Regulation of seed size and interactions between auxin and cytokinin signalling pathways

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Abstract

Seeds make up over 50% of the world’s agricultural calories, thus discovering genes that regulate seed size is of paramount importance in a world with a rapidly growing population. Several genes that regulate seed size are involved in auxin and cytokinin signalling pathways. Classic experiments by Skoog and Miller demonstrated that auxin and cytokinin in particular ratios could determine cell fate, despite this, no in-depth transcriptome analysis of auxin+cytokinin treated tissues has been performed.

In this work, we identify a new regulator of seed size, ARGONAUTE10 (AGO10), and demonstrate that much of its regulation of seed development is dependent on the overexpression of INDEHISCENT (IND). IND has been linked with both the auxin and cytokinin signalling pathways, thus we performed a transcriptome analysis to investigate the regulation of genes by IND, auxin and cytokinin, and all combinations of those treatments. In this analysis we discover the existence of a set of genes regulated only in the presence of auxin+cytokinin, which we call the Dual Hormone Response (DHR). The DHR contains 518 genes, which is a gene set of comparable size to the gene set regulated by auxin or cytokinin treatment alone. We demonstrate preliminary motif and qRT-PCR data that suggests that the bHLH transcription factor, SPATULA, a binding partner of INDEHISCENT, is involved in regulating the DHR. Further analysis of the transcriptome data revealed that regulation of genes by auxin may be dominant over the regulation of genes by cytokinin, and that this response is dependent on TRANSPORT INHIBITOR RESPONSE 1 (TIR1). Lateral root development in seedlings was more sensitive to auxin than cytokinin, which was abolished in tir1 mutants. Auxin amplified the expression of cytokinin-regulated genes that auxin itself does not regulate in isolation, and vice-versa, and these responses were more sensitive to auxin concentration than cytokinin. Finally, we present preliminary evidence that suggests that cytokinin acts as a direct inhibitor of TIR1 action, diminishing subsequent auxin signalling. Treatment with cytokinin inhibited fluorescence in DII-VENUS root tips within 15 minutes, and also inhibited the auxin-promoted degradation of the Auxin Inducible Degron (AID) in mammalian cells.

This work opens up the field of hormones. Our data suggests that multi-hormone transcriptomes will reveal large sets of genes that are only regulated in the presence of multiple hormones. Such transcriptomes may also reveal the coveted existence of a common set of signalling components to regulate growth. The prioritisation of auxin signalling over cytokinin signalling in regulating genes and lateral root development, suggests the possibility of hormone hierarchies. Finally, the possibility that cytokinin directly inhibits the action of TIR1 represents an important discovery in the interaction between auxin and cytokinin.
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Chapter 1 – General Introduction

1.1 INDEHISCENT, ARGONAUTE10 and seed size

1.1.1 Agronomic benefits of increased seed size

Seeds make up over 50% of the world’s agricultural calories (Tilman et al., 2011). Seeds from maize, rice and wheat are staple foods that together feed all regions of the world, and are the top three annually produced crops in the world (Tilman et al. et al., 2011). As seeds play such an important role in feeding communities and nations, it is unsurprising that seed size is one of the primary traits selectively bred for over thousands of years by farmers (Kesavan et al., 2013). Wild relatives of modern crops reveal the large increases in seed size attained through millennia of selective breeding; particularly striking is the comparison between modern maize (Zea mays ssp. mays) and its wild relative teosinte (Zea mays ssp. parviglumis) (Figure 1.1). As well as calorie intake, seeds are important sources of proteins, oils, sugars and essential amino acids, which benefit the consumer (Garcia et al., 2003; Fatihi et al., 2013). Increasingly, seeds are used as a source of renewable oil; seeds from Rapeseed (Brassica napus), a member of the same Brassicaceae family as the model organism Arabidopsis thaliana, are an important source of rapeseed oil which is harvested for production of vegetable oil and for use as a biodiesel fuel (Fröschle et al., 2017). Seed size increases are not only beneficial for calorie intake; they have also been linked with improved viability and vitality of young seedlings (Milberg & Lamont, 1997). Modern breeding and genetic modification techniques now allow for the rapid discovery of seed size regulating genes, particularly within Arabidopsis thaliana, and for their utilization within crops. The hunt for genes that can increase seed size grows more important in our time, as a growing population requires an increased amount of food to be produced. The FAO estimates that by 2100, food production will need to increase by 70% to stave off mass hunger.

1.1.2 Seed anatomy

Seeds comprise of three major tissues, the seed coat, embryo and endosperm. All of these compartments constitute important sources of nutrition in different crops. Upon fertilisation of the embryo sac, proliferation of the seed coat cells, two layers of integument cells, allows more room for endosperm and embryo development, and resource storage (Li & Li, 2015). The endosperm provides the embryo with the relevant nutrition to enable its morphological development (Berger et al., 2003). Before 4 days after pollination (DAP), endosperm contents move freely inside the seed vacuole, at 4 DAP the endosperm cellularizes and differentiates.
into the chalazal, micropylar and peripheral endosperm, occupying a large portion of the seed cavity (Gehring et al., 2004). Aberrant endosperm development impairs the development of the seed coat, as seen in mini-seed and haiku mutants (Garcia et al., 2003). Endosperm also signals the initiation of seed coat development via auxin movement coordinated by AGAMOUS-LIKE 62 (AGL62) (Sun et al., 2010). The embryo undergoes several major stages of development: globular, heart, torpedo and mature (Figure 1.2) (Graham and Wareing). Although seed development is a continuous process seed development has been characterised into different stages. During the pre-globular and globular stages (1-4 DAP), suspensor and embryo proper differentiate, and there is early cell and tissue type differentiation. During the heart stage (5-6 DAP), cell and tissue differentiation continues, and shoot/root meristem specification occurs. During the torpedo stage (or linear and bent stages) (7-12 DAP), cotyledon and axis development occurs, and storage reserves begin to accumulate. Towards the end of torpedo stage (bent) the endosperm begins to be absorbed by the embryo that is now occupying the majority of the seed cavity. During the mature stage (13-19 DAP), the embryo grows to occupy almost all of the cavity, the endosperm continues to be absorbed, final storage reserves are deposited, and cells switch from division to expansion. Once the seed is fully mature (post 19 DAP), it enters a state of dormancy during which water is lost to form a dry seed ready for long term dormancy (Boscá et al., 2011).

The developing seed is attached to the mother plant via the funiculus and the suspensor, which is the sole route of parent-offspring communication and resource allocation to seeds. The funiculus is outside the developing seed and connects it to the silique wall (Larsson, 2017). The funiculus contains vascular tissues in its centre to transport metabolites; these vascular tissues, particularly the phloem, increases in size significantly during 1-4 DAP to facilitate metabolite, signal and storage reserve transport. The suspensor is inside the developing seed, and connects the embryo to the seed coat and therefore signals and nutrients from the funiculus. Throughout the development of the seed, increasing amounts of sugars, proteins and oils are stored to allow proper germination and early seedling viability, they can make up to 90% or more of dry seed weight (Baud et al., 2008). These storage reserves are required to provide nutrients and energy in germinating seeds that undergo energy-intensive processes whilst being unable to create their own energy with photosynthesis. Occasionally, defects in seed development cause spontaneous abortions, if this happens early in development, the seed shrivels and turns brown, if it occurs late in development, the seed turns a translucent white (Agorio et al., 2017; Andreuzza et al., 2010).
Figure 1.1. Modern maize (*Zea mays* ssp. *mays*) (bottom) and its wild relative teosinte (*Zea mays* ssp. *parviglumis*) (top). Credit: John Doebley

Figure 1.2. Developmental stages of *Arabidopsis thaliana* embryo. Credit: Graham, C.F., and Wareing, P.F.
1.1.3 Seed size regulators

Genes that regulate seed size have been the recipient of some study due to their agronomical importance. Table 1.1 contains a list of genes shown to regulate seed size that we investigate in this work. Many genetic loci regulate seed size by controlling the development of the seed coat, altering the cavity size for the endosperm and embryo to grow into. Primarily this is achieved by influencing, directly or indirectly, the division and/or expansion of the integument cells that make up the seed coat. AUXIN RESPONSE FACTOR 2 (ARF2) negatively regulates seed size by down-regulating genes that promote integument cell division such as CYCLIN D3;1 and AINTEGUMENTA; “CYTOCHROME P450, FAMILY 78, SUBFAMILY A, POLYPEPTIDE 5” (KLUH) promotes integument cell proliferation and regulates oil content of the seed; and TRANSPARENT TESTA GLABRA 2 (TTG2) promotes integument cell growth (Garcia et al., 2005; Adamski et al., 2009; Lim et al., 2010). The endosperm provides many developmental signals to the integument cells, directing growth, differentiation and division, thus genes that act primarily in the endosperm can have indirect effects on the growth of the integument and resulting cavity. Many genes are active both in integument cells and endosperm to control seed size, and whether integument growth regulated by these genes is direct or indirect can be difficult to delineate. APETALA 2 (AP2) and FERONIA (FER) negatively regulate the length of integument cells and endosperm development, limiting seed size; HAiku1 (IKU1), HAiku2 IKU and MINISEED 3 (MINI3) act in the same pathway to positively regulate endosperm development and cavity size (Luo et al., 2005; Ohto et al., 2009; Yu et al., 2014). Seed size regulators are often parent of origin specific (imprinted); the above genes represent maternally imprinted genes, however paternally regulated genes can also regulate seed size. FERTILIZATION INDEPENDENT SEED 1 (FIS1), FERTILIZATION INDEPENDENT SEED 2 (FIS2), MULTICOPY SUPPRESSOR OF IRA1 (MSI1), SWINGER (SWN), DNA METHYLTRANSFERASE 1 (MET1) and FERTILIZATION-INDEPENDENT ENDOSPERM (FIE) regulate seed development via paternally-imprinted control over endosperm development (Sun et al., 2010).

It is also interesting to note that none of the genes described above are cell cycle factors such as cyclins, cyclin-dependent kinases, or cyclin-dependent kinase inhibitors, though some may regulate such genes, which is surprising as the division of integument cells is a key determinant of seed size. Additionally, when discussing genes that regulate seed size, it is important to note that many genes might affect seed size indirectly, such as by altering photosynthesis, source-sink storage, and general regulators of cell size of division, all of which might affect seed size incidentally. Thus, relative localisation of the protein in question should be taken into
consideration when determining whether a gene directly or indirectly regulates seed size. Subsequently, when considering how to utilise these genes in the improvement of crops, indirect regulators of seed size may have a number of other phenotypes that are undesirable. One way to avoid this is with seed specific promoters, such as those isolated by Jeong et al., which can be used to drive gene expression in specific compartments of the seed (Jeong et al., 2014). Alternatively, determining the direct regulator of seed size acted upon by the above genes would provide a more specific and targetable set of genes for improving crops. Finally, the fertility of the plant, as well as nutritional content of the seed (oil, protein, and sugars) can all be manipulated as well, indeed several of the above described genes do modify these characteristics. These should also be taken into account when improving crop seeds.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutant</th>
<th>Seed size phenotype</th>
<th>Tissue of action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>APETALA2</td>
<td>ap2-7</td>
<td>Increase size</td>
<td>Integument and endosperm</td>
<td>(Ohto et al. et al., 2009)</td>
</tr>
<tr>
<td>ARF2</td>
<td>Salk_108995</td>
<td>Increase size</td>
<td>Integument</td>
<td>(Schruff et al. et al., 2005)</td>
</tr>
<tr>
<td>BIG BROTHER</td>
<td>da1-1</td>
<td>Increase size</td>
<td>Integument</td>
<td>(Xia et al. et al., 2013)</td>
</tr>
<tr>
<td>CKX2</td>
<td>oeCKX2/iku2-2</td>
<td>Decrease size</td>
<td>Endosperm</td>
<td>(Li et al. et al., 2013)</td>
</tr>
<tr>
<td>EOD3</td>
<td>eod3-1D</td>
<td>Decrease size</td>
<td>Integument</td>
<td>(Fang et al. et al., 2012)</td>
</tr>
<tr>
<td>FERONIA</td>
<td>fer-4</td>
<td>Increase size</td>
<td>Integument and endosperm</td>
<td>(Yu et al. et al., 2014)</td>
</tr>
<tr>
<td>KLUH</td>
<td>klu-2</td>
<td>Decrease size</td>
<td>Integument</td>
<td>(Adamski et al. et al., 2009)</td>
</tr>
<tr>
<td>MET1</td>
<td>met1-6</td>
<td>Increase size</td>
<td>Endosperm</td>
<td>(Xiao et al. et al., 2006)</td>
</tr>
<tr>
<td>MYB56</td>
<td>myb56-1</td>
<td>Decrease size</td>
<td>Integument</td>
<td>(Zhang et al. et al., 2013)</td>
</tr>
<tr>
<td>TTG2</td>
<td>ttg2-1</td>
<td>Decrease size</td>
<td>Integument</td>
<td>(Garcia et al. et al., 2005)</td>
</tr>
</tbody>
</table>

Table 1.1. Seed size regulators used in this study, covering known pathways for regulating seed size.

1.1.3.1 Seed size regulators from the auxin and cytokinin signalling pathways

Several seed size regulating genes have been found in the auxin and cytokinin signalling pathways, demonstrating the important roles of these hormones in the development and growth of seeds. Further details of the auxin and cytokinin signalling pathways can be found below; here we focus on seed size regulators. The gene (ARF2) encodes a transcription factor (TF) involved in the regulation of auxin-responsive genes. ARF2 suppresses the cell division of integument cells to restrict the growth cavity, arf2 mutant seeds are dramatically larger (Schruff et al., 2005). ARF2 is a direct target of the brassinosteroid (BR) signalling TF
**BRASSINAZOLE-RESISTANT 1** (BZR1), thus ARF2 represents a focal point for auxin and BR regulation of seed size (Vert et al., 2008). *arf2* mutants have reduced fertility, which has been shown to increase seed size, though the mechanism by which infertility results in larger seeds has not been explored (Hughes et al., 2008). *arf2* mutant seeds also exhibit improved drought tolerance (Hughes et al., 2008). Transgenic overexpression of **AUXIN RESPONSE FACTOR 19** (JcARF19) in the perennial *Jatropha curcas*, grown for biofuel from their seeds, resulted in a 30% increase in seed size (Sun et al., 2017). The **CYTOKININ OXIDASE 2** (*CKX2*) gene encodes a protein that irreversibly degrades active cytokinin. *CKX2* has been shown to be a direct transcriptional target of the *IKU* pathway, and to then regulate seed size by control of endosperm growth (Li et al., 2013). Overexpression of *CKX2* in the *iku2* background rescued the reduced seed size phenotype, indicating that *CKX2* is a positive regulator of seed size. *CKX2* is also regulated by epigenetic maternal imprinting via MET1 (Li et al., 2013). The **ARABISOPSIS HISTIDINE KINASE** (*AHK*) family of genes encodes membrane-bound cytokinin receptors. *ahk1 ahk2 ahk3* triple mutant seeds had a 250% greater volume vs. wild type, with embryo cell number and size increased by ~15 and 30%, respectively (Riefler et al., 2006). The physiological cause of the seed size increase was not investigated further.

### 1.1.4 Causes of infertility in *Arabidopsis thaliana*

In searching for genes that increase seed size or nutrition, it is important that plant fertility is not negatively altered by the manipulation of that gene, lest gains in size and nutrition be lost on the whole plant scale. *Arabidopsis thaliana* is self-fertilising. Pollen grains from the anthers are deposited onto the head of the pistil. The pollen grains germinate in response to signals from the stigma, and a pollen tube forms, tunnelling through the transmitting tract in the stigma, style and ovary towards the ovules at the pistil base. This process is mediated by complex interactions between the pollen tube cell and the female reproductive tissues, mediated by extracellular signalling molecules designed to discriminate among pollen grains. The male gamete cells from the pollen grain then travel through the pollen tube and fertilises the ovary. (Joseph et al., 1993). Common causes of infertility phenotypes include: automatic self-pollination defects that limit pollen grain deposition, defects in pollen tube formation, aberrant signalling between the pollen tube and the female reproductive tissues. Post-fertilisation infertility arises from defects on proper seed development, resulting in spontaneous abortion of the seed.
1.1.5 INDEHISCENT

The INDEHISCENT (IND) gene encodes a member of an atypical class of bHLH transcription factors (TFs) which contain an alanine residue at site 9 rather than the usual glutamic acid critically required for DNA binding (Groszmann et al., 2010). IND can still bind DNA, however (Liljegren et al., 2004). bHLH TFs bind DNA via a basic DNA-binding region, and also contain a helix-loop-helix domain which allows them to homo- or hetero-dimerize with other bHLH TFs; this dimerization is necessary for regulation of transcription (Liljegren et al., 2004). Dimerization allows bHLH TFs to have a range of binding partners and thus a diverse set of gene targets. IND is commonly known for its regulation of fruit dehiscence and seed dispersal (Liljegren et al., 2004). Fruit indehiscence is another useful agronomic trait which has been selectively bred into modern crops to prevent seed dispersal by ear-shattering, allowing for easier harvest of the crop (Sonnante et al., 2009). In Arabidopsis thaliana, IND promotes the proper formation of valve margin, which primes the matured siliques for shattering and seed dispersal (dehiscence), and is highly expressed at the valve margin (Liljegren et al., 2004). IND coordinates the generation of an auxin minimum at the valve margin to promote the formation of a lignified separation layer, which will be digested by cell-wall degrading enzymes upon maturity, allowing dehiscence (Sorefan et al., 2009). The separation layer of valve margins doesn’t develop in ind mutants, and their siliques fail to shatter upon reaching maturity.

IND has been shown to bind and function with another bHLH TF, SPATULA (SPT), to coordinate auxin distribution for fruit development (Girin et al., 2011). IND-SPT jointly regulate the expression of PINOID (PID) and WAG2, which act as binary switches to coordinate the polarity of PIN proteins, which control auxin efflux (discussed further below) (Girin et al., 2011). IND can homodimerise, but it’s interaction with SPT is hypothesised to be stronger due to a HEC domain that flanks the bHLH domain on IND, which stabilises the interaction (Girin et al., 2011). IND also positively and directly regulates the expression of SPT (Girin et al., 2011). SPT is discussed further below. IND is also positively regulated by AGAMOUS-LIKE 1 (AGL1) and AGAMOUS-LIKE 2 (AGL2), which are negatively regulated by AP2, a seed size regulator; in ap2 mutants, IND is overexpressed in the valve margin (Bomblies et al., 1999; Ripoll et al., 2011). Low levels of IND have been detected in pollen grains and mature seeds using a GUS insertion line (Girin et al., 2011). Loss of IND function partially rescued infertility phenotypes observed in a line overexpressing PsGA 2-OXIDASE2, a gibberellic acid (GA) degrading enzyme, which exhibited reduced pollen tube growth (Kay et al., 2013). IND did not, however, rescue GA-
independent pollen tube growth mutants. *ind spt* double mutants have severe transmitting tract defects (Groszmann *et al.*, 2010).

### 1.1.5.1 INDEHISCENT and auxin

IND has been demonstrated to interact with the auxin signalling pathway. The IND-SPT heterodimer controls fruit and gynoecium development by controlling the distribution of auxin. This is achieved by regulating members of auxin transport machinery, such as *PIN-FORMED 3 (PIN3)* and *PID* and *WAG2* proteins, to create auxin minima for valve margin specification, though this regulation is unlikely to be direct (Sorefan *et al.*, 2009). IND can also form homodimers with AUXIN RESPONSE FACTOR (ARF) TFs. The IND- AUXIN RESPONSE FACTOR 3 (*ARF3*) complex mediates gynoecium patterning via regulation of *PID*, and the interaction between them is reported to be disrupted by high levels of auxin. Interestingly, ARF3 lacks the C-terminal PB1 required for inhibition of Aux/IAAs (Marsh-Martínez & de Folter, 2016). Instead, ARF3 dimerises with IND, and auxin, specifically IAA, may directly disrupt this interaction, possibly constituting a novel auxin-sensing mechanism (Simonini *et al.*, 2016). *IND* has been shown to genetically interact with *AUXIN RESPONSE FACTOR 6 (ARF6)* and *AUXIN RESPONSE FACTOR 8 (ARF8)*, to regulate development of reproductive tissues and promote flower maturation. Aberrant patterning of *ARF6/8* can result in a reduced ability to attract pollen tubes and misshapen ovules (Kay *et al.*, 2013). *ind* was shown to rescue of *arf8* infertility, though *IND* expression is not altered in the *arf8* background.

### 1.1.6 SPATULA

The *SPT* gene encodes a bHLH TF related to the PHYTOCHROME INTERACTING FACTORS (PIFs), a group of light-sensitive TFs (Ichihashi *et al.*, 2010). Importantly, SPT lacks an APB-like domain, which is allows phytochrome interaction, distinguishing it from the PIFs (Ichihashi *et al.*, 2010). SPT has been demonstrated to regulate the development of the stigma, style, embryo, and root tips, seed dormancy, cotyledon expansion, circadian rhythm, and repression of growth in cold temperatures (Groszmann *et al.*, 2010; Heisler *et al.*, 2001; Makkena & Lamb, 2013b; Penfield *et al.*, 2010; Penfield *et al.*, 2005). SPT has a broad expression throughout plant development, and it has been suggested that SPT functions through interactions with more specifically expressed TFs, such as IND, to regulate gynoecium development (Groszmann *et al.*, 2010). Interestingly, SPT also has ecotype-specific phenotypes; SPT promotes seed dormancy in the Col-0 background, and represses seed dormancy in the Ler background, exhibiting lower germination rates in Ler background *spt* mutants (Vaistij *et al.*, 2013). Mutant *spt* seeds show
an increase in size in the Col-0 and the Ler background, as well as altered storage amount and ultrastructure (Liu et al., 2017).

1.1.6.1 SPATULA, auxin and cytokinin

Spt mutants exhibit gynoecia apical closure defects, which can be rescued by application of cytokinin, suggesting that SPT may promote cytokinin biosynthesis to coordinate this development (Reyes-Olalde et al., 2017). SPT also regulates the expression of the type-B ARRs RESPONSE REGULATOR 1 (ARR1) and RESPONSE REGULATOR 12 (ARR12), demonstrated by reduced expression of ARR1 and ARR12 in spt mutants (Reyes-Olalde et al., 2017). Regulation of ARR1 is predicted to be direct, whilst regulation of ARR12 is likely to be indirect. SPT is also proposed to be negatively regulated by ARF3 (Xigang et al., 2009). During gynoecium development, cytokinin regulates the TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAA1) and PIN3 to control auxin biosynthesis and efflux respectively (Reyes-Olalde et al., 2017). This process appears to be dependent on SPT. Loss of SPT function also results in a broad auxin maximum at the root tip due to altered expression of PIN-FORMED 4 (PIN4), demonstrating the role of SPT in regulating auxin transport (Makkena & Lamb, 2013a). Whether these roles require IND has not been explored.

1.1.7 ARGONAUTE10

The ARGONAUTE (AGO) family of proteins perform RNA-mediated post-transcriptional gene silencing. 21 nucleotide small non-coding RNAs (miRNA and ta-siRNA) complementary to specific genes are recruited by AGOs into the RNA-induced silencing complex (RISC), and target specific mRNA molecules for cleavage and resultant down-regulation (Höck & Meister, 2008). The AGO family contains a conserved C-terminal PA2-MID-PIWI domain. The PAZ and MID domains recognise the 3’ and 5’ ends of the miRNA and ta-siRNA, and the PIWI domain possesses an RNase H-like fold structure which performs endonuclease activity to cleave targeted mRNA (Höck & Meister, 2008).

Arabidopsis thaliana has 10 AGO proteins, AGO1-10 (Ji et al., 2011). ARGONAUTE 10 (AGO10) possesses slicer activity in vitro, but is thought to predominantly recruit miR165/166 and not use it in order to target mRNA, in order to prevent its use by ARGONAUTE 1 (AGO1), which AGO10 bears a strong relation to (Vaucheret et al., 2008). Pulldown and sequencing experiments have demonstrated that miR165/166 makes up 90% of the small non-coding
RNAs bound to AGO10, though it can recruit an array of other miRNAs and ta-siRNAs (Zhu et al., 2014), and is thus theorised to control the silencing of many genes.

*ago10* mutants have revealed the role of AGO10 in leaf polarity, shoot apical meristem (SAM) development, and floral stem cell termination (Liu et al., 2009; Roodbarkelari et al., 2015). Particularly interesting is the role of AGO10 in leaf polarity, where AGO10 appears to act as a decoy of AGO1; miRNA165/166 preferentially binds AGO10, preventing its use by AGO1 to cleave mRNA of *HDZIP III* genes, allowing correct patterning of leaf abaxial and adaxial identity (Liu et al., 2009). AGO10 also recruits miR168, which leads to downregulation of AGO1 (Minoia et al., 2014). AGO10 recruitment of miR172 represses the expression of AP2, a seed size regulator (Ji et al., 2011).

Interestingly, many *ago10* phenotypes are ecotype-specific; the premature termination SAM stem cells, aberrant silique development, increased organ size, stunted growth, and increased number of primary inflorescences observed in mutants in the Ler background, such as *zll-3*, have no or minimal occurrence in mutants in the Col-0 background, such as *ago10-3* (Tucker et al., 2013). Furthermore, the stem cell phenotypes of *ago10* mutants in the Ler background display variable penetrance, including wild-type SAM, a pin- or cup-shaped structure in place of the SAM, or no SAM; even the strongest mutant alleles exhibit incomplete penetrance of 90% (Tucker et al., 2013). Tucker et al. have suggested that differential expression of *SQUINT*, a cyclophilin-40 orthologue, between Ler and Col-0 may be responsible for the ecotype-specific phenotypes.

AGO10 expression has been detected in the globular stage of embryo development, which then dissipates at later developmental stages and becomes restricted to embryo vasculature (Roodbarkelari et al., 2015). *Ago1 ago10* double mutants in the Col-0 led to embryo arrest at the late globular stage, single mutants were unaffected, suggesting a redundancy in function between AGO1 and AGO10 (Mallory et al., 2009). Overexpression of *IND* in the Ler background has been demonstrated to phenocopy the *zll-3* mutant SAM phenotypes (Moubayidin & Østergaard, 2014). Unpublished data from the Sorefan lab has demonstrated that AGO10 and IND negatively regulate each other (Figure 1.3).
Figure 1.3. Relationship between AGO10, IND and SPT. AGO10 disrupts the post-transcriptional silencing of HDZIP IIIs by preferentially binding miR165/166 preventing its use by AGO1. Unpublished data from the Sorefan lab has demonstrated that AGO10 and IND negatively regulate each other, AGO10 possible performs this role via post-transcriptional silencing with miR165/166. The IND-SPT dimer regulates the expression of PIN3, PID and WAG2 to generate auxin maxima.

1.2 Auxin and cytokinin

1.2.1 History of auxin and cytokinin

Auxin and cytokinin are plant phytohormones that coordinate and regulate numerous growth, development and environmental response processes. The first effects of auxins were observed by Charles Darwin in 1881, when he and his Son, Francis Darwin, performed experiments that demonstrated phototropism in plants, and that this response was dependent on light reaching the apical tip (Holland et al., 2009). This discovery was built upon by Frits Went and Nicolai Cholodny in 1928, who both independently showed that phototropism was controlled by a mobile growth-promoting hormone, which Went named ‘auxin’ (Enders & Strader, 2016). The Cholodny-Went theory proposes that in response to tropic stimuli, accumulation of auxin into asymmetric gradients stimulates a growth response. This is the earliest demonstration of the importance of auxin gradients, which would later be discovered to be vital for a range of developmental processes. Later research implicated auxin in the control of organ patterning, plant organisation, root and fruit growth and development, flowering, wound responses and ethylene biosynthesis, among many roles.

Cytokinins were discovered by a series of coincidental discoveries by Skoog and Miller in the 1950s whilst trying to create a more effective growth medium for plants, including adding old yeast extract and old herring sperm DNA to the growth media. They noted that media with
these special ingredients added had improved cell proliferation (by cytokinesis) and shoot formation. Purification of the active factor from these special ingredients identified it as an adenine derivate, which they tested and later called kinetin. This was the first identified cytokinin. Later, Miller also discovered the first naturally occurring cytokinin, zeatin (Miller et al., 1956). Later research has demonstrated that cytokinins also regulate various cell growth and differentiation processes, leaf senescence, root/shoot balance control, and transduction of nutritional signals, among many roles.

Further work performed by Skoog and Miller, in classic experiments demonstrated that particular ratios of auxin:cytokinin in nutrient media could determine cell fate (Skoog and Miller et al., 1957). At high auxin:cytokinin, undifferentiated callus tissues could be induced to form roots, and at high cytokinin:auxin ratios, shoot formation was induced. These early experiments began a wealth of research investigating the interactions between auxin and cytokinin. It is interesting to note that despite the important role auxin and cytokinin plays together, few studies have performed experiments with the hormones in combination.

Auxin and cytokinin are 2 of 10 known plant phytohormones; the others are: abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), gibberellic acid (GA), brassinosteroid (BR), strigolactone (SL), ethylene (ETH) and nitric oxide (NO). Each phytohormone has a distinct signalling pathway, though there is crosstalk between pathways. Comparative genomic and phylogenetic analysis performed by Wang et al., 2015, has revealed that canonical auxin, cytokinin and SL signalling pathways evolved first, before the division of Charophyceae and land plants (Wang et al., 2015). ABA, JA and SA signalling pathways probably evolved next, in the last common ancestor of land plants. GA originated after land plants diverged from bryophytes, BR and ETH evolved before and after the emergence of angiosperms, respectively. Not all parts of the signalling pathways evolved simultaneously, for example, the auxin receptor family of genes (AFBs and TIR1) evolved into 3 clades with duplication events before the angiosperm and gymnosperm lineage diversion, “TRANSPORT INHIBITOR RESPONSE 1/ AUXIN SIGNALING F-BOX 2” (“TIR1/AFB2”), “AUXIN SIGNALING F-BOX 4” (“AFB4”) and “AUXIN SIGNALING F-BOX 6” (“AFB6”), and separate “TIR1” and “AFB2” clades formed before the split between eudicot plants and monodicot plants. Early and relatively close evolution of auxin and cytokinin core signalling machinery may help to understand the large, complex and often synergistic relationship between these two hormones.
1.2.2 Auxin signalling pathway

1.2.2.1 Auxin biosynthesis

Indole-3-acetic acid (IAA) is the primary active auxin in plants (Zhao et al., 2010). IAA is synthesised via four well-characterised tryptophan-dependent pathways, named for their intermediates: the indole-3-pyruvic acid (IPA), indole-3-acetamide (IAM), indole-3-acetaldoxime (IAOx) and tryptamine (TAM) pathways. In the best studied tryptophan-dependent pathway, the IPA pathway (Figure 1.4), the TRYPOTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) family of enzymes remove the amino group of the amino acid tryptophan to produce IPA (Zhao et al., 2010). The 11-member YUCCA (YUC) family of flavin monooxygenases then catalyse the oxidative decarboxylation of IPA to generate IAA (Won et al., 2011). The existence of a tryptophan-independent pathway remains controversial (Woodward & Bartel, 2005). yuc quadruple and taa mutants result in reduced IAA biosynthesis, and developmental defects related to reduced auxin signalling, which phenocopy other strong auxin signalling pathway mutants (Hofmann et al., 2011; Nishimura et al., 2014).
Figure 1.4. The IPA pathway of auxin biosynthesis. Tryptophan is converted to IPA by TAA proteins, which is then converted to IAA by YUCCA proteins. NADPH and oxygen are utilised by YUCCA, and carbon dioxide and water are released. Source: (Zhao, 2012).

