CHEMICAL DISSECTION OF HORMONE SIGNALLING IN ARABIDOPSIS

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ABSTRACT

The plant hormone gibberellin (GA) regulates many developmental processes during a plant's life cycle, including root and hypocotyl growth. Bioactive GAs promote GA-responsive growth and development by targetting DELLA proteins for degradation. Whilst the early steps of GA signalling are well understood it is not yet clear how the DELLA proteins alter the expression of GA-responsive genes. As other steps of the signalling pathway are encoded by multi-gene families it is possible that genetic redundancy is masking the transcription factors that act downstream of DELLAs. Using a chemical screen based on DELLA protein's control of GA biosynthesis, 28 chemicals which blocked the GA-mediated downregulation of *GA20ox1::GUS* activity were identified. Using GA-mediated RGA degradation as a marker, 11 chemicals were identified as acting downstream of DELLAs in the GA signalling pathway.

One of the chemicals (N23) identified in the screen was found to induce agravitropic root growth, a response more often associated with perturbation of auxin signalling. However, N23 had no effect on auxin signalling based on the characterisation of its effect on auxin-inducible genes and AUX/IAA degradation. The mode of action of N23 requires further investigation. However, N23 represents a potential for studying the role of GA in modulating gravitropism.

The compound N16 potentially perturbs GA signalling by altering GA transport. It was found to block the uptake of both radiolabelled and fluorescent labelled GA into the root. Five days of exposure to N16 was required before any inhibition was observed on Col-0 roots but root elongation in *ga1-3* seedlings was inhibited after only 24 hours suggesting that roots of wild type plants are saturated for GA. The site of action of N16 was not identified, but a putative oligopeptide transporter OPT6 was which is rapidly downregulated in the roots in response to GA application was investigated as a potential novel GA transporter. However, GA uptake assays in yeast strains overexpressing OPT6 proved inconclusive.

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ABBREVIATIONS

ABA	abscisic acid
BR	brassinosteroids
$[^3H]GA_4$	tritiated GA_4
µ	micro
3AT	3-amino-1,2,4-triazole
ABCB/PGP	ATP-binding cassette transporter B subfamily/PGLYCOPROTEIN MULTIDRUGRESISTANCE
ABP1	AUXIN BINDING PROTEIN 1
AD	activation domain
ARE	auxin-response elements
ARFs	auxin response factors
AUX/IAA	AUXIN/INDOLE-3-ACETIC ACID
bHLH	basic helix-loop-helix
BOI	BOTRYTIS SUSCEPTIBLE1 INTERACTOR
BRI1	BRASSINOSTEROID INSENSITIVE 1
BZR1	BRASSINAZOLE RESISTANT1
CAND1	CULLIN-ASSOCIATED and NEDD8-DISSOCIATED1
cDNA	complementary DNA
Col-0	Columbia-0
CPS	ent-copalyl diphosphate synthase
DB	DNA-binding domain
dH2O	deionised water
DMSO	Dimethyl sulfoxide
DREBs	dehydration-responsive element-binding proteins
DWF4	DWARF 4
EIN3/EIL1	ETHYLENE INSENSITIVE 3/EIN3-LIKE 1
ent-CDP	ent-copalyl diphosphate
ENY	ENHYDROUS
EtOH	ethanol
EUI	ELONGATED UPPER INTERNODE
Fl-GA_3	Flourescent tagged GA3
GA	gibberellin
GA20ox	GA 20-oxidase
GA2ox	GA 2-oxidase
GA3ox	GA 3-oxidase
GAI	GIBBERELLIN INSENSITIVE
GAMT	GA methyl transferases
GFP	green fluorescent protein
GGDP	trans-geranylgeranyl diphosphate
GGPP	geranylgeranyl-pyrophosphate
GID1	GA INSENSITIVE DWARF1
GSK	glycogen synthase kinase
GUS	β-Glucuronidase
HLS1	HOOKLESS1

IAA	indole-3-acetic acid
IAM	indole-3-acetamide
IAOX	indole-3-acetaldoxime
IDD1	INDETERMINATE DOMAIN1
JAZ	JASMONATE ZIM-DOMAIN
KAO	ent-kaurenoic acid oxidase
KO	ent-kaurene oxidase
KS	ent-kaurene synthase
m	Milli
n	Nano
PIF	phytochrome interacting factors
PIN	PINFORMED
PYR	PYRABACTIN RESISTANCE
RAM	root apical meristem
RGA	REPRESSOR OF GA1-3
RGL	RGA-LIKE
RSG	REPRESSION OF SHOOT GROWTH
RUB	RELATED TO UBIQUITIN
SABATH	salicylic acid MT, benzoic acid MT and theobromine synthase
SAM	shoot apical meristem
SCF	SKP-Cullin F-box
SCL3	SCARECROW-LIKE 3
SLY1	SLEEPY1
SNE/SLY2	SNEEZY
SPL	SQUAMOSA PROMOTER BINDING-LIKE
SPT	SPATULA
TAA	TRYPTOPHAN AMINOTRANSFERASE of ARABIDOPSIS
TIR1	TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX
trp	Tryptophan
VP1/ ABI3	VIVIPAROUS1/ABSCISIC ACID INSENSITIVE 3
YAB1	YABBY1
YFP	yellow fluorescent protein
YUC	YUCCA

CHAPTER 1. INTRODUCTION

1.1. THE PLANT HORMONES

Plants exhibit a large degree of flexibility in both growth and development and this is in a large part due to the various endogenous phytohormones (hormones). Abscisic acid (ABA), auxins, brassinosteroids (BR), cytokinins, ethylene, gibberellins (GAs), jasmonate and strigolactones have all demonstrated a role in the coordination and control of plant development and growth in response to environmental cues. The major roles of ABA in development are in seed dormancy and response to environmental stress (Kermode, 2005). Auxins have a role in numerous plant development processes including, stem elongation, gravitropism, lateral root and shoot branching and vascular development (Perrot-Rechenmann and Napier, 2005; Sauer et al., 2013). Brassinosteroids promote cell elongation, are involved in vascular development, photomorphogenesis and senescence, whilst also being implicated in improving tolerance to salt and mild drought stress in crops (Clouse and Sasse, 1998). Cytokinin has been shown to regulate shoot growth, shoot apical meristem (SAM) size, leaf cell production and root growth through its promotion of cell division (Werner et al., 2001). Ethylene is a gaseous hormone required for root hair development, seedling growth, leaf and petal abscission, fruit ripening, seed germination and organ abscission (De Paepe and Van der Straeten, 2005). Ethylene also has a role in mediating the plant's response to wounding as a result of pathogen attack or to stress (De Paepe and Van der Straeten, 2005). GAs promote seed germination, stem and root elongation, flowering, flower, leaf expansion, fruit growth, juvenile to adult transition and seed development (Achard and Genschik, 2009). Jasmonate is the plant hormone most commonly associated with the plant's response to wounding as a result of pathogen and insect attack (Browse, 2005), whilst strigolactone has been implicated in the inhibition of shoot branching, root elongation and lateral root density (Ruyter-Spira et al., 2011; Umehara, 2011). Each hormone has been shown to have a distinct and complex metabolism and signalling pathway.

Despite the numerous advances made in modern genetics there is still much to learn about the hormone signalling pathways, progress possibly being hampered by genetic redundancy in the favoured model species, *Arabidopsis thaliana* (hereafter referred to as Arabidopsis). Additionally various studies have shown that specific plant phenotypes are the result of signalling from numerous hormones. Generally it has been found that auxin orchestrates growth and development whereas other hormones, e.g. GA, are required for the modulation of these growth processes (Teale et al., 2008). This project will focus on the study of the hormone GA and IAA, which interact with each other to control plant growth (Fu and Harberd, 2003).

Auxin and GA are known to control similar aspects of plant development, such as organ elongation. With recent insights into their respective signalling cascades the existence of similarities between these pathways has become apparent. In both cases, activation of the signalling cascade results in the targeted degradation of repressor proteins. In the case of GA signalling, binding of GA with its receptor GID1 (GA INSENSITIVE DWARF1) initiates the formation of a complex between GA, GID1, the F-box protein SLY1 and a member of the DELLA family of proteins, resulting in the ubiquitination and subsequent degradation of the DELLA proteins by the 26S-proteasome. As DELLA proteins are transcriptional regulators that repress GA responses, their removal results in the initiation of GA-responsive growth, such as root elongation. The components of GA signalling that have been discovered so far have predominantly been identified by forward genetics-based approaches (reviewed in Hedden and Thomas, 2012). Despite these achievements in elucidating aspects of the GA signalling pathway, there has previously been limited success in the identification of components acting downstream of the DELLA proteins using these approaches. It is known that DELLA proteins are transcriptional regulators but they are unlikely to function as transcription factors due to the absence of a conserved DNA binding domain (Zentella et al., 2007). Recent work suggests that DELLAs regulate transcription through their association with transcription factors. For example, DELLA proteins control Arabidopsis hypocotyl cell expansion through their interaction

with the phytochrome interacting factors (PIFs) (de Lucas et al., 2008). One possible explanation for the early lack of success in identifying components downstream of DELLAs could be functional redundancy in the transcription factors. The components and mechanisms of the auxin signalling pathway are now well understood. Auxin binds to its receptor TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1), which also acts as an F-box protein, leading to the formation of a complex with the AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins which are subsequently ubiquitinated and degraded via the 26S-Proteasome. In the absence of auxin the AUX/IAA proteins bind to and inhibit the action of the auxin response factors (ARFs) which are transcription factors which can either activate or inhibit the expression of auxin responsive genes. As with GA signalling most of the components of auxin signalling have been identified by forward genetic approaches, particularly the study of mutants such as *iaa* and *tir1* (Reed, 2001; Dharmasiri et al., 2005).

1.2. GIBBERELLIN

1.2.1. Gibberellins history and use

Gibberellin was initially isolated from the fungus *Gibberella fujikuroi* which causes stem overgrowth in rice plants (Stowe and Yamaki, 1957). Despite some bacteria and fungi producing GAs, comparisons between the *Gibberella fujikuroi* and the higher plant biosynthesis indicate there is no common ancestral origin (Hedden et al., 2002). Currently 136 different GA structures have been characterised in plants, fungi and bacteria (www.plant-hormone.info/gibberellins.htm). GAs comprise a group of tetracyclic diterpenoid carboxylic acids, a small number of which have bioactivity in higher plants. All identified GAs are given the name gibberellin $A_{1\text{-}136}$ (shortened to $GA_{1\text{-}136}$), depending on the order they were discovered. Only a limited number of GAs have intrinsic biological activity, the principal ones being GA_1, GA_3, GA_4 and GA_7 (structures of GA_1 and GA_4 shown in Figure 1.1., page 13), of which only GA_1 and GA_4 are ubiquitous in plants. Other GAs are known to have bioactivity but these only

occur in selected plants and tissues. The first GA to be structurally characterised was GA_3, which was isolated from *Gibberella fujikuroi* (Takahashi et al., 1955). Since the characterisation of this GA numerous others have been identified, particularly in immature seeds where the reasons for the high levels of accumulation and their roles are currently unclear. There is evidence of a role for GA in cell expansion, proliferation and seed germination (Swain et al., 1993; AitAli et al., 1997; Cowling and Harberd, 1999; Achard and Genschik, 2009). This raises the possibility that immature seeds require high level of bioactive GA to induce growth but must inactivate these GAs before germination is induced. Such a theory would explain the high levels of numerous inactive GAs observed in immature seeds. GA signalling comprises the biosynthesis and deactivation of bioactive GAs from their precursors, their perception by the GA receptor GID1, and the resulting signal transduction. Each of these stages is subject to regulation by spatial, developmental and environmental factors. The concentration of GA within plant tissues is determined by their rates of biosynthesis and deactivation. GA metabolism has been studied for over half a century with numerous enzymes within the pathway being identified by varied approaches including enzyme purification, functional screening of cDNA expression libraries and molecular genetic approaches using dwarf mutants defective in GA biosynthesis (Yamaguchi, 2008). More recently genomic tools have led to the identification of more enzymes (Yamaguchi, 2008). The discovery of the GA receptor GID1 has allowed the elucidation of the GA signalling pathway and the intricate role GA has in plant development (Ueguchi-Tanaka et al., 2005). As with most advances in understanding of GA signalling, GID1 was cloned following the identification and characterization of GA-insensitive dwarf mutant, in this case in rice. Famously, the varieties of wheat and rice that were important elements of the Green Revolution contain dwarfing alleles of the *Rht1* and *SD1* genes, respectively These mutations were shown to compromise GA signalling and biosynthesis, respectively (Peng et al., 1999; Ashikari et al., 2002; Monna et al., 2002; Spielmeyer et al., 2002).

1.2.2. The role of GA in development

Possibly the first characterization of a GA mutant was the identification of a mutation in the gene encoding the *ent*-kaurene synthase (KS) enzyme in the *dwarf5* maize mutant (Hedden and Phinney, 1979). Whilst many GA-response mutations studied in other plant species arose naturally, the early GA-responsive Arabidopsis mutants, *ga1*, *ga2*, *ga3*, *ga4* and *ga5*, were produced by chemical mutagenesis (Koornneef and Vanderveen, 1980). These mutants can be divided into two categories: those with mutations in single copy genes (*ga1, ga2, ga3*) which produced seeds that did not germinate without exogenous GA application and produced plants that did not bolt and were infertile, and those (*ga4, ga5*) that resulted in semi-dwarfism, with fully fertile flowers and seeds that germinated normally. It was found subsequently that the phenotype of this latter group was due to genetic redundancy, with the mutant genes members of multigene families (Plackett et al., 2012). Further studies of these mutants led to the demonstration that *GA1* encoded the enzyme *ent*-copalyl diphosphate synthase (CPS) which catalyzes the first step in the GA-biosynthetic pathway (Sun et al., 1992; Sun and Kamiya, 1994). Due to the negligible levels of GA produced by severe *ga1* mutants, the *ga1-3* allele is regularly used as a positive control for experiments where GA production is inhibited. Additionally, studies using a promoter::GUS reporter line and RT-PCR to analyse *GA1* expression have demonstrated that GA biosynthesis occurs in specific cell types of developing tissues including shoot apices, root tips, developing flowers and seeds (Silverstone et al., 1997). *GA2* encodes the second enzyme in the GA biosynthetic pathway KS (Yamaguchi et al., 1998) whilst *GA3* encodes the enzyme for the following step, *ent*-kaurene oxidase (KO) (Helliwell et al., 1999). *GA4* and *GA5* encode *GA 3-oxidase1* (*GA3ox1*) and *GA 20-oxidase1* (*GA20ox1*), respectively (Chiang et al., 1995; Phillips et al., 1995; Xu et al., 1995). Together the GA20ox and GA3ox enzyme families catalyze the final steps in the conversion of GA-precursors to bioactive GAs. GA biosynthesis is described in full in Chapter 1.3.1. From this initial starting point in

Arabidopsis many other GA mutants have been produced and characterized to fine tune our understanding of the roles of GA in various developmental processes.

1.2.3. Role of GA in flowering

GA-deficient mutants of Arabidopsis, which is a facultative long-day species, exhibit delayed flowering in long days, whilst requiring exogenous application of GA to flower in short-days (Wilson et al., 1992). Later reports showed that this induction of flowering is a result of primarily GA_4 on the floral pathway integrators and under long day conditions can be bypassed by the light-dependent flowering pathway (Blazquez et al., 1998; Moon et al., 2003; Eriksson et al., 2006; Tan and Swain, 2006). *ga1-3* also demonstrates the role of GA in floral development. This GA-deficient mutant is infertile due to poorly developed stamens, which do not dehisce, and severely reduced fertility of the pistil (Goto and Pharis, 1999). Whilst this review focuses primarily on Arabidopsis it should be noted that GA-deficient mutations can also delay or even abolish flowering in other species (Plackett, 2011).

1.2.4. Role of GA in seed germination

GAs are known to have a wide variety of roles within Arabidopsis germination. To date GA has been implicated in the production of hydrolytic enzymes to weaken the seed coat, mobilization of seed nutrients, induction of plant embryo expansion and hypocotyl elongation upon germination (Gubler and Jacobsen, 1992; Saibo et al., 2003; Penfield et al., 2006). As the GA deficient seeds (*ga1-3*) can still germinate and produce normal plants after GA application it can be concluded that GA is essential for germination (Koornneef and Vanderveen, 1980). However plants overexpressing the GA deactivating *GA 2-oxidase2* (*GA2ox2*) enzyme exhibit a high level of seed abortion suggesting GA does have a role in the early aspects of seed development in addition to germination (Singh et al., 2002).

Due to their importance to agriculture the effect of GA on seed germination have been studied extensively in the cereal aleurone layer. During germination of the cereal grain, GA is produced in the embryo from where it is transported or diffuses to the aleurone layer induces the expression of hydrolytic enzymes. These are secreted into the endosperm to produce metabolites to nourish the germinating embryo and promote seedling growth. (Gubler and Jacobsen, 1992). GA also acts by counteracting the effects of the germination inhibiting hormone ABA. The ratio of ABA to GA signalling and the amount of the hormones present influences germination (Finch-Savage and Leubner-Metzger, 2006).

1.2.5. GAs role in growth and elongation

Restricted stem elongation is a useful agricultural trait that has been selected for in many crops, most famously in the semi-dwarf *Rht-1* alleles in wheat and *sd-1* in rice varieties that gave rise to the Green Revolution (Harberd et al., 1999). Further analysis of these mutants identified *sd-1* as being a mutation in the rice *GA20ox2* gene whilst *Rht-1* mutations affect the wheat DELLA protein that mediates GA signalling (Peng et al., 1999; Ashikari et al., 2002; Sun and Gubler, 2004; Pearce et al., 2011). Similarly the GA deficient Arabidopsis mutant *ga1-3* displays severe dwarfism as a result of reduced leaf expansion and root elongation, and the elimination of bolting (Koornneef and Vanderveen, 1980). It has been shown that GA promotes organ growth by stimulating cell division and/or cell elongation (Sauter and Kende, 1992; Inada et al., 2000; Fu and Harberd, 2003; Ubeda-Tomas et al., 2008; Ubeda-Tomas et al., 2009). Application of GA to rice plants resulted in the increased expression of the cyclin *cycA1;1* and the cyclin dependent kinase *cdc2Os-3* which have been suggested to contribute to the progression of a cell from the G2 phase to mitosis during cell division. *cycD1;1*, which is involved in the transition of cells from G1 to the DNA synthesis stage of cell cycling is also known to be up-regulated in Arabidopsis seeds after a 12 hour GA treatment (Ogawa et al., 2003). Taken together these results go some way to explaining the role of GA in cell division. Promotion of

cell elongation by GA involves loosening of cell walls, through the induction of the cell wall loosening enzymes such as xyloglucan endotransxyloglucosylase and expansins (Chen et al., 2002). Additionally GA can promote cell expansion by increasing cell turgor through the stimulation of osmolytes production by aquaporins (Cosgrove and Sovonick-Dunford, 1989).

In recent years there have been important advances in the understanding of the regulation of GA-mediated root growth. The expression of a non-degradable DELLA protein, *gai*, specifically in the endodermal cells of Arabidopsis seedlings blocked cell expansion in the root resulting in reduced root growth (Ubeda-Tomas et al., 2008). In a second study by the same group, *gai* expressed in the endodermis of the root meristem blocked division of all cells within the meristem (Ubeda-Tomas et al., 2009). The authors suggest that GA controls cell division via this tissue by degrading the DELLA proteins which in turn allows cell expansion. Cell division is governed by the size of neighbouring cells therefore the expansion and division of endodermal cells in response to GA allows the expansion and division of cells in the neighbouring tissues.

It is becoming increasingly clear that GA achieves a control of root growth as a result of interactions with numerous other signalling pathways. Fu and Harberd (2003) demonstrated that shoot apex produce auxin is required for the DELLA protein degradation within the root and thus regular root growth.

1.2.6. The role of GA mutants in agriculture

The study of GA signalling and biosynthesis in Arabidopsis furthers the scientific communities' understanding of the mechanisms of plant growth. Due to the importance of GA mutants within agriculture this information could potentially lead to more manageable and productive crops. As mentioned previously the wheat *Rht-1* and rice *sd-1* mutants played an integral role in improving crop yields during the Green Revolution. Both these varieties produced shorter plants that were less likely to

lodge in wet or windy weather and produced higher yields upon fertiliser application as more biomass was partitioned to the grain as opposed to the stem (Harberd et al., 1999; Peng et al., 1999; Pearce et al., 2011). *The Rht-1* mutations occur in the wheat DELLA gene and result in mutant proteins that constitutively repress GA signalling (Peng et al., 1999; Pearce et al., 2011). There are numerous *Rht-1* alleles producing plants of varying heights (Ellis et al., 2005b). The limitation of the *Rht-1* varieties is due to the pleiotropic effects caused by altered GA sensitivity, within the plant resulting in alterations to GA controlled process, including, fertility and root growth, and not just the desired stem elongation (Bai et al., 2013). For example, the inhibition of stem elongation in *Rht* may be of benefit to reduce lodging but the accompanying reduced root elongation may limit nutrient capture (Lynch, 2007). It is therefore important to understand GA control of different aspects of organ growth which may allow us to uncouple the responses allowing a more targeted manipulation of GA-responsive growth. Additionally the targeted control of separate development processes may be possible with the identification of GA signalling components downstream of the DELLA proteins.

1.3. GA BIOSYNTHESIS AND SIGNALLING

1.3.1. GA biosynthesis

The major components of GA biosynthesis have been identified in both monocot and dicot plants and indicate that there is a conserved pathway in higher plants (Hedden and Phillips, 2000; Yamaguchi, 2008). GA biosynthesis can be sub-divided into three specific stages. The first stage is a methylerythritol phosphate pathway which produces the hydrocarbon intermediate *ent*-kaurene from geranylgeranyl-pyrophosphate (GGPP) (Kasahara et al., 2002). Initially it was believed that GA biosynthesis competed with other pathways, such as carotenoid biosynthesis, for GGPP as a substrate. However, van Schie et al. (2007) showed that Arabidopsis and tomato plants lacking geranyl diphosphate synthase had reduced GA levels but

carotenoid levels were unaffected, indicating that *ent*-kaurene is synthesised from a separate pool of GGPP by a GGPP synthase that requires GPP as a substrate. The production of *ent*-kaurene from GGPP requires the conversion of *trans*-geranylgeranyl diphosphate (GGDP) to *ent*-copalyl diphosphate (*ent*-CDP) via a proton-initiated cyclisation catalysed by the class II terpene cyclase, CPS (also known as *ent*-kaurene synthase A). The conversion of *ent*-CDP to *ent*-kaurene, initiated by phosphate ionization, is catalysed by the class I cyclase, KS; both reactions occur in the plastid (Figure 1.2.) (Sun and Kamiya, 1994; Hedden and Phillips, 2000; Sakamoto et al., 2004). Both CPS and KS are encoded by single genes in Arabidopsis (Koornneef and Vanderveen, 1980).

The second stage of GA biosynthesis involves the conversion of *ent*-kaurene to GA_{12}, which is a common precursor for all GAs in plants (Hedden and Phillips, 2000), is catalysed by two cytochrome P450 monooxygenases: KO and *ent*-kaurenoic acid oxidase (KAO) in the endoplastic reticulum (Figure 1.2.) (Helliwell et al., 2001; Yamaguchi, 2008). KO has also been shown to localise to the plastidic outer envelope (Helliwell et al., 2001). KO catalyses the C-19 hydroxylation of *ent*-kaurene→*ent*-kaurenol→*ent*-kaurenal→*ent*-kaurenoic acid, with the initial step being rate limiting and the subsequent intermediates being retained at the enzyme active site (Morrone et al., 2010). Again KO is encoded by one gene in Arabidopsis whilst rice possess five KO-like genes with only one (OsKO2) genuinely encoding a functional KO enzyme (Sakamoto et al., 2004). Conversion of *ent*-kaurenoic acid to GA_{12} by KAO is a three step process via the intermediates *ent*-7α-hydroxykaurenoic acid and GA_{12}-aldehyde that requires the successive oxidations of C-7β, C-6β and C-7 (Figure 1.2.) (Hedden and Kamiya, 1997). As only one gene encodes the KO enzyme in Arabidopsis (*GA2*), mutants lacking this gene display a severe GA-deficient phenotype, e.g. severely dwarfed and require GA application to germinate (Koornneef and Vanderveen, 1980). Using a KAO encoding gene sequence from barley, *GRD5*, two homologous sequences in Arabidopsis were identified, *KAO1* and *KAO2* (Helliwell et al., 2001). Due to genetic redundancy Arabidopsis KAO mutants have not been identified. KAO

loss-of-function mutations in pea, sunflower and rice result in severe dwarfism (Davidson et al., 2003; Sakamoto et al., 2004; Fambrini et al., 2011). Interestingly a pumpkin 2-oxoglutarate-dependent dioxygenase (ODD) has been shown to convert GA_{12}-aldehyde to GA_{12}, although this has not been observed in other species (Lange, 1997).

After GA_{12} the biosynthesis pathway branches into two separate oxidation pathways, which form the final stage in the synthesis of bioactive GAs (GA_1, GA_3, GA_4) (Figure 1.2.). The production of GA_1 and GA_3 (in monocots) first requires the C-13 hydroxylation of GA_{12} to GA_{53} (Yamaguchi, 2008). Both P450 and ODD enzymes have been shown to catalyse the 13-hydroxylation of GA_{12} (Sponsel and Hedden, 2004). In rice two P450s have been shown to catalyse the conversion of GA_{12} to GA_{53}, but in Arabidopsis and *Stevia rebaudiana* these enzymes catalyse the 13-hydroxylation of *ent*-kaurenoic acid (Sponsel and Hedden, 2004; Brandle and Richman, 2008; Yamaguchi et al., 2008; Magome et al., 2013). The second branch in the pathway from GA_{12} is the C-20 oxidation which ultimately results in the bioactive GA_4. GA20ox, an ODD, performs a series of 20-oxidations that converts $GA_{12}\rightarrow GA_{15}\rightarrow GA_{24}\rightarrow GA_9$ (Figure 1.2.) (Hedden and Kamiya, 1997). In the 20-hydroxylation reaction the C-20 methyl group is oxidised to form an alcohol then an aldehyde which leads to the loss of C-20 when a γ-lactone is formed between C-19 and C-10 (Figure 1.1.) (Hedden and Thomas, 2012). The aldehyde intermediates of the C-20 oxidation accumulate to high levels in the plant, as do the alcohol intermediates to a lesser extent (Webb et al., 1998), suggesting the intermediates have to rebind to GA20ox for oxidation to continue. The oxidation of the alcohol intermediate can be prevented by the lactonisation with the C-19 carboxylic acid, although some tissues contain a GA20ox capable of oxidising the C-20 lactone form (Ward et al., 1997). There is little information as to how the C-20 is lost. There is some evidence that the formation of a free radical on C-10 that reacts with C-19 carboxylic acid to form a lactone is involved (Ward et al., 2002). Another study has shown that C-20 is lost directly as carbon dioxide, although this would require an as yet unidentified intermediate after the C-20

aldehyde (Kamiya et al., 1986). The conversion of GA_{53} to GA_{20} via GA_{44} and GA_{19} also requires C-20 oxidation by GA20ox. (Figure 1.2.).

The final oxidation step of the GA biosynthesis pathway, the conversion of GA_9 to GA_4 and GA_{20} to GA_1, is the 3β-hydroxylation by the ODD, GA3ox enzyme (Lester et al., 1997; Martin et al., 1997; Williams et al., 1998). Additionally in monocots GA_3 is formed from GA_{20} via GA_5, with all the reactions being catalysed by GA3ox (Spray et al., 1996; Itoh et al., 2001; Appleford et al., 2006). Both classes of ODD are encoded by multiple genes in all plant species with Arabidopsis possessing five *GA20ox*s and four *GA3ox*s (Mitchum et al., 2006; Plackett et al., 2011). Multiple genes for the GA ODDs may be a consequence of them being the primary sites of regulation of GA biosynthesis, allowing a fine control of the pathway (Hedden and Thomas, 2012). Due to multiple genes encoding *GA20ox* and *GA3ox*, a level of genetic redundancy is observed in mutants of these genes. *ga4* (*ga3ox1*) and *ga5* (*ga20ox1*) only display mild GA deficient phenotypes, such as semi-dwarf stature (Koornneef and Vanderveen, 1980). Further studies of mutants lacking multiple paralogues from each family have shown that the different *GA20ox* and *GA3ox* genes have distinct roles in development. For example, analysis of various *GA20ox* combination knock-outs demonstrated the redundant role of *GA20ox1, -2 and -3* in the floral transition and only minor roles for *GA20ox4 and -5* (Plackett et al., 2012).

The regulation of the biosynthesis pathway is a complex process responding to cues from environmental, homeostatic and developmental factors resulting in GA intermediates existing at higher levels than their bioactive forms and biosynthesis proceeding when bioactive GAs are required (Kobayashi et al., 1988). Other hormones also regulate GA biosynthesis with auxin, ABA and ethylene signalling affecting GA biosynthetic gene transcription (Ross, 1998; Ross et al., 2000; Gomez-Cadenas et al., 2001; Xie et al., 2006; Bjorklund et al., 2007; Oh and Wehner, 2007; Zentella et al., 2007; Hattori et al., 2009). Whilst the levels of bioactive GAs in some tissues are controlled by synthesis there is also evidence that GA mobility is

necessary in some cases. Expression patterns of *GA20ox* and *GA3ox* in both the roots and floral tissues do not show complete overlap (Silverstone et al., 1997; Mitchum et al., 2006; Hu et al., 2008), suggesting some level of GA mobility. As a result the transcription of early and late GA biosynthesis genes may be spatially distributed. In the roots early GA biosynthesis gene *CPS* has been shown to be expressed in the root apical meristem (RAM), cell division zone and the cell elongation zone (Silverstone et al., 1997). *GA3ox*, a late GA biosynthesis gene, was shown to be expressed in the vasculature above the cell elongation zone as well as in the RAM, cell division zone and the cell elongation zone (Mitchum et al., 2006).

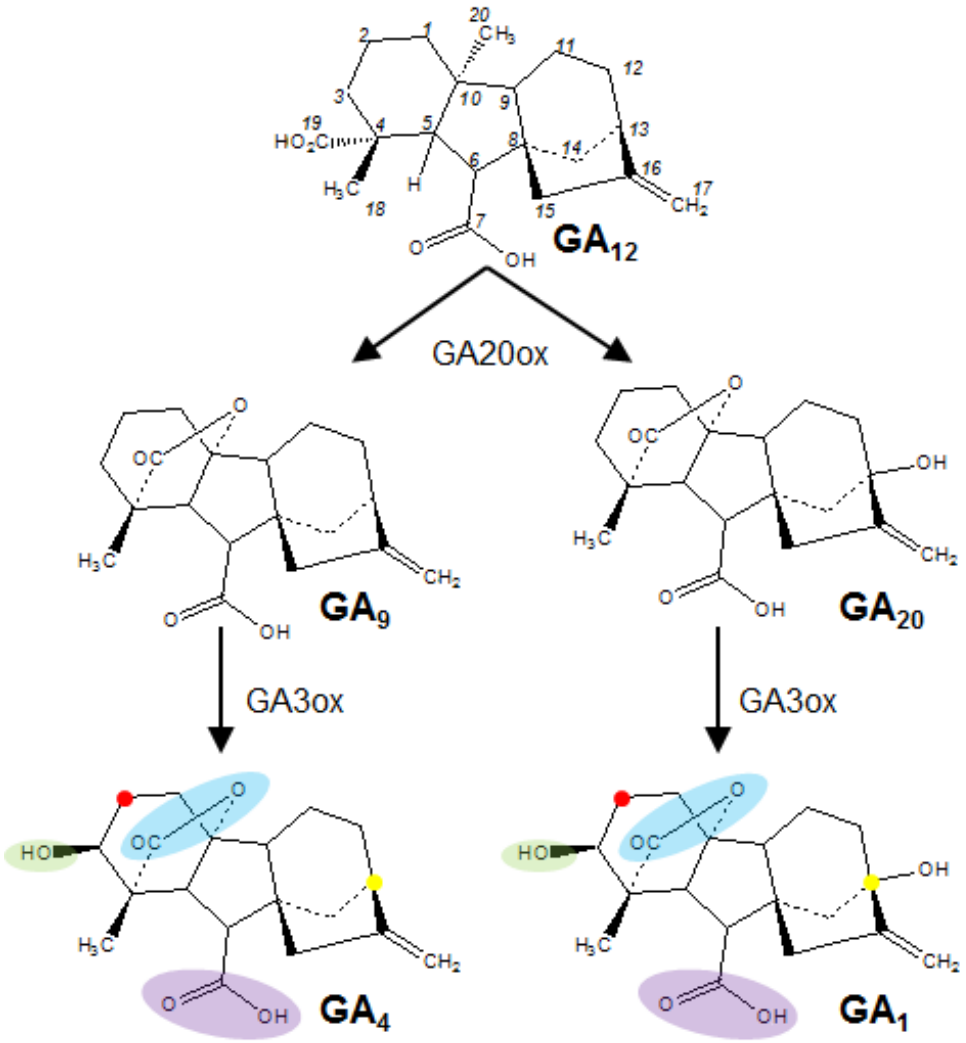


Figure 1. 1. Molecular structure of GA in biosynthesis pathway. Italicised numerals shown of GA_{12} structure denote carbon position in GA structure. Coloured regions on GA_1 and GA_4 represent common structural features that confer bioactivity: green (3 hydroxyl group), blue (lactone group) and purple (6 hydroxyl group). Red (C2) and yellow (C13) circles represent carbon positions with structural or biological significance.

1.3.2. Deactivation of GA

As with all plant hormones it is essential for the plant to be able to rapidly regulate the levels of bioactive GA in response to environmental cues. GA inactivation provides the primary method for control of bioactive GA levels in plants. The most prevalent

method of GA deactivation is the 2β-hydroxylation of bioactive GAs by the GA2ox enzyme (Figure 1.2.). Whilst the *GA2ox* genes encode a large gene family it is possible to divide them into two subfamilies based on function and sequence phylogeny (Han and Zhu, 2011). One group of these ODD enzymes oxidises the C_{19}-GAs, including the bioactive GAs. These enzymes can act as 2β-hydroxylases but can also oxidise the 2β-hydroxy group to a ketone (Sponsel and Macmillan, 1978). The second group of GA2ox enzymes acts on the C_{20}-GA intermediates. In Arabidopsis five *GA2ox* genes are known: *GA2ox-1, -2, -3, -4, -6* with *GA2ox5* being a pseudo gene (Thomas et al., 1999; Hedden and Phillips, 2000; Jasinski et al., 2005). Loss of function mutants for each gene displayed no distinct phenotype, suggesting functional redundancy, but a loss-of-function *GA2ox* quintuple mutant showed an increased level of bioactive GA and phenotypic characteristics consistent with GA overdose (Rieu et al., 2008a). Exogenous application of GA to Col-0 had a greater effect on plant development than seen in the quintuple *GA2ox* mutant (Rieu et al., 2008a) suggesting that GA2oxs are the most important enzymes for reducing levels of bioactive GAs. However, the fact that the quintuple *ga2ox* mutant only displayed a partial GA overdose phenotype indicates that there are still other bioactive GA deactivating enzymes working within the plant.

Several other mechanisms that lead to the inactivation of bioactive GAs have been identified in plants. *ELONGATED UPPER INTERNODE* (EUI) identified in rice encodes a P450 mono-oxygenase that converts GAs into their 16α,17-epoxides, although this conversion was less effective with the 13-hydroxylated GAs (Zhu et al., 2006). The GA methyl transferases 1 and 2 (GAMT1, 2) were also shown to abolish biological activity of C_{19}-GAs by methylating the 6-carboxy group (Varbanova et al., 2007; Xing et al., 2007). GAMT1 and 2 are members of the salicylic acid MT, benzoic acid MT and theobromine synthase (SABATH) family of methyl transferase, but GAMT1 and -2 are specific to GAs.

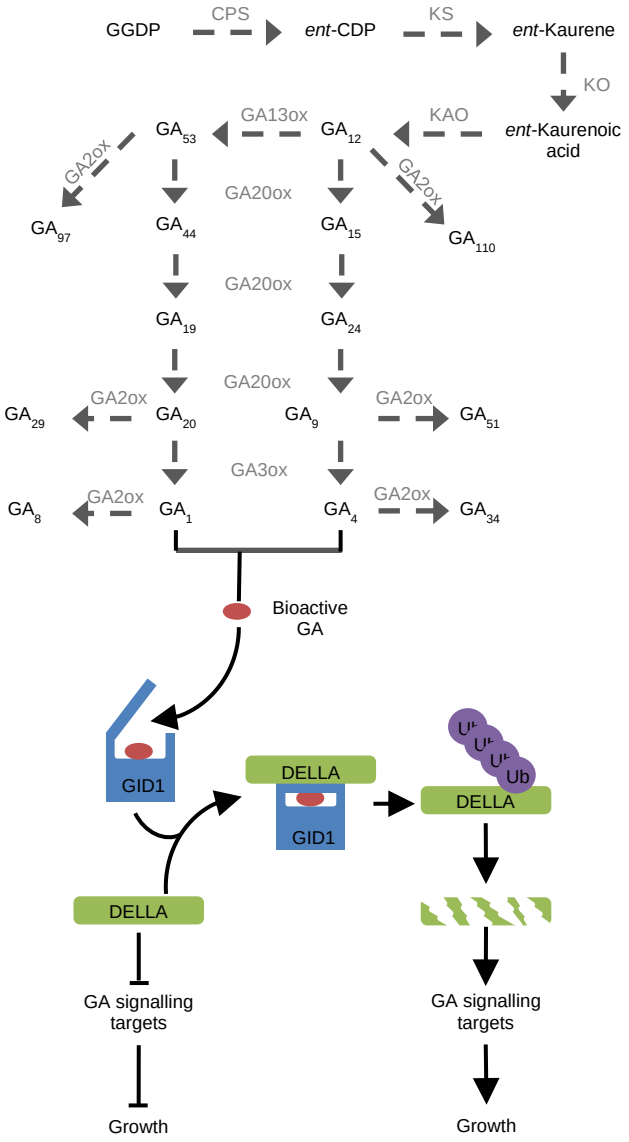


Figure 1. 2. Schematic of GA biosynthesis and signalling pathways. GA biosynthesis from early stage in the plastid (GGDP to *ent*-Kaurene) to the production of bioactive GA by GA3ox enzymes and its deactivation by GA2ox enzymes. Reactions catalysed by enzyme in GA biosynthesis pathway represented by grey, dashed arrows. GA induced phosphorylation of DELLA proteins via the GA receptor ,GID1, and SCF^{SLY1} complex (GID1) interacting with the GRAS domain of DELLA proteins resulting in polyubiquitination (Ub) and degradation via the ubiquitin-26S proteasome pathway.GA signalling represented by black, solid line.

1.3.3. GA signalling

For a biological response to occur in response to a plant hormone a pathway involving the perception and signal transduction of this signal is necessary. In the last decade our understanding of the GA signalling pathway has improved dramatically. First the GA receptor GID1 must perceive the bioactive GA in the cell. The binding of GA to GID1 results in the degradation of a transcriptional regulator, the DELLA protein, by a 26S-proteasome which ultimately changes the transcriptional output of the cell and causes a physiological response (summarised in Figure 1.2.).

1.3.3.1. GID1

The first stage of the GA signalling cascade is initiated through the interaction of bioactive GAs with the GID1 receptor (Figure 1.2.) (Ueguchi-Tanaka et al., 2005). Initially identified in rice, the *GID1* gene encodes a soluble nuclear protein which has some homology to a human hormone sensitive lipase (Ueguchi-Tanaka et al., 2005; Gallego-Bartolome et al., 2010). Arabidopsis was shown to have three paralogues of the GID1 receptor *GID1a, GID1b* and *GID1c* (Griffiths et a., 2006). In Arabidopsis the three GID1s can function redundantly (Nakajima et al., 2006). The crystal structures of the rice GID1 and Arabidopsis GID1a show a GA binding pocket and a flexible N-terminal extension (Murase et al., 2008; Shimada et al., 2008). GA binds to GID1 by the C-3 hydroxyl group hydrogen-bonding to the Tyr31 residue in the GA binding pocket (Murase et al., 2008; Shimada et al., 2008). This brings about a conformational change in GID1 that causes the N-terminal extension to close over the GA binding pocket. Once bound, the N-terminal region of the GA-GID1 complex has increased binding affinity for the DELLA protein at a domain containing the DELLA, TVHYNP and LExLE motifs (Griffiths et al., 2006a; Murase et al., 2008; Shimada et al., 2008). DELLA proteins lacking the DELLA and TVHYNP domains lose the ability to bind GID1 (Griffiths et al., 2006a; Willige et al., 2007).

Arabidopsis GID1b shows a slight GA-independent interaction with DELLA (Griffiths et al., 2006a). The *gid1* triple mutant in Arabidopsis displays a dwarf phenotype more severe than the GA biosynthesis mutants, and the application of GA does not rescue growth (Griffiths et al., 2006a). *gid1* single and double mutants show only small differences in vegetative growth when compared to wild-type and expression analysis shows all *GID1* genes are expressed to some degree across all tissues. This evidence indicates a level of functional redundancy for the *GID1* gene in Arabidopsis. Rice *gid* results in a severely dwarfed plant and a similar phenotype is observed in the barley loss-of-function mutants; *gse1* (Ueguchi-Tanaka et al., 2005; Chandler et al., 2008). The phenotypes of the *gse1* and *gid1* triple mutants suggest that GID1 is the primary GA receptor.

1.3.3.2. DELLA proteins

The DELLA proteins that form a complex with GA and GID1 are a class of proteins defined by a C-terminal GRAS domain (Figure 1.2.) (Pysh et al., 1999). Like all GRAS proteins DELLAs contain two leucine heptad repeats (LHR1 and -2) and three conserved motifs VHIID, PFYRE and SAW (Bolle, 2004). Mutations within the C-terminal GRAS domain often result in a loss of DELLA function (Silverstone et al., 1998). DELLA proteins possess two distinct regions that separate them from other members of the GRAS family of proteins, the DELLA and TVHYNP regions which as previously discussed are essential for GID1 binding. DELLA loss-of-function mutations display constitutively active GA signalling resulting in GA independent growth. This is illustrated by their rescue of GA biosynthetic mutants (Silverstone et al., 1997; Silverstone et al., 1998).

In Arabidopsis there are 5 DELLA paralogues: *GIBBERELLIN INSENSITIVE (GAI)*, *REPRESSOR OF GA1-3* (*RGA*), *RGA-LIKE1* (*RGL1*), *RGL2* and *RGL3* (Dill and Sun, 2001). These 5 proteins have both specific and overlapping functions. RGL3 has a role in environmental stress response (Achard et al., 2008a; Wild et al., 2012). RGA,

RGL1 and RGL2 are all required for floral development (Cheng et al., 2004a; Tyler et al., 2004) whereas repression of vegetative growth is a result of GAI and RGA function (Dill and Sun, 2001; King et al., 2001a). The DELLA proteins are conserved across plant species, such as in wheat (Rht-1), rice (Slender Rice1, SLR1), barley (SLN1) and Brassica (BnRGA) (Peng et al., 1997; Fu et al., 2002; Sun and Gubler, 2004; Gao et al., 2012).

