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Defective ABA-mediated sugar signalling pathway in an established Arabidopsis thaliana cell suspension culture explains its stay-green phenotype at high sugar concentrations

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Abstract

An unusual sugar insensitive phenotype was identified in an established cell suspension culture of *Arabidopsis thaliana*. We characterized the physiology, biochemistry and genetics of the sugar insensitive cell culture, in order to identify factors contributing to the phenotype. Chlorophyll levels of the cell suspension culture were insensitive to high sucrose (6-15% w/v) and maintained a green phenotype. Immunoblotting indicated that levels of key photosynthetic proteins (PsaA, Lhcb2 and Rubisco) increased as a function of external sucrose concentration. The green cell culture was photosynthetically competent based on light-dependent, CO_2-saturated rates of O_2 evolution as well as Fv/Fm and P700 oxidation. Transcript profiling indicated that key sugar signalling transcripts *ABI3* and *ABI4* were not detectable in the cell suspension culture, and this was confirmed by qPCR. Because the transcription factors *ABI3* and *ABI4* are essential for WT sugar signalling, the lack of these transcripts resulted in a sugar insensitive phenotype that mimicked the phenotype of *abi3* and *abi4* mutant seedlings. Addition of ABA to the external medium failed to rescue the suspension cells from its sucrose insensitivity. We sequenced the genome at over 300-fold coverage, and identified three hundred thousand genetic variants unique to the cell culture, not present in the genomes of twenty-seven *Arabidopsis thaliana* ecotypes with WT sugar sensitivity. Despite widespread genetic changes in the genome, there were no variations in the promoter regions or regulatory regions of *ABI3* or *ABI4* that could explain the lack of expression of these transcription factors. We conclude that the genetic change has impacted an unknown regulator of *ABI3* or *ABI4* impairing their expression resulting in a sugar insensitive phenotype. Because of the genetic variation that has occurred, and because of the unusual sugar and photosynthetic responses, caution must be exercised in the interpretation of physiological and biochemical data obtained from experimental use of this culture in any comparison with wild-type *Arabidopsis* seedlings.

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Kisiala A. completed the GC/MS analysis of plant hormones

Kurepin L. guided analysis of plant hormones

Maxwell D.P. guided and reviewed research

Grbic V. guided and reviewed research, and provided critical comments on the manuscript

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Table of Contents

Abstract i

Acknowledgments iv

Table of Contents v

List of Figures xi

List of Abbreviations xiv

List of Appendices xviii

Chapter 1 1

1 General Introduction 1

1.1 Photosynthesis 1

1.1.1 The chloroplast 3

1.1.2 The light reactions of photosynthesis 4

1.1.3 The Calvin-Benson-Bassham cycle 7

1.1.4 Chlorophyll biosynthesis 9

1.1.5 The biosynthesis of sucrose and starch in photosynthetic tissue 11

1.1.6 The degradation of sucrose and starch 14

1.1.7 Sugar transport and source-sink relationships 15

1.1.8 *Arabidopsis thaliana* 18

1.1.9 Plant cell suspension cultures 18

1.1.10 Isolation and maintenance of cell suspension cultures 19

1.1.11 Phenotypes of cell suspension cultures 21

1.1.12 Somaclonal variation 22

1.2 End product inhibition of photosynthesis by sugar 22

1.2.1 The metabolic cost of photosynthesis 23

1.3 Sugar signalling pathways in plants that regulate photosynthetic gene expression 25

1.3.1 Sugar insensitive mutants 25
1.3.2 The SnRK1 sugar signalling pathway 26
1.3.3 Sucrose induced repression of translation 26
1.3.4 The hexokinase sugar signalling pathways 29
1.3.5 The ABA sugar signalling pathway 33
1.3.6 Trehalose metabolism and signalling 37
1.4 Other sugar insensitive mutants 39
1.5 Thesis objectives 40
1.6 References 41
1.7 Supplementary Tables 55
Chapter 2 56
2 An established *Arabidopsis thaliana* var. Landsberg *erecta* cell suspension culture accumulates chlorophyll and exhibits a stay-green phenotype in response to high external sucrose concentrations 56
2.1 Introduction 57
2.2 Methods 59
2.2.1 Growth of the cell suspension cultures and seedlings 59
2.2.2 Transmission electron microscopy 60
2.2.3 Confocal microscopy 60
2.2.4 Gas exchange 61
2.2.5 Chlorophyll a fluorescence 61
2.2.6 Redox state of P700 62
2.2.7 77K chlorophyll fluorescence emission spectra 62
2.2.8 Transcript abundance from RNA-Seq 62
2.2.9 Immunoblotting 63
2.2.10 HPLC analysis of pigments 64
2.2.11 Enzymatic assay of sugar content 65

2.3 Results 65

2.3.1 The effects of sucrose concentration and light on cell phenotype 65

2.3.2 The effects of sucrose concentration on cell and chloroplast ultrastructure 67

2.3.3 The effects of carbon source, sugar concentration, and light on exponential growth rates 69

2.3.4 Sucrose utilization 71

2.3.5 The effects of sucrose concentration on the structure and function of the photosynthetic apparatus 71

2.3.6 The effects of sucrose concentration on transcript abundance of genes associated with the Chl a biosynthesis 80

2.3.7 The effects of sucrose concentration on gene expression associated with thylakoid protein complexes 81

2.4 Discussion 83

2.5 References 87

2.6 Supplementary Tables 87

2.7 Supplementary Figures 91

Chapter 3 92

3 Transcriptome profiling indicates that sugar-insensitive phenotype of a cell suspension culture generated from wild-type *Arabidopsis* var. Landsberg *erecta* is due to the absence of *ABI3* and *ABI4* transcripts which impairs the ABA-mediated sugar signalling pathway 92

3.1 Introduction 92

3.2 Methods 95

3.2.1 Growth conditions 95

3.2.2 Sugar and starch assays 96

3.2.3 ABA and cytokinin assays 97

3.2.4 RNA-Seq transcript profiling 98

3.2.5 Meta-analyses 99

3.3 Results 99

3.3.1 Sugar and starch accumulation 99

3.3.2 Global transcriptional response of the cell suspension culture to sucrose 102

3.3.3 Sucrose metabolism genes 104

3.3.4 Sugar signalling pathways 109

3.3.5 ABA content of the cell suspension cultures 113

3.3.6 Cytokinin content of the cell suspension culture 116

3.4 Discussion 119

3.5 References 124

3.6 Supplementary Tables 126

Chapter 4 128

4 The genome of an established *Arabidopsis thaliana* cell suspension culture reveals widespread genetic change 128

4.1 Introduction 128

4.1.1 WT *Arabidopsis* genome 128

4.2 Methods 129

4.2.1 Growth of the cell suspension cultures and seedlings 129

4.2.2 Whole Genome Sequencing 130

4.2.3 Bioinformatics 130

4.3 Results 131

4.3.1 Genetic variability in key sugar signalling genes 131

4.3.2 Genetic variability in ABA biosynthesis genes 137

4.3.3 Evaluation of Candidate genes identified by RNA-Seq: Genetic variation in genes strongly upregulated by sugar in WT *Arabidopsis* which were not expressed in the cell suspension culture 139

4.3.4 Genetic variability in the cell suspension culture 141

4.4 Discussion 141

4.5 References 143

4.6 Supplementary Tables 143
4.7 Appendices 147
Chapter 5 148
5 Summary and Perspectives 148
5.1 References 151
Curriculum Vitae 152

List of Tables

Table 1.1. Mutants of the three key sugar signalling pathways that have a sugar insensitive phenotypes. 27

Table 1.2 Mutants and transgenic seedlings with sugar insensitive phenotypes. 28

Table 2.1 Rate of sucrose uptake from the medium by the *Arabidopsis* cell suspension cultures during exponential growth in batch culture..... 72

Table 2.2 The effect of sucrose concentration on the maximum photochemical efficiency of PSII, the functional activity of PSI, the respiration rate, and the light-saturated rate of photosynthesis..... 75

Table 2.3. PSI:PSII ratios of the *Arabidopsis* cell suspension culture determined by 77K chlorophyll fluorescence emission spectra. 76

Table 4.1 Genetic variation in genes required for WT sugar signalling..... 134

Table 4.2 Genetic variations in *NCED9*, the only gene in the ABA biosynthesis pathway with a high impact variation..... 137

Table 4.3 Genetic variation in the promoter region of genes which were not expressed in the cell suspension culture, but which were strongly upregulated by sugar in WT *Arabidopsis*. 140

List of Figures

Figure 1.1 The light reactions of photosynthesis and the Calvin-Benson-Bassham cycle. 2

Figure 1.2 Components of the linear light-dependent reactions of photosynthesis. 5

Figure 1.3 The Calvin-Benson-Bassham cycle. 8

Figure 1.4 The chlorophyll biosynthesis pathway in plants. 10

Figure 1.5 *Arabidopsis thaliana* var. Col-0 and Ler seedlings have a non-green phenotype of at 6% (w/v) glucose, and green phenotype at 6% (w/v) mannitol. 12

Figure 1.6 Key steps of the starch and sucrose biosynthetic pathways in illuminated photosynthetic tissue. 13

Figure 1.7 Sugar transport from source to sink in *Arabidopsis thaliana*. 17

Figure 1.8 *Arabidopsis* and tobacco cell suspension cultures (Left image source: Barkla et al. 2014; Right image source: Mayo, K. J. et al., 2006) 20

Figure 1.9 The SnRK1 sugar signalling pathway. 30

Figure 1.10 Sucrose induced repression of translation (SIRT). 31

Figure 1.11 Hexokinase-dependent sugar signalling in *Arabidopsis*..... 32

Figure 1.12 The ABA sugar signalling pathways. 35

Figure 1.13 Trehalose metabolism in plants. The reactions are catalyzed by the enzymes trehalose-6-phosphate synthase (TPS), trehalose phosphate phosphatase (TPP), and Trehalase (TRE). Trehalose-6-phosphate is a key sugar signalling molecule in plants. 38

Figure 2.1 Phenotypes of the *Arabidopsis thaliana* var. Landsberg *erecta* cell suspension culture contrasted with the phenotypes of Landsberg *erecta* (Ler-0) seedling leaves in response to sucrose. 66

Figure 2.2 Microscope images of the *Arabidopsis* cell suspension culture. 68

Figure 2.3 Specific growth rates of the *Arabidopsis* cell suspension culture in response to (A) Sucrose concentration, (B) Mannitol-induced osmotic stress, (C) Sugar type, (D) CO_2 supplementation, and (E) Light. 70

Figure 2.4 Immunoblots for polypeptides from the major photosynthetic proteins: D1, A core subunit of PSII, PsaA, A core subunit of photosystem I polypeptide, Lhcb2, A chloroplhyll-a/b binding proteins of PSII, and RbcL, the large subunit of Rubisco. 77

Figure 2.5 The effects of sucrose concentration on the photosynthetic response of cell suspension cultures. 79

Figure 2.6 Heat map illustrating the effect of sucrose and mannitol on transcript abundance of (A) Chl a biosynthesis genes, (B) genes encoding subunits of the light-dependent reactions associated with PSII and PSI, (C) genes encoding components of electron transport including the Cyt b6f complex and PSI acceptor-side electron carriers and (D) subunits of the ATP synthase complex. 82

Figure 3.1 Summary of the major sugar signalling pathways in plants that repress photosynthetic gene expression. 94

Figure 3.2. Intracellular (A-C) sugar content and (D) starch content of *Arabidopsis* cell suspension cultures and WT seedlings leaves grown at 0-9% (w/v) sucrose. 100

Figure 3.3 Transcript profiling of the sugar insensitive *Arabidopsis* cell suspension culture grown at 0%, 3%, 6%, 9% (w/v) sucrose and at 9% (w/v) mannitol (n=3). 103

Figure 3.4 Transcript profile heatmaps of (A-C) sugar transport genes and (D-E) trehalose-6-phosphate metabolism genes of the *Arabidopsis* cell suspension culture 106

Figure 3.5 (A) Expression heatmaps of the genes required for WT sugar responses in *Arabidopsis*. 111

Figure 3.6 Meta-analysis examining the sugar response of genes regulated by SnRK1 (KIN10) and ABI4. 112

Figure 3.7 ABA content, the sugar response of ABA metabolism genes, and the effects of exogenous ABA treatment on phenotype. 115

Figure 3.8 Cytokinin content and the sugar response of cytokinin metabolism genes..... 118

Figure 3.9 The response of key sugar signalling pathways in the cell suspension culture to high exogenous sucrose concentrations (≥6% w/v)..... 123

List of Abbreviations

1,3-BPG	1,3-Bisphosphoglycerate
1-qP	PSII excitation pressure
3-PGA	3-phosphoglyceric acid
ABA	Abscisic acid
ABA1	Gene encoding zeaxanthin epoxidase; *AT5G67030*
ABA2	Gene encoding aba deficient 2; *AT1G52340*
ABA3	Gene encoding aba deficient 3; AT1G16540
ABAR	ABA-BINDING PROTEIN
ABF2	Gene encoding abscisic acid responsive elements-binding factor 2; *AT1G45249*
ABI3	Gene encoding abscisic acid insensitive 3; *AT3G24650*
ABI4	Gene encoding glucose insensitive 4; *AT1G52340*
ABI5	Gene encoding abscisic acid insensitive 5; *AT2G36270*
ABI8	Gene encoding abscisic acid insensitive 8; *AT3G08550*
AF	Allele frequencies
AGPase	ADP-glucose pyrophosphorylase
ALA	5-aminolevulinic acid
ALT	Alternative allele (variation differing from reference)
ANOVA	Analysis of variance
ARIA	Gene encoding arm repeat protein interacting with ABF2; *AT5G19330*
arm	Armadillo
ATP	Adenosine triphosphate
ATP-δ	Thylakoid ATPase
BAMs	β-amylases
BSA	Bovine serum albumin
ca	Carbonic anhydrase
cab	Chlorophyll a/b binding protein
CEF	Cyclic electron flow
Chl a	Chlorophyll a
Chlide b	Chlorophyllide b
CO_2	Carbon dioxide
DBEs	Debranching enzymes
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DNA	Deoxyribonucleic acid
DP	Read depth
DV Pchlide	3,8-Divinyl protochlorophyllide a
ETR	Electron transport rate
F_M	Dark-adapted maximal fluorescence
F_M'	Light-adapted maximum fluorescence

F_O	Dark-adapted basal fluorescence
F_O'	Light-adapted minimum florescence
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
F_S	Steady-state fluorescence
FS	Fisher strand
FW	Fresh weight
G1P	Glucose-1-phosphate
G3P	Glyceraldehyde 3-phosphate
GA	Gibberellic acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GGPP	Geranylgeranyl-pyrophosphate
GIN1	Glucose insensitive 1; *Arabidopsis ABA2* mutant
GIN2	Glucose insensitive 2; *Arabidopsis HXK1* mutant
GIN5	Glucose insensitive 5; *Arabidopsis ABA3* mutant
GIN6	Glucose insensitive 6; *Arabidopsis ABI4 m*utant
GLU	Glutamate
H^+	Proton
HMB	Hydroxymethylbilane
HPLC	High-performance liquid chromatography
HUP2	19S regulatory particle of proteasome complexes, encoded by RPT5B
HXK	Hexokinase
HXK1	Gene encoding hexokinase; *AT4G29130*
INV	Invertase
Invertase	Beta-fructofuranosidase
KIN10	Gene encoding SNF1-related protein kinase; *AT3G01090*
LEF	Linear electron flow
LHCB	The light harvesting complex of photosystem II
MEL	C4 malic enzyme
MEP	Methylerythritol phosphate
Mg-proto	Mg-protoporphyrin IX
Mg-proto ME	Mg-protoporphyrin IX monomethyl ester
MQ	A measure of mapping quality
MS medium	Murashige and Skoog medium
MV Chlide a	Monovinyl chlrophyllide a
MV Pchlide	Monovinyl Protochlorophyllide
NAA	Naphthaleneacetic acid
NADH	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NPQ	Nonphotochemical quenching
O_2	Molecular oxygen

OEC	Oxygen evolving complex
$P680^+$	Oxidized reaction centre chlorophyll a of photosystem II
P700	Reaction centre chlorophyll a of photosystem I
PAM	Pulse amplitude fluorometry
PBG	Porphobilinogen
pc	Plastocyanin
PEPC	C4 phosphoenolpyruvate carboxylase
PET	Photosynthetic electron transport
PGK	Phosphoglycerate kinase
pmf	Proton motive force
POR	Protochlorophyllide oxidoreductase
PPDK	Pyruvate phosphodikinase
PQ	Plastoquinone
PRK	Phosphoribulokinase
Proto IX	Protoporphyrin IX
PSBA	D1 protein of PSII
PSI	Photosystem I
PT	Triose phosphate translocator
PWD	Phosphoglucan water dikianse
PYL	ABA receptor
QD	Quality by depth
qP	Photochemical quenching
R5P	Ribulose-5-phosphate
RBCS	Rubisco small subunit
RCA	Rubisco activase
RCAR	ABA receptor
REF	Reference sequence
RGS1	Gene encoding regulator of G-protein signalling 1; *AT3G26090*
RIN	RNA integrity number
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing; whole transcriptome shotgun sequencing
RPT5B	Gene encoding 19S regulatory particle of proteasome complexes; *AT1G09100*
SBE	Starch branching enzymes
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIRT	Sucrose-induced inhibition of translation
SNP	Single-nucleotide polymorphism
SnRK1	SNF1-related kinase 1
SPP	Sucrose-phosphate phosphatase
SPS	Sucrose-phosphate synthase

SS	Starch synthase
SuSy	Sucrose synthase
SWEET	Sugar transport proteins
T6P	Trehalose-6-phosphate
TAIR	The *Arabidopsis* Information Resource
TBS	Tris-buffered saline
TCA cycle	The tricarboxylic acid cycle; Krebs cycle
TPP	Trehalose phosphate phosphatase
TPS	Trehalose-6-phosphate synthase
TRIS	2-Amino-2-(hydroxymethyl)propane-1,3-diol
TSS	Transcriptional start site
UGPase	UDP-glucose pyrophosphorylase
UTR	Untranslated region
VAB1	Gene encoding a vacuolar H+-ATPase; *AT1G76030*
WRKY	WRKY transcription factor family
WT	Wild type
XE	Xenon flash lamp
Φ_{PSII}	Proportion of light energy absorbed by photosystem II used to drive photochemistry
Φf,D	Sum proportions of light energy absorbed by photosystem II dissipated as fluorescence and constitutive thermal dissipation

List of Appendices

Appendix 4.1. Genome sequencing quality statistics, ecotype identification, and analysis of genetic variants identified in the cell suspension culture genome. 147

Chapter 1

1 General Introduction

There is nothing more interesting in biology than when an organism behaves in a way that conflicts with our current understanding. The objective of this thesis is to examine an established *Arabidopsis thaliana* cell suspension culture that responds differently to sugar than the wild-type seedlings from which it was generated. This specific plant cell culture has been used as a model system in plant biology for over two decades, and is often assumed to be wild-type. However, the cells have an unusual response to sugar. When supplied with high concentrations of sugar exogenously, the cells have a dark green phenotype. This conflicts with what we know about sugar signalling in plants. In response to an abundant supply of sugar, plants normally downregulate photosynthesis and chlorophyll biosynthesis, resulting in a non-green phenotype. The primary goal of this thesis was to identify factors contributing to the unusual sugar insensitive phenotype of this plant cell suspension culture by examining sugar signalling, sugar transport, photosynthetic regulation, and genetic variation.

1.1 Photosynthesis

Plants are photoautotrophs that capture light energy to fix carbon dioxide into sugar molecules (Fig.1.1). Photosynthesis can be divided into two parts: (i) the light reactions of photosynthesis capture light energy and convert it to chemical energy, producing the energy-storage molecules ATP and NADPH, and (ii) the Calvin-Benson-Bassham cycle which is a series of biochemical reactions that utilize the energy of ATP and NADH to fix CO_2 into high-energy sugar molecules. The end-product sugar molecules fulfill many roles in plants: (A) sugars are high-energy molecules that can be catabolized to meet cellular energy demands, (B) sugars can be polymerized to starch to store excess energy, (C) sugars can be used to synthesize cellulose or hemicellulose which are structural components of cell walls, (D) sugars can be exported from source tissue to support sink tissue metabolism, and (E) sugars are signalling molecules that regulate the expression of myriad genes and the activity of many enzymes.

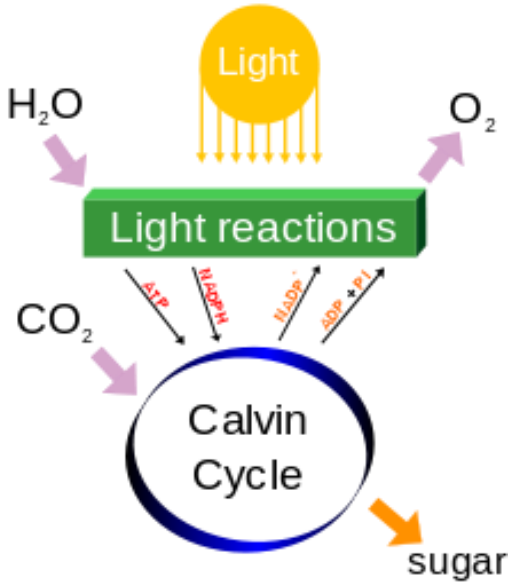


Figure 1.1 The light reactions of photosynthesis and the Calvin-Benson-Bassham cycle. Photosynthesis can be divided into two parts: (i) The light reactions of photosynthesis capture light energy and convert it to chemical energy, producing the energy storage molecules ATP and NADPH, and (ii) The Calvin-Benson-Bassham cycle is a series of biochemical reactions that utilize the energy from ATP and NADH to fix CO_2 into high-energy sugar molecules. (Mayer and Yerpo, 2008)

1.1.1 The chloroplast

The chloroplast, which hosts the process of photosynthesis, was acquired by eukaryotic cells over a billion years ago in an endosymbiotic event that provided plants and green algae with autotrophic capacity (Margulis, 1971). Coevolution between the photosynthetic symbiont and the plant cell resulted in the transfer of many genes from the plastid to the nucleus (Table 1.1) (Huang *et al.,* 2004). Therefore, the plastid genome encodes around 85 protein coding genes, and 95% of the proteins required for plastid function are encoded in the nucleus (Joyard *et al.,* 2009; Richly and Leister, 2004; Surpin and Chory, 1997; Zybailov *et al.,* 2008). These nuclear-encoded plastid proteins (NEPPs) are imported into the plastid post-translation (Li and Chiu, 2010). Metabolic pathways synthesizing starch, amino acids, fatty acids, vitamins, hormones, and other secondary metabolites also occur in the plastid (Gould *et al.,* 2008). The nucleus and chloroplast coordinate their activities through anterograde (nucleus to chloroplast), and retrograde (chloroplast to nucleus) regulatory pathways.

Chloroplasts have three membrane systems: the outer chloroplast membrane, the inner chloroplast membrane, and the thylakoid membrane system. The chloroplast membranes strictly regulate the transport of metabolites and ions and in and out of the chloroplast stroma through a variety of transport systems: porines, solute channels, ion-specific cation and anion channels and various active transport systems (Pottosin and Shabala, 2016). There is a thin intermembrane space of 10-20 nanometers between the inner and outer chloroplast membranes. (Joyard *et al.,* 1998). The chloroplast stroma is located inside the inner chloroplast membrane, and constitutes a large proportion of the chloroplast's total volume. The stroma contains chloroplast DNA, ribosomes, thylakoid membranes, starch granules, Rubisco, and other proteins and metabolites. The light reactions of photosynthesis are localized to the thylakoid membranes, which are suspended in the chloroplast stroma and are arranged in helical stacks called grana (Austin and Staehelin, 2011).

1.1.2 The light reactions of photosynthesis

The photosynthetic pigments chlorophyll and carotenoids, absorb photons, and this energy is used to either: (A) generate NADPH and ATP through a series of linear electron flow reactions (Hill and Bendall, 1960), or (B) generate ATP through cyclic electron transport (Endo *et al.*, 2004; Fork and Herbert, 1993; Heber and Walker, 1992). The linear electron flow reactions typically dominate, except when reactions are $NADP^+$-limited and cyclic electron transport occurs. The linear and cyclic electron flow reactions are mediated by the thylakoid protein complexes. The thylakoid electron transport complexes include: photosystem II, photosystem I, cytochrome b6f, and ATP synthase (Fig. 1.2). The thylakoid proteins – plastocyanin, ferredoxin, and ferredoxin-$NADP^+$ reductase – are also important components of thylakoid electron transport. The genes encoding these key photosynthetic proteins are listed in Table 1. Some of the light reaction protein subunits are encoded in the chloroplast, but the majority of them are encoded in the nucleus (Joyard *et al.*, 2009; Richly and Leister, 2004; Surpin and Chory, 1997; Zybailov *et al.*, 2008).

The net reactions of photosynthetic electron transport:

(A) Linear electron flow (LEF):

$$\text{Light} + 2\ NADP^+ + 3\ ADP + 3\ P_i + 2\ H_2O \rightarrow 2\ NADPH + 3\ ATP + 2\ H^+ + O_2$$

(B) Cyclic electron flow (CEF):

$$\text{Light} + ADP + Pi \rightarrow ATP$$

In LEF, the two photosystems – photosystem II (PSII) and photosystem I (PSI) – capture light energy, and this energy is used to (i) oxidize H_2O to form O_2 and H^+, (ii) pump H^+ ions from the stroma to the lumen, and (iii) to reduce $NADP^+$ to NADPH. The thermodynamics of LEF is typically described by the Z-scheme (Hill and Bendall, 1960). PSI absorbs light energy, and this energy photo-oxidizes the PSI reaction centre chlorophyll a, P700 ($P700 \rightarrow P700^+ + e^-$). The resulting electron is used to reduce ferredoxin, which in turn donates it to Ferredoxin-$NADP^+$ reductase, which then

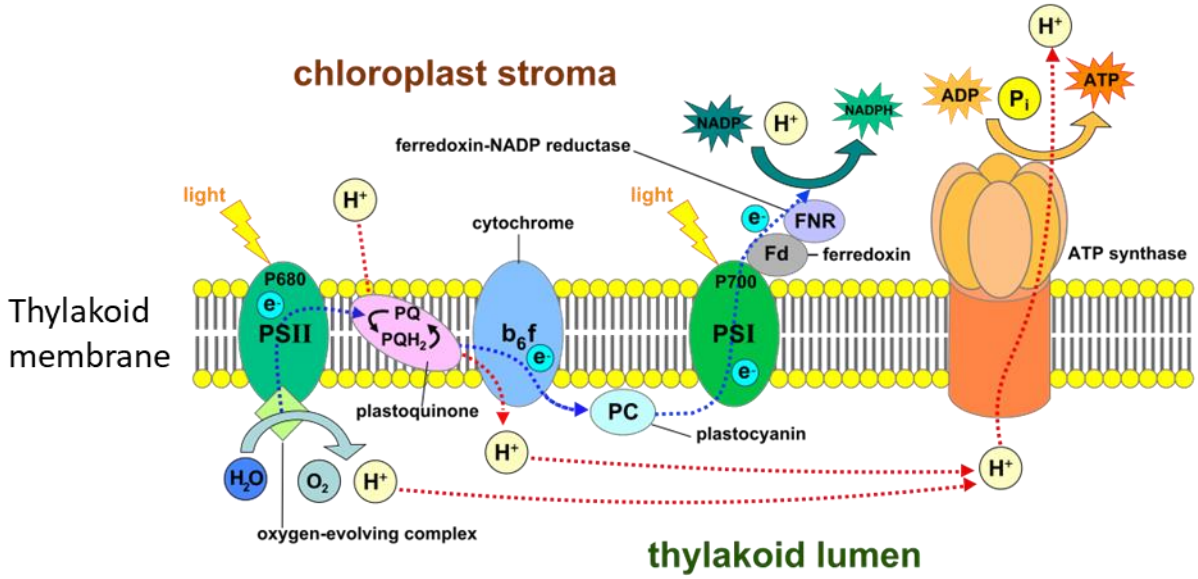


Figure 1.2 Components of the linear light-dependent reactions of photosynthesis. The light reactions take place across the thylakoid membranes in the chloroplast. The blue arrows show the flow of electrons through the electron transport chain from H_2O to $NADP^+$. (Somepics, 2015)

catalyzes the reduction of $NADP^+$ to NADPH. NADPH is then consumed in the reduction of CO_2 in the stroma. Concomitantly, light photo-oxidizes the reaction centre chlorophyll a of PSII reaction centres, P680 (P680 $\rightarrow P680^+ + e$). Through a series of exergonic, coupled redox reactions associated with the intersystem electron transport chain [plastoquinone pool (PQ), cytochrome b6/f and plastocyanin (PC)], the electron generated by PSII photo-oxidation is used to reduce $P700^+$. The free energy released is used to generate a trans-thylakoid ΔpH or proton motive force (pmf) between the stroma and the chloroplast lumen. $P680^+$ is an exceptionally strong oxidizing agent that catalyzes the oxidation of water ($H_2O \rightarrow 1/2O_2 + 2H^+ + 2e$) through the luminal oxygen evolving complex (OEC) associated with PSII and reduces $P680^+$ ($2P680^+ + 2e \rightarrow 2P680$). The protons generated by the OEC also contribute to the establishment of the light-dependent pmf (Fig. 1.2). Because of the pmf across the thylakoid membrane, the H^+ ions diffuse from the lumen to the stroma through ATP synthase, which utilizes the pmf to phosphorylate ADP to ATP. The ATP synthesized in the stroma from the light reactions can then be used to fuel primarily the reactions of the Calvin-Benson-Bassham cycle (Fig. 1.2). Although energy needed for photosynthesis is considered to be produced primarily through LEF, this process alone is not sufficient to provide the energy requirements for CO_2 assimilation. Thus, in addition to LEF, photosynthesis also generates energy through a series of cyclic reactions known as cyclic electron flow (CEF). (Allen, 2003; Joliot and Joliot, 2006; Rochaix, 2011). CEF is specifically associated with PSI. In CEF, electrons generated by the photo-oxidation of P700 are donated to ferredoxin, which subsequently donates the electrons to the cytochrome b6f complex via the PQ pool. These electrons are used to reduce $P700^+$ through a series of oxidation-reduction reactions (plastoquinone → cytochrome b6f → plastocyanin → PSI). Thus CEF generates a trans-thylakoid H^+ gradient used by ATP synthase to generate ATP with no concomitant biosynthesis of NADPH. LEF and CEF regulate the chloroplast ATP/NADPH ratio to ensure optimum rates of CO_2 assimilation by the Calvin-Benson-Bassham Cycle.

1.1.3 The Calvin-Benson-Bassham cycle

The reactions of the Calvin-Benson-Bassham cycle are a series of biochemical reactions that occur in the stroma of the chloroplast (Bassham *et al.*, 1954). The Calvin-Benson-Bassham cycle is tightly coupled to the light reactions, which provide reducing power (NADPH) and energy (ATP), to the Calvin-Benson-Bassham cycle reactions are required for the continuous regeneration of ribulose-1,5-bisphosphate (RuBP).

The enzyme Rubisco catalyzes the first step of the Calvin-Benson-Bassham cycle. Rubisco fixes CO_2 into sugar molecules (Ellis, 1979). Rubisco reacts with ribulose-1,5-bisphosphate generating an Rubisco-enediol complex that captures CO_2 or O_2. The CO_2 captured by enediol formation produces a six carbon intermediate that immediately splits in half forming two molecules of 3-phosphoglycerate. Rubisco has catalytic limitations that can limit the rate of the Calvin-Benson-Bassham cycle (Parry *et al.*, 2007). Therefore large amounts of Rubisco must be present to sustain photosynthesis (Ellis, 1979). Even though Rubisco can account for 50% of soluble leaf protein (Spreitzer and Salvucci, 2002) in C3 plants and 20-30% of total nitrogen (Evans and Seemann, 1989; Kumar *et al.*, 2002; Makino, 2003), its activity can still be limiting under some conditions (Stitt and Schulze, 1994). The activity of Rubisco is the primary rate-limiting factor of the Calvin-Benson-Bassham cycle during the day. During the night, Rubisco is usually substrate limited, as RuBP is not regenerated in the dark by the Calvin-Benson-Bassham Cycle. The reactions of the Calvin-Benson-Bassham cycle are illustrated in Fig. 1.3, and detailed in Supplemental Table S1.1. The net reaction of the Calvin-Benson-Bassham cycle is:

$$3\ CO_2 + 6\ NADPH + 5\ H_2O + 9\ ATP \rightarrow \text{glyceraldehyde-3-phosphate (G3P)} + 2H^+ + 6\ NADP^+ + 9\ ADP + 8\ Pi$$

The Calvin-Benson-Bassham cycle is regulated by the light because the cycle requires light-derived NADPH and ATP. Additionally, the Rubisco enzyme is activated by Rubisco activase, which improves CO_2 binding to the Rubisco active site by removing a proton from lysine. The activity of Rubisco activase is activated by high ATP concentrations which typically arise from the light reactions of photosynthesis. Finally, when light is available, ferredoxin reduces thioredoxin, which activates these five key

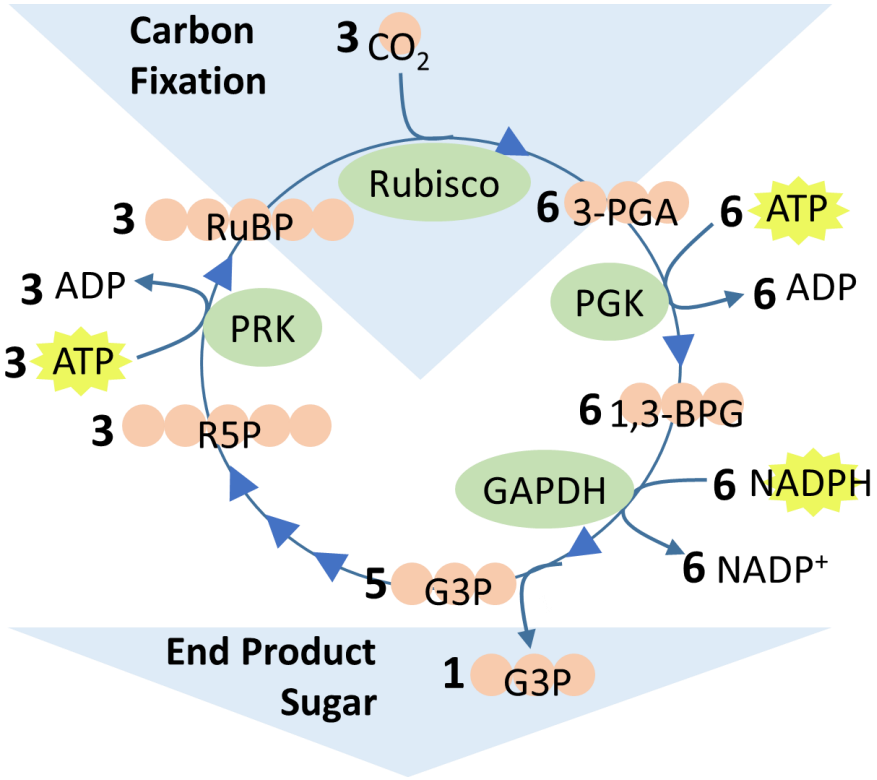


Figure 1.3 The Calvin-Benson-Bassham cycle. Rubisco catalyzes the carboxylation of ribulose-1,5-bisphosphate (RuBP) by carbon dioxide, producing a six carbon intermediate that breaks down to two molecules of 3-phosphoglyceric acid (3-PGA). The enzyme phosphoglycerate kinase (PGK) catalyzes the phosphorylation of 3-PGA by ATP producing 1,3-Bisphosphoglycerate (1,3-BPG). 1,3-BPG is then reduced by the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) generating glyceraldehyde 3-phosphate (G3P). G3P is a sugar end-product of the Calvin-Benson-Bassham cycle. Finally, the cycle is completed by a series of reactions that regenerate RuBP from G3P. A notable enzyme in this regeneration is phosphoribulokinase (PRK), which consumes ATP to convert ribulose-5-phosphate (R5P), to RuBP.

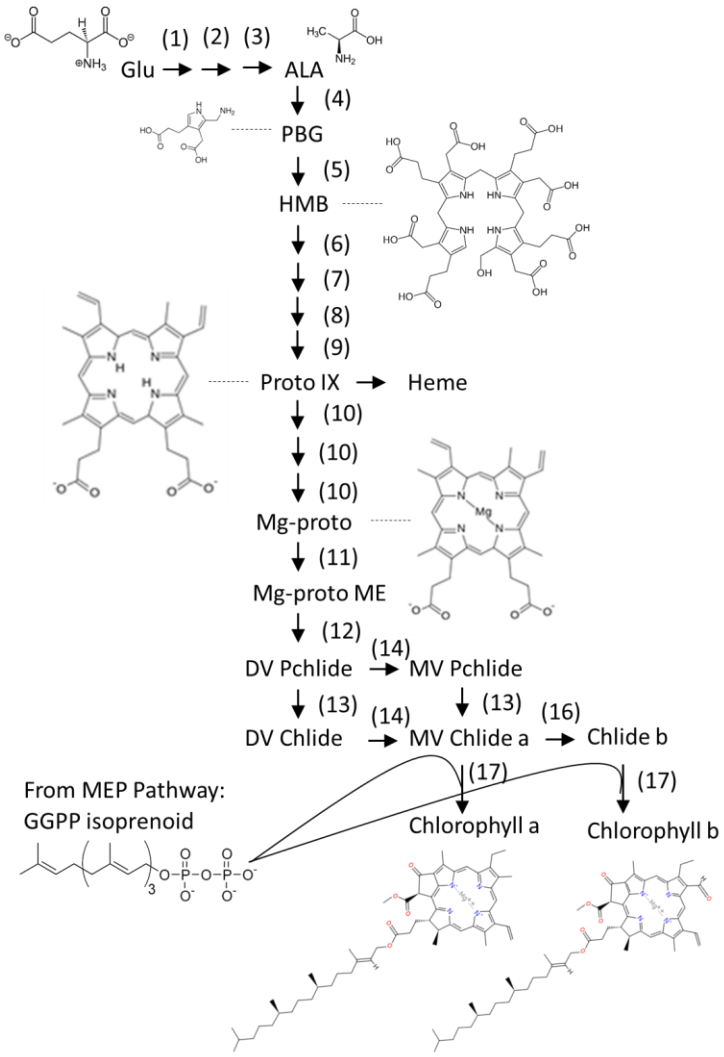
Calvin-Benson-Bassham cycle enzymes: glyceraldehyde-3-P dehydrogenase, glyceraldehyde-3-P phosphatase, fructose-1,6-bisphosphatase, sedoheptulose-1,7 bisphosphatase, and ribulose-5-phosphate kinase.