1.2.2.2 Auxin transport

1.2.2.2.1 AUX1/LAX family

IAA is predominantly synthesised in stem apices, and is transported to other tissues via bulk flow in mature phloem (Petrasek & Friml, 2009), however, more precise cell-to-cell transport of IAA is mediated by the AUX1/LIKE AUX1 (AUX1/LAX) family and PIN-FORMED (PIN) family of transmembrane proteins, which control influx and efflux of IAA respectively (Petrasek & Friml, 2009). AUX1 belongs to a small gene family which includes LAX1-3, and non-redundantly transport auxin into the cell. AUX1 expressed in *Xenopus laevis* oocytes allowed the influx of IAA (Yang *et al.*, 2006), and IAA binds to AUX1 in a pH-dependent manner (Rubery & Sheldrake, 1974). AUX1 is essential for proper gravitropic response; aux1 mutants show severely agravitropic roots (Bennett *et al.*, 1996). LAX3 is essential for development of lateral roots,
creating an auxin sink; lax3 mutants had reduced numbers of lateral roots (Swarup et al., 2008). LAX3 is also auxin-inducible (Swarup et al., 2008).

1.2.2.2 PIN family

The PIN family of proteins contains 8 members (PIN1-8), and serve to transport auxin across membranes utilising electrochemical $H^+$ gradients (Forestan & Varotto, 2012). PIN1-4,7 transport auxin across the cell membrane out of the cell, and differ in expression location rather than auxin transport function; PIN5 and PIN8 are localised to the endoplasmic reticulum, rather than the cell membrane, and regulate intracellular transport of auxin (Mravec et al., 2009; Dal Bosco et al., 2012). PIN1-4,7 are asymmetrically localised on cell membranes, enabling them to regulate intercellular flow of auxin, which is particularly important in the generation of auxin maxima and minima (Wisniewska et al., 2006). Their gene expression, polar localisation, transport activity and protein stability can all be modulated to tightly regulate the efflux of auxin. Clathrin-dependent endocytosis and recycling of plasma membrane PINs is a key mechanism for the rearrangement of PIN polarity, as well tightly controlling the rate of auxin efflux (Dhonukshe et al., 2007). Such rearrangements are important for gravitropism and organogenesis, among other processes (Forestan & Varotto, 2012; Paciorek et al., 2005). PIN proteins are phosphorylated by kinases such as PID, which mark them for specific trafficking pathways (Christensen et al., 2000). PIN protein transport can also be inhibited by chemicals such as naphthylphthalamic acid (NPA) which blocks the transport cavity (Scanlon et al., 2003).

1.2.2.3 ABCB family

Auxin can also be transported by members of the ATP-binding cassette B (ABCB)family, which are localised uniformly across the membrane (Cho & Cho, 2013). The phenotypes of the mutants of these transporters are milder than pin mutants, suggesting that basal auxin transport is important, but less integral for developmental processes than the formation of auxin gradients and maxima (Blakeslee et al., 2007).
1.2.2.3 Auxin regulation of transcription

Figure 1.5. Briefly, inside the cell, active auxins are detected by auxin receptors, TIR1/ the AFB family (Dharmasi et al., 2005). TIR1 and AFBs are components of the Skp, Cullin, F-box containing complex, SCF$^{TIR1/AFB}$, an E3 ubiquitin ligase complex (Cardozo & Pagano, 2004). Auxins act as a molecular glue between TIR1/AFBs and INDOLE-3-ACETIC ACID INDUCIBLE (Aux/IAA) proteins (Tan et al., 2007). Aux/IAAs bind AUXIN RESPONSE FACTOR (ARF) proteins which are TFs responsible for regulation of auxin-responsive genes. When bound to Aux/IAAs, ARFs are unable to bind DNA and regulate transcription. In the presence of auxin, TIR1/AFBs are able to bind Aux/IAAs and mark them for 26S proteasome-mediated degradation by ubiquitination. This releases the ARFs to regulate gene expression. Source: (Leyser, 2018).

1.2.2.3.1 $TIR1/AFBs$

The auxin receptor TIR1 is part of an F-box containing family, which also includes 5 AFBs, all of which are localised to the nucleus (Salehin et al., 2015). AFBs show some functional redundancy with each other, but tir1 mutants show strong auxin-signalling-deficient phenotypes (Dharmasiri et al., 2005). Expression levels of $TIR1$ and $AFB1-3$ is fairly broad and uniform, however, protein accumulation of TIR1, AFB2 and AFB3 is highly localised to regions of growth, suggesting a mechanism of posttranscriptional regulation (Parry et al., 2009). TIR1 contains an 18 Leu-rich repeat (LRR) domain, which is the binding pocket for Aux/IAAs, in
which auxin acts as the molecular glue (Tan et al., 2007). TIR1 has a large range of binding affinities for the different Aux/IAAs presumed to be dependent on small differences in structure between the Aux/IAA family DII domain, which is the recognition domain bound by the TIR1 LRR domain (Irina et al., 2012). The interaction of TIR1 with Aux/IAAs is dependent on the concentration of auxin (Dharmasiri et al., 2005), as well as the auxin affinity of individual Aux/IAAs, which varies (Irina et al., 2012). Binding affinities between TIR1 and Aux/IAAs can be altered/abolished by the modification of the LRR and DII domains (Liao et al., 2015; Yu et al., 2013). The crystal structure of auxin bound to TIR1 has been uncovered, revealing that auxin fills a hydrophobic surface in the LRR domain, enhancing TIR1–Aux/IAA interactions without altering the conformation of TIR1 (Tan et al., 2007). This analysis also revealed that the auxin-binding site is partially promiscuous, allowing the binding of auxin analogues. This promiscuity has led to the development of synthetic auxins and TIR1 inhibitors, such as auxinole, which can strongly bind the auxin pocket but also contains a phenyl ring which prevents Aux/IAA binding by blocking access to the Phe82 in TIR1 crucial for that interaction (Hayashi et al., 2012).

TIR1 is able to bind the DII domains of Aux/IAAs in isolation from the rest of that protein, which has led to the development of several important modern tools. One such tool is the auxin expression reporter line DII-VENUS, in which the DII domain of IAA28 is bound to YFP-VENUS, and degradation of fluorescence indicates auxin signalling activity (Brunoud et al., 2012). Another tool is the Auxin Inducible Degron (AID) system, in which an operational TIR1 complex is cloned into the desired system, and the DII domain of an Aux/IAA is fused to the protein of interest, which can then be knocked down with relative precision by treatment with auxin (K. Nishimura et al., 2009).

1.2.2.3.2 Aux/IAAs

*Arabidopsis thaliana* has 29 INDOLE-3-ACETIC ACID INDUCIBLE (Aux/IAA) proteins. Although there is some functional redundancy, most aux/iaa mutants display similar auxin-deficient phenotypes, such as defected embryo, flower, hypocotyl, lateral root development, aberrant tropism response (Overvoorde et al., 2005). Aux/IAAs have 4 domains (I–IV) mostly conserved in the family. Domain I (DI) recruits co-repressor proteins such as TOPLESS (TPL), Domain II (DII) is necessary for binding of Aux/IAAs to TIR1 in the physical presence of an auxin molecule, Domain III (DIII) binds ARFs to repress their transcriptional activity and also allow homo- and hetero-dimerisation between Aux/IAAs (Bargmann & Estelle, 2014). The DII domain is largely conserved, and contains a 13 amino acid degron motif which confers the instability
characteristic of the high turnover rate of these proteins (Dharmasiri et al., 2005). Ubiquitylation sites flanking the degron motif vary between Aux/IAAs, leading to variation in auxin-binding efficiencies, and subsequent variation in degradation (Irina et al., 2012). It is unknown whether Aux/IAAs – co-receptor complexes prevent ARFs from initiating gene transcription via physical blocking of the ARF DNA-binding domain, or by recruiting chromatin remodellers to the DNA to maintain ‘tightness’. Interactome studies have revealed the complexity of Aux/IAA binding (Luo et al., 2018). All 29 Aux/IAAs can form heterodimers with other Aux/IAAs in a total of 253 interactions, making up 47% of Aux/IAA interactions. The 29 Aux/IAAs also formed heterodimers with 20 ARFs in a total of 544 interactions, making up 45% of Aux/IAA interactions. Remaining interactions were with a number of other TFs, TPL and TIR1/AFBs. This response is dependent on the type I/II Phox and Bem1p (PB1) domain in the DIII/DIV region of many Aux/IAAs which allows protein-protein interaction (Guilfoyle et al., 2015). Aux/IAAs have been shown to be regulated at multiple levels; epigenetic modifications via PICKLE (PKL) and GENERAL CONTROL NONDEREPRESSIBLE 5 (GCNS) (Fukaki et al., 2006; Weiste and Dröge-Laser et al., 2014); transcriptional regulation via PHYTOCHROME INTERACTING FACTOR 3 (PIF3) (Sellaro et al., 2011), post-translational regulation via PHYTOCHROME B (PhyB) and miR847 (Hrtyan et al., 2015; Xu et al., 2018); and protein turnover regulation via RGA-LIKE PROTEIN 3 (RGL3) and UBQUITIN-CONJUGATING ENZYME 13 (UBC13) (Shi et al., 2017; Wen et al., 2014), represent the complexity of Aux/IAA regulation and resultant diversity of responses.

1.2.2.3.3 Auxin Response Factors (ARFs)

The AUXIN RESPONSE FACTOR (ARF) family contains 23 members, which are released upon the degradation of the Aux/IAA that is binding and inhibiting them. ARFs regulate distinct and overlapping functions. For example, ARF1 and ARF2 regulate leaf senescence (Ellis et al., 2005), ARF6 and ARF8 overlap in regulating flower maturation (Nagpal et al., 2005), arf10 arf16 double mutants exhibit an auxin-deficient phenotype not shown by single mutants (J.-W. Wang et al., 2005), and ARF3 is responsible for leaf polarity (Vial-Pradel et al., 2018). Like Aux/IAAs, ARFs also contain DIII/DIV domains similar to Aux/IAAs, which allows the formation of Aux/IAA-ARF dimers, but these domains also allow the formation of ARF-ARF dimers, though these appear less common (Luo et al., 2018). ARFs also contain a DNA-binding domain (DBD) and a middle domain (MD) which determines activity (Angelica & Fong., 2008). ARF5-8,19 are thought to be transcriptional activators, and the other ARFs transcriptional repressors, based on motifs in the MD (Tiwari et al., 2003). How ARFs mediate transcriptional repression is not
known; repression by forming dimers with activator ARFs, recruitment of co-repressors such as TPL, or promoter site competition, have all been suggested, but further investigations are required. Activator ARFs bind DNA at auxin-responsive elements (AuxREs) the canonical AuxRE is TGTCTC via the B3 domain in the DBD (Ulmasov et al., 1997). The canonical AuxRE is TGTCTC, which is enriched in the promoter regions of auxin-responsive genes and is sufficient for ARF binding. AuxRE spacing and repetition is important for binding specificities of different ARFs, and is also important for recognising real auxin-responsive genes, as the TGTCTC motif occurs in approximately 50% of Arabidopsis thaliana promoters (Mironova et al., 2014). Once bound to the AuxRE, the MD of ARF5 has been demonstrated to recruit the SWI/SNF chromatin remodellers BRAHMA (BRM) and (SPLAYED) SYB, which ‘loosen’ the chromatin and allow transcription of the target gene (Yamamuro et al., 2016). This model is proposed to be a general mechanism for activator ARFs. Chromatin remodelling is discussed further below. ARFs can also form heterodimers with other TFs. The ARF-IND interaction has been discussed above, ARF7-MYB77 regulates lateral root development (Shin et al., 2007), and ARF8- BIG PETAL UB (BPEp) regulates petal growth (Varaud et al., 2011). ARFs can also be phosphorylated to suppress their interaction with Aux/IAAs and promote their transcriptional activity (Cho et al., 2014). Additionally, many ARFs are targets of post-transcriptional modification; miR167 targets ARF6,8 (Wu et al., 2006); miR160 targets ARF10,16,17 (Lin et al., 2015); TAS3 ta-siRNAs target ARF2-4 (Fahlgren et al., 2006).

### 1.2.2.4 Auxin degradation

Included in the auxin-induced transcriptome are genes involved in the negative feedback of the auxin signalling pathway, such as upregulation of particular Aux/IAAs and the GRETCHEH HAGEN 3 (GH3) protein family which catalyses the conjugation of amino acids to IAA, rendering them inactive (Park et al., 2007). The GH3 family contains 19 members, 7 of which are able to conjugate amino acids to IAA (Staswick et al., 2005). The GH3s appear largely redundant, with single mutants bearing no obvious phenotypes; however overexpression causes strong auxin-deficient phenotypes, such as short hypocotyles (Park et al., 2007). Upregulation of GH3 genes has been observed within 5 minutes of auxin treatment, making them a good marker for auxin response (Ding et al., 2008). Overexpression of GH3.5 improved resistance to abiotic stresses such as drought, freezing and high salt concentrations (Westfall et al., 2016).
1.2.3 Cytokinin signalling pathway

1.2.3.1 Cytokinin biosynthesis

Isoprenoid cytokinins are the most abundant class of the cytokinin family in plants; the major isoprenoid derivate is trans-zeatin (tZ) (Figure 1.6); cis-zeatin (cZ), isopentenyladenine (iP) and dihydrozeatin (DZ) are other isoprenoid cytokinins (Kudo et al., 2012). Another class of cytokinins are aromatic cytokinins, found in only some plants, which includes: 6-Benzyaminopurine (BAP), the most widely used experimental cytokinin aside from tZ, orthotopolin (oT), meta-topolin (mT), ortho-methoxytopolin (MeoT), and meta-methoxytopolin (MemT) (Bajguz & Piotrowska, 2009). Kinetin is a commonly used synthetic cytokinin, and the first discovered active cytokinin (Miller et al., 1956).

![Figure 1.6. trans-Zeatin (tZ) biosynthesis pathway. tZ is biosynthesised from ATP, ADP and AMP in a series of enzyme-catalysed steps. First, ATP, ADP or AMP are bound to isoprenoid molecules by ARABIDOPSIS THALIANA ISOPENTENTLTRANSFERASE (IPT) proteins, this is the rate-limiting step (Werner & Schmülling, 2009). The resulting cytokinin-nucleotides are converted to corresponding tZ-nucleotides by the CYTOCHROME P450 (CYP735A) (Takei et al., 2004). tZ-nucleotides are dephosphorylated, converted to tZR by 5’-ribonucleotide phosphohydrolase action, then finally converted to tZ by adenosine nucleosidase action. Source: Adapted from (Mou, et al., 2013)](image)

The IPT family contains 8 members, of which IPT1,3,8 are involved in tZ biosynthesis (Sakakibara et al., 2006). IPT1,3,8 are located in plastids, ostensibly the primary subcellular
compartment for biosynthesis of cytokinin (Kasahara et al., 2004). The IPT family exhibits distinct expression patterns; IPT1 is expressed in root tips, ovules and immature seeds; IPT3 is expressed in phloem companion cells; IPT8 is expressed in immature seeds, with high expression in the chalazal endosperm (Li et al., 2013; Miyawaki et al., 2004; Takei et al., 2004). The ipt1 3 5 7 quadruple mutant has severely reduced levels of tZ, resulting in classic cytokinin-deficient phenotypes, such as growth retardation, diminished activity of apical meristems, and rapid root growth (Matsumoto-Kitano et al., 2008). IPT overexpressing lines have increased tZ levels and form shoots from calli independent of cytokinin treatment (Miyawaki et al., 2006).

1.2.3.2 Cytokinin transport

As the 8 IPTs are expressed in specific tissues, but cytokinins are required across the plant, the cytokinins must be transported. For long distance transport, cytokinins travel through xylem and phloem in the sap in bulk flow (Sakakibara et al., 2006). This allows iP synthesised in the aerial parts of the plant to affect a cytokinin response in the root, and tZ synthesied in the roots to affect a cytokinin response in the aerial parts of the plant (Kiba et al., 2013; Nordström et al., 2004). Less is known about cellular uptake of cytokinin than auxin, but 3 cytokinin transporter types have been reported to date: purine permeases (PUPs), equilibrative nucleoside transporters (ENT), and G subfamily ATP-binding cassette (ABCG) transporters (Bürkle et al., 2003; Li et al., 2000; Zhang et al., 2014).

PUP1,2,14 proteins have cytokinin uptake transport activity in Arabidopsis thaliana, PUP7 cytokinin transport activity has been shown in Oryza sativa (Davière & Achard, 2017). PUP proteins show overlapping and distinct expression patterns, suggesting non-redundant roles (Bürkle et al., 2003; Zürcher et al., 2013). PUPs have been suggested to only be present in vascular plants, and can also transport other molecules, such as nicotine (Hildreth et al., 2011). PUP14 is suggested to transport cytokinins into the cell from the apoplast, reducing the extracellular detection of cytokinin signalling to ensure proper cotyledon development (Bürkle et al., 2003).

ENT3,6,8 proteins have cytokinin uptake transport activity in Arabidopsis thaliana, ENT2 cytokinin transport activity has been shown in Oryza sativa (Zuo et al., 2005; Wormit et al., 2004). Like PUP proteins, ENT3,6,8 have overlapping and distinct expression patterns, suggesting non-redundant roles, however ENT3 and ENT8 share expression patterns (Zuo et
al., 2005; Wormit et al., 2004). ENT transporters show similarity with the human transporters hENT1 and hENT2 (Li et al., 2000).

ABCG14 is the only cytokinin exporter found to date. It is mostly expressed in the roots, and abcg14 mutants have defects in long-distance transport of root-synthesized tZ, and phenocopy cytokinin biosynthesis mutants (Zhang et al., 2014). ABCG transporters are abundant in plants, and more investigation is required to determine their role in cytokinin transport.

1.2.3.3 Cytokinin regulation of transcription

Figure 1.7. Cytokinin signalling pathway. Cytokinin regulation of gene expression is coordinated by a two-component signalling system. Cytokinin is detected on the extracellular side of the membrane by the ARABIDOPSIS HISTIDINE KINASE (AHK) family of proteins acting as cytokinin receptors. The extracellular CHASE domain of AHKs binds cytokinin, prompting phosphotransfer from the Kinase domain to the Receiver domain of the AHKs. This phosphotransfer prompts the phosphorylation of HISTIDINE-CONTAINING PHOSPHOTRANSMITTERS (AHPs), which are then translocated from the cytoplasm to the
nucleus, whereupon they phosphotransfer ARABIDOPSIS RESPONSE REGULATORs (ARRs), which are TFs that upon phosphotransfer, regulate the expression of cytokinin-responsive genes. Source: (El-Showk et al., 2013)

1.2.3.3.1 Histidine Kinases (AKHs)
The HISTIDINE KINASE (AHK) family of proteins contains 3 members, AHK2-4, discovered in mutant screens searching for resistance to cytokinin treatment in callus tissue culture (Inoue et al., 2001). ahk mutants are resistant to cytokinin treatment, and phenocopy cytokinin biosynthesis mutants, and overexpression of these genes increased sensitivity to cytokinin (Higuchi et al., 2004; Nishimura et al., 2004). AHKs have different expression patterns and cytokinin ligand binding affinities, contributing to the diversity and specificity of cytokinin signalling (Higuchi et al., 2004). Phenotypes of single and triple mutants suggest some redundancy between the AHKs, but also distinct roles such as inhibition of root growth in ahk4 not observed in ahk2 ahk3 double mutants (Tanaka et al., 2004). AHK4 is capable of a directional phosphorelay, in the presence of cytokinin it phosphorylates AHP, and in the absence of cytokinin it dephosphorylates AHP (Mähönen, et al., 2006). AHKs can also be localised to endoplasmic reticulum membranes (Lomin et al., 2011).

1.2.3.3.2 Histidine-Containing Phosphotransmitters (AHPs)
The Arabidopsis thaliana HISTIDINE-CONTAINING PHOSPHOTRANSMITTER (AHP) family of proteins contains 5 true members, AHP1-5, AHP6 is related but considered a pseudo AHP (Mähönen, et al., 2006). AHPs receive the phosphoryl group from AHKs and translocate to the nucleus to transfer the group to ARRs. Overexpression of AHPs in Arabidopsis caused cytokinin sensitivity, and triple/quadruple ahp mutants phenocopy cytokinin biosynthesis mutants (Hutchison et al., 2006; Suzuki et al., 2002). AHPs have overlapping expression patterns, and single and double mutants show no cytokinin response defects, indicating large functional redundancy (Hutchison et al., 2006). AHK-AHP interaction is promiscuous (Skerker et al., 2007). AHPs contain a conserved His residue that receives the phosphoryl group from AHKs, AHP6 lacks this His residue (Mähönen, et al., 2006). AHP6 is able to inhibit phosphoryl group transfer from AHP to ARRs (Mähönen, et al., 2006). AHPs can also interact with the TCP family of TFs (Suzuki et al., 2001).
1.2.3.3 Response Regulators (ARRs)

RESPONSE REGULATORS (ARRs) are the transcriptional regulators of the cytokinin signalling pathway. There are 23 ARRs which fall into two groups; type-B ARRs receive a phosphate group from AHPs, become transcriptionally active and regulate the expression of genes, including the type-A ARRs which are thought to negatively feedback the cytokinin signalling pathway by competing with type-B ARRs without initiating transcription. Type-A ARRs include ARR3-9, 15-17, type B-ARRs include ARR1,2,10-14,18-21,23 (Lee et al., 2007; Mason et al., 2005; Tajima et al., 2004; To et al., 2007; To et al., 2004).

Type-B ARRs regulate cytokinin-responsive genes. Loss of function mutants exhibited cytokinin insensitivity, and ARR1,2,10-12,18 appear to have overlapping functions (Mason et al., 2005). Type-B ARRs contain a GARP domain which binds DNA and a conserved Asp residue for phosphorylation by AHPs (Hosoda et al., 2002). The GARP domain preferentially binds spaced repetitions of the 5’-AGATT-3’ motif in promoters, though the motif in isolation cannot be used to predict cytokinin-responsive genes due to its high occurrence frequency (Imamura et al., 2003). Concatenation of this motif is utilised in the cytokinin reporter line TCSn::GFP, in which Type-B ARRs promote the expression of GFP in areas of cytokinin signalling (Liu et al., 2017).

Type-A ARRs are cytokinin primary-response genes, acting to negatively feedback the cytokinin signalling pathway. Expression of type-A ARRs generally overlaps, and most appear to be functionally redundant (To et al., 2004). Some tissue-specific ARR roles exist but require further investigation. AHPs can also transfer phosphoryl groups to Type-A ARRs, possibly to increase protein stability (To et al., 2007). Some type-A ARRs can still positively influence gene expression; ARR4 can interact with phytochrome B to regulate gene expression (Salome et al., 2006), and the octuple arr3,4,5,6,7,8,9,15 mutant altered levels of PIN4 proteins, which influences regulation of genes by auxin (Carolina & Hill, 2011).

1.2.3.4 Cytokinin Response Factors (CRFs)

Another group of genes that can regulate cytokinin-responsive genes are the CYTOKININ RESPONSE FACTORS (CRFs). There are 6 CRFs (CRF1-6), which are also activated by the AHK-AHP two-component signalling system (Cutcliffe et al., 2011). Upon activation, they are transported into the nucleus (Rashotte et al., 2006). crf mutants show low phenotypic penetrance and functional redundancy, possibly partly explained by CRF regulation of gene expression overlapping with that of type-B ARRs (Rashotte et al., 2006).
1.2.3.4 Cytokinin degradation

Cytokinins are degraded by the CYTOKININ OXIDASE (CKX) family of proteins, which irreversibly degrade active cytokinins into adenosine or adenine and side chains (Hwang et al., 2012). There are 7 CKXs (CKX1-7) which show mostly distinct expression patterns (Werner et al., 2003). CKX overexpression results in a dramatic reduction in isoprenoid cytokinins, and cytokinin insensitivity phenotypes (Köllmer et al., 2014). CKXs from many plant sources are heavily glycosylated, which has been suggested to regulate pH-dependent localisation and enzyme activity (Schlüter et al., 2007). Translocation sequences in CKXs appear to direct CKX2,4-6 for secretion, presumably to the apoplast where cytokinins interact with the CHASE domain of AHKs, and CKX1,3 are predicted to be imported into the mitochondria (Kowalska et al., 2010; Schmülling et al., 2003). However, CKX3 excretion has been reported (Bilyeu et al., 2001). CKX7 lacks a locational signal peptide, and may be cytoplasmic (Schmülling et al., 2003).

1.2.3.4.1 CKX3

CYTOKININ OXIDASE 3 (CKX3) is preferentially expressed in xylem, and expression can also be observed in the central zone of the SAM; work with a CKX3-GUS reporter line occasionally observed very weak expression in young shoot tissues ~2 weeks post-germination (Werner et al., 2003). Arabidopsis CKX3 overexpressing lines exhibit longer roots with more lateral roots, a reduction in apical dominance, more auxiliary branches, decreased rosette area, and retarded growth ((Köllmer et al., 2014; Vercruyssen et al., 2011). Tobacco CKX3 overexpressing lines showed enhanced drought and salt tolerance (Qin et al., 2011). ckx3 mutants only show phenotypes in ckx3 cxx5 double mutants, so there is functional redundancy between them (Bartrina et al., 2011). SHORT ROOT (SHR) and HANABA TANARU (HAN) have been shown to directly and positively regulate CKX3 (Cui et al., 2011; Ding et al., 2015).

1.2.4 Auxin and cytokinin crosstalk

Crosstalk between hormone signalling pathways means that one hormone regulates genes involved in the biosynthesis, transport, signalling or degradation of another hormone. Extensive study of auxin and cytokinin research, coupled with transcriptome data has showed that there is considerable crosstalk between auxin and cytokinin, much of which is involved in the correct maintenance and development of specific tissues.
Cytokinin has been demonstrated to upregulate TAA1, as thus presumably increase the auxin signalling pathway (Müller et al., 2017). Treatment of cytokinin onto the inflorescences of TAA1::GFP lines showed strong increases in florescence in the media domains of gynoecia (Müller et al., 2017). The 35S::ARR1ΔDDK-GR line allows the upregulation of ARR1 in the absence of cytokinin upon treatment with dexamethasone (DEX); treatment with DEX was sufficient to increase TAA1 promoter fragment expression when measured with qRT-PCR (Reyes-Olalde et al., 2017). Cytokinin can also induce the upregulation of YUC8 expression, an auxin biosynthesis protein involved in root growth and development; cytokinin treatment in pif4 mutants revealed that PIF4 is necessary for this induction (Di et al., 2016). Elevated cytokinin levels increase auxin biosynthesis, and decreased synthesis of cytokinins in ipt mutants also led to decreased biosynthesis of auxins, demonstrating the connected nature of these hormones (Jones et al., 2010). Müller et al., 2007, showed that cytokinin can also regulate the efflux of auxin by upregulation of PIN7. Fluorescence in PIN7::GFP roots increased upon treatment with cytokinin, and the promoter of PIN7 was shown to contain a PIN CYTOKININ RESPONSE ELEMENT (PCRE); truncation of that promoter resulted in aberrant root growth, meristem size and lateral root initiation. crf TF mutants also exhibited altered lower expression of PIN1 and PIN7 in the root (Šimášková et al., 2015). qRT-PCR analysis of cytokinin treated tissues has also been shown to reduce expression of PIN1-3 (Laplaze et al., 2007).

Auxin influx can also be regulated by cytokinin. Roots treated with cytokinin show inhibited cell expansion; aux1 mutant roots were insensitive to cytokinin-induced cell expansion (Street et al., 2016). NanoString analysis of cytokinin treatment root tips showed a reduction in AUX1 and LAX2 expression (Street et al., 2016). Work by Dello Ioio et al. showed that ARR1 can induce the expression of Aux/IAA3; the Aux/IAA3 promoter contains an ARR1-binding consensus sequence which ARR1 has been shown to interact with in vivo, and overexpression of ARR1 in aux/iaa3 mutants does not trigger the meristem size decrease observed in the wild type background (Dello Ioio et al., 2008). Aux/IAA3 represses the auxin-induced expression of PIN proteins, presumably by binding the ARF that directly regulates them, thus cytokinin induction of Aux/IAA3 organises auxin efflux and patterning (Tian et al., 2002; Dello Ioio et al., 2008).

qRT-PCR analysis has revealed that IPT5 and IPT7 are upregulated upon treatment with auxin (Miyawaki et al., 2004). IPT5 upregulation by auxin was later shown to be mediated by Aux/IAA3 (Dello Ioio et al., 2008). Interestingly, qRT-PCR analysis of another cytokinin biosynthesis gene, CYP735A, showed downregulation after treatment with auxin (Takei et al.,
The pseudo AHP, AHP6, promoter can be bound and upregulated by ARF5 to regulate phyllotaxis (Bishopp et al., 2011). The promoters of type-A ARRs, ARR7 and ARR15 contain auxin-responsive elements and are responsive to auxin treatment (Bruno et al., 2008). Auxin was thus shown to downregulate specific parts of the cytokinin signalling pathway via upregulation of ARR7 and ARR15, to regulate root-stem cell specification in early embryogenesis (Bruno et al., 2008). qRT-PCR analysis of CKX gene expression has also revealed that auxin weakly downregulated CKX2, 4, 7 and upregulated CKX1, 6 (Werner et al. 2006).

1.2.4.1 Auxin, cytokinin and abiotic stress

Many parts of both the auxin and cytokinin signalling pathways are responsive to abiotic stress and/or modulate responses to abiotic stress. Single mutants of yuc6 and yuc10 have enhanced drought tolerance (Lee et al., 2012; Cha et al., 2015). pin1 pin3 pin7 triple mutants show increased tolerance against salt-induced reduction of root meristem length and cell number (Wen et al., 2015). tir1 afb2 double mutants have increased salinity tolerance (Iglesia et al., 2014). Overexpression of Aux/IAA6 in Oryza sativa increases drought tolerance (Jung et al., 2015). gh3.13 mutants in Oryza sativa increased drought tolerance (Zhang et al., 2009). ipt3 mutants showed improved salt tolerance (Nishiyama et al., 2012), and ipt1 ipt3 ipt5 ipt7 quadruple mutants showed increased salt and drought tolerance (Werner et al., 2010). ahk2 and ahk3 single mutants exhibited increased salt and drought tolerance, ahk2 ahk3 double mutants showed enhanced freezing tolerance (Tran et al., 2007). ahp2 ahp3 ahp5 triple mutants have greatly increased drought-tolerance (Nishiyama et al., 2013). arr1 arr12 double mutants were less sensitive to salt stress (Mason et al., 2010).

1.2.5 Chromatin remodellers and regulation of genes by auxin and cytokinin

Chromatin remodelling represents a crucial level of gene expression regulation. Chromosomal DNA is wrapped about histone protein complexes to form nucleosomes. The ‘tightness’ or ‘looseness’ of this wrapping increases or decreases, respectively, the availability of the DNA for binding of transcriptional machinery. The tightness and looseness of DNA wrapping can be modulated by chromatin remodelers, which post-transcriptionally modify histone proteins with a range of complex signals to modify their DNA-binding affinity. There are 4 chromatin remodelling complex (CRC) subfamilies, named for their central ATPase subunit; the best understood is the SWI/SNF (Switch/Sucrose non-fermenting). Yeast SWI/SNF CRCs are composed of 12 subunits, and the complexity of Arabidopsis CRCs has not been fully explored (Jégu et al., 2015; Lee & Young, 2000). Chromatin remodelling does appear to play an important role in the signalling pathways of auxin and cytokinin. ARF5 recruits SWI/SNF
complexes to the promoters of *PIN1*-3,7 to regulate their expression (Wu et al., 2015). *switch/sucrose nonfermenting 3c* (*swi3c*) mutants show altered expression of auxin-responsive genes involved in the regulation of gravitropism (Sarnowska et al., 2013). SWI/SNFs are recruited by TCP FAMILY TRANSCRIPTION FACTOR 4 (TCP4), a bHLH TF, to upregulate *ARR16*, a type-B ARR, and reduce cytokinin sensitivity in the leaf (Efroni et al., 2014). SWI/SNFs are also necessary for proper regulation of *IPT3* and *IPT9*, and thus proper cytokinin biosynthesis (Jégu et al., 2015). Auxin has been recently shown to decrease chromatin accessibility via the TIR1/AFB pathway (Hasegawa et al., 2018).

### 1.2.6 Lateral root development

Lateral roots (LRs) are a critical part of root architecture, providing mechanical stability and a greater ability to find and take up water and nutrients. Lateral root development (LRD) begins when two adjoining protoxylem pericycle founder cells of the same file divide asymmetrically and anticlinally to create shorter and longer daughter cells. Mature LR primordium is generated by a series of tightly organised cell divisions and organisations, beginning with the rapid and expansive division of the shorter daughter cell to produce inner and outer layers. Expansive division of the LR primordium pushes the tissue through the endodermis, cortex and epithelial cells, and emerges as a functional LR (Bishopp et al., 2011).