1.3.3.3. DELLA degradation

It is known that the DELLA proteins repress all developmental processes that are regulated by GA, e.g. germination and cell elongation (Achard and Genschik, 2009). GA therefore acts by relieving DELLA repression of these processes. The process promoted by DELLA proteins are downstream negative aspects of GA signalling (Zentella et al., 2007).

As described earlier the presence of GA in the cell leads to the formation of the DELLA-GA-GID1 complex in the nucleus. The formation of this complex allows for association with an F-box protein, which forms part of a Skp-Cullin-F-box (SCF) E3 ubiquitin ligase; the resulting ubiquitination of the DELLA targets it for degradation (Figure 1.2.) (Silverstone et al., 2001). In Arabidopsis there are two closely related SCF E3 Ub ligases involved in DELLA degradation: SLEEPY1 (SLY1) and SNEEZY (SNE/SLY2) (McGinnis et al., 2003; Strader et al., 2004). In the *sly1* mutant, overexpression of *SNE* partially represses the mutant phenotype by reducing RGA and GAI accumulation, but not RGL2 (Strader et al., 2004; Ariizumi et al., 2011). This suggests that the two SCF proteins have different DELLA targets. Whilst the SCF-GID1 complex is required for DELLA ubiquitination and degradation it is possible for just the binding of the GID1 protein to inactivate the DELLAs (Ariizumi et al., 2008; Ueguchi-Tanaka et al., 2008). Whilst DELLA degradation is likely the main form of control on the DELLA proteins other studies have shown that phosphorylation/dephosphorylation also plays an important role in DELLA function (Fu

et al., 2002; Sasaki et al., 2003; Gomi et al., 2004; Hussain et al., 2005; Itoh et al., 2005; Hussain et al., 2007).

1.3.3.4. Regulation of developmental processes by DELLA proteins

It has been postulated by Ogawa et al. (2000) that DELLAs can function as transcription factors and studies showing association of RGA to chromatin would support this proposal (Zentella et al., 2007; Zhang et al., 2011). The lack of any obvious DNA-binding domain within DELLAs would suggest that they primarily function through transactivation and interaction with other regulatory proteins and not as transcription factors (Daviere and Achard, 2013). Recent studies in rice have demonstrated the potential of DELLAs as transactivators (Hirano et al., 2012). Fusion of the rice SLR1 with the activation domain of the herpes simplex virus protein VP16 resulting in decreased plant growth, whereas fusion of SLR1 with the repressor domain had no effect on plant growth. The transactivation ability of SLR1 was suppressed by binding to GID1. These results indicate that DELLAs are repressing plant growth and potentially other GA responses by activating the transcription of downstream genes. This is supported by the findings of transcriptional profiling studies which demonstrate that many genes are upregulated by DELLAs (Zentella et al., 2007).

Important findings in our understanding of DELLA functionality have been the demonstration that they directly interact with and regulate the activity of transcription factors. In all cases the DELLAs act by binding to and inhibiting the activity of transcription factors. The first of these to be identified were the PIFs which mediate phototropic growth of the hypocotyl (Figure 1.3.) (de Lucas et al., 2008; Feng et al., 2008; Gallego-Bartolome et al., 2010). PIF4 is a basic helix-loop-helix (bHLH) transcription factor, which modulates light-regulated genes involved in photomorphogenesis. PIF4 previously had been shown to up-regulate cell elongation but this upregulation can be inhibited by its destabilisation through phytochrome B. de

Lucas et al. (2008) demonstrated *in vitro* and *in planta* that RGA bound to PIF4 preventing it from binding to its target promoter sequence and thus suppressing cell elongation. RGA binds to the PIF4 at its bHLH DNA-binding domain. Another closely related PIF, SPATULA (SPT), has been shown to interact with RGA and RGL2. Unlike PIF4 the DELLA regulation appears to act post-transcriptionally on the SPT transcript accumulation (Josse et al., 2011). The DELLA-PIF interaction is also known to contribute to the accumulation of chloroplasts in etiolated tissue through the inhibition of PIF transcriptional activity (Cheminant et al., 2011).

Since the discovery of the PIFs as DELLA-interacting proteins, numerous other DELLA partners have been identified. DELLAs have a role in plant defence due to their competitive binding to JASMONATE ZIM-DOMAIN (JAZ). DELLAs have been shown to compete with MYC2 for JAZ binding, the binding of JAZ-DELLA allowing MYC2 to activate JA response (Figure 1.3.) (Hou et al., 2010). The DELLA RGA has also been shown to bind MYC2 to prevent its regulation of the sesquiterpene biosynthesis pathway (Hong et al., 2012). The zinc finger C2H2 protein INDETERMINATE DOMAIN1 (IDD1)/ENHYDROUS (ENY) has also been shown to interact with DELLAs (Figure 1.3.) (Feurtado et al., 2011). It is thought that ENY mediates the effect of GA on ABA accumulation during seed maturation through its interaction with the DELLAs. ALCATRAZ is reported to block DELLA function in defining the separation layer responsible for fruit opening (Arnaud et al., 2010). HOOKLESS1 (HLS1) regulates apical hook formation in dark grown seedlings and is promoted by ETHYLENE INSENSITIVE 3/EIN3-LIKE 1 (EIN3/EIL1). DELLAs have been shown to bind directly to the DNA binding region of EIN3/EIL1 thus repressing up-regulation of *HLS1* expression by this transcription factor (Figure 1.3.) (An et al., 2012). Overexpression of *BRASSINAZOLE RESISTANT1* (*BZR1*) is known to restore cell elongation in GA deficient plants; DELLA proteins can repress this function by direct binding to BZR1 thus preventing its DNA-binding capability (Bai et al., 2012). The microRNA156 (miR156)-targeted SQUAMOSA PROMOTER BINDING-LIKE (SPL) transcription factors activate the expression of MADS box and miRNA172

genes and are also bound by DELLAs thus inactivating their transcriptional activation and delaying floral transition (Figure 1.3.) (Yu et al., 2012). Park et al. (2013) showed that the BOTRYTIS SUSCEPTIBLE1 INTERACTOR (BOI) binds to DELLAs, the complex binding to and repressing expression from the promoters of GA responsive genes. DELLA proteins are also known to bind to the prefoldin protein, sequestering them to the nucleus thus preventing their role in the complex for chaperoning tubulin folding which controls the direction of cellular elongation (Figure 1.3.) (Locascio et al., 2013). To date the prefoldin protein is the only know example of a DELLA protein function that does not involve gene expression.

Some members of the MYB gene family are transcription factors that are known to act downstream of GA signalling (GAMYB) (Gubler et al., 1995; Gocal et al., 2001; Achard et al., 2004). In rice it appears that the GAMYB is solely responsible for transmitting the GA signal during stamen development (Aya et al., 2009). In the barley aleurone it has been shown that constitutive expression of GAMYBs can simulate GA regulated gene expression (Gubler et al., 1995). Despite a role for GAMYB in relaying the GA signal it has not yet been demonstrated that they are under DELLA regulation. In fact, transcriptomic studies demonstrate DELLA regulation of the two Arabidopsis GAMYBs (*MYB33, MYB65*). Microarray data has shown SCARECROW-LIKE 3 (SCL3) to be a direct target of DELLAs in Arabidopsis seedlings. However, *scl3* null mutants show increased signalling of GA biosynthetic genes and reduced GA responses (Figure 1.3.) (Zentella et al., 2007; Zhang et al., 2011). These results indicate that SCL3 is a positive regulator of GA signalling, and has a role in the homeostatic regulation of GA signalling. Furthermore, SCL3 regulates its own expression through interaction with DELLA and acts antagonistically with DELLA in the control of downstream GA responses and upstream GA biosynthesis genes.

1.3.3.5. DELLA proteins control the homeostasis of the GA signalling pathway

As mentioned previously one of the major stages in GA signalling homeostasis is the deactivation of bioactive GAs by the GA2ox enzymes. Additionally homeostasis of bioactive GAs is maintained through the regulations of GA biosynthesis genes (Hedden and Phillips, 2000; Olszewski et al., 2002; Yamaguchi, 2008). Mutants with reduced bioactive GA have increased expression of some *GA3ox* and *GA20ox* biosynthetic genes and reduced *GA2ox* expression, and the converse is observed in plants with high levels of bioactive GA (Ogawa et al., 2003). Zentella et al. (2007) showed using microarrays of Arabidopsis that the presence of DELLA proteins increases expression of the *GA3ox1* and *GA20ox2* genes. To date SCL3 is the only GA biosynthesis regulatory protein known to interact with DELLA but other proteins have been implicated. In rice YABBY1 (YAB1) has been shown to bind to the promoter region of *GA3ox* and repress its expression (Dai et al., 2007). Additionally comparison of the expression patters of *GA3ox, EUI, YAB1* and *SLR1* between the *YAB1* and *slr1-1* mutants indicate that GA signaling is required for GA-mediated repression of *YAB1.* The bZIP transcription factor RSG (REPRESSION OF SHOOT GROWTH) has also been shown to bind the DELLA proteins and control the expression of GA20ox and GA3ox genes in tobacco (Fukazawa et al., 2006).

Other proteins have been shown to control GA biosynthesis although as yet there is no known interaction between them and the DELLA proteins. *knotted1*-like homeobox (KNOX) proteins suppress *GA20ox1* expression in tobacco (Figure 1.3.) (Sakamoto et al., 2001). Additionally the expression of other GA signaling genes was effected, including SLR1 and GID1. Whilst studying the light dependent hypocotyl elongation of the *cryptochrome1/cryptochrome2 mutant in Arabidopsis, Zhao et al. (2007) demonstrated that* cryptochromes were required for the upregulation of *GA2ox* and down-regulation of *GA20ox* and *GA3ox* under blue light (Figure 1.3.). Similar results were observed in rice (Hirose et al., 2012). This result is a further example of the many environmental cues involved in the regulation of GA biosynthesis and signalling.

More recently dehydration-responsive element-binding proteins (DREBs) have been shown to reduce levels of bioactive GAs in tomatoes by specifically binding to the dehydration-responsive element/C-repeat elements of the *CPS* promoter and reducing *CPS* expression (Figure 1.3.) (Li et al., 2012). Ethylene response factors and C-repeat binding factors have also been shown to regulate the expression of GA2ox genes in response to cold and salt stress (Jung et al., 2010; Kurepin et al., 2013).

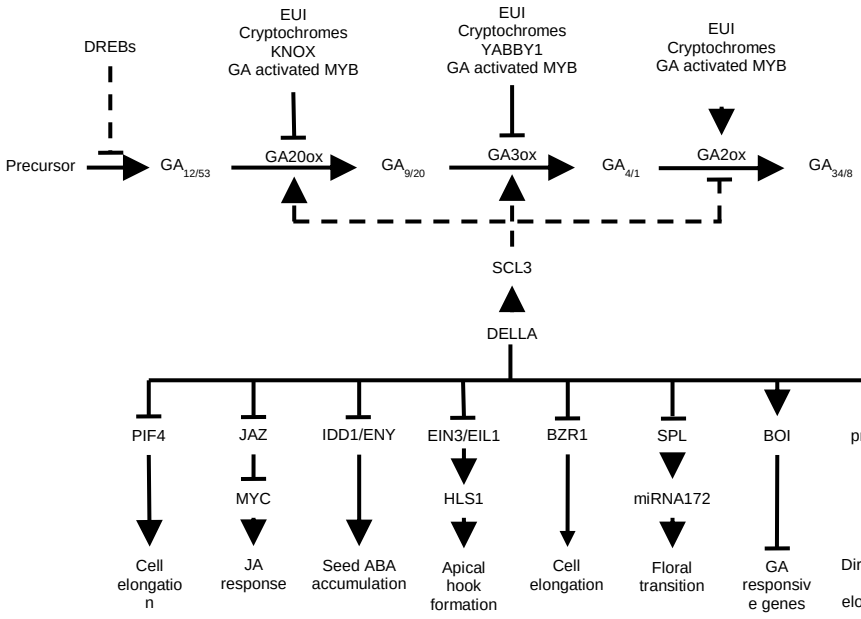


Figure 1. 3. DELLA protein regulation of GA biosynthesis and Arabidopsis development through their interaction with transcriptional regulators.

Genetic pathways showing transcriptional regulators that regulate the late steps of GA biosynthesis. Also shown are components which regulate Arabidopsis developmental processes and whose activity is directly controlled through the binding of DELLA proteins. Solid arrows indicate direct interactions whilst dotted arrows represent indirect interactions.

1.3.3.6. GA transport

There is some evidence that bioactive GAs are produced within the required tissues with *GA3ox* and *GA20ox* being expressed in the elongating organs of rice and tobacco (Itoh et al., 1999; Kaneko et al., 2003). However, the expression of these enzymes at the sites of GA action does not rule out the transport of early intermediates in the GA biosynthesis pathway. *ent*-kaurene, GA_4, GA_6, GA_{15} GA_{20} and GA_{24} have all been postulated as forms of transported GA (Proebsting et al., 1992; King and Ben-Tal, 2001; Yamaguchi et al., 2001; King, 2003; King, 2006; Kramer, 2006). One example of where GA transport is required is in germinating cereal seeds as bioactive GAs are synthesised in the scutellum epithelial cells of the embryo and move to the aleurone to drive production of α-amylase and other hydrolytic enzymes (Kaneko et al., 2002). The aleurone does not have the capacity to produce GAs and is therefore dependent on this external source of bioactive GA (Kaneko et al., 2002). Additionally, petal are non-autonomous for GA, requiring the hormone to be supplied from the anthers and receptacle (Weiss and Halevy, 1989). Other studies have shown that GA_1, GA_3, and GA_4 are all transported between different tissues (Drake and Carr, 1979; Eriksson et al., 2006; Shani et al., 2013)

A more in-depth review of GA transport is provided in Chapter 5.1.

1.4. AUXIN

1.4.1. The Auxin Receptors

To date three classes of proteins have been shown to bind and potentially perceive bioactive auxin (indole-3-acetic acid or IAA): AUXIN BINDING PROTEIN 1 (ABP1), TIR1 and the recently identified SKP2A (Figure 1.4.). ABP1, unlike the nuclear localised SKP2A and TIR1, is thought to be active in the extracellular space. The binding affinity of ABP1 was found to be highest at pH 5.5, the pH found in the

extracellular space, whilst ABP1 was shown to have no binding at pH 7 which is the pH of the endoplastic reticulum where ABP1 is predominantly localised (Tian et al., 1995). As ABP1 is a soluble protein a carrier protein would be required to transport IAA the signal into the nucleus and as yet no obvious candidates have been identified. A glycosylphosphatidylinositol (GPI)-anchored protein in maize has been suggested as a potential ABP1 interactor but as this also lacks a transmembrane domain it is unlikely to be a facilitator of the IAA signal (Shimomura, 2006). Both leucine-rich repeat receptor-like protein kinase and phospholipases have been postulated as downstream elements of ABP1 signalling, but as yet there is no direct evidence for this (Sauer et al., 2013). As little is known about how ABP1 functions there is little information of how it controls development. T-DNA insertion mutants of ABP1 in Arabidopsis are embryo lethal, showing the integral role of this protein in plant development (Chen et al., 2001). There is some evidence that ABP1 is required for auxin-dependent cell cycle and cell expansion (David et al., 2007; Braun et al., 2008) and auxin-triggered ion fluxes (Sauer and Kleine-Vehn, 2011). ABP has also been implicated in the control of (PINFORMED) PIN localisation to the plasma membrane due to its role in auxin induced inhibition of clathrin-mediated endocytosis (Robert et al., 2010) and a cell polarity-generating mechanism which activates the Rho-GTPases ROP2 and -6. These ROPs control endocytosis and cytoskeleton reorganization via the effectors RIC4 and RIC1 (Xu et al., 2010). ABP1 has been shown to act upstream of ROP6 and RIC1 regulated clathrin-mediated endocytosis of the auxin efflux transporters (PINs) (Chen et al., 2012). These results indicate that ABP1 may have an involvement in the control of auxin transporter membrane proteins and suggest ABP1 could act purely on the protein level rather than on transcriptional activity. As such ABP1 could be controlling cellular responses to local auxin gradients, such as those observed in the leaf (Sauer et al., 2006; Scarpella et al., 2006).

Recently it has been shown that SKP2A can bind directly to auxin (Jurado et al., 2010) resulting in the promotion of the interaction of this receptor with the cell cycle factors, DPB and E2FC, leading to their degradation (del Pozo et al., 2006; Jurado et

al., 2008). Interestingly SPK2A is also degraded under high auxin conditions (del Pozo et al., 2006; Jurado et al., 2008). SKP2A shares many similarities with the best understood auxin receptor TIR1, since both are nuclear localised and the binding site of auxin in SKP2A was discovered using the TIR1 binding site as a template. TIR1 was postulated as an auxin receptor after the application of auxin to crude plant extracts promoted the interaction between SCF^{TIR1} and the AUX/IAAs. The presence of radio-labeled IAA in a purified SCF^{TIR1}-AUX/IAA complex later confirmed TIR1's role as an auxin receptor (Figure 1.4.) (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Further analysis of the X-ray crystal structure of TIR1 showed a core ring-like structure for IAA binding (Tan et al., 2007). TIR1, like SLY1 in the GA signalling pathway, is an F-box protein in a SKP-Cullin F-box (SCF) type ubiquitin E3 ligase. As with SLY1 and GA TIR1 requires the presence of the other components of the SCF complex and a 26S-proteasome to have any downstream function, which ultimately leads to the degradation of the AUX/IAAs and activation of auxin-responsive gene expression (Figure 1.4.). Unlike the GA signalling pathway, where the SLY1 subunit of the SCF^{SLY1} E3 ubiquitin ligase binds to DELLA proteins in association with the GID1-GA complex, TIR1 binds directly to AUX/IAAs in the presence of auxin (Figure 1.4.). SCF^{TIR1} does not require post-translational modification after auxin binding as is the case in many other SCF ubiquitin E3 substrate interactions (Petroski and Deshaies, 2005). Similar to other components of hormone signaling pathways there is a level of functional redundancy mediating auxin signalling, with the presence of five TIR1 paralogues in Arabidopsis (ABF1 to -5), that bind auxin with different affinities (Villalobos et al., 2012).

1.4.2. TIR1-auxin binding induces AUX/IAA degradation

Auxin induces the formation of a TIR1-AUX/IAA receptor-ligand interaction therefore implicating AUX/IAA as an auxin co-receptor (Figure 1.4.). AUX/IAA domain II binds auxin, the co-factor inositol hexakisphosphate and TIR1 upon auxin binding to TIR1, essentially forming a lid over the TIR1 auxin binding site (Dharmasiri et al., 2005;

Kepinski and Leyser, 2005; Tan et al., 2007). Aux/IAAs are nuclear localised proteins with four domains: domain I represses transcription, domain II regulates protein stability and domains III and IV at the C-terminus allow for the binding with other components of the IAA signalling pathway (Ulmasov et al., 1999; Worley et al., 2000; Ouellet et al., 2001; Tiwari et al., 2004). The demonstration that AUX/IAAs are stabilised by the presence of the proteasome inhibitor MG132, highlights the importance of the 26S-proteasome in controlling their degradation (Ramos et al., 2001).

The study of gain-of-function AUX/IAA mutants in Arabidopsis has demonstrated the complexity of auxin-mediated AUX/IAA degradation. The CUL1 subunit of the SCF complex requires covalent modification by conjugation to the RELATED TO UBIQUITIN (RUB) protein. The RUB conjugation in turn requires the action of E1, E2 and Ring box 1 which appears to be an E3 enzyme. Loss-of-function mutations in any of the genes encoding these enzymes results in reduced SCF^{TIR1} effectiveness (del Pozo and Estelle, 1999; Gray et al., 2001; Gray et al., 2002; Dharmasiri et al., 2003). Furthermore if the RUB protein is not cleaved by COP9 SIGNALOSOME post-modification of CUL1, the SCF effectiveness is reduced (Schwechheimer et al., 2001; Petroski and Deshaies, 2005). CULLIN-ASSOCIATED and NEDD8-DISSOCIATED1 (CAND1) bind unmodified CUL1 mutually exclusively of SKP1 (Petroski and Deshaies, 2005), inhibiting SCF activity and AUX/IAA degradation. Interestingly, *cand1* Arabidopsis mutants show decreased AUX/IAA degradation, potentially due to a requirement for CAND1 in SCF cycling (Cheng et al., 2004b; Chuang et al., 2004; Feng et al., 2004; Petroski and Deshaies, 2005).

1.4.3. AUX/IAA-ARF interaction represses ARFs role as transcriptional regulators of auxin-responsive genes

In Arabidopsis there are 29 Aux/IAA proteins (IAA1-20 and IAA26-34). With this level of genetic redundancy it is unsurprising that there has been limited success assessing

loss-of-function AUX/IAA mutations (Overvoorde et al., 2005). In contrast gain-of-function mutants have provided important information on the role of AUX/IAAs in auxin signalling (Woodward and Bartel, 2005). For example the gain-of-function *IAA12/BODENLOS (BDL) mutant confers the same embryo and seedling lethal phenotype as the arf5/monopteros (arf5/mp) (Hamann et al., 2002),* indicating the antagonistic relationship between the AUX/IAAs and the ARFs (Figure 1.4.). Later studies revealed that IAA12 interacts with ARF5, negatively regulating ARF5 activity (Hardtke et al., 2004). Both ARFs and AUX/IAAs contain C-terminal domains III and IV which are the sites of heterodimerization (Kim et al., 1997) and subsequent repression of ARF transcriptional activation (Tiwari et al., 2003). Whether an ARF acts as a repressor or activator is dependent on its middle region (MR) domain. A Q-rich MR (e.g. ARF19) will result in activation and an S-rich region (ARF9-18) will result in repression (Hagen and Guilfoyle, 2002; Okushima et al., 2005). Genetic analysis has shown that specific ARFs are involved in specific auxin controlled developmental processes including; *ARF5/MP* and *ARF17 in embryogenesis* (Hardtke and Berleth, 1998; Mallory et al., 2005); *ARF7, ARF10, ARF16* and *ARF19 in root development* (Mallory et al., 2005; Okushima et al., 2005; Wang et al., 2005a), *ARF2, ARF3/ETTIN (ETT)*, *ARF6* and *ARF8* in flower development (Sessions et al., 1997; Ellis et al., 2005a; Nagpal et al., 2005; Schruff et al., 2006), and *ARF1* and *ARF2* in senescence (Ellis et al., 2005a).

The ARFs control transcription through the VIVIPAROUS1/ABSCISIC ACID INSENSITIVE 3 (VP1/ ABI3) like DNA binding domain at the N-terminus (Ulmasov et al., 1997a). This domain interacts with the auxin-response elements (ARE) of promoters of certain auxin-inducible genes, including *AUX/IAAs* and *GH3s* (Ulmasov et al., 1995) (Figure 1.4.). These promoters contain a 6 nucleotide motif, GAGACA, although as there is variability in the 1st two nucleotides, the consensus sequence being GACA (Ulmasov et al., 1995; Abel et al., 1996; Ulmasov et al., 1997a). Differing binding affinities of TIR1/ABFs, AUX/IAA and ARFs allow for fine tuning of the auxin signalling pathway. As mentioned previously, there is extensive functional redundancy

within the three important central components within the auxin signalling pathway; TIR1/ABFs (6), AUX/IAA (29) and ARFs (23). In the TIR1-AUX/IAA interaction the auxin interaction surface is not strikingly conserved giving rise to certain TIR1/ABF-AUX/IAA pairs with specific auxin affinities. Using a yeast heterologous system it was shown that TIR1-AUX/IAA7 had a high affinity of Kd ≈ 10 nM whilst TIR1-AUX/IAA12 had a much lower affinity of around 300 nM (Villalobos et al., 2012). Sauer et al. (2013) postulated that differences in TIR1/ABF-AUX/IAA interaction could explain the large dynamic range of auxin responses and the variety of developmental processes under auxin control. Similarly the homologues of TIR1/ABF show differing auxin affinity and AUX/IAA degradation rates. ABF4 and -5 show a higher binding affinity for certain synthetic auxins. Yeast assays have shown that TIR1 and ABF2 induce a more rapid degradation of AUX/IAAs than ABF1 and -3 due to their higher affinity for IAA (Parry et al., 2009; Havens et al., 2012). The AUX/IAA-ARF interactions also possess another level of complexity and control due to the 667 possible interactions that can occur. Furthermore, the 23 ARFs can compete with each other for ARE binding sites (Sauer et al., 2013).

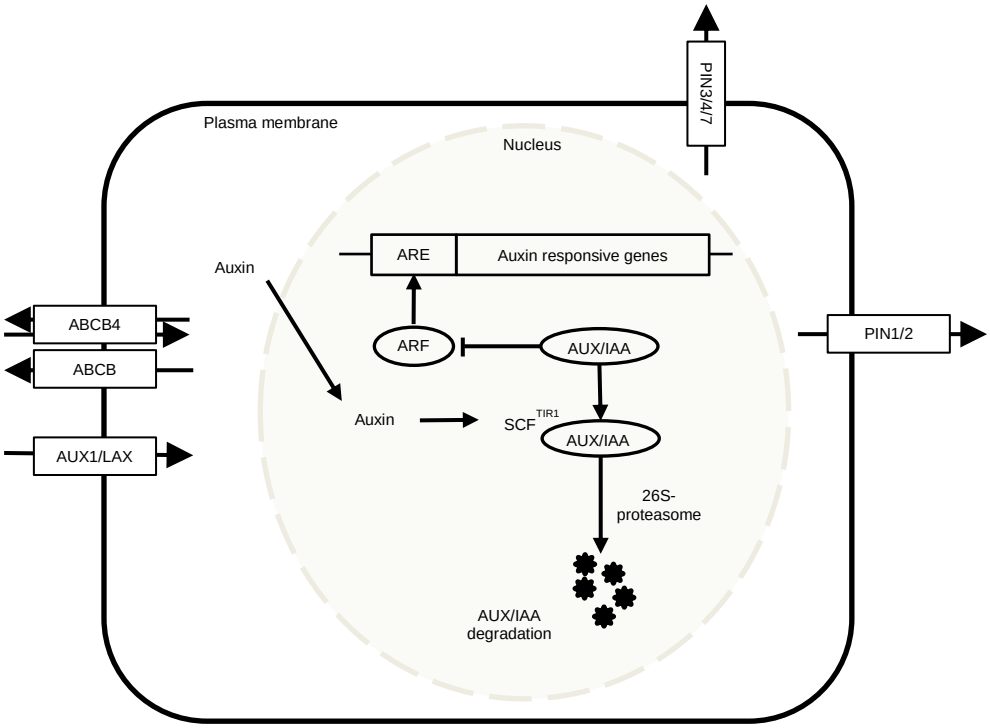


Figure 1. 4. Summary model of auxin transport and nuclear signalling via TIR/AFB.

IAA maintained in plant cells through auxin transport. IAA can diffuse through the lipidic plasma membrane or be transported by the AUX1/LAX influx carriers into the cell. IAA can exit cells by the action of PGP- or PIN-type efflux carriers. PIN1/2 contributes to polar auxin transport. PIN3/4/7 allow for asymmetric auxin distribution and tropic response. In cell IAA binds TIR1 receptor in the SCF^{TIR1} complex, recruiting the Aux/IAA repressors to TIR1 resulting in AUX/IAA destruction and the subsequent activation of the auxin-response genes by the ARFs.

1.4.4. Auxin Transport

Auxin is one of the few hormones for which the mechanism of intercellular transport is well understood. To date auxin transport and the auxin gradient it creates have been implicated in root gravitropism, shoot branching, phototropism and leaf shape (Chen et al., 1985; Emery et al., 1998; Miguel et al., 1998; Friml et al., 2002a; Nordstrom et al., 2004; Morita, 2010). The process of auxin transport can be separated into three

main components: auxin efflux, auxin influx and ATP-dependent auxin transport (Figure 1.4). Auxin efflux is maintained by the PIN proteins, first identified in Arabidopsis plants with the PINFORMED inflorescence phenotype of *pin* mutants (Okada et al., 1991; Galweiler et al., 1998). To date eight PIN proteins have been identified, which fall into two different classes: five complete PINs (PIN1, -2, -3, -4, and -7) and three short PINs (PIN5, -6 and -8) which lack the long hydrophobic loop found in the other PIN proteins (Mravec et al., 2009). It is believed these shorter PINs have a function in the compartmentalisation of auxin within the cell (Mravec et al., 2009). Whilst all PINs function as efflux transporters, the polarity of their localisation can differ. PIN1 and -2 are predominantly polar localised (shootward and rootward ends of the cell) whilst PIN3, -4 and -7 have apolar as well as polar localisation (Friml et al., 2002b; Friml and Palme, 2002; Friml et al., 2002a; Blilou et al., 2005; Wisniewska et al., 2006) (Figure 1.4.). The localisation of the PINs is usually related to their specific control of plant development. PIN2's primary role is in root gravitropsim, PIN3 redirects auxin for directional growth whilst PIN1, -4 and -7 are required for a variety of developmental processes, including organogenesis (Chen et al., 1998; Muller et al., 1998; Friml et al., 2002b; Friml et al., 2004; Zazimalova et al., 2007; Rahman et al., 2010). Recent studies have shown that PIN localisation within the cell can be altered by phosphorylation (Michniewicz et al., 2007; Zhang et al., 2010).

In Arabidopsis auxin influx is performed by an amino acid permease family of plasma membrane (PM) H+-symporters which consists of AUX1, LAX1, -2 and -3 with AUX1 and LAX3 being the high affinity transporters (Bennett et al., 1996; Parry et al., 2001; Yang et al., 2006; Kerr and Bennett, 2007; Swarup et al., 2008; Yang and Murphy, 2009) (Figure 1.4.). The auxin transport model suggests that the AUX1/LAX transporters function to create auxin sinks from which they can then be transported to specific areas of the plant to induce developmental processes (Marchant et al., 2002; Kramer, 2004; Swarup et al., 2005; Kramer and Bennett, 2006; Bainbridge et al., 2008; Swarup et al., 2008; Ugartechea-Chirino et al., 2010; Vandenbussche et al., 2010). *aux1* and *lax3* mutants show a distinct lack of gravitropism or lateral root

formation, respectively (Mirza et al., 1984; Bennett et al., 1996; Swarup et al., 2008), showing that certain paralogues have roles in specific developmental processes.

The ATP-binding cassette transporter B subfamily/PGLYCOPROTEIN MULTIDRUGRESISTANCE (ABCB/PGP) P-glycoproteins use energy from ATP to transport auxin. ABCB1, -4 and -19 have been shown to transport auxin in Arabidopsis and are implicated in long distance transport (Figure 1.4.) (Noh et al., 2001; Multani et al., 2003; Petrasek et al., 2006; Blakeslee et al., 2007; Cho et al., 2007; Lewis et al., 2007; Peer and Murphy, 2007; Wu et al., 2007; Yang and Murphy, 2009; Knoller et al., 2010). As with the other auxin transporters, each ABCB transporter has a role in specific developmental processes. ABCB1 controls the movement of auxin out of apical tissues (Bandyopadhyay et al., 2007). ABCB4 also has roles in shootward auxin transport, root hair elongation, and light/sucrose-dependent primary root growth (Santelia et al., 2005; Terasaka et al., 2005; Cho et al., 2007). Mutants lacking ABCB4 show increased rates of gravitropic bending (Lewis et al., 2007). The *abcb19/pgp19/mdr1/fby-1* mutant shows increased rates of phototropic bending (Noh et al., 2003; Lin and Wang, 2005; Rojas-Pierce et al., 2007; Nagashima et al., 2008). Interestingly the mutants lacking ABCB4 and -9 show an increase in tropic responses that require auxin, suggesting ABCB transporters may have an important role in maintaining precise hormone levels in the cells. This would explain why ABCB4 can function as an auxin import transporter under low auxin concentrations and as a much more effective exporter under high auxin conditions (Figure 1.4.) (Terasaka et al., 2005; Peer and Murphy, 2007; Yang and Murphy, 2009; Kim et al., 2010).

1.4.5. Auxin biosynthesis

Despite the extensive data on both auxin signalling and transport the understanding of auxin biosynthesis in plants is still quite poor. It has been postulated that there are two separate types of auxin biosynthesis pathways (Figure 1.5.): the tryptophan (trp)-

independent pathway which may have indole-3-glycerol phosphate or indole as early precursors but the route of the biochemical pathway to IAA is still poorly understood (Jian et al., 2000; Zhang et al., 2008); and the Trp-dependent pathway for which several different pathways have been suggested (Mashiguchi et al., 2011; Won et al., 2011) (Figure 1.5.). Little is known about the localization of the auxin biosynthesis enzymes within the cell, although tryptophan is known to be synthesized within the chloroplast (Radwanski and Last, 1995). A complete trp-dependent auxin biosynthesis has been identified in the bacteria *Agrobacterium rhizogenes, Agrobacterium tumefaciens* and *Pseudomonas syringae*. *Agrobacterium rhizogenes,* which induces the hairy roots, contains a large root inducing plasmid with both the *AUX1*/*IAAM*/*TMS1* and *AUX2*/*IAAH*/*TMS2* genes (Comai and Kosuge, 1982; Schroder et al., 1984; Thomashow et al., 1984; Yamada et al., 1985; Camilleri and Jouanin, 1991). Initially *AUX1* encodes the tryptophan-2-monooxygenase enzyme which catalyzes the conversion of Trp to indole-3-acetamide (IAM) which is subsequently converted to IAA by an indole-3-acetamide hydrolase which is encoded by *AUX2* (Figure 1.5.) (Yamada et al., 1985; Camilleri and Jouanin, 1991; Gaudin et al., 1993; Nemoto et al., 2009; Mano et al., 2010). Subsequently it has been shown that IAM is present in numerous plant species suggesting the IAM pathway as a conserved auxin biosynthesis pathway within plants as well as bacteria (Saotome et al., 1993; Rajagopal et al., 1994; Lemcke et al., 2000; Pollmann et al., 2002; Sugawara et al., 2009). Furthermore Arabidopsis and tobacco were both shown to possess genes (*AtAMI1* or *NtAMI1*) that encode a cytoplasm localized indole-3-acetamide hydrolase (Figure 1.5.) (Pollmann et al., 2003; Pollmann et al., 2006; Nemoto et al., 2009). There is still little evidence on how IAM is produced within plants. Studies into ethylene response (Stepanova et al., 2008) and shade avoidance (Tao et al., 2008) identified a gene (*TRYPTOPHAN AMINOTRANSFERASE of ARABIDOPSIS 1 or TAA1)* encoding a cytoplasm localized aminotransferase that converts Trp to indole-3-pyruvic acid (IPA) (Figure 1.5.). Mutation in the *TAA1* gene lead to a severe reduction in plant IAA levels, additionally wild-type plants transferred to shade experience an increase in IAA synthesis whereas *taa1* plants do not (Tao et

al., 2008), indicating a role for IPA in the rapid production of IAA in response to environmental stimuli. There are four genes closely related to *TAA1*, *TRYPTOPHAN AMINOTRANSFERASE RELATED 1* to *4*, whilst *TRANSPORT INHIBITOR RESPONSE 2 has been shown to be identical to TAA1 (Stepanova et al., 2008; Yamada et al., 2009). TAA1*-overexpressing lines show no increase in IAA levels indicating TAA1 is unlikely to be the rate-limiting enzyme in this pathway (Stepanova et al., 2008; Tao et al., 2008).

Similar to IAM the IPA auxin biosynthesis pathway is also present in the bacterial kingdom where *Azospirillum brasilense*, *Enterobacter cloacae*, and *Pseudomonas putida* convert Trp→IPA→IAD→IAA (Koga et al., 1991; Costacurta et al., 1994; Patten and Glick, 2002). It is not known if IPA is converted to (indole-3-acetaldehyde (IAD) in plants. More recently it has been shown that IPA can be converted directly to IAA in plants by a family of YUCCA (YUC) proteins (Mashiguchi et al., 2011; Stepanova et al., 2011; Won et al., 2011). Using [$^{13}C_{11}$,$^{15}N_2$]-Trp it was shown that Trp can converted to both IPA and IAD, but *TAA1ox* plants showed increased levels of IPA and not IAD, indicating IAD may function in a different auxin biosynthesis pathway to IPA (Mashiguchi et al., 2011). By blocking the action of TAA1 Stepanova et al. (2011) showed that this removed the high IAA phenotypes of *YUC1ox* plants, suggesting YUC1 required TAA1 to function. YUC2 was shown to convert IPA to IAA in *E. coli* (Mashiguchi et al., 2011). Additionally *taa* mutants are deficient in IPA whereas *yuc* mutants have low IAA, but high IPA (Mashiguchi et al., 2011; Won et al., 2011). Taken together these results indicate a pathway for IAA biosynthesis through the conversion of Trp to IAA via IPA by the TAA1 and YUC enzymes (Figure 1.5.).

Two additional Trp-dependent IAA biosynthesis pathways have been proposed: the TAM and IAOX/IAD pathways. The cytosol-localized tryptophan decarboxylase is known to convert Trp to TAM (Di Fiore et al., 2002). In Arabidopsis, maize and tomato it has been reported that YUCCA proteins catalyze the conversion of TAM to *N*-hydroxytryptamine (Zhao et al., 2001; LeClere et al., 2010; Exposito-Rodriguez et al.,

2011). However, *N*-hydroxytryptamine has never been identified in plants bringing into question its role as an IAA precursor. The indole-3-acetaldoxime (IAOX)/IAD auxin biosynthesis pathway is specific to *Brassica* species (Nafisi et al., 2007). Two homologous cytochrome P450 enzymes, CYP79B2 and CYP79B3 can convert Trp to IAOX (Hull and Celenza, 2000; Hull et al., 2000; Mikkelsen et al., 2000), which in turn is converted to IAN by another cytochrome P450, CYP71A13 (Figure 1.5.) (Nafisi et al., 2007). How IAN is converted to IAA is still unclear. The Arabidopsis nitrilase genes *AtNIT1–AtRNIT4* have been shown to hydrolyze IAN *in vitro*, although this reaction is inefficient when compared to the hydrolysis of phenylpropionitrile, allylcyanide, phenylthio acetonitrile, and methylthio acetonitrile by these enzymes (Vorwerk et al., 2001). There is some evidence that the DELLA protein interactor PIF4 regulates the expression of some auxin biosynthesis genes, such as TAA1 (Franklin et al., 2011).

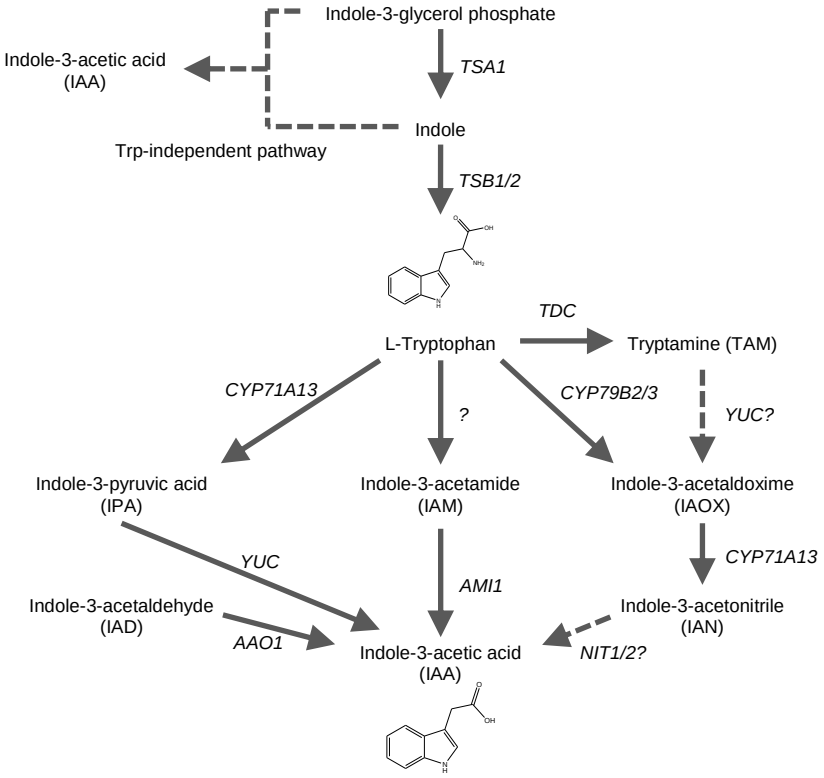


Figure 1. 5. IAA biosynthesis in plants.

Proposed synthesis of IAA from indole-3-glycerol phosphate via numerous trypotophan-dependent pathways. Solid black text represents chemical steps in pathway, italicised grey text represents enzyme/gene, solid grey arrows represent reaction where gene function is known, dashed grey arrows depict reactions where gene/enzyme function is not well known.

1.5. CHEMICALS SCREENS FOR ELEMENTS CONTROLLING PLANT DEVELOPMENT

The basis of chemical screens is the use of small molecules to alter protein function and therefore biological function of the target organism. Whilst chemical screens have been utilised for many years (Macey and Barber, 1970) it was not until the mid-1990s that their true advantages over conventional genetic approaches for probing biochemical pathways was initially realised (Mitchison, 1994; Schreiber, 1998). A major advantage of chemical screens is their ability to overcome genetic redundancy.

It is believed that around 65% of Arabidopsis genes are in gene families containing more than two members (The Arabidopsis Genome Initiative, 2000) , and therefore the importance of genetic functional redundancy in research cannot be underestimated. There are numerous example of this within both GA signalling and biosynthesis. In Arabidopsis there are three GA receptors (GID1a-c) and five DELLA proteins (RGA, GAI, RGL-1, -2, -3) (Hedden and Thomas, 2012). Mutations in the individual GID1 genes have a negligible effect on plant phenotype as a result of redundancy (Griffiths et al., 2006). Similarly, single loss-of-function mutations in the biosynthesis gene, *AtGA20ox2* results in a largely wild-type phenotype due to the presence of the other four *GA20o*x genes (Rieu et al., 2008b). In chemical screens genetic redundancy is overcome when small molecules bind and inhibit multiple components of the network as a general antagonist, or act as a specific agonists by activating specific components (Toth and van der Hoorn, 2010). An example of a small molecule acting as general antagonist is bikini, which was uncovered in a screen of compounds aimed at identifying those which produced a constitutive BR response, similar to plants overexpressing the BR biosynthetic gene *DWARF4* or the BR receptor gene *BRASSINOSTEROID INSENSITIVE 1 (BRI1)* (De Rybel et al., 2009). Using BR signalling mutants it was shown that bikinin acts on BIN2, *a* glycogen synthase kinase (GSK)3-like kinase that mediates phosphorylation of the transcription factors bri1-EMS-SUPRESSOR 1 and BRASSINAZOLE RESISTANT 1. Bikinin was also shown to bind to the ATP pocket to inhibit another subset of GSKs along with the BIN2 and thus triggering the complete BR response. This paper demonstrates the potential of small molecules to overcome genetic redundancy as T-DNA insertions or specific GSK3 inhibitors would not have displayed a phenotype.