The Calvin-Benson-Bassham cycle reactions of photosynthesis produce triose phosphates, and these carbohydrates can fulfill many roles in the cell. Triose phosphates can be converted to ADP-glucose in the chloroplast and can subsequently be polymerized into starch to store energy. Triose phosphates can also be exported to the cytosol and used in glycolysis to fuel growth and metabolism. Triose phosphates in the cytosol can be used for the biosynthesis of sucrose in the cytosol, which can be exported from the mesophyll cells of leaves to support the growth and metabolism of heterotrophic sink tissues. The sugars can also be used as building blocks in the biosynthesis of other biomolecules like cellulose. To help fulfill such a diverse range of functions, sugars also act as signalling molecules to help regulate plant growth and metabolism (Ramon *et al.*, 2008; Rolland *et al.*, 2006).

1.1.4 Chlorophyll biosynthesis

Chlorophyll is a green light harvesting pigment that is essential for photosynthesis. The chlorophyll pigment is localized to the chloroplast and is responsible for the green colour of plants. Chlorophyll absorbs light in the blue and red portions of the electromagnetic spectrum, and can transfer the light energy to nearby pigment molecules. The antennae of photosystem II and photosystem I contain two types of chlorophyll: chlorophyll a and chlorophyll b, as well as carotenoids. Chlorophyll is composed of a chlorin ring and a geranylgeranyl pyrophosphate (GGPP)-derived isoprenoid. These components are synthesized through two separate metabolic pathways: the tetrapyrrole pathway (Tanaka and Tanaka, 2007) and the methylerythritol phosphate (MEP) pathway (Kim *et al.*, 2013).

The tetrapyrrole biosynthetic pathway (Fig. 1.4; Supplementary Table S1.2) produces the chlorophyll precursor chlorophyllide. Tetrapyrrole biosynthesis can be broken down into two parts. First, a series of nine reactions convert glutamate to protoporphyrin IX (Fig. 1.4). Then, magnesium chelatase adds a magnesium ion to protoporphyrin IX (Fig. 1.4). At this key step, protoporphyrin IX could alternatively bind an Fe ion to form a heme



1. Glutamyl-tRNA synthetase (GTS)
2. Glutamyl-tRNA reductase (HEMA)
3. GSA aminomutase (GSA)
4. ALA dehydratase (HEMB1)
5. Porphobilinogen deaminase (HEMC)
6. Uroporphyrinogen III synthase (HEMD)
7. Uroporphyrinogen III decarboxylase (HEME2)
8. Oxygen-dependent coproporhyrinogen III oxidase (HEMF1)
9. Protoporphyrinogen IX oxidase (PPO)
10. *Magnesium chelatase* (MgCh). Subunits: CHLD, CHLH, GUN4, CHLI
11. Mg-protoporphyrin IX methyltransferase (MgMT)
12. Mg-protoporphyrin IX monomethyl ester cyclase (CRD1)
13. Protochlorophyllide oxidoreductase (POR)
14. Divinylchlorophyllide reductase (DVR)
15. Chlorophyll Synthase
16. Chlorophyllide a oxygenase (CAO)
17. Chlorophyll Synthase

Figure 1.4 The chlorophyll biosynthesis pathway in plants. Abbreviations of precursors and **i**ntermediates: Glutamate (Glu), 5-aminolevulinic acid (ALA), Porphobilinogen (PBG), Hydroxymethylbilane (HMB), Protoporphyrin IX (Proto IX), Mg-protoporphyrin IX (Mg-proto), Mg-protoporphyrin IX monomethyl ester (Mg-proto ME), 3,8-Divinyl protochlorophyllide a (DV Pchlide), Monovinyl Protochlorophyllide (MV Pchlide), Geranylgeranyl-pyrophosphate (GGPP), Monovinyl chlrophyllide a (MV Chlide a), Chlorophyllide b (Chlide b).

precursor, and this step commits the molecule to either the heme biosynthetic pathway or the chlorophyll biosynthetic pathway. Magnesium chelatase is the committed first step of chlorophyll biosynthesis, and is therefore highly regulated both transcriptionally and post-transcriptionally (Ikegami *et al.*, 2007). Next, Mg-protoporphyrin IX methyltransferase transfers a methyl group from S-adenosyl-L-methionine to Mg-protoporphyrin IX (Table 3K). Monomethyl ester cyclase catalyzes the formation of the fifth ring, to form protochlorophyllide *a* (Fig. 1.4). Protochlorophyllide oxidoreductase (POR) catalyzes a key reaction forming the chlorin ring of the chlorophyll molecule (Table 3M). In *Arabidopsis*, tetrapyrrole biosynthesis is light-dependent because angiosperms only contain a light-dependent protochlorophyllide oxidoreductase (POR).

Chlorophyll biosynthesis is a highly regulated process because the generation of free chlorophyll molecules or precursors like protochlorophyllide generates single oxygen ($^1O_2$) producing significant photooxidative stress (Kim *et al.*, 2012; Kruse *et al.*, 1995; Menon *et al.*, 1989; Meskauskiene *et al.*, 2001; op den Camp *et al.*, 2003). Sugars negatively regulate chlorophyll biosynthesis and consequently, addition of exogenous sugars typically decreases chlorophyll accumulation in plant tissue cultures (Edelman and Hanson, 1971; Kaul and Sabharwa, 1971). In *Arabidopsis* seedlings, sucrose, hexoses, and sugar analogs strongly repress chlorophyll biosynthesis, which results in a yellow-white phenotype (Solfanelli *et al.*, 2006), or red-purple anthocyanin phenotype (Fig. 1.5; Cho *et al.*, 2010).

1.1.5 The biosynthesis of sucrose and starch in photosynthetic tissue

During the day, the chloroplasts of photosynthetic tissue capture light energy to fix carbon dioxide producing triose-phosphate sugars. This triose phosphate can then be synthesized into sucrose and starch. Sucrose is synthesized in the cytosol and starch is synthesized in chloroplasts and amyloplasts (Jiang et al. 2015).

Stromal triose phosphate generated by the Calvin-Benson-Bassham Cycle is exported to the cytosol by the triose phosphate translocator localized in the inner chloroplast envelope membrane (Fig. 1.6) (Walters *et al.*, 2004). Triose phosphate is then converted

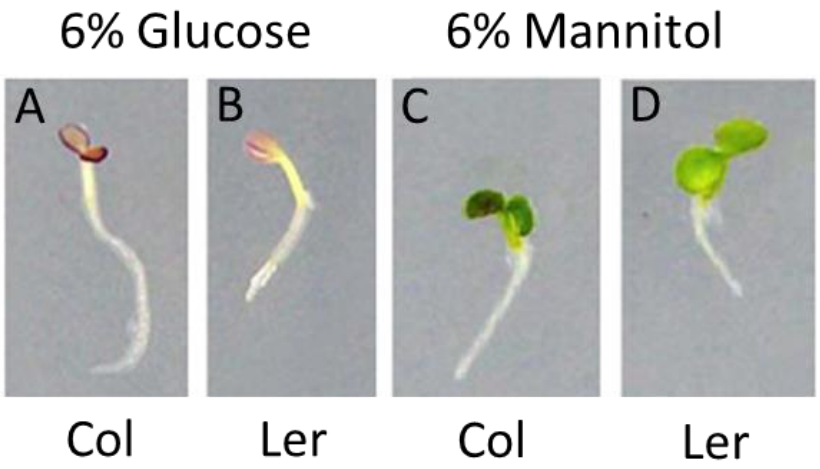


Figure 1.5 *Arabidopsis thaliana* var. Col-0 and Ler seedlings have a non-green phenotype of at 6% (w/v) glucose, and green phenotype at 6% (w/v) mannitol. Mannitol is a non-metabolizable sugar. (Image source: Cho *et al.*, 2010; Copyright American Society of Plant Biologists)

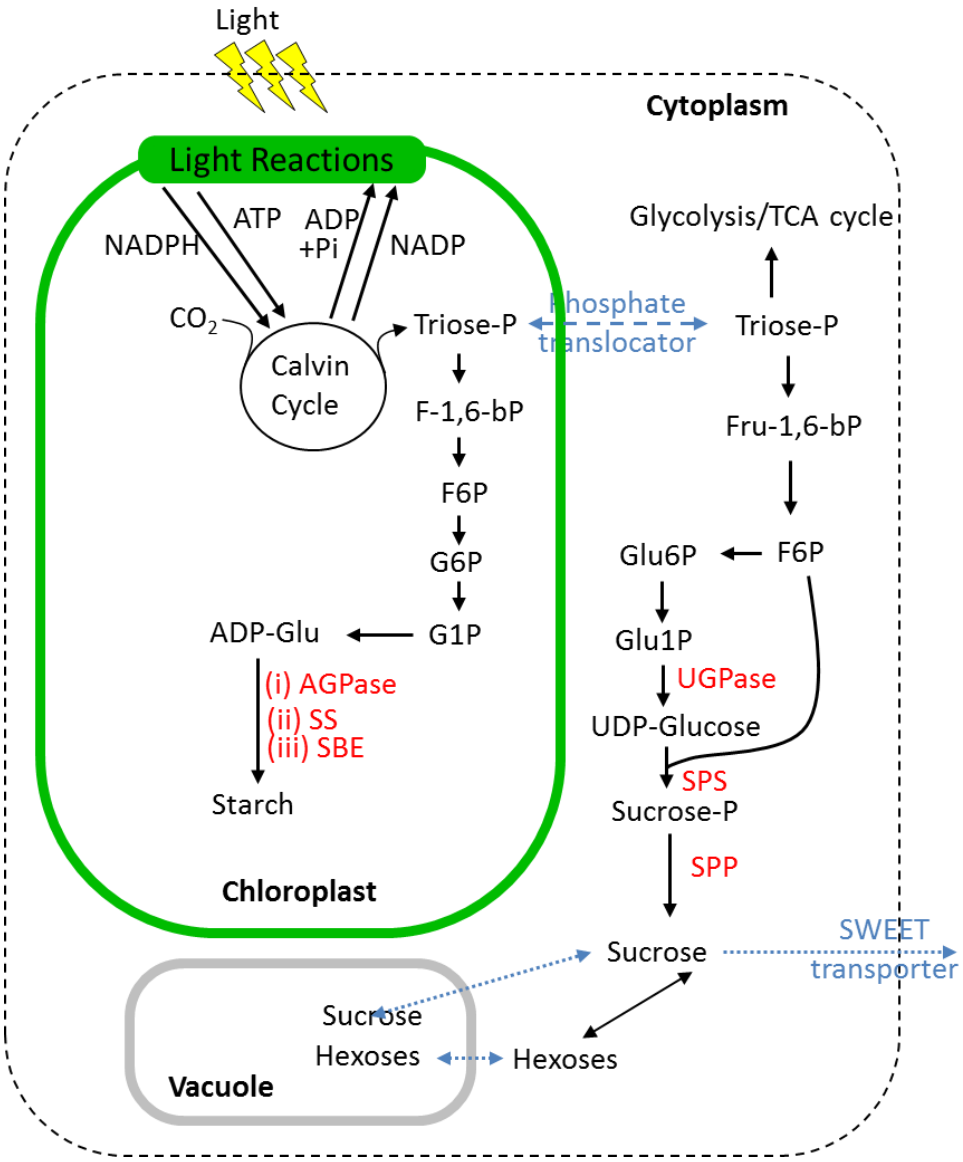


Figure 1.6 Key steps of the starch and sucrose biosynthetic pathways in illuminated photosynthetic tissue. Key enzymes of starch biosynthesis are ADP-glucose pyrophosphorylase (AGPase), starch synthase enzymes (SS), and starch branching enzymes (SBE). Key enzymes of sucrose biosynthesis are sucrose-phosphate synthase (SPS), Sucrose-phosphate phosphatase (SPP), and UDP-glucose pyrophosphorylase (UGPase). Abbreviations: fructose 1,6-bisphosphate (Fru-1,5-bP), fructose 6-phosphate (F6P), glucose 6-phosphate (Glu6P), gluocose 1-phosphate (Glu1P).

to glucose-1-phosphate through five reactions that are catalyzed by the following enzymes: (i) Triose phosphate isomerase, (ii) Fructose-bisphosphate adolase, (iii) Fructose-1,6-bisphosphatase, (iv) Phosphoglucose isomerase, and (v) Phosphoglucose mutase. The key enzymes of sucrose biosynthesis are sucrose-phosphate synthase (SPS), Sucrose-phosphate phosphatase (SPP), and UDP-glucose pyrophosphorylase (UGPase) (Jiang *et al.*, 2015). These key enzymes catalyze the reactions, converting Glucose-1-phosphate to sucrose (Fig. 1.6). The *Arabidopsis* nuclear genome encodes four SPS genes, four SPP genes, and six UGPase genes (Jiang *et al.*, 2015).

Starch is an important storage form of carbon and its subsequent catabolism and mobilization can affect intracellular sugar concentrations, thereby influencing sugar signalling. Starch is a polymer of glucose molecules joined by α-1,4- and α-1,6-glycosidic bonds. This polysaccharide is produced by plants in chloroplasts and amyloplasts to store energy (Fig. 1.6) (Geigenberger, 2011; Kötting *et al.*, 2010). The key enzymes of starch biosynthesis are ADP-glucose pyrophosphorylase (AGPase), starch synthase enzymes (SS), and starch branching enzymes (SBE). AGPase catalyzes the reaction combining glucose-1-phosphate (G1P) and ATP to produce ADP-glucose and P_i. Typically AGPase is the rate limiting step of starch biosynthesis (Cross *et al.*, 2004). ADP-glucose is then the substrate for starch synthase (SS) enzymes that add glucose to the end of a starch polymer chain and release ADP. Starch branching enzymes (SBEs) hydrolyze α-1,4-glycosidic bonds and replace them with α-1,6- bonds between glucose units. In the *Arabidopsis* nuclear genome, there are six genes encoding AGPases (*APL1-4, APS1-2*), five genes encoding starch synthases (*SS1-4, GBSS*), and three starch branching enzymes (*BE1-3*).

1.1.6 The degradation of sucrose and starch

The first step in the degradation of sucrose is catalyzed by the enzymes invertase and sucrose synthase (SuSy) (Jiang *et al.*, 2015). Invertase (Beta-fructofuranosidase) catalyzes the hydrolysis of sucrose to glucose and fructose, and SuSy reversibly degrades sucrose into UDP-glucose and fructose-6-phosphate. The *Arabidopsis* nuclear genome encodes six SuSy genes (*SUS1-6*), four cell wall invertases (*CWINV1-2, CWINV4-5*), two

vacuolar invertases (*β-FRUCT3-4*), and at least four cytoplasmic invertase genes (*INVB, INVD, INVG, INVI*).

Starch is catabolized in a series of reactions (Streb and Zeeman, 2012) by which glucan is phosphorylated by the enzymes α-glucan water dikinase (GWD1) and phosphoglucan water dikinase (PWD). Hydrolytic enzymes then degrade the glucosidic bonds. The linear glucans are hydrolyzed by β-amylases (BAMs), while the branch points are hydrolyzed by debranching enzymes (DBEs). There are nine genes encoding BAMs in *Arabidopsis* (*BAM1-9*), and *BAM1*, *BAM2*, and *BAM3* are localized to the chloroplast and catalyze the hydrolysis of glucans. There are four DBE genes in *Arabidopsis*: *ISA3* and *LDA* are involved in starch breakdown whereas *ISA1* and *ISA2* are involved in starch biosynthesis.

1.1.7 Sugar transport and source-sink relationships

The diffusion of sugars between neighboring cells occurs through plasmodesmata (Turgeon and Medville, 1998). This passive symplastic transport allows cells to exchange sugars without expending energy. However, transport of sucrose across cell membranes requires sucrose transporters and/or hexose transporters.

Plants consist of photosynthetic 'source' tissue which produce and export sugar, and 'sink' tissue that depend on sugar import to meet cellular energy demands. Source tissues are parts of the plant that produce excess sugars and have low energy demand (e.g. mature leaves), and sink tissues are parts of the plant where carbohydrate is needed to meet energy demands (e.g. roots). The non-reducing disaccharide sucrose is the major form of energy and soluble carbon transported long-distances in *Arabidopsis*. Sucrose is transported over long distances in the phloem tissue, along with a small amount of the trisaccharide raffinose (Haritatos *et al.*, 2000). Sugar transport must be tightly controlled to ensure sufficient energy is supplied to sink cells from source tissue.

Sieve element cells are responsible for transporting sugars throughout the plant (Raven *et al.*, 1992). Mature sieve elements lack a nucleus and have few organelles to ensure fluids can move through the cells freely (Oparka and Turgeon, 1999) and rely on companion cells for their metabolic needs. Companion cells, therefore, have a large number of

ribosomes and mitochondria to support the metabolic needs of sieve elements. Plasmodesmata facilitate sucrose transport between these two cell types (Raven *et al.*, 1992).

In plant tissue, the symplast is a continuous route for water and low-molecular-weight solutes to diffuse from one cell to another. The symplast consists of plasmodesmata connecting the cytoplasm of neighboring cells. Another continuum of space, the apoplast, is formed outside the plasma membrane and within the cell wall. The apoplast provides a route for water and solutes to be transported across tissues or organs.

Sucrose is synthesized in the cytosol of photosynthetic cells and is transported to the apoplast by active transport or passive diffusion (Fig. 1.7; Shiratake, 2007). Sucrose transporters in the membranes of companion cells import sucrose where it is loaded into sieve elements through plasmodesmata (Fig. 1.7). This process is called “phloem loading” (Giaquinta, 1983; Lalonde *et al.*, 2003). Phloem transport occurs as a pressure-driven bulk flow through specialized cells named sieve elements. Active transport of sugar into the sieve elements causes water to move into the sieve tube by osmosis, which increases the hydrostatic pressure of the sieve tube, thereby forcing sucrose through the sieve tube by mass flow (Fig. 1.7). In sink tissue, sucrose diffuses from the sieve elements into the apoplast and it is also unloaded to some cells symplastically through plasmodesmata (Fig. 1.7; Shiratake, 2007). Sink cells that import sucrose from the apoplast utilize sucrose transporters and a combination of cell wall invertases and hexose transporters (Fig. 1.7).

There are nine sucrose transporter homologues in *Arabidopsis* (SUC1-SUC9), and 17 SWEET sugar transport proteins in *Arabidopsis* (SWEET1-SWEET17) (Chen *et al.*, 2015). SWEET9-15 are the sucrose transporters of the SWEET family that are responsible for sucrose efflux and vacuolar transport (Chen *et al.*, 2015). The transporters SUC2, SWEET11 and SWEET12 are responsible for phloem loading (Fig. 1.7; Chen *et al.*, 2012a; Lalonde *et al.*, 2004; Sauer, 2007; Durand *et al.*, 2018). SUC2 is a high-affinity sucrose transporter with a Km from 0.1-2.0 mM (Kühn 2003) that is localized in the plasma membrane and is responsible for the majority of phloem loading

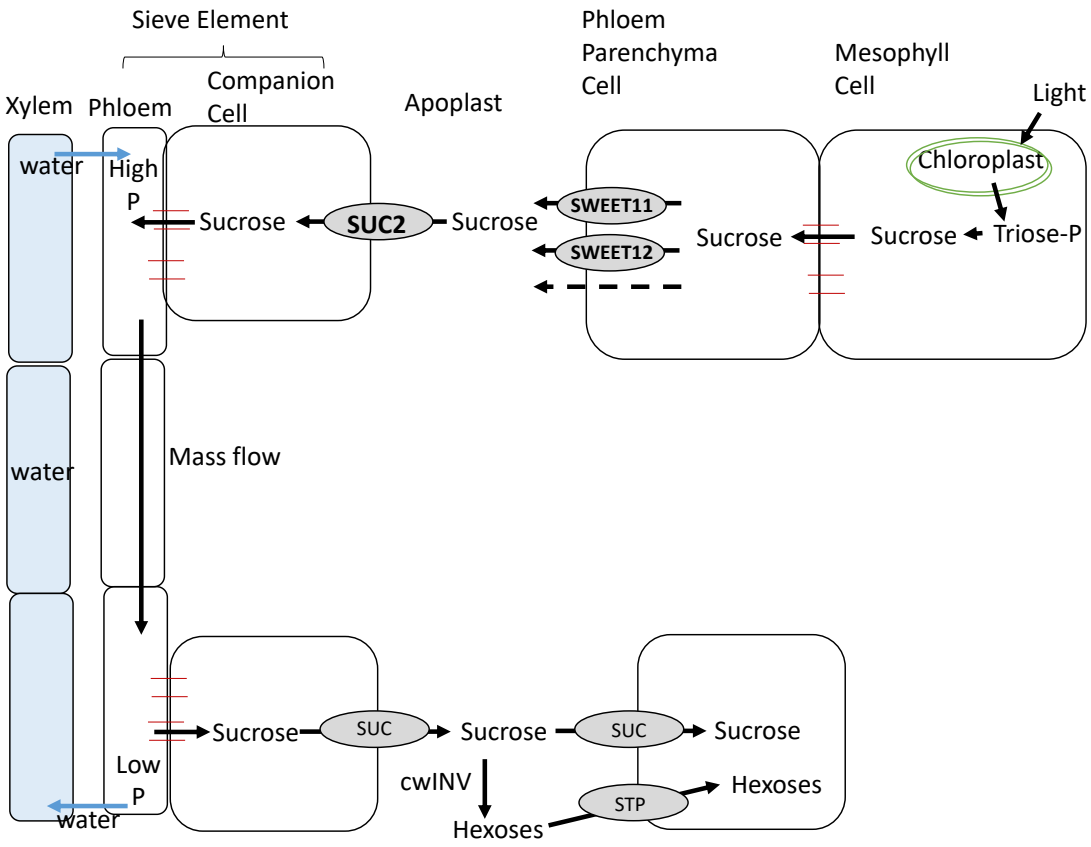


Figure 1.7 Sugar transport from source to sink in *Arabidopsis thaliana*. Sucrose synthesized in the cytosol of photosynthetic cells is moved to phloem parenchyma cells through plasmodesmata (red lines) where it is exported to the apoplast by sucrose transporters (SWEET12, SWEET13) or by simple diffusion (dotted line). Sucrose in the apoplast is actively loaded into companion cells by sucrose transporters (SUC2), in a process called phloem loading. Sucrose in companion cells moves to the sieve elements through plasmodesmata (red lines). Active transport of sucrose into the sieve elements causes water to move into the sieve tube by osmosis (blue arrow), increasing the hydrostatic pressure of the sieve tube and forcing sucrose through the sieve tube by mass flow. In sink tissues, sucrose diffuses from the sieve elements into the apoplast and it is also unloaded to some cells symplastically through plasmodesmata. Sink cells that import sucrose from the apoplast utilize sucrose transporters and a combination of cell wall invertases and hexose transporters (e.g. STP glucose transporters).

(Gottwald et al., 2000; Srivasta et al., 2008; Srivasta et al., 2009). SWEET11 and SWEET12 are expressed in leaf phloem tissue and the *sweet11/sweet12* double mutant exhibits reduced phloem loading, as indicated by high levels of starch in the source leaf tissue, as well as by delayed root development (Chen *et al.*, 2012b).

In *Arabidopsis*, sucrose is transported into cells by SUC1, SUC3, SUC5, SUC8 and SUC9, which are the sucrose transporters localized to the plasma membrane (Wippel and Sauer, 2012). Sugar can also be broken down in the apoplasm by cell wall invertases and imported into cells as glucose and fructose. In addition to the sucrose transporters, *Arabidopsis* has about 60 genes encoding other sugar transport proteins (Shiratake, 2007). For example, there are 14 glucose transport genes (*STP1-14*). Sugar transport can impact sugar signalling in plant cells because the signalling pathways sense intracellular sugar levels.

1.1.8 *Arabidopsis thaliana*

Arabidopsis thaliana is a small flowering plant from the mustard family (Brassicaceae). *Arabidopsis* has a fast growth rate, prolific seed production, and a small genome, which has made it an excellent model organism for genetics and molecular biology research. Of the nine *Arabidopsis* species, *Arabidopsis thaliana* has gained the most scientific interest and is commonly used as a model organism for research in plant biology. The 135 Mbp *Arabidopsis thaliana* genome was first published in 2000 by the *Arabidopsis* Genome Initiative (Kaul *et al.*, 2000). The 1001 genomes project has sequenced 1135 *Arabidopsis* genomes to date, highlighting the genetic variability between ecotypes (Alonso-Blanco *et al.*, 2016). This thesis focuses on an established cell suspension culture derived from wild type *Arabidopsis thaliana* Landsberg *erecta* by May and Leaver (1993).

1.1.9 Plant cell suspension cultures

The *in vitro* culture of plant cells has many important applications in biology and biotechnology. In cell suspension cultures, plant cells grow as single cells and small clusters of cells in an artificial liquid medium (Fig. 1.8; Barkla et al. 2014; George *et al.*, 2007; Mayo, K. J. et al., 2006). Cell suspension cultures are ideal for studying biological processes as they are axenic, homogenous, and because the artificial medium can be

strictly controlled and easily manipulated. Cell cultures facilitate studies at the cellular levels while removing the structural complexity of a multicellular plant. Cell suspension cultures are also ideal for generating large amounts of biomass for quantitative or qualitative analyses. Additionally, cell suspension cultures are ideal for the isolation of protoplasts used in transient gene expression assays and in *Agrobacterium* mediated transformation (Forreiter *et al.*, 1997; Mathur *et al.*, 1998). Because of these traits, cell suspension cultures have found many uses in biotechnology, including secondary metabolite production (Hellwig *et al.*, 2004; Rao and Ravishankar, 2002). Plant cell cultures are totipotent and are able to regenerate the whole plant (Nagata and Takebe, 1971; Steward *et al.*, 1958), although totipotentcy can be lost after prolonged culturing (Gaspar *et al.*, 2000). In addition, plant cell cultures are also subject to high rates of genetic change, which has been useful in creating plants with new desirable traits (Jain, 2001; Larkin and Scowcroft, 1981).

1.1.10 Isolation and maintenance of cell suspension cultures

The first step in isolating cell suspension cultures is to generate callus from a plant or seed (Mathur and Koncz, 1998b). Callus is a coherent and unstructured tissue formed when plant cells grow in a disorganized way (George *et al.*, 2007), which can be induced from small sections of tissues excised from a developed plant or seedling. The explants are surface sterilized, and grown in a petri dish containing a callus induction medium. Complete surface sterilization is important to the viability of the cell culture, as the *in vitro* conditions are also favourable for microbial growth. Callus induction media are artificial media (e.g., Murashige and Skoog medium) supplemented with high concentrations of auxin and sugar, and solidified with a gelling agent such as agar. The high concentration of auxin induces cells to dedifferentiate, generating unorganized cell masses, known as callus, that can be propagated on the artificial medium. For *Arabidopsis*, the auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) is commonly used as an auxin as it provides the high efficiency of callus formation, and is more stable and less expensive than endogenous auxins (Mathur and Koncz, 1998a; Sauer *et al.*, 2013). Different kinds of callus, differing in appearance, colour, compaction, and regeneration potential can arise from a single explant (George *et al.*, 2007; Northmore *et al.*, 2016).

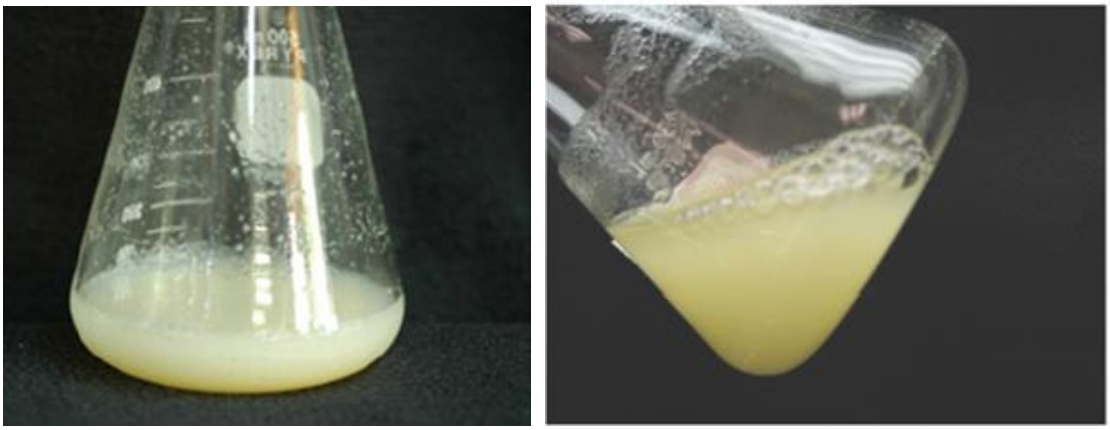


Figure 1.8 *Arabidopsis* and tobacco cell suspension cultures (Left image source: Barkla et al. 2014; Right image source: Mayo, K. J. et al., 2006)

Once callus is induced from the sterilized seed or plant tissue, it is transferred to a liquid maintenance medium. Friable callus is typically selected to inoculate the liquid medium, as this reduces the formation of large clumps (George *et al.*, 2007). The maintenance medium is an artificial medium (e.g., MS medium) supplemented with sucrose, auxin, and cytokinin. The liquid medium is aerated by continuous shaking and is subcultured periodically (George *et al.*, 2007). During subculture, single cells and small groups of cells are transferred by pipetting to fresh medium using a minimum inoculation medium that is empirically determined (George *et al.*, 2007; Mustafa *et al.*, 2011).

Cell suspension cultures can be maintained *in vitro* indefinitely. However, the regeneration capability can be lost after prolonged subculture (Gaspar *et al.*, 2000), and genetic stability of the cells also decreases over time (Larkin and Scowcroft, 1981). Therefore, newly initiated cell suspension cultures should be used in applications that require regeneration of diploid fertile plants. For long term maintenance, cell suspension cultures can be frozen using cryopreservation techniques (Menges and Murray, 2004; Mustafa *et al.*, 2011).

1.1.11 Phenotypes of cell suspension cultures

Typically, callus cells have a non-green phenotype (George et al., 2008). When plant cells containing chloroplast are transferred to callus induction medium, they dedifferentiate, which initially results in the loss of thylakoids and grana, and after some time, the chloroplasts change shape and degenerate (George et al., 2008). Typically, newly developed callus contain only plastids containing starch grains and under-developed lamellar structures. However, callus cultures can turn green after continuous exposure to light, although chlorophyll levels are usually minimal compared to those of mesophyll cells of leaves (George et al., 2008). When green cell cultures are obtained, they are typically photomixotrophic and growth is partially dependent on sugar in the medium.

Strategies to generate photoautotrophic cell cultures were reviewed by Widholm (1992). One would assume that because these cells originate from plant tissue, the establishment of green photoautotrophic cell cultures would be straightforward. However, it is a slow

and difficult process (Widholm, 1992). Widholm hypothesized that the generation of photosynthetic cultures may involve selection for a mutant cell type, however no genetic studies were performed (Widholm, 1992).

1.1.12 Somaclonal variation

While plant cell suspension cultures can be maintained indefinitely *in vitro*, the cells experience genetic variation. Somaclonal variation describes variability brought about by *in vitro* culturing (Larkin and Scowcroft, 1981) including genetic, epigenetic, and karyotypic changes. Genetic changes occur at a much faster rate in cell cultures than in intact plants (Noro *et al.*, 2007). Transposable elements become active during *in vitro* culturing (Hirochika, 1993) and often there are changes in chromosome numbers (Chen and Chen, 1980). In addition, epigenetic modifications appear to be more variable and less temporally stable in cell cultures (Kaeppler *et al.*, 2000)

1.2 End product inhibition of photosynthesis by sugar

Feedback inhibition of photosynthesis by sugars is a mechanism that plants use to conserve valuable resources. When sugar builds up to high levels in plant tissues, photosynthesis is no longer required to produce sugar, and photosynthesis is downregulated. The cells can then optimize the use of available nutrients and micronutrients by utilizing them for other processes such as growth. End product inhibition of photosynthesis was first proposed by Boussingault (1868). An early study that provided evidence for this hypothesis showed that the buildup of photosynthetic end products (e.g. sucrose) in wheat leaves negatively regulates photosynthetic rate (Neales and Incoll, 1968). The removal of sink tissue in bean plants was also shown to reduce photosynthetic rates (Geiger, 1976). Furthermore, photosynthesis was shown to be inhibited in experiments inducing end product accumulation in wheat leaves through leaf base chilling, low temperatures (Dahal et al. 2012; 2014), high CO_2, and low O_2 concentrations (Azconbieto, 1983). Girdling leaves to completely prevent translocation of assimilates yielded similar results (Krapp *et al.*, 1993). The accumulation of end products has been shown to inhibit the levels of Rubisco accumulation and the activities of Calvin cycle enzymes (Besford, 1990).

The addition of external sugars suppressed chlorophyll levels in plant tissue cultures of tobacco (Kaul and Sabharwa, 1971) and carrot (Edelman and Hanson, 1971). This implicated sugars as the molecules mediating the inhibition of photosynthetic activity. Further evidence was provided when photosynthetic activity was correlated with the activity of invertase (Goldschmidt and Huber, 1992; Huber, 1989). In subsequent studies on transgenic plants overexpressing a yeast invertase, the plant leaves accumulated hexose intracellularly, which inhibited photosynthetic activity (Dickinson *et al.*, 1991; Vonschaewen *et al.*, 1990).

The accumulation of sugars in many plant species has been shown to repress numerous photosynthetic genes such as: (i) thylakoid ATPase, ATP-δ (ii) carbonic anhydrase, ca (iii) chlorophyll a/b binding protein, cab (iv) D1 protein of PSII, PsbA (v) the light harvesting complex of photosystem II, lhcb, (vi) C4 malic enzyme, MEL (vii) plastocyanin, pc (viii) C4 phosphoenolpyruvate carboxylase, PEPC (ix) triose phosphate translocator, PT (x) pyruvate phosphodikinase, PPDK (xi) Rubisco small subunit, RBCS (xii) Rubisco activase, RCA. (Cheng *et al.*, 1992; Criqui *et al.*, 1992; Dijkwel *et al.*, 1996; Harter *et al.*, 1993; Knight and Gray, 1994; Krapp *et al.*, 1993; Raines *et al.*, 1992; Sheen, 1990; Vanoosten and Besford, 1994, 1995). The accumulation of intracellular sugar is required for sugar signalling and sugars which aren’t substrates for hexokinase (6-deoxyglucose and 3-O-methylglucose) do not repress photosynthetic genes (Jang and Sheen, 1994).

1.2.1 The metabolic cost of photosynthesis

There is a large metabolic cost to photosynthesis due to the requirements for large amounts of carbon, nitrogen and other macronutrients (e.g., magnesium), as well as micronutrients (e.g., iron) for the development of the photosynthetic machinery. Consequently, when carbohydrates are abundantly available to cells, photosynthesis is not required, and generation of the metabolically expensive photosynthetic complexes is a waste of resources. When sugars are abundantly available, these resources could be better allocated to other metabolic processes, such as growth.

Photosynthesis requires a lot of nitrogen. For example, Rubisco is the most abundant protein in photosynthetic tissue, accounting for up to 50% of soluble leaf protein (Spreitzer and Salvucci, 2002) in C3 plants and 20-30% of total nitrogen (Evans and Seemann, 1989; Kumar *et al.*, 2002; Makino, 2003). Therefore Rubisco imposes a huge nitrogen cost to photosynthetic cells. The synthesis of light reaction proteins also requires a lot of nitrogen, as they are highly abundant proteins in photosynthetic cells (Evans and Seemann, 1989; Raven, 1984). Chlorophyll and chloroplast-related nucleic acids also contain significant amounts of nitrogen. Because of the high nitrogen cost of photosynthesis, if sugar is abundantly available, photosynthetic gene expression is repressed since sugar synthesis is not required (Sheen, 1994).

Magnesium is a constituent of the chlorophyll molecule. The biosynthesis of chlorophyll and the formation of thylakoid membrane stacks require magnesium (Tanaka and Tanaka, 2007). Approximately 10% of the plant's magnesium supply is bound to chlorophyll (Mayland, 1990). Sulfur is a component of the amino acids cysteine and methionine, and is therefore ubiquitous in proteins, including the photosynthetic proteins. Sulfur is also present in the chloroplast membrane lipids, which represent 4-7% of total leaf lipids (Shimojima, 2011).

Iron and manganese are trace elements necessary for photosynthesis. Iron is required for some of the electron transfer reactions of photosynthetic electron transport (van Oijen *et al.*, 2004). Photosystem II, photosystem I, cytochrome b6f, and ferredoxin all contain iron. Iron is a constituent of cytochromes, other heme molecules, as well as Fe-S clusters. Iron is also a structural component of metallo-enzymes that are involved in nitrate uptake. Manganese is an essential component of the photosystem II OEC (Fischer *et al.*, 2015). The OEC of photosystem II contains a Mn_4CaO_5 cluster, a locus of charge accumulation that couples the four-electron oxidation of water with the one-electron photochemistry at PSII.

Despite the high metabolic costs of the biosynthesis and assembly of the photosynthetic apparatus and Calvin cycle enzymes, plants typically benefit greatly from the investment when light is converted into chemical energy through photosynthesis. However, under

sugar-replete conditions, photosynthesis is downregulated, allowing these resources to be allocated to other biological processes.

1.3 Sugar signalling pathways in plants that regulate photosynthetic gene expression

There are three major sugar signalling pathways in *Arabidopsis* that regulate photosynthetic gene expression: the SNF1-related kinase 1 (SnRK1) pathway, the hexokinase (HXK) signalling pathway, and the abscisic acid (ABA) pathway. Additionally, specific parts of ethylene signalling, gibberellin signalling, and auxin signalling interact with the sugar signalling pathways and are required for plants to repress photosynthesis in response to sugar (Ramon *et al.*, 2008; Rolland *et al.*, 2006).

1.3.1 Sugar insensitive mutants

Many of the pathways involved in sugar signalling were discovered in genetic screens identifying mutant seedlings with a green phenotype when grown on a high sucrose medium (≥6% w/v) (Laby *et al.*, 2000; Zhou *et al.*, 1998). The dark green phenotype of seedlings grown under high sucrose (≥6% w/v) is regarded as a sugar-insensitive phenotype since high external sugar availability typically represses chlorophyll accumulation and photosynthetic gene expression in *Arabidopsis* (Laby *et al.*, 2000; Zhou *et al.*, 1998). Further work with these mutants elucidated the details of the plant sugar signalling pathways (Reviewed in Rolland et al., 2006; Ramon et al., 2008).