#### 1.2.6.1 Auxin and lateral root development

Auxin has been demonstrated to be a promoter of lateral root development (LRD), decreasing the spacing of pericycle founder cells (Casimiro et al., 2001). This spacing is coordinated by an oscillating auxin response which primes periodically pericycle founder cells for LRD at a regular interval of ~15 hours (Smet et al., 2007). Formation of an auxin maximum in the two primed pericycle cells signals the asymmetric, anticlinal division into longer and shorter cells, the latter maintaining the auxin maxima (Smet et al., 2007), (Figure 1.8). Auxin maxima at the outer layer of the lateral roots continue to promote the patterning, development and emergence of the LR. Treatment with exogenous auxin increases LRD, whilst auxin transport inhibitors decreases LRD (Casimiro et al., 2001). Auxin promotes LRD by stimulating the division of pericycle cells (Dubrovsky et al., 2008). Proper control of auxin influx and efflux is also necessary for LRD; *aux1* and *pin1* mutants show reduced LRD (Marchant et al., 2002; KXu et al., 2016). The polar transport of auxin creates an auxin gradient necessary for gene regulation of LRD. Many components of the auxin signalling pathway coordinate various aspects of LRD. At the basal meristem, xylem pole pericycle cells are primed to become lateral root founder cells via PIN-
regulated auxin efflux to increase auxin signalling; this process is coordinated by auxin-induced degradation of Aux/IAA28, leading to the release of ARF5-8,19 (Rybel et al., 2010). Aux/IAA8 and Aux/IAA19 may have redundant roles with Aux/IAA28 in this process (Groover et al., 2003; Tatematsu et al., 2004). At the nuclear migration zone, the lateral root founder cells, still with increased auxin concentrations begin to initiate lateral root formation. Here, auxin-induced degradation of Aux/IAA14 releases ARF7 and ARF19 to promote cell cycle activation, and cell polarity and identity specification (Swarup et al., 2008). At the LR initiation zone, the asymmetrical, anticlinal divisions are promoted by the Aux/IAA14-ARF7/-ARF19 module, as well as the Aux/IAA12-ARF5 module (Swarup et al., 2008; Smet et al., 2010). LBD16 and LBD29 are direct targets of ARF7 and ARF19, which positively contribute to LR formation (Okushima et al., 2007). Cell specification of the shorter cells vs. the longer flanking cells is dependent on Aux/IAA14-dependent upregulation of ACT DOMAIN REPEAT 4 (ACRA4) (De Smet et al., 2008). LOB DOMAIN-CONTAINING PROTEIN 18 (LBD18) is also involved in regulation of the cell cycle during these processes (Kim et al., 2012). Lateral root growth and emergence is controlled by auxin-induced cell wall remodellers (Péret et al., 2012). Aux/IAA3-ARF7 module also coordinates LR emergence (Swarup et al., 2008).

Figure 1.8. Roles of auxin signalling pathway in the development of lateral roots (LR). Firstly, auxin promotes the degradation of Aux/IAA28 to release ARF5,6,7,8,19 to prime the root for LR initiation. LR founder cells are polarised by ARF7,19 action as auxin promotes the degradation of Aux/IAA14 which sequesters them. Early patterning for LRs is coordinated by ARF7,ARF9 and ARF5 released by Aux/IAA14 and Aux/IAA12, respectively. Finally, in the emergence zone Aux/IAA14 and Aux/IAA3 are degraded by auxin action to release ARF7,19 and ARF7 respectively to begin LR emergence. Source: (Lavenus et al., 2013).
1.2.6.2 Cytokinin, auxin and lateral root development

Cytokinin plays an antagonistic role to auxin in LRD. Treatment with exogenous cytokinin reduces the formation of lateral roots (Li et al., 2006). Cytokinins act on xylem pole pericycle cells to block LR-initiating asymmetric, anticlinal division (Laplaze et al., 2007). *ahk* and *type-B arr* mutants, and CKX overexpressing lines have increased LRD (Riefler et al., 2006; Werner and Schmülling, 2009; Chang et al., 2015). These mutants also have increased rates of abnormally close LRs, suggesting cytokinin regulates the oscillation of auxin levels. Cytokinin repression of *PINs*, particularly *PIN1*, efflux carriers prevent the accumulation of auxin, and thus prevent auxin-induced LR patterning and organisation (Marhavy et al., 2014). Cytokinin treatment reduced PIN1 signalling at the membrane via regulation of PIN1 endocytic trafficking; this response continued upon co-treatment with cytokinin + cycloheximide (CHX), an inhibitor of translation, which suggests that this process is not dependent on transcription and biosynthesis of new proteins (Marhavy et al., 2011).

1.3 Hypothesis and Objectives

The studies in Chapter 1 discuss the relationship between seed size and the auxin and cytokinin signalling pathways, but there are many gaps in the understanding of these relationships. In particular there is a notable absence of in depth analyses of concurrent auxin and cytokinin treatment. We hypothesised that: the seed size increase observed in ago10 mutants was due to overexpression of *IND*, and that this constituted a novel part of an existing seed size regulation pathway; and that dual treatment of auxin and cytokinin would regulate a novel transcriptome. The following results chapters will investigate these hypotheses. Chapter 3 will investigate how IND and AGO10 regulate seed development, including: size, fertility, and storage contents, and will examine IND’s involvement in the auxin and cytokinin signalling pathways, summarised in Section 3.8. Chapter 4 will analyse the transcriptome of auxin and cytokinin dual-hormone treatment to investigate the dual hormone response, and will discuss the possibility of the auxin signalling pathway being dominant over the cytokinin signalling pathway, summarised in Section 4.4. Chapter 5 will investigate an unexpected line of enquiry: that cytokinin is a direct inhibitor of TIR1, summarised in Section 5.5. Chapters 4 and 5 come together to create a novel model of the interaction between the auxin and cytokinin signalling pathways, shown in Chapter 6.
Chapter 2 – Materials and Methods

2.1 Materials

2.1.1 Plant materials

All lines used were in the Columbia (Col-0) and Landsberg erecta (Ler) ecotype backgrounds. 2.1 lists the transgenic and mutant lines used in this study, and were purchased from the NASC seed stock centre (Nottingham, UK), unless otherwise stated to be donated in the Acknowledgements.

Table 2.1 Plant lines

<table>
<thead>
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<th>Line</th>
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<th>Gene</th>
<th>Gene ID</th>
<th>Mutation</th>
<th>Reference</th>
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<td>ago10 (Col-0)</td>
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<td>AT5G43810</td>
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<td>(Endrizzi et al., 1996)</td>
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2.1.2 Hormones and chemicals

General laboratory analytical grade chemicals were purchased from Alfa Aesar, Duchefa Biochemie, Fisher, Sigma Aldritch, and TAAB. Table 2.2 lists the hormones and chemicals used in this study. Plants were treated with or grown on plant agar medium or liquid media containing: Indole-3-acetic acid (IAA), 6-Benzylaminopurine (BAP), trans-zeatin (tZ), Dexamethasone (DEX), naphthylphthalamic acid (NPA), cycloheximide (CHX). Dimethyl sulfoxide (DMSO) was used for mock treatments. All treatments within an experiment were performed from the same stocks.
### Table 2.2 Chemical stocks and storage

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<th>Storage conditions</th>
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<td>tZ</td>
<td>Sigma Aldrich Z0876</td>
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<td>-20°C</td>
</tr>
<tr>
<td>DEX</td>
<td>Alfa Aesar #A17590</td>
<td>DMSO</td>
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<td>NPA</td>
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<td>DMSO</td>
<td>-20°C</td>
</tr>
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<td>CHX</td>
<td>Acros Organics, China (AC35742)</td>
<td>DMSO</td>
<td>-20°C</td>
</tr>
</tbody>
</table>

#### 2.2 Plant methods

##### 2.2.1 Plant growth conditions

For soil-based plant experiments seedlings were sown on Levington® Advance Seed and Modular F2+S compost plus horticultural grade sand mixture, pH 5.3-6.0 (ICL, Ipswich, UK). Seeds were stratified at 4°C for 3 days. For agar-based experiments, seeds were washed briefly with 70% ethanol, surface-sterilised with 20% bleach for 5 minutes and washed 3 times with autoclaved water, then stratified for 3 days at 4°C. Seeds were then sown onto 1% agar supplemented with 1% (w/v) sucrose (Sigma Aldrich S0389), and ½ Murashige and Skoog salts (Murashige and Skoog et al., 1962) plus vitamins (MS; Duchefa Biochemie, The Netherlands, #20222), in sterile plates, and sealed with micropore tape. In lateral root and root growth experiments, the plates were positioned vertically. For liquid-media based experiments, seeds were sterilised and stratified as described above, then sown in ½ MS, 1% sucrose medium in a 50mL Falcon tube, aerated by constant upright shaking at 60 rotations per minute (rpm). Plants were illuminated for 16 hours with light every 24 hours delivered at 120µmol m-2 sec-1 at a constant temperature of 23°C in a Versatile Environmental Test Chamber MLR 350-HT (Sanyo, Japan). All plants studied within an experiment were grown simultaneously and watered equally.
2.2.2 Size and weight measurements

Mature seed size length was initially measured by capturing images under a dissecting microscope (ZEISS, Germany, 10W bulb), imaged with a CCD camera, and analysed with ImageJ software. 1000 seed weight was measured by manually counting sets of 1000 mature seeds, and measuring the mass of a 1.5mL Eppendorf tube on an analytical balance before and after holding 1000 seeds.

To measure embryo sizes, mature seeds were imbibed in water for 60 minutes then the embryo was excised from the seed coat with fine forceps. Embryos were mounted in 50% (v/v) glycerol and observed under a light microscope (Leica). Images were captured with a CCD camera, and measured with ImageJ. Cotyledon length from tip to the start of petiole, and longest width perpendicular to the length, were measured and area was calculated using \((\text{length}/2)*\text{width}^2*\pi\), the formula for calculating the area of an oval. Root length from tip to base of petiole, and longest width perpendicular to the length were measured and volume was calculated using \((\text{width}/2)*\text{length}^2*\pi\), the formula for calculating the volume of a cylinder.

To measure embryo mesophyll cell size, mature, dry seeds were imbibed in water overnight, the embryos excised from the seed coat and then cleared in Hoyer’s solution (chloral hydrate:water:glycerol; 8:3:1; w/v/v) for 30 minutes (Hughes et al., 2008). Cleared embryo mesophyll cells were visualised under a light microscope (Leica), imaged with a CCD camera, and analysed with ImageJ.

To measure developing seed size, unfertilised siliques were made accessible by removal of petals and sepals, and were manually fertilised with the anthers of another flower. 1 day after pollination (DAP), fertilised developing seeds were dissected from the siliques, cleared with Hoyer’s solution (chloral hydrate:water:glycerol; 8:3:1; w/v/v), imaged under a light microscope (Leica), imaged with a CCD camera, and area was measured with ImageJ.

Volume of mature dry seeds was determined using \(\text{length}^2*\pi*(4/3)\), the formula for calculating the volume of a prolate ellipsoid.

2.2.3 Fertility analysis

Total seed yield was determined by combining 1000 seed weight data with weight of total seed produced by an individual plant. Mature silique length was measured manually with a standard
ruler. Limited silique fertility was achieved by removing petals and sepals of an unopened flower with fine forceps to reveal the unfertilised silique, which was then fertilised manually with anthers from another flower with varying degrees of contact, to create siliques with a variable number of developing seeds. In other experiments, this process was repeated but with large amounts of pollen deposition on the stigma to examine fertility mechanical defects. Unfertilised ovules within siliques with developing seeds were imaged by dissection of siliques (7 DAP) and imaged under a dissecting microscope.

2.2.4 Imprinting analysis
Cross-fertilisation between Ler and zll-3 lines was achieved by removing petals and sepals of an unopened flower with fine forceps to reveal the unfertilised silique, which was then fertilised manually with anthers other flowers, ensuring large amounts of pollen deposition.

2.2.5 Protein content analysis
Protein content of seeds was determined as described by (Fatihi et al., 2013). 80 mature, dry seeds were manually counted then homogenised by vortexing with a 4mm steel ball bearing in a 2mL Eppendorf tube. 250μL acetone was added and the solution was centrifuged at 16,000 x g and the supernatant was discarded. The pellet was dried with vacuum infiltration. The pellet was re-suspended in 250μL extraction buffer (50mM Tris-HCL, pH 8.0, 250mM NaCl, 1mM EDTA, 1% (w/v) SDS). 50μL protein extract in 950μL Bradford solution, was used to measure protein content using a Bradford protein assay (Sigma-Aldrich).

2.2.6 Sugar content analysis
Sucrose and glucose content of seeds was determined as described by (Fatihi et al., 2013). 50 mature, dry seeds were manually counted then homogenised by vortexing with a steel pellet in a 2mL Eppendorf tube. 250μL 80% (v/v) ethanol was added, and the solution was incubated at 70°C for 90 minutes. The solution was centrifuged at 16,000 x g for 5 minutes, and the supernatant was transferred to a new Eppendorf tube. Centrifuge and transfer steps were repeated 3 times in total. The combined supernatant was vacuum dried off at 16,000 x g at room temperature. The remaining residue was dissolved in 50μL water. 10μL of the sample was treated with invertase (5 units/ μL) (Sigma Aldrich I4504), for 20 minutes to convert sucrose to glucose. 10 μL of the sample (invertase treated and untreated), were added to 990μL glucose assay reagent (Glucose HK Assay Kit, Sigma Aldritch GAHK20), mixed and
incubated for 15 minutes at room temperature. Absorbance at 340nM was measured with deionised water as a control, with a Cary 60 UV-Vis Spectrophotometer, Agilent Technologies.

2.2.7 Lateral root development and root length
Seeds were sterilised, stratified, sown on hormone/chemical-treated agar in plates positioned vertically, as described above. At 9 days after germination (DAG), the number of lateral roots was manually counted under a dissecting microscope. Root length was determined by imaging the roots and measuring them using ImageJ. Lateral roots/ cm was determined from those measurements.

2.3 Imaging, microscopy and analysis
2.3.1 Confocal microscopy
All confocal imaging was performed on an Olympus FV1000 confocal microscope. YFP was excited with an argon laser beam at 514 nm and emission at 520-530 nm was measured. GFP was excited with an argon laser beam at 488 nm and emission at 495-515 nm was measured. FV10-ASW software was used to control the laser settings, and images were processed in ImageJ. All tissues were mounted on microscope slides in 50% (v/v) glycerol (+ hormone treatment in PIN2::GFP root tissues). Tissues examined were: developing seeds (excised from siliques with fine forceps) at 20x magnification, and root tips at 40x magnification.

Fluorescence levels in the root tip of the auxin expression reporter DR5::GFP were quantified using ImageJ, generating a threshold for fluorescence values to find the area and measuring the mean fluorescence within that area, to generate fluorescence intensity. Fluorescence levels in the root tip of the auxin expression reporter TCSn::GFP were quantified using ImageJ, measuring the fluorescence mean in the root tip and the area of the root tip (up to 30µm from the very root tip), to generate fluorescence intensity. Fluorescence levels in the root tip of the auxin expression reporter DII-VENUS and mDII-VENUS were quantified using ImageJ to measure the mean fluorescence of nuclei in the root tip. Fluorescence levels in the cortex and epithelium root cells in the pPIN2::PIN2::GFP reporter were quantified using Cellset software (Pound et al., 2012).

2.3.2 B-Glucuronidase (GUS) assay
7 DAG seedlings of the HS::AXR3-GUS and HS::axr3-GUS lines were heat shocked at 37°C for 2 hours, to upregulate Aux/IAA17 (AXR3) fused to the GUS enzyme, allowed to recover at room
temperature for 30 minutes, and treated at room temperature with 1µM IAA, 1µM BAP, 1µM IAA + 1µM BAP, or mock (DMSO) in liquid media for 15 minutes. Seedlings were then incubated with GUS assay buffer (0.1M phosphate buffer [pH 7], 10mM EDTA, 0.1% Triton X-100, 1mg/mL X-Glue A, 2mM potassium ferricyanide) for an hour, and cleared in 50% (v/v) ethanol. The GUS enzyme converts bromo-4-chloro-3-indolyl glucuronide (X-Gluc) to a blue precipitate, which was observed under a light microscope (Leica) and imaged with a CCD camera.

2.4 Nucleic acid techniques

2.4.1 RNA extraction from plants

Plant tissue was collected in a 2mL Eppendorf tube containing a 4mm steel ball bearing. Tissues were snap-frozen in liquid nitrogen and vortexed until the tissue was reduced to a pale green powder. Total nucleic acid (TNA) was extracted using a phenol-chloroform extraction procedure adapted from (White and Kaper, 1989). Ground samples are transferred to ice and 600µL of ice-cold extraction buffer (100mM Glycine, 10mM EDTA, 100mM NaCl, 2% SDS, pH 9.5), freshly prepared from a 10x stock, was added. The solutions are vortexed to ensure proper homogenisation.

The homogenised solution was transferred to a 1.5mL Eppendorf tube containing 600µL phenol (pH 4), and vortexed for 10 seconds. The solutions were centrifuged for 10 minutes at 16,000 x g at 4°C. The upper phase was transferred to an ice-cold 1.5mL Eppendorf containing 600µL 25:24:1 phenol:chloroform:isoamyl alcohol. This was vortexed for 10 seconds then centrifuged for 10 minutes at 16,000 x g at 4°C. The upper phase is transferred to an ice-cold 1.5mL Eppendorf containing 500µL 24:1 chloroform:isoamyl alcohol, vortexed for 10 seconds, and centrifuged for 5 minutes at 16,000 x g at 4°C. The upper phase was transferred to an ice-cold 1.5mL Eppendorf containing 800µL absolute ethanol and 40µL 4M sodium acetate, mixed by inversion, and left on ice for 15 minutes to precipitate the TNA. TNA was pelleted by centrifuging for 15 minutes at 16,000 x g at 4°C, and supernatant was removed by aspiration. The pellet was washed gently with 70% (v/v) ethanol, and allowed to air-dry at room temperature for 10 minutes. The TNA pellet was re-suspended in ice-cold 30-50µL RNase-free water and stored at -80°C.

TNA was extracted from developing and mature seeds using the method described by (Oñate-Sánchez & Vicente-Carbajosa., 2008). Ground tissue was added to ice cold 550µl of extraction
buffer (0.4 M LiCl, 0.2 M Tris pH:8, 25 mM EDTA, 1% SDS) and 550µl chloroform, vortexed for 10 seconds, and centrifuged for 5 minutes at 16,000 x g at 4°C. The supernatant was transferred to an ice-cold 1.5mL Eppendorf containing 500µl of water-saturated acidic phenol, vortex thoroughly, add 200µl of chloroform, vortexed for 10 seconds, and centrifuged for 5 minutes at 16,000 x g at 4°C. Supernatant was transferred to an ice-cold 1.5mL Eppendorf and 1/3 volume 8 M LiCl was mixed in, and was left to precipitate TNA for 1 hour at -20°C. TNA was pelleted by centrifuging for 15 minutes at 16,000 x g at 4°C, and supernatant was removed by aspiration. The pellet was re-suspended in 470µl DEPC-water, 7µl 3 M NaAc pH:5.2 and 250µl ethanol, mixed well and spun 10 min at 4°C to precipitate carbohydrates. Supernatant was transferred to an ice-cold 1.5mL Eppendorf containing 43µl 3 M NaAc pH:5.2 and 750µl ethanol, and was left to precipitate TNA for 1 hour at -20°C. TNA was pelleted by centrifuging for 15 minutes at 16,000 x g at 4°C, and supernatant was removed by aspiration. The pellet was washed gently with 70% (v/v) ethanol, and allowed to air-dry at room temperature for 10 minutes. The TNA pellet was re-suspended in ice-cold 30-50µL RNase-free water and stored at -80°C.

2.4.2 Agarose gel electrophoresis
Total RNA yield and purity was determined with NanoDrop (Thermofisher); samples with a 260/280 ratio of ~2.0 were accepted as ‘pure’ and taken forward. The quality of RNA from the extracted TNA was examined with agarose gel electrophoresis. ~1µg TNA extract was added to an equal volume of 2x gel-loading solution (10ml deionized formamide, 200µl 0.5M EDTA, pH 8.0, 1mg xylene cyanol FF and 1 mg bromophenol blue), and denatured at 65°C for 5 minutes. The TNA-loading buffer solution was loaded onto a 1% TBE agarose gel and visualised under a UV light, looking for distinct bands of 18S and 28S ribosomal RNA. Samples with distinct bands were taken forward.

2.4.3 cDNA synthesis
DNA was then degraded from the TNA extract using the Ambion DNA-free™ kit or SIGMA Dnase I Kit (AMPD1). ~2µg TNA was incubated with 2U rDNasel in DNase I buffer for 20 minutes at 37°C. The DNase was degraded by addition of 2µL Stop Solution and incubation at 70°C for 10 minutes. Complementary DNA (cDNA) was then generated from the RNA in the DNase I treated TNA extract using a High Capacity cDNA Reverse Transcription Kit (Invitrogen, #4374966). The DNase I treated TNA extract was added to a PCR tube containing 4nM dNTPs, 1xRandom primers, 1xRT buffer, and 5U Multiscribe Reverse Transcriptase, and mixed by
pipetting. After brief centrifugation, the tubes were transferred to a thermal cycler set to: 10 minutes 25°C → 120 minutes 37°C → 5 minutes 85°C. cDNA was diluted in analytical grade water (1:4) and stored at -20°C.

### 2.4.4 Primer design

Primers for analysis of gene expression with qRT-PCR were designed to hybridise either side of an intron where possible, to avoid amplification from DNA contaminants. Table 2.3 contains a list of primers used for qRT-PCR analysis. Primers were designed with AtRTPrimer (Han & Kim, 2006).

#### Table 2.3 Primers for qRT-PCR

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2.4.5 Semi-quantitative Real-Time PCR (qRT-PCR)
cDNA template, qRT-PCR primers and SYBR Green Jump-startTM Taq Ready-MixTM (Sigma-Aldrich S5193), were pipetted into the wells of 96 well plates on ice to a final concentration of: ~500ng cDNA template, 300nM forward and reverse primers, 1.23U Taq DNA polymerase, 10mM Tris-HCl, 50 mM KCl, 2.5mM MgCl2, 0.2 mM dNTP, and 0.01% ROX reference dye. The 96-well plates are clear, Non-Skirted PCR Plates (STARLAB), and were sealed with a Microseal ‘B’ Adhesive seal (Bio-Rad MSB-1001). Each experiment contained 3-5 biological replicates, and 2-3 technical replicates to control for biological variation and pipetting error, respectively. The plate was centrifuged at 2000rpm briefly to eliminate air bubbles in a 3K15 centrifuge (Sigma, Germany). qRT-PCR cycling and fluorescence detection was performed in a Mx3005PTM Multiplex Quantitative PCR System (Stratagene, U.S.A. #401455) qRT-PCR machine operated through MxProTM software (Mx3005P v 4.10, Stratagene, U.S.A.). The thermal cycler was set to: 2 minutes 94°C → 40 cycles of (15 seconds 94°C → 1 minute 60°C) → 2 minutes 94°C → 1 minute 60°C → 2 minutes 94°C. ACTIN2 was the normalisation control in Chapter 4, UBQ5 was the normalisation control in Chapters 5 and 6. Amplification plots, dissociation curves and threshold fluorescence were viewed in MxPro software. Gene expression change between 2 samples (control vs. treated/mutant) was calculated using the (2 –ΔΔCT) method (Schmittgen and Livak, 2008), where CT = the threshold cycle determined by MxProTM software which uses the following equation:

\[ ΔCT = (CT \text{ gene of interest} – CT \text{ normalisation control}) \]
\[ −ΔΔCT = -[(ΔCT) \text{ sample 1} – (ΔCT) \text{ sample 2}] \]

Fold change = \( 2^{-ΔΔCT} \)

2.5 Microarray methods
2.5.1 Microarray (Dr. Manoj Valluru)
The microarray experiment was performed by Dr Manoj Valluru, another member of the Sorefan lab. 35S::IND:GR seeds were sterilised, stratified and germinated in liquid media as described above. At 7DAP, biological triplicates of seedlings were treated with a final concentration of 10µM DEX, 1µM BAP, 10µM IAA, 1µM BAP + 10µM IAA, 10µM DEX + 1µM BAP, 10µM DEX + 10µM IAA, 10µM DEX + 1µM BAP + 10µM IAA, or DMSO (mock) for 6 hours. TNA was extracted as described above. The microarray (Arabidopsis Gene 1.0 ST Array,
Thermofisher, #901915) was performed by Dr Paul Heath at the University of Sheffield core facility for microarray and next generation sequencing. RNA integrity and concentration was determined with an Aligent 2100 bioanalyser. Hybridization and scanning procedures were performed according to the manufacturer (Affymetrix) using the Affymetrix Gene Chip hybridisation system.

2.5.2 Microarray analysis

Analysis of the microarray data was performed by myself. Affymetrix® Expression Console™ software was used to process and normalise the Arabidopsis Gene 1.0 ST Array CEL files. Affymetrix® Transcriptome Analysis Console (TAC) software was used to analyse the Arabidopsis Gene 1.0 ST Array CHP files, and perform statistical analysis to create a list of differentially expressed genes. TAIR ID and protein annotation from Arabidopsis Gene 1.0 ST library files, were imported into Affymetrix® (TAC) software. Fold change (FC) was determined for Treatment1 vs. Treatment2 using $2^{\text{Condition1 Bi-weight Avg Signal (log2) - Treatment2 Bi-weight Avg Signal (log2)}}$. Treatment Bi-weight Avg Signal (log2) is the Tukey's Bi-weight average of exon intensity of all the samples in a condition. A threshold of FC > 1.5, FC < -1.5, and a One-Way Between-Subject ANOVA ($p$-value < 0.05) was used to filter differentially expressed genes. Further analysis was performed in Microsoft Excel.

Hierarchical CLICK clustering was performed using EXPression ANalyzer and DisplayER (EXPANDER) software. Gene Ontology (GO) analysis was performed with DAVID and PANTHER tools, the TAIR GO bulk data tool was also used for hunting for genes with a specific GO term. Promoter regions were gathered from TAIR, and searched for enriched motifs with the DREME tool. Pearson Correlation of microarray data was performed in Excel.

2.5.3 Analysis of published datasets

Published datasets were analysed either with tables of differentially expressed genes provided alongside the research, or by direct analysis of the datasets submitted to the Gene Expression Omnibus (GEO) database, using the GEO2R tool. Table 2.4 contains a list of public data sets analysed in this study.
Table 2.4. Data sets analysed

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2.6 Mammalian cell culture methods

2.6.1 Cell growth conditions, antibiotics and treatments

The hnRNP U Auxin-Inducible Degron (AID) cell-line was generated by Ms Catherine Heath from the HCT116-TIR1 human colorectal carcinoma cell-line that contains a functional Oryza sativa TIR1 degradation-promotion system. The hnRNP U AID and HCT116-TIR1 cell-lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies), supplemented with 10% foetal calf serum (FCS, Life Technologies).

hnRNP AID cells were selected in maintenance media supplemented with 800µg/ml Neomycin, 10µ/ml Blasticidin, 1µg/ml Puromycin and 150µg/ml Hygromycin (Thermo Fischer). IAA, BAP and tZ were added to the cell-line at a final concentration of 500µM for the hours stated in results.

2.6.2 SDS page

Gels for SDS-PAGE (resolving – 10% acrylamide, stacking 5% acrylamide) were prepared from a 30% acrylamide stock (Bio-Rad) with the following buffers (Resolving – 375mM Tris, pH8.8, 0.0375% (w/w) SDS; Stacking – 125mM Tris, pH6.8, 0.0375% (w/w) SDS. Gel polymerisation within Bio-Rad Mini-Protean II gel cast was facilitated with 0.1% (w/v) ammonium persulphate and 0.2% (v/v) TEMED. Cells were lysed in IP lysis buffer supplemented with 1x RiboSafe RNase Inhibitor (Bioline), 1mM DTT, and protease inhibitors (SIGMAFAST protease
inhibitor cocktail tablets), and centrifuged for 5 minutes at 13200rpm and 4°C. Bradford protein assay (Sigma-Aldrich). Samples for SDS-PAGE were boiled with 10% (v/v) β-mercaptoethanol and 1x SDS loading buffer (50mM Tris pH6.8, 100mM DiThioThreitol, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, 2% (w/v) SDS) at 95°C for 5 minutes, and loaded alongside a protein ladder. Gels were run in SDS-PAGE running buffer (25mM Tris, 250mM Glycine, 0.1% (w/v) SDS) at 25mA for an hour.

2.6.3 Western Blotting

A Bio-Rad fast transfer machine, set at 25V for 15 minutes, was used to transfer SDS-PAGE gels to nitrocellulose membranes. Membranes were blocked with milk solution (5% milk powder (w/vol), 0.2% Tween-20, 1x TBS) for 1 hour, and then primary antibodies diluted in milk solution were added for 1 hour. Table 2.5 contains the antibodies used in this study. After washing with TBST (20mM Tris, 0.137M NaCl, 0.2% Tween 20), the membranes were probed with secondary antibodies diluted in milk for 30 minutes, and then washed again in TBST. Antibody-bound proteins were detected by Enhanced ChemiLuminescence (ECL), using equal volumes ECL1 (100mM Tris-HCL pH8.5, 2.5mM Luminol, 400μM p-coumaric acid) and ECL2 (100mM Tris pH8.5, 5.3mM Hydrogen Peroxide), and the membrane was exposed and imaged using a Bio-Rad Chemidoc system. Spot densitometry was performed in ImageJ.

Table 2.5. Antibodies

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Chapter 3 – ARGONAUTE10 and INDEHISCENT regulation of seed development

3.1 Introduction

Seeds are an important source of calories and nutrition for the global population, and are excellent targets for genetic manipulation to improve agronomic and nutritional traits. Seeds comprise of three major regions, the embryo, endosperm, and seed coat (integument). Several genes that regulate seed size have been uncovered, many of which regulate the growth of the seed coat, generating a physical limit for growth within that capsule. Other genes regulate both integument and endosperm development, though it is unclear if the development of one is causal of the other. Finally, some genes regulate seed growth via endosperm development alone. Many of these seed size regulators are also parent-of-origin specific (imprinted) (Luo et al., 2005; Ohto et al., 2009; Yu et al., 2014; Sun et al., 2010). Genes that regulate seed size via endosperm development alone are usually paternally imprinted, and those that regulate seed size via the seed coat, or seed coat and endosperm, are typically maternally imprinted. Some genes involved in the signalling pathways of the major plant hormones, auxin and cytokinin, can also regulate seed size. The TF ARF2 from the auxin signalling pathway, the cytokinin oxidase CKX2, and cytokinin receptors AHKs, all contribute to the regulation of seed size (Schruft et al., 2006; Li et al., 2013; Riefler et al., 2006). Finally, infertility can also increase seed size, as the plant invests the same resources into fewer plants. The seed size increase from infertility is modest, ~15% (Hughes et al., 2008). Infertility in Arabidopsis thaliana can be caused by automatic self-pollination defects, aberrant pollen tube formation, and spontaneous abortion.

The INDEHISCENT (IND) gene encodes a member of an atypical class of bHLH TFs. IND is best known for its role in the regulation of fruit dehiscence, where it promotes proper valve margin formation to allow the mature silique to shatter and disperse its seeds. IND is able to form functional heterodimers with another bHLH TF, SPATULA (SPT). The IND-SPT heterodimer can coordinate the distribution of auxin via regulating the expression of PID and WAG2, which coordinate PIN protein polarity (Sorefan et al., 2009). Thus the IND-SPT heterodimer regulates auxin efflux. AP2, a seed size regulator negatively regulates IND (Bomblies et al., 1999; Ripoll et al., 2011). IND has not thus far been implicated in the regulation of seed size.