Park et al. (2009) demonstrated the power of chemical screens to identify specific agonists when they identified the elusive ABA receptors and their role in ABA signalling using pyrabactin. Using a chemical screen to isolate ABA agonists, they identified pyrabactin. This compound then led to the identification, of *PYRABACTIN RESISTANCE 1* (*PYR1*), using an Arabidopsis suppressor screen against pyrabactin.

PYR1 was then confirmed to encode an ABA receptor. PYR1 is a member of the START domain superfamily which also contains 13 similar genes (*PYR-Like* 1-13, *Pyl1-13*). In a related paper Ma et al. (2009) showed that this 14-member gene family are ABI1-interacting proteins, RCAR1-14 (Regulatory Components of ABA Receptor). Interaction studies showed that PYR1 bound to members of the PP2C subfamily in the presence of ABA. Using enzyme kinetics it was shown that in the presence of ABA and PYR1, PP2C (an ABA signalling inhibitor) and phosphatase activity was decreased. This study shows how chemical genetics can be used as a starting point to identify potentially novel aspects of a hormone signalling pathway that would otherwise be masked from conventional genetic screens by functional redundancy.

The structures of small molecules to bind specific proteins can be extremely diverse, requiring specific contours, charge, hydrogen bonding, hydrophobicity, salt bridges, van der Walls interactions and other factors associated with protein binding pockets (Robert et al., 2009). The composition of the chemical library for the screen is therefore particularly important. In essence the formation of chemical libraries falls into two categories: 'focused libraries' and 'diversity orientated libraries' (Young and Ge, 2004), each with its own advantages and disadvantages. Diversity-orientated libraries, due to their wide scope, are more likely to bind a new class of protein and overcome genetic redundancy, but produce fewer hits and the identified compound is unlikely to possess the potency of a compound identified by a focused library screen (Robert et al., 2009). Literature searches have shown around 10 million pure compounds of a molecular mass of around 1000 have been identified as having a biological effect as compared to the 10^{60} that have the potential to have an effect (Dobson, 2004). Due to the advent of combinatorial chemistry it has become possible for the commercial production of libraries containing numerous previously unknown chemicals. The identification of pyrabactin (discussed previously) provides a good example of the successful use of diversity-orientated libraries. Triazoles inhibit the activity of cytochrome P450s, which catalyse many reactions in the BR biosynthesis pathway (Kaschani and van der Hoorn, 2007). Using a small focus library of 10 synthetic

triazoles. Min et al. (1999) identified brassinazole as a candidate BR biosynthesis inhibitor in rice and *Lepidium sativum*. Later papers on Arabidopsis confirmed that brassinazole was inhibiting BR biosynthesis by binding the cytochrome P450 monooxygenase, DWF4 (Asami et al., 2001).

It is possible to combine the large amount of genetic data for model species and the information from chemical screens to help identify the role of the identified chemicals on plant processes. This form of chemical screen has been termed 'chemical genetic screens' (McCourt and Desveaux, 2010). Two genetic approaches used regularly to identify the target of chemicals identified in a chemical screen are transcriptome analysis and a mutagenesis-based suppressor screens. Once a chemical is identified by a chemical screen, transcriptome analysis can be utilised to identify previously identified chemicals that induce similar transcriptional changes; for example microarray analysis demonstrated that bikinin and BR had a 88% overlap in inducing transcriptional changes, whilst brassinazole has almost the opposite effect to BR (De Rybel et al., 2009; Park et al., 2009). A suppressor screen with mutants displaying agravitropism discovered that *pgp19* displayed a positive gravitropic response when treated with gravacin, thus identifying this process as the target of the compound (Rojas-Pierce et al., 2007).

1.6. AIMS AND OBJECTIVES

A chemical screen was developed to identify chemicals which blocked the GA-mediated feedback regulation of the *GA20ox*1 using a transgenic line containing a *GA20ox1* promoter::GUS reporter. The screen, which was performed at the University of Ghent, identified 28 commercially available chemicals. Using these chemicals the two main aims of this project can be characterised as below:

(1) To identify compounds which perturb GA signalling:

- Confirmation of the effect of the compounds on *GA20ox1* expression

Initial work will focus on characterising the 28 chemicals to confirm that they block GA mediated downregulation of *GA20ox1*, determine their optimal concentration and analysis their effect on endogenous *GA20ox1* expression.

- Determining the effect of compounds on GA-regulated growth responses

Alterations in GA-responsive growth may provide potential clues as to how chemicals are altering GA signalling, therefore hypocotyl and root elongation will be analysed.

- Location of compound activity within the GA signalling cascade

As a main objective of the project is to identify novel components of the GA signalling pathways, we will focus on chemicals which do not GA regulated DELLA degradation in *in planta* GA-mediated DELLA degradation assays.

(2) Use chemicals to identify novel components effecting GA signalling components:

- Chemicals effect on DELLAs binding to downstream TFs

Yeast 2-hybrid screens carried out within our group have identified many DELLA interactors that have a potential role controlling Arabidopsis root development. Chemicals blocking these interactions will be analysed further, with genes encoding these interactors targeted using reverse genetics based approaches.

- Suppressor screening

Suppressor screens have proven a highly successful tool in the identification of chemical targets. A suppressor screen will be used to create and identify mutants in the *GA20ox1::GUS* reporter line that are insensitive to the action of a chemical of interest. Mutated genes in insensitive mutants will then be characterised further.

Refocusing of aims and objectives during the project

During the course of the project two chemicals of interest were chosen for further study. N23 induced agravitropism indicating the chemical was acting on both GA and auxin. As there is limited knowledge of the interactions between these two hormones it was decided to analyse N23's role in auxin signalling using auxin signalling reporter lines and yeast-2-hybrid interactions. Using a novel fluorescent gibberellin N16 was identified as blocking GA uptake. As a result N16 was tested in detail for its role in GA uptake, whilst a punitive GA transport and N16 target was also analysed via radiolabelled GA uptake assays in yeast.

CHAPTER 2. METHODS

2.1. PLANT MATERIAL

All *in vivo* assays in this project were carried out with the model species *Arabidopsis thaliana* in the ecotype Col-0 unless stated otherwise. All Arabidopsis lines used are listed in Table 2.1. The *GA20ox1::GUS* transgenic reporter line was produced by transforming Col-0 with an expression construct in which a sequence coding for the GUS reported protein was fused to the translational sequence (promoter and coding gene sequence) of the *GA20ox1* gene (Desgafne-Penix et al., 2005). *pRGA::GFP-RGA* (GFP-RGA) was produced by transforming Col-0 with a fusion of a green fluorescent protein (GFP*) to the coding region of the RGA* gene, flanked by 8-kb 5′ upstream and 5.8-kb 3′ downstream sequences around the RGA locus to represent the promoter (Silverstone et al., 2001). The *ga1-3* mutant allele was originally generated in the *Landsberg erecta* ecotype, but has been introgressed into the Col-0 background (Tyler et al., 2004). Col-0, *GA20ox1::GUS*, *ga1-3* and *pRGA::GFP-RGA* seeds were all provided by Dr S. Thomas of Rothamsted Research. The VENUS protein is a fast cycling YFP that has been linked to a sequence containing six duplicates of the ARE to create a reporter for auxin responsive genes, *DR5::VENUS* (Brunoud et al., 2012). The VENUS coding sequence has also been fused to the auxin-TIR1 interacting domain (DII) of IAA28 to provide a reporter that is rapidly degraded in response to auxin and therefore provides an *in vivo* fluorescent marker for monitoring auxin response (DII-VENUS ; Brunoud et al., 2012). Both DII-VENUS and *DR5::VENUS* seeds were provided by Dr D. Wells of CPIB, University of Nottingham.

Table 2. 1. Summary of Arabidopsis lines used during this project.

Line	Gene	Locus	Publication
Col-0	-	Wild-type	-
GA20ox1::GUS	*GA20ox1*	At4g25420	Desgagné-Penix et al., 2005
pRGA::GFP-RGA	*RGA-17*	Atg01570	Dill et al., 2001
ga1-3	*CPS/GA1*	Atg02780	Koornneff et al., 1980
DR5::VENUS	*DR5*	Synthetic reporter	Brunoud et al., 2012
DII-VENUS	*DII*	Synthetic reporter	Brunoud et al., 2013

2.2. PLANT GROWTH CONDITIONS

Unless otherwise stated seeds were sterilised by a 5 min treatment in 20% (2.48-2.52 g mL^{-1}) sodium hypochlorite (BDH Lab Supplies, Poole, U.K.) solution with 0.1% Tween20 (Sigma-Aldrich Company Ltd., Dorset, UK) followed by six washes with sterilised deionised water (dH_2O, Milli-Q purification system, Millipore Corp., MA, USA). Sterilised seeds were imbibed in dH_20 at 4^0C for a minimum of two days before sowing. To induce germination imbibed *ga1-3* seeds were imbibed for 8 hours at room temperature in 50 µM GA_4 (provided by Prof. P. Hedden, Rothamsted Research) in dH_2O. GA_4 treated seeds were washed a minimum of six times in dH_2O prior to sowing to reduce GA_4 contamination in the surrounding media during germination. Seeds were sown by pipette in dH_2O. After sowing of seeds, plates were sealed with micropore tape (3M Health Care, Neuss, Germany) and incubated. All plant lines were incubated under 16 hour photoperiod with day/night temperatures of $23/18^0$C. Light levels were maintained at a minimum of 250 µmol m^2 s^{-1}.

2.3. PLANT GROWTH MEDIA

Unless otherwise stated plants were germinated and grown on 1x concentration Murashige and Skoog growth media (Duchefa Biochemie, Haarlem, The Netherlands) with 1% sucrose (Fisher Scientific, Loughborough, U.K.) adjusted to a final pH of 6 using 1 M potassium hydroxide (Fisher Scientific, Loughborough, U.K.). For semi-solid plant media 7 g L^{-1} of Agar type A (Sigma-Aldrich Company Ltd., Dorset, UK) was added. All media was autoclaved for sterilisation. Any addition of chemicals was performed when media was molten (55ºC) after autoclaving.

2.4. CHEMICAL SCREEN FOR CHEMICALS THAT AFFECT GA SIGNALLING

A commercial 10,000-chemical library (Hit2Lead, CA, USA) was screened for maintenance of high *GA20ox1::GUS* staining in the presence of GA. Chemicals were tested at a final concentration of 50 µM, to achieve a biological response but with the aim of reducing toxicity. The screen was performed at VIB, University of Ghent. Three to four *GA20ox1::GUS* seeds were sown in each well of 96 well plates (Multiscreen HTS MSBVS1210; Millipore, Watford, U.K.) containing 0.5x liquid MS medium. Plants were grown in a growth chamber under continuous light (110 $\mu E.m^{-2}s^{-1}$) at 22°C. After 5 days of growth, media was replenished and paclobutrazol (PAC) added to a final concentration of 1 µM. PAC is an inhibitor of the GA biosynthesis enzyme KO, resulting in reduced bioactive GA levels and a subsequent upregulation of *GA20ox1* through the GA signalling-mediated feedback pathway. Twenty-four hours later chemical was added followed by the addition of GA_3 to a concentration of 1 µM two hours later. Histochemical β-glucuronidase (GUS) staining was performed 24 hours later (Chapter 2.4.2.).

2.4.1. Confirmation of effect of identified chemicals and identification of their active concentration

Chemicals selected based on the screen performed at VIB were re-screened at Rothamsted for their effect on *GA20ox1::GUS*, to confirm the effect/rule out false positives. Three to four *GA20ox1::GUS* seeds were sown in each well of 24 well plates (Corning, NY, USA) containing 1x MS liquid medium. After 5 days of growth media was replenished and PAC added to a final concentration of 1 μM. Twenty-four hours later chemical was added followed by the addition of 5 μM GA_3 two hours later. GUS staining was performed 24 hours later. To ascertain the active concentration of each chemical, the *GA20ox1::GUS* screen was repeated using the chemicals at four concentrations: 1, 5, 10 and 50 μM. For each chemical concentration three biological repeats were performed.

2.4.2. Histochemical GUS staining

GUS staining was performed on whole *GA20ox1::GUS* seedlings. After MS media was removed by pipetting, histochemical GUS activity was observed by staining with 1mL of 0.5mg/ml X-Gluc (Melford, Suffolk, U.K.) in a phosphate buffer. The concentration of potassium ferricyanide (an oxidation catalyst) necessary in this assay was determined empirically to be 0.5 μM. Chlorophyll was removed from stained seedlings by washing in 70% ethanol (Sigma-Aldrich Company Ltd., Dorset, UK) over a minimum of 48 hours. Ethanol percentage was then decreased in a stepped series (60%, 50%, 40%, 30%, 20%, 10%, 0%) until seedlings were stored in dH_2O in preparation for analysis. Seedlings were mounted in dH_2O and analysed using a Leica DFC 300FX (Leica Microsystems, Wetzlar, Germany) camera system mounted on a stereomicroscope and the Leica IM50 image capture software package (Leica Microsystems, Wetzlar, Germany).

GUS phosphate staining buffer:	100 mM Sodium Phosphate (pH 6.7)
	100 mM EDTA (pH 7.0)
	0.005% Tween

2.5. PHENOTYPIC ANALYSIS

2.5.1. Root elongation

To test the effect of selected chemicals on root elongation, phenotypic analysis was performed on five day old seedlings over a 24 hour period. Col-0 seeds were germinated and grown on semi-solid 1x MS + 1% sucrose. After 5 days eight to ten seedlings were transferred to 1x MS + 1% sucrose containing one chemical at a concentration of 1, 5, 10 or 50 µM. As chemical stocks were produced by dissolving solid chemicals into pure dimethylsulfoxide (DMSO, Sigma-Aldrich Company Ltd., Dorset, UK) a DMSO treatment was applied as a control (mock). The order in which each treatment was applied to seedlings was randomised by randomised block design prior to the experiment. Seedlings were photographed at time 0 (immediately after transfer) and time 24 (24 hours after transfer) using a Canon G9 camera (Canon, Surry, U.K.). Root length of seedlings at time 0 and 24 was measured using the ImageJ software. Root elongation over 24 hours was obtained by subtracting root length at time 0 from time 24 for each seedling.

2.5.2. Root agravitropism

Phenotypic analysis was performed on five day old seedlings to test the effect of N23 on root gravitropism over a 24 hour period. Col-0 seeds were germinated and grown on semi-solid 1x MS + 1% sucrose. After 5 days 10-15 seedlings were transferred to 1x MS + sucrose containing either N23 (5 µM) or IAA (1 µM). A DMSO (mock) control was also tested. Seedlings were photographed at time 0, 5, 8, 10, 15 and 24 hours

after transfer using the CPIB Rhizotron (University of Nottingham). Root tip angle was measure using the ImageJ software (http://rsbweb.nih.gov/ij/). Root elongation at 0, 5, 8, 10, 15, 24 hours was also analysed using the method described in Chapter 2.5.1.

2.5.3. Hypocotyl length on vertical plates

Phenotypic analysis was performed on five day old seedlings to test the effect of N16 on hypocotyl length after a 48 hour period. Eight to ten Col-0 seeds were germinated and grown on semi-solid 1x MS + 1% sucrose containing N16 at a concentration of 1, 5, 10 or 50 µM. A DMSO (mock) control was also tested. After 8 hours plates were wrapped in multiple layers on aluminium foil to simulate dark growth conditions. After 48 hours seedlings were photographed using a Canon G9 camera (Canon, Surry, U.K.). Hypocotyl length of seedlings was measured using the ImageJ software.

2.5.4. Hypocotyl length in liquid media

Phenotypic analysis was performed on five day old seedlings to test the effect of N16 on hypocotyl length after 5 days in liquid media. Three to five Col-0 seeds were germinated in liquid 1x MS + 1% sucrose in six well plates. After 8 hours N16 was added to a concentration of 1, 5, 10 or 50 µM and plates were wrapped in multiple layers of aluminium foil to simulate dark growth conditions. Plates were grown at 25˚C in the dark for 5 days. A DMSO (mock) control was also tested. After 5 days seedlings were mounted on slides in dH_2O and photographed using Canon G9 (Canon, Surry, U.K.). Hypocotyl length was measured using the ImageJ software.

2.6. CONFOCAL MICROSCOPY TECHNIQUES

2.6.1. Monitoring of *pRGA::GFP-RGA* degradation in response to chemical treatments

pRGA::GFP-RGA seeds (eight to ten) were germinated and grown on filter paper on semi-solid 1x MS + 1% sucrose. After 5 days filter paper and seedlings were transferred to 1x MS + 1% sucrose containing one chemical at a previously identified dose (Chapter 3.2.3.). After 6 hours seedlings were sprayed with 100 μM GA_3 and left for 2 hours, before GFP fluorescence was visualised in 3 seedling root tips per treatment. Roots were mounted in water for visualisation. GFP excitation peak is 488nm and emission peak is 507nm. Images were processed using the Carl Zeiss Zen 2011 (Zeiss, Cambridgeshire, U.K.) software and Adobe Photoshop (Adobe, Berkshire, U.K.).

2.6.2. Visualisation of *DR5::VENUS* and DII-VENUS in the presence of N23 or IAA.

Visualisation and quantification was performed at CPIB in the University of Nottingham, using the method outlined in Brunoud et al. (2012). *DR5::VENUS* and DII-VENUS seeds were germinated and grown on semi-solid 1x MS + 1% sucrose for 5 days, before transferred to medium containing N23 at concentrations of 5, 10, 25, 50 μM, with or without 1 μM IAA.

For visualisation seedling root tips were analysed using a Nikon Eclipse Ti 2000 laser-scanning confocal microscope (Nikon, Surry, U.K.). Roots were mounted in water and seedling cell walls were stained with 0.5% propidium iodide solution. VENUS excitation peak is 515nm and emission peak is 528nm. Images were processed using the Carl Zeiss Zen 2011 (Zeiss, Cambridgeshire, U.K.) software and Adobe Photoshop (Adobe, Berkshire, U.K.).

For live quantification of fluorescence the seedlings were scanned either every 5 mins for 60 mins (DII-VENUS) or every 30 mins for 8 hours (*DR5::VENUS*) immediately after the beginning of the treatment to follow the evolution of the VENUS signal. A similarly sized portion of the root, corresponding approximately to the first 200 µM from the root tip, was scanned. To quantify fluorescence the average fluorescence intensity over each portion of the root was extracted using Fiji software (http://fiji.sc/Fiji) and the values analyzed using Microsoft Excel. For *DR5::VENUS* quantification all levels of fluorescence were measured as relative to the final time point of the IAA treatment. DII::VENUS quantification represents levels of fluorescence of each treatment relative to the fluorescence at the initial time point of that treatment.

2.7. WESTERN BLOT TO CONFIRM RESULTS OF *PRGA::GFP-RGA* ASSAY

To confirm the effect of on GA mediated DELLA protein degradation (as observed on the *pRGA::GFP-RGA* reporter line), *in vivo* GA mediated RGA degradation was analysed using Western blot analysis.

2.7.1. Protein extraction

Col-0 seedlings were cultured in Gamborg B5 liquid media in 250 mL flasks for 4 weeks in the dark to give a root culture. Three hours before harvest seedlings were treated with 10 µM of chemical or DMSO. One hour prior to harvest, 100 nM GA_4 was added to the culture that already contained either the chemical or DMSO. To harvest, root cultures were rinsed with ice cold dH_2O three times before being flash frozen in liquid nitrogen. For protein extractions frozen root cultures were ground in liquid nitrogen and crushed material was re-suspended in 8 mL of extraction buffer EB1 (0.4 M sucrose, 10mM Tris-HCL, pH 8.0, 5 mM β-mecaptoethanol. 0.1 mM phenylmethanesulfonylfluoride (PMSF), 1X Sigma protease inhibitors). Suspended

material was filtered through a microcloth filter into a falcon tube and centrifuged at 2800 g for 20 mins at 4ºC. The resulting pellet was resuspended in 1 mL of EB2 (0.25 M sucrose, 10mM Tris-HCL, pH 8.0, 10 mM MgCl2, 1% Triton X-100, 5 mM βm-ME. 0.1 mM PMSF, 1X Sigma protease inhibitors) and centrifuged at 12000 g for 10 mins at 4ºC. Pellet was resuspended in 500 µL EB3 (1.7 M sucrose, 10mM Tris-HCL, pH 8.0, 2 mM MgCl2, 0.15% Triton X-100, 5 mM βm-ME. 0.1 mM PMSF, 1X Sigma protease inhibitors) and transferred to a fresh Eppendorf before 500 µL more EB3 was added and centrifuged at 16000 g for 30 mins at 4ºC. Pellet was resuspended in 50 µL of 6X SDS sample buffer and boiled at 95ºC for 10 mins.

2.7.2. SDS-PAGE

Sample in SDS buffer (20 µL) and 5 µL PageRuler Prestained Ladder (Thermo, Northumberland, U.K.) were run on a Bio-Rad 4-20% precast gel. Gel was run at 170 V for 40 mins.

2.7.3. Western blot

SDS-PAGE gel was rinsed with dH_2O and shaken for 15 min in blotting buffer. Prior to transfer, a PVDF membrane was briefly soaked in methanol, while six sheets of filter paper were soaked in blotting buffer (500 mL, 48 mM Tris, 39 mM glycine, 20% methanol). Gel and membrane were sandwiched between two stacks of three sheets of filter paper, then run for 15 V for 1 hour. Following transfer, membrane was soaked in methanol for 2 mins before shaking in blocking buffer (48 mM Tris, 39 mM glycine, 20% methanol, dehydrated milk) for 1 hour. Blocking buffer was replenished with 5 mL of fresh blocking buffer was added to membrane with 10 µL of anti-RGA. Membrane was shaken overnight in buffer plus anti-RGA antibody at 4ºC followed by three rinses in TBS-$T_{0.05}$ (0.5 M sodium chloride). The second antibody was added to membrane in 5 mL and shaken for 1 hour, followed by rinses in TBS-$T_{0.05}$ (0.5 M sodium chloride). Femto (3 mL; 300 µL peroxide, 300 µL luminol, 2.4 mL dH_2O) was applied to

membrane by constant pipetting for 1 min. Membrane was placed between x-ray film and two sheets of clear film and placed in developing cassette for 30 secs. X-ray film was developed in developer solution then fixed in fixer solution.

2.8. EFFECT OF CHEMICAL N16 ON GA UPTAKE

2.8.1. Fluorescent tagged GA_3 (Fl-GA_3) uptake assay

Seven chemicals were sent to the University of San Diego for analysis of their effect on GA uptake using the Fl-GA_3 method described in Shani et al. (2012). Six to eight day old Col seedlings were transferred to agar media containing the Fl-GA_3 (5 µM) and the chemical (at active concentration defined in Chapter 3.2.2.) for two to three hours. Prior to imaging, roots were washed in dH_2O and stained with propidium iodide. Images were obtained using a Zeiss LSM 710 confocal microscope, with argon laser set at 488 nm for excitation, 566-617 nm filter for propidium iodide emission, and 493-543 nm filter for Fl derivative emission. Images were processed using Zeiss Zen 2011 software.

2.8.2. Radiolabelled GA_4 uptake assay

To confirm effects of N16 on GA uptake, radiolabelled GA_4 ($[^3H]GA_4$) uptake was analysed in plants exposed to the chemical. Col-0 and *ga1-3* seeds were germinated and grown on agar (0.7%) 1x MS+ 1% sucrose for 6 days. 15-20 seedlings were transferred to 3 cm petri dishes with water containing 30 µM N16 or DMSO (mock) and 1 µM GA_4 and ≈10 kBq $[^3H]GA_4$ (provided by P. Hedden). Following 24 hours in water, roots of seedlings were harvested into 1.5 mL Eppendorfs and frozen in liquid nitrogen before grinding with a micropestle. $[^3H]GA_4$ was extracted by adding 330 µL of methanol (Sigma-Aldrich Company Ltd., Dorset, UK) to ground material, before samples were vortexed and pelleted using a centrifuge (3 mins, 10,000 rpm). Methanol was removed to a scintillation vial. A second methanol extraction was then

performed. A final methanol extraction transferred remaining methanol and tissue to a scintillation tube. Two mL of scintillation fluid (Ultima Gold, Perkin Elmer, MA, USA) was added to 1 mL of methanol extract before samples were analysed using a Tri-Carb 2100 TR scintillation counter (Perkin Elmer, Ma, USA) giving results in degradations per min (DPM). 1000 Becquerels (Bq) of radioactivity in root was viewed as 60,000 DPMs.

2.9. ANALYSIS OF GENE ACTIVITY USING QRT-PCR

The effect of chemicals on *GA20ox1* expression was analysed using quantitative real time PCR (qRT-PCR) analysis. RNA was extracted from whole seedling tissue using the QIAgen RNeasy plant RNA extraction kit (QIAgen), following the manufacturers protocol. Purified RNA was DNase-treated using Ambion Turbo DNase (Invitrogen) and quantified using a Nanodrop^{TM} ND-1000 spectrophotometer (LabTech international Ltd.). cDNA was synthesised from RNA using the Invitrogen Superscript III First Strand cDNA synthesis kit (Oligo[dt] method), according to the manufacturers protocol.

All qRT-PCR reactions were performed using Sigma SYBR Green Jumpstart^{TM} Taq Readymix (Sigma-Aldrich):

cDNA (diluted 1/10 using dH_2O)	3.6 µL
Forward primer (10 µM)	0.4 µL
Reverse Primer (10 µM)	0.4 µL
2x Sybr Green (+ ROX dye, 2 µL per 1 mL)	10 µL
$MgCl_2$ (25 mM)	5.6 µL
Final Volume	20 µL

qRT-PCR reactions were run on an Applied Biosystem 7500 Real-Time PCR system (Life Technologies Corporation). The qRT-PCR reaction was as follows:

Initial denaturation	95ºC	2 mins
Thermocycling	95ºC	15 secs
	60ºC	1 min
	X 40 cycles	
Dissociation analysis	95 ºC	15 secs
	60 ºC	1 min
	95 ºC	15 secs
	60 ºC	15 min

All experiments comprised of 4 biological and two technical replicates run on 96-well plates with water controls. For each experiment three reference genes were used, *YLS8*, *UBQT* and *PP2A* which have been shown to be stable under hormone treatment (Czechowski et al., 2005; Rieu et al., 2008a) (Table 2.2.). qRT-PCR data was analysed using method outlined by Pfaffl (2001).

Table 2. 2. Primers used in *GA20ox1* expression analysis.

Primer name	Sequence	Target	From
GA20ox1 F	GATCCATCCTCCACTTTAGA	*GA20ox1*	Reiu et al. 2008a
GA20ox1 R	GTGTATTCATGAGCGTCTGA	*GA20ox1*	Reiu et al. 2008a
AT5G08290 qF	TTACTGTTTCGGTTGTTCTCCATTT	*YLS8*	Czechowski et al. 2005
AT5G08290 qR	CACTGAATCATGTTCGAAGCAAGT	*YLS8*	Czechowski et al. 2005
AT1G13320 qF	TAACGTGGCCAAAATGATGC	*PP2A*	Czechowski et al. 2005
AT1G13320 qR	GTTCTCCACAACCGCTTGGT	*PP2A*	Czechowski et al. 2005
AT3G53090 qF	TTCAAATACTTGCAGCCAACCTT	*UBQT*	Czechowski et al. 2005
AT3G53090 qR	CCCAAAGAGAGGTATCACAAGAGACT	*UBQT*	Czechowski et al. 2005

2.10. YEAST-2-HYBRID

Yeast-2-hybrid was used in this project to identify the effect of the 28 identified chemicals on the interaction between a DELLA protein, GAI, and known interactors, SLY, gar (a version of SLY), and GID1.

2.10.1. Growth conditions

Unless otherwise stated yeast colonies were grown on either semi-solid (10 g L^{-1} Bacto agar^{TM}, Becton, Dickinson and Company, Oxford, U.K.) YPD (Yeast Extract

Peptone Dextrose, Formedium, Hunstanton, U.K.) prior to transformation or semi-solid DOB (Drop-out bases, MP Biochemicals, OH, USA) media lacking tryptophan and leucine (Sigma-Aldrich Company Ltd., Dorset, UK) post transformation. Plates were incubated in the dark at 30ºC for 2-3 days to yield colonies.

2.10.2. Yeast transformation

Yeast transformation was performed using an adaptation of the lithium acetate method (Becker et al., 1993). Overnight liquid culture (100ml YPD, 30°C, 210 rpm) of yeast strain MaV203 (Lifetechnologies, Paisley, UK) or L40 (provided by S. Thomas) was diluted to an OD_{600} of 0.2 and returned to incubator until OD_{600} reached 0.8. Cells were harvested (3000 rpm, 3 mins) and washed with dH_2O. Cells were re-suspended in 10 mL 0.1 M lithium acetate at 30°C, 190 rpm for 1 hour. Each plasmid (1 µg) was incubated at 30°C with 150 µL of cells in lithium acetate and 350 µL polyethylene glycerol 3350 (Sigma-Aldrich Company Ltd., Dorset, UK) for 1 hour. Cells were then subjected to heat shock at 42°C for 5 min before a 3 min recovery period on ice. Each transformation (200 µL) was plated on minimal media lacking leucine and tryptophan. Transformations were incubated at 30°C for 2-3 days to give colonies.

2.10.3. Yeast-2-hybrid of DELLA degradation machinery.

To determine the strength of the interaction between the bait and prey plasmids the HIS3 (Invitrogen, 2005) L40 yeast strain containing: pACT2 vs pLEXA; pACT2-*GAI* vs pLEXA; pACT2-*GAI* vs pLEXA-*SLY*; pACT2-*GAI* vs pLEXA-*gar*; pACT2-*GAI* vs pLEXA-*GID1b*; and pACT2-*GAI* vs pLEXA-*GID1c*, were plated on media lacking leucine, trpyptophan and histidine (Sigma-Aldrich Company Ltd., Dorset, UK) and containing one chemical at 50 µM, and 0 or 20 mM 3AT (Bio 101 Systems, UK). A DMSO (mock) control and 0 or 20 mM 3AT only were also tested. Each yeast strain colony was diluted in 100 µL dH_2O. Each colony dilution (5 µL) was spotted on each

plate. Five colonies per interaction were spotted on each plate. After 72 hours yeast growth indicated an interaction in the presence of the chemical.

2.10.4. Yeast-2-hybrid of IAA-ARF interactions.

MaV203 yeast strain containing: pDEST22-*IAA14* vs pDEST32-*ARF19 dD3*; pDEST22-*IAA14* vs pDEST32-*ARF19 dQR*; pDEST22-*IAA14* vs pDEST32-*ARF19 2-15*; pDEST22-*IAA14* vs pDEST32-*ARF19 FL*; and pDEST22-*IAA14* vs pDEST32, were plated on media lacking leucine, trpyptophan and histidine and containing N23 at 50 µM, and also containing either 0, 20, 50, or 100 mM 3AT. A DMSO (mock) control and 0 or 20 mM 3AT only were also tested. Each yeast strain colony was diluted in 100 µl dH_2O. Each colony dilution (5 µL) was spotted on each plate. Five colonies per interaction were spotted on each plate. After 72 hours yeast growth indicated an interaction in the presence of the chemical.

2.10.5. OPT6 yeast GA transport assay.

To analyse oligopeptide 6 (OPT6) role as a GA transporter a yeast uptake assay was performed based on the method by (Kanno et al., 2012). *OPT6* was initially amplified from seven day old whole seedling cDNA with forward (TAGGATCCACGATGGGAGAGATAGCAAC) and reverse (TAGAATTCCTAGAAGACGGGACAGCCTT) primers, which inserted *BamH1* (GGATCC) and *EcoR1* (GAATTC) sites, respectively, into the 5'- and 3'- ends of the amplified sequence. Following confirmation by sequencing that the amplified fragment had the correct sequence (primers: T3, T7, AGAGCTCACTGTACCCAAG) the *OPT6* clone was ligated into the *pSCB* vector and amplified in DH5α. After excision of *OPT6* from *pSCB* with *BamH1* and *EcoR1*, it was ligated into the *pPH3* vector. The *pPH3-OPT6* plasmid was cloned into the YMM-ABC8 yeast strain for [^{3}H]GA_4 uptake analysis (Benton et al., 1994; Schuetzer-Muehlbauer et al., 2003). It was therefore decided to use *pPH3-AIT3* in the same YMM-ABC8 yeast system (produced by Steve

Thomas) as a positive control for the *OPT6* [^{3}H]GA_4 uptake assay, with *pPH3* being the negative control. The YMM-ABC8 yeast strain containing *pPH3*, *pPH3-OPT6* or *pPH3-AIT3* was grown in liquid culture (DOB lacking tryptophan and uracil) to an OD_{600} of 0.7 to 1.0 before being concentrated to an OD_{600} of 6.0 by centrifugation and dilution with DOB lacking tryptophan and uracil. Yeast was cultured at this concentration for 60 and 180 minutes before [^{3}H]GA_4 uptake was quantified by centrifuging yeast cells and suspending in 1 mL 100% methanol. 2 mL of scintillation fluid (Ultima Gold, Perkin Elmer, MA, USA) was added to 1 mL of methanol extract before samples were analysed using a Tri-Carb 2100 TR scintillation counter (Perkin Elmer, Ma, USA) giving results in degradations per min (DPM). 1000 Becquerels (Bq) of radioactivity in root was viewed as 60,000 DPMs.

PCR reactions for *OPT6* amplification from cDNA was performed using:

cDNA	4 µL
dNTP (10 mM)	0.5 µL
Forward primer (10 µM)	1.25 µL
Reverse Primer (10 µM)	1.25 µL
5x Phusion buffer	5 µL
Phusion Taq Polymerase (5 U/µL)	1 µL
dH2O to a final Volume	25 µL

PCR reactions were run on a C100 Thermal Cycler (BioRad, Hertfordshire, U.K.). The PCR reaction was as follows:

Initial denaturation	98ºC	30 secs
Thermocycling	98ºC	10 secs
	55ºC	30 sec
	72ºC	1 min
	X 25 cycles	
Final extension	72 ºC	5 mins

CHAPTER 3: A CHEMICAL SCREEN TO IDENTIFY COMPOUNDS WHICH PERTURB GA SIGNALLING

3.1. INTRODUCTION

The advent of reverse genetics-based tools such as T-DNA insertion lines (Parinov et al., 1999; Sessions et al., 2002; Alonso, 2003; Rosso et al., 2003; Kuromori et al., 2004), RNA interference (Mello and Conte, 2004) and transgenic overexpression lines (Holtorf et al., 1995) has allowed for a greater understanding of the role of candidate genes in controlling growth and development in model plants such as Arabidopsis. Despite these tools and resources, genetic redundancy can often hamper the characterisation of novel genes (McCourt and Desveaux, 2010). For example in Arabidopsis around 65% of the identified genes belong to gene families containing more than two members (Arabidopsis genome initiative, 2000). This is clearly highlighted through recent studies of the GA signalling pathway in Arabidopsis, where there are three and five genes encoding GA receptors and DELLA proteins, respectively (Ueguchi-Tanaka et al., 2005; Zentella et al., 2007). Loss-of-function mutations in the individual *GID1* genes have very little effect on plant phenotype due to functional redundancy, whereas the *gid1* triple mutant is a severe GA-insensitive dwarf (Griffiths et al., 2006b). To date no components of the GA signalling pathway that are downstream of DELLAs have been identified by forward genetic-based approaches. One plausible explanation for this lack of success is the possibility of genetic redundancy within these components. A potential strategy to overcome genetic redundancy is a chemical screen which has the potential to alter the activity of whole gene families.

Chemical screens provide an alternative approach to classical genetic techniques, instead using small organic molecules to target protein activity as opposed to altering a DNA sequence as in classical genetics (McCourt and Desveaux, 2010). Typical chemical screens utilise large libraries of organic chemicals (>10,000) to test their

effect on a given pathway in either a cell line or a whole organism (McCourt and Desveaux, 2010). The libraries utilised can vary dramatically. Initial screens utilised libraries biased towards target-related activity, for example the use of auxin and TIR1 has led to the discovery of auxin agonists and antagonists (Hayashi et al., 2008). As the technology developed it was possible to utilised libraries with diverse chemical structures potentially identifying core chemical structures that perturb a plant process causing a phenotype (McCourt and Desveaux, 2010). Arabidopsis seedlings provide an ideal system for performing chemical screens due to its small size which allows high-throughput screening. There are numerous examples of chemical screens that have been used successfully to identify novel components of hormone signalling cascades, most notably one which recently led to the identification of the ABA receptor (De Rybel et al., 2009; Park et al., 2009). Using a chemical screen Park et al. (2009) isolated an ABA agonist pyrabactin. Microarray data confirmed a highly correlated transcriptional response to either pyrabactin or ABA in seeds and seedlings, further confirming pyrabactin's role as an ABA agonist. A subsequent Arabidopsis suppressor screen using pyrabactin led to the identification of PYR1, a cyclase subfamily of the START domain superfamily which possess hydrophobic ligand–binding pocket. Further analysis using the *pyr/pyl* mutants confirmed PYR1 as a receptor for ABA. In the same study it was also discovered, using yeast-2-hybrid, that the downstream elements of the PYRs/PYLs are the PP2Cs and a model for ABA reception was proposed whereby ABA receptors functioning at the apex of a negative regulatory pathway that controls ABA signaling by inhibiting PP2Cs.

The application of exogenous GA to Arabidopsis results in a rapid reduction in the expression of some members of the *GA20ox* and *GA3ox* gene families indicating a feedback regulatory response mediated by GA signalling (Chiang et al., 1995; Phillips et al., 1995; Mitchum et al., 2006; Rieu et al., 2008b). This transcriptional response mediated by the GA signalling pathway acts to maintain GA homeostasis, essentially ensuring that GA levels are optimal for controlling plant growth and development. Feedback regulation of GA signalling revolves around the DELLA protein. The

presence of bioactive GA results in the degradation of the DELLA protein which in turn removes their transcriptional regulation of the GA biosynthetic genes thus reducing bioactive levels of GA. In the cases of *GA20ox1* and *GA20ox2*, the transcriptional changes are observed within 15 minutes resulting in a 10-20 fold decrease in expression (Zentella et al., 2007). In contrast to the feedback regulation of the biosynthesis genes expression of the *GA2ox* genes, which encode enzymes that inactivate bioactive GA and their precursors, are up-regulated by GA (Thomas et al., 1999; Rieu et al., 2008a; Weston et al., 2008). The study of GA signalling mutants has demonstrated that a functional GA signal transduction cascade is necessary for the feedback regulation of GA biosynthesis (Silverstone et al., 1998; Ueguchi-Tanaka et al., 2005; Griffiths et al., 2006b; Chandler et al., 2008). For example, in DELLA loss-of-function mutants the transcription of these feedback-regulated genes, including *GA20ox1* is reduced and is not sensitive to changes in the levels of bioactive GAs (Dill et al., 2001; Dill and Sun, 2001; King et al., 2001a; Silverstone et al., 2001; Weston et al., 2008). This demonstrates that DELLAs are required for this transcriptional control (Figure 3.1.), although the mechanisms are not fully understood. In recent years, multiple classes of transcription factors have been found to interact with the DELLA proteins (review in Chapter 1.3.3.4.), including SCL3, which has been shown to directly affect the transcription of GA biosynthetic genes and DELLA genes (Zhang et al., 2011). The homeostatic regulation of GA metabolism by the GA signalling pathway presents an ideal target for a chemical screen aimed at identifying novel components of GA signalling.

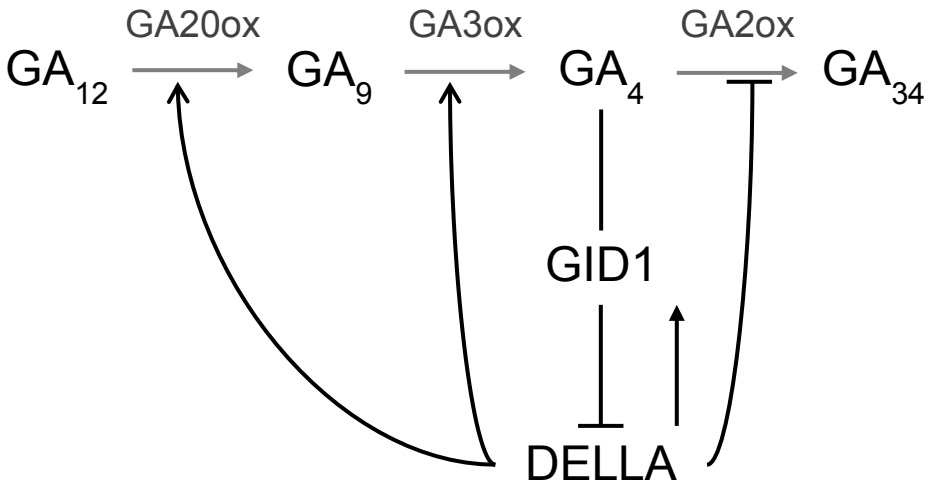


Figure 3. 1. Summary of GA homeostasis

DELLAs increase expression of GA biosynthesis genes leading to an increase in GA-mediated DELLA degradation. Grey arrows indicate enzyme-mediated reactions. Black arrows indicate transcriptional control. (Adapted from Hedden and Thomas (2012))

3.1.1. The potential of chemical screens to identify novel components of the GA signalling pathway

Screening a diverse chemical library represents an alternative strategy to identify novel components of GA signalling, potentially those which are acting downstream of DELLA proteins. As mentioned previously few elements downstream of the DELLA proteins have been identified, possibly as a result of genetic redundancy. Global transcriptomic studies of GA signalling mutants have identified numerous genes that are regulated by DELLAs and which control GA-responsive growth and development (Ogawa et al., 2004; Cao et al., 2006; Zentella et al., 2007; Hou et al., 2010). Early GA-responsive genes provide the opportunity to develop potential reporter gene constructs which can be used to screen for chemicals that perturb GA signalling. For example, chemicals which perturb the interaction of DELLA proteins with transcription factors would be expected to affect the expression of downstream GA-responsive genes. The primary aim of this project is to use a chemical screen to identify Arabidopsis GA signalling components, potentially novel transcription factors, which

are involved in the homeostatic transcriptional regulation of GA biosynthetic genes, although other interesting targets are also likely to be identified. The basis for this chemical screen is the feedback-regulated GA biosynthesis gene, *GA20ox1*. Whilst DELLA proteins are known to up-regulate the expression of *GA20ox1* the mechanisms by which they do this are currently unknown (Figure 3.2.). However, there is evidence that DELLAs do not bind directly to the *GA20ox1* promoter (Zentella et al., 2007). Our group has generated a transgenic *GA20ox1::GUS* reporter line in the Arabidopsis Col-0 ecotype (Hay et al., 2002; Clark, 2005) (Figure 3.3.). This line has been demonstrated to provide an effective reporter for GA-mediated downregulation of *GA20ox1* expression by monitoring GUS activity. Furthermore, it highlights the distinct temporal and spatial expression patterns of *GA20ox1*, which include expression within the meristem and elongation zones of the root (Figure 3.3.). Interestingly, the spatial expression pattern within the root elongation zone is found exclusively within the cortical cells (Clark, 2005). Using the *GA20ox1::GUS* reporter line a high throughput chemical screen was established to identify compounds which block the GA-mediated down-regulation of GUS activity in Arabidopsis roots. In addition to identifying compounds that affect transcriptional feedback regulation of *GA20ox1*, the screen may also lead to the identification of components that control GA-responsive root growth. For the chemical screen only the roots were observed as Ubeda-Tomas et al. (2008) had demonstrated the integral role of GA in root elongation, showing roots provide an ideal tissue for studying GA signalling. Roots, as opposed to whole seedlings, also allowed for a higher throughput screen as Arabidopsis *GA20ox1::GUS* root staining can be visually analysed after 8 hours staining with X-gluc whereas hypocotyls require clearing with ethanol.

