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Exploring plant hormonal signalling through chemical perturbation

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Submitted for the Degree of Doctor Philosophy by Research

Biosciences Department

February 2018

“All progress takes place outside the comfort zone.”

-Michael John Bobak

“The important thing is not to stop questioning. Curiosity has its own reason for existing.”

-Albert Einstein

Abstract

Plant growth and development is tightly regulated by a set of plant hormones that includes abscisic acid (ABA) and gibberellic acid (GA). Understanding how this is achieved is challenging due to a complex interplay between the various signalling pathways involved. A chemical genetics approach was used in this study to explore this hormonal crosstalk with a focus on plant growth. A small library of analogues, modified from the calmodulin inhibitor W7, was generated and the effect of these compounds was tested in root growth assays. One particular compound, eW5, was identified as providing enhanced and prolonged root growth even when plants were subsequently removed from the compound. Further analysis suggested that the phenotype induced upon eW5 application was due to modification of DNA methylation. Therefore it was hypothesized that eW5 might affect gene expression, which was tested using a RNA-seq experiment. Results from this suggested that eW5 regulates hormone signalling pathways, with a particular positive correlation to the GA signalling pathway observed.

Interestingly, eW5 binds to the ABA receptor PYR1, thus potentially functioning as an ABA antagonist and also promotes DELLA (a negative regulator of plant growth) degradation, in a similar fashion to that observed with GA. Further work was performed to investigate the effect of eW5 on ABA and GA independent pathways. In ABA signalling, eW5 showed inhibition of some ABA responses such as stomatal opening, however no recovery in PP2C phosphatase activity suggests that it does not promote growth by inhibiting ABA perception. In addition, due to the specific GA-mediated response in hypocotyl growth, the eW5 effect was further explored in this particular process. With regard to eW5's potential role in GA signalling, it was found to enhance sensitivity to GA that leads to DELLA protein degradation and growth promotion.

Moreover, it was suggested that the promotion of eW5 in stomatal opening occurs through GA signalling.

Having established the positive effect of eW5 on root and hypocotyl growth and stomatal opening, a further small library of analogues of eW5 was generated to further explore its mode-of-action. The position of sulfonamide was identified as a potential site that is responsible for hypocotyl growth promotion.

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Statement of Authorship

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Statement of Copyright

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List of abbreviations

The standard scientific conventions for protein and gene naming have been followed: wild type genes and proteins are in capitals and mutants are denoted by lower case, gene names are italicized whereas protein names are not.

Standard scientific abbreviations have been used for units of weight, length, amount, molarity, temperature and time.

Standard chemical element symbols, nucleic acid and amino acid codes are used.

ABA	Abscisic acid
GA	Gibberellic acid
JA	Jasmonic acid
SA	Salicylic acid
ACC	1-aminocyclopropane-1-carboxylate
PIN	PINFORMED
START	Steroidogenic acute regulatory related lipid transfer
Y2H	Yeast two hybrid
ABI	ABA INSENSITIVE
RCAR	Regulatory component of ABA receptor
HAB	HOMOLOGY TO ABI1
PYR	PYRABACTIN RESISTANCE
PP2Cs	Protein phosphatase type C
SnRK	SNF1-related protein kinases
AMPK	5' adenosine monophosphate-activated protein kinase
ABF	ABA-responsive element binding factors
ROS	Reactive oxygen species
GGDP	Geranylgeranyl diphosphate
TPs	Terpene synthase
CPS	ent-copalyl diphosphate synthase
KS	ent-kaurene synthase
KO	ent-kaurene oxidase

KAO	ent-kaurenoic acid oxidase
2-ODD	2-oxoglutarate-dependent dioxygenases
GA20ox	GA 20-oxidase
GA3ox	GA 3-oxidase
PIF	PHYTOCHROME INTERACTING FACTOR
SLN	SLENDER
RGA	REPRESSOR OF GIBBERELLIC ACID
GAI	GA-INSENSITIVE
RGL	RGA LIKE
SLY	SLEEPY
bHLH	Basic helix-loop-helix
phyB	Phytochrome B
PGP19	P-glycoprotein19
BIN	BRASSINOSTEROID INSENSITIVE
BES	BRI1-EMS-SUPPRESSOR1 (BES1)
BZR1	BRASSINAZOLE RESISTANT1
GSK	GLYCOGEN SYNTHASE KINASE
TAA1/TARs	TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1/TRYPTOPHAN AMINOTRANSFERASE RELATEDs
EIN3	ETHYLENE INSENSITIVE3
EBF	EIN3 BINDING F-BOX PROTEIN
MES	2-(N-morpholino)-ethane-sulfonic acid
TSA	Thermal shift assay
MST	MicroScale Thermophoresis
pNPP	para-nitrophenylphosphate
CaM	Calmodulin
CBK	CaM-binding protein kinase
HSF	Heat shock transcription factors
MLCK	Myosin light-chain kinase
HDA	Histone deacetylase
DNMT	DNA methyltransferase
RAPD	Random Amplified Polymorphic DNA

5-mdC	5-methyldeoxycytidine
5-AC	5-aza-29 deoxycytidine
PTMs	Post-translational modofocations
bZIP	Basic leucine zipper
ABRE	ABA-responsive promoter element
DREB	dehydration-responsive element binding protein

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First and foremost, I would like to express my deepest gratitude to my awesome pair of supervisors, Prof. Marc Knight and Prof. Patrick Steel for their unlimited support, untiring encouragement, constructive criticisms, help and support throughout my entire PhD journey. Their guidance and encouragement has helped me to grow as a research scientist. I have very much enjoyed working with them and could not ask for better supervisors.

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CHAPTER 1

Introduction

1.1 Introduction

The main objective of this project is to explore hormone signalling pathways in plants through perturbation with small molecule. This thesis will introduce the series of chemicals that has been tested, followed by the investigation of these compounds in hormonal signalling pathways based on the phenotype observed. This chapter will discuss plant hormones in detail focusing particularly on gibberellic acid and abscisic acid, introduce what hormone signalling is: i.e. the perception and action of hormones, introduce the chemical genetics approach, and specifically the application of a chemical genetic approach to understand plant hormonal signalling.

1.2 Hormones in plant growth

Plant growth and development involves the integration of many environmental signals and growth regulators, called plant hormones (Gray, 2004). Further to this, as sessile organisms, plants need to survive by adjusting various biological activities when encountering biotic and abiotic stresses. Plant survival under stress conditions requires morphological and physiological adaptations. During these situations, plant hormones cooperate to modify biological responses for the formation and maintenance of plant stress tolerance (De Smet et al., 2015).

Plant hormones including gibberellic acid (GA), abscisic acid (ABA), cytokinin, ethylene and brassinosteroids regulate many aspect of plant growth and development at relatively low concentrations (Rigal et al., 2014; Gray, 2004). Cytokinin, auxin, GA and brassinosteroids are considered essential for plant growth, as gauged by the

phenotype of mutants with disrupted hormone biosynthesis or perception (Depuydt and Hardtke, 2011). GA promotes important processes in plant growth and development such as seed germination, cell elongation and cell division, as well as floral transition (Richards et al., 2001). Auxin and cytokinin also regulates cell elongation and cell division, and play a major role in establishment, maintenance of meristems, and apical dominance (Dello Ioio et al., 2008; Mockaitis and Estelle, 2008; Peng et al., 2009; Vert et al., 2005).

While GA, auxin, cytokinin and brassinosteroid are important in plant growth and development, other hormones like abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and ethylene are classified as plant stress hormones (Loake and Grant, 2007; Seo et al., 2001; Tuteja, 2007; Ecker and Davis, 1987). ABA, SA, JA and ethylene production are all increased when plants are exposed to stress therefore play major roles in mediating plant defense response against biotic and abiotic stress (Bari and Jones, 2009; Nakashima and Yamaguchi-Shinozaki, 2013). ABA levels are higher in response to various stresses such as drought, cold and heat stress therefore it is important for plant defense against such abiotic stresses (Lata and Prasad, 2011; Zhang et al., 2006).

With a role in plant development and plant adaptation to the stresses, hormones constitute a signalling network and regulate several signalling and metabolic systems. Often, hormones cross talk to each other in order to contribute to plant growth and development, as shown in Figure 1.1. For example, in root growth there are a lot of hormones that interact with each other, either by forming a synergistic effect or antagonising each other during plant development. ABA and GA always antagonize each other's activity especially in the case of seed germination where ABA is needed for seed dormancy to avoid germination of the seed until the seeds are ready to germinate based on the environment conditions. A higher ratio of GA:ABA will promote seed germination (Kermode, 2005). Auxin and brassinosteroid act synergistically in

cell elongation and cell division, vascular differentiation, senescence, reproductive development, lateral root development, and hypocotyl elongation (Bao et al., 2004; Nemhauser et al., 2004; Hardtke et al., 2007).

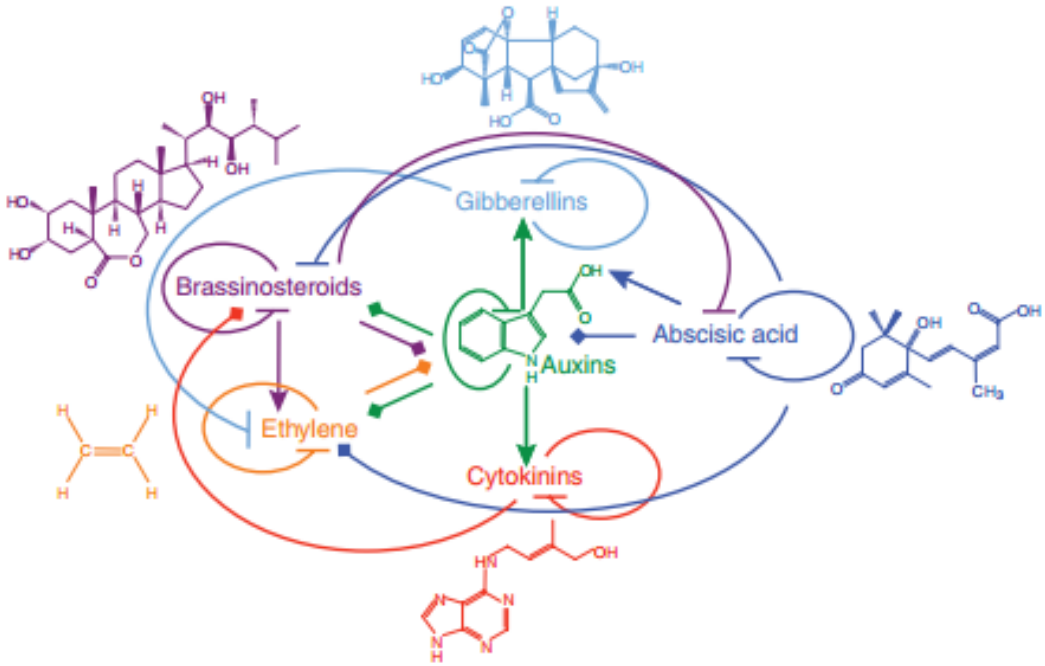


Figure 1.1: The interaction between hormones in plant development. The arrow heads and blocked arrows show the upregulation and downregulation of hormone biosynthetic pathway, respectively, whilst diamond arrows represent synergistic effects between the hormones. Figure is taken from Jaillais and Chory (2010).

Hormone interaction can involve changes in hormone level or response. Several genes required for auxin biosynthesis are under transcriptional control by ethylene (Stepanova et al., 2005; Stepanova, 2008; Tao, 2008), while auxin can influence ethylene biosynthesis by inducing expression of genes encoding ACC synthase (Tsuchisaka and Theologis, 2004). During root development, auxin promotes lateral root initiation while cytokinin opposes this response. Cytokinin influences the expression of the PINFORMED (PIN) auxin efflux carrier genes. By reducing PIN expression, cytokinin disrupts local auxin gradient formation in lateral root founder, thereby inhibiting lateral root initiation (Laplaze, 2007).

Hormonal signalling pathways are also known to interact at the level of gene expression. For example, studies show that there is significant overlap between auxin- and brassinosteroids- responsive gene sets. Generally, common target genes repressed by auxin are also repressed by brassinosteroids, and genes induced by auxin are also induced by brassinosteroids, which suggests coordination between the signalling pathways (Goda, 2004; Nemhauser et al., 2004).

1.2.1 Abscisic acid (ABA)

ABA is a sesquiterpenoid molecule, one of plant hormones that regulates diverse processes including seed germination, dormancy, and seedling growth (Finkelstein, 2002). ABA is also a central regulator of plant adaptation to biotic and abiotic stress (Fujita et al., 2006; Zhu, 2002; Cutler et al., 2010). Under stressful environmental conditions such as water shortage, high salinity and temperature extreme, the ABA content is increased, stimulating stress-tolerance effects that help plants adapt and survive under those condition. Under drought or osmotic stress, ABA promotes stomatal closure which prevents water loss through transpiration (Ng et al., 2014).

1.2.1.1 ABA receptor

There are a three different classes of ABA receptor; G-protein coupled receptors (GCR2, GTG1/2) (Liu et al., 2007; Pandey et al., 2009), H-subunit of Mg-chelatase (ABAR) (Shen et al., 2006) and PYR/PYL that belong to steroidogenic acute regulatory related lipid transfer (START) superfamily (Park et al., 2009). Among these classes, START group are the most recently discovered and widely studied. The identification of PYR/PYLs ABA receptors was achieved through two independent studies, using yeast two hybrid (Y2H) and a chemical genetics approach. In the Y2H approach, ABA receptor was identified as a protein that interacts with *Arabidopsis* protein phosphatase, ABA INSENSITIVE 2 (ABI2), a negative regulator in ABA signalling (Ma et al., 2009). Through this approach, one of proteins identified is regulatory component

of ABA receptor 1 (RCAR1), which was identified to have a similar sequence to PYL9 (Ma et al., 2009). The interacting Y2H partners of another protein phosphatase, HOMOLOGY TO ABI1 (HAB1) were also discovered to be PYL5, PYL6 and PYL8, receptors that belong to START protein family (Santiago et al., 2009).

The other approach was using a chemical genetics approach, involving the application of a small molecule that helped in dissecting protein function. This approach identified mutations in the PYR1 gene leading to insensitivity to the synthetic ABA agonist pyrabactin, based on the screening for mutants that able to germinate in the presence of pyrabactin (Park et al., 2009). Thirteen PYR1-LIKE (PYL) proteins encoded in *Arabidopsis* genome were identified by sequence analysis (Park et al., 2009; Klingler et al., 2010). Simultaneous discovery of these particular ABA receptors using two very different approaches have created two name sets of this family as shown in Table 1.1.

Table 1.1. Nomenclature of ABA receptor

Nomenclature	
PYLs	RCAR
PYR1	RCAR11
PYL1	RCAR12
PYL2	RCAR14
PYL3	RCAR13
PYL4	RCAR10
PYL5	RCAR8
PYL6	RCAR9
PYL7	RCAR2
PYL8	RCAR3
PYL9	RCAR1
PYL10	RCAR4

PYL11	RCAR5
PYL12	RCAR6
PYL13	RCAR7

Analysis of the *Arabidopsis* genome indicates the presence of 14 members of the PYR/PYL/RCAR gene family with high homology at the amino acid sequence level (Figure 1.2). All members encode small proteins, between 159 and 211 amino acid residues and this receptor family is homologous to the Bet v 1-fold superfamily, which is characterized by the presence of the START domain (Ponting and Aravind, 1999; Iyer et al., 2001; Klingler et al., 2010). This superfamily have a seven-stranded β-sheet and two α-helices enfolding a long, carboxy-terminal α-helix, which collectively form a helix-grip fold structural motif that creates a hydrophobic ligand-binding pocket (Iyer et al., 2001; Radauer et al., 2008; Santiago et al., 2012).

Figure 1.2: Amino acids sequence alignment for all member of START family. The yellow highlight indicate the conserved region for all the member, except for PYL13. Magenta domain represent the domain that responsible PYL13-PP2C binding and the cyan colour shows the unique residue of PYL10 and PYL13. The figure was modified from Li et al., 2013.

Biochemical and structural data indicates that ABA receptors can be distinguished on the basis of their oligometric states; dimeric conformation (PYR1,

PYL1, PYL2) and monomeric receptors (PYLs 4-12) (Hao et al., 2011; Dupeux et al., 2011). The classification based on this conformation can be seen in Figure 1.3. Dimeric receptors display lower affinities for ABA than the monomeric forms, with the binding constant of >50 μM, while the K_d value for monomeric receptors are 1 μM (Santiago et al., 2009, Miyazono et al., 2009).

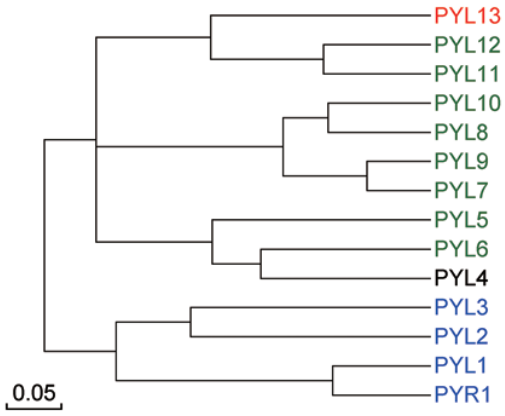


Figure 1.3: The classification PYR/PYLs ABA receptors based on phylogenetic tree analysis. The receptors classified in green colour can interact with PP2Cs in the absence of ABA, while receptors in blue colour are ABA-dependent to interact with PP2C.

The mutation of a single receptor does not show any reduction in ABA response due to genetic redundancy. However, Antoni et al. (2013) has discovered that a *pyl8* mutant is ABA-insensitive in terms of root growth inhibition after ABA treatment. This finding suggests that root growth is mainly regulated by PYL8. Triple *pyr1/pyl1/pyl4* and quadruple *pyr1/pyl1/pyl2/pyl4* mutants were found to reduce sensitivity in germination and root growth in response to ABA (Park et al., 2009). Furthermore, quadruple mutants also reduced the ABA-sensitivity in stomatal closure and ABA-induced gene expression (Nishimura et al., 2010; Park et al., 2009). More recently, the mutation of 6 receptors, *pyr1/pyl1/pyl2/pyl4/pyl5/pyl8* showed dramatic ABA insensitive effect in seed germination, root growth and the stomatal closure showing that there is a redundancy of the receptor's function (Gonzalez-Guzman et al., 2012).

While the mutation of the receptors causes and ABA-insensitive phenotype, the over expression of PYL5 causes a higher sensitivity to ABA-mediated seed

germination inhibition and root growth inhibition. Such a transgenic line was found to enhance drought tolerance by reducing water loss under drought stress condition (Santiago et al., 2009). This suggested that these receptors are responsible in controlling ABA signalling and physiology (Gonzalez-Guzman et al., 2012).

1.2.1.2 ABA signalling pathway

The main components in the ABA signalling pathway are its positive regulators, SNF1-related protein kinases (SnRK2) and its negative regulators, Clade A protein phosphatase type C (PP2Cs) and the ABA-responsive element binding factors (ABFs). There are 76 genes encode PP2Cs in *A. thaliana* which are divided into 10 groups (A-J), where members of Group A have roles in ABA signalling pathway (Schweighofer et al., 2004). The phosphatases that belong to this group include ABA insensitive 1 (ABI1), ABI2 (homolog to ABI1), hypersensitive to ABA1 (HAB1) and HAB2 are members of the group A (Saez et al., 2004; Schweighofer et al., 2004; Yoshida et al., 2006). PP2Cs act as negative regulators of ABA signalling, supporting by the finding that *abi1-1* and *abi2-1* mutants show ABA insensitivity in seed germination and root growth responses (Leung et al., 1997; Rodriguez et al., 1998; Umezawa et al., 2010). *hab1-1* mutants showed enhanced ABA responsive gene expression, enhanced ABA-mediated stomatal closure, and ABA hypersensitivity that leads to the inhibition of seed germination and growth (Saez et al., 2004). The phenotype of ABA hypersensitivity can also be observed in loss-of-function mutants of PP2Cs, supporting a role as major negative regulators of ABA (Umezawa et al., 2010; Hirayama and Shinozaki 2007).

SnRKs are serine/threonine protein kinases classified under the SNF1 family, which also includes yeast SNF1 kinases and AMPKs from mammals. SnRKs can be divided into three classes: SnRK1, SnRK2 and SnRK3 (Kulik et al., 2011), with SnRK2 and SnRK3 kinases are involved in plant development and environmental stress signalling (Boudsocq and Lauriere, 2005; Coello et al., 2011). Three members of

SnRK2 subgroup III namely SnRK2D/SnRK2.2, SnRK2.6/OST1 and SnRK2.3 are major regulators of plant responses to ABA. SnRK2.6/OST1 are expressed in guard cells leading to stomatal closure in the presence of ABA, while SnRK2.2. and SnRK2.3 are expressed in seeds and vegetative tissues (Yoshida et al., 2002; Yoshida et al., 2006; Fujita et al., 2009). In addition, *snrk2.6* mutants show severely impaired stomatal regulation and lose water rapidly (Yoshida et al., 2002; Mustilli et al., 2002). The SnRK2.2/2.3/2.6 triple mutants are insensitive to ABA, showing impaired in growth and reproduction, as well as losing drought stress tolerance. These phenotypes suggesting that most of the molecular actions of ABA are triggered by SnRK2-mediated phosphorylation of substrate proteins (Wang et al., 2013).

To activate the ABA signalling, all of the core components of ABA signalling interact with each other; discovered through co-immunoprecipitation experiments (Nishimura et al., 2010). Consistent with this, studies from Ma et al. (2009) and Santiago et al. (2009) also indicated that the interaction between all of the component in the pathway is important to activate it. In the presence of ABA, the interaction between the receptor and phosphatase protein was enhanced. ABSCISIC ACID INSENSITIVE1 (ABI1) is one of the PP2Cs and its mutation, *abi1* failed to activate ABA signalling indicating that it is important in the process.

In the absence of ABA, the activation of SnRK2 is inhibited by PP2Cs protein phosphatases. The inhibition of SNRK2 in this way prevents the signal from ABA to be transduced. In contrast, with a higher level of ABA, ABA binds to its receptor which enables them in turn to bind to, and inhibit, PP2C activity. The inhibition of these phosphatases leads to the activation of SnRKs kinases that will phosphorylate downstream effectors and induce gene expression (Figure 1.4). With this activation, the ABA signal is also activated and ABA effect can be observed in physiological responses such as the inhibition of seed germination, the breaking of dormancy, the inhibition of root length and the closure of stomatal aperture.

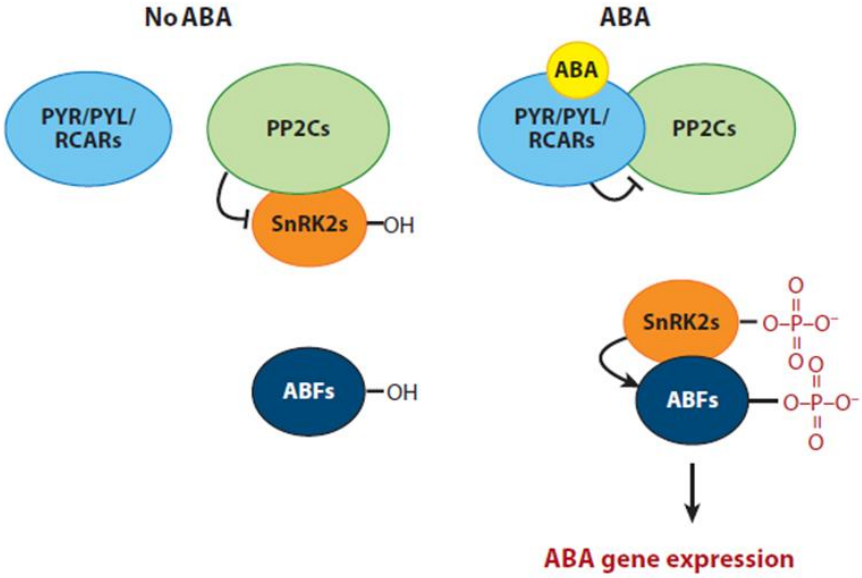


Figure 1.4: The main components and their mechanism in ABA signalling pathway. ABA signalling activation occur after the interaction between ABA and PYR/PYL/RCAR which suppresses the inhibition effect of PP2Cs on SnRK2s. The graph was taken from Cutler et al., 2010.

Structural studies have discovered a conserved gate-latch-lock mechanism that is important in ABA perception and signal transduction. The apo ABA receptor induces an open gate conformation due to its conformation that is flanked by two highly conserved loops that serve as a gate and latch. Conformational rearrangement in these two loops upon ABA binding triggers the closure of ligand entry. The closed gate conformation induces the surface that enables the receptor to interact with PP2Cs and inhibit its activity (Melcher et al., 2009; Nishimura et al., 2010). A conserved tryptophan from the PP2C active site inserts between the gate and latch and locks the receptor-PP2C complex into a closed conformation. This tryptophan also enables PP2C to sense the presence of ABA from its water-mediated interaction with ABA in the ligand-binding pocket (Figure 1.5) (Melcher et al., 2009).

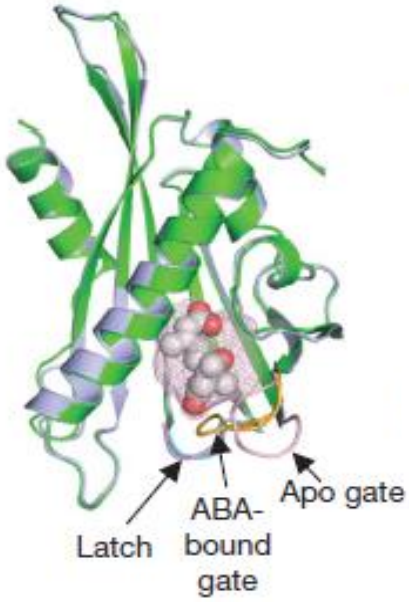


Figure 1.5: The conformation of gate-latch-lock for ABA signalling. Apo receptor leads to open gate conformation (pink) while in the presence of ABA, the closed conformation is induced (yellow). The image is taken from Melcher et al., 2009.

1.2.1.5 ABA in plant physiology

ABA has several functions in plant development. The production of ABA in plants is increased when plants are exposed to stress, which indicates that ABA acts as a plant stress hormone. The importance of ABA in plant development and physiology is showed by the phenotypes of ABA-deficient mutants, which includes loss of dormancy, reduced size and wilting (Koornneef and Jorna, 1982). To avoid germination under unfavourable conditions, seed germination has to be tightly controlled. ABA acts as a repressor for seed germination, where it will delay the germination until the seeds are fully formed and have achieved the favourable conditions to germinate and allow seedling establishment (Kang et al., 2015; Kermode, 2005).

The equilibrium between seed dormancy and germination is controlled by a dynamic balance of synthesis and catabolism of two antagonistic hormones, ABA and GA. The transition between seed dormancy and germination is known as an important stage in a plant's life cycle (Rodriguez-Gacio et al., 2009). Germination is promoted

when the ratio of GA/ABA is relatively higher. Other than hormones, seed germination is also regulated by external environmental signals such as water, low temperature and light (Finch-Savage and Leubner-Metzger, 2006; Holdsworth et al., 2008). Evidence regarding the contribution of ABA biosynthesis and catabolism to dormancy status has come from transgenic studies. In tobacco, overexpression of the gene encoding the ABA biosynthetic enzyme zeaxanthin epoxidase (ABA2) produces transgenic lines that increased ABA levels in mature seeds and exhibited delayed germination. Meanwhile, lower expression of this enzyme reduced the production of ABA therefore reduced the dormancy (Frey et al., 1999).

