The Integration of Metabolite and Hormone Signalling Drives Seedling Development

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Abstract

Sugars have a profound impact on plant biology, acting as structural components, signalling molecules, and sources of energy. As such, the availability of sugars has important implications for plant growth and development. Sugar levels rise and fall as part of a daily cycle, while cues from other environmental stimuli are also in flux. As sessile organisms in an ever-changing environment, plants must integrate signals from multiple pathways in order to promote the appropriate developmental responses.

To uncover pathways that interact with sugars during seedling development, a chemical screen was performed in search of compounds that modify responses to sucrose. This screen identified an interaction between the folate inhibitor sulfamethoxazole (SMX) and sucrose that resulted in changes to auxin signalling and distribution. Synergy between sucrose and SMX was used to explore the effect of metabolic cues on
auxin signalling during hypocotyl elongation.

A second line of investigation explored whether sucrose and folates influence root meristem activity. Sucrose induced hormone signalling pathways that promote cell division and differentiation. Treatment with SMX perturbed the effect of sucrose on hormone networks that mediate growth, and resulted in a loss of meristem integrity. This study highlights the influence of metabolism on hormone signalling at the root apex, and its role in maintaining balance of a complex signalling network that drives root growth.

These studies characterise an interaction between metabolic pathways that is integrated with hormone signalling during plant development. Taken together, they highlight a mechanism through which plant growth might be regulated by metabolism.
Acknowledgements

Firstly, I would like to thank my supervisor, Dr Malcolm Campbell, for his generous support and guidance throughout the time I have spent in his lab. He has been a great mentor, and I am grateful for having had the opportunity to work with his group. I would also like to thank Joan Ouellette, who has put in countless hours keeping the lab running smoothly. Without her support over the years, this thesis would not have been possible. I would also like to thank the members of my Advisory Committee, Dr Darrell Desveaux and Dr Peter McCourt, for guiding my research efforts, their insight and thoughtful advice is truly appreciated. Thank you to Dr Eiji Nambara and Dr Philip Benfey, who have devoted their valuable time to review my thesis and take part in my final defence.

I would like to acknowledge the guidance and friendship of my labmates, who have helped with brainstorming new ideas, and then with troubleshooting. It has been great working with you, especially Erin Hamanishi, Mike Prouse, Rohan Patel, Heather Wheeler, and Joseph Skaf. Special thanks to the graduate students and postdocs in other labs that have taken time to help me with protocols or to offer advice, especially Dr Steven Chatfield, Dr Amy Lee, Dr Karl Schreiber and Dr Wenzi Ckurshumova.

I would like to thank my parents Kevin and Rose, and my sisters Adrienne and Jacqueline, for their encouragement, love and support. Without them I would not be here. I would like to thank my friends, especially Aarie Glas and Evan Mackenzie, who offered comic relief at the times it was needed. Above all, I would like to thank Charlotte Burke for her warmth, kindness, and understanding. Her love has made all the difference.
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<td>%</td>
<td>per cent</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
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<td>microgram</td>
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<tr>
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<td>abscisic acid</td>
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<tr>
<td>ABF</td>
<td>Auxin-binding F-box</td>
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<td>Arabidopsis Biological Resource Centre</td>
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<tr>
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<td>silver ion</td>
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<td>AGI</td>
<td>Arabidopsis Genome Initiative</td>
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<td>ANOVA</td>
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<td>ARF</td>
<td>AUXIN-RESPONSE FACTOR</td>
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<td>ARABISOPSIS RESPONSE REGULATOR</td>
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<td>dH₂O</td>
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<tr>
<td>DHFR</td>
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<td>dihydrofolate synthetase</td>
</tr>
<tr>
<td>DHPS</td>
<td>dihydropteroate synthase</td>
</tr>
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<td>DIC</td>
<td>differential interference contrast</td>
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<tr>
<td>DMSO</td>
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<td>DPG</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EMS</td>
<td>ethyl methanesulfonate</td>
</tr>
<tr>
<td>eto2</td>
<td>ethylene overproducer2</td>
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FA  folic acid
FAA  Formalin-acetic-alcohol
FBP  FRUCTOSE-1,6-BISPHOSPHATASE
FC  fold change
FPGS  folylpolyglutamate synthetase
FW  forward
GA  gibberellic acid
gai  gibberellic acid insensitive
GAL  galactose
gcrma  GC-robust multi-array analysis
GEO  Gene Expression Omnibus
GFP  green fluorescent protein
gim1  galactose insensitive1
gin  glucose-insenstitive
GL2  GLABRA2
Glu  glutamate
GSK  GLYCOGEN SYNTHASE KINASE
GUS  beta-glucoronidase
H2Folate  dihydrofolate
H2O  water
H2Pterin  hydroxydihydropterin
H2Pterin-PPi  hydroxymethylidihydropterin pyrophosphate
H2Pteroate  dihydropteroate
HCl  hydrochloric acid
HLB  hydrophilic-lipophilic balance
HPPK-DHPS  hydroxymethylidihydropterin pyrophosphokinase-dihydropteroate synthase
hr  hour
hy2  elongated hypocotyl2
HXX1  HEXOKINASE1
IAA  indole-3-acetic acid
JA  jasmonic acid
K3Fe(CN)6  Potassium hexacyanoferrate(III)
K4Fe(CN)6  Potassium hexacyanoferrate(II)
LATCA  Library of Active Compounds in Arabidopsis
LC  liquid chromatograph
LC-ESI-MS/MS  liquid chromatograph electrospray ionization tandem mass spectrometry
LC-MS  liquid chromatography-mass spectrometry
limma  linear model for microarray
LRC  lateral root cap
m^2 s^-1  square meter-per-second
m/z  mass-to-charge ratio
mDHPS  mitochondrial dihydropteroate synthase
min  minute
mL  millilitre
mM  millimolar
MS  Murashige and Skoog
msg2  *massugu2*
MTX  methotrexate
NaAc  sodium acetate
NaOH  sodium hydroxide
NASC  Nottingham Arabidopsis Stock Centre
ng/gFW  nanograms-per-gram-fresh-weight
nm  nanometre
NPA  naphthylphthalamic acid
OAS  O-Acetyl serine
pABA  *para*-aminobenzoic acid
PAC  paclobutrazol
PCR  polymerase chain reaction
phyB  *phytochromeB*
PI  propidium iodide
PIF  PHYTOCHROME INTERACTING FACTOR
PIN  PIN-FORMED
PLT  PLETHORA
*plt1;plt2*  *plethora1;plethora2*
pg  picogram
QC  quiescent centre
qPCR  quantitative polymerase chain reaction
RAM  root apical meristem
RNA  ribonucleic acid
ROS  reactive oxygen species
RT-PCR  reverse transcriptase polymerase chain reaction
RV  reverse
SAIL  Syngenta *Arabidopsis* Insertion Library
SAM  shoot apical meristem
SCF  Skp, Cullin, F-box containing
SD  standard deviation
SDZ  sulfadiazine
SE  standard error
*shy2*  *short hypocotyl2*
SKP1  S PHASE KINASE-ASSOCIATED PROTEIN 1
SLR  single lens reflex
SMP  sulfamethoxypyridazine
SMT  sulfameter
SMX  sulfamethoxazole
SMZ  sulfamerazine
SNA  sulfanilamide
*ssr*  *sucrose and sulfonamide resistant*
Suc  sucrose
Sul1  sulfonamide-resistant1
TCS  two-component-output-sensor
<table>
<thead>
<tr>
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<th>Full Form</th>
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<tr>
<td>T-DNA</td>
<td>Transfer DNA</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofolate</td>
</tr>
<tr>
<td>THF-Glu₁</td>
<td>tetrahydrofolate monoglutamate</td>
</tr>
<tr>
<td>THF-Gluₙ</td>
<td>tetrahydrofolate polyglutamate</td>
</tr>
<tr>
<td>TIR1</td>
<td>TRANSPORT INHIBITOR RESPONSE1</td>
</tr>
<tr>
<td>UBQ10</td>
<td>POLYUBIQUITIN10</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine diphosphate</td>
</tr>
<tr>
<td>Vasc</td>
<td>vasculature</td>
</tr>
<tr>
<td>v/v</td>
<td>volume-to-volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight-to-volume</td>
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<tr>
<td>WAX</td>
<td>weak anion exchange</td>
</tr>
<tr>
<td>WOX5</td>
<td>WUSCHEL-RELATED HOMEOBOX5</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>X-GLUC</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-glucuronide</td>
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Chapter 1

Introduction
1 Introduction

1.1 Sugars Have a Profound Impact on Plant Development

As sessile organisms in an ever-changing environment, plants must be able to sense their surroundings in order to promote the appropriate responses, which may include changes to directional growth (Esmon et al., 2005; Ding et al., 2011). This developmental plasticity allows plants to optimise growth and development in response to their environment (De Kroon et al., 2009). Some stimuli, such as light, can be sensed directly through the activity of receptors (Chory, 1994; Quail, 1998, 2007). Alternatively, plants can indirectly sense changes in their environment through the perception of metabolites that accumulate under certain conditions (Kim et al., 1999; Stevenson et al., 2000; Bouche and Fromm, 2004; Vergara et al., 2012).

Sugars are one example of a metabolite that can modify growth and development (Smith and Stitt, 2007). Playing a multifaceted role as structural components, sources of energy, and as signalling molecules, the effect of sugar signalling can be seen across all stages of the plant life cycle (Rolland et al., 2006). At the earliest stages of plant growth, germination and establishment are inhibited by sugars (Dekkers et al., 2004; Gibson, 2005; Yuan and Wysocka-Diller, 2006). As the seedling matures, sugars promote primary root elongation and the emergence of lateral roots, giving rise to root system architecture (Nieuwland et al., 2009; Sanz et al., 2011; Kircher and Schopfer, 2012). Vegetative growth and developmental state are influenced by sugars, as the application of sugar to the shoot apex can induce floral transition (Corbesier et al., 2002; Smith and Stitt, 2007; Coneva et al., 2012). Sugars influence leaf senescence as well (Quirino et al., 2000; Parrott et al., 2005), though the exact role they play in this process is contested (van Doorn, 2008). Taken together, it is apparent that sugar signals play an influential role across the life of a plant.
Some striking effects of sugar on plant development can be observed by exposing dark-grown plants to sugar (Roldan et al., 1999; Baier et al., 2004; Casson and Lindsey, 2006). In the absence of light, seedlings enter into skotomorphogenic development that is characterized by elongated hypocotyls, inhibition of root growth, and an apical hook that is believed to protect the shoot apical meristem as the plant pushes through soil to reach light (Zadnikova et al., 2010). Treatment with sucrose to the aerial tissues is sufficient to break the etiolated seedling from skotomorphogenic development (Roldan et al., 1999). In these dark-grown seedlings, sugars can promote the development of true leaves, and plants are able to grow right through to flowering and seed set (Roldan et al., 1999). This implies that light itself is dispensable in breaking etiolated growth, and that the products of photosynthesis are sufficient to induce traits typically associated with photomorphogenesis (Roldan et al., 1999).

In addition to promoting the development of true leaves, sucrose is sufficient to induce root elongation in dark-grown seedlings (Kircher and Schopfer, 2012). Though there is evidence that photoreceptor-mediated light signalling plays a role during primary root elongation (Dyachok et al., 2011), it appears that light is dispensable so long as sugar signalling is maintained (Kircher and Schopfer, 2012). It has been proposed that photosynthetically-derived sucrose acts as a long-distance signal to induce root elongation once the cotyledons have established photosynthetic competency (Kircher and Schopfer, 2012). In this manner, sucrose acts as a metabolic signal to the root that favorable above-ground conditions have been met (Kircher and Schopfer, 2012).

1.2 Sugar Levels Rise and Fall Throughout the Day, With Widespread Consequences

Sugar levels rise and fall on a daily cycle, reaching maximal levels toward the end of the day, and decreasing throughout the night (Blasing et al., 2005). During the day, sucrose produced through photosynthesis is converted to starch, which is used at
night to support growth in the absence of light (Caspar et al., 1985; Geiger and Servaites, 1994). Studies using extended night treatments (Osuna et al., 2007; Usadel et al., 2008) or genetic mutants with reduced sugar levels (Blasing et al., 2005) have shown that the availability of sucrose during the night is important for maintaining growth. Plant growth becomes arrested in the absence of sucrose, highlighting the importance of sugars in supporting plant development.

Sugars influence gene transcription and other metabolic processes, therefore daily fluctuations in carbohydrate abundance have profound influences on plant biology (Blasing et al., 2005; Smith and Stitt, 2007; Usadel et al., 2008). Sugars induce widespread changes to the transcriptome, and the daily flux of sugar accounts for a large portion of daily transcript cycling that are part of the circadian rhythms in plants (Blasing et al., 2005). Whereas the circadian rhythm is entrained by numerous environmental stimuli, such as light and temperature (McClung, 2006), cellular sugar abundance is a dominant influence in daily cycling of the transcriptome (Blasing et al., 2005). Sugar signaling also feeds directly into clock machinery that governs the circadian rhythm (Dalchau et al., 2011). Through transcriptome activity, the signalling architecture of the cell is changed and results in altered sensitivity to environmental stimuli (Hotta et al., 2007). Known as gating, responsiveness to a stimulus can be modulated based on the status of the circadian rhythm (Hotta et al., 2007; Harmer, 2009). As daily flux of sugars moderate transcriptome activity, the sugar status of the plant can gate responses to environmental and endogenous stimuli.

Sugars modulate responses to inorganic nutrients, offering an example of how endogenous metabolic signals can gate plant responses to their environment (Price et al., 2004; Muller et al., 2005). By analyzing the transcriptome of seedlings treated with sugars and nitrate or phosphate independently, then comparing this response to seedlings given both treatments, interactions between inorganic nutrients and sugars were explored (Price et al., 2004; Muller et al., 2005). Using this experimental design, cluster analysis identified sets of transcripts that responded synergistically to glucose and nitrate (Price et al., 2004). Whereas previous experiments studying
nitrate responses were performed in the presence of sucrose (Wang et al., 2000; Wang et al., 2003). Price et al identified that many of these responses were dependent on the sugar (Price et al., 2004). Similar trends were observed in studies looking at transcriptome-wide interactions between sucrose and phosphate (Muller et al., 2005), suggesting that sugars influence plant responses to a variety of other nutrients.

To put this into a biological context, as the levels of carbohydrate availability rise and fall throughout the day (Blasing et al., 2005), pathway interactions may allow plants to fine-tune signalling and responses. In this manner, endogenous metabolic signals are integrated with inorganic nutrient pathways to influence plant responses to the environment.

1.3 Sugars are perceived, resulting in changes to gene expression

As sugars are an important factor for growth, plants have evolved numerous sensing pathways to detect sugar abundance (Moore et al., 2003; Rolland et al., 2006; Smith and Stitt, 2007; Smeekens et al., 2010; Cho and Yoo, 2011). These sensing pathways often invoke changes in gene expression that allow plants to capitalise on carbohydrate resources (Koch, 2004; Wiese et al., 2005; Cho et al., 2006). In this way the sugar can act not only to provision growth, but also acts as a signal that informs development.

The main glucose sensor in plants is HEXOKINASE1 (HXK1), an enzyme that functions in the conversion of phosphorylated hexose sugars, and is a key component of a sugar signalling pathway (Jang et al., 1997; Moore et al., 2003). HXK1 phosphorylates glucose as part of glycolysis, playing a functional role in plant primary metabolism (Moore et al., 2003; Rolland et al., 2006). The metabolic function of HXK1 can be uncoupled from its signalling role by introducing point mutations to the catalytic domain (Moore et al., 2003). Catalytically-inactive mutants maintain glucose signalling function, supporting the hypothesis that HXK1 plays a
dual role in metabolism and sugar sensing (Moore et al., 2003). In keeping with this, nuclear-localised HXK1 interacts with other signalling components to mediate the transcription of glucose-responsive genes (Cho et al., 2006). The combined activity of sugar metabolism and signalling enables tight coordination of plant development with resource availability (Rolland et al., 2006).

Other metabolic enzymes play a dual role in sensing sugars and regulating transcriptional responses. The fructose metabolism enzyme FRUCTOSE 1,6 BISPHOSPHATASE (FBP), which produces fructose-6-P during sucrose synthesis (Rolland et al., 2006), mediates fructose signalling during seedling development (Cho and Yoo, 2011). Similar to experiments with HXK1 (Moore et al., 2003), catalytically-dead alleles of FBP maintain gene regulatory functions (Cho and Yoo, 2011). These studies highlight a mechanism through which sugars act both as metabolic constituents and as signalling molecules. Sugars provide energy as nutrients to support the growth of new tissues. Metabolic flux is detected by enzymes involved in the metabolism of sugars, resulting in changes in gene expression that affect resource demand.

Though sucrose induces widespread changes to gene expression, it is unclear to what extent this effect is due to the degradation of sucrose into its constituent hexoses. Both glucose and fructose induce changes in gene expression, and can be generated from sucrose through invertases (Pego and Smeekens, 2000; Price et al., 2004; Rolland et al., 2006; Cho and Yoo, 2011). There is evidence of disaccharide-sensing mechanisms in plants that supports the role of sucrose as a signalling molecule independently of glucose and fructose (Barker et al., 2000; Huijser et al., 2000; Loreti et al., 2000; Solfanelli et al., 2006; Mudgil et al., 2009). As sucrose is the main transported sugar in plants, it would make sense for plants to possess mechanisms through which they can be detected. The extent to which glucose and fructose are necessary for plant responses to sucrose is an issue of ongoing research. Sugars are readily interconverted in plant cells, making this an issue that is difficult to address experimentally.
1.4 Transcriptome Analysis to Uncover Pathway Interactions

By comparing the transcriptome-level responses to two individual treatments relative to their combined treatment, interactions between signalling pathways can be uncovered (Price et al., 2004; Muller et al., 2005). Though this has proven successful in a variety of contexts, different methods of microarray analysis have been implemented. The analysis by Price et al used k-means clustering to identify groups of transcripts that responded synergistically or antagonistically to glucose and nitrate treatments (Price et al., 2004). Synergy was defined as any changes in response to the cotreatment that was greater than the added responses of both treatments individually (Price et al., 2004). Similarly, Muller et al defined interaction as a change in abundance level in response to the two factors together, which is significantly different than expected from either treatment individually (Muller et al., 2007). Hierarchical clustering of transcripts fitting this definition identified three clusters, one of which was enriched for transcripts involved in growth and development that exhibited a positive interaction between phosphate and sucrose treatments (Muller et al., 2007). Pathways that promote growth were activated only when the metabolic and nutrient resources were available to support growth (Muller et al., 2007).

1.5 Sugars and Hormones

Many responses to sugar are mediated through hormone signalling, adding to the complexity of sugar signalling networks in plants (Gazzarrini and McCourt, 2001; Gibson, 2005; Rolland et al., 2006). Forward-genetic screens have identified sugar-insensitive mutants to be novel alleles of hormone signalling components, highlighting the extensive crosstalk between sugars and hormones in regulating plant development (Gibson, 2005). For example, by screening for mutants resistant to glucose, a set of glucose-insensitive (gin) mutants were isolated (Zhou et al., 1998), many of which were later found to be novel alleles of known abscisic acid (ABA) and ethylene hormone pathways (Zhou et al., 1998; Arenas-Huertero et al., 1998).
Similar findings were uncovered by screening for altered sensitivity to sucrose (Gibson et al., 2001; Rook et al., 2001; Baier et al., 2004), suggesting that responses to sugar are generally mediated through hormone signalling, and may not be sugar-specific.

Anthocyanin production in seedlings exemplifies the complex interplay that exists between sucrose and hormone signalling, and highlights the manner in which hormones can either enhance or negate the effect of sugars (Loreti et al., 2008; Jeong et al., 2010). Growth in the presence of sucrose causes an increase in anthocyanin accumulation in seedlings (Loreti et al., 2008; Jeong et al., 2010), that is associated with increased expression of genes encoding biosynthetic enzymes (Solfanelli et al., 2006; Loreti et al., 2008). When seedlings were treated with sucrose in the presence of ABA or jasmonic acid (JA), the effect of sucrose was approximately twice as high as it had been in the absence of the hormones (Loreti et al., 2008). Intriguingly, ABA and JA induced no effect on anthocyanins when treated independently of sucrose, indicating that they are acting to modulate the effect of sucrose rather than exerting an additive effect (Loreti et al., 2008). Contrary to ABA and JA, the presence of GA completely inhibited the inductive effect of sucrose on anthocyanin accumulation, yet again induced no change when treated independently (Loreti et al., 2008). Taken together, these studies highlight the manner in which sugars and hormone signalling converge to shape plant growth. The nature of this interaction, whether inhibitory or synergistic, can be hormone dependent.

1.6 Sugars and Auxin Coordinate Plant Development

Auxin is a plant hormone that plays an important role in establishing patterning and shaping morphology (Bilou et al., 2005; Woodward and Bartel, 2005; Vanneste and Friml, 2009). Given that sugars promote some morphological traits, it is plausible that auxin signalling mediates some developmental responses to sugar. For example, in the absence of light, treatment with exogenous sucrose is sufficient to promote the development of true leaves, restricts hypocotyl elongation, and promotes root development (Figure 1.1) (Roldan et al., 1999; Kircher and Schopfer,
Auxin is involved in all three of these processes by acting as an instructive signal during phyllotaxy (Reinhardt et al., 2003), promoting cell expansion in the hypocotyl (Tatematsu et al., 2004; Saito et al., 2007), and regulating cell division and patterning in the root meristem (Aida et al., 2004; Perilli et al., 2012). Despite this, very few sugar-insensitive mutants have been identified as disrupted in auxin signalling, as have been found in other hormone signalling pathways (Gibson, 2005). This may be a result of the extensive redundancy that exists in the auxin signal transduction pathway (Okushima et al., 2005; Overvoorde et al., 2005). The extent to which auxin mediates responses to sugars remains poorly understood.

There is evidence that sugars and light signalling pathways interact downstream of the photoreceptors to promote auxin accumulation (Lilley et al., 2012; Sairanen et al., 2012). A modest increase in auxin abundance was observed in response to sucrose, yet this was diminished in the pitQ mutant, which is deficient in four members of the PHYTOCHROME INTERACTING FACTOR (PIF) family of transcription factors (Shin et al., 2009; Lilley et al., 2012). Treatment with high levels of glucose promoted the accumulation of auxin, and induced the expression some genes in the auxin biosynthetic pathway (Sairanen et al., 2012). Taken together, these studies suggest that sugars may direct plant development by inducing auxin biosynthesis, or by influencing the distribution of auxin across plant tissues.