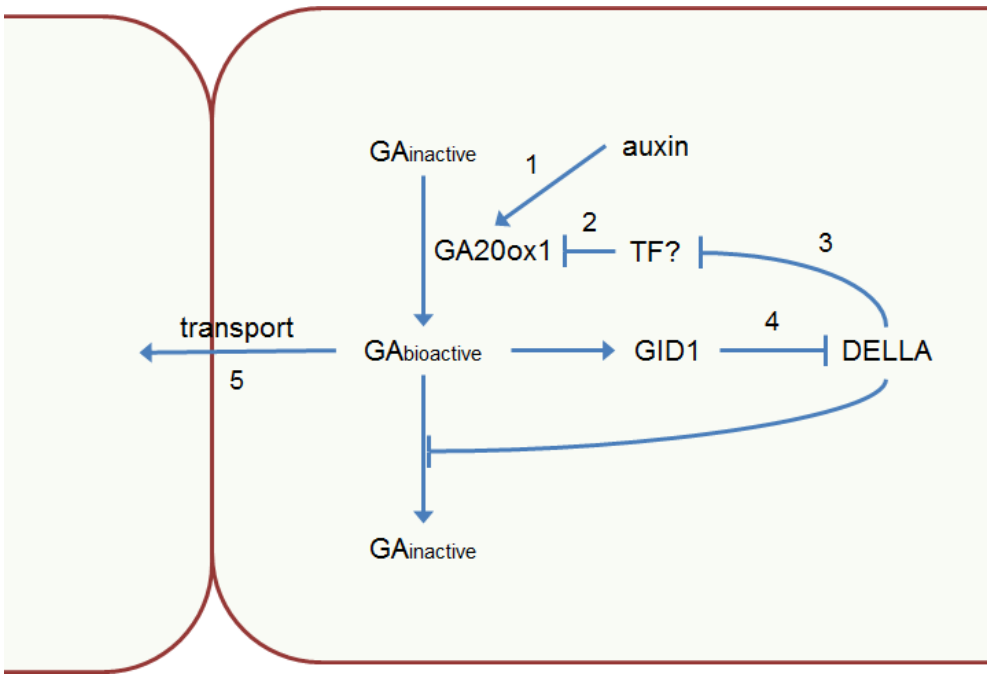


Figure 3. 2. Potential sites of actions of chemicals identified by GA20ox1::GUS assay carried out at VIB.

1. Chemicals could be affecting aspects of other hormone signalling pathways known to regulate *GA20ox1* (e.g. auxin). 2. Chemicals could be affecting unknown transcription factors which regulate *GA20ox1*. 3. Chemicals could be affecting DELLA regulation of putative transcription factors. 4. Chemicals could be preventing DELLA degradation by affecting interactions between components of the DELLA degradation machinery. 5. Chemicals could be blocking the transport of GA to required sites in the root.

This chapter describes an extensive characterisation of the chemicals identified in the initial screen, focussing specifically on their effects on GA signalling and root elongation. A preliminary characterisation of the compounds demonstrated that all of the available 28 chemicals resulted in an increase in GUS activity in the roots of the *GA20ox1::GUS* line when treated with GA_3 compared to the mock treatment. Subsequent dose-response experiments were used to establish the effective concentrations at which the chemicals actively blocked GA-mediated suppression of *GA20ox1::GUS* expression in the reporter line. To establish whether the compounds have a physiological effect on root development, root elongation assays were

performed. These assays demonstrated that some of the compounds acted to inhibit root elongation. However, in some cases this effect was caused by toxicity to the root cells. As an approach to narrow down where the chemicals are acting within the GA signalling cascade, their effects on GA-mediated DELLA degradation was monitored using the *pRGA::GFP-RGA* reporter line. Of the 28 chemicals 11 were demonstrated to block GFP-RGA degradation, suggesting that they act upstream of DELLA degradation. In contrast, 11 chemicals did not block GFP-RGA degradation suggesting that they may act downstream of DELLAs within the GA signalling cascade. In an attempt to establish if the chemicals acting upstream of DELLA degradation are affecting protein-protein interactions that control this process, yeast-2-hybrid assays were performed in which GID1-DELLA and SLY1-DELLA interactions were assessed in the presence of the compounds. Only one of the compounds, N11 appeared to have any effect on the DELLA interactions. In this case it appeared to block the interaction of DELLA with both the GID1 and SLY1 proteins.

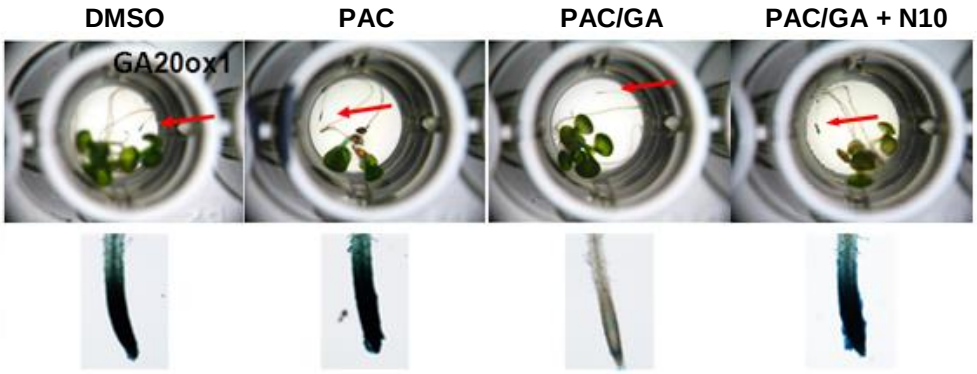


Figure 3. 3. Initial chemical screen performed at Ghent. Screen aimed to find chemicals that blocked GA-mediated down-regulation of GUS activity in roots of the GA20ox1::GUS seedlings.

Top panel shows *GA20ox1::GUS* seedlings after treatment with, DMSO (mock), PAC (positive control), PAC/GA (negative control) and PAC/GA + N10 (one of positive compounds tested) in the cells of a 96-well plate. Bottom panel shows root tips from seedlings in the 96-well plate at a higher magnification (x20). *GA20ox1::GUS* seeds grown for 5 days in liquid culture are treated with 1 µM PAC. After 24 hours the library of chemicals (10,000) are added to the media to a concentration of 50 µM followed two hours later by a GA application of 1 µM. 24 hours after GA application seedlings are GUS stained. (Screen performed at University of Gent by Bert De Rybel, Dominique Audenaert, Tom Beeckman)

3.2. RESULTS AND DISCUSSION

3.2.1. Chemical screen to identify compounds which inhibit GA-mediated transcriptional regulation of a *GA20ox1::GUS* reporter

From the 28 chemicals selected from the screen it was possible to identify common core structural features such that the chemicals could be divided into four structural groups: Group 1 (secondary amides), Group 2 (coumarins), Group 3 (sulphonamides) and Group 4 (compounds that do not fit into the three main groups) (structures shown in Figure 3.4.). Each chemical was coded N1 to N28 (Table 3.1.) and will be referred to as such for the remainder of the thesis. An amide is a chemical with a core

functional group of $R_nE(O)_xNR'R''$ where R, R' and R'' represent either a hydrogen or an organic group whilst E can be one of many elements including, carbon (C), phosphorous (P) or sulphur (S). All the chemicals within Group 1 are "organic amides", termed as such because of the presence of a C in the E position of the amide forming an acyl group (RCONHR'). Another common structural component of all chemicals belonging to Group 1 is the presence of H at the R'' position, such that they are secondary amides. Coumarins belong to the benzopyrone class of chemicals. As the name suggests, benzopyrones consist of a benzene ring fused to a pyrone. In the case of coumarins the pyrone takes a 2-pyrone form with the ketone group being in position two of the unsaturated six membered ring containing one oxygen atom. The structure can therefore be represented as $RC_9H_6O_2R'$. Additionally all chemicals in Group 2 possess a methyl group at position 4 on the pyrone ring and an acetic or propanoic acid group (R') at position 3. An ether function (R) is attached at position 2 of the benzene ring. Coumarins and their derivatives are naturally produced in many plants but tonka bean, vanilla grass, sweet woodruff, mullein, sweet grass, cassia cinnamon, sweet-clover, and deertongue are known to have particularly high levels of these chemicals. Sulphonamides are in essence amides where E = S and $O_x = O_2$ (RSO_2NHR'). The chemical library used for the *GA20ox1::GUS* assay performed in Ghent consisted of novel synthetic chemicals constructed from chemical building blocks (e.g. simple acids and amides), and in most cases the chemicals identified from the screen have to date no known biological function.

Whilst the core structure of any chemical identified is integral to its shape and conformation and will therefore decide the domain, motif or fold of a protein the chemical can interact with, it is the outlying functional groups of the structure that will determine if a chemical can bind the protein. For example the presence of a pyridine instead of a benzene is integral for pyrabactin's activity as an ABA transport and seed germination inhibitor (Park et al., 2009). Additionally the location of the N in the pyridine ring is important for activity, while the presence of additional bromine or methyl groups can also decrease the efficacy of the molecule. It is therefore possible

that any of the functional groups on the chemical structures of the 28 identified chemicals could be necessary biological activity. Group 1 consists of nine compounds. For N3 E is a carbon and R is a phenoxy group. In the R' position N3 possesses a methylbenzene coupled at the 2-position to a benzothiazol moiety. R' in N4 is a thiazolyl moiety linked to a dichlorophenol whilst at the R position there is a bicycloheptane with a carboxylic acid. The R' position of N5 is a bicycloheptene carboxylic acid, which is similar in structure to the R' of N4. A methyltetrahydrobenzothienyl with an ethoxycarbonyl on the C_3 is in the R position of N5. N6 is a relatively simple amide with a chlorobenzene in the R position and a benzoic acid in the R' position. N8 also has a benzoic acid in the R' position but the R position is filled by a furan with methyl and isobutyl at the C_2 and C_5 positions, respectively. A dichlorobenzene fills the R position of N14 with a dimethoxybenzoic acid in the R' position. N18 has a biphenylcarboxylic acid at the R position and trifluoromethoxy benzene group in the R' position. At the R' position of N19 is a chlorobenzoic acid with an ethoxybenzene in the R position. N24 is also a relatively simple amide with 3-chlorophenol in the R position and toluene in the R' position.

The constituent groups decorating the core coumarin molecules in Group 2 are simpler structures than most those linked to the amides, potentially as the coumarins are larger, more complex core structures than the amides. N20 has a fluorobenzyloxy group at the R position with propanoic acid at the R'. N22, N23 and N26 all have acetic acid at the R' position, but have phenylpropenyloxy, 2-bromobenzyloxy and 4-bromo-2-fluorobenzyloxy, respectively, at the R position. N27 has propanoic acid at the R' position and a butoxy chain at the R position. Despite also being derived from amides, the sulphonamides identified in the initial *GA20ox1::GUS* screen also have simpler R and R' groups than the amides that were identified. N2 has a benzene ring at the R position and a pentafluorophenol at the R' position. Both N10 and N11 have a nitrophenol in the R' position. N10 has 3-chloro-4-fluorobenzene at the R position whilst N11 has a bromothiophene. N12 is the most complex sulphonamide in Group 3. In the R position it has a simple bromothiophene and at R' there is benzyl-2-

benzamide. N16 differs from the other sulphonamides in Group 3 as both positions on N are substituted by cyclohexyl and acetic acid moieties. In the R position there is trimethylbenzene. Of the 28 identified chemicals seven did not have a core structure in common with any of the other identified chemicals. N1 is a tetrafluorobenzene para substituted with a trifluoromethyl and phenoxypyrrolidinedione groups. The core structure of N7 is 5-methylthiotetrazole, substituted at the 1-position with benzoic acid and with 2,4,6-trimethylbenzene on the methyl group. N9 is a 2-keto-3-hydroxy-2,5-dehydro pyrrole substituted at the 1 position with 2-methylpyridine, on C-4 with 2-formylthiophene and at C-5 with 4-isopropylbenzene. N13 also contains a 5-methylthiotetrazole, substituted on N-1 with benzene and on the S-methyl group with 5-keto-1-benzylpyrazole. N17 has a 2,3-dihydropyrazole moiety at its core, to which is substituted 4-oxobutyric acid on N-1, 5-bromophenyl on C-3 and phenylvinyl on C-5. N21 is a thiourea derivative, substituted with 3,4-dichlorobenzyl and 4-nitrophenyl groups. N25 is similar to N21 with cyclohexene linked to the thiourea via an ethyl group in place of the dichlorobenzyl group.

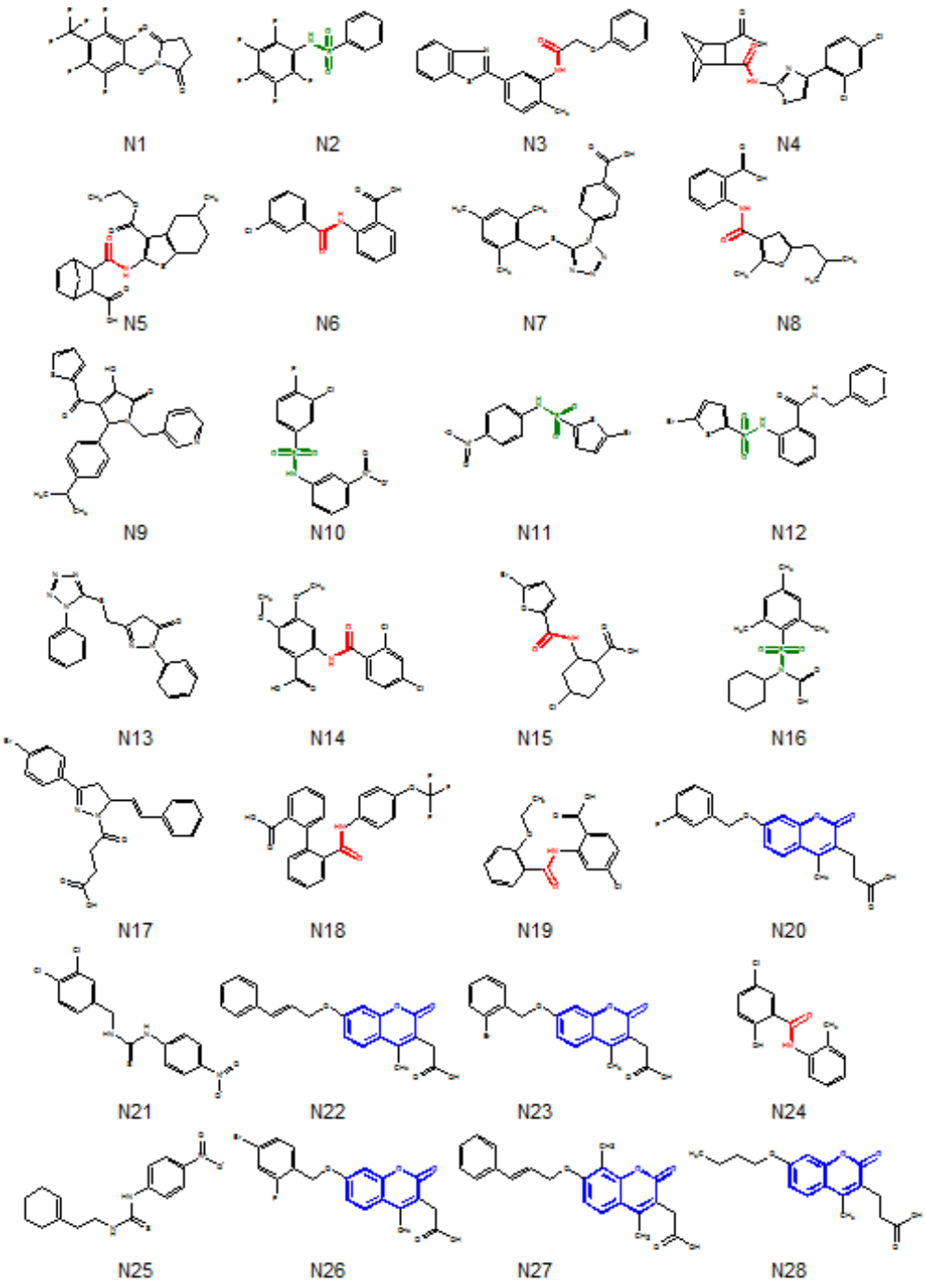


Figure 3. 4. Structures of 28 chemicals identified by the initial chemical screen.

Chemicals are divided into four groups by their core chemical structure (represented by the highlighted colour): Group 1 are the amides (red); Group 2 are coumarins (blue); Group 3 are sulphonamides (green); Group 4 are chemicals that have no common core structure.

Table 3. 1. Searchable chemical IDs and important information.

Information provided includes searchable Chemspider ID (CS ID, http://www.chemspider.com/Chemical-Structure), chemical ID used for this project, formula weight, chemical formula, chemical name and logP.

Group 1

CS ID	Chemical ID	Formular wt	Chemical formula	Chemical Name	logP
1144038	N3	374.5	$C_{22}H_{18}N_2O_2S$	N-[5-(1,3-benzothiazol-2-yl)-2-methylphenyl]-2-phenoxyacetamide	4.73
2161571	N4	411.3	$C_{18}H_{16}Cl_2N_2O_3S$	3-({[4-(2,4-dichlorophenyl)-1,3-thiazol-2-yl]amino}carbonyl)bicyclo[2.2.1]heptane-2-carboxylic acid	4.61
2168160	N5	403.5	$C_{21}H_{25}NO_5S$	3-({[3-(ethoxycarbonyl)-5-methyl-4,5,6,7-tetrahydro-1-benzothien-2-yl]amino}carbonyl)bicyclo[2.2.1]hept-5-ene-2-carboxylic acid	4.39
1707015	N6	275.7	$C_{14}H_{10}ClNO_3$	2-[(3-chlorobenzoyl)amino]benzoic acid	4.09
518782	N8	301.3	$C_{17}H_{19}NO_4$	2-[(5-isobutyl-2-methyl-3-furoyl)amino]benzoic acid	4.54
1080835	N14	370.2	$C_{16}H_{13}Cl_2NO_5$	2-[(2,4-dichlorobenzoyl)amino]-4,5-dimethoxybenzoic acid	3,593
846892	N15	344.5	$C_{12}H_7BrClNO_4$	2-[(5-bromo-2-furoyl)amino]-4-chlorobenzoic acid	4,214
4671966	N18	401.3	$C_{21}H_{14}F_3NO_4$	2'-({[4-(trifluoromethoxy)phenyl]amino}carbonyl)-2-biphenylcarboxylic acid	4,164
847644	N19	319.7	$C_{16}H_{14}ClNO_4$	4-chloro-2-[(2-ethoxybenzoyl)amino]benzoic acid	4,703

Group 2

CS ID	Chemical ID	Formular wt	Chemical formula	Chemical Name	logP
1384374	N20	356.3	$C_{20}H_{17}FO_5$	3-{7-[(3-fluorobenzyl)oxy]-4-methyl-2-oxo-2H-chromen-3-yl}propanoic acid	3.87
1409718	N22	350.4	$C_{21}H_{18}O_5$	{4-methyl-2-oxo-7-[(3-phenyl-2-propen-1-yl)oxy]-2H-chromen-3-yl}acetic acid	3.84
1409581	N23	403.2	$C_{19}H_{15}BrO_5$	{7-[(2-bromobenzyl)oxy]-4-methyl-2-oxo-2H-chromen-3-yl}acetic acid	4.1
840557	N24	261.7	$C_{14}H_{12}ClNO_2$	5-chloro-2-hydroxy-N-(2-methylphenyl)benzamide	4
1088900	N26	421.2	$C_{19}H_{14}BrFO_5$	{7-[(4-bromo-2-fluorobenzyl)oxy]-4-methyl-2-oxo-2H-chromen-3-yl}acetic acid	4.24
1385704	N27	364.4	$C_{22}H_{20}O_5$	{4,8-dimethyl-2-oxo-7-[(3-phenyl-2-propen-1-yl)oxy]-2H-chromen-3-yl}acetic acid	4.34
1410536	N28	304.3	$C_{17}H_{20}O_5$	3-(7-butoxy-4-methyl-2-oxo-2H-chromen-3-yl)propanoic acid	3.54

Group 3

CS ID	Chemical ID	Formular wt	Chemical formula	Chemical Name	logP
1685618	N2	323.2	C_{12} H_8 F_5 N O_2 S	N-(pentafluorophenyl)benzenesulfonamide	3.05
608006	N10	330.7	C_{12} H_8 Cl F N_2 O_4 S	3-chloro-4-fluoro-N-(3-nitrophenyl)benzenesulfonamide	3,742
2216296	N11	363.2	C_{10} H_7 Br N_2 O_4 S_2	5-bromo-N-(4-nitrophenyl)-2-thiophenesulfonamide	3,441
870065	N12	451.4	C_{18} H_{15} Br N_2 O_3 S_2	N-benzyl-2-{[(5-bromo-2-thienyl)sulfonyl]amino}benzamide	4.87
829391	N16	339.5	C_{17} H_{25} N O_4 S	N-cyclohexyl-N-(mesitylsulfonyl)glycine	4,775

Group 4

CS ID	Chemical ID	Formular wt	Chemical formula	Chemical Name	logP
2105951	N1	331.1	C_{11} H_4 F_7 N O_3	1-[2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenoxy]-2,5-pyrrolidinedione	1.65
892211	N7	354.4	C_{18} H_{18} N_4 O_2 S	4-{5-[(mesitylmethyl)thio]-1H-tetrazol-1-yl}benzoic acid	4.63
2186347	N9	418.5	C_{24} H_{22} N_2 O_3 S	3-hydroxy-5-(4-isopropylphenyl)-1-(3-pyridinylmethyl)-4-(2-thienylcarbonyl)-1,5-dihydro-2H-pyrrol-2-one	4.63
1080660	N13	350.4	C_{17} H_{14} N_6 O S	2-phenyl-5-{[(1-phenyl-1H-tetrazol-5-yl)thio]methyl}-2,4-dihydro-3H-pyrazol-3-one	2,519
4671966	N17	427.3	C_{21} H_{19} Br N_2 O_3	4-[3-(4-bromophenyl)-5-(2-phenylvinyl)-4,5-dihydro-1H-pyrazol-1-yl]-4-oxobutanoic acid	4.3
2228099	N21	356.2	C_{14} H_{11} Cl_2 N_3 O_2 S	N-(3,4-dichlorobenzyl)-N'-(4-nitrophenyl)thiourea	4.35
2229751	N25	305.4	C_{15} H_{19} N_3 O_2 S	N-[2-(1-cyclohexen-1-yl)ethyl]-N'-(4-nitrophenyl)thiourea	3.97

3.2.2. Confirmation of effect of the 28 chemicals identified by chemical screen on *GA20ox1::GUS* reporter line

To rule out the possibility of false positives from the initial chemical screen, fresh batches of the identified chemicals were purchased (Chembridge, San Francisco) and the *GA20ox1::GUS* screen was repeated. Identical growth conditions and chemical treatments were used with the exception that 5 µM GA_3 was added as opposed to 1 µM which was used in the initial screen. A higher concentration of GA_3 was used as it was found to be more effective in reducing GUS activity in the *GA20ox1::GUS* line.

All 28 chemicals induced increased staining in the root tips of nine *GA20ox1::GUS* seedlings when compared to the control, suggesting that they are blocking feedback regulation of *GA20ox1* by GA signalling (Figures 3.5.). Under control conditions (DMSO, Figure 3.5) the *GA20ox1::GUS* line exhibits strong GUS activity at the root tip with a reduction in staining towards the basal end of the root elongation zone. Clark (2005) showed from the staining of *GA20ox1::GUS* seedlings that in the root tip the activity of GA20ox1 is predominantly localised to the meristem whilst in the elongation zone it can be observed in the cortical cells. When GA_3 is applied a dramatic reduction in GUS activity is observed, such that the staining is barely detectable in the root (GA, Figure 3.5.) (Clark, 2005). Nine of the chemicals (N4, N5, N12, N14, N15, N19, N20, N21, N28) identified by the initial screen maintained the level of staining observed in *GA20ox1::GUS* control seedlings even in the presence of GA suggesting a complete block in GA signalling. The remaining 19 chemicals resulted in loss of GUS activity in the elongation zone but with some remaining in the root tip. The two remaining chemicals (N10 and N16) displaying low levels of GUS activity throughout the root. *GA20ox1::GUS* staining in the presence of GA was maintained by all chemicals when applied at a concentration of 50 µM, which may be well in excess of their active concentration in some cases.

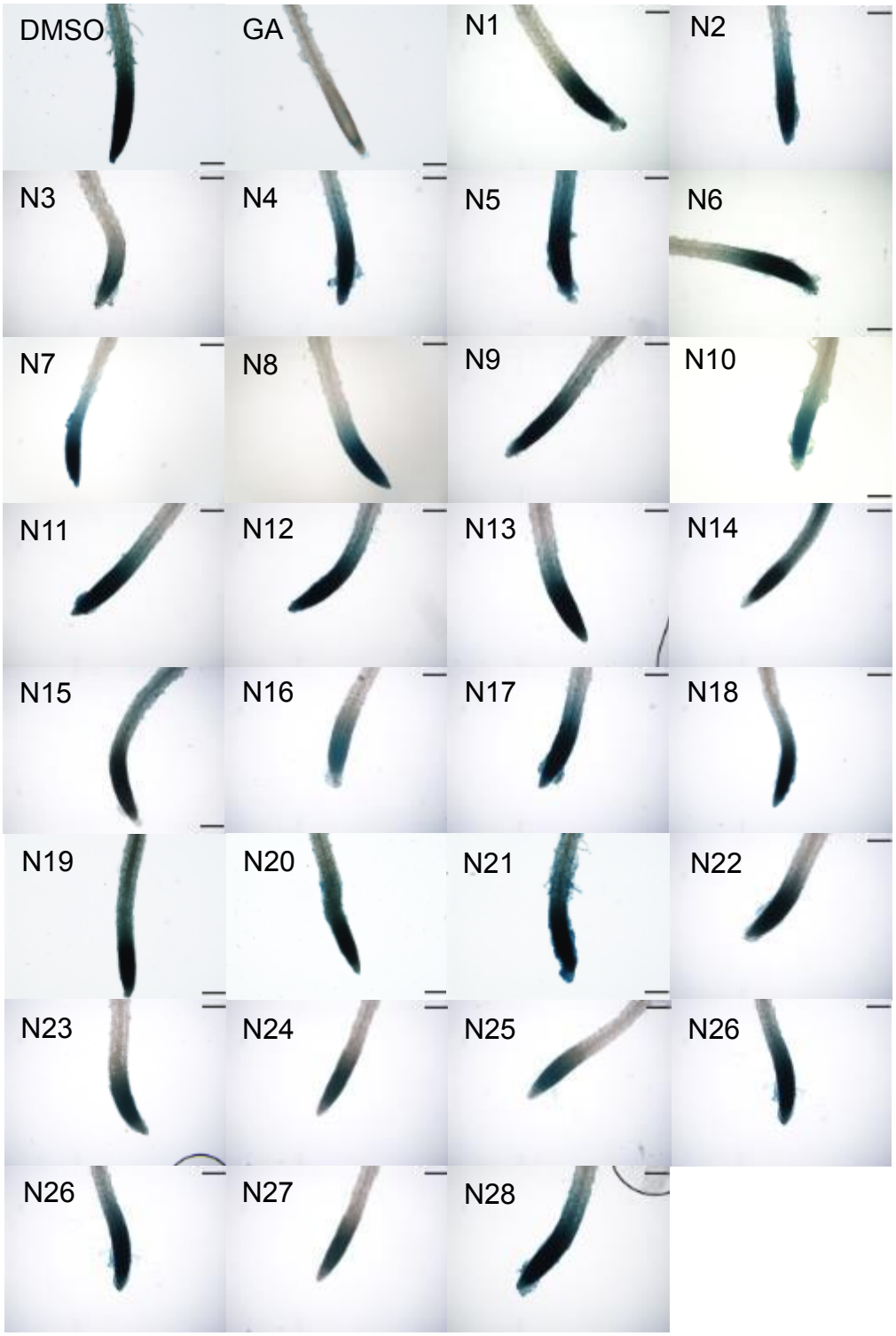


Figure 3. 5. Chemical inhibition of GA-mediated down-regulation of *GA20ox1::GUS* expression.

Effect of each chemical (N1 to N28) on *GA20ox1::GUS* staining was compared to the mock and GA-treated controls.1 µM PAC was applied at day 5, 50 µM of each chemical was applied at day 6 and 5 µM GA was applied 2 hours after the chemical application. Seedlings were analysed for GUS activity at 7 days old. Similar staining was observed in all nine seedlings analysed. Scale bar = 1 mm.

Published chemical screens for compounds which have biological activity demonstrate that the active concentration of the identified compounds can vary between approximately 50 µM to 200 nM (Armstrong et al., 2004; Rojas-Pierce et al., 2007). To determine the concentrations at which these compounds inhibit GA-mediated *GA20ox1::GUS* down-regulation, a dose response study was performed. The assay was performed using all chemicals at 50, 10, 5 and 1 µM (summarised in Figure 3.6.). None of the chemicals tested produced increased staining at 1 µM when compared to the GA_3 treated control. Chemicals N7, N9, N10, N11, and N20 maintained staining at 5 µM. N2, N3, N4, N5, N6, N8, N13, N16, N17, N18, N21, N22, N23, N24, N25, N26, N27 and N28 were active at 10 µM and N1, N14, N15, N19 were active only at 50 µM (Figure 3.6.) (summarised in Table 3.2.).

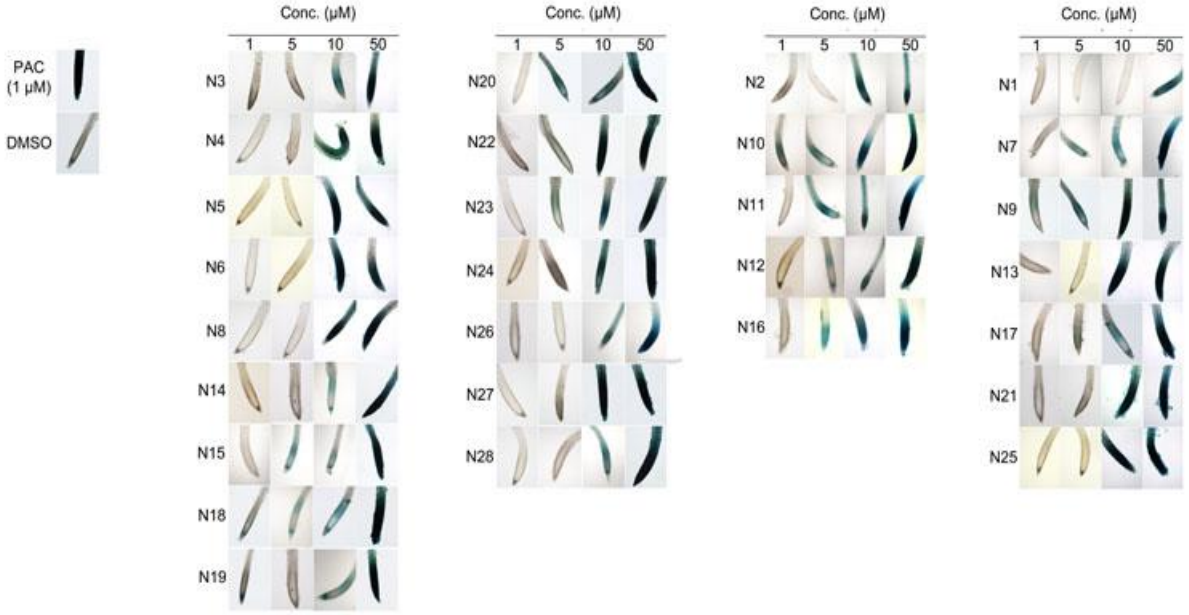


Figure 3. 6. Histochemical β-glucuronidase staining of *GA20ox1::GUS* seedlings grown in the presence of PAC, GA and identified chemicals.

Root tips of *GA20ox1::GUS* seedlings were grown in liquid MS media for 5 days before 1 µM PAC application. At day 6 chemicals were added to the specified concentration. Two hours after chemical application GA was added to a concentration of 5 µM. Seven-day old seedlings were stained for >8 hours. Figure shows no treatment (DMSO, negative control), just PAC treatment (positive control) and treatment with the 28 chemicals at 1, 5, 10 and 50 uM. n = 9.

Table 3. 2. Minimum concentration at which chemicals prevent GA mediated reduction of *GA20ox1::GUS* staining in the root tip, grouped by chemical class.

PAC (1 µM) was applied to 5-day old Col-0 seedlings grown in liquid MS, followed 24 hours later by the chemical at 1, 5, 10 or 50 µM. GA_3 (5 µM) was applied 2 hours after the chemical application and 24 hours prior to GUS staining. (a) Group 1; (b) Group 2; (c) Group 3; (d) Group 4

Group 1

Chemical	Active conc. (µM)
N3	10
N4	10
N5	10
N6	10
N8	10
N14	50
N15	50
N18	10
N19	50

Group 2

Chemical	Active conc. (µM)
N20	5
N22	10
N23	10
N24	10
N26	10
N27	10
N28	10

Group 3

Chemical	Active conc. (µM)
N2	10
N10	5
N11	5
N16	10

Group 4

Chemical	Active conc. (µM)
N1	50
N7	5
N9	5
N13	10
N17	10
N21	10
N25	10

The *GA20ox1::GUS* reporter line used in the initial screen and experiments described above contains a *GA20ox1* translational fusion to the GUS reporter gene (Plackett, 2011). This raises the possibility that the effects of the chemicals leading to increased GUS activity in the presence of GA may not be caused by effects on the transcription from the *GA20ox1* promoter. For example, they may also be the result of increased GA20ox1 protein stability. Therefore to establish whether the chemicals are acting at the level of transcription, qRT-PCR analysis was performed to analyse changes in the expression of the endogenous *GA20ox1* gene. Plant growth and chemical treatments were carried out as described previously for the *GA20ox1::GUS* assays, using the active concentrations established from the *GA20ox1::GUS* dose-response experiment (Table 3.2.). Due to problems associated with isolating RNA from roots, the assays were performed on whole Col-0 seedlings. A representative sample of nine chemicals (N2, N3, N6, N10, N11, N13, N14, N16, N23) as used. qRT-PCR analysis was performed using the *GA20ox1* forward and reverse primers described in Rieu et al.

(2008b) and primers for YLS8 (*At5g08290*), PP2A (*At1g13320)* and UBQT (*At4g33380*) as control genes (Czechowski et al., 2005). As the screen was for chemicals that prevented *GA20ox1* down-regulation, transcript levels were calculated relative to those from the PAC/GA control treatment (e.g. treatment divided by [PAC/GA])

Plants that were not treated with GA (DMSO mock treatment) exhibited around nine-fold higher *GA20ox1* expression than those treated with GA (PAC/GA, Figure 3.7.). Five of the chemicals (N3, N6, N13, N14, N16) increased expression of *GA20ox1*, whereas two chemicals (N2 and N23) had no effect compared to the PAC/GA control treatment. Treatment with N10 (95% of PAC/GA treatment) and N11 (68%). produced a slight decrease in expression when compared to the PAC/GA control. Only two chemicals (N13 and N16) produced higher expression than the DMSO control. Two (N6 and N14) of the three (N2) Group 1 chemicals tested induced a significant increase in *GA20ox1* expression compared to PAC/GA, whilst N2 produced a small, but non-significant increase (Figure 3.7.). Group 1 chemicals are therefore likely acting on signalling elements that induce a change in transcription. Four of the nine chemicals tested belonged to Group 3 (N2, N10, N11 and N16). Interestingly all Group 3 chemicals apart from N16 induced a similar response, with no significant change in *GA20ox1* expression (Figure 3.7.). N16 induced a large change in expression (Figure 3.7.). Only one chemical from Group 2 (N23) and Group 4 (N13) was tested. It is important to note that the *GA20ox1::GUS* used in the initial screen is a translational fusion whereas the qRT-PCR assay analyses transcription. It is therefore possible that those chemicals showing no increase in *GA20ox1* transcription could still be perturbing GA signalling by affecting the stability of the GA20ox1 proteins. There is some evidence that GA regulates the stability of the GA20ox1 enzymes, as GA application results in the loss of fluorescence in the Arabidopsis *ga20ox1,2,3:SCR::YFP::AtGA20ox1* line (Barker, 2011). As the SCR (scarecrow) promoter is not GA regulated it is possible that GA is affecting the enzyme stability in an as yet unidentified manner as part of the feedback mechanism. Additionally

ethylene has been shown to alter the stability of its biosynthetic enzyme, 1-aminocyclopropane-1-carboxylic acid synthase (Chae et al., 2003). It is therefore possible that the chemicals that do not alter *GA20ox1* expression but maintain *GA20ox1::GUS* staining in the presence of GA could present a useful tool for the study of the control of hormone biosynthetic enzymes by plant hormones. Alternatively the differences in phenotypes induced by the same chemicals in the *GA20ox1* qRT-PCR and GUS assays could be contributed to the differences in time scale for response to GA Application of exogenous GA to plants results in the GA signalling feedback loop reducing GA20ox expression within 60 minutes (Middleton et al., 2012). One of the main limitations of a GUS assays is the high stability of the GUS-fusion proteins meaning intensities of GUS staining can increase over time even if expression levels are relatively low (Mantis and Tague, 2000). In contrast qRT-PCR data shows the exact expression of the gene at the time of harvest. It is therefore possible that the 24 hour GA treatment in both the GUS and qRT-PCR assays was long enough to reduce *GA20ox1* expression in the presence of some chemicals but GUS staining remained high as a result of the increased stability of the GUS-fusion protein.

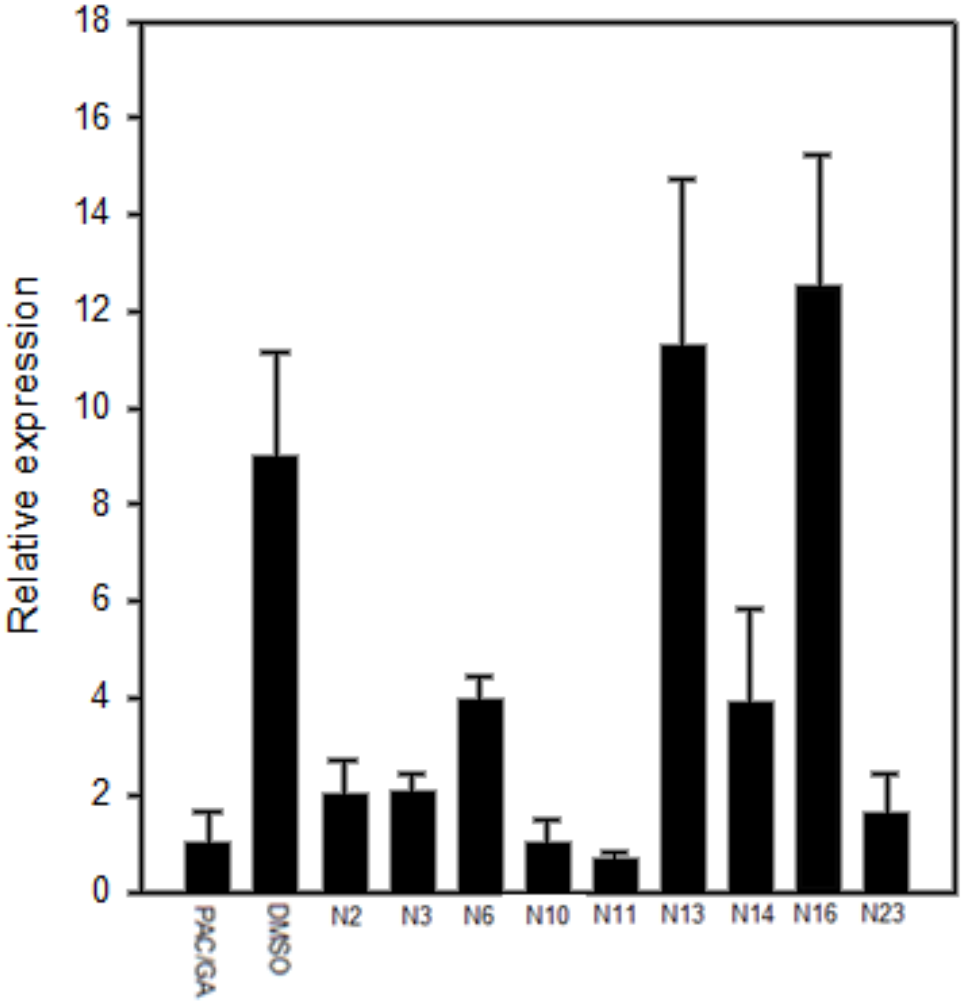


Figure 3. 7. Expression analysis of *GA20ox1* in Col-0 seedlings treated with nine chemicals.

Relative expression of *GA20ox1* in Arabidopsis seedlings treated with nine chemicals and a DMSO negative control relative to PAC/GA treatment. Six-day old Col-0 seedlings treated with each chemical at their previously identified effective concentrations (N2, 10 µM; N3, 50 µM; N6, 10 µM; N10, 10 µM; N11, 5 µM; N13, 10 µM; N14, 50 µM; N16, 50 µM; N23, 10 µM) and 1 µM PAC for 24 hours were treated with GA_3 (1 µM) for a further 24 hours before *GA20ox1* expression was analysed by qRT-PCR. *YSL*, *UBQT* and *PP2A* were used as control genes. Error bars represent standard errors, n = 4.

3.2.3. Effect of chemicals on Arabidopsis root elongation

Gibberellins have been demonstrated to have an important role in controlling root elongation in many plants including Arabidopsis (Tanimoto, 1987; Fu and Harberd, 2003; Ubeda-Tomas et al., 2008; Ubeda-Tomas et al., 2009). Based on the finding that compounds were identified that blocked GA-mediated down-regulation of *GA20ox1* expression and therefore potentially GA signalling, their effect on root elongation was investigated. The physiological effect of blocking feedback regulation of *GA20ox1* expression is not predictable as the chemicals could be regulating numerous aspects of the GA-signalling pathway. For example, it could indicate a block in the GA signalling cascade resulting in an accumulation of DELLAs which act to increase the expression of *GA20ox1*, but also inhibit root elongation. Alternatively chemicals could be up-regulating *GA20ox1* expression resulting in increased levels of bioactive GA and thus increased root elongation. Therefore, to test the effect of these compounds on root elongation, five-day old Col-0 seedlings were transferred to medium containing a compound at four concentrations (1, 5, 10 and 50 µM) and the increase in root elongation was measured after a 24 hour period. All 28 chemicals were tested. Five-day old seedlings were used as their root length was unlikely to exceed the size of the petri dish (10 cm) after a further 24 hours of growth even if the chemicals promoted root elongation. No GA control application was used for this experiment as it has previously been shown that GA induces a minimal increase in the root elongation of Arabidopsis roots (Griffiths, 2007).