In the early seedling development, ABA reduces the transcript levels of the auxin inducible gene, *AXR/IAA7* and *AXR3/IAA17* therefore reducing auxin signalling and repressing growth (Belin et al., 2009). ABA also inhibits root growth through ethylene biosynthesis, whereby ABA promotes the production of ethylene. The treatment with ethylene biosynthesis inhibitors and ethylene perception inhibitors increased root growth indicating that both ethylene production and signalling reduces root growth (Luo et al., 2014). Ethylene biosynthesis is catalyzed by ACC synthase which converts S-adenosylmethionine to ACC. Ethylene was found to delay the degradation of the growth repressors, DELLA proteins (Achard et al., 2003). Therefore, higher levels of ABA increase the production of ethylene thus inhibits root growth. ABA also regulates polar auxin transport by targeting PIN-FORMED 1 (PIN1) and PIN2 auxin efflux transporters to inhibit root elongation in *Arabidopsis* (Thole et al., 2014; Yuan et al., 2014; Shkolnik-Inbar and Bar-Zvi, 2010; He et al., 2012).

Guard cells perceive the environmental signal, including biotic and abiotic stress. In response to the signals, guard cells will convert them into appropriate turgor pressure changes (Kim et al., 2010b). Guard cell are specialized epidermal cells that are located in pairs on the aerial organs of plants. Each pair of guard cells forms a pore or stoma that closes and opens in response to osmotic shrinking and swelling of

the guard cells, respectively. In guard cells, ABA promotes the closure of stomatal pores to restrict transpiration to prevent plants losing water especially during drought stress (Rodriguez-Gacio et al., 2009; Acharya and Assmann, 2009; Melotto et al., 2008; Schroeder et al., 2001).

Calcium is an important second messenger in guard cells ABA signalling. Higher concentration of ABA induces ROS formation and increase the concentration of cytosolic [Ca^{2+}] in guard cells and enhances [Ca^{2+}] sensitivity (Siegel et al., 2009), which activates two different type of anion channels; slow (S-type) activating sustained and rapid-transient (R-type) (Hedrich et al., 1990; Linder and Raschke, 1992; Schroeder and Hagiwara, 1989; Schroeder and Keller, 1992). Activation of anion channels at the plasma membrane of guard cells is regarded as a critical step in stomatal closure (Grabov et al., 1997; Pei et al., 1997; Schmidt et al., 1995). Anion efflux via anion channels block K^{+}_{in} channel and promote Cl^{-}_{out} channel, causing membrane depolarization (Hosy et al., 2003; Schroeder et al., 1984; Schroeder et al., 1987; Thiel et al., 1992). Membrane depolarization activates K^{+}_{out} channels on plasma membrane (Figure 1.6). K^{+} and anions to be released across the plasma membrane are first released from vacuoles into the cytosol. K^{+} channel mediates K^{+} uptake for net solute accumulation that drives water influx, guard ell swelling and pore opening (Thiel et al., 1992).

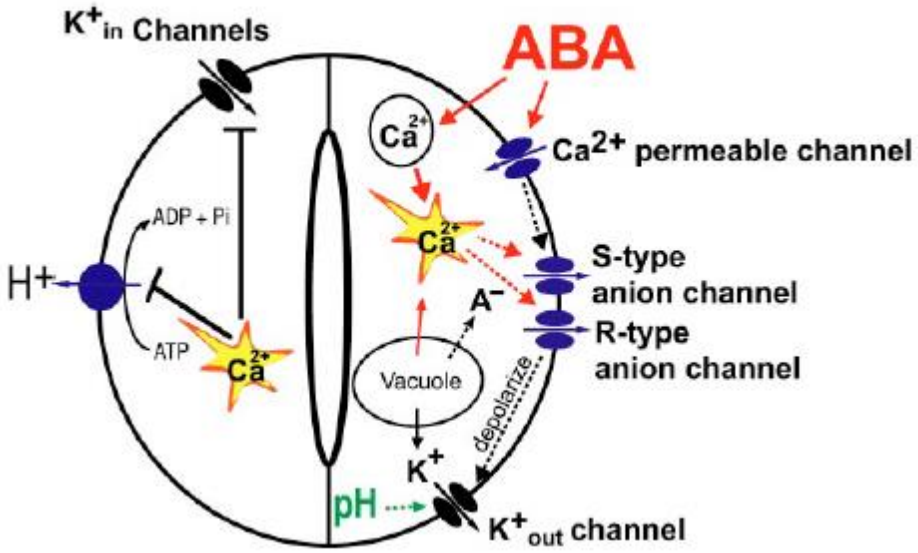


Figure 1.6: A guard cell model. ABA increases the concentration of [Ca^{2+}]. The influx of Ca^{2+} initiates the intracellular Ca^{2+} transient and release Ca^{2+} from vacuoles. Higher [Ca^{2+}] activates anion channel and caused depolarization.

1.2.2 Gibberellic acid (GA)

Bioactive gibberellic acids (GAs) are diterpene phytohormones that modulate plant growth and development throughout the plant life cycle (Sun, 2010). The major function of GAs is to stimulate organ growth through enhancement of cell elongation and cell division (Hedden and Phillips, 2000; Gupta and Chakrabarty, 2013). This section will discuss in detail the GA signalling pathway which consists 4 major components: GA, the receptors, DELLA proteins and F box protein, as well as the role of GA in plant development.

1.2.2.1 GA biosynthesis

GA biosynthesis starts from a common C20 precursor for diterpenoids, geranylgeranyl diphosphate (GGDP). The biosynthesis requires three different classes of enzymes; terpene synthase (TPs) which include ent-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS), cytochrome P450 monooxygenase (P450s) which include *ent*-kaurene oxidase (KO) and ent-kaurenoic acid oxidase (KAO), and 2-oxoglutarate-

dependent dioxygenases (2ODDs) which include GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox). TPs, P450s and 2ODDs are responsible in three stages: biosynthesis of ent-kaurene, conversion of ent-kaurene to GA12 and the formation of C20- and C19-GAs, respectively.

Biosynthesis of ent-kaurene from GGDP occurs in two steps via CPS and KS and located in plastids (Aach et al., 1997; Helliwell et al., 2001; Sun and Kamiya, 1994; Sun and Kamiya, 1997). ent-kaurene is then oxidised to ent-kaurenoic acid in three reaction catalyzed by one of P450s enzyme, KO before further converted to GA12 catalyzed by another P450s enzyme, KAO in three steps (Nelson et al., 2004). GA12 is a non-hydrolxylated GA which can be converted to GA53 by 13-hydroxylation. GA12 and GA53 are then converted to their bioactive form, GA4 and GA1, respectively by two parallel pathways. The conversion include a series of oxidation step catalyzed by 2ODDs, *GA20ox* and *GA3ox*, where *GA20ox* is responsible for the production of inactive precursors, GA9 and GA20 (from GA12 and GA53, respectively) before converted into active forms catalyzed by *GA3ox*.

A hydroxyl group on 3β position, a carboxyl group on C-6 and a lactone between C-4 and C-10 on ring A are the common feature of the bioactive GAs-GA1, GA3, GA4 and GA7. These bioactive GAs can be converted to inactive form through the introduction of 2β-hydroxylation catalyzed by *GA20x.* The homeostasis is important in order to maintain the level of GA. (Figure 1.7).

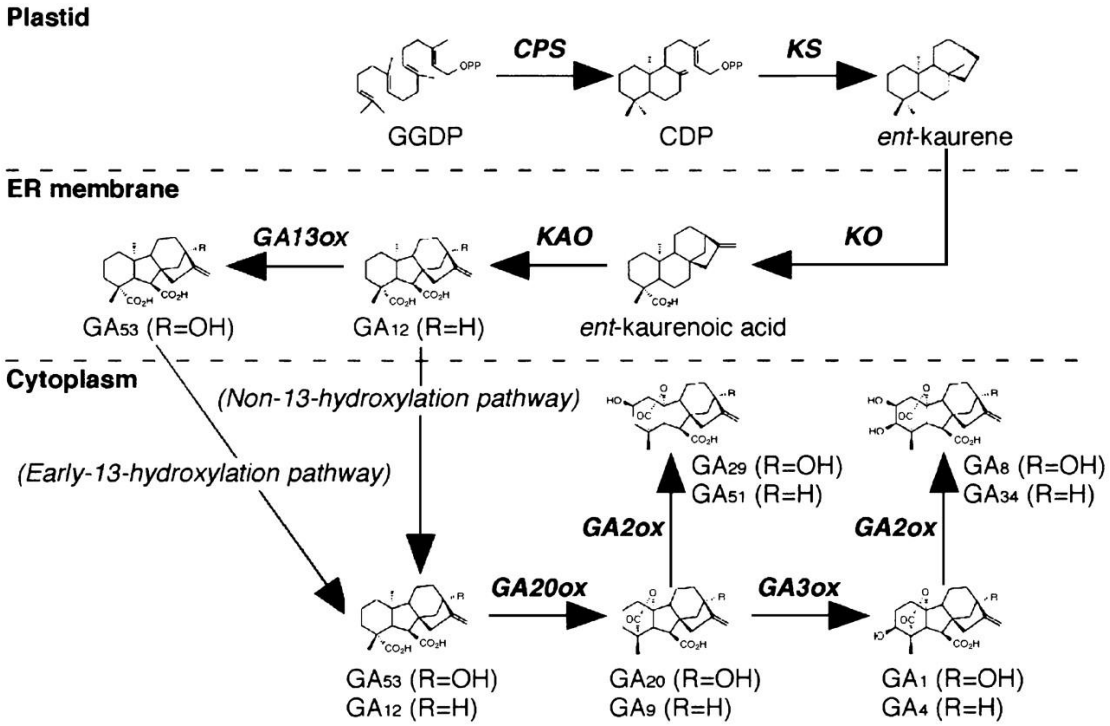


Figure 1.7: The biosynthesis pathway of bioactive GA. The biosynthesis is divided into three stages and involved 5 enzymes; CPS, KS, KO, GA20ox and GA3ox. The figure is taken from (Grennan, 2006).

Bioactive GAs homeostasis is maintained by feedback regulation of genes involved in GA metabolism (Achard and Genschik, 2009). Alteration in GA level cause changes in expression in some of the GA biosynthesis genes (*GA20ox* and *GA3ox*) that catalyze the final steps in the synthesis of bioactive GAs as well as GA catabolism genes (*GA2ox*). The transcript level of GA biosynthetic genes, *GA20ox* and *GA3ox* is found high in GA deficient mutants, while the expression of catabolic genes (*GA2ox*) is low. In contrast, transcript level of *GA20ox* and *GA3ox* is down regulated by applied GA and the expression of GA catabolism genes is up-regulated by GA application (Hedden and Phillips, 2000).

1.2.2.2 GID1: the receptors

The GA receptor was first identified in rice where *OsGID1* gene encodes a protein that has an interaction with GA, and its mutation results in a severe dwarf phenotype that

does not respond to GA in either stem elongation or seed germination (Ueguchi-Tanaka et al., 2008). The identification of the receptors in *Arabidopsis* was found through map based cloning and it was demonstrated that there are three homologs of the receptors. In *Arabidopsis*, the database search of several genes orthologous to *OsGID1* identified three genes, *AtGID1a*, *AtGID1b* and *AtGID1c* (Nakajima et al., 2006). Sequence alignment generated from ClustalW is shown in Figure 1.8. The deduced amino acid sequences show 67-85% similarity to each other and 60-63% to *OsGID1*, with *AtGID1a, AtGID1b* and *AtGID1c* encode 345 (39 kDa), 358 (40 kDa) and 344 (38 kDa) amino acids, respectively (Nakajima et al., 2006).

These genes were expressed in *Escherichia coli (E. coli)* and all of these proteins found to interact with GA with similar affinities to GID1. Loss of GID1 function in rice caused failure to respond to GA stimulation of leaf and cell elongation showing that the interaction between OsGID1 and GA is important in plant growth (Ueguchi-Tanaka et al., 2008; Ueguchi-Tanaka et al., 2005). In *Arabidopsis*, biochemical analyses revealed that *AtGID1b* bind with GA the strongest with the K_d value of 4.8×10^{-7} M, while the K_d value of *AtGID1a* and *AtGID1c* is 2.0×10^{-6} M and 1.9×10^{-6} M, respectively (Nakajima et al., 2006).

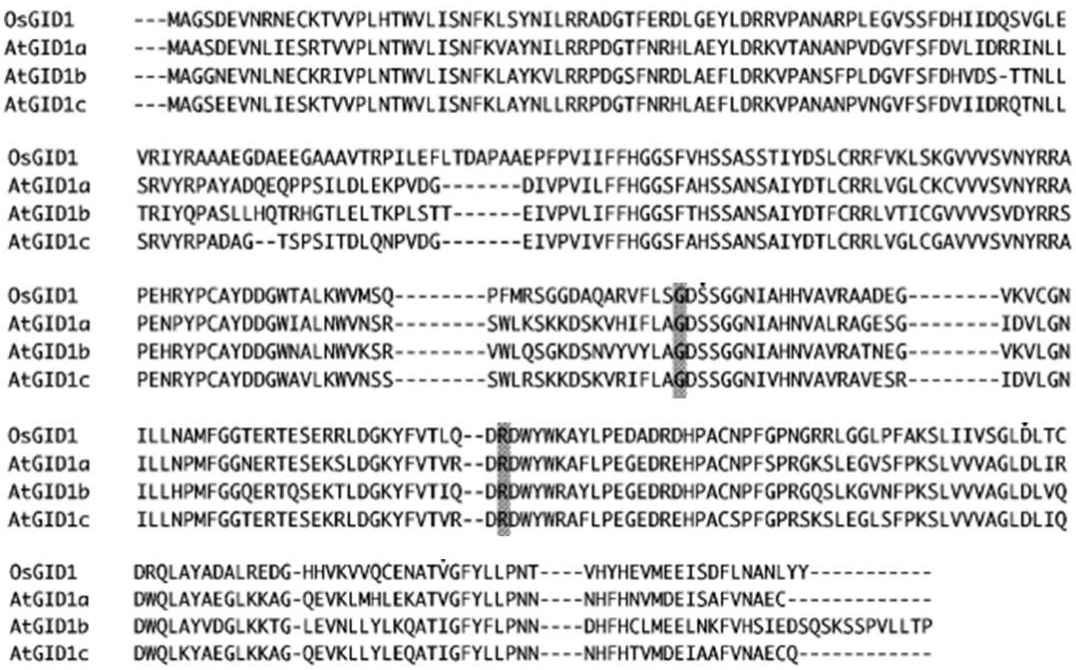


Figure 1.8: Amino acid alignment of *OsGID1* and its three homologs in *Arabidopsis*. The shaded amino acids show the residue that is responsible for GA-binding, G (Gly-196) and R (Arg-251). The figure is modified from Nakajima et al., 2006.

Single mutation of *GID1a*, *GID1b* and *GID1c* results in the same phenotype as wild type in terms of stem elongation and root length. This suggests that the receptors have a redundant function in plants. However, the specificity of GID1 homologs function can be observed from double mutants (Suzuki et al., 2009; Iuchi et al., 2007). *gid 1a-1gid1b-1* and *gid1b-1gid1c-1* mutants show no difference in stem elongation to wild type, however a *gid1a-1 gid1c-1* double mutant has a dwarfed phenotype, suggesting that GID1a and GID1c play a more prominent role in stem elongation, while GID1b has a minor influence (Griffiths et al., 2006). This finding was supported by quantitative real time measurement where GID1b was found to express the least as compared to GID1a and GID1c. While a clear difference can be observed from the double mutants in stem elongation, there is no difference in flower development. However, it was found that GID1a is responsible for flower development as there is a decrease in silique number for the *gid1a-1*, *gid1a-1gid1b-1*, *gid1a-1gid1c-1* mutants. (Griffiths et al., 2006). The triple receptor mutant shows a severe GA-insensitive

phenotype including seed germination, severe dwarfism and complete infertility and it cannot be rescued by the application of GA showing that the interaction between GID1 and GA is important to activate GA signalling (Griffiths et al., 2006; Willige et al., 2007; Iuchi et al., 2007).

1.2.2.3 The GA signalling pathway

In the GA signalling pathway, the most important mechanism is that GA represses DELLA protein function. DELLA proteins are negative regulators of plant growth which belong in GRAS protein superfamily of transcriptional regulators. The GRAS protein was named after the first three member identified, GAI, RGA and SCARECROW (SCR) and have a diverse role in regulating plant growth and development (Alvey and Boulton, 2008). This transcriptional regulator family has a high sequence similarity at the C-terminus whilst they differ at the N-terminus. Therefore, the N-terminus is responsible for their biological function. DELLA proteins are characterized by amino acids sequence Asp-Glu-Leu-Leu-Ala (DELLA) in the N-terminus (Peng et al., 1997; Silverstone et al., 1998; Pysh et al., 1999) (Figure 1.9). They have no DNA binding activity themselves, hence they are acting as a growth repressors by interacting with the transcription factor such as PHYTOCHROME INTERACTING FACTOR3 (PIF3), PIF4 and SCARECROW-LIKE3 (SCL3) and inhibit their activity (de Lucas et al., 2008; Feng et al., 2008; Arnaud et al., 2010). As a result, the degradation of these protein is considered as a major event in plant growth (Hauvermale et al., 2012).

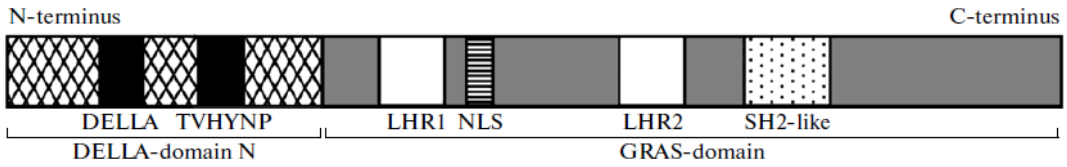


Figure 1.9: Typical DELLA protein structure showing the domain layout. DELLA is a subset of GRAS transcriptional regulator family. DELLA and TVHYNP domains are responsible for GID1 binding. Figure from Hauvermale et al., 2012.

While there is only one DELLA protein in rice (SLENDER RICE 1 (SLR1)), in barley (SLENDER1 (SLN1) and maize (DWARF8 (DWF8), there are five DELLA repressor genes in *Arabidopsis*. They are REPRESSOR OF GIBBERELLIC ACID (RGA), GA-INSENSITIVE (GAI), RGA-Like Protein (RGA1), RGA2, RGA3. The genes have redundant function in plant development, however the genetic evidence showed that some of them have distinct functions in plant growth (Tyler et al., 2004). The roles of these proteins have been determined based on the ability of loss-of-function alleles to rescue phenotypes of the GA biosynthesis mutant, *ga1-3*. Mutations of GAI, RGA and RGL1 rescue plant height, mutations in RGA, RGL1, RGL2 rescue flowering while mutations in RGL2, RGA, GAI and RGL3 rescue seed germination (Dill and Sun, 2001; King et al., 2001; Lee et al., 2002a; Tyler et al., 2004; Cao et al., 2005; Lee et al., 2002b). Mutants that lack four out of five DELLA gene function show GA overdose phenotype which is characterized by longer stems and early flowering (Alvey and Boulton, 2008).

Mutation in the DELLA motifs of the regulatory domain interfere with the interaction between DELLA protein and GID1 receptor hence causing the inability to down regulate the DELLA repressor, leading to a semi-dwarfed phenotype (Dill and Sun, 2001; Sun et al., 2010). Interestingly, the application of GA cannot rescue this phenotype, showing that this mutation is insensitive to GA. This finding indicates that DELLA motifs are important in GA signal perception. The deletion of both Ser/Thr/Val and TVHYNP motifs blocks DELLA phosphorylation, indicating that these two domain are responsible for DELLA phosphorylation (Itoh et al., 2005; Silverstone et al., 2007). It was suggested that DELLA phosphorylation promotes ubiquitination and proteasomal degradation (Gomi et al., 2004; Fu et al., 2002; Qin et al., 2014). The mutation in the GRAS functional domain resulted in slender phenotype with a longer stem tall phenotypes (Chandler et al., 2002; Itoh et al., 2005; Itoh et al., 2002).

Numbers of studies has been performed indicates that GA-induced degradation of RGA and GAI requires the SLEEPY1 (SLY1) protein, a positive regulator of GA signalling in *Arabidopsis* (Dill et al., 2004). SLY1 encodes a protein highly homologous to F-box proteins, where F-box protein functions as a part of SCF (SKP1, CULLIN, F-box)-type E3 ubiquitin ligase complexes. This protein catalyzes the polyubiquitylation of DELLA proteins which resulted a subsequent degradation of the DELLA proteins (Wang and Deng, 2011). The loss-of-function *sly1-10* mutant accumulates high levels of RGA and GAI proteins therefore has a GA-insensitive dwarfed phenotype (Steber et al., 1998; Dill et al., 2004).

Activation of the GA signalling pathway is initiated by the interaction between bioactive GAs and GID1 which will promote the conformational changes of the receptor. X-ray crystallography suggests that the N-terminal of the receptor act as a lid and trap GA inside the pocket (Murase et al., 2008). The formation of GA-GID1-DELLA complex triggers the protein-protein interaction between DELLA and the F-box protein SLY1 before undergoing ubiquitination and degradation of DELLA protein (Griffiths et al., 2006; Hirano et al., 2010). The degradation of DELLA protein activates transcription factors downstream hence the response of GA signalling can be observed through the manifestation of this gene expression: seed germination, cell elongation and flowering. DELLA protein induces expression of upstream GA biosynthetic genes and GA receptor genes suggesting that DELLA functions in maintaining GA homeostasis via a feedback mechanism (Hauvermale et al., 2012).

1.2.2.4 Antagonism between GA and light

Light is one of the most important environmental factors that regulates plant growth and development. Seedlings undergo photomorphogenesis, which is characterized by open and expanded cotyledons and short hypocotyls, when grown under light conditions. This is due to the higher light that induces the activity of phytochrome,

whereby the activity of this protein inhibits the accumulation of bioactive GAs. On the other hand, seedlings undergo skotomorphogenesis when grown in the dark, which is characterized by closed cotyledons and longer hypocotyls (Achard et al., 2007; Alabadi et al., 2004; Li et al., 2015). Etiolated seedlings are regulated by phytochrome interacting factors (PIFs), a subset of basic helix-loop-helix (bHLH) transcription factors (Li et al., 2016). PIFs mediate cell elongation and their activity is negatively regulated by the red light photoreceptor phyB and by DELLA proteins that act as a repressor in GA signalling pathway (de Lucas et al., 2008). The activation of phyB in the light leads to destabilization of PIFs while the accumulation of DELLA proteins block PIFs activity by binding the DNA-recognition domain of this factor (Figure 1.10). In contrast, PIF proteins accumulate and directly regulate genes to maintain skotomorphogenesis in the dark (Li et al., 2016).

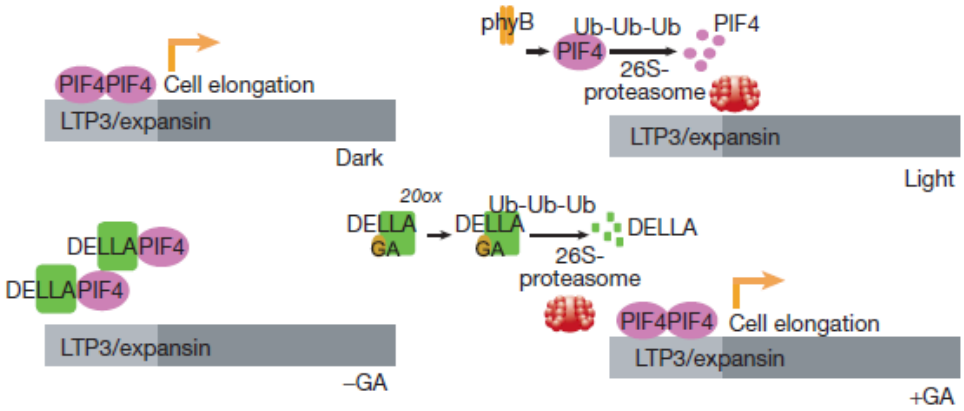


Figure 1.10: The mechanism of cell elongation under different condition. PIF4 mediates cell elongation in the dark condition. When exposed to the light, phyB degrades PIF4 and inhibits cell elongation. Due to DELLA protein accumulation in the absence of GA, PIF4 is bound to DELLA protein and no expansion can be observed. However, the addition of GA degrades DELLA protein and increases the free PIF4, hence cell elongation can be promoted. Figure was taken from de Lucas et al., 2008.

Transcript levels of GA biosynthetic enzymes (*GA20ox1* and *GA3ox1*) is high while the transcript encoding GA catabolism enzymes, *GA2ox1* is low in dark grown hypocotyls. In contrast, when grown in light, hypocotyls contain low and high amounts of *GA20ox1* and *GA2ox1* transcript, respectively. Therefore, when plants are grown in the dark, the DELLA degradation takes place due to a higher level of bioactive GAs

hence the hypocotyl growth can be promoted (Achard et al., 2007). In addition, *ga1-3* mutants can be suppressed by *rga* and *gai* null alleles, indicating that RGA and GAI are the two main DELLA members involved in GA-dependent repression of photomorphogenic growth in seedlings (Alabadi et al., 2004; Achard et al., 2007).

1.2.2.5 Function of GA in plant development

It is well established that GAs modulate several functions in plant growth and development. GAs positively regulate seed germination, cell elongation, root growth and flowering. Conversely to ABA function in seed germination, GAs inhibit seed dormancy in order to promote seed germination. Unlike ABA that promotes seed dormancy, GA overcoming seed dormancy and causing rapid seed germination. This is proven by the defect in seed germination of GA-deficient mutants (Piskurewicz et al., 2008).

Germination is under tight control by the environment, being affected by light, temperature and water potential. Environmental factors determine the relative levels of GA and ABA, which act antagonistically to each other in this development process. In seed germination, the balance between ABA and GA is important. Higher levels of GA and lower levels of ABA is the favourable condition for seed germination. In contrast, unfavourable condition for seed germination is high levels of ABA and low levels of GA (Olszewski et al., 2002; Nambara and Marion-Poll, 2005). ABA insensitive mutants, *abi1*, *abi2* and *abi3* reduce seed dormancy and allow germination even at higher concentration of ABA. On the other hand, GA-deficient mutants like *ga1-3* fail to germinate in the absence of exogenous GA (Koornneef and van der Veen, 1980; Koornneef and Karssen, 1994). This phenotype can be suppressed in *ga1-3 rgl2* double mutants, which suggests that RGL2 is involved in inhibiting seed germination (Cao et al., 2005; Lee et al., 2002b; Penfield et al., 2006). Interestingly, the high levels of RGL2 leads to ABA accumulation by the regulation of RING-zinc finger protein-

encoding gene XERICO, which has been shown to promote the accumulation of ABA (Ko et al., 2006; Zentella et al., 2007; Piskurewicz et al., 2008).

It is well known that GA promotes growth through cell expansion by stimulating the degradation of DELLA protein. GA biosynthesis mutant, *ga1-3* shows a severe dwarfed phenotype, resulting from the accumulation of DELLA protein due to the low levels of GA. This severe dwarfed phenotype can be rescued by the application of GA, confirming its function in cell elongation (Achard and Genschik, 2009).

1.3 Chemical genetics in plant hormonal signalling

1.3.1 Introduction of chemical genetics

Classical genetics has been an important approach in order to understand systems biology by investigating the relationship between genes and phenotypes. Forward genetic identifies target genes and pathways based on the study of phenotypes induced by random mutagenesis. The identification leads to the understanding or discovery of novel function of genes and proteins (Burdine and Kodadek, 2004). While reverse genetics involves the mutation/alteration of the gene of interest to identify the phenotype (Kawasumi and Nghiem, 2007). However, due to the similar function of some of the genes that responsible for a particular phenotype, this approach often leads to genetic redundancy problem. Further to this, the complexity of hormonal cross link also contributes to the difficulty of identifying the function of specific protein. For example, the redundancy can be seen from *Arabidopsis* GID1 single mutation, where no growth defects were observed from single mutation. However, *gid1a-1 gid1c-1* double mutant has a dwarfed phenotype, indicates that GID1a and GID1c have a role in stem elongation (Griffiths et al., 2006).