ARGONAUTE10 (AGO10) is a member of a family of RNA-mediated post-transcriptional gene silencers. AGO10 regulates floral stem cell termination, leaf polarity, SAM development. ago10 phenotypes are ecotype-specific. ago10 mutants in the Ler background, zll-3, show increased
organ size, stunted growth, aberrant silique development, premature termination of SAM stem cells, and an increased number of primary inflorescences. Such phenotypes show little or no prevalence in ago10 mutants in the Col-0 background, such as ago10-3. Furthermore, the stem cells phenotypes observed in zll-3 show variable penetrance, with even the strongest mutant alleles exhibiting 90% penetrance (Tucker et al., 2013). AGO10 expression has been detected in the globular stage of embryo development (Roodbarkelari et al., 2015), but has thus far not been implicated in the regulation of seed size. Unpublished data from the Sorefan lab has demonstrated that AGO10 and IND negatively regulate each other.

In this chapter, we investigate the role of AGO10 and IND in the regulation of seed size. We explore the nature of the seed size increase observed in zll-3 mutants, including physical characteristics, effects on fertility, and nutritional content. We also compile a list of candidate genes that may play a role in the AGO10-IND regulation of seed size worth further investigation.

3.2 Seed size

3.2.1 AGO10 regulates seed area
Unpublished data from the Sorefan lab demonstrated that the resultant overexpression of the bHLH transcription factor INDEHISCENT (IND) in zll-3 may cause many of those defects, and that a major role of AGO10 is to regulate the expression levels of IND. Whilst working with zll-3, we observed that it had noticeably larger seeds. To confirm this, we collected the mature, dry seeds of Ler and zll-3 plants grown in identical conditions, imaged them under a dissecting microscope and measured their length using ImageJ. zll-3 seeds were 23% longer than Ler seeds (Figure 2.1B). This increase in seed size also appeared to be ecotype-specific, as ago10-3 was 10% shorter in length (Figure 2.1A). The ind allele, ind-6, is an enhancer trap line which carries a GUS reporter gene and a Ds insertion. ind-6 and the zll-3 ind-6 double mutant line were used to determine whether the seed size phenotype observed in zll-3 required IND overexpression, as is the case with many zll-3 phenotypes (Moubayidin & Østergaard, 2014). ind-6 and ind-6/zll-3 seeds showed no increase in length, and so appeared to mostly rescue the zll-3 seed size phenotype (Figure 2.1B).

3.2.2 AGO10 and IND regulate seed 1000 seed weight
The most common method of detecting seed size phenotypes is 1000 seed weight (Liu et al., 2017). Here, mature dry seeds were harvested from Ler, zll-3, ind-6, zll-3 ind-6, Col-0, ago10-3
and ago10-4 plants grown in identical conditions. Sets of 1000 seeds were manually counted and weighed on an analytical balance (n=3-5). In the Ler ecotype, zll-3, ind-6 and zll-3 ind-6 seeds were 93%, 32% and 15% heavier than wild type, respectively (Figure 2.2B). In the Col-0 ecotype, ago10-3 was 13% % lighter (Figure 2.2A). This demonstrated that the AGO10 seed size phenotype is ecotype-specific, and, at least in part, requires IND overexpression. Interestingly, a decrease in IND expression in ind-6 increased seed size, but seed size also increased in zll-3, partially dependent on an increase in IND expression. This suggests that IND expression levels are regulated to minimise seed size.

3.2.3 AGO10 and IND regulate embryo size

In many crops, the seed embryo constitutes an important source of nutrition. To determine whether the zll-3 seed size phenotype may be of use to crop breeders, we measured the embryo size of mature Ler, zll-3, ind-6, zll-3 ind-6 seeds. Dry, mature seeds were imbibed in water for 60 minutes, and the embryo was excised from the seed coat (Fang et al., 2012). Embryos were imaged under a light microscope and measured with ImageJ. Cotyledon length from tip to start of petiole, and longest width perpendicular to the length, were measured and area was calculated using \((\text{length}/2)\times(\text{width}/2)\times\pi\), the formula for calculating the area of an oval. Root length from tip to base of petiole, and longest width perpendicular to the length were measured and volume was calculated using \((\text{width}/2)\times\text{length}\times\pi\), the formula for calculating the volume of a cylinder. Cotyledon area of zll-3 was increased, and was not rescued in the zll-3 ind-6 embryos (Figure 3.3A). Root volume was significantly increased in zll-3 ind-6 embryos, possibly additively (Figure 3.3B).

3.2.4 AGO10 and IND regulate cotyledon and root cell size

We then asked whether the increased cotyledon and root area seen in zll-3, ind-6 and zll-3 ind-6 was due to an increased cell size or cell number. Mature seeds were imbibed in water overnight, the embryos excised from the seed coat and then cleared in Hoyer’s solution (chloral hydrate:water:glycerol; 8:3:1; w/v/v) buffer for 30 minutes (Fang et al., 2012). Cleared embryos were visualised under a light microscope. Meosphyll cells were imaged and measured using ImageJ. Cotyledon mesophyll cell size was unchanged in zll-3 embryos, thus the increased cotyledon area must be due to an increased cell number (Figure 3.4). The cotyledon mesophyll cell size of ind-6 was 22% smal Ler (Figure 3.4A). Thus, the unchanged cotyledon area in ind-6 must be due to an increase in cell number large enough to compensate for the reduced cell size. zll-3 rescued the cell size phenotype of ind-6 in the double mutant.
Therefore, both zll-3 and ind-6 appear to increase cell number independently of each other, though there is no additive effect. In the root, mesophyll cell size was not significantly different in zll-3 ind-6 or zll-3 ind-6 embryos, suggesting that the size increase observed in zll-3 ind-6 roots is due to an additive increase in cell number operating in different pathways (Figure 3.4B).

3.2.5 AGO10 regulation of seed size occurs early in development

We then asked at which stage of seed development the zll-3 seed size phenotype appears. Unfertilised siliques were made accessible by removal of petals and sepals, and were manually fertilised. 1 day after pollination (DAP), fertilised developing seeds were dissected from the siliques, cleared with Hoyer’s solution (chloral hydrate:water:glycerol; 8:3:1; w/v/v) and imaged under a dissecting microscope. Unfertilised ovules were similarly tested. Stage 0 unfertilised embryos in zll-3 plants were ~40% larger than wildtype (Figure 3.5A). At 1 DAP, the developing zll-3 seeds were 43% larger than wild type (Figure 3.6). This is in line with the seed size area increases seen in the preliminary data. Thus the increase in seed size occurs very early in seed development. As the seed size increase in observed before fertilisation, it suggests this phenotype is maternally imprinted, which will be discussed further below.

3.2.6 AGO10 regulates seedling size

As zll-3 mature embryos are larger, we asked whether size was also increased in seedling early development. Seed size has been shown to be positively correlated with size-related seedling fitness and survival (Milberg and Lamont, 1997), so the zll-3 seed size phenotype may have a further agricultural benefit. Seeds from Ler, zll-3, ind-6 and zll-3 ind-6 were germinated and allowed to grow for 7 days, then seedling fresh weight was measured. zll-3 seedlings were 73% heavier than wild type, and this was not rescued by ind-6 (Figure 3.7).
Figure 3.1. **AGO10** regulates seed size. (A) *ago10* mutant in the Col-0 background reduced seed length. (B) *ago10* mutant in Ler background increased seed length, which was rescued in zll-3 ind-6 double mutants. Student t-test, 1 way ANOVA and Tukey’s multiple comparison test (letter = p<0.05, **p<0.01), n=30-40.

Figure 3.2. **AGO10** and **IND** regulate seed weight. 1000 seed weight was measured. (A) *ago10* mutant in the Col-0 background reduced seed weight. (B) *ago10* mutant in Ler background increased seed weight, ind-6 also increased seed weight, and the double mutant largely rescued the phenotype. Student t-test, 1 way ANOVA and Tukey’s multiple comparison test (letter = p<0.05, ***p<0.005), n=3.
Figure 3.3. AGO10 regulates embryo size. (A) zll-3 cotyledons were larger, not rescued by ind-6 (B) root volume was increased in zll-3 ind-6, possibly additively. (C-F) Representative embryo dissected from mature Ler, zll-3, ind-6 and zll-3 ind-6, respectively. 1 way ANOVA and Tukey’s multiple comparison test, (letter = p<0.05), n=25.
Figure 3.4. AGO10 and IND regulate cell size in the embryo. (A) *ind-6* cotyledon cell area was reduced, rescued in *zll-3 ind-6* double mutant. (B) *zll-3* root cell area was increased, rescued in *zll-3 ind-6* double mutant. 1 way ANOVA and Tukey’s multiple comparison test, (letter = p<0.05), n=10x10.

Figure 3.5. *AGO10* regulates ovule size. (A) *zll-3* ovule size was ~40% larger than wild type. Student t-test (***p<0.005), n=15.
Figure 3.6. AGO10 regulates size at early stages of seed development. (A) zll-3 globular stage seed size was ~40% larger than wild type. (B-C) Cleared globular stage seed of Ler and zll-3, respectively. Student t-test (***p<0.005), n=30.
Figure 3.7. AGO10 regulates seedling size. Seedling fresh weight was increased in zll-3, not rescued in the zll-3 ind-6 double mutant. 1 way ANOVA and Tukey’s multiple comparison test, (letter = p<0.05), n=25.

3.3 Seed yield and infertility

3.3.1 zll-3 mutants have reduced total fertility

We then asked whether the increased seed size led to an increase in total seed yield. Total seed was harvested from individual Ler and zll-3 plants, grown in identical conditions, and weighed. Combined with the 1000 seed weight data, we can estimate the number of seeds produced per plant. On average, Ler plants produced 48mg of seed (~4000 seeds), whilst zll-3 plants produced 37mg of seed (~1500 seeds), 60% fewer seeds than wild type (Figure 3.8A). Thus, although zll-3 seeds are almost twice as large as wild type, there is a small decrease (p = 0.053) in total seed yield (Figure 3.8B). Therefore, if zll-3 is to be useful in crops, the source of the reduced seed yield must be explored.

3.3.2 Altered expression of IND reduced fertility

One way that total seed yield can be decreased is by a reduction in fertility. Many seed size phenotypes are linked to infertility, as the plant divests the same resources into fewer seeds (Hughes et al., 2008; Jofuku et al., 2005; Ye et al., 2010). Reduced fertility in lines with increased seed size nullifies the seed size gains in terms of total seed yield. Therefore, we investigated fertility of zll-3 plants. Mature, but not dried, siliques from Ler, zll-3, ind-6 and zll-3 ind-6 plants, grown in identical conditions, were manually measured, and the mature seeds
dissected and counted under a dissecting scope. Silique length was halved in zll-3 plants, ind-6 siliques were unchanged, and ind-6 partially rescued the zll-3 silique length phenotype in the double mutant (Figure 3.9B). Interestingly, all 3 mutant lines showed reduced seed number/silique, and ind-6 again partially rescued the zll-3 phenotype in the double mutant (Figure 3.9A). As zll-3 and ind-6 both lead to decreased fertility, with the zll-3 phenotype dependent on IND overexpression, it suggests again that IND expression level is maintained to allow maximum fertility. Silique length is strongly correlated with seed number in wild type plants (Bac-Molenaar et al., 2015), we found that silique length was also strongly correlated with seed number in zll-3, ind-6 and zll-3 ind-6 plants (Figure 3.9 C-E). Therefore, for ease; silique length was used as a proxy of fertility in a later experiment.

3.3.3 Silique-specific infertility does not affect seed size

Previous studies have shown that whole-plant infertility can maximally increase seed size by 15%, as resources are shared between fewer seeds (Hughes et al., 2008). However, silique-specific fertility (number of seeds in the silique) has not been tested for effect on seed size. To test this, we created an assay in which unfertilised Ler siliques were manually fertilised with varying contact to pollen-bearing anthers, in order to create siliques with a broad fertility range. Siliques with between 2-47 seeds were yielded from the assay. Dry seeds were collected, imaged under a dissecting scope, and their volume estimated using \( \text{length} \times \text{width}^2 \times \pi \times (4/3) \), the formula for calculating the volume of a prolate ellipsoid. No significant trend was seen to link silique-specific fertility and seed size (Figure 3.10). Therefore, the zll-3 seed size phenotype is not linked to silique-specific fertility, and increasing silique-specific fertility in zll-3 plants would be a viable method of increasing total seed yield.

3.3.4 Ovule number is not reduced in zll-3 mutants

Reduced ovule number, failure to automatically self-pollinate, pollen tube defects, and spontaneous abortion can all reduce fertility in Arabidopsis thaliana. Ovule number was examined by excising ovules from siliques of Ler and zll-3 plants, and manually counting them under a light microscope. No difference in ovule number was observed (Figure 3.11).

3.3.5 The zll-3 mutant has aberrant automatic self-pollination

We then tested whether the zll-3 infertility phenotype was caused by automatic self-pollination failure, whereby there are physical development defects preventing proper anther-stigma positioning. Unfertilised zll-3 siliques were exposed and hand pollinated. For ease,
silique length was used as a proxy for fertility. Hand pollinated siliques saw a 15% increase in silique length, bringing the average length to 80mm, though they were still far shorter than wild type siliques (Figure 3.12). This suggests that automatic self-pollination failure accounts for some of the reduced fertility in zll-3 plants. This is probably caused by the deformation seen in zll-3 siliques and flowers (Ji et al., 2011).

3.3.6 zll-3 may have pollen tube defects

When dissecting maturing zll-3 siliques, we noted that many ovules appeared not to have been fertilised at all. They were not aborted, as early aborted seeds shrivel and turn brown, and late-aborted seeds turn a translucent white (Andreuzza et al., 2010; Agorio et al., 2017). The ovules of siliques that were mature but in which no seeds were developing were dissected from siliques, cleared with Hoyer’s solution (chloral hydrate:water:glycerol; 8:3:1; w/v/v) and imaged under a dissecting microscope. The size of these ovules was the same as unfertilised ovules from nascent siliques, and none contained developing embryos (Figure 3.13). Therefore we concluded that the zll-3 infertility phenotype is caused by a combination of automatic self-pollination failure, and pollen tube defects resulting in limited fertilisation.
Figure 3.8. *ago10* mutants have reduced yield. (A) *zll* plants developed a lower total seed mass than wild type. (B) Combined with 1000 seed weight data, *zll* plants produced less than half of the number of seeds per plant than wildtype. Student t-test (**p<0.005), n=8.

Figure 3.9. *ago10* mutants have reduced fertility. (A) *zll* plants grew fewer seeds per silique, not rescued by *zll* *ind*-6 double mutant. (B) Silique length of *zll* was reduced, partially rescued in the *zll* *ind*-6 double mutant. (C-E) Correlation of number of seeds per silique vs. length of silique in *zll*, *ind*-6, and *zll* *ind*-6, respectively. 1 way ANOVA and Tukey’s multiple comparison test, (letter = p<0.05), Pearson correlation coefficient, n=10.
Figure 3.10. Fertility of an individual silique does not affect the seed size. Dotted line is SE around the line of best fit. Pearson correlation coefficient, n=10.

Figure 3.11. zll-3 has the same number of ovules as wild-type. Student t-test, n=8.
Figure 3.12. Artificial pollination of *zll*-3 increased silique length, a proxy for fertility. Student t-test, n=30.

Figure 3.13. Nascent vs. mature siliques unfertilised ovules. In *zll*-3, non-developing ovules in mature siliques were the same size as unfertilised ovules in nascent siliques. Student t-test, n=10.
3.4 The zll-3 fertility phenotype is maternally imprinted

We then sought to determine whether the infertility phenotype was parent-of-origin specific, in order to identify the mechanism of this fertility defect. Ler and zll-3 plants were reciprocally crossed and seeds per silique were manually counted. Ler plants fertilised with zll-3 pollen showed no significant change in fertility vs. Ler plants fertilised with Ler pollen, and zll-3 plants fertilised with Ler pollen showed no significant change in fertility vs. zll-3 fertilised with zll-3. This result as well as the increased size of zll-3 ovules vs. Ler, suggests that the seed size phenotype in zll-3 is maternally imprinted.

Figure 3.14. The zll-3 fertility phenotype is maternally imprinted. (A) zll-3 plants fertilised with wild type pollen are less fertile than wild type, Ler plants fertilised with zll-3 pollen did not reduce fertility. 1 way ANOVA and Tukey’s multiple comparison test, (letter = \( p<0.05 \)), n=10.
3.5 Seed nutrition content

3.5.1 AGO10 and IND regulate seed protein content

Seed protein content is an important agricultural trait, providing consumers around the world with healthy sources of calories. The main role of seed storage proteins is to create a source of rapidly-accessible carbon, sulphur and nitrogen for the developing seedling. We investigated whether the increased seed size phenotype in zll-3 contained an increased amount of storage proteins. We extracted the total protein from 50 dry, mature seeds of Ler, zll-3, ind-6 and zll-3 ind-6 plants, grown in identical conditions, to determine their protein content, as described by Fatihi et al., 2013.

Only zll-3 showed a very small increase in protein content per seed (Figure 3.14A-B). Combined with 1000 seed weight data, zll-3 seeds contained slightly less than half of the protein as a proportion of seed mass (Figure 3.15A-B). ind-6 and zll-3 ind-6 showed a similar trend, but their reduced 1000 seed weight vs. zll-3 meant that they had a higher concentration of protein in the seed (Figure 3.15). Despite a lower concentration of protein in the seed, improving the fertility of zll-3 plants could provide a slight increase in total protein yield.

3.5.2 AGO10 and IND regulate seed sugar content

Sugar content of seeds is another important agricultural trait. As Arabidopsis thaliana seeds develop, the levels of insoluble (non-bio-accessible sugars), such as starch drop, and levels of soluble (bio-accessible sugars), such as glucose and sucrose, increase. The levels of these forms of sugars have different physiological effects. In early stages of development, higher starch levels are thought to promote the establishment of the embryo as a sink organ; further on in development, the dominant form is sugar is glucose, which promotes cell division; and towards maturation, higher levels of sucrose promotes the synthesis of various storage products. (Weber et al., 1997; Angelovici et al., 2006). It is interesting to note that inhibition of starch synthesis in the seed did not affect final sucrose content, thus the degradation of starch is unlikely to contribute to the ultimate increase in sucrose content, and sugar transporters are more likely to play the primary role (Vigeolas & Geigenberger, 2004). We extracted total soluble sugars from 50 dry, mature seeds of Ler, zll-3, ind-6 and zll-3 ind-6 plants, grown in identical conditions, as described by Fatihi et al., 2013.
zll-3 seeds contained 25% more sucrose than wild type (Figure 3.14E-F), however, when combined with 1000 seed weight data, zll-3 plants produce less sucrose as a proportion of seed mass. Ind-6 showed no change in seed sucrose content, and did not rescue zll-3 (Figure 3.14E-F). Glucose per seed was increased in ind-6 (Figure 3.14C-D). zll-3 showed no change in seed glucose content and did not rescue ind-6 (Figure 3.14C-D). Thus, sucrose appears to be regulated by AGO10, independently of IND, and seed glucose content appears to be regulated by IND, independently of AGO10. As glucose is converted to sucrose in the developing seed, and zll-3 seeds show a sucrose content increase without a corresponding decrease in glucose, it is probable that AGO10 promotes the transport of additional glucose into the seed, the excess of which is converted into sucrose.
Figure 3.15. AGO10 and IND regulate seed storage content. (A) zll-3 slightly increased seed protein content, but (B) as a proportion of seed mass, protein content was reduced in zll-3, ind-6, and zll-3 ind-6. (C) ind-6 increased seed glucose content, not rescued in the zll-3 ind-6 double mutant, but (D) glucose as a proportion of seed mass is unchanged in ind-6 and zll-3.
ind-6, in zll-3 glucose as a proportion of mass is reduced. (E) zll-3 increased seed sucrose content, rescued by the zll-3 ind-6 double mutant, but (F) sucrose content as a proportion of seed mass was reduced in zll-3 and ind-6. 1 way ANOVA and Tukey’s multiple comparison test, (letter = p<0.05), n=5.

3.6 AGO10 is expressed in the globular embryo and funiculus

We then asked where AGO10 is expressed in the developing seed. The pZLL::ZLL-YFP transgenic line was used to characterise AGO10-YFP expression in the developing seed. An argon laser (Ex. 514 nm) was used to detect YFP expression under a confocal microscope. Globular stage seeds were imaged, later stages of development were difficult to image due to obscuring material that accumulates in the seed. In the globular-stage seed, YFP was observed in the funiculus, embryo and chalazal endosperm (Figure 3.16). Combined with our seed sucrose content data, we predict that AGO10 plays a role in the movement of glucose into developing seeds via the funiculus.

Figure 3.16. Expression of AGO10-YFP in the globular stage developing seed. Fluorescence is localised to the funiculus, embryo and chalazal endosperm. Imaged with confocal fluorescence microscopy. False coloured in ImageJ. Representative image of 10 samples.
3.7  Seed size regulators

3.7.1  AGO10 does not consistently regulate known seed size regulators

Many genes have been reported to control seed growth and development, operating in various pathways (see Table 1.1 in Chapter 1). As AGO10 is involved in post-transcriptional gene silencing, we asked whether AGO10 regulated seed size via an already discovered pathway. Using qRT-PCR, we examined the gene expression of seed size regulating genes in: 7 day old seedlings, dry seeds, developing seeds 10 days after pollination (DAP), and developing seeds 5 DAP. Ler, zll-3, ind-6, and zll-3 ind-6 lines were used. Total RNA was extracted from dry and developing seeds as described by (Oñate-Sánchez & Vicente-Carbajosa, 2008). cDNA was synthesised from total RNA as described in Chapter 2. However, given the difficulty of cleanly extracting RNA from developing and mature seeds due to the high amount of carbohydrate, protein and lipid content, not all genotypes were able to be examined in all developmental stages. Apart from CKX2 and KLUH (Figure 3.17), no obvious and consistent change in the expression was seen in any seed size regulating gene (Supplementary Figure 1). CKX2 showed consistent downregulation in zll-3, ind-6, and zll-3 ind-6, at 5 DAP; and KLUH showed consistent upregulation in dry seeds; but these results are not consistent across developmental stages, and do not match the seed size phenotype relationship between zll-3, ind-6, and zll-3 ind-6 (Figure 3.17). The majority of changes observed were small in magnitude, and many changes would appear to decrease seed size based on previous studies. Finally, few changes are rescued by ind-6. Therefore, it is unlikely that AGO10 regulates seed size via a known seed size regulating pathway.

3.7.2  IND regulates a number of seed size candidate genes

Previously in the Sorefan lab, microarray analysis of the effect of IND upregulation on global gene expression was explored. The microarray itself and validity analysis was performed by Manoj Valluru, another member of the Sorefan lab, but they experiment will be briefly detailed here. All subsequent analysis was performed by myself.

7 DAG 35S::IND::DR seedlings were treated with: 10µM DEX (to upregulate IND) or DMSO (mock), for 6 hours in liquid media (n=3). Normalisation with Affymetrix® Expression Console™ software, and differential gene expression analysis with Affymetrix® Transcriptome Analysis Console (TAC) software was performed by myself. A linear fold change of >1.5 or <1.5 and a per-gene variance (ANOVA) p-value of <0.05 threshold was set to classify genes as differentially expressed by the treatments. A total of 2017 genes were differentially expressed
by the treatments. The microarray results were compared with a published IND overexpression dataset, and the expression patterns seen in the microarray were confirmed with qRT-PCR.

Compared to mock treatment, DEX treatment regulated 952 genes (380 upregulated and 572 downregulated). Gene ontology (GO) term analysis of the DEX regulated genes using the TAIR database, Table 3.1 contains 19 genes regulated by DEX treatment that have a GO term related to seed development, and are candidates for further investigation of how IND regulates seed size. Of particular interest is EOD3, a known seed size regulator, was regulated by IND in this microarray, though this was not confirmed in our qRT-PCR experiments (Figure 3.12E).

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Table 3.1. Candidate genes for regulating seed size in zll-3, regulated by IND, with GO terms related to seed development.

3.7.3 CESA9 and TZF1 expression patterns suggest they may regulate ecotype-specific seed size phenotypes

As the phenotypes of AGO10 knockout are ecotype-specific between Col-0 and Ler (Tucker et al., 2013), and the only genotypic difference between Col-0 and Ler is an ERECTA knockout in Ler (Tucker et al., 2013), which is not responsible for the zll-3 phenotypes, the phenotypes must be caused by differential expression of genes between the ecotypes. To examine this, we analysed the processed microarray files (GEO: GSE18482), which included data comparing Ler and Col-0 seedlings. Differentially expressed genes were calculated with NCBI’s GEO2R program (Smyth et al., 2004), with a threshold of >1.5 or <-1.5 and a per-gene variance (ANOVA) p-value of <0.05. 3566 genes were differentially expressed between Col-0 and Ler. This list of genes was then compared with our 952 IND-regulated genes. 208 candidate genes were identified that were differentially regulated between Col-0 and Ler, and regulated by IND overexpression. Of these, 22 genes had GO terms related seed development.

To test these candidates, we looked at their expression levels between Ler vs. Col-0, Ler vs. zll-3 vs. ind-6, and Col-0 vs. ago10-3 vs. ind-2. We hypothesized that any gene involved in the regulation of seed size in zll-3 and ind-6, and regulated by IND, would show altered expression levels in zll-3 mutants, which is oppositely altered in ind-6 mutants. They would also then
either show a different response in ago10-3 and ind-2, or be differentially expressed between Col-0 and Ler. Of the candidates tested, CELLULOSE SYNTHASE A9 (CESA9), TANDEM CCCH ZINC FINGER PROTEIN 5 (TZF5), and WRKY DNA-BINDING PROTEIN 60 (WRKY60) all showed opposing regulation of gene expression between zll-3 and ind-6, and different expression profiles between Col-0, ago10-3 and ind-2 (Figure 3.18). CESA9 showed reduced expression, and TZF1 expression was increased in Ler vs. Col-0. These genes are good candidates for further investigation of seed size regulation by IND.

3.7.4 IND, auxin and cytokinin transcriptomes reveal seed size regulator candidates

Genes involved in the signalling pathways of the phytohormones auxin and cytokinin have been demonstrated to regulate seed size. AUXIN RESPONSE FACTOR 2 (ARF2), a transcription factor in the auxin signalling pathway, and ARABIDOPSIS HISTIDINE KINASE proteins (AHK2, AHK3, and AHK4), cytokinin receptors, have been shown to negatively regulate seed size. CYTOKININ OXIDASE 2 (CKX2), a protein which catalyses the irreversible degradation of active cytokinins, has been shown to positively regulate seed size (Schruff et al., 2006; Li et al., 2013; Riefler et al., 2006). Thus, both auxin and cytokinin are important regulators of seed size.

It has recently been demonstrated that SPATULA (SPT), a bHLH transcription factor and important binding partner of IND, is important for the interaction between auxin and cytokinin, modulating the signalling of both pathways (Reyes-Olalde et al., 2017). Although the role of IND was not explored in that research, IND has been shown to modulate ARF3 and ARF8 (Marsch-Martinez and de Folter et al., 2016; Kay et al., 2013). IND has also been shown to be responsible for coordinating auxin effluxes to form an auxin minimum via the regulation of PINOID (Girin et al., 2011). IND has also been shown to regulate the cytokinin signalling pathway in the valve margins of developing fruit. Thus, the relationship between IND, and the phytohormones auxin and cytokinin was investigated to explore whether IND and AGO10 regulate seed size via the auxin and cytokinin pathways.

In addition to the microarray treatments performed by Dr. Manoj Valluru and detailed in Chapter 3.8.2, treatments with auxin and cytokinin were performed to investigate the role of IND in auxin and cytokinin signalling. 7 DAG 35S::IND::DR seedlings were treated with: 10µM DEX (to upregulate IND), 10µM IAA + 1µM BAP, 10µM DEX + 10µM IAA + 1µM BAP, or DMSO (mock), for 6 hours in liquid media (n=3). A linear fold change of >1.5 or <-1.5 and a per-gene
variance (ANOVA) p-value of <0.05 threshold was set to classify genes as differentially expressed by the treatments. Further quality control is detailed in Chapter 4.

Compared to mock treatment, DEX treatment regulated 952 genes (380 upregulated and 572 downregulated), auxin+cytokinin treatment regulated 2302 genes (1773 upregulated and 529 downregulated), and DEX+auxin+cytokinin treatment regulated 2356 genes (1351 upregulated and 1005 downregulated) (Figure 3.19). Of interest is the group of 774 genes regulated only in the presence of DEX+auxin+cytokinin, in this group either IND can only regulate these genes when auxin and/or cytokinin are present, or IND is required for the regulation of these genes by auxin and/or cytokinin. Gene ontology of this group of genes revealed a further 11 genes that have a gene ontology term related to seed development (Table 3.2), and are candidates for further investigation of how IND regulates seed size. Of particular interest is the regulation of RESPONSE REGULATOR 10 (ARR10), a positive regulator of the cytokinin signalling pathway, by IND only in the presence of auxin+cytokinin, which suggests that where auxin and cytokinin overlap, IND promotes a part of the cytokinin pathway, potentially to regulate seed size, as other parts of the cytokinin signalling pathway regulate seed size.

<table>
<thead>
<tr>
<th>ATG</th>
<th>GO term</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1G01070</td>
<td>Seed development</td>
<td>USUALLY MULTIPLE ACIDS MOVE IN AND OUT TRANSPORTERS 28</td>
</tr>
<tr>
<td>AT1G52880</td>
<td>Seed morphogenesis</td>
<td>ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 18</td>
</tr>
<tr>
<td>AT1G73030</td>
<td>Embryo development ending in seed dormancy</td>
<td>CHARGED MULTIVESICULAR BODY PROTEIN/CHROMATIN MODIFYING PROTEIN1A</td>
</tr>
<tr>
<td>AT2G40900</td>
<td>Seed development</td>
<td>USUALLY MULTIPLE ACIDS MOVE IN AND OUT TRANSPORTERS 11</td>
</tr>
<tr>
<td>AT3G15510</td>
<td>Seed morphogenesis</td>
<td>ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 56</td>
</tr>
<tr>
<td>AT3G46330</td>
<td>Embryo development ending in seed dormancy</td>
<td>MATERNAL EFFECT EMBRYO ARREST 39</td>
</tr>
<tr>
<td>AT3G48740</td>
<td>Seed maturation</td>
<td>SWEET11</td>
</tr>
<tr>
<td>AT4G31920</td>
<td>Regulation of seed growth</td>
<td>RESPONSE REGULATOR 10</td>
</tr>
<tr>
<td>AT5G17800</td>
<td>Regulation of seed growth</td>
<td>BRASSINOSTEROIDS AT VASCULAR AND ORGANIZING CENTER</td>
</tr>
<tr>
<td>AT5G53550</td>
<td>Seed development</td>
<td>YELLOW STRIPE LIKE 3</td>
</tr>
</tbody>
</table>
Table 3.2. Candidate genes for regulating seed size in zll-3, regulated by DEX+auxin+cytokinin only, with GO terms related to seed development.