When applied at a concentration of 1 µM none of the compounds inhibited root elongation. All chemicals except N3 and N16 produces a severe inhibition of root elongation at 50 µM and in some cases complete growth arrest was observed (Figure 3.8. and 3.11.). To establish whether the compounds were causing growth inhibition due to toxicity, cell viability was assessed using propidium iodide staining. In dead or dying cells propidium iodide would stain the cytoplasm which is indicative of cell death, as propidium iodide is membrane impermeable and thus cannot enter viable

cells. Using similar growth conditions, five-day old Col-0 seedlings grown in liquid MS media were treated with the compounds for 24 hours followed by staining with propidium iodide and visualisation using a confocal microscopy. One chemical that blocked root elongation at a concentration of 50 μM was selected from each of four groups (N6, N10, N23 N25) for this experiment. The effect of these four compounds on cell viability at the RAM was assessed. In all cases treatment with the chemicals resulted in propidium iodide staining of the cytoplasm (Figure 3.12). In contrast, treatment with N16, which did not inhibit root elongation at 50 μM, did not cause propidium staining of the cytoplasm (Figure 3.12.). This suggests that inhibition of root elongation observed when using higher concentrations of the chemicals is due to toxicity. Interestingly the initial screen was performed by treatment with the chemicals at a concentration of 50 μM which led to increased GUS activity in the *GA20ox1::GUS* line when compared to the GA treated control. This is somewhat surprising given their apparent toxicity. However, it has been demonstrated that *GA20ox1* expression is increased in Col-0 plants exposed to cold stress (Achard et al., 2008b), suggesting that this induction in expression level could be caused by toxicity-induced stress.

Four chemicals (N3 [Figure 3.8.], N9 [Figure 3.11.], N16 [Figure 3.10.], N27 [Figure 3.9.]) did not reduce root elongation when compared to control even at 50 μM (Figures 3.8. to 3.11.). The remaining 15 chemicals all showed a dose dependent inhibition of root elongation over 24 hours. Of the 15, five (N2, N11, N13, N22, N26) inhibited root elongation at the relatively low concentration of 5 μM and eight (N1, N7, N10, N12, N17, N21, N23, N25) reduced elongation at 10 μM (Figures 3.8. to 3.11.). Two chemicals (N4, N24) decreased root elongation severely at 50 μM, but also produced a small, but not significant decrease in root elongation compared to the control when applied at 1 μM to 10 μM (Figure 3.8., Figure 3.9.). Interestingly one chemical (N23) induced agravitropism in this assay (discussed further in Chapter 4) which is a processes more readily associated with auxin signalling.

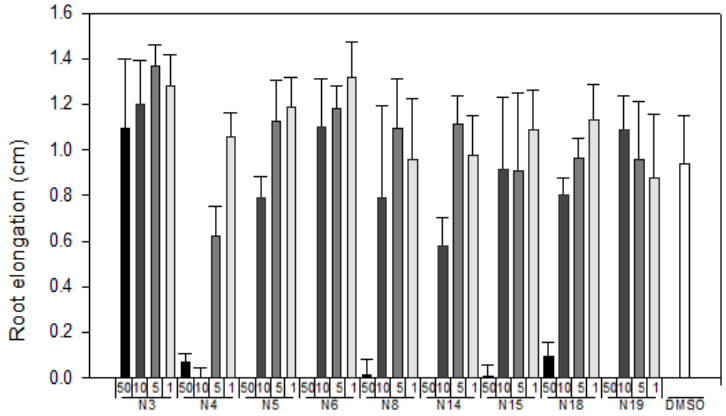


Figure 3. 8. Effect of Group 1 chemicals on root elongation at 1, 5, 10 and 50 µM.

Eight to ten five-day old seedlings were transferred to MS-medium containing the chemical (N3, N4, N5, N6, N8, N14, N15, N18, N19) at varying concentrations (1 (black bars), 5 (dark grey bars), 10 (medium grey bars) and 50 (light grey bars) µM). Root elongation was measured over 24 hours using ImageJ software. Error bars represent S.E.

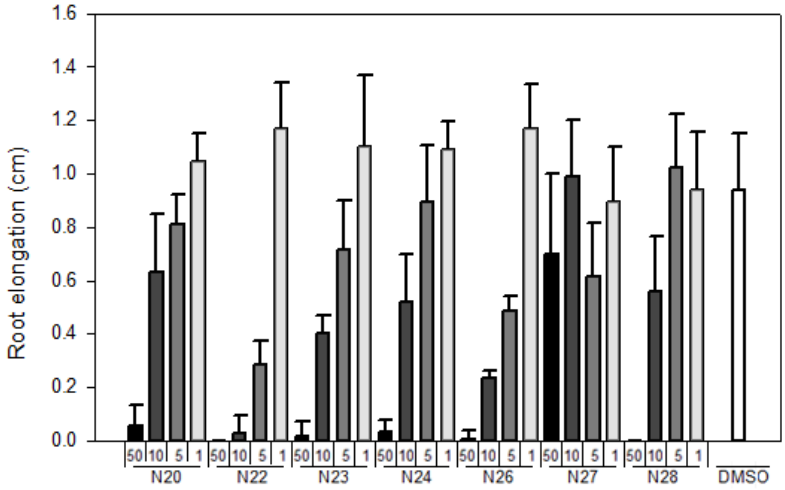


Figure 3. 9. Effect of Group 2 chemicals on root elongation at 1, 5, 10 and 50 µM.

Eight to ten five-day old seedlings were transferred to MS-medium containing the chemical (N20, N22, N23, N24, N26, N27, N28) at varying concentrations (1 (black bars), 5 (dark grey bars), 10 (medium grey bars) and 50 (light grey bars) µM).. Root elongation was measured over 24 hours using ImageJ software. Error bars represent S.E.

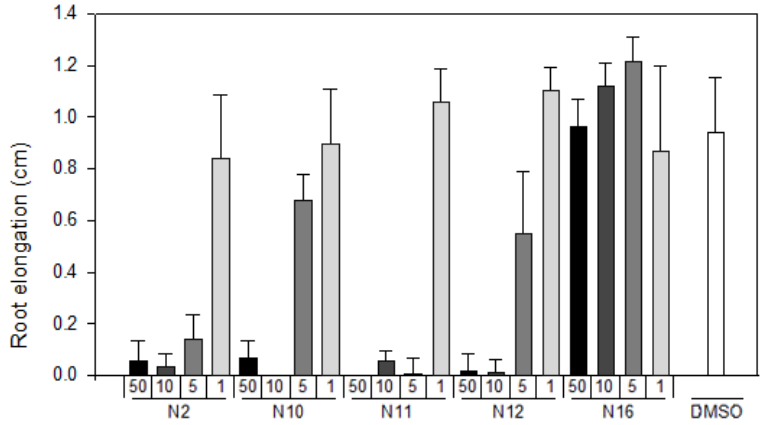


Figure 3. 10. Effect of Group 3 chemicals on root elongation. Eight to ten five-day old seedlings were transferred to MS-medium containing the chemical (N2, N10, N11, N12 and N16) at varying concentrations (1 (black bars), 5 (dark grey bars), 10 (medium grey bars) and 50 (light grey bars) µM). . Root elongation was measured over 24 hours using ImageJ software. Error bars represent S.E.

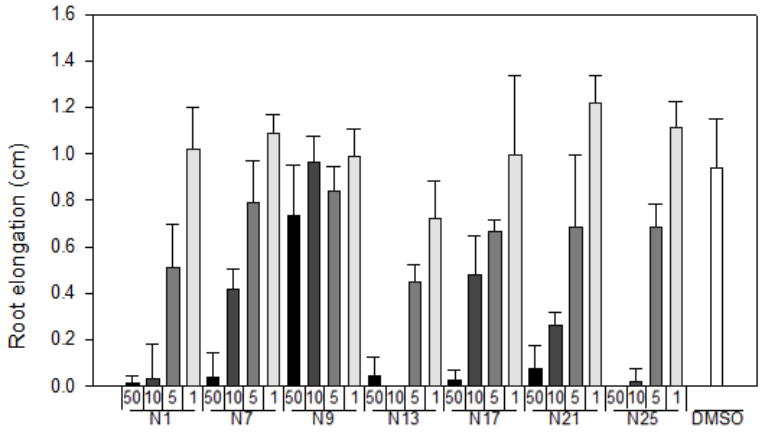


Figure 3. 11. Effect of other chemicals on root elongation. Eight to ten five-day old seedlings were transferred to MS-medium containing the chemical (N1, N7, N9, N13, N17, N21 and N25) at varying concentrations (1 (black bars), 5 (dark grey bars), 10 (medium grey bars) and 50 (light grey bars) µM). . Root elongation was measured over 24 hours using ImageJ software. Error bars represent S.E.

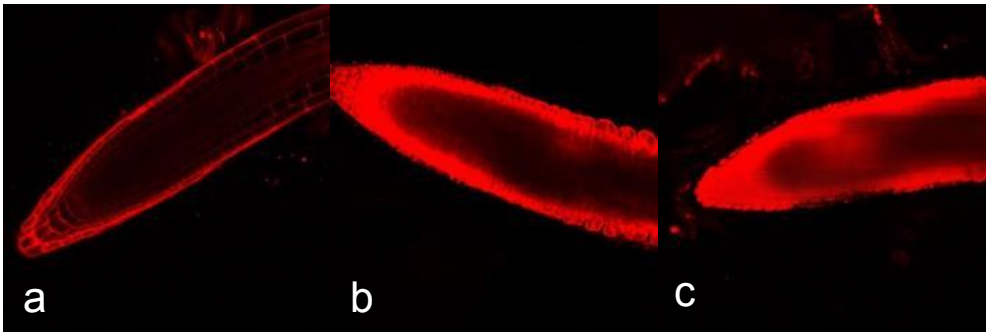


Figure 3. 12. Viability of Arabidopsis roots in the presence ofN6 and N25.

N6 (b) and N25 (c) treatment resulted in loss of cell viability in the root tip. DMSO treatment shows regular propidium iodide training. Four-day old Col-0 seedlings were transferred to dH_2O containing (a) 0.33 % v/v DMSO (mock), (b) N6 (50 µM) or (c) N25 (50 µM) for 24 hours before staining with propidium iodide and imaging with a confocal microscope. n = 3

3.2.3.1. Statistical modelling of root elongation data to establish the active concentration of chemicals

From the raw root elongation data it was possible to provide a basic model for the effective dose on root phenotype (50% reduction in root elongation). Here we use either a logistic dose response curve or an exponential decay curve to model the root elongation (cm) data and predict the effective dose value for chemicals actually inducing a response with increasing concentration (Table 3.3.). The trends observed in the root elongation data over all the chemicals could be summarised in two possible ways. Some chemicals provided a sigmoidal trend, with slow decrease in root elongation with increasing concentration initially and at high concentration, and with a more linear decrease in the middle concentration range. The others gave a more rapid, instantaneous decreasing trend with increasing concentration. Two models, the logistic for the sigmoidal trend and the exponential for the rapid decrease, were therefore chosen as appropriate empirical representations to describe the trends seen in the data in a biologically sensible manner. Moreover, these models contain parameters that can be used to draw conclusions about the process being modelled,

reducing a large amount of data to a small set of parameters without loss of information (Bates and Watts, 1988).

The logistic model is:

$y = A/(1 + \exp(B(\log(\text{Concentration})-M))$

where *y* is root elongation (increase in root length over a 24 hour period), *A* is the control response, *B* is the measure of increasing response caused by the increasing concentration (*i.e.* the 'slope' of the curve), *M* is the log(Concentration) which reduces *y* by 50%, *i.e.* the log (LC50), which we back-transform to the raw scale.

The exponential decay model is:

$y = A\exp(-B(\text{Concentration}))$

where *y* is root elongation, *A* is the control response and *B* is the exponential rate of decrease. Parameter *B* is used to calculate the effective concentration (EC50) as $\log(2)/B$.

These models were fitted using the method of nonlinear least squares, as implemented in the GenStat (2011, 14th edition, (c) VSN International Ltd, Hemel Hempstead, UK) FITNONLINEAR routine, which also allows output of the functions of the parameters from the exponential decay model, *i.e.* estimated LC50 values with standard errors.

The majority of the chemicals modelled (N1, N2, N4, N5, N7, N11, N12, N13, N17, N20, N21, N22, N23, N24, N25, N26) had an LC50 or EC50 of under 10 µM. Six chemicals (N5, N6, N8, N14, N15, N28) have EC50s or LC50s of between 10 and 20 µM. Only N18 and N19 had an LC50 of over 20 µM. The models suggest that four

chemicals (N3, N9, N16 and N27) had no effect on root elongation. N11 has a particularly low EC50s of 0.55 µM. Such a low EC50 demonstrates the limitation of the statistical model which should not yield a EC50 below the lowest concentration tested (1 µM). The major drawback of the model is the limited number of data points (4 different concentrations; 1, 5, 10 and 50 µM) which prevents the statistical software from accurately differentiating between a poor straight exponential plot and a noisy sigmoidal plot. Whilst only four concentrations were analysed to allow comparisons to be drawn with other experiments in this chapter a larger number of data points (e.g. 10 concentrations between 1 and 50 µM) would have provided better plots to define a model. With a higher number of data points it is more likely that more sigmoidal models would have been apparent for the chemicals tested.

When compared to the active concentration determined from the *GA20ox1::GUS* assay the modelled results show high similarity (when standard errors were taken into account) (Table 3.3.). Chemicals that produced no effect on root elongation (N3, N9, N16, N27) had the maximum active concentration of 50 µM in the *GA20ox1::GUS* assay. Chemicals with an EC50 or LC50 higher than 10 µM had an active concentration of 50 µM as no concentrations between 10 and 50 µM were tested. N4 was an exception as its active concentration in the *GA20ox1::GUS* assay (10 µM) was lower than its LC50 for inhibition of root elongation (17.7 µM). Chemicals with an LC50 of 5 to 10 µM and 0 to 5 µM had active concentrations of 10 µM and 5 µM, respectively. N20 was the only chemical with an LC50 (6.56 µM) that greatly differed from its active concentrations (50 µM).

Table 3. 3. Prediction of the EC50 or LC50 values for inhibition of root elongation by the 28 chemicals and active concentrations identified from the *GA20ox1::GUS* assay.

Either an exponential or logistic model was used to determine the dose at which each chemical reduced root elongation to 50% of control. Figure in parenthesis represent SE. LC50 relates to logistic model. EC50 relates to exponential model.

Chemical	**Model**	**Parameters**			**LC50 or EC50 µM**	**s^2**	**R^2 (%)**
		A	***B***	***M***			
1	Logistic	1.021 (0.107)	5.04 (5.26)	1.609 (0.093)	5 (0.465)	0.0228	89.2
2	Exponential	1.304 (0.245)	0.44 (0.14)	-	1.58 (0.52)	0.0143	89.7
3*	-	-	-	-	-	-	-
4	Logistic	1.055 (0.368)	13.50 (80.90)	1.637 (0.298)	5.14 (1.58)	0.1781	47.3
5	Logistic	1.172 (0.080)	4.13 (4.84)	2.480 (0.202)	11.94 (2.41)	0.0134	95.4
6	Logistic	1.259 (0.102)	4.00 (5.88)	2.776 (0.691)	16.10 (11.10)	0.035	89.8
7	Logistic	1.106 (0.208)	1.99 (1.52)	2.058 (0.336)	7.83 (2.64)	0.0605	72.5
8	Exponential	1.151 (0.148)	0.04 (0.02)	-	16.49 (7.26)	0.0502	77.7
9*	-	-	-	-	-	-	-
10	Exponential	1.131 (0.177)	0.17 (0.05)	-	4.03 (1.21)	0.0299	82.5
11	Exponential	3.730 (8.030)	1.26 (2.12)	-	0.55 (0.98)	0.0152	93.9
12	Exponential	1.122 (0.278)	0.15 (0.07)	-	4.62 (2.29)	0.0811	65.5
13	Exponential	1.058 (0.291)	0.20 (0.10)	-	3.48 (1.79)	0.0725	62.5
14	Logistic	1.019 (0.119)	4.20 (7.51)	2.870 (1.040)	17.70 (18.50)	0.0509	79.1

15	Exponential	1.214 (0.156)	0.04 (0.02)	-	17.80 (7.92)	0.0594	77.8
16*	-	-	-	-	-	-	-
17	Logistic	1.17 (0.672)	1.26 (0.16)	1.989 (0.084)	7.31 (0.61)	0.0045	97.5
18	Logistic	1.095 (0.195)	4.68 (5.48)	3.195 (0.880)	24.40 (21.50)	0.0434	84
19	Logistic	1.054 (0.053)	3.80 (1.80)	3.191 (0.361)	24.32 (8.78)	0.0099	95.3
20	Exponential	1.224 (0.105)	0.11 (0.02)	-	6.56 (1.29)	0.0138	92.2
21	Exponential	1.602 (0.162)	0.30 (0.06)	-	2.30 (0.42)	0.014	94.6
22	Exponential	1.670 (0.120)	0.36 (0.05)	-	1.94 (0.25)	0.0057	97.9
23	Exponential	1.233 (0.201)	0.11 (0.04)	-	6.27 (2.30)	0.0495	78.1
24	Logistic	1.099 (0.211)	2.23 (2.02)	2.256 (0.344)	9.55 (3.29)	0.0757	68.4
25	Logistic	1.113 (0.042)	6.32 (2.84)	1.685 (0.043)	5.392 (0.233)	0.0036	98.6
26	Exponential	1.420 (0.207)	0.20 (0.05)	-	3.45 (0.94)	0.0364	85.4
27*	Exponential	-	-	-	-	-	-
28	Exponential	1.103 (0.129)	0.06 (0.02)	-	12.37 (4.60)	0.0297	84.9

* No discernible dose-response relationship.

There are obvious correlations between the EC50 or LC50 of chemicals with similar core structures (Table 3.3.). With the exception of N28 in Group 2 all the chemicals that induced an effect on root elongation with an EC50 or LC50 of greater than 10 µM belong to Group 1 (Table 3.3.). As mentioned previously, N28 (12.37 µM) of Group 2 had a EC50 of over 10 µM, while all other members of Group 2 have a EC50 or LC50 of between 1.94 and 6.56 µM, apart from N27 which had no effect on root elongation (Table 3.3.). The lack of effect of N27 when compared to an EC50 of 1.94 µM for N22 demonstrates how small changes in chemical structure can have large effects on biological effect, as the only difference in structure of the two chemicals is the

presence of a methyl group in the C9 position of the coumarin in N27 (Table 3.3.). With the exception of N16, all Group 3 chemicals had EC50 or LC50 values of less than 5 μM. Interestingly N16 is the only member of Group 3 that does not have a H atom at one of the R' positions of the sulphonamide, instead possessing a carboxylic acid group. Additionally N16 is the only chemicals identified by the initial *GA20ox1::GUS* screen that induces an agravitropic response in roots.

Taken together the results for all four groups of chemicals shows that the core chemical structure has a major influence on the biological activity of the molecule, likely as a result of the tertiary structure of the molecule deciding the potential binding pocket of the target protein. The outlying chemical groups are more likely to determine the binding of the molecule to the amino acid residues within the target protein and would therefore cause the variation in efficacy observed here. Additionally the data also indicate at the concentration at the chemicals may be affecting GA signalling to an extent that they will inhibit a GA regulated developmental process.

3.2.4. Eleven chemicals prevent GA-mediated degradation of a *GFP-RGA* reporter

DELLA proteins represent a central step of the GA signalling pathway. Whereas they act downstream of GA biosynthesis and perception (Silverstone et al., 2001; Griffiths et al., 2006a), they are upstream of transcription factors that control GA-responsive growth and development (de Lucas et al., 2008; Feng et al., 2008). Based on the fact that DELLA proteins are degraded in response to GAs, it is therefore possible to broadly narrow down where the chemicals are acting within the GA signalling cascade. For example, those which are acting to interfere with DELLA-transcription factor interactions that control *GA20ox1* expression would not be expected to perturb DELLA protein stability. In contrast, those compounds which affect GA perception by GID1 or DELLA binding to GID1/SLY1 would be expected to block GA-mediated DELLA protein degradation. Additionally chemicals could also be increasing

endogenous levels of the DELLA proteins under normal GA conditions, potentially by increasing transcription of the DELLA genes. To establish the effect of the 28 chemicals on DELLA protein levels and GA-mediated DELLA protein degradation a *RGAp*::*GFP-RGA* transgenic reporter line was utilised (Silverstone et al., 2001). Fluorescent proteins, such as GFP, provide a qualitative means by which to visualise genetically encoded proteins in living cells in real time (Dundr et al., 2002). The *RGAp*::*GFP-RGA* line provides a convenient reporter for monitoring GA-induced degradation of DELLA proteins *in planta*. It has been shown to be functional in the GA signalling pathway through the demonstration that the *GFP-RGA* transgene is capable of complementing the *rga/ga1-3* mutant (Silverstone et al., 2001). The GFP-RGA protein has been demonstrated to be nuclear localised and is degraded in the presence of GA within in two hours of treatment, visualised by a loss of fluorescence.

There are three potential scenarios for the effect of the chemicals on the *RGAp*::*GFP-RGA* reporter line: firstly chemicals that act upstream of the DELLA proteins in GA signalling (e.g. binding of GA to GID1) would prevent DELLA protein degradation and result in constitutive GFP-RGA fluorescence in the presence of GA; alternatively chemicals acting downstream of DELLA proteins would not be expected to affect GA-mediated DELLA protein degradation which would result in the loss of GFP-RGA fluorescence in the presence of GA. Additionally chemicals could be increasing endogenous levels of DELLA proteins in the absence of GA. To establish the effect of the chemicals on GFP-RGA fluorescence in the presence or absence of GA, five-day old *RGAp*::*GFP-RGA* seedlings were transferred to semi-solid medium containing the chemical for 6 hours, after which the seedlings were treated with either dH_2O (as a mock treatment) or GA_3. Two hours later GFP fluorescence was analysed in the root tips using a confocal microscope (Figures 3.13. to 3.16.). Chemicals were applied to the media at the lowest active concentration identified from the *GA20ox1::GUS* assay (Chapter 3.2.2.).

In contrast to the root elongation assays, there was no consistency in the disappearance of GFP-RGA in response to GA within most of the chemical classes, with the exception of Group 3. In this case, all compounds, with the exception of N11 which was toxic to roots at 5 µM, blocked RGA degradation in the presence of GA (Figure 3.15., Table 3.4.). Within Group 1, four chemicals (N3, N5, N6, N19) blocked GFP-RGA degradation, whereas five (N4, N8, N14, N15, N18) had no effect on degradation (Figure 3.13., Table 3.4.). Toxicity was observed in the roots treated with several of the Group 1 and Group 2 chemicals (N20, N24, N26, N28), based on propidium iodide staining observed within the cytoplasm. In these cases, the toxicity prevented visualisation of nuclear expressed GFP-RGA and characterisation of its response to GA. The three remaining Group 2 chemicals (N22, N23, N27) had no effect on degradation (Figure 3.14., Table 3.4.). Four chemicals belonging to Group 4 (N9, N13, N17, N25) had no effect on GFP-RGA degradation whereas two from this group (N7, N21) partially blocked degradation (Figure 3.16., Table 3.4.). N1 was found to be toxic to the roots. Some chemicals appeared to increase (N5, N7, N23, N27) or decrease (N4, N13, N25) the basal level of GFP-RGA fluorescence. Whilst this may be due to the chemicals affecting of GFP-RGA abundance, it is also conceivably due to an artefact of the confocal microscopy and cannot be quantified as GFP fluorescence provides qualitative information (Dundr et al., 2002).

In total 11 of the chemicals had no effect on GA-mediated GFP-RGA degradation. It is conceivable that these compounds block the action of transcription factors acting downstream of DELLA proteins that are involved in the homeostatic regulation of *GA20ox1*. Several transcription factors which act downstream of the DELLA proteins have been identified and therefore it is possible that these chemicals could be affecting any number of putative DELLA protein interactions. For example, the interaction of DELLA proteins with SCL3 may also present a potential target for these chemicals as this transcription factor is involved in GA homeostasis (Zhang et al., 2011).

Based on the DELLA protein degradation studies, the other class of compounds (11 chemicals) are those that block GA-mediated degradation of GFP-RGA. The process of DELLA protein degradation in response to GA signalling is now well understood: when bioactive GAs are produced in the plant they bind to GID1 causing a conformational change which allows binding of DELLA protein to form a DELLA-GID1-GA complex, which, in Arabidopsis, is recognised by the F-Box subunit SLY1, of an SCF^{SLY1} E3 ubiquitin ligase allowing ubiquitination and subsequent degradation by the 26S proteasome (Silverstone et al., 2001; Griffiths et al., 2006b; Harberd et al., 2009). These chemicals could be blocking any of these interactions. However GID1 receptors that either cannot perceive GA or bind to the DELLA protein also lead to the stabilisation of DELLA proteins (Hirano et al., 2010). Additionally plants with mutations in the *SLY1* gene (*sly1*) that prevent the SLY1 F-box protein binding to the DELLA proteins and mutations in the *DELLA* gene that prevent the protein forming a complex with GID1 result in a similar DELLA protein accumulation (Dill et al., 2001; Silverstone et al., 2001; Fleck and Harberd, 2002; Itoh et al., 2002; Dill et al., 2004). It is therefore possible that the chemicals that prevent DELLA protein degradation in the presence of GA could be perturbing any of the GA-mediated DELLA interactions. The DELLA protein degradation assay described is focussed on RGA, which is only one of the five Arabidopsis DELLA paralogues and. it is therefore possible that other chemicals could affect the stability on one or more of the other four DELLA proteins.

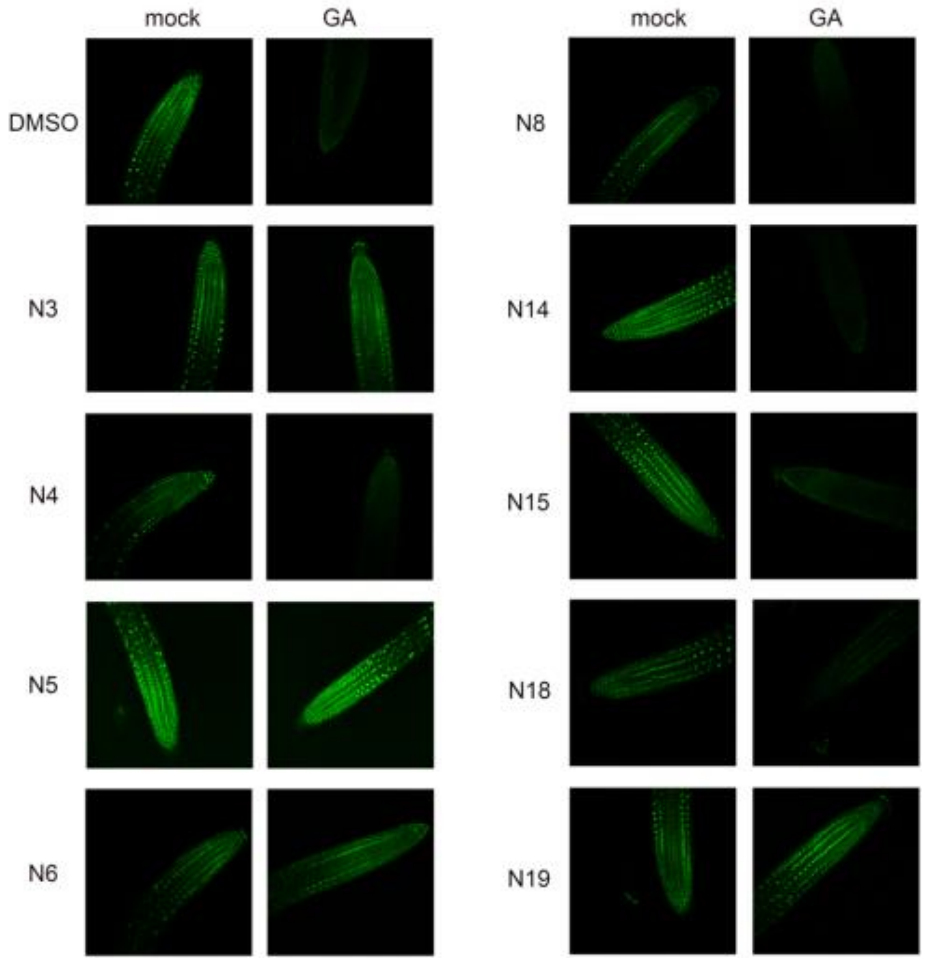


Figure 3. 13. Effect of Group 1 chemicals on the fluorescence in the roots of transgenic plants expressing the GFP-RGA in the presence and absence of GA_3.

Five-day old *GFP-RGA* seedlings were transferred to MS-medium containing the chemical (N3, N4, N5, N6, N8, N14, N15, N18, N19) at its active concentration (shown in Figure 3.2). After 6 hours seedlings were treated with dH_2O (mock) or 100 µM GA_3 (GA) for 2 hours and and then fluorescence in root tips was visualized by confocal laser microscopy under an identical setting for all images. n=4.

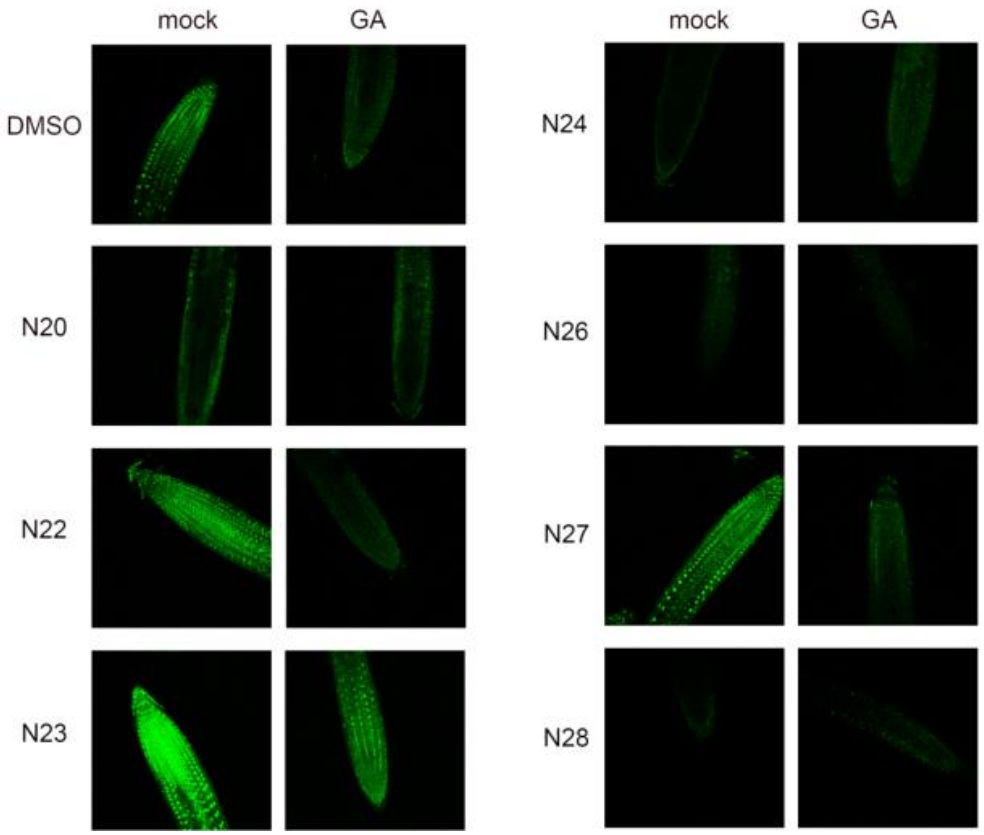


Figure 3. 14. Effect of Group 2 chemicals on the fluorescence in the roots of transgenic plants expressing the GFP-RGA in the presence and absence of GA_3.

Five-day old *GFP-RGA* seedlings were transferred to MS-medium containing the chemical (N20, N22, N23, N24, N26, N27, N28) at its active concentration (shown in Figure 3.2). After 6 hours seedlings were treated with dH_2O (mock) or 100 µM GA_3 (GA) for 2 hours and and then fluorescence in root tips was visualized by confocal laser microscopy under an identical setting for all images. n = 4.

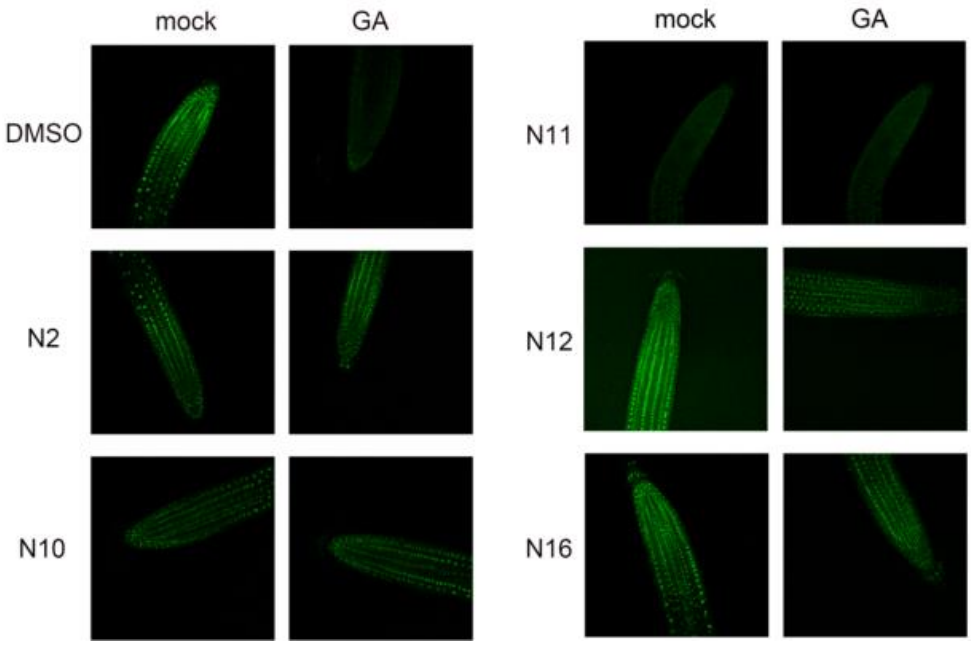


Figure 3. 15. Effect of Group 3 chemicals on the fluorescence in the roots of transgenic plants expressing the GFP-RGA in the presence and absence of GA_3.

Five-day old *GFP-RGA* seedlings were transferred to MS-medium containing the chemical (N2, N10, N11, N12 and N16) at its active concentration (shown in Figure 3.2). After 6 hours seedlings were treated with dH_2O (mock) or 100 µM GA_3 (GA) for 2 hours and and then fluorescence in root tips was visualized by confocal laser microscopy under an identical setting for all images. n = 4.

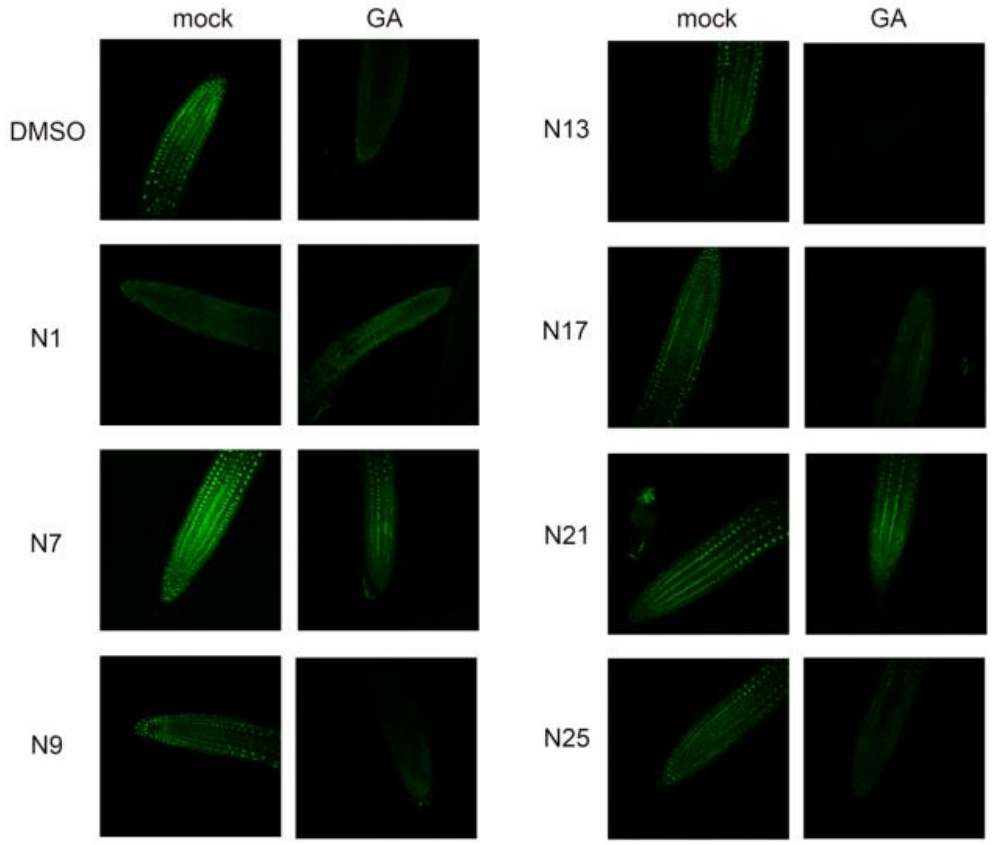


Figure 3. 16. Effect of Group 1 chemicals on the fluorescence in the roots of transgenic plants expressing the GFP-RGA in the presence and absence of GA_3.

Five-day old *GFP-RGA* seedlings were transferred to MS-medium containing the chemical (N1, N7, N9, N13, N17, N21 and N25) at its active concentration (shown in Figure 3.2). After 6 hours seedlings were treated with dH_2O (mock) or 100 µM GA_3 (GA) for 2 hours and and then fluorescence in root tips was visualized by confocal laser microscopy under an identical setting for all images. n = 4.

Table 3. 4. Summary of the effect of the chemicals on GFP fluorescence in the *RGAp::GFP-RGA* reporter line.

Summary of effect of chemical on RGA degradation in the presence or absence of GA. Chemicals were divided into their defined groups. Results show if the chemical prevents GA-mediated RGA degradation (Stabilised), has no effect on RGA degradation (Degraded), or is toxic to the root tip (Toxic). Tables also show if chemical increases (Increased), decreases (Decreased) or has no effect (None) on the basal level of RGA abundance. GFP-RGA was used to represent RGA degradation. Five-day old seedlings were treated with the chemical for 6 hours followed by GA treatment for 2 hours.

Group 1

Chemical	GA mediated RGA-degradation	Basal level of RGA compared to control
N3	Stabilised	None
N4	Degraded	Decreased
N5	Stabilised	Increased
N6	Stabilised	None
N8	Degraded	None
N14	Degraded	None
N15	Degraded	Decreased
N18	Degraded	None
N19	Stabilised	None
N24	Toxic	-

Group 2

Chemical	GA mediated RGA-degradation	Basal level of RGA compared to control
N20	Toxic	-
N22	Degraded	None
N23	Stabilised	Increased
N26	Toxic	-
N27	Degraded	Increased
N28	Toxic	-

Group 3

Chemical	GA mediated RGA-degradation	Basal level of RGA compared to control
N2	Stabilised	None
N10	Stabilised	None
N11	Stabilised	None
N12	Stabilised	None
N16	Stabilised	None

Group 4

Chemical	GA mediated RGA-degradation	Basal level of RGA compared to control
N1	Toxic	-
N7	Stabilised	Increased
N9	Degraded	None
N13	Degraded	Decreased
N17	Degraded	None
N21	Stabilised	None
N25	Degraded	None

Transcript analysis by qRT-PCR would identify if chemicals were increasing or decreasing transcription of the *DELLA* genes which could potentially explain the differences in protein level. Western blots could be used to identify changes in protein levels and confirm the effect of the chemicals on the stability of the endogenous RGA protein. Therefore, western blots were performed on root extracts using polyclonal α-RGA antibodies. Col-0 seeds were grown in liquid culture for four weeks to produce root cultures, which were then treated with either DMSO (mock), N6, N16 or N25 at 50 µM for 24 hours before application of 10 µM GA_4. N6 was shown to prevent GA-mediated GFP-RGA degradation (Figure 3.13.), N25 had no effect on its degradation (Figure 3.17.) while N16 treatment resulted in only a minor decrease in GFP-RGA levels following GA treatment (Figure 3.15). In order to assess the effects of these chemicals on native RGA levels, protein was extracted from root cultures using the method outlined in Bolwer et al. (2004) and a western blot was performed. Figure 3.17. shows that in the DMSO (mock) control native RGA accumulated to high levels, but following GA application the levels were undetectable. This demonstrates that GA-mediated RGA degradation occurs in root cultures as has been demonstrated in many other Arabidopsis tissues (Dill et al., 2001; Dill et al., 2004). The presence of N6 was found to block GA-mediated degradation of RGA whereas N25 had no effect (Figure 3.17.). These observations support the findings of the GFP-RGA confocal assays using the same compounds. Somewhat surprisingly, in the presence of N16 a slight increase in the levels of RGA was observed following GA treatment, which is in contrast to the GFP-RGA assays. However, these results largely confirm the effects of these compounds on DELLA protein degradation established from the GFP-RGA assays. Interestingly, the basal level of RGA in the control root cultures was much higher than in those treated with N6, N16 or N25 (Figure 3.17.). This reduction was not observed when monitoring GFP-RGA accumulation in the presence of N16 or N25. However, N6 did appear to reduce the basal levels of GFP-RGA (Figure 3.13.). The reasons for these differences are not currently clear.

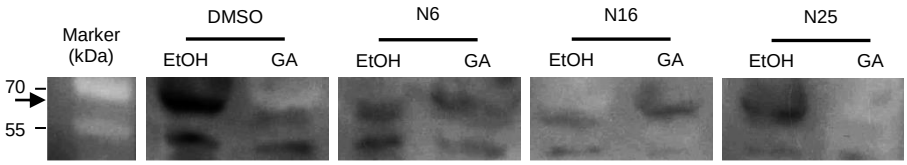


Figure 3. 17. The effect of three chemicals on GA mediated RGA degradation in a western blot using anti-RGA antibodies.
Preliminary data (n=2) showing levels of RGA in plants treated with chemical and GA or ethanol (EtOH, mock GA treatment,).The blot contained 25 ug of total protein extracted from four-week old root cultures in liquid Gamborg B5 treated with either DMSO (-ve control), N6, N16 or N25 at 50 µM for 24 hours before application of ethanol (EtOH, 0.0033 % v/v) or 10 µM GA_4 10 µM. A rabbit anti-RGA antiserum and a goat anti-rabbit IgG were used as primary and secondary antibodies, respectively. Protein marker shows bands at 70 and 55 kDa and arrow displays expected position of RGA protein band (64 kDa). Coomassie blue was used as loading dye. For clarity, only RGA bands and section of marker have been shown. Both original western blots are shown in Appendix 1.