As a result, one approach that can be used to achieve this is through a chemical genetics approach. Chemical genetics employs small molecule compounds to

understand biological processes due to their ability to bind and activate or inhibit that can alter protein functions (Stockwell, 2000b; Stockwell, 2000a). Therefore chemical genetics is widely studied as it can reduce the redundancy problem. This is due to the ability of the small molecule to specifically interact with the protein and act as a general antagonist by inhibiting multiple components in the network, or acting as a specific agonist by activating a specific component, subsequently allowing identification of protein function through biochemical approach (Toth and van der Hoorn, 2010).

Chemical genetics has many similarity with mutational analysis. From a chemical genetics perspective, compounds that inhibits any particular protein function are equivalent to loss-of-function alleles. Alternatively, chemicals that acts as agonist rather than antagonists are analogous to gain-of-function phenotype (Mccourt and Desveaux, 2010). The ultimate objective for the chemical genetics approach is to identify the target protein of the compounds. To achieve this, affinity based approaches are often used which involved conjugating the compound with a suitable moiety such as biotin or directly immobilized on a resin through a linker before identifying the protein attached (Liu et al., 1991; Usui et al., 2004; Nicodeme et al., 2010).

Although the previous approach has been proven to be the most efficient approach to pull out the target protein of the compound, there is some constraint to perform it. The compound must retain its activity or target recognition properties during immobilization. Therefore, the modification of the compound is the crucial step in order to maintain the biological activity of the modified compound (Kawatani and Osada, 2014). To achieve this, structural activity relationship is a common approach which involves the synthesis of structural related compounds and investigations on their bioactivity (Toth and van der Hoorn, 2010).

1.3.2 Chemical genetics approach in plant hormones signalling

Due to its wide application that can be used to reduce gene redundancy problem, one of its approaches is the discovery of activators or inhibitors of different hormone signalling pathways (Fonseca et al., 2014; Rigal et al., 2014). For auxin signalling, some compounds have been identified acting as auxin agonist (Christian et al., 2008), while gravicin and rootin were identified as an auxin transport antagonists (Rojas-Pierce et al., 2007; Jeong et al., 2015), where these identification was achieved by phenotypic screens. Gravicin inihibits P-glycoprotein19 (PGP19), which has a major role in gravitropism. The mutation of PGP19 was found to resist the effect of gravicin on hypocotyl gravitropism. On the other hand, rootin modulates cell division and elongation to inhibit root development. The accumulation of PINFORMED proteins such as PIN1 and PIN3 which resulted in root development defect is due to the application of rootin (Overvoorde et al., 2010; Aida et al., 2004; Benjamins and Scheres, 2008).

Chemicals screens also helped reveal aspects of auxin signalling and biosynthesis (Kim et al., 2010a; Nishimura et al., 2014). The specific auxin-signailing inhibitors yokonolide A, yokonolide B and terfestatin A has been discovered through a screening of natural library of fermentation products from the soil bacterium *Streptomyces diastatochromogenes* (Hayashi et al., 2001; Hayashi et al., 2003; Yamazoe et al., 2005). Yokonolide B prevents auxin-induced degradation of the AUX/IAA transcription factors without inhibiting proteasome activity, indicating that its targets act upstream of the degradation of AUX/IAA proteins (Hayashi et al., 2003).

On the other hand, a screening for brassinosteroid responses such as hypocotyl and petiole elongation has identified bikinin as an example of general antagonist (De Rybel et al., 2009). Bikinin treatment mediates phosphorylation of transcription factors BES1 and BZR1, acting through BIN2 (BRASSINOSTEROID INSENSITIVE 2). BIN2 is a GSK3 (GLYCOGEN SYNTHASE KINASE3) kinase that

phosphorylates and inactivates the key transcription factors in the BR pathway, BZR1 and BES1/BZR2 (He et al., 2002). The interaction between bikinin and BIN2 specifically inhibits seven of the 10 GSK3 kinases (Vert and Chory, 2006; De Rybel et al., 2009; Yan et al., 2009).

Chemical genetics was also used to discover the interaction between ethylene and auxin. He et al. (2011) has revealed the interaction between ethylene and auxin pathways by exploring the key enzymes in the indole-3-pyruvic acid pathway of auxin biosynthesis, TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1/TRYPTOPHAN AMINOTRANSFERASE RELATEDs (TAA1/TARs). They demonstrated that L-kynurenine (Kyn) is an alternate substrate for the auxin biosynthetic enzymes TAA1/TAR. Kyn represses the ETHYLENE INSENSITIVE3 (EIN3) transcription factor thus inhibits ethylene responses in *Arabidopsis thaliana* root tissues. Further to this, ethylene-induced auxin biosynthesis in roots and TAA1/TARs expression was decreased with the application of this compound (Stepanova et al., 2008). It was suggested that there is a feedback loop between auxin biosynthesis and ethylene signalling, which was discovered through the accumulation of EIN3 nuclear in an EIN3 BINDING F-BOX PROTEIN1 (EBF1)/EBF2-dependent manner upon Kyn treatment (He et al., 2011).

Chemical genetics was instrumental in uncovering the ABA signalling pathway, and is one of the best examples of its use. The identification of the ABA receptors was based on this approach, as mentioned in Section 1.2.1. Consistent with the idea that chemical genetics can overcome the problem of genetic redundancy, the application of pyrabactin selectively activates only a subset of PYR/PYL ABA receptors. Pyrabactin shows a similar effect to ABA in inhibiting seed germination, but not in the ABA response in the vegetative phase (Park et al., 2009).

Structural biology increases the understanding of the interaction between the protein and the ligand. The X-ray and modelling approach of the PYR1-pyrabactin interaction has revealed that the gate-latch-lock mechanism is important to activate ABA signalling. The interaction between pyrabactin and PYR1 changes the conformation of the receptor and hence induces the closure of the gate and the loops surrounding ligand-binding pocket (Melcher et al., 2010). The closed conformation provides a surface for the interaction with the phosphatase, a negative regulator in ABA signalling (Nishimura et al., 2010). While pyrabactin is an agonist for PYR1 and PYL1, it is an antagonist for PYL2, where their interaction failed to induce the closed conformation of the receptor. This finding is supported by phosphatase assays, where PP2Cs activity was found higher in PYL2 as compared to PYL1 (Melcher et al., 2010).

Unlike pyrabactin, another ABA agonist, named quinabactin has a similar ABA-like physiological effect which includes ABA responses in vegetative tissue and promoting drought tolerance (Okamoto et al., 2013; Cao et al., 2013). Interestingly, these two agonists have similarity in their structure where both of them have a sulfonamide moiety (Figure 1.11). Crystal structure of quinabactin- and pyrabactin-receptor-PP2C has revealed that quinabactin-receptor closely mimics ABA-receptor interaction, which is consistent with the ABA response seen in response to quinabactin application (Cao et al., 2013).

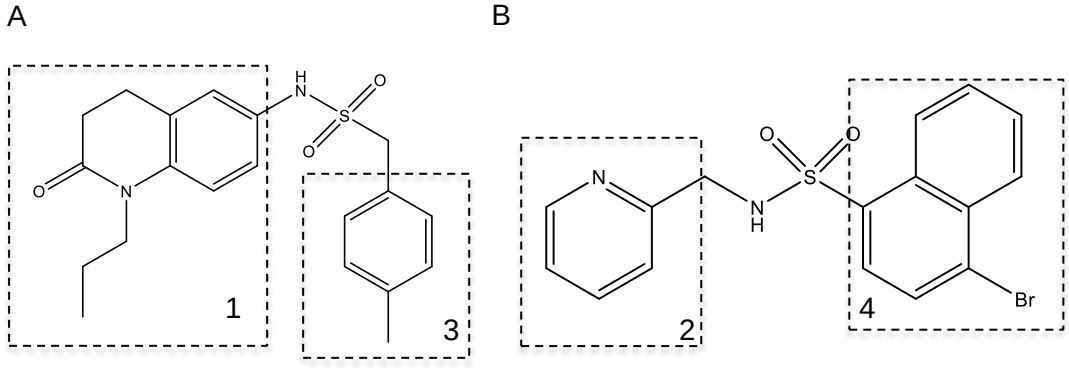


Figure 1.11: The structure of A) quinabactin and B) pyrabactin. Dihydro-quinolinone ring (1) replaced pyridine ring (2) of pyrabactin and benzyl ring (3) replaced naphthalene double ring (4). The figure is adapted from Rigal et al., 2014.

In structural biology, the interaction of the compound and its target protein is a crucial finding. Molecular modelling, X-ray crystallography and biophysical approach are the main approaches used to achieve this objective. Determining this interaction can help develop analogues of compound based on their predicted interaction obtained from molecular modelling. This approach has been widely used in auxin signalling where the analogues of auxin has been synthesized and tested on plants. The bioactivity of the compound was then determined based on the phenotypic analysis and molecular mechanism. Structural studies involving TIR1 and auxin show that the hormone fits deeply into a specific hydrophobic pocket on the surface of the TIR1 protein with high affinity (Tan et al., 2007). A series of chemically designed auxin analogues has been synthesized and a modification made on the alkyl chain at the position of α-alkyl auxin. The lengthening of the chain has found to interrupt the TIR-auxin interaction and resulted in losing auxin activity (Hayashi et al., 2008a).

1.4 Summary

Plant growth regulators or plant hormones are important for plant growth as well as plant adaptation to the environment. Therefore it is necessary to understand their function and signalling pathways, especially to improve the crop quality and yield. However, there is a limitation to achieving this objective by using classical genetic approaches, due to the redundancy of protein function and the complexity of hormone cross talk. With the discovery that chemical genetics has an advantage to overcome this limitation, this approach has been considered as a versatile approach to explore hormone signalling. Furthermore, structural biology is an important approach to increase the understanding of the molecular mechanism and mode of action of the small molecules.

1.5 Thesis aim

The aim of the study includes:

1) To investigate a series of compounds that has been developed from calmodulin inhibitor, W5/W7 on calcium signal and plant phenotype (Chapter 3).
2) To investigate whether there are changes in gene expression and transcript levels after the application of the compound (Chapter 4).
3) To investigate the effect of the eW5 compound on plant hormone signalling due to the ability of eW5 to promote growth. This question is divided into two different hormones; ABA (Chapter 5) and GA (Chapter 6).
4) To further understand the mode of action of eW5 in promoting growth. Structural activity relationship approach has been used by generating a series of compounds based on eW5 structure (Chapter 7).

CHAPTER 2

Materials and Methods

2.1 Chemical synthesis

2.1.1 General Notes

Chemicals: All chemicals were purchased from commercial suppliers and were used without further purification unless otherwise stated.

TLC: TLC analysis was performed on a pre-coated aluminium-backed plate (Silica gel 60 F254, Merck). Signals were visualized with UV-light (254nm and 36nm) or by staining with potassium permanganate in water where necessary.

The characterization was performed using the instruments listed below. The purity of the compounds was not performed.

Column chromatography: Flash column chromatography was performed on a CombiFlash System from Teledyne Isco equipped with an UV-light detector using prepacked silica RediSep Rf cartridges with the stated solvent gradient. Crude mixture to be purified were dried loaded onto silica prior loading on the column.

Elemental analysis: CHN analysis were conducted on an Exeter CE-440 Elemental Analyser. The elemental characterization of the product was confirmed upon ±0.4% of the result.

LC-MS: LC-MS analysis were conducted using a TQD mass spectrometer (Waters Ltd, UK) which equipped with an Acquity UPLC, using Acquity UPLC BEH C18 1.7 µm (2.1 mm x 50 mm) column, and an electrospray ion source. Absorbance data were acquired from 210 to 400 nm using an Acquity photodiode array detector.

HRMS: The analysis were carried out using QToF Premier mass spectrometer (Waters Ltd, UK) with an electrospray ion source.

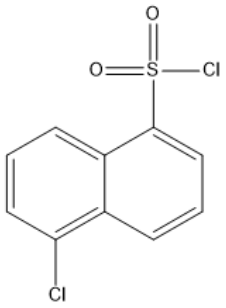
IR spectroscopy: IR spectra were recorded on a Perkin-Elmer RX I FT-IR spectrometer via use of a Diamond ATR accessory (Golden Gate) in the range of 3500-600 cm-1. Assigned peaks are recorded in wavenumber (cm-1).

Melting points: Melting points were measured in open capillary tubes using a Thermo Scientific Melting Point Apparatus and are uncorrected.

NMR-spectroscopy: NMR-spectra were recorded from $CDCl_3$ or D_2O solutions on a Bruker Advance-400 (400 MHz) spectrometer. Chemical shift values are reported in parts per million (ppm) and coupling constant (J) are given in Hertz (Hz). The multiplicity is indicated by singlet (s), doublet (d), triplet (t), quartet (q), broad (br) or a combination thereof.

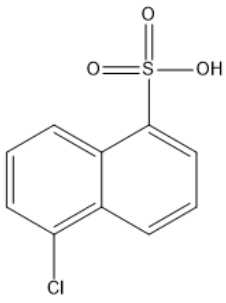
2.1.2 Synthesis of chemicals

2.1.2.1 5-chloro-naphthalenesulfonic acid



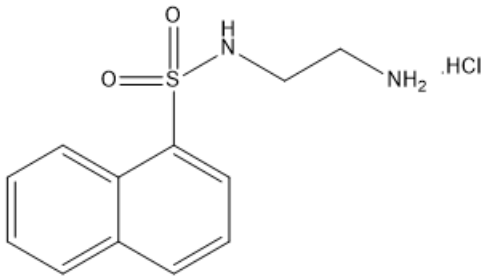
5-amino-naphthalenesulfonic acid (10g, 44.8mmol) was added to 0.55M sodium hydroxide solution (0.96 equiv.). 40 mL (0.45mL/mmol sulfonic acid) of 6M aqueous hydrochloric acid was then added at room temperature. The resulting mixture was cooled to below 2°C and 7M aqueous sodium nitrite (1.09 equiv.) solution was added dropwise maintaining the temperature below 2°C. The mixture was stirred for a further 30 minutes at this temperature. Urea (0.13 equiv.) was then added. The mixture was then added to freshly prepared and heated (80°C) copper chloride (CuCl) solution (1 equiv) in 20 mL of 6M HCl. After stirring the mixture at 80°C for 1h, it was cooled to room temperature and concentrated in vacuo. The product was washed with hexane and then dried in a vacuum dessicator overnight to afford the title sulfonic acid as a grey solid (9.8g, 91.2%). ν_{max} (ATR): (O-H): 3591, 1373. LRMS (ES^+): m/z 241 ((M+H), ^{35}Cl), 243 ((M+H), ^{37}Cl).

2.1.2.2 5-chloronaphthalenesulfonylchloride (1)



5-chloronaphthalenesulfonic acid (3g, 12.39 mmol) was dissolved in excess thionyl chloride (60 mL). Dimethyl formamide (1.8 mL) was added and the mixture then heated under reflux for 2 hours. Upon cooling, the mixture was added to an excess ice and the product extracted with DCM (3 x 10 mL). The combined organic extracts were dried over $MgSO_4$ and concentrated to give the title sulfonyl chloride as a brownish yellow solid (2.6g, 81.6%). LRMS (ES^+): m/z 261 ((M+H), ^{35}Cl), 263 ((M+H), ^{37}Cl).

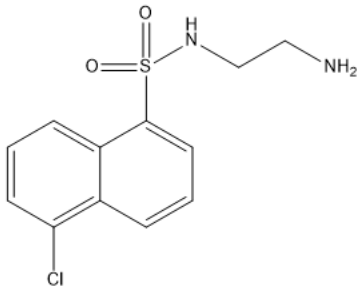
2.1.2.3 2'-Amino-1'-(1-naphthylsulfonylamino)ethane hydrochloric acid salt (2)



Naphthalene sulfonylchloride (1.00g, 4.41 mmol) was dissolved in 15 mL of dry DCM and added dropwise to a solution of ethylene diamine (5.9 mL, 88.2 mmol, 20 equiv) in 10 mL of dry DCM. After stirring at room temperature for 1h, the reaction was quenched by addition of 10 mL of H_2O. The mixture was extracted with DCM (3 x 10 mL) and the combined organic layers were dried over $MgSO_4$. The mixture was concentrated to afford a crude product as a light yellow oil (0.88g, 80 %). Without further purification this product (0.88 g, 3.5 mmol) was dissolved in 10 mL of dry DCM and added to a solution of di-tert-butyl dicarbonate (1.08 g, 4.94 mmol, 1.4 equiv) in 10 mL of dry DCM. The mixture was stirred at room temperature for 16h when TLC analysis confirmed complete consumption of the amine. The reaction was then quenched with 10 mL of H_2O and the reaction mixture extracted with DCM (3 x 10 mL). The combined organic layers were dried over $MgSO_4$, concentrated to afford a white powder (1.15 g, 94%). Without further purification, this product (0.65 g, 1.8 mmol) was dissolved in 10 mL of dry DCM and HCl (1 mL of a 4.0M solution in dioxane (excess)) added. The mixture was then stirred at room temperature for 16h when TLC analysis

(hexane:ethyl acetate, 2:1) showed complete consumption of starting material. After concentrating under vacuum, the solid obtained was washed with diethyl ether, filtered and dried under vacuum overnight to afford the title salt as a white solid (0.92g, 65%). M.p: 178.8-179.3, $\tilde{V}_{max}$ (ATR): (N-H): 3022, 1154, 1130, 1021, 777 cm^{-1}. δ_H (400 MHz, D_2O): 8.55 (d, 1H, *J*=8.0 Hz, Ar-*H*), 8.28 (d, 1H, *J*=7.8 Hz, Ar-*H*), 8.25 (d, 1H, *J*=7.8 Hz, Ar-*H*), 8.13 (d, 1H, *J*=8.0 Hz, Ar-*H*), 7.81 (t, 1H, *J*=8.0 Hz, Ar-*H*), 7.74 (t, 1H, *J*=8.0Hz, Ar-*H*), 7.68 (t, 1H, *J*=7.8 Hz, 3-*H*), 3.15 (m, 2H, CH_2), 3.10 (m, 2H,CH_2). δ_C (D_2O, 400 MHz): 135.3 (C-Ar), 134.1 (C-Ar), 132.1 (C-Ar), 129.9 (C-Ar), 129.5 (C-Ar), 128.8 (C-Ar), 127.3 (C-Ar), 127.1 (C-Ar), 124.4 (C-3), 123.2 (C-Ar), 39.8 (CH_2), 39.14 (CH_2). LRMS (ES^+): m/z 251 (M+H), HRMS ($ASAP^+$): Found M+H, 251.0854, $C_{12}H_{15}N_2O_2S$, requires *M* 251.0856. Elemental analysis: Calculated for $C_{12}H_{15}ClN_2O_2S$ C, 50.26; H, 5.27; N, 9.77. Measured C, 50.32; H, 5.27; N, 9.69.

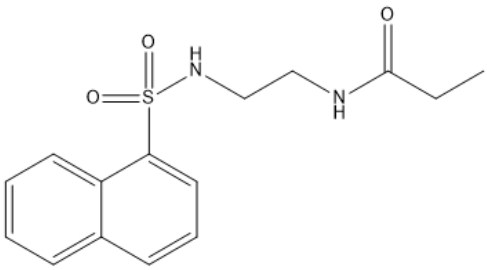
2.1.2.3 5-chloro -1'-(1-naphthylsulfonylamino)-2'-amino ethane (3)



Without further purification, Compound (1) (4.69g, 18 mmol) was dissolved in dry DCM (40 mL) and added dropwise to a solution of ethylene diamine (20.06 mL, 360 mmol, 20 equiv.) in 30 mL dry DCM. The mixture was stirred at room temperature for 1h before being quenched with 15 mL H_2O. The reaction mixture was then extracted with DCM (3 x 10 mL) and the combined organic layers were dried over $MgSO_4$ and concentrated. Recrystallization from MeOH then afforded the title ethane as a light brown solid (1.64g, 32%). M.p: 168.9-179.3, $\tilde{V}_{max}$ (ATR): (N-H): 3350, 1306, 1131, 1102, 784 cm^{-1}. δ_H (400 MHz, d^6-DMSO): 8.68 (d, 1H, *J*=8.0Hz, Ar-*H*), 8.50 (d, 1H, *J*=7.9 Hz, Ar-*H*), 8.24 (d, 1H, *J*=7.9 Hz, Ar-*H*), 7.87 (d, 1H, *J*=8.0 Hz, Ar-*H*), 7.82 (t,

1H, *J*=7.9 Hz, Ar-*H*), 7.70 (t, 1H, *J*=8.0 Hz, Ar-*H*), 2.76 (t, 2H, *J*=6.5 Hz, CH_2), 2.43 (t, 2H, *J*=6.5 Hz, CH_2). δ_C (d^6-DMSO, 400 MHz): 136.6 (C-Ar), 131.5 (C-Ar), 130.7 (C-Ar), 129.4 (C-Ar), 129.1 (C-Ar), 129.0 (C-Ar), 128.0 (C-Ar), 127.5 (C-Ar), 126.3 (C-Ar), 124.3 (C-Ar), 46.1 (CH_2), 41.4 (CH_2). LRMS (ESI^+): m/z 285 ((M+H), ^{35}Cl), 287 ((M+H), ^{37}Cl). HRMS ($ASAP^+$): Found M+H, 285.0465, $C_{12}H_{14}N_2O_2S^{35}Cl$, requires *M* 285.0459. Elemental analysis: Calculated for $C_{12}H_{13}ClN_2O_2S$; C, 50.62; H, 4.60; N, 9.84. Measured C, 50.56; H, 4.61; N, 9.75.

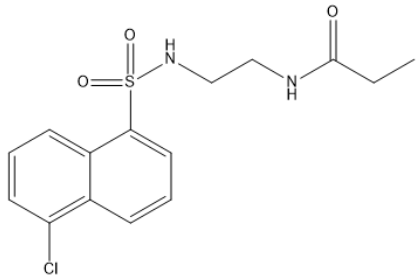
2.1.2.4 N-[(napthylsulfonylamino)ethyl]-propanamide (4)



2'-Amino-1'-(1-naphthylsulfonylamino)ethane was prepared as described above. Without further purification, the crude product (1.6g, 6.4 mmol) was dissolved in dry DCM (20 mL) before added dropwise to a solution of propionic anhydride (0.82 mL, 6.4 mmol, 1 equiv) and triethylamine (2.23 mL, 16.0 mmol 2.5 equiv) in dry DCM (5 mL). The reaction was stirred for 16h at room temperature when it was quenched with H_2O (10 mL) and the reaction mixture extracted with DCM (3 x 10 mL). The combined organic layers were dried ($MgSO_4$) and concentrated. Flash column chromatography (hexane:EtOAc; 3:1) yielded the title amide as a white solid (1g, 51 %). M. p.: 118.1-118.8, $\tilde{V}_{max}$ (ATR): (N-H): 3393, 3044, 1742, 1618, 1540 cm^{-1}. δ_H (400 MHz, $CDCl_3$): 8.62 (d, 1H, *J*=8.0 Hz, Ar-*H*), 8.25 (d, 1H, *J*=7.7 Hz, Ar-*H*), 8.08 (d, 1H, *J*=7.7 Hz, Ar-*H*), 7.96 (d, 1H, *J*=8.0 Hz, Ar-*H*), 7.70 (t, 1H, *J*=8.0 Hz, Ar-*H*), 7.62 (t, 1H, *J*=8.0 Hz Ar-*H*), 7.55 (t, 1H, *J*=7.7 Hz, Ar-*H*), 5.71 (s, 1H, NH), 5.41 (s, 1H, NH), 3.30 (t, 2H, *J*=5.6 Hz, CH_2), 3.04 (t, 2H, *J*=5.6 Hz, CH_2), 2.03 (q, 2H, *J*=7.7 Hz, 2'-*H*), 1.04 (t, 3H, *J*=7.7 Hz, 3'-*H*). δ_C ($CDCl_3$, 400 MHz): 175.0 (C=O), 134.4 (C-Ar), 134.3 (C-Ar), 134.2 (C-

Ar), 129.7 (C-Ar), 129.2 (C-Ar), 128.4 (C-Ar), 128.0 (C-Ar), 127.0 (C-Ar), 124.3 (C-Ar), 124.1 (C-Ar), 43.8 (CH_2), 39.3 (CH_2), 29.4 (C-2'), 9.80 (C-3'). LRMS (ES^+): m/z 307 (M+H), HRMS ($ASAP^+$): Found M+H 307.1116, $C_{15}H_{19}N_2O_3S$, requires *M* 307.1113. Elemental analysis: Calculated for $C_{15}H_{18}N_2O_2S$ C, 58.80; H, 5.92; N, 9.14. Measured C, 58.69; H, 5.90; N, 9.07.

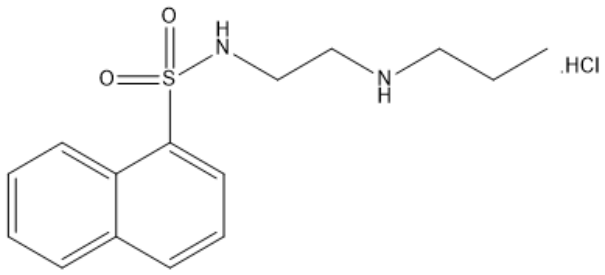
2.1.2.5 N-[(5-chloronapthylsulfonylamino)ethyl]-propanamide (5)



5-chloro -1'-(1-naphthylsulfonylamino)-2'-amino ethane (3) (2.25 g, 7.9 mmol) was dissolved in dry DCM (20 mL) and added to the a solution of propionic anhydride (1.01 mL, 7.9 mmol, 1 equiv) and triethylamine (2.76 mL, 19.81 mmol, 2.5 equiv) in dry DCM (5 mL). The solution was stirred at room temperature for 16h and then quenched with H_2O (10 mL). The reaction mixture was extracted with DCM (3 x 10 mL) and the combined organic layers dried over $MgSO_4$ and concentrated. Flash column chromatography (hexane: EtOAc; 1:3) afforded the title amide as a light brown solid (1.62 g, 60%). M. p.: 145.9-146.6, ν_{max} (ATR): (N-H): 3414, 1746, 1621, 1539, 1320, 1138 cm^{-1}. δ_H (400 MHz, $CDCl_3$): 8.60 (d, 1H, *J*=4.6 Hz, Ar-*H*), 8.59 (d, 1H, *J*=4.6 Hz, Ar-*H*), 8.31 (d, 1H, *J*=7.5 Hz, Ar-*H*), 7.71 (d, 1H, *J*=7.5 Hz, Ar-*H*), 7.66 (t, 1H, *J*=7.5 Hz, Ar-*H*), 7.60 (t, *J*=4.6 Hz, Ar-H), 5.74 (s, 1H, NH), 5.62 (s, 1H, NH), 3.31 (t, 2H, *J*=5.5 Hz, CH_2), 3.04 (t, 2H, *J*=5.5 Hz, CH_2), 2.07 (q, 2H, *J*=7.6 Hz, 2'-*H*), 1.06 (t, 3H, *J*=7.6, 3'-*H*). δ_C ($CDCl_3$, 400 MHz): 174.8 (C=O), 134.4 (C-Ar), 134.2 (C-Ar), 129.7 (C-Ar), 129.1 (C-Ar), 128.5 (C-Ar), 128.0 (C-Ar), 126.9 (C-Ar), 124.2 (C-Ar), 124.1 (C-Ar), 43.6 (CH_2), 39.1 (CH_2), 30.9 (C-2'), 29.3 (C-3'). LRMS (ES^+): m/z 341 ((M+H), ^{35}Cl), 343 ((M+H), ^{37}Cl) HRMS ($ASAP^+$): Found M+H, 341.0718, $C_{15}H_{17}N_2O_3S^{35}Cl$, requires

M 341.0715. Elemental analysis: Calculated for $C_{15}H_{17}ClN_2O_2S$ C, 52.86; H, 5.03; N, 8.22. Measured C, 52.91; H, 5.03; N, 8.19.