Many of these mutants are part of major sugar signalling pathways that affect photosynthetic gene expression: the SnRK1 pathway, the hexokinase (HXK) signalling pathway, and the ABA pathway (Table 1.1). The other sugar insensitive mutants (Table 1.2) are still under investigation; many of them belong to hormone signalling pathways that interact with the sugar signalling pathways (Ramon *et al.*, 2008). Transgenic plants overexpressing *trehalose-6-phosphate synthase* (*TPS1*) or *multiprotein bridging factor 1A* (*MBF1*) also had sugar insensitive phenotypes (Avonce *et al.*, 2004; Kim *et al.*, 2007). The details of these sugar signalling pathways are discussed in more detail in Sections 1.3.2-1.3.7.

1.3.2 The SnRK1 sugar signalling pathway

The SnRK1 pathway is a central signalling pathway in plants that activates photosynthesis in response to stressors such as darkness, hypoxia, and the presence of herbicides (Baena-Gonzalez *et al.*, 2007). Microarrays from cells overexpressing KIN10 revealed that the SnRK1 pathway responds strongly to sugars and regulates photosynthetic gene expression (Baena-Gonzalez *et al.*, 2007). SnRK1 is a protein kinase which, through a signal transduction cascade, reprograms the plant transcriptome by altering the expression of over a thousand genes involved in energy homeostasis, growth, stress response, survival, development, reproduction and senescence (Baena-Gonzalez *et al.*, 2007). Characterization of the *kin10*/*kin11* double knockout mutant confirmed the role of SnRK1 in the regulation of these genes, as the *kin10*/*kin11* double mutant no longer regulated this broad set of genes in response to darkness, hypoxia or 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) treatment (Baena-González *et al.*, 2008).

Notable elements of this signal transduction cascade are the S-class bZIP transcription factors (e.g. bZIP1, bZIP2, bZIP11, bZIP53), which are activated by SnRK1 and alter photosynthetic gene expression (Baena-Gonzalez *et al.*, 2007). Glucose-1-phosphate, glucose-6-phosphate, and trehalose-6-phosphate strongly inhibit the activity of SnRK1 (Nunes *et al.*, 2013b), which in turn represses photosynthetic gene expression by regulating the activity of the bZIP transcription factor family (Baena-Gonzalez *et al.*, 2007) as illustrated in Fig. 1.9.

1.3.3 Sucrose induced repression of translation

The five transcription factors – bZIP1, bZIP2, bZIP11 bZIP44, and bZIP53 – are regulated through sucrose-induced inhibition of translation (SIRT; Fig. 1.10) (Hummel *et al.*, 2009; Weltmeier *et al.*, 2009). While the full mechanism of SIRT remains unknown, it is related to the 5’ region of these bZIP mRNAs, which encodes a sucrose control peptide that is required for SIRT (Rook *et al.*, 1998; Wiese *et al.*, 2004). Many of the genes regulated at the translational level by sucrose through SIRT (Fig. 1.10) are also regulated by the SnRK1 signalling pathway (Fig. 1.9), including photosynthetic genes (Hummel *et al.*, 2009).

Table 1.1. Mutants of the three key sugar signalling pathways that have a sugar insensitive phenotypes. Knockout of a single one of these genes causes a sugar insensitive phenotype in *Arabidopsis* (green phenotype at ≥6% sugar)

Sugar signalling pathway	Mutants (protein)
SnRK1	*kin10* (SnRK1 subunit)
Hexokinase	*gin2* (HXK1), *vab1* (vacuolar H+-ATPase-B1), *hup2* (RPT protein 5B)
ABA	*aba1* (ABA1), *abf2* (ABF2), *abi3* (ABI3), *abi5* (ABI5), *abi8* (ABI8), *gin1* (ABA2), *gin5* (ABA3), *gin6* (ABI4), *rgs1* (RGS1), *aria (*ARIA*)*

Table 1.2 Mutants and transgenic seedlings with sugar insensitive phenotypes. For the mutants, knockout of a single one of these genes causes a sugar insensitive phenotype in *Arabidopsis* (green phenotype at ≥6% sugar). For the transgenic seedlings, overexpression of a single one of these genes can cause a sugar insensitive phenotype in *Arabidopsis* (green phenotype at ≥6% sugar).

Transgenic seedlings	Mutant seedlings
35S::TPS1, 35S:MBF1, 35S:ARR2, 35S::CKI1	*axr1, axr2, ctr1, eto1, fin219, lin1, rcd1, rgl2, spy, tir1, wox5*

1.3.4 The hexokinase sugar signalling pathways

Hexokinase (HXK1) regulates nuclear gene expression in response to sugars (Cho *et al.*, 2006) (Fig. 1.11). When stimulated by sugar, HXK1 migrates to the nucleus and binds to cis-regulatory elements of photosyntethic genes, repressing their expression (Cho *et al.*, 2006). The nuclear signalling functions of hexokinase were shown to be independent of its catalytic activity (Moore *et al.*, 2003). HXK1 is encoded by the gene *GIN2 (AT4G29130*). When grown at high glucose (6% w/v glucose), transgenic plants with the antisense construct of *HXK1* had a green sugar insensitive phenotype, while plants overexpressing *HXK1* had a white sugar hypersensitive phenotype (Jang *et al.*, 1997). The HXK1 loss-of-function, glucose insensitive mutants (*gin2-1* and *gin2-2*), grew with a green phenotype at 6% w/v glucose, which is a sugar insensitive phenotype (Moore *et al.*, 2003). Furthermore, seedlings with two catalyticially inactive HXK1 alleles were still able to repress photosynthesis despite lacking the capacity to participate in the metabolism of glucose (Moore *et al.*, 2003), thus uncoupling the roles of HXK1 in sugar signalling and sugar metabolism. Further metabolism of sugars, beyond hexokinase phosphorylation, is not necessary to repress photosynthesis; the non-metabolizable sugars 2-deoxyglucose (2-dG) and mannose, which cannot be metabolized after phosphorylation, repress photosynthesis (Jang and Sheen, 1994).

HXK1 requires two partners to bind to the cis-regulator elements in the nucleus (Fig. 1.11): a vacuolar H+-ATPase (VAB1) encoded by the *VAB1* gene (AT1G76030) and the 19S regulatory particle of proteasome complexes (HUP2) encoded by the gene *RPT5B* (AT1G09100) (Sze *et al.*, 2002; Yang *et al.*, 2004). Similar to the *gin2* mutant, the mutants *vab1* and *rbt5b* had glucose insensitive phenotypes, and had green phenotypes at high glucose concentrations (Moore *et al.*, 2003).

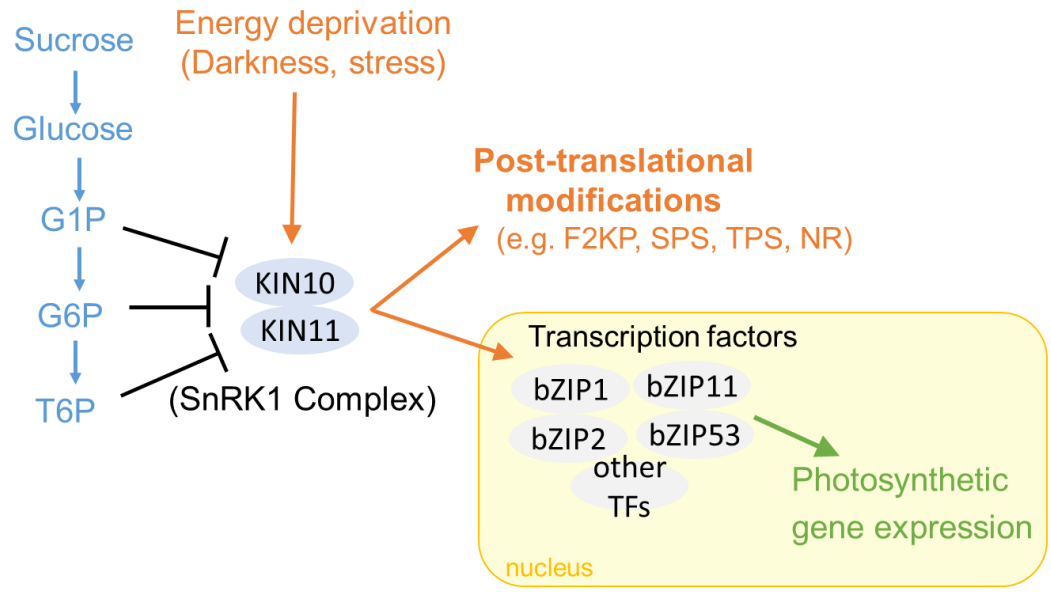


Figure 1.9 The SnRK1 sugar signalling pathway. In response to energy deprivation signals like darkness or stress, the SnRK1 complex upregulates photosynthesis. The SnRK1 complex regulates proteins and transcription factors through post-translational modifications. The repression of photosynthesis by SnRK1 is thought to be mediated through the bZIP class of transcription factors. The sugars glucose-1-phosphate, glucose-6-phosphate, and trehalose-6-phosphate repress the activity of the SnRK1 complex.

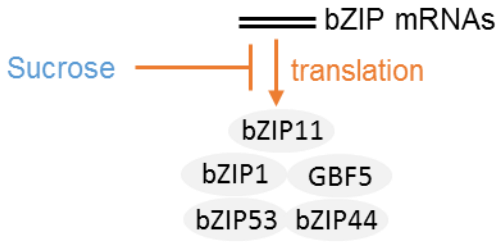


Figure 1.10 Sucrose induced repression of translation (SIRT). Sucrose represses the translation of the transcription factors bZIP1, bZIP11, GBF5, bZIP53, and bZIP44. While the exact mechanism is still unknown, the 5' region of these bZIP mRNAs encode a sucrose control peptide that is required for SIRT.

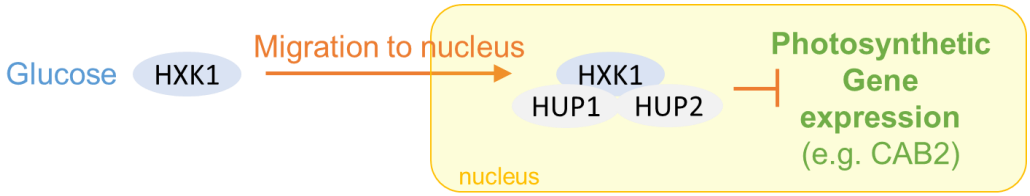


Figure 1.11 Hexokinase-dependent sugar signalling in *Arabidopsis*. In response to intracellular glucose, HXK1 migrates to the nucleus and recruits two partners, VAB1 and HUP2, to bind cis-regulatory elements in the nucleus, repressing photosynthetic genes.

In response to sugar, ABA, a potent regulator of photosynthetic gene expression accumulates in cells (Arenas-Huertero *et al.*, 2000; Ramon *et al.*, 2008; Zhou *et al.*, 1998). While transgenic plants overexpressing *HXK1* had a sugar hypersensitive phenotype (Jang *et al.*, 1997), over expression of *HXK1* in mutants with impaired ABA biosynthesis (*aba2* and *aba3* mutants) did not relieve their sugar insensitive phenotype (Zhou et al., 1998; Arenas-Huertero et al., 2000). While this is often interpreted as ABA acting downstream of hexokinase in a signalling pathway, there has not been sufficient evidence for that conclusion. ABA is a potent regulator of photosynthetic gene expression (Section 1.3.5), and the hexokinase sugar signalling pathway cannot repress photosynthetic gene expression when ABA levels are low.

1.3.5 The ABA sugar signalling pathway

Sugars also inhibit photosynthetic gene expression through the ABA signalling pathway (Dekkers *et al.*, 2008; Gibson, 2004) (Fig. 1.12) as a consequence of ABA accumulation (Cheng *et al.*, 2002; Rolland *et al.*, 2006; Seo and Koshiba, 2002). The ABA biosynthesis genes *ABA1*, *ABA2*, *ABA3*, are required for sugar signalling through the ABA pathway, as the mutants of the ABA biosynthesis genes *aba1*, *aba2* (*gin1*), *aba3* (*gin5*) exhibit a green, sugar-insensitive phenotype when exposed to high sugar concentrations (Arenas-Huertero *et al.*, 2000; Zhou *et al.*, 1998). For example, glucose is unable to repress either of the genes encoding the chlorophyll a/b binding protein (*CAB1*) or plastocyanin (*petE*) in the *aba2* and *aba3* backgrounds (Zhou *et al.*, 1998).

In response to sugar-induced ABA accumulation, the ABA receptors PYL/RCAR inhibit PP2C proteins, which in turn inhibit the activity of SnRK2.2, SnRK2.3, and SnRK2.6 (Fig. 1.12) (Cutler *et al.*, 2010; Ma *et al.*, 2009; Park *et al.*, 2009; Umezawa *et al.*, 2009; Yoshida *et al.*, 2002). The SnRK2 proteins regulate the transcription of several ABA-responsive transcription factors including ABI3, ABI4, ABI5, ABI8, and ABF2 in response to ABA (Cutler *et al.*, 2010; Ramon *et al.*, 2008). These ABA-regulated transcription factors regulate a wide variety of genes including photosynthetic genes (Huang *et al.*, 2007). All of the knockout mutants *abf2*, *abi3*, *abi4*, *abi5*, and *abi8* had green sugar-insensitive phenotypes (Arenas-Huertero *et al.*, 2000; Brocard-Gifford *et al.*, 2004; Kim *et al.*, 2004b; Yuan and Wysocka-Diller, 2006). Mutants of *gin6* (*abi4)* and

abi5 were insensitive to 7% w/v glucose (Arenas-Huertero *et al.*, 2000). The *abi3* mutants differed slightly in their response to sugar, as they were only insensitive to glucose at high ABA concentrations (1 μM) (Nambara *et al.*, 2002). The *abi8* mutants also had a green sugar insensitive phenotype at 6% w/v glucose, as well as severely stunted growth in low glucose media (Brocard-Gifford *et al.*, 2004). Overexpression of bZIP transcription factors ABF2 and ABI5 caused hypersensitivity to sugar (Brocard *et al.*, 2002; Kang *et al.*, 2010). The armadillo (arm) repeat protein encoded by *ARIA* is a positive regulator of the ABA responsive transcription factor ABF2.The *aria* mutant had a sugar insensitive phenotype at 5% w/v glucose (Kim *et al.*, 2004a). Knockout of other ABA effectors, *abi1-1*, and *abi2-1* did not cause glucose insensitivity, suggesting that there is a distinct ABA signalling pathway for sugars (Arenas-Huertero *et al.*, 2000).

In addition to the PYL/RCAR ABA receptors, there is also a secondary ABA signalling pathway in *Arabidopsis* that is mediated by the chloroplast-localized ABA receptor ABAR (Fig. 1.12) (Leon *et al.*, 2013). In response to ABA, ABAR recruits WRKY transcription factors from the nucleus to the cytoplasm (Rushton *et al.*, 2012; Shen *et al.*, 2006). WRK18, WRK40 and WRKY60 repress the transcription of *ABI3*, *ABI4*, *ABF4* and other transcription factors in the nucleus (Fig. 1.12), and this repression is alleviated when WRKY transcription factors are recruited to the cytoplasm, in response to ABA (Rushton *et al.*, 2012).

The plasma membrane localized GPCR-type G proteins, GTG1 and GTG2, are a third type of ABA receptor in plants (Fig 1.12) (Cutler *et al.*, 2010; Pandey *et al.*, 2009). The G-alpha subunit GPA1 negatively regulates the GTPase activity of GTG1 and GTG2 (Cutler *et al.*, 2010), and influences chloroplast development (Zhang *et al.*, 2009a). The *gpa1* mutants were hypersensitive to sugar and ABA (Pandey *et al.*, 2006; Ullah *et al.*, 2002). The details of this ABA signalling pathway are still unclear. RGS1 is a glucose sensor in the plasma membrane that interacts with GPA1, and this interaction is modified by glucose (Chen *et al.*, 2003; Johnston *et al.*, 2007). The *rgs1* mutant is insensitive to sugar and has a green phenotype on 6% w/v glucose (Chen and Jones, 2004).

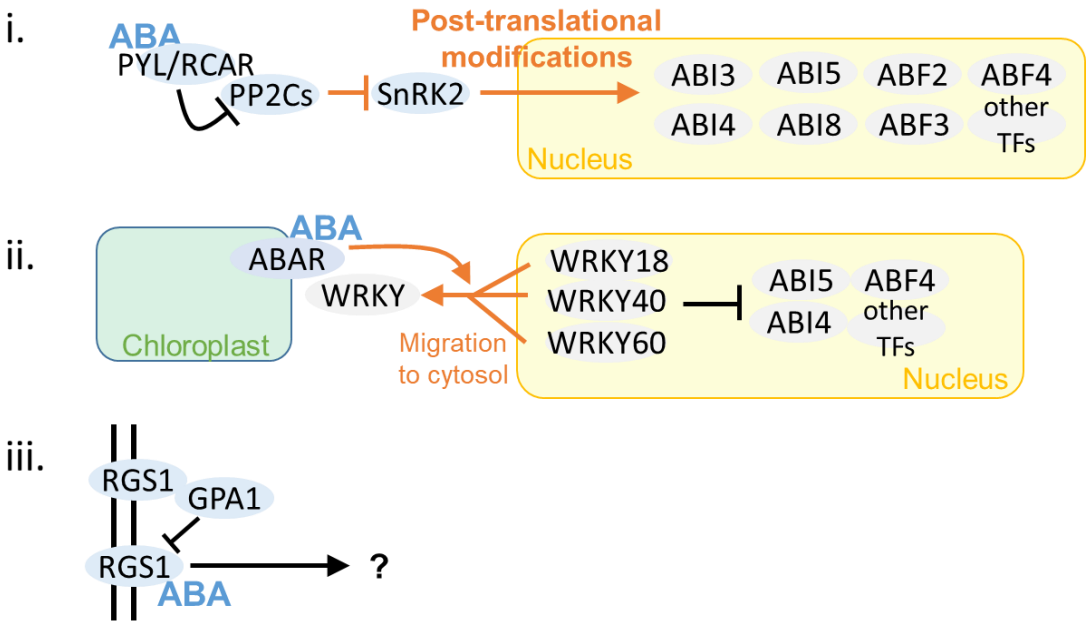


Figure 1.12 The ABA sugar signalling pathways. High intracellular sugar concentrations cause ABA to accumulate intracellularly. (i) ABA is sensed by the ABA receptors PYL/RCAR, which inhibit PP2C proteins, which in turn inhibit the activity of SnRK2 proteins. The SnRK2 proteins regulate the transcription of several ABA-responsive transcription factors including ABI3, ABI4, ABI5, ABI8, and ABF2, which regulate photosynthetic gene expression. (ii) A second ABA signalling pathway is mediated by the chloroplast-localized ABA receptor ABAR. In response to ABA, ABAR recruits WRKY transcription factors from the nucleus to the cytoplasm where they can no longer repress *ABI3*, *ABI4*, *ABF4*, or other transcription factors in the nucleus. (iii) The plasma membrane localized GPCR-type G proteins, GTG1 and GTG2, also sense and respond to ABA. The G-alpha subunit GPA1 negatively regulates the GTPase activity of GTG1 and GTG2. RGS1 is a sugar sensor of the plasma membrane that interacts with GPA1, modifying the regulation of GTG1 and GTG2. The GTG signalling pathway has not been fully characterized.

1.3.5.1 Interactions between ethylene and ABA signalling

Knockout mutation of two genes – *ETO1* and *CTR1* – that negatively regulate the ethylene pathway also caused a green sugar-insensitive phenotype in seedlings. ETO1 is a negative regulator of the ethylene biosynthesis gene *1-aminocyclopropane-1-carboxylate synthase 5* (*ACS5*) that catalyzes the first reaction in ethylene biosynthesis. Knockout of *eto1* caused a green sugar insensitive phenotype in seedlings (Zhou *et al.*, 1998). CTR1 (a.k.a. GIN4) is a MAPKKK protein that negatively regulates ethylene responsive transcription factors. Knockout of c*tr1* caused a green sugar insensitive phenotype at 6% w/v glucose (Cheng *et al.*, 2002). Further evidence for the role of ethylene in sugar signalling is provided by evaluation of the sugar response of the mutants, *ein2* and *ein3*, which were hypersensitive to glucose (Cheng *et al.*, 2002; Yanagisawa *et al.*, 2003). Evidence suggests that ethylene participates in sugar signalling by negatively regulating ABA accumulation, thereby influencing photosynthetic gene expression (León and Sheen, 2003; Salazar *et al.*, 2015; Tholen *et al.*, 2008; Zhou *et al.*, 1998).

1.3.5.2 Retrograde regulation of ABA signalling

ABI4 is intricately linked to regulation of photosynthetic gene expression in the *gun* retrograde signalling pathways (Koussevitzky *et al.*, 2007; Leon *et al.*, 2013; Sun *et al.*, 2011) The *gun* retrograde signalling pathway regulates nuclear encoded photosynthetic genes in response to defects in plastid gene expression. Defects in plastid gene expression can be caused by inhibition of plastid transcription or translation (e.g., lincomycin treatment), inhibition of tetrapyrrole biosynthesis (e.g., norflurazon treatment), altered chloroplast redox status, or by the accumulation of reactive oxygen species production. The *gun* retrograde signalling pathway regulates *ABI4* expression in response to plastid signals, and ABI4 regulates the expression of nuclear encoded photosynthetic genes (Koussevitzky *et al.*, 2007; Leon *et al.*, 2013; Sun *et al.*, 2011). The *gun* retrograde signalling pathway also regulates nuclear encoded photosynthetic genes through other ABI4-indpendent pathways (Koussevitzky *et al.*, 2007; Leon *et al.*, 2013; Sun *et al.*, 2011).

1.3.6 Trehalose metabolism and signalling

The sugar trehalose-6-phosphate (T6P) regulates many aspects of plant metabolism and development (Gómez *et al.*, 2006; Kolbe *et al.*, 2005; Paul *et al.*, 2008; Schluepmann *et al.*, 2003), by inhibiting SnRK1 in the SnRK1 sugar signalling pathway (Debast *et al.*, 2011; Delatte *et al.*, 2011; Martínez-Barajas *et al.*, 2011; Nunes *et al.*, 2013a; Zhang *et al.*, 2009b). In *Arabidopsis*, trehalose-6-phosphate (T6P) is synthesized from glucose-6-phosphate and UDP-glucose by the enzyme trehalose-6-phosphate synthase (TPS) (Fig. 1.13). The enzyme trehalose phosphate phosphatase (TPP) can then convert T6P to trehalose (Fig. 1.13). The *Arabidopsis* genome contains 11 TPS (*TPS1-11*) and 10 TPP (*TPPA-J*) homologues. In *Arabidopsis*, T6P is present at low concentrations (Delatte *et al.*, 2009) from 37 ng g^{-1} DW in whole plants to 100 ng g^{-1} DW in flowers (Müller *et al.*, 2001; Roessner *et al.*, 2000; Suzuki *et al.*, 2008; Vogel *et al.*, 2001).

T6P levels respond quickly to changes in sugar availability. Trehalose-6-phosphate levels were 27-fold lower in *Arabidopsis* seedlings that were carbon starved, compared to seedlings supplied with sucrose (Lunn *et al.*, 2006). Feeding *Arabidopsis* seedlings with trehalose also caused T6P to accumulate (Schluepmann *et al.*, 2004). TPS and TPP gene expression responded rapidly and dramatically to changes in carbon status (Liu *et al.*, 2005; Osuna *et al.*, 2007; Piippo *et al.*, 2006; Price *et al.*, 2004), in effect, controlling the T6P levels. TPS is also regulated post-translationally in response to changes in carbon status (Glinski and Weckwerth, 2005; Harthill *et al.*, 2006). Knockout of *tps1* was embryo lethal (Eastmond *et al.*, 2002; Gomez *et al.*, 2006). Overexpression of *TPS1* in *Arabidopsis* resulted in a dark green phenotype on high sugar media (≥6% w/v glucose), suggesting a link between T6P and sugar signalling (Avonce *et al.*, 2004).

T6P inhibits the SnRK1 regulatory kinases (Zhang *et al.*, 2009b). Two transgenic lines of *Arabidopsis* over-expressing *KIN10* (SnRK1) were insensitive to external application of trehalose (Delatte *et al.*, 2011). Furthermore, *Arabidopsis* lines overexpressing a bacterial TPP had very low levels of T6P, and had phenotypes identical to *KIN10* overexpressors (Wingler *et al.*, 2012). The sugar trehalose-6-phosphate (T6P) regulates photosynthetic gene expression by repressing the activity of SnRK1 (Debast *et al.*, 2011; Delatte *et al.*, 2011; Martínez-Barajas *et al.*, 2011; Nunes *et al.*, 2013a; Zhang *et al.*, 2009b).

$$\text{Glucose-6-phosphate} + \text{UDP-Glucose} \xrightarrow{\text{TPS}} \text{Trehalose-6-phosphate} \xrightarrow{\text{TPP}} \text{Trehalose} \xrightarrow{\text{TRE}} \text{Glucose}$$

Figure 1.13 Trehalose metabolism in plants. The reactions are catalyzed by the enzymes trehalose-6-phosphate synthase (TPS), trehalose phosphate phosphatase (TPP), and Trehalase (TRE). Trehalose-6-phosphate is a key sugar signalling molecule in plants.

1.4 Other sugar insensitive mutants

Aside from the three major sugar signalling pathways (Sections 1.3.2-1.3.5), other mutants have been identified with sugar insensitive phenotypes (Table 1.2). While the details of many of these pathways are unclear, the expression of each of these genes is required for photosynthetic repression by sugar.

The auxin resistant mutants, *tir1, axr1,* and *axr2* had a green sugar insensitive phenotype at 6% (w/v) glucose (Moore *et al.*, 2003). *TIR1* encodes an auxin receptor, *AXR1* encodes a subunit of RUB1 activating enzyme that influences auxin responses, and *AXR2* is a transcription factor that represses auxin-inducible genes. AAR2 and CKI1 are both involved in cytokinin signalling: *CKI1* encodes a putative plasma membrane cytokinin sensor, and *ARR2* encodes a cytokinin response regulator (Hejátko *et al.*, 2009). Transgenic plants overexpressing cytokinin type B response regulator protein (*35S::ARR2*), and transgenic plants overexpressing the cytokinin histidine kinase 1 protein (*35S:CKI1*) had green phenotypes at 6% w/v glucose (Moore *et al.*, 2003).

RGL2 encodes a DELLA transcription factor that is degraded in response to gibberellic acid. DELLA transcription factors are key negative regulators of gibberellin signalling. *SPY* encodes a glucosamine transferase that represses gibberellic acid responses by glycosylating molecules involved in Gibberellic acid (GA) signalling. SPY activates DELLA transcription factors by glycosylation (Sun, 2010). The *rgl2* and *spy* mutants had increased germination rates on 1.5% w/v glucose (Yuan and Wysocka-Diller, 2006).

Transgenic plants that overexpressed the Multiprotein bridging factor 1a protein (*35S::MBF1*) were insensitive to 5% w/v glucose (Kim *et al.*, 2007). MBF1 functions by bridging bZIP and TATA-box binding protein, enhancing the transcription of genes under the control of bZIP transcription factors.

The mutants *lin1*, *rcd1*, *fin219,* and *wox5* also had a sugar insensitive phenotype (Ramon *et al.*, 2008). However, their phenotypes differed from those of the other sugar signalling mutants. *LIN1* encodes a high-affinity nitrate transporter. The *lin1* mutant is green when grown at high sucrose and low nitrogen (Malamy and Ryan, 2001). However, the

phenotype may be due to an altered carbon:nitrogen ratio. The mutant *rcd1* is defective in programmed cell death control, and *rcd1* does not undergo programmed cell death in response to reactive oxygen species. RCD1 interacts with 21 transcription factors and regulates many genes, including chloroplast antioxidant genes (Hiltscher *et al.*, 2014). The *rcd1* mutant had reduced sensitivity to glucose, but had a non-green phenotype at 6% w/v glucose (Ahlfors *et al.*, 2004). *FIN219* encodes a jasmonate synthase that catalyzes the formation of biologically active jasmonyl-isoleucine. FIN219 is part of the phytochrome A signalling pathway and the jasmonic acid signalling pathway. The FIN219 phenotype was sensitive to sucrose, as hypocotyl elongation was increased in *fin219* mutants at 1% w/v sucrose (Hsieh *et al.*, 2000). WOX5 is a transcription factor involved in root stem cell maintenance, which is induced by turanose and auxin. The *wox5* mutants developed normally on 90mM turanose, a turanose-insensitive phenotype (Gonzali *et al.*, 2006).

1.5 Thesis objectives

As discussed in Section 1.2, typically, sucrose causes feedback inhibition of photosynthesis (Neales and Incoll, 1968; Geiger, 1976; Vonschaewen et al., 1990; Dickinson et al., 1991). The objective of this thesis was to determine why the commonly used *Arabidopsis thaliana var.* Landsberg *erecta* cell suspension culture isolated by May and Leaver (1993) exhibits a sucrose insensitive phenotype. Three hypotheses are tested :

1. The cells do not accumulate high intracellular sugar concentrations, despite the high sucrose concentration of the medium. For sugar-induced feedback inhibition of photosynthesis to occur there must be accumulation of sugars intracellularly. Exogenous sucrose treatment may not cause intracellular sugar accumulation in the sugar-insensitive cell culture, like it does in wild type seedlings.

2. There is dysfunction in a sugar sensing or sugar signalling pathway. Therefore, despite high intracellular sugar levels, there is no inhibition of photosynthesis. At high sucrose, the green cell culture may not express high levels of key sugar signalling transcription factors that are strongly upregulated in response to sugar in wild type seedlings.

3. There is dysfunction of a novel gene or genes that regulate photosynthetic gene expression in response to sugar. Comparison of the sucrose-regulated genes from the transcriptional profiles of the cell culture, to the sucrose-regulated genes from transcriptional profiles of wild type seedlings may uncover novel genes that regulate photosynthetic gene expression in response to sugar.

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1.7 Supplementary Tables

Supplemental Table S1.1. The enzymes of the Calvin-Benson-Bassham cycle.

Supplemental Table S1.2. The enzymes of the tetrapyrrole biosynthesis pathway.

Chapter 2

2 An established *Arabidopsis thaliana* var. Landsberg *erecta* cell suspension culture accumulates chlorophyll and exhibits a stay-green phenotype in response to high external sucrose concentrations

An established cell suspension culture of *Arabidopsis thaliana* var. Landsberg *erecta* was grown in liquid media containing 0 to 15% (w/v) sucrose. Exponential growth rates of about 0.40 d^{-1} were maintained between 1.5 to 6% (w/v) sucrose, which decreased to about 0.30 d^{-1} between 6 and 15% (w/v) sucrose. Despite the presence of external sucrose, cells maintained a stay-green phenotype at 0 to 15% (w/v) sucrose. Sucrose stimulated transcript levels of genes involved in the chlorophyll biosynthetic pathway (*ChlH*, *ChlI2*, *DVR*). Although most of the genes associated with photosystem II and photosystem I reaction centers and light harvesting complexes as well as genes associated with the cytochrome b6f and the ATP synthase complexes were downregulated or remained unaffected by high sucrose, immunoblotting indicated that protein levels of PsaA, Lhcb2 and Rubisco per gram fresh weight increased as a function of external sucrose concentration. The green cell culture was photosynthetically competent based on light-dependent, CO_2-saturated rates of O_2 evolution as well as Fv/Fm and P700 oxidation. Similar to *Arabidopsis* WT seedlings, the suspension cells etiolated in the dark but remained green in the light. However, the exponential growth rate of the cell suspension cultures in the dark (0.45 ± 0.07 d^{-1}) was comparable to that in the light (0.42 ± 0.02 d^{-1}). High external sucrose levels induced feedback inhibition of photosynthesis as indicated by the increase in excitation pressure measured as a function of external sucrose concentration. Regardless, the cell suspension culture still maintained a stay-green phenotype in the light at sucrose concentrations from 0 to 15% (w/v) due, in part, to a stimulation of photoprotection through nonphotochemical quenching. The stay-green, sugar-insensitive phenotype of the cell suspension contrasted with the sugar-dependent, non-green phenotype of *Arabidopsis* Landsberg *erecta* WT seedlings grown at comparable external sucrose concentrations. It appears that the commonly used *Arabidopsis thaliana* var. Landsberg *erecta* cell suspension culture has undergone

significant genetic change since its initiation in 1993. We suggest that this genetic alteration has inhibited the sucrose sensing/signalling pathway coupled with a stimulation of chlorophyll a accumulation in the light with minimal effects on the composition and function of its photosynthetic apparatus. Therefore, caution must be exercised in the interpretation of physiological and biochemical data obtained from experimental use of this culture in any comparison with wild-type *Arabidopsis* seedlings.

2.1 Introduction

Cell suspension cultures are often used as a model system in plant biology because they can be easily manipulated and grown under uniform conditions. Established cell suspension lines such as the T87 cell culture of *Arabidopsis thaliana* var. Columbia (Axelos, 1992), and the *Arabidopsis thaliana* var. Landsberg *erecta* cell culture used in the present study (May and Leaver, 1993) have been exploited for more than two decades for the study of the physiology and biochemistry of *Arabidopsis* (Supplemental Table S2.1). Cell suspension cultures are typically initiated on a medium containing high concentrations of both sucrose and auxin, as this medium provides the highest efficiency of callus formation (Mathur and Koncz, 1998; Mustafa et al., 2011). This medium induces dedifferentiation of cells, which has specific effects on chloroplasts. Dedifferentiation initially results in the loss of thylakoids and grana, and after some time the chloroplasts change shape and degenerate (George *et al.*, 2008). Although these de-differentiated cells contain plastids with some under-developed lamellar structures, chlorophyll levels are minimal compared to those of mesophyll cells of leaves (George *et al.*, 2008). This suppression of chlorophyll biosynthesis and chloroplast biogenesis is considered to be a normal plant response to the high external concentrations of sucrose and auxins in the growth medium (Hüsemann, 1995). Suspension cultures initiated under these conditions usually have a light green or non-green phenotype (Mustafa et al 2011). The establishment of green photoautotrophic cell suspension cultures from these cells usually requires additional treatments over a prolonged period of time, and even under these conditions, it is not always successful (Widholm, 1992). One would assume that because these cells originate from plant tissue that establishment of photoautotrophic cell cultures would be straightforward. However, this conversion from heterotrophic to

photoautotrophic growth appears to require selection over long periods of time (Widholm, 1992).

Feedback inhibition of photosynthesis by sugars represents a plant's response to optimize the use of resources, such as carbon and nitrogen, as they grow (Paul and Pellny, 2003). In vegetative tissue, the accumulation of sucrose inhibits photosynthesis due to end product accumulation and is generally referred to as feedback inhibition of photosynthesis (Krapp *et al.*, 1993; Martins *et al.*, 2013; Sharkey *et al.*, 1986; Smith and Stitt, 2007; Stitt *et al.*, 1987; Stitt, 1992). In most plants, prolonged exposure to sucrose applied exogenously results in an inhibition of photosynthesis which is associated with an inhibition of chlorophyll biosynthesis and photosynthetic gene expression (Sheen, 1994; Rolland et al., 2006; Ramon et al. 2008). For example, when *Arabidopsis thaliana* seedlings are grown in a high sugar medium (≥ 6% w/v), photosynthesis is completely repressed, resulting in leaves with a white phenotype (Zhou *et al.*, 1998). It is generally accepted that these changes in phenotype as well as photosynthetic rates occur as a consequence of the shift from photoautotrophy to heterotrophy when a supply of carbon, such as sucrose, is abundantly available.

Our historical research interest has been focussed on chloroplast redox regulation of photosynthetic performance in response to acclimation to environmental stress in cyanobacteria, green algae and terrestrial plants (Hüner et al., 1993, 1998; 2013; Bode et al. 2016). In contrast to whole plant studies, plant cell cultures potentially represent a convenient and simpler model system to investigate the cellular and molecular basis of plant responses to environmental stress (Mustafa et al. 2011). Consequently, an *Arabidopsis thaliana* Landsberg *erecta* cell suspension culture originally isolated by May and Leaver in 1993 was obtained with the goal of elucidating the role(s) of chloroplast retrograde regulation in retailoring the structure and function of the photosynthetic apparatus in response to irradiance and low temperature stress.

To our surprise, we discovered that the *Arabidopsis thaliana* Landsberg *erecta* cell suspension culture remained green not only at 3% (w/v) sucrose but also at higher sucrose concentrations. Thus, this suspension culture appeared to exhibit an abnormal

response to the presence of external sucrose, a component generally required in the growth medium for the maintenance and growth of plant cell suspension cultures (Mustafa et al., 2011). In the present study, we provide an initial physiological and biochemical characterization of the abnormal phenotype and the photosynthetic response of this established *Arabidopsis* Landsberg *erecta* cell suspension culture to varying growth irradiance and external sucrose concentrations. We note with interest that this suspension culture has been used as a model system for more than 140 published studies (Supplementary Table S2.1). Although photosynthesis and respiration are inhibited with increasing sucrose concentrations, this culture continues to grow, accumulates chlorophyll and maintains a stay-green phenotype at external sucrose concentrations as high as 15% (w/v). Furthermore, we report that sensitivity to sucrose-induced feedback inhibition of photosynthesis in this culture has been uncoupled from the expected sucrose-induced changes in phenotype. We suggest that prolonged maintenance of this established cell culture line *in vitro* has resulted in an inhibition of the sucrose sensing/signalling pathways that regulate photosynthetic gene expression and phenotype (Sheen 1994; Rolland et al. 2006; Ramon et al. 2008).

2.2 Methods

2.2.1 Growth of the cell suspension cultures and seedlings

The cell suspension culture of *Arabidopsis thaliana* var. Landsberg *erecta* (May and Leaver, 1993) was grown in liquid Murashige and Skoog medium (MS, Sigma M5524) supplemented with 3% (w/v) sucrose, 0.5 mg/L naphthaleneacetic acid (NAA), 47 μg/L kinetin, 0.1X of Vitamin B solution, and an additional 2.5 mM of K_2HPO_4. The cell cultures were maintained on an orbital shaker (150 rpm) in an incubator set at 25^{o}C and illuminated with 30 μmol photons m^{-2} s^{-1} of continuous white light. The culture was sustained in exponential growth phase by diluting the 75 mL batch cultures (1:15) with fresh media every 7 days. This subculturing routine ensured cells were always in log phase of growth. For sucrose treatments, the MS medium was prepared with the desired concentration of sucrose. Growth curves for suspension cultures were generated by making daily measurements of OD_{750}, chlorophyll a (Chl a) levels and fresh weight. To quantify Chl a, the tissue was ground in liquid nitrogen and the pigments were extracted

with 100% acetone. The absorbance at 663, 645 and 750 nm was used to calculate the Chl a concentration according to the equations of Arnon (1949).