3.2.5. Effect of the chemicals on DELLA protein interactions with SLY and GID1 in a yeast-2-hybrid system

To establish whether the chemicals are blocking the interaction of DELLA proteins with SLY1 (F-box protein) and/or GID1 (GA receptor), yeast-2-hybrid assays were performed (Dill et al., 2004). These assays have been previously used to demonstrate that DELLA proteins interact with GID1 and SLY1 as part of the evidence to establish the role of these proteins in GA signalling (Dill et al., 2004; Griffiths et al., 2006a; Willige et al., 2007; Hirano et al., 2010). To identify the effect of the compounds on the DELLA-SLY1 interaction, a yeast-2-hybrid assay was performed in which SLY1 (or *sly1-d*) was expressed as a LexA DNA binding domain fusion (DB) protein and GAI as a GAL4 activation domain (AD) fusion. GAI was used as the representative DELLA protein because an established yeast-2-hybrid system containing thisDELLA protein already existed and had been optimised. As GA signalling results in the degradation

of both RGA and GAI through the same mechanism it is highly likely that chemicals blocking RGA degradation will also have the same effect on GAI.*sly1-d* is a gain-of-function mutation which results in a predicted Glu-to-Lys substitution at amino acid 138 near the C terminus of SLY1 protein (Dill et al., 2004). This mutation has the effect of greatly enhancing the interaction with DELLA proteins, thereby causing increased degradation and enhanced GA-responsiveness (Dill et al., 2004). In addition, to investigate the effect of the compounds on the GID1-DELLA interaction, GID1B was expressed in these assays as LexA DB fusions and their interaction with a GAI GAL4 AD fusion analysed in the presence or absence of GA. All yeast strains harboured the *HIS3* reporter gene, expression of which is induced when AD and DB fusion proteins interact, allowing growth on histidine drop-out media. Interaction of AD and DB fusion proteins was assessed in yeast strains by visualising growth on SD medium lacking leucine, tryptophan and histidine and supplemented with 20 mM 3-amino-1,2,4-triazole (3AT) and a chemical (N1-N28) at 50 µM or DMSO (mock). GA_3 (10 µM) was also added to the media to promote the interaction of GAI-GID1B. 3AT is a competitive inhibitor of the HIS3 enzyme and therefore functions as an indicator of the strength of interaction between the DB and AD protein fusions (Durfee et al., 1993).

Initial experiments analysing the growth of the yeast in the presence of the chemicals on basic medium with no selection pressure showed that three chemicals N21, N24, N25 prevented growth of *GAI-pLEXA, GAI-GID1, GAI-SLY1, GAI-gar* (*sly1-d*) yeast (Figure 3.18.), suggesting that these chemicals are toxic to the L40 yeast strain containing the interactors at 50 µM. The *pLEXA-pACTII* negative control L40 strain showed growth on N21, N24, N25, although these colonies showed more growth than the GAI containing colonies on all chemical treatments, suggesting yeast transformed with *pLEXA-pACTII* has less of a growth cost than yeast transformed with plasmids containing *GAI*.

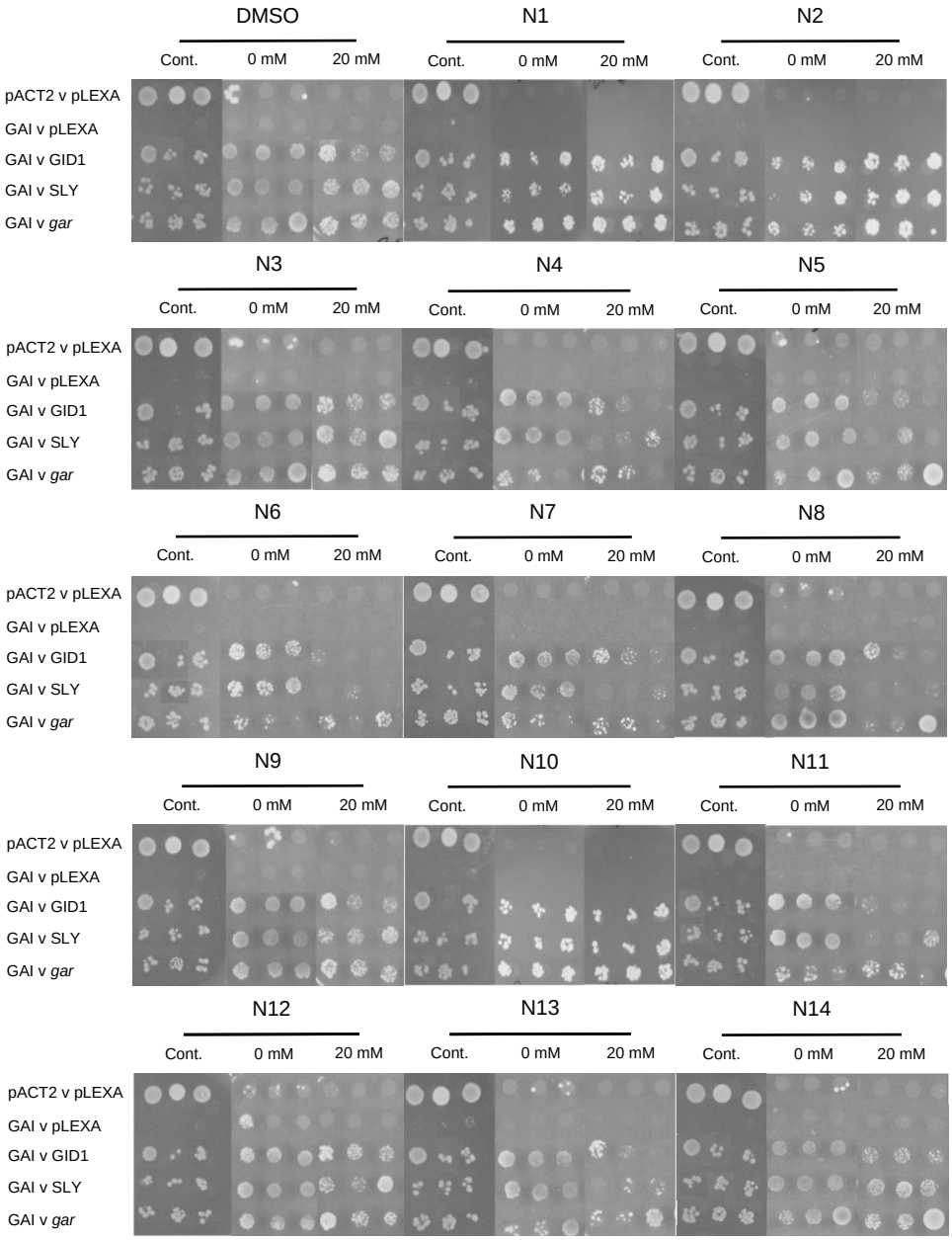
It has been demonstrated previously in yeast-2-hybrid studies using the same LexA-based system that the interaction between GAI and both sly1-d and GID1 allows growth at a maximum 3-AT concentration of 60 mM 3AT (Griffiths et al., 2006a). In contrast, the interaction between GAI and SLY1 in these assays is weaker, allowing growth at only 30 mM 3-AT (Dill et al., 2004; Griffiths et al., 2006b). Pilot tests for the current experiment showed that SLY-GAI and GID1-GAI would both interact and allow growth of yeast at 20 mM 3AT and growth would start to be inhibited above this concentration (data not shown); therefore 3AT was added to the plates at 20 mM. Numerous chemicals (N6, N8, N11 and N20) inhibit the interaction between GAI and both SLY1 and GID1 as illustrated by the lack of growth at 20 mM 3AT concentrations, whereas growth is observed on minimal media containing histidine, whilst N7 appears to inhibit only the GAI-GID1a interaction (Figure 3.18.). N6 (Figure 3.13.) and N7 (Figure 3.16.) block GA mediated *GFP-RGA* degradation but not N8 (Figure 3.13.). N11 is a sulphonamide, of which all the other chemicals belonging to this class block GA-mediated DELLA protein degradation. N20 was toxic to the root tips of the *GFP-RGA* at 50 µM and therefore its effect on GA mediated degradation could not be analysed (Figure 3.14.). It is possible that N6 and N11 are also blocking the degradation of RGA by blocking DELLA protein interactions with SLY1 and/or GID1, whilst N7 is directly blocking the GAI-GID1a interaction. *sly* mutants in Arabidopsis do not respond to exogenous GA because DELLA protein degradation is blocked leading to constitutive repression of GA responses (Strader et al., 2004). There is also evidence to suggest that solely SLY1 is affecting all DELLA proteins (Dill et al., 2004; Tyler et al., 2004). If these chemicals are blocking the DELLA-SLY1 or DELLA-GID1 interactions this would result in higher endogenous levels of DELLA protein which would cause up-regulation of *GA20ox1* expression. For the chemicals to block these interactions they would need to bind a specific protein. A similar chemical screen for the ABA receptor identified the location of chemical binding to a protein complex by performing analysis of the tertiary crystalline structure (Melcher et al., 2010). In the *GFP-RGA* degradation assay N11 was the only chemical of its class that was toxic to the seedlings. It is therefore possible that it could potentially be blocking

yeast growth, which is only apparent when the strains are grown on minimal medium lacking histidine

Chemicals which did not block either the GAI-SLY or GAI-GID1 interaction are potentially affecting other interactions within the GA signalling pathway. For example, they could be affecting the three-way formation of the DELLA-GID1-SLY1 complex, the binding of GA to the GID1 receptor or the interaction of the ubiquitinated DELLA proteins with the 26S-proteasome. To affect the interactions described above the chemical would either have to interact directly with the binding regions of the SLY1, GID1 or DELLA proteins or cause a conformational change in the protein. The use of pyrabactin to identify the ABA receptor was enabled by the chemical binding directly to the ABA binding site (Cutler et al., 2009).

SUMOylation and DELLA transactivation are two other DELLA related processes that could be altered by the chemicals resulting in changes in DELLA protein activity or degradation. Hirano et al. (2012) demonstrated that suppression of stem elongation by the rice DELLA protein SLR1 is dependent on its transactivation activity, in which the TVHYNP motif plays a major role. Furthermore, the GID1 receptor inhibits SLR1 transactivation activity by directly binding to the TVHYNP motif. Potentially these chemicals could alter DELLA transactivation directly by influencing the TVHYNP motif, a process that would be independent of DELLA protein degradation. Small ubiquitin-related modifiers (SUMOs) are ubiquitin like polypeptides that conjugate covalently to cellular proteins similar to ubiquitination (Johnson, 2004). SUMOylation of proteins has numerous functions: such as nuclear-cystolic transport (Matunis et al., 1996) or transcriptional activity (Gill, 2005). SUMOs can also be deconjugated from proteins (Hay, 2007; Mukhopadhyay and Dasso, 2007). It has been reported that one major target for SUMOs in plants are the DELLA proteins (Woodcock et al., 2012). RGA and GAI overexpressing Arabidopsis mutants lacking the site of SUMOylation are not dwarfed, indicating that SUMOylation is required for DELLA transcriptional control, although it is not clear as to why DELLA proteins lacking SUMO have little effect in

reducing plant height (Woodcock, 2012). It is therefore possible that chemicals that do not block GA-mediated DELLA protein degradation could be facilitating de-SUMOylation of DELLA proteins.



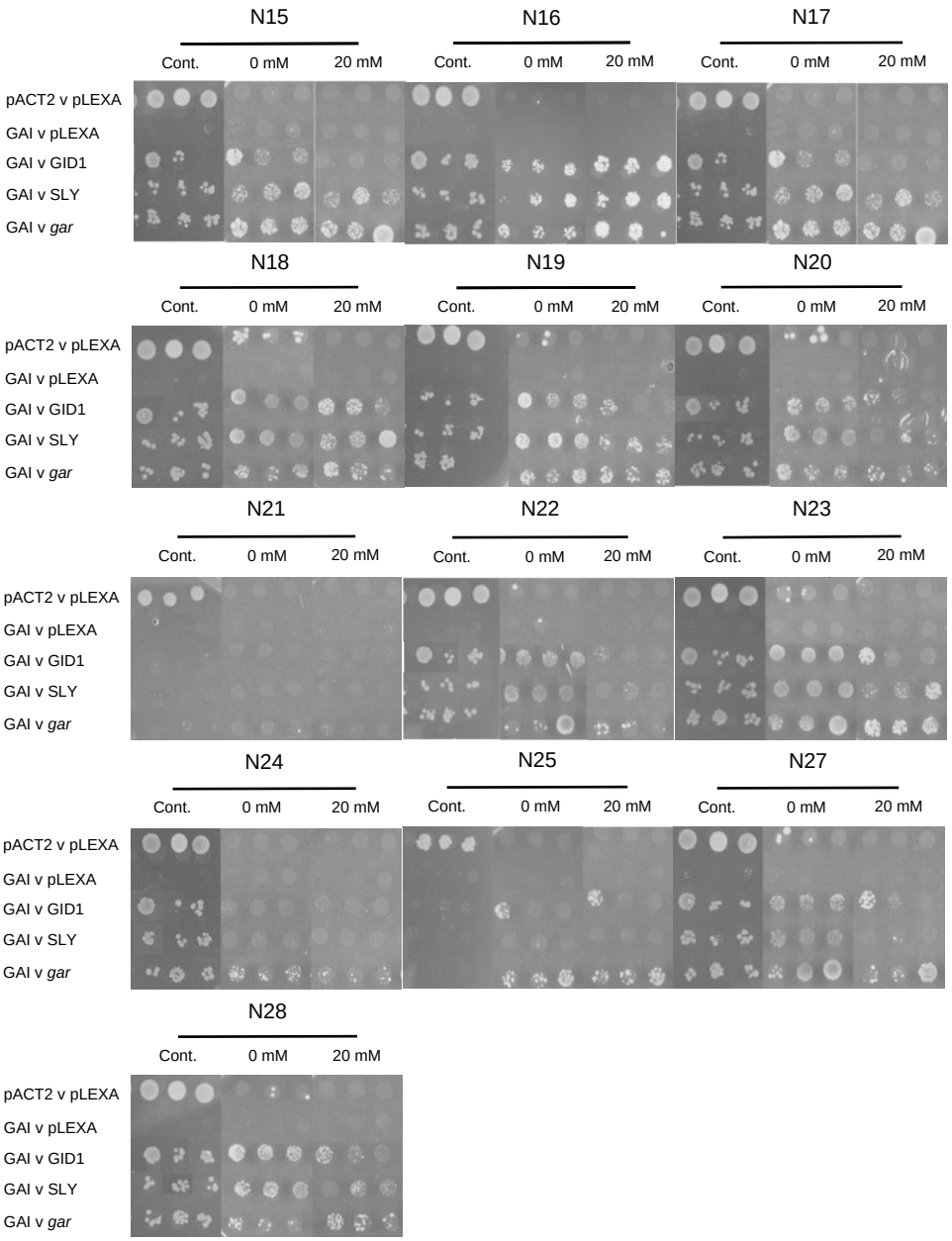


Figure 3. 18. Yeast-2-hybrid assays assessing effects of the chemicals on interactions between GAI and SLY1 or GID1.

All chemicals were tested for GAI vs GID1 interactions (pACT-GAI vs pLEXA-GID1c) and DELLA vs SLY1 interactions (pATC-GAI vs pLEXA-*gar*, pATC-GAI vs pLEXA-SLY). All chemicals apart from N26 were tested at 50 µM and compared to the negative (DMSO) control. Interactions were compared in control -leu-trp medium (Cont.) to show the effect of the chemicals on yeast growth and on in the presence of interaction inhibitor 3AT in –leu-trp-his media (0 mM, 20 mM) to demonstrate their effect on protein interactions. All media contained 10 µM GA_3 to facilitate the DELLA-GID interaction. n = 3, all repeats shown.

3.2.6. Other potential sites of action of chemicals identified by the *GA20ox1::GUS* screen

The higher level of GUS staining of *GA20ox1::GUS* in the presence of GA and the chemicals as compared to GA alone could be caused by other factors than those discussed above. GA crosstalk with other hormone signalling pathways, GA transport and GA metabolism may all influence *GA20ox1* expression. GA regulates numerous developmental processes alongside other plant hormones. ABA and GA play a key antagonistic role in germination (Koornneef et al., 1982), although Ross et al. (2011) show no interaction between the hormone metabolism pathways in pea stems. There is direct interaction between the auxin and GA biosynthesis pathways. Evidence has shown that auxin positively affects GA biosynthesis and conversely limiting auxin results in lower GA levels in some tissues (Ross, 1998). Furthermore, GA and auxin crosstalk has been shown to be integral to lateral root formation in *Poplus* (Gou et al., 2010), distribution of PIN proteins for gravitropism in Arabidopsis (Willige et al., 2011) and fruit set in tomato and peas (Ross et al., 2000; Serrani et al., 2008). How one of the chemicals (N23) affects crosstalk between GA and auxin is explored further in Chapter 4. To date the mode of GA transport and the forms of GA that are being transported is not well understood. Indeed numerous forms of GA and precursors have been implicated in transport, including CDP, *ent*-kaurene, GA_1, GA_3, GA_4, GA_5, GA_6, GA_{15}, GA_{19}, GA_{20} and GA_{24} (Drake and Carr, 1979; Kazama and Katsumi, 1983; Proebsting et al., 1992; King et al., 2001b; Yamaguchi et al., 2001; King et al., 2003; Eriksson et al., 2006; King et al., 2006; Kramer, 2006).

3.3. CONCLUSIONS

The aim of this project was to identify and characterise chemicals that perturb GA signalling, with a view to identifying novel components of this pathway. The screen for potential chemicals involved the use of a *GA20ox1::GUS* reporter line in a chemical screen which aimed to identify compounds which blocked the GA-mediated

downregulation of *GA20ox1* expression. Twenty eight chemicals, from a non-biased library of 10,000 compounds, were identified in the screen. Further analysis using the *GA20ox1::GUS* reporter line identified the active concentration of the chemicals as being between 5 and 50 µM (Figure 3.6.). It was demonstrated that seven out of nine compounds tested also partially blocked the GA-mediated down-regulation of the endogenous *GA20ox1* genes, supporting their roles in perturbing the GA signalling pathway. The other two chemicals which were tested by qRT-PCR study had no effect on *GA20ox1* expression. In these two cases it is conceivable that the compounds are affecting post-transcriptional regulation of *GA20ox1::GUS* activity. It was possible to divide the 28 identified chemicals into four groups based on a core structural motif (Figure 3.4.). However, while no clear relationship between these classes and their effects on GA signalling could be clearly established, it was possible to group some of the compounds based on their effects on DELLA proteins degradation, with one group acting to block this process and others having no effect. The former group therefore potentially act to perturb GA signalling by affecting components that are upstream of DELLA proteins within this pathway. Yeast 2-hybrid assays suggest that one member of this group (N11) may block GA-mediated degradation of DELLA proteins by inhibiting their interactions with SLY1 and GID1. Interestingly one chemical (N23) identified as altering GA signalling was shown to induce agravitropism in the roots of Col-0 seedlings. As the detailed mechanism of crosstalk between the GA and auxin signalling pathways has not yet been identified it is possible this chemical could be used as a tool for dissecting this process.

CHAPTER 4. N23 – A CHEMICAL AFFECTING GA AND IAA

4.1. INTRODUCTION

There is considerable evidence demonstrating that crosstalk between the GA and auxin has an important a role in controlling many physiological processes including stem and root elongation (Ross et al., 2000; Wolbang and Ross, 2001; Fu and Harberd, 2003; Ross et al., 2010; Willige et al., 2011). As yet the molecular mechanism mediating this hormonal crosstalk has yet to be elucidated. This chapter describes the characterisation of a chemical, N23 which affects both GA and auxin signalling, potentially providing a novel tool for dissecting the mechanism of crosstalk between the two hormones. In a recent study Saini et al. (2013) provided evidence that auxin-GA cross talk is partially mediated by the effect of GA on the localisation of the PIN proteins. This results in a higher abundance of PIN proteins at the plasma membrane which allows an influx of auxin into a cell, resulting in the degradation of the AUX/IAAs and activation of auxin responses. Saini et al. (2013) proposed that this frees the ARF7 transcription factors to activate expression of GA biosynthesis genes, which ultimately promote GA-responsive development and produces asymmetric growth during gravitropic stimulation. Willige et al., (2011) demonstrated that GA controlled auxin-regulated gravitropism through increasing levels of PIN proteins in the plasma membranes of cells, resulting in the efflux of auxin into surrounding cells and thus an asymmetric auxin gradient. Willige et al., (2011) suggested that GA does not directly control auxin mediated gravitropism, but merely indirectly modifies it. As N23 was identified by a screen for chemicals affecting GA signaling it is possible that it produces an agravitropic phenotype through fine tuning the auxin response.

This chapter shows how one chemical (N23) that was identified in the screen for chemicals that alter GA-mediated expression of *GA20ox1::GUS* induces

agravitropism when applied to growing roots, indicating an additional auxin related role. Evidence from experiments using the *DR5::VENUS* and the DII-VENUS reporter lines indicates that N23 does not enhances the expression of auxin response genes or AUX/IAA degradation. Yeast-2-hybrid assays suggest that N23 does not alter auxin signalling by affecting the binding of AUX/IAAs to ARFs, suggesting that N23 is not acting on auxin signalling at all. A more likely scenarios is that N23's target protein exists within the auxin transport mechanism which ultimately leads to an effect on GA signalling.

4.2. RESULTS AND DISCUSSION

4.2.1. N23 induces root agravitropism

Whilst performing the root elongation assays described in Chapter 3.2.3., it was noticed that the chemical N23, at concentrations between 1 and 10 µM, induced agravitropism in the roots of seedlings. As this chemical was identified from a screen for chemicals affecting GA signalling this was unexpected as the phytohormone more commonly associated with control of gravitropism is IAA (Went, 1928; Mirza and Maher, 1987). Until recently it was not thought that GA had a role in gravitropism. In the past decade our understanding of how auxin controls gravitropism has increased dramatically. Three recent studies have demonstrated that root columella cell asymmetrically release auxin in response to a gravity stimulus, resulting in an auxin gradient which inhibits elongation of cells on the lower side of the root, leading to curvature of the root towards the gravitropic stimulus (Rashotte et al., 2001; Boonsirichai et al., 2003; Ottenschlager et al., 2003). While auxin is the major player in controlling gravitropic responses, there is recent evidence for the involvement of GAs. It has been shown that GA is asymmetrically distributed in the leaf pulvini during the gravitropic response (Wolbang et al., 2007). Further studies have highlighted a role for GA in auxin controlled root gravitrospim (Lofke et al., 2013). Gallego-Bartolomé et al., (2010) demonstrated that GA-insensitive mutants showed enhanced

root gravitropic reorientation. They subsequently identified *IAA19*/*MSG2* as a potential transcriptional target of GA signalling that mediates this response.

To confirm the effect of N23 on the root gravitropic response a root bending assay was performed following the method described by Marchant et al. (1999). Five-day old Col-0 seedlings grown on vertical standing agar plates were transferred to medium containing 1 µM N23 (the lowest concentration at which agravitropism was observed in the elongation assay, Chapter 3.2.2.). Plates were turned so roots were growing horizontally and then they were imaged 5, 8, 10, 15 and 20 hours later (Figure 4.1.a.). As auxin treatment has been demonstrated to block root gravitropic reorientation (Ottenschlager et al., 2003), the effect of IAA (1 µM) was also analysed. In the absence of IAA or N23 treatment, curvature of the root tips was observed within 5 hours and complete gravitropic reorientation was achieved (a reorientation of 90º ±10º) within 15 hours (Figure 4.1.c.). When the seedlings were treated with N23 or IAA no reorientation was observed within the first 10 hours (Figure 4.1.a.). Between 10 to 20 hours after turning of the plates there was some curvature (≈10%) observed in the roots treated with either N23 or IAA (Figure 4.1.a.). To confirm that this lack of gravitropic reorientation in the presence of N23 is not simply due to inhibition of root elongation, the growth of the roots was also measured. During the course of the root bending experiment, the root lengths of seedlings treated with DMSO, N23 and IAA were measured after 5, 8,10, 15 and 20 hours (Figure 4.1.b.). Treatment with N23 did not appear to inhibit root elongation when compared to the DMSO (mock) control, whereas 1 µM IAA produced severe growth inhibition as has been previously demonstrated (Rahman et al., 2007). This experiment confirms that N23 is blocking root gravitropic reorientation and raises the possibility that it is acting by perturbing auxin signalling or altering auxin transport. However, the lack of effect of N23 on root elongation suggests that it is not acting simply as an auxin agonist. *aux1* mutants (lacking auxin influx carriers) exhibit a similar phenotype to that induced by N23 (Maher and Martindale, 1980). Previously other small molecules 1-NOA, 2-NOA and CHPAA have also been shown to phenocopy *aux1* (Parry et al., 2001). It was later

confirmed that 2-NOA and CHPAA blocked auxin influx whilst 1-NOA blocked both auxin influx and efflux (Lankova et al., 2010). It is therefore possible that N23, like 2-NOA and CHPAA, is affecting auxin transport and thus inducing the *aux1* like phenotype observed in Figure 4.1.

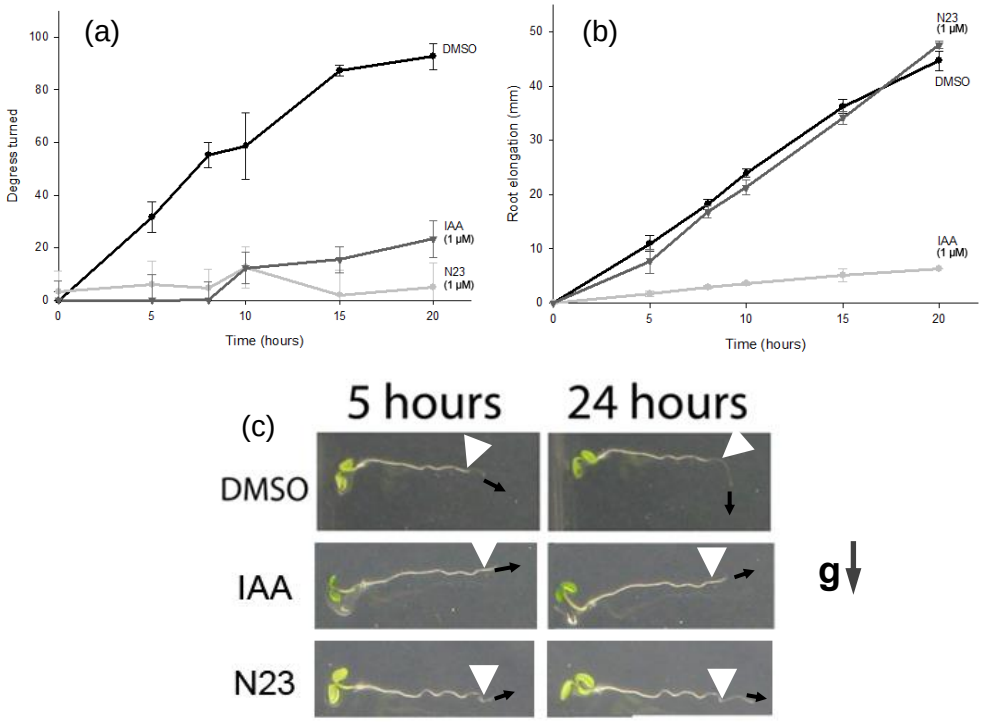


Figure 4. 1. Effect of N23 on root gravitropism.

(a) N23 and IAA prevent gravitropic response in five-day old Col-0 roots turned through 90º and grown for 24 hours, (b) N23 has no effect on root elongation of seedlings whilst IAA inhibits root elongation. [DMSO (mock), N23 (1 µM) or IAA (1 µM)], (c) Col-0 seedlings, with the original site of measurement indicated (white arrow), grown on medium containing DMSO (mock), N23 (1 µM) or IAA (1 µM) and photographed at 5 and 25 hours after reorientation. Black arrows show direction of root growth. Grey arrow shows direction of gravity (g). For (a) and (b) ≈20 roots were measured. Error bars represent standard error.

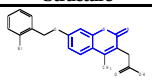
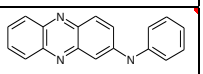
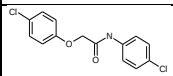
4.2.2. N23 does not induce auxin responsive genes.

Surpin et al (2005) used a chemical screen to identify gravicin as an inhibitor of Arabidopsis hypocotyl gravitropism. This screen of a ChemBridge library of 10,000 chemicals (a library from the same supplier as the screen in this project) initially identified 219 chemicals which affected gravitropism in the Arabidopsis hypocotyl. Of these 219 chemicals, 199 inhibited gravitropism whereas 20 appeared to enhance the gravitropic response. Some of the chemicals identified had structures similar to that of synthetic auxins and could therefore, in theory, be metabolised *in planta* to produce bioactive auxin. Following a repeat of the first screen, 34 chemicals were identified as being active (Table 4.1.). Further analysis showed that these 34 chemicals were affecting root gravitropism as well as hypocotyl gravitropism, altering also vacuole morphology and acting on either auxin signalling or transport or independently of auxin. None of the chemicals identified by Surpin et al. (2005) had any structural relation to N23 (Table 4.1.). No further details on the effect of this compound are currently available. A detailed characterisation of gravacin coupled with a genetic suppressor screen identified the ABC transporter P-GLYCOPROTEIN19 (PGP19) as a potential target of this compound (Rojas-Pierce et al., 2007). To assess whether these chemicals altered the gravitropic response by perturbing auxin signalling, Surpin and colleagues (2005), investigated their effect on the auxin-responsive gene reporter line, *DR5::GUS*. *DR5* is a synthetic promoter that contains auxin-responsive *cis*-elements. In response to auxin signalling an increase in GUS expression is observed, which can then be detected by histochemical staining (Ulmasov et al., 1997b). To establish whether N23 is affecting auxin signalling, a similar assay was performed using the auxin-responsive *DR5::VENUS* transcriptional reporter system which has recently been developed (Brunoud et al., 2012). Similar to other auxin-responsive genes (GH3, AUX/IAA), *DR5* contains eight copies of the GAGACA 6 nucleotide *ARE* motif that is bound by ARF1 (Abel et al., 1996). The VENUS reporter is a fast maturing YFP which allows for the auxin response to be monitored in real-time instead of at the end point, as is the case for the GUS reporter (Heisler et al., 2005). A recent

study by Band et al. (2012) demonstrated how it was possible to visualise and quantify the real-time spatial localisation of these fluorescent proteins during root gravitropism using confocal microscopy.

Table 4. 1. Chemspider identification numbers of N23 and chemicals that affected the gravitropic response in Arabidopsis seedlings.

Table shows chemical ID number, whether the chemical is auxin- or non-auxin-like (unique), or shares structural similarities to the synthetic auxin 2,4-dichlorophenoxy acetate. Information reproduced from Surpin et al., 2005.

chemspider ID	Category	Effect	Structure
1409581	N23	-	
74030	unique	enhancer	
4495457	unique	inhibitor	
260497	auxin	inhibitor	
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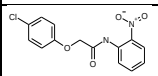
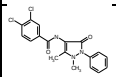
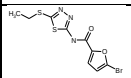
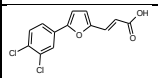
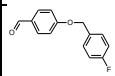
4195609	auxin	inhibitor	
868318	unique	inhibitor	
646529	unique	inhibitor	
678393	unique	inhibitor	
643014	unique	inhibitor	

chemspider ID	Category	Effect	Structure
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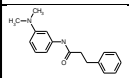
766639	auxin	inhibitor	
595691	unique	inhibitor	
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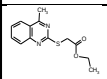
605783	unique	inhibitor	
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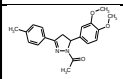
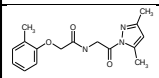
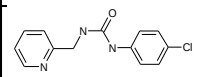
2975273	unique	inhibitor	
2396772	auxin	inhibitor	
745619	unique	enhancer	

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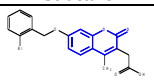
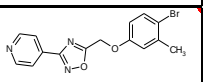
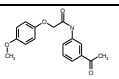
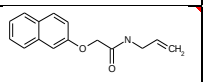
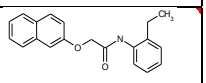
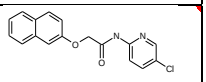
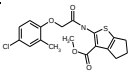
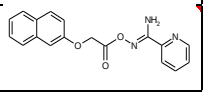
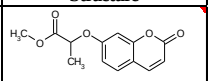
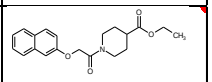
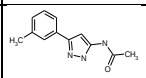
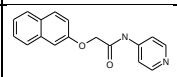
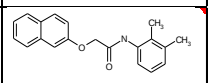
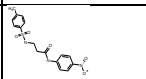
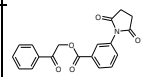
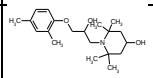
1409581	N23	-	
763562	unique	inhibitor	
1553677	auxin	inhibitor	
1597704	auxin	inhibitor	
1365122	auxin	inhibitor	
645248	auxin	inhibitor	
848101	auxin	inhibitor	
1696949	auxin	inhibitor	

chemspider ID	Category	Effect	Structure
2162562	auxin	inhibitor	
739370	auxin	inhibitor	
739132	unique	inhibitor	
719469	auxin	inhibitor	
764173	auxin	inhibitor	
4409829	unique	enhancer	
595817	unique	inhibitor	
2166143	unique	inhibitor	

To obtain quantitative data on *DR5::VENUS* induction by N23 and IAA treatments, 5-day old *DR5::VENUS* seedlings were transferred to media containing either N23 (5 µM), IAA (1 µM) or DMSO (mock) and total fluorescence was measured by confocal imaging over a 390-minute period and quantified as described by Brunoud et al. (2012). N23 was used at a concentration of 5 µM as this was the lowest concentration at which root inhibition was observed previously (Figure 3.8.). Fluorescence was then measured using Fiji software. Over a 390-minute period DMSO had no statistical effect on *DR5::VENUS* fluorescence levels (Figure 4.2.). Despite imaging numerous DMSO treated roots (5) only two were viable for analysis and as a result of this small sample size (n= 2) large error bars were observed. The high degree of variability which was observed may also have been due to movement of the Arabidopsis roots whilst being imaged. As the root moves the confocal microscope will image slightly different layers at each time point and at low fluorescence (as is observed in DMSO treatments) these changes in layers lead to relatively large changes in fluorescence observed. In contrast to the control treatment, IAA induced a large increase in *DR5::VENUS* fluorescence. Due to the large error bars observed in the DMSO treatment it is difficult to judge the exact time at which IAA starts inducing an effect

although the fluorescence starts to increase around 150 minutes which is similar to the findings of Brunoud et al. (2012).

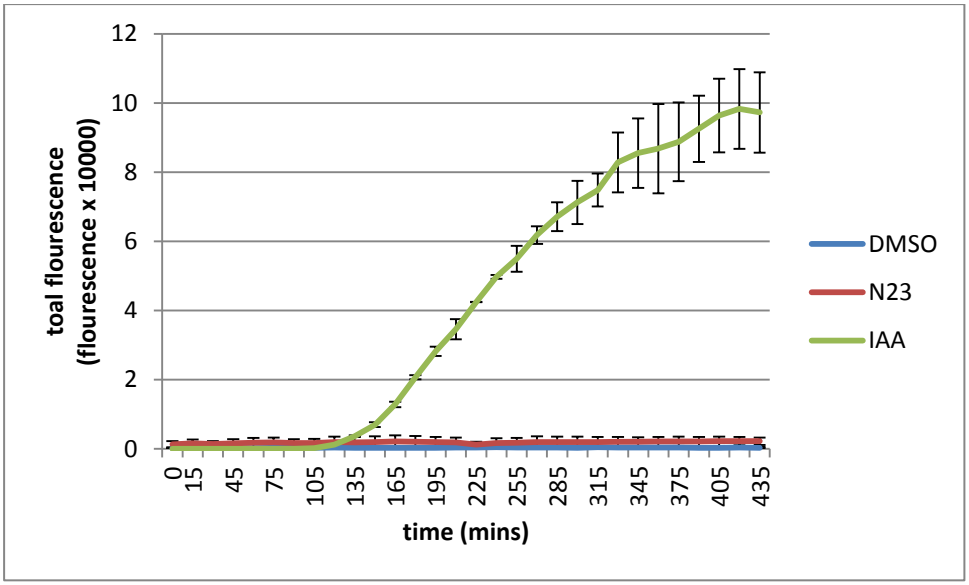


Figure 4. 2. Induction kinetics of absolute YFP fluorescence by N23 in the *DR5::VENUS* reporter line.

DR5::VENUS fluorescence in response to DMSO, IAA or N23 treatment was quantified. Five-day-old seedlings were transferred to medium containing IAA (1 µM, n=4), N23 (5 µM, n=5) or DMSO (mock, n=2) and imaged over 6.5 hours using a confocal microscope. Fluorescence was quantified using Fiji software as described in Brunoud et al. (2012). All fluorescence values shown are of absolute fluorescence. Error bars represent SE.

In the previous chapter it was shown that N23 inhibited GA-mediated down-regulation of GUS activity in the *GA20ox1::GUS* line (Chapter 3.2.2.), but had no effect on the transcriptional down-regulation of the endogenous *GA20ox1* gene expression by GA. In addition, N23 was found to prevent the GA-mediated degradation of RGA (Figure 3.14.), indicating that it acts upstream of DELLA degradation within the GA signalling cascade. Fu and Harberd (2003) provided a potential mechanism for the crosstalk between auxin and GA that was responsible for the regulation of Arabidopsis root growth and which involved regulation of DELLA stability by auxin signalling. They demonstrated that the removal of the shoot apex of Arabidopsis seedlings resulted in

the restriction of the primary root growth. This inhibition was overcome by the application of IAA to the site of the shoot apex, indicating that shoot apex-derived auxin promotes root growth by enabling the response of root cells to GA. Based on the study of a transgenic line expressing a GFP-RGA reporter, Fu and Harberd (2003) provided evidence that this control of root growth is mediated by the polar transport of auxin to the roots where it promotes GA-mediated DELLA protein degradation, thus relieving inhibition of root growth by DELLA. However, a recent study has shown that whilst IAA needs to be transported to the root to allow proper root growth, GA is also required for the vacuolar trafficking of the auxin efflux transporters (PINs), indicating another mechanism of crosstalk between these two phytohormones (Willige et al., 2011). Root gravitropism involves the establishment of an auxin gradient. When Arabidopsis seedlings are turned through 90^0, an auxin gradient forms, increasing from the bottom to top of the root, causing differential cells growth that results in downward bending of the root (Boonsirichai et al., 2002; Morita, 2010; Band et al., 2012). The auxin is transported to the desired location by the efflux carriers, the PIN proteins (PINs). PIN3 and PIN7 form on the lower side of the vascular root cells to allow auxin flux into the root tip (Friml et al., 2002a; Harrison and Masson, 2008; Kleine-Vehn et al., 2010). PIN2 then transports the auxin towards the elongation zone where an auxin gradient can be formed. The cycling of these PINs between the membrane and the vacuole allow for the formation of an auxin gradient (Friml et al., 2002a; Kleine-Vehn et al., 2010; Rakusova et al., 2011; Ding et al., 2012). This localization of PIN2 is regulated by auxin and GA (Baster et al., 2012). From the results observed in Figure 4.2., where N23 has no obvious effect on the auxin responsive genes, together with the information available regarding GAs role in modulating the localization of the IAA transport proteins (Baster et al., 2012) it is conceivable that N23 is affecting the transport of auxin.

In pea it has been shown that auxin signalling induces GA biosynthesis and reduces expression of GA deactivation enzyme *GA2ox* (Ross et al., 2000). In Arabidopsis and tobacco the GA-biosynthesis gene *GA20ox1* is up-regulated in the presence of auxin

(O'Neill and Ross, 2002). N23 has no effect on *GA20ox1* expression, but does maintain *GA20ox1::GUS* activity after GA application (Figure 3.6. and 3.7.). These results are consistent with N23 altering GA signalling through an inhibition of GA20ox1 enzyme turnover, which could be investigated by Western blotting with antibodies raised against AtGA20ox1. Although such antibodies are currently not available, it may be possible to screen the reporter line with antibodies for the GUS protein. *GA20ox1::GUS*, GFP-RGA and *GA20ox1* expression results also indicate that whilst the N23 mimic's auxin's control DELLA protein degradation it does not directly control transcription of the GA biosynthesis genes as auxin does but rather acts post-transcriptionally.

4.2.3. N23 acts downstream of AUX/IAA degradation in auxin signalling

The effect N23 on the *DR5::VENUS* reporter line provides evidence that this compound is not activating the expression of auxin-responsive genes. However, it is not clear where N23 is acting within the auxin-signalling pathway. The auxin signalling pathway is now well understood and is defined by several key steps (Figure 4.3.). The primary transcriptional output of this pathway, which leads to auxin-responsive growth, is directly regulated by the ARF transcription factors. In the absence of auxin signalling, the AUX/IAAs bind to and inhibit the activity of the ARFs. Binding of auxin to the auxin receptor leads to targeted degradation of the AUX/IAAs which frees the ARFs to activate downstream response genes. An important reporter has recently been developed which allows the monitoring of AUX/IAA degradation in response to auxin signalling (Brunoud et al., 2012). This reporter line, DII-VENUS provides an important tool to narrow down where N23 is acting within this pathway. The DII-VENUS line consists of a VENUS (YFP) reporter protein fused to the domain-II (DII) of an AUX/IAA protein, expression of which is under the control of a constitutive 35S promoter. The DII region of the AUX/IAAs is a highly conserved region that binds, along with auxin, to the auxin receptor allowing ubiquitination and subsequent 26S proteasome-mediated degradation of the DII-YFP fusion protein (Tan et al., 2007).

This auxin-mediated degradation of the reporter protein allows convenient real-time monitoring of auxin signalling status *in vivo*. For the construction of DII::VENUS the domain I of IAA28 starting from the conserved lysine up to the end of domain II (28–61), which has a basal half-lives of between 15 and 20 minutes (Brunoud et al., 2012) was used. The recent study by Brunoud et al., (2012) demonstrated the power of DII::VENUS in the mapping of the spatio-temporal response of Arabidopsis roots to auxin. If N23 does remove DII::VENUS fluorescence in the root it would therefore be acting upstream of AUX/IAA degradation, possibly as an auxin agonist. However, if it has no effect then it will indicate that it is acting downstream, potentially through the regulation of the ARFs.