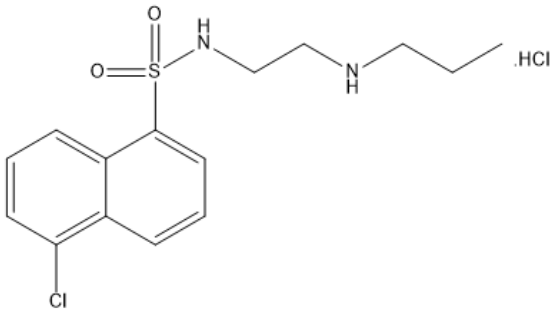
2.1.2.6 N-propyl-N-naphthylsulfonyl-1,2-ethanediamine hydrochloric acid salt(6)



Amide (4) (0.46 g, 1.50 mmol) was dissolved in dry THF (10 mL) and added to BH_3.THF (4.50 mL, 4.50 mmol, 3 equiv) solution. The mixture was stirred at 55-60°C for 16 h when TLC analysis (DCM:MeOH; 9:1) showed complete consumption of starting material. The reaction was quenched by adding MeOH (1.3 mL) and the mixture heated under refluxed for 3h. The mixture was then added to a solution of di-tert-butyl dicarbonate (0.46 g, 21 mmol, 1.4 equiv) in dry DCM (5 mL). The mixture was stirred at room temperature for 16h and then quenched with H_2O. The mixture extracted with DCM (3 x 10 mL) and combined organic layers were dried over $MgSO_4$ and concentrated to give a white solid. Without further purification, this product (0.34, 0.86 mmol) was dissolved in dry DCM (5 mL) and 1 mL HCl in 4.0 dioxane (excess) was added. The mixture was stirred for 16h at room temperature when TLC analysis (hexane: EtOAc; 2:1) showed complete consumption of starting material. After concentration in vacuo, the solid obtained was washed with diethyl ether and dried under vacuum to afford the title amine salt as a white solid product (0.11g, 44%). M. p.: 159.5-159.9, $\tilde{V}_{max}$ (ATR): (N-H): 3142, 1315, 1169, 769 cm^{-1}. δ_H (400 MHz, D_2O): 8.56 (d, 1H, *J*=7.2 Hz, Ar-*H*), 8.30 (d, 1H, *J*=7.7, Ar-*H*), 8.27 (d, *J*=7.7 Hz, Ar-*H*), 8.14 (d, 1H, J=7.2 Hz, Ar-*H*), 7.82 (t, 1H, *J*=7.2 Hz, Ar-*H*), 7.75 (t, 1H, *J*=7.2 Hz, Ar-*H*), 7.69 (t, 1H, *J*=7.7 Hz, Ar-*H*), 3.20 (t, 2H, *J*=5.6, CH_2), 3.12 (t, 2H, *J*=5.6 Hz, CH_2), 2.94 (t, 2H, *J*=7.4 Hz, CH_2), 1.63 (sex, 2H, *J*=7.4 Hz, 2'-*H*), 0.93 (t, 3H, *J*=7.4, 3'-*H*). δ_C (D_2O,

400 MHz): 135.3 (C-Ar), 134.3 (C-Ar), 132.2 (C-Ar), 130.0 (C-Ar), 130.0 (C-Ar), 128.9 (C-Ar), 127.5 (C-Ar), 127.0 (C-Ar), 124.4 (C-Ar), 123.2 (C-Ar), 49.0 (CH_2), 46.5 (CH_2), 38.8 (CH_2), 18.9 (C-2'), 10.0 (C-3'). LRMS (ES^+): m/z 293 (M+H). HRMS ($ASAP^+$): Found M+H, 293.1324, $C_{15}H_{21}N_2O_2S$, requires *M* 293.1307. Elemental analysis: Calculated $C_{15}H_{21}ClN_2O_2S$; C, 54.79; H, 6.44; N, 8.52. Measured C, 54.62; H, 6.44; N, 8.25.

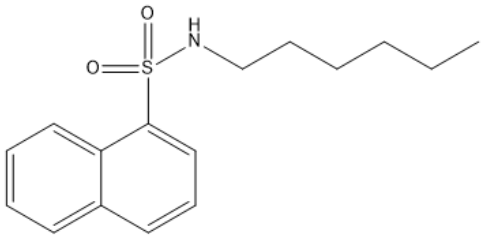
2.1.2.7 N-propyl-5-Chloro-N-naphthylsulfonyl-1,2-ethanediaminehydrochloric salt (7)



Amide 5 (0.7 g, 2.06 mmol) was dissolved in dry THF (15 mL) and added to borane BH_3.THF (6.18 mL, 6.18 mmol, 3 equiv) solution. The mixture was stirred at 55-60°C for 16 hours when TLC analysis (DCM:MeOH; 9:1) showed the complete consumption of starting material. The reaction was quenched by adding MeOH (1.3 mL) and the mixture heated under refluxed for 3h. The mixture was then added to a solution of di-tert-butyl dicarbonate (0.63g, 2.88 mmol, 1.4 equiv.) in dry DCM (5 mL). The mixture was stirred at room temperature for 16h and then quenched with H_2O (10 mL). The mixture was extracted with DCM (3 x 10 mL) and the combined organic layers were dried over $MgSO_4$ and concentrated. Without further purification, this product (0.5 g, 1.17 mmol) was dissolved in dry DCM (5 mL). 1 mL of HCl in 4.0M dioxane (excess) was added. The mixture was stirred for 16h at room temperature when TLC analysis (DCM;MeOH; 9:1) showed the complete consumption of starting material. After concentrating in vacuo, the product obtained was washed with diethyl ether and dried under vacuum to afford the title amine as a white solid (0.35g, 83%). M. p.: 178.9-179.6, ν_{max} (ATR): (N-H): 2968, 1742, 1328, 1135, 1010, 786 cm^{-1}. δ_H (400 MHz, D_2O):

8.60 (d, 1H, *J*=8.2 Hz, Ar-*H*), 8.47 (d, 1H, *J*=8.1 Hz, Ar-*H*), 8.29 (d, 1H, *J*=8.2 Hz, Ar-*H*), 7.80 (d, 1H, *J*=8.1 Hz, Ar-*H*), 7.73 (t, 1H, *J*=8.2 Hz, Ar-*H*), 7.68 (t, 1H ,*J*=8.1 Hz, Ar-*H*), 3.18 (t, 2H, *J*=5.6, CH_2), 3.12 (t, 2H, *J*=5.6 Hz, CH_2), 2.95 (t, 2H, *J*=7.4 Hz, CH_2), 1.64 (sex, 2H, *J*=7.4 Hz, 2'-*H*), 0.94 (t, 3H, *J*=7.4 Hz, 3'-*H*). δ_C (D_2O, 400 MHz): 132.7 (C-Ar), 132.6 (C-Ar), 131.2 (C-Ar), 131.1 (C-Ar), 130.5 (C-Ar), 128.6 (C-Ar), 128.4 (C-Ar), 127.7 (C-Ar), 125.5 (C-Ar), 122.6 (C-Ar), 49.0 (CH_2), 46.5 (CH_2), 38.6 (CH_2), 18.7 (C-2'), 9.9 (C-3'). LRMS (ES^+): m/z 327 ((M+H), ^{35}Cl), 329 ((M+H), ^{37}Cl), HRMS ($ASAP^+$): Found M+H, 327.0934, $C_{15}H_{20}N_2O_2S^{35}Cl$, requires *M* 327.0936. Elemental analysis: Calculated for $C_{15}H_{20}Cl_2N_2O_2S$; C, 49.59; H, 5.55; N, 7.71. Measured C, 49.25; H, 5.62; N, 7.84.

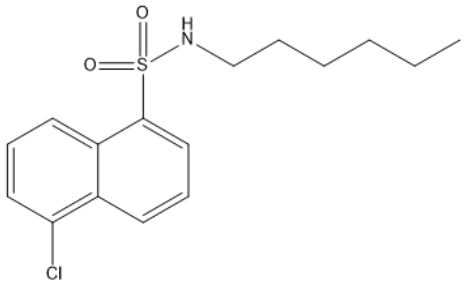
3.1.2.8 (1-Naphthylsulfonyl)hexylamine (8)



Naphthalenesulfonamide (1 g, 3.85 mmol) was dissolved in dry DCM (15 mL) and added dropwise to a solution of hexylamine (0.76 mL, 5.77 mmol, 1.5 equiv) and triethylamine (1.34 mL, 9.63 mmol, 2.5 equiv) in dry DCM (15 mL) at 0°C. The reaction was stirred at room temperature for 1h and then quenched with 10 mL of H_2O. The mixture was extracted with DCM (3 x 10 mL) and combined organic layers dried over $MgSO_4$ and concentrated. Column chromatography (hexane:ethyl acetate (9:1)) yielded the title hexylamine as a light yellow oil (1.05g, 94%). $\tilde{V}_{max}$ (ATR): 2927, 2855, 1315, 1158, 1130, 768 cm^{-1}. δ_H (400 MHz, $CDCl_3$): 8.65 (d, 1H, *J*=7.7 Hz, Ar-*H*), 8.27 (d, 1H, *J*=8.0 Hz, Ar-*H*), 8.07 (d, 1H, *J*=8.0 Hz, Ar-*H*), 7.96 (d, 1H, *J*=7.7 Hz, Ar-*H*), 7.68 (t, 1H, *J*=7.7 Hz, Ar-*H*), 7.61 (t, 1H, *J*=7.7 Hz, Ar-*H*), 7.55 (t, 1H, *J*=8.0 Hz, Ar-*H*), 4.53 (s, 1H, NH), 2.90 (q, 2H, *J*=7.1 Hz, CH_2), 1.35 (p, 2H, *J*=7.1 Hz, CH_2), 1.12 (m,

2H, CH_2), 1.05 (m, 2H, CH_2), 0.77 (t, 3H, *J*=7.1 Hz, CH_3). δ_C ($CDCl_3$, 400 MHz): 135.0 (C-Ar), 134.6 (C-Ar), 134.5 (C-4), 130.1 (C-2), 129.5 (C-Ar), 128.7 (C-Ar), 128.5 (C-Ar), 127.2 (C-Ar), 124.6 (C-Ar), 124.5 (C-3), 43.7 (CH_2), 31.4 (CH_2), 29.8 (CH_2), 26.5 (CH_2), 22.7 (CH_2), 14.2 (CH_3). LRMS (ES^+): m/z 292, HRMS ($ASAP^+$): Found M+H, 292.1371, $C_{16}H_{22}NO_2S$, requires *M* 292.1379. Elemental analysis: Calculated for $C_{16}H_{21}NO_2S$; C, 65.95; H, 7.26; N, 4.81. Measured C, 65.78; H, 7.15; N, 4.66.

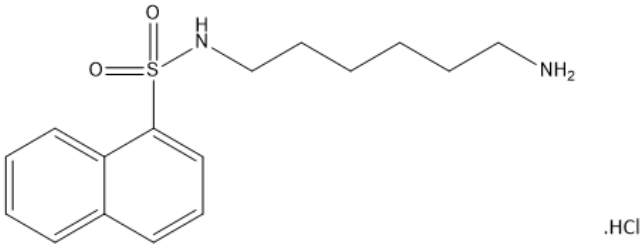
2.1.2.9 5-Chloro-(1-naphthylsulfonyl)hexylamine (9)



Compound 1 (1.5 g, 5.77 mmol) was dissolved in dry DCM (15 mL) and added dropwise to a solution of hexylamine (1.42 mL, 8.65 mmol, 1.5 equiv), triethylamine (2.01 mL, 14.43 mmol, 2.5 equiv) in dry DCM (5 mL) at 0°C. The mixture was stirred at room temperature for 1h. The reaction was quenched with H_2O (10 mL) and extracted with DCM (3 x 10 mL). The combined organic layers dried over $MgSO_4$ and concentrated. Column chromatography (hexane:EtOAc; 9:1) afforded the title hexylamine as a light brown solid (1.38 g, 74%). M. p.: 99.5-100.3, V_{max} (ATR): (N-H): 2941, 2855, 1422, 1315, 1136, 1100, 779. δ_H (400 MHz, $CDCl_3$): 8.62 (d, 1H, *J*=7.9 Hz, Ar-*H*), 8.59 (d, 2H, *J*=7.9 Hz, Ar-*H*), 8.34 (d, 1H, *J*=7.9 Hz, Ar-*H*), 7.71 (d, 1H, *J*=7.9 Hz, Ar-*H*), 7.67 (t, 1H, *J*=7.9 Hz, Ar-*H*), 7.58 (t, 1H, *J*=7.9 Hz, Ar-*H*), 4.50 (s, 1H, NH), 2.91 (q, 2H, *J*=5.5 Hz, CH_2), 1.35 (p, 2H, *J*=5.5 Hz, CH_2), 1.12 (m, 4H, CH_2), 1.07 (m, 2H, CH_2), 0.78 (t, 3H, *J*=5.5 Hz, CH_3). δ_C ($CDCl_3$, 400 MHz): 135. 7 (C-Ar), 133.5 (C-Ar), 132.0 (C-Ar), 130.8 (C-Ar), 130.7 (C-Ar), 129.9 (C-Ar), 128.4 (C-Ar), 127.7 (C-Ar), 125.6 (C-Ar), 123.9 (C-Ar), 43.7 (CH_2), 31.4 (CH_2), 29.8 (CH_2), 26.4 (CH_2), 22.7 (CH_2), 14.2 (CH_3). LRMS (ES^+): m/z 326 ((M+H), ^{35}Cl), 328 ((M+H), ^{37}Cl). HRMS ($ASAP^+$):

Found M+H, 326.0982, $C_{16}H_{21}NO_2S^{35}Cl$, requires *M*, 326.0981. Elemental analysis: Calculated for $C_{16}H_{21Cl}NO_2S$; C, 58.98; H, 6.19; N, 4.30. Measured C, 59.04; H, 6.09; N, 4.05.

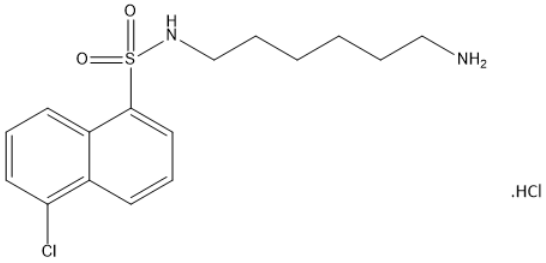
2.1.2.10 6'-Amino-1'-(1-naphthylsulfonylamino)hexane hydrochloric acid salt (10)



Naphthalenesulfonamide (0.5 g, 2.21 mmol) was dissolved in dry DCM (10 mL) and added dropwise to a solution of 1,6-diaminehexane (5.14g, 44.2 mmol, 20 equiv.) in dry DCM (10 mL) at 0°C. The mixture was stirred at room temperature for 2h and quenched with H_2O (10 mL). The mixture extracted with DCM (3 x 10 mL). The combined organic layers dried over $MgSO_4$ and concentrated. This product (0.5 g, 1.6 mmol) was dissolved in dry DCM (20 mL) and added to a solution of di-tert-butyl dicarbonate (0.5 g, 2.28 mmol, 1.4 equiv.) in dry DCM (5 mL). The mixture was stirred for 16h at room temperature and quenched with H_2O (10 mL). The mixture extracted with DCM and the combined organic layers were dried over $MgSO_4$ and concentrated and give a light yellow oil. Without further purification, this product (0.6g, 1.48 mmol) was dissolved in dry DCM (5 mL). 1 mL (excess) of HCl in 4.0M dioxane was added and the mixture was stirred for 16h at room temperature. After concentrating under vacuum, the solid obtained was washed with diethyl ether, filtered and dried under vacuum overnight to afford the title salt as a white solid (0.23g, 45%). $\tilde{V}_{max}$ (ATR): (N-H): 3262, 2938, 2182, 1737, 766 cm^{-1}. δ_H (400 MHz, D_2O): 8.60 (d, 1H, *J*=7.8 Hz, Ar-*H*), 8.28 (d, 1H, J=7.7 Hz, Ar-*H*), 8.26 (d, 1H, *J*=7.7 Hz, Ar-*H*), 8.14 (d, 1H, *J*= 7.8 Hz, Ar-*H*), 7.81 (t, 1H, *J*= 7.8 Hz, Ar-*H*), 7.74 (t, 1H, *J*= 7.8 Hz, Ar-*H*), 7.68 (t, 1H, *J*=7.7 Hz, Ar-*H*), 2.95 (t, 2H, *J*= 7.2 Hz, CH_2), 2.76 (t, 2H, *J*=7.2 Hz, CH_2), 1.34 (p, 2H, *J*= 7.2

Hz, CH_2), 1.28 (p, 2H, *J*=7.2 Hz, CH_2), 1.03 (m, 4H, CH_2). δ_C (400 MHz; D_2O): 135.0 (C-Ar), 134.1 (C-Ar), 133.3 (C-Ar), 129.7 (C-Ar), 129.4 (C-Ar), 128.6 (C-Ar), 127.3 (C-Ar), 127.2 (C-Ar), 124.5 (C-Ar), 123.5 (C-Ar), 42.3 (CH_2), 39.1 (CH_2), 28.0 (CH_2), 26.3 (CH_2), 24.8 (CH_2), 24.7 (CH_2). LRMS (ES^+): m/z 305 (M-H), HRMS ($ASAP^+$): Found M+H, 307.1480, $C_{16}H_{23}N_2O_2S$, requires *M* 307.1473. Elemental analysis: Calculated for $C_{16}H_{23}ClN_2O_2S$ C, 56.05; H, 6.76; N, 8.17. Measured C, 56.00; H, 6.72; N, 8.01.

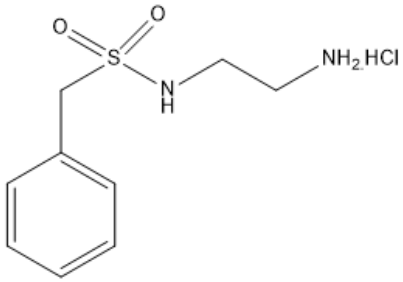
2.1.2.11 N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloric acid salt (11)



5-chloronaphthalene sulfonamide (1.5 g, 4.62 mmol) was dissolved in dry DCM (10 mL). The solution was then added dropwise to a solution of 1,6-diaminehexane (10.73 mL, 92.3 mmol, 20 equiv) in dry DCM (10 mL) at 0°C. The mixture was stirred at room temperature for 2h and quenched with H_2O (10 mL). The mixture extracted with DCM (3 x 10 mL) and the combined organic layers were dried over $MgSO_4$. The solution was concentrated and yielded brown solid as a product. This product (1.26 g, 3.7 mmol) was dissolved in dry DCM (40 mL). The solution was added to a solution of di-tert-butyl dicarbonate (1.13 g, 5.18 mmol, 1.4 equiv) in dry DCM (10 mL). The mixture was stirred for 16h at room temperature. The reaction was then quenched with H_2O (10 mL) and extracted with DCM (3 x 10 mL). The combined organic layers were dried over $MgSO_4$ and concentrated. Without further purification, the product (1 g, 2.2 mmol) was dissolved in 5 mL dry DCM. 1 mL (excess) of HCl in 4.0M dioxane was added. The mixture was then stirred at room temperature for 16h when TLC analysis (DCM:MeOH; 9:1) showed complete consumption of starting material. After

concentrated in vacuo, the solid obtained was washed with diethyl ether and dried under vacuum overnight to afford the title sulfonamide as a light brown solid (0.27g, 35 %). $\tilde{V}_{max}$ (ATR): 3290, 2924, 1734, 1133, 701 cm^{-1}. δ_H (600 MHz, D_2O): 8.69 (d, 1H, *J*=7.6 Hz, Ar-*H*), 8.58 (d, 1H, *J*= 8.4 Hz, Ar-*H*), 8.34 (d, 1H, *J*= 7.6 Hz, Ar-*H*), 7.87 (d, 1H, *J*=8.4 Hz, 8-*H*), 7.80 (t, 1H, *J*= 7.6 Hz, Ar-*H*), 7.73 (t, 1H, *J*=8.4 Hz, Ar-*H*), 2.96 (t, 2H, *J*= 7.5 Hz, CH_2), 2.77 (t, 2H, *J*= 7.5 Hz, CH_2), 1.32 (p, 2H, *J*= 7.5 Hz, CH_2), 1.26 (p, 2H, *J*= 7.5 Hz, CH_2), 0.98 (m, 4H, CH_2). δ_C (400 MHz; D_2O) 134.0 (C-Ar), 132.5 (C-Ar), 131.22 (C-Ar), 130.7 (C-Ar), 130.4 (C-Ar), 128.7 (C-Ar), 128.4 (C-Ar), 127.6 (C-Ar), 125.7 (C-Ar), 123.0 (C-Ar), 42.2 (CH_2), 39.2 (CH_2), 27.8 (CH_2), 26.3 (CH_2), 24.8 (CH_2), 24.7 (CH_2). LRMS (ES^+): 341 ((M+H), ^{35}Cl), 343 ((M+H), ^{37}Cl). HRMS (ASAP+): Found M+H, 341.1091 $C_{16}H_{22}N_2O_2S^{35}Cl$, requires *M* 341.1081. Elemental analysis: Calculated for $C_{16}H_{22}Cl_2N_2O_2S$; C, 50.93; H, 5.88; N, 7.42. Measured C, 51.07; H, 5.82; N, 7.18.

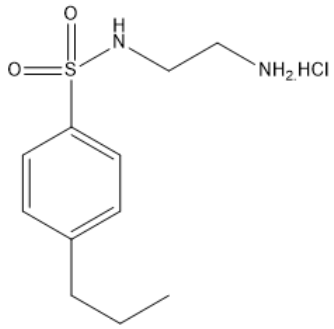
2.1.2.12 Alpha-toluenesulfonylethylene diamine hydrochloric acid salt



Alpha-toluenesulfonyl chloride (0.25g, 1.31. mmol) was dissolved in dry DCM (2mL) before added dropwise into a solution of N-Boc-ethylene diamine (0.21 mL, 1.31. mmol, 1 equiv.) and trimethylamine (0.37 mL, 2.62 mmol, 2 equiv.) in dry DCM (5 mL). The reaction was stirred for 1h at room temperature. The reaction was quenched with water (5 mL) after the complete consumption of starting material by TLC (hexane: ethyl acetate; 3:2). The reaction mixture was extracted with DCM (3 x 5mL). The combined organic layers were washed with NaHCO3, dried (MgSO4) and concentrated. Flash column chromatography (hexane: EtOAc; 3:2) yielded the Boc-protected compound

as white solid (0.13g, 31%). The Boc-protected compound (0.13g, 0.41 mmol) was then dissolved in dry DCM (5mL). 1 mL (excess) of HCl in 4.0M dioxane was added dropwise. The mixture was then stirred at room temperature for 16h when TLC analysis (DCM:MeOH; 9:1) showed complete consumption of starting material. After concentrated in vacuo, the solid obtained was washed with diethyl ether and dried under vacuum overnight to afford the title sulfonamide as a white solid (0.06g, 75%).The M. p.: 191.3-191.8, V_{max} (ATR): (N-H): 3410, 1587, 777, 695 cm^{-1}. δ_H (400 MHz, D_2O): 7.51 (m, 5H, Ar-*H*), 4.58 (s, 1H, *J*=7.7 Hz, N-*H*), 3.31 (t, 2H, *J*=5.7 Hz, N-CH_2), 3.08 (t, 2H, *J*=5.7 Hz, N-CH_2), 1.20 (t , 1H, *J*=7.1 Hz, CH_2). δ_C ($CDCl_3$, 400 MHz): 130.7 (C-Ar), 129.1 (C-Ar), 128.8 (C-Ar), 128.4 (C-Ar), 57.5 (C-1), 40.3 (CH_2), 39.6 (CH_2). LRMS (ES^+): m/z 215 (M+H), HRMS ($ASAP^+$): Found M+H 215.0852, $C_{15}H_{19}N_2O_3S$, requires *M* 215.0854.

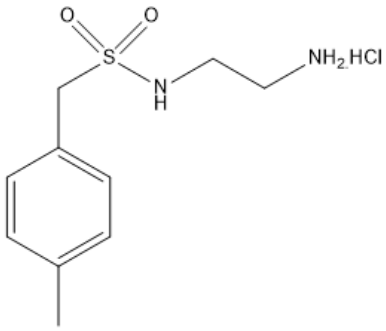
12.1.2.13 4-n-propylbenzenesulfonylethylene diamine hydrochloric acid salt



4-n-propylbenzenesulfonyl chloride (0.25g, 1.14 mmol) was dissolved in dry DCM (3 mL) before added dropwise to a solution of N-Boc-ethylene diamine (0.18 mL, 1.14 mmol, 1 equiv) and triethylamine (0.32 mL, 2.28 mmol, 2 equiv) in dry DCM (7 mL). The reaction was stirred for 1h at room temperature when it was quenched with H_2O (5 mL) and the reaction mixture extracted with DCM (3 x 5 mL). The combined organic layers were washed with $NaHCO_3$ (5mL), dried ($MgSO_4$) and concentrated. Flash column chromatography (hexane:EtOAc; 3:2) yielded the Boc-protected compound as a white solid (0.22 g, 55.5 %). The Boc-protected compound (0.22g, 0.64 mmol) was

then dissolved in dry DCM (5mL). 1 mL (excess) of HCl in 4.0M dioxane was added dropwise. The mixture was then stirred at room temperature for 16h when TLC analysis (DCM:MeOH; 9:1) showed complete consumption of starting material. After concentrated in vacuo, the solid obtained was washed with diethyl ether and dried under vacuum overnight to afford the title sulfonamide as a white solid (0.13g, 89 %). The M. p.: 158.8-159.4, V_{max} (ATR): (N-H): 3044, 1598, 730, 695 cm^{-1}. δ_H (400 MHz, D_2O): 7.78 (d, 2H, *J*=7.6 Hz, Ar-*H*), 7.20 (d, 2H, *J*=7.6 Hz, Ar-*H*), 3.37 (t, 2H, *J*=7.7 Hz, CH_2), 3.28 (t, 2H, *J*=7.7 Hz, Ar-*H*), 2.55 (t, 1H, *J*=7.6 Hz, C-1), 1.58 (t, 2H, *J*=7.6 Hz, C-2), 0.88 (t, 3H, *J*=7.6 Hz, CH_3). δ_C (D_2O, 400 MHz): 148.1 (C-Ar), 136.2 (C-Ar), 129.2 (C-Ar), 127.3 (C-Ar), 40.4 (CH_2), 37.8 (CH_2), 24.3 (C-1), 15.3 (C-2), 13.7 (CH_3). LRMS (ES^+): m/z 244 (M+H), HRMS ($ASAP^+$): Found M+H 243.1167, $C_{11}H_{19}N_2O_2S$, requires *M* 243.1170.