Arabidopsis thaliana var. Landsberg *erecta* (Ler-0) seeds were obtained from The *Arabidopsis* Information Resource (TAIR). The seedlings were grown on MS medium, solidified with 0.8% (w/v) agar, supplemented with sucrose, 0.5 mg/L naphthaleneacetic acid (NAA), 47 µg/L kinetin, 0.1X of Vitamin B solution, and an additional 2.5 mM of K_2HPO_4. The sucrose concentration was varied for each treatment. The seeds were treated with a solution containing 20% (v/v) bleach and 0.1% (v/v) Tween for 10 minutes, and then rinsed five times with sterile water. The seeds were cold treated by placing the plates in the dark at 4^{o}C for 2 days and then shifted to growth at 25^{o}C with a 8h light:16h dark short day cycle at 250 µmol photons m^{-2} s^{-1}. All seedlings were sampled at mid-day.

2.2.2 Transmission electron microscopy

Samples of cells and leaf tissue were fixed in 2.5% (w/v) glutaraldehyde in 0.02M phosphate buffer for 2 h, and then washed three times with 0.02 M phosphate buffer. Subsequently, the samples were fixed for 1h in 2% (v/v) osmium tetraoxide in 0.02 M phosphate buffer, washed three times with 0.02 M phosphate buffer, and left in the phosphate buffer overnight. The tissue was then washed 4 times with ddiH_2O, stained with 5% (w/v) uranyl acetate for 30 minutes, and then washed again twice with ddiH_2O. Subsequently, the sample was mixed 1:1 with 2% (w/v) liquid agarose and left to solidify. The agarose was cut into small cubes and dehydrated through an ethanol series of 20% (v/v) to 100% ethanol and then infiltrated with Spurr's plastic (2:1 ethanol:Spurr's plastic for 7h, 1:1 overnight, 1:2 for 7 hours, 100% Spurr's plastic overnight, and then 100% Spurr's plastic for 7h). The embedded samples were cut into small pieces and then sectioned with an ultramicrotome (Ultracut-E, Reichert-Jung). Images were then captured using a Philips CM 10 Transmission Electron Microscope.

2.2.3 Confocal microscopy

Confocal images of live cells were taken with a Zeiss LSM5 Duo microscope (Carl Zeiss AG, Oberkochen Germany) at the Biotron Imaging Facility (University of Western

Ontario, London, Canada). For the imaging of chlorophyll autofluorescence, a 488 nm argon laser was used for excitation and fluorescence was detected at 630 to 690 nm.

2.2.4 Gas exchange

Gas exchange measurements were made using a DW2 oxygen electrode with a LH11/R light probe (Hansatech Instruments, Norfolk, England). Cells were suspended in 1.5 mL of fresh MS medium supplemented with 5 mM sodium bicarbonate and exposed to an irradiance ranging from 0-800 μmol photons m^{-2} s^{-1} for 2 min at each irradiance while rates of oxygen evolution were measured.

2.2.5 Chlorophyll a fluorescence

A XE-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany) was used for Chl a fluorescence measurements on the cell suspension culture. The Xe-PAM was equipped with an optical unit (ED-101US/M), a photodiode detector unit (XE-PD) consisting of a 10x10 mm PIN-photodiode (Hamamatsu) and a pulse preamplifier and a PAM data acquisition system (PDA 100). Prior to measurements, samples were dark adapted for 15 minutes and then sodium bicarbonate was added to a final concentration of 5 mM. A xenon flash lamp (XE-MF, Hamamatsu xenon discharge lamp, Type L4641) with a BG39 blue-glass filter (Schott) provided the modulated excitation light (650 nm, 0.12 μmol photons m^{-2} s^{-1}) used to measure basal fluorescence (F_O). A saturating flash (800 ms, 2800 μmol photons m^{-2} s^{-1}) was then applied with a white actinic light to measure dark-adapted maximal fluorescence (F_M). An actinic light (0-500 μmol photons m^{-2} s^{-1}) was then applied and F_M', F_S and F_O' were measured as described in van Kooten and Snel (1990).

PSII excitation pressure was estimated as 1-qP, where qP = $(F'_m-F_S)/ (F'_m-F'_o)$ (Hüner et al. 1998; Rosso et al. 2009; Bode et al. 2016). Energy partitioning of light energy absorbed by PSII was calculated as follows: (A) The fraction utilized by PSII photochemistry $\Phi_{PSII} = 1 - F_S/F_M'$, (B) the fraction dissipated by non-photochemical quenching $\Phi_{NPQ} = F_S/F_M' - F_S/F_M$, and (C) the fraction dissipated by constitutive energy dissipation and fluorescence $\Phi_{f,D} = F_S/F_M$.

2.2.6 Redox state of P700

The far-red light-induced oxidation of P700 was determined *in vivo* on dark-adapted cells under ambient O_2 and CO_2 conditions at 20ºC using a PAM-101 modulated fluorometer. The procedure followed was from Klughammer and Schreiber (1991) as described in detail by Ivanov et al. (1998). The fluorometer was equipped with a dual wavelength emitter-detector (ED-P700DW), and a PAM-102 unit (Heinz Walz, Germany). Far red light was provided by an FL-101 light source (max =715 nm, 10 W m^{-2}, Schott filter RG 715) to fully oxidize P700 to $P700^+$. Single turnover (half-peak width of 14 μs) and multiple turnover flashes (50 ms) were then applied with XST-103 and XMT-103 power control units to reduce $P700^+$. From these traces, $\Delta A_{820-860}$ gave a reliable measure of the functional activity of PSI.

2.2.7 77K chlorophyll fluorescence emission spectra

Corrected low temperature (77K) Chl fluorescence emission spectra were collected using a PTI QM-7/2006 spectrofluorometer (Photon Technology International, South Brunswick, NJ, USA) equipped with a double monochromator, R928P red-sensitive photomultiplier tube (Hamamatsu Photonics, Shizuoka-ken, Japan) and a liquid nitrogen cell. The PSI/PSII ratios were calculated from the corresponding spectra by dividing the area of the PSI fluorescence peak (λ_{emPSI} = 735 nm) by the area of the PSII peak (λ_{emPSII} = 680 nm) after background correction. The background was corrected by subtraction of the spectrum from the frozen medium without cells.

2.2.8 Transcript abundance from RNA-Seq

Arabidopsis thaliana cell suspension cultures were grown at external sucrose concentrations that varied from 0 to 9% (w/v) as well as 9% (w/v) mannitol with three biological replicates per treatment. RNA was extracted from each replicate following the Qiagen RNeasy Plant Mini Kit. RNA quality was assessed with a Nanodrop 2000 spectrophotometer and RNA integrity was assessed by size distribution analysis with an Agilent 2100 Bioanalyzer. RNA integrity number (RIN) scores of all samples were higher than 9.0. The sample library was prepared with the Illumina TruSeq Stranded Total RNA Sample Preparation Kit with Ribo-Zero Plant. RNA sequencing was

performed on the Illumina HiSeq2000 using 75-cycle paired-end protocol with 5 samples per lane (approx. 40M reads per sample).

Overall read quality was checked using FASTQC v.0.10.1. Sequencing adaptors were trimmed using cutadapt version 1.4.2. The sequence “AGATCGGAAGAGC” was used for both adaptors and only the sequences with overlap length longer than 7, with the adaptor sequence, were modified. Trimmed sequences with a length shorter than 20 were discarded. The trimmed sequences were aligned against *Arabidopsis thaliana* genome Ler-0 (downloaded from http://mus.well.ox.ac.uk/19genomes/) by Tophat2 version 2.0.11 with Bowtie2 version 2.2.2 and samtools version 0.0.19. One hundred and thirty used for the mean inner distance between mate pairs (mate-inner-dist). Two hundred was chosen to be the minimum intron size (min-intron-length). Transcripts were assembled using the accepted hits for each sample from the previous step as input using Cufflinks version 2.2.1. The reference annotation file from the 19 genomes of *Arabidopsis thaliana* (consolidated_annotation.Ler_0.gff3) was used as a guide, and output included all reference transcripts as well as any novel genes and isoforms that were assembled. An upper-quartile normalization method was used. Cuffdiff was used to calculate transcript abundances from RNA-seq and to test statistical significance between treatments.

2.2.9 Immunoblotting

Immunoblotting was used to detect subunits of key photosynthetic complexes. *Arabidopsis* tissue was ground using a mortar and pestle in 100% acetone. The acetone and mortar and pestle were pre-chilled on ice. After centrifugation at 16000 g the pellet was resolubilized in solubilization buffer [4% (w/v) SDS, 0.3M Tris, pH 7.8]. Prior to loading, samples were mixed with an equal volume of loading buffer [13% (v/v) glycerol, 0.5% (w/v) bromophenol blue, 2% (v/v) β-mercapto-ethanol], heated to 80^{o}C for 10 minutes and then centrifuged at 16000 g for 5 minutes. Eight μg of Chl were loaded per lane on a 15% (w/v) polyacrylamide gel (6M urea, 0.66M Tris, pH 8.8). In addition to the cell suspension culture, as a control, a thylakoid sample was prepared and processed as described above from leaves of *Arabidopsis thaliana* var. Columbia plants grown in soil at 20^{o}C and 250 μmol photons m^{-2} s^{-1} on a 8h light : 16h dark short day cycle.

Nitrocellulose membranes were blocked overnight at 4°C in Tris-buffered saline (TBS) (20mM Tris (pH 7.5), 150mM NaCl) containing 5% (w/v) milk powder and 0.01% (v/v) Tween 20. The polypeptides separated by SDS-PAGE were transferred electrophoretically to the nitrocellulose membranes (Bio-rad, 0.2μm pore size) at 100V for 1h on ice. The membranes were blocked overnight at 4^{o}C in Tris-buffered saline containing milk powder and Tween (20 mM Tris, 150 mM NaCl, 5% (w/v) milk powder, 0.01% (v/v) Tween 20, pH 7.5). Membranes were probed with primary antibodies obtained from Agrisera: Lhcb1 (1:2000 dilution), Lhcb 2 (1:2000 dilution), D1 (1:5000 dilution); PsaA (1:1000 dilution); PsaB (1:2000 dilution) PsaD (1: 10000 dilution). Additionally, an RbcL primary antibody was generated by Dr. Norman Hüner (Wilson *et al.*, 2003). Membranes were then incubated with a horseradish peroxidase secondary antibody (Sigma, 1:10000 dilution). After washing the membranes, bound secondary antibody was visualized with the Amersham Biosciences ECL detection system (GE Healthcare) exposed on X-ray film (Fuji Film). The immunoblots were quantified using ImageJ by comparing background-subtracted band intensities (Table S2.2). Chlorophyll-normalized protein levels were converted to a FW basis using Chl a FW^{-1} measurements.

2.2.10 HPLC analysis of pigments

Pigments were separated and quantified by HPLC following the method of Gilmore and Yamamoto (Gilmore and Yamamoto, 1991) with minor modifications (Gray *et al.*, 1996). Briefly, pigments were extracted from leaf and cell culture samples by homogenization in 100% acetone containing 0.3 mg mL^{-1} $CaCO_3$ in dim light. Pigments were separated using a reverse-phase column (5-μm particle size; 25 × 0.46 cm i.d.; CSC-Spherisorb ODS-1, Beckman) and detected with a diode-array detector (module 168, Beckman). Twenty microliters of sample was injected using a sample-injection valve with a 20 μL loop (model 210A, Beckman). The solvent module (System Gold 126, Beckman) was programmed to begin with a 148:22:7 (v/v) mixture of acetonitrile:methanol:0.1M Tris-HCl (pH 8.0) for 6 minutes followed by a 2 minute linear gradient to a 17:8 (v/v) mixture of methanol:ethylacetate that continued for the final 4 minutes. The total run time was 12 mins with a flow rate of 2 mL min^{-1}. The epoxidation state of xanthophyll pigments were

calculated from HPLC quantifications of the xanthophyll pigments as described in detail previously (Krol *et al.*, 1999).

2.2.11 Enzymatic assay of sugar content

Cells were filtered and a sample of the medium was collected for immediate analysis. Enzymatic assays were adapted for microplate analyses from (Kunst, 1988). A solution containing hexokinase (HXK) and glucose-6-phosphate dehydrogenase (G6PDH) was prepared in a Tris buffer (1.0 U/mL HXK, 1.0 U/mL G6PDH, 0.1M Tris buffer pH 7.8, 1.5 mM NAD, 1.0 mM ATP, 10 mM $MgCl_2$, 0.5 mg/mL BSA).

To measure sucrose, 15 µL of sample was pre-treated for 1 hour with 15 µL of an invertase solution (0.8 M Acetate buffer pH 4.5, 150 U/mL Invertase). Three hundred microliters of the HXK/G6PDH solution was then added to the well and the absorbance at 340 nm was measured after 15 minutes. To measure glucose, a separate well was used. Thirty microliters of sample was added to 300 µL of HXK/G6PDH solution and the absorbance at 340 nm was measured after 15 minutes. Sample blanks and reagent blanks were prepared with ddiH_2O. Standard curves were prepared and measured for each of the sugars and used to determine the sugar concentrations in the experimental samples.

2.3 Results

2.3.1 The effects of sucrose concentration and light on cell phenotype

The *Arabidopsis* suspension cells maintained a dark green phenotype at all external sucrose concentrations tested from 3 to 15% (w/v) (Fig. 2.1A) and Chl a levels, measured on a per g FW basis, increased with an increasing external sucrose concentration in the growth media (Fig. 2.1B, closed symbols). Although the suspension culture exhibited a dark green phenotype when grown in the light, the suspension culture was etiolated in the dark and exhibited a yellow phenotype (Fig. 2.1C, dark). The absence of chloroplasts in etiolated cells (Fig. 2.1D, dark) and their presence in cells grown in the light (Fig. 2.1D, light) was confirmed by confocal microscopy. This etiolated phenotype was reversible since cells grown in darkness for several weeks quickly regained their dark green

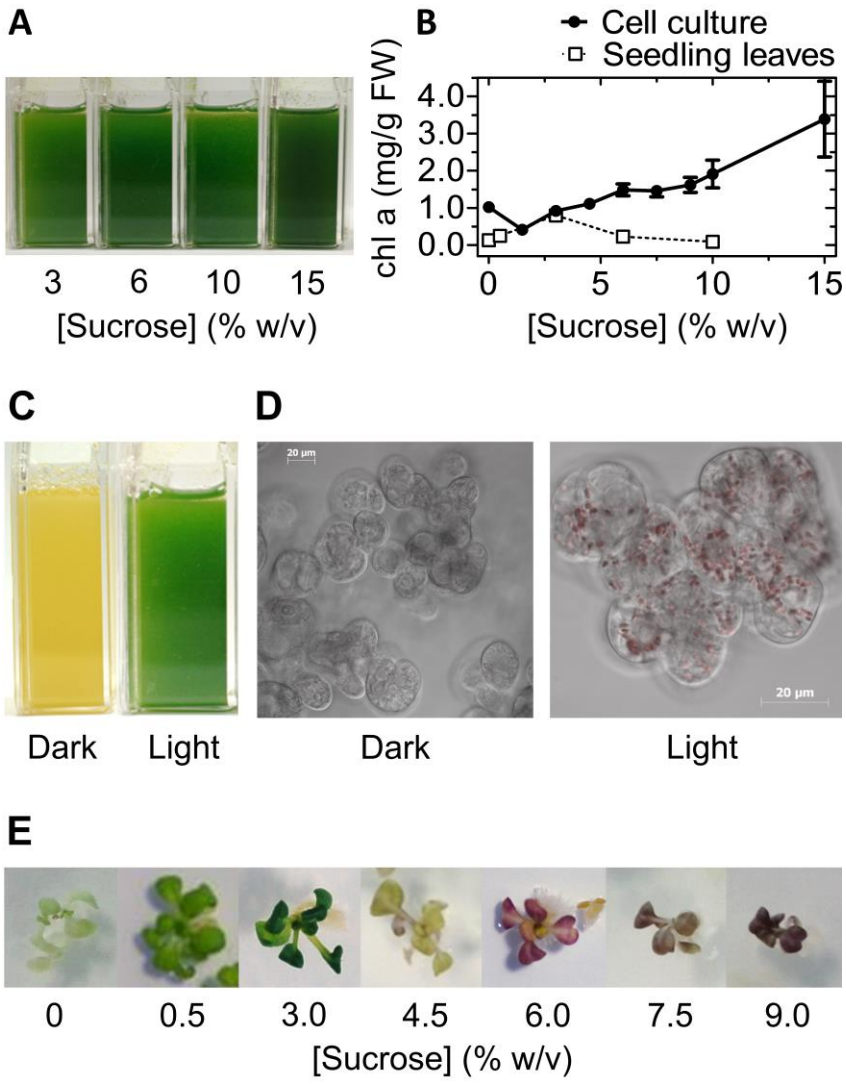

Figure 2.1 Phenotypes of the *Arabidopsis thaliana* var. Landsberg *erecta* cell suspension culture contrasted with the phenotypes of Landsberg *erecta* (Ler-0) seedling leaves in response to sucrose. (A) Cell suspension cultures grown in MS medium containing 3, 6, 10 and 15% (w/v) sucrose. These are qualitative data since the different cuvettes do not necessarily have precisely equal cell densities. (B) Chl a content of the cell suspension culture and leaves of WT seedlings measured on a per gm fresh weight of cells and grown in MS medium containing 0-15% (w/v) sucrose. n = 3 and error bars represent standard error. (C) Phenotypes of light and dark grown cell cultures. (D) Confocal microscope images of cells either grown in the dark or the light. (E) *Arabidopsis thaliana* Lansberg *erecta* seedlings grown aseptically in MS medium containing 0, 0.5, 3.0, 4.5, 6.0, 7.5, and 9.0% (w/v) sucrose solidified with 0.8% (w/v) agar.

phenotype when shifted back to the light (Fig. 2.1C, light). The dark green phenotype of the cells grown under high sucrose (≥ 6% w/v) is regarded as a sugar-insensitive phenotype since high external sugar availability typically represses chlorophyll accumulation in *Arabidopsis* (Laby *et al.*, 2000; Zhou *et al.*, 1998).

As a positive control, we examined the response of WT *Arabidopsis thaliana* var. Landsberg *erecta* (Ler-0) seedlings, from which the suspension culture was originally derived (May and Leaver 1993), to external sucrose concentrations. In contrast to the suspension cultures, the WT seedlings exhibited a decrease in Chl a per g FW with increasing sucrose above 3% (w/v) (Fig. 2.1B open symbols). At 6% (w/v) sucrose, the cells exhibited 7-fold higher levels of Chl a than the leaves of seedlings, and at 10% (w/v) sucrose, Chl a levels were 18-fold higher in the suspension cells than WT leaves (Fig. 2.1B). Furthermore, *Arabidopsis* WT seedlings grown at 0% to 9% (w/v) sucrose displayed a change in phenotype from dark green at 3% (w/v) sucrose to non-green at sucrose concentrations of 6% (w/v and higher (Fig. 2.1E), which represents a typical phenotypic response of *Arabidopsis thaliana* to high external sucrose concentrations (Laby *et al.*, 2000; Zhou *et al.*, 1998).

2.3.2 The effects of sucrose concentration on cell and chloroplast ultrastructure

The cell suspension cultures grown in 3% (w/v) sucrose were composed primarily of small groups as well as single green cells (Fig. 2.2A). Electron microscopy was used for a more detailed examination of chloroplasts within the cells (Fig. 2.2B) using a mesophyll leaf chloroplast for comparison (Fig. 2.2C). The electron micrographs indicated that chloroplasts from the cell suspension culture were intact and appeared to exhibit decreased granal stacking as well as more distended thylakoids (Fig. 2.2B) compared to leaf mesophyll chloroplasts (Fig. 2.2C).

Confocal images of the cells from the suspension culture were obtained for cells grown in media containing varying sucrose concentrations from 3 to 15% (w/v) sucrose (Fig. 2.2D-G). The confocal images showed an increasing chlorophyll fluorescence intensity

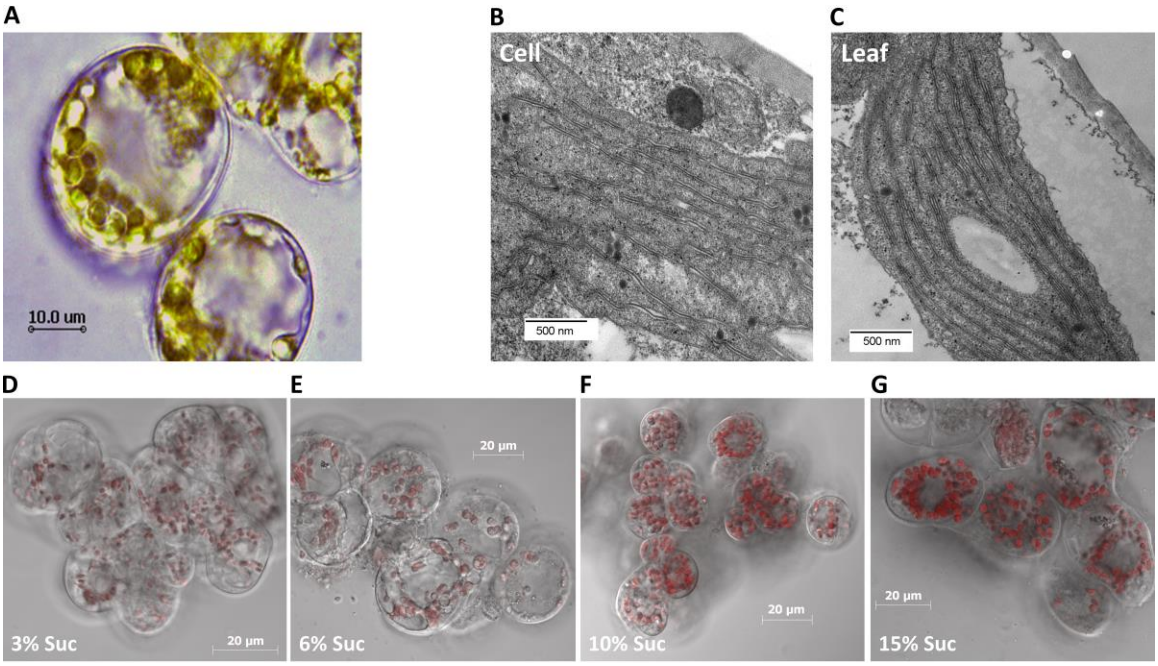

Figure 2.2 Microscope images of the *Arabidopsis* cell suspension culture. The cell suspension culture was maintained in exponential growth phase at 25°C in Murashige and Skoog (MS) medium and illuminated with 30 µmol photons m^{-2} s^{-1} of continuous light. (A) Light microscope images of a representative cell grown in MS medium containing 3% (w/v) sucrose. (B) Electron microscopy image of a chloroplast from the cell suspension culture. (C) Electron microscope image of a chloroplast from an *Arabidopsis* seedling leaf. (D-G) Confocal microscopy images of small clusters of cells from cultures grown in MS medium containing 3, 6, 10 and 15% (w/v) sucrose.

emanating from the many chloroplasts within each cell (Fig. 2.2D-G). The number of fluorescing chloroplasts per biovolume appeared to increase with increased sucrose concentrations (Fig. 2.2D-G), which is consistent with the observed sugar insensitive phenotype illustrated in Fig. 1A and the increased Chl a content on a fresh weight basis (Fig. 2.1B, closed symbols). Since normal cell structure was observed regardless of the external sucrose concentration, we concluded that aberrant cell and chloroplast ultrastructure could not account for the sugar insensitive phenotype (Fig. 2.2D-G).

2.3.3 The effects of carbon source, sugar concentration, and light on exponential growth rates

Preliminary experiments showed that growth rates determined by OD_{750} were similar to those obtained from fresh weight and chlorophyll a concentrations (Supplemental Fig. S1). Optimal growth rates for the cell suspension culture were achieved when the medium contained 3-6% (w/v) sucrose (Fig. 2.3A). At 3% (w/v) sucrose, the growth rate was 0.42 ± 0.02 d^{-1}. Cells grown in media containing 7.5 to 15% (w/v) sucrose showed reduced growth rates compared to 3% (w/v) sucrose (Fig. 2.3A). This decline was not simply due to osmotic pressure since cells grown in 3% (w/v) sucrose supplemented with 7% (w/v) mannitol did not exhibit significantly reduced growth rates compared to the treatment at 3% (w/v) sucrose (Fig. 2.3B). However, minimal cell growth was detected in the presence of 10% (w/v) mannitol alone (Fig. 2.3B).

Since the disaccharide, sucrose, is composed of glucose and fructose, we tested whether these monosaccharides could maintain growth by growing cells in MS medium supplemented with either 3% (w/v) glucose or 3% (w/v) fructose (Fig. 2.3C). Growth rates of cells grown in the presence of fructose were not significantly lower than cells grown in 3% (w/v) sucrose (Fig. 2.3C). However, growth of cells in the presence 3% (w/v) glucose inhibited growth by 57% compared to sucrose at the same concentration (Fig. 2.3C).

No growth was detected when cells were grown in a sugar-free medium where ambient CO_2 was the only carbon source (Fig. 2.3A). The growth rate in the absence of sucrose was not negative however, and the green cells rapidly resumed growth when shifted back

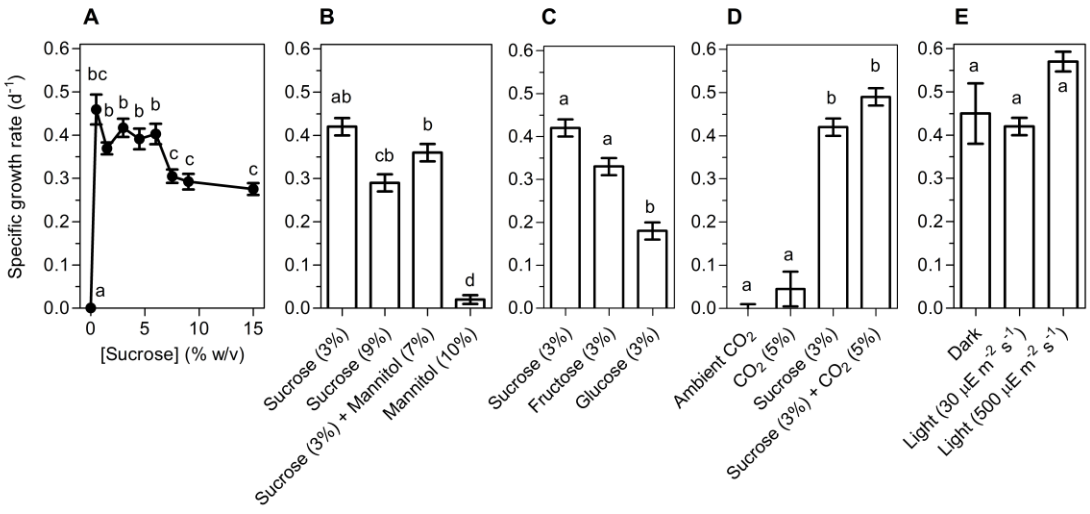

Figure 2.3 Specific growth rates of the *Arabidopsis* cell suspension culture in response to (A) sucrose concentration, (B) mannitol-induced osmotic stress, (C) sugar type, (D) CO_2 supplementation, and (E) light. All of the cell suspension cultures were kept in exponential growth phase at 25^{o}C in Murashige and Skoog (MS) medium and growth rates were determined based on absorbance at 750 nm. (A-D) The cell suspension cultures were illuminated with 30 µmol photons m^{-2} s^{-1} of continuous light (E) The cell suspension cultures were grown in the dark and in the light at 30 µmol photons m^{-2} s^{-1} and 500 µmol photons m^{-2} s^{-1}. Results were averaged and error bars represent standard error ($n \geq 3$). One-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$).

to a sucrose-containing medium. Neither bubbling the 3% (w/v) sucrose medium with 5% CO_2 (Fig. 2.3D) nor supplementing a sucrose-free medium with 5% CO_2 significantly enhanced growth rates (Fig. 2.3D). Therefore, we conclude that growth rates were not CO_2-limited during growth under the various sucrose concentrations employed.

Surprisingly, cells grown in the dark exhibited an exponential growth rate (0.45 ± 0.07 d^{-1}) that was comparable to cells grown in the light at 30 μmol photons m^{-2} s^{-1} (0.42 ± 0.02 d^{-1}) (Fig. 2.3E). A higher light intensity of 500 μmol photons m^{-2} s^{-1} did not significantly improve growth rates (Fig. 2.3E).

2.3.4 Sucrose utilization

During growth in the light, cells grown at 1.5 to 6% (w/v) sucrose imported extracellular sucrose at a rate of 0.12-0.16 g suc · g FW^{-1} · day^{-1} (Table 2.1). However, cells grown at sucrose concentrations of 7.5% (w/v) or higher exhibited the greatest uptake rates between 0.24 and 0.35 g suc · g FW^{-1} · day^{-1}. This increase in sucrose uptake at high sucrose concentrations coincided with a decrease in growth rate (Fig. 2.3A). While cells initially grew well at sucrose concentrations of 0.5% (w/v), growth of the culture was arrested at day 5, before the end of the typical 7-day subculture period. Thus, growth arrest at 0.5% (w/v) sucrose appeared to occur when sucrose was depleted from the medium.

2.3.5 The effects of sucrose concentration on the structure and function of the photosynthetic apparatus

The light saturated rates of gross photosynthesis was 220 ± 20 μmol O_2 evolved hr^{-1} mg Chl a^{-1} for cells grown at 3% (w/v) sucrose (Table 2.2). Increasing the external sucrose concentrations resulted in a 70% inhibition of light saturated rates of photosynthesis and a concomitant 67% inhibition of respiration (Table 2.2). This inhibition by elevated sucrose levels was correlated with a 40% inhibition of exponential growth rates (Fig. 2.3A).

Table 2.1 Rate of sucrose uptake from the medium by the *Arabidopsis* cell suspension cultures during exponential growth in batch culture. In triplicate, flasks containing 0 to 9% (w/v) sucrose were inoculated with the cell suspension culture (5 mg FW/mL). The batch cultures were then grown at 30 µmol photons m^{-2} s^{-1} continuous light for 7 days. The sucrose content of the medium was assayed enzymatically to calculate uptake rates using the formula d[Suc]/dt = -k*FW. The cultures were sub-cultured weekly for four weeks and uptake rates were determined each week. The table presents the average uptake rate of batch cultures grown at 0-9% (w/v) sucrose over 4 weeks of exponential growth. n = 12 and error bars represent SE. One-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$).

[Sucrose] (%w/v)	Sucrose Uptake Rate (g Suc · g FW^{-1} · day^{-1})
0%	0.008 ± 0.004 a
1.5%	0.15 ± 0.03 b
3.0%	0.12 ± 0.01 b
4.5%	0.14 ± 0.01 b
6.0%	0.16 ± 0.02 b
7.5%	0.24 ± 0.04 bc
9.0%	0.35 ± 0.05 c

To elucidate the basis of this inhibition of light saturated rates of photosynthesis, the polypeptide composition of the photosynthetic apparatus of the cell suspension cultures were assessed by immunoblots used to probe for the abundance of the following key thylakoid components of photosynthetic electron transport as well as the Calvin Benson cycle: D1, a core subunit of PSII reaction centres, PsaA, a core subunit of photosystem I reaction centres, Lhcb2, a chloroplhyll-a/b light harvesting protein of PSII, and RbcL, the large subunit of Rubisco, the stromal CO_2 fixing enzyme (Fig. 2.4). Chloroplast polypeptides isolated from the leaves of WT *Arabidopsis thaliana* var. Landsberg *erecta* (Ler-0) seedlings were used as a reference (Fig. 2.4, leaf). All of these key thylakoid and stromal proteins were present in cell suspension cultures. Increasing sucrose concentrations only caused a small decrease in the chlorophyll-normalized levels of D1, PsaA and Rubisco and a minor increase in Lhcb2 (Fig. 2.4). On a fresh weight basis, increasing sucrose caused a 50-150% increase in the levels of D1, PsaA, Lhcb2, and Chlorophyll fluorescence emission spectra generated at 77K (Fig. S2.2) were used to examine the PSI:PSII ratio of cells grown as a function of increasing sucrose concentration (Table 2.3). As expected, two emission bands associated with PSII were detected at approximately 684 and 691nm as well as the PSI emission maximum at 735nm. However, higher sucrose concentrations generally induced a higher PSI:PSII ratio relative to growth in the absence of sucrose (PSI:PSII = 2.7 ± 0.1) (Table 2.3). This is consistent with the differential sensitivity of D1 versus the PsaA content as a function of external sucrose concentration (Fig. 2.4). Furthermore, the PSI:PSII ratio was light dependent (Table 2.3). The dark-adapted cells had a higher PSI:PSII ratio than those that were kept in the light, which indicates that the green cells are in state 2 in the dark and are able to adjust energy distribution between PSII and PSI as indicated by the light dependent decrease in the PSI:PSII ratio (Table 2.3).

Compared to leaves, the cell cultures grown at 3% (w/v) sucrose had lower levels of D1, Lhcb2, PsaA and Rubisco per unit chlorophyll (Fig. 2.4). Regardless of the polypeptide composition, sucrose concentrations between 3-10% (w/v) had no significant effects on either maximum photochemical efficiency of PSII (Fv/Fm) or the photochemical activity of PSI measured as P700 photo-oxidation ($\Delta A_{820\text{-}860}$). However, growth at 15% (w/v) sucrose inhibited Fv/Fm by 20% but had no effect on P700 photo-oxidation (Table 2.2).

To gain further insight on the sucrose-dependent inhibition of light saturated photosynthesis (Table 2.2), light response curves for net O_2 evolution were examined in cells grown at increasing sucrose concentrations (Fig. 2.5A). Measurements made in darkness (0 µmol photons m^{-2} s^{-1}) represent oxygen consumption rates associated with dark cellular respiration (Fig. 2.5A). At 3% (w/v) sucrose, the cells exhibited a respiration rate of 270 ± 20 µmol O_2 consumed hr^{-1} mg Chl a^{-1} which decreased by 70% between 10-15% (w/v) sucrose, consistent with the data presented in (Table 2.2). Remarkably, at all sucrose concentrations tested, the rate of net photosynthesis never exceeded zero (Fig. 2.5A), that is, the rate of photosynthesis never exceeded the rate of respiration. This indicates that despite the presence of the major thylakoid and stromal components necessary for photosynthetic competency, the cell suspension cultures grew below their light compensation point (Fig. 2.5A).

The sucrose sensitivity of O_2 gas exchange was matched by the light response curves for photosynthetic electron transport (PET) rates measured by Chl a fluorescence (Fig. 2.5B). Although PET was similar in cells grown at either 3 or 6% (w/v) sucrose (Fig. 2.5B), PET was reduced by up to 50% in cells grown in either 10 or 15% (w/v) sucrose (Fig. 2.5B). As expected, excitation pressure, measured as 1-qP, increased with increasing measuring irradiance (Fig. 2.5C) irrespective of external sucrose concentration. However, cells grown at either 10 or 15% (w/v) sucrose exhibited excitation pressures that were 50% higher than cells grown at the lower sucrose concentrations (Fig. 2.5C), which was consistent with the inhibition of growth rates (Fig. 2.3A) and light saturated rates of photosynthesis at the high sucrose concentrations (Table 2.2). This inhibition of electron transport rates and increased excitation pressure appeared to be compensated by a greater induction of photoprotection through NPQ, especially at light intensities between 0 and 200 µmol photons m^{-2} s^{-1}, in cells exposed to either 10 or 15% (w/v) sucrose compared to cells grown in either 3 or 6% (w/v) sucrose (Fig. 2.5D).

Xanthophyll cycle activity is an important component of NPQ which can be measured as changes in the epoxidation state of the xanthophyll cycle pigments (Demmig-Adams and Adams, 1996; Demmig-Adams et al. 2014). The rate of change in the epoxidation state in the green cells was comparable to that observed for WT *Arabidopsis* leaves when

Table 2.2 The effect of sucrose concentration on the maximum photochemical efficiency of PSII, the functional activity of PSI, the respiration rate, and the light-saturated rate of photosynthesis. *Arabidopsis* cell suspension cultures were grown under continuous light (30 μmol photons m^{-2} sec^{-1}) from 3 to 15% (w/v) sucrose. Maximum photochemical efficiency of PSII (F_V/F_M) was calculated as $(F_M - F_O)/F_M$ from chlorophyll a induction traces measured at 100 μmol photons m^{-2} sec^{-1}. The functional activity of PSI was determined from the increase in absorbance induced by far red light ($\Delta A_{820-860}$). Rates of respiration were calculated as rate of oxygen consumption in complete darkness. Light saturated rates of photosynthesis were determined by subtracting respiration rates from oxygen evolution rates at saturating light intensities. For all measurements n = 3 and error bars represent SE. One-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$).

[Sucrose] (%w/v)	F_V/F_M	$\Delta A_{820-860}$	Respiration rate (μmol O_2 hr^{-1} mg chl^{-1})	Light saturated rate of photosynthesis (μmol O_2 hr^{-1} mg chl^{-1})
3%	0.73 ± 0.01 a	58 ± 7 a	270 ± 20 a	220 ± 20 a
6%	0.74 ± 0.01 a	79 ± 10 a	250 ± 20 ac	190 ± 20 a
10%	0.74 ± 0.02 a	87 ± 22 a	110 ± 20 b	60 ± 20 b
15%	0.59 ± 0.06 b	91 ± 23 a	90 ± 20 bc	60 ± 20 b

Table 2.3. PSI:PSII ratios of the *Arabidopsis* cell suspension culture determined by 77K chlorophyll fluorescence emission spectra. *Arabidopsis thaliana* cells were grown in medium containing either 0%, 3%, 6%, or 9% (w/v) sucrose and 77K emission spectra were obtained. *Arabidopsis thaliana* cells grown in 6% (w/v) sucrose were split into two portions. One was dark adapted for 15 minutes, and the other was treated with the growth light of 30 µmol photons m^{-2} s^{-1} for 15 minutes. 77K Emission spectra of these two aliquots were obtained (Fig. S2B) and the PSI/PSII ratios were calculated from the corresponding spectra by dividing the area of the PSI fluorescence peak (λ_{emPSI} = 735 nm) by the area of the PSII peak (λ_{emPSII} = 680 nm) after background correction. n = 3; AV ± SE. One-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$).