Auxin signal transduction is mediated by a core set of transcription factors, called AUXIN-RESPONSE FACTORs (ARFs), that are bound to the promoters of early auxin-responsive genes (Ulmasov et al., 1999; Hagen and Guilfoyle, 2002). These ARFs interact with the Aux/IAA family of transcriptional repressors that bind to the ARFs in the absence of auxin (Liscum and Reed, 2002; Tiwari et al., 2003). Auxin interacts with its receptor family of F-BOX proteins, promoting an interaction between the receptor and other members of an SCF ubiquitin-ligase complex (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). This complex targets the Aux/IAA repressors for degradation via the 26S proteasome (Dharmasiri et al., 2005; Santner et al., 2009). ARFs can then regulate the
Figure 1.1. Sucrose induces pleiotropic developmental responses in dark-grown seedlings. Seedlings grown in liquid media in the absence (left panels) of presence (right panels) of 30mM sucrose. (A) Whole seedlings after seven days of growth. (B) Shoot apical meristem and true leaves imaged after fourteen days of growth. (C) Detail of hypocotyls after seven days of growth. (D) Primary roots after seven days of growth.
expression of their target genes (Dharmasiri and Estelle, 2004; Dharmasiri et al., 2005). The accumulation of auxin in plant tissues is fundamental to initiating auxin signaling (Gray et al., 2001; Vanneste and Friml, 2009). The active transport of auxin between cells and tissues is facilitated by the PIN-FORMED (PIN) family of auxin efflux carriers (Bliilou et al., 2005; Feraru and Friml, 2008; Robert and Friml, 2009). PINs focus auxin to create concentration gradients that are fundamental to auxin signalling (Grieneisen et al., 2007; Robert and Friml, 2009; Vanneste and Friml, 2009). The accumulation of auxin in is under control of developmental and environmental cues, which is facilitated by the regulation of PIN abundance and subcellular localisation (Feraru and Friml, 2008; Vanneste and Friml, 2009). Sugars can promote the accumulation of auxin in specific tissues (Mishra et al., 2009; Mudgil et al., 2009; Lilley et al., 2012); however, the role that sugars play in influencing PIN abundance is not known. Pathways that modify PIN activity can direct auxin localization and may offer a mechanism through which development is influenced by environmental conditions (Vanneste and Friml, 2009).

Glucose induces lateral root formation, a trait typically associated with auxin signalling (Dubrovsky et al., 2008; Mishra et al., 2009; Sanz et al., 2011; Muraro et al., 2013). This similarity suggested a connection between glucose and auxin signalling, and prompted a study into the nature of this interaction (Mishra et al., 2009). Rationalising that glucose might influence auxin signalling during root development, transcriptome analysis was performed using seedlings treated with glucose, auxin, or a combination of the two (Mishra et al., 2009). This design enabled the authors to explore the extent to which glucose can influence auxin-responses at the transcript level (Mishra et al., 2009). A large subsets of genes were identified as responding synergistically to glucose and auxin, including many core auxin signalling components (Mishra et al., 2009). This interaction between glucose and auxin highlights crosstalk between metabolic and hormone signalling as a driver of plant development.
1.7 Sugars Impact the Plant Metabolome; Metabolites Can Act as Signalling Molecules That Regulate Gene Expression

Sugars influence the metabolic profile of the plant cell (Gibon et al., 2006; Osuna et al., 2007; Usadel et al., 2008). As carbohydrate reserves become depleted, decreases in several fatty acids and organic acids, like fumarate and malate, occurs (Usadel et al., 2008). Depletion of carbohydrates also causes an increase in tocopherol biosynthesis and elevated amino acid pools that occurred as a result of protein degradation (Usadel et al., 2008). Within three hours of resupplying sucrose, many of the metabolic effects of sucrose starvation are reversed, indicating a rapid recovery of biosynthetic metabolism (Osuna et al., 2007). Cellular sugar abundance influences the metabolic profile of the plant, and suggests that the daily cycling of sugar abundance could affect a wide range of metabolic pathways.

Metabolites can act as signalling molecules to regulate gene expression (Kim et al., 1999; Stevenson et al., 2000; Vergara et al., 2012). For example, in times of sulfate deficiency the cysteine precursor O-Acetyl serine (OAS) accumulates (Kim et al., 1999), which is detected by plants and regulates the expression of genes involved in sulfur acquisition (Hirai et al., 2003). In this sense, a metabolite can act as a signalling molecule to inform the plant of its environmental condition and activate the appropriate response. Given the affect that sugars have on plant metabolism, it is possible that sugar exerts an effect on plant growth and development through its influence on various plant metabolite pools.

1.8 Folates Effect Plant Development and Productivity

Folate biosynthesis is a branch of primary metabolism that is important for plant productivity (Storozhenko et al., 2007; Mehrshahi et al., 2010). The tetrahydrofolate (THF) molecule comprises three components: pteridine, para-aminobenzoate (pABA), and a polyglutamate chain that can vary in length (Figure 1.2A) (Hanson and Gregory, 2002; Sahr et al., 2005). Folates are classified based on the
methylation state at N-5 and N-10 of the THF molecule (Figure 1.2 B) (Ravanel et al., 2011). THF acts as a co-factor in various single-carbon transfer reactions, collectively termed one-carbon (C1) metabolism (Hanson and Roje, 2001; Hanson and Gregory, 2002), by donating methyl units from these sites (Ravanel et al., 2011). These C1 reactions are necessary for the synthesis of secondary metabolites such as lignin and choline, and are important for the production of serine and glycine (Hanson and Gregory, 2002). Folates are a core component of primary metabolism and are involved in many facets of plant metabolism.

Given the importance of folates in a number of metabolic processes, it is not surprising that mutants deficient in folate metabolism exhibit developmental phenotypes (Goyer et al., 2005; Storozhenko et al., 2007; Mehrshahi et al., 2010; Srivastava et al., 2011). In a screen for mutants with defective root growth, Srivastava et al identified a folylpolyglutamate synthetase (FPGS)-deficient line with restricted root elongation (Srivastava et al., 2011). The quiescent centre of the fpgs mutants were disorganised, leading to differentiation of the stem cell pool at the root meristem (Srivastava et al., 2011). These findings highlight the importance of folates in supporting root growth, and the maintenance of meristematic potential at the root apex.

Polyglutamylation of the tetrahydrofolate molecule is important for maintaining folate stability and homeostasis (Akhtar et al., 2010; Mehrshahi et al., 2010). Folylpoly glutamate synthetase (FPGS) exists in three isoforms in Arabidopsis, each isoform is active in a different organelle to support compartmentalisation of folate metabolism, and is involved in the addition of the polyglutamate tail to the THF molecule (Mehrshahi et al., 2010). Loss-of-function mutant analysis indicated that each of the three isoforms was dispensable, as each individual mutant exhibited no morphological phenotype (Mehrshahi et al., 2010). In contrast, the double-mutant fpgs1 fpgs3 exhibited a severely dwarfed phenotype, and the mutant combinations of fpgs1 fpgs2 and fpgs2 fpgs3 resulted in embryo and seedling lethality, respectively (Mehrshahi et al., 2010). Folate metabolism is necessary for seedling
Figure 1.2. Structure of the tetrahydrofolate molecule. (A) Tetrahydrofolate (THF) consists of three components, a Pterin ring, p-aminobenzoate, and a polyglutamate sidechain. (B) Folates are classed by the methylation state of N-5 and N-10. These locations act as the methyl donor sites in C1 metabolism. (Modified from Ravanel et al. 2011).
Figure 1.3. Tetrahydrofolate biosynthesis in plants. Abbreviations: HPPK, hydroxymethyl dihydropterin pyrophosphokinase; DHPS, dihydropteroate synthase; DHFS, dihydrofolate synthetase; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthetase; pABA, para-aminobenzoic acid; ATP, adenosine triphosphate; Glu, glutamate; H₂Pterin-PPi, hydroxymethyl dihydropterin pyrophosphate; H₂Pterin, hydroxy dihydropterin; H₂Pteroate, dihydropteroate; H₂Folate, dihydrofolate; THF, tetrahydrofolate; THF-Glu₁, tetrahydrofolate monoglutamate; THF-Gluₙ, tetrahydrofolate polyglutamate.
vitality, and supports normal plant growth and development. The study by Mehrshahi et al offers insight into the redundancy that exists within the folate biosynthetic pathway (Ravanel et al., 2001; Storozhenko et al., 2007; Mehrshahi et al., 2010). Despite the subcompartmentalisation of each isozyme, individual loss-of-function mutants exhibited little phenotype, suggesting that the compartmentalisation of polyglutamilation may be redundant (Mehrshahi et al., 2010). Genetic redundancy is present at multiple steps in folate metabolism, in that multiple isoforms exist to perform the same enzymatic step (Ravanel et al., 2001; Storozhenko et al., 2007; Akhtar et al., 2010; Mehrshahi et al., 2010).

In addition to highlighting the importance of folates in plant growth and reproductive development, the study by Mehrshahi et al also eluded to a connection between folate metabolism and sucrose during seedling development (Mehrshahi et al., 2010). Double fpgs2 fpgs3 mutants, which typically failed to grow beyond germination, were able to become established when growing on media containing sucrose (Mehrshahi et al., 2010). To date, this represents the only established connection between folate metabolism and sugar signalling in plants.

1.9 Chemical Inhibition of THF Biosynthesis as a Tool for Plant Sciences

Chemical inhibition of THF biosynthesis can be used to study the effect of folates on plant biology (Ravanel et al., 2011). There are two main targets of chemical inhibition, the bifunctional enzyme hydroxymethylidihydropterin pyrophosphokinase-dihydropteroate synthase (HPPK-DHPS) and dihydrofolate reductase (DHFR) (Ravanel et al., 2011). DHPS uses para-aminobenzoic acid (pABA) and dihydropterin as substrates in the synthesis of Dihydropteroate, which is then combined with glutamate to generate dihydrofolate (Hanson and Gregory, 2002; Storozhenko et al., 2007). Dihydrofolate is reduced by DHFR to create tetrahydrofolate, which then goes on to facilitate C1-metabolism in plants (Hanson and Roje, 2001; Ravanel et al., 2011).
DHFR can be targeted by a number of inhibitors differing in structural class, though methotrexate (MTX) is most commonly used (Ravanel et al., 2011). As some cancers rely on folates to support rapid rates of cell division, MTX has been used extensively in chemotherapy (Bertino, 2009). MTX has also been used in plants to explore the role of folates in amino acid metabolism (Prabhu et al., 1997; Prabhu et al., 1998), root development (Srivastava et al., 2011), and chlorophyll biosynthesis (Van Wilder et al., 2009). This is not an ideal approach, as MTX is highly toxic and produces xenobiotic stress responses in plants (Loizeau et al., 2008).

Alternatively, folate biosynthesis can be restricted by the inhibition of DHPS, which is targeted by the sulfonamide family of compounds (Brown, 1962). Sulfonamides bind and inhibit the DHPS active site by acting as a competitive inhibitor to pABA (Brown, 1962; Prabhu et al., 1997; Prabhu et al., 1998). Sulfonamides have been used in plants to measure the effect of folate-dependent metabolic processes, such as glycine metabolism (Prabhu et al., 1997). Treatment with sulfonamide has uncovered the involvement of folates in processes such as DNA methylation (Zhang et al., 2012), and plant defence (Schreiber et al., 2008; Noutoshi et al., 2012). These novel roles for folates in plant development were indentified in high-throughput chemical-genetic screens. The fact that inhibitors of folate synthesis can induce disparate phenotypes speaks to the far reaching influence of this central metabolic pathway on plant growth and development.

Due to the existence of DHPS in microbes and plants, but not in humans, sulfonamides have a long history of use as antimicrobials (Wise and Abou-Donia, 1975). As a result, bacteria have developed resistance to sulfonamides at the site of inhibition, by evolving a DHPS protein that shows reduced affinity for the compound (Wise and Abou-Donia, 1975). This has been exploited by plant researchers, who have cloned this gene from *Escherichia coli* and expressed it in plants to confer resistance to sulfonamides (Guerineau et al., 1990). This resistance trait is now used as the *sulfonamide resistant1* (*Sul1*) selectable marker during genetic transformation (Guerineau et al., 1990; Hadi et al., 2002), and was used in the creation of the GABI-KAT collection of T-DNA insertion lines (Rosso et al., 2003).
1.10 Overcoming Redundancy: Chemical Genetics

Plants have evolved multiple sugar-sensing pathways, and it is likely that there is genetic redundancy within many of these pathways (Smith and Stitt, 2007). Though forward-genetic screening has proven useful in uncovering some sugar signalling components (Gibson, 2005), this approach is limited by an inability to uncover mutant phenotypes that are masked by genetic redundancy (McCourt and Desveaux, 2010; Toth and van der Hoorn, 2010). It is necessary to employ alternate methods that circumvent functional redundancy in order to establish a more complete picture of sugar signalling networks that govern plant development.

One method that can be used to overcome functional redundancy is to probe biological systems with chemical perturbation, rather than genetic mutation (Stockwell, 2004; Robert et al., 2009). Termed chemical genetics, this approach involves screening chemical libraries for compounds that induce a phenotype of interest, then working to uncover how the chemical is modifying protein function (Stockwell, 2000, 2004). When used in tandem with classical genetic approaches, this technique has proven to be a powerful tool for elucidating biological function in plants (Dai et al., 2005; Zheng et al., 2006; Park et al., 2009).

Chemical genetics can be used to overcome functional redundancy associated with multimember gene families (De Rybel et al., 2009; Park et al., 2009). For example, a chemical that inhibits the active site of an enzyme may block that enzyme from functioning and thus induce a phenotype (McCourt and Desveaux, 2010). This chemical inhibitor may act as a general antagonist to the enzyme, and overcome functional redundancy by binding the common active site of the whole family of enzymes (De Rybel et al., 2009; McCourt and Desveaux, 2010; Toth and van der Hoorn, 2010). In this way, the chemical inhibitor acts analogously to a higher-order genetic loss-of-function mutation (McCourt and Desveaux, 2010). Alternatively, functional redundancy may be overcome by using a selective agonist to activate a single member of a closely-related family (Park et al., 2009; Toth and van der Hoorn, 2010). The role of that gene, and by extension that gene family, can be uncovered.
by screening for mutants resistant to the selective agonist (Park et al., 2009).

Another shortfall of classical genetics is that some mutations are lethal when in a homozygous state, making them difficult to study and impossible to maintain as homozygous, non-segregating plant lines (McCourt and Desveaux, 2010; Candela et al., 2011). Instead, chemical inhibitors can be added or removed at different time points so as not to inhibit a critical developmental state (Toth and van der Hoorn, 2010; Hicks and Raikhel, 2012). This tunability of the chemical system allows the investigation into genes that were once difficult to explore.

The choice of chemical library used for screening can dictate the success of the experiment (Walters and Namchuk, 2003; Shelat and Guy, 2007). Some chemical libraries are collections of known compounds, such as natural products and bioactive molecules (Stockwell, 2000; Smukste and Stockwell, 2005; Shelat and Guy, 2007). These libraries often have high levels of success in chemical-genetic screening, owing to the wide range of molecular structures present in the collection (Shelat and Guy, 2007). These diversity-oriented libraries increase the range of protein structures that can be targeted in the screen, increasing the chance of success in high-throughput chemical screening (Stockwell, 2004).

Focused libraries are generally created with a priori knowledge of the protein target or with the goal of increasing the efficacy of a known active molecule (Sodeoka et al., 2001; Jimonet and Jager, 2004; Stockwell, 2004). These libraries can be synthesized from a core structure, or scaffold, and various functional moieties attached (Stahura et al., 1999; Sodeoka et al., 2001; Stockwell, 2004; Welsch et al., 2010). Through slight modifications to chemical structure, the activity of the molecule is altered (Stockwell, 2004). The type of library chosen for the chemical screen depends on the goal of the experiment, whether to identify the new components in biological phenomena, or to target specific proteins of interest (Stockwell, 2004; Shelat and Guy, 2007).

The efficacy of a compound in eliciting a biological response is dependent on
properties that enable the chemical to enter the living cell and maintain bioactivity (Lipinski, 2000). The cellular membrane is a semi-permeable barrier that prohibits many compounds from entering the cell, and therefore limits the number of proteins that can be targeted in a chemical screen (Hopkins and Groom, 2002). Compounds that are successful in passing through the cell membrane, and thus most useful in chemical genetics experiments, generally conform with Lipinski’s Rule of Five (Lipinski, 2000). These physico-chemical properties highlight factors influencing compound solubility and permeability, two determinants of drug bioavailability (Lipinski, 2000; Hopkins and Groom, 2002). Chemicals become less effective as useful drugs owing to poor absorption or membrane permeability when they have a molecular mass above 500 Da, when there are more than five hydrogen-bond donors, when more than ten hydrogen-bond acceptors, and when they exhibit a high level of lipophilicity (Lipinski, 2000). These traits influence the ability of the chemical to enter the cell and maintain activity, and inherently limit the number of different types of structures that can be useful for chemical screening.

Limitations on the number and breadth of chemical structures that are useful for inclusion in a chemical library impacts the number of proteins that can be targeted by chemical screening (Lipinski, 2000; Hopkins and Groom, 2002). The term “druggable genome” was coined to reflect the total number of proteins that can be targeted by chemical screening (Hopkins and Groom, 2002). Estimates have put the number of possible targets that can be hit with Lipinski-rule-of-five-complaint compounds at around 10% in various model organisms (Hopkins and Groom, 2002). This number is derived based on assumptions that most drug targets contain active sites for competitive inhibition, yet only a portion of proteins of an organism have an active site that can be targeted (Hopkins and Groom, 2002). The fact that only a subset of molecular pathways can be targeted by chemical biology is an important consideration when designing a high-throughput chemical screen.

When assessing potential leads from a chemical screen, it is important to consider that many compounds become degraded or metabolized upon entry to the cell (Eichelbaum and Burk, 2001; He et al., 2011). Chemical degradation may result in
lower abundance of that compound and reduced efficacy, or may result in the generation of a new molecule with bioactivity (Zhao et al., 2007). This latter scenario may confound results and, in the case of pharmaceuticals, render the chemical non-viable as a drug lead (Eichelbaum and Burk, 2001; He et al., 2011). Sometimes the metabolism of the compound in vivo is necessary to introduce bioactivity, and can be used to uncover biological processes. This was the case in a screen that identified hypostatin as the target for glycosylation by a UDP glycosyltransferase in Arabidopsis (Zhao et al., 2007). In this instance, the metabolism of the compound was in fact causative for the phenotype of the compound, which was used to uncover processes in plant biology (Zhao et al., 2007).

1.11 Combination Chemical Genetics

Distinct signalling pathways can converge to modulate the activity of one another, adding to the complexity to biological systems (Segre et al., 2005; Lehar et al., 2007). A comprehensive understanding of pathway interactions is necessary to fully appreciate the response of biological systems to their environment (ten Tusscher and Scheres, 2011). To uncover novel interactions, chemical combination screens can be performed in search of pairs of compounds that induce synergistic or antagonistic effects (Lehar et al., 2007; Farha and Brown, 2010; Piotrowski et al., 2010). Depending on the nature of the interaction, and the effect that each compound has on its protein target, information related to the topology of the underlying signalling pathway can be inferred (Lehar et al., 2007; Yeh and Kishony, 2007). Chemical combination screens have been used extensively in the pharmaceutical industry to discover drug combinations for use in syncretic drug therapies (Keith et al., 2005). This approach is now becoming an important tool to explore the complex interactions between signalling pathways that underpin organism-level responses to their environment (Lehar et al., 2007; Yeh and Kishony, 2007; Falconer et al., 2011; ten Tusscher and Scheres, 2011).

Chemical combination screens are performed by systematically screening through pairs of chemicals for responses that deviate from what can be expected from each
compound individually (Lehar et al., 2008; Falconer et al., 2011). Synergistic responses to combined treatments can indicate an interaction between pathways, though the best measure of synergy is debated (Keith et al., 2005). One common measure of synergy is Bliss Independence, which describes a null hypothesis of two compounds affecting the same phenotype through distinct, non-interacting pathways (Bliss, 1939; Keith et al., 2005). In this scenario, if two compounds each induce a 50% inhibition when applied individually, then cotreatment should cause a 75% inhibition; deviation from this is considered an interaction (Bliss, 1939). The Bliss Independence model is widely considered to be the most relevant to signal pathway analysis in biological systems (Keith et al., 2005). Screening for synergistic responses to treatments is a method through which pathway interactions can be uncovered (Lehar et al., 2007; Yeh and Kishony, 2007). This approach can glean new insight into the manner through which organisms fine-tune sensitivity to endogenous and environmental stimuli.

1.12 Research Questions and Hypotheses

Plant responses to sugars are mediated in part through interactions with other signaling pathways. Transcriptome studies have uncovered both antagonistic and synergistic responses between sugars and inorganic nutrients (Price et al., 2004; Muller et al., 2007), and genetic screens found sugar insensitivity in hormone signalling mutants (Gibson, 2005). The nature of these interactions is difficult to tease apart, but is an important component in our understanding of how the plant system uses these signals to shape development. To uncover novel pathways that interact with sucrose, a combination chemical genetic screen was undertaken. Using hypocotyl elongation as the phenotypic output, it was reasoned that chemicals exhibiting synergy with sucrose were affecting pathways that interact with sugar signalling. This screen identified that the sulfonamide family of compounds exhibited synergistic responses with sucrose during etiolated hypocotyl elongation. Transcriptome analysis found that cotreatment of sucrose and sulfamethoxazole (SMX) resulted in changes in the transcript abundance of core auxin signalling
components.

**Hypothesis 1: Sulfonamides modulate endogenous auxin signalling during sucrose-mediated development.**

To address this hypothesis, a series of experiments were undertaken to explore the effect of sulfonamides on sugar signalling, and to understand the molecular basis for this interaction. This study identified a specific auxin signalling pathway that mediates the interaction between folates and sugars, providing insight into the regulation of seedling development by primary metabolism.

By exploring the pleiotropic effects of sucrose on plant development, it was found that sugars promoted meristematic activity that ultimately shapes root growth. In the absence of sucrose, very little meristem activity was observed. Treatment with sucrose promoted cell-cycle progression and hormone signalling that resulted in root elongation. This effect of sucrose was perturbed by sulfonamide, causing a misexpression of cell-cycle markers and a disruption in hormone signalling that was partially phenocopied by treatment with a cell-cycle inhibitor. The presence of either the sulfonamide or the cell-cycle inhibitor resulted in meristem differentiation, yet only when treated in the presence of sucrose.

**Hypothesis 2: Metabolism influences cell division and differentiation at the root apex through hormone signalling networks.**

Taken together, these studies provide insight into the metabolic cues that govern plant development, highlighting the interaction between metabolic pathways as a unique feature through which hormone signalling can be modulated. It is postulated that the integration of metabolic and hormone signalling allows plants to fine-tune sensitivity to a complex, and ever-changing environment.
Chapter 2

Interplay between sucrose and folate modulates auxin signalling in Arabidopsis.

This chapter has been accepted for publication in Plant Physiology as:


Contributions: MES, MMC designed research; MES, AC, OW, EN performed research; MES, AC, OW, EN, MMC analysed data; MES, MMC wrote manuscript with editorial assistance from AC, OW, EN.
2 Interplay between sucrose and folate modulates auxin signalling in Arabidopsis.