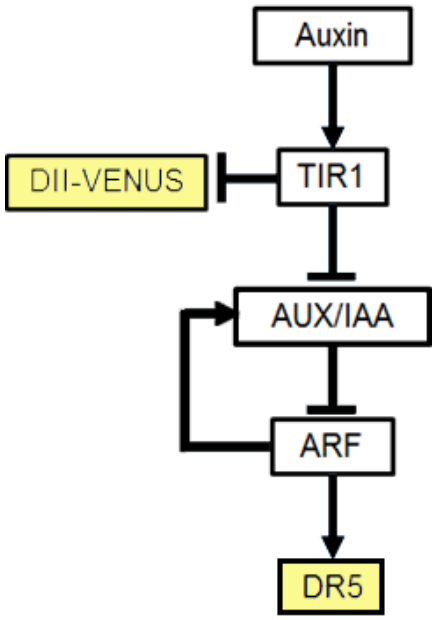


Figure 4. 3. *DR5::VENUS* and DII::VENUS are reporters of auxin signalling. Auxin binds to its receptor TIR1 which results in the degradation of AUX/IAA by the 26S-proteasome pathway. In the absence of auxin, AUX/IAAs bind to ARFs preventing their activation of auxin-responsive genes. Auxin-induced degradation of AUX/IAAs relieves their expression of ARFs allowing the up-regulation of the auxin-responsive genes. DII and *DR5* report on AUX/IAA degradation and auxin responsive genes, respectively. Adapted from Band et al. (2012).

To analyse the effects of N23 on DII-VENUS fluorescence, 5-day old seedlings were transferred to MS containing N23 (5 µM), IAA (1 µM) or DMSO and YFP fluorescence within the root was detected using a confocal microscope over a 1 hour time period. Treatment with 1 µM IAA caused a rapid decrease in fluorescence of DII:VENUS (Figure 4.4.). Within 15 minutes IAA had reduced fluorescence by 50% and within 30 minutes the fluorescence had almost disappeared. This loss of fluorescence is slightly quicker than that reported by Brunoud et al. (2012) where fluorescence was reduced by 50% within 30 minutes and absent in under one hour. In contrast, N23 (5 µM) had no observed effect on DII-VENUS levels (Figure 4.4.). This suggests that N23 is not promoting AUX/IAA degradation and is therefore acting downstream of this process in the auxin-signalling cascade (Figure 4.4.). These results, in addition to those from the *DR5* assays, indicate that N23 is unlikely to be affecting auxin signalling. Therefore, it is feasible that N23 is instead affecting IAA transport.

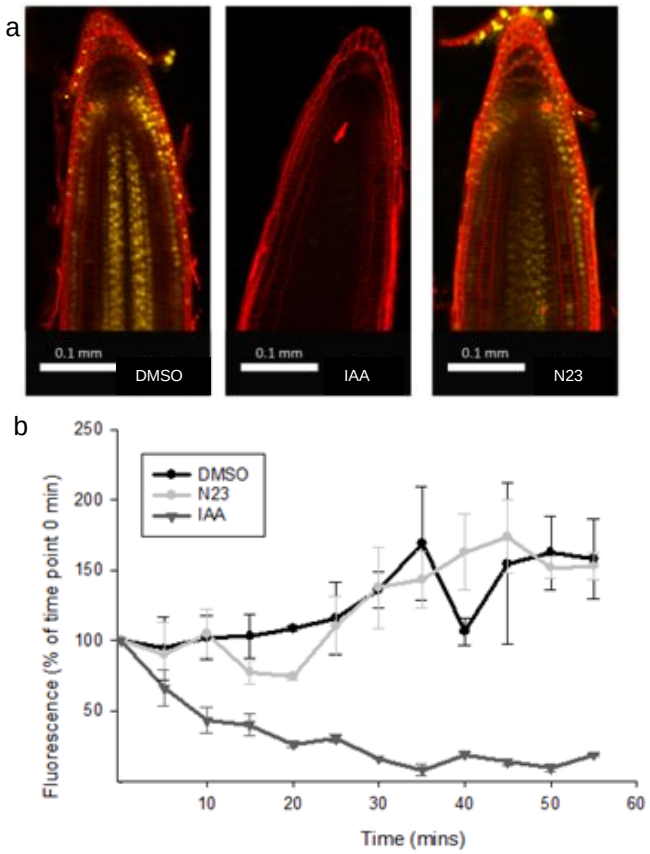


Figure 4. 4. N23 does not induce DII:VENUS degradation.

(a) DII::VENUS fluorescence in the root tip 60 mins after treatment with DMSO (mock), IAA (1 µM) and N23 (5 µM). (b) Quantification of DII::VENUS fluorescence following treatment with DMSO, IAA or N23. Five-day old seedlings were transferred to media containing IAA, N23 or mock (DMSO) and imaged over 1 hour using a confocal microscope. Fluorescence was quantified using Fiji software. As described in Brunoud et al. (2012). Values are the average of 3 replicates (±SE).

4.2.4. Does N23 block AUX/IAA interactions with ARF?

Characterisation of the *DR5::VENUS* and DII-VENUS transgenic reporter lines has demonstrated that N23 has no effect on the expression of auxin-responsive genes or AUX/IAA degradation. Based on a knowledge of the auxin signalling cascade (Figure 4.5.), there are a number of possible scenarios in which N23 could potentially be altering this pathway and causing agravitropsim. Studies using the *DR5::GUS* reporter

line have demonstrated that some ARFs are repressors (ARF1, -2, -3, -4, and -9) whereas others are activators (ARF5, -6, -7, and -8) (Tiwari et al., 2003). As N23 does not induces an increase in *DR5::VENUS* fluorescence it is possible that the chemical is either promoting the ARF repression or blocking ARF transcriptional activation. Tiwari et al. (2003) suggested that nuclear proteins could be increasing the stability of ARFs whilst bound to ARE *in vivo*. It is conceivable that N23 could also be decreasing ARF stability, which would result in decreased activation of auxin-responsive genes, such as *DR5*. Another potential mode of action for N23 is that it blocks the binding of AUX/IAA proteins with the repressor ARFs. This would prevent the inhibition of ARFs by the AUX/IAA proteins leading to constitutive deactivation of auxin-responsive gene expression (Figure 4.5.c.). An additional component of AUX/IAA-ARF binding is the protein TOPLESS (TPL) which has been shown to be required for IAA12/BDL repression of ARF5/MONOPTEROS (Szemenyei et al., 2008). TPL is known to bind both IAA12/BDL and ARF5/MONOPTEROS. If N23 was blocking ARF-AUX/IAA interaction it would also explain why it does not mimic IAA and lead to increased AUX/IAA degradation. As chemicals identified by previous chemical screens were predominantly found to be blocking protein-protein interactions (Armstrong et al., 2004; Kim et al., 2011), it was therefore decided to analyse the effect of N23 on ARF-AUX/IAA interactions.

Yeast-2-hybrid assays provide a convenient heterologous system for monitoring ARF and AUX/IAA interactions (Tiwari et al., 2003). Previous studies of ARF7 and ARF19 have demonstrated that they are involved in positive transcriptional regulation of the auxin responsive genes through their interaction with the ARE, including *DR5* (Tiwari et al., 2003; Wang et al., 2005b). They have also been demonstrated to have a role in regulating root gravitropism (Okushima et al., 2005). The AUX/IAA gene *IAA14* also has an important role in controlling gravitropism, and has been demonstrated to interact with ARF7 and ARF19 in yeast 2-hybrid assays (Fukaki et al., 2002). These findings suggest that IAA14 may act to block ARF7 and ARF19 activity *in planta* to regulate gravitropism in response to auxin signalling (Fukaki et al., 2002). Given that

N23 blocks gravitropism it was decided to analyse whether it perturbs the interaction between ARF19 and IAA14 in yeast-2-hybrid assays.

Numerous other ARF-IAA interactions including ARF1 and IAA12 and -13 have been identified using the yeast-2-hybrid system (Ulmasov et al., 1997b), IAA14 with ARF7 and -19 (Fukaki et al., 2005) and more recently to test >1200 possible interactions among ARFs and Aux/IAA proteins (Vernoux et al., 2011). To test the interaction of ARF19 and IAA14 in the presence of N23 the Invitrogen ProQuest© yeast-2-hybrid system was used. This consists of the MaV203 yeast strain with the *pDEST32* bait vector and the *pDEST22* prey vector. In the case of this experiment the IAA14 was tested as a GAL4DB fusion and ARF19 as a GAL4AD fusion. This was because ARF19 self-activates expression of the reporter genes when it is expressed as a GAL4DB fusion protein due to its intrinsic transcriptional activation activity. Two ARF19 clones were used in these assays: a full length ARF19 (ARF19 FL) clone and one containing an N-terminally truncated clone, but which contained AUX/IAA interaction domains (ARF19 2-15). The reporter gene for the yeast-2-hybrid assays is *HIS3*, which allows the yeast strain to grow on plates lacking histidine. The competitive inhibitor of HIS3, 3AT is included in the assays as it provides some idea about the strength of the interactions based on the level of *HIS3* expression.

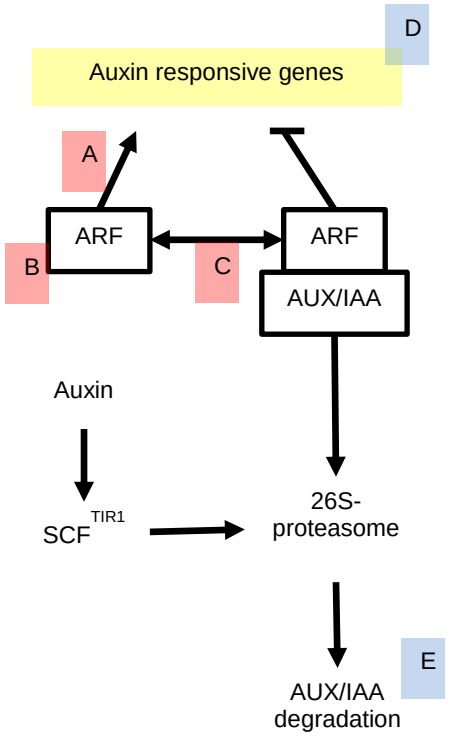


Figure 4. 5. Auxin signalling with possible sites of action of N23 highlighted in red.

The auxin responsive genes are up-regulated by the ARF which in turn are inhibited by binding to the AUX/IAAs. This inhibition can be overcome by the degradation of the AUX/IAA by the 26S-proteasome. Degradation is mediated by auxin binding to the F-box component (TIR1) of SCF^{TIR1} which forms a complex with the AUX/IAAs. Possible modes of action of N23 are: (A) N23 increases the interaction of ARF with the responsive genes, (B) N23 stabilises ARFs leading to increased auxin responsive gene expression, (C) N23 blocks interaction of ARFs with AUX/IAAs allowing ARFs to up-regulate DR5 expression without affecting AUX/IAA degradation, (D) N23 up-regulates auxin responsive genes (DR5), (E) N23 does not affect AUX/IAA degradation.

Yeast strains containing the IAA14 bait and both ARF19 prey constructs demonstrated growth on media lacking histidine with 50-100 mM 3AT, whereas the control strain (IAA14 bait and empty prey vector) only grew on those containing 10 mM 3AT (Figure 4.6.). This confirmed the previously report demonstrating an interaction between IAA14 and ARF19 in yeast-2-hybrid assays (Fukaki et al., 2005).

When the assays were repeated in the presence of 50 µM N23 all of the strains continued to demonstrate growth at the same concentrations of 3AT (Figure 4.6). This indicates that N23 is not affecting the interaction between IAA14 and ARF19 in these assays. Whilst this assay implies N23 has no effect on IAA14-ARF19 interaction, the possibility that this chemical may be perturbing specific AUX/IAA-ARF interactions cannot be discounted. For example, IAA14 has also been demonstrated to interact with ARF7 which has been implicated in gravitropism (Fukaki et al., 2002; Fukaki et al., 2005). Taken together the results from the *DR5::VENUS*, DII-VENUS and ARF-IAA yeast screen rule out the likelihood of N23 acting primarily on components of the auxin signalling pathway.

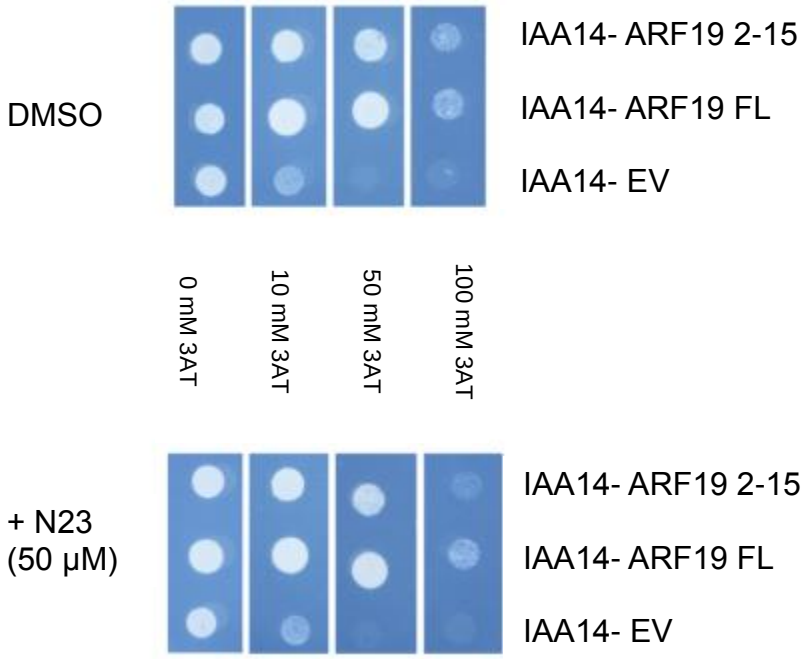


Figure 4. 6. Yeast-2-hybrid assay showing N23 does not block IAA14-ARF19 interaction

Yeast two-hybrid study on the interaction between IAA14 and ARF19 FL and ARF19 2-15. Yeast colonies diluted in dH_2O were grown on plates containing growth inhibitor 3AT ± N23 (1 µM). On control medium IAA14 interacts with both full length ARF19 (FL) and ARF19 (2-15), which only contains coding regions.

4.2.5. Potential sites of action of the chemical N23

Whilst the site of action of N23 was not identified there are still numerous potential target sites. Auxin is transported around the plant and root in a polar manner by the PIN proteins and the AUX1 protein (Muller et al., 1998; Marchant et al., 1999). *GA20ox1* expression was found to be increased in Arabidopsis mutants with reduced auxin transport or plants treated with the auxin transport inhibitor NPA (Desgagne-Penix et al., 2005; Desgagne-Penix and Sponsel, 2008). It is therefore possible that N23 is increasing *GA20ox1::GUS* activity in the presence of GA by blocking auxin transport. Interestingly the expression pattern of *DR5* in the presence of N23 was localised to the epidermis of the root (Figure 4.2.) in a region associated with acropetal auxin transport (Mitchell and Davies, 1975; Tsurumi and Ohwaki, 1978). N23 did not induce expression of *DR5::VENUS* down the central axis of the root, a region more associated with transport of auxin from the aerial parts of the plant (basipetal transport) (Lewis et al., 2007). N23 could conceivably be blocking this acropetal transport. Additionally, application of N23 to *DR5::VENUS* plants removed fluorescence in the RAM where an auxin maxima is usually created as a result of auxin transport. This further suggests a role of N23 in transport.

Another potential scenario is that N23 could be affecting elements downstream of the AUX/IAAs. AUX/IAAs function as transcriptional activators mostly through their interaction with the transcriptional repressor TOPLESS and ARFs (Long et al., 2006; Szemenyei et al., 2008). As auxin application increases *GA20ox1* expression and TOPLESS is a transcriptional repressor it is unlikely that TOPLESS binds directly to the *GA20ox1* promoter. A more likely scenario is that TOPLESS represses another downstream gene that is necessary for the repression of *GA20ox1*. N23 could be promoting this by either increasing AUX/IAA binding to TOPLESS or by increasing TOPLESS binding to the promoter.

Villalobos et al. (2012) showed that the AUX/IAAs also form a complex with TIR1 that is essential for the perception of IAA. The combination of any of the 6 TIR1/AFB auxin receptors with any of the 29 AUX/IAA is integral to the affinity of the complex to auxin. Another potential scenario for N23 enhancement of auxin signalling is that it may increase the affinity of the TIR1/AFB-AUX/IAA complex for auxin binding leading to increased AUX/IAA degradation. However, this scenario is not supported by the effect of N23 on DII-VENUS degradation, which would be expected to be increased.

4.3. CONCLUSION

When the 28 chemicals were tested for their effect on root elongation one chemical, N23, induced an agravitropic response commonly associated with auxin mutants (Figure 4.1.). Upon further study N23 was shown to not to affect expression of the auxin responsive gene reporter *DR5*, AUX/IAA degradation or AUX/IAA-ARF interactions, indicating that N23 is unlikely to be acting on a component of auxin signalling. Results from the previous chapter, where N23 blocks GA mediated degradation of RGA, further confirm the auxin-like effect of N23, which also inhibits DELLA protein degradation (Fu and Harberd, 2003). It has been suggested that GA fine-tunes the control of gravitropism by auxin (Willige et al., 2011) and it is possible that N23 induces agravitropism by altering GA signalling as a result of its effect on the auxin. Figure 4.7. shows a model indicating where of where N23 may be acting in the auxin transport and GA signalling pathways N23 based on the findings of Chapters 3 and 4.

As N23 has been shown to interact with both auxin and GA signalling pathway, but not exactly mimic either hormone it presents a potentially useful tool for identifying possible novel components regulating the crosstalk between these two important hormones. A useful method for narrowing down the site of action of the chemical would be a microarray to identify transcriptional changes brought about by the chemical; such results can be compared to publically available data to identify which

mutation-induced transcriptional changes are mimicked by treatment with chemical. Additionally a suppressor screen of mutants could also identify a potential target for N23.This approach was used to determine the target of gravicin, identified in a chemical screen of compounds altering the gravitropic response (Rojas-Pierce et al., 2007). From the screen of 220,000 M_2 mutagenised plants gravacin was shown to be inhibiting an ABCB transporter of auxin, PGP19. Similarly to identify the binding partner of N23 a mutaganised population could be screened for mutants that are resistant to the gravitropic phenotype induced by N23 treatment.

From the experiments detailed in this chapter it can be concluded that the chemical N23 represents a tool for the study of crosstalk between the auxin and GA.

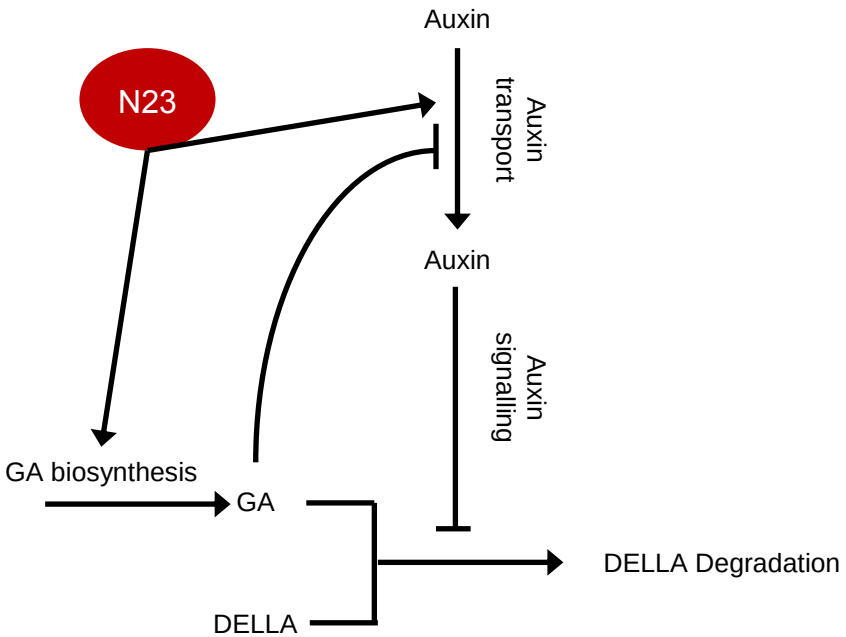


Figure 4. 7. Scheme showing potential sites of action of N23 in crosstalk between GA and auxin signalling pathways.
The model is based on information from the literature and results described in Chapters 3 and 4. N23 may act on the auxin transport. The effect of N23 on auxin signalling results in inhibition of GA-mediated DELLA protein degradation.

CHAPTER 5. N16: A POTENTIAL INHIBITOR OF GA TRANSPORT

5.1. INTRODUCTION

There is considerable evidence in the literature suggesting that transport of GAs is important for controlling specific plant growth processes. In general, the highest levels of bioactive GA are found in the actively growing organs which contain high levels of GA biosynthetic gene expression, indicating that GA is synthesised at and not transported to the required site (Smith et al., 1992; Kaneko et al., 2003; Jin et al., 2011). However there are numerous examples of developmental processes requiring the transport of GAs between neighbouring or even distant tissues. The expression of *CPS* in the provasculature of germinating Arabidopsis seeds whilst *KO* and *GA3ox* were expressed in the cortex and endodermis suggest that a GA intermediate is being transported between the tissues (Yamaguchi et al., 2001). In cereal seeds bioactive GA is required to be transported from the scutellum to the non-GA-producing aleurone to induce the synthesis of hydrolytic enzymes during germination (Fath et al., 2001). While the role of GA transport is well known, to date, the mechanism of GA transport is poorly understood. This lack of knowledge stems largely from the absence of identified molecular components that are involved in this process and the assumption that GAs move by diffusion and thus do not require transport. With the recent identification of an ABA transporter in Arabidopsis by a chemical screen (Park et al., 2009), there is renewed interest in identifying GA transport components and investigating the mechanisms involved in this process. This knowledge will be essential in establishing a better understanding of how GAs control plant growth and development.

There have been some conflicting suggestions as to the forms of GA transported. The *GA3ox* and *GA20ox* genes are expressed in the dividing cells of tobacco and

elongating cells of rice, indicating that bioactive GAs are produced at the sites at which they are required (Itoh et al., 1999; Kaneko et al., 2003). The physical separation of steps of the GA biosynthesis pathway in the developing embryo, where *CPS* is expressed in the provasculature and *KO* is expressed in the cortex, has suggested that an intermediate of the biosynthesis pathway must be transported, possibly CDP or *ent*-kaurene (Yamaguchi et al., 2001). Interestingly, in Arabidopsis over-expressing *CPS/KS* and thus *ent*-kaurene, *ent*-kaurene is emitted and converted to bioactive GA by the surrounding plants (Otsuka et al., 2004) suggesting a role for GA in signalling between plants, although this is unlikely as *ent*-kaurene is not usually a rate limiting intermediate. One argument against *ent*-kaurene being transported is the localization of the enzyme KO, that uses *ent*-kaurene as a substrate, to the plastid envelope (Olszewski et al., 2002) suggesting the formation and oxidation occur in the same cell. Numerous precursors of bioactive GA have been postulated as the transported forms. Reid et al. (1983) showed that when shoots of pea mutants blocking the early steps in GA biosynthesis were grafted to WT stocks then elongation in the mutant stems occurred, while mutant shoots lacking later steps in the biosynthetic pathway were not rescued by grafting to WT stocks, suggesting that precursors, but not the active GAs were mobile from roots to shoots. Proebsting et al. (1992) used grafting experiments to identify inactive GA_{20} as being the major form of GA transported in pea lines. The same study also identified GA_{19} as an unlikely candidate for transport.

Ross et al. (2006) suggested that there may be a difference in GA transport between monocots and dicots, with monocots transporting bioactive GA and its precursors whilst dicots only transport the precursors. This was based on findings by Katsumi et al. (1983) in maize that showed that grafting of *dwarf-1* stems, which lack GA 3β-hydroxylation, the final step in the pathway, to WT stocks lead to the elongation of the mutants stems, indicating that the bioactive GA is transported. When deuterium-labelled GA_4, another bioactive GA, is applied to the leaves of the dicot Arabidopsis it has been detected in the shoot apex, suggesting this form may also be a transported

(Eriksson et al., 2006), which is not consistent with the theory that dicots only transport precursors. As the mechanism (e.g. phloem, xylem, cell-cell) for the transport of the deuterium-labelled GA_4 is not known, although it is likely to be phloem transport, it is possible that Ross et al. (2006) theory may still hold true. There are numerous claims that GAs are one of the transported florigens (King et al., 2001b). GA_5 and GA_6, were shown to move from the leaf to the shoot apex of *Lolium temulentum* where they are involved in floral initiation (King et al., 2001b; King et al., 2003; King et al., 2006). King et al. (2001) showed that after floral induction radiolabelled GA_5 (a bioactive form in *Lolium temulentum*) is transported from the leaf to the apex at a speed of 1-2 cm h^{-1}, over a distance of 10 to 12 cm. This is similar to IAA transport, which is active transport, in Arabidopsis which occurs at a speed of 0.5 to 2 cm h^{-1} (Lomax et al., 1995). Eriksson et al. (2006) also showed that bioactive GA_4 in Arabidopsis is transported from the rosette to the shoot apex to initiate flowering. Further studies of this subject have been difficult to perform due to the challenges of isolating specific vascular tissue and measuring potentially small changes in GAs (King and Evans, 2010). Without knowing the form of GA transported it is difficult to identify the method of transport within the plant. Two possible modes of transport for GA are through the xylem and phloem (Sponsel., 1986; Arteca, 1996). Kramer (2006) suggested that GA_{15} and GA_{24} could diffuse out of the cell but these GAs could not re-enter the cells without specific transporters. A study by Drake and Carr (1979) in oat coleoptiles showed that azides reduced longitudinal transport of GA_1 and GA_3. This result indicated to the authors that GA is transported symplastically, possibly via the plasmodesmata. A recent study by Shani et al. (2013) has shown that fluorescent labelled GA_3 (Fl-GA_3) accumulates in the endodermis of Arabidopsis roots and the authors postulate that the bioactive GA is actively transported from the meristem, cortical cells and epidermal cells to the endodermis where it induces root elongation by reducing DELLA protein levels.

This chapter presents evidence to suggest that one chemical identified in the initial chemical screen (Chapter 3.2.1), N16, may block GA transport. Root and hypocotyl

growth assays in the presence of N16 and 1 µM GA_3 show that Col-0 plants are saturated for GA and that N16 is blocking the GA response in roots whilst having no effect on hypocotyls. N16 was shown to reduce the uptake of tritiated GA_4 [^{3}H]GA_4 by the root of Col-0 and *ga1-3* seedlings. Furthermore, the uptake of the Fl-GA_3 molecule developed by Shani et al. (2013) into the root endodermis was blocked by N16. Microarray data have identified OPT6 as a transporter rapidly down-regulated in the presence of GA, and a potential candidate for a GA transporter. However, [^{3}H]GA_4 uptake assays in yeast expressing the transporter could not confirm a role for OPT6 in GA transport.

5.2. RESULTS AND DISCUSSION

5.2.1. The chemical N16 blocks the uptake of Fl-GA_3

Recently, Shani et al. (2013) demonstrated that Col-0 seedlings grown on media containing Fl-GA_3 accumulated fluorescence in the endodermis, indicating the uptake and movement of the Fl-GA_3 from outside the root specifically into this tissue. Further studies using un-tagged GA_3 as a competitor and comparison of plants cultured at 4ºC and 22ºC in the presence of Fl-GA_3 indicated that the molecule was actively taken up by the plant and transported to the epidermis. If the compounds alter GA transport then this may be apparent by blocking the accumulation of Fl-GA_3 in the endodermis. To test this hypothesis, seven chemicals that blocked GA-mediated DELLA degradation were analysed at the University of San Diego by Dr. E. Shani for their effect on the uptake of Fl-GA_3 at their active concentrations previously determined in the *GA20ox1::GUS* assay in Chapter 3.2.2. (N3: 50 µM, N4: 5 µM, N6: 50 µM, N10: 10 µM, N16: 50 µM, N21: 10 µM). To investigate the effect of the compounds on the uptake of GA_3-Fl, six to eight day old Arabidopsis seedlings were transferred to agar plates containing the Fl-GA_3 with the seven chemicals (at active concentrations determined in Chapter 3.2.2.) and allowed to grow for 2-3 hours. Seedlings were then stained with propidium iodide, to highlight cell walls, and imaged using a confocal

microscope. Three of the chemicals were toxic to the roots at the concentration tested (N4, N10, N21). Chemicals N3, N6 and N25 had no effect on Fl-GA_3 fluorescence in the endodermis. As there is conflicting evidence on which forms of GA are transported and that only GA_3 was tested here, these three chemicals cannot be ruled out from blocking GA transport. Based on the lack of fluorescence observed, one of the chemicals (N16) appeared to block the accumulation of the Fl-GA_3 in the endodermal cells of the elongation zone (Figure 5.1.). This observation raises the possibility that N16 is blocking GA uptake and possibly transport to the endodermis. Further studies by Shani et al. (2013) showed that Fl-GA_3 accumulation in the endodermal cells was a result of active uptake. It is therefore possible that N16 prevents Fl-GA_3 accumulation by inhibiting this active transport. This compound could therefore provide an important tool for investigating the process of GA transport within plants.

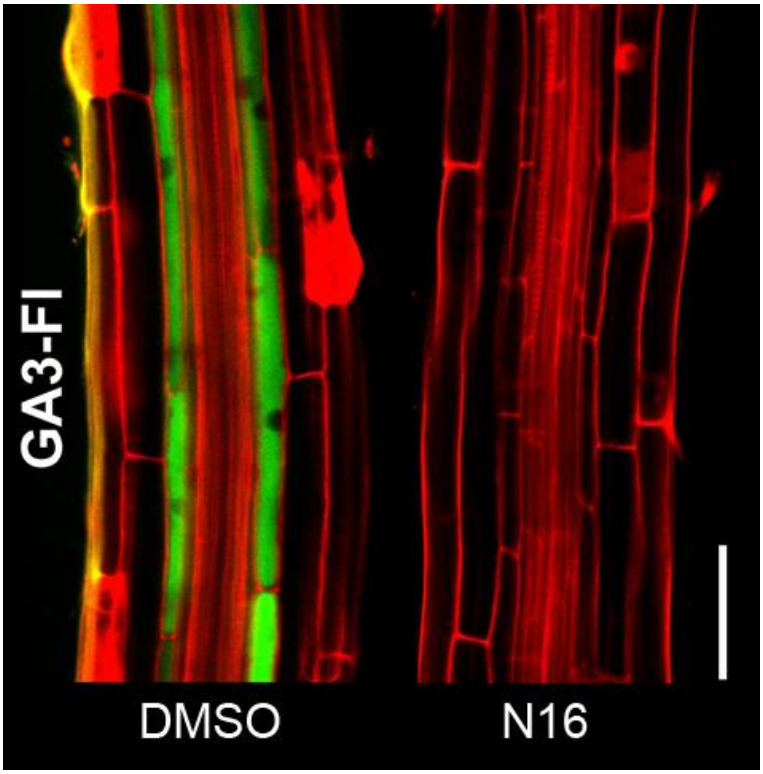


Figure 5. 1. N16 blocks uptake of Fl-GA_3 in root elongation zone. Seedling roots treated with N16 and Fl-GA_3 show no sign of fluorescence in the endodermis, which is observed in the mock control. Seven day-old seedlings exposed to GA_3-FL and either mock (DMSO) or N16 (50 µM) for 3 hours. Roots were stained with propidium iodide and analysed using a confocal microscope. Experiment carried out by Dr. E. Shani (University of San Diego) using method outlined in Shani et al., 2013.

5.2.2. N16 reduces root elongation over a 5-day period

GA is known to drive root elongation through the elongation of cells in the elongation zone and cell division in the root apical meristem (Ubeda-Tomas et al., 2008; Ubeda-Tomas et al., 2009). It is difficult to predict what effect inhibition of GA transport in the root is likely to have on elongation growth as it is uncertain if the elongating cells in the root endodermis synthesise GA or require GA from other sources. When five day old Col-0 seedlings were transferred to media containing 1, 5, 10 or 50 µM N16 for 24 hours there was no reduction in root elongation observed over this period (see Figure

3.14. in Chapter 3.6.). It is conceivable that effects on GA transport may only have a subtle effect on growth in these assays. It was therefore decided to analyse root growth over a longer period. Four day-old Col-0 seedlings were transferred to media containing N16 at 10, 20 or 30 µM and grown for a further 5 days. The final root lengths of the seedlings were then measured using ImageJ software. Roots grown on 20 µM N16 were on average 96% the length of the mock treatment ($t=2.16$, $p=0.036$) whilst at 30 µM roots were 86% the length of mock treated roots ($t=7.68$, $p=<0.001$) showing N16 inhibited root growth over this period (Figure 5.2.). This inhibition of root elongation is consistent with reduced GA transport, if GA action is separate from the site of synthesis; it is however possible that N16 may inhibit other processes. Reduced root elongation is also observed when GA biosynthesis inhibitors are applied to Col-0 seedlings, although this effect is usually more severe, with roots of Col-0 seedlings grown on media containing 0.05 µM PAC reduced to ≈57% the length of seedlings grown on media lacking PAC (Griffiths, 2007), and rapid [within 6 hours (Koizumi et al., 2012)]. The length of time required for N16 treatment to reduce root elongation in Col-0 may be a result of slow chemical uptake by the plant, although effects on *GA20ox1::GUS* staining were observed in just eight hours after N16 was applied at 50 µM in the presence of GA (Chapter 3.2.1.).

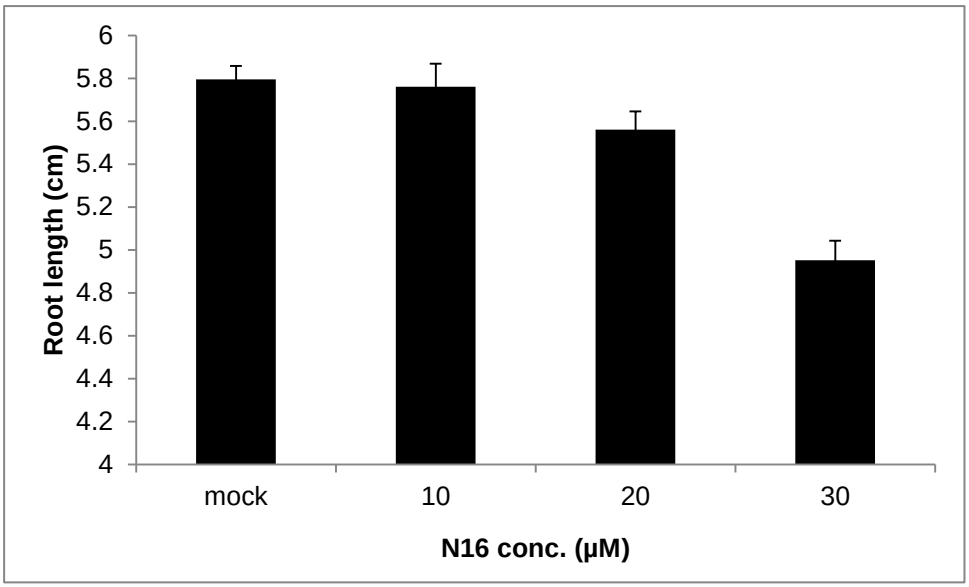


Figure 5. 2. Inhibition of root growth by N16.

N16 inhibits root growth at 20 and 30 µM but not 10 µM. Four day-old Col-0 seedlings transferred to mock, 10, 20 or 30 µM N16 for five days and roots measured using ImageJ software. 22 to 27 roots analysed per treatment. The vertical axis starts at 4 cm as no root was shorter than 4 cm. Error bars represent standard error.

The *GA20ox1::GUS* screen with N16 showed enhanced staining after eight hours in the presence of GA. Given the evidence suggesting that N16 blocks GA uptake it was decided to assess the effect of N16 on root elongation in the presence of applied GA. Four-day-old Col-0 seedlings were transferred to semi-solid medium containing either DMSO (mock), N16 (30 µM), DMSO + GA_3 (1 µM) or N16 (30 µM) + GA_3 (1 µM). After 5 days root length was measure using ImageJ software. Roots of seedlings grown in the presence of N16 and GA were shorter compared to DMSO and DMSO + GA treatments and similar to those after treatment with N16 alone, suggesting that N16 is blocking uptake and possible transport of GA (Figure 5.3). As for treatment with N16 in the absence of applied GA (Figure 5.2), the effect on root elongation was observed only after 5 days (Figure 5.3.). There is little information on the rate of GA transport in the literature. King et al. (2001b) showed that a bioactive GA is transported from the leaf at a speed of 1-2 cm h^{-1} over a distance of 10-12 cm in *Lolium temulentum*. Additionally exogenous application of Fl-GA_3 results in accumulation in the

endodermis within 15 mins (Shani et al., 2012). If GA was travelling this quickly in Arabidopsis you would expect to see a more pronounced effect if N16 was blocking GA transport, suggesting the store of GA within root cells is high enough to allow normal root growth for 4 days before being depleted. It is unlikely that there is a prolonged period of time before N16 reaches an active concentration within the root as N16 was shown to block Fl-GA_3 uptake into the root endodermis within 3 hours (Figure 5.1.). The lack of growth in roots treated with GA alongside N16 (Figure 5.3.) rules out the possibility that N16 is functioning as an anti-GA in the same way that PAC does, by inhibiting GA production. Were N16 acting in a similar way to PAC GA application would rescue root growth. As N16 prevents the GA mediated degradation of RGA (Figure 3.15.) it is possible that N16 is functioning as an anti-GA by blocking the binding of GA with GID1. To test this hypothesis a similar assay to that initially used to characterise the binding of GA to GID1 could be performed (Ueguchi-Tanaka et al., 2005). In these assays, the effect of N16 on the binding of radiolabelled-GA to recombinant GST–GID1 could be assessed. If N16 acts as an anti-GA it would prevent the binding of GA with the GST-GID1. However, the demonstration that N16 prevents FL-GA_3 uptake (Figure 5.1.) indicates that it is more likely to affect GA transport.

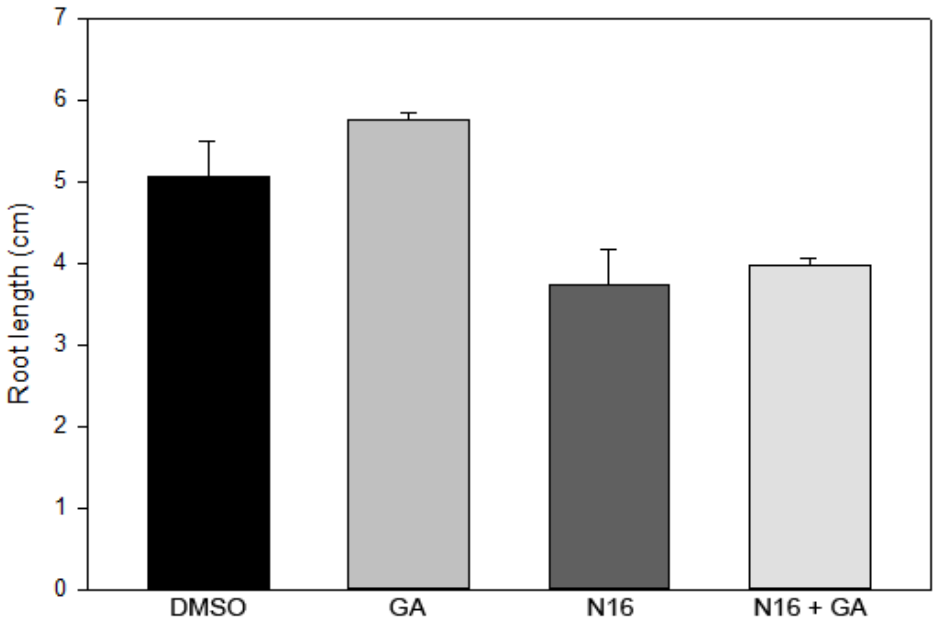


Figure 5. 3. Root length of Col-0 seedlings cultured with GA, N16 and N16 + GA.

GA marginally increase seedling root length compared to DMSO treatment. N16 treatment inhibits natural root growth and GA induced root growth. Five-day-old Col-0 seedlings transferred to medium containing DMSO: DMSO (N16 mock) + EtOH (GA mock), GA: 10 µM GA_3 + DMSO, N16: 30 µM GA_3 + EtOH (mock) or N16 (30 µM) + GA (10 µM). Root length was measured after 5 days using ImageJ. Error bars show standard error.

There is some evidence for GA saturation in the Col-0 root. Griffiths et al. (2006) showed that Col-0 roots are 90% saturated for GA_3 at 1 µM. As we used GA_3 at 1 µM it is possible that an effect of N16 is only observed on Col-0 after 5 days as there is already sufficient bioactive GA at the sites required for root growth. *ga1-3* is a gibberellin biosynthesis mutant with reduced levels of bioactive GA (Koornneef et al., 1983). Application of GA_3 to *ga1-3* resulted in an increase in root elongation over 24 hours (Figure 5.4.). As Col-0 roots grow at between 0.7 and 0.95 cm a (Ubeda-Tomas et al., 2009), application of GA_3 to *ga1-3* plants (which increased root elongation to 0.55 cm a day) did not completely recover wild-type root growth. This effect of GA on promoting root elongation was clearly visible following a 24 hour treatment; therefore it was decided to observe the effect of N16 over a similar time period. When seedlings

were treated with GA and N16 there was no increase in root elongation (Figure 5.4.), suggesting N16 is blocking uptake of GA and indicating the delayed effect of N16 on Col-0 root elongation is a result of GA saturation in the root, rather than the slow uptake rate of N16.

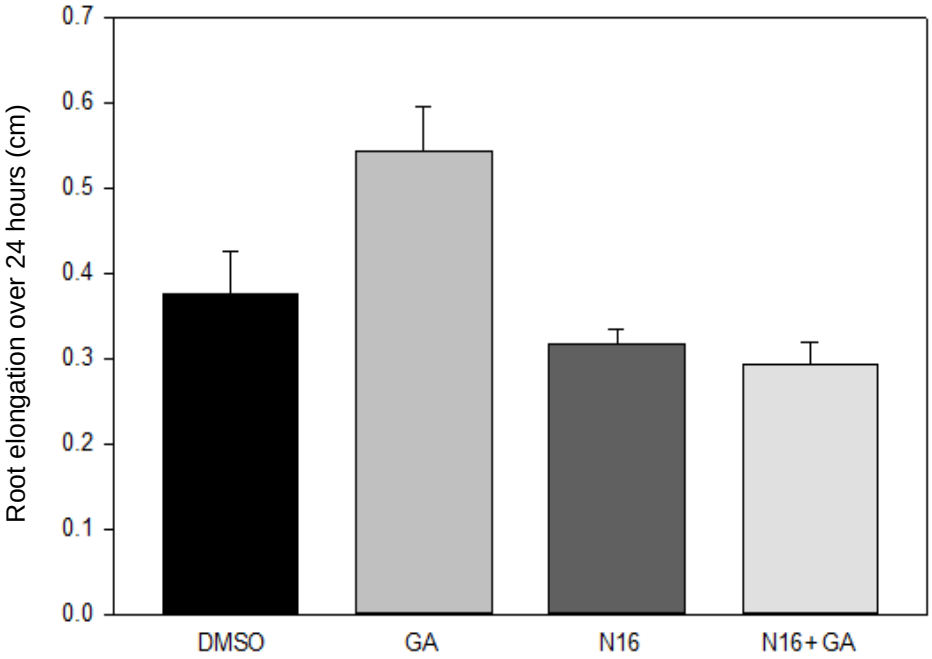


Figure 5. 4. Root elongation of *ga1-3* seedlings cultured with GA, N16 and N16 + GA.