[Sucrose]	PSI:PSII ratio	
(%w/v)	Growth light	Dark-adapted
0%	2.7 ± 0.1 a	
3%	4.0 ± 0.3 bc	
6%	3.3 ± 0.2 ab	4.7 ± 0.1 c
9%	4.3 ± 0.2 c	

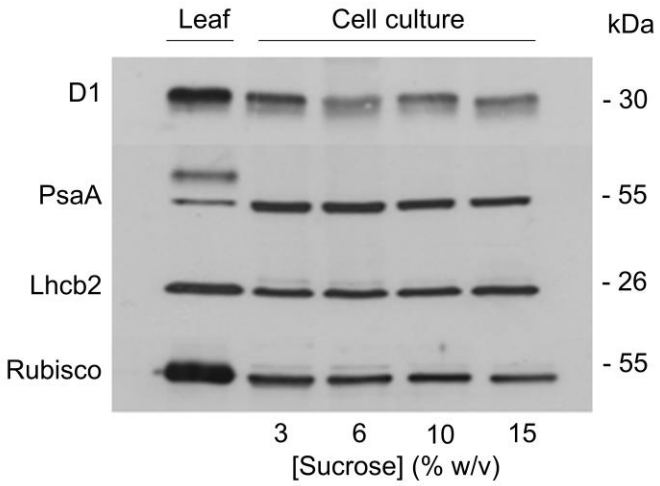

Figure 2.4 Immunoblots for polypeptides from the major photosynthetic proteins: D1, A core subunit of PSII, PsaA, A core subunit of photosystem I polypeptide, Lhcb2, A chloroplhyll-a/b binding proteins of PSII, and RbcL, the large subunit of Rubisco. The cell suspension culture was grown in MS medium containing 3, 6, 10, or 15% (w/v) sucrose for 1 week. *Arabidopsis* leaves were used as a control. The extracted proteins were loaded on an equal chlorophyll basis (8 μg Chl per lane).

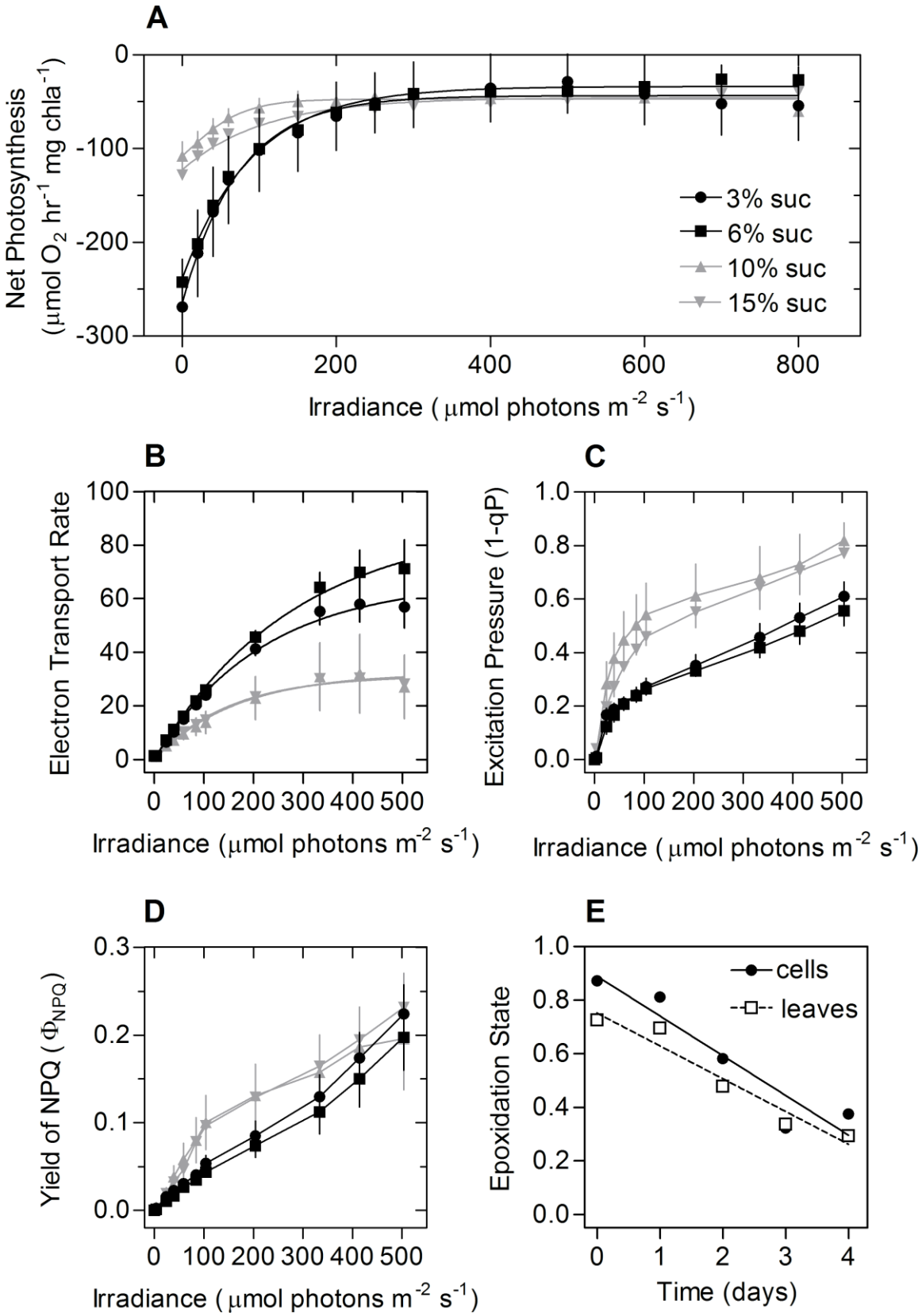
A
Net Photosynthesis (μmol O2 hr-1 mg chla-1)
0
-100
-200
-300
0
200
400
600
800
Irradiance (μmol photons m-2 s-1)
3% suc
6% suc
10% suc
15% suc
B
Electron Transport Rate
100
80
60
40
20
0
0 100 200 300 400 500
Irradiance (μmol photons m-2 s-1)
C
Excitation Pressure (1-qP)
1.0
0.8
0.6
0.4
0.2
0.0
0 100 200 300 400 500
Irradiance (μmol photons m-2 s-1)
D
Yield of NPQ (ΦNPQ)
0.3
0.2
0.1
0.0
0 100 200 300 400 500
Irradiance (μmol photons m-2 s-1)
E
Epoxidation State
1.0
0.8
0.6
0.4
0.2
0.0
cells
leaves
0
1
2
3
4
Time (days)

Figure 2.5 The effects of sucrose concentration on the photosynthetic response of cell suspension cultures. (A) Light response curves for net photosynthesis estimated by CO_2 saturated rates of oxygen evolution normalized to Chl a content. (B) Light response curves for electron transport rates estimated by room temperature Chl a fluorescence induction. (C) Light response curves for excitation pressure measured as 1-qP by room temperature Chl a fluorescence induction (D) Light response curves for non-photochemical quenching (NPQ) estimated by room temperature Chl a induction. Cell suspension cultures were grown under continuous light (30 µmol photons m^{-2} s^{-1}) in MS medium at 3% (squares), 6% (circles), 10% (inverted triangles) and 15% (triangles) sucrose. (E) Time –dependent change in the epoxidation state of xanthophyll pigments in response to high light. Cells from the cell suspension culture and leaves were exposed to high light (1100 µmol photons m^{-2} s^{-1}) over a 4 day period. The concentrations of xanthophyll pigments violaxanthin, antheraxanthin and zeaxanthin were measured by HPLC from which the epoxidation state was calculated as described in detail previously (Krol et al. 1999). For all panels n = 3 and error bars represent SE.

exposed to 3% (w/v) sucrose (Fig. 2.5E), which indicates that the cells exhibit an active xanthophyll cycle comparable to attached leaves, consistent with their capacity to induce photoprotection through NPQ as a function of increased sucrose concentration (Fig. 2.5D).

2.3.6 The effects of sucrose concentration on transcript abundance of genes associated with the Chl a biosynthesis

A heat map illustrating transcript abundance of key genes of the Chl a biosynthetic pathway was constructed from cells grown at 0% sucrose, 9% (w/v) sucrose and 9% (w/v) mannitol (Fig. 2.6). Of the 4 genes involved in the conversion of Proto IX to Mg-Proto (Fig. 2.6A), growth at 9% (w/v) sucrose stimulated mRNA levels of *ChlH* and *ChlI2* by 40-90% compared to cells grown in the absence (0%) sucrose. Only *ChlD* was down regulated under 9% (w/v) sucrose conditions and the expression level of *ChlI1*remained unchanged. Since the expression levels of all four genes involved in the conversion of Proto IX to Mg-Proto were insensitive to 9% (w/v) mannitol, we conclude that modulation of the expression of these genes was not due to an osmotic effect but rather due to sucrose concentration. Six genes were detected that were involved in the conversion of Mg-Proto to Chl a (Fig. 2.6A). *DVR* was upregulated 4.8-fold in the 9% (w/v) sucrose treatment whereas the expression levels of the other 5 genes (*ChlM*, *CRD1*, *PorB, PorC* and *ChlG*) were unaffected by 9% (w/v) sucrose. However, *ChlM*, *CRD1 and PorC* were downregulated by mannitol. Thus, of the 10 genes detected and associated with the Chl a biosynthetic pathway only one gene (*ChlD*) appeared to be down-regulated in response to high sucrose levels. These data for the mRNA levels associated with genes of the Chl biosynthetic pathway are consistent with the phenotypic data illustrated in Fig. 1A and the data for Chl a accumulation in suspension cells exposed to increasing external sucrose concentrations (Fig. 2.1B). Thus, increasing external sucrose concentrations did not repress the Chl biosynthetic pathway in this cell suspension culture.

2.3.7 The effects of sucrose concentration on gene expression associated with thylakoid protein complexes

A heat map was generated to summarize the effects of exposure to high sucrose on the mRNA levels of genes encoding subunits of the PSII and PSI supercomplexes (light harvesting complex + reaction center core) involved in light absorption and the initiation of photosynthetic electron transport as well as the intersystem Cyt b6f complex (Fig. 2.6B). Of the 24 genes detected for the PSII supercomplex, the expression levels of 12 genes were down regulated (red), 3 were upregulated (green) and 9 genes (white) remained unaffected by 9% (w/v) sucrose (Fig. 2.6B). However, of the 12 genes that were down-regulated by 9% (w/v) sucrose, 11 exhibited a comparable down-regulation by 9% (w/v) mannitol (red) (Fig. 2.6B).

The expression levels of a majority of the 18 genes associated with the PSI supercomplex were down-regulated (12, red) while 6 genes (white) were unaffected by exposure to 9% (w/v) sucrose. However, down-regulation of genes by high sucrose was matched by down regulation by 9% (w/v) mannitol (red). No upregulation of genes associated with the PSI supercomplex by either high sucrose, or high mannitol concentrations, was observed (Fig. 2.6B).

The heatmap in Figure 2.6C illustrates the gene expression results for genes associated with the following electron transport components: 2 genes associated with subunits of the the Cyt b6f complex, the major redox complex that mediates photosynthetic electron transport between the two photosystems to generate a trans-thylakoid ΔpH; 9 genes associated with electron carriers on the PSI-acceptor-side involved in the PSI-dependent reduction of $NADP^+$; 6 genes associated with the non-pigmented, chloroplast ATP Synthase complex used conversion of the trans-thylakoid ΔpH into ATP (Fig. 2.6D)(Hopkins and Hüner 2009). Of the 17 genes involved in electron transport and ATP synthesis (Fig. 2.6C and D), the transcript levels of 5 genes decreased (red)(*PetC*, *Fd2*, *PetE1*, *ATPD*, and *ATPaseFo*), 10 remained unaffected (white) and two genes (*FNR 2*, *Fd1*) were upregulated (green) by 40-50% when cells were exposed to 9% (w/v) sucrose. However, the 5 genes that were down-regulated by high sucrose were also down-regulated to a similar extent by 9% (w/v) mannitol (Fig. 2.6C and D). Thus, in contrast to

transcript levels of genes associated with the Chl a biosynthetic pathway, it appears that the down-regulation of most photosynthetic genes that encode polypeptide subunits of the major thylakoid protein complexes is not a consequence of external sucrose concentration *per se*, but rather due to increased osmotic pressure.

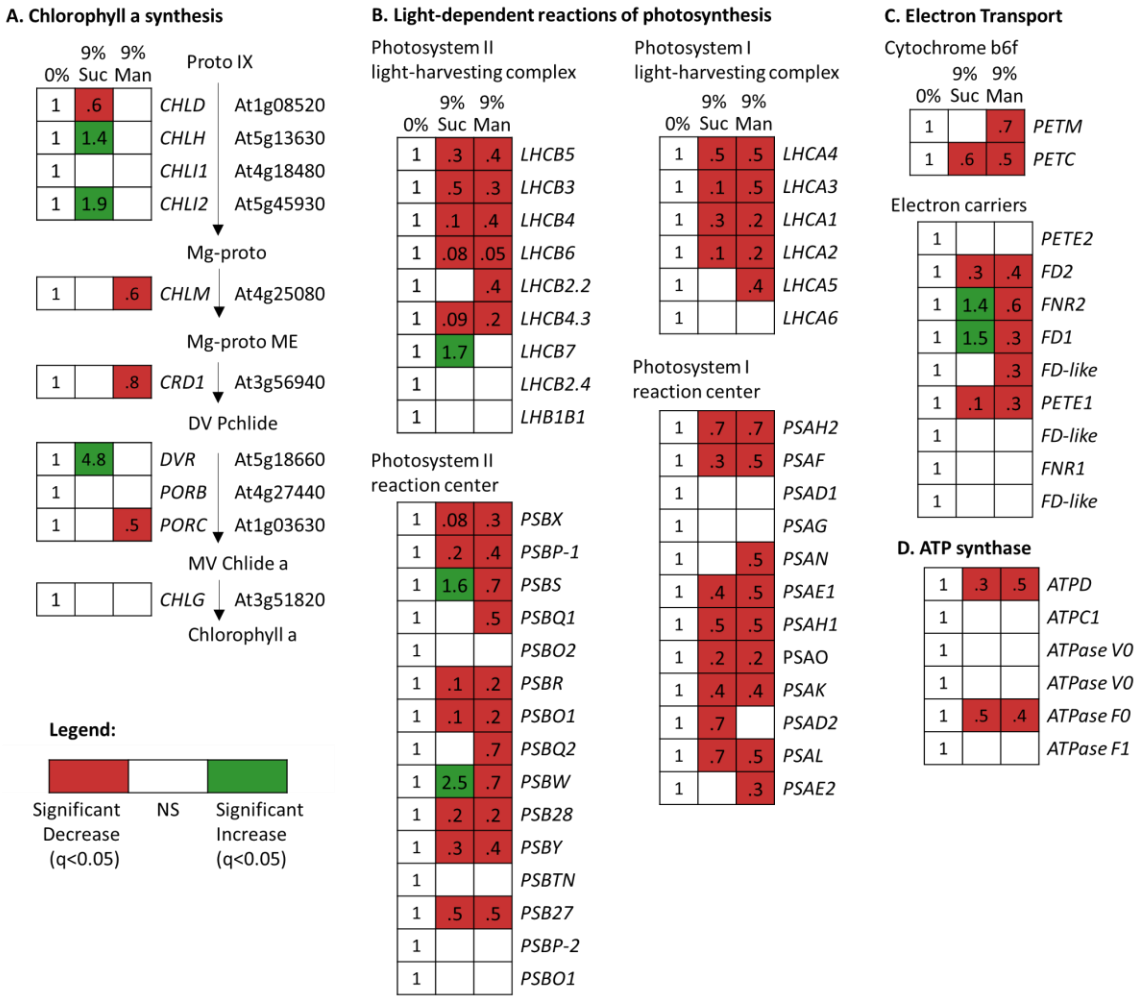

Figure 2.6 Heat map illustrating the effect of sucrose and mannitol on transcript abundance of (A) Chl a biosynthesis genes, (B) genes encoding subunits of the light-dependent reactions associated with PSII and PSI, (C) genes encoding components of electron transport including the Cyt b6f complex and PSI acceptor-side electron carriers and (D) subunits of the ATP synthase complex. *Arabidopsis thaliana* cell suspension cultures were grown in triplicate at 0% sucrose/mannitol, 9% (w/v) sucrose, and 9% (w/v) mannitol. Cuffdiff was used to calculate transcript abundances from RNA-seq and to test statistical significance between treatments. Estimates of transcript abundance (FPKM-based) are shown relative to the 0% treatment. Red shading indicates that there

was a significant decrease in transcript abundance compared to 0% sucrose/mannitol ($q < 0.05$). Green shading indicates that there was a significant increase in transcript abundance compared to 0% sucrose/mannitol ($q < 0.05$). White shading indicates there was no significant difference in transcript abundance compared to 0%. Numbers in the squares indicate the relative change in transcript abundance normalized to 0% sucrose.

2.4 Discussion

The cell suspension culture originally derived from *Arabidopsis thaliana* var. Landsberg *erecta* by May and Leaver (1993) not only exhibits a stay-green phenotype when grown in the light at concentrations of external sucrose varying from 0 to 15% (w/v) but actually increases Chl concentrations as external sucrose concentrations increase. (Fig. 2.1A and B). As expected, this green phenotype is light dependent since, in the dark, these cells exhibit no Chl accumulation and remain yellow unless shifted to the light (Fig. 2.1C). This unusual stay-green phenotypic response to high external sucrose is in stark contrast to the response of intact WT *Arabidopsis thaliana* var. Landsberg *erecta* seedlings, which exhibited the expected inhibition of Chl accumulation with increasing external sucrose concentrations (Fig. 2.1B and E) (Sheen 1994; Rolland et al. 2006; Ramon et al. 2008). Magnesium chelatase, consisting of 3 subunits, *CHLD*, *CHLH*, *GUN4*, and *CHLI* (Papenbrock et al. 2000; Muller and Hansson 2009) is the committed first step of chlorophyll biosynthesis, and is therefore highly regulated both transcriptionally and post-transcriptionally (Ikegami *et al.*, 2007). Based on the upregulation in the expression of *CHLH* and *CHLI2* in response to elevated sucrose, and no evidence for a general downregulation of the Chl a biosynthesis pathway in response to elevated sucrose (Fig. 2.6A), we infer an increased flux through the Chl a biosynthesis pathway at high sucrose in the cell suspension culture. The data for transcript abundance are consistent with increased Chl a accumulation observed as a function of increased external sucrose concentrations (Fig. 2.1A and B).

Similar to the well documented repression of photosynthetic gene expression by sugar in WT plants (Cheng *et al.*, 1992; Criqui *et al.*, 1992; Dijkwel *et al.*, 1996; Harter *et al.*, 1993; Knight and Gray, 1994; Krapp *et al.*, 1993; Raines *et al.*, 1992; Sheen, 1990, 1994; Vanoosten and Besford, 1994, 1995), most of the transcript levels of genes encoding the

light harvesting polypeptides of PSII and PSI, their component reaction center polypeptides as well as the redox components of the intersystem photosynthetic electron transport chain and the ATP synthase also appeared to be down regulated by high sucrose concentration in the *Arabidopsis thaliana* var. Landsberg *erecta* cell suspension culture (Fig. 2.6). However, the mannitol control generally mimicked the repression of photosynthetic gene expression by sucrose (Fig. 2.6). Thus, we conclude that the repression of transcript abundance of genes associated with the protein complexes of thylakoid membranes is not due to sucrose *per se* but rather is due to osmotic repression of these sets of genes in the cell suspension cultures. The osmotic gene repression appears to be specific for genes encoding protein complexes of the thylakoid membrane but not for genes encoding enzymes of the Chl a biosynthetic pathway.

Despite the inhibition of general transcript abundance of genes encoding subunits of the thylakoid membrane, we observed minimal effects of sucrose concentration on the chlorophyll-normalized levels of the major Chl a-binding proteins (PsaA and Lhcb2) or Rubisco (Fig. 2.4). On a FW basis, the levels of these Chl a-binding proteins and Rubisco increased with sucrose concentration (Table S2.2). The results for thylakoid protein levels are consistent with the fact that the suspension cells accumulate Chl a, the bulk of which is bound to Lhcb2 and PsaA (Hopkins and Hüner, 2009), as a function of increased sucrose. Although our global analyses of transcript abundance for genes encoding subunits of thylakoid protein complexes indicate a general repression, protein levels of these major complexes increased with external sucrose concentrations (Table S2.2). Thus, we conclude that the structure and composition of the photosynthetic apparatus of the cell suspension culture is not regulated at the transcriptional level in response to either sucrose or mannitol but rather must be regulated at the translational level. This contrasts with studies that have shown up to 90% reduction of Rubisco protein levels, in response to external sugar, in various plant species (Krapp *et al.*, 1991; Krapp *et al.*, 1993; Stitt, 1991).

Functionally, the green suspension cultures are photosynthetically competent as indicated by the light response curves for O_2 evolution and ETR based on *in vivo* Chl a fluorescence measurements (Fig. 2.5A-B), the light-dependent modulation of PSI/PSII

ratios (Table 2.3; Fig. S2B) and the capacity to modulate the epoxidation state of its xanthophyll cycle pigments (Fig. 2.5E). However, since the maximum light saturated rates of photosynthesis do not exceed the rates of respiration, suspension cells grow below their light compensation points (Fig. 2.5A; Table 2.1). This, in part, explains why light has a minimal impact on exponential growth rates of the cell suspensions (Fig. 2.3E).

Our data clearly show that gross photosynthesis, ETR and respiration in cells of the green suspension culture are inhibited by increasing external sucrose concentrations (Fig. 2.5A-B; Table 2.1). Given that sucrose is an end product of photosynthesis, these data are consistent with the hypothesis that the cells had sucrose-induced feedback inhibition of photosynthesis (Sharkey *et al.*, 1986; Sharkey and Vanderveer, 1989). End product inhibition of photosynthesis is further supported by the fact that sucrose concentrations that exceed 6% (w/v) also result in a significant increase in excitation pressure (Fig. 2.5C), which reflects an accumulation of closed PSII reaction centers at the highest sucrose concentrations, due to an inability to process PSII-generated electrons (Hüner et al. 1998; Rosso et al. 2009; Hüner et al. 2013). Thus, at the highest sucrose concentrations, the cells are exposed to excess light energy. To compensate, the cells exposed to sucrose levels greater than 6% (w/v) respond by elevating their levels of energy dissipation through NPQ (Fig. 2.5D) in order to protect their photosynthetic apparatus from photodamage (Demmig-Adams and Adams 1996; Demmig-Adams et al. 2014). This allows these cell cultures to remain green despite the fact that they grow below their light compensation points (Fig. 2.6A). Thus, a novel characteristic of this Landsberg *erecta* cell suspension culture is that, despite suffering from feedback-limited photosynthesis, the cells exhibit a stay-green, sucrose-insensitive phenotype and accumulate Chl as external sucrose concentrations increase (Fig. 2.1A-B). Thus, this cell suspension culture has circumvented the effects of feedback-limited photosynthesis on pigment accumulation, and consequently, the limitations typically imposed by feedback inhibition of photosynthesis have been uncoupled from the expected effects on phenotype.

Percent inhibition of respiration as a function of sucrose concentration (Table 2.1) is positively correlated with the percent inhibition of growth rate (Fig. 2.3A). Since green cells grow at or below their light compensation point (Fig. 2.5A), and at 3% (w/v) sucrose exhibit rates of growth (0.42 ±0.02 d^{-1}) at 30 μmol photons m^{-2} s^{-1} that are not significantly different from suspension culture cells grown in the dark (0.45 ± 0.07 d^{-1}), the energy requirement to maintain rates of growth of suspension culture cells is primarily provided through the uptake, respiration and metabolism of external sucrose.

If this is the case, why do the suspension culture cells maintain a green phenotype and a functional photosynthetic apparatus regardless of the external supply of sucrose? The scientific literature contains abundant evidence that links end product inhibition of photosynthesis (Azconbieto, 1983; Besford, 1990; Geiger, 1976; Krapp *et al.*, 1993; Neales and Incoll, 1968; Sharkey *et al.*, 1986) with the inhibition of Chl accumulation and photosynthetic gene expression (Cheng *et al.*, 1992; Criqui *et al.*, 1992; Dijkwel *et al.*, 1996; Harter *et al.*, 1993; Knight and Gray, 1994; Krapp *et al.*, 1993; Raines *et al.*, 1992; Sheen, 1990, 1994; Vanoosten and Besford, 1994, 1995), which led to a non-green phenotype in *Arabidopsis* seedlings grown at 6% (w/v) sucrose and higher. Sensors and genetic elements associated with sugar sensing/signalling pathways have been identified through genetic screening (Ramon *et al.*, 2008). In contrast to WT, glucose insensitive (*gin*) and sucrose insensitive (*sis*) mutants exhibit a characteristic stay-green phenotype when seedlings are germinated on media containing high sugar levels (Laby *et al.*, 2000; Zhou *et al.*, 1998). Thirty stay-green sugar insensitive lines of *Arabidopsis* have been identified to date (Ramon *et al.*, 2008), which have helped elucidate the main sugar signalling pathways that include: the hexokinase (HXK) signalling pathway, the ABA pathway and the SnRK1 pathway (Reviewed in: Ramon *et al.*, 2008; Rolland *et al.*, 2006; Sheen, 2014). Thus, the WT Landsberg *erecta* cell suspension culture used in the present study appears to mimic the response to external sucrose of known sugar-insensitive mutants of *Arabidopsis* (Laby *et al.*, 2000; Zhou *et al.*, 1998). Thus, the *Arabidopsis thaliana* Lansberg *erecta* cell suspension culture generated by May and Leaver in 1993 appears to lack a normal sugar sensing/signalling pathway since the cells no longer exhibit the expected sugar-sensitive, WT phenotype in response to high external sucrose concentrations.

Somaclonal variation describes variability brought about by *in vitro* culturing (Larkin and Scowcroft, 1981) including genetic, epigenetic and karyotypic changes. Genetic changes occur at a much faster rate in cell cultures than in intact plants (Noro *et al.*, 2007). Transposable elements become active during *in vitro* culturing (Hirochika, 1993) and often there are changes in chromosome numbers (Chen and Chen, 1980). In addition, epigenetic modifications appear to be more variable and less stable temporally in cell cultures (Kaeppler *et al.*, 2000). These modifications may be, in part, caused by a high selective pressure to maximize growth rate, as growth rate is the main determinant of competitiveness of cells in an axenic culture. Cell suspension cultures are under specific artificial growth conditions, and newly isolated cultures have slow growth rates under these conditions compared to established cell lines. Often the variation of cell cultures is leveraged as a tool to improve desirable plant traits (Larkin *et al.*, 1989). We suggest that decades of propagation of this *Arabidopsis thaliana* var. Landsberg *erecta* cell suspension culture has resulted in some genetic modification(s) of its sucrose sensing/signalling pathway, rendering the cells insensitive to external sucrose. Thus, caution should be exercised in the use of this *Arabidopsis thaliana* var. Landsberg *erecta* cell suspension culture as a model system for comparison with *Arabidopsis* WT seedlings.

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2.6 Supplementary Tables

Table S2.1. Studies which have used the cell suspension *Arabidopsis thaliana* var Landsberg *erecta* that was isolated by May and Leaver in 1993.

Table S2.2. Immunoblot quantification and normalization of the polypeptide levels to fresh weight. The immunoblots (Fig. 2.4) probed for polypeptides from the major photosynthetic proteins: D1, A core subunit of PSII, PsaA, A core subunit of photosystem I polypeptide, Lhcb2, A chlorophyll-a/b binding proteins of PSII, and RbcL, the large subunit of Rubisco.

2.7 Supplementary Figures

Figure S2.1. Growth curves of the *Arabidopsis* cell suspension culture grown in MS media containing 3% sucrose at 30 μmol photons m-2 s-1 of continuous light.

Figure S2.2. 77K chlorophyll fluorescence emission spectra of the *Arabidopsis* cell suspension culture.

Chapter 3

3 Transcriptome profiling indicates that the sugar-insensitive phenotype of a cell suspension culture generated from wild-type *Arabidopsis* var. Landsberg *erecta* is due to the absence of *ABI3* and *ABI4* transcripts which impairs the ABA-mediated sugar signalling pathway

We reported that a cell suspension culture generated from wild-type *Arabidopsis* var. Landsberg *erecta* unexpectedly exhibits a green, sucrose-insensitive phenotype in the presence of external sucrose concentrations as high as 15% (w/v). We employed a global transcriptome profiling approach coupled with meta-analyses of Arabidopsis wild type seedlings to assess whether this sucrose-insensitive phenotype of the cell suspension culture was due to either a deficiency in cellular sugar transport and/or impaired sugar signalling. The cells readily accumulated sucrose, and the intracellular sucrose content of the suspension cells grown at ≥ 6% (w/v) sucrose were comparable to the levels observed in WT seedlings grown at ≥ 6% (w/v) sucrose. Although the high levels of intracellular sucrose repressed chlorophyll levels in WT seedlings, chlorophyll levels of the cell suspension culture were insensitive to high sucrose and maintained a green phenotype. Transcript profiling indicated that key sugar signalling transcripts *ABI3*, *ABI4*, *WOX5*, and *LIN1* were not detectable in the cell suspension culture, which was confirmed by qPCR. Furthermore, addition of ABA to the external medium failed to rescue the suspension cells from its sucrose insensitivity. We conclude that the sugar-insensitive phenotype of the cell suspension culture is due to the absence of *ABI3* and *ABI4* expression, which impairs the ABA-mediated sugar signalling pathway.

3.1 Introduction

We previously characterized an established cell suspension culture of *Arabidopsis* var. Landsberg *erecta* and concluded that it has a sucrose-insensitive phenotype (McCarthy et al., 2016). The cell culture accumulated chlorophyll and exhibited a stay-green phenotype in response to external sucrose concentrations as high as 15% (w/v). High external sucrose treatment inhibited photosynthetic gene expression in the suspension cells

primarily through its osmotic effects rather than due its role in sucrose signalling *per se* (McCarthy et al., 2016). The cell suspension grown at elevated sucrose accumulated normal levels of the major photosynthetic proteins PsaA, Lhcb2, and Rubisco compared to WT *Arabidopsis* leaves and were photosynthetically competent even though a large proportion of their energy requirements was supplied through the uptake of sucrose (McCarthy et al., 2016). Our physiological, biochemical, and molecular results for the effects of external sucrose on the photosynthetic characteristics of the cell suspension culture are incongruent with literature indicating that high sugar concentrations typically result in a reduction of chlorophyll levels, repression of photosynthetic gene expression and greatly reduced photosynthetic activity compared to an osmotic control (Sharkey et al., 1986; Stitt et al., 1987; Stitt, 1992; Krapp et al., 1993; Sheen, 1994; Rolland et al., 2006; Smith and Stitt, 2007; Ramon et al., 2008; Martins et al., 2013). Because the phenotype of the *Arabidopsis* cell culture closely resembled that of sugar-insensitive mutant *Arabidopsis* seedlings, we suggested that the observed phenotype may reflect genetic changes that occurred as a consequence of prolonged *in vitro* culturing of the *Arabidopsis* cell culture (McCarthy et al. 2016).

Since this *Arabidopsis* cell suspension culture is considered comparable to wild type seedlings, it has been used as a model, single-cell system for more than 140 published studies and is still widely used today (McCarthy *et al.*, 2016). The cell suspension culture used in our study was originally isolated over two decades ago by May and Leaver (1993). Since then, several additional cell suspension cultures including MM1, MM2D, PSBD, and PSBL have been generated from this original cell suspension culture (Menges and Murray, 2002). Identifying the cause of the sugar insensitivity in this cell suspension culture is imperative since sugar signalling pathways regulate myriad gene networks within plant cells (Price et al., 2004; Müller et al., 2007; Osuna et al., 2007). If any of the sugar signalling pathways are shown to be dysfunctional (Fig. 3.1), the cell suspension culture should no longer be considered wild-type for experimental purposes. In addition, we suggest that elucidation of molecular basis for sugar-insensitive phenotype in these suspension cells may also reveal novel aspects of plant sugar signalling.

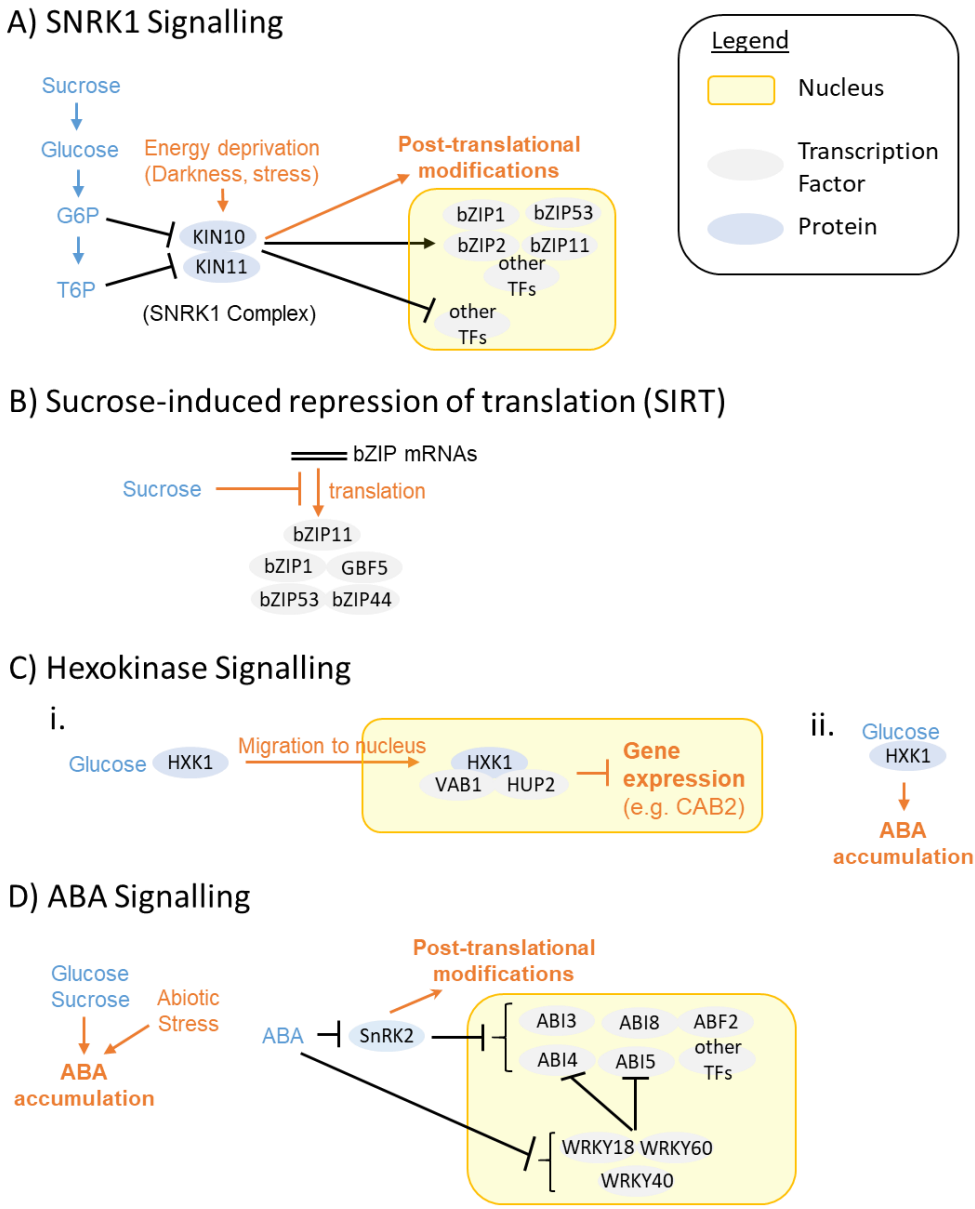


Figure 3.1 Summary of the major sugar signalling pathways in plants that repress photosynthetic gene expression. A) The SnRK1 signalling pathway. The SnRK1 complex is composed of two subunits KIN10 and KIN11. The activity of the SnRK1 complex is enhanced by energy deprivation signals and repressed by the sugars glucose-6-phosphate and trehalose-6-phosphate. These sugars inhibit the SnRK1 complex from modifying proteins and transcription factors post-translationally, which has wide-ranging effects on metabolism. B) Sucrose represses the translation of bZIP transcription factors through a mechanism known as sucrose-induced repression of translation. C) The two hexokinase signalling pathways. (i) In response to sugars, HXK1 migrates to the nucleus and associates with binding partners

VAB1 and HUP2. This complex represses the expression of genes in the nucleus. (ii) Hexokinase also promotes the accumulation of ABA in response to high sugar levels through an unknown mechanism. D) The ABA signalling pathway. High sugar levels cause cells to accumulate ABA. High levels of ABA activate the SnRK2 complex, which modifies proteins and transcription factors post-translationally.

We hypothesized that the unusual sucrose-insensitive phenotype of this cell suspension culture is due to either (1) an impairment of sugar transport limiting intracellular sugar concentrations resulting in the lack of sugar signalling, or (2) inhibition or impairment of a sugar signalling pathway that regulates photosynthetic gene expression. We test these hypotheses using a non-targeted, global transcriptomic approach and show that, although the cell suspension culture retains the capacity for sucrose and sugar accumulation, the sucrose-insensitive phenotype is due to the impairment of *ABI3* and *ABI4*, combined with an insensitivity to ABA.

3.2 Methods

3.2.1 Growth conditions

The *Arabidopsis* cell suspension culture was originally generated by May and Leaver in 1992 (May and Leaver, 1993). The stock cell suspension cultures were grown in liquid Murashige and Skoog medium (MS, Sigma M5524), supplemented with 3% (w/v) sucrose, 0.5 mg/L naphthaleneacetic acid (NAA), 47 μg/L kinetin, 0.1X of Vitamin B solution, and an additional 2.5 mM of K_2HPO_4. The cell cultures were maintained on an orbital shaker set at 150 rpm in an incubator set at 25^{o}C and illuminated with 30 μmol photons m^{-2} s^{-1} of continuous white light. The culture was sustained in exponential growth phase by diluting the 75 mL batch cultures (1:15) with fresh media every 7 days. For sucrose treatments, the medium was prepared with the desired concentration (0-9% w/v). Cell cultures were grown for twelve days at the desired sucrose concentration prior to RNA or metabolite extraction.

Arabidopsis thaliana var. Landsberg *erecta* (Ler-0) seedlings were grown from seeds on MS medium, solidified with 0.8% (w/v) agar, supplemented with sucrose, 0.5 mg/L NAA, 47 μg/L kinetin, 0.1X of Vitamin B solution (Sigma), and an additional 2.5 mM of

K_2HPO_4. The sucrose concentration was varied for each treatment as indicated. The seeds were sterilized with a solution containing 20% (v/v) bleach and 0.1% (w/v) Tween for 10 minutes, and then rinsed five times with sterile water. The seeds were then cold treated by placing the plates in the dark at 4°C for 2 days. Finally, the seedlings were grown at 25°C on a 8:16 short day cycle at 250 μmol photons m^{-2} s^{-1}. The seedlings were sampled at mid-day.