2.1 ABSTRACT

As sessile organisms growing in an ever-changing environment, plants must integrate multiple regulatory inputs to promote the appropriate developmental responses. One such nutritional signal is cellular sugar levels, which rise and fall throughout the day and affect a variety of developmental processes. To uncover signalling pathways that modulate sugar perception, compounds from the library of active compounds in Arabidopsis (LATCA) were screened for the ability to perturb developmental responses to sucrose in Arabidopsis thaliana seedlings. This screen found that sulfonamides, which inhibit folate biosynthesis in plants, restrict hypocotyl elongation in a sugar-dependent fashion. Transcriptome analysis identified a small set of transcripts that respond to the interaction between sulfonamide and sucrose, including a number of transcripts encoding Aux/IAAs, negative regulators of auxin signal transduction. Chemical inhibition of auxin transport or genetic disruption of auxin signalling relieved this interaction, suggesting that responses to these two nutritional stimuli are mediated by auxin. Reporter systems used to track auxin signalling and distribution showed enhanced activity in the vascular region of the hypocotyl in response to co-treatment of sucrose and sulfonamide, yet no change in auxin abundance was observed. Taken together, these findings suggest that the interplay between sucrose and folates acts to fine-tune auxin sensitivity and affects auxin distribution during seedling development.
2.2 INTRODUCTION

Sugars play a multifaceted role in plant biology, acting as structural components, sources of energy, and signalling molecules (Rolland et al., 2006). The effect of sugars on development can be seen across all stages of the plant life cycle, from germination and establishment (Zhou et al., 1998; Rognoni et al., 2007), through vegetative growth and floral transition (Ohto et al., 2001; Corbesier et al., 2002) until senescence (Wingler and Roitsch, 2008). At the earliest stages of plant growth, the perception of sugars influences a number of seedling traits, including primary root growth (Freixes et al., 2002; Yazdanbakhsh et al., 2011) and hypocotyl elongation (Stevenson and Harrington, 2009). As carbohydrate availability fluctuates on a diurnal cycle (Blasing et al., 2005), other metabolic and environmental stimuli are in flux as well (Gibon et al., 2006). It is therefore necessary for sugar signals to be integrated with other signalling pathways to promote the appropriate developmental responses.

Studies into the effect of sugars on plant growth have revealed a connection between carbohydrates, nutrient status, and primary metabolism (Gibon et al., 2006; Usadel et al., 2008). Transcriptome analysis has uncovered many overlapping and synergistic responses between treatment with sugars and with inorganic nutrients, such as nitrate and phosphate, suggesting both similar response pathways as well as interactions between signalling components (Price et al., 2004; Muller et al., 2007). Sugar availability has also been linked to the transcriptional regulation of many components of primary and secondary metabolism, including the oxidative pentose-phosphate pathway (Lejay et al., 2008), the anthocyanin biosynthetic pathway (Solfanelli et al., 2006), and starch metabolism (Sokolov et al., 1998), among others. It is apparent that sugars impact many facets of plant metabolism, and that some of these changes may be regulated at the transcriptional level.

Many plant responses to sugar are mediated by the interaction between metabolic and hormone signalling pathways (Gazzarrini and McCourt, 2001; Rolland et al., 2006; Rook et al., 2006). Forward genetic screens have uncovered sugar-resistant
mutants to be novel alleles of known hormone mutants, highlighting the extensive crosstalk between sugar- and hormone-signalling pathways (Zhou et al., 1998; Arenas-Huertero et al., 2000; Laby et al., 2000; Gibson et al., 2001). Crosstalk between hormone pathways is a well-explored phenomenon, and there are many examples of hormone pathways that modify, impinge upon or promote the signalling of other hormones (Depuydt and Hardtke, 2011). In instances where points of crosstalk do exist, interplay between metabolite and hormone signalling may offer a point through which primary metabolism can direct plant growth and development (Falkenberg et al., 2008).

Given the level of redundancy that exists within metabolic and hormone signalling pathways, it is likely that there are pathways that interact with and modify sugar perception that have not been elucidated thus far by classical genetic methods (Smith and Stitt, 2007). As an alternate approach to forward genetic screening, chemical genetics can be used to overcome issues of functional redundancy (McCourt and Desveaux, 2010; Toth and van der Hoorn, 2010). For example, a chemical could bind and inhibit the common active site of a group of redundant proteins (De Rybel et al., 2009), or activate a single member of a family of closely-related receptors (Park et al., 2009). In either scenario, chemical genetics may be used to illuminate biological function that is masked by functional redundancy and likely unattainable through classical forward-genetic approaches.

In addition to overcoming functional redundancy, interconnectivity between biological processes can be inferred by screening for compounds that act synergistically on a phenotypic output (Lehar et al., 2007; Yeh and Kishony, 2007; Owens et al., 2010). Termed “combination chemical genetics”, this approach entails screening chemical libraries in the presence of a compound of interest to uncover secondary chemicals that enhance or negate its effect (Lehar et al., 2008). Hits from these screens can act antagonistically, additively, or synergistically, based on the manner in which their targets interact biologically (Lehar et al., 2007; Lehar et al., 2008). When chemicals act synergistically on a given phenotype, it is believed that the chemicals affect interacting molecular pathways (Yeh et al., 2006; Lehar et al., 2007).
In the present study, a combination chemical genetic screen was used to identify pathways that modify plant responses to sugar. This screen uncovered sulfonamides, known to inhibit tetrahydrofolate (THF) biosynthesis in plants (Prabhu et al., 1998), to act synergistically with sucrose to inhibit etiolated hypocotyl elongation. The pool of folates produced by the THF biosynthetic pathway are required for a variety of metabolic processes and provides the cofactors for the methyl-transfer reactions that constitute one-carbon (C1) metabolism in plants (Hanson and Roje, 2001). Though folate metabolism is important for normal plant growth and development (Van Wilder et al., 2009; Mehrshahi et al., 2010; Srivastava et al., 2011), the existence of any specific interaction between sugar signalling and folates is unknown. Here, the folate inhibitor sulfamethoxazole was used to explore the role of folates in mediating developmental responses to sucrose.

2.3 RESULTS

2.3.1 Sulfonamides alter seedling responses to sucrose

Chemicals were screened from the Library of Active Compounds in Arabidopsis (LATCA), and were selected as positive “hits” based on the ability to act synergistically with sucrose to inhibit hypocotyl elongation (Figure 2.1A). Of the 2100 compounds screened, 33 positive hits were reconfirmed in a secondary screen (Supplemental Table S2.1). Though positive hits were derived from a variety of structural classes and functions, the largest group of successful hits belonged to the sulfonamide family of compounds, which were chosen for further study (Figure 2.1B). Dose-response curves for five sulfonamides showed a saturable, dose-dependent inhibition of hypocotyl elongation that was enhanced by the presence of sucrose (Figure 2.1C, Supplemental Figure S2.1). The interaction between sucrose and sulfonamides was not related to the osmotic potential of the growth media (Supplemental Figure S2.2). The core structure for this family of compound is sulfanilamide (Figure 2.1B) (Prabhu et al., 1997; Prabhu et al., 1998), a compound not found in the LATCA library. When tested, sulfanilamide did not exert as strong of an effect compared to other sulfonamides, and required a much higher concentration
to exert an effect on hypocotyl elongation (Figure 2.1C, Supplemental Figure S2.1). This indicates that the R-group plays a role in determining the efficacy of the sulfonamide. Since sulfamethoxazole (SMX) exerted the strongest effect, it was chosen for use in subsequent experiments. This enabled experiments to be carried out at lower working concentrations of the chemical, which helped reduce any off-target effects of the sulfonamide.

2.3.2 Sulfamethoxazole impinges on sucrose-mediated development through inhibition of Dihydropteroate Synthase (DHPS)

Sulfonamides are a class of compounds known to inhibit the tetrahydrofolate (THF) biosynthetic pathway by competing with para-amino benzoic acid (pABA) for the active site of dihydropteroate synthase (DHPS; Figure 2.2A) (Prabhu et al., 1997; Prabhu et al., 1998; Storozhenko et al., 2007). DHPS is an enzyme that combines dihydropterin and pABA to form dihydropteroate, which is then polyglutamylated and reduced to form THF (Hanson and Gregory, 2002). THF exists bound to C1 units at various oxidation states, collectively termed folates, and acts as a cofactor in a variety of single-carbon transfer reactions (C1 metabolism) (Hanson and Roje, 2001; Hanson and Gregory, 2002).

Though sulfonamides have previously been shown to inhibit DHPS (Prabhu et al., 1997), it is possible that the compounds affected other processes as well, or that the sulfonamides were metabolized to new compounds with alternate modes of action (Zhao et al., 2007). It was therefore necessary to confirm that the phenotypes observed were related to the inhibition of DHPS, rather than secondary effects of the compound. To this end, chemical complementation was performed by supplementing the growth medium with either pABA, or folic acid (FA). The addition of the DHPS substrate (pABA) or product (FA) negated the inhibitory effect of SMX, and rescued hypocotyl elongation (Figure 2.2, B and C). This was consistent with
Figure 2.1. Sucrose conferred hypersensitivity to sulfonamides. (A) Chemicals selected as positive hits based on the ability to act synergistically with sucrose to inhibit hypocotyl elongation in dark-grown seedlings. (B) Structures of five sulfonamides identified in the chemical screen, plus the core structure sulfanilamide. SNA, sulfanilamide; SMZ, sulfamerazine; SMX, sulfamethoxazole; SDZ, sulfadiazine; SMT, sulfameter; SMP, sulfamethoxypyridazine. (C) Dose-response curves of five sulfonamides identified in original chemical screen and the core structure, sulfanilamide, indicate that the effect of sulfonamide on hypocotyl elongation is augmented by the presence of sucrose. Data represent the mean ±SE of three biological replicates from a representative experiment; each data point represents at least 36 individuals.
Figure 2.2. Overview of C1 metabolism in plants. (A) Sulfamethoxazole inhibits dihydropteroate synthase (DHPS), an early step in tetrahydrofolate (THF) biosynthesis. (B-C) Supplementing media with either Folic acid or pABA rescues hypocotyl elongation. Dark-grown seedlings were raised in the presence of 30mM sucrose and SMX, as indicated. Data represent the mean ± SE of three biological replicates from a representative experiment; each data point represents at least 36 individuals. Letters indicate values with statistically significant differences (P<0.05; ANOVA followed by Tukey’s b).
the fact that FA supplements the folate pool downstream of DHPS, and therefore compensates for the effect of the compound. In the case of pABA, the effect of SMX was likely reversed by out-competing SMX at the DHPS active site.

To verify that the inhibition of DHPS was modifying sensitivity to sucrose, the effect of SMX was tested on plants with elevated DHPS activity. It was reasoned that if DHPS is the true target of SMX, then elevating DHPS abundance would confer resistance to the compound. The Arabidopsis genome contains two DHPS isoforms, one mitochondrial (At4g30000) and one cytosolic (At1g69190) (Storozhenko et al., 2007); each was constitutively expressed under the control of the cauliflower mosaic virus (CamV) 35S promoter in planta. Activity of the construct was confirmed by measuring the expression of DHPS in three independently-transformed lines using qPCR (Supplemental Figure S2.3A). Although constitutive expression of either isoform attenuated the inhibitory effect of SMX at a lower concentration, higher concentrations of sulfonamide still inhibited hypocotyl elongation at a level similar to wild type (Figure 2.3, Supplemental Figure S2.3B). The need for higher concentrations of SMX to produce an effect equivalent to wild type in plants over-expressing DHPS was consistent with DHPS being the primary target for the sulfonamide. As a means of explaining the heightened sensitivity to SMX conferred by sucrose, it was hypothesised that sucrose negatively regulates DHPS and that when growing in sucrose the seedlings simply have lower DHPS abundance. To test this, the expression of both isoforms was measured in response to sucrose using qPCR, and it was found that sucrose did not affect transcript abundance of either DHPS isoform (Supplemental Figure S2.3C).

As plants that constitutively expressed the wild-type DHPS alleles maintained sensitivity to higher concentration of SMX, an additional test was undertaken to confirm that the binding of DHPS was necessary to confer sensitivity to sucrose. Plants expressing a microbial sulfonamide-resistant (sul1) DHPS were used to corroborate the mild resistance conferred by over-expressing the wild-type plant DHPS. Consistent with the hypothesis that inhibition of DHPS by the sulfonamide
Figure 2.3. Enhanced DHPS activity attenuated the interaction between sucrose and SMX. Hypocotyls were measured from seven-day-old dark-grown seedlings raised in the presence of sucrose and SMX, as indicated. Plants constitutively expressing either the mitochondrial (35S::mDHPS) or the cytosolic (35S::cDHPS) isoform exhibited mild resistance to the synergistic effect of sucrose and SMX, compared to WT. Plants expressing the bacterial SMX-resistant DHPS (sul1) exhibited greater resistance than plants expressing wild-type plant alleles. Data represent the mean ± SE of three biological replicates from a representative experiment; each data point represents at least 36 individuals. Letters indicate values with statistically-significant differences (P<0.05; ANOVA followed by Tukey’s b).
caused hypersensitivity to sucrose, plants harbouring the sul1 marker were highly resistant to SMX (Figure 2.3, Supplemental Figure S2.3B). These plants exhibited a markedly higher resistance to the sulfonamide, compared to the plant harboring the wild-type DHPS alleles. Taken together, the chemical complementation assays and resistance conferred by enhanced DHPS activity supported the notion that the inhibition of DHPS caused heightened sensitivity to sucrose.

2.3.3 Changes in transcript abundance of specific auxin signal transduction components occurs in response to the combined action of SMX and sucrose

The transcriptome-level responses of seedlings to sucrose and SMX were examined by microarray analysis. Concentrations of sucrose and SMX were identified that had no effect on hypocotyl elongation when administered separately (10mM sucrose, 0.2µM SMX), but inhibited elongation when they were present together in the growth media (Figure 2.4A). When the relationship between these treatments was tested using a two-way analysis of variance (ANOVA), it was determined that the effect sucrose and SMX exhibited an interaction (P<0.01). These treatments were then used as the basis for a microarray experiment aimed at uncovering changes in the transcriptome that may underpin this interaction. Total RNA was extracted from three-day-old dark-grown seedlings that were cultured in MS growth media alone, or in media supplemented with sucrose, SMX, or a combination of sucrose and SMX. Thus, the experiment was designed as a 2 X 2 complete randomized factorial ANOVA (two sucrose treatments and two SMX treatments). This enabled the identification of genes with significant differential transcript accumulation in response to either sucrose or SMX, independently of the other compound (main effects) or whose response to SMX treatment was dependent on the presence or absence of sucrose in the media (interaction).

Growth in the presence of 10mM sucrose promoted widespread changes to the transcriptome, whereas treatment with 0.2µM SMX induced many fewer changes
**Table I: Number of probe sets with significant main effect or interaction determined by ANOVA\(^a\)**

<table>
<thead>
<tr>
<th>Effect</th>
<th>Number of probe sets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfamethoxazole main effect</td>
<td>12</td>
</tr>
<tr>
<td>Sucrose main effect</td>
<td>9292</td>
</tr>
<tr>
<td>Sucrose-sulfamethoxazole interaction</td>
<td>19</td>
</tr>
</tbody>
</table>

\(^a\)Benjamini–Hochberg (BH) adjusted P < 0.1 (Benjamini and Hochberg, 1995).
Figure 2.4
Figure 2.4. Synergy between sucrose and SMX restricts the expression of a set of Aux/IAAs. (A) Concentrations of sucrose and SMX were determined which when administered independently induced no effect on hypocotyl elongation, yet inhibited it when administered together (10mM sucrose, 0.2µM SMX). Two-way ANOVA indicated an interaction between the two treatments (P<0.01); these conditions formed the basis of the microarray experiment to explore transcriptome-level changes that may underpin the interaction. (B) Three Aux/IAAs identified by ANOVA exhibited sucrose-dependent responses to SMX (IAA3, IAA6, and IAA29). IAA19 transcript abundance responded synergistically to co-treatment with sucrose and SMX. Transcript abundance values are displayed in log$_2$ scale. (C) Heatmap includes 117 probe sets that correspond to transcripts identified for which the effect of sucrose and SMX together (columns 9-12) was at least two-fold greater than the additive effect of treatment with SMX alone (columns 4-6) and sucrose alone (columns 7-9). Each column corresponds to a discrete biological sample and all treatments are presented as biological triplicate replicates. Red indicates higher, and blue indicates lower, levels of transcript abundance. Hierarchical clustering performed using Pearson correlation coefficients, and data are row-normalized to identify trends in transcript abundance across treatments irrespective of absolute abundance. (D-E) In the absence of SMX, IAA6::GUS and IAA19::GUS expression is observed throughout the hypocotyl tissues of seven-day-old dark grown seedlings; co-treatment of sucrose and SMX caused a restriction of reporter expression. Images were taken along the centre of the hypocotyl, midway between the shoot apical meristem and the root-hypocotyl junction. Scale bar, 50µm.
transcript abundance profile (Table 2.1). A total of 9292 transcripts had significant changes in abundance in response to sucrose, independently of the presence of SMX. In contrast, the effect of SMX was modest: 12 transcripts exhibited altered abundance in response to the chemical and there was no apparent trend towards a specific signalling pathway or developmental response. The sucrose-dependent effect of SMX (interaction) was significant for 19 transcripts (Supplemental Table S2.2), including a number of cell-wall-related enzymes and three members of the Aux/IAA family of transcriptional regulators: IAA3, IAA6, and IAA29. All three Aux/IAAs exhibited decreased transcript abundance in response to co-treatment with sucrose and SMX (Figure 2.4B).

Given the inhibitory effect that the combined treatment of sucrose and SMX had on the hypocotyl length, compared to the effects of SMX alone, it was striking that so few transcripts were identified for which this interaction was significant. We hypothesized that the magnitude of the changes induced by the co-treatment may differ from that induced by either sucrose or SMX alone; however, because of the low power of the ANOVA and the stringency of corrections for multiple testing, these effects may have been missed (Wilkins et al., 2010). For this reason, a second approach was used to identify transcripts that exhibited synergistic responses to the co-treatment with sucrose and SMX. Linear models were used to identify genes with differential transcript accumulation in the presence of sucrose and SMX together compared with their expression in the absence of either compound. From this set of differentially accumulating transcripts, a subset of transcripts were identified for which the effect of sucrose and SMX together was at least two-fold greater than the additive effect of treatment with sucrose alone and SMX alone (synergy); 117 transcripts met this criteria (Supplemental Table S2.3). The abundance values for these transcripts were row-normalised and visualised using a heat map, so that trends in transcript accumulation across the four treatments could be observed, irrespective of the absolute abundance (Figure 2.4C). Within this group, a fourth Aux/IAA was identified that has previously been shown to regulate auxin responses in the hypocotyl, IAA19/MSG2 (Tatematsu et al., 2004). Similar to the three
Aux/IAAs identified by the two-way ANOVA, IAA19 was characterised by decreased transcript abundance in response to the co-treatment of sucrose and SMX (Figure 2.4B).

Promoter::GUS fusions were generated to assess the spatial regulation of each of the four Aux/IAAs identified in the microarray analysis. The promoter region of each gene was fused upstream of the uidA sequence, and GUS staining was assessed. IAA6::GUS and IAA19::GUS showed expression patterns that were consistent with the microarray analysis and supported the hypothesis that these Aux/IAAs may regulate responses to SMX and sucrose in the hypocotyl. When grown in the absence of SMX, the IAA6::GUS reporter showed a high level of expression throughout the hypocotyl tissues (Figure 2.4D). The presence of SMX restricted expression of the reporter when administered in the presence of sucrose, and the interaction between sucrose and SMX can be seen in the expression of the reporter at the four concentrations used in the microarray analysis (10mM sucrose, 0.2µM SMX). Treating seedlings with both 10mM sucrose and 1µM SMX together completely inhibited expression of the reporter in the hypocotyl, whereas treatment with 1µM SMX in the absence of sucrose still allowed moderate expression. The IAA19::GUS reporter exhibited expression throughout the hypocotyl, similar to the IAA6::GUS line (Figure 2.4E). Treatment with SMX did not restrict IAA19::GUS expression independently of the presence of sucrose; however, co-treatment with sucrose resulted in expression of the construct exclusively in the vascular cylinder, and inhibited expression in other tissues of the hypocotyl. Neither the IAA3::GUS nor the IAA29::GUS reporter showed any change in expression within the hypocotyl tissues in response to the treatments (Supplemental Figure S2.4). Changes in the expression profiles of IAA3 and IAA29 were observed in other tissues, suggesting that the sucrose-SMX interaction is not limited to the hypocotyl, and may be a more general response throughout the plant.
2.3.4 Interaction between sucrose and SMX is dependent on auxin transport and signal transduction

To test whether auxin transport is necessary to mediate the interaction between sucrose and SMX, plants were grown in the presence of sucrose, SMX and/or the inhibitor of polar auxin transport, naphthylphthalamic acid (NPA) (Morgan, 1964). The presence of 1µM NPA in the growth media attenuated the interaction between sucrose and SMX, and rescued hypocotyl elongation (Figure 2.5A). NPA also affected hypocotyl elongation differently depending on whether sucrose was present in the media or not. NPA restricted elongation in the absence of sucrose, yet exerted no effect in its presence, suggesting that despite equal hypocotyl lengths, auxin transport is regulated differentially between these two treatments. These data support the findings of the microarray experiment, and suggest that the interaction between folates and sucrose is dependent on polar auxin transport.

Given that SMX perturbs plant metabolism, it was hypothesised that the compound affects auxin homeostasis, which might explain the changes in Aux/IAA transcript abundance uncovered by the microarray experiment. To test this hypothesis, indole-3-acetic acid (IAA) was extracted from dark-grown seedlings raised in the presence of sucrose, SMX, or a combination of the two treatments, and quantified using liquid chromatography-mass spectrometry (LC-MS). Seedlings were sampled after three or seven days of growth, to assess changes in IAA abundance in response to growth treatments across time. Though IAA levels were lower in samples taken after seven days of growth, there was no change in IAA abundance in response to the growth treatments (Figure 2.5B). Auxin was also quantified from seedlings grown in higher concentrations of sucrose and SMX (30mM and 1µM, respectively) to test whether there were concentration-dependent effects not observed at lower concentrations. Similar to the trends observed at lower concentrations, higher levels of sucrose and SMX did not affect IAA abundance (Supplemental Figure S2.5). It was concluded that any changes in auxin activity suggested by the transcriptome analysis were occurring independently of auxin levels.
As an alternate hypothesis, the role of auxin signal transduction in mediating crosstalk between sucrose and SMX was explored. This genetic analysis made use of mutants lacking the functional auxin signalling components IAA19 and ARF7. IAA19 has an established role in mediating hypocotyl elongation in response to auxin, and has been shown to interact with AUXIN RESPONSE FACTOR7 (ARF7; Tatematsu et al., 2004). A dominant-negative allele of IAA19, msg2, is able to stably block auxin signal transduction, overcoming redundancy in the signalling pathway (Tatematsu et al., 2004). Similarly, the ARF7-dependent signalling pathway can be shut down by loss-of-function mutations in ARF7. Though many arf mutants do not exhibit mutant phenotype due to functional redundancy, arf7 loss-of-function mutants display reduced auxin sensitivity and tropic responses in the hypocotyl, suggesting that ARF7 may promote specific and partially non-redundant functions in auxin responses (Okushima et al., 2005). Both the dominant-negative msg2 allele and the arf7 loss-of-function mutants were resistant to the inhibition of hypocotyl elongation induced by the action of SMX and sucrose (Figure 2.5C). These findings support the hypothesis that the interaction between sucrose and SMX is dependent on auxin signalling, and that phenotypes observed in the hypocotyl are likely related to the activity of the ARF7-dependent auxin response pathway.