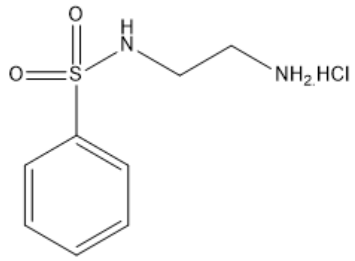
2.1.2.14 Alpha-p-xylenesulfonylethylene diamine hydrochloric acid salt



Alpha-p-xylenesulfonyl chloride (0.2g, 0.98 mmol) was dissolved in dry DCM (3 mL) before added dropwise to a solution of N-Boc-ethylene diamine (0.16 mL, 0.98 mmol, 1 equiv) and triethylamine (0.27 mL, 1.95 mmol, 2 equiv) in dry DCM (7 mL). The reaction was stirred for 1h at room temperature when it was quenched with H_2O (5 mL) and the reaction mixture extracted with DCM (3 x 5 mL). The combined organic layers were washed with $NaHCO_3$, dried ($MgSO_4$) and concentrated. Flash column chromatography (hexane:EtOAc; 3:2) yielded the Boc-protected compound as a white solid (0.21 g, 67 %). The Boc-protected compound (0.17g, 0.58 mmol) was then

dissolved in dry DCM (7mL). 1 mL (excess) of HCl in 4.0M dioxane was added dropwise. The mixture was then stirred at room temperature for 16h when TLC analysis (DCM:MeOH; 9:1) showed complete consumption of starting material. After concentrated in vacuo, the solid obtained was washed with diethyl ether and dried under vacuum overnight to afford the title sulfonamide as a white solid (0.09g, 89 %). The M. p.: 191.3-192.0, $\tilde{V}_{max}$ (ATR): (N-H): 3293, 1624, 795 cm^{-1}. δ_H (400 MHz, D_2O): 7.22 (d, 2H, *J*=8.0 Hz, Ar-*H*), 7.17 (d, 2H, *J*=7.7 Hz, Ar-*H*), 4.36 (s, 2H, 1-*H*), 3.12 (d, 2H, *J*=6.2 Hz, CH_2), 2.92 (d, 2H, *J*=6.2 Hz, CH_2), 2.23 (s, 3H, CH_3). δ_C (D_2O, 400 MHz): 139.6 (C-Ar), 130.1 (C-Ar), 129.6 (C-Ar), 125.3 (C-Ar), 57.2 (C-1), 40.3 (CH_2), 39.5 (CH_2), 20.2 (CH_3). LRMS (ES^+): m/z 230 (M+2H), HRMS ($ASAP^+$): Found M+H 229.1011, $C_{10}H_{17}N_2O_2S$, requires *M* 229.1022.

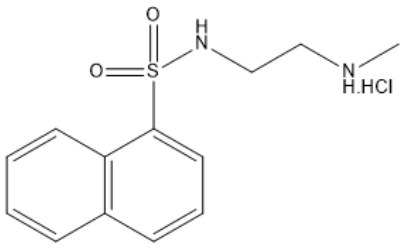
2.1.2.15 Benzenesulfonylethylene diamine hydrochloric acid salt



Benzenesulfonyl chloride (0.2g, 1.13 mmol) was dissolved in dry DCM (3 mL) before added dropwise to a solution of N-Boc-ethylene diamine (0.18 mL, 1.13 mmol, 1 equiv) and triethylamine (0.32 mL, 2.26 mmol, 2 equiv) in dry DCM (5 mL). The reaction was stirred for 1h at room temperature when it was quenched with H_2O (5 mL) and the reaction mixture extracted with DCM (3 x 5 mL). The combined organic layers were washed with $NaHCO_3$, dried ($MgSO_4$) and concentrated. Flash column chromatography (hexane:EtOAc; 3:2) yielded the Boc-protected compound as a white solid (0.17 g, 50 %). The Boc-protected compound (0.17g, 0.58 mmol) was then dissolved in dry DCM (7mL). 1 mL (excess) of HCl in 4.0M dioxane was added dropwise. The mixture was then stirred at room temperature for 16h when TLC analysis

(DCM:MeOH; 9:1) showed complete consumption of starting material. After concentrated in vacuo, the solid obtained was washed with diethyl ether and dried under vacuum overnight to afford the title sulfonamide as a white solid (0.32g, 99 %). The M. p.: 179.2-179.9, V_{max} (ATR): (N-H): 3000, 1677, 1632, 1540 cm^{-1}. δ_H (400 MHz, D_2O): 7.75 (m, 2H, Ar-*H*), 7.61 (m, 1H, Ar-*H*), 7.53 (m, 2H, Ar-*H*), 3.05 (d, 2H, *J*=6.2 Hz, CH_2), 2.99 (d, 2H, *J*=6.2 Hz, CH_2). δ_C (D_2O, 400 MHz): 137.4 (C-Ar), 133.8 (C-Ar), 129.9 (C-Ar), 126.6 (C-Ar), 39.9 (CH_2), 39.1 (CH_2). LRMS (ES^+): m/z 201 (M+H), HRMS ($ASAP^+$): Found M+H 201.0698, $C_8H_{13}N_2O_2S$, requires *M* 201.0697.

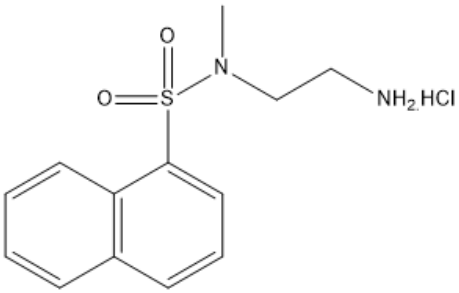
2.1.2.16 N-methyl-N-naphthylsulfonyl-1,2-ethanediamine hydrochloric acid salt



Naphthalenesulfonyl chloride (0.3g, 1.33 mmol) was dissolved in dry DCM (3 mL) before added dropwise to a solution of N-(2-Aminoethyl)-N-methyl-carbamic acid tert-butyl ester (0.24 mL, 1.33 mmol, 1 equiv) and triethylamine (0.37 mL, 2.66 mmol, 2 equiv) in dry DCM (7 mL). The reaction was stirred for 1h at room temperature when it was quenched with H_2O (5 mL) and the reaction mixture extracted with DCM (3 x 5 mL). The combined organic layers were washed with $NaHCO_3$, dried ($MgSO_4$) and concentrated. Flash column chromatography (DCM;MeOH; 9:1) yielded the Boc-protected compound as a white solid (0.31 g, 64 %). The Boc-protected compound (0.21g, 0.57 mmol) was then dissolved in dry DCM (8mL). 1 mL (excess) of HCl in 4.0M dioxane was added dropwise. The mixture was then stirred at room temperature for 16h when TLC analysis (DCM:MeOH; 9:1) showed complete consumption of starting material. After concentrated in vacuo, the solid obtained was washed with diethyl ether and dried under vacuum overnight to afford the title sulfonamide as a

white solid (0.15g, 73 %). The M. p.: 182.8-183.5, V_{max} (ATR): (N-H): 3251, 1613, 891, 775 cm^{-1}. δ_H (400 MHz, D_2O): 8.43 (d, 1H, *J*=8.7 Hz, Ar-*H*), 8.14 (m, 2H, Ar-*H*), 8.00 (d, 1H, *J*=8.7 Hz, Ar-*H*), 7.69 (d, 1H, *J*=8.7 Hz, Ar-*H*), 7.62 (d, 1H, *J*=8.3 Hz, Ar-*H*), 7.54 (t, 1H, *J*=8.3 Hz Ar-*H*), 3.46 (t, 2H, *J*=7.0 Hz, CH_2), 3.04 (t, 2H, *J*=7.0 Hz, CH_2), 1.08 (d, 3H, *J*=7.1 Hz, CH_3). δ_C (D_2O, 400 MHz): 135.3 (C-Ar), 134.0 (C-Ar), 131.9 (C-Ar), 129.9 (C-Ar), 129.5 (C-Ar), 128.8 (C-Ar), 127.3 (C-Ar), 127.1 (C-Ar), 124.4 (C-Ar), 123.2 (C-Ar), 48.3 (CH_2), 38.6 (CH_2), 32.7 (CH_3). LRMS (ES^+): m/z 266 (M+2H), HRMS ($ASAP^+$): Found M+H 265.1011, $C_{13}H_{17}N_2O_2S$, requires *M* 265.1009.

2.1.2.17 N-methylamino-N-naphthylsulfonyl-ethylamine hydrochloric acid salt



Naphthalenesulfonyl chloride (0.2g, 0.89 mmol) was dissolved in dry DCM (3 mL) before added dropwise to a solution of N-Boc-2-methylamino-ethylamine (0.3 g, 1.77 mmol, 2 equiv) and triethylamine (0.37 mL, 1.77 mmol, 2 equiv) in dry DCM (7 mL). The reaction was stirred for 1h at room temperature when it was quenched with H_2O (5 mL) and the reaction mixture extracted with DCM (3 x 5 mL). The combined organic layers were washed with $NaHCO_3$, dried ($MgSO_4$) and concentrated. Flash column chromatography (hexane:EtOAc; 3:2) yielded the Boc-protected compound as a white solid (0.06 g, 19 %). The Boc-protected compound (0.06 g, 0.11 mmol) was then dissolved in dry DCM (5mL). 1 mL (excess) of HCl in 4.0M dioxane was added dropwise. The mixture was then stirred at room temperature for 16h when TLC analysis (DCM:MeOH; 9:1) showed complete consumption of starting material. After concentrated in vacuo, the solid obtained was washed with diethyl ether and dried under vacuum overnight to afford the title sulfonamide as a white solid (0.02g, 74 %).

The M. p.: 118.1-118.8, V_{max} (ATR): (N-H): 3025, 1651, 814, 774 cm^{-1}. δ_H (400 MHz, D_2O): 8.54 (d, 1H, *J*=8.3 Hz, Ar-*H*), 8.19 (d, 1H, *J*=7.4 Hz, Ar-*H*), 8.07 (d, 1H, *J*=7.4 Hz, Ar-*H*), 8.02 (d, 1H, *J*=8.3 Hz, Ar-*H*), 7.70 (t, 1H, *J*=8.3 Hz, Ar-*H*), 7.64 (t, 1H, *J*=7.4 Hz Ar-*H*), 7.58 (t, 1H, *J*=7.4 Hz, Ar-*H*), 3.44 (t, 2H, *J*=5.8 Hz, CH_2), 3.14 (t, 2H, *J*=5.8 Hz, CH_2), 2.89 (s, 3H, CH_3). δ_C (D_2O, 400 MHz):.135.3 (C-Ar), 134.3 (C-Ar), 131.4 (C-Ar), 129.6 (C-Ar), 129.4 (C-Ar), 128.8 (C-Ar), 127.8 (C-Ar), 127.3 (C-Ar), 124.5 (C-Ar), 123.8 (C-Ar), 47.3 (CH_2), 36.8 (CH_2), 34.5 (CH_3). LRMS (ES^+): m/z 266 (M+2H), HRMS ($ASAP^+$): Found M+H 265.1011, $C_{13}H_{17}N_2O_2S$, requires *M* 265.1017.

2.2 Biological methods

2.2.1 Materials

2.2.1.1 Chemicals

All chemicals and media were supplied by one of the following companies unless otherwise stated: Sigma-Aldrich Ltd (Poole, UK), Fischer Scientific UK Ltd (Loughborough, UK), Bioline (London, UK)

2.2.1.2 Plant material

Arabidopsis thaliana wild-type seeds were from laboratory lab stocks of the Columbia (Col-0) and *Landsberg erecta* (Ler-0). The *A. thaliana* line constitutively expressing 35S::apoaequorin was also available in the lab (Knight et al., 1991). The *A. thaliana* line expressing RGA-GFP seeds were obtained from Prof. Keith Lindsey (Durham University, UK), the mutants of *gid1* (Griffiths et al., 2006), *sly1-10* (McGinnis et al., 2003) and *ga20ox* (Rieu et al., 2008) were obtained from Dr. Steve Thomas (Rothamsted Research, UK), whilst *della* (Achard et al., 2007), *ga1-5* (Zentella et al., 2007) mutants were purchased from Nottingham Arabidopsis Stock Centre (NASC) stock.

2.2.1.3 Enzymes

All DNA and RNA modifying enzymes were purchased from Bioline, Fisher Scientific UK Ltd (London, UK), Applied Biosystems (Forster City, USA), Qiagen (Crawley, UK), Promega (Southampton, UK) or New England Biolabs Ltd. (NEB, Hitchin, UK).

2.2.1.4 Oligonucleotides

Oligonucleotides were purchased from Life Technologies (Paisley, UK) and Integrated DNA Technologies (IDT, Leuven, Belgium).

2.2.1.5 Antibiotics

All antibiotics were purchased from Melford Laboratories Ltd (Ipswich, UK). Antibiotics were filter sterilized using a 0.22 µm filter (Milipore Corporation, Bedford, USA) attached to a syringe (VWR International Ltd, Lutterworth, UK), to make stocks, conserved at -20 °C, or directly prior to addition to liquid media. The concentrations used are listed in Table 2.1.

Table 2.1: Concentrations of antibiotics used

Antibiotic	**Stock concentration (mg/ml)**	**Working concentration (µg/ml)**	**Stock solvent**
Kanamycin	100	100	Water
Chloramphenicol	34	34	MeOH

2.2.2 Plant growth conditions

2.2.2.1 Seed sterilization

Arabidopsis seeds were sterilized in 1 mL 70% ethanol (v/v) by shaking (Labnet Vortex Mixer, Labnet International Inc., Woodbridge, New Jersey, USA) for 5 minutes before

being transferred on Whatman filter paper (Whatman™ International Ltd, Kent, UK). The seeds were air-dried in a laminar flow hood before being spread onto solid MS growth medium (see section 2.2.2.2 below). After sowing the seeds, the plates were sealed with micropore tape (3M Health Care, Neuss, Germany).

2.2.2.2 Plant growth media

Arabidopsis seeds were grown on solid 1 X MS medium agar plates (Murashige and Skoog, 1962). The medium adjusted to pH 5.8 by using 0.1M potassium hydroxide before adding 8 g of plant tissue culture agar (Sigma-Aldrich, Poole, UK). The medium was then sterilized by autoclaving for 20 minutes at 121°C, with 10^5 Pa pressure.

For the chemical plates, 1.2% (w/v) plant agar was used, with the addition of the chemicals was performed when MS media was cooling (50 °C) after autoclaving.

2.2.2.3 Plants growth condition

Sterilised seeds were stratified at 4°C for a minimum of 48 h to achieve synchronous germination. The seeds were then grown in a Percival (CU-36L5D, CLF plant climatics, Emersacker, Germany) with a photoperiod of 16/8 h at a light intensity of 150 µmol m^2 s^{-1} and a temperature of 20±1°C. Root growth analysis (see 2.2.4.1) was performed on 8-day-old seedlings; RNA extraction (see 2.2.5.7) was performed on 10-day-old seedlings; biochemical analyses (see 2.2.10) was performed on two week-old seedlings; imaging experiments (see 2.2.8) were performed on 7-day-old seedlings. For guard cell assays (see 2.2.4.4), 7-day-old seedlings were transferred onto hydrated 44mm peat plugs (Jiffy Products International AS, Moerdijk, Norway) and grown at 20 °C with a photoperiod of 12/12 h for another 3 weeks. Seedlings transferred to peat plugs were covered in cling film for two days. Peat plugs were watered until the siliques had developed. If seeds needed to be collected from individual plants, the Aracon system (Beta tech, Ghent, Belgium) was used to contain each plant.

2.2.2.4 Chemical treatment

For root growth (see 2.2.4.1), hypocotyl growth and seed germination, chemical treatments were conducted by adding the chemical with appropriate concentration into 1.2 % (w/v) agar after autoclaving and cooling (50 °C).

For stomatal aperture (see 2.2.4.4) and RNA extraction (see 2.2.5.7) experiments, chemical pre-treatments were performed by floating plants on water with the addition of the chemical overnight unless otherwise stated. Appropriate controls were applied by adding an equal concentration of the solvent to a control set of plants.

For calcium measurements (see 2.2.3), chemicals were applied directly to the plants by injection with a syringe inside the luminometer cuvette, where the plants were housed. As the plant was in 0.5 mL of water, 0.5 mL of chemical at double concentration needed was added in each case.

2.2.3 Calcium measurements

2.2.3.1 Reconstitution of aequorin

Aequorin reconstitution was performed by floating *Arabidopsis* seedlings on water containing 10 μM coelenterazine (Biosynth Srl, Staad, Switzerland) 1% (v/v) methanol. All plants were left in the dark from 12 to 24h at 20 °C before calcium measurement.

2.2.3.2 Luminescence measurement

Arabidopsis seedlings were individually transferred to a cuvette containing 0.5 mL of water (Sarstedt, Numbrecht, Germany). Following a 30 mins resting period, the cuvettes were individually inserted into a luminometer chamber and luminescence levels were recorded every 1 sec using a digital chemioluminometer with discriminator and cooled housing unit (Electron Tubes Limited, Middlesex, UK) in order to reduce background noise (Knight et al., 1996; Knight et al., 1991). Luminescence was

recorded for 10 sec before injection of any chemical. Discharge was performed at the end of the experiment by injection of an equal volume of 2 M $CaCl_2$, 20% (v/v) ethanol (final concentration 1 M $CaCl_2$, 10% (v/v) ethanol).

2.2.3.3 Aequorin luminescence calibration

Calibration was performed as previously described (Knight et al., 1996), following this logarithmic equation: pCa = 0.332588(-logk) + 5.593, where k = counts luminescence counts per second/ total remaining counts. The number of total counts is calculated as the amount of data collected from the beginning of the experiment to the end of the discharge.

2.2.4 Plant physiology

2.2.4.1 Root elongation

To test the effect of selected chemicals on root elongation, phenotypic analysis was performed on 7 days old seedlings. Col-0 seeds were germinated and grown on 0.8% agar plates. After 7 days, the seedlings were transferred to 1.2% agar plates containing one chemical at a final concentration of 25, 50, 100 and 200µM. The end of the roots at this point was marked on the petri dish using pen. As chemical stocks were produced by dissolving solid chemicals in dimethylsulfoxide (DMSO, Sigma-Aldrich Company Ltd, Dorset, UK), a DMSO treatment was applied as a control. The plants were subsequently grown vertically. After 5 days of chemical treatment, images of the plates were scanned and the root length was measured using ImageJ software. The measurement value starts from the marks that have been put right after seedlings transferring step.

2.2.4.2 Hypocotyl length

The assay performed was adapted from de Lucas et al. (2008). Hypocotyl length was performed under reduced intensity of light on 2 days old seedlings. Col-0 seeds were

germinated on nylon mesh, on 1.2% agar plates. The plates were placed in a vertical orientation and under fluorescent white light (fluence rate of 40-60 μmol m^{-1} s^{-1}). After 2 days, the nylon mesh was transferred across to the plate containing chemical. The plates were then continue to place in vertical orientation for three days under reduced light intensity before the plates were scanned. The measurement of hypocotyl was done using ImageJ software. At least 15 seedlings were measured for each plate.

2.2.4.3 Seed germination assay

After seed sterilization, seeds were sowed on chemical plates (55mm) at 100 μM concentration, with or without the addition of 1 μM of ABA. To score seed germination percentage, the radical emergence was analyzed after 48h and 72h.

2.2.4.4 Stomatal aperture assay

Arabidopsis thaliana at the age of four weeks were used in this assay. Epidermal peels were incubated in stomatal opening buffer, 10mM KCl and 50mM 2-(N-morpholino)-ethane-sulfonic acid (MES), pH 6.15 at 20 °C (Gonzalez-Guzman et al., 2012). For the competition assay (Chapter 5), the peels were incubated for an hour before pre-treatment with eW5 (final concentration of 100 μM) for another an hour. The peels were then incubated for 2h in the same buffer supplement with or without 5 μM of ABA. The stomatal aperture was observed under light microscope and the image was captured using StreamCatcher software, with 10 images for every peel. The measurement was performed using ImageJ software.

For the assay presented in Chapter 6, the experiment was conducted in minimal light and the pre-treatment with the chemicals was not performed. The peels were incubated in MES buffer described above for 2h before adding the chemical with the final concentration of 100 μM. The treatment was take place for another 2h before imaging.

2.2.5 Plant molecular biology

2.2.5.1 DNA extraction

2.2.5.1.1 Total genomic extraction

The extraction method was adapted from Edwards et al. (1991). A single leaf from 2-3 week old plants was transferred to a microfuge tube and flash frozen in liquid nitrogen. The sample was ground in 400 µL of Edwards extraction buffer (200mM Tris HCl pH 7.5, 250mM NaCl, 25mM EDTA). The tube was then spun for 1 min in a centrifuge at full speed (15000*g*). An aliquot of the supernatant (300 µL) was transferred to a fresh tube. The supernatant was then mixed with 300 µL of isopropanol and incubated at room temperature for 2 minutes. The tube was spun again for 5 mins and the supernatant was removed and discarded. The pellet was dried using a vacuum dessicator (5031 eppendorf UK Ltd, Stevenage, UK) before resuspension in 50 µL of TE buffer.

2.2.5.1.2 Plasmid DNA extraction from *E. coli*

Small scale bacterial plasmid DNA extraction was performed using the Wizard® Plus SV Minipreps DNA Purification System (Promega), according to the manufacturer's instructions. Briefly, 5 mL bacterial cultures were centrifuged and the bacterial pallet was resuspended and lysed in the presence of alkaline phosphatase. DNA was bound to the column supplied. The column was then washed in an ethanol-based buffer and the DNA was eluted in nuclease-free water.

2.2.5.1.3 Extraction of DNA from an agarose gel

DNA fragments separated by gel electrophoresis were excised from the gel using a scalpel blade whilst visualizing on a UV trans-iluminator (Ultra-Violet products Ltd, Cambridge, UK). The DNA was then purified using a QIAquick gel extraction kit (Qiagen) according to manufacturer's manual. In this method, the agarose gel slices

were first dissolved in a buffer and the mixture was applied to a column with a silica membrane. Nucleic acids adsorbed to the membrane and impurities were washed away. The DNA was finally eluted in a low-salt buffer.

2.2.5.2 Polymerase Chain Reaction (PCR)

2.2.5.2.1 DNA polymerases and reaction mixes

For general PCR reactions either BioTaq polymerases (Bioline) were used. For high fidelity applications, a proof reading Phusion DNA polymerase was used (Finnzymes, Keileranta, Finland).

Reaction mixes were made up according to the manufacturer's instructions using the buffers and $MgCl_2$ provided.

For random amplification polymorphism DNA (RAPD), the details for the reaction is shown in Table 2.2.

Table 2.2: The details for one reaction of RAPD

Component	Volume in one reaction (µL)
My Taq Reaction Buffer	10
Template DNA	2
Primer (20mM)	1
My Taq DNA Polymerase	0.5
Water	36.5
Total per reaction	50

2.2.5.2.2 Oligo nucleotides

Primers were designed to be a minimum of 18 bp in length and to have a GC content of 40-60 % for optimal annealing. The full list of oligo nucleotides used for PCR can be found in Appendix A.

2.2.5.2.3 Cycling conditions

PCR was performed using a 96 well Px2 thermocycler (Thermo Electron Corporation, Waltham, Massachusetts, USA). The PCR conditions are listed in Table 2.3. The resulting PCR products were analysed using gel electrophoresis (see 2.2.5.3).

Table 2.3: PCR conditions for different Taq polymerases

Cycle steps	Time and temperature			No. of cycles
	Bio Taq	Phusion	RAPD	
Initial denaturation	95 °C; 5 min	98 °C; 30 s	95 °C; 10 min	1
Denaturation	95 °C; 30 s	98 °C; 10 s	95 °C; 30 s	25-35
Annealing	50-60 °C; 30 s	50-60 °C; 20 s	35 °C; 15 s	
Extension	72 °C; 2 min	72 °C; 30 s	72 °C; 45s	
Final extension	72 °C; 2 min	72 °C; 10 min	72 °C; 45s	1

2.2.5.3 Gel electrophoresis

DNA was separated by size using agarose gel electrophoresis. Gels were prepared by melting 1% (w/v) electrophoresis grade agarose (Sigma) in 0.5 x TBE buffer (0.11 M Tris, 90 mM borate, 2.5 mM EDTA, pH 8.0) in a microwave oven. After cooling to 50 °C, Midori Green (NIPPON Genetics EUROPE, Dueren, Germany) was added to a final concentration of 5 µg/mL. The molten gel was poured into a gel tank containing a comb and allowed to set.

TBE (0.5X) was used as a running buffer and 5 X DNA sample-loading buffer (Bioline) was added to DNA samples before loading into wells. Gels were run at 35 mA for approximately 1 h. Nucleic acid bands were visualized using a UV trans-illuminator

(Uvitech Limited, Cambridge, UK) at a wavelength of 254 nm. Fragment size was determined by comparing to a 1 kb molecular size standard (Bioline Hyperladder 1).

2.2.5.4 Cloning

2.2.5.4.1 Plasmid

The pET24a plasmid (Addgene) used was from lab stock, with the map is provided in Appendix B.

2.2.5.4.2 Restriction digests

Restriction digests were carried out to obtain fragments for cloning. Digests were carried out using NEB restriction enzymes and buffers and these were incubated for a minimum of 2 hr at the temperature recommended by the manufacturer (usually 37 °C). Digests were then run on a gel to determine the size of the insert (see 2.2.5.3).

For epigenetic analysis, a mixture of 2µL of gDNA, 1µL of *HpaII* or *MspI* enzyme (20,000 unit/mL each), 1µL of CutBuffer and 16µL of nuclease free water was incubated at 37°C overnight followed by PCR reaction (see 2.2.5.2.3).

2.2.5.4.3 Ligation

DNA fragments were ligated into a linearized vector using T4 DNA ligase (Promega) in the supplied buffer. A 1:3 molar ratio of linearized vector to insert was used. Ligation reactions were incubated overnight at 16 °C.

2.2.5.5 Transformation

Aliquots (25 µL) of α-select silver cells (Bioline) were transformed with plasmid DNA. DNA (2.5 µL) was added to thawed cells on ice and incubated for 20 min. The cells were then heat shocked at 42 °C for 30 s before being returned to ice for a further 2 min. SOC media (250 µL) (Life Technologies) was added to the cells and they were

then incubated with shaking at 220 rpm for 1 h at 37 °C. Cell were then plated onto LB containing 100 µg/mL Kanamycin and incubated overnight at 37 °C.

For cells used for protein expression, BL21 cells were used and 300 µL of LB media was added to the cells before shaking for an hour at 37 °C for 1 h. Cells were then plated onto LB containing 100 µg/mL Kanamycin and 34 µg/mL chloramphenicol.

2.2.5.6 Sequencing

2.2.5.6.1 Sequencing reactions

For sequencing of plasmids containing cloned fragments, DNA was isolated using the Miniprep method (see 2.2.5.1.2). All sequencing reactions were carried out by the DNA sequencing laboratory (Department of Biosciences, Durham University).

2.2.5.6.2 Sequence alignments

Analysis of chromatograms was carried out using SnapGene (www.snapgene.com).

2.2.5.7 RNA extraction

Total RNA was extracted from about 25 seedlings that had been treated with a final concentration of 100 µM eW5 using the protocol supplied from the RNeasy Plant Total RNA kit (Qiagen, Crawley, UK). DNase digestion using RNase free DNase (Qiagen, Crawley, UK) was carried out. RNA was eluted into 50 µL nuclease-free water and RNA concentration was measured using NanoDrop ND-1000 Spectrophotometer. The RNA was eluted in RNase free water and stored at -80 °C.

2.2.5.8 Nucleic acid quantification

2.2.5.8.1 UV-Vis spectrophotometer

DNA or RNA concentrations were determined using a ND-1000 UV-Vis spectrophotometer (Labtech International Ltd.). Water or elution buffer was used as a zero reference.

2.2.5.8.2 Bioanalyzer

The quality and integrity of the RNA to be used for RNA-seq experiments was determined using the Agilent 2100 bioanalyzer (Palo Alto, CA) according to manufacturer's instructions.

2.2.5.9 cDNA synthesis

cDNA was produced from RNA using the Applied Biosystems High Capacity cDNA synthesis kit according to manufacturer's instructions. A total volume of 10 µL was made up with 1 µg total RNA and nuclease-free water. A master mix was made up containing (per reaction): 2 µL 10 x RT buffer, 2 µL 10 x RP buffer, dNTP Mix (100mM), 1 µL Multiscribe^{TM} Reverse Transcriptase and 4.2 µL of nuclease free water. Aliquots of this master mix (10 µL) were added to each diluted RNA sample to give a total volume of 20 µL. Controls with no RNA and no reverse transcriptase enzyme were all set up in parallel. The samples were then transferred to a Px2 thermocycler and run on the following program: 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 s. The resulting cDNA was diluted 1:50 with nuclease free water before use in qPCR and then stored at -20 °C until needed.