3.2.2 Sugar and starch assays

Sugar and starch were quantified by enzymatic assays. Cells were filtered from the medium and seedling leaves were collected for analysis. Cells and leaves were rinsed quickly with 50 mL of distilled water to ensure all traces of medium were washed away. The tissue was then suspended in 80% (v/v) ethanol, heated for 10 minutes at 80°C, and sonicated. The samples were then centrifuged at 8000 x g for 1 minute, and the supernatant was collected for sugar analysis. The pellet was re-suspended in 80% (v/v) ethanol and the extraction was repeated two additional times without heating. The remaining starch pellet was resuspended in water for starch analysis.

Enzymatic microplate assays were adapted from Bergmeyer and Bernt (1974). A solution containing hexokinase (HXK) and glucose-6-phosphate dehydrogenase (G6PDH) was prepared in a Tris buffer (1.0 U/mL HXK, 1.0 U/mL G6PDH, 0.1M Tris buffer pH 7.8, 1.5 mM NAD, 1.0 mM ATP, 10 mM $MgCl_2$, 0.5 mg/mL BSA). To measure sucrose, 15 μL of sample was pre-treated for 1 hour with 15 μL of an invertase solution (0.8M Acetate buffer pH 4.5, 150 U/mL invertase). Three hundred microliters of the HXK/G6PDH solution was then added to the well and the absorbance at 340 nm was measured after 15 minutes. To measure glucose, a separate well was used. Thirty microliters of sample was added to 300 μL of HXK/G6PDH solution and the absorbance at 340 nm was measured after 15 minutes. To measure fructose, 2 μL of 1000 U/mL was added to the same well, and absorbance at 340 nm was measured after 15 minutes. For all sugar assays, sample blanks were prepared by diluting the sample in ddiH2O. Reagent blanks were prepared by substituting H_2O in place of samples. Standard curves for each of the sugars (glucose, fructose, and sucrose) were prepared and analyzed. The standard

curves were used to quantify the sugar concentrations of the cell extracts. Each sample was measured by averaging three technical replicates.

Two milliliters of DMSO and 0.5 mL 11.65M HCl were added to the starch pellet (20-100 mg) and heated at 60ºC for 30 minutes to completely solubilize the starch. Subsequently, 5 mL ddiH2O was added, and then the sample was titrated to pH 4.5 and brought to a final volume of 10 mL. The samples were then treated with thermostable α-amylase (1000 U/mL) at 80ºC for 1h and subsequently treated with amyloglucosidase (200 U/mL). The sample blank consisted of the same procedure with ddiH_2O substituted in place of the sample. The assay reagent blank consisted of the same procedure with ddiH_2O substituted for amyloglucosidase. Starch standards were prepared using corn starch (Sigma). The glucose of these starch samples was then measured using the protocol described in the previous paragraph.

3.2.3 ABA and cytokinin assays

Cells were grown at 3% and 6% (w/v) sucrose and seedlings were grown at 3% (w/v) sucrose as a control. After two weeks under these conditions, the cells were filtered and flash frozen, and the seedling leaves were excised and flash frozen. All tissues were stored at -20ºC. ABA and cytokinins were then extracted and purified following the protocol of Noble et al. (2014). Hormone levels were then quantified by electrospray ionization liquid chromatography-tandem mass spectrometry, HPLC-(ESI) MS/MS (Agilent HPLC connected to Sciex Applied Biosystem 5500 API Mass Spectrometer). Twenty microliters of sample volume was injected on a Luna reverse-phase C18 column (3 µm, 150 × 2.1 µm; Phenomex, Torrance, California, USA). Cytokinins were eluted with (A) a gradient of 0.08% (v/v) acetic acid in acetonitrile, mixed with (B) 0.08% (v/v) acetic acid in DDW, at a flow rate of 0.2 ml min^{-1}. A linear gradient program was used as follows: (1) Start at 50% A and 50% B, then over 1 min. change to (2) 95% A and 5%B, then over 20 mins return to (3) 50% A and 50% B. The effluent was introduced to the electrospray source (block temperature at 700ºC) using conditions specific for each cytokinin, with multiple reaction monitoring (MRM) of the protonated intact cytokinin molecule and the specific production ion $[M+H]^+$. ^2H-labelled internal standards were used for quantitation via isotope dilution analysis.

3.2.4 RNA-Seq transcript profiling

Arabidopsis thaliana cell suspension cultures were grown at external sucrose concentrations that varied from 0 to 9% (w/v) as well as 9% (w/v) mannitol with three biological replicates per treatment. RNA was extracted following the Qiagen RNeasy Plant Mini Kit. RNA quality was assessed with a Nanodrop 2000 spectrophotometer and RNA integrity was assessed by size distribution analysis with an Agilent 2100 Bioanalyzer. RIN scores of all samples were higher than 9.0. The sample library was prepared with the Illumina TruSeq Stranded Total RNA Sample Preparation Kit with Ribo-Zero Plant. RNA sequencing was performed on the Illumina HiSeq2000 using 75-bp paired-end protocol with 5 samples per lane (approximately 40 M reads per sample). Overall read quality was checked using FASTQC v.0.10.1. Sequencing adaptors were trimmed using cutadapt version 1.4.2. The sequence "AGATCGGAAGAGC" was used for both adaptors and only the sequences with overlap length longer than 7, with the adaptor sequence, were modified. Trimmed sequences with a length shorter than 20 were discarded. The trimmed sequence was aligned against *Arabidopsis thaliana* genome Ler-0 (downloaded from http://mus.well.ox.ac.uk/19genomes/) by Tophat2 version2.0.11 with Bowtie2 version 2.2.2 and samtools version 0.0.19.130 used for mate-inner-dist. Two hundred was chosen to be min-intron-length. Transcripts were assembled using the accepted hits for each sample from the previous step as input using Cufflinks version 2.2.1. The reference annotation file from the 19 genomes of *Arabidopsis thaliana* (consolidated annotation.Ler 0.gff3) was used as a guide, and output included all reference transcripts as well as any novel genes and isoforms that were assembled. Upper-quartile normalization method was used. Cuffdiff was used to calculate transcript abundances from RNA-Seq and to test statistical significance between treatments. The transcript levels of the photosynthetic genes from this experiment have been previously published and analyzed (McCarthy et al., 2016). Taqman gene expression assays with pre-designed probes for *ABI3*, *ABI4,* and *ACT2* were amplified with TaqMan Fast Advanced Master Mix on the Applied Biosystems Viia7.

3.2.5 Meta-analyses

Meta-analyses examined genes regulated by KIN10 of the SnRK1 sugar signalling pathway, and genes regulated by ABI4 of the ABA-mediated sugar signalling pathway. A list of genes regulated by KIN10 were obtained from Baena-González et al. (2007). Baena-González et al. identified genes regulated by KIN10 by comparing *Arabidopsis* seedlings transiently overexpressing *KIN10* to WT seedlings. Additionally, Baena-González et al. (2007) showed that these KIN10-regulated genes were also regulated by sucrose, by comparing the expression profiles of KIN10-regulated genes of seedlings at 0% and 3% (w/v) sucrose. The expression profiles of these KIN10-regulated genes identified by Baena-González et al. (2007) were examined in sugar in the sugar insensitive cell culture by comparing differential expression between 0% and 3% (w/v) sucrose. Heatmaps were generated summarizing the log2 fold-changes, and hierarchical clustering was performed using Spearman's correlation.

A list of genes regulated by ABI4 were obtained from Reeves et al. (2011). Reeves et al. identified genes regulated by ABI4 by comparing *Arabidopsis* seedlings transiently overexpressing *ABI4* to WT seedlings. The expression profiles of these ABI4-regulated genes were examined in sugar in the sugar insensitive cell culture by comparing differential expression between 0% and 9% (w/v) sucrose. Heatmaps were generated summarizing the log2 fold-changes, and hierarchical clustering was performed using Spearman correlation.

3.3 Results

3.3.1 Sugar and starch accumulation

The cell suspension culture exhibited a green phenotype at all sucrose concentrations (0 – 9% w/v) as well as 9% (w/v) mannitol. Although WT seedlings exhibited a green phenotype when grown at 0 to 4.5% (w/v) sucrose, at high sucrose concentrations (6 – 9% w/v) the WT seedlings exhibited a non-green phenotype, as expected (McCarthy et al. 2016). Exponential growth rates of the cell suspension cultures were maximal at 3% and 6% (w/v) sucrose ($0.38 \pm 0.03\ d^{-1}$) but reduced by 24% at 9% (w/v) sucrose ($0.29 \pm 0.02\ d^{-1}$). Minimal cell growth was detected during growth either in the absence of external sucrose ($0.01 \pm 0.01\ d^{-1}$) or in the presence on 9% (w/v) mannitol ($0.02 \pm 0.01\ d^{-1}$) but the cell suspension retained a green phenotype. These results for growth and

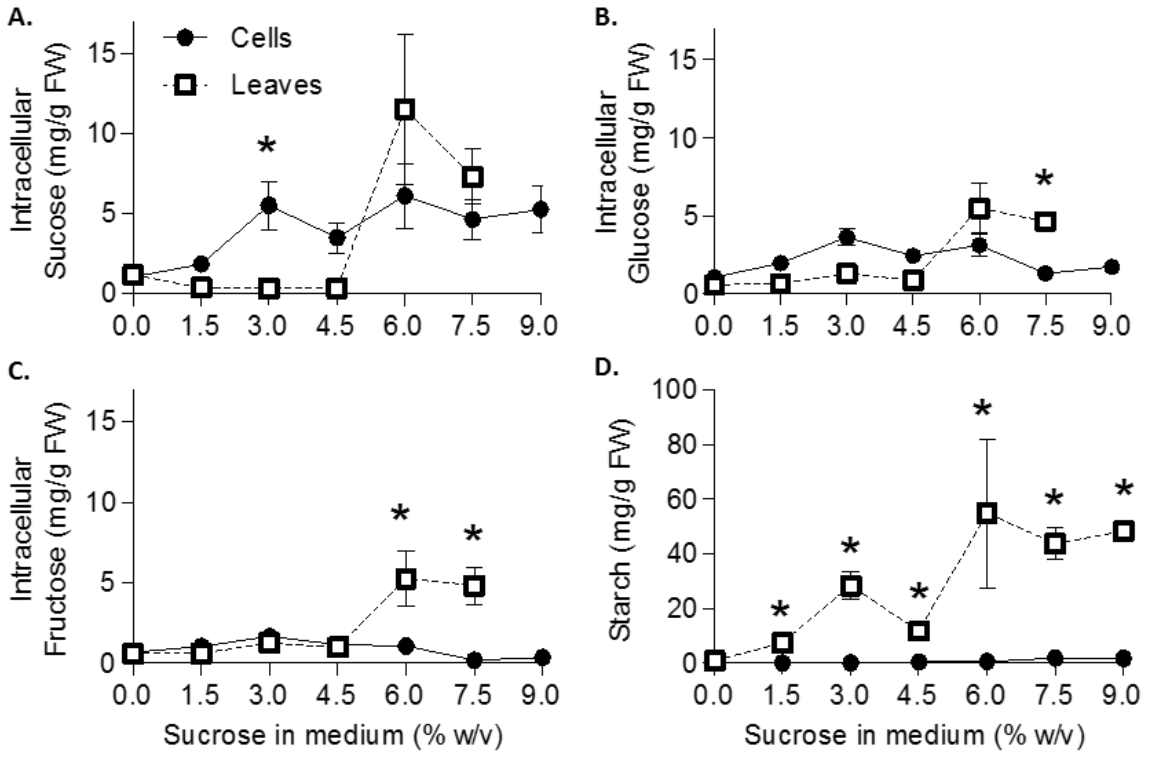


Figure 3.2. Intracellular (A-C) sugar content and (D) starch content of *Arabidopsis* cell suspension cultures and WT seedlings leaves grown at 0-9% (w/v) sucrose. Sugar and starch content was assayed with enzymatic assays. Results were analyzed with 2-way ANOVAs with pairwise Tukey's post-hoc tests (n=3 for seedlings and n=12 for cell cultures). There was a significant interaction between sucrose treatment and tissue type for all four dependent variables ($p<0.001$). The results of the post hoc tests comparing cells to leaves tissue are displayed on the graphs with asterisks ($p<0.05$).

phenotype of the suspension culture are consistent with our previously published results (McCarthy et al. 2016).

As a control, the intracellular levels of sucrose, glucose, and fructose were measured in WT *Arabidopsis* seedlings (Fig. 3.2A-C). Despite large increases in exogenous sucrose between 0 and 4.5% (w/v), intracellular levels of sucrose, glucose and fructose remained low and stable in the suspension cultures. However, when seedlings were grown at 6.0% (w/v) and higher, intracellular glucose and fructose levels spiked to 400% ($p=0.01$) and intracellular sucrose levels increased by 2600% ($p<0.001$). These spikes in intracellular sugar concentrations coincided with a marked change in phenotype, as the seedling turned from green (<6% w/v sucrose) to non-green (≥6% w/v sucrose).

We hypothesized that the cell suspension cultures did not accumulate sugar intracellularly at high sucrose (≥6% w/v sucrose) as observed for the WT seedlings, which would explain the apparent sugar-insensitive phenotype, as a high concentration of intracellular sugar is required to repress photosynthetic gene expression. Despite large changes in external sucrose concentrations, the cells maintained relatively stable levels of intracellular sucrose (Fig. 3.2A). There was a significant interaction ($p<0.001$) in the 2-way ANOVA comparing the intracellular sucrose content of cells and seedlings, indicating that the intracellular sucrose content of cells responded differently to exogenous sugar treatment compared to the WT seedlings. Sucrose concentrations were much higher in the suspension cells than in seedlings at 3% (w/v) exogenous sucrose treatment. Since increasing the exogenous sucrose treatment from 4.5% to 9% (w/v) did not further increase sucrose levels, we conclude that the suspension cells tightly controlled their intracellular sucrose concentrations.

The intracellular glucose levels in the cells were maximal between 3% and 6% (w/v) sucrose, and were 2- to 3-fold lower at 0%, 7.5% and 9% (w/v) sucrose (Fig. 3.2B). The cells grown at 3% and 6% (w/v) sucrose exhibited similar levels of intracellular glucose (Fig. 3.2B), and transcriptional profiles for photosynthetic genes have been previously shown to have similar physiological characteristics (McCarthy et al., 2016). Increasing the sucrose concentration from 6% to 9% (w/v) led to decreased intracellular glucose

(Fig. 3.2B), and has previously been shown to reduce rates of growth, photosynthesis, respiration and to induce an increase in excitation pressure (McCarthy et al., 2016). When levels of intracellular glucose in the suspension cells were compared to the WT seedlings (Fig. 3.2B), there was a significant interaction ($p<0.001$) in the 2-way ANOVA comparing the intracellular glucose content of suspension cells and seedlings, indicating that the intracellular glucose content of cells responded differently to exogenous sugar treatment compared to the WT seedlings. The intracellular glucose levels were not significantly different than the WT seedlings except at the highest sucrose concentration, 7.5% (w/v) sucrose (Fig. 3.2B). The low levels of intracellular glucose at 7.5% and 9% (w/v) sucrose were surprising given the high levels of sucrose supplied exogenously, and they contrasted with the high levels of intracellular glucose observed in WT seedlings grown at high sucrose. The trends observed in intracellular fructose levels (Fig. 3.2C) were similar to those observed for intracellular glucose (Fig. 3.2B). At high sucrose concentrations (≥6% w/v) there was a spike in intracellular fructose in WT leaves, while in the cells, intracellular fructose levels remained low.

Starch is a major sink for carbon metabolites and when WT seedlings were supplied with exogenous sucrose, starch accumulated to high levels in the seedling leaves (Fig. 3.2D). Conversely, the cell suspension culture accumulated very little starch despite growing in sucrose replete media (Fig. 3.2D). At 6% (w/v) sucrose, starch levels were 90-fold lower in cells than in leaves and at 9% (w/v) sucrose, starch levels were 30-fold lower in cells.

3.3.2 Global transcriptional response of the cell suspension culture to sucrose

Transcriptional profiles were obtained for the sugar-insensitive cell suspension culture grown at 0%, 3%, 6%, and 9% (w/v) sucrose, as well as for a 9% (w/v) mannitol osmotic control. The cuffdiff output is available in supplementary Table S3.1 and includes FPKM estimates of transcript abundance for each gene at every treatment, as well as statistical tests comparing the treatments. A heatmap of the transcriptional profiles is provided in Fig. 3.3A, and a fully annotated heatmap with FPKM transcript estimates is provided in supplementary Table S3.2. The 3%, 6% and 9% (w/v) sucrose treatments had very different transcriptional profiles than the 0% and 9% (w/v) mannitol controls (Fig. 3.3A),

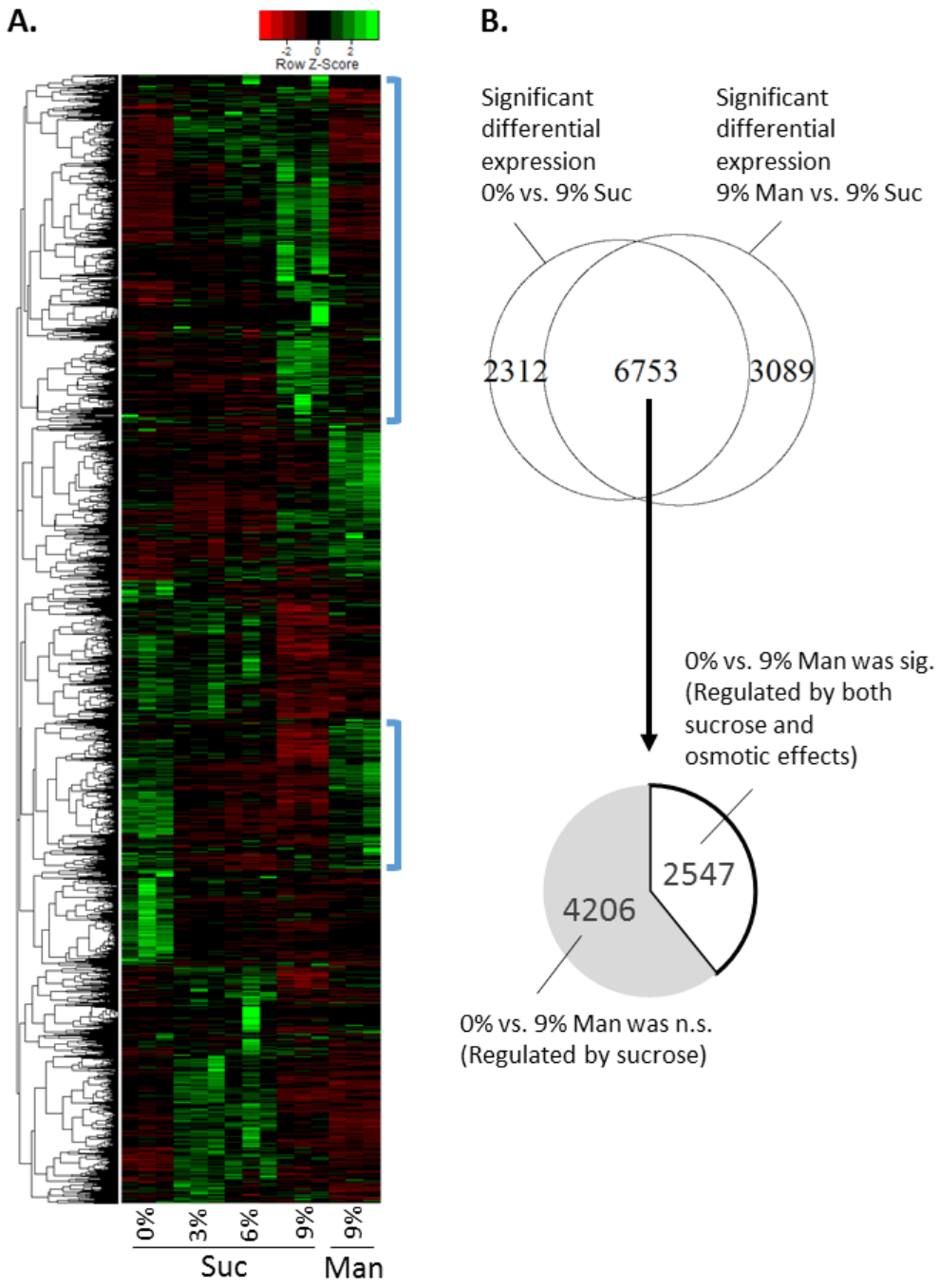


Figure 3.3 Transcript profiling of the sugar insensitive *Arabidopsis* cell suspension culture grown at 0%, 3%, 6%, 9% (w/v) sucrose and at 9% (w/v) mannitol (n=3). (A) Heatmap of *Arabidopsis* transcriptome. Blue brackets highlight genes differentially regulated by sugar in the cell suspension culture. Heatmap colours are based on z-scores. (B) 6753 genes were differentially regulated by 9% sucrose, compared to 0% sucrose and 9% (w/v) mannitol control. 6753 genes were regulated by sucrose and 2547 of these genes also responded to osmotic pressure.

indicating that many genes in the cells were regulated by sucrose. While the 3% and 6% (w/v) sucrose treatments had similar effects on the expression of genes across the transcriptome, the expression profiles of the 9% (w/v) sucrose treatments varied widely from the 3% and 6% (w/v) treatments (Fig. 3.3A). This is consistent with previous findings which showed that cells grown at 3% and 6% (w/v) sucrose had similar physiological characteristics, while those grown at 10% (w/v) had reduced growth rates, lower rates of photosynthesis and respiration, contained more chloroplasts per biovolume, and experienced higher excitation pressures (McCarthy et al., 2016).

To identify genes regulated by sugar, we first excluded genes regulated solely by osmotic effects. The 6620 genes regulated by osmotic pressure (Table S3.3) were identified by comparing the 9% mannitol treatment to the 0% treatment. To identify genes regulated by sugar and exclude genes regulated by osmotic pressure, the genes upregulated by 9% (w/v) sucrose were compared to (a) a 0% sucrose control and (b) a 9% (w/v) mannitol control (Fig. 3.3B). The intersection of these lists provided a list of 6753 genes that were differentially regulated by sucrose (Fig. 3.3B). From this list, 2547 genes were regulated by sucrose alone and 4206 genes were regulated by both sucrose and osmotic effects (Fig. 3.3B, Supplemental Tables S3.4 and S3.5). These 6753 genes were then grouped by their GO terms to identify whether any groups were over-represented (Supplemental Table S3.6). Sucrose treatment affected the expression of genes across the entire transcriptome, generally affecting 10-20% of genes from each category of biological processes (Supplemental Table S3.6). There was a higher percentage of genes upregulated by sucrose associated with pigmentation (50%), DNA metabolic processes (25%), cell cycle (29%), ribosome biogenesis (25%), cell division (28%), mitotic nuclear division (34%), and chromosome segregation (32%) (for more detail see supplemental Table S6). This is consistent with the physiology, as cells grown at 0% or 9% (w/v) mannitol had growth rates near zero, while those grown in sucrose grew rapidly and maintained a green phenotype.

3.3.3 Sucrose metabolism genes

Sucrose must be actively transported into the cells, so we examined the expression of sucrose transport genes (Fig. 3.4A). Sucrose transport genes that import sucrose into the

Sucrose and glucose import

A. Sucrose transporters - influx

B. Invertases - cell wall

C. Glucose transporters - influx

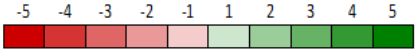
		0%	Suc 3%	Suc 6%	Suc 9%		Man 9%	
A.	*SUC3*	72	75	75	38	*	76	At2g02860
	SUC1	22	15	19	7.2	*	4.8	At1g71880
	SUC5	27	11	17	4.5	*	8.3	At1g71890
	SUC6	28	0.93	1.3	2.8	*	6.7	At5g43610
	SUC2	11	0.24	0.79	0.18	*	26	At1g22710
	SUC4	2.4	2	2.6	2.6	*	5.2	At1g09960
	SUC8	0	0	0.028	0.026		0.077	At2g14670
	SUC9	0	0	0.041	0		0	At5g06170
	SUC7	0	0	0	0		0	At1g66570
B.	*CWINV1*	120	3.8	0.28	2.3	*	4.4	At3g13790
	CWINV2	2.3	4	5.4	12		7.4	At3g52600
	CWINV5	0.068	0.099	0.23	0.19		0.14	At3g13784
	CWINV4	0	0	0	0.051		0.037	At2g36190
C.	*ATSTP13*	280	57	32	29	*	160	At5g26340
	ATSTP1	200	11	4.5	1.6	*	13	At1g11260
	ATSTP4	120	10	4.5	11	*	40	At3g19930
	ATSTP2	45	0.095	0.011	0.036		24	At1g07340
	ATSTP14	46	0.21	0.041	1.1	*	7.1	At1g77210
	STP7	12	1.9	1.8	6.4		9.8	At4g02050
	ATSTP9	0.41	0.57	0.49	1.1		0.86	At1g50310
	STP12	0.91	0.59	0.97	0.19		0.43	At4g21480
	ATSTP11	0.035	0	0.02	0.037		0	At5g23270
	ATSTP6	0	0	0	0		0	At3g05960

Trehalose-6-phosphate metabolism

D. Trehalose-6-phosphate synthases

E. Trehalose-6-phosphate phosphatases

D.	*TPS11*	160	28	16	15	*	85	At2g18700
	TPS7	78	64	61	26	*	64	At1g06410
	TPS8	110	11	4.2	10	*	100	At1g70290
	TPS10	68	19	18	29	*	78	At1g60140
	TPS9	88	9.5	9	37		46	At1g23870
	TPS5	12	31	40	58	*	27	At4g17770
	TPS1	15	17	16	12		17	At1g78580
	TPS6	6.5	5.7	4.4	3.7	*	9.1	At1g68020
	TPS4	0	0.026	0.038	0.17		0.06	At4g27550
	TPS3	0	0	0	0.011		0	At1g17000
	TPS2	0	0	0	0		0	At1g16980
E.	*TPPE*	18	30	18	55	*	4.8	At2g22190
	TPPB	2.3	33	20	9.5	*	1.5	At1g78090
	TPPA	4.7	13	12	14	*	1.9	At5g51460
	TPPC	0	1.3	4.3	0.03		0	At1g22210
	TPPI	0.13	0.81	0.32	0.74		0.028	At5g10100
	TPPH	0.023	0.16	0.28	0.022		0.46	At4g39770
	TPPJ	0	0	0	0.022		0	At5g65140
	TPPD	0	0	0	0		0	At1g35910



n.s. (q>0.05) compared to 0% treatment

* Significant difference between 9% suc and 9% man

Numbers in cells denote FPKM estimates of transcript abundance

Figure 3.4 Transcript profile heatmaps of (A-C) sugar transport genes and (D-E) trehalose-6-phosphate metabolism genes of the *Arabidopsis* cell suspension culture. The *Arabidopsis* cell suspension culture was grown in triplicate at 0%, 3%, 6%, 9% (w/v) sucrose and at 9% (w/v) mannitol. Cuffdiff was used to calculate expression in samples (FPKM) and test the statistical significance (q-values). The numbers in the cells indicate the average FPKM estimate of transcript abundance. Green shading indicates a significant increase ($q<0.05$) with sugar or mannitol treatment compared to 0% and red shading indicates a significant decrease ($q<0.05$). An asterisk indicates a significant difference between 9% (w/v) sucrose and 9% (w/v) mannitol treatments ($q<0.05$), highlighting genes regulated by sucrose compared to the osmotic control.

cells were strongly downregulated by exogenous sucrose (Fig. 3.4A). For example, in response to increasing exogenous sucrose from 0% to 3% (w/v) sucrose, transcript levels of the high affinity sucrose transporters decreased: *SUC1* decreased 32% and levels of *SUC2* decreased 46-fold (Fig. 3.4A). Increasing exogenous sucrose further from 3% to 9% (w/v) resulted in an additional 50% decrease in the expression of the *SUC1* and 25% decrease in *SUC2* (Fig. 3.4A). We also observed that the expression of sucrose transporters genes, *SUC2* and *SUC4*, increased with osmotic pressure. The cells were able to limit their intracellular sucrose uptake by downregulating sucrose import proteins. However, even with fewer sucrose transporters, the cells accumulated high levels of intracellular sucrose at 3%-9% (w/v) sucrose, comparable to the high levels of sucrose in WT seedlings grown at exogenous sucrose concentrations of 6-9% (w/v) (Fig. 3.2A). Because sucrose is a major signalling molecule in cells, the high levels of intracellular sucrose present at 3-9% (w/v) exogenous sucrose should have repressed photosynthetic gene expression, similar to the repression we observed in seedlings at 6-9% (w/v) sucrose. However, the cells remained green at high sucrose concentrations.

Glucose is sensed by hexokinase (Fig. 3.1C) and also converted to the signalling sugars glucose-1-phosphate, glucose-6-phosphate, and trehalose-6-phosphate. In addition to the repression of sucrose import genes, exogenous sucrose treatments also strongly downregulated cell wall invertase and hexose transporter genes (Fig. 3.4B-C). Exogenous sucrose can be hydrolized by extracellular cell wall invertases and subsequently imported as glucose by hexose transporters. The 3% (w/v) exogenous sucrose treatments reduced the expression of cell wall invertase 1 (cwINV1) 30-fold compared to the 0% treatment (Fig. 3.3B). Additionally, compared to the 0% treatment, transcript levels of the hexose transporters STP1, STP2, STP4, STP13 and STP14 were reduced by 5 to 480 fold in response to 3% (w/v) sucrose, and there was a further 2-10 fold reduction in response to 9% (w/v) sucrose (Fig. 3.4C). Therefore, in addition to downregulating sucrose uptake, the cells also limited hexose uptake when high exogenous sucrose was available.

Sucrose biosynthesis genes were upregulated by exogenous sucrose treatment compared to the 0% and mannitol controls (Supplemental Table S3.2). The levels of these sucrose biosynthesis genes were maximal and 3% and 6% (w/v) and comparatively reduced at

9% (w/v) sucrose (Supplemental Table S3.2). Similarly, many genes from glycolysis, the TCA cycle, and mitochondrial electron transport processes were also maximal at 3% and 6% (w/v) sucrose, and reduced at 9% (w/v) sucrose (Supplemental Table S3.2). These expression patterns paralleled the trends of intracellular glucose content (Fig. 3.2B) and growth rates (Chapter 2 Fig. 2.3). The SWEET proteins SWEET11 and SWEET12 are active sucrose efflux transporters of plant cell membrane (Chen et al., 2012). SWEET11 was not expressed in the cell suspension culture, and SWEET12 was not expressed in the cell suspension cultures at 0%, 3% or 6% (w/v) sucrose (Supplemental Table S3.2).

Transcript profiling indicated that the trehalose-6-phosphate synthases were downregulated by sucrose (Fig. 3.4D) whereas the treholose-6-phosphate phosphatases were upregulated by sucrose (Fig. 3.4E). This suggests that sucrose treatment caused a decrease in trehalose-6-phosphate levels in the cells. Low levels of trehalose-6-phosphate at high sucrose would be unusual, as trehalose-6-phosphate typically accumulates under high sucrose treatment in *Arabidopsis* plants (Yadav et al., 2014). Low levels of trehalose-6-phosphate at high sucrose would result in higher SnRK1 activity, thereby increasing photosynthetic gene expression, and potentially causing a sugar insensitive phenotype.

The starch biosynthesis genes were all expressed in the cells, and many were upregulated 2- to 4-fold by 3% (w/v) sucrose compared to the 0% control Supplemental Table S3.2). The lack of starch accumulation with exogenous sucrose treatments is further evidence that the response of these cells to sucrose differs dramatically from WT seedlings. Although other studies have shown that the expression of the starch biosynthesis gene *APL3* is upregulated by sucrose (Wingler et al., 2000; Rook et al., 2001), *APL3* was not upregulated by sucrose in these cells. *APL3* is regulated by the transcription factor ABI4 from the ABA sugar signalling pathway (Rook et al., 2001), and the sucrose-insensitive response of this gene indicates potential dysfunction of the ABA sugar signalling pathway. While there was no upregulation of *APL3*, sucrose treatment generally upregulated starch biosynthesis genes (Supplemental Table S3.2). Increasing sucrose from 0% to 3% (w/v) resulted in upregulation AGPases (ADG1, APL2, APL4 increased 3-4 fold), starch synthases (SS1 and GBSS1 increased 2-4 fold), and starch branching

enzymes (SBE2.2 and EMB2729 increased 2-fold). However, the cells accumulated very little starch at all sucrose treatments, and starch levels were much lower in the suspension cells, compared to WT seedlings (Fig. 3.2D).

3.3.4 Sugar signalling pathways

Studies on wild-type *Arabidopsis* seedlings have identified 27 genes that are required for sugar signalling (Zhou et al., 1998; Arenas-Huertero et al., 2000; Moore et al., 2003; Brocard-Gifford et al., 2004; Yuan and Wysocka-Diller, 2006); these were summarized and reviewed by Ramon et al. (2008). The expression of each of these genes was quantified by RNA-Seq for suspension cells grown at 0-9% (w/v) sucrose and at 9% (w/v) mannitol (Fig. 3.5A). All of these sugar signalling genes were expressed in the cell suspension culture except *ABI3, ABI4, WOX5,* and *LIN1,* which had undetectable expression in all of the treatments (Fig. 3.5A). The lack of these transcripts is not simply due to inadequate sequencing depth since as we had performed deep sequencing with three lanes of a HiSeq 2500 (approx. 40 Million reads per sample), and rRNA had been removed from the samples. There was effectively no detectable expression of *ABI3, ABI4, WOX5,* and *LIN1* transcription factors in these cells (Fig. 3.5A). The genes *RGL2* and *AXR2* also had extremely low expression levels in the cells (Fig. 3.5A). *RGL2* and/or *AXR2* could play a role in the sugar insensitive phenotype. The expression of each of these genes is required for competent sugar signalling, and that the knockout of any one of these genes would produce a sugar insensitive phenotype in wild-type seedlings.

The lack of *ABI3* and *ABI4* expression was confirmed by qPCR. In seedlings, high sugar treatments typically result in an increase in the expression of the ABA-responsive transcription factors, *ABI3* and *ABI4*. This was confirmed by qPCR, which revealed that *ABI3* and *ABI4* were strongly induced by 6% and 9% (w/v) sucrose treatments in *Arabidopsis* seedlings (Fig. 3.5C-D). However, the 6% and 9% (w/v) sucrose treatments did not induce *ABI3* or *ABI4* in the suspension cells (Fig. 3.5C-D).

The lack of *ABI3* and *ABI4* expression indicated dysfunction in the ABA sugar signalling pathway. Because many elements of the signalling pathways are regulated post-translationally, it is difficult to infer whether the sugar signalling pathways were

		0%	Suc 3%	Suc 6%	Suc 9%		Man 9%	
A.	*RCD1*	310	240	220	150	*	330	At1g32230
	ARIA	200	170	160	80	*	160	At5g19330
	ABA2	320	78	32	26	*	100	At1g52340
	RGS1	95	77	68	110	*	71	At3g26090
	KIN10	65	52	51	47	*	65	At3g01090
	FIN219	75	34	37	37	*	74	At2g46370
	VAB1	55	57	47	45		46	At1g76030
	SIS8	47	51	57	31	*	61	At1g73660
	SPY	35	37	37	18	*	42	At3g11540
	ABI8	32	36	36	23	*	40	At3g08550
	TIR1	52	29	40	12	*	19	At3g62980
	HXK1	31	33	39	7.3	*	31	At4g29130
	CTR1	47	21	22	20	*	28	At5g03730
	RPT5B	32	38	29	14	*	22	At1g09100
	AXR1	31	28	22	23		28	At1g05180
	ETO1	21	23	19	36		31	At3g51770
	LBA1	23	21	24	14	*	24	At5g47010
	ABA1	22	16	16	15		18	At5g67030
	ABF2	9.7	9.6	9.5	9.3		12	At1g45249
	ABA3	6.9	5.4	6.1	6.5	*	9.7	At1g16540
	ABI5	0.084	1.2	1.4	1.5		0.24	At2g36270
	AXR2	0.21	0.42	0.12	0		0	At3g23050
	RGL2	0	0.058	0.03	0.018		0.024	At3g03450
→	*WOX5*	0	0	0.026	0		0	At3g11260
→	*LIN1*	0.025	0	0	0		0	At1g08090
→	*ABI3*	0	0	<0.01	0.018		<0.01	At3g24650
→	*ABI4*	0	0	0	0		0	At2g40220
B.	*MBF1A*	120	210	190	260	*	120	At2g42680
	TPS1	15	17	16	12		17	At1g78580
	ARR2	23	6.7	5.1	4.5	*	13	At4g16110
	CKI1	0	0.012	0.077	0.055		<0.01	At2g47430

Log2 fold change from 0% treatment

-5 -4 -3 -2 -1 1 2 3 4 5

Significant decrease (q<0.05) Significant increase (q<0.05)

n.s. (q>0.05) compared to 0% treatment

* Significant difference between 9% suc and 9% man

Numbers in cells denote FPKM estimates of transcript abundance

→ Genes not expressed (or with minimal expression)

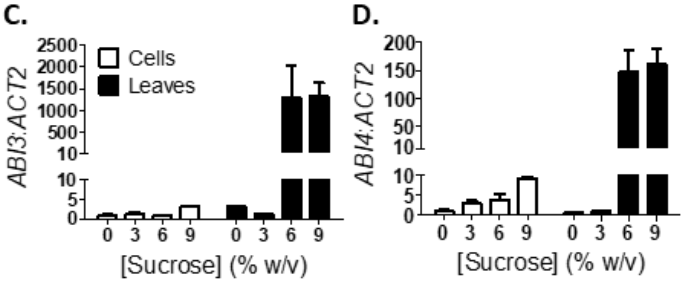


Figure 3.5 (A) Expression heatmaps of the genes required for WT sugar responses in *Arabidopsis*. Knockout of any one of these genes causes a sugar insensitive phenotype in *Arabidopsis* seedlings. (B) Expression heatmaps of genes that produce a sugar insensitive phenotype when overexpressed in WT plants. (A-B) The *Arabidopsis* cell suspension culture was grown in triplicate at 0%, 3%, 6%, 9% (w/v) sucrose and at 9% (w/v) mannitol. Cuffdiff was used to calculate expression in samples (FPKM) and test the statistical significance (q-values). The numbers in the cells indicate the average FPKM estimate of transcript abundance. Green shading indicates a significant increase with sugar or mannitol treatment compared to 0% and red shading indicates a significant decrease. An asterisk indicates a significant difference between 9% (w/v) sucrose and 9% (w/v) mannitol treatments ($q<0.05$), highlighting genes regulated by sucrose compared to the osmotic control. (C) qPCR measurement of the expression of *ABI3* in *Arabidopsis* cell suspension cultures and WT seedling leaves at 0, 3, 6 and 9% (w/v) sucrose. (D) qPCR measurement of the expression of *ABI4* in *Arabidopsis* cell suspension cultures and WT seedling leaves at 0, 3, 6 and 9% (w/v) sucrose. (C-D) Expression was normalized to the *ACT2* housekeeping gene (n=3).