Having established that co-treatment with sucrose and SMX perturbed auxin signalling, it was hypothesised that treatment with folic acid would alter seedling responses to auxin. Treatment with exogenous IAA inhibits hypocotyl elongation in dark grown seedlings (Tatematsu et al., 2004). This hypothesis was not supported, in that supplementing the growth media with concentrations of FA up to 50µM did not alter responses to exogenous auxin (Supplemental Figure S2.6). Similar findings were observed using lines with enhanced DHPS activity, where heightened DHPS expression had no effect on exogenous auxin (Supplemental Figure S2.7), indicating that DHPS itself is not directly involved in auxin signalling. Taken together, it is concluded that though folates may be necessary to maintain normal endogenous auxin signalling, they are not sufficient to alter seedling responses to exogenous IAA.
Figure 2.5
Figure 2.5. Auxin transport and signalling mediate synergy between sucrose and SMX. (A) Blocking polar auxin transport with NPA attenuated the synergistic effect of sucrose and SMX on hypocotyl elongation. Data represent the mean ± SE of three biological replicates from a representative experiment; each data point represents at least 36 individuals (P<0.01; ANOVA followed by Tukey’s b). (B) Indole-3-acetic acid (IAA) was extracted from three- and seven-day-old dark-grown seedlings, and quantified by LC/MS. No change in auxin abundance was observed in response to the sucrose or SMX treatments, though seedlings sampled after seven days of growth exhibited reduced auxin levels, on a per-gram fresh weight (FW) basis. Data represent the mean ± SD from three independent experiments (P<0.05; ANOVA followed by Tukey’s b). (C) The IAA19/ARF7 auxin signalling pathway is necessary for the interaction between sucrose and SMX. msg2 is a stable IAA19 allele resistant to degradation in the presence of auxin (Tatematsu et al., 2004), whereas arf7 is a loss-of-function mutant (Okushima et al., 2005). In both mutant backgrounds, synergy between sucrose and SMX is attenuated and hypocotyl elongation is rescued. Data represent the mean ± SE of three biological replicates from a representative experiment; each data point represents at least 36 individuals. Letters indicate values with statistically significant differences (P<0.01; ANOVA followed by Tukey’s b).
2.3.5 Sucrose and SMX promote auxin signalling in vasculature tissues of the hypocotyl

Two auxin-responsive reporter systems were used to assess changes in auxin signalling and accumulation within the hypocotyl: DII-VENUS and DR5. The DII-VENUS system was created by fusing the degron motif of Aux/IAA28 to the VENUS fluorescent protein (Brunoud et al., 2012). Due to the auxin-sensitive degron, the reporter is degraded in the presence of auxin. Consequently, cells with greater auxin concentrations have lower abundance of the reporter (Brunoud et al., 2012). Thus, the DII-VENUS reporter system inversely tracks the distribution of auxin in plant tissues (Brunoud et al., 2012). By contrast, intensity of the reporter in the DR5 system is greater in cells where auxin is being perceived. The DR5 system consists of tandem repeats of the Auxin-responsive element (AuxRE) fused to a minimal promoter (Ulmasov et al., 1997), driving the expression of a reporter gene (Sabatini et al., 1999).

When raised in the absence of sucrose, the DII-VENUS lines showed expression of the reporter in the nuclei of cells along the centre of the hypocotyl, in what appears to be the vasculature. In the absence of sugar, the reporter was observed along the vasculature region irrespective of the presence of SMX. Seedlings grown in the presence of both sucrose and SMX exhibited less expression of the reporter in the vascular region, and at higher concentrations of SMX, expression of the reporter was greatly restricted (Figure 2.6A).

Fluorescence intensity was quantified to assess tissue-specific changes in auxin distribution in response to sucrose and SMX. The fluorescence intensity of nuclei from vascular cells relative to those of ground tissue cells was represented as a ratio, where a value of 1 indicated equal distribution across vascular and ground tissues. In the absence of sucrose, the ratio of fluorescence remained close to 1, irrespective of the presence of SMX. This indicated that auxin remained evenly distribution between the tissues of the hypocotyl. SMX caused a decrease in fluorescence in the vasculature, indicated by a reduced ratio of intensity between
vasculature and ground tissues when sucrose was present. These data support the qualitative assessment that auxin appears to accumulate preferentially in the vascular tissues in response to sucrose and SMX.

Auxin signalling as indicated by the DR5 reporter system was consistent with the trends observed using the DII-VENUS system. Treatment with either SMX or sucrose individually did not induce expression of the DR5::GUS reporter, nor was expression observed in response to the combined treatments of sucrose and SMX at lower concentrations (Figure 2.6B). In contrast, expression of the reporter appeared within the vascular region of plants treated with 1µM SMX, but only in the presence of sucrose. Taken together, the reporter systems indicate that the interaction between sucrose and SMX promotes auxin accumulation in the vasculature of the hypocotyl.

Based on these data, a model is proposed in which folate deprivation caused by SMX results in changes to auxin distribution. Treatment with SMX resulted in auxin becoming localised to the vasculature, where IAA19/ARF7-dependent signalling mediates responses to auxin and inhibits hypocotyl elongation (Figure 2.6C). The accumulation of auxin in the vasculature is likely the result of transport from other tissues, as evidenced by the attenuation of this affect by treatment with NPA.

2.4 DISCUSSION

Combination chemical genetics can be used to overcome genetic redundancy and probe molecular pathways for interaction (Lehar et al., 2007; Lehar et al., 2008). By screening for compounds that act synergistically on a given phenotype, connectivity between targeted pathways can be explored (Lehar et al., 2007; Lehar et al., 2008). As exogenous sucrose exerts a mild restrictive effect on etiolated hypocotyl elongation, it was reasoned that sucrose could be used as the base compound in a combination chemical screen to identify pathways that modify sugar perception. Through this screen, an interaction between folate biosynthesis and sucrose signalling was uncovered. These two pathways appeared to influence auxin
Figure 2.6

(A) DII-VENUS

(B) DR5::GUS

(C) No Sucrose-SMX Interaction: Hypocotyl Elongation

Sucrose and SMX Together: Shortened Hypocotyl
Figure 2.6. Co-treatment of sucrose and SMX promotes auxin signalling in the hypocotyl. The DII-VENUS reporter system exhibited high levels of fluorescence in the vascular region of the hypocotyl. Treatment with SMX and sucrose caused a decrease in VENUS fluorescence, yet this effect was not observed when seedlings were treated with SMX or sucrose alone. White numbering indicates the ratio of fluorescence intensity in the vascular region relative to the ground tissues, mean±SD for at least ten individuals. Fluorescence decreased preferentially in the vasculature compared to the ground tissues (A). Scale bar, 100µm. DR5::GUS expression was observed in the hypocotyl vascular region during growth in the presence of both sucrose and SMX, suggesting auxin signalling is enhanced by the combined action of these two treatments (B). Scale bar, 50µm. Images were taken along the centre of the hypocotyl, midway between the shoot apical meristem and the root-hypocotyl junction. A proposed model depicting changes in auxin distribution that may underpin the sucrose-SMX interaction (C). In the absence of SMX, auxin is evenly distributed at low concentrations throughout the hypocotyl. SMX causes auxin accumulation in the vasculature, resulting in hypocotyl inhibition via IAA19/ARF7 signalling.
signalling through the regulation of a subset of Aux/IAAs. In this sense, the metabolic system has been perturbed by restricting folate biosynthesis, and a small and clearly defined signalling mechanism was changed as a result. Metabolic, hormonal and developmental pathways represent a web of interconnected signalling processes that are integrated to shape growth and development, and it is possible that during normal seedling development folates and sucrose act in concert to shape auxin distribution and sensitivity.

Forward genetic studies have been widely implemented to uncover the genetic basis for plant responses to sugars, and have proven successful in uncovering multiple sugar perception and signalling pathways (Jang et al., 1997; Zhou et al., 1998; Xiao et al., 2000; Rook and Bevan, 2003; Rook et al., 2006). Despite these successes, this approach is often limited by an inability to uncover mutant phenotypes masked by genetic redundancy, where a loss in the activity of one gene through mutation can be compensated by the activity of a related gene (McCourt and Desveaux, 2010; Toth and van der Hoorn, 2010). The Arabidopsis genome contains two isoforms of DHPS (Storozhenko et al., 2007), which may explain why dhps mutants have not been uncovered in screens for sugar-sensitive mutants, as it is possible that either isoform is sufficient to maintain wild-type levels of metabolic flux through the THF biosynthetic pathway. The general inhibition by sulfamethoxazole is believed to restrict the activity of both isoforms equally, and thus promote a phenotypic response while maintaining seedling viability. In this sense, treatment with sulfamethoxazole promoted a phenotype analogous to a double “knockdown” mutation that uncovered a role for DHPS in mediating sucrose responses.

Though this study highlights a novel role for DHPS during seedling development, the effects of the folates in shaping plant growth have been well documented (Storozhenko et al., 2007; Van Wilder et al., 2009; Mehrshahi et al., 2010; Srivastava et al., 2011). A direct connection between folate and sucrose was observed by Mehrshahi et al. (2010), who uncovered an important role for folylpolyglutamate synthetase (FPGS) across many facets of plant development, including hypocotyl and root elongation (Mehrshahi et al., 2010). Double fpgs2
fpgs3 mutants were seedling lethal, but could be partially rescued by the presence of sucrose (Mehrshahi et al., 2010). To our knowledge, this finding represents the only reported connection directly linking sucrose to the folate pool; however, most studies involving folates were undertaken using light-grown materials, and it is possible that the interaction between folates and photosynthetically-derived sugars underpin some phenotypes observed in these studies.

Treating the aerial tissues of dark-grown seedlings with sucrose can induce profound changes in morphology, such as the initiation of leaf development and root elongation (Roldan et al., 1999). Though the promotion of these traits is generally thought to be underpinned by changes in local auxin distribution (Reinhardt et al., 2003; Grieneisen et al., 2007), no change in total auxin abundance was observed during growth in the presence of sucrose. It is therefore possible that the promotive effect of sucrose on the development of these traits is a result of a redistribution of auxin, rather than de novo biosynthesis. Though auxin has been proposed not to play a dominant role during etiolated hypocotyl elongation (Jensen et al., 1998), our data suggest that the effect of sucrose and SMX on hypocotyl elongation is dependent on polar auxin transport. If sucrose is indeed promoting a change in auxin distribution, then this may explain why blocking polar auxin transport attenuated the interaction between sucrose and SMX.

Consistent with the notion that sugars promote auxin activity, sucrose conferred hypersensitivity to exogenous IAA (Supplemental Figure S2.5). As sugars can promote the development of traits often associated with auxin, such as leaf development and root elongation (Roldan et al., 1999; Kircher and Schopfer, 2012), this hypersensitivity to auxin may underpin some developmental responses to sucrose. Though the treatments used in this study did not affect total auxin abundance, recent studies highlight the influence of sugars on auxin accumulation during seedling development (Lilley et al., 2012; Sairanen et al., 2012). The notion that sucrose promotes auxin activity in etiolated seedlings is supported by the observation that transcript abundance of many auxin-related signalling components was changed by growth in the presence of sucrose (Supplemental Table S2.4).
Folate deprivation resulted in changes to auxin distribution, resulting in the inhibition of hypocotyl elongation. Quantifying DII-VENUS expression indicated a preferential accumulation of auxin in the vascular tissues, relative to ground tissues, in response to SMX. It is possible that folates are especially important in the vasculature, and that phenotypic responses to folate deprivation are a result of tissue-specific signalling. Blocking the effect of SMX with NPA suggests that auxin may be transported from neighbouring tissues to the vasculature in response to the sulfonamide, though the possibility that differential rates of auxin synthesis and metabolism between tissue types is contributing to this distribution cannot be ruled out. Though folates are not sufficient to alter responses to exogenous IAA, they are necessary to maintain normal auxin signalling during etiolated hypocotyl elongation. It may be that auxin inhibits hypocotyl elongation by signalling in the ground tissues, or through signalling pathways not influenced by folates.

Aux/IAAs are core components of auxin signal transduction that negatively regulate auxin signalling through their interaction with Auxin Response Factors (ARFs) (Hagen and Guilfoyle, 2002; Liscum and Reed, 2002; Tiwari et al., 2003). In the presence of auxin, Aux/IAAs are degraded, allowing ARFs to mediate transcription of auxin-responsive genes (Tiwari et al., 2001; Liscum and Reed, 2002). The four Aux/IAAs identified in the microarray analysis have previously been shown to regulate hypocotyl elongation (Kim et al., 1996; Reed et al., 1998; Tatematsu et al., 2004; Koini et al., 2009). IAA3/SHY2 and IAA6/SHY1 were originally identified in genetic screens for suppressors of the long-hypocotyl phenotypes of phyB and hy2, respectively (Kim et al., 1996; Reed et al., 1998). In both cases, dominant alleles were isolated that suppress hypocotyl elongation, and were posited to impinge on light-mediated hypocotyl elongation (Kim et al., 1998; Reed et al., 1998). IAA19/MSG2 was identified in a screen for mutants exhibiting auxin insensitivity in the hypocotyl, and has been shown to mediate tropic responses (Tatematsu et al., 2004; Saito et al., 2007). Though less is known about the specific role of IAA29, a study into plant growth at high temperatures found that IAA29 mediated hypocotyl elongation in response to heat stress (Koini et al., 2009). In a number of different
contexts, these Aux/IAAs have been found to regulate hypocotyl development in response to both environmental and endogenous stimuli, suggesting that perhaps they might serve as a point of integration for multiple regulatory inputs.

Transcriptional reporters for each of the four Aux/IAAs identified by the microarray experiment supported the hypothesis that interplay between sucrose and SMX impinged on the expression of these Aux/IAAs. The *IAA6::GUS* reporter exhibited marked changes in activity throughout the hypocotyl, suggesting this regulator may play a more prominent role in the hypocotyl phenotype observed by treatment with SMX and sucrose. Similarly, altered *IAA19::GUS* expression was observed in the ground tissues of the hypocotyl. These changes were similar to those reported by Saito et al. (2007), who observed that during tropic responses, *IAA19* expression was observed in expanding cells of the hypocotyl ground tissue layers, and inhibited in cells that were restricted in elongation (Saito et al., 2007). Perhaps this regulation underpins the short-hypocotyl phenotype, as a restriction of *IAA19* expression may result in an inhibition of cell expansion throughout the ground tissues of the hypocotyl. Given that the transcription of Aux/IAAs is activated by auxin (Abel et al., 1995), it may be that the decrease in *IAA6::GUS* and *IAA19::GUS* expression in the ground tissue, and the concomitant increase in *IAA19::GUS* expression in the vascular tissues, indicated a change in auxin distribution (Figure 2.6C). In this model, auxin becomes transported from the ground tissue to the vasculature, where IAA19/ARF7-dependent signalling occurs to restrict hypocotyl elongation. The genetic and pharmacogenetic analyses supported this hypothesis, as either the inhibition of polar auxin transport or the inactivation of the IAA19/ARF7 signalling pathway resulted in insensitivity to the sucrose-SMX interaction.

Given the high level of redundancy within the Aux/IAA gene family (Overvoorde et al., 2005), it is possible that all four Aux/IAAs uncovered by the array analysis act in concert to modify plant growth in response to metabolic cues. Changes to Aux/IAA transcript abundance has been observed in response to treatment with hormones (Nakamura et al., 2003), as well as changes to nutrient status (Falkenberg et al., 2008), and may offer a point of crosstalk through which other pathways may modify
auxin signalling (Nakamura et al., 2003). By adjusting the abundance of these negative regulators, it may be possible that nutrient status can adjust auxin sensitivity. Currently, the mechanism through which SMX promotes changes to Aux/IAA abundance remains unclear, and whether the compound directly regulates Aux/IAA transcript levels, or whether the changes in overall transcript abundance are a result of changed auxin distribution in the seedling, remains to be tested. Future work will explore the mechanisms through which auxin signalling and distribution are regulated in response to metabolic cues during seedling development.

2.5 MATERIALS AND METHODS

2.5.1 Plant material and growth conditions

Wild-type Arabidopsis thaliana, ecotype Columbia-0 (Col-0), seeds were obtained from the Nottingham Arabidopsis Stock Centre (NASC); all transgenic lines and mutants are of the Columbia ecotype. Plants harbouring the DR5::GUS construct were described previously (Sabatini et al., 1999), as were the dominant-negative IAA19 allele (msg2) and the arf7 loss-of-function mutants (Tatematsu et al., 2004; Okushima et al., 2005). All plant materials were grown in Conviron growth cabinets (Conviron, Winnipeg, Canada) at 21ºC in a 16/8hr photoperiod at 135µmol m⁻² s⁻¹.

2.5.2 Chemical screen

Seeds were sown in 96-well microtiter plates containing 200µL 1X Murashige and Skoog (MS) liquid growth media (Murashige and Skoog, 1962), with alternate rows supplemented with 30mM sucrose. Gamborg’s vitamin solution (Sigma-Aldrich, Oakville, Canada) was added (0.1% v/v) after autoclaving the media (Gamborg et al., 1968). Chemicals were drawn from the Library of Active Compounds in Arabidopsis (LATCA), provided by Dr Sean Cutler (UC Riverside), and adjusted to a final concentration of 2.5µM in each well. Approximately twenty seeds were added to each well in the microtiter plate. The plates were wrapped in aluminium foil and cold stratified at 4ºC, after which they were exposed to six hours of light to promote
germination, rewrapped in the foil, and then transferred to the growth chamber. After seven days in the growth cabinet, the microtiter plates were unwrapped, the growth media was drawn from the wells, and the seedlings were fixed by adding approximately 250µL Farmer’s Fixative (3:1, ethanol:acetic acid) to each well, ensuring that all seedlings were entirely submerged in the fixative. Upon rehydration using autoclaved dH₂O, seedlings were aligned on agarose plates and photographed using a Canon EOS Rebel XT EF-S 18-55 digital camera. Hypocotyl lengths were measured using ImageJ software (rsbweb.nih.gov/ij/).

2.5.3 Dose response curves and chemical treatments

Dose-response curves and chemical complementation analyses made use of a concentrated stock solution of each chemical, dissolved in dimethylsulfoxide (DMSO). Experimental concentrations were adjusted from this stock solution by dilution in sterile liquid growth media. An equivalent volume of DMSO was added to MS medium as a control. Seeds were sterilized as described above, and sown in 24-well microtiter plates in 2mL full strength MS growth media supplemented with the experimental treatments (Murashige and Skoog, 1962). Plates were then wrapped in light-blocking foil, and cold-stratified for three days at 4ºC. After stratification, plates were unwrapped and exposed to light for six hours to promote germination. Plates were wrapped in foil, and transferred to a Conviron growth cabinet (Conviron, Winnipeg, Canada) at 21ºC in a 16/8hr photoperiod at 135µmol m⁻² s⁻¹. After seven days of growth in the cabinet, seedlings were fixed, aligned on agarose plates, and measured as described above.

2.5.4 Tissue preparation, RNA extraction, and microarray analysis

Seedlings (Col-0) were grown in 24-well microtiter plates in MS liquid media (Murashige and Skoog, 1962), supplemented with 10mM sucrose, 0.2µM sulfamethoxazole, or both 10mM sucrose and 0.2µM sulfamethoxazole together. Four plates, comprising three experimental treatments and one non-treatment control, were prepared together using sterilized seeds as described above. The four
plates were each individually wrapped in light-blocking foil, and cold-stratified at 4°C for three days. The plates were then unwrapped and exposed to light for six hours to promote germination, after which they were wrapped and placed in a Conviron growth chamber (Conviron, Winnipeg, Canada) as described above. Together, these four plates comprise one biological replicate; the experiment was repeated three times to generate three biological replicates for the microarray experiment. After three days of growth, hundreds of seedlings were harvested from each plate under a green safelight, snap frozen in liquid nitrogen, and ground to a fine powder in a mortar and pestle. RNA was extracted using the RNeasy RNA extraction kit (Qiagen, Mississauga, Canada), and was precipitated overnight in 3M NaAc at -80°C to achieve concentrations necessary for prehybridization procedures. RNA quality was determined electrophoretically. For each sample, 5μg of total RNA was reverse transcribed (SuperScript II; Invitrogen), labeled and hybridized to the Arabidopsis ATH1 Genome Array according to manufacturer’s protocols (Affymetrix) at the Centre for the Analysis of Genome Evolution & Function at the University of Toronto, Canada.

GeneChip data analysis was performed using the Bioconductor suite (Gentleman et al., 2004) in R statistical programming language (R Development Core Team, 2009; http://www.R-project.org) using the affy package (Gautier et al., 2004). All 12 arrays were pre-processed together using GC-robust multi-array analysis (gcrma) (Wu et al., 2004). Expression data were filtered to remove probe sets which reported low transcript abundance and low variance across all arrays (minimum intensity of 100 on at least two arrays, minimum inter-quartile range of 0.5 on the log2-scale). The pre-processed data were analysed as a 2 X 2 factorial complete randomized ANOVA using the linear model for microarray (limma) package in R (R Development Core Team, 2009). The linear model was parameterized by group means with a manually defined sum-to-zero contrast matrix to test directly for the contrasts of interest: the main and interaction effects overall, as well as the effect of the co-treatment with sucrose and SMX. A Benjamini-Hochberg false discovery rate of 0.1 was applied to the output of all tests (Benjamini and Hochberg, 1995). Data,
description of the experimental design and methods are available for download as GEO accession GSE37484.