2.2.5.10 Real time quantitative PCR analysis (qRT-PCR)

The relative transcript level of genes of interest was determined by qPCR using the Applied Biosystems 7300 real time PCR machine and Go Taq qPCR master mix

(Promega). Diluted cDNA (5 µL) (see 2.2.5.9) was added to 10 µL of SYBR green master mix (see Table 2.4). The GoTaq qPCR master mix contains ROX reference dye to account for optical differences between the wells. The diluted cDNA and master mix were added to wells of a 96-well plate (STARLAB UK, Milton Keynes, UK). For each sample to be tested, three replicate wells were set up to give three technical replicates. At least three biological replicates were also carried out for each experiment. *PEX4* (At5g25760) was used as an endogenous control (Moffat et al., 2012). A full list of qPCR primers can be found in Appendix A. All qPCR primers were designed using Primer3 (http://primer3.ut.ee) with an amplicon size of 80-120 bp. Relative quantification was performed by the $\Delta\Delta C_t$ method (Applied Biosystems).

Table 2.4: The reaction mixture for qRt-PCR

Component	Volume in one reaction (µL)
2xSYBR master mix	7.5
5 µM forward primer	0.9
5 µM reverse primer	0.9
Water	0.7
Template cDNA	0.5
Total per reaction	15

qRT-PCR reactions were run on an Applied Biosystem 7300 Real-Time PCR system (Life Technologies Corporation). The cycling condition for qRT-PCR reaction was as follows (Table 2.5).

Table 2.5: The cycling condition for qRT-PCR

Cycle steps	Time and temperature	No. of cycles
Initial denaturation	50 °C; 2 min	1
Denaturation	95 °C; 10 min	25-35
Annealing	°C; 30 s	
Extension	72 °C; 2 min	
Final extension	72 °C; 2 min	1

2.2.5.11 Protein expression

A colony from BL21 cells transformed (see 2.2.5.5) was growing in the LB with 100 µg ml^{-1} Kan was incubated overnight at 37 °C, with shaking (250 rpm). The cells were then incubated in a 500 mL of medium (40 g/L yeast extract, 30 g/L glycerol, 1 g/L NaCl, 13.1 g/L K_2HPO_4 and 1.88 g/L $(NH_4)H_2HPO_4$; pH 7.0) containing 100 µg/ml Kan and 34 µg/ml Chl at 37 °C, with shaking (250 rpm). The culture was incubated to an optimal density of 0.6-0.8 at 600nm. The culture was induced to express protein by adding IPTG with the final concentration of 1 mM and the induction was carried out overnight at 37°C. Cells were collected by centrifuging the cultures and collected the product spun down. The cell paste was stored at -80°C before lysis and sonication step (see 2.2.5.12).

2.2.5.12 Lysis and sonication of the bacteria

The cells (see 2.2.5.11) was resuspended in 5 mL of lysis buffer (20mM Tris, 200mM NaCl, 5mM EDTA; pH 7.5) and sonicated for 5 min. The cells were then centrifuged at 20,000 rpm and 4 °C for 20 min before the clear lysate was collected. The purification was performed using Akta Purifier system (see 2.2.5.13).

2.2.5.13 Protein purification

The protein was purified using Akta Purifier system with the filtered running buffer (20mM Tris pH 7.5, 200mM NaCl, 5mM EDTA). The protein was loaded by injecting 5 mL of lysate solution. The bound protein was eluted using desthiobiotin and collected. The purification of the protein can be monitored under 280 nm wavelength. The concentration of the protein was determined using NanoDrop (see 2.2.5.8.1).

2.2.6 Identification of purified protein

2.2.6.1 Protein size separation

Protein was separated using sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The separating gel (390 mM Tris-HCl pH 8.8; 0.1% (w/v) electrophoresis grade SDS; 10% (w/v) acrylamide:bis (37.5:1); 0.01% (w/v) ammonium persulphate (APS), 0.05% (v/v) TEMED) was cast, overlaid with methanol and allowed to set. Methanol was removed and the stacking gel (125 mM Tris-HCl pH 6.8; 0.1% electrophoresis grade SDS, 5% (w/v) acrylamide:bis (37.5:1); 0.01% (w/v) APS; 0.01% (v/v) TEMED) was added, the comb inserted and the gel allowed to set. Fractionated samples were diluted in 2x SDS-loading buffer (125mM Tris-HCl pH 6.8; 20% glycerol; 4% SDS; 10% β-mercaptoethanol, 0.0025% bromophenol blue). Samples were denatured at 95 °C for 5 minutes. The gel was electrophoresed at 30mA in 1x running buffer (25mM Tris-HCl; 192mM glycine; 0.1% (w/v) SDS, pH 8.3). Gels were then stained with Coomassie blue (see 2.2.6.2).

2.2.6.2 Coomassie staining of gels

Gels were stained for 2h in Coomassie (10% methanol; 10% acetic acid; 0.025% (w/v) Coomassie Brilliant Blue R-250). Gels were then destained in water overnight. Fragment size was determined by comparing to a 1 kDa molecular size standard (Color Prestained Protein Standard).

2.2.7 Biophysical analysis

2.2.7.1 Thermal Shift Assay (TSA)

The experiment was conducted using a range of protein concentration and chemical. For every concentration of protein (1mL), 4µL of 10X SYPRO orange was added and 10 µL the solution was pipetted into each well followed by 10 µL of chemical. Analysis was carried out using an Applied Biosystems 7500 Real Time PCR System, melt curve

program, with a ramp rate of 1°C, a temperature range of 25-90 °C, and 450nm as wavelength (Grøftehauge et al., 2014). Analysis was performed using a Phyton program, NAMI software (https://www.dur.ac.uk/chemistry/academic-groups/ehmke.pohl/nami/). The software generated a heat map table that can be referred to colour coded with respect to the reference temperature (usually 24 °C). White colour indicates that the difference is not significant. Lower melting temperatures from reference temperature are coloured from yellow to red, while the wells with higher temperature are coloured from light to dark blue as the temperature increased.

2.2.7.2 Microscale Thermophoresis (MST)

MST was performed using Monolith N.T. 115 (Jerabek-Willemsen et al., 2014). Approximately 10 µM recombinant protein was labeled with red fluorescent dye (NT-647-NHS, Alexa). The range of concentrations of the required ligand (ranging from 6 nm to 100 µM for ABA, and 12nM to 200 µM for eW5) was incubated with 300 nM of purified protein for an hour in assay buffer (20mM HEPES/150mM NaCl/1mM EDTA; pH 7.5). The samples were loaded into NanoTemper glass capillaries and microthermophoresis was carried out using 20% LED power and 80% MST. The K_d was calculated using the mass action equation via the NanoTemper software.

2.2.8 Confocal laser scanning microscopy techniques

GFP:RGA seeds were germinated and grown on 1.2% MS vertically. After 7 days, the seedlings were incubated in chemical solution (at the final concentration of 100 µM). The seedlings were then imaged according to time point of 2h and 24 h. The microscope used was Leica SP5 CLSM FLIM FCCS (Leica Microsystems, Wetzlar, Germany). The laser was Argon, excitation 488 nm (blue) and used at 50% intensity. Emission spectra were collected through a 585 nm long pass filter. Images were processed using Leica software, LAS AF Lite.

2.2.9 Assay of PP2Cs phosphatase activity

This assay was adapted from Melcher et al. (2009). Phosphatase assay was performed by colorimetric determination by phosphate release. Para-nitrophenylphosphate (pNPP) was used as a substrate and read at 405nm. The PP2C used in this experiment is HAB1 expressed in pETM11 (provided by Prof. Pedro Rodriguez, Valencia). This experiment was performed in 96-well plate. $MnCl_2$ (final concentration of 1µM) was added in each well before adding eW5 (final concentration of 100 µM). The solution of protein mixture of PYR1/PYL and HAB1 (1:2 molar ratio) and water was added and the reaction was incubated for 10 minutes before adding ABA (final concentration of 1µM). The reaction was incubated at room temperature for 10 minutes. pNPP was added before immediately reading absorbance at 405 nm.

2.2.10 Quantification of gibberellic acid (GA)

GA levels were quantified using UPLC (Waters Acquity H-Class UPLCR system with fluorescence and photodiode array detectors) (Waters, Wilmslow, UK). About 500 mg of two week old seedlings were ground in a mortar and pestle with 5 mL solvent composed of 75% (v/v) MeOH, 20% (v/v) H_2O and 5% (v/v) formic acid. The material was transferred to a 50 mL Falcon tube and the mortar was rinsed with 1 mL of extraction solvent, added to the previous solution. The tube was vortexed for 5 min before mixed on a rotater overnight at 4 °C. The solution was centrifuged at 1000*g* for 10 min at 4 °C, the supernatant was collected and the pallet was re-extracted twice with 1 mL MeOH. The combined supernatant was concentrated using a nitrogen evaporator, followed by freeze-drying and re-dissolved in 2 mL H_2O at pH 2.5 (previously acidified with 1N HCl). The GAs were extracted into 3 x 2 mL diethyl ether and the upper layer was further dried under nitrogen. The samples were then resuspended in 1 mL of 50% MeOH. The analysis was performed using a LC system (Shimadzu Nexera UHPLC system), with a Waters HSS T3 C18 1.7 µm 1 mm x 100 mm column (Waters, Elstree, UK) at a flow rate of 100 µL/min. Mobile phase A was

made up of 0.1% (v/v) formic acid in water. Mobile phase B was made up of acetonitrile (initial 5% B, held for 2 minutes, with a linear gradient to 95% at 9 min, held for 3 min). A flow rate of 400 µL/min was used Automated LC injection added 2 µL of the sample for analysis (Forcat et al., 2008). Compounds were identified by multiple-reaction-monitoring (MRM) analysis in the negative mode. The quantification of GAs was determined using a standard curve with standards of 0, 10, 100 and 1000 µM concentrations.

2.2.11 RNA-Seq data analysis

2.2.11.1 Sample preparation

10 days old seedlings were incubated in water overnight before adding eW5 or W5 with the final concentration of 100 µM. Chemical treatment was performed for 6 hrs, with DMSO (10%) treatment as a control. RNA extraction was then performed on the resultant tissue samples (see 2.2.5.7) before confirming the integrity of the RNA using a bioanalyzer as described in Section 4.3. The samples were then sent to Illumina Services, Belgium for whole transcriptome analysis (https://emea.illumina.com/).

2.2.11.2 Data analysis

The raw data was pre-processed and normalized using the DESeq2 program through its standardized workflow (Love et al., 2014). The data was then tested for significance by Wald test (Engle, 1984), and the false discovery rate was determined using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) before performing Principal Component Analysis (PCA). Genes that were differentially expressed by >2 fold when compared to the respective control samples were selected for further analysis. Singular Enrichment Analysis (SEA) of Gene Ontology (GO) terms was carried out using agriGO (http://bioinfo.cau.edu.cn/agriGO/). GO terms for upregulated and downregulated genes for specifcally-eW5 treatment was performed. However, this approach did not give any significant GO terms specific for eW5 treatment only.

Therefore, as an alternative, a more targeted approach was taken: namely generating correlation graphs between eW5 and W5 treatment for genes of specific function. As the obvious difference between the effect of eW5 and W5 on plants is the root/shoot growth phenotype, genes related to plant hormones were extracted from the main data and plotted this way for analysis.

CHAPTER 3

Chemical screening to identify the phenotype induced

3.1 Introduction

Biologists often used classical genetics in order to characterize and dissect plant processes. However, as discussed in Section 1.3, this approach can be challenged by gene redundancy problem, which prevents the isolation of suitably functional mutants (Serrano et al., 2015). Therefore to overcome this problem, a more versatile approach, chemical genetics has been widely used. The basic principle of this approach is disturbing the biology system by applying a small molecules. Small molecules screening often lead to the discovery of the specificity of the protein, due to their specific binding to protein targets (Hicks and Raikhel, 2009; Serrano et al., 2015). In this approach, structure activity relationships (SAR) are used to understand the correlation between the chemical structure and biological activity (Hayashi et al., 2008b).

In earlier work in the group, a small scale chemical genetics study was undertaken to explore the role of the known calmodulin inhibitor, N-(6-Aminohexyl)-1-naphthalenesulfonamide (W5) and N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) in plants. For example, introducing W7 and W5 to the plants generated specific $[Ca^{2+}]_{cyt}$ transients in *Arabidopsis* seedlings and enabling the identification of Ca^{2+}-responsive genes in plants such as touch-responsive genes, *TOUCH3* (*TCH3*), *TCH4-like* and dehydration-responsive gene, *ERD15* (Kaplan et al., 2006). In addition, CaM inhibitors have been reported to increase cytosolic Ca^{2+} in carrot protoplast due to the inhibition of Ca^{2+} pumping from the cytosol used normally to maintain the low resting Ca^{2+} levels (Gilroy et al., 1987). W5 and W7 are both naphthalene sulfonamide compounds, with the only difference between them being

the C-5 chlorine substituent in W7 (Figure 3.1). Despite their high degree of structural similarity, the activity of these antagonists are significantly different with W5 showing reduced activity as compared to W7. For example, the application of W7 inhibits *Arabidopsis* root growth at a concentration of 10 μM while W5 only shows the same degree of inhibition at a concentration of 100 μM (Sinclair et al., 1996).

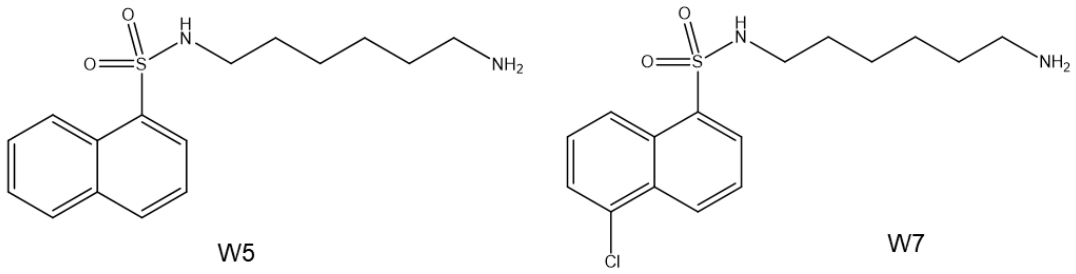


Figure 3.1: The structural similarity between W5 and W7 where W7 has a chlorine substituent at its C-5 naphthalene ring position.

To attempt to better understand this difference, a small set of structurally related analogues were prepared (Figure 3.2A). These compounds were then tested for effect on calcium signalling and growth. From the phenotypic (root growth) analysis, it was shown that one of the compounds (Compound 4) significantly induced root growth promotion (Figure 3.2B). Intriguingly this root growth promotion appear to be long term effect with seeds that have been soaked with the chemical prior to germination in the absence of the chemical.

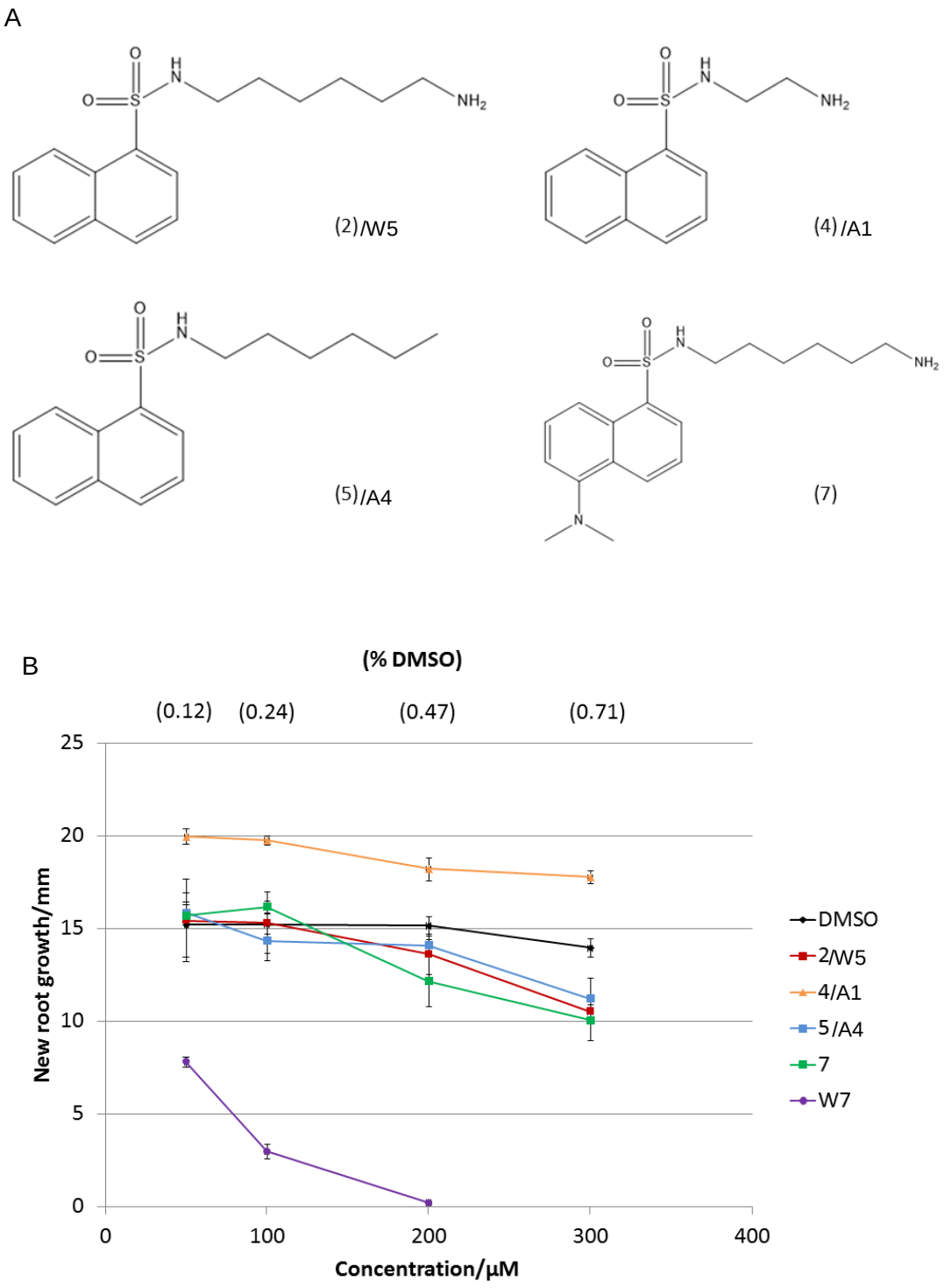


Figure 3.2: Previous study of the group. A) The list of compounds synthesized based on modification of W5 (2). B) The root growth assay after the treatment of the compounds. The figures were taken from the previous thesis.

This selected enhanced root growth was interesting and merited a further investigation. An initial question was whether this was a structure related effect and a study to answer this question was the initial objective of this work which is discussed

below. The explanation of the long term effect of this compound is discussed in the following chapter.

3.2 Introduction of the analogue

As discussed above simple replacement of the diamino hexane with an ethylene diamine unit led to a dramatic enhancement of growth (A1). The reason for this could be due to many factors including the sorter chain length and the position of the amino substituent. A simple analogue which reflected the hexyl chain of W5/W7 but had the nitrogen atom positioned at the same side as found in A1 was designed (A3). At the same time since having the chlorine substitution naphthalene at C-5 ring position renders W7 10 times more active than W5, the corresponding analogues with the same chlorine position were also synthesized with the hypothesis that the chlorinated compounds will show higher activity than their corresponding non-chlorinated compounds (Table 3.1). These compounds could be simply achieved from the parent sulfonyl chloride with standard techniques (Figure 3.3) (see Section 2.1 for reaction details). Each compound was purified by column chromatography.

However, the purification of the amino compounds was more challenging and was achieved by using a tert-butyloxycarbonyl (t-Boc)-protection procedure. t-Boc derivatives are one of the most widely used amino protecting group in organic synthesis because of its ease of installation and stability towards hydrolysis under most basic conditions and nucleophilic reagents (Theodoridis, 2000). Each amines was reacted with di-*tert*-butyl dicarbonate $(Boc)_2O$ (Basel and Hassner, 2000). After purification of the carbamate deprotection of Boc group was simply achieved under mild acidic conditions such as hydrochloric acid HCl (Ragnarsson and Grehn, 2013). Concentration of the product afford the pure amine protected as its hydrochloric salt.

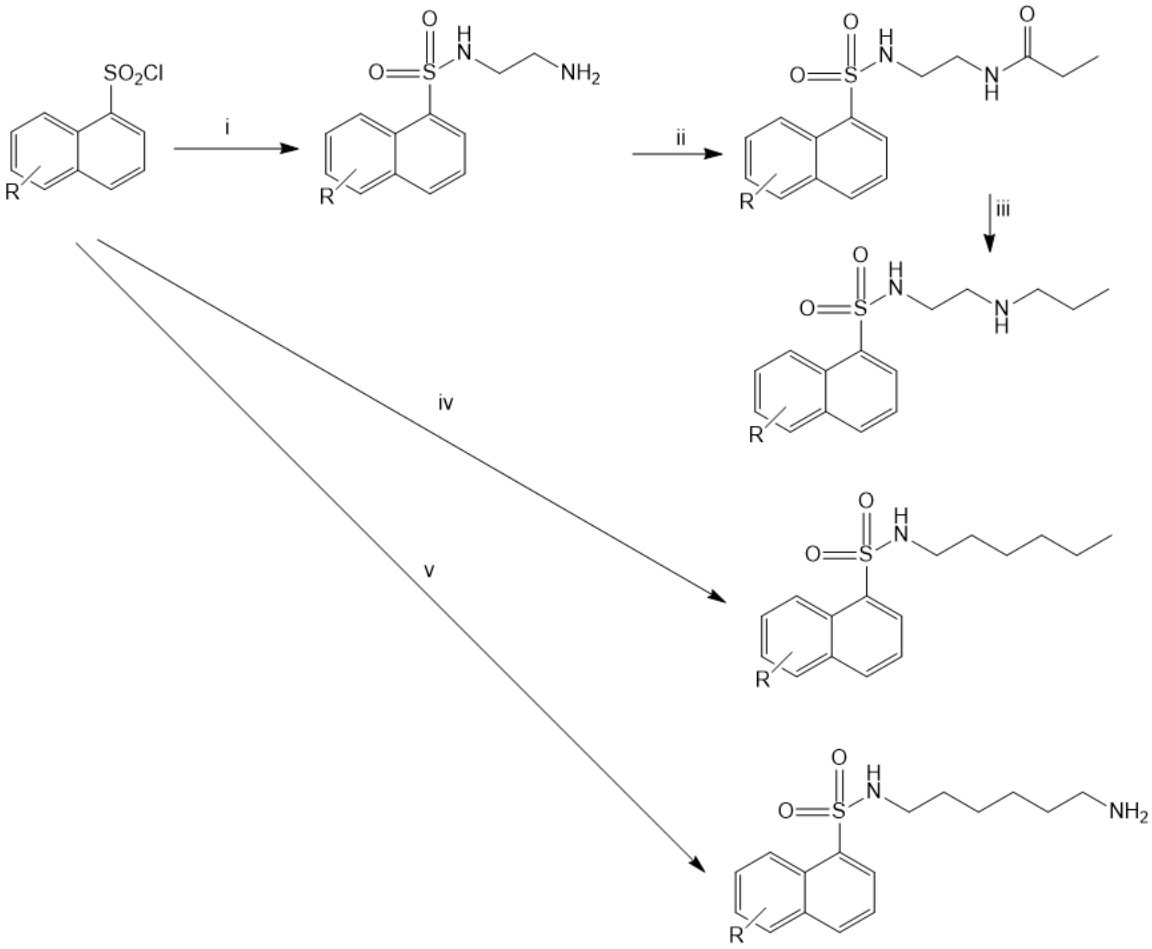
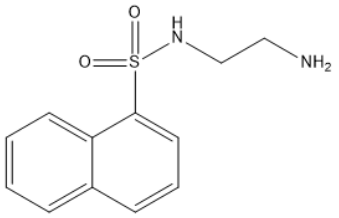
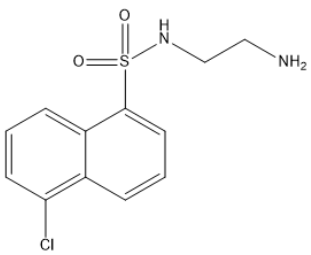
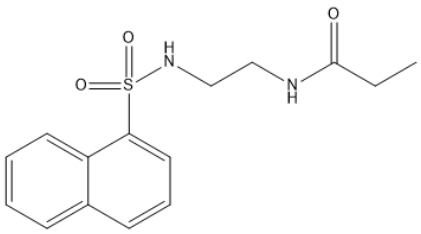


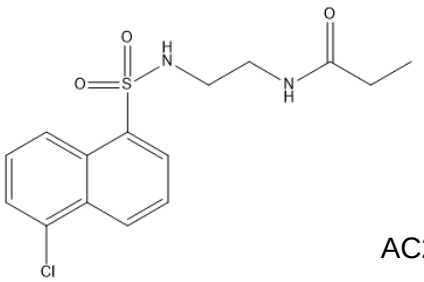
Figure 3.3: The schematic reaction for the synthesis of the analogues. i) ethylene diamine, CH_2Cl_2; ii) propionic anhydride, Et_3N, CH_2Cl_2; iii) BH_3.THF, THF; iv) hexylamine, Et_3N, CH_2Cl_2; v) 1,6-diaminehexane, CH_2Cl_2.

Table 3.1: The series of compounds that have been modified from calmodulin inhibitors, W7 and its less active analogue, W5. The series is divided into two groups: Non-chlorinated and chlorinated compounds.

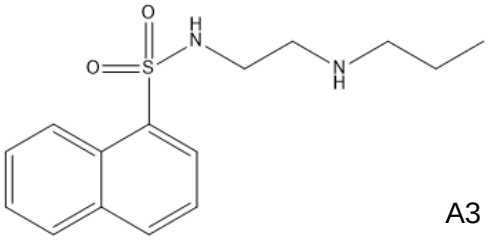
Non-clorinated compound		Chlorinated-compound	
	A1		AC1



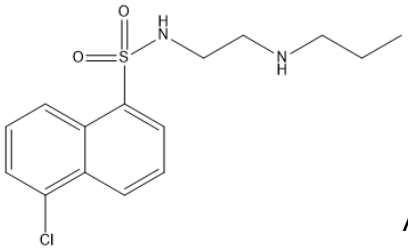
A2



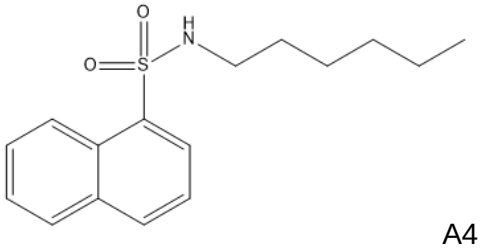
AC2



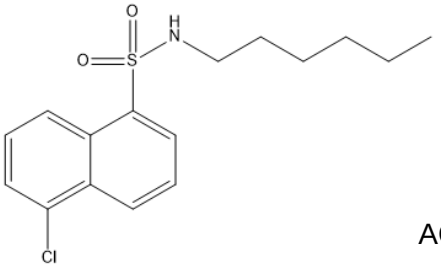
A3



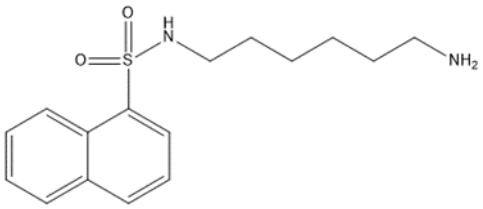
AC3



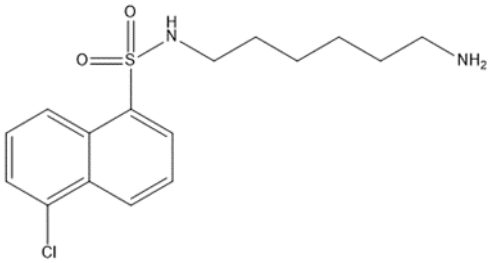
A4



AC4



W5



W7

With the compounds in hands the next objective was to explore was to explore effect of these in the plants. The main objectives to be discussed in this chapter include:

- Investigate the phenotype induced in response to the application of these compounds.
- Observing the response of these compounds on cellular calcium concentration.