GA treatment quickly increases root elongation when compared to the DMSO mock treatment. N16 treatment inhibits natural root growth and GA induced root growth. Five-day-old *ga1-3* seedlings were transferred to medium containing DMSO: DMSO (N16 mock) + EtOH (GA mock), GA: 10 µM GA_3 + DMSO, N16: 30 µM GA_3 + EtOH (mock) or N16 (30 µM) + GA (10 µM). Root length was measured at 0 and 24 hours using ImageJ and elongation calculated. Error bars show standard error.

GA signalling is also known to be a driver of hypocotyl elongation through cell elongation, in which it plays an integral role in mediating the plants phototropic response (Cowling and Harberd, 1999; de Lucas et al., 2008). To further analyse the effects on growth of N16, a hypocotyl elongation assay was performed in dark grown seedlings. GA signalling has an important role in controlling Arabidopsis hypocotyl elongation in the dark (Alabadi et al., 2004) and therefore alterations in transport are

likely to have a pronounced effect if transport is required for normal hypocotyl elongation. Arabidopsis seeds were spotted onto agar plates containing N16 at 0, 1, 5, 10 and 50 µM. Seeds were allowed to germinate in light for 24 hours before plates were sealed in aluminium foil and hypocotyl elongation was measured after 5 days. No differences in hypocotyl elongation were observed between any of the N16 treatments and controls (Figure 5.5.). As it has been reported that exogenous GA application does greatly affect dark-grown hypocotyl length a GA control was not used in this experiment (Cowling and Harberd, 1999).

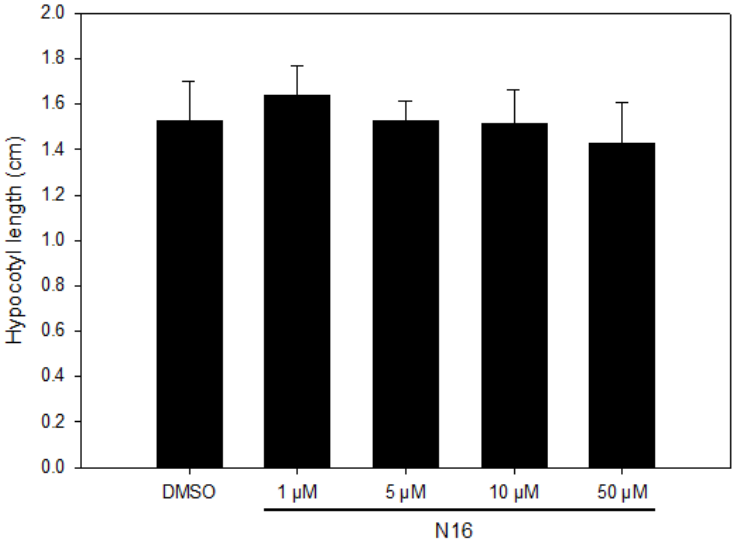


Figure 5. 5. N16 has no effect on hypocotyl elongation when grown on semi-solid agar media.

Col-0 seeds were germinated on medium containing N16 at 1, 5, 10 or 50 µM and grown in the dark for 5 days. Hypocotyl elongation was measured using ImageJ software. 10 to 15 hypocotyls were measured per treatment. Error bars represent standard error.

One possible explanation as to why N16 did not affect hypocotyl elongation is that N16 is not transported to the hypocotyl to inhibit GA transport. In an attempt to circumvent this problem, the hypocotyl elongation assay was repeated growing the seedlings submerged in liquid media. Seedlings were germinated in liquid medium in the light for 8 hours, then N16 was applied at concentrations of 0, 1, 5, 10 or 50 µM

and plates were sealed in aluminium foil. After 5 days of growth in the dark hypocotyl length was measured. As found previously, no difference in hypocotyl elongation was observed between the N16 treatments and the controls. Interestingly N16 had no effect on hypocotyl length over 5 days on semi-solid media containing N16 at 50 µM whereas under the same conditions but with a lower concentration of N16 (30 µM) root elongation was reduced, indicating that the lack of effect on hypocotyl elongation by N16 is not due to GA saturation or slow uptake of N16. As GA is known to promote hypocotyl elongation (Cowling and Harberd, 1999; de Lucas et al., 2008), inhibition of GA transport by N16, as was indicated by the Fl-GA_3 results (Figure 5.1), should result in a reduction of hypocotyl elongation. Indeed grafting of stems lacking in GA to WT root stocks induces stem elongation showing GA is transported from the roots to the stems in pea (Proebsting et al., 1992). In wheat *GA20ox1* was shown to be expressed in the intercalary meristem whereas *GA3ox1* was expressed in the internodes. This distribution of GA biosynthetic activity suggests that at the very least there must be transport of a GA precursor around the stem. In Arabidopsis it has been reported that *CPS, KS* and *GA3ox1* are expressed in virtually all tissues whilst *GA20ox1::GUS* show staining in the hypocotyl (Mitchum et al., 2006; Plackett, 2011), indicating that GA can be synthesised at the site of hypocotyl cell elongation and does not require transport. The transport of GA and its precursors within the hypocotyl cannot be ruled out, but the synthesis of bioactive GA in the hypocotyl is a possible reason for N16 having no effect on hypocotyl elongation. Taken together the differential effect of N16 on hypocotyl and root growth may indicate that the transport of bioactive GA is only relevant to root growth.

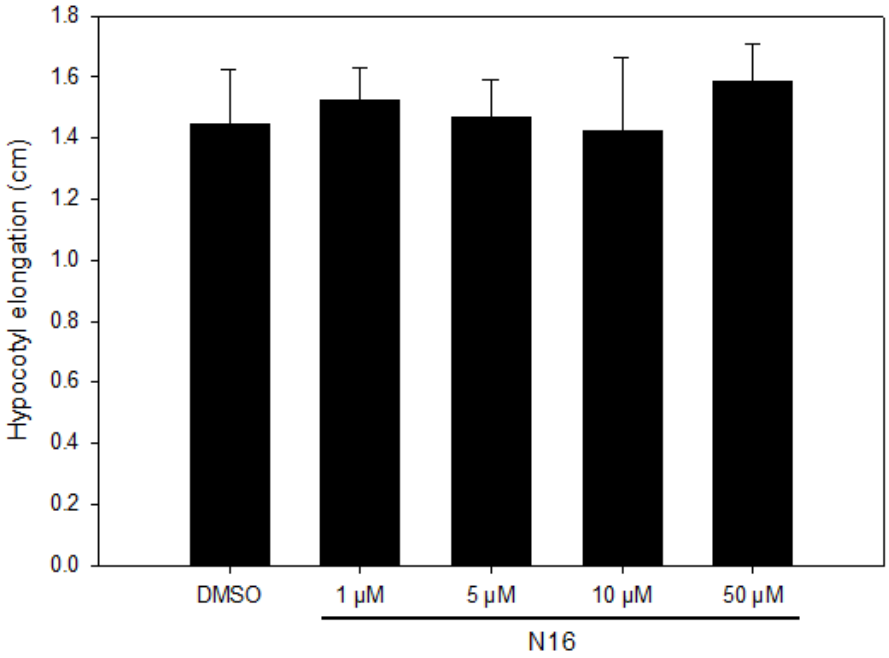


Figure 5. 6. N16 has no effect on hypocotyl length when grown in liquid media.

N16 added at 1, 5, 10 or 50 µM to liquid medium containing two day-old Col-0 seedlings. After 3 days seedlings were imaged and hypocotyl length measured using ImageJ software. 10 to 15 hypocotyls measured per treatment. Error bars represent standard error.

5.2.3. N16 reduces uptake of [^{3}H]GA$_4$

Numerous papers have shown that various forms of GA are taken up into plants (King et al., 2001b; Eriksson et al., 2006; Shani et al., 2013). At present there is little data on the rate at which exogenous sources of GA are taken up. To determine the rate of uptake of GA, four-day old Col-0 seedlings were cultured in dH$_2$O containing [^{3}H]GA$_4$ for 6, 12 and 24 hours and uptake into the roots quantified by scintillation counting. After preliminary tests no difference in either GA uptake or plant health was observed between plants cultured in MS or dH$_2$O, therefore dH$_2$O was chosen as the media for all GA uptake experiments. Figure 5.7. shows that longer treatment with [^{3}H]GA$_4$ results in higher uptake. The results also show that the rate of uptake does not vary

over time, suggesting equilibrium between exogenous and endogenous GA_4 is not reached within 24 hours.

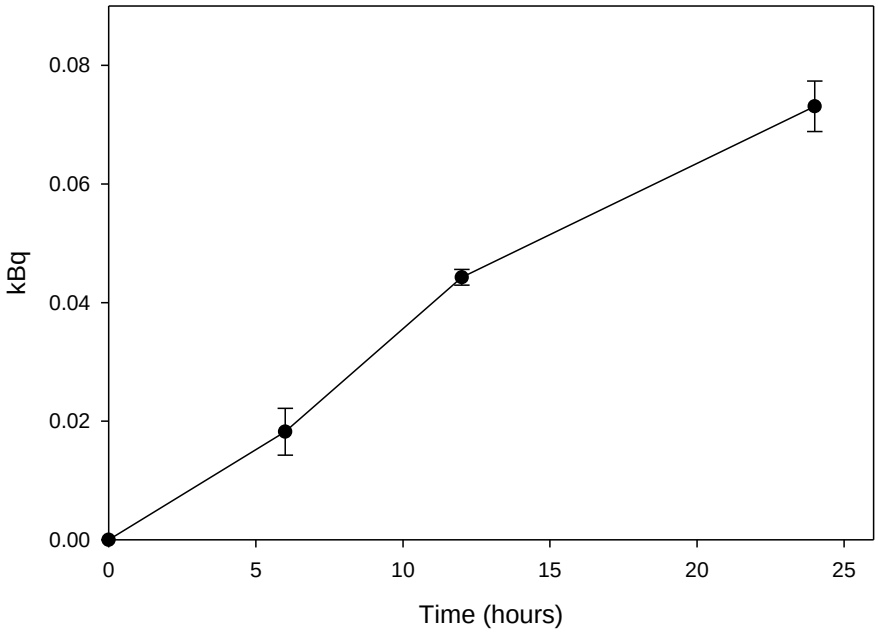


Figure 5. 7. [^{3}H]GA_4 uptake by Col-0 roots over a 24 hour period.
Col-0 roots continue to take up GA over a 24 hour period. Four day old Col-0 seedlings were cultured in dH_2O with 10 kBq of [^{3}H]GA_4 (equating to 1 e^{-11} M of GA) for 6, 12 and 24 hours. Roots harvested and uptake characterised by scintillation counting (kBq). 15 roots measured per treatment. Error bars represent standard error. Background reading counts were 0.002 kBq.

N16 has previously been shown to block the uptake of the Fl-GA_3 produced by Shani et al. (2013). To confirm this effect with a GA endogenous to Arabidopsis, four day-old Col-0 and *ga1-3* seedlings were grown for 24 hours in dH_2O containing [^{3}H]GA_4 with and without 30 µM N16 before flash freezing in liquid N_2 and grinding. [^{3}H]GA_4 was extracted from ground tissue in 1 mL of 100% ethanol before transfer to a 5 mL plastic vial with 2 mL of scintillation fluid and measuring the amount of radioactivity taken up by the roots by scintillation counting. In both Col-0 and *ga1-3* N16 reduced GA uptake (Figure 5.8.). *ga1-3* plants are deficient in the production of bioactive GA, lacking the enzyme CPS (Figure 5.8.). In the *ga1-3* mutants treated with N16 GA uptake is similar

to that of the N16-treated Col-0 plants (Figure 5.8.). This suggests that if GA is being actively taken up into the plant and N16 is blocking this uptake then there may be still a base level of diffusion of GA into the roots. N16 appears to be blocking the uptake of at least two forms of bioactive GA, Fl-GA_3 into the root endodermis and [^{3}H]GA_4 into the roots cultured with the radiolabelled GA_4 (Figure 5.1. and 5.8.) suggesting there may be a generic transporter for all four bioactive forms of GA (GA_1, GA_3, GA_4, GA_7). As the effect of N16 on non-bioactive forms has not been tested it is impossible to say if the chemical is blocking the transport of all GAs. LAX3 (an auxin transporter from the same family as AUX1) has been shown to perform a similar role in auxin transport by transporting two forms of auxin (IAA and IBA) in Xenopous oocytes (Swarup et al., 2008).

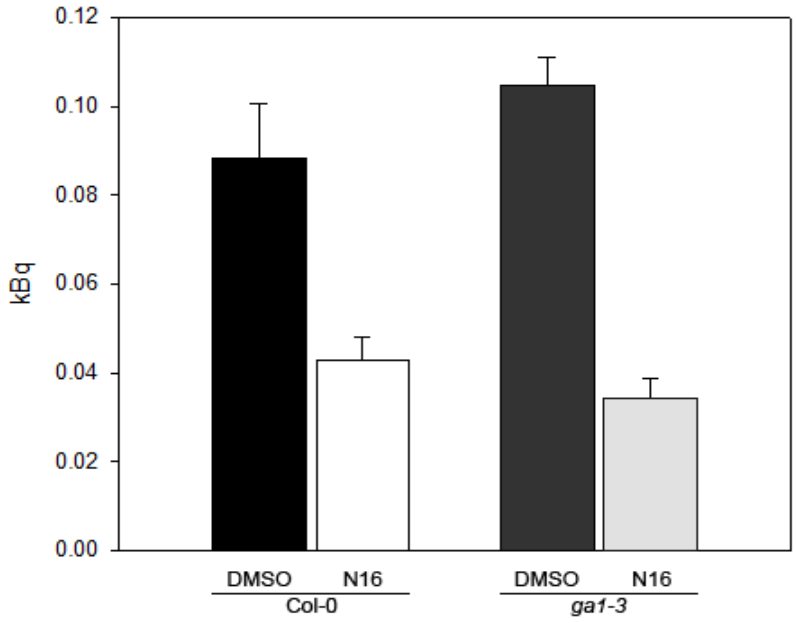


Figure 5. 8. [^{3}H]GA_4 uptake in Col-0 and *ga1-3* in the absence or presence of N16.Figure 5.8.

N16 inhibits GA uptake to the same extent in roots of both Col-0 and *ga1-3* seedlings. Four-day-old Col-0 or *ga1-3* seedlings were cultured in dH_2O containing 10 kBq of [^{3}H]GA_4 (equating to 1 e^{-11} M of GA) and DMSO (mock) or N16 (30 µM) for 24 hours. Roots were harvested and the uptake of [^{3}H]GA_4 was characterised by scintillation counting. 15 roots were measured per treatment. Error bars represent standard error. Background reading counts were 0.002 kBq.

As described in the introduction there is little agreement on the form of GA transported, potentially the mechanism of transport varies depending on the route of transport and plant type. The chemical N6 blocked DELLA degradation (Chapter 3.2.3.) but, unlike N16, had no effect on Fl-GA_3 uptake (Section 5.2.1.). To asses the validity of the Fl-GA_3 assay the [^{3}H]GA_4 assay was performed with 10 µM N6, which was the active concentration identified in the *GA20ox1::GUS* assay (Section 3.2.2.). When four-day-old Col-0 seedlings were cultured with [^{3}H]GA_4 for 24 hours GA uptake was reduced in the presence of N6 (Figure 5.9.) suggesting Fl-GA_3 uptake is not a totally reliable assay for GA transport. It is possible that N6 is specifically acting on GA_4 transport. Such specificity is uncommon in chemical screens although it was observed by Park et al. (2009) with their discovery of the ABA transporter. As most of the forms of C19-GA stipulated to be transported have a similar core structure you would expect there to be a generic transporter for all forms, as observed in auxin transport, with LAX3 transporting both IBA and IAA (Xu et al., 1997; Galweiler et al., 1998)*, although some auxin transporters display structural specificity.* In *pin2* mutants (plants lacking an IAA efflux transporter) basipetal transport of IBA is unaffected and mammalian cells expressing *PIN2* and *PIN7* do not transport IBA (Poupart and Waddell, 2000; Zolman et al., 2000; Ruzicka et al., 2010). Similarly IBA does not competitively inhibit deuterium labelled IAA uptake in Xenopous cells expressing *AUX1* (Xu et al., 1997), again suggesting distinct carriers for the two auxins. It is therefore possible that there are multiple transporters of GA; with N16 inhibiting a transporter showing limited specificity, while N6 inhibits a more specific transporter. Furthermore in auxin transport the efflux carriers display more specificity in the forms of auxin transported than the influx proteins. It is possible that in GA transport also there are separate transporters for import and export. If this is the case N16 and N6 could be blocking different types of transporters.

Any further test on the effect of N6 on GA transport would first require a more in depth analysis of the chemicals bioactivity. It has previously been shown that over a 24 hour

period that 1 µM to 10 µM N6 had a negligible effect on root elongation whilst 50 µM completely stopped root elongation (Figure 3.8). Additionally, N6 at 50 µM prevented GA mediated DELLA degradation (Figure 3.13.). Both these results indicate that N6 is bioactive at a concentration between 10 and 50 µM. To identify the exact concentration of bioactivity a germination assay could be performed at varying concentrations.

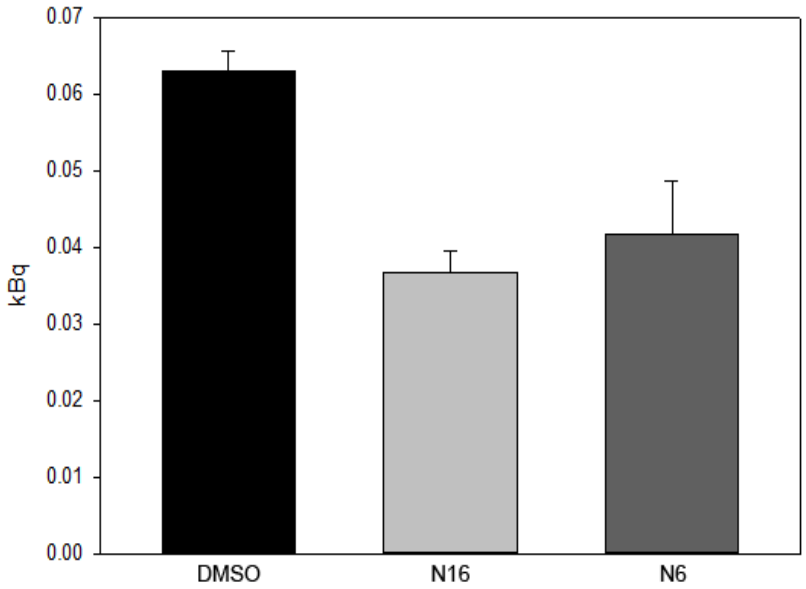


Figure 5. 9. [^{3}H]GA$_4$ uptake in Col-0 roots cultured with N16 and N6. Both N16 and N6 inhibit uptake of GA into roots. Four-day-old Col-0 seedlings were transferred to dH$_2$O with 10 kBq of [^{3}H]GA$_4$ (equating to 1 e^{-11} M of GA) containing DMSO: DMSO (N16 mock), N16 (30 µM) or N6 (10 µM). GA$_4$ uptake was measured by scintillation counting. Error bars show standard errors. Background reading counts were 0.002 kBq.

5.2.4. OPT6 a potential GA transporter

Microarray data for Arabidopsis roots treated with GA identified *At4g22730* as being downregulated (S. Thomas, unpublished data). *At4g22730* encodes an oligopeptide transporter (OPT6) which is thought to be expressed at high levels throughout the plant during its lifecycle (http://bar.utoronto.ca). *OPT6* was first identified as part of a

9-membered oligopeptide transporter gene family (*OPT1-9*) by screening the GenBank database for genes with sequence similarity to *Candida albicans OPT1 (Koh et al., 2002). This study also showed that OPT6 was predominantly expressed in the floral tissue and possessed the ability to transport the peptide KLLLG. Further expression analysis in Arabidopsis identified OPT6 expression in the* embryonic cotyledons prior to root radicle emergence, the post-germinated seedling and the ovules (Stacey et al., 2006), all tissues that require GA for development. Transport analysis in *Xenopus laevis* oocytes demonstrated OPT6 has the ability to transport peptides up to ten amino acids in length, including many tetra- and pentapeptides (Pike et al., 2009). The specificity of OPT6 was also demonstrated from its ability to transport reduced glutathione but not the oxidized form. With OPT6's role in the early stages of plant development (Stacey et al., 2006) and its up-regulation in response to GA treatment it was considered as a potential putative GA transporter. As GA is a diterpenoid acid and OPT6 has to date only been shown to transport peptides, GA could potentially be transported by OPT6 using a carrier peptide.

In the absence of readily available *opt6* mutant is Arabidopsis the role of OPT6 as a GA transporter was investigated using yeast-based GA uptake assay, similar to the approach used by Kanno et al., (2012) and Kang et al. (2010) or the characterisation of the ABA transporter. The *OPT6* gene was cloned into a yeast vector, which was incubated with [^{3}H]GA_4. Initially this required the amplification of the *OPT6* gene from the cDNA of seven-day old whole seedlings using forward (TAGGATCCACGATGGGAGAGATAGCAAC) and reverse (TAGAATTCCTAGAAGACGGGACAGCCTT) primers, which inserted *BamH1* and *EcoR1* sites, respectively, into the 5'- and 3'- ends of the amplified sequence. The *OPT6* clone was ligated into the *pPH3* vector, The *pPH3-OPT6* plasmid was then transformed into the YMM-ABC8 yeast strain for [^{3}H]GA_4 uptake analysis (Benton et al., 1994; Schuetzer-Muehlbauer et al., 2003). Kanno et al. (2012) reported that the ABA transporter *ABA-IMPORTING TRANSPORTER 3* (*AIT3*) can also transport GA_3 in a yeast-based assay. It was therefore decided to use *pPH3-AIT3* in the same YMM-

ABC8 yeast system (produced by Steve Thomas) as a positive control for [^{3}H]GA_4 uptake, with *pPH3* empty vector being the negative control.

The [^{3}H]GA_4 uptake assay was based on similar assays performed by Kanno et al., (2012) and Kang et al. (2010) for analysis of the ABA transporter. The YMM-ABC8 yeast strain containing *pPH3*, *pPH3-OPT6* or *pPH3-AIT3* was grown in liquid culture to an OD_{600} of 0.7 to 1.0 before being concentrated to an OD_{600} of 6.0 by centrifugation and dilution with DOB lacking tryptophan and uracil. The yeast was cultured at this concentration for 60 and 180 minutes before [^{3}H]GA_4 uptake was quantified by centrifuging yeast cells and suspending in 100% methanol before scintillation counting as with the Col-0/*ga1-3* root uptake assays. *pPH3-AIT3* and *pPH3* showed similar levels of GA uptake after both 60 and 180 minutes indicating (Figure 5.10.), in this experiment at least, that *AIT3* is not transporting GA into the cell. Yeast expressing *OPT6* showed slightly reduced levels of GA uptake when compared to both *pPH3* and *pPH3-AIT3* (Figure 5.10.) suggesting it may function as an efflux transporter of GA. One potential approach to establish if OPT6 acts as a GA exporter would be by loading yeast cells (expressing OPT6) with [^{3}H]GA_4 and then analysing its export into the media. Generating an OPT6 inducible expression construct would allow greater control in monitoring the export activity from the yeast strains loaded with [^{3}H]GA_4. However, if the rapid down-regulation of *OPT6* after GA application observed in the microarray data is part of the GA homeostasis mechanism, this would be inconsistent with its function as an efflux transporter, as cells with high levels of GA would be expected to export excess hormone to maintain GA levels.

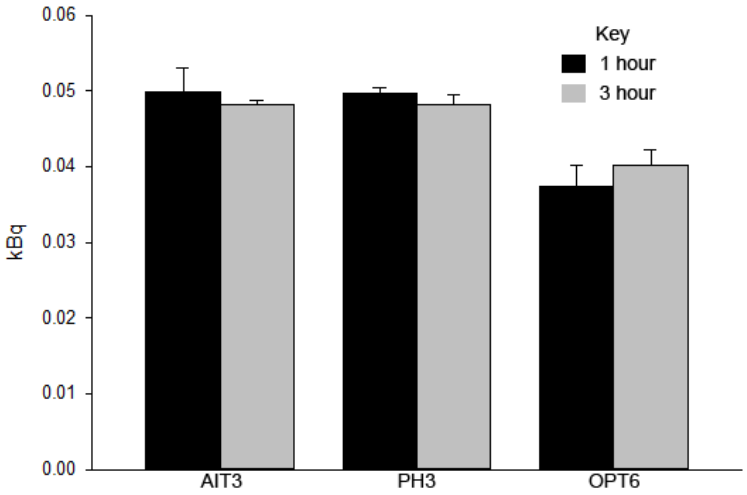


Figure 5. 10. GA uptake by yeast strains expressing the putative GA transporter *OPT6*.

Yeast stain YMM-ABC8 expressing the putative GA transporter pPH3-*OPT6, pPH3-AIT3* (positive control) and *pPH3* (negative control) cultured in S.D. –ura –trp containing 5 kBq of [^{3}H]GA_4 (0.5e^{-11} M of GA_4). Yeast was cultured for 1 (black) and 3 (grey) hours before GA uptake was assessed by liquid scintillation counting. Error bars show standard error from three replicate incubations. Background reading counts were 0.002 kBq.

Due to the anion trapping of GA by cells it is possible that over a 60 min period the yeast cells in these assays become saturated for GA by purely diffusion alone. O'Neill et al. (1986) have shown that cowpea vesicles become saturated for GA after one hour. This result means that any experiment assessing GA uptake is time critical. Additionally Kang et al. (2010) established that uptake of ABA by the PDR transporters was rapid (within 15 seconds). To ascertain if this was the case for GA uptake we measured [^{3}H]GA_4 uptake after 1, 30 and 60 minutes with *pPH3* and *pPH3-AIT3* (Figure 5.11). The results showed no difference between *pPH3* and *pPH3-AIT3* at any of the time points (Figure 5.11). Additionally cells were not shown to be saturated for GA within 60 mins suggesting an anion trap did not exist in Figure 5.10. The failure to obtain enhanced uptake by yeast expressing *pPH3-AIT3* may indicate that the assay conditions are not optimal and require improvement. Firstly as the yeast strains grow at different rates the final number of cells at the end of the

experiment may vary (Bergman, 2001). Kanno et al. (2012) overcame this problem by dividing the amount of ABA taken up by the final OD of the culture. Secondly due to time constraints the concentration of GA_4 (1 µM) used in the assay was not optimised. For yeast-2-hybrid assay comparing the interaction of GID1 with RGA in the presence of GA_3 100 µM of GA_4 is applied, which is around 100 times more than is required to produce a phenotype in Col-0 roots (Griffiths et al., 2006a; Griffiths, 2007), suggesting that higher levels of GA may be required to produce a response by the OPT6 transporter. Additionally the high starting OD_{600} of the yeast culture results in the precipitation out of the yeast during the assay resulting in some yeast cells not being constantly in contact with the [^{3}H]GA_4 solution.

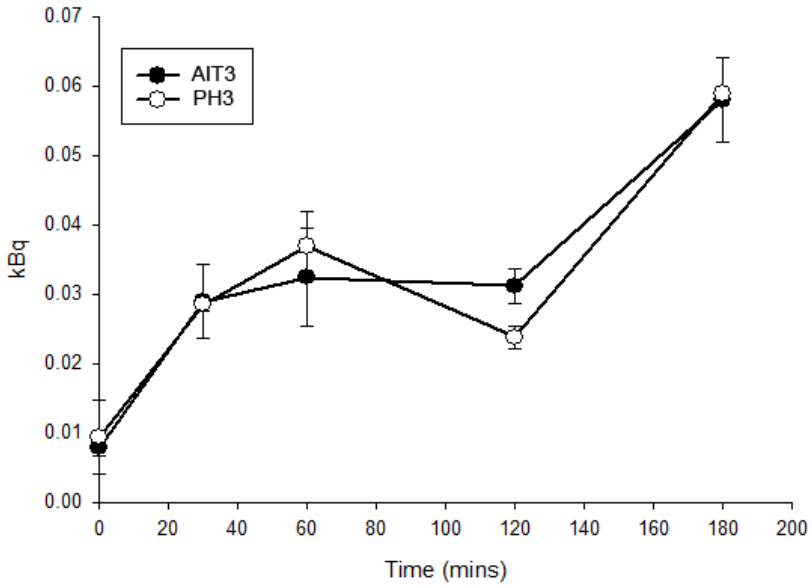


Figure 5. 11. GA uptake over time by yeast transformed with *pPH3* and *pPH3-AIT3*.

The yeast stains, *pPH3-AIT3* expressing a known GA importer (positive control) and the empty vector *pPH3* (negative control), were cultured in S.D. –ura –trp containing 5 kBq of [^{3}H]GA_4 ($0.5e^{-11}$ M of GA_4) for 1, 30, 60, 120 and 180 mins before GA uptake was assessed by liquid scintillation counting. Medium was conditioned prior to experiment. Error bars show standard error of three replicates. Background reading counts were 0.002 kBq.

5.3. CONCLUSIONS

The results of the [^{3}H]GA_4 assays with Col-0 and *ga1-3* seedlings presented in this chapter indicate that the chemical N16 reduces the uptake of GA by the roots. This confirms the result of an assay carried out at the University of San Diego showing that N16 blocked the uptake and transport of Fl-GA_3 into the endodermis of Col-0 roots. Analysis of root and hypocotyl growth suggested that by blocking GA transport N16 inhibits root elongation but has no effect on hypocotyl growth. Interestingly N16 only induced a reduction of root elongation in Col-0 after 5 days but reduced root elongation in *ga1-3* after 24 hours, indicating that it may take 4 to 5 days for the pool of GA in Col-0 root cells to be depleted when GA transport is blocked. Another chemical identified in the chemical screen, N6, was shown to have no effect on Fl-GA_3 uptake but appeared to block uptake of [^{3}H]GA_4 indicating that there may be separate transporter for different forms of GA.

Despite microarray data indicating that OPT6 was rapidly down-regulated by GA and Kanno et al. (2012) showing that the ABA transporter AIT3 can also transport GA there was no difference in [^{3}H]GA_4 taken up by yeast strains expressing these transporters from the negative control. Ideally this assay needs to be optimised in order to replicate the data observed by Kanno et al. (2012), unless the results described in this paper are not reproducible. Before the potential function of OPT6 as a GA transporter can be properly tested or to identify potential targets for N16 suitable controls need to be identified.

CHAPTER 6. GENERAL DISCUSSION.

Using a chemical screen of GA signalling this project managed to generate novel insight into the GA signalling pathway's homeostatic feedback regulation of GA biosynthesis, auxin transport and potentially identified a novel GA transporter (results are summarised in Table 6.1.).

Table 6. 1. Summary of results.

Summary of results obtained during thesis. Information shown: chemical ID (Chem), Effect of chemical on GA-mediated down regulation of GA20ox1 (all chemicals blocked this), Concentration at which chemical blocked GA mediated down regulation of GA20ox1 (Active conc.), if chemical inhibited root elongation over a 24 hour period (Effect on root elongation), other information obtained on action of chemical (Other information), likely site of action of the chemical.

Chem	Effect on GA mediated downreg of GA20ox1	Active conc. (μM)	Effect on root elongation	Effect on GA-mediated DELLA degradation	Other information	Likely site of action
N1	Blocks	50	Inhibited	NA		
N2	Blocks	10	Inhibited	Stabilised		Upstream of DELLA protein
N3	Blocks	10	None	Stabilised		Upstream of DELLA protein
N4	Blocks	10	Inhibited	Degraded		Downstream of DELLA protein
N5	Blocks	10	Inhibited	Stabilised		Upstream of DELLA protein
N6	Blocks	10	Inhibited	Stabilised		Upstream of DELLA protein
N7	Blocks	5	Inhibited	Stabilised		Upstream of DELLA protein
N8	Blocks	10	Inhibited	Degraded		Downstream of DELLA protein
N9	Blocks	5	None	Degraded		Downstream of DELLA protein
N10	Blocks	5	Inhibited	Stabilised		Upstream of DELLA protein
N11	Blocks	5	Inhibited	Stabilised		Upstream of DELLA protein
N12	Blocks	10	Inhibited	Stabilised		Upstream of DELLA protein

N13	Blocks	10	Inhibited	Degraded		Downstream of DELLA protein
N14	Blocks	50	Inhibited	Degraded		Downstream of DELLA protein
N15	Blocks	50	Inhibited	Degraded		Downstream of DELLA protein
N16	Blocks	10	None	Stabilised	Blocks GA uptake	GA transport
N17	Blocks	10	Inhibited	Degraded		Downstream of DELLA protein
N18	Blocks	10	Inhibited	Degraded		Upstream of DELLA protein
N19	Blocks	50	Inhibited	Stabilised		Upstream of DELLA protein
N20	Blocks	5	Inhibited	Stabilised		Upstream of DELLA protein
N21	Blocks	10	Inhibited	NA		
N22	Blocks	10	Inhibited	Stabilised		Upstream of DELLA protein
N23	Blocks	10	Inhibited	Degraded	induces *aux1* like phenotype	auxin transport
N24	Blocks	10	Inhibited	Stabilised		Upstream of DELLA protein
N25	Blocks	10	Inhibited	NA		
N26	Blocks	10	Inhibited	Degraded		Downstream of DELLA protein
N27	Blocks	10	None	NA		
N28	Blocks	10	Inhibited	Degraded		Downstream of DELLA protein

Using the *GA20ox1::GUS* reporter line an initial screen at VIB in Ghent identified 28 commercially available chemicals were identified as preventing the GA-mediated downregulation of GUS activity (Figure 3.4.). The research in this project confirmed the effect of these chemicals on *GA20ox1::GUS*. Whilst the limited time frame of the project did not allow for the target identification of all the 28 chemicals they do present useful tools for identifying novel molecular components that are regulating GA biosynthesis. Performing a genetic suppressor screen is one potential approach to identify the targets of these chemicals. Such a screen would involve screening a mutagenized population for mutants which rescue growth defects under the chemical treatment. Clearly, for such a screen it is necessary that the chemical treatment

produces a phenotype. From the 28 chemicals identified in the initial screen 15 inhibited root elongation, a GA controlled developmental process (Figure 3.8.-3.11.). Therefore a potential screen would involve the identification of mutants that showed enhanced root growth in the presence of the compound. From this starting point the mutant gene could be cloned and functional studies performed to identify the function of the chemical.

When the effect of nine chemicals on the expression of the endogenous *GA20ox1* gene were analysed, four of the compounds did not inhibit the GA-mediated transcriptional downregulation (Figure 3.7.). This observation raises the intriguing possibility that these four chemicals are not maintaining *GA20ox1::GUS* staining in the presence of GA by altering *GA20ox1* gene expression but are instead enhancing the stability of the GA20ox1 enzyme. Such a result would indicate a novel process whereby GA regulates GA metabolism by directly targeting the enzymes of the pathway for degradation. Previous work by our group has shown that GA application results in the loss of fluorescence in the Arabidopsis *ga20ox1,2,3:SCR::YFP::AtGA20ox1* line, further suggesting that there is some GA mediated regulation of GA20ox1 (Barker, 2011) To confirm the effect of these four compounds it will be necessary to directly analyse the effect of GA signalling on GA20ox1 protein levels using western blotting. Initially, the stability of the GA20ox1-GUS fusion protein could be monitored in the *GA20ox1::GUS* line using commercially available GUS antibodies. However, it will also be necessary to confirm this effect on the endogenous GA20ox1 enzyme, which will require specific antibodies to be raised against this protein. Rice mutants lacking the GA20ox gene (*sd-1*) have been instrumental in increasing crop yields since the 1960s (Harberd et al., 1999). This has been achieved through their effects on reducing stem height which prevents lodging of the crop and allows the application of higher levels of nitrogen fertiliser (Harberd et al., 1999). If these chemicals do affect novel components that target the degradation of GA biosynthetic enzymes, they represent a new chemical target for commercially fine tuning the stem height of agricultural plants to maximise yields. A recent study

demonstrated that DELLA proteins regulate the direction of microtubule orientation via their interaction with a protein (prefoldin), a process that does not involve the control of gene expression (Locascio et al., 2013). It is therefore possible that DELLA proteins have a non-transcriptional role in the GA control of GA biosynthesis enzymes stability. To establish the role of DELLA proteins in this process, western blots of the GA20ox1 protein and qRT-PCR analysis of *GA20ox1* could be performed in Arabidopsis mutants lacking DELLA proteins (*rga-24* and *gai-t6* mutants which lack RGA and GAI) and GA-insensitive lines with high DELLA protein content (*gai-1*, gain of function GAI mutant). Such research may provide insight into how the chemicals maintaining *GA20ox1::GUS* staining but not blocking GA mediated DELLA protein degradation are working.

The *Rht-1* wheat mutants, along with the *sd-1* mutants, were a major component of the massive yield increases obtained during the green revolution (Harberd et al., 1999). Later studies identified the *Rht-1* mutants as lacking a degradable DELLA protein (Peng et al., 1999; Ashikari et al., 2002; Sun and Gubler, 2004; Pearce et al., 2011). For nearly two decades it has been known that DELLA proteins mediate the transcriptional feedback control of GA biosynthesis and are therefore essential to GA's control of plant development. This project identified 11 chemicals were shown to block GA-mediated RGA degradation (Figure 3.13.-3.16.). Five (N6, N7, N8, N11 and N20) of these compounds are potentially achieving this by blocking interaction of the DELLAs with GID1 or SLY1 and thus conceivably blocking SCF^{SLY1}-mediated ubiquitination (Figure 3.18.). Such chemicals maintaining DELLA protein levels in the presence of GA again present potential tools for agriculture in controlling stem height, and other developmental processes. Another 11 chemicals identified in this project had no effect on DELLA degradation indicating they are affecting potentially novel proteins acting downstream of the DELLA proteins degradation (Figure 3.13.-3.16.). These protein targets potentially present novel breeding targets for plant breeder to control aspects of development. These 11 chemicals also have the possibility to function as plant growth regulators targeting specific GA regulated processes as

opposed to chemicals blocking DELLA degradation which would have a more global effect on the plant. Whilst most these chemicals do have potential uses in agriculture as either chemical treatments or discovery of breeding targets considerable work is required to identify the protein targets of each chemical. To develop these chemicals a plant growth regulators would require the identification of their active site, their effect on commercial crops, toxicity and production method.

The chemical screen used in this project not only identified numerous chemical with potential commercial agricultural use but also identified chemicals which could be used as tools in fundamental plant research. During the assessment of the chemicals effect on root elongation it was noticed that one chemical treatment (N23) induced root agravitropism. Further studies identified that this chemical was potentially acting on auxin transport but was unlikely to be acting on auxin signalling. Interestingly, this chemical did not affect the transcriptional regulation of the endogenous *GA20ox1* gene (Figure 3.7.), suggesting that it may alter protein stability instead. This provides a potentially novel mechanism by which auxin interacts with the GA signalling pathway. Such a chemical represents an ideal tool for the study of the mechanism of GA-auxin crosstalk, a poorly understood process.

Another chemical (N16), which blocked RGA degradation but had no effect on GAI's interaction with SLY1 or GID1, was identified as blocking the uptake of both Fl-GA_3 and [^{3}H]GA_4 into Col-0 roots (Figure 5.1. and 5.8.). Microarray data identified OPT6 as a putative GA transporter with GA uptake assays in yeast expressing OPT6 suggesting OPT6 functions as a GA exporter (Figure 5.10.). It is therefore possible that N16 could be used for the study of this novel GA transporter and AIT3, the other reported GA transporting protein, or alternatively N16 could be a tool for identifying new novel GA transporters. As AIT3 transports both GA and ABA it is conceivable the N16 could be used to identify novel transport proteins of numerous other hormones.

In summary, this project characterising and analysing 28 chemicals identified in a chemical screen of GA signalling has identified numerous chemicals that alter DELLA protein degradation and therefore have potential as agricultural chemical regulators of stem height. In addition two chemicals could provide useful tools in the study of two poorly understood processes; GA and auxin crosstalk (N23), and GA transport (N16).

CHAPTER 7. REFERENCES

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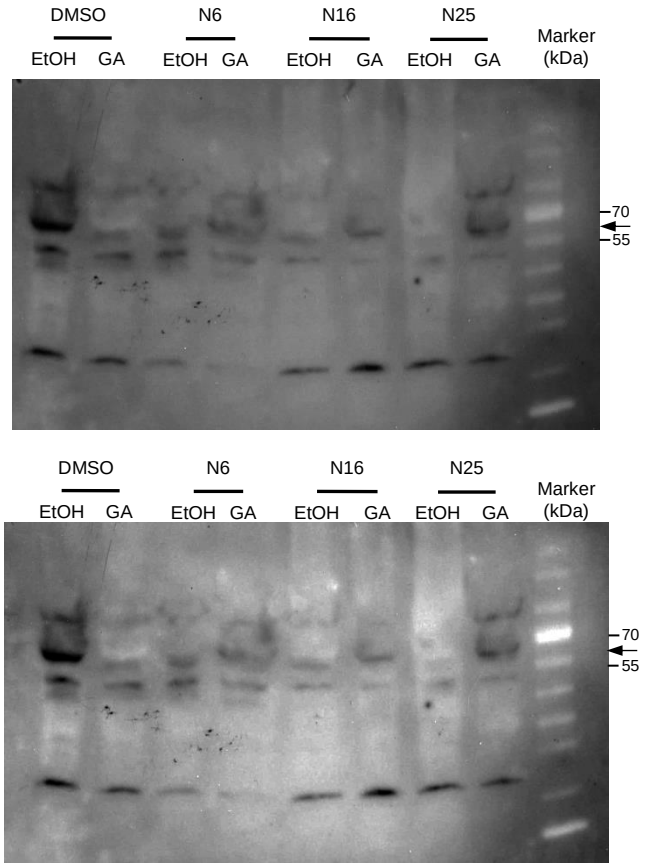
CHAPTER 8. APPENDIX.



Appendix 1. The effect of three chemicals on GA mediated RGA degradation in a western blot using anti-RGA antibodies – full western blots used to make Figure 3.17.
Preliminary data (n=2) showing levels of RGA in plants treated with chemical and GA or ethanol (EtOH, mock GA treatment). Both western blots shown.The blot contained 25 ug of total protein extracted from four-week old root cultures in liquid Gamborg B5 treated with either DMSO (-ve control), N6, N16 or N25 at 50 µM for 24 hours before application of ethanol (EtOH) or 10 µM GA_4 10 µM. A rabbit anti-RGA antiserum and a goat anti-rabbit IgG were used as primary and secondary antibodies, respectively. Protein marker shows bands at 70 and 55 kDa and arrow displays expected position of RGA protein band.