Figure 3.17. Multiple seed size regulators have altered expression in zll-3, ind-6, and zll-3 ind-6 at various stages of development, however few are consistently changed. (A,B) expression profiles of CKX2 and KLUH, respectively, in Ler, zll-3, ind-6, and zll-3 ind-6 at 5 Days After Pollination (DAP), 10 DAP, dry seed and seedling stages of development. CKX2 showed consistent downregulation in zll-3, ind-6, and zll-3 ind-6, at 5 DAP; and KLUH showed consistent upregulation in dry seeds; but these results are not consistent across developmental stages, and do not match the seed size phenotype relationship between zll-3, ind-6, and zll-3 ind-6. Student t-test (*p<0.05, **p<0.01, ***p<0.005), n=2-4 biological replications, and 2 technical replications.
Figure 3.18. *TZF5* and *WRKY60* show ecotype-specific expression patterns in *ago10* and *ind* mutants. Expression profiles of genes in *ago10* and *ind* mutants: (A) *TZF5* in *Ler* background, (B) *TZF5* in *Col* background, and vs. *Ler* expression, (C) *WRKY60* in *Ler* background, (D) *WRKY60* in *Col* background, and vs. *Ler* expression. Student t-test, 1 way ANOVA and Tukey’s multiple comparison test (letter/*p*<0.05), n=4 biological replicates, and 2 technical replicates.
Figure 3.19. Venn diagram showing the number of genes regulated by treatment with DEX, Auxin+Cytokinin, DEX + Auxin+Cytokinin, and the overlapping genes which are regulated by multiple of those treatments. Importantly, 774 genes are regulated only in the presence of DEX+auxin+cytokinin, and 915 genes are regulated only in the presence of auxin+cytokinin, which suggests that in the presence of IND, the regulatory targets of auxin+cytokinin treatment change dramatically. Similarly, 395 genes are regulated only in the presence of DEX, but in DEX+auxin+cytokinin, those 395 genes are no longer regulated, in favour of a different 774 genes. This suggests that the presence of auxin+cytokinin can alter the regulatory targets of IND, presumably by either modulation of chromatin architecture of the differential regulation of different IND binding partners.
3.8 Discussion
3.8.1 Seed size
Seed size is an important agronomical trait, demonstrated by the gradual increase of crop seed size over thousands of years by selective breeding (Kesavan et al. 2013). Seeds provide >50% of the world’s agricultural calories (Tilman et al., 2011), and are essential sources of calories and nutrition for billions of humans. Increases in seed size have also been linked with seedling viability and vigour (Milberg and Lamont et al., 1997). As such, developments in seed size research provide new avenues for crop production which can have big impacts on food security and global malnutrition.

Here, we have shown that IND is a negative regulator of seed size. The expression levels of IND appear to be maintained to minimise seed size, as both knockout and overexpression of IND results in an increase in seed size. The use of IND in future crop development poses an interesting conundrum. One of the earliest agronomical traits believed to be introduced into crops by selective breeding is prevention of dehiscence (Sonnante et al., 2009). This means the seed-bearing ear of the crop does not shatter upon reaching maturity, seed is not scattered to the soil and the seed is more easily harvested. IND, as its name suggests, is an important gene in regulating this process, and may already mutated in many crops. If the seed size increase in ind-6 we observed also occurs in crop plants, it is possible that this ancient selection for indehiscence also coincided with a seed size increase, making it a powerful agronomic trait for early farmers. However, we have demonstrated that although knockout of IND can increase seed size, zll-3 can increase seed size even further, dependent on the overexpression of IND. Unfortunately, plants overexpressing of IND still dehisce, thus an important agronomic trait is lost. However, future studies may be able to delineate the genetic control of seed size and indehiscence by IND, which might enable us to develop crops with the seed size increase of overexpression of IND and the indehiscence of IND knockout. Having seen no obvious candidate from known seed size regulators, we have highlighted several more candidates through which IND may be regulating seed size for future investigators to pursue.

Interestingly, spt mutants have also been shown to have ecotype specific phenotypes. SPT promotes seed dormancy in the Col-0 background, and represses seed dormancy in the Ler background, exhibiting lower germination rates in Ler background spt mutants (Vaistij et al., 2013). Perturbed interaction between IND and SPT, the latter of which is already prone to ecotype-specific phenotypes, may be involved in the ecotype-specific phenotypes observed in
3.8.2 Fertility

We observed that although *zll-3* results in increased seed size dependent on the overexpression of *IND*, the total seed yield is decreased due to infertility. Once again, whilst *zll-3* greatly reduced fertility, dependent on *IND* overexpression, *IND* knockout also reduced fertility, suggesting that *IND* levels are maintained to maximise fertility in wild type plants. The *zll-3 ind-6* double mutant fertility was partially rescued, meaning that *AGO10* controls fertility two pathways, only one of which is *IND*-dependent. Nonetheless, the fertility decreases seen in *IND* overexpressing plants need to be understood and removed if *IND* overexpression is to be useful for crop development.

We concluded that fertility in *zll-3* plants, dependent on *IND* overexpression, reduces fertility in two ways. Firstly, the deformation of siliques (Ji et al., 2011) limits automatic self-pollination mechanically. The mechanical defects are reduced, though still present, in the double mutant, suggesting that the silique structural defects arise from a modified *IND*-AGO10 interplay. Secondly, as manual pollination only partly rescued infertility, and dissected siliques with developing seeds contain unfertilised rather than aborted seeds, pollen tube formation and growth towards the ovules is likely to be defected. Low levels of *IND* have been detected in pollen grains with a GUS insertion line, thus it is possible that *IND* plays a role in signalling between the pollen and the ovules (Girin et al., 2011). In a line overexpressing PsGA 2-OXIDASE2, a gibberellic acid (GA) degrading enzyme, loss of *IND* function partially rescued aberrant pollen tube growth that led to infertility, which seems like the likely mechanism occurring in *zll-3* plants (Kay et al., 2013). The *ind spt* double mutant has severe transmitting tract defects, thus a disrupted interaction between *IND* and SPT in *zll-3* may also be responsible for the reduced fertility. The fertility phenotype is maternally imprinted, thus genes regulating chemical signals that guide pollen tube formation are suitable candidates for future investigation. Of the 952 *IND*-regulated genes identified from our transcriptome data, 6 genes have GO terms relating to pollen tube formation and guidance (Table 3.3) Further investigation of the relationship between these genes and *IND* may explain the fertility phenotype.
Table 3.3 Candidate genes for regulating pollen tube guidance in zll-3, regulated by IND, with GO terms related to pollen tube development.

<table>
<thead>
<tr>
<th>ATG</th>
<th>GO term</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1G51800</td>
<td>Pollen tube</td>
<td>IMPAIRED OOMYCETE SUSCEPTIBILITY 1</td>
</tr>
<tr>
<td>AT4G08850</td>
<td>Pollen tube guidance</td>
<td>MDIS1-INTERACTING RECEPTOR LIKE KINASE2</td>
</tr>
<tr>
<td>AT5G04950</td>
<td>Pollen tube growth</td>
<td>ARABIDOPSIS THALIANA NICOTIANAMINE SYNTHASE 1</td>
</tr>
<tr>
<td>AT5G43285</td>
<td>Pollen tube guidance</td>
<td>ATLURE1.1</td>
</tr>
</tbody>
</table>

3.8.3 Nutrition

The sugar and protein contents of seeds are important agronomical traits that contribute to food security and nutrition. In early seed development sucrose is converted into hexose sugars by the enzyme invertase to maintain embryo division and expansion. Later, invertase activity disappears, hexose levels fall and sucrose becomes the main sugar in the endosperm (Barratt et al., 2009). Seeds overexpressing IND (zll-3) had increased levels of sucrose, and seeds with IND knocked out (ind-6) had increased levels of glucose. Thus, IND may regulate genes that downregulate the synthesis of invertase, reducing the conversion rate of sucrose to hexose sugars. Of the 952 IND-regulated genes identified from our transcriptome data, 13 genes have GO terms relating to glucose, sucrose and sugars. Further investigation of the relationship between these genes and IND may explain the sugar storage phenotype (Table 3.4). Several of the genes identified are sugar transporters, e.g. SWEET15 and SWEET16. As AGO10 appears to be expressed in the funiculus, the sole channel between the maternal plant and the developing seed (Larsson et al., 2017), it is also possible that IND plays a role in the regulation of these sugar transporters.
<table>
<thead>
<tr>
<th>ATG</th>
<th>GO term</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1G32900</td>
<td>ADP-glucose-starch glucosyltransferase activity</td>
<td>GRANULE BOUND STARCH SYNTHASE 1</td>
</tr>
<tr>
<td>AT1G56650</td>
<td>Sucrose mediated signaling</td>
<td>ARABIDOPSIS THALIANA PRODUCTION OF ANTHOCYANIN PIGMENT 1</td>
</tr>
<tr>
<td>AT1G78570</td>
<td>dTDP-glucose 4,6-dehydratase activity</td>
<td>ARABIDOPSIS THALIANA RHAMNOSE BIOSYNTHESIS 1</td>
</tr>
<tr>
<td>AT2G18950</td>
<td>Phloem sucrose loading</td>
<td>HOMOGENTISATE PHYTYLTRANSFERASE</td>
</tr>
<tr>
<td>AT2G22980</td>
<td>Sinapoylglucose-malate O-sinapoyltransferase activity</td>
<td>SERINE CARBOXYPEPTIDASE-LIKE 13</td>
</tr>
<tr>
<td>AT2G22990</td>
<td>Sinapoylglucose-malate O-sinapoyltransferase activity</td>
<td>SERINE CARBOXYPEPTIDASE-LIKE 8</td>
</tr>
<tr>
<td>AT2G43820</td>
<td>UDP-glucose:4-aminobenzoate acylglucosyltransferase activity</td>
<td>ARABIDOPSIS THALIANA SALICYLIC ACID GLUCOSYLTRANSFERASE 1</td>
</tr>
<tr>
<td>AT3G16690</td>
<td>Glucose transmembrane transporter activity</td>
<td>SWEET16</td>
</tr>
<tr>
<td>AT4G34590</td>
<td>Sucrose induced translational repression</td>
<td>RABIDOPSIS THALIANA BASIC LEUCINE-ZIPPER 11</td>
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<tr>
<td>AT4G36670</td>
<td>Glucose transmembrane transporter activity</td>
<td>POLYOL TRANSPORTER 6</td>
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<tr>
<td>AT5G13170</td>
<td>Sucrose transport</td>
<td>SWEET15</td>
</tr>
<tr>
<td>AT5G20830</td>
<td>Response to glucose</td>
<td>SUCROSE SYNTHASE 1</td>
</tr>
<tr>
<td>AT5G27350</td>
<td>Glucose transmembrane transporter activity</td>
<td>SFP1</td>
</tr>
</tbody>
</table>

Table 3.4 Candidate genes for regulating glucose and sucrose storage in zll-3 and ind-6, regulated by IND, with GO terms related to glucose and sucrose.

### 3.8.4 New candidates

The AGO10-IND did not appear to regulate tested known seed size regulators. AP2 is a seed size regulator and is known to down-regulate IND, it is possible that the seed size increase in ap2 mutants is due to an upregulation in IND. Although expression of ARF2 and CKX2 were not consistently altered, it remains possible that disrupting IND expression may alter auxin patterning via PID and WAG2, which may lead to a change in expression pattern of ARF2 and CKX2, if not total expression level. We did not explore whether the differential expression of
*SQUINT* between Col-0 and Ler played a role the ecotype-specific seed size phenotype; this should be explored further alongside our other candidates.
Chapter 4 – The Dual Hormone Response and Auxin Dominance

4.1 Introduction

Auxin and cytokinin are 2 of 10 known plant phytohormones, and have both been subjects of intense research. Classic experiments with auxin and cytokinin demonstrated that auxin and cytokinin have ratio-specific developmental effects (Skoog and Miller et al., 1957). Calli treated with a greater proportion of auxin to cytokinin developed into root tissues, and a greater proportion of cytokinin to auxin led to the development of shoot tissues. Despite this phenomenon, there is a dearth of research examining auxin + cytokinin treatments. Such research has typically been limited to crosstalk, where one hormone regulates genes involved in the biosynthesis, transport, signalling or degradation of another hormone. Between auxin and cytokinin, there is extensive crosstalk (Moubayidin et al., 2009).

Briefly, auxin is synthesised from Trp → IPA via TAA proteins, from IPA → IAA (the major active auxin) via YUCCA proteins. Auxins are transported into cells (influx) via proteins from the AUX1/LAX family, and out of cells (efflux) via PIN proteins. Inside cells, auxins act as a molecular glue between TIR1/SCF complexes and AUX/IAAs, allowing TIR1 to ubiquitinate and promote the degradation of the AUX/IAA releasing the ARF TF it was bound to. That ARF is then free to regulate the expression of auxin-responsive genes. Auxins are conjugated by GH3 proteins, irreversibly inactivating them. (Woodward & Bartel, 2005)

Briefly, the major cytokinin trans-zeatin (tZ) is synthesised from ATP or ADP in a series of biochemical steps, of which, the rate-limiting step is ATP/ADP binding to isoprenoid molecules catalysed by IPT proteins. PUP, ENT and ABCG transporter families are responsible for cytokinin influx and efflux, though little is known about them. AHKs are cytokinin receptors which, once bound by cytokinin, promote the phosphorylation of AHP proteins, which are translocated into the nucleus. In the nucleus, AHPs transfer the phosphate to ARRs, which are TFs that, upon phosphotransfer, regulate the expression of cytokinin-responsive genes. Cytokinins are irreversible degraded by CKX proteins. (Sakakibara et al., 2006)

In the development of lateral roots, crosstalk between auxin and cytokinin signalling pathways has been well characterised. Here, auxin promotes the formation of lateral roots, and cytokinin inhibits formation of lateral roots. Exogenous treatment with cytokinin disrupts the formation of auxin maxima via repression of PINs and regulation of PIN1 localisation, preventing proper lateral root formation (Marhavy et al., 2011).
The question of the existence of hormone hierarchies has been an enduring question in plant hormone studies. To date, no candidates for a central regulating mechanism of hormones have been found, and little dual-hormone treatment research has been undertaken to determine whether some hormone signalling pathways take precedence over others (Nemhauser et al., 2006).

In this chapter, we explore the transcriptome of Arabidopsis seedlings that have been treated with auxin+cytokinin, and examine the existence of a hormone hierarchy between auxin and cytokinin.

### 4.2 The Dual Hormone Response

#### 4.2.1 Auxin and cytokinin single hormone microarray

Whilst exploring the relationship between INDEHISCENT, auxin and cytokinin, we became interested in investigating further the relationship between auxin and cytokinin. The microarray experiment was performed by Manoj Valluru as detailed in Chapter 3.9. Normalisation with Affymetrix® Expression Console™ software, and differential gene expression analysis with Affymetrix® Transcriptome Analysis Console (TAC) software was repeated by myself. The treatments examined further in this chapter were: 10 µM IAA (auxin), 1 µM BAP (cytokinin), 10 µM IAA + 1 µM BAP (auxin+cytokinin), and DMSO (mock), for 6 hours on 7 DAG 35S::IND:GR seedlings. A basic examination of the transcriptome data as well as the full dataset have been published (Simonini et al., 2016). Previous microarrays analyses have used these treatment concentrations to elicit strong transcriptional responses (Zhao et al., 2003; Bishopp et al., 2011). The 6 hour time point was chosen to identify mid-late regulated genes (Vanneste et al., 2005).

A linear fold change of >1.5 or <-1.5 and a per-gene variance (ANOVA) p-value of <0.05 threshold was set to classify genes as differentially expressed by the treatments. A total of 2017 genes were differentially expressed by the treatments. Compared to mock treatment, auxin regulated 1005 genes (428 upregulated and 577 downregulated), and cytokinin regulated 691 genes (364 upregulated and 327 downregulated) (Figure 4.1A). These represent silos of genes that would be identified using a single hormone transcriptome analysis.
4.2.2 Comparisons with published datasets validate our transcriptome data

Our data overlapped with existing hormone treatment experiments which used a single hormone treatment. Nemhauser *et al.* identified a set of robust hormone-specific reporter genes after 30, 60 and 180 minute treatments of either auxin (1μM) or cytokinin (1μM) (Nemhauser *et al.*, 2006). 60% of their auxin reporter genes and 50% of their cytokinin reporter genes are equivalently up or down regulated in our data. In analyses by the AtGenExpress Consortium and Brenner, 30% of our auxin-responsive genes and 20% of our cytokinin-responsive genes respectively were equivalently regulated (60 minute treatment with auxin 1μM or 15/120 minute treatment with cytokinin 2μM) (Brenner *et al.*, 2005). Differences between the transcriptome results likely derive from different treatment times and concentrations used in each study. These results support the validity of the single-hormone-regulated transcriptome from the microarray analysis.

4.2.3 Auxin+Cytokinin treatment regulates a novel silo of genes

We also investigated the transcriptomic effect of treatment with auxin+cytokinin. Compared to mock treatment, the combined auxin+cytokinin treatment differentially regulated 1473 genes (794 upregulated and 679 downregulated) (Figure 4.1B). Of these 1473 genes, 964 were also regulated by either auxin or cytokinin treatment alone. The remaining 518 genes (~25% of total responsive genes) were only significantly regulated by auxin+cytokinin treatment, and would not have been identified by single-hormone treatments. We named this large transcriptional response to the combined auxin+cytokinin treatment the Dual-Hormone Response (DHR). Given the importance of auxin and cytokinin in determining cell fate, we hypothesized that the DHR would have a role in the formation of calli, coordination of cell differentiation factors; and regulating genes that maintain the stem cell niche. We also hypothesised that a group of TFs was responsible for the regulation of this silo of genes, and was responsive, in some manner, to the co-presence of auxin and cytokinin. Finally, we hypothesised that in lieu of obvious TF candidates, there would be evidence of chromatin structure alterations specific to the presence of auxin and cytokinin together. We also hoped to find individual, key genes that may provide a clear, distinct function for genes regulated by auxin and cytokinin together.
Figure 4.1. Venn diagrams of genes regulated by treatment with (A) Auxin and Cytokinin, and genes regulated by both treatments, (B) Auxin, Cytokinin, Auxin+Cytokinin, and genes regulated by multiple treatments. Importantly, 518 genes are regulated only in the presence of auxin+cytokinin, showing no regulation in single hormone treatments, this silo of genes represents the Dual Hormone Response. Table 4.1 shows the significance criteria for genes that fall into each area of the Venn diagram shown in (B).

<table>
<thead>
<tr>
<th>Venn diagram group – Genes regulated by treatment of:</th>
<th>Expression change after auxin treatment</th>
<th>Expression change after cytokinin treatment</th>
<th>Expression change after auxin+cytokinin treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Auxin</td>
<td>( p &lt; 0.05 )</td>
<td>( p &gt; 0.05 )</td>
<td>( p &gt; 0.05 )</td>
</tr>
<tr>
<td>Cytokinin</td>
<td>( p &gt; 0.05 )</td>
<td>( p &lt; 0.05 )</td>
<td>( p &gt; 0.05 )</td>
</tr>
<tr>
<td>Auxin+Cytokinin</td>
<td>( p &gt; 0.05 )</td>
<td>( p &gt; 0.05 )</td>
<td>( p &lt; 0.05 )</td>
</tr>
<tr>
<td>Auxin and Cytokinin</td>
<td>( p &lt; 0.05 )</td>
<td>( p &lt; 0.05 )</td>
<td>( p &gt; 0.05 )</td>
</tr>
<tr>
<td>Auxin and Auxin+Cytokinin</td>
<td>( p &lt; 0.05 )</td>
<td>( p &gt; 0.05 )</td>
<td>( p &lt; 0.05 )</td>
</tr>
<tr>
<td>Cytokinin and Auxin+Cytokinin</td>
<td>( p &gt; 0.05 )</td>
<td>( p &lt; 0.05 )</td>
<td>( p &lt; 0.05 )</td>
</tr>
<tr>
<td>Auxin and Cytokinin and Auxin+Cytokinin</td>
<td>( p &lt; 0.05 )</td>
<td>( p &lt; 0.05 )</td>
<td>( p &lt; 0.05 )</td>
</tr>
</tbody>
</table>

Table 4.1. Table of ANOVA values from the GeneChip data (generated in Transcriptome Analysis Console (TAC) software) used to allocate the regulated genes into the Venn diagram groups in Figure 4.1. \( p \)-values in bold show < 0.05 (our threshold for significance).
4.2.4 **Comparisons with published datasets validate our Dual Hormone Response data**

We then compared our DHR gene group to existing transcriptome analyses that indirectly treated with auxin and cytokinin. To our knowledge no transcriptome analysis after treatment with auxin+cytokinin has been performed to compare against. Ditt *et al.* performed a transcriptome analysis of the *Agrobacterium tumefaciens* tumour, which is known to increase auxin and cytokinin biosynthesis (Ditt *et al.*, 2006); 20% of our DHR gene group are equivalently regulated. Che *et al.* treated undifferentiated tissues with auxin and cytokinin to form a callus and analysed the callus transcriptome (Che *et al.*, 2006); 38% of our DHR gene group are equivalently regulated. These results support the validity of the DHR transcriptome from the GeneChip analysis.

4.2.5 **qRT-PCR validates our transcriptome data**

The total responsive genes in this experiment can thus be divided into a total of 7 categories (Figure 4.1A): 240 were regulated only by the auxin treatment, 276 by only the cytokinin treatment, 518 by only the auxin+cytokinin treatment, 29 by the auxin and the cytokinin treatments, 568 by auxin and auxin+cytokinin treatments, 218 by cytokinin and auxin+cytokinin treatments, and 168 by all three treatments. Table 4.1 shows which ANOVA values from the GeneChip data were used to allocate the regulated genes into the above groups. To further validate the microarray data, we confirmed the expression patterns of 18 genes, covering all the segments of the Venn diagram, using qRT-PCR. Col-0 seedlings were grown in 0.5% MS liquid media for 7 days, then transferred to 0.5% MS liquid media containing: 10µM IAA, 1µM BAP, 10µM IAA + 1µM BAP, or DMSO (mock). RNA was extracted and cDNA synthesised as described in Chapter 2. The results agreed with the microarray data (Supplementary Figure 2).

4.2.6 **The DHR includes several interesting genes**

Within the DHR gene group, several genes of interest were regulated. *ABA REPRESSOR1 (ABR1), RELATED TO AP2 6L (RAP2.6L)* and *ETHYLENE RESPONSE FACTOR 107 (ERF107)* are transcription factors involved in the ethylene signalling pathway, suggesting that some ethylene-responsive genes can also be regulated by the combination of auxin+cytokinin. *INDOLE-3-ACETIC ACID INDUCIBLE 20 (IAA20)*, a negative regulator of the auxin response, and *ARABIDOPSIS PIN-FORMED (PIN4)*, an auxin transporter, are also regulated only in the
presence of auxin+cytokinin, which suggests the existence of an auxin feedback loop restricted to tissues with localisation of auxin and cytokinin. The cell expansion regulators EXPANSIN A1 (EXPA1) and ARABIDOPSIS THALIANA EXPANSIN 10 (EXPA10) are also in the DHR gene group, suggesting a role for auxin and cytokinin cooperative regulation in cell wall modification.

4.2.7 GO term analysis reveals potential role for DHR in abiotic stress

To determine possible functions of the DHR, we performed Gene Ontology (GO) analysis using DAVID (Huang et al., 2008). The only significantly enriched functional categorisation was abiotic stress. To confirm this result, we compared our DHR gene group with those of studies looking at transcriptome changes in Arabidopsis under abiotic stresses. 11% of our genes were equivalently regulated by drought stress, 3% by cold stress, 7% by high light stress, and 15% by hypoxia; for all these stresses, our genes were enriched 2-4x more than the background genomic level (Liu et al., 2005; Huang et al., 2008; Rasmussen et al., 2013). The full list of genes can be found in Supplementary Table 1, but particular genes of interest include: the jasmonic acid (JA)–induced TF, RELATED TO AP2 6L; JASMONATE-ZIM-DOMAIN PROTEIN 1, a nuclear-localized protein involved in jasmonate signalling; ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 1, a TF within the ethylene signalling pathway; CYTOKININ RESPONSE FACTOR 4, a TF involved in the cytokinin signalling pathway and SCARECROW-LIKE 3 which promotes gibberellin signalling. Together, these suggest that the DHR cross-talks with other signalling pathways during abiotic stress. This validates the GO result and suggests that the DHR plays a broad role in responding to abiotic stress. A possible explanation is that Arabidopsis thaliana responds to abiotic stress by increasing biosynthesis of auxin and cytokinin, resulting in tissue localisation overlap between these hormones and subsequent regulation of the DHR, some genes of which can improve resistance to the abiotic stress. Future experiments examining whether auxin+cytokinin treatment can provide resistance to abiotic stresses, could be used to determine whether such treatment could be helpful in the improvement of crops.

We also examined the functional classification of the DHR using PANTHER (Mi et al., 2018) (Figure 4.2). A large proportion of the DHR was involved in metabolic processes; cytoskeletal proteins (mostly proteases) and transporter proteins (including ABCs, and transporters of amino acids, carbohydrates and cations) are among the highest represented classes; and the DHR also contains genes from several pathways, including the ubiquitin proteasome pathway. (Figure X). None of these categories are statistically enriched, but they may provide insight into DHR functions. For example, both auxin and cytokinin can be transported by ABC transporters,
ABC5 and ABC13 are downregulated in the DHR, whilst ABC3 is upregulated, suggesting the DHR could regulate the localisation of auxin and cytokinin. *UBQUITIN-CONJUGATING ENZYME 12* (UBC12) is also downregulated, and as the TIR1 pathway requires ubiquitination of Aux/IAAs, it’s possible that the DHR modulates the auxin pathway.

### 4.2.8 The DHR contains 6 distinct clusters

To further investigate the DHR gene group, we used CLICK clustering to group the DHR gene silo for further examination (Sharan and Shamir, 2000). CLICK clustering generated six groups with two singletons (Figure 4.3). Cluster 1 contains genes that may have a sub-threshold upregulation by auxin and cytokinin treatments which increase additively in auxin+cytokinin treatment. Cluster 2 contains genes that may have a sub-threshold downregulation by auxin and cytokinin treatments which decreased additively in auxin+cytokinin treatment. Cluster 3 contains genes which show sub-threshold upregulation by auxin, no change by cytokinin, and upregulation by auxin+cytokinin. Cluster 4 contains genes which show sub-threshold downregulation by auxin, no change by cytokinin, and downregulation by auxin+cytokinin. Cluster 5 contains genes which show no change by auxin, sub-threshold downregulation by cytokinin, and downregulation by auxin+cytokinin. Cluster 6 contains genes which show no change by auxin, sub-threshold upregulation by cytokinin, and upregulation by auxin+cytokinin. It is interesting that although many genes in the transcriptome data showed little or no changes in expression in single hormone treatments, no resultant cluster formed. Instead, Clusters 3-6 are characterised by one of the hormones having no transcriptional effect, but amplifying the sub-threshold transcriptional change of the second hormone to create an above-threshold transcriptional change in the dual treatment. In Clusters 3 and 4, cytokinin appears to amplify the regulatory effect of auxin, whilst itself having no transcriptional effect. In Clusters 5 and 6, auxin appears to amplify the regulatory effect of cytokinin, whilst itself having no transcriptional effect. This amplification of gene expression will be examined more thoroughly below. The generation of clusters can also be useful when searching for TFs that regulate the DHR, as genes with similar expression profiles are more likely to be regulated by the same TF(s).

### 4.2.9 DHR motif analysis with DREME and TOMTOM reveal potential role for SPT

We then asked if the DHR was regulated by a transcription factor(s) (TFs). To explore this, we downloaded the upstream sequences of the DHR genes from TAIR and used DREME software to look for enriched DNA motifs which might be used to regulate the DHR. Five sequences of
interest were generated: CCACGT(G/C), CAAAACA(G/A), ATATAT(T/G)(T/G), TATAT(A/G)TA, and (A/C)CACGT(G/C) (Figure 4.4). The resulting motifs were then put into TOMTOM software to search for transcription factors that may bind these enriched motifs. The CCACGT(G/C) motif could be bound by a series of bHLH transcription factors, of which, *SPATULA* (*SPT*), a binding partner of INDEHISCENT (*IND*) involved in flower development and seed dormancy, and *BRASSINAZOLE-RESISTANT 1* (*BZR1*), a positive regulator of the brassinosteroid signalling pathway, were upregulated in our transcriptome. These TFs represent potential candidates for control of the DHR worth exploring. (Figure 4.4). It is possible that although SPT is not regulated in the DHR, one of its binding partners may be; alternatively, the presence of auxin+cytokinin and/or differential regulation DHR genes could promote the differential binding of SPT from one TF to another. We know, for example, that IAA can modulate the binding between ETTIN and IND, a binding partner of SPT. Thus exploring the role of IAA+BAP/tZ in modulating bHLH TF binding partners would be interesting to examine, especially in the context of regulating the DHR. In the future, concatenated motifs found here could be used with a reporter gene so determine specific sensitivity to auxin+cytokinin treatment.
Figure 4.2. Results GO term and pathway analysis of the Dual Hormone Response silo of genes in Panther. (A) Categorisation of DHR by biological process, dominated by ‘Metabolic process’. (B) Categorisation of DHR by protein class, largely represented by cytoskeletal proteins and transporter proteins. (C) Further breakdown of the cytoskeletal protein class of proteins, dominated largely by proteases. (D) Further breakdown of the transporter protein class of proteins, containing ABCs and amino acid transporters. (E) List of known pathways with genes represented in the DHR.

Figure 4.3. Dual-hormone regulated genes cluster into 6 Clusters. Cluster 1 (171 genes) is upregulated when treated with auxin+cytokinin. Cluster 2 (128 genes) is downregulated when treated with auxin+cytokinin. Cluster 3 (77 genes) is upregulated when treated with auxin+cytokinin, and cytokinin is slightly downregulated. Cluster 4 (57 genes) is downregulated when treated with auxin+cytokinin, and auxin is slightly downregulated. Cluster 5 (46 genes) is downregulated when treated with auxin+cytokinin, and cytokinin is slightly downregulated. Cluster 6 (43 genes) is upregulated when treated with auxin+cytokinin, and cytokinin is slightly upregulated. Clusters were generated with CLICK clustering, with 2 singletons that could not be clustered. Importantly, Clusters 3&4 show genes which cytokinin does not itself regulate,
but the presence in dual-hormone treatments may amplify the below-threshold regulation by auxin, and Clusters 5&6 show vice-versa.

Figure 4. SPT and BZR1 binding motifs are enriched in the DHR gene set. Enriched motifs were generated from the promoter regions of the DHR gene set using DREME software. TOMTOM software was used to match these motifs to SPT and BZR1 binding sites.

4.2.10 SPT may play a small role in regulating the DHR
We then asked whether SPT played a role in regulating the DHR. Col-0 and spt-12 seedlings were grown in 0.5% MS liquid media for 7 days, then transferred to 0.5% MS liquid media containing: 10µM IAA, 1µM BAP, 10µM IAA + 1µM BAP, and DMSO (mock), for 6 hours. RNA extraction and cDNA synthesis was performed as described in Chapter 2. The expression levels of genes identified as DHR-responsive was measured in spt-12 after the dual hormone treatment. The expression levels of PHOT2 and KNAT3 are significantly downregulated in both Col-0 and spt-12, however, the downregulation of KNAT3 is significantly reduced in spt-12 (Figure 4.5). Together, this suggests that SPT plays a role in regulating the DHR gene set, probably among various other TFs.

4.2.11 Auxin+cytokinin treatment alters regulatory targets of IND
The possibility of SPT regulating the DHR prompted us to also examine the role of INDEHISCENT (IND), a bHLH TF and binding partner of SPT, in regulating the DHR. Comparison of DEX, auxin+cytokinin (in which auxin and cytokinin treatments alone did not alter expression, hereafter referred to as auxin+cytokinin-DHR), and DEX + auxin+cytokinin-DHR responsive transcriptomes, revealed a fascinating interaction between IND and the DHR silo.
949 genes were regulated by DEX treatment, 531 genes by auxin+cytokinin-DHR, 150 genes by DEX + auxin+cytokinin-DHR. Importantly, of the 531 genes regulated by auxin+cytokinin-DHR, only 46 continue to be regulated when IND is overexpressed (Figure 4.6). This means that the majority of the DHR silo stops being regulated when IND is overexpressed, and a new, smaller silo of 104 genes is preferentially regulated. Thus, IND appears to inhibit the regulation of the DHR silo.

Figure 4.5. SPT plays a small role in the regulation of DHR genes. Gene expression profiles after treatment with 10µM IAA, 1µM BAP, 10µM IAA + 1µM BAP, or DMSO (mock) for 6 hours in 7 DAG Col-0 and spt-12 seedlings. (A) auxin+cytokinin downregulation of KNAT3 is reduced in spt-12 vs. Col-0. (B) There is no difference in the expression of PHOT2 between Col-0 and spt-12 in any hormone treatments. (C) SPT is required for upregulation of CKX1 by cytokinin treatment. (D) SPT increases the upregulation of CKX3 by auxin and cytokinin treatments, but doesn’t affect regulation by auxin+cytokinin treatment. Student t-test (*p<0.05, **p<0.01, ***p<0.005), n=4 biological replicates and 2 technical replicates.
4.2.12 The DHR may be regulated by altered chromatin architecture

A possible method by which the DHR is regulated is by the modulation of chromatin architecture. Genes can be hidden or exposed from transcription factors by condensation or opening of the surrounding chromatin region. Chromatin remodellers post-translationally modify histone proteins to promote the tighter or looser binding of DNA to the histones. Modifications to histone proteins include: methylation, acetylation, phosphorylation, and ubiquitination (Clapier & Cairns, 2009). We hypothesized that the DHR genes may be in regions of condensed chromatin that are only opened in the presence of auxin+cytokinin regulated chromatin remodellers.