2.5.5 Quantitative RT-PCR

Total RNA was prepared as described above, except that RNA was treated with DNase to reduce risk of amplification from genomic DNA. A total of 4µg RNA was used for cDNA synthesis from oligo(dT)18 with SuperScript II Reverse Transcriptase (Invitrogen), following the manufacturer’s instructions. Real-time quantitative PCR (qRT-PCR) was performed using the iCycler iQ real-time PCR detection system (Bio-Rad, Toronto, Canada). The relative abundance of cDHPS and mDHPS transcripts were determined by the Pfaffl method (Pfaffl, 2001), using ACTIN2 as a reference gene. A melting curve was performed to assess primer specificity. The primers used were as follows: mDHPS FW5'TGTTGATAATGATACAGTTGC3', RV5'CTCAAGTAGAGTTGAGAAGCA3', cDHPS FW5'GACTATGGGATCAGTTGAAACC3', RV5'CCTTTGTATAATACCCTTTT3', ACT2 FW5'GGCTCCTCTCAAACCACAGGC3', RV5'CACACCATCCACGGAATCCAGC3'.

2.5.6 Creation of GUS reporter constructs and histochemical analysis

Four IAA promoter::GUS constructs were created using the Clontech In-Fusion reporter system (Clontech). For each construct, primers were designed to amplify 2kb upstream of the transcriptional start site from genomic Columbia DNA, as follows: IAA3 (At1g04240), FW5'CCGGCGCCGCAAAGCTGACCTGATGTTACACGTAGTACG3', RV5'GATCTACCAGTTCACATTTGAGGAAAGGTGTG3', IAA6 (At1g52830), FW5'CCGGCGCCGCAAAGCTTCTCTCCTCCCTCTCGAAATGTCTT3', RV5'GATCTACCAGTTCACATTTGAGGAAAGGTGTG3', IAA19 (At3g15540), FW5'CCGGCGCCGCAAAGCTATCGACTGATGTTACACGTAGTACG3', RV5'GATCTACCAGTTCACATTTGAGGAAAGGTGTG3', IAA29 (At4g322290), FW5'CCGGCGCCGCAAAGCTAATGTTGAGGAAAGGTGTG3'.
RV5\'GATCTACCATGTGCCGAGAATTAGGATCATGAGTAT3\'. These PCR products were incorporated into a modified pCAMBIA1390 by homologous recombination, directly upstream of the \textit{uidA} sequence. These plasmids were introduced into \textit{E. coli} by heat shock for amplification of plasmids, which were then extracted using a miniprep kit (Qiagen, Mississauga, Canada) and sequenced to ensure accuracy. These plasmids were transformed into \textit{Agrobacterium tumefaciens} strain GV3101 by heat shock method. Arabidopsis ecotype Columbia was then inoculated using the floral dip method (Clough and Bent, 1998). Successful T1 transformants were selected by growth on medium containing 25µg/mL hygromycin.

To facilitate GUS staining, seedlings were immersed in 90% Acetone at -20°C for 20 minutes, followed by two rinses in 50mM sodium phosphate buffer to remove acetone. GUS expression was assessed by incubating seedlings at 37°C in GUS buffer containing 50mM sodium phosphate, pH7, 1mg/mL (w/v) 5-bromo-4-chloro-3-indolyl-β-D-glucoronic acid (X-GLUC; Sigma-Aldrich, Oakville, Canada), 0.5mM K$_3$Fe(CN)$_6$, 0.5mM K$_4$Fe(CN)$_6$, 10mM EDTA. Staining times vary between constructs. \textit{IAA6::GUS} was incubated for 16hrs; \textit{IAA19::GUS} and \textit{DR5::GUS} were incubated for 24hrs. After staining, seedlings were rinsed in autoclaved dH$_2$O, followed by fixation in FAA (50% ethanol, 10% acetic acid, 10% formaldehyde). Tissues were cleared using a chloral hydrate solution (125g chloral hydrate in 50mL 30% glycerol solution) as described previously (Willemsen et al., 1998). Seedlings were observed using an Olympus BX5 microscope (Olympus), and images were captured with a QImaging MicroPublisher 3.3RTV digital camera using QCapture version 2.7 software.

2.5.7 Confocal microscopy

The DII-VENUS reporter was visualised using a Leica SP5 confocal microscope (Leica, Mannheim, Germany). An argon laser at 514nm was used for excitation while emittance was measured at wavelengths between 518nm and 560nm (Yellow Channel/VENUS) and between 595nm and 690nm (Red Channel/PI). Hypocotyls were counter-stained with propidium iodide by transferring seedlings to the
appropriate growth media containing 10μg/mL prodiam iodide (PI; Sigma-Aldrich, Oakville, Canada). This was performed under a green safe light, while being cautious to disturb the seedlings as little as possible. Images were prepared using Leica Application Suite Advanced Fluorescence – Lite (Leica). Fluorescence was quantified using ImageJ. The intensities of at least ten randomly-selected nuclei were quantified from both the vasculature and the ground tissue. For each nucleus, a neighbouring region was also measured for intensity to subtract background noise. A ratio of intensity of vasculature cells and the ground tissue cells was generated for ten individuals and averaged for each treatment.

2.5.8 Auxin quantification

Indole-3-acetic acid (IAA) was extracted from whole dark-grown seedlings after three or seven days of growth using solid phase extraction as described by Preston et al. (2009) with minor modifications (Preston et al., 2009). Approximately 10-15 mg of frozen tissue was submerged in 1 mL of 80% (v/v) methanol containing 1% (v/v) glacial acetic acid along with 300 pg of d2-IAA (Sigma-Aldrich) as an internal standard. Tissues were disrupted in a TissueLyser II (Qiagen, Mississauga, Canada), and stored at 4ºC overnight. The samples were centrifuged to remove debris, and the pellet was washed twice. The supernatant was evaporated in a SpeedVac, reconstituted in 1 mL of 1% (v/v) acetic acid, and passed through a pre-equilibrated Oasis HLB column (Waters, Ireland) according to the manufacturer's instruction. The IAA fraction was washed with 1 mL of water containing 1% (v/v) acetic acid, and eluted with 1 mL methanol containing 1% (v/v) acetic acid. The sample was evaporated in a SpeedVac and reconstituted in 1 mL of water containing 1% (v/v) acetic acid. The resultants were applied to preconditioned Oasis WAX columns (Waters, Ireland), washed with 1 mL of methanol, and eluted with 1 mL of methanol containing 1% (v/v) acetic acid. The solvent was removed under vacuum and subjected to the liquid chromatograph electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS; Agilent 6410 TripleQuad LC/MS system). An LC (Agilent 1200 series) equipped with a 50 mm x 2.1 mm, 1.8-μm Zorbax SB-
Phenyl column (Agilent) was used with a binary solvent system comprising 0.01% (v/v) acetic acid in water (Solvent A) and 0.05% (v/v) acetic acid in acetonitrile (Solvent B). Separations were performed using a gradient of increasing acetonitrile content, and an initial flow rate of 0.2 mL min\(^{-1}\). The gradient was increased linearly from 97% A, 3% B to 50% A, 50% B over 15 min, then acetonitrile content was increased linearly to 98% over 1 min and held for 1 min. This condition was held for an addition 0.5 min with an increased flow rate of 0.3 mL min\(^{-1}\). After 1 min, the initial condition was restored and allowed to equilibrate for 7.5 min for the next analysis. The retention time of IAA and d\(_2\)-IAA was 11.6 min. MS/MS conditions were as follows: capillary 4.0 kV; source temperature, 100 °C; desolvation temperature, 350 °C; cone gas flow, 0 L/min; desolvation gas flow, 12 L/min; fragmentor, 110; collision energy, 18; polarity, positive; MS/MS transition, 178/132 m/z for d\(_2\)-IAA and 176/130 m/z for endogenous IAA. A calibration curve was made using MassHunter software.

### 2.6 ACKNOWLEDGEMENTS

We are grateful to Joan Ouellette and Thanh Nguyen for excellent technical assistance. We would also like to thank Hilda Doan and Ray Persaud for assistance with data collection. We are grateful to Dr Kotaro Yamamoto for sharing previously-published materials. The authors are also very appreciative for the thoughtful and helpful commentary given by the anonymous reviewers.
Supplemental Figure S2.1. Sucrose confers hypersensitivity to sulfonamides. Dose-response curves for five sulfonamides identified in the chemical screen, plus the core structure sulfanilamide. Shown here are the effects of the compounds at higher concentrations than shown in the main body of the text.
Supplemental Figure S2.2. Osmotic environment does not affect sensitivity to SMX. Seven day-old etiolated seedlings grown in liquid media supplemented with sugar at 30mM. Data represent the mean ± SE of three biological replicates from a representative experiment; each data point represents at least 36 individuals.
Supplemental Figure S2.3. Plants with enhanced \textit{DHPS} activity exhibit resistance to the sucrose-SMX interaction. (A) Plants were transformed with constructs expressing either the cytosolic (cDHPS) or mitochondrial (mDHPS) isoform of Dihydropteroate synthase under the control of the 35S cauliflower mosaic virus promoter. Three independently-transformed lines were tested by qPCR to confirm elevated \textit{DHPS} transcript abundance. (B) Plant lines expressing either the 35S::cDHPS or 35S::mDHPS construct were resistant to lower concentrations of SMX, yet succumbed to wild-type levels of inhibition at higher concentrations. Only the plant line expressing the microbial sulfonamide-resistant allele of DHPS (sul1) was resistant to SMX at all concentrations tested. Note: Only lines 35S::mDHPS-1 and 35S::cDHPS-1 were shown in the main body of the text. (C) The affect of sucrose on \textit{DHPS} transcript abundance was assessed using qPCR; no significant change was observed (ANOVA, p<0.01).
Supplemental Figure S2.4. Sucrose and SMX do not affect IAA3::GUS and IAA29::GUS expression in the hypocotyl. Seven-day-old dark-grown seedlings were raised in the presence of sucrose and SMX, as indicated. IAA3::GUS did not exhibit staining in the hypocotyls in response to etiolated growth in presence of sucrose and SMX (A). IAA29::GUS was expressed faintly throughout the vascular region and was unaffected by presence of sucrose or SMX. Images were taken along the centre of the hypocotyl, midway between the shoot apical meristem and the root-hypocotyl junction. Scale bar, 50μm.
Supplemental Figure S2.5. Auxin abundance is unchanged by growth in the presence of sucrose and SMX. Indole-3-acetic acid (IAA) was extracted from seedlings grown in the presence of sucrose and SMX, and quantified by LC/MS. Seedlings were harvested after three or seven days of growth. Though seedlings harvested after seven days of growth tended to contain less auxin on a per-gram fresh weight (FW) basis, the treatments did not induce a significant change in auxin abundance (P<0.05; ANOVA followed by Tukey’s b).
Supplemental Figure S2.6. Folic acid is not sufficient to alter sensitivity to exogenous auxin, but does affect hypocotyl elongation in the absence of sucrose. Seedlings were grown in media containing sucrose and SMX, and supplemented with folic acid (FA), to explore whether treatment with FA alters responses to exogenous auxin. Data represent the mean ± SE of three biological replicates from a representative experiment; each data point represents at least 36 individuals.
Supplemental Figure S2.7. Enhanced *DHPS* activity does not alter the effect of exogenous auxin on hypocotyl elongation. Transgenic lines with elevated *DHPS* expression were treated with indole-3-acetic (IAA) acid to explore whether DHPS plays a direct role in response to exogenous IAA. Data represent the mean ± SE of three biological replicates from a representative experiment; each data point represents at least 36 individuals. (P<0.01; ANOVA followed by Tukey’s b).
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Supplemental Table S2.1 (Cont'd)
**Supplemental Table S2.1.** Chemical structures and seedling phenotypes. Chemicals were screened for inhibitory effects in the sucrose treatment that were less severe in the absence of sucrose. All chemicals were added at 2.5µM, in either the absence or presence of 30mM sucrose. One chemical, methacycline HCl, seemed to negate the restrictive effect of the sucrose, and is included here.
### Supplemental Table S2.2. Probe sets with significant sucrose-SMX interaction.

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**Supplemental Table S2.3.** Probe sets exhibiting synergy to co-treatment with sucrose and SMX. Values represent log₂ fold change.

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**Supplemental Table S2.4.** Probe sets corresponding to core auxin signalling components with significant sucrose main effect. Included are all ARFs, PINs, Aux/IAAs, and TIR1/ABF receptors that exhibit significantly different transcript abundance as sucrose main effect.

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Chapter 3
Metabolite and hormone signalling is integrated at the meristem to drive root growth
3 Metabolite and hormone signalling is integrated at the meristem to drive root growth

3.1 ABSTRACT

Nutrient sensing at the root apex helps drive the meristematic activity that ultimately shapes root growth. Given the sensitivity of the root meristem to changes in nutrient abundance, as well as the established role that hormone signalling plays in meristem development, the root meristem offers a unique platform through which the interaction between metabolite- and hormone-signalling pathways can be explored. Root elongation is inhibited in dark-grown seedlings, a characteristic of skotomorphogenic development. Treatment with sucrose is sufficient to release this inhibition and promote primary root elongation in dark-grown seedlings. This effect of sucrose was associated with changes in hormone signalling at the meristem, resulting in altered rates of cell division and differentiation that determine meristem productivity. Recently, the folate biosynthesis inhibitor, sulfamethoxazole (SMX), was found to perturb seedling responses to sucrose. This effect was related to changes to auxin signalling and distribution. Here, SMX and sucrose were used to probe the effect of metabolism on hormone signalling at the root meristem. SMX treatment of dark-grown seedlings induced pleiotropic effects at the root meristem, including a disruption of cell division and changes to hormone signalling. Prolonged exposure to sucrose and SMX resulted in a complete differentiation of the meristem. Taken together, these findings indicate that metabolic cues influence hormone signalling pathways to promote division and differentiation, and in the absence of metabolic inputs required to support division, the meristem succumbs entirely to differentiation.
3.2 INTRODUCTION

Root growth is dependent on the activity of a small group of rapidly dividing cells at the tip, called the root apical meristem (RAM (Beemster and Baskin, 1998; Scheres, 2007). As new cells are created through division at the RAM, other cells are displaced from this region and enter into a phase of differentiation and elongation (Scheres, 2007; Perilli et al., 2012). As displaced cells mature, they give rise to the tissues that constitute a functional root organ (Perilli et al., 2012). The relative rates of cell division and differentiation at the meristem determine its overall productivity (Dello Ilio et al., 2007; Dello Ilio et al., 2008; Tsukagoshi et al., 2011), which is tightly controlled by developmental and environmental cues (Bennett and Scheres, 2010; Ubeda-Tomas and Bennett, 2010; Tsukagoshi et al., 2011). The integration of multiple signalling inputs enables plants to maintain optimal growth rates, and establish root system architecture in response to an ever-changing environment (Teale et al., 2008).

The high level of cellular activity at the root apex is dependent on nutrient acquisition and metabolism, which support the rapidly dividing tissues (Francis and Halford, 2006). Consistent with this, supplying roots with metabolic products, such as sugars, promotes root elongation (Baskin et al., 2001; Freixes et al., 2002). Sugars play important roles in plant development as both structural components in cell walls, and as sources of energy (Rolland et al., 2006); therefore, it is necessary for the plant to detect the abundance of sugars to match growth rates with carbohydrate availability (Smith and Stitt, 2007). Sugar perception and signalling pathways can modulate the activity of other regulatory pathways (Moore et al., 2003; Mudgil et al., 2009). In this manner, sugar signalling is integrated with environmental or endogenous signals to mediate growth and development.

Sucrose can influence plant growth by promoting cellular proliferation through the regulation of D-type and B-type cyclins, connecting primary metabolism to growth and development (Riou-Khamlichi et al., 1999; Riou-Khamlichi et al., 2000; Skylar et al., 2011). D-type cyclins are also under hormonal control and may offer a potential
point of crosstalk between hormone signalling and metabolic cues (Riou-Khamlichi et al., 1999; Riou-Khamlichi et al., 2000). In addition, cyclinB1;1 is regulated by sugar signalling in the shoot apical meristem (SAM), linking sugars to multiple stages of cell-cycle progression, and across diverse tissue types (Skylar et al., 2011). In the root, cyclinB1;1 also regulates rates of cell division in response to hormone signalling, and contributes to the size of the root meristem (Ubeda-Tomas et al., 2009; Gonzalez-Garcia et al., 2011; Hacham et al., 2011). Given that multiple inputs regulate cyclin activity, cyclins may offer a point at which hormone signalling and metabolic cues may be integrated to regulate plant growth.

A complex interaction of hormone signalling pathways converge at the root apex to dictate meristematic activity (Ubeda-Tomas and Bennett, 2010; Perilli et al., 2012). The regulation of cell division and differentiation is mediated through crosstalk between auxin and cytokinin (Dello Ioio et al., 2008; Moubayidin et al., 2010). Auxin is transported through the root vascular tissues by the concerted action of the PIN-FORMED (PIN) family of auxin efflux carriers, resulting in a maximum at the root tip (Sabatini et al., 1999; Aida et al., 2004; Blilou et al., 2005; Grieneisen et al., 2007). The auxin maxima are important for establishing the quiescent centre, which maintains the pool of stem cells in this location through positional cues (vandenBerg et al., 1997; Aida et al., 2004; Blilou et al., 2005; Sarkar et al., 2007). Additionally, the auxin gradient promotes cell division in the meristem, resulting in the proliferation of cells that contribute to root growth (Galinha et al., 2007; Grieneisen et al., 2007).

Cytokinin signalling antagonises auxin at the transition zone, promoting differentiation as cells mature and exit the meristematic region (Dello Ioio et al., 2007; Moubayidin et al., 2010). Cytokinin signalling is mediated by the Arabidopsis Response Regulators (ARRs) that act redundantly to promote (Type B) or antagonise (Type A) cytokinin signalling pathways (To et al., 2004; Mason et al., 2005). Cytokinin promotes differentiation at the transition zone by inducing the expression IAA3/SHY2 (Dello Ioio et al., 2008), a negative regulator of auxin signal transduction (Tian and Reed, 1999; Tian et al., 2002). IAA3/SHY2 inhibits the expression of PIN1, PIN3, and PIN7 to restrict auxin flow to the root tip.
reduced rates of cell division (Dello Ioio et al., 2008; Perilli et al., 2012). In this manner, crosstalk between cytokinin and auxin signalling influences the relative rates of cellular division and differentiation that ultimately give rise to meristem size and productivity (Dello Ioio et al., 2008; Moubayidin et al., 2009; Moubayidin et al., 2010).

The elevated rates of cell division at the meristem represents a major investment of energy and nutrients (Francis and Halford, 2006). To ensure that these resources are sufficient to support growth, it is plausible that metabolite and hormonal regulatory pathways are integrated to match rates of cell division with resource availability (Francis and Halford, 2006). The joint regulation of cyclins by sugars and hormones supports the notion that these two pathways are integrated to coordinate growth. Consistent with this, recent findings have alluded to inputs derived from primary metabolism in mediating root meristem activity (Srivastava et al., 2011). A recent screen for mutants exhibiting reduced primary root elongation uncovered that folates are necessary to maintain proper meristem organisation (Srivastava et al., 2011), drawing a connection between folates and the regulation of root growth.

We recently reported on an interaction between sulfamethoxazole (SMX), a folate biosynthesis inhibitor, and sucrose (Stokes, 2013). This interaction perturbed auxin signalling and distribution during hypocotyl elongation (Stokes, 2013). Folates are necessary for a wide variety of cellular processes including amino acid metabolism and nucleotide biosynthesis, and are cofactors in one-carbon metabolism (Hanson and Roje, 2001; Hanson and Gregory, 2002). Given the regulatory role of auxin at the root meristem, it is hypothesised that metabolism may influence root growth through auxin signalling. Here, the folate inhibitor SMX is used to probe the effect of folates and sucrose in shaping hormone signalling at the meristem.
Figure 3.1. Sucrose promotes root elongation in dark-grown seedlings, which is restricted by SMX. (A) Seedlings were grown for seven days in the dark in the presence of sucrose and SMX, as indicated. (B) Root measurements were taken from seven-day-old dark-grown seedlings. Data represent mean±SE of three independent experiments; each data point represents at least thirty-six individuals (B; p<0.01, ANOVA followed by Tukey’s b).
3.3 RESULTS

3.3.1 Sulfamethoxazole inhibits the effect of sucrose on root elongation

In the absence of a carbon source, dark-grown *A. thaliana* seedlings exhibit minimal root elongation (Kircher and Schopfer, 2012). A recent report by Kircher and Schopfer (2012) suggested that sucrose can act as a long distance signal to promote root elongation upon developing photosynthetic competency (Kircher and Schopfer, 2012). Consistent with previous reports (Kircher and Schopfer, 2012; Lilley et al., 2012), sucrose was sufficient to induce primary root elongation in dark-grown seedlings (Figure 3.1). As folate inhibition can alter plant responses to sucrose (Stokes, 2013), root elongation was measured in the presence of SMX, to test whether folates influence the effect of sucrose on root development. Growth in the presence of SMX restricted root elongation, which was completely inhibited at 1µM (Figure 3.1). This suggests that the promotion of root elongation by sucrose is a folate-dependent process.

3.3.2 Sucrose promotes auxin accumulation at the root tip, which is inhibited by folate deprivation

The interplay between sucrose and SMX perturbs auxin signalling and distribution during seedling development (Stokes, 2013). Auxin signalling was assessed during growth in the presence of sucrose and SMX, to test whether changes in auxin distribution underpin responses to SMX during root growth. Two auxin-responsive reporter systems were used to explore the effect of sucrose on auxin accumulation at the root tip. The DII-VENUS reporter comprises the Aux/IAA28 degron motif fused to the VENUS fluorophore (Brunoud et al., 2012). The reporter becomes degraded in the presence of auxin and inversely tracks auxin accumulation in plant tissues (Brunoud et al., 2012). The DR5 synthetic promoter consists of tandem repeats of the auxin responsive element (AuxRE) fused to the 35S minimal promoter, and serves to track auxin signalling in plant tissues
Figure 3.2. SMX inhibits auxin accumulation at the root apex, resulting in ectopic signalling throughout the meristem. (A) Dark-grown DII-Venus seedlings grown for seven days in media supplemented to sucrose and SMX, as indicated. Scale: 50µm. Tissues counter-stained with propidium iodide to aid visualisation of root structures. (B) Dark-grown DR5::GUS seedlings grown for fourteen days in media supplemented with sucrose and SMX, as indicated.
Both auxin-responsive reporters indicated that sucrose promotes the accumulation of auxin at the root tip (Figure 3.2). The DII-VENUS reporter exhibited decreased fluorescence at the root tip and the ground tissue layers of the meristem; whereas, the DR5::GUS reporter showed stronger staining in the root tip during growth in the presence of sucrose. Taken together, these reporters indicate sucrose promoted the accumulation of auxin at the root tip, which is consistent with previous work into the role of sugar signalling on plant development (Mudgil et al., 2009; Lilley et al., 2012).