3.3 Chemical screening using a root growth assay

As mentioned, earlier studies in the group suggested that the difference in bioactivity of the compounds might be correlated with the structural difference. The obvious effect was shown from the compound with the replacement of diamine hexane with the ethylene diamine chain, being the only compound that promoted root growth. It was hypothesized that there will be difference on root growth in response to different compound. On this basis that similar effects may arise the analogues in this study were initially explored in the same phenotypic analysis.

This assay was performed on 7 day old seedlings, where the seedlings were transferred onto chemical plates and grown vertically for five days. To identify the concentration that would be used for the rest of the compounds in these assays, the effect of a series of concentration of W7 (25, 50, 100 and 200 µM) on the root growth was performed. As shown in Figure 3.4A, root growth inhibition by W7 was dose-dependent, with 100 µM showing a significant difference to control. Therefore a concentration of 100 µM was chosen for the rest of the compounds in the series for testing. Overall, compounds containing chlorine inhibited the root growth more as compared to the non-chlorine series, except for A4 and AC4. Using W5 and W7 as references, all compounds inhibited root growth more than W5, but there was no compound that showed higher inhibition than W7, as new root length when plants were treated with W7 was the shortest for all the compounds (Figure 3.4B). In the series, there was one compound that showed a different effect from other compounds, where this compound was found to actually *promote* root growth.

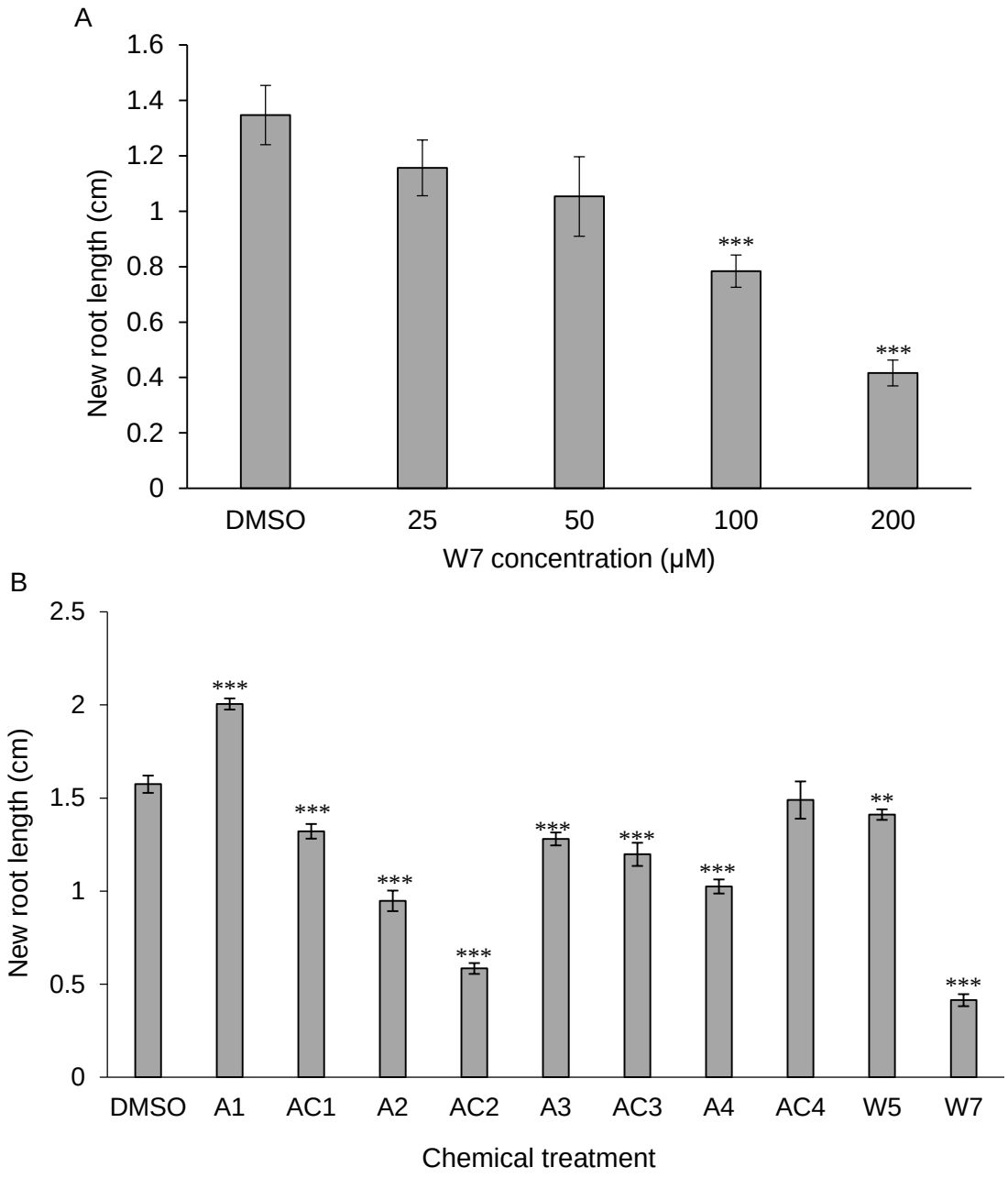


Figure 3.4: Root growth analysis of 7th days old seedlings after 5 days of chemical treatment. Error bars represent standard error of 18 seedlings. A) The experiment was performed at different concentration of W7 to choose the concentration to be used for the rest of the chemicals. B) The screening of the compounds in the series at the concentration of 100 μM. Asterisks indicate statistically significant differences (independent t-test, ** P<0.005, *** P<0.001) between DMSO and chemical treatment.

With different effect on root growth observed from the analogues, the compounds were further investigated to determine whether they are regulating the phenotype through calcium signalling. Investigation on calcium signalling was specifically performed due to the inhibition activity of W5/W7 in this particular

signalling. Therefore, the measurement of calcium concentration was then undertaken, which will be discussed in the following section.

3.4 Chemical screening on calcium signals

Calcium is important in plant development and is an essential plant nutrient. It is required in the cell wall and membrane, as well as an intracellular messenger in the cytosol where it has a major role in sensing abiotic and biotic stress of the plants. Although calcium has these multiple function in plants, it is only needed in a very low concentration (Sanders et al., 2002). At resting levels, the free concentration of Ca^{2+} is 100-200nM in the cytosol. However, at times it can reach to milimolar level notably when plants are exposed to stress (Reddy, 2001). Since at higher concentrations Ca^{2+} can cause toxicity (Sanders et al., 2002), cells actively pump Ca^{2+} to the apoplast or organelles to maintain the low concentration of the calcium (van der Luit et al., 1999).

Calcium has been implicated in mediating various developmental processes such as root hair and lateral root development as well as hormone-regulated cellular activities like cell division and elongation (Reddy and Reddy, 2004). With the finding that the structural differences led to differences in root growth as mentioned in the previous section, therefore the effect of the compounds was further explored to determine if the phenotypic effect is through calcium signalling. The investigation of the compounds on the cytosolic concentration of Ca^{2+} was then undertaken, achieved by using an aequorin expressing transgenic *Arabidopsis thaliana* line.

Aequorin is a bioluminescent protein that emits light on binding Ca^{2+} and was originally isolated from the coelenterate *Aequoria Victoria*. Aequorin has both protein (apoaequorin) and cofactor components (coelenterazine). The protein contains three EF-hand Ca^{2+} binding sites and aequorin undergoes conformational change when the binding sites are occupied by Ca^{2+}. In the bioluminescent reaction with Ca^{2+},

coelenterazine is converted into excited coelenteramide in a decarboxylation reaction, which later the relaxation of this leads to emission of light at 468 nm (Knight et al., 1996).

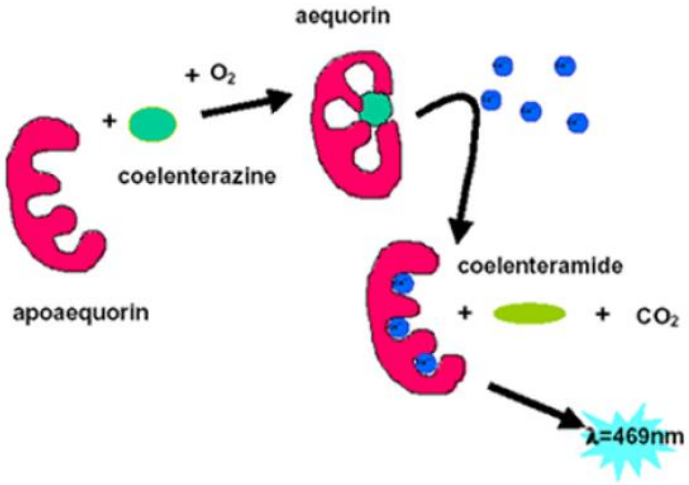


Figure 3.5: Reaction of coelentrazine and apoaequorin that lead to the emission of blue light that will be detected by luminometer. Figure was adapted from Mithofer and Mazars (2002).

With the obvious difference observed in root growth assay from a range of W7 concentration, a similar approach was used to determine at what concentration of W7 there was an effect on cellular Ca^{2+} concentration. Using three different concentrations (25, 50 and 100 µM), it was shown that the treatment of W7 increased $[Ca^{2+}]_{cyt}$ as compared to control (DMSO). However, there was no significant difference observed from all three W7 concentrations as shown in Figure 3.6. Therefore, the lowest W7 concentration, 25 µM was chosen to proceed with other chemicals.

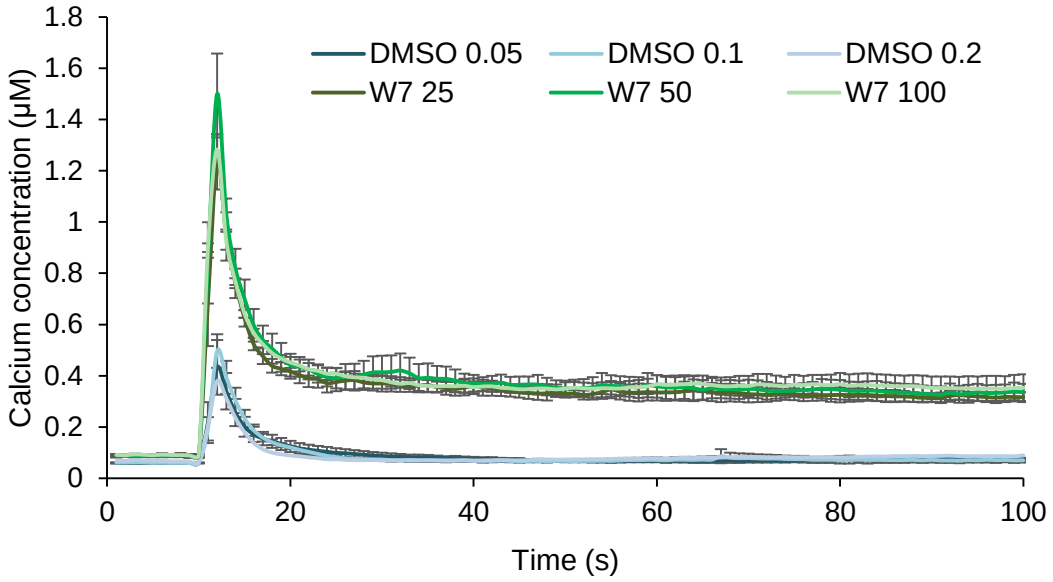


Figure 3.6: Cellular calcium concentration in response to W7 and its control, DMSO at different concentrations to see if the effect of chemical treatment on calcium concentration is dose-dependent.

The application of a CaM inhibitor will increase the cellular concentration of Ca^{2+}, due to the interaction of these antagonists with CaM, and inhibit cation channel activity. Hence, Ca^{2+} release from inositol triphosphate receptor channel was inhibited (Khan et al., 2001). This is consistent with this study where the application of W5 and W7 increased cytosolic Ca^{2+} concentration (Figure 3.7). This finding is however is not similar with the study conducted by Kaplan et al. (2006), where they showed that Ca^{2+} signal of W5 was lower than its chlorinated analogue, W7, suggesting that W5 is an less active analogue of W7. Unexpectedly, all of the compounds in the series increased cytosolic Ca^{2+} concentration, suggesting that all of the compounds might act as calcium antagonists.

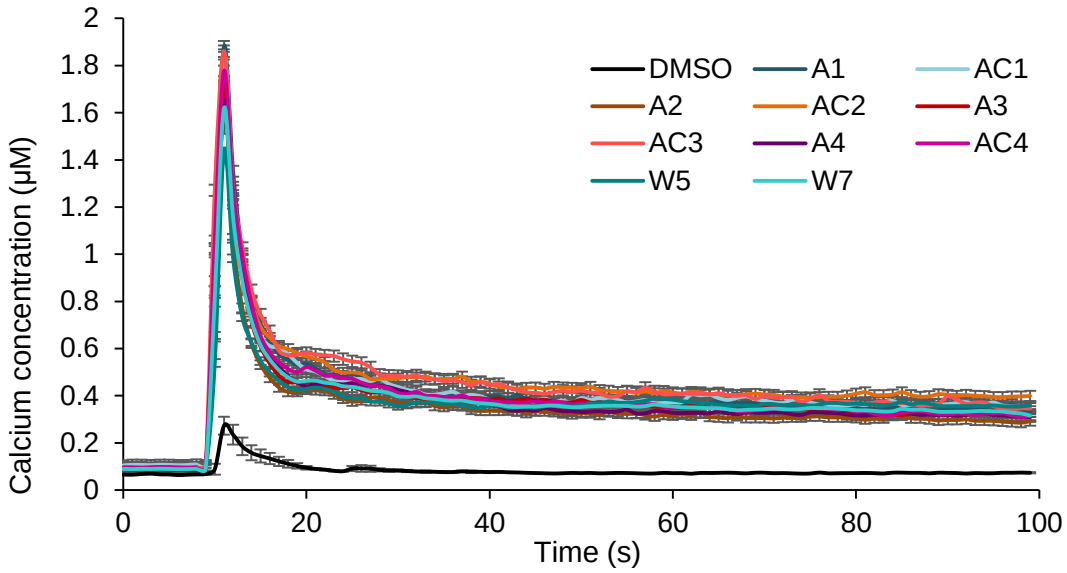


Figure 3.7: Cytosolic calcium response upon chemicals application at the concentration of 25 µM. DMSO was used as a control and the data presented was based on three biological replicates.

3.5 Discussion

Calcium is a universal second messenger that modulates stimulus-response coupling to regulate plant growth, development and response to environmental stresses (Tuteja, 2007). Calcium is very important in plant development being needed in root growth, including gravitropism, as well as maintaining the cell wall and membrane (Hepler, 2005). In addition, various stimuli such as cold, heat shock and drought triggers changes in the cytosolic Ca^{2+} concentration (Yang and Poovaiah, 2003; Yang et al., 2004). The higher concentration of Ca^{2+} are detected by sensors such as calmodulin (CaM). CaM has no enzymatic activity of its own, however upon Ca^{2+} binding, the complex induces conformational changes that enables it to interact with and activate a number of target proteins include kinases/phosphatases and metabolic enzymes regulate the function of many genes observed through physiological responses, such as cell growth and differentiation, and stress tolerance (Yang and Poovaiah, 2003; Snedden and Fromm, 2001; Bouché et al., 2005; Tuteja and Mahajan, 2007; DeFalco et al., 2010; Batistič and Kudla, 2012). For example CaM3 activates the CaM-binding

protein kinase (CBK3) which then phosphorylates the heat shock transcription factors (HSF), *HSFA1a*. This modulates the binding of the HSF to regulatory promoter elements thereby promoting the expression of heat shock proteins and mediating heat stress response (Liu et al., 2008; Zhang et al., 2009).

The presence of chloride in W7 C-5 naphthalene ring enhances its interaction with CaM with higher affinity than W5 (K_d for W7: 27μM and W5: 8.87 mM), and this is supported by the finding that W7 activity is 10 times higher than W5 in root growth inhibition (Sinclair et al., 1996). The possible regulation of the compounds as inhibitor of calcium signalling was investigated through phenotypic analysis to see the effect of the compounds on plant development. Different activity between W5 and W7 suggests that different functional groups affect this biological activity differently, based on how the interaction of the compounds with their targets (Stinemetz et al., 1992). Consistent with this, the different functional group in the compounds showed different degree of inhibition, as shown in Figure 3.4B. As W5 is less active than W7 (Gong et al., 1997; Yang and Poovaiah, 2003), the chlorinated versions of the applied chemicals were expected to have more profound effect on the plants compared to the non-chlorinated chemicals.

The interaction of Ca^{2+}-CaM with the target proteins through its deep hydrophobic binding pocket on both domains (Ikura et al., 1992; Meador et al., 1993). Osawa et al. (1998) determined the solution structure of Ca^{2+}-CaM complexed with W7 using an NMR approach, finding that one molecule of W7 has an interaction with each of the two domains of CaM. In each domain, the chloronaphthalene ring binds to the hydrophobic pocket, with the chlorine atom located in the deepest part of the hydrophobic pocket, completely blocking the key hydrophobic site in each CaM domain (Figure 3.8) (Osawa et al., 1998). Both sites are crucial for binding of target proteins such as myosin light-chain kinase (MLCKs) and CaM kinase IIα (Ikura et al., 1992; Meador et al., 1993).

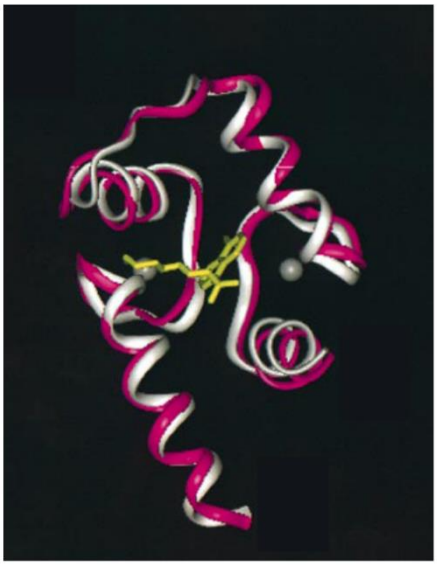


Figure 3.8: Crystal ctructure of the interaction between Ca^{2+}-CaM complex with W7. W7 and Ca^{2+} is shown in yellow stick model and grey ball, respectively. Figure was adapted from Osawa et al., 1998.

With different modifications of the compounds, it was suggested that the compounds bind to CaM and affect Ca^{2+} concentration or signal differently by blocking the interaction between Ca^{2+}-CaM, probably with the similar mechanism as described above, thus affect Ca^{2+} concentration or signal differently. This effect can be observed from the increased of Ca^{2+} response upon the chemicals treatment, and their root growth inhibition effect. However, there was an exception for A1 that shows root growth promotion, which suggest that there might be a different interaction between eW5 and Ca^{2+}-CaM complex, therefore it was suggested that A1 promotion effect is not through calcium signalling.

3.5.1 Conclusion

Taken together, all of the compounds in the series shows an increase in cellular calcium concentration and potentially acting as CaM antagonists, which supported by their phenotypic responses. However, one of the compound, A1 (later is called eW5) was found to have an opposite effect and it was suggested that this root growth promotion effect might not through calcium signalling. Therefore, the rest of the thesis

will focus on this compound to discover its mode-of-action in order to promote growth, probably through hormone signalling pathway which will be discussed in Chapter 5 and Chapter 6.

CHAPTER 4

The application of eW5 changes plant gene expression and transcript level

4.1 Introduction

As a sessile organism, plants have to adapt to their environment. Adverse environmental conditions can interrupt growth, development and productivity of plants (Sahu et al., 2013). As a result, plants develop flexible mechanisms to re-programme gene expression in order to respond and acclimatize to stresses (Golldack et al., 2011). Transcriptional responses of plants to environmental stress factors have been widely studied, and include genome-wide transcript profiling, determining specific signalling pathways and identification of specific protein function (Singh and Laxmi, 2015; Tang et al., 2015). There are groups of genes that belong to transcription factor families which have been shown to regulate stress-responsive genes, therefore play an important role in plant adaptation to their environment (Chinnusamy and Zhu, 2009).

It was found that epigenetic mechanisms can regulate various genetic functions including translation, transcription, DNA repair and cell differentiation (Sahu et al., 2013). It has been reported that epigenetics is responsible for heritable phenotypic differences (Miura, 2009). The best-known epigenetic mechanisms involve DNA methylation, histone modification and histone variants, where these mechanisms lead to enhanced or reduced gene expression and RNA transcription (Richards, 2006; Holeski et al., 2012). For DNA methylation, methylated cytosine residues in promoter and enhancer region may directly prevent the binding of transcription factors, but in most cases the presence of methylated cytosine is thought to attract methylcytosine-binding proteins, which recruit histone deacetylase and chromatin modelling proteins

that in turn compact the chromatin and restrict access of the transcriptional machinery (Cao and Jacobsen, 2002).

This chapter will discuss more about the effect of the application of eW5 that induces a longer root phenotype as mentioned in Chapter 3. The main objectives dealt with in this chapter includes:

- Determining if the phenotypic growth effect due to eW5 application is correlated to a specific epigenetic mechanism, namely DNA methylation.
- Further explore the effect of eW5 at the transcript level after its application.

4.2 Does eW5 promote growth through epigenetics?

As previously mentioned, plants are sessile organism and in order to adapt to the changing environment, they perceive and integrate the environmental signals and change the gene expression in response to these signals. Such responses are highly influenced by chromatin modifications, nucleosome positioning and DNA methylation (Lamke and Baurle, 2017). This process often known as epigenetics, which refer to heritable alterations in gene expression that may lead to a variation of phenotype without a change in DNA sequence (Morris, 2001). Epigenetics provide a molecular memory that supports the adaptation to the response by allowing the changed states to be prolonged through cell divisions (Baulcombe and Dean, 2014).

The structure of chromatin regulates the accessibility of genes to the transcriptional machinery, and is thus is an integral part of regulated gene expression and plant development (Zentner and Henikoff, 2013; Struhl and Segal, 2013). The basic unit of chromatin is the nucleosome, consisting of histone octamers of two molecules each of histone H2A, H2B, H3 and H4, around which 147 bp of DNA are wrapped in almost two turns. Histones are responsible for protection of DNA as well

as maintaining the shape and structure of nucleosome (Figure 4.1) (Behe, 1990). Nucleosomes act as physical barriers to transcription factors that bind to certain regions of DNA. However, specific acetylation can remove the positive charge on the lysine amino group that is acetylated, so that the nucleosome becomes loosened from the DNA (Goodsell, 2003). Histone N-termini subjected to posttranslational modifications that alter their interaction with DNA and nuclear proteins, such modifications include methylation, acetylation, phosphorylation, sumoylation, ubiquitination and ADP-ribosylation determine the interaction between the histone and other proteins, which may in turn regulate chromatin structure and transcription (Bird, 2002; Shahbazian and Grunstein, 2007; Loury and Sassone-Corsi, 2003; Nathan et al., 2003; Gibney and Nolan, 2010).

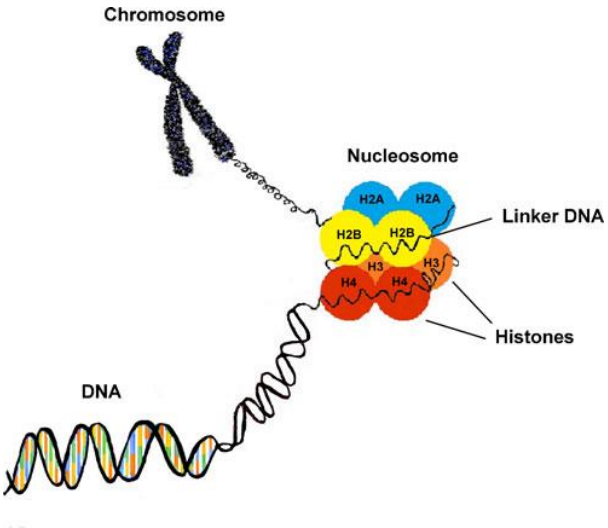


Figure 4.1: Diagram showing how DNA is wrapped around a cluster of histone proteins to form nucleosomes. Nucleosomes are composed from octamers that contain four histone homodimers. The image is taken from (Anier and Kalda, 2012).

In epigenetics research, DNA methylation is the most widely studied of parameter, since it has been the easiest to study. It is a crucial epigenetic modification of the genome that involves the covalent transfer of a methyl group to the C-5 position of the cytosine ring of DNA (Ponger and Li, 2005). DNA methylation is catalyzed by DNA methyltransferase (DNMTs) that transfer a methyl group to DNA by using S-adenosyl methionine (SAM) as the methyl donor. Operation of DNMTs leads to

conversion of cytosine to 5-methyl cytosine (Giannino et al., 2003). Generally methylation of some lysine and arginine residues of histones leads to gene suppression (Rosenfeld et al., 2009; Berr et al., 2010). This is due to DNA inaccessibility to transcription factors and other proteins due to the methyl group addition to the DNA or histone tail. In contrast, demethylation causes DNA accessibility by removing the methyl group. Methylation mostly take place at CpG sites. CpG islands are rich in CpG sites and are often located in the promoter region of genes.

A similar mechanism can be observed with histone acetylation. The activity of the target genes can be understood from histone modifications mechanisms. Some of the marker for active genes include histone H3 Lys9 (H3K9) acetylation, histone H3 Lys14 (H3K14) acetylation, and histone H3 Lys 4 (H3K4) trimethylation, while H3K9 deacetylation, H3K14 deacetylation and H3K4 dimethylation are known as the markers for silenced genes (Chen and Tian, 2007). The amino terminal tails of histones are highly basic due to a high content of lysine and arginine amino acids (Luger and Richmond, 1998). The neutralization of the positive charge of the histone tails will take place by the acetylation of lysine residues and therefore the interaction of the histone tails for negatively charged DNA is decreased. This promotes the accessibility of chromatin to transcriptional regulators thus activate the transcription (Kuo and Allis, 1998). In contrast, deacetylation through histone deacetylase (HDA) complexes is associated with gene repression (Chen and Tian, 2007).

The adaptation of plants towards environmental stress is influenced by histone modification, including methylation and acetylation which interrupt the binding of transcription factors thus bringing changes in phenotype (Lamke and Baurle, 2017). A big discovery came from studies of *Arabidopsis thaliana* methylation mutants such as methyltransferase 1 (*met1*) and decreased DNA methylation 1 (*ddm1*) (Stroud et al., 2013). *ddm1* mutants lead to the decrease in cytosine methylation throughout the genome (Vongs et al., 1993) and the decrease resulted in abnormalities in plant growth

and development such as dwarfed, late flowering and floral structure alteration (Stokes et al., 2001). Similarly, abnormalities in plant growth can be observed in *met1* mutants, also showing that the methylation is important in plant growth and development. In this mutant, the plant growth includes abnormalities in floral development, alteration in flowering time and reduced apical dominance (Fujimoto et al., 2012). Two genes that are responsible for flower development, *SUP* (*SUPERMAN*) and/or *AG* (*AGAMOUS*) were found to be inactive in the *met1* mutants that contribute to its floral abnormalities (Jacobsen and Meyerowitz, 1997; Jacobsen et al., 2000).