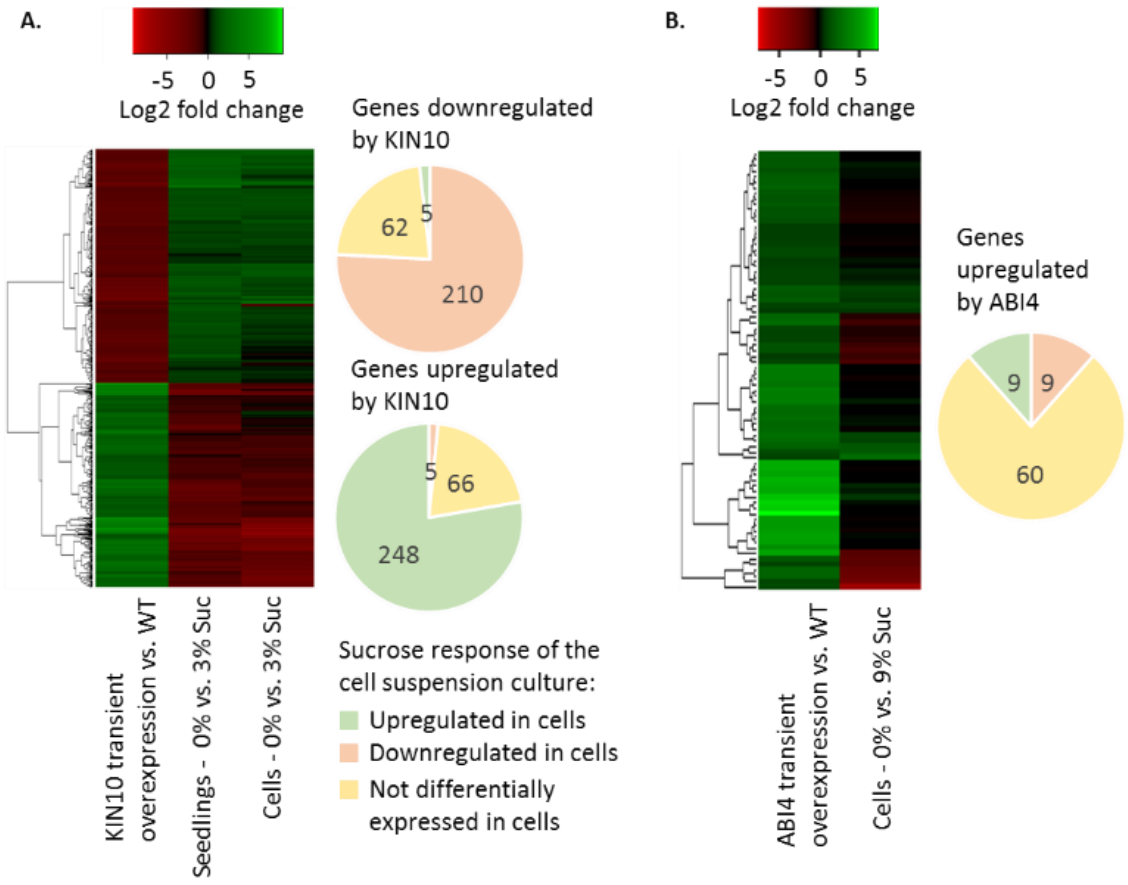


Figure 3.6 Meta-analysis examining the sugar response of genes regulated by SnRK1 (KIN10) and ABI4. (A) Genes regulated by KIN10 were identified by Baena-González et al. (2007) by comparing *Arabidopsis* seedlings transiently overexpressing *KIN10* to WT seedlings (left column). Baena-González et al. also showed that the genes regulated by KIN10 are also regulated by 3% (w/v) sucrose in WT seedlings (centre column). Many of the genes regulated by KIN10 were also differentially regulated by 3% (w/v) sucrose in the sugar insensitive cell suspension culture of this study (right column and pie charts). This indicated that the sugar insensitive cell suspension culture has a functional SnRK1 sugar signalling pathway. (B) Genes upregulated by ABI4 were identified by Reeves et al. (2011) by comparing *Arabidopsis* seedlings transiently overexpressing *ABI4* to WT seedlings (left column). Many genes upregulated by ABI4 did not respond to 3% (w/v) sucrose in the sugar insensitive cell suspension culture of this study (right column and pie charts). This indicated a possible dysfunction of the ABA sugar signalling pathway in the cell suspension culture.

functional from the expression of the signalling elements alone. Therefore, to further examine the function of the sugar signalling pathways, we examined marker genes regulated by these signalling pathways.

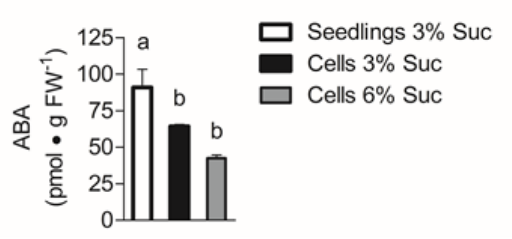
Baena-González et al. (2007) examined the genes regulated by SnRK1. By examining *Arabidopsis* protoplasts overexpressing *KIN10* (a subunit of SnRK1), they published a list of 278 genes upregulated by SnRK1 and 319 genes downregulated by SnRK1 (Fig. 3.6A, see the left column of the heatmap). They showed that all of these genes were also regulated by sucrose in the opposite direction (Fig. 3.6A, centre column). When we examined the expression of these SnRK1-regulated genes in the cell suspension culture, they were also regulated by sucrose (Fig. 3.6A, right column). For example, all of the dark inducible genes (DINs), which are regulated by SnRK1, were strongly downregulated by sucrose treatment. This meta-analysis indicates that the SnRK1 sugar signalling pathway appears to be functional in these suspension cells.

Reeves et al. (2011) examined the genes regulated by the bZIP transcription factor ABI4. Using *Arabidopsis* protoplasts overexpressing *ABI4*, they published a list of 78 genes upregulated by ABI4. When we examined the expression of these ABI4-regulated genes in the suspension cells, only 9 of the 78 genes were significantly upregulated with sucrose treatment (Fig. 3.6B). Furthermore, 9 of the 78 genes were significantly downregulated with sucrose treatment in these cells. Therefore, sucrose does not appear to regulate gene expression through the ABI4 transcription factor in the cell suspension culture. This agrees with the lack of *ABI4* expression observed at high sucrose in the cell suspension culture (Fig. 3.5C), and further supports the hypothesis that there is dysfunction of the ABA sugar signalling pathway.

3.3.5 ABA content of the cell suspension cultures

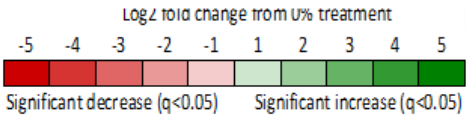
The lack of expression of these ABA-responsive transcription factors could be due to a lack of ABA accumulation in response to sugar in these cells, or it could be due to a genetic variation that has affected the expression of these transcription factors. Therefore, ABA levels were assessed via GC-MS (Fig. 3.7A). Suspension cells had lower levels of ABA than seedlings at 3% (w/v) sucrose (Fig. 3.7A). Increasing sucrose from 3% to 6%

A.



B. ABA Biosynthesis
C. ABA Degradation
D. ABA Glucosylation
E. ABA Deconjugation

		0%	Suc 3%	Suc 6%	Suc 9%		Man 9%	
B.	*ABA2*	320	78	32	26	*	100	At1g52340
	AAO2	54	66	69	51		56	At3g43600
	ABA4	16	13	24	32		42	At1g67080
	ABA1	22	16	16	15		18	At5g67030
	AAO3	15	12	10	10	*	39	At2g27150
	ABA3	6.9	5.4	6.1	6.5	*	9.7	At1g16540
	AAO4	3.1	2.2	2.3	2.9	*	9.2	At1g04580
	NCED3	1.5	0.41	0.15	0.012		0.39	At3g14440
	NCED5	1.3	0.5	0.19	0.12		0.03	At1g30100
	NCED2	0.16	0.62	0.066	0.037		0.028	At4g18350
	NCED9	0.04	0.12	0.098	0.057		0.14	At1g78390
	NCED6	0	0	0	0		0	At3g24220
C.	*NUGWD1*	21	55	55	55	*	25	At5g11240
	CYP707A3	2.5	3.5	0.86	0.55	*	1.3	At5g45340
	CYP707A1	3	0.84	0.23	0.025		2.8	At4g19230
	UGT71B6	3	0.19	0.14	0.14	*	0.79	At3g21780
	CYP707A4	0.079	1.5	0.56	0.028		0.06	At3g19270
	CYP707A2	0	0	0	0		0	At2g29090
D.	*UGT73B1*	12	2.1	0.85	1.7	*	8.3	At4g34138
	UGT71C5	12	2.9	2.4	0.5		4.6	At1g07240
	UGT84B2	0	0	0	0		0	At2g23250
	UGT84B1	0	0	0	0		0	At2g23260
E.	*BGLU18*	4.5	15	25	12	*	46	AT1G52401



n.s. (q>0.05) compared to 0% treatment
* Significant difference between 9% suc and 9% man
Numbers in cells denote FPKM estimates of transcript abundance

F.

0 10 100 500

[ABA] (µM)

Figure 3.7 ABA content, the sugar response of ABA metabolism genes, and the effects of exogenous ABA treatment on phenotype. (A) Intracellular ABA content of cells from the *Arabidopsis* cell suspension culture and from seedling leaves at 3% (w/v) sucrose. Results were analyzed with a one-way ANOVA with Tukey's post-hoc tests ($p<0.05$, $n=3$ for seedlings and $n=4$ for cell cultures). (B-E) Transcript profile heatmaps of the ABA metabolism genes and ABA conjugation genes of the *Arabidopsis* cell suspension culture. The *Arabidopsis* cell suspension culture was grown in triplicate at 0%, 3%, 6%, 9% (w/v) sucrose and at 9% (w/v) mannitol. Cuffdiff was used to calculate expression in samples (FPKM) and test the statistical significance (q-values). The numbers in the cells indicate the average FPKM estimate of transcript abundance. Green shading indicates a significant increase with sugar or mannitol treatment compared to 0% and red shading indicates a significant decrease. An asterisk indicates a significant difference between 9% (w/v) sucrose and 9% (w/v) mannitol treatments ($q<0.05$), highlighting genes regulated by sucrose compared to the osmotic control. (F) Phenotypes of the cell suspension cultures grown at 6% (w/v) sucrose supplemented with 0, 10, 100, and 500 µM ABA.

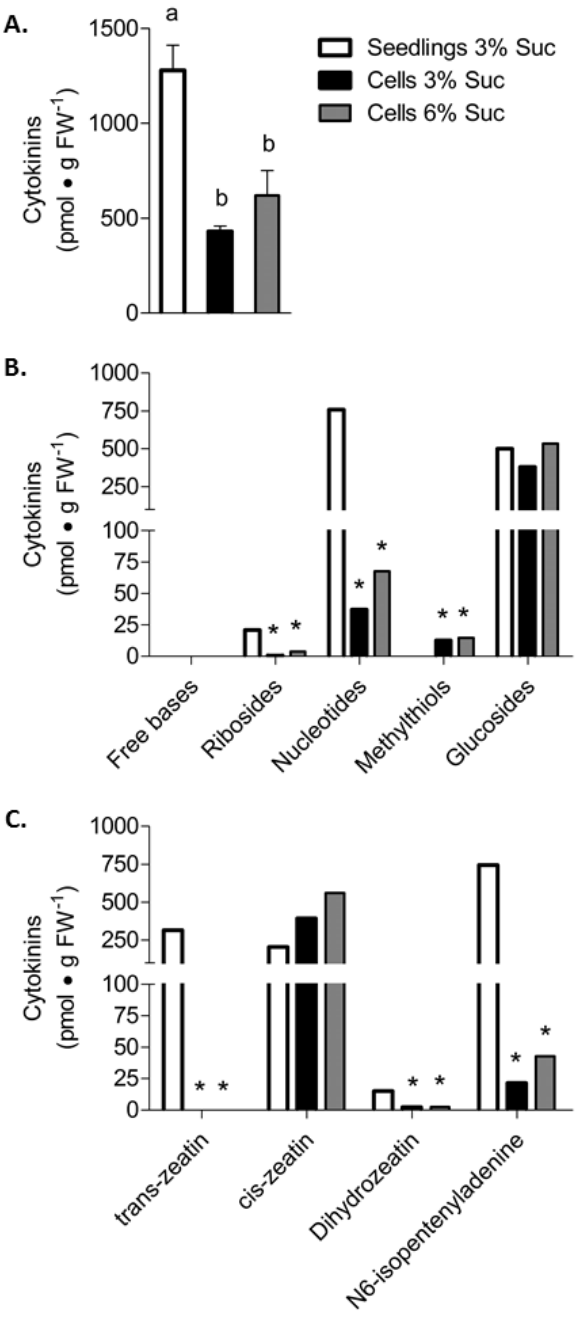
(w/v) did not result in significantly higher ABA levels in suspension cells. This is consistent with the low levels of *ABI3* and *ABI4* observed at 3% and 6% (w/v) sucrose in cells (Fig. 3.5C-D). The lack of ABA accumulation was surprising, as high concentrations of sugar typically enhance ABA biosynthesis and cause ABA to accumulate in WT seedlings (Cheng et al., 2002).

With increased sucrose treatment, we observed a decrease in the expression of ABA biosynthesis genes *ABA1* (32% decrease) and *ABA2* (92% decrease), *NCED3* (99% decrease), *NCED5* (91% decrease), and sucrose did not affect the expression of *ABA3* in these cells (Fig. 3.7B). There was also a decreased expression of ABA degradation genes: *CYP707A1* (99% decrease), *CYP707A3* (78% decrease), and *UGT71B6* (95% decrease). *NUGWD1* was upregulated 160% by sucrose and it is a negative regulator of ABA degradation (Fig. 3.7C). While both ABA biosynthesis and ABA degradation genes were downregulated with sucrose in the suspension cells (Fig. 3.7B-C), there was no net change in ABA levels between 3% and 6% (w/v) sucrose (Fig. 3.7A). These results contrast with the typical sugar response of WT seedlings where ABA biosynthesis genes are typically upregulated in response to sugar (Cheng et al., 2002).

As the suspension cells did not express *ABI3* or *ABI4* at any sucrose treatments, or accumulate ABA in response to 6% (w/v) sucrose treatment, we hypothesized that the cell suspension culture may also be ABA-insensitive. Suspension cells were grown in media supplemented with 0-500µM ABA (Fig. 3.7F) and remained green when ABA was supplied to the growth medium (Fig. 3.7F). Since the suspension cells retained a green phenotype at 10-100µM ABA, this suggests that the suspension cells exhibit a sucrose-insensitive phenotype that is also ABA-insensitive.

3.3.6 Cytokinin content of the cell suspension culture

In freshly isolated cell cultures, cytokinin is required for cell proliferation. The cells of this study were grown without cytokinin to test whether the cells required cytokinin for growth. The cells grew for one month without any cytokinin in the medium. The loss of cytokinin dependency has been observed in other old cell suspension cultures and has been termed cytokinin habituation (Meins, 1989; Pischke et al., 2006).



D. Cytokinin biosynthesis
E. Cytokinin glucosylation
F. Cytokinin activation
G. Cytokinin degradation

		0%	Suc 3%	Suc 6%	Suc 9%		Man 9%	
D.	*IPT9*	17	26	25	18		23	At5g20040
	IPT2	6.7	11	11	7.7		8.9	At2g27760
	IPT1	3.7	2.8	1.5	0.78	*	2	At1g68460
	IPT3	0	0	0.046	0.022		0.02	At3g63110
	IPT7	0	0	0.038	0.037		0	At3g23630
	IPT6	0	0	0.019	0.028		0	At1g25410
	IPT4	0	0	0	0		0.028	At4g24650
	IPT5	0	0	0	0		0	At5g19040
	IPT8	0	0	0	0		0	At3g19160
E.	*CKX1*	16	20	24	24		23	At2g41510
	CKX3	49	7.8	9.5	4.5	*	9.5	At5g56970
	CKX5	3.2	1.4	0.8	0.96		2	At1g75450
	CKX6	1.9	0.57	0.49	0.28		0.46	At3g63440
	CKX7	0.23	0.58	0.68	0.14	*	1.19	AT5G2148
	CKX2	0.016	0	0.019	0		0	At2g19500
	CKX4	0	0	0	0		0	At4g29740
F.	*LOG8*	29	26	25	42	*	26	At5g11950
	LOG4	1.1	1.5	1.7	2		3.3	At3g53450
	LOG3	3.7	0.39	0.24	0.26		0.46	At2g37210
	LOG7	2	0.081	0.027	0.065		0.53	At5g06300
	LOG1	0.11	0	0	0		0.13	At2g28305
	LOG2	0.056	0	0	0.02		0	At2g35990
	LOG5	0	0	0.016	0.057		0	At4g35190
	LOG6	0	0	0	0		0	At5g03270
G.	*UGT76C1*	28	13	18	14		13	At5g05870
	UGT76C2	26	12	17	11		11	At5g05860
	UGT85A1	0.044	0	0.071	0.092		0.071	At1g22400
	UGT73C1	0	0	0	0.051		0	At2g36750

Log2 fold change from 0% treatment

-5 -4 -3 -2 -1 1 2 3 4 5

Significant decrease (q<0.05) Significant increase (q<0.05)

n.s. (q>0.05) compared to 0% treatment

* Significant difference between 9% suc and 9% man

Numbers in cells denote FPKM estimates of transcript abundance

Figure 3.8 Cytokinin content and the sugar response of cytokinin metabolism genes. (A-C) Intracellular cytokinin content of WT seedling leaves grown at 3% (w/v) sucrose, and of *Arabidopsis* cell suspension cultures at 3% and 6% (w/v) sucrose. Results were analyzed with one-way ANOVAs with Tukey's post-hoc tests. ($p<0.05$, $n=3$ for seedlings and $n=4$ for cell cultures, asterisks indicate a significant difference between cells and seedlings) (D-G) Transcript profile heatmaps of the cytokinin metabolism genes from the *Arabidopsis* cell suspension culture. The *Arabidopsis* cell suspension culture was grown in triplicate at 0%, 3%, 6%, 9% (w/v) sucrose and at 9% (w/v) mannitol. Cuffdiff was used to calculate expression in samples (FPKM) and test the statistical significance (q-values). The numbers in the cells indicate the average FPKM estimate of transcript abundance. Green shading indicates a significant increase with sugar or mannitol treatment compared to 0% and red shading indicates a significant decrease. An asterisk indicates a significant difference between 9% (w/v) sucrose and 9% (w/v) mannitol treatments ($q<0.05$), highlighting genes regulated by sucrose compared to the osmotic control.

The cytokinin levels of the cell suspension cultures and of WT seedling leaves were measured by LC-MS. The medium for both the cells and seedlings contained 47 μg/L kinetin. Suspension cells exhibited lower levels of cytokinin (Fig. 3.8A) as well as riboside-conjugated and nucleotide-conjugated cytokinin, which did not change as a function of external sucrose concentrations (Fig. 3.8B). At 3% (w/v) sucrose, the riboside-conjugated cytokinin levels were 30-fold lower in suspension cells than in WT leaves.

We examined the transcript levels of cytokinin biosynthesis genes grown at 0-9% (w/v) sucrose. There are two classes of isopentenyltransferases (IPTs), the ATP/ADP IPTs (IPT1, IPT3, and IPT4-8) and the tRNA IPTs (IPT2 and IPT9). In general, the ATP/ADP IPTs are responsible for isopentenyladenine and trans-zeatin biosynthesis, and the tRNA IPTs are responsible for the cis-zeatin biosynthesis. The suspension cells did not express most of the ATP/ADP IPTs, with the exception of IPT1 (Fig. 3.8D), and consequently had extremely low levels of trans-zeatin and N6-isopentenyladenine (Fig. 3.8C) compared to seedling leaves. However, the suspension cells did express the tRNA IPTs (IPT2 and IPT9) and had comparable levels of cis-zeatin to seedling leaves (Fig. 3.8C).

3.4 Discussion

Recently, we reported that a widely studied *Arabidopsis* cell suspension culture, previously presumed to be wild type in nature exhibits a green, sucrose-insensitive phenotype in the presence of high external sucrose concentrations (McCarthy et al., 2016). We hypothesized that the unexpected sucrose-insensitive phenotype of this cell suspension culture is due to either (1) an impairment of sugar transport limiting intracellular sugar concentrations resulting in the lack of sugar signalling, or (2) impairment of a sugar signalling pathway that regulates photosynthetic gene expression. In this report, we employed a global transcriptome profiling approach to test these hypotheses through a comparison of the gene expression profiles of the *Arabidopsis* suspension cells and WT *Arabidopsis* seedlings in response to increasing external sucrose concentrations.

At the transcriptional level, sugar transport was highly regulated in the suspension cells: the sucrose transporters, cell wall invertases, and hexose transporters of the plasma membrane were strongly downregulated by exogenous sucrose (Fig. 3.4A,B,C). However, despite this repression of the sugar import genes, high levels of sucrose accumulated in the cells. The intracellular sucrose content of the suspension cells grown in the presence of ≥3% (w/v) sucrose were comparable to the levels observed in WT seedlings grown at ≥6% (w/v) sucrose. While these high levels of intracellular sucrose repressed chlorophyll levels in WT seedlings resulting in a non-green phenotype, the chlorophyll levels of the cell suspension culture were insensitive to these high levels of intracellular sucrose and consequently maintained a green phenotype.

The sugar insensitive phenotype of the cell suspension culture mimicked the phenotype of sugar insensitive *Arabidopsis* mutants. Twenty-seven mutants with sugar insensitive phenotypes have been identified in *Arabidopsis* seedlings and their genes are listed in Fig. 3.5A (Reviewed in Ramon et al., 2008). Because the knockout of a single one of these genes causes a sugar insensitive phenotype in WT *Arabidopsis* seedlings, we examined the expression of these genes in the cell suspension culture. Expression of four of these genes, *ABI3*, *ABI4*, *WOX5*, and *LIN1* was not detectable by global transcriptomics of the cell suspension culture (Fig. 3.5A). qPCR confirmed that the expression level of *ABI3* was at 200-fold lower, and that of *ABI4* at least 20-fold lower, in cells than WT seedlings grown at high sucrose (Fig. 3.5C, D). Since the *Arabidopsis* mutants, *abi3* and *abi4*, have a green phenotype on high sucrose media (Arenas-Huertero et al., 2000; Yuan and Wysocka-Diller, 2006) and *wox5* mutants have a green phenotype upon growth on high turanose, a non-metabolized sugar analogue (Gonzali et al. 2005), which matches the phenotypic response of the *Arabidopsis* suspension cells (McCarthy et al. 2016), we suggest that the lack of expression of *ABI3*, *ABI4*, and *WOX5* in the cell suspension culture appears to govern the sucrose-insensitive phenotype of the suspension cells.

In WT seedlings, *ABI3* and *ABI4* regulate photosynthetic gene expression in response to ABA. The lack of *ABI4* expression indicates significant dysfunction in the ABA sugar signalling pathway (Fig. 3.9A; Fig. 3.1D), which was confirmed by meta-analyses of

genes typically regulated by ABI4 in WT seedlings (Fig. 3.6B). Seventy-seven percent of the genes strongly upregulated by ABI4 in WT seedlings were unaffected by external sucrose in the suspension cell culture (Fig. 3.6B, yellow sector). This absence of *ABI4* transcript abundance has significant repercussions on the phenotype of the suspension cells since *ABI4* regulates the expression of a wide variety of genes in plant cells, including photosynthetic genes (Wind et al., 2013).

The low levels of the *ABI3* and *ABI4* transcript abundance suggested that ABA concentrations may be low in the suspension cells, and that ABA levels may be insensitive to external sucrose. When comparing the ABA levels in the suspension cells to those in WT seedlings, we observed that the suspension cells exhibited about 40% lower ABA on a FW basis than WT seedlings when grown at 3% (w/v) sucrose (Fig. 3.7A). In addition, increasing the sucrose level to 6% (w/v) reduced ABA levels in the suspension culture, which contrasts with the typical sugar-response: an increase in ABA levels (Fig 3.9A; Cheng et al. 2002). Furthermore, since supplying the cell suspension with exogenous ABA up to 500 μM did not alleviate the sucrose-insensitive phenotype (Fig. 3.7F), the signalling pathway is also malfunctioning downstream of ABA (Fig. 3.9A).

In addition to the sugar-insensitive phenotype, the suspension cells also appeared to be habituated to cytokinin, and therefore, no longer required exogenous cytokinin for growth. The loss of cytokinin dependency has been observed in other old cell suspension cultures and has been termed cytokinin habituation (Meins, 1989; Pischke et al., 2006). Furthermore, we have found that modifying auxin and cytokinin levels does not induce differentiation in these cells. This loss of organogenic totipotentcy has also been observed in other longstanding cell cultures (Heinz et al., 1977; Gaspar et al., 2000).

While the ABA-mediated signalling pathway was insensitive to sucrose in the cell suspension culture, the SnRK1 sugar signalling pathway (Fig. 3.1A) appears to be functional (Fig. 3.9B). The *KIN10* and *KIN11* genes, encoding components of the SnRK1 complex, were expressed in the cell suspension culture. Genes regulated by the SnRK1 sugar signalling pathway had the same sugar response in the cell suspension culture as in

WT *Arabidopsis* seedlings (Fig. 3.6A). For example, the DIN genes, known to be regulated by SnRK1 (Baena-Gonzalez et al., 2007), were strongly downregulated by sucrose (Fig. 3.9B). The similarities between the sugar response of SnRK1-regulated genes of the suspension cells and of WT leaves confirm a functional SnRK1 sugar signalling pathway (Fig. 3.6A), despite a dysfunctional ABA sugar signalling pathway (Fig. 3.9).

We conclude that, although the SnRK1 signalling pathway is functional in the *Arabidopsis* var. Landsberg *erecta* suspension culture, and this culture is deficient in the ABA signalling pathway, which results in the observed sucrose-insensitive, stay-green phenotype.

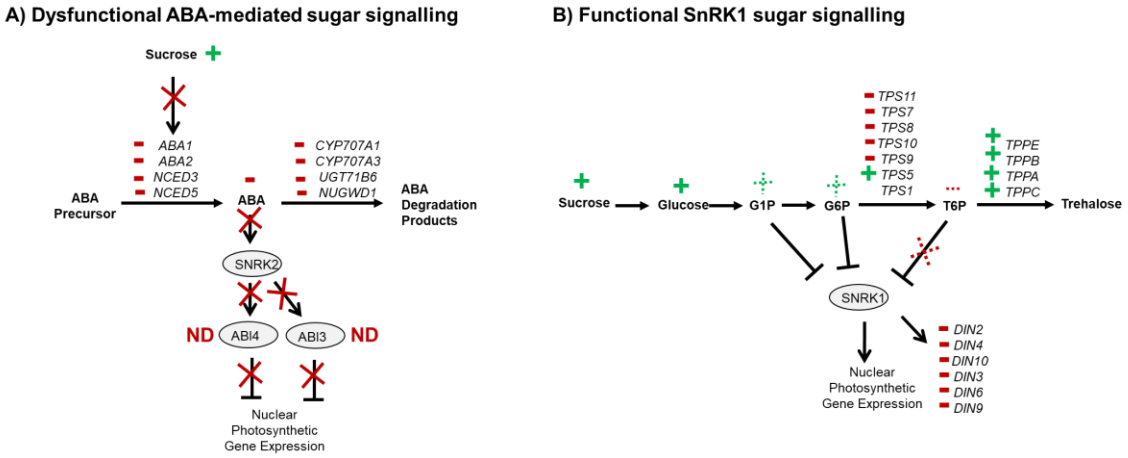


Figure 3.9 The response of key sugar signalling pathways in the cell suspension culture to high exogenous sucrose concentrations (≥6% w/v). (A) The ABA-mediated sugar signalling pathway does not function normally at high sucrose. At high sucrose, ABA biosynthesis genes are strongly downregulated, and ABA does not accumulate in the cells. The transcripts *ABI3* and *ABI4* are essentially not expressed in the cell suspension culture. ABI3 and ABI4 regulate nuclear photosynthetic gene expression, and the lack of ABI3 and ABI4 at high sucrose causes the cells to have a green sugar-insensitive phenotype. (B) The SnRK1 sugar pathway is functional at high sucrose, as indicated by the repression of the dark inducible genes (DIN). Other SnRK1-regulated genes also had a typical response to high exogenous sucrose concentrations. In response to high sucrose concentrations, trehalose-6-phosphate synthases (TPS) were repressed and trehalose-6-phosphate phosphatases (TPP) were upregulated, suggesting trehalose-6-phosphate accumulates in the cells, but this did not alleviate the overall inhibition of SnRK1 activity by high sugar concentrations. Symbols: A red (X) shows when a pathway in the cells differs from seedlings with WT sugar sensitivity. A green (+) shows analytes that increased in concentration at high sucrose (6% w/v) compared to 0% sucrose, and a red (-) shows a decrease. Dashed lines were inferred and not measured directly. The red 'ND' denotes no detection of *ABI3* or *ABI4* by RNA-Seq. G1P glucose-1-phosphate; G6P glucose-6-phosphate.

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3.6 Supplementary Tables

Table S3.1. Cuffdiff estimates of gene expression (FPKM) with statistical comparisons (q-values).

Table S3.2. Annotated heatmap of transcript profiles from the cell suspension cultures grown at 0%, 3%, 6%, and 9% (w/v) sucrose, and at 9% (w/v) mannitol.

Table S3.3. Genes differentially regulated by osmotic pressure in the cell suspension culture.

Table S3.4. Genes differentially regulated by sucrose in the cell suspension culture which were NOT regulated by osmotic effects.

Table S3.5. Genes differentially regulated by both sucrose and osmotic effects in the cell suspension culture.

Table S3.6. Gene ontology analysis of genes differentially regulated by sucrose in the cell suspension culture.

Chapter 4

4 The genome of an established *Arabidopsis thaliana* cell suspension culture reveals widespread genetic change

Plant cell cultures are commonly used in research as model systems of plant cells, in biotechnology to produce important compounds or proteins, or as an important tool in advanced plant breeding protocols. Long-term *in vitro* culturing of cell cultures is known to induce genetic changes that can lead to heritable changes in phenotype. We present a whole-genome investigation of the genetic variability present in an established cell suspension culture of *Arabidopsis thaliana* with an unusual sugar insensitive phenotype. The complete genome of the cell suspension culture was sequenced at over 300-fold coverage, and was used to identify genetic variations predicted to impact the expression and function of encoded proteins. Previous transcript profiling experiments had linked the sugar insensitive phenotype of the cells with their inability to express *ABI3* and *ABI4* transcription factors. Despite widespread genetic changes in the genome, there were no variations in the promoter regions or regulatory regions of *ABI3* or *ABI4* that could explain the lack of expression of these transcription factors. We conclude that genetic change has impacted an unknown regulator of *ABI3* or *ABI4* impairing their expression resulting in a sugar insensitive phenotype. Complete characterization of genetic variation in this established cell culture will benefit those who use established plant cell systems in biology and biotechnology.

4.1 Introduction

4.1.1 WT *Arabidopsis* genome

Arabidopsis thaliana is a small flowering plant with five chromosomes and a genome size of about 135 Mbp. The complete genome sequence for *Arabidopsis* was first published in 2000 by the *Arabidopsis* Genome Initiative (Kaul *et al.*, 2000). Since then, the genome assembly has been frequently updated, and the most recent assembly is named TAIR10. The current genome assembly currently contains 27,655 annotated coding genes.

Genetic variation in *Arabidopsis* has been heavily surveyed (Alonso-Blanco *et al.*, 2016; Cao *et al.*, 2011; Horton *et al.*, 2012; Long *et al.*, 2013; Nordborg *et al.*, 2005; Schmitz *et al.*, 2013). The 1001 genomes project has sequenced 1135 genomes with the Illumina platform, and called variants with MPI-SHORE and GMI-GATK pipelines (Alonso-Blanco *et al.*, 2016). The RegMap panel was another major project that surveyed genetic variation in 1179 *Arabidopsis* strains using Affymetrix 250k *Arabidopsis* SNP chips (Horton *et al.*, 2012). The EnsemblPlants *Arabidopsis* variation database currently includes the data from the RegMap panel (Horton *et al.*, 2012), 392 strains from the 1001 genomes project (Alonso-Blanco *et al.*, 2016), 18 *Arabidopsis* strains (Clark *et al.*, 2007), 80 strains from the Cao pilot study (Cao *et al.*, 2011), 132 strains from the Salk institute, and 180 strains from the Nordborg group (Nordborg *et al.*, 2005).

Genetic, epigenetic and karyotypic changes occur at a much faster rate in plant cell cultures than in intact plants (Larkin and Scowcroft, 1981; Noro et al., 2007). The increased genetic variation has been well documented, and this trait of cell cultures has been utilized to improve plant traits (Larkin and Scowcroft, 1981). Epigenetic modifications are more variable and less stable over time in cell cultures (Kaeppler et al., 2000). Transposable elements become active during *in vitro* culturing (Hirochika, 1993) and often there are changes in chromosome numbers (Chen and Chen, 1980).

We hypothesize that prolonged maintenance of this established cell culture line *in vitro* has resulted in genetic variation that has affected the sucrose sensing/signalling pathways that regulate photosynthetic gene expression, chlorophyll biosynthesis, and phenotype.

4.2 Methods

4.2.1 Growth of the cell suspension cultures and seedlings

The cell suspension culture of *Arabidopsis* used in this study was provided by Dr. Greg Moorhead (University of Calgary, Canada). This cell suspension culture was thought to be a culture of *Arabidopsis thaliana* var. Landsberg *erecta* originally initiated by May and Leaver (1993). The stock cell suspension cultures were grown in liquid Murashige and Skoog medium (MS, Sigma M5524), supplemented with 3% (w/v) sucrose, 0.5 mg/L naphthaleneacetic acid (NAA), 47 μg/L kinetin, 0.1X of Vitamin solution (final

concentrations of 0.2 µg/mL glycine, 10 µg/mL myo-inositol, 0.05 µg/mL nicotinic acid, 0.05 µg/mL pyridoxine hydrochloride, and 0.01 µg/mL thiamine hydrochloride), and an additional 2.5 mM of K_2HPO_4. The cell cultures were maintained on an orbital shaker set at 150 rpm in an incubator set at 25°C and illuminated with 30 µmol photons m^{-2} s^{-1} of continuous white light. The culture was sustained in exponential growth phase by diluting the 75 mL batch cultures (1:15) with fresh media every 7 days.

4.2.2 Whole Genome Sequencing

Cell cultures were grown in three separate flasks for 1 week. DNA was extracted from each flask using the DNeasy Plant Maxi Kit. Genomic DNA was fragmented using a Covaris focused ultrasonicator. The sample library was prepared using the Illumina TruSeq DNA Sample Preparation Kit. The quality of the library was assessed using TapeStation, Qubit and qPCR. Sequencing was performed using 101-cycle pair-end protocol with all three samples in a single lane (~100X coverage per sample) and 0.5% PhiX was spiked-in. DNA sequencing was performed on the Illumina HiSeq 2000 using a paired-end protocol with three samples multiplexed in a single lane.

4.2.3 Bioinformatics

Quality control analysis was generated by Qualimap v.2.1. The data was aligned and mapped to the TAIR10 *Arabidopsis* genome using bwa (v0.7.12-r1039) and 96.99% of the reads mapped to the genome. GATK (v3.3-0-g37228af) was used for small variant calling. A filter set of DP > 10, QD > 2.0, MQ > 30.0, and FS < 80.0 was applied in GATK for small variant calling. DP is the read depth. Quality by depth (QD) is the variant confidence divided by the unfiltered depth of the samples. MQ is a measure of mapping quality. MQ is the root mean square of the mapping quality of all the reads. Fisher strand (FS) is a measure of strand bias used to filter variants when the variation is seen only on either the forward or the reverse strand. High FS values indicate false positive calls. The Ensembl Variant Effect Predictor (release 34) was used to evaluate variants and predict their effects on protein coding genes.

The Strain ID tool from the 1001 genome project was used to calculate the concordance of the SNPs in the cell culture with the SNPs detected in the 1179 previously sequenced *Arabidopsis* ecotypes (Alonso-Blanco *et al.*, 2016).

The genomes of WT *Arabidopsis* ecotypes with WT sugar responses were downloaded from the 1001 genomes project (Alonso-Blanco *et al.*, 2016). Twenty-seven genomes were chosen to represent *Arabidopsis* ecotypes with WT genomes: RLD-1, Col-0, Cvi-0, Es-0, Kas-1, Ler-0, Mv-0, Tsu-0, Yo-0, Gr-1, Hn-0, Hs-0, Kil-0, Le-0, Lm-2, Mnz-0, Nc-1, NFA-8, Ob-0, Or-0, Phw-3, Phw-34, Pi-0, Pt-0, Sg-1, Si-0, and Zu-1 (Appendix A1). These ecotypes with WT sugar responses were used to reduce the list of variants identified in the sugar-insensitive cell culture. Variations associated with the sugar insensitive phenotype would not occur in the ecotypes with WT sugar responses.

4.3 Results

4.3.1 Genetic variability in key sugar signalling genes

The key genes of sugar signalling pathways were examined for deleterious variations (Table 4.1. Twenty-six genes are required for plants to respond normally to sugar. The knockout mutants for these genes have a sugar insensitive phenotype (Arenas-Huertero *et al.*, 2000; Baena-Gonzalez *et al.*, 2007; Ramon *et al.*, 2008; Yuan and Wysocka-Diller, 2006; Zhou *et al.*, 1998). Four other genes cause a sugar insensitive phenotype when overexpressed (Ramon *et al.*, 2008). The DNA sequences of these sugar signalling genes from the sugar insensitive cells were compared to 27 WT *Arabidopsis* ecotypes with WT responses to sugar. When examining these unique variants, we identified significant genetic changes affecting the following sugar signalling genes: *ABA2*, *ABF2*, *CTR1*, *ETO1*, *VAB1*, *SIS8*, *WOX5*, *LIN1*, and *TPS1*.