DII-VENUS and DR5::GUS expression was monitored to explore whether auxin plays a role in mediating the interaction between sucrose and SMX at the root meristem. When seedlings were treated with SMX in the presence of sucrose, the DII-VENUS system showed a high level of fluorescence throughout the root tip, indicating a reduction in auxin abundance. In the absence of sucrose, SMX induced no change in DII-VENUS expression (Figure 3.2A). Treatment with SMX appeared to induce ectopic auxin signalling throughout the meristem, primarily in the vascular region, as evidenced by the increased expression domain of the DR5::GUS reporter (Figure 3.2B). Similar to the trends observed with the DII-VENUS system, the expression of DR5::GUS indicated that in the absence of sucrose, there is a low level of auxin found at the root tip, which was unaffected by treatment with SMX. The sulfonamide perturbed auxin distribution only when administered in the presence of sucrose.

3.3.3 PIN expression may underpin changes in auxin distribution caused by sucrose and SMX

The transport of auxin to the root apex is facilitated by the function of PIN auxin efflux carriers, especially PIN1, PIN3, and PIN7 (Blilou et al., 2005; Dello Ioio et al., 2008). Microarray data mining uncovered PIN transcripts that had significant changes in transcript abundance in response to sucrose (Stokes, 2013). Four PINs were identified that exhibited elevated transcript abundance in response to 10mM
Figure 3.3. Sucrose promotes auxin accumulation at the root tip through increased PIN abundance and polar localisation. (A-B) Sucrose promoted auxin accumulation at the root tip. (C) Mining microarray data identified four PIN transcripts that exhibit elevated abundance in response to sucrose. (D-F) PIN translational fusions grown in the dark for seven days in the presence of sucrose. (G-I) Scale bar, 50µm. Detail shows PIN subcellular localisation. Scale: 25µm.
sucrose, including PIN1, PIN3, and PIN7 (Figure 3.3C). Translational fusions were used to track protein abundance and localisation of these three PINs in response to sucrose and SMX. Consistent with the increased auxin accumulation induced by sucrose, all three of the PIN-GFP reporter lines tested showed increased PIN abundance during growth in the presence of sucrose (Figure 3.3D-I). In the absence of sucrose, GFP signal was faint and evenly distributed throughout the expression domain. Sucrose promoted stronger GFP signal, as well as greater protein localisation to the plasma membrane (Figure 3.3G-I).

The interaction between sucrose and SMX does not affect auxin abundance under these conditions (Stokes, 2013), therefore phenotypes associated with these treatments are likely the result of changes to auxin distribution or signalling. Changes in auxin activity indicated by the DII-VENUS and DR5::GUS reporters may be underpinned by changes in PIN abundance or localization. PIN translational fusions were used to test whether changes in PIN abundance might explain the differences in auxin distribution at the root tip. Treatment with SMX decreased PIN abundance in the meristem, but only when administered in the presence of sucrose (Figure 3.4A-C). This trend was observed for all three PIN proteins tested, though variable levels of inhibition were observed between reporter lines. SMX also caused a sucrose-dependent decrease in PIN3 and PIN7 transcript abundance, consistent with the trends observed with the translational fusions (Figure 3.4D). PIN1 did not respond to SMX at the transcriptional level.

3.3.4 Folate deprivation causes ectopic cytokinin signalling

The transport of auxin to the root tip is restricted by cytokinin signalling, which inhibits PIN expression through the SHY2/IAA3 signalling pathway (Dello Ioio et al., 2008; Moubayidin et al., 2010). Consequently, changes in auxin distribution observed in response to sucrose and SMX could be the result of cytokinin signalling. The cytokinin-responsive TCS::GFP reporter was used to track cytokinin signalling in vivo (Muller and Sheen, 2008). The TCS::GFP reporter indicated that cytokinin
Figure 3.4. SMX decreases PIN protein abundance at the root tip. (A-C) PIN translational fusions grown in the dark for seven days in presence of 10mM sucrose and SMX, as indicated. Scale bar, 50µm. (D) Transcript abundance of PIN1, PIN3 and PIN7 was measured in seven day-old etiolated seedlings grown in presence of 10mM sucrose and SMX, as indicated. Data represent mean ±SD of three independent experiments.
Figure 3.5. Sucrose promotes cytokinin signalling at the root tip; ectopic signalling induced by SMX. Dark-grown TCS::GFP lines grown for seven days in media supplemented with sucrose and SMX, as indicated. Scale bar, 50µm.
signalling was strongest in the columella cells at the distal root tip during etiolated root growth. The presence of sucrose increased reporter intensity, suggesting that cytokinin signalling was enhanced by sucrose, yet there was no change in the spatial distribution (Figure 3.5). In contrast, SMX induced strong TCS::GFP expression throughout the lateral root cap, in addition to the expression in the columella cells. SMX also promoted expression of the reporter in some vascular cells in the apical meristem. The TCS::GFP reporter system indicated that co-treatment of sucrose and SMX resulted in ectopic cytokinin signalling.

3.3.5 Interaction between sucrose and SMX perturbs cell-cycle progression at the meristem

As sucrose was sufficient to promote root elongation in dark-grown plants, sucrose might promote meristematic activity at the root tip. Sucrose induces the expression of cyclinD2 (cycD2) and cyclinD3 (cycD3) in A. thaliana cell cultures (Riou-Khamlichi et al., 2000; Richard et al., 2002). D-type cyclins promote G₁-to-S-phase transition in plants (De Veylder et al., 2007), and are potential regulatory checkpoints in response to environmental cues during cell-cycle progression (Riou-Khamlichi et al., 2000; Dewitte and Murray, 2003). The later G₂-to-M phase is regulated, in part, by another member of the cyclin family, cyclinB1;1 (De Veylder et al., 2007). CyclinB1;1 regulates cell division at the meristem, and contributes to changes in overall size of the organ in response to hormonal cues (Ubeda-Tomas et al., 2009; Gonzalez-Garcia et al., 2011; Hacham et al., 2011).

Two cell-state transition markers (cycD2::GUS and cycB1;1::GUS) were used to assess whether sucrose promotes cell-cycle progression at the root tip (Donnelly et al., 1999; Riou-Khamlichi et al., 2000; Masubelele et al., 2005). The cycD2::GUS reporter was induced by sucrose in a concentration-dependent manner, supporting the hypothesis that sucrose promotes meristematic activity (Figure 3.6A). CycD2 transcript abundance, also increased in response to sucrose, as determined by RT-qPCR (Figure 3.6B). Contrary to this, cycB1;1::GUS was not induced by sucrose
Figure 3.6. Sucrose and SMX promote cell-cycle progression markers in the meristem. (A, C) Seven-day-old dark-grown cycD2::GUS and cycB1;1::GUS seedlings grown in presence of sucrose and SMX, as indicated. Scale bar: 50µM. (B, D) Transcript abundance of cyclinD2 and cyclinB1;1 quantified with qPCR. Data represent mean±SD of three independent experiments.
alone, and no change in cycB1;1 transcript abundance was observed (Riou-Khamlichi et al., 2000), the mechanism through which this is accomplished, and how this relates to overall meristematic activity, is not known. The effect of sucrose on cell-cycle progression might be buffered by input from other nutritional cues. To test whether folate status modifies the effects of sucrose on meristematic activity, seedlings were grown in the presence of both sucrose and SMX, and expression of the cell-cycle reporters was observed. Surprisingly, SMX enhanced the expression of both the cycD2::GUS and cycB1;1::GUS reporters (Figure 3.6A,C). The action of SMX on the cell cycle reporters was sucrose-dependent, as SMX exerted no effect on the reporters when administered in the absence of sucrose. The sucrose-dependent effect of SMX on the expression of the cell markers is reminiscent of previous reports of synergy between auxin, cytokinin, and sucrose in the regulation of cyclin gene expression (Riou-Khamlichi et al., 1999; Riou-Khamlichi et al., 2000; Richard et al., 2002). In many cases, the induction of gene expression by hormone treatments was dependent on the presence of sucrose, with little response to the hormone observed when sucrose was not present (Riou-Khamlichi et al., 1999; Riou-Khamlichi et al., 2000; Richard et al., 2002).

3.3.6 Meristem size increases in sucrose, but differentiates in response to folate deprivation

Meristem size was measured by counting the number of unexpanded cortex cells at the root tip (Figure 3.7A-B; black arrows)(Dello Ioio et al., 2008). Sucrose increased meristem size in a concentration-dependent manner, consistent with the sucrose-induced changes in root elongation and cell-cycle progression. By contrast, SMX decreased meristem size, leading to a complete loss of meristematic cells at higher concentrations (Figure 3.7). As in earlier experiments, SMX only had an effect in the presence of sucrose, and induced no change in the meristem when applied on its own. The disruptive effect of SMX on meristem organisation became more pronounced as seedlings matured, and resulted in approximately 80% of individuals
Figure 3.7. Sucrose increases meristem size, yet cotreatment with SMX results in complete differentiation. (A) Meristem size determined by counting cortex files in cleared root tips of fourteen-day-old dark grown seedlings. Data represent mean ±SD of a representative experiment (p<0.01, ANOVA followed by Tukey's b). (B) DIC images of cleared root tips of fourteen-day-old dark-grown seedlings treated with sucrose and SMX, as indicated. (C) Cleared root tips visualised using confocal microscopy. Scale bar, 25 µm.
Figure 3.8. Meristem integrity diminishes across time. Dark-grown seedlings presence of 30mM sucrose and 1µM SMX were sampled at indicated time points. Meristems assessed using brightfield microscopy. Values represent mean±SD of three independent experiments, each with at least thirty individuals.
exhibiting differentiated meristems by fourteen days of growth (Figure 3.8). The cellular organisation of the root meristem, which usually follows a well-defined pattern (Scheres et al., 2005; Scheres, 2007), appeared to be lost in these seedlings.

Seedlings grown in presence of 30mM sucrose and 1µM SMX had differentiated meristems that appeared to lack a quiescent centre (QC; Figure 3.7C). The QC is established and maintained by the auxin maxima at the root tip (Aida et al., 2004), and provides positional information to neighbouring stem cells to maintain them in an undifferentiated state (van den Berg et al., 1995; Scheres, 2007). The expression of *WUSCHEL-RELATED HOMEOBOX5* (*WOX5*) is confined to the QC, and can be used as a marker to assess QC specification (Sarkar et al., 2007; Sena et al., 2009). The loss of *WOX5* expression indicates differentiation of the QC and loss of the stem cell niche (Sena et al., 2009; Hernandez-Barrera et al., 2011).

The differentiation state of the QC in roots treated with SMX was tracked using the *WOX5::GFP* reporter gene. After seven days of growth, *WOX5::GFP* was consistently expressed in the QC of all individuals (Figure 3.9). Higher concentrations of sucrose and SMX, which caused some individuals to appear disrupted when observed using bright field microscopy, still maintained *WOX5::GFP* expression at the QC. This suggests that despite their appearance, these meristems maintained QC specification. After fourteen days of growth, a higher proportion of meristems appeared differentiated, and the effect of folate deprivation on the meristems was more prominent. In these individuals, sucrose and SMX diminished *WOX5::GFP* expression, indicating differentiation of the stem cell niche. Meristems that maintained organised structure had elevated levels of *WOX5::GFP* expression, which was observed in a broader domain than in the non-treated samples (Figure 3.9).
**Figure 3.9. Differentiated meristems lose QC specification.** WOX5::GFP seedlings grown in the dark for either seven or fourteen days, in the presence of sucrose and SMX, as indicated. After seven days of growth, some individuals appeared disrupted when viewed under the brightfield microscope (5/20). After fourteen days of growth, a far greater proportion appeared disrupted (21/24). Scale bar, 50µM.
Figure 3.10

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Figure 3.10. Blocking cell division with aphidicolin partially phenocopies SMX.

(A) Seven-day-old dark-grown cycD2::GUS seedlings were raised in the presence of sucrose and aphidicolin (APH), as indicated. Scale bar, 50µM. (B-C) Fourteen-day-old seedlings grown in 30mM sucrose and 1µM SMX and seven-day-old seedlings grown 30mM sucrose and 8mg/mL aphidicolin exhibited root hair development at the distal root tip (black arrows). (D) TCS::GFP grown for seven days in dark in presence of sucrose and aphidicolin, as indicated. Aphidicolin caused the intake of PI into some cells (white arrows).
3.3.7 Phenotypic responses to SMX may be due to restricted cell division

As folates are necessary for purine biosynthesis (Bertino, 2009), it is possible that SMX restricts cell division. Cell division was inhibited with aphidicolin to assess similarities to SMX treatments. Aphidicolin is an inhibitor of DNA replicase that blocks cell-cycle progression at the G₁-S transition (Ikegami et al., 1978; Planchais et al., 2000). Similar to SMX, aphidicolin induced the cycD2::GUS reporter, and caused severe morphological defects at the root meristem only when administered in the presence of sucrose (Figure 3.10A-D). Cells at the meristem were swollen and differentiated, as evidenced by the development of root hairs at the distal root tip. This effect of aphidicolin on meristem organisation was similar to fourteen-day-old seedlings grown in the presence of 30mM sucrose and 1µM SMX (Figure 3.10C-D).

Despite the similarities in phenotype, some important differences suggested the effect of SMX is not strictly related to the inhibition of cell division. Propidium iodide (PI) does not pass the membrane of living cells, and can be used as a vital stain (Truer nit and Haseloff, 2008). Treatment with aphidicolin caused the accumulation of PI into the cytosol of some cells in the meristem, indicating cell death (Figure 3.10B). In response to DNA damage, mitotic checkpoints invoke nonapoptotic programmed cell death destroy the cell (Fulcher and Sablowski, 2009). By blocking replication rather than continue with mitosis, this mechanism ensures stability of the genome (Fulcher and Sablowski, 2009). Prolonged exposure to aphidicolin caused cell death in the meristem at concentrations that did not induce differentiation (4mg/mL), as indicated by the entry of PI into the dead cells (Figure 3.10D). In contrast, cell death has not been observed in response to high concentrations of SMX. Multiple pathways exist that sense genome integrity, and they converge to induce the expression of \textit{WEE1}.
Figure 3.11. Sucrose and SMX does not promote *WEE1* transcript abundance. *WEE1* transcript abundance in seven-day-old dark grown seedlings was measured using qPCR. Data represent mean±SD of three independent experiments.
(De Schutter et al., 2007; Fulcher and Sablowski, 2009). WEE1 is a kinase that arrests cell division in response to DNA damage (De Schutter et al., 2007). While aphidicolin induced expression of WEE1 (De Schutter et al., 2007), no change in transcript abundance was observed in response to SMX (Figure 3.11). From these observations it was concluded that SMX does not invoke DNA repair pathways that inhibit cell division and cause cell death.

To determine if aphidicolin induced similar hormone signalling pathways as SMX, the TCS::GFP reporter was grown in the presence of aphidicolin. In contrast to treatment with SMX, aphidicolin did not broaden the expression domain of the cytokinin reporter, and instead inhibited its expression (Figure 3.10B). This suggests that despite the fact that both treatments result in differentiated meristems, the mechanism through which this occurs is different. There is a signalling response to SMX that does not mediate responses to aphidicolin.

3.3.8 Stabilising IAA3/SHY2 signalling rescues meristem disruption

The complex interplay between auxin and cytokinin converges on IAA3/SHY2 (Dello Ioio et al., 2008; Moubayidin et al., 2010), a canonical Aux/IAA repressor of auxin signalling (Tian and Reed, 1999; Tian et al., 2002; Tiwari et al., 2004). Aux/IAAs are degraded in the presence of auxin to allow the expression of early auxin-responsive genes (Tiwari et al., 2001; Liscum and Reed, 2002). Aux/IAAs are themselves auxin induced, forming a negative feedback loop (Liscum and Reed, 2002). By promoting the expression of IAA3/SHY2 in the transition zone, cytokinin can influence auxin sensitivity and enable crosstalk between the two hormone signalling pathways (Dello Ioio et al., 2008; Moubayidin et al., 2009). The current model meristem development proposes that IAA3/SHY2 inhibits PIN expression in the transition zone to restrict auxin transport to the root apex (Dello Ioio et al., 2008; Perilli et al., 2012).

Transcript accumulation of four Aux/IAAs is perturbed by the interplay between sucrose and SMX, including IAA3/SHY2 (Stokes, 2013). Given the importance of
Figure 3.12. IAA3/SHY2 signalling mediates responses to sucrose and SMX. (A) Seven-day-old dark-grown IAA3::GUS seedlings raised in the presence of sucrose and SMX. (B) DIC microscopy images of shy meristems after fourteen days of growth in 30mM sucrose and 1µM SMX. Scale bar, 50µM. (C) Proportion of shy seedlings grown in 30mM sucrose and 1µM SMX that exhibit intact meristems after fourteen days of growth.
IAA3/SHY2 in regulating meristem activity, an IAA3::GUS transcriptional reporter was used to explore the regulation of IAA3/SHY2 in response to sucrose and SMX in the root. In contrast with previous reports that IAA3/SHY2 is expressed in the transition zone (Dello Ioio et al., 2008; Moubayidin et al., 2010), IAA3::GUS expression was observed throughout the meristem (Figure 3.12A). Sucrose promoted IAA3::GUS expression, likely the result of increased auxin abundance at the root. SMX inhibited IAA3::GUS expression throughout the meristem, resulting in complete inhibition at 1 µM SMX when sucrose was present. SMX induced no effect on IAA3::GUS expression when administered in the absence of sucrose.

Mutants have been isolated in which the signalling capacity IAA3/SHY2 is impaired (Tian and Reed, 1999; Tian et al., 2002). The shy2-2 allele is stable in the presence of auxin, shutting down the IAA3/SHY2-dependent pathway (Tian and Reed, 1999; Tian et al., 2002). Consistent with the role of IAA3/SHY3 in repressing auxin signalling, shy2-2 mutants exhibit smaller meristems, whereas the meristems of the null shy2-31 alleles are enlarged (Dello Ioio et al., 2008; Moubayidin et al., 2010). These mutants were used to test whether IAA3/SHY2 mediates responses to sucrose and SMX at the meristem.

If reduction in auxin abundance underpins the meristem differentiation, then the shy2-2 mutant should be hypersensitive to SMX. Contrary to this hypothesis, a greater proportion of shy2-2 mutants exhibited normal meristem morphology compared to WT (Figure 3.12B-C). The shy2-31 alleles responded similarly to WT. By shutting down the IAA3/SHY2 pathway, meristem organisation was rescued, implicating IAA3/SHY2 in the response to folate deprivation. The nature of this role is unclear, as the presumed role of IAA3/SHY2 during meristem development is to reduce PIN abundance and restrict auxin flow to the root tip. SMX induced both a reduction in PIN expression as well as a reduction in IAA3/SHY2 expression, an anomaly that requires further investigation.
3.4 DISCUSSION

A growing body of evidence reveals a crucial interplay between metabolic and hormone signalling in the control of plant growth and development. In the current study, sucrose and folates interact with two hormone signalling pathways, suggesting that sugars lie upstream of hormones in the regulation of root cell division and differentiation. This mechanism may ensure that adequate metabolic energy and structural molecules are available to support growth of the root tissues. SMX application highlights the importance of folates during this proliferation of tissues. Inputs from primary metabolism support growth, and in their absence, cell proliferation is restricted. SMX uncovered a relationship between folates and the maintenance of hormonal interactions at the meristem.

Based on these findings, a model of meristem regulation by metabolism is proposed (Figure 3.13). During etiolated growth, basal levels of hormone signalling maintain the root meristem in a dormant state. Sucrose induced both auxin and cytokinin signalling at the root tip, leading to increased division and differentiation at the root apex (Figure 3.13A-B). Together, these two processes drive primary root elongation. Treatment with SMX uncovered the importance of folates in balancing hormone signalling at the root meristem. Folate deprivation inhibited PIN expression and restricted auxin accumulation at the root apex (Figure 3.13C). This decrease in auxin abundance may have impinged on division rates at the meristem. Ectopic cytokinin signalling was also observed in the lateral root cap. As cytokinin promotes cellular differentiation at the root meristem (Dello Ioio et al., 2007; Dello Ioio et al., 2008; Perilli et al., 2012), it is possible that this induction of cytokinin contributed to the differentiated meristems observed in response to SMX and sucrose.

Fluctuations in metabolite pools can induce signalling pathways and regulate gene expression to influence growth. For example, sucrose is believed to induce root elongation by acting as a proxy indicator for favourable above-ground conditions
Figure 3.13. Model: Sucrose and folates promote hormone signalling at the meristem to drive root growth. (A) In the absence of sucrose, basal hormone signalling maintains limited rates of division and differentiation. (B) Sucrose induces hormone networks that promote growth. (C) Maintenance of the hormone balance is dependent on folates, as the addition of SMX resulted in reduced auxin transport and ectopic cytokinin signalling at the lateral root cap.
(Kircher and Schopfer, 2012). In this manner, metabolite signalling can act analogously to hormone pathways by enabling plants to respond to changes in their environment. It is possible that metabolite signalling is integrated into hormone pathways as a form of crosstalk. Plant growth and development is orchestrated through hormone signalling, and though it is accepted that metabolism is necessary to support growth, there are but a few known instances where metabolism inputs directly into hormone networks. To this end, progress has been made toward an understanding of how hormone and metabolic cross-talk influences meristem productivity, offering insight into meristem function in a fluctuating environment.

In the established model of meristem development, IAA3/SHY2 is transcription is regulated by cytokinin in the transition zone to inhibit auxin flow to the root tip (Dello Ioio et al., 2008; Perilli et al., 2012). Though shy2-2 has reduced meristem size (Dello Ioio et al., 2008; Moubayidin et al., 2010), suppressing the pathway by stabilising IAA3/SHY2 increased resistance to SMX. It is unclear how SMX induced ectopic cytokinin signalling and yet inhibited IAA3/SHY2 expression to disrupt meristem potential. Current models are based on observations made in the transition zone, and it is not uncommon for hormones to have distinct effects based on tissue type and developmental context. If ectopic cytokinin signalling is causing the differentiated phenotype, it is possible that cytokinin is acting in the LRC through uncharacterised pathways. Tissue-specific hormone signalling can influence the activity of the entire root meristem (Ubeda-Tomas et al., 2008; Ubeda-Tomas et al., 2009; Hacham et al., 2011). The lateral root cap is not typically associated with cytokinin signalling, and future work will address whether these changes to cytokinin signalling are necessary for the phenotypes observed at the meristem in response to sucrose and SMX.

Meristem collapse may be caused by the reduced auxin accumulation at the root tip (Benjamins et al., 2001; Friml et al., 2004). Inhibition of PIN expression was observed in response to SMX, which was also associated with a reduction in auxin accumulation at the root tip. The inability to focus auxin at the tip, as evidenced by
the changes in DII-VENUS distribution, may cause the loss of QC specification at the stem cell niche. The diminished auxin signalling at the apex may also be the cause the reduction in \textit{IAA3/SHY2} expression, as the transcription of \textit{Aux/IAA}s is induced by auxin. Rescuing meristem potential by shutting down IAA3/SHY2 is in contradiction to this hypothesis, in that the \textit{shy2-2} mutant should be hypersensitive to SMX. Further experimentation using auxin signalling and biosynthesis mutants can explore this hypothesis further.