A list of chromatin remodellers was generated using GO term data from TAIR (Berardini et al., 2015), and compared with microarray data to see if any were regulated by auxin, cytokinin or
auxin+cytokinin. 10 candidates were found and their expression profiles were confirmed with qRT-PCR. ACTIN-RELATED PROTEIN 9 (ARP9), AT4G38495, BUSHY GROWTH (BSH) and SWITCH/SUCROSE NONFERMENTING 3C (CHB) were all significantly downregulated in auxin+cytokinin treatment, and not significantly changed by auxin or cytokinin treatment alone (Figure 4.7). Auxin+cytokinin treatment was not significantly different from cytokinin treatment alone. ARP9, BSH and CHB are present in SWI/SNF and RSC complexes, which can be recruited to chromatin via post-transcriptional histone and nucleosome modifications (Jégu et al., 2015). SWI/SNF and RSC complexes both act as transcriptional activators (Jégu et al., 2015). Therefore, the downregulation of SWI/SNF and RSC complex protein components might promote condensation of particular regions of chromatin. This may be a pathway of regulation of the down-regulated set of genes in the DHR.
Figure 4.7. Chromatin remodellers are downregulated after treatment with auxin+cytokinin. Gene expression profiles after treatment with 10μM IAA, 1μM BAP, 10μM IAA + 1μM BAP, or DMSO (mock) for 6 hours in 7 DAG Col-0 seedlings. (A-D) expression levels of ARP9, AT4G38495, BSH, and CHB, respectively, are downregulated after treatment with auxin+cytokinin, which is significantly different from mock and auxin treatments, but not cytokinin treatments. 1 way ANOVA and Tukey’s multiple comparison test (letter/*p<0.05), n=4 biological replicates and 2 technical replicates.
4.3 Auxin dominance

4.3.1 Auxin+cytokinin treatment is more closely correlated with auxin treatment than cytokinin.

An enduring question in hormone research has been whether hierarchies of hormones exist, in which particular hormones exert an unequally reciprocated regulatory control over another hormone, or hormone regulation of a developmental process takes precedence over than of another hormone. During our analysis, we observed that the transcriptome of auxin+cytokinin treatment appeared more closely related to the regulation pattern of auxin closer than it did of cytokinin. Pearson Correlation between auxin and auxin+cytokinin revealed a very strong positive relationship, with a correlation coefficient of 0.87 (Figure 4.8A). The correlation coefficient between cytokinin and auxin+cytokinin demonstrates a strong but markedly weaker relationship, 0.60 (Figure 4.8B). This analysis constitutes an early examination pointing us in a direction of investigation rather than firm evidence, as it is possible that this relationship is caused by the 10:1 auxin:cytokinin ratio used in the microarray, and a 1:1 ratio might show a markedly different relationship. It is also possible that a number of “outliers” with very high levels of expression in auxin and auxin+cytokinin treatments skew the data, creating a stronger correlation. Later evidence, however, does point towards the concentration of auxin setting the expression level of genes regulated both by auxin and cytokinin.

In addition, of the genes regulated by cytokinin treatment, 40% were differentially expressed when auxin is also present (cytokinin vs. auxin+cytokinin), whereas the additional presence of cytokinin differentially affects only 30% of auxin-regulated genes (auxin vs. auxin+cytokinin). This suggests that although auxin and cytokinin can each influence the regulatory silos of the other, auxin may be slightly dominant, having more control over cytokinin than cytokinin has over auxin.
Whole transcriptome expression changes after treatment with auxin+cytokinin are more similar to the expression changes after treatment with auxin than cytokinin. Relative fold change expression of the examined transcriptome after auxin+cytokinin treatment vs. (A) relative fold change expression after auxin treatment, and (B) relative fold change expression after cytokinin treatment. Pearson’s correlation coefficient.

4.3.2 Expression of genes under dual-hormone concentration gradients reveals amplified regulation between auxin and cytokinin regulation of genes

We then sought to dissect the hierarchical relationship between auxin and cytokinin at an individual gene expression level. To explore this, Col-0 seedlings were grown in 0.5% MS liquid media for 7 days, then transferred to 0.5% MS liquid media containing: IAA (10nM, 100nM, 1μM, 10μM, and 100μM) +/- 1μM BAP, and BAP (10nM, 100nM, 1μM, 10μM, and 100μM) +/- 1μM IAA, and DMSO (mock) for 6 hours. RNA was extracted and cDNA synthesised as described in Chapter 2. We then measured the changes in expression levels under these treatments of 5 genes from the A-AC group of the Venn diagram, meaning they are auxin-responsive and not cytokinin-responsive, and 5 genes from the C-AC group of the Venn diagram, meaning they are cytokinin-responsive and not auxin-responsive. From the A-AC group *INDOLE-3-ACETIC ACID INDUCIBLE 1 (AUX/IAA1), INDOLE-3-ACETIC ACID INDUCIBLE 5 (AUX/IAA5), INDOLE-3-ACETIC ACID INDUCIBLE 19 (AUX/IAA19), AUXIN RESPONSE FACTOR 19 (ARF19), and LOB DOMAIN-CONTAINING PROTEIN 18 (LBD18)* were investigated. From the C-AC group *RESPONSE REGULATOR 3 (ARR3), RESPONSE REGULATOR 4 (ARR4), ARABIDOPSIS HISTIDINE KINASE 4 (AHK4), NAP1-RELATED PROTEIN 1 (NRP1), and CYTOKININ OXIDASE 4 (CKX4)* were investigated.
This analysis revealed several trends which are demonstrated by the Aux/IAA5, ARR4 and ARR15 (Figure 4.9), the full data set can be found in Supplementary Figure 3. Firstly, for Aux/IAA5 and the A-AC genes, cytokinin treatment alone did not alter expression; however, when combined with auxin, cytokinin tended to amplify gene upregulation vs. auxin treatment alone (Figure 4.9A; Supplementary Figure 3A,C,E,G,I,K,M,O,Q,S,U,W). Pearson correlation coefficients revealed that Aux/IAA5 showed a very strong relationship between IAA concentration and expression level, and the strength of this relationship showed a modest decrease upon addition of 1μM BAP (Figure 4.9B; Supplementary Table 2; Supplementary Figure 3B,F,J,K,N,R,V). An increasing concentration of BAP + stable concentration of IAA did not further amplify expression, and Pearson correlation revealed low correlation between the concentration of BAP and gene expression levels (Figure 4.9C,D; Table 4.3; Supplementary Table 2; Supplementary Figure 3D,H,L,P,T,X). Thus, expression levels of genes under the transcriptional control of auxin can be: amplified by additional cytokinin; remain sensitive to auxin concentrations; and are mostly insensitive to cytokinin concentrations.

A similar trend appeared for C-AC genes, focusing on ARR4 and ARR15, with the remaining data set found in Supplementary Figure 4. Auxin treatment alone did not alter expression; however, when combined with cytokinin, auxin tended to amplify gene upregulation vs. cytokinin treatment alone, such as for ARR15 (Figure 4.9I; Supplementary Figure 4A,E,I,M,Q,U). Indeed, the presence of auxin served to enable expression of a cytokinin-regulated gene to a level equivalent to a high concentration of cytokinin, when the cytokinin concentration was in fact orders of magnitude lower. Interestingly, the amplified expression curve in auxin+cytokinin treatments appears to be dependent on the stable auxin concentration, rather than the increasing cytokinin concentration. Pearson correlation coefficients revealed that all 6 genes analysed showed a very strong relationship between BAP concentration and expression level. The strength of these relationships is dramatically reduced upon addition of 1μM IAA, such as for (Table 4.3; Supplementary Table 3; Supplementary Figure 4A,C,E,G,I,K,M,O,Q,S,U,W). Fascinatingly, Pearson correlation analysis of gene expression levels upon treatment with 1μM BAP + increasing IAA concentrations showed a strong relationship with IAA concentration levels (Table 4.3; Supplementary Table 3). Thus, genes whose expression is sensitive to cytokinin concentrations, upon addition of auxin become desensitised to cytokinin concentrations and sensitive to auxin concentrations.
Together, this suggests that both auxin and cytokinin can amplify the expression of genes that are unresponsive to their own signalling pathways, presumably by interacting with the activity of the other hormone’s signalling pathway; and that the concentration of auxin is dominant in setting the expression level, both for genes responsive and unresponsive to auxin treatment alone. This dominance is highlighted by the drop in expression levels at high concentrations of BAP (100μM), which retains the consistent expression level set the addition of 1μM IAA (Figure 4.9E,I).

In support of these findings, in our analysis of the transcriptome we found that 35% of the cytokinin-responsive genes, were differentially regulated upon the addition of auxin treatment (cytokinin vs auxin+cytokinin), but not differentially regulated by auxin alone. This means that auxin alters the expression of 354 genes only through the cytokinin signalling pathway. Conversely, 25% of the auxin-responsive genes were so altered by the addition of cytokinin; this means that cytokinin alters the expression of 331 genes only through the auxin signalling pathway. Thus, both hormones appear to alter and reinforce the regulation of genes through the other, but auxin appears to do so in a greater proportion of the cytokinin-responsive gene set than vice-versa.

<table>
<thead>
<tr>
<th>Gene</th>
<th>$r^2$ – IAA [range]</th>
<th>$r^2$ – 1μM BAP + IAA [range]</th>
<th>$r^2$ – 1μM IAA + BAP [range]</th>
<th>$r^2$ – 1μM IAA + BAP [range]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aux/IAA5</td>
<td>0.9958</td>
<td>0.8413</td>
<td>0.7391</td>
<td>0.3671</td>
</tr>
<tr>
<td>Gene</td>
<td>$r^2$ – BAP [range]</td>
<td>$r^2$ – 1μM IAA + BAP [range]</td>
<td>$r^2$ – 1μM BAP + IAA [range]</td>
<td>$r^2$ – 1μM BAP + IAA [range]</td>
</tr>
<tr>
<td>ARR15</td>
<td>0.8692</td>
<td>0.7416</td>
<td>0.6708</td>
<td>0.6189</td>
</tr>
<tr>
<td>ARR4</td>
<td>0.9674</td>
<td>0.5626</td>
<td>0.6871</td>
<td>0.8418</td>
</tr>
</tbody>
</table>

Table 4.3. Pearson correlation values for C-AC genes under various hormone concentration gradients, and the probability of statistical difference between BAP [range] and 1μM IAA + BAP [range]. Importantly, examining Aux/IAA5 from left to right, the expression of Aux/IAA5 is strongly correlated with the concentration of auxin, a correlation only modestly reduced by the additional presence of cytokinin, with no significant difference between the correlations; finally, the amplified expression of Aux/IAA5 shows little correlation with the concentration of...
cytokinin. Examining ARR4 from left to right, the expression of ARR4 is strongly correlated with the concentration of cytokinin, and the strength of this correlation is reduced, though not significantly, and finally, the amplified expression of ARR4 shows a strong correlation with the concentration of auxin. Together, the trends of the data presented here, and in Supplementary Figures 3 & 4 and Supplementary Tables 2 & 3, suggest that indirectly regulates the expression of genes under the control of cytokinin in an auxin-concentration-responsive manner; but cytokinin does not do the same. Data excludes BAP 100µM which breaks the cytokinin responsiveness trend and skews the data.
Figure 4.9. Treatment with cytokinin modifies the auxin-response of auxin-responsive genes which are unresponsive to cytokinin alone, and vice-versa, and this occurs in an auxin-dominant manner. In sets of 4 from left to right, graphs show: 1) gene expression profiles after treatment with IAA (10nM, 100nM, 1µM, 10µM, 100µM) +/− 1µM BAP, 1µM BAP or DMSO (0); 2) scatterplot of aforementioned data + regression line; 3) gene expression profiles after treatment with BAP (10nM, 100nM, 1µM, 10µM, 100µM) + 1µM IAA, or DMSO (mock); 4) scatterplot of aforementioned data + regression line. Treatment is for 6 hours in 7 DAG Col-0 seedlings. (A-D) Aux/IAA5, (E-H) ARR4, (I-L) ARR15. Importantly, (A) shows that cytokinin amplified expression of Aux/IAA5 by auxin (cytokinin does not itself regulate the gene, and auxin+cytokinin treatments show a higher expression response than vs. auxin treatment alone). See table for Pearson’s correlation coefficient. 1 way ANOVA and Tukey’s multiple comparison test (letter = p < 0.05), and Pearson’s correlation coefficient, n=4 biological replicates and 2 technical replicates.

4.3.3 Auxin is dominant over cytokinin in the formation of lateral roots

We then asked if the auxin dominance seen in the regulation of genes translated to a physiological function. Treatment with auxin has been shown to promote lateral root formation, whilst treatment cytokinin inhibits lateral root formation, this therefore provides a good model for which to investigate auxin dominance. We germinated and grew seedlings for 10 days on plant growth media with IAA (1nM, 10nM and 25nM) +/- 25nM BAP, BAP (1nM, 10nM and 25nM) +/- 25nM IAA, and DMSO (mock), and measured lateral root density (LRD), which is the number of lateral roots / root length. When investigating lateral root density, cell size can also be investigated to give a clearer picture of the underlying processes, but in large, complex experiments such as these this is often omitted. Future investigation focusing on more precise experiments should include an analysis of cell size. Lateral roots were counted manually under a dissecting microscope; roots were imaged under a dissecting microscope and root length quantified using ImageJ.

As expected, low concentrations of auxin increased LRD, and higher concentrations showed no change, and low concentrations of cytokinin reduced LRD, eliminating all lateral root formations in many seedlings at higher concentrations (Figure 4.10A). Treatment of 25nM IAA + 25nM BAP resulted in no change in LRD (Figure 4.10B). Importantly, the addition of 1nM IAA is sufficient to significantly alter the LRD reduction caused by 25nM BAP (25nM BAP vs. 1nM
IAA + 25nM BAP), whereas it requires 25nM BAP to alter the LRD increase caused by 25nM IAA (25nM IAA vs. 1nM, 10nM and 25nM BAP + 25nM IAA) (Figure 4.10B). Similarly, 25nM BAP has no effect on LRD in the presence of 50nM IAA, but 25nM IAA rescues LRD in the presence of 50nM BAP (Figure 4.10C). Together, this demonstrates that although both auxin and cytokinin regulate the formation of lateral roots, auxin appears to be the dominant coordinator; at low IAA:BAP ratios being able to counteract inhibition of LRD by BAP, and requiring high BAP:IAA ratios to overcome IAA-promotion of LRD.

4.3.4 Auxin is dominant over cytokinin in the regulation of root growth

Using the LRD data, we also tested whether auxin dominance affected root growth. Increasing concentrations of both IAA and BAP reduced root growth, and the inhibitory effect of BAP was significantly greater (Figure 4.11A). It is interesting to note that combined treatments did not show additive inhibition of root growth; instead, combined treatment actually diminished the inhibition of root growth exhibited in single treatments, suggesting that IAA and BAP regulate root growth via distinct competing pathways (Figure 4.11B).
Figure 4.10. Lateral root development is more sensitive to auxin than cytokinin. Number of lateral roots of Col-0 seedlings measured 9 DAG grown on media containing (A) 1nM, 10nM, 25nM BAP or IAA, and mock, (B) 25nM IAA + (0nM, 1nM, 10nM or 25nM) BAP, and 25nM BAP+ (0nM, 1nM, 10nM or 25nM) IAA, and (C) 50nM IAA (+/- 25nM BAP), 50nM BAP(+/- 25nM IAA), and mock. Importantly, (A) Auxin treatments increase lateral root development (LRD), cytokinin treatments decrease LRD, (B) LRD is more sensitive to auxin concentration than cytokinin, showing a significant increase in LRD from 0:25nM IAA:BAP vs. 1:25nM IAA:BAP; and
a 1:1 (or 25:25nM) IAA:BAP is required to see a significant decrease in LRD vs. 25:0 IAA:BAP. Student t-test (*p<0.05, **p<0.01, ***p<0.005), n=20-55.

Figure 4.11. Root growth is more sensitive to auxin than cytokinin. Root growth of Col-0 seedlings measured 9 DAG grown on media containing (A) 1nM, 10nM, 25nM BAP or IAA, and mock, and (B) 25nM IAA + (0nM, 1nM, 10nM or 25nM) BAP, and 25nM BAP+ (0nM, 1nM, 10nM or 25nM) IAA. (A) Single hormone treatments reduce root growth, (B) double hormone treatments increase root growth vs. single hormone treatments; and auxin treatment rescues cytokinin inhibition of root growth, but not vice-versa (25:25nM treatment increases LRD significantly vs. 0:25nM IAA:BAP, but not 25:0nM IAA:BAP. Student t-test (*p<0.05, **p<0.01, ***p<0.005), n=20-35.
4.3.5 **GH3s and CKXs are differentially regulated by auxin+cytokinin treatment**

We then explored how auxin dominance might be physically established. The GH3 family of proteins (GH3.1, GH3.2 and GH3.3 tested here) reduce auxin signalling by irreversibly conjugating aspartate and other amino acids to active auxins, disabling their signalling action (Staswick et al., 2005). These proteins are downregulators of auxin signalling. The CYTOKININ OXIDASE (CKX) family of proteins (CKX1-7) catalyse the degradation of active cytokinin. These proteins are downregulators of cytokinin signalling. Col-0 seedlings were grown in 0.5% MS liquid media for 7 days, then transferred to 0.5% MS liquid media containing: 10μM IAA, 1μM BAP, or 10μM IAA + 1μM BAP or DMSO (mock) for 6 hours. The RNA was extracted and cDNA synthesised as described in Chapter 2. Changes in expression levels of the GH3 and CKX genes after these treatments were examined using qRT-PCR.

Expression levels of GH3.1, GH3.2 and GH.3 were all dramatically upregulated by auxin treatment, as demonstrated by many studies (Ding et al., 2008) (Figure 4.12; Supplementary Figure 5). GH3.2 was slightly downregulated by cytokinin treatment, whilst GH3.1 and GH3.3 were unchanged. GH3.1, GH3.2 and GH.3.3 were all dramatically upregulated by auxin+cytokinin treatment, however, the upregulation was significantly reduced vs. treatment with auxin alone (Figure 4.12; Supplementary Figure 5). These represent more examples of genes that cytokinin exerts transcriptional control over in the presence of auxin, despite having little or no transcriptional effect in isolation. Here, cytokinin in the presence of auxin reduces levels of GH3 expression, reducing the conjugation of active auxins, and increasing of the auxin signal. This may explain the amplification of expression seen in our dual hormone concentration gradient qRT-PCR experiments.

Expression levels of CKX1, CKX3, CKX5 and CKX6 increased in response to auxin treatment (Figure 4.13; Supplementary Figure 6). Expression levels of CKX1-6 increased in response to cytokinin and auxin+cytokinin treatments Expression levels of CKX2, CKX4 and CKX5 in auxin+cytokinin treatments appears to be additive, and CKX1, CKX3 and CKX6 appear to be synergistic. Particularly striking is the 35 fold upregulation of CKX3 in auxin+cytokinin treatments, vs. the 4 fold upregulation by auxin or cytokinin treatments (Figure 4.13). Here, auxin and cytokinin both regulate CKX negative feedback loops of the cytokinin signalling pathway, and in combination do so both additively and synergistically. Taken together with the GH3 data, this suggests that where auxin and cytokinin signals overlap, levels of molecular cytokinin and thus cytokinin signalling is reduced, and levels of molecular auxin and thus auxin
signalling is increased. This may explain some aspects of auxin dominance genetically and physiologically.

Figure 4.12. Cytokinin reduces regulation of GH3s by auxin. Gene expression profiles after treatment with 10µM IAA, 1µM BAP, 10µM IAA + 1µM BAP, or DMSO (mock) for 6 hours in 7 DAG Col-0 seedlings. GH3.1 is upregulated by auxin, and showed disproportionately reduced upregulation after auxin+cytokinin treatment. Student t-test (*p<0.05, **p<0.01, ***p<0.005), n=4 biological replicates and 2 technical replicates.
Figure 4.13. Auxin+cytokinin treatment synergistically upregulates **CKX3**. Gene expression profiles of **CKX3** after treatment with 10µM IAA, 1µM BAP, 10µM IAA + 1µM BAP, or DMSO (mock) for 6 hours in 7 DAG Col-0 seedlings. Both auxin and cytokinin increase expression of **CKX3**, and treatment with auxin+cytokinin results in a dramatic, synergistic increase in expression. Student t-test (a = p<0.05 vs. auxin, c= p<0.05 vs. cytokinin), n=4 biological replicates and 2 technical replicates.

### 4.3.6 **CKX3 plays a role in the development of lateral roots and auxin dominance**

As CKX3 showed a large upregulation in auxin+cytokinin treatments, we asked whether CKX3 also played a role in auxin dominance in lateral root development. As with Col-0, we germinated and grew **ckx3-1** (a **ckx3** mutant line) seedlings for 10 days on plant growth media with IAA (10nM and 25nM) +/- 25nM BAP, BAP (10nM and 25nM) +/- 25nM IAA, and DMSO (mock), and measured lateral root density (LRD), which is the number of lateral roots / root length. Lateral roots were counted manually under a dissecting microscope; roots were imaged under a dissecting microscope and root length quantified using ImageJ.
DMSO and 10nM IAA treatments showed no change in LRD between Col-0 and ckx3-1 seedlings (Figure 4.14). Counter-intuitively, ckx3-1 rescued the cytokinin reduction in LRD phenotype, whereas we might have expected a stronger response due to the reduced ability to degrade active cytokinins. LRD in ckx3-1 was also reduced vs. Col-0 in 10nM IAA + 25nM BAP and 10nM BAP + 25nM IAA treatments. This suggests that CKX3 plays a role in establishing auxin dominance, and that it possibly does so by acting through the cytokinin LRD pathway.

Figure 4.14. CKX3 is required for cytokinin inhibition of lateral root development (LRD) and auxin dominance in LRD. Number of lateral roots of Col-0 seedlings measured 9 DAG grown on media containing 10nM IAA (+/- 25nM BAP), 10nM IAA (+/- 25nM BAP), and DMSO (mock). Importantly: in ckx3 seedlings, LRD was not reduced by cytokinin treatment; LRD is not reduced between 10nM IAA vs. 10nM IAA + 25nM BAP in Col-0, but in ckx3-1 LRD is
significantly reduced between these treatments, thus CKX3 is necessary for auxin dominance over cytokinin in LRD. Student t-test (*p<0.05, **p<0.01, ***p<0.005), n=15-30.

4.4 Discussion

4.4.1 Dual Hormone Response
To our knowledge, we have performed the first in-depth analysis of a transcriptome after simultaneous treatment with two hormones, in any organism. Interestingly, transcriptome studies of multiple stresses in combination are fairly common (Kreps et al., 2002), and the gap in knowledge of multiple hormone treatments is substantial, especially considering that no cell is ever only in the presence and under the transcriptional control of only one hormone at a time.

Auxin and cytokinin are important plant phytohormones that regulate a numerous aspects of plant growth and development. In classic experiments performed by Skoog and Miller in 1957, auxin and cytokinin were shown to coordinate the formation of undifferentiated callus tissues, and commit cells to root or shoot lineages, when applied in particular ratios (Skoog and Miller et al., 1957). These experiments demonstrated that auxin and cytokinin could act together to be greater than the sum of their parts. Despite these classic experiments, there had been no in-depth transcriptome investigation of simultaneous treatment with auxin and cytokinin. Some studies exist that have indirectly looked at this transcriptome by studying the transcriptome of Agrobacterium tumefaciens infected plants which upregulate auxin and cytokinin levels, or studying the transcriptome of calluses initiated by auxin+cytokinin treatment, but none have looked directly (Che et al., 2006; Ditt et al., 2006).

We have shown for the first time the existence of a Dual Hormone Response (DHR), a set of genes that are only regulated in the presence of both auxin and cytokinin together. Classic thought in hormone studies have concluded that for the most part, plant hormones regulate a distinct silo of genes (Nemhauser et al., 2006). Within these silos are genes that regulate the signalling pathways of other hormones, so-called crosstalk genes, as well as genes that can be regulated by several different hormones, though these genes tend to be in the minority. We have demonstrated that another silo of genes exists which is only regulated when auxin and cytokinin act together, and is of comparable size to the silos regulated by single hormones: in single hormone treatments 808 genes were differentially expressed upon treatment with auxin alone, and 494 with cytokinin alone; and 518 genes were differentially expressed upon
treatment with auxin+cytokinin. This represents a new area of investigation into auxin-cytokinin callus formation, a completely new way of understanding gene regulation by hormones, and opens up the field of hormone research to include combinatory hormone responses in both plant and animal research.

Unfortunately, the role of the auxin-cytokinin DHR in Arabidopsis thaliana proved difficult to elucidate. Examination of particular DHR genes suggests specific roles for growth and development, such as positive and negative feedback of both auxin and cytokinin, or regulation of cell expansion. However, gene ontology (GO) term analysis revealed only a small enrichment of genes involved in responses to abiotic stress. An obvious DHR function is the ability to form calluses, which requires both auxin and cytokinin, however further testing of the role of the DHR in callus formation requires investigation of individual DHR genes. Further responses of auxin+cytokinin treated plants to abiotic stresses needs to be performed to confirm that role of the DHR.

We then searched for transcription factors (TFs) than may regulate the DHR. Motif enrichment searches in the promoter regions of the DHR genes with DREME and subsequent motif-TF matching with TOMTOM revealed that the bHLH TF SPATULA (SPT) may regulate a subset of the DHR silo. As no motif was highly enriched, we concluded that the DHR silo is likely regulated by a number of TFs which bind to distinct motifs. We are also limited in our investigation by the 6 hour time point used in our microarray, which means the DHR silo will include genes that are indirectly and downstream regulated, complicating our search for enriched motifs. The enrichment of the canonical Auxin Response Element (TGTCTC) was examined, but the motif is presence in almost all promoters. The cytokinin response element, 5’-(G/A)GGAT(T/C), is equally frequent, and was not enriched in our gene sets, but should also not be considered a universal cytokinin response element, as few cytokinin-responsive genes contain the element. Recently, it has been showed that cytokinin and SPT work together to promote the expression of TAA1 and PIN3 (Reyes-Olalde, et al., 2017). It is possible that cytokinin regulates the expression of genes that show modest responses to auxin via SPT, and in combinatory treatments, the exogenous auxin + increased endogenous auxin (from cytokinin and SPT) additively combines modest expression responses to create expression levels that would classify it as a DHR gene.
The possibility of SPT regulating the DHR prompted us to also examine the role of INDEHISCENT (IND), a bHLH TF and binding partner of SPT, in regulating the DHR. Comparison of DEX, auxin+cytokinin and DEX + auxin+cytokinin treatments, revealed a fascinating interaction between IND, auxin and cytokinin. 450 genes were regulated by auxin+cytokinin treatment only, and 104 by DEX + auxin+cytokinin treatment only, but importantly, only 46 genes were regulated by both of those treatments. This means that the majority of the DHR silo stops being regulated when IND is upregulated, and a new, smaller silo of genes is preferentially regulated. This supports the possibility of SPT being a regulator of the DHR, as upregulation of one of SPT’s binding partners, IND, results in abolishment of much of the DHR regulation, possibly because SPT is now more likely to bind IND, rather than other binding partners that might coordinate the DHR. Pull-down and binding partner analysis of both IND and SPT after treatment with auxin, cytokinin and auxin+cytokinin may yield further insights into this relationship.

Finally, a possibility remains that the DHR may represent a set of genes that are in areas of condensed DNA, which are only opened up upon treatment with auxin+cytokinin. Our preliminary experiments show that some proteins involved in chromatin remodelling complexes can be downregulated when treated with auxin+cytokinin. However, DNase I footprinting, which identifies the openness of chromosomes, after treatment with auxin, cytokinin and auxin+cytokinin would be conclusive in this matter.

4.4.2 Auxin Dominance

An enduring question in plant phytohormones research is whether there is an overarching hormone-regulatory system, either in the form of genes regulated by all phytohormones which then coordinate general responses, or in the form of some hormones taking precedence and priority over others (Nemhauser et al., 2006). Nemhauser et al. demonstrated that there is no common set of signalling components that integrate multiple hormones to regulate growth. Furthermore, the lack of multi-hormone studies has prevented the investigation of hormone hierarchies. Studies have shown that auxin and cytokinin signalling pathways were among the earliest phytohormones signalling pathway to evolve (Wang et al., 2015). This could suggest that signalling pathways that evolved later might be under more regulatory control of these older hormones, than they are able to exert control upon them. Crosstalk genes between hormone signalling pathways can promote both negative and positive regulation of another hormone signalling pathway, sometimes simultaneously. Often, the role of auxin and cytokinin
appears to be to inhibit the action of the other by altering the expression of biosynthesis, transport, signalling component, and degradation proteins (Chandler & Werr, 2015).

After observing the close correlation between auxin vs. auxin+cytokinin transcriptome values, we were prompted to further dissect the relationship between auxin and cytokinin regulation of genes. Our dual hormone concentration gradient qRT-PCR experiments demonstrated that for many genes, auxin can amplify the expression of a gene, it itself does not regulate, in the presence of cytokinin, and vice-versa. As with the existence of the DHR, this points to a much more cooperative relationship between auxin and cytokinin than has previously been observed. This also raises the possibility that many of the developmental processes ostensibly coordinated by auxin or cytokinin exclusively might be reinforced by the presence of the other hormone via indirect amplification of gene expression. Further dissection of these developmental processes is needed to confirm this. Further analysis of this data revealed that the amplified response was more correlated with the concentration of auxin rather than cytokinin, even in genes auxin treatment alone didn’t regulate. This suggests that amplification of gene expression between auxin and cytokinin is auxin-dependent, and auxin exerts more control over regulation of these genes than cytokinin does. This prompted us to hypothesize that auxin is dominant in the regulation of gene expression.

We then asked whether auxin was dominant over cytokinin in the initiation of developmental processes. We demonstrated that low levels of auxin were required to overcome cytokinin inhibition of lateral roots, and that relatively high levels of cytokinin were required to overcome auxin inhibition of lateral roots. Co-treatment with auxin and cytokinin also showed that root length was preferentially determined by auxin over cytokinin. Altogether, this represents early evidence of auxin dominance over cytokinin. With regards to auxin dominance in lateral root development, much further and closer analysis needs to be performed to examine how lateral root formation is altered by auxin, cytokinin and auxin+cytokinin. One possibility is that as auxin maxima are required for the early initiation of expansion of lateral roots, is that auxin+cytokinin promotes the formation of auxin maxima, whereas cytokinin treatment disrupts their formation.

Finally, we asked whether the amplification of gene expression and auxin dominance observed in qRT-PCR and lateral roots was due to alterations in gene expression of auxin and cytokinin metabolism genes. This could suggest that the levels of active auxin and cytokinin may be
altered by in these long treatment periods of 6 hours or 7 days. The GH3 family of proteins irreversibly conjugate active auxins, inactivating them. GH3 upregulation by auxin was diminished by the additional presence of cytokinin. The CKX family of proteins irreversibly degrades active cytokinins. CKX1-6 were upregulated by auxin, cytokinin or auxin+cytokinin treatments. The upregulation in auxin+cytokinin was additive for CKX2, CKX4 and CKX5, and synergistic for CKX1, CKX3 and CKX6. CKX3 was 35 fold upregulated in response to auxin+cytokinin. Taken together, these results suggest that in areas where auxin and cytokinin overlap, regulation of these genes is altered to reduce the inactivation of auxin, and increase the degradation of cytokinin. This raises the possibility than auxin dominance is physically established in areas of hormone overlap by the increase in active auxin and decrease in active cytokinin. This also may explain the amplification of auxin-regulated genes by cytokinin, where cytokinin alone does not regulate the gene. Cytokinin may instead serve to increase active auxin, amplifying the expression of that gene. Finally, this relationship may enable the formation of auxin maxima, as the inactivation of auxin is diminished in the presence of cytokinin, which may create auxin dominance in the formation of lateral roots. Examination of LR initiation in auxin and cytokinin reporters would be useful in determining if significant overlap of auxin and cytokinin occurs where auxin maxima will later develop.

