   StDev ---------+---------+---------+-------
   1      6  0.3500  0.1381 (-----*------)
   2      6  0.7917  0.1564                (------*-----)
   3      6  0.4167  0.1726    (-----*-----)
   4      6  0.6717  0.1120              (------*-----)
                          ---------+---------+---------+-------
Pooled StDev =  0.1465             0.40      0.60      0.80

Tukey's pairwise comparisons

   Family error rate = 0.0100
Individual error rate = 0.00202

Critical value = 5.02

Intervals for (column level mean) - (row level mean)

          1       2       3

   2  -0.7419
      -0.1414

   3  -0.3669   0.0747
      0.2336   0.6753

   4  -0.6219  -0.1803  -0.5553
     -0.0214   0.4203   0.0453


MTB > Erase C1-C1000
MTB > Erase K1-K1000
```

```
MTB > Erase M1-M100
MTB > Let K998 = '*'
MTB > Let K999 = 2.7182818
MTB > Let K1000 = 3.14159265
MTB > Oneway 'cab' 'code';
SUBC>   Tukey 5.

One-Way Analysis of Variance

Analysis of Variance on cab
Source   DF      SS      MS      F      p
code      3  10.136   3.379  14.50  0.000
Error    20   4.661   0.233
Total    23  14.797
                          Individual 95% CIs For Mean
                          Based on Pooled StDev
Level    N    Mean   StDev ----------+---------+---------+------
   1     6  1.8733  0.7205                (-----*-----)
   2     6  0.4717  0.0578  (-----*-----)
   3     6  1.8750  0.6321                (-----*-----)
   4     6  0.6967  0.1003     (-----*-----)
                          ----------+---------+---------+------
Pooled StDev =  0.4827              0.70      1.40      2.10

Tukey's pairwise comparisons

   Family error rate = 0.0500
Individual error rate = 0.0111

Critical value = 3.96

Intervals for (column level mean) - (row level mean)

          1       2       3

   2   0.6213
       2.1821

   3  -0.7821  -2.1837
       0.7787  -0.6229

   4   0.3963  -1.0054   0.3979
       1.9571   0.5554   1.9587


MTB > Oneway 'cab' 'code';
SUBC>   Tukey 1.

One-Way Analysis of Variance
```

Analysis of Variance on cab

Source	DF	SS	MS	F	p
code	3	10.136	3.379	14.50	0.000
Error	20	4.661	0.233		
Total	23	14.797			

Individual 95% CIs For Mean
Based on Pooled StDev

Level	N	Mean	StDev	----------+---------+---------+------
1	6	1.8733	0.7205	(-----*-----)
2	6	0.4717	0.0578	(-----*-----)
3	6	1.8750	0.6321	(-----*-----)
4	6	0.6967	0.1003	(-----*-----)

Pooled StDev = 0.4827 ----------+---------+---------+------
0.70 1.40 2.10

Tukey's pairwise comparisons

Family error rate = 0.0100
Individual error rate = 0.00202

Critical value = 5.02

Intervals for (column level mean) - (row level mean)

	1	2	3
2	0.4124 2.3910		
3	-0.9910 0.9876	-2.3926 -0.4140	
4	0.1874 2.1660	-1.2143 0.7643	0.1890 2.1676

MTB > Oneway 'rbcS' 'code';
SUBC> Tukey 5.

One-Way Analysis of Variance

Analysis of Variance on rbcS

Source	DF	SS	MS	F	p
code	3	465.0	155.0	10.08	0.000
Error	20	307.6	15.4		
Total	23	772.6			

Individual 95% CIs For Mean
Based on Pooled StDev

Level	N	Mean	StDev	----------+---------+---------+------
1	6	13.817	5.947	(------*-----)

```
   2    6    3.798    1.555  (------*-----)
   3    6   12.868    4.605                (------*-----)
   4    6    5.495    1.591     (------*------)
                      ----------+---------+---------+------
Pooled StDev =    3.922                5.0      10.0      15.0

Tukey's pairwise comparisons

   Family error rate = 0.0500
Individual error rate = 0.0111

Critical value = 3.96

Intervals for (column level mean) - (row level mean)

          1       2       3

   2    3.678
       16.358

   3   -5.392  -15.410
        7.288   -2.730

   4    1.982   -8.037    1.033
       14.662    4.643   13.713


MTB > Oneway 'rbcS' 'code';
SUBC>  Tukey 1.