In support of the hypothesis that reduced auxin activity at the root tip resulted in a loss of meristem potential, this phenotype is also seen in mutants with reduced auxin signalling and accumulation at the root apex (Aida et al., 2004; Hernandez-Barrera et al., 2011). Loss of the auxin-responsive PLETHORA (PLT) transcription factors, which help establish and maintain the stem cell niche (Aida et al., 2004), resulted in differentiated meristems that closely resemble the meristems of seedlings treated with SMX. The inability to transduce auxin signals in the \textit{plt1;plt2} double mutants resulted in stem cell differentiation (Aida et al., 2004). This effect has also been observed in mutants with reduced auxin accumulation at the distal root tip (Hernandez-Barrera et al., 2011). The \textit{moots koom1} mutation causes a decrease in meristem potential that appears to be the result of reduced auxin maxima. Similar to SMX-treated seedlings, the expression of \textit{WOX5} is became diminished well after the meristems exhibited the differentiated phenotype (Hernandez-Barrera et al., 2011).

Folates are involved in many different metabolic processes, and a specific branch of metabolism that is important for meristem growth remains unknown. The inhibition of folate synthesis, or the downstream products of this pathway, may become limiting for the replication capacity of rapidly dividing meristem tissues. It is possible that an inhibition of division occurs as a result of metabolic deficiency, and that this is the cause of the differentiated meristem. This notion is supported by the fact that the disrupted meristems could be rescued by the application of folic acid, indicating that folates are required to maintain meristematic potential (Figure 3.14).

The phenotypic response of the meristem was also partially phenocopied by the cell-
**Figure 3.14. Folic acid rescues disrupted meristem and promotes cell-cycle progression.** Dark-grown seedlings treated with sucrose and SMX were grown in media supplemented with 25µM folic acid (FA). FA rescued the effect of SMX on meristem organisation, as all individuals exhibited intact meristems when FA present. Ratios indicate proportion of individuals exhibiting the phenotype shown. Scale bar, 50µm.
cycle inhibitor aphidicolin. In both cases, complete differentiation of the meristem was induced by the chemical treatment. The experiments using aphidicolin supported the hypothesis that a reduction in cell division relative to the rate of differentiation resulted in complete differentiation of the meristem. The dependency of the cell-division inhibitor on sucrose highlights the role of sucrose in inducing both division and differentiation as two distinct processes. It is noteworthy that aphidicolin did not perturb the hormone signalling pathways that SMX induced, suggesting that the signalling effects of SMX at the meristem are not related solely to the inhibition of cell division. This finding also supports the notion that there are perception pathways that sense folate metabolism and input into canonical hormone signalling networks that govern meristem development.

3.5 CONCLUSION

By integrating metabolic pathways with hormone signalling at the meristem, plants can coordinate growth to match resource availability. Rapidly-dividing tissues require investment of energy and nutrients; metabolite sensing at the meristem ensures resources are available to support growth. Metabolite accumulation can be detected by plants and acts as a signal to induce changes in plant behaviour. In this capacity, it is likely that metabolite signalling is integrated with other developmental and environmental cues. Crosstalk such as this would enable plants to fine-tune responsiveness to a fluctuating environment. That SMX perturbs plant sugar responses at the root highlights a role for folates in supporting meristematic potential, and suggests that this metabolic pathway feeds into hormone signalling in the control of meristem productivity.

3.6 MATERIALS AND METHODS

3.6.1 Plant Material and Growth Conditions

Wild-type *Arabidopsis thaliana*, ecotype Columbia-0 (Col-0), seeds were obtained from the Nottingham Arabidopsis Stock Centre (NASC); all transgenic lines and
mutants are of the Columbia ecotype. Plant materials were grown in Conviron growth cabinets (Conviron, Winnipeg, Canada) at 21°C in a 16/8hr photoperiod at 135µmol m⁻² s⁻¹. Plants harbouring the auxin reporter DR5::GUS (Sabatini et al., 1999) and DII-VENUS (Brunoud et al., 2012) were described previously, as were the PIN translational fusions: PIN1-GFP (Benkova et al., 2003), PIN3-GFP (Zadnikova et al., 2010), PIN7-GFP (Blilou et al., 2005).

Seeds were surface sterilized using a 10% bleach, 1% Triton X-100 solution and sown in 1X Murashige and Skoog (MS) liquid growth medium (Murashige and Skoog, 1962), in 24-well microtiter plates as previously described (Stokes, 2013). Filter-sterilized stock solutions of SMX dissolved in dimethyl sulfoxide (DMSO) were added to the growth media after the media was autoclaved. Equivalent volumes of DMSO were added to control treatments to ensure equal addition of DMSO across all treatments. Plates were wrapped in light-blocking foil, and cold stratified at 4°C for three days. After cold treatment, plates were exposed to light for six hours to promote germination. The plates were then wrapped again in foil and transferred to a growth cabinet (Conviron, Winnipeg, Canada) at 21°C.

3.6.2 Root elongation measurements

After seven days of growth, seedlings were fixed by submersion in 70% Ethanol, 10% Acetic acid solution at 4°C overnight. Fixed seedlings were rehydrated by submersion in autoclaved water for 24 hours, after which seedlings were aligned on agarose plates and photographed using a Canon Rebel SLR camera. Photographed roots were measured using ImageJ software (rsbweb.nih.gov/ij/).

3.6.3 Histochemical assay of GUS reporter activity

Seedlings harboring transgenic GUS reporters were sown in liquid growth media and grown in the dark for specified periods of time, as described above. Upon harvesting, seedlings were permeabilised by submersion in ice-cold 90% acetone and incubated at -20°C for 20 minutes. Seedlings were rinsed twice for ten minutes in 50mM sodium phosphate buffer, then infiltrated with GUS buffer containing 50mM
sodium phosphate, pH7, 1mg/mL (w/v) 5-bromo-4-chloro-3-indolyl-β-D-glucoronide (X-GLUC), 5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆, 10mM EDTA. Staining times vary between constructs. DR5::GUS and cycB1;1::GUS were incubated for 2hrs; cycD2::GUS was incubated for 16hrs. After staining, seedlings were rinsed in autoclaved dH₂O, followed by fixation in FAA (50% ethanol, 10% acetic acid, 10% formaldehyde). Tissues were cleared using a chloral hydrate solution (125g chloral hydrate in 50mL 30% glycerol solution) as described previously (Willemsen et al., 1998). Seedlings were observed using an Olympus BX5 microscope (Olympus, Tokyo, Japan), and images were captured with a QImaging MicroPublisher 3.3RTV digital camera using QCapture version 2.7 software, as described previously (Stokes, 2013).

3.6.4 Quantitative real-time PCR (qPCR)

Seedlings were germinated and grown in liquid culture as described above. After seven days of growth, seedlings were collected and rinsed with sterile distilled water under a green safe light before being snap frozen in liquid nitrogen. Frozen tissue was ground with a mortar and pestle to a fine powder, and total RNA was extracted using an RNeasy kit from Qiagen, following the manufacturer’s instructions (Qiagen, Mississauga, Canada). RNA quality was determined spectrophotometrically, and 1µg RNA was used for cDNA synthesis using the iSELECT cDNA synthesis kit following the manufacturer’s instruction (Bio-Rad, Mississauga, Canada). Real-time quantitative PCR (qRT-PCR) was performed using the iCycler iQ real-time PCR detection system (Bio-Rad, Mississauga, Canada). The relative transcript abundance was determined by the Pfaffl method (Pfaffl, 2001), using UBQ10 as a reference gene. A melting curve was performed to assess primer specificity. The primers used were as follows: UBQ10 (At4g05320) FW5’GGCCTTGTATAATCCCTGATGAAATA3’, RV5’AAAGAGATAACAGGAACGGAACATA3’; PIN1 (At1g73590), FW5’AAACCACACCCGCATTACT3’, RV5’TTCCTGAGGTACAGAGATCTTAAC3’; PIN3 (At1g70940),
FW5’TCCGATCAGCTGGTTTACTAC3’, RV5’GGCATCAGCATTCTAAATCA3’;
PIN7 (At1g23080) FW5’CGGTGAGATTTCTTTACTGGACCA3’, RV5’CGGTGAGATTTCTTTACTGGACCA3’;
RV5’CTGTGGAGGACTTCTGACCA3’; CYCB1;1 (At4g37490) FW5’CTGATCCTGGTGGAGTGTT3’, RV5’CTGATCCTGGTGGACCA3’;
CYCD2 (At2g22490) FW5’GCTGCTGCAGTGTCGGTT3’, RV5’GCTGCTGCAGTGTCGGTT3’;
WOX5 (At3g11260) FW5’ACAGCTCTTACCGACAACCTCG3’;
WOX5’GCGAAGAAGATTGTCAAGAGGA3’, RV5’GATGTTCCATTTCAGCTCCACA3’;
WEE1 (At1g02970) FW5’CAAGAGCTTGCACTCCACATCATG3’, RV5’TGGTGTGGACACATTTCAGCTCGG3’.

3.6.5 Confocal microscopy

PIN-GFP and DII-VENUS reporter lines were visualized using a Leica SP5 confocal microscope (Leica, Mannheim, Germany). Seedlings were counterstained with propidium iodide (PI) as described previously (Stokes, 2013). To visualize GFP, fluorophores were excited using an argon laser at 488nm; emission was measured between 500nm and 550nm (green channel/GFP), and between 600nm and 710nm (red channel/PI). To visualise VENUS, excitation was accomplished using an argon laser at 514nm; emission was measured between 518nm and 560nm (yellow channel/VENUS) and between 595nm and 690nm (red channel/PI), as previously described (Stokes, 2013). Gain and laser intensity were kept constant between treatment groups to allow equal comparison across the experiment.

3.6.6 Root meristem measurements

After seven days of growth, seedlings were fixed, rehydrated, and tissues were cleared as described above. Seedlings were mounted in clearing solution and imaged using differential interference contrast (DIC) microscopy on a Zeiss Axio Imager AX10 microscope.
Chapter 4

General conclusions and future directions
4 General Conclusions and Future Directions

4.1 GENERAL CONCLUSIONS

Sugars provide energy and carbohydrate resources that support plant growth, yet also inform development. For example, sucrose is transported throughout the plant where it is used in the production of new cellular components. The sugar molecules themselves function as signals that regulate gene expression to capitalize on these resources, allowing the plant to balance carbon supply with demand. There are regulatory factors that respond directly to sugar, enabling sugars to influence gene expression (Cho et al., 2006; Hummel et al., 2009). Changes in gene expression that allow the cell to respond to sugars may not be sufficient themselves to regulate the development of new organs. The induction of hormone signalling networks may be necessary to orchestrate development on a whole-organ level, such as during root elongation and leaf production.

Sugars promote the development of traits typically associated with photomorphogenesis, such as true leaves, and hypocotyl inhibition, and the induction of primary root elongation. This developmental state represents a form of “glycomorphogenesis”, a term coined here to describe the developmental traits induced by sugars. These changes result in plant morphology that is distinct from either skoto- or photomorphogenic developmental patterns. Glycomorphogenic development can describe not only the developmental state of dark-grown seedlings treated to sugars, but rather to encapsulate the total contributions sugar metabolism and signalling on plant morphology.

It is believed that sugars are perceived through a metabolite sensing mechanism that results in changes to hormone signalling networks (Figure 4.1). Other metabolites likely share similar functions as signalling molecules that inform plant development. Blocking folate biosynthesis with SMX has uncovered such a role for folates. By restricting the effects of sugars on morphology, SMX treatment revealed
that these processes are not only dependent on folates, but that folates provide signalling information related to the state of metabolism. It is also possible that folates are synthesized in response to sucrose, and constitute part of the glycomorphogenic response. These metabolic cues manage the balance between auxin and cytokinin that drives development. In this manner, folates act not only as rate-limiting metabolites, but as “checkpoint” molecules during plant development, ensuring that sufficient metabolic support is available for the proliferation of new tissues.

Two lines of investigation were used to explore the role of folates in shaping developmental responses to sucrose. The inhibitory effect of sucrose on hypocotyl elongation was enhanced by SMX, suggesting that folates temper this effect of sucrose (Figure 4.1B). A different form of interaction was observed at the root apex, in which folates acted in concert with sucrose to promote root growth. Folate deprivation resulted in an imbalance in hormone activity that ultimately resulted in meristem differentiation (Figure 4.1C). Together, these studies highlight the role of sucrose and folates in promoting seedling development through hormone signalling.

The first line of experimentation explored the interaction between folates and sucrose during hypocotyl elongation. Synergy between SMX and sucrose indicated pathway interaction, and was used to interrogate the interplay between signalling pathways (Lehar et al., 2007; Yeh and Kishony, 2007). Transcriptome analysis uncovered that the interaction between sucrose and SMX resulted in changes to auxin signalling, as a number of Aux/IAA transcripts exhibited changes in transcript abundance in response to cotreatment of sucrose and SMX. The interaction between sucrose and SMX acted through an Aux/IAA19 – ARF7 signalling pathway to regulate hypocotyl growth, as evidenced by attenuated responses to SMX in the mutants. Changes in the expression profiles of auxin-responsive reporters indicated that auxin distribution was affected by sucrose and SMX. This hypothesis was supported when blocking polar auxin transport attenuated the interaction between sucrose and SMX. Taken together, is concluded that:
Figure 4.1. Metabolite sensing modulates hormone interactions during seedling development. (A) Metabolite sensing integrates signals from sucrose and folates to mediate hormone and developmental cues. These signalling events result in “glycomorphogenesis”, a term used here to describe the effects of sugars on plant morphology. (B) The interplay between folates and sucrose mediates hypocotyl development. SMX enhanced the effect of sucrose on hypocotyl elongation, suggesting that folates temper the inhibitory effect of sucrose. (C) Sucrose promotes hormone signalling at the root apex, with folates acting as “checkpoint” molecules. In the absence of folates, auxin flow is inhibited resulting in meristem differentiation.
The interplay between sucrose and folates fine-tune auxin signalling during hypocotyl development, a process that is mediated through changes in auxin distribution.

The aim of this second line of experimentation was to explore the effect of sucrose on the meristem, and how this regulation may be integrated with other metabolic signals to dictate plant growth. In the absence of a carbon source, dark-grown seedlings exhibit minimal root elongation, and very little meristematic activity is observed. Treatment with sucrose promoted root growth and meristem activity, as indicated by cell-cycle reporters and increases in meristem size. These changes in activity are likely the result of hormone signalling, as treatment with sucrose promoted auxin and cytokinin signalling at the root tip. These hormones regulate cell division and differentiation at the root apex (Perilli et al., 2012). Following on from the first line of investigation, work outlined in the latter half of this thesis explored the extent to which folates are integrated into sucrose signalling at the root tip. SMX disrupted hormone signalling and inhibited PIN expression, restricting auxin accumulation at the root apex. Growth in the presence of sucrose and SMX also led to ectopic cytokinin signalling throughout the lateral root cap. Together, these changes in hormone signalling likely contributed to a disruption in meristem integrity and complete differentiation of the meristem. These findings suggest that:

Sucrose promotes root growth by inducing hormone signalling networks that govern meristem activity. This is dependent on folates, as SMX disrupted hormone signalling and caused meristem collapse.

Together, these two lines of investigation uncover a connection between folates and sucrose that modulates hormone signalling during seedling development. Metabolism is fundamental to the growth of the living plant, yet there are few instances where metabolic cues feed into signalling pathways that regulate growth rates. This thesis sought to understand how metabolism can shape the growth behaviour of the developing plant by influencing hormone signalling pathways.
The approach used in this study highlights the utility of the chemical genetic approach in uncovering biological function. Treatment with SMX inhibited DHPS activity to highlight the role of folates during seedling development. The presence of multiple DHPS isoforms likely masked this phenotype in other forward-genetic screens aimed at dissecting the effects of sugars on plant development (Mehrshahi et al., 2010). In this way, chemical inhibition was able to overcome genetic redundancy and highlight a role for folates in balancing hormone signalling during development.

Finding this interaction in disparate organs suggests that the interplay between folates and sucrose is a general theme across plant development. These metabolic pathways converge to shape plant growth through auxin distribution. In both studies, tissue-specific changes in hormone signalling were observed. In the hypocotyl, auxin distribution shifted to become more concentrated in the vascular region, where auxin signalling restricted hypocotyl elongation. In the root, auxin became less focused at the tip and it appeared that diffuse auxin signalling occurred in the vasculature. Changes in auxin distribution have a profound impact on plant development (Blilou et al., 2005; Grieneisen et al., 2007; Vanneste and Friml, 2009), and may enable metabolism to exert an influence on plant growth.

4.2 FUTURE DIRECTIONS

This thesis made inroads into an understanding of how metabolic processes inform plant development. Despite these initial steps toward characterising this interaction, an understanding of how these pathways regulate seedling growth is far from complete. There are a number of research endeavours that could illuminate the mechanism through which these pathways interact. These efforts may provide insight into the manner in which metabolic pathways influence hormone signalling and plant growth.
4.2.1 Exploring the role of other hormone signalling pathways in mediating responses to sucrose and SMX at the meristem

Current models of root meristem development emphasise the interaction between auxin and cytokinin (Dello Ioio et al., 2007; Dello Ioio et al., 2008; Moubayidin et al., 2009; Moubayidin et al., 2010; Perilli and Sabatini, 2010; Dello Ioio et al., 2012; Perilli et al., 2012). This, along with earlier findings that SMX perturbed auxin signalling in the hypocotyl, encouraged a focus on these two hormones in the study of SMX on root meristem activity. This view of hormonal interactions at the meristem is incomplete, as gibberellins (GA) and brassinosteroid (BR) have also been implicated in regulating the root meristem (Achard et al., 2009; Ubeda-Tomas et al., 2009; Gonzalez-Garcia et al., 2011; Hacham et al., 2011). Exploring the role of these other hormones in mediating responses to sugars and SMX may provide a more fulsome understanding of the intricate signalling network that integrates metabolism into developmental cues.

Tools are available that can be used to dissect the functional role of GA and BR at the meristem. These can be used to assess the extent of their involvement in response to sucrose and SMX. For example, GA signalling in the endodermis regulates cell expansion that is believed to influence division rates in neighbouring tissues (Ubeda-Tomas et al., 2008; Ubeda-Tomas et al., 2009). The *gibberellic acid insensitive* (*gai*) mutant shuts down GA signalling (Ubeda-Tomas et al., 2008), and exhibits decreased root meristem size (Ubeda-Tomas et al., 2008; Ubeda-Tomas et al., 2009). It would be interesting to explore the role of GA in the response to sucrose, and whether the mutant is hypersensitive to SMX. Whether *de novo* biosynthesis of GA is necessary for sucrose to promote growth can be tested by treating seedlings to sucrose in the presence of paclobutrazol (PAC), a chemical inhibitor of GA biosynthesis (Olszewski et al., 2002). Through chemical and genetic inhibition of GA, the role of this hormone in developmental responses to sugars can be explored.

Similar tools are available to dissect BR signalling during plant development.
Mutants that are deficient in BA perception and signal transduction have been isolated, which can be used to study BR signal transduction during root development (Belkhadir and Chory, 2006). For example, the *brassinosteroid insensitive1 (bri1)* mutant is deficient in BR perception and exhibits smaller root meristems due to reduced cell division (Clouse et al., 1996; Li and Chory, 1997; Gonzalez-Garcia et al., 2011; Hacham et al., 2011). Auxotrophic BR mutants can also be used to explore the necessity of BR biosynthesis in responses to sucrose and SMX (Chory et al., 1991; Noguchi et al., 1999). Seedlings can be hypersensitised to BR through chemical treatments that inhibit negative regulators signal transduction (De Rybel et al., 2009). Exposing plants to bikinin inhibits the GLYCOGEN SYNTHASE KINASE (GSK) family of negative regulators, and caused heightened responses to BR (De Rybel et al., 2009). A combination of chemical and genetic approaches can assess the role of BR in mediating responses to sucrose and SMX during meristem development.

4.2.2 Exploring tissue-specific hormone signalling in response to sucrose and SMX

Recent studies highlight the role of tissue-specific hormone signalling in governing root meristem activity (Dello Ioio et al., 2007; Ubeda-Tomas et al., 2008; Ubeda-Tomas et al., 2009; Hacham et al., 2011). By expressing a dominant mutant allele under the control of a tissue-specific promoter, signalling pathways can be inhibited exclusively in that tissue (Ubeda-Tomas et al., 2009; Hacham et al., 2011). Similarly, by expressing hormone metabolism enzymes, hormone abundance can be changed in specific tissue types (Dello Ioio et al., 2007). For example, by expressing the cytokinin oxidase-dehydrogenase1 (CKX1) gene under a transition zone-specific promoter, the activity of cytokinin in promoting differentiation was uncovered (Werner et al., 2003; Dello Ioio et al., 2007). This approach to tissue-specific hormone activity is facilitated by the identification of promoter elements that confer tissue-specific gene expression.

Treatment with sucrose and SMX induced tissue-specific hormone responses. The
TCS::GFP reporter exhibited elevated levels of expression in the lateral root cap (LRC) and epidermis in response to SMX. Whether this signalling in the LRC plays a role in mediating responses to SMX is a hypothesis that would be interesting to address. To this end, it should be possible to reduce cytokinin abundance in the LRC and epidermis by expressing CKX1 under the control of the GL2 promoter (Lin and Schiefelbein, 2001). The GL2 promoter expresses exclusively in the epidermis and LRC, and has previously been used to activate signalling pathways specifically in those tissues (Lin and Schiefelbein, 2001; Hacham et al., 2011). As a control, this construct could be transformed into the TCS::GFP background to confirm the reduction in cytokinin activity at the LRC. In this way, the dependence of cytokinin signalling in the LRC in response to SMX can be investigated.

Stabilising the IAA3/SHY2 pathway with the dominant shy2-2 allele conferred resistance to the differentiating effect of sucrose and SMX. The specificity of this response could also be explored by using tissue-specific promoters driving expression of the shy2-2 allele. This would allow hypotheses related to specific auxin signalling pathways to be addressed. Changes in DR5::GUS expression indicate that auxin signalling was occurring in throughout the meristem in response to SMX. By shutting down IAA3/SHY2-dependent signalling in single tissue types, the role of this ectopic auxin signalling can be explored further.

4.2.3 Assessing the oxidative state of the meristem

Reactive oxygen species (ROS) are produced as a by-product of metabolism, and can have profound implications for cellular behaviour (Mittler et al., 2004). Redox signalling has been implicated as a regulator of cell cycle progression in both plants and animals (Mittler et al., 2004). ROS signalling plays an important role in root growth and development, influencing the rates of cellular proliferation and differentiation at the meristem (Tsukagoshi et al., 2011; Tsukagoshi, 2012). It could be that ROS proliferation occurs as a result of sucrose metabolism, which helps drive root growth. This may explain why little meristematic activity occurs in the absence of sucrose. To test this hypothesis, the proliferation of ROS can be
observed in roots using staining techniques that discriminate between different types of redox signals (Dunand et al., 2007; Tsukagoshi et al., 2011). Mutants that are deficient in cellular redox regulators or in metabolising ROS could also be tested to see whether responses to sucrose are impaired by an inability to manage ROS accumulation. If folates are required as antioxidants, or to buffer ROS signalling, then that might explain the sucrose-dependent effect of SMX on the meristem. An excess of ROS proliferation results in senescence and cell death (Mittler et al., 2004), which is not observed in response to SMX, therefore the likelihood of ROS signalling mediating responses to SMX is questionable.