CKX3 also appears to be essential for the inhibition of lateral root development by cytokinin, raising the possibility, that cytokinin inhibits lateral root development by increasing the expression of CKX3, leading to defects in the delicate patterning of cytokinin concentrations, and auxin by extension of the above observations, during lateral root initiation. CKX3 expression was not readily observable in CKX3-GUS lines (Werner et al., 2003), presumably because it is primarily responsive to auxin+cytokinin treatment, thus, expression of CKX3 needs to be revisited in areas of auxin and cytokinin overlap, and under auxin+cytokinin treatment. CKX3 is believed to be secreted into the apoplast, where cytokinins bind AHKs, but is also predicted to be imported into the mitochondria, the relevance of this in the formation of lateral roots should be examined.
Chapter 5 – TIR1, auxin and cytokinin

5.1 Introduction

The auxin receptor TIR1 is part of an F-box containing family, which also includes 5 AFBs, all of which are localised to the nucleus (Salehin et al., 2015). TIR1 contains an 18 Leu-rich repeat (LRR) domain, which is the binding pocket for Aux/IAAs, in which auxin acts as the molecular glue (Tan et al., 2007). TIR1 has a large range of binding affinities for the different Aux/IAAs presumed to be dependent on small differences in structure between the Aux/IAA family DII domain, which is the recognition domain bound by the TIR1 LRR domain (Irina et al., 2012). Binding affinities between TIR1 and Aux/IAAs can be altered/abolished by the modification of the LRR and DII domains (Yu et al., 2013; Liao et al., 2015). The crystal structure of auxin bound to TIR1 has been uncovered, revealing that auxin fills a hydrophobic surface in the LRR domain, enhancing TIR1-Aux/IAA interactions without altering the conformation of TIR1 (Tan et al., 2007). This analysis also revealed that the auxin-binding site is partially promiscuous, allowing the binding of auxin analogues. A number of such analogues have been generated, and small modifications to their chemical structure can alter their binding affinity to TIR1 and AFBs, which typically vary between each other, and whether they promote or block the pathway. Auxinole is an example of an auxin analogue which binds the auxin binding site and blocks the formation of the TIR1-auxin-Aux/IAA complex, inhibiting the auxin signalling pathway. Specifically, the phenyl ring in auxinole strongly interacts with the TIR1 Phe82 crucial for recognition of Aux/IAAs (Hayashi et al., 2012). It is interesting to note, and will become relevant, that the molecular structure of the cytokinins 6-Benzylaminopurine (BAP) and trans-zeatin (tZ) are not dissimilar to auxinole and IAA respectively (Figure 5.1).

TIR1 is able to bind the DII domains of Aux/IAAs in isolation from the rest of that protein, which has led to the development of several important modern tools. One such tool is the auxin expression reporter line DII-VENUS, in which the DII domain of IAA28 is bound to YFP-VENUS, and degradation of fluorescence indicates auxin signalling activity (Brunoud et al., 2012). Another tool is the Auxin Inducible Degron (AID) system, in which an operational TIR1 complex is cloned into the desired system, and the DII domain of an Aux/IAA is fused to the protein of interest, which can then be knocked down with relative precision by treatment with auxin (Nishimura et al., 2009).

For brief explanations of the biosynthesis, signalling pathways and degradation of auxin and cytokinin, please see Section 4.1; for a more detail review, please refer to Section 1.2.
In this chapter, we explore the role of TIR1 in the establishment of dominance of the auxin signalling pathway over the cytokinin signalling pathway. We also explore the mechanism by which cytokinin may inhibit TIR1 activity, thus downregulating the auxin signalling pathway.

5.2 TIR1 and auxin dominance

5.2.1 TIR1 plays a role in the development of lateral roots and auxin dominance

Following from our analysis of auxin dominance in the formation of lateral roots, we explored whether the dominance of the auxin signalling pathway over the cytokinin signalling pathway we observed operated through TIR1, an important auxin receptor that begins a cascade of auxin signalling. We began by measuring the lateral root development (LRD), which is the number of lateral roots / root length, of the tir1 mutant line tir1-1, under different hormone treatments, vs. Col-0. As with Col-0 and cxx3-1, and detailed in Chapter 5, we germinated and grew seedlings for 10 days on plant growth media with IAA (10nM and 25nM) +/- 25nM BAP, BAP (10nM and 25nM) +/- 25nM IAA, and DMSO (mock), and measured lateral root density (LRD). Lateral roots were counted manually under a dissecting microscope; roots were imaged under a dissecting microscope and root length quantified using ImageJ.

As expected, tir1-1 seedlings had reduced LRD compared to Col-0 under mock treatment (Figure 5.1). Interestingly, 10nM IAA treatment further reduced LRD in tir1-1 seedlings. 10nM BAP, 10nM BAP + 25nM IAA, and 10nM IAA + 25nM BAP treatments removed almost all LRD in tir1-1 seedlings, and the auxin dominance of the combinatory treatments in Col-0 seedlings was not observed in tir1-1 seedlings. Thus, TIR1 appears to play a role in establishing auxin dominance. It is important to remember the important role of TIR1 in auxin signalling pathways. The perturbed nature of auxin signalling in tir1-1 mutants means that results can be difficult to interpret in isolation, and other methods of dissecting the role of TIR1 are required.
Figure 5.1. *TIR1* is required for auxin promotion of lateral root development (LRD), limiting cytokinin inhibition of LRD, and auxin dominance in LRD. Number of lateral roots of Col-0 seedlings measured 9 DAG grown on media containing 10nM IAA (+/-25nM BAP), 10nM IAA (+/-25nM BAP), and DMSO (mock). Importantly: in *tir1-1* seedlings, LRD was reduced in all treatments; and in *tir1-1* auxin was unable to overcome the inhibitory effect of cytokinin (10nM BAP vs. 10nM BAP + 25nM IAA), and cytokinin was able to overcome the promoting effect of auxin (10nM IAA vs. 10nM IAA + 25nM BAP), thus auxin dominance over cytokinin in LRD has been abolished. Student t-test (*p*<0.05, **p*<0.01, ***p*<0.005), n=30.
5.2.2 TIR1 plays a role in auxin dominance over cytokinin in regulating genes

We then investigated the role of TIR1 in the regulation of genes from the A-AC and C-AC groups of the Venn diagram in Chapter 4, particularly looking at whether the amplification of gene expression observed in Chapter 4 was coordinated by the TIR1 signalling pathway.

Col-0 and tir1-1 seedlings were grown in 0.5% MS liquid media for 7 days, then transferred to 0.5% MS liquid media containing: 10μM IAA, 1μM BAP, 10μM IAA + 1μM BAP, 1μM IAA, 100nM BAP, 1μM IAA + 100nM BAP, or DMSO (mock), for 6 hours. RNA was extracted and cDNA synthesised as described in Chapter 2. We then measured the changes in expression levels under these treatments of 6 genes from the A-AC group of the Venn diagram, meaning they are auxin-responsive and not cytokinin-responsive, and 5 genes from the C-AC group of the Venn diagram, meaning they are cytokinin-responsive and not auxin-responsive. From the A-AC group *INDOLE-3-ACETIC ACID INDUCIBLE 1* (Aux/IAA1), *INDOLE-3-ACETIC ACID INDUCIBLE 5* (Aux/IAA5), *INDOLE-3-ACETIC ACID INDUCIBLE 19* (Aux/IAA19), *AUXIN RESPONSE FACTOR 2* (ARF2), *AUXIN RESPONSE FACTOR 19* (ARF19), and *LOB DOMAIN-CONTAINING PROTEIN 18* (LBD18) were investigated. From the C-AC group *RESPONSE REGULATOR 3* (ARR3), *RESPONSE REGULATOR 4* (ARR4), *ARABIDOPSIS HISTIDINE KINASE 4* (AHK4), *NAP1-RELATED PROTEIN 1* (NRPI), and *CYTOKININ OXIDASE 4* (CKX4) were investigated.

Of the A-AC group of genes examined, Aux/IAA1, best demonstrates several trends. Firstly, Aux/IAA1 was still upregulated by auxin in tir1-1, so the regulation is not dependent on the TIR1 signalling pathway (Figure 5.2A-F). However, in Col-0 auxin+cytokinin amplified expression, the same treatments showed no amplification in tir1-1 (Figure 5.2A; Supplementary Figure 7). Suggesting that TIR1 may not be necessary for expression of these genes, but TIR1 may be necessary for cytokinin to amplify the expression of these genes. It is interesting that rather than a removal of the amplified expression in auxin+cytokinin treatments, the response to auxin is instead increased to similar levels, suggesting that the expression is sensitised to auxin in tir1-1. LBD18 also shows a unique expression profile; here, the large reduction of expression caused by the addition of cytokinin treatment in Col-0 is abolished in tir1-1 seedlings, suggesting that cytokinin is operating through the TIR1 signalling pathway (Supplementary Figure 7F).
Of the C-AC group of genes examined, CKX4 best demonstrates several trends. Firstly, in tir1-1, CKX4 showed an increased gene expression response to cytokinin treatment, suggesting that the TIR1 signalling pathway may antagonise the expression of these genes, or that the lack of the TIR1 signalling pathway predisposes tir1-1 seedlings to cytokinin sensitivity (Figure 5.B). Also, the amplification of CKX4 expression between cytokinin treatment vs. auxin+cytokinin treatment in Col-0 seedlings, by the addition of auxin which itself has no regulatory effect, a, is abolished in tir1-1 seedlings (Figure 5.2G-K).

In combination with the results from the A-AC genes, this suggests again that TIR1 plays a role in the amplification of gene expression by auxin on cytokinin and vice-versa. In the C-AC genes, the loss of amplification is due to the increase of gene expression by cytokinin treatment to a level equivalent to auxin+cytokinin treatment, which could mean that tir1-1 is sensitive to cytokinin treatment, or possibly that tir1-1 seedlings accumulate auxin which can then amplify the exogenous cytokinin in the same way that the auxin+cytokinin treatment works.

The further exploration of sensitivity to cytokinin was not pursued, as it is difficult to experimentally verify and delineate from gene-specific phenotypes. However, not all genes that respond to cytokinin showed an increased sensitivity to cytokinin in tir1-1 seedlings (Figure 5.2L-P). Expression of GH3s showed some differences in tir1-1 in response to auxin and auxin+cytokinin. ARR6 and CKX5 exhibited a reduction in gene expression after cytokinin treatment in tir1-1 seedlings vs. Col-0, and CKX3 was unchanged. The expression profile of CKX3 in tir1-1 seedlings was also interesting because whilst regulation by cytokinin was unchanged, the regulation by auxin was eliminated, and the regulation by auxin+cytokinin was only slightly reduced. This suggests that TIR1 may be necessary for auxin regulation of CKX3 it plays only a small role in the large synergistic response seen in auxin+cytokinin treatments.
Figure 5.2. *tir1-1* mutants have altered gene expression responses to auxin, cytokinin and auxin+cytokinin treatments. Gene expression profiles of *Aux/IAA1* and *CKX4* genes after treatment with 10µM IAA, 1µM BAP, 10µM IAA + 1µM BAP, or DMSO (mock) for 6 hours in 7 DAG Col-0 and *tir1-1* seedlings. Importantly: in Col-0 dual-treatments amplify the expression of *Aux/IAA1* and *CKX4* by auxin and cytokinin, respectively, but in *tir1-1*, the amplified expression is abolished (10nM IAA vs. 10nM IAA + 1µM BAP in *Aux/IAA1*, and 100nM BAP vs. 1µM IAA + 100nM BAP for *CKX4*), achieved by an increase in sensitivity to the single hormone treatments; additionally, sensitivity to all treatments is increased in *tir1-1*. Student t-test (*p<0.05, **p<0.01, ***p<0.005), n=4 biological replicates and 2 technical replicates.

5.2.3 Auxin dominance over cytokinin in regulation of some genes is specific to the TIR1 pathway

The *wei8-1 tar1-1* mutant line is a double mutant of *WEAK ETHYLENE INSENSITIVE 8* (*WEI8*) and *TYRPTOPHAN AMINOTRANSFERASE RELATED 1* (*TAR1*) (Stepanova et al., 2008). Both are proteins involved in the biosynthesis of bioactive auxin. Both *tir1-1* and *wei8-1 tar1-1* exhibit a reduction in auxin signalling, but whilst *tir1-1* eliminates TIR1-specific auxin signalling, leaving the AFB signalling pathways functional (though with potentially higher frequency of stimulation), *wei8-1 tar1-1* has an overall reduction in both the TIR1 and AFB signalling pathways. Thus, to examine the role of TIR1 in the amplified expression of genes in auxin+cytokinin treatments, and the apparent auxin dominance of those expression levels, comparison between the *tir1-1* and *wei8-1 tar1-1* lines allows us to determine whether the apparent role of TIR1 can be explained by a reduction in total auxin signalling rather than a more direct role or pathway.
Col-0, *tir1-1*, *wei8-1-tar1-1* seedlings were grown in 0.5% MS liquid media for 7 days, then transferred to 0.5% MS liquid media containing: 10μM IAA, 1μM BAP, 10μM IAA + 1μM BAP, 1μM IAA, 100nM BAP, 1μM IAA + 100nM BAP, or DMSO (mock), for 6 hours. RNA was extracted and cDNA synthesised as described in Chapter 2. We then measured the changes in expression levels of *ARF2*, *ARF19*, *Aux/IAA1*, *Aux/IAA5*, *Aux/IAA19*, *LBD18*, *ARR6*, *CKX3*, and *CKX5* (Figure 5.3). Shown here are the expression profiles of *Aux/IAA1* and *ARF19*, the remaining data can be found in Supplementary Figure 8.

The expression profiles of *Aux/IAA1* and *Aux/IAA19*, in *wei8 tar1* seedlings mimic those of *tir1-1* seedlings: auxin+cytokinin treatment did not cause an amplification of expression vs. the auxin treatment alone, instead, the seedlings became more sensitive to the single treatment, increasing expression to the same level as the dual treatment (Figure 5.3A; Supplementary Figure 8C,E). This suggests that TIR1’s apparent role in these expression phenomena for these genes is due to a reduction in overall auxin signalling, rather than a specific function of TIR1 or its signalling pathway. However, the expression profiles of *ARF2*, *ARF19* and *CKX3* in *wei8-1 tar1-1* seedlings do not mimic those of *tir1-1*: suggesting that TIR1 plays a role in the auxin-cytokinin relationship that determines expression levels of these genes which is beyond a simple reduction in auxin signalling (Figure 5.3B; Supplementary Figure 8A,B,H). That there is a familial split in response between the Aux/IAAs and ARFs tested here should also not go unnoticed, and deserves further examination. The *CKX5* seedling expression profile mimics *tir1-1* in cytokinin and auxin+cytokinin treatments, but not auxin treatments, suggesting a more complex regulation (Figure 5.3I).

To summarise, auxin and cytokinin show complex co-regulation of particular genes. Most clearly, the amplification of expression in dual treatments, when one of the single treatments has no effect on expression, but also that this level of expression correlates more closely with the levels of auxin rather than cytokinin, even for cytokinin-regulated genes, which we have called auxin dominance. It appears that for some of the genes tested, TIR1 is necessary for these expression phenomena, but for other genes, a reduction in overall auxin signalling is sufficient to observe the same expression phenomena.
Figure 5.3. The altered expression responses to hormones in *tir1-1* mutants is mimicked in *wei8-1 tar1-1* mutants for some genes. Gene expression profiles of (A) *Aux/IAA1* and (B) *ARF19* after treatment with 10µM IAA, 1µM BAP, 10µM IAA + 1µM BAP, or DMSO (mock) for 6 hours in 7 DAG Col-0, *tir1-1*, and *wei8-1 tar1-1* seedlings. Importantly, expression profile of *Aux/IAA1* is unchanged between *tir1-1* vs. *wei8-1 tar1-1* (A), and the expression profile of *ARF19* is significantly different between *tir1-1* vs. *wei8-1 tar1-1*. This suggests that TIR1 is required for the amplified expression of some genes, but not all. Student t-test (*p*<0.05, **p**<0.01, ***p**<0.005), n=4 biological replicates and 2 technical replicates.

5.3 Dual-hormone global signalling activity

5.3.1 Auxin modulates the cytokinin signalling pathway in the TCSn::GFP reporter

We then asked whether auxin could influence cytokinin signalling pathways and resulting in a change in total expression levels. To determine this, we used the cytokinin expression reporter *TCSn::GFP*, which expresses GFP under a cytokinin-responsive promoter, to detect and estimate levels of cytokinin signalling activity (Liu et al., 2017). *TCSn::GFP* seeds were grown on 0.5% MS agar for 7 days, then transferred to 0.5% MS agar containing 100nM tZ (trans-Zeatin, a biological cytokinin), 100nM IAA, 10µM IAA, 100nM tZ + 100nM IAA, 100nM tZ + 10µM IAA, or mock (DMSO), for 2 hours. GFP fluorescence in the root tip was imaged under a confocal microscope and quantified using ImageJ.

As expected, treatment with tZ increased fluorescence (Figure 5.4). Treatment with 100nM or 10µM IAA did not significantly increase fluorescence. Dual treatment showed an increase in fluorescence when tZ was combined with the lower 100nM IAA, however, when tZ was combined with the higher treatment of 10µM IAA there was no significant increase in fluorescence. This suggests that at higher ratios of auxin:cytokinin, auxin can partially inhibit cytokinin signalling.
5.3.2 Cytokinin modulates the auxin signalling pathway in the DR5::GFP reporter

We then asked whether cytokinin could influence auxin signalling pathways and resulting in a change in total expression levels. To determine this, we used the auxin expression reporter DR5::GFP, which expresses GFP under an auxin-responsive promoter (Brunoud et al., 2012). DR5::GFP seeds were grown on 0.5% MS agar for 7 days, seedlings were then transferred to 0.5% MS agar containing 1μM IAA, 1μM BAP, 1μM IAA + 1μM BAP, or mock (DMSO), for 2 hours. Fluorescence in the root tip was imaged with a confocal microscope and analysed. Fluorescence mean and area were measured, from which intensity (mean/area) was determined.

Our analysis showed that auxin treatment did not significantly increase fluorescence intensity (Figure 5.5). Interestingly, treatment with cytokinin showed a 50% increase in fluorescence intensity, and combined auxin + cytokinin treatment did not increase fluorescence intensity. Therefore, cytokinin appears able to promote the auxin signalling pathway, but this effect can be inhibited by additional treatment of auxin. Thus, auxin is dominant over cytokinin’s regulation of the auxin signalling pathway.

5.3.3 Cytokinin modulates the auxin signalling pathway in the DII-Venus reporter

We then tested the effect of cytokinin treatment on auxin signalling with the more modern auxin expression reporter, DII-VENUS. DII-VENUS responds to auxin with a reduction in nucleus-localised fluorescence, as the DII domain of Aux/IAA28 fused to the VENUS fast maturing yellow fluorescent protein (YFP) is tagged for degradation by TIR1 in the presence of auxin. DII-VENUS is a useful tool as responses to auxin can be detected within minutes, rather than hours with DR5::GFP (Brunoud et al., 2012). To confirm the response seen in DR5::GFP, DII-VENUS seeds were grown on 0.5% MS agar for 7 days, seedlings were then transferred to 0.5% MS agar containing 100nM IAA, 100nM BAP, 100nM tZ, 100nM IAA + 100nM BAP, 100nM IAA + 100nM tZ, or mock (DMSO), for 1 hour. The fluorescence of cell nuclei in the epidermis of root tip was imaged under a confocal microscope and quantified using ImageJ.

As expected, IAA treatment dramatically reduced the cell nuclei fluorescence (Figure 5.6). Interestingly, treatment with BAP and tZ significantly increased fluorescence. This suggests that treatment with cytokinin reduced signalling by endogenous auxin, at least via the TIR1-IAA28 specific pathway. Combined treatment of IAA+BAP was unchanged vs. IAA treatment
alone, and combined IAA+tZ treatment appeared to slightly inhibit IAA reduction of fluorescence ($p = 0.08$). This suggested that cytokinin may play a role in inhibiting auxin signalling, at the very least via the TIR1-AUX/IAA28 specific pathway.

Figure 5.4. High auxin levels inhibit cytokinin induction of cytokinin signalling pathway. Fluorescence in root tips of TCSn::GFP seedlings treated for 2 hours with 100nM tZ (+/- 100nM IAA, or 10µM IAA), 100nM IAA, 10µM IAA, and DMSO (mock). Cytokinin treatment upregulates cytokinin signalling pathway, auxin treatment has no effect, but higher levels of auxin inhibit cytokinin induction of cytokinin signalling pathway (100nM tZ vs. 100nM tZ + 10µM IAA). 1 way ANOVA and Tukey’s multiple comparison test (letter = $p<0.05$), n=10-15.
Figure 5.5. BAP positively regulates auxin signalling pathway, and auxin can inhibit that positive regulation. Fluorescence in root tips of DR5::GFP seedlings treated for 2 hours with 1µM IAA, 1µM BAP, 1µM IAA + 1µM BAP, and DMSO (mock). Cytokinin treatment upregulates auxin signalling pathway, auxin treatment has no effect, combined treatment stops cytokinin promotion of auxin signalling pathway. 1 way ANOVA and Tukey’s multiple comparison test (letter = p<0.05), n=10.
Figure 5.6. Cytokinin inhibits TIR1 action and auxin signalling pathway. Fluorescence in nuclei of root tips of DII::VENUS seedlings treated for 1 hour with 100nM IAA (+/- 100nM BAP/tZ), 100nM BAP, 100nM tZ, and DMSO (mock). Importantly, cytokinin treatment increased fluorescence vs. control (Mock vs. 100nM BAP and vs. 100nM tZ), presumably through inhibition of endogenous auxin action, and in combined treatments, reduced the action of exogenous auxin (100nM IAA vs. 100nM IAA + 100nM tZ). 1 way ANOVA and Tukey’s multiple comparison test (letter = p<0.05), n=10.
5.4 Direct inhibition of TIR1 by cytokinin

5.4.1 Cytokinin inhibits the auxin signalling pathway within 15 minutes

Next, we explored the possibility of cytokinin acting as a direct inhibitor of TIR1. If cytokinin is acting directly, then the inhibitory response will be detectable soon after treatment. To begin testing this, *DII-VENUS* seeds were grown on 0.5% MS agar for 7 days, seedlings were then transferred to 0.5% MS agar containing: 1μM IAA, 1μM BAP, 10μM BAP, 1μM tZ, 10μM tZ, 1μM IAA + 1μM BAP, 1μM IAA + 10μM BAP, 1μM IAA + 1μM tZ, 1μM IAA + 10μM tZ, or mock (DMSO), for 15 minutes. The fluorescence of cell nuclei in the epidermis of root tip was imaged under a confocal microscope and quantified using ImageJ.

As expected, treatment with IAA dramatically decreased fluorescence (Figure 5.7). Treatment with tZ and BAP, at both 1μM and 10μM, resulted in largely similar increases in fluorescence. That both concentrations of tZ and BAP equally inhibited the response, suggests that the response is saturated at 1μM. Combined treatments of IAA+BAP/tZ resulted in a small inhibition of IAA-induced fluorescence degradation. Thus, cytokinin is able to influence an auxin signalling pathway within 15 minutes.

5.4.2 Key auxin and cytokinin genes respond transcriptionally within 15 minutes

We then asked whether plant tissues could respond transcriptionally to auxin and cytokinin within 15 minutes. Col-0 seedlings were grown in 0.5% MS liquid media for 7 days, then transferred to 0.5% MS liquid media containing: 10μM IAA, 1μM BAP, 10μM IAA + 1μM BAP, or mock (DMSO) for 15 minutes. RNA was extracted and cDNA synthesised as described in Chapter 2. The GH3 family of proteins (GH3.1, GH3.2 and GH3.3 studied here) reduce auxin signalling by irreversibly conjugating aspartate and other amino acids to active auxins, disabling their signalling action (Park et al., 2007). Expression of GH3 proteins is known to increase quickly to auxin treatment (Ding et al., 2008). *PIN1-3* code for auxin transport proteins, known to be downregulated by cytokinin (Laplaze et al., 2007). GH3.1, GH3.2 and GH3.3 all showed an increase in expression after 15 minutes of treatment with IAA (Figure 5.8B-D). *PIN1-7* both showed a decrease in expression after 15 minutes of treatment with BAP (Figure 5.8E-J). CKX3 also responded within 15 minutes (Figure 5.8A). Thus, the response observed in *DII-VENUS* may be transcriptionally controlled.
5.4.3 Cytokinin inhibition of the auxin signalling pathway may be transcriptional

To test whether the inhibitory effect of cytokinin seen in Dii-VENUS at 15 minutes was controlled by a transcriptional response, we pre-treated seedlings with cycloheximide (CHX), an inhibitor of translation (Schneider-poetsch et al., 2010), and then repeated the 15 minute treatment experiment. Dii-VENUS seedlings were grown on 0.5% MS agar for 7 days, then transferred to 0.5% MS agar containing 5μM CHX or mock (DMSO) for 45 minutes, then transferred CHX-treated seedlings to agar containing: 5μM CHX + 1μM IAA/ 1μM BAP/ 1μM IAA + 1μM BAP/ DMSO (mock). The fluorescence of cell nuclei in the epidermis of root tip was imaged under a confocal microscope and quantified using ImageJ.

Treatment with CHX for 1 hour caused a decrease in cell nuclei fluorescence (Figure 5.9). We take this to mean that CHX is inhibiting translation effectively, but the precise method by which CHX reduced fluorescence in Dii-VENUS was not further explored. Pre-treatment with CHX did not prevent the IAA treatment-induced fluorescence decrease, nor the BAP treatment-induced fluorescence increase (in terms of the trend, rather than intensity specifically, which was not compared here). CHX pre-treatment did however prevent the BAP partial inhibition of the IAA treatment-induced decrease. This suggests that cytokinin inhibition of endogenous auxin action is not a transcriptional response, but the inhibition of exogenous auxin may be.
Figure 5.7. Cytokinin inhibits TIR1 action and auxin signalling pathway within 15 minutes. Fluorescence in nuclei of root tips of DII::VENUS seedlings treated for 15 minutes with 1µM IAA (+/- 1µM/10µM BAP/tZ), 1µM BAP, 10µM BAP, 1µM tZ, 10µM tZ, and DMSO (mock). Importantly, cytokinin treatment increased fluorescence vs. control, presumably through inhibition of endogenous auxin action (Mock vs. 1µM BAP; 10µM BAP; 1µM tZ; and 10µM tZ), and in combined treatments, reduced the action of exogenous auxin (1µM IAA vs. 1µM IAA + 1µM BAP; 1µM IAA + 10µM BAP; 1µM IAA + 1µM tZ; and 1µM IAA + 10µM tZ). Student t-test (***p<0.005), n=10.
Figure 5.8. Genes can be transcriptionally regulated within 15 minutes of treatment with auxin or cytokinin. Expression profiles of 10 genes after treatment with 10µM IAA, 1µM BAP, 10µM
IAA + 1µM BAP, or DMSO (mock) for 15 minutes in 7 DAG Col-0 seedlings. Student t-test (*p<0.05, **p<0.01, ***p<0.005), n=4 biological replicates and 2 technical replicates.

Figure 5.9. Cytokinin inhibition of endogenous auxin action is independent of transcription. Fluorescence in nuclei of root tips of DII::VENUS seedlings treated for 1 hour with 50µM CHX followed by 15 minutes on 100nM IAA/BAP/IAA+BAP/DMSO, and DMSO (mock) for 65 minutes. Importantly, cytokinin inhibition of endogenous auxin is maintained after treatment with CHX (50µM CHX vs. 50µM CHX → 100nM BAP); Cytokinin promotes auxin signalling pathway when combined with auxin after treatment with CHX (50µM CHX → IAA vs. 50µM CHX → 100µM IAA + 100nM BAP. Student t-test (***p<0.005), n=10.
5.4.4 Auxin, cytokinin and dual treatments modulate PIN2 localisation within 15 minutes

PIN proteins transport auxin molecules across cell membranes, and play key roles in allowing auxin to perform its developmental roles. PIN2 has been shown to be regulated by cytokinin (Street et al., 2016), and unlike PIN1, PIN2 has been shown to be resistant to cytokinin-mediated intracellular trafficking to modulate polarity after cytokinin treatment for 1.5 hours (Marhavy et al., 2014). We have already demonstrated that PINs can be transcriptionally downregulated within 15 minutes of cytokinin treatment. We asked whether PIN2 can be directed to membranes within 15 minutes by cytokinin, and might therefore be responsible for the inhibited auxin signalling seen in DII-VENUS, via transport of active auxin away from the tissues measured. pPIN2::PIN2::GFP seeds were grown on 0.5% MS agar for 7 days, then seedlings were mounted with 50% glycerol containing, 1μM IAA, 1μM BAP, 1μM IAA + 1μM BAP, or DMSO (mock). GFP fluorescence in cell membranes was imaged under a confocal microscope immediately after mounting (0 minutes) and after 15 minutes of treatment, then was quantified using Cellset software (Pound et al., 2012).

Treatment with IAA or BAP alone did not change the fluorescence observed in the cytoplasm or membranes of cortex or epithelium cells (Figure 5.10). However, combined treatment of IAA + BAP significantly increased fluorescence in both the cortex and epithelium cell membranes and cytoplasm. This appears to be additive. Thus, cytoplasmic and membrane-bound PIN2 levels can be altered by auxin+cytokinin treatment within 15 minutes, but not cytokinin alone, and it seems unlikely that the inhibitory effect of cytokinin on auxin signalling seen in our DII-VENUS experiments is explained by modulation of PIN2.

5.4.5 PIN proteins may play a role in cytokinin inhibition of the auxin signalling pathway

To test whether the inhibitory effect of cytokinin seen in DII-VENUS at 15 minutes was controlled by a PIN protein activity, we pre-treated seedlings with naphthylphthalamic acid (NPA), which blocks PIN proteins to inhibit auxin efflux, and then repeated the 15 minute treatment experiment. DII-VENUS seedlings were grown on 0.5% MS agar for 7 days, then transferred to 0.5% MS agar containing 100μM NPA or DMSO (mock) for 30 minutes, then transferred NPA-treated seedlings to agar containing 100μM NPA + 1μM IAA/ 1μM BAP/ 1μM IAA + 1μM BAP/ DMSO (mock) for 15 minutes. The fluorescence of cell nuclei in the epidermis of root tip was imaged under a confocal microscope and quantified using ImageJ.
Pre-treatment with NPA reduced IAA-induced fluorescence loss, presumably because NPA blocked the efflux of the exogenous auxin (Figure 5.11). Interestingly, pre-treatment with NPA caused BAP and tZ to further reduce fluorescence, but BAP and tZ inhibition of exogenous auxin-induced fluorescence loss was not prevented by NPA. Thus, cytokinin may inhibit auxin activity via PIN proteins, but also, where auxin efflux is inhibited, cytokinin may induce fluorescence loss. Further investigations are required to determine if cytokinin induction of fluorescence loss occurs in areas where auxin has built up or depleted from being by the auxin efflux-inhibiting action of NPA.

Figure 5.10. Localisation of PIN2-GFP can be altered by auxin+cytokinin treatment within 15 minutes. Fluorescence in membrane and cytoplasm of cortex and epithelium cells in root of PIN2::GFP seedlings treated for 15 hours with 100nM IAA, 100nM BAP, 100nM IAA + 100nM
BAP, or DMSO (mock). Importantly, combined auxin+cytokinin treatment increased fluorescence in all regions measured, possibly additively. 1 way ANOVA and Tukey's multiple comparison test (letter = $p<0.05$), $n=30-40$.