The genes *ABA1*, *ABA2*, and *ABA3* encode ABA biosynthesis enzymes. *ABA1* did not have any unique variations in the promoter or protein coding regions. The variant effect predictor tool incorrectly called a two-base insertion and two base deletion in *ABA1* as a unique variation. However, manual inspection of the *ABA1* sequence revealed that WT *Arabidopsis* ecotypes had 4 SNPs in this region which were equivalent to these two INDELs. *ABA2* had a one base deletion in its promoter region. However, this did not

appear to affect the expression of *ABA2* (See Chapter 3), as transcriptome analysis showed that the gene was highly expressed in the cell culture at 0% sucrose, and strongly downregulated by sucrose treatment. *ABA3* had a twenty-seven base deletion in its 3' UTR region which could affect the localization of the *ABA3* transcript, translation efficiency or stability of the mRNA.

The genes *ABI3*, *ABI4*, *ABI5*, *ABI8* and *ABF2* encode transcription factors involved in ABA signal transduction. The genes *ABI3*, *ABI5*, and *ABI8* did not contain any unique genetic variations compared to WT. The *ABF2* gene encodes an ABA-responsive transcription factor. *ABF2* had a fifteen base inframe insertion in the exon region, as well as multiple SNPs and INDELs in the 5' UTR and 3' UTR regions (Table 4.1). Previous RNA Sequencing experiments showed that *ABF2* is expressed in the cells (see Chapter 3), however the deletion in the exon could have affected the function of this transcription factor, and the change in the 3' UTR region could have impacted the localization of the *ABF2* transcript.

ABI3 and *ABI4* were key genes of interest, because previous transcriptomic analysis showed that these key sugar signal transducers (*ABI3* and *ABI4*) were not expressed in these cells (See Chapter 3). Since ABI3 and ABI4 are required for sugar signalling, the lack of expression of these genes is a probable cause of the sugar insensitive phenotype of this cell culture. *ABI3* did not contain any unique variations compared to WT *Arabidopsis* ecotypes. *ABI4* contained a three base inframe deletion in the exon region (Table 4.1). However, since the gene was not expressed in the cells, this deletion in the exon region is inconsequential. There were no variants in the promoter regions of *ABI3* or *ABI4* that could explain the total lack of expression of these two genes in the cells. There were variants over 2 kb upstream of the transcriptional start sites of *ABI3* and *ABI4* but more work is required to see whether these variations could have contributed to the complete repression of *ABI3* and *ABI4*.

CTR1 is a negative regulator of ethylene responsive transcription factors. The *CTR1* gene had a two-base insertion in the 3' UTR region, and two deletions in the 5' UTR (two bases and sixteen bases in length; Table 4.1). ETO1 is also a negative regulator of

ethylene signalling. The *ETO1* gene contained a one base pair frameshift deletion in the exon region (Table 4.1). This variant had an allelic frequency of 0.5, and therefore half of all transcripts differed from WT at this position. The *ETO1* gene also contained a missense SNP in the exon region, but the frequency of this allele was only 33% (Table 4.1). The variant calling software also found a twelve-base deletion in the exon region of *ETO1*, but manual inspection determined this was not actually unique, because other WT ecotypes contained two equivalent deletions at that position in the gene (an 11 base deletion and a one base deletion).

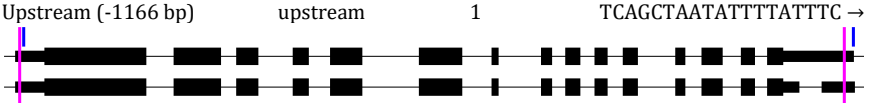
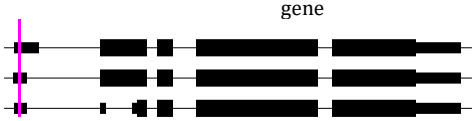
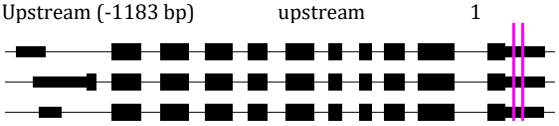
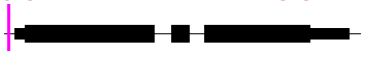
LIN1 encodes a high affinity nitrate transporter (Little *et al.*, 2005). The *LIN1* gene had a unique SNP in its promoter region, thirty-eight bases upstream of the transcriptional start site (Table 4.1). This SNP had an allele frequency of 0.5, and may have affected the transcription of this gene. Previous transcriptome profiling experiments showed that *LIN1* was not expressed in the cells, independent of sucrose treatment.

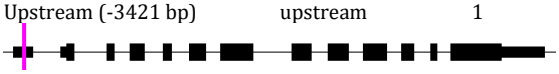
SIS8 encodes a mitogen-activated protein kinase kinase kinase, and *UGT72E1* encodes a UDP-glucose:glycosyltransferase. SIS8 and UGT72E1 are interacting proteins that are both required for a wild-type sugar response (Huang *et al.*, 2014). The *SIS8* gene had a missense SNP in the exon region (Table 4.1). This missense SNP had an allele frequency of 0.5, and could affect the function of half of the SIS8 proteins. *SIS8* also had a unique synonymous SNP in the exon region that is unlikely to affect protein function (Table 4.1). The *UGT72E1* gene did not contain any significant unique genetic variations in the promoter or protein coding regions.

TPS1 encodes a key trehalose phosphate synthase that can cause a sugar insensitive phenotype when overexpressed (Avonce *et al.*, 2004). The gene did not have any genetic variations that could cause increased expression. There was a high impact frameshift two base insertion in the exon region that had an allele frequency of 0.5, which affected the protein sequence of half of the TPS1 transcripts, and there were two SNPs in introns (Table 4.1).

Table 4.1 Genetic variation in genes required for WT sugar signalling. Pink, red and grey lines indicate genetic variations unique to the cell culture, not found in twenty-seven WT *Arabidopsis* ecotypes. The impact of the variations were predicted using the Ensembl Variant Effect Predictor tool tool, and are coloured purple (moderate-impact variations), red (high-impact frameshift exon variations), and grey (low-impact synonymous variations). Blue lines indicate the locations of variations compared to the WT RLD-1 *Arabidopsis* strain. The allelic frequency (AF) of each variation is given in the description. Gene models are drawn 5' (left) to 3' (right). Continued in Table S4.1.

Gene (Locus)	Variant Region	Variant Type	Allele Frequency	Reference Sequence → Cells Sequence
ABI3 (AT3G24650)				
	Upstream (-4303 bp)	upstream	0.5	T → TA
	Upstream (-2622 bp)	upstream	1	T → TATATACAC
ABI4 (AT2G40220)				
	Exon (Moderate Impact)	**inframe deletion**	**1**	**TTTG → T**
	Upstream (-1919 bp)	upstream	1	TC → T
	Upstream (-1922 bp)	upstream	1	TTC → T
	Upstream (-2156 bp)	upstream	1	T → A
ABA2 (AT1G52340)				
	Promoter (-55bp)	**upstream**	**1**	GT → G
	Intron	Intron	1	TGATCTGGGCCACAAAATAAAAAAAAA AA → T
	Upstream (-1904 bp)	upstream	1	C → T
	Upstream (-1922 bp)	upstream	1	GA → G
ABA3 (AT1G16540)				
	3' UTR	3' UTR	1	ATCAAAAGTATAAACATATGGAGAAGC G → A
	Upstream (-4942 bp)	Upstream	1	AGCC → A
	Upstream (-4939 bp)	Upstream	1	C → CTAAT
	Upstream (-4860 bp)	Upstream	1	G → GA
	Upstream (-4834 bp)	Upstream	1	A → G
	Upstream (-4812 bp)	Upstream	0.66	C → CAAACAAAAAAACAAGTAATGCACTAA ACAGAGGAAT CAAAG
	Upstream (-4805 bp)	Upstream	0.66	A → AGAAACAAATAATAATACACT
	Upstream (-4802 bp)	Upstream	1	G → GCAGAGGAATCAAACAAGCAAAGAA
	Upstream (-4127 bp)	Upstream	1	A → T
	Upstream (-1694 bp)	Upstream	1	AAAACATTC → A
	Upstream (-1429 bp)	Upstream	0.16	T → C
ABF2 (AT1G45249)				
	Exon (Moderate Impact)	**inframe insertion**	**1**	**A → ATTGTTGTTCTTGAAG**
	5' UTR	5' UTR	1	AAT → A
	5' UTR	5' UTR	1	A → C
	5' UTR	5' UTR	1	A → C
	5' UTR	5' UTR	1	G → A
	3' UTR	3' UTR	1	AC → A

	3' UTR	3' UTR	1	GA → G
	3' UTR	3' UTR	1	A → ATG
	3' UTR	3' UTR	1	A → AG
	Upstream (-477 bp)	upstream	0.33	C → CATATAT,CATATATAT
	Upstream (-941 bp)	upstream	1	G → GA
	Upstream (-1158 bp)	upstream	1	TAAAAAAA → T
	Upstream (-1166 bp)	upstream	1	TCAGCTAATATTTTATTTC → T
CTR1 (AT5G03730)				
	5' UTR	5' UTR	1	GTCTCTCTCTCTCTCTC → G
	5' UTR	5' UTR	1	TTG → T
	3' UTR	3' UTR	1	T → TAG
	Upstream (-873 bp)	upstream	1	A → T
	Upstream (-1230 bp)	upstream	1	C → CAAA
	Upstream (-1250 bp)	upstream	1	A → T
	Upstream (-1298 bp)	upstream	1	CAA → C
	Upstream (-1862 bp)	upstream	1	TA → T
	Upstream (-3661 bp)	upstream	0.16	G → GT,GTT
	Intron	Intron	1	TAAA → T
	Intron	Intron	0.83	A → T
	Intron	Intron	1	CT → C
ETO1 (AT3G51770)				
	Exon (High Impact)	**frameshift**	**0.5**	**GA → G**
	Exon (Moderate Impact)	**missense**	**0.33**	**C → T**
	Exon (Moderate Impact)	**inframe deletion**	**1**	**TACCACCACCACC → T**
	3' UTR	3' UTR	1	GA → G
	Exon (Low Impact)	synonymous	0.5	A → T
	Exon (Low Impact)	synonymous	0.5	G → A
	Exon (Low Impact)	synonymous	0.5	C → A
	Exon (Low Impact)	synonymous	0.5	C → T
	Exon (Low Impact)	synonymous	0.5	G → T
	Exon (Low Impact)	synonymous	0.33	G → A
	Downstream (3889 bp)	downstream gene	0.5	AAT → A
FIN219 (AT2G46370)				
	5' UTR	5' UTR	1	TTC → T
	Upstream (-885 bp)	upstream	1	GATATGAAGAATTGTTAACC → G
	Upstream (-847 bp)	upstream	1	T → A
	Upstream (-1183 bp)	upstream	1	GT → G
KIN10 (AT3G01090)				
	3' UTR	3' UTR	1	GAAAGAGAAGAAGAAAA → G
	3' UTR	3' UTR	0.5	GAA → G,GA
LIN1 (AT1G08090)				
	Promoter (-38 bp)	**upstream**	0.5	G → A
	Upstream (-2387 bp)	upstream	0.83	C → A
	Upstream (-2377 bp)	upstream	1	G → A
	Downstream (550 bp)	downstream gene	1	C → T
SIS8 (AT1G73660)				
	Exon (Moderate Impact)	**Missense**	**0.5**	C → G
	Exon (Low Impact)	synonymous	0.5	T → A
	Upstream (-285 bp)	upstream	1	A → AT
	Upstream (-1400 bp)	upstream	0.5	CA → C
TIR1 (AT3G62980)				
	5' UTR	5' UTR	1	T → TAG

	Upstream (-2952 bp)	upstream	1	A → AGT
	Upstream (-2957 bp)	upstream	1	CAA → C
	Upstream (-4643 bp)	upstream	1	AC → A
	Upstream (-4651 bp)	upstream	1	AAAACATAACATAACATAAAATTTG → A
UGT72E1 (AT3G50740)				
	5' UTR	5' UTR	0.33	T → TG,TTTG
	Upstream (-465 bp)	upstream	1	G → GA
	Upstream (-2604 bp)	upstream	0.5	CAA → C,CA
	Upstream (-3215 bp)	upstream	1	G → A
	Upstream (-3219 bp)	upstream	1	C → T
	Upstream (-3237 bp)	upstream	1	C → T
	Upstream (-3421 bp)	upstream	1	G → A
VAB1 (AT1G76030)				
	5' UTR	5' UTR	1	TCTCTCTCC → T
TPS1 (AT1G78580)				
	Exon (High Impact)	**frameshift**	**0.5**	**T → TGA**
	5' UTR	5' UTR	1	T → TA
	Intron	intron	0.5	T → A
	Upstream (-3445 bp)	upstream	1	GAA → G
	Upstream (-3447 bp)	upstream	1	T → TCC

4.3.2 Genetic variability in ABA biosynthesis genes

The accumulation of ABA in response to sugar is the first step in the ABA sugar signalling pathway. Previous experiments have suggested possible dysfunction of ABA biosynthesis and/or catabolism in the sugar insensitive cell suspension culture. When WT *Arabidopsis* seedlings are treated with high sucrose (≥6% w/v), the leaves accumulate ABA (Cheng *et al.*, 2002; Rolland *et al.*, 2006; Seo and Koshiba, 2002). However, the sugar insensitive cell culture did not accumulate ABA in response to sugar (See Chapter 3). The genes involved in ABA biosynthesis were therefore examined for deleterious genetic variations.

The ABA biosynthesis genes of the cells were compared to those from twenty-seven WT *Arabidopsis* ecotypes (Table S4.2). The ABA biosynthesis genes *AA04, ABA4, CED1, NCED2, NCED5, NCED6,* and *TOR* did not have any unique variations compared to WT *Arabidopsis* (Table S4.2). The ABA biosynthesis genes *AAO1, AAO2,* AAO4, *ABA3,* and NCED3 had 3' UTR variations which could affect transcript localization, translation efficiency or stability of these mRNA. The ABA biosynthesis gene *ABA2* had a one base deletion in its promoter region. However, this did not appear to affect the expression of *ABA2* (See Chapter 3), as transcriptome analysis showed that the gene was highly expressed in the cell culture at 0% sucrose, and strongly downregulated by sucrose treatment.

The only ABA biosynthesis gene with significant genetic variation in exon regions was the *NCED9* gene, which contained a missense SNP in the exon region with an allele frequency of 0.5 (Table 4.2). Previous RNA-Seq data showed that this gene was only expressed at very low levels in the cell suspension culture (See Chapter 3).

Table 4.2 Genetic variations in *NCED9*, the only gene in the ABA biosynthesis pathway with a genetic variations in the exon region. This genetic variation did not occur in twenty-seven WT *Arabidopsis* ecotypes. The pink line indicates a genetic variation unique to the cell culture, not found in twenty-seven WT *Arabidopsis* ecotypes.

Gene (Locus)	Variant Region	Variant Type	Allele Frequency	Reference Sequence → Cells Sequence
NCED9 (AT1G78390)				
	Exon (Moderate Impact)	**Missense variant**	**0.5**	**C → T**
	Upstream (-1234 bp)	Upstream variant	1	T → TA
	Upstream (-1856 bp)	Upstream variant	1	C → CATCATTAATTAT
	Upstream (-3785 bp)	Upstream variant	1	A → AGTCT

4.3.3 Evaluation of Candidate genes identified by RNA-Seq: Genetic variation in genes strongly upregulated by sugar in WT *Arabidopsis* which were not expressed in the cell suspension culture

Lists of candidate genes that may be responsible for the sugar insensitive phenotype of the cell suspension culture, were generated from meta-analysis comparing the RNA-Seq transcriptome profiles of the cell cultures grown at 0%, 3%, 6%, and 9% (w/v), to transcriptional profiles of WT *Arabidopsis* from the literature. Müller et al. (2007) identified hundreds of genes up-regulated by sucrose by comparing the transcriptional profiles of *Arabidopsis* seedlings grown at 0% and 3% (w/v) sucrose. Fifty-two genes strongly upregulated by sucrose (0% to 3% w/v) in wild-type seedlings were not expressed at all in the cell suspension culture at 0%, 3%, 6%, or 9% (w/v) sucrose (Table S4.3). A second study by Han et al. (2015) identified 558 genes upregulated by high sugar treatments by comparing the transcriptional profiles of WT seedlings grown at 1% and 5% (w/v) glucose. One hundred and seventy-three of these genes were not expressed at all in the cell suspension culture, independent of sucrose treatment (Table S4.4). The promoter region of each of these key genes was examined to look for variations unique the cell culture that could contribute to the absence of expression.

Of the 225 genes strongly upregulated by sugar in WT *Arabidopsis* but not expressed in the cell suspension culture (Table S4.3 and S4.4), only six genes had variations in the promoter regions: *AT5G54060, AT1G62540, AT4G09600, AT3G41762, AT3G04290, AT5G24660* (Table 4.3). *AT5G54060* encodes a UDP-glucose:flavonoid 3-O-glucosyltransferase located in the chloroplast. *AT1G62540* encodes flavin-monooxygenase glucosinolate S-oxygenase 2, which is involved in glucosinolate biosynthesis. *AT4G09600* encodes the GASA1 protein which is involved in the gibberellic acid signalling pathway. *AT3G04290* encodes Li-tolerant lipase 1, which is involved in lipid catabolism and is also involved in the response to hyperosmotic salinity. *AT5G24660* encodes the response to low sulfur 2 (LSU2) nuclear protein involved in response to stress and chloroplast organization.

Table 4.3 Genetic variation in the promoter region of genes which were not expressed in the cell suspension culture, but which were strongly upregulated by sugar in WT *Arabidopsis*. These genetic variations are unique to the cell suspension culture and have not been recorded in any of the *Arabidopsis* strains sequenced in the 1001 genomes project. 'REF' refers to the reference TAIR10 sequence, and 'ALT' shows the genetic variation. The distance upstream of the transcriptional start site (TSS), and the allele frequencies (AF) of the variations are also shown.

	Encoded protein	REF	ALT	Distance upstream of TSS	AF
AT5G54060	UF3GT	C	CTTTGTTATT	5	1.0
AT1G62540	FMO	AATG	A	65	1.0
AT4G09600	GASA3	A	G	28	1.0
AT3G41762	unknown	TCC	T	4	0.5
AT3G04290	ATLTL1	TGACAA	T	12	1.0
AT5G24660	LSU2	G	C	51	0.5

4.3.4 Genetic variability in the cell suspension culture

Genomic sequencing revealed that the sugar insensitive *Arabidopsis* cell suspension culture is derived from the RLD-1 ecoytpe, not Landsberg *erecta* (Appendix 4.1). The RLD-1 ecotype of the cell culture was determined using the StrainID tool of the 1001 Genomes Consortium (2016). The genome of the cell suspension culture was compared to the genomes from 27 *Arabidoipsis* ecotypes with WT sugar responses (Appendix 4.1). The cells had 342,528 genetic variations (SNPs/INDELs) that did not occur in RLD-1, nor in 26 other *Arabidopsis* ecotypes with WT sugar responses (Appendix 4.1). Within this list of variations, 2,733 variations occurred in exon regions, and were predicted to have a high impact on encoded protein function (frameshift, stop-gained, start-lost, and stop-lost), and 14,173 variations in exon regions were predicted to have a moderate impact on encoded protein function (missense, in-frame nonsynonymous deletions, and in-frame nonsynonymous insertions; See Appendix 4.1). The genetic variations unique to this cell culture occurred in across all cellular processes (Appendix 4.1). The genome of the cells has changed considerably during two decades of *in vitro* culturing, and we have identified many novel and deleterious genetic changes (Appendix 4.1).

4.4 Discussion

The purpose of this work was to examine whether the genetic variation brought about by *in vitro* culturing was related to the unusual sugar insensitive phenotype observed in this *Arabidopsis* cell suspension culture. We have previously shown that the cell suspension culture was insensitive to sugar, as it has a dark green phenotype on high sugar media (McCarthy *et al.*, 2016). Independent of sugar concentrations, the cells had high levels of chlorophyll, expressed the full set of photosynthetic genes, and accumulated high levels of key photosynthetic proteins (McCarthy *et al.*, 2016). Transcript profiling identified ABA biosynthesis and the ABA-sugar signalling pathways as pathways that malfunctioned in this cell suspension culture (See Chapter 3). We therefore explored genetic variability affecting genes of the ABA biosynthesis and ABA-sugar signalling pathways. The ABA biosynthesis genes *NCED3* and *NCED9*, and the sugar signalling genes *ABF2, CTR1, ETO1, LIN1,* and *SIS8*, had significant variations that could affect their expression, function, or transcript localization. Although these variations were

significant, there were too many variations in the cell suspension culture to identify a single variation as the cause of the unusual sugar insensitive phenotype.

From the variants identified in sugar signalling genes, a few stand out as potential contributors to the sugar insensitive phenotype. The inframe insertion in the *ABF2* exon region could have affected the function of this ABA-responsive transcription factor, an essential component of the sugar signalling pathway. Variants in the 3' UTR regions of *ABA3*, *ABF2*, and *CTR1* could affect cellular localization of these transcripts that are involved in ABA biosynthesis, ABA signalling, and regulation of ethylene signalling. *ETO1* and *SIS8* contain unique missense SNPs that could affect the function of ETO1 and SIS8 proteins, two proteins required for WT sugar responses. Finally, *LIN1* contained a SNP in the promoter region that may have blocked the expression of this gene in the cell culture. While LIN1 is essential for WT sugar responses in roots, there is no known link between LIN1 to photosynthetic regulation. Previous transcript profiling experiments showed that the cell suspension culture did not express *LIN1*, independent of sugar treatment. While some of these highlighted variations may have caused or contributed the sugar insensitive phenotype of the cell culture, given the abundance of genetic variations throughout the genome, further experiments are required to conclusively identify the cause of the sugar insensitive phenotype.

Transcript profiling associated the sugar insensitive phenotype with the lack of *ABI3* and *ABI4* expression in the cell suspension culture (Chapter 3). However, neither *ABI3* nor *ABI4* had variations in their promoter regions that could explain the complete lack of expression. Future experiments should look for novel regulators of *ABI3* and *ABI4*. Given the abundance of genetic variations in the cells, we suspect that there could have been impairment of an unknown regulator of *ABI3* or *ABI4* causing the sugar insensitive phenotype.

Transcriptome profiling experiments identified 225 genes that were strongly upregulated by sugar in WT, but not expressed under any conditions in the cell suspension cultures. Of these, the genes *UF3GT, FMO, GASA3, AT3G41762, ATLTL1,* and *LSU2* had significant genetic variations in their promoter regions that could be related to their lack

of expression. These variations were unique to the cell suspension culture and may be related to the sugar insensitive phenotype of the cells. However, we again caution readers that the high number of genetic variations in these cells make these associations weak, and much more analysis is required.

This work has provided the first complete genome sequence of a plant cell suspension culture. If the unique genetic variants observed in this cell suspension culture reappear in other plant cell suspension cultures in the future, they could potentially be associated with enhanced growth or fitness of plant cell cultures *in vitro*. Factors affecting growth of plant cell cultures have important implications in biology and biotechnology. Although cell suspension cultures are often used as model systems for plant biology research, this work indicates that established cell culture lines should be used with caution as their genome can differ significantly from that of wild-type *Arabidopsis*.

Characterization of genetic variations in this *Arabidopsis* cell suspension culture will provide a useful reference to researchers using this established plant cell culture line. As a result of prolonged *in vitro* culturing, this cell culture has undergone significant genetic change; enough to perhaps be considered a new ecotype of *Arabidopsis*. This study provides strong rationale for the sequencing of other prominent plant cell suspension cultures. The utility of plant cell cultures has grown considerably recently, and it is critical to understand the effects of *in vitro* culturing on the genome and ultimately phenotype.

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4.6 Supplementary Tables

Table S4.1 Genetic variation in genes required for WT sugar signalling (Continuation of Table 4.1)

Table S4.2 Genetic variation in genes involved in ABA biosynthesis.

Table S4.3 Genes upregulated by sucrose in WT leaves which were not expressed at all in the cell suspension culture. These genes were identified through meta-analysis comparing transcript profiles from WT seedlings from Müller et al. (2007), with the transcript profiles of the cell suspension cultures.

Table S4.4 Genes upregulated by sucrose in WT leaves which were not expressed at all in the cell suspension culture. These genes were identified through meta-analysis comparing transcript profiles from WT seedlings from Han et al. (2015), with the transcript profiles of the cell suspension cultures.

Table S4.5. The VCF file listing all genetic variants called by GATK compared to the TAIR10 reference genome. The list has been filtered for read depth (DP > 10), quality by depth (QD > 2.0), mapping quality (MQ > 30.0), and strand bias (FS < 80.0).

Table S4.6. The variants identified in the sugar insensitive cell culture genome (vs. TAIR10) compared to the natural variants that occur in the 27 WT strains of Arabidopsis with WT sugar insensitivity: RLD-1, Col-0, Cvi-0, Es-0, Kas-1, Ler-0, Mv-0, Tsu-0, Yo-0, Gr-1, Hn-0, Hs-0, Kil-0, Le-0, Lm-2, Mnz-0, Nc-1, NFA-8, Ob-0, Or-0, Phw-3, Phw-34, Pi-0, Pt-0, Sg-1, Si-0, and Zu-1.

Table S4.7. The list of cell culture genetic variants that that did not occur in the 27 WT genomes with WT sugar sensitivity. These unique genomic variants that were sorted by the regions where they occur (e.g exon, intron, promoter, 3’ UTR, 5’ UTR, upstream, downstream). Variants in the exon regions were further categorized based on the predicted impact on the protein product.

4.7 Appendices

Appendix 4.1. Genome sequencing quality statistics, ecotype identification, and analysis of genetic variants identified in the cell suspension culture genome.

Chapter 5

5 Summary and Perspectives

Arabidopsis cell suspension cultures are commonly used as model systems in plant biological research. The cell line examined in this thesis has been utilized in over 140 published studies to date. Prior to this work, no genomic reference existed for this established *Arabidopsis* cell culture, and the genomic reference is important because the cell line has developed considerable genomic variation since its initiation decades ago. The cell line has also developed an impaired ABA-mediated sugar signalling pathway resulting in an unusual stay-green phenotype at high sugar concentrations. The striking genome-wide variations, coupled with the unusual sugar responses, has important implications for designing and interpreting experiments with this *Arabidopsis* cell suspension culture line.

This work has linked the sugar insensitive phenotype of the cells to the lack of *ABI3* and *ABI4* expression, two essential transcription factors in the ABA-mediated sugar signalling pathway. These transcription factors were not expressed at high sugar in these cells, and as a result, the sugar insensitive phenotype of the cell suspension culture mimicked that of mutant *abi3* and *abi4* seedlings. The cell suspension culture also had a stay-green phenotype when high levels of ABA were added to the culture medium, and this ABA insensitivity provided further evidence of the dysfunction of the ABA-mediated sugar signalling pathway. Transcriptional responses to sugar showed that transcripts typically regulated by *ABI4* in WT plants did not respond to sugar in the cell suspension culture. Future experiments could examine whether transfecting the cells to express *ABI3* and/or *ABI4* transcription factors reverts the cells to a WT phenotype. Future work should also examine whether dysfunction of the ABA-mediated sugar signalling pathway imparts the cell suspension culture with a competitive advantage over new freshly isolated cell lines.

Given the abundance of genetic variations in the cells, and the lack of *ABI3* and *ABI4* expression at high sucrose, the sequences of the promoter and regulator regions of *ABI3*

and *ABI4* were examined. Remarkably, the promoter and regulatory regions of *ABI3* and *ABI4* did not have any genetic variations compared to WT. The lack of *ABI3* and *ABI4* expression impacts other biosynthetic processes, in addition to altering photosynthetic regulation, and this should be taken into consideration when designing or interpreting experiments with this established *Arabidopsis* cell suspension culture.

This thesis examined how these cells with impaired ABA-mediated sugar signaling respond to sugar through characterization of cell physiology, biochemistry, transcriptomics, and genomics, advancing our understanding of sugar signaling, cellular sugar responses, and photosynthetic regulation. Dysfunction of the ABA-mediated sugar signalling pathway caused the cells to continue producing high levels of photosynthetic machinery when the cells already have high levels of sugars intracellularly. Sucrose stimulated transcript levels of genes involved in the chlorophyll biosynthetic pathway (*ChlH*, *ChlI2*, *DVR*), and chlorophyll levels in the cells were insensitive to sucrose. As a result, the cells maintained a dark green phenotype at high sucrose concentrations (≥ 6% w/v). Although most of the genes associated with photosynthesis (photosystem II and photosystem I reaction centers and light harvesting complexes as well as genes associated with the cytochrome b6f and the ATP synthase complexes) were downregulated or remained unaffected by high sucrose, immunoblotting indicated that protein levels of PsaA, Lhcb2, and Rubisco per gram fresh weight increased as a function of external sucrose concentration. Photosynthesis is a metabolically expensive process, so synthesizing these photosynthetic complexes and pigments when sucrose is abundantly available is a waste of nutritional resources.

The cells were photomixotrophic, combining energy from photosynthesis with energy from sucrose obtained from the medium. When the growth medium of the cell suspension culture contained moderate or high sugar (3-9% w/v sucrose), the cells had high rates of sugar uptake (0.15-0.35 g suc $\cdot$ g FW^{-1} $\cdot$ day^{-1}), and this translated to high levels of intracellular sucrose (approx. 5 mg suc $\cdot$ g FW^{-1}). Despite high levels of intracellular sucrose, there was no repression of chlorophyll biosynthesis, and we also observed very little accumulation of starch in the cells. The cell culture was photosynthetically competent based on light-dependent, CO_2-saturated rates of O_2 evolution, as well as

Fv/Fm and P700 oxidation. The cells had high rates of cellular respiration and the maximum light saturated rates of photosynthesis were always lower than the rates of respiration. Future studies should examine whether photosynthesis imparts the cell suspension culture with a competitive advantage over new freshly isolated cell lines with WT sugar sensitivity.

The *in vitro* culturing of plant cells has many important applications in biology and biotechnology. Cell suspension cultures are ideal for studying biological processes as they are axenic, homogenous, and because the artificial medium can be strictly controlled and easily manipulated. Cell cultures facilitate studies at the cellular levels while removing the structural complexity of a multicellular plant. However, given the abundance of genetic variations in this cell suspension culture, and the unusual responses to sugar, caution is warranted when designing experiments using this cell suspension culture to study biological processes. This thesis detailed the genetic, biochemical, and physiological consequences of long-term cultivation of an established plant cell culture. This is an important resource for other groups maintaining long term plant cell cultures for biological research or biotechnology applications.

This unusual sugar insensitive phenotype is a consequence of the *in vitro* culturing of the cell cultures. Genetic analysis has shown that there have been 342,528 genetic variations unique to this cell line that have occurred since it was first induced from a WT plant. This agrees with studies that have shown that cell cultures undergo rapid genetic variation (Larkin and Scowcroft, 1981; Noro *et al.*, 2007). Analysis of the variations unique to this cell culture showed that 2,733 of the variations likely impair proteins. While the cells have undergone significant genetic changes, some of these genetic changes have likely been adaptive, helping the cells to grow more rapidly *in vitro*. The cell culture has a high growth rate of 0.42 d^{-1}, which is faster than the typical growth rate of newly initiated cell cultures. Variations and traits benefiting *in vitro* growth rates would have important biotechnology applications. Future work should explore whether *in vitro* culturing selects for similar traits in other established plant cell cultures.

The cell culture has undergone significant genetic variations since its induction. We have identified thousands of variations that were predicted to impair the function of encoded proteins. Consequently, the cells have some unusual traits and caution is warranted when using established cell cultures as a model system. The genome of the cells will serve as a resource to those who continue to use this cell suspension culture, and will allow further genetic variations to be quantified in the future. This study provides strong rationale for the sequencing of other established plant cell cultures including the T87 *Arabidopsis* cell culture, the BY-2 tobacco culture, and established cell cultures of other plant taxa. Plant cell suspension cultures will continue to grow as an important tool in plant biology, and it is important to understand the genetic consequences of *in vitro* culturing.

5.1 References

Larkin PJ, Scowcroft WR. 1981. Somaclonal variation - a novel source of variability from cell cultures for plant improvement. *Theoretical and Applied Genetics* **60**, 197-214.

Noro Y, Takano-Shimizu T, Syono K, Kishima Y, Sano Y. 2007. Genetic variations in rice *in vitro* cultures at the EPSPs-RPS20 region. *Theoretical and Applied Genetics* **114**, 705-711.

Curriculum Vitae

Avery McCarthy

Education

September 2011 – Current
Ph.D. in Biology
University of Western Ontario
London, ON

September 2008 – October 2010
M.Sc. in Chemistry
Mount Allison University
Sackville, NB

September 2004 – April 2008
B.Sc. in Chemistry and Biochemistry
Mount Allison University
Sackville, NB

Research Experience

February 2017 – Current
Genomics Laboratory Technologist
eDNAtec Inc.

- Used DNA metabarcoding to characterize biodiversity of specific areas and to monitor changes over time.
- Designed and tested primers to target specific taxa in DNA metabarcoding.
- Increased throughput of DNA metabarcoding workflow by testing and implementing high throughput methods, and automating protocols
- Purchased equipment and consumables to set up a new next generation DNA sequencing lab with two Illumina MiSeqs and a NovaSeq
- Wrote SOPs for DNA metabarcoding methods

September 2011 – Current
Doctoral Research
Biology Department, University of Western Ontario

- Designed experiments to identify genes responsible for an unusual sugar insensitive phenotype we observed in a model plant cell culture.

- Produced a detailed biochemical and physiological survey of the sugar insensitive cell suspension culture.
- Sequenced plant genomes and transcriptomes and analyzed hundreds of gigabytes of sequencing data. The sequencing was carried out on the Illumina HiSeq 2500 next generation sequencing platform. The plant of interest, *Arabidopsis thaliana*, has a relatively small genome of 135 megabase pairs. However, the nuclear genome encodes over 25,000 genes.
- Successfully identified two genes (*ABI3* and *ABI4*) responsible for the sugar insensitive phenotype.

September 2008 – October 2010
Masters Research
Biochemistry Department, Mount Allison University

- Designed experiments to determine the influences of increased atmospheric CO_2 on the growth, physiology, pigmentation, and key protein levels of selected marine phytoplankton.
- Set up and developed operating procedures for new photobioreactors and mass flow controllers. The bioreactors were set up to maintain constant light intensity, temperature, cell density, pCO_2, and pH while the axenic cultures grew exponentially.
- Helped to build an understanding of the adaptation strategies and capacities of two major phytoplankton groups, diatoms and coccolithophores, which are responsible for about 40% of marine primary production and produce about 20% of the world's oxygen.

September 2010 – September 2011
Research Assistant
Full Time Contract Position
Biochemistry Department, Mount Allison University

Meta-analyses and publication of 10 years of physiological and molecular data on phytoplankton biology. Developed a MYSQL database with website interface to facilitate data analysis and publication. http://phytoplankton.mta.ca/Search.php

May 2008 – August 2008
Research Assistant
Full Time Contract Position
Biochemistry Department, Mount Allison University

Collaborated on a study investigating how cell size trade-offs govern light exploitation strategies in marine phytoplankton. I was responsible for aspects of the study that involved quantitative immunoblotting, HPLC, and *in vivo* fluorometry.

Laboratory Skills

Whole genome sequencing	HPLC	Aseptic technique
RNA-Seq	LCMS	Cell culturing
Metabarcoding	Automated protocols	Primer design and testing
qPCR	Enzyme assays	Bioreactors
Immunoblotting	Kinetics measurements	NMR
Northern blotting	IRGA	AAS
in vivo fluorescence	Coulter Counter	Light microscopy
77K fluorescence	CHN analysis	Confocal microscopy
Bioanalyzer	Molecular cloning	Electron microscopy

Data Analysis and Programming Tools

R, Perl, Excel, SQL, PHP, HTML, Javascript, MATLAB, SPSS

Research Dissemination

Peer-Reviewed Publications

McCarthy, A., Chung, M., Ivanov, A. G., Krol, M., Inman, M., Maxwell, D. P., & Hüner, N. P. A. (2016). An established *Arabidopsis thaliana* var. Landsberg *erecta* cell suspension culture accumulates chlorophyll and exhibits a stay-green phenotype in response to high external sucrose concentrations. *Journal of Plant Physiology*, 199, 40-51.

Li, G., Brown, C. M., Jeans, J. A., Donaher, N. A., **McCarthy, A.**, & Campbell, D. A. (2015). The nitrogen costs of photosynthesis in a diatom under current and future pCO_2. *New Phytologist*, 205(2), 533-543.

Farhat N., Rabhi M., Krol M., Barhoumi Z., Ivanov A. G., **McCarthy A.**, Abdelly C., Smaoui A., & Hüner N. P. A. (2014). Starch and sugar accumulation in *Sulla carnosa* leaves upon Mg^{2+} starvation. *Acta Physiol Plant*, 36, 2157-2165.

McCarthy, A., Rogers, S. P., Duffy, S. J., & Campbell, D. A. (2012). Elevated carbon dioxide differentially alters the photophysiology of T*halassiosira pseudonana* (Bacillariophyceae) and *Emiliania huxleyi* (Haptophyta). *Journal of Phycology*, *48*(3), 635-646.

Wu, H., Cockshutt, A. M., **McCarthy, A.**, & Campbell, D. A. (2011). Distinctive photosystem II photoinactivation and protein dynamics in marine diatoms. *Plant Physiology*, *156*(4), 2184-2195.

Key T., **McCarthy A.**, Campbell D. A., Six C., Roy S., Finkel Z.V. (2009). Cell size tradeoffs govern light exploitation strategies in marine phytoplankton. *Environmental Microbiology*, 12, 95–104.

Total citations: 244, h-index: 5, i10-index: 4

Academic Awards

2014/2015
Queen Elizabeth II Graduate Scholarship in Science and Technology
University of Western Ontario, London, ON

2011 - 2014
Ann Oaks Doctoral Fellowship
Canadian Society of Plant Physiologists

2009/2010
Robert Lewis Rice Memorial Graduate Fellowship
Mount Allison University, Sackville, NB

2009/2010
New Brunswick Graduate Scholarship
Post-Secondary Education, Training and Labour

2004 - 2008
Mount Allison University Entrance Scholarship
Mount Allison University, Sackville, NB