One-Way Analysis of Variance

Analysis of Variance on rbcS
Source    DF      SS      MS      F      p
code       3   465.0   155.0   10.08  0.000
Error     20   307.6    15.4
Total     23   772.6
                        Individual 95% CIs For Mean
                        Based on Pooled StDev
 Level     N    Mean    StDev ----------+---------+---------+------
   1       6  13.817    5.947                (------*-----)
   2       6   3.798    1.555  (------*-----)
   3       6  12.868    4.605                (------*-----)
   4       6   5.495    1.591     (------*------)
                        ----------+---------+---------+------
Pooled StDev =    3.922                5.0      10.0      15.0

Tukey's pairwise comparisons

   Family error rate = 0.0100
```

Individual error rate = 0.00202

Critical value = 5.02

Intervals for (column level mean) - (row level mean)

	1	2	3
2	1.981 18.055		
3	-7.089 8.985	-17.107 -1.033	
4	0.285 16.359	-9.734 6.340	-0.664 15.410

```
MTB > Oneway 'chS' 'code';
SUBC>  Tukey 5.
```

One-Way Analysis of Variance

Analysis of Variance on chS

Source	DF	SS	MS	F	p
code	3	0.03818	0.01273	2.45	0.094
Error	20	0.10398	0.00520		
Total	23	0.14216			

```
                          Individual 95% CIs For Mean
                          Based on Pooled StDev
Level     N    Mean   StDev --------+---------+---------+--------
   1      6  0.60167  0.08773             (--------*--------)
   2      6  0.55833  0.04834          (--------*--------)
   3      6  0.49833  0.07782  (--------*--------)
   4      6  0.51667  0.06861     (--------*--------)
                     --------+---------+---------+--------
Pooled StDev =  0.07211              0.490     0.560     0.630
```

Tukey's pairwise comparisons

Family error rate = 0.0500
Individual error rate = 0.0111

Critical value = 3.96

Intervals for (column level mean) - (row level mean)

	1	2	3
2	-0.07324		

```
      0.15990

   3 -0.01324 -0.05657
     0.21990  0.17657

   4 -0.03157 -0.07490 -0.13490
     0.20157  0.15824  0.09824


MTB > Oneway 'nr' 'code';
SUBC>  Tukey 5.

One-Way Analysis of Variance

Analysis of Variance on nr
Source   DF      SS      MS      F      p
code      3  1.6439  0.5480  11.45  0.000
Error    20  0.9570  0.0479
Total    23  2.6009
                          Individual 95% CIs For Mean
                          Based on Pooled StDev
Level    N    Mean   StDev --------+---------+---------+--------
  1      6  0.3667  0.2255     (-----*-----)
  2      6  0.8933  0.3248                (-----*-----)
  3      6  0.2667  0.0937  (-----*-----)
  4      6  0.7600  0.1620            (-----*------)
                          --------+---------+---------+--------
Pooled StDev =  0.2187            0.30      0.60      0.90

Tukey's pairwise comparisons

   Family error rate = 0.0500
Individual error rate = 0.0111

Critical value = 3.96

Intervals for (column level mean) - (row level mean)

           1       2       3

   2  -0.8803
      -0.1730

   3  -0.2536   0.2730
       0.4536   0.9803

   4  -0.7470  -0.2203  -0.8470
      -0.0397   0.4870  -0.1397
```

```
MTB > Oneway 'nr' 'code';
SUBC>  Tukey 1.

One-Way Analysis of Variance

Analysis of Variance on nr
Source   DF      SS      MS      F      p
code      3   1.6439  0.5480  11.45  0.000
Error    20   0.9570  0.0479
Total    23   2.6009
                          Individual 95% CIs For Mean
                          Based on Pooled StDev
Level    N    Mean   StDev --------+---------+---------+--------
   1     6  0.3667  0.2255    (-----*-----)
   2     6  0.8933  0.3248                (-----*-----)
   3     6  0.2667  0.0937  (-----*-----)
   4     6  0.7600  0.1620            (-----*------)
                          --------+---------+---------+--------
Pooled StDev =  0.2187            0.30      0.60      0.90

Tukey's pairwise comparisons

   Family error rate = 0.0100
Individual error rate = 0.00202

Critical value = 5.02

Intervals for (column level mean) - (row level mean)

          1       2       3

   2  -0.9750
      -0.0784

   3  -0.3483   0.1784
       0.5483   1.0750

   4  -0.8416  -0.3150  -0.9416
       0.0550   0.5816  -0.0450
```

APENDIX B: Statistical analysis chlorophyll levels in response to starvation of cell cultures

This analysis was performed by Dr. Peter Dominy:

```
Worksheet size: 100000 cells

MTB > Retrieve  'C:\SHARE\OLIVER.MTW'.
Retrieving worksheet from file: C:\SHARE\OLIVER.MTW
Worksheet was saved on  4/27/2000
MTB > Oneway 'Data' 'StvCode';
SUBC>   Tukey 5.