4.2.4 Genetic screening to identify novel components of metabolite signalling

Though auxin signalling is perturbed by SMX, the precise cause of this effect is unknown. Folates are metabolised into secondary products, and one-carbon metabolism influences a wide range of secondary processes (Ravanel et al., 2011). It is unclear as to which downstream process is affecting sucrose and hormone signalling. Screening for mutants with altered sensitivity to SMX may uncover pathways that modify auxin signalling in response to metabolic cues. A study such as this may shed light on the branch of metabolism that is necessary, or on signalling proteins that mediate the cross talk. To this end, an M₂ population of ethyl methanesulfonate (ems)-mutagenised seedlings was grown in the presence of sucrose and SMX at concentrations that inhibit hypocotyl elongation (10mM and 1µM, respectively). Mutant seedlings that exhibited hypocotyls longer than wild type were identified. Twenty-two sucrose-and-sulfonamide resistant (ssr) mutant lines were isolated (Figure 4.2A).

This screen can also be used to identify signalling components involved in maintaining meristem integrity. These mutants were rescreened at concentrations of sucrose and SMX that induce meristem collapse (30mM and 1µM, respectively). This screen identified nine lines that exhibit higher proportions of intact meristems compared to WT (Figure 4.2B). Future work will characterise these mutants in hopes
Figure 4.2. Forward-genetic screen identifies SMX-resistant mutants. (A) Hypocotyl measurements of twenty-two sucrose and sulfonamide resistant (ssr) mutants grown in the dark for seven days in the presence of 10mM sucrose and 1µM SMX. (B) Brightfield images of ssr meristems after seven days of etiolated growth in the presence of 30mM sucrose and 1µM SMX.
of identifying novel genetic elements that govern meristem activity in response to metabolic cues.

Alternatively, chemical complementation could be used to assess which aspect of folate metabolism is causal for sucrose interactions. Downstream products of the folate metabolic pathway could be supplemented into the growth medium, similar to experiments presented in Chapter 2. These experiments would address hypotheses related to the role of specific metabolites in the interaction with sucrose. Negative results would need to be viewed with caution, and controls are necessary to show that the added compounds are reaching sufficient levels in the seedlings. For example, ethylene biosynthesis is a downstream product in one branch of folate metabolism (Figure 2.2A). Blocking ethylene perception with 20µM silver thiosulfate (Ag⁺) did not rescue hypocotyl elongation, suggesting ethylene production was not causing the inhibition of hypocotyl elongation (Figure 4.3). Rescuing hypocotyl elongation of the *ethylene overexproducer2* (*eto2*) mutant verified that the chemical treatment was effective at blocking ethylene perception (Kieber et al., 1993). Chemical complementation experiments, in conjunction with proper controls, will test hypotheses related to the influence of specific metabolite pools during hypocotyl elongation.

4.2.5 Uncovering regulators of shoot meristem responses to sucrose

Leaf development at the shoot apical meristem (SAM) is mediated by auxin and cytokinin (Shani et al., 2006; Barton, 2010). The hormones have reversed roles at the SAM compared to the root; auxin promotes differentiation, and cytokinin promotes division (Shani et al., 2006; Barton, 2010). As treating aerial tissues of the etiolated seedling with sucrose is sufficient to induce the development of true leaves (Roldan et al., 1999), there are likely hormonal responses mediating sugar signalling at the SAM. The extent to which sugars influence hormone signalling during leaf growth is not known.
Figure 4.3. Ethylene perception does not mediate interaction between sucrose and SMX. Seven-day-old dark-grown seedlings raised in the presence of sucrose and SMX, as indicated. Media supplemented with Ag⁺ to inhibit ethylene perception. The ethylene overproducer2 (eto2) mutant was used as a control (inset). Data represent the mean ± SE of three biological replicates from a representative experiment; each data point represents at least 36 individuals.
To test whether auxin and cytokinin mediate responses to sucrose at the meristem, some preliminary experiments made use of mutant and pharmacogenic analyses. The cytokinin hypersensitive arr3;4;5;6 quadruple mutant is deficient in four Type A ARRs, negative regulators of cytokinin signalling (To et al., 2004). Dark-grown arr3;4;5;6 do not induce SAM activation when treated with sucrose, suggesting that cytokinin inhibits sucrose signalling at the SAM (Figure 4.4B). To test whether auxin is necessary for sucrose effects at the SAM, seedlings were grown in sucrose and NPA (Morgan, 1964). Seedlings treated with NPA were impaired in the ability to produce true leaves in response to sucrose (Figure 4.4C). Taken together, these preliminary experiments hint that interplay between cytokinin and auxin mediates the induction of leaf development by sucrose.

It would be interesting to explore the interaction of folates and sucrose at the SAM by performing a set of experiments similar to those used to explore root development. Hormone markers and mutant lines are available to test hypotheses related to the interplay between folates and sucrose at the SAM. Given that the cell proliferation was inhibited at the root apex by SMX, it is likely that similar effects will be observed at the SAM.

The principles that were used to identify the interaction between sucrose and folates could be employed to uncover novel regulators of sugar signalling at the SAM. The activation of the SAM causes seedlings to grow too large for high-throughput chemical screening in 96-well plates. Since the arr3;4;5;6 mutant does not induce true leaf development, the mutant can be used to screen for chemicals that suppress this phenotype. Screening for chemicals that induce true leaf development in the arr3;4;5;6 mutant in response to sucrose may identify compounds that antagonize cytokinin signalling during sucrose treatment. A similar screen could be performed in search of chemicals that negate the inhibitory effect of NPA. In this way, modulators of the crosstalk between sugars and hormones can be identified.
Figure 4.4. Auxin and cytokinin mediate shoot meristem activation by sucrose.
(A) As a reference, two-week-old dark-grown seedlings grown in liquid media supplemented with 30mM sucrose, as indicated. (B) Wild-type and the cytokinin-hypersensitive mutant arr3;4;5;6 grown for two weeks in media supplemented with 30mM sucrose. (C) Wild-type seedlings grown in presence of 30mM sucrose supplemented with NPA, as indicated.
4.3 Closing Remarks

The work presented in this thesis uncovered a previously-unknown relationship between sucrose and folates that direct plant development. Metabolites such as sucrose and folates are sensed, and these signalling cues are integrated to modulate hormone signalling during seedling development. By restricting tetrahydrofolate biosynthesis with SMX, a role for folates as a metabolic checkpoint was discovered. The importance of metabolism in supporting plant growth is well established and accepted as a fundamental aspect of plant biology. It is less clear, however, how metabolites are sensed and the mechanisms through which metabolite signalling influences development. The nature of such a metabolite-sensing pathway remains to be elucidated, and future efforts will explore signalling pathways that regulate development in response to metabolic cues.
Appendix

A novel F-box that mediates galactose sensitivity
5 Appendix: A novel F-box that mediates galactose sensitivity

5.1 INTRODUCTION

Sugars play key roles as structural molecules, sources of energy, and signaling molecules in plants (Rolland et al., 2006). As products of photosynthesis, sugar levels rise and fall throughout the day in response to light (Blasing et al., 2005). The daily flux of carbohydrate pools has profound impacts on plant biology (Blasing et al., 2005; Osuna et al., 2007). It is important for plants to detect the availability of carbohydrates before activating carbon-rich developmental processes, such as cell-wall biosynthesis (Koch, 1996). To accomplish this, plants have evolved multiple sugar-perception pathways that enable them to coordinate growth and development in response to resource availability (Smith and Stitt, 2007).

Sugar-sensing pathways can be integrated with developmental cues through bi-functional proteins that possess catalytic and signalling functions (Moore et al., 2003; Cho et al., 2006; Cho et al., 2012). For example, the metabolic function of HEXOKINASE1 (HXK1) is to phosphorylate glucose during the initial steps of glycolysis. Upon binding glucose HXK1 undergoes a conformational change that causes the protein to translocate to the nucleus to direct gene expression (Cho et al., 2006). By coupling the catalytic function of HXK1 with a signalling role, HXK1 acts as a glucose sensor to coordinate transcriptional responses to glucose (Moore et al., 2003; Cho et al., 2006).

Other sugars are sensed through coupled metabolic and signalling mechanisms. For example, FRUCTOSE-1,6-BISPHOSPHATASE (FBP) is involved in fructose metabolism, and mediates developmental responses to the sugar (Cho and Yoo, 2011). By rescuing \textit{fbp} mutants with catalytically-inactive FBP constructs, the metabolic and signalling functions of FBP were uncoupled (Cho and Yoo, 2011). The parallels between glucose and fructose perception suggests that coupling of catalytic
and signalling functions may be a conserved mechanism through which development can be coordinated with resource availability.

Upon sensing sugars, pathways that utilise these resources are induced at the transcriptional level (Koch, 1996). For example, lignin biosynthesis is a carbon-intensive process regulated by sugar signalling (Amthor, 2003; Rogers et al., 2005). Lignin is a rigid structural polymer found in the secondary cell walls of specialised cell types (Sederoff et al., 1999; Rogers and Campbell, 2004). The deposition of lignin provides strength to the plant to support vegetative structures (Boerjan et al., 2003), guards against pathogen attack (Uppalapati et al., 2009; Bi et al., 2011), and endows hydrophobic properties to the cell wall (Hose et al., 2001). Lignin deposition is under strict developmental and environmental control, as lignification restricts further cell expansion (Rogers et al., 2005). Genes encoding lignin biosynthetic enzymes are induced by sugars, enabling the activation of this carbon-intensive process only when the resources are available to support it (Rogers et al., 2005).

A wide variety of developmental responses are induced by sugars (Rolland et al., 2006), highlighting the important role that carbohydrates play in shaping plant development. Some sugars, such as galactose, are associated with detrimental effects on seedling growth (Rosti et al., 2007; Grossmann et al., 2011). The “toxic” effect of has been known for a long time (Knudson, 1917; Burstrom, 1948; Hughes and Street, 1974); however, the molecular underpinnings and the biological significance of these responses remain unknown. Here, the inhibitory effect of galactose was used as the basis for a genetic screen to uncover the mechanism through which plants detect galactose, and to explore the implication of this sugar signalling pathway during seedling development.

5.2 RESULTS AND DISCUSSION

5.2.1 Galactose induces pleiotropic effects on seedling development

Dark-grown seedlings were treated with exogenous galactose to explore the effect of
the sugar on seedling development. Consistent with earlier reports, galactose inhibited root and hypocotyl elongation, and induced a stunted morphology (Figure 5.1, A and B). Vegetative tissues were dwarfed, and leaf expansion was restricted in seedlings grown in the presence of galactose.

Some sugars induce lignification in dark grown seedlings (Rogers et al., 2005). Since lignin deposition restricts cell expansion, it was hypothesised that changes in cell wall composition may occur in response to galactose. To test this hypothesis, seedlings were stained with phloroglucinol to assess changes in lignin distribution. In the absence of galactose, mild phloroglucinol staining was observed along the vascular cells of the hypocotyl (Figure 5.1E), indicating the presence of lignin. Treatment with galactose induced ectopic lignification throughout the vascular cylinder, as indicated by intense red staining (Figure 5.1H).

It was hypothesised that the changes in lignin distribution may be associated with abnormalities in the cellular organisation of the hypocotyl. To explore the effect of galactose on vascular development, cellular organisation was examined using semi-thin cross-sections of hypocotyl tissues stained with toluidine blue. Treatment with galactose disrupted vascular cellular organisation (Figure 5.1C-F). The vasculature of galactose-treated seedlings had enlarged, densely stained cells. Dark clusters were also observed in cortex cells of seedlings treated with galactose, which may have been starch granules (Figure 5.1C,F).

5.2.2 Forward genetic screen identifies a galactose-insensitive mutant

To uncover the genetic basis for galactose sensitivity, a population of randomly-inserted T-DNA mutants was screened for resistance to galactose. Mutants were screened on the basis of root morphology, and several mutants were identified that exhibit resistance to galactose during root growth. A second, high-throughput mutant screen took advantage of the autofluorescent properties of lignin. Seedlings were examined under fluorescent light to identify mutants that had a lignification pattern that was equivalent to untreated seedlings, even in the presence of galactose.
Figure 5.1. Galactose inhibits root elongation and vascular development. (A-B) Light-grown seedlings exhibit reduced elongation when growing on media supplemented with galactose. (C, F) Galactose induces the formation of dark clusters in the cortical layer of the hypocotyl. (D, G) When viewed at higher magnification, defects in vascular development can be observed. (E-H) Lignification observed throughout the hypocotyl vasculature cylinder in response to galactose, whereas normally lignin restricted to the xylem.
This screen identified a galactose-insensitive mutant (gim1) with reduced lignification in the presence of galactose. The mutant was identified based on reduced autofluorescence in the hypocotyl compared to wild type (Figure 5.2). The reduction in lignin fluorescence was confirmed with phloroglucinol staining, which showed lignin deposition in the hypocotyl akin to untreated controls, even in the presence of galactose (Figure 5.3A). Root elongation was also rescued in this mutant, which was resistant to galactose-mediated inhibition of root growth (Figure 5.3C). When the root tissues were stained with phloroglucinol, gim1 exhibited a drastic reduction in lignin content compared to WT (Figure 5.3B).

The galactose-induced changes to lignin deposition were confirmed by quantifying lignin abundance. Consistent with the phloroglucinol staining, galactose caused an increase in lignin abundance in wild-type seedlings that was not observed in the gim1 mutant (Figure 5.3D). The effect of galactose on lignin production is not related to the osmotic potential of the medium, as adding equivalent amounts of the non-metabolisable sugar mannitol induced no change in lignin abundance or distribution.

The inhibition of root elongation by galactose has been known for decades (Endo et al., 1968; Hughes and Street, 1974), but the underlying cause of this effect is unclear. Given that lignin restricts cell expansion, it may be that premature lignification of cells restricts their ability to elongate. In support of this hypothesis, the gim1 mutant had a reduction in lignification in the root tissues, and exhibited greater elongation than WT.

5.2.3 gim1 disrupts the activity of an F-box with galactose-oxidase activity

The gim1 mutant was isolated from a population of randomly-inserted T-DNA mutants, each of which could contain multiple DNA insertion events (Alonso et al., 2003). The gim1 genome was sequenced to locate any T-DNA insertions that may
Figure 5.2. Genetic screen identifies a *galactose insensitive mutant1* (*gim1*). Using fluorescence microscopy, lignin deposition can be viewed in vivo. Lignin observed along the hypocotyl vasculature in seedlings treated with galactose. Screening for mutants with reduced lignin deposition identified a *galactose-insensitive mutant1* (*gim1*).
Figure 5.3. Galactose resistance of the *gim1* mutant. (A-B) The *gim1* mutant was isolated based on reduced lignification of the hypocotyl vasculature, yet exhibits resistance in root tissues as well. (C) Roots of *gim1* are resistant to galactose, exhibiting greater elongation compared to WT. (D) Treatment with galactose induces lignin biosynthesis, which did not occur in the *gim1* mutant.
Figure 5.4: gim1 deficient in At4g39560, encoding a putative galactose oxidase/F-box. (A) Sequenceing gim1 genome identified one T-DNA insertion between two genes encoding galactose oxidases with F-box motifs. (B) Only an insertional mutation in At4g39560 phenocopied gim1.
be responsible for the mutant phenotype. This approach successfully identified a single T-DNA sequence on chromosome 4, located between two loci annotated as having galactose oxidase activity, as well as containing canonical F-box motifs (Figure 5.4A). Genome analysis identified three other F-box-containing galactose oxidases neighbouring these two, totaling five paralogous genes in tandem. These F-box-containing galactose oxidases are members of a large gene family in Arabidopsis (Andrade et al., 2001), comprising 131 members that share at least 76% similarity at the genetic level.

As the gim1 mutant contains a T-DNA insertion that resides between two distinct loci, it may be that the phenotype was related to altered expression of either, or both, of the two neighbouring genes. To test whether a loss of either neighbouring gene was sufficient to phenocopy gim1, T-DNA insertion lines corresponding to each of the two genes were tested for resistance to galactose in a root elongation assay (Figure 5.4B). The insertion line disrupted at At4g39550 responded to galactose like WT, suggesting that this locus was not responsible for the gim1 phenotype. Contrary to this, an insertion in At4g39560 conferred resistance to galactose, similar to gim1. It is likely that the gim1 phenotype is caused by a disruption to At4g39560 activity, despite the insertion not residing in the coding region of the gene.

5.3 FUTURE DIRECTIONS

5.3.1 Confirming the Galactose oxidase and F-box activity of At4g39560

The At4g39560 locus has been annotated as a kelch-repeat galactose oxidase/F-box (Andrade et al., 2001), but this functional activity remains to be confirmed. It is important to test whether this gene product is involved in galactose metabolism, which can be confirmed in vitro. Assays are commercially available that couple galactose oxidation with the creation of a colorful product, which can be used to verify galactose-oxidase activity.

Galactose-oxidase/F-boxes comprise a large gene family in Arabidopsis (Andrade et
al., 2001). Early studies into the function of this family used truncated versions of the protein in which the N-terminal F-box domain had been removed. These truncated versions of the protein lost the ability to interact with ASK1 (Andrade et al., 2001), and orthologous SKP1 protein that forms part of the SCF ubiquitin ligase complex (Gray et al., 1999). This supports the functional significance of the motif, suggesting that the F-box is still functional. Using a truncated version of At4g39560, hypotheses related to the importance of the F-box motif in galactose signalling can be tested.

5.3.2 Uncoupling catalytic activity from signalling function

Provided that At4g39560 retains both metabolic function and signalling capacity, it would be interesting to explore whether the galactose sensitivity is mediated through metabolism or signalling. To test this, catalytically-dead alleles with reduced galactose-oxidase activity would be generated and transformed into gim1. If the catalytically-dead allele restores wild-type phenotype, than the signalling function of At4g39560 is necessary for galactose sensitivity. Additionally, truncated versions of At4g39650 that lack the functional F-box can be transformed into gim1to assess whether the signalling role of the protein is necessary for galactose sensitivity. In both cases, the mutant allele needs to be checked to confirm that the secondary function has not been affected. For example, the truncated protein that lacks an F-box will still be tested to ensure that the galactose oxidase activity is still present prior to transformation.

5.4 MATERIALS AND METHODS

5.4.1 Plant materials and growth conditions

Wild-type Arabidopsis thaliana, ecotype Columbia-0 (Col-0) and T-DNA insertion lines were obtained from the Arabidopsis biological resource centre (ABRC). Plants were grown in Conviron growth cabinets (Conviron, Winnipeg, Canada) at 21°C in a 16/8hr photoperiod at 135μmol m⁻² s⁻¹. For growth assays and morphological studies, seedlings were grown in full strength liquid MS growth media containing
30mM sucrose (Murashige and Skoog, 1962). Galactose or mannitol was added to the media prior to filter sterilization, after which Gamborg's vitamins were added to the media. After the indicated period of growth, seedlings were fixed by submersion in FAA solution (50% ethanol, 5% formaldehyde, 10% acetic acid) for overnight. After rinsing with autoclaved distilled H$_2$O to remove fixative, seedlings were aligned on agar plates and photographed using a Canon EOS Rebel XT EF-S 18-55 digital camera, as previously described (Stokes et al., Submitted). Root and hypocotyl measurements were generated using ImageJ software (rsbweb.nih.gov/ij/).

5.4.2 Microscopy and staining techniques

Prior to staining, full tissues were cleared using a chloral hydrate solution (125g chloral hydrate in 50mL 30% glycerol solution) as described previously (Willemsen et al., 1998). Phloroglucinol stain was prepared by dissolving 20mg/mL phloroglucinol in 16% ethanol, 10% hydrochloric acid solution. To facilitate phloroglucinol staining, tissues were submerged in staining solution for 10 minutes in darkness, after which they were observed under an Olympus BX5 microscope (Olympus). Images were captured with a QImaging MicroPublisher 3.3RTV digital camera using QCapture version 2.7 software.

Semi-thin sections were created by fixing dissected hypocotyls in 4% paraformaldehyde in 0.1M Sorensen’s phosphate buffer, and rotating overnight. Tissues were washed twice with Sorensen’s phosphate buffer to remove paraformaldehyde, then dehydrated through a graded ethanol series. After three ten minute incubations with 100% EtOH to ensure complete dehydration, the hypocotyl tissues were infiltrated with Spurr’s epoxy resin. Infiltration was accomplished by incubating in 3:1 ethanol:Spurr’s for 30 minutes, followed by 1:1 ethanol:Spurr’s for 30 minutes, 1:3 ethanol:Spurr’s for 30 minutes, then incubation in 100% Spurr’s resin overnight on a rotator. The following day, tissues were infiltrated with fresh resin three times for sixty minutes each. Hypocotyls were embedded in a flat mold and polymerized in an oven. Semi-thin sections were cut 0.5µm thick on an
ultramicrotime, then transferred and heat fixed to a glass slide. Toluidine blue staining was performed by submersion for ten minutes.

5.4.3 Lignin quantification

The rapid extraction by washing and microscale lignin extraction method was adapted from Change et al, 2008. Briefly, air-dried Arabidopsis seedlings were ground using a mortar and pestle and sieved to 150-mesh. The ground samples were heated in MilliQ water at 65°C for 30 min with occasional mixing. The residues were filtered through a 0.45 μm nylon membrane and were washed with MilliQ water, acetone, ethanol, and diethyl ether. The extracted samples were dried in a vacuum oven at 40°C for 48 h followed by an overnight incubation over P2O5 in a vacuum dessicator. The extracted Arabidopsis samples (5 ± 0.1 mg) were incubated with 1 mL of 25% acetyl bromide in acetic acid in a tube with Teflon-sealed cap at 70°C for 30 min with shaking at 10 min intervals. The solution was then cooled in ice-cold water for 120 min. During the cooling, 5 mL of acetic acid was added to each tube. After cooling, 30 μL of the mixture, 40 μL of 1.5 M NaOH, 30 μL of 0.5 M hydroxylamine hydrochloride, and 150 μL of acetic acid were sequentially added to a 96-well quartz plate. The absorbance at 280 nm was measured and the extinction coefficient of 23.29 g⁻¹ L cm⁻¹ (Chang et al., 2008) was used to calculate the lignin content.
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Copyright Acknowledgements

Statement of Publications

The research presented in this thesis has appeared or has been submitted as a series of original publications in refereed journals.

Chapter 2