Other than DNA methylation, histone acetylation/deacetylation is important for plants to activate/repress gene activity during plant adaptation. For example, the attack by the plant pathogen, *Alternaria brassicicola* or by wounding increased the expression of histone deacetylases HDA19 and HDA6. It was suggested that the regulation of gene expression in plant responses to abiotic and biotic stress is due to the interaction between HDA19 and AtERF7, a member of the ethylene-responsive element binding family (Song et al., 2005; Song and Galbraith, 2006; Kim et al., 2008; Zhou et al., 2005b).

There are a number of methods to detecting methylation in the genome, Methylation Sensitive-Random Amplified Polymorphic DNA-Polymerase Chain Reaction (MS-RAPD-PCR) and bisulfite sequencing. The former technique involves restriction enzyme digestion and PCR based method that can be easily performed. This method needs a very small amount of DNA and can detect the methylation difference at the genome level (Singh, 2014). Methylation-sensitive restriction endonucleases are classic tools for locus-specific DNA methylation analysis (Bird and Southern, 1978; Lindsay and Bird, 1987) and were used in first genome-wide profiling approach which was based on two-dimensional separation of differently digested DNA fragments (Hatada et al., 1991). The most commonly used methylation-sensitive restriction enzymes are the isoschizomers *HpaII* and *MspI*.

There have been many reports indicating that epigenetic changes can cause phenotypic variation, thus epigenetics can be considered as an important factor in understanding phenotypic change (Fujimoto et al., 2012). Therefore, due to the specific phenotype observed upon eW5 application: promoting root growth as mentioned in Chapter 3, eW5 was hypothesized to potentially alter the histone arrangement. To investigate this hypothesis, MS-RAPD-PCR technique was performed. RAPD is a multiplex marker system that can be used to amplify random DNA fragments by using single-primer PCR (Kumar and Gurusubramanian, 2011). The amplification of DNA segment will take place on positions that are complementary to the primers' sequence (Williams et al., 1990).

For sample preparation, genomic DNA of *Arabidopsis* that has been grown on chemical plates (DMSO as a control and 100 µM of eW5) for two weeks was extracted. Enzyme cleavage was performed using *HpaII* and *MspI* where both of these enzymes cleave the sequence 5'-CCGG-3'. *MspI* is a methylation insensitive enzyme, therefore it will cleave at both methylated and unmethylated sites. While *HpaII* will only cleave unmethylated sites because it is methylation sensitive (Cedar et al., 1979). The digestion was conducted for 16h (overnight) followed by PCR amplification. Amplification products are separated by size on an agarose gel, and the band profile between DMSO and eW5 treatment was observed. The absence or presence of bands between samples leads to a rapid discovery of differentially methylated DNA fragments (Tryndyak et al., 2006). As shown in Figure 4.2, eW5 might have a potential to regulate epigenetic changes. However, before advancing this possibility any further, to confirm this result, further study such as bisulfite sequencing could be implemented, to both confirm and identify effects in the whole genome. Moreover, since epigenetic changes are often related to heritable phenotypes, a test of possible heritable effects of eW5 could be performed to further explore this.

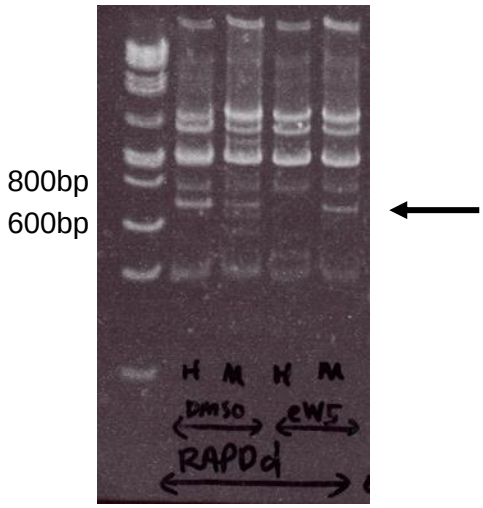


Figure 4.2: Agarose gel of PCR products after Random Amplified Polymorphic DNA primers amplification. H and M represent *HpaII* and *MspI*, respectively. *HpaII* is methylation sensitive thus it only cleaves the unmethylated site. The comparison between the methylated or unmethylated site are observed from the bands of the *HpaII* and *MspI* products. The experiment was performed using genomic DNA.

4.3 The changes in transcript level upon eW5 application

The discovery of RNAs role as the key intermediate between the genome and proteome makes the identification and quantification of the transcript a core facet of molecular biology. As discussed in the previous section, changes in gene expression can be due to the histone modification. Whilst not proven yet, the possible effect of eW5 in modifying histone arrangement by promoting DNA methylation led to the proposal that eW5 might affect gene expression.

The current technique to identify the genes that are affected by chemical treatment is RNA-seq which an approach to transcriptome profiling that uses next-generation sequencing to reveal the presence and quantity of RNAs, even low-abundance genes with sufficient sensitivity (Wang et al., 2009; Bellin et al., 2009). Understanding the transcriptome is important to understand the functional elements of the genome as well as the molecular constituents of cells and tissues (Wang et al., 2009). Transcriptomics is a powerful technique to catalogue all species of transcript,

including mRNA, non-coding RNAs and small RNAs; as well as to quantify the transcript level that adapt during development and under different conditions. This technique is widely used for gene discovery and differential gene expression analysis (Wang et al., 2009; Wang et al., 2010).

To investigate changes in gene expression, 10-days old seedling were either treated with eW5 or DMSO (as a control). Due to the chemical modification of eW5 relative to the calmodulin inhibitor, W5 as discussed in Chapter 3, it (W5) was used as a negative control in this experiment. The final concentration of chemical treatment was 100 μM for both eW5 and W5, with a 24h incubation time. Total RNA was extracted from the tissue and samples were run on a bioanalyzer to check the quality of the RNA. A typical bioanalyzer result is shown in Figure 4.3 and high quality RNA was confirmed by the presence of defined peaks of ribosomal RNA. The proportion of ribosomal bands (28S:18S) is considered as the primary indicator of RNA integrity, with a ratio of 2 being considered as an indicator of high quality RNA (Sambrook et al., 1989).

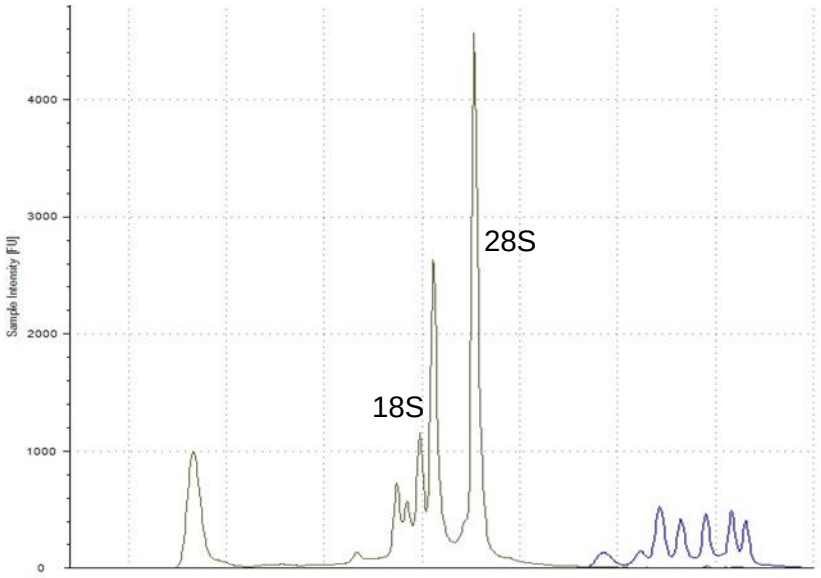


Figure 4.3: An electrophoreogram of an RNA sample used for RNA-seq analysis, which showed a high quality of RNA. The x-axis and y-axis indicate time and fluorescence signal, respectively.

RNA samples passing quality-control were sent to Illumina Services, Belgium for sequencing experiment. The raw data (Appendix C.1) was analysed using DESeq2 (Dr. David Dolan, Durham University) and Principle Component Analysis (PCA) was used to summarize the data set and to identify predominant gene expression profiles. The samples showed distinct clustering, with samples that had been treated in the same way clustering together (Figure 4.4). There was a consistency across biological replicates, except for one replicate, Sample 5.

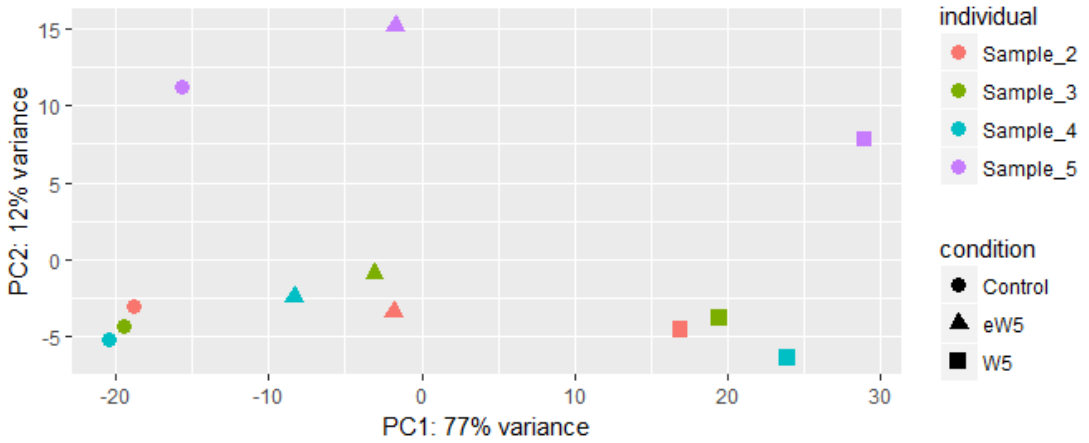


Figure 4.4: PCA plot showing the predominant gene expression profile of each sample. The different shape represent the different treatments. The x-axis represents 77% of the variance while the y-axis represents 12%.

The RNA-seq raw data was pre-processed and normalized, and the lists of genes that were different in expression by >2 fold when compared to control were submitted to genevenn.sourceforge.net to generate a Venn Diagram. The difference between the genes affected by these two compounds is shown in Figure 4.5. There are 1195 transcripts which were found to be upregulated and 1193 downregulated by eW5 treatment. While for W5 treatment, there are 1610 and 3185 transcripts that upregulated and downregulated, respectively.

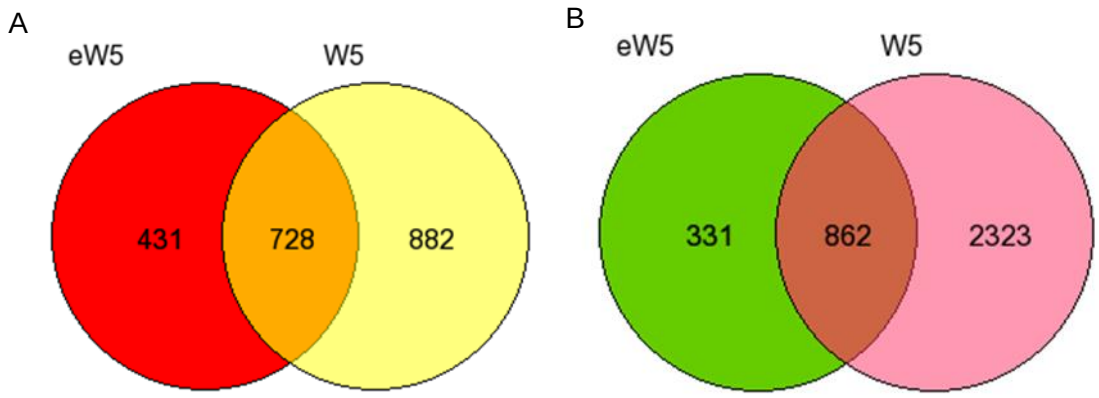


Figure 4.5: Venn diagram showing the number of transcript that A) upregulated and B) downregulated by eW5 and W5. The diagram was generated from genevenn.sourceforge.net. The data represents transcripts with a change of >2 fold.

In order to explore the biological processes and molecular functions that are enriched within the differentially expressed gene sets, gene ontology (GO) analysis was carried out. To perform this analysis, pairwise comparisons; eW5 vs DMSO (Appendix C.2), W5 vs DMSO (Appendix C.3) and W5 vs eW5 (Appendix C.4) were done first. The AgriGO gene ontology tool (http://bioinfo.cau.edu.cn/agriGO/) was then used to group genes into broad functional categories based on their GO annotations. The up- and down-regulated gene in eW5 and W5 treatment (as compared to DMSO) was assembledto extract out the unique genes for eW5 treatment, as shown in Table 4.1 and Table 4.2.

Table 4.1: Gene Ontology (GO) terms enriched ($p<0.05$) for upregulated gene after eW5 treatment

GO accession	Term	p-value
GO:0009791	post-embryonic development	0.00012
GO:0048856	anatomical structure development	0.0011
GO:0010154	fruit development	0.0046
GO:0003700	transcription factor activity	0.0055

GO:0032502	developmental process	0.0099
GO:0007275	multicellular organismal development	0.012
GO:0048316	seed development	0.012
GO:0016757	transferase activity, transferring glycosyl groups	0.014
GO:0048608	reproductive structure development	0.016
GO:0048513	organ development	0.016
GO:0003006	reproductive developmental process	0.016
GO:0048731	system development	0.016
GO:0030528	transcription regulator activity	0.016
GO:0032501	multicellular organismal process	0.017
GO:0009793	embryonic development ending in seed dormancy	0.021
GO:0022891	substrate-specific transmembrane transporter activity	0.025
GO:0022857	transmembrane transporter activity	0.036
GO:0009790	embryonic development	0.038
GO:0022414	reproductive process	0.043
GO:0003677	DNA binding	0.043
GO:0000003	reproduction	0.048
GO:0022892	substrate-specific transporter activity	0.057

Table 4.2. Gene Ontology (GO) terms enriched ($p<0.05$) for downregulated gene after eW5 treatment.

GO accession	Term	p-value
GO:0005732	small nucleolar ribonucleoprotein complex	0.0002
GO:0042254	ribosome biogenesis	0.0052
GO:0006355	regulation of transcription, DNA-dependent	0.0056
GO:0051252	regulation of RNA metabolic process	0.0059

GO:0022613	ribonucleoprotein complex biogenesis	0.0063
GO:0045449	regulation of transcription	0.0079
GO:0032774	RNA biosynthetic process	0.008
GO:0006351	transcription, DNA-dependent	0.008
GO:0016070	RNA metabolic process	0.0085
GO:0010468	regulation of gene expression	0.0094
GO:0010556	regulation of macromolecule biosynthetic process	0.0096
GO:0003700	transcription factor activity	0.0097
GO:0019219	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0.0098
GO:0060255	regulation of macromolecule metabolic process	0.012
GO:0031326	regulation of cellular biosynthetic process	0.012
GO:0009889	regulation of biosynthetic process	0.012
GO:0051171	regulation of nitrogen compound metabolic process	0.012
GO:0030528	transcription regulator activity	0.013
GO:0042221	response to chemical stimulus	0.014
GO:0006350	transcription	0.014
GO:0080090	regulation of primary metabolic process	0.016
GO:0044085	cellular component biogenesis	0.016
GO:0050794	regulation of cellular process	0.017
GO:0015291	secondary active transmembrane transporter activity	0.021
GO:0031323	regulation of cellular metabolic process	0.022
GO:0019222	regulation of metabolic process	0.023
GO:0050789	regulation of biological process	0.026
GO:0003677	DNA binding	0.037

Despite the obvious growth phenotype shown after eW5 application, there was no indication from GO analysis that indicated that eW5 affected the expression of

genes involved with plant hormones. Therefore, a targeted analysis of the RNA-seq data was adopted, focusing on genes that are involved in plant growth and development in particular hormone signalling pathway. To achieve this, the list of the gene involved in these pathway were derived from *Arabidopsis* database (www.tair.arabidopsis). All genes listed in eW5 and W5 treatment (as compared to DMSO) in particular hormone pathway with P<0.05 were extracted from the main data. Using the gene lists for each of the hormone, correlation graphs were derived to observe any effect of eW5. Among 4 classes of hormones, which are gibberellic acid (GA), abscisic acid (ABA), auxin and ethylene, eW5 only showed an effect on the ethylene (Figure 4.6). From here, there was a few attempts to measure the transcript level of specific individual genes that showed a difference between eW5 and W5 treatments (Appendix C.5). However, no difference could be detected from these gene expression measurements, suggesting that the growth promotion effect of eW5 is through different mechanism such as protein activity rather than gene expression.

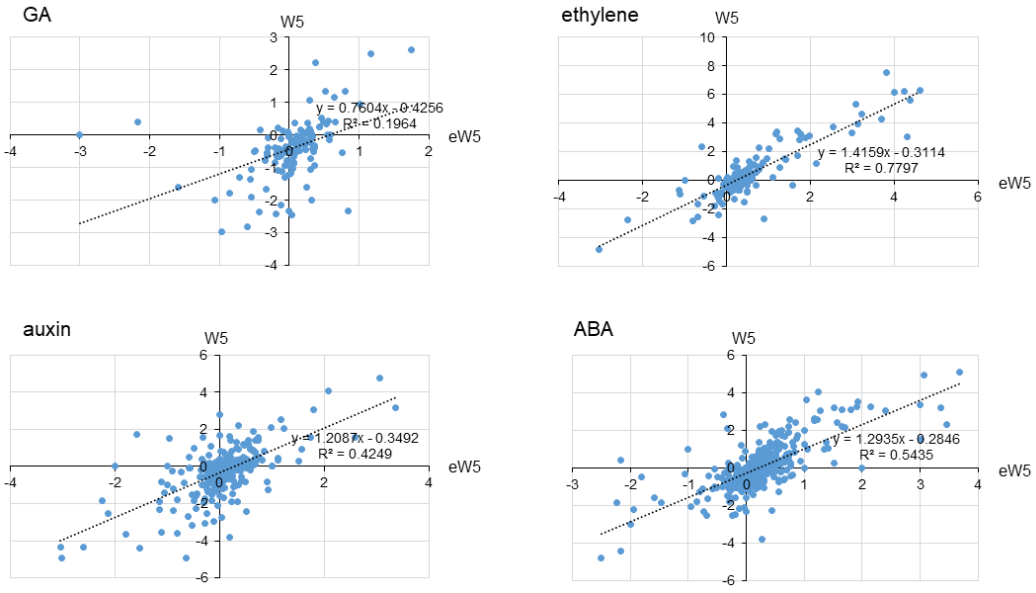


Figure 4.6: The correlation graph of the genes after eW5 (x-axis) and W5 (y-axis) treatment. The correlation graph was made based on hormone-regulated genes after treatment with eW5 or W5.

4.4 Discussion

Plants are exposed to a lot of environmental factors that influence their growth. They have to merge the information from their environment into different phenotypic or growth responses via the epigenome. Chromatin modifications contribute at multiple levels, including the expression of the gene and cell type differentiation. Epigenetic changes include chemical modifications that are heritable, thereby influencing the chromatin structure and gene expression without altering the DNA sequence (Berger et al., 2009). Methylation will also influence the plant phenotype through genome-wide changes in DNA methylation in response to changes in the environment which have been increasingly shown in recent years for plants (Chinnusamy and Zhu, 2009). There are numbers of studies that showed the differential regulation of genes encoding epigenetic regulators as well as local chromatin and DNA methylation changes in response to a variety of abiotic stresses (Su et al., 2015; Li et al., 2014; Fang et al., 2014).

It is well known that epigenetics regulates the level of transcripts and brings about changes in phenotype. One hypothesis is that environmental stress can result in targeted epigenetic changes that result in adaptive characteristics (Springer, 2013). Therefore, it was suggested that eW5 induces DNA methylation before showing its growth promoting effect. This is due to the methylation pattern that can be seen from the agarose gel of PCR product which indicates that there are differences between the band profile of control and eW5 treatment.

The result obtained from this experiment was consistent with the finding of Latzel et al. (2012), where the application of jasmonic acid and salicylic acid caused altered gene expression and DNA methylation levels. In addition, there are effect of the application of GA biosynthesis inhibitor, paclobutrazol and DNA methylation inhibitor in flowering as well as in DNA methylation content (Campos-Rivero et al.,

2017). The levels of 5-mdC (5-methyldeoxycytidine) decreased and affected the GAs level during floral transition (Meijon et al., 2011). The modification of histones upon chemical treatment is also supported by the study that has been performed by Hudson et al. (2011) where they discovered that the genes up- or down- regulated after the treatment of the inhibitor of methylation, 5-aza-29 deoxycytidine (5-AC) appear to change the expression by up to 100-fold. In contrast, in the decrease in DNA methylation (*ddm*) mutants, there are only a few genes that changed in their expression (Hudson et al., 2011). These findings suggest that upon the application of chemicals or hormones, the phenotype is changed due to the changes in gene expression. This was hypothesized due to the tendency of the gene expression to be change resulted from the interruption of the methyl/ acetyl group with the transcription factor binding (Lauria and Rossi, 2011). Therefore, it is suggested that the application of eW5 might alter the histone modification thus changing the gene expression.

The suggestion that eW5 can alter histone modification was supported by the finding from RNA-seq, a sensitive technique that offers a complete transcriptome analysis. From this technique, it was found that eW5 changed the levels of transcripts in *Arabidopsis*, particularly transcripts that are involved in hormone signalling pathway, due to the root elongation effect upon its application. However, it did not show any such correlation for any other hormones. This finding suggested that eW5 specifically regulates the GA signalling pathway in order to show its growth promoting effect. Therefore, the effect of eW5 on GA signalling pathway was investigated and will be further discussed in Chapter 5.

Additionally, plants undergo epigenomic reprogramming when exposed to stresses, due to the DNA methylation as well as post-translational modifications. Trimethylation at lysine 4 on histone 3 (H3K4me3) showed to be positively correlated with the drought induced genes, when the analysis was performed on Arabidopsis that has been exposed to drought stress. (Dijk et al., 2010; Zong et al., 2013).

Overall, the application of eW5 changed gene expression, observed from DNA methylation and RNA-seq analysis. For DNA methylation, alternatively bisulfite profiling could be performed in order to confirm the modification of histone after eW5 application. In this technique, DNA is treated with sodium bisulfite which will convert the unmethylated cytosine to uracil, but does not affect methylated cytosine, has also been employed for high-throughput sequencing, followed by mapping of the sequenced reads to a reference genome (BS-seq) (Cokus et al., 2008; Lister et al., 2008; Feng et al., 2010).

4.4.1 Conclusion

The phenotype induced by eW5 treatment results was suggested due to the epigenetic modification. Although there was a difference between eW5 and DMSO treatment from a DNA methylation-detection method, the possibility that eW5 promotes growth through epigenetics could be supported from another study such as bisulfite sequencing. The finding from RNA-seq analysis however did not give an absolute conclusion on where pathway that eW5 specifically regulates on. However, due to the root growth promotion effect upon its application, it has led to the subsequent direction of this study, namely to focus on the regulation of eW5 in plant hormonal signalling pathways, which is discussed in the following chapters.

CHAPTER 5

Effect of eW5 in ABA signalling pathway

5.1 Introduction

ABA is a stress-induced phytohormone, which is important in regulating seed dormancy, germination, plant senescence and abiotic stress responses. ABA signalling is normally repressed by group-A protein phosphatases 2C (PP2Cs) at an early stage of the pathway. PP2Cs are vital phosphatases that play an important role in ABA signalling. At low concentrations of ABA in plants, PP2Cs inhibit the activity of SnRK2 kinases, positive regulators of ABA signalling, hence no ABA response can be observed. However, when the level of ABA is increased, ABA binds the PYR/PYLs/RCAR (PYL) receptors which undergo conformational rearrangement leading to the formation of PYL-PP2Cs heterodimers which then inhibit PP2Cs (Park et al., 2009). With the inhibition of PP2Cs, SnRK2 kinases are phosphorylated leading to the activation of ABA downstream signalling.

As discussed in Chapter 1, pyrabactin and quinabactin which have sulfonamide moieties in their structure, display different ABA responses. While pyrabactin only interacts with a subset of ABA receptors and inhibits seed germination, the interaction of quinabactin with the receptors leads to effects in vegetative growth (Okamoto et al., 2013). This finding highlights the specificity of two different agonists. Considering the structural similarity between eW5 and pyrabactin (Figure 5.1), eW5 was hypothesized to promote growth by regulating the ABA signalling pathway.

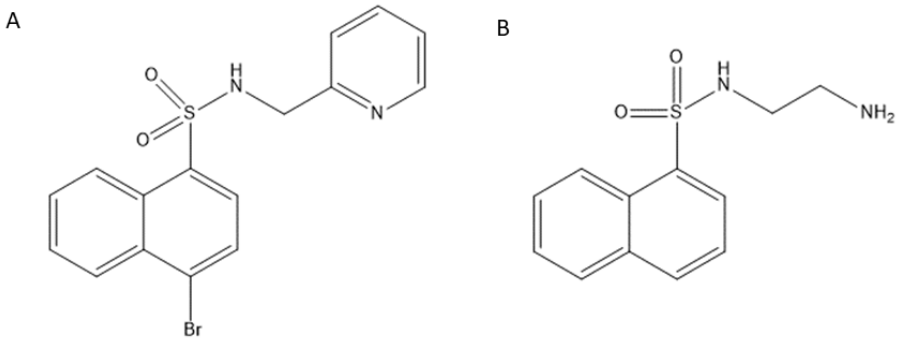


Figure 5.1: Structural similarity between A) pyrabactin and B) eW5. Both of the compounds have sulfonamide moieties that attached to naphthalene ring.

This chapter will discuss more about the possible role of eW5 as an antagonist in the ABA signalling pathway. The main objectives to be discussed in this chapter include:

- The interaction between eW5 and the ABA receptor, PYR1, using two different techniques.
- Exploration of the antagonistic effect of eW5 on ABA responses in plant physiology.
- The investigation of eW5 inhibition of ABA-responsive gene expression.
- The confirmation of the antagonistic effect of eW5 in restoring phosphatase activity *in vitro*.

5.2 Interaction between eW5 and ABA receptor

As mentioned in the previous section, the structural similarity between eW5 and pyrabactin has led to a hypothesis that eW5 promoting growth by interacting with ABA receptors and inhibiting ABA perception. To investigate this hypothesis, it was necessary to determine the interaction between eW5 and an ABA receptor. Two techniques were used; Thermal Shift Assay (TSA) and Microscale Thermophoresis (MST) and the details are discussed below.

5.2.1 Sample preparation

The *Arabidopsis* PYR1 coding was cloned into the pET24a expression vector. The final product of cloning (Appendix B.2) was analyzed using DNA sequencing and the sequence was checked using the DNASeq software against the full sequence of PYR1. The protein was then cloned into *E.coli* and expressed before purification using an Akta Purifier. To confirm the purification of the protein, SDS-PAGE gel electrophoresis was performed and the gel was stained using Coomassie blue to visualise the band (Appendix B.4). A mass identification of PYR1 was performed using ESI-MS, with the value of 24734.00 Da (Appendix B.5).

5.2.2 Thermal Shift Assay (TSA)

The first technique used to investigate the interaction between eW5 and PYR1 was Thermal shift assay (TSA). TSA is used to study thermal stabilization of proteins upon ligand binding (Jafari et al., 2014). By using this technique, protein is denatured upon heating and the temperature shift will be measured, which is translated from the thermal melting curve that derived from fluorescence changes. SYPRO orange is the normal fluorescent dye used, where it will bind the hydrophobic regions of a protein, and increasingly exposed during protein denaturation (Grøftehauge et al., 2014). As shown in Figure 5.2, the melting temperature, Tm, is the temperature when 50% of the protein is denatured (Krishna et al., 2013). A ligand bound to the active site of the protein has the ability to increase its thermal stability (Layton and Hellinga, 2011; Huynh and Partch, 2016).