ANALYSIS OF VARIANCE ON Data
SOURCE     DF         SS         MS         F         p
StvCode     9     2255.6      250.6      6.72     0.000
ERROR      20      745.5       37.3
TOTAL      29     3001.1

                                        INDIVIDUAL 95% CI'S FOR MEAN
                                        BASED ON POOLED STDEV
 LEVEL      N       MEAN      STDEV  --------+---------+---------+------
--
     0      3     45.250      6.602            (-----*-----)
     1      3     40.550      1.519       (-----*-----)
     2      3     53.050      3.592                 (-----*-----)
     4      3     59.167      4.696                      (-----*-----)
     8      3     58.073      3.306                    (-----*------)
    12      3     58.920      4.150                     (-----*-----)
    24      3     57.277      3.181                    (-----*-----)
    48      3     55.203      6.716                  (-----*-----)
   486      3     40.557     12.138       (-----*-----)
  4812      3     34.720      7.823  (-----*-----)
                                        --------+---------+---------+------
--
POOLED STDEV =    6.105                      36        48        60

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.00204

Critical value = 5.01
```

Intervals for (column level mean) - (row level mean)

	0	1	2	4	8	12	24	48	486
1	-12.96 22.36								
2	-25.46 9.86	-30.16 5.16							
4	-31.58 3.74	-36.28 -0.96	-23.78 11.54						
8	-30.48 4.84	-35.18 0.14	-22.68 12.64	-16.57 18.75					
12	-31.33 3.99	-36.03 -0.71	-23.53 11.79	-17.41 17.91	-18.51 16.81				
24	-29.69 5.63	-34.39 0.93	-21.89 13.43	-15.77 19.55	-16.86 18.46	-16.02 19.30			
48	-27.61 7.71	-32.31 3.01	-19.81 15.51	-13.70 21.62	-14.79 20.53	-13.94 21.38	-15.59 19.73		
486	-12.97 22.35	-17.67 17.65	-5.17 30.15	0.95 36.27	-0.14 35.18	0.70 36.02	-0.94 34.38	-3.01 32.31	
4812	-7.13 28.19	-11.83 23.49	0.67 35.99	6.79 42.11	5.69 41.01	6.54 41.86	4.90 40.22	2.82 38.14	-11.82 23.50

MTB >

The output from Minitab is not particularly intuitive. The 'ANALYSIS OF VARIANCE ON Data' table reports a probability (p) of 0.000 (this means < 0.001 of course) and tells you that there is a significant difference between at least one pair of averages at the probablility of <0.001 (>99.9% certainty.) What ANOVA does NOT tell you is which pair(s) are different. From the table presented just under the ANOVA table it should be clear to you that the largest (4 hours) and smallest (48 hours re-fed for 12) are generating a significant difference, but what about the others, for instance, are 2 hours and 4 hours different?

To determine this you need to conduct another test (here I've used TUKEY's). Now, the TUKEY's output from Minitab is not very helpful in my opinion and I wouldn't worry too much about it. What I have done is construct a 'Tables of Orthoganol Contrasts' in your original Excel spreadsheet, and pasted the important table below.

This is the important table of 'orthoganol contrasts'. The critical value from Minitab (5.01) is calulated from the 'pooled standard deviations' and for the given level of probability (here p= 0.05) represents the minimum difference between two averages that must be exceeded before a significant difference can be inferred. It is constructed by ranking in a table (from highest to lowest) the averages. So, from the table below, the '4 hours starvation' sample is significantly different (*) from the following samples only: 2 hour, Pre wash, 48 hour re-fed for 6 hours, Post Wash and 48 hours re-fed for 12 hours. Similarly, 48 hours is NOT significantly different from 4 hours, 12 hours, 8 hours, 24 hours (from the 48 hour row) and 2 hours (from the 48 hour column).

			4 Hours	12 Hours	8 Hours	24 Hours	48 Hours	2 Hours	Pre Wash	48 Hours / 6 Re-Fed	Post Wash	48 Hours / 12 Re-Fed
			4	12	8	24	48	2	0	486	1	4812
			59.17	58.92	58.07	57.27	55.20	53.05	45.25	40.56	40.55	32.83
4 Hours	4	59.17	-									
12 Hours	12	58.92	-	-								
8 Hours	8	58.07	-	-	-							
24 Hours	24	57.27	-	-	-	-						
48 Hours	48	55.20	-	-	-	-	-					
2 Hours	2	53.05	*	*	*	-	-	-				
Pre Wash	0	45.25	*	*	*	*	*	*	-			
48 Hours / 6 Re-Fed	486	40.56	*	*	*	*	*	*	-	-		
Post Wash	1	40.55	*	*	*	*	*	*	-	-	-	
48 Hours / 12 Re-Fed	4812	32.83	*	*	*	*	*	*	*	*	*	-

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