Figure 5.11. Cytokinin inhibition of exogenous auxin action is independent of PIN activity. Fluorescence in nuclei of root tips of DII::VENUS seedlings treated for 30 minutes DMSO or 100µM NPA, followed by 15 minute treatment of 1µM IAA, 1µM BAP, 1µM tZ, 1µM IAA + 1µM BAP, 1µM IAA + 1µM tZ, or DMSO (mock). Importantly, action of exogenous auxin was partly inhibited (1µM IAA vs. 100µM NPA → 1µM IAA); NPA treatment prevented cytokinin inhibition of endogenous auxin (100µM NPA → Mock vs. 100µM NPA → 1µM BAP, and vs. 100µM NPA → 1µM tZ), but cytokinin continued to inhibited the action of exogenous auxin (100µM NPA → IAA vs. 100µM NPA → 1µM IAA + 1µM BAP, and vs. 100µM NPA → 1µM IAA + 1µM tZ). Student t-test (***, $p<0.005$), $n=10$. 
5.4.6  Cytokinin does not inhibit the auxin signalling pathway in the mDII-VENUS reporter

To test whether the inhibitory effect of cytokinin seen in DII-VENUS at 15 minutes was dependent on TIR1-DII domain interaction, we performed the 15 minute hormone treatment with the mDII-VENUS line. mDII-VENUS has a P53L mutation in the DII domain which dramatically reduces its efficiency of binding to TIR1 (Brunoud et al., 2012). This means the reporter tool in this line is auxin-resistant, and in conjunction with DII-VENUS can be used to determine auxin signalling intensity. Here, it enabled us to ask whether cytokinin inhibited auxin action by preventing the interaction between auxin, TIR1 and the DII domain of Aux/IAA28. mDII-VENUS seedlings were grown on 0.5% MS agar for 7 days, then transferred to 0.5% MS agar containing 1µM IAA, 1µM tZ, 1µM IAA + 1µM tZ, or DMSO (mock) for 15 minutes. The fluorescence of cell nuclei in the epidermis of root tip was imaged under a confocal microscope and quantified using ImageJ.

Cell nuclei fluorescence was slightly decreased upon IAA treatment, as mDII-VENUS has strongly reduced, but not totally removed, binding efficiency (Brunoud et al., 2012) (Figure 5.12). Treatment with tZ had no effect on fluorescence, and combined treatment of IAA+tZ was not statistically different from IAA treatment alone. Thus, cytokinin inhibition of this auxin signalling pathway is dependent on proper binding between TIR1 and the DII domain of Aux/IAA28. As the combined treatment is unchanged vs. IAA treatment alone, it is unlikely that cytokinin acts through modulating proteasome or PIN protein activity. This suggests that cytokinin can bind TIR1 directly as an auxin inhibitor.
Figure 5.12. Cytokinin does not inhibit auxin action in mDII::VENUS. Fluorescence in nuclei of root tips of mDII::VENUS seedlings treated for 1 hour with 100nM IAA, 100nM tZ, 100nM IAA + 100nM tZ, and DMSO (mock). Importantly, cytokinin did not inhibit the action of endogenous auxin (Mock vs. 1µM tZ,) or exogenous auxin (1µM IAA vs. 1µM tZ) in mDII::VENUS. This suggests that cytokinin inhibition of auxin on TIR1 is dependent on the proper formation of a TIR1-IAA-Aux/IAA complex, which supports the hypothesis of cytokinin acting as a direct inhibitor of the formation of this complex. 1 way ANOVA and Tukey’s multiple comparison test (letter = p<0.05), n=10.
5.4.7 Cytokinin may inhibit the auxin signalling pathway in the HS::AXR3NT-GUS reporter

Another tool for visualising auxin activity is the HS::AXR3NT-GUS line, in which GUS-tagged AXR3 expression can be induced, and its degradation upon hormone treatments can be comparatively visualised (Mishra et al., 2009). AXR3 is also known as Aux/IAA17. 7 day old HS::AXR3NT-GUS seedlings grown on 0.5% MS agar were incubated at 37°C for 2 hours, recovered at room temperature for 30 minutes, then treated with 1µM IAA, 1µM BAP, 1µM IAA + 1µM BAP, or DMSO (mock) in liquid media for 15 minutes, and then GUS-stained for approximately 45 minutes.

IAA treatment had no qualitatively determinable effect (Figure 5.13). BAP treatment appeared to have more GUS staining than mock treated, suggesting that BAP protected AXR3-GUS from degradation by endogenous auxin. Combined treatment of IAA+BAP appeared to have reduced expression vs. mock, raising the possibility that BAP enhanced the IAA-induced degradation of AXR3-GUS.

The same treatment in HS::axr3-1NT-GUS, in which the mutated axr3-1 cannot bind TIR1 and thus be tagged for degradation (Mishra et al., 2009), showed no qualitative difference in GUS staining between treatments (Figure 5.14), further suggesting that cytokinin acts to inhibit auxin signalling via regulating the TIR1-Aux/IAA binding relationship, rather than by modulating downstream degradation.
Figure 5.13. Cytokinin inhibits degradation of AXR3-GUS. 9 DAG *HS::AXR3NT-GUS* seedlings were heat shocked for 2 hours, recovered for 30 minutes, treated with 1µM IAA, 1µM BAP, 1µM IAA + 1µM BAP, or mock (DMSO) for 15 minutes, and then GUS-stained. Cytokinin treatment qualitatively appears to inhibit degradation of AXR3-GUS, whilst promoting degradation in auxin+cytokinin treatment vs. auxin treatment. Images are representative of 2 repeats, n=12-14.
**HS::axr3NT-GUS**

DMSO

Auxin

Cytokinin

Auxin+Cytokinin

Figure 5.14. Cytokinin doesn’t inhibit degradation of AXR3-GUS in *HS::axr3-GUS*. 9 DAG HS::axr3-GUS seedlings were heat shocked for 2 hours, recovered for 30 minutes, treated with 1µM IAA, 1µM BAP, 1µM IAA + 1µM BAP, or mock (DMSO) for 15 minutes, and then GUS-stained. No qualitative change between treatments was observed. Images are representative of 2 repeats, n=13-14.
5.4.8 Cytokinin inhibits TIR1 in a mammalian system

We then sought to confirm whether cytokinin directly interacted with the TIR1-Aux/IAA system to inhibit auxin-induced degradation of Aux/IAA proteins. The Auxin Inducible Degron (AID) system has been developed in mammalian cells to enable the rapid degradation of target AID-tagged proteins upon treatment with auxin (Nishimura et al., 2009). This system enables us to examine the TIR1-Aux/IAA-auxin (and perhaps cytokinin) system, in an organism with no endogenous auxin, cytokinin or plant genes to interact with the system, excepting a proteasome. The hnRNP U-FLAID cell line contains an AID-tagged hnRNP (a ribosomal protein), which can be rapidly degraded by treatment with auxin. The levels of hnRNP can then be measured with a Western blot, and the activity of TIR1 determined. hnRNP U-FLAID cells system were cultured overnight, then treated with 500µM IAA, 500µM BAP, 500µM tZ, 500µM IAA + 500µM BAP, 500µM IAA + 500µM BAP or DMSO (mock) for 6 hours. Total protein was extracted from the cells, and levels of hnRNP and alpha-tubulin (loading control) were visualised with Western blotting.

As predicted, IAA treatment dramatically reduced the levels of hnRNP, demonstrating the responsiveness of the cell line (Figure 5.15). Treatment with BAP and tZ alone had no effect on hnRNP levels, which was expected as there is no endogenous auxin present to inhibit the action of. Finally, combined treatments of IAA + BAP/tZ had reduced levels of hnRNP, but those levels were higher vs. IAA treatment alone, suggesting that cytokinin is having an inhibitory effect on the TIR1-AUX/IAA-auxin system. Whether cytokinin affects the mammalian proteasome or transcription was not tested, though the lack of cytokinin signalling machinery in mammalian cells would suggest that it does not. This adds further evidence to the hypothesis that cytokinins can directly interact with the TIR1-Aux/IAA-auxin system.

5.4.9 Cytokinin inhibition of TIR1 in mammalian system is independent of transport across the cell

As mammalian cells lack plant phytohormone signalling machinery, the mammalian U-FLAID cell line is an excellent tool for measuring the potential cytokinin inhibition of TIR1 activity. Being mammalian cells, they also lack classic plant phytohormone transport proteins. However, auxins and cytokinin can cross cell membranes through ABC transporters in plants (Cho and Cho, 2013; Zhang et al., 2014), of which mammalian cells have homologues (Coleman et al., 2001). Thus, it is possible that the cytokinin inhibition of auxin-induced degradation is due to competing access to limited transport proteins. To test this hypothesis, we set up time-
course treatments in which some samples were treated with media containing IAA, which was later removed and replaced media containing BAP, or vice-versa, to remove any transporter competition. See Table 5.1 for full details.

2 hour treatment with IAA reduced levels of hnRNP by around 40% (Figure 5.16), while pre-treatment with BAP followed by 2 hours treatment with IAA caused only a 30% reduction in hnRNP levels. This suggests that the BAP already transported into the cell, and not competing with IAA for transport, is able to inhibit IAA action. Treatment with IAA for 2 hours, followed by 1 hour of blank treatment, reduced hnRNP levels by around 60%. Treatment with IAA for 2 hours, followed by 1 hour of BAP treatment, only reduced hnRNP levels by around 50%. Similarly, treatment with IAA for 2 hours, followed by 2 hours of blank treatment, reduced hnRNP levels by around 70%. Treatment with IAA for 2 hours, followed by 2 hours of BAP treatment, only reduced hnRNP levels by around 60%. This suggests that BAP was able to be transported into the cells, with no IAA competition, and inhibit the action of IAA already transported into the cells. Thus, we concluded that the observed inhibition of IAA-inducible degradation of hnRNP by cytokinin is not due to competition for transport into the cell between auxin and cytokinin.
Figure 5.15. Cytokinin inhibits auxin-induced TIR1 action in mammalian cells. hnRNP U-FLAID cells system were cultured overnight, then treated with 500µM IAA, 500µM BAP, 500µM tZ, 500µM IAA + 500µM BAP, 500µM IAA + 500µM BAP or DMSO (mock) for 6 hours. (A) Western blot probed for α-hnRNPU and α-Tubulin as a loading control from which normalisation was performed. Blot representative of 3 repeats. (B) Spot densitometry of representative blot. Cytokinin inhibits action of exogenous auxin.
Figure 5.16. Cytokinin inhibition auxin-induced TIR1 action in mammalian cells independent of transport into cell. hnRNP U-FLAID cells system were cultured overnight, then treated as shown in (A). (B) Western blot probed for α-hnRNPU and α-Tubulin as a loading control from which normalisation was performed. Blot representative of 2 repeats. (C) Spot densitometry of representative blot.
Table 5.1. Treatments for mammalian AID transport control, results shown in Figure 5.16.

<table>
<thead>
<tr>
<th>Treatment (as shown in Figure 5.16)</th>
<th>Explanation</th>
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<tbody>
<tr>
<td>Mock</td>
<td>4 hours blank treatment</td>
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<tr>
<td>2A</td>
<td>2 hours treatment with auxin</td>
</tr>
<tr>
<td>2C-2A</td>
<td>2 hours treatment with cytokinin, followed by 2 hours treatment with auxin</td>
</tr>
<tr>
<td>2A-1B</td>
<td>2 hours treatment with auxin, followed by 1 hour blank treatment</td>
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<tr>
<td>2A-1C</td>
<td>2 hours treatment with auxin, followed by 1 hour treatment with cytokinin</td>
</tr>
<tr>
<td>2A-2B</td>
<td>2 hours treatment with auxin, followed by 2 hours blank treatment</td>
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<tr>
<td>2A-2C</td>
<td>2 hours treatment with auxin, followed by 1 hour treatment with cytokinin</td>
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5.5 Discussion

5.5.1 TIR1 and auxin dominance

We hypothesised that the auxin receptor TIR1 played a role in establishing auxin dominance and amplifying gene expression. *tir1-1* mutants did not exhibit auxin dominance in lateral root development, and generally showed no amplification of gene expression between auxin and auxin+cytokinin treatment. The initiation of lateral roots requires is complex and requires precise patterning of auxin and cytokinin (Péret et al., 2009). Thus, the disruption in LRD observed in *tir1-1* mutants was expected. The inhibition of LRD by auxin in *tir1-1* mutants was unexpected, and this response deserves further study. As expression of *TIR1*, *AFB1*, and *AFB2*, overlap around LR initiation sites (Parry et al., 2009), it is possible that they coordinate the regulation of different genes that cooperate for proper LRD. Thus, exogenous auxin in *tir1-1* may promote the regulation of a subset a genes via AFB1 and AFB2, but TIR1-regulated genes can’t respond to cooperatively organise LRD, thus exogenosus auxin further disorganises patterning required for LRD vs. endogenous auxin.

The amplification of gene expression by auxin on cytokinin-responsive genes and vice-versa was altered or abolished in *tir1-1*. Of the 12 genes examined, 9 genes exhibited amplified expression in Col-0; in *tir1-1* mutants the expression amplification was abolished. Largely, this was achieved by increased single hormone sensitivity, raising expression levels under single hormone treatments to match auxin+cytokinin treatments. Thus, *tir1-1* mutants exhibited increased sensitivity to both auxin and cytokinin. It is interesting to note that the increased sensitivity mostly only occurred in single hormone treatments, and raised expression to a level
equivalent to auxin+cytokinin treatments in Col-0. Therefore, it is possible that the amplified expression observed in auxin+cytokinin treatments operates through increased hormone sensitivity via TIR1. The substantial decrease in LBD18 expression in auxin+cytokinin vs. auxin is abolished in tir1-1, suggesting that cytokinin inhibits this response via TIR1, which deserves further exploration regarding the formation of lateral roots, in which LBD18 plays an important role (Kim et al., 2012). Repeating this experiment with AFB mutants would shed further light on the TIR1-specificity of this response, though as AFBs can have redundant roles, double and perhaps triple mutants may need to be examined (Dharmasiri et al., 2005).

5.5.2 Direct inhibition of TIR1 by cytokinin

We have performed preliminary experiments that point towards cytokinin being a direct inhibitor of the TIR1 complex. Cytokinin inhibition of auxin-induced degradation of hnRNP in the mammalian FLAID cell system, cytokinin inhibition of Aux/IAA28 DII domain-YFP degradation in the DII-VENUS line, lack of said inhibition in the mDII-VENUS line, the elimination of a transcription-based response, and the limited effect of PIN proteins in Arabidopsis, come together to point towards cytokinin inhibiting the auxin-induced TIR1 signalling pathway. However, we were only able to examine PIN2 localisation; the localisation of other PINs deserves examination. It is also worth noting that because of the scale of the DII-VENUS experiments and the short time frame of the early-responses measured, the loss of fluorescence due to tissue absorption was not estimated with an internal fluorescent stain, such as propidium iodide; given more time this should be rectified. In addition, localisation of AUX1/LAX protein family members and ABCB family transporters than can transport auxin might be explored. Physical confirmation of this hypothesis would best be achieved with x-ray crystallography, much in the same way that auxin has been shown to bind TIR1 (Tan et al., 2007). Such an experiment might also uncover whether cytokinin binds to TIR1 or another part of the TIR1 complex. Here, we do not completely exclude other possibilities of cytokinin inhibition of TIR1 activity, however our data suggests cytokinin acts as a direct inhibitor of TIR1.

It remains a possibility that cytokinin inhibition of the TIR1 signalling pathway is specific to the TIR1-Aux/IAA interaction. TIR1 has a large range of binding affinities for the different Aux/IAAs presumed to be dependent on small differences in structure between the Aux/IAA family DII domain, which is the recognition domain bound by the TIR1 LRR domain (Irina et al., 2012). The interaction of TIR1 with Aux/IAAs is dependent on the concentration of auxin (Dharmasiri
et al., 2005), as well as the auxin affinity of individual Aux/IAAs, which varies (Irina et al.,
2012). Furthering the complexity, interactome studies have revealed the complexity of
Aux/IAA binding. Luo et al., 2018, showed that the 29 Aux/IAAs van form heterodimers with 20
ARFs in a total of 544 interactions, all of which may have different affinities to TIR1 and
different auxin and perhaps cytokinin sensitivities. Zenser et al. have developed a tool to
observe the degradation rates of Aux/IAAs by measuring enzyme activity in Aux/IAA luciferase
(LUC) fusions after treatment with auxin (Zenser et al., 2001). Using this tool with LUC fused to
a variety of Aux/IAAs would enable us to determine whether inhibition of TIR1 by cytokinin is
specific to a subset of Aux/IAAs. Of particular interest would be Aux/IAA3 (SHY2) which is a
crosstalk gene between auxin and cytokinin, being a transcriptional target of ARR1 that
decreases the expression of PIN proteins and regulates the formation of lateral roots (Dello
Ioio et al., 2008; Swarup et al., 2008). Cytokinin inhibition of Aux/IAA28 DII-YFP degradation in
DII-VENUS and mDII-VENUS lines, and inhibition of Aux/IAA17 fragment degradation in the
FLAID mammalian cell line is unambiguous. However, some evidence points towards cytokinin
activation of the TIR1 signalling pathway. ARR6 and CKX3 regulation by cytokinin was altered in
tir1-1, suggesting cytokinin acts through TIR1 to promote expression of ARR6 and CKX3. It is
also possible that cytokinin acts as a cofactor to increase the binding efficiency of specific
Aux/IAAs to TIR1, resulting in the amplified expression seen in auxin+cytokinin treatments.
Further investigations are also required to determine if cytokinin induction of fluorescence loss
occurs in areas where auxin has built up or depleted from being by the auxin efflux-inhibiting
action of NPA. TIR1 and other AFBs also show different dose responses to auxin levels
(Dharmasiri et al., 2005), if they show similar dose-responsiveness to cytokinin levels, this
would add complexity, and is worth examining further.
Chapter 6 - General discussion of key findings

6.1 ARGONAUTE10 and INDEHISCENT regulation of seed development

In this work, we have showed that zll-3 mutants have an increased seed size and a reduced fertility, creating a total seed yield lower than wild type. These phenotypes were partially rescued in zll-3 ind-6 double mutants, and previous work from the Sorefan lab has demonstrated that AGO10 and IND negatively regulate each other. Thus, much of the phenotypes observed in zll-3 mutants are presumably dependent on overexpression of IND. Interestingly, seed size increase was observed in both ind-6 and zll-3 mutants, suggesting that expression of IND is maintained to minimise seed size. ind spt double mutants have severe defects in pollen tube formation; combined with data from Chapter 4, it is therefore probable that infertility observed in zll-3 mutants is due largely to aberrant pollen tube formation, which represents a new role for AGO10.

We observed expression of AGO10 in the funiculus and seed coat zone around the funiculus of the developing seed. Combined with our seed sucrose content data, we predict that AGO10 plays a role in the development of the funiculus to allow movement of glucose into developing seeds.

Expression levels of known seed size regulators were not consistently perturbed in zll-3, ind-6, or zll-3 ind-6 mutants. However, AP2 is a direct negative regulator of IND, thus, the seed size increase observed in ap2 mutants may operate through the same pathway as AGO10. We have generated lists of candidates that may also be involved in this pathway by looking at transcriptome data between Ler and Col-0 ecotypes, as well as auxin and cytokinin responsive genes that are regulated by IND. This list provides a set of genes which will be valuable for the further dissection of the AGO10-IND pathway and the processes it regulates.

6.2 Dual Hormone Response and Auxin Dominance

We have performed the first in-depth analysis of a transcriptome after simultaneous treatment with two hormones, in any organism. Crosstalk between hormones signalling pathways has been the subject of extensive research, comparing the regulation of genes between transcriptomes regulated by single hormones treatments. Such studies, whilst useful, show an incomplete picture of the complexity of hormone interaction. Nemhauser et al. compared the transcriptomes of seven single-hormone treatments, to look for an overarching regulator of hormone responses. Such a regulatory-hub was not found, and was considered to
not exist. They also noted that hormones regulated a low number of common genes, and that each hormone largely regulated non-overlapping transcriptional responses.

Here, we have shown the existence of a dual-hormone response, observed by the co-treatment of auxin+cytokinin. This large set of genes is comparable in size to the gene sets regulated by treatment with a single hormone alone. In addition, a large number of genes regulated by auxin can have their expression pattern under auxin treatment significantly changed by the co-presence of cytokinin, despite cytokinin alone having no transcriptional effect, and vice-versa. In other words, in the presence of auxin, cytokinin can regulate an increased set of genes, many of which it could not regulate on its own, and vice-versa. This adds several layers of complexity to the story of hormone regulation, and reopens the search for a set of core regulatory genes. It also demonstrates the need for other dual-hormone and even multi-hormone transcriptome studies. Although we were unable to determine a clear function of the DHR gene set, the overlap of genes between our transcriptome and the transcriptome of calli, suggests the expected role in the maintenance of an undifferentiated state. Other dual-hormone responses may yield clearer responses.

We were unable to fully explore the possibility of SPATULA being a regulator of the DHR gene set, or to confirm the enriched motif. A reporter system with a concatenated SPT binding motif would represent a good first step in searching for a DHR response element. It seems likely that the DHR is regulated by a set of TFs. The search for response elements and regulating TFs could be helped by a dual-hormone transcriptome experiment with an earlier time-point, to remove secondary and tertiary regulators and responders from the pool of study.

Finally, we have shown preliminary evidence demonstrating that although auxin and cytokinin appear to mutually reinforce each other’s regulation of genes, auxin appears to do so in a dominant fashion. This was also demonstrated physiologically, as auxin was more dominant in regulating the development of lateral roots. This hints at a hormone hierarchy, in which the colliding signalling pathways eventually work out in favour of one of the pathways, and in that balance allow for proper development.
6.3 TIR1, auxin and cytokinin

In this work, we demonstrate that the auxin dominance in gene expression and lateral root formation is dependent on the proper function of TIR1. The role of the AFB family requires similar research. In terms of gene expression, tir1-1 mutants exhibited an increased sensitivity to both auxin and cytokinin, highlighting the interconnectedness of the auxin and cytokinin signalling pathways. The data also suggested that the amplified expression observed in auxin + cytokinin treatments was due to increased sensitivity to the hormone that primarily regulates that gene. As with investigations of other dual-hormone treatments, the effect of combinatory hormone treatments on gene expression may provide insights into the complexity of the relationship between plant hormones.

Our data also suggests that cytokinin is a direct inhibitor of TIR1 or the TIR1 complex, though it does not necessarily rule out non-direct options for said inhibition. This hypothesis would best be confirmed through x-ray crystallography. It would also be worth exploring specific dynamics of this interaction by altering: auxin molecule, cytokinin molecule, concentrations of hormone, AFBs, Aux/IAA-ARF combinations. The localisation of PINs deserves more thorough attention in answering this question, as does the immediate effect of cytokinin on proteasome activity. Further investigations are also required to determine if cytokinin induction of fluorescence loss in NPA-treated tissues occurs in areas where auxin has built up or depleted from being by the auxin efflux-inhibiting action of NPA.

6.4 A model for auxin and cytokinin interaction

Taken together with the amplification of gene expression, auxin dominance, expression of GH3s and CKXs, we propose a model of gene regulation by auxin and cytokinin (Figure 6.1). For auxin regulated genes, in wild type, auxin promotes gene expression via TIR1, amplification of gene expression in auxin+cytokinin treatments is achieved by diminished GH3 expression by the presence of cytokinin, increasing proportionally increasing auxin levels and signalling. This amplification disappears in tir1-1 mutants because auxin, acting through AFBs, expresses less GH3s in response to auxin, thus increasing levels of active auxin and amplifying expression in single hormone treatments. This single hormone sensitivity amplification does not occur in auxin+cytokinin treatment as the expression of those genes has been saturated. Cytokinin inhibits the auxin-induced TIR1 signalling pathway, but either does so to a lesser effect than GH3 diminishment increases signalling, or cytokinin inhibits specific TIR1-Aux/IAA interactions.
Cytokinin regulation of genes is downregulated by a TIR1-dependent process, explaining the amplification of gene expression after treatment with cytokinin in tir1-1 mutants. This amplification does not occur in tir1-1 mutants treated with auxin+cytokinin as the gene expression is saturated. Auxin+cytokinin treatment upregulates CKXs, this should decrease active cytokinin, and therefore reduce cytokinin inhibition of TIR1, resulting in gene amplification, but may also reduce diminishment of auxin-induced GH3 downregulation by cytokinin. How auxin amplifies cytokinin regulation of genes is not explained by this model and remains unknown. Here, we do not completely exclude other possibilities of cytokinin inhibition of TIR1 activity, however our data suggests cytokinin acts as a direct inhibitor of TIR1.
Figure 6.1. A model for auxin and cytokinin interaction. For auxin regulated genes, in wild type, auxin promotes gene expression via TIR1, amplification of gene expression in auxin+cytokinin treatments is achieved by diminished GH3 expression by the presence of cytokinin, increasing proportionally increasing auxin levels and signalling. This amplification disappears in tir1-1 mutants because auxin, acting through AFBs, expresses less GH3s in response to auxin, thus increasing levels of active auxin and amplifying expression in single hormone treatments. This single hormone sensitivity amplification does not occur in auxin+cytokinin treatment as the expression of those genes has been saturated. Cytokinin inhibits the auxin-induced TIR1 signalling pathway, but either does so to a lesser effect than GH3 diminishment increases signalling, or cytokinin inhibits specific TIR1-Aux/IAA interactions. Cytokinin regulation of genes is downregulated by a TIR1-dependent process, explaining the amplification of gene expression after treatment with cytokinin in tir1-1 mutants. This amplification does not occur in tir1-1 mutants treated with auxin+cytokinin as the gene expression is saturated. Auxin+cytokinin treatment upregulates CKXs, this should decrease active cytokinin, and therefore reduce cytokinin inhibition of TIR1, resulting in gene amplification, but may also reduce diminishment of auxin-induced GH3 downregulation by cytokinin.
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Supplementary figures

A. **AP2**

B. **ARF2**

C. **BB**

D. **CKX2**

E. **EOD3**

F. **FER**

G. **KLUH**

H. **MET1**

Genotype

Legend:
- 5 DAP
- 10 DAP
- Dry Seed
- Seedling
Supplementary Figure 1. Multiple seed size regulators have altered expression in zll-3, ind-6, and zll-3 ind-6 at various stages of development, however none are consistently changed. (A-J) expression profiles of: AP2, ARF2, BB, CKX2, EOD3, FER, KLUH, MET1, MYB56, and TTG2, respectively, in Ler, zll-3, ind-6, and zll-3 ind-6 at 5 DAP, 10 DAP, dry seed and seedling stages of development. Student t-test (*p<0.05, **p<0.01, ***p<0.005), n= 2-4 biological replications, and 2 technical replications.
Supplementary Figure 2. qRT-PCR of genes to confirm results of microarray. Gene expression profiles after treatment with 10µM IAA, 1µM BAP, 10µM IAA + 1µM BAP, or DMSO (mock) for 6 hours in 7 DAG Col-0 seedlings. Expression profiles of genes confirmed to respond to: (A) auxin treatment alone, (B) cytokinin treatment alone, (C) auxin+cytokinin treatment alone, (D) auxin treatment and cytokinin treatment, (E) auxin treatment and auxin+cytokinin treatment, (F) cytokinin treatment and auxin+cytokinin treatment, and (G) auxin treatment, cytokinin treatment, and auxin+cytokinin treatment. Student t-test (*p<0.05, **p<0.01, ***p<0.005).
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Supplementary Table 1. Dual Hormone Response genes involved in abiotic stress responses. Found by comparing the DHR silo of genes with transcriptomes from *Arabidopsis thaliana* subjected to various stresses: cold, drought, high light and hypoxia. Source data: (Liu et al., 2005; Huang et al., 2008; Rasmussen et al., 2013).

<table>
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<th>Gene</th>
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<th>$p$-value – are the lines different?</th>
<th>$r^2$ – 1μM IAA + BAP [range]</th>
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Supplementary Table 2. Pearson correlation values for A-AC genes under various hormone concentration gradients, and the probability of statistical difference between IAA [range] and 1μM BAP + IAA [range].

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Supplementary Table 3. Pearson correlation values for C-AC genes under various hormone concentration gradients, and the probability of statistical difference between BAP [range] and 1µM IAA + BAP [range]. Data excludes BAP 100µM which breaks the cytokinin responsiveness trend and skews the data.
Supplementary Figure 3. Treatment with cytokinin modifies the auxin-response of auxin-responsive genes which are unresponsive to cytokinin alone in an auxin-dominant manner. In sets of 4, graphs show: gene expression profiles after treatment with IAA (10nM, 100nM, 1µM, 10µM, 100µM) +/- 1µM BAP, 1µM BAP or DMSO (0); scatterplot of aforementioned data + regression line; gene expression profiles after treatment with BAP (10nM, 100nM, 1µM, 10µM, 100µM) + 1µM IAA, or DMSO (mock); scatterplot of aforementioned data + regression line. Treatment is for 6 hours in 7 DAG Col-0 seedlings. (A-D) ARF2, (E-H) ARF19, (I-L) Aux/IAA1, (M-P) Aux/IAA5, (Q-T) Aux/IAA19, (U-X) LBD18. See table for Pearson’s correlation coefficient. 1 way ANOVA and Tukey’s multiple comparison test (letter = p<0.05), and Pearson’s correlation coefficient.
Supplementary Figure 4. Treatment with auxin modifies the cytokinin-response of cytokinin-responsive genes which are unresponsive to auxin alone in an auxin-dominant manner. In sets of 4, graphs show; gene expression profiles after treatment with BAP (10nM, 100nM, 1µM, 10µM, 100µM) +/- 1µM IAA, 1µM IAA or DMSO (0); scatterplot of aforementioned data + regression line; gene expression profiles after treatment with IAA (10nM, 100nM, 1µM, 10µM, 100µM) + 1µM BAP, or DMSO (mock); scatterplot of aforementioned data + regression line. Treatment is for 6 hours in 7 DAG Col-0 seedlings. (A-D) ARR3, (E-H) ARR4, (I-L) ARR15, (M-P) CKX4, (Q-T) NRP1, (U-X) WOL. See table for Pearson’s correlation coefficient. 1 way ANOVA and Tukey’s multiple comparison test (letter = p<0.05), and Pearson’s correlation coefficient.

Supplementary Figure 5. Cytokinin reduces regulation of GH3s by auxin. Gene expression profiles after treatment with 10µM IAA, 1µM BAP, 10µM IAA + 1µM BAP, or DMSO (mock) for 6 hours in 7 DAG Col-0 seedlings. GH3.1, GH3.2 and GH3.1 are upregulated by auxin, and show disproportionately reduced upregulation after auxin+cytokinin treatment, whilst only GH3.1 and GH3.2 show downregulation by cytokinin. Student t-test (*p<0.05, **p<0.01, ***p<0.005).
Supplementary Figure 6. Auxin+cytokinin treatment additively and synergistically upregulates CKXs. Gene expression profiles of CKX1-7 after treatment with 10μM IAA, 1μM BAP, 10μM IAA + 1μM BAP, or DMSO (mock) for 6 hours in 7 DAG Col-0 seedlings. Student t-test (a = p<0.05 vs. auxin, c= p<0.05 vs. cytokinin).
Supplementary Figure 7. *tir1-1* mutants have altered gene expression responses to auxin, cytokinin and auxin+cytokinin treatments. Gene expression profiles of 17 genes after treatment with 10µM IAA, 1µM BAP, 10µM IAA + 1µM BAP, or DMSO (mock) for 6 hours in 7 DAG Col-0 and *tir1-1* seedlings. (A-F) A-AC genes, (G-K) C-AC genes, (L-P) other genes. Student t-test (*p<0.05, **p<0.01, ***p<0.005).
A. ARF2

B. ARF19

C. Aux/IAA1

D. Aux/IAA5

E. Aux/IAA19

F. LBD18

Hormone treatment
Supplementary Figure 8. The altered expression responses to hormones in tir1-1 mutants is mimicked in wei8-1 tar1-1 mutants for some genes. Gene expression profiles of 9 genes after treatment with 10µM IAA, 1µM BAP, 10µM IAA + 1µM BAP, or DMSO (mock) for 6 hours in 7 DAG Col-0, tir1-1, and wei8-1 tar1-1 seedlings. (A-F) A-AC genes, (G-I) other genes. Student t-test (*p<0.05, **p<0.01, ***p<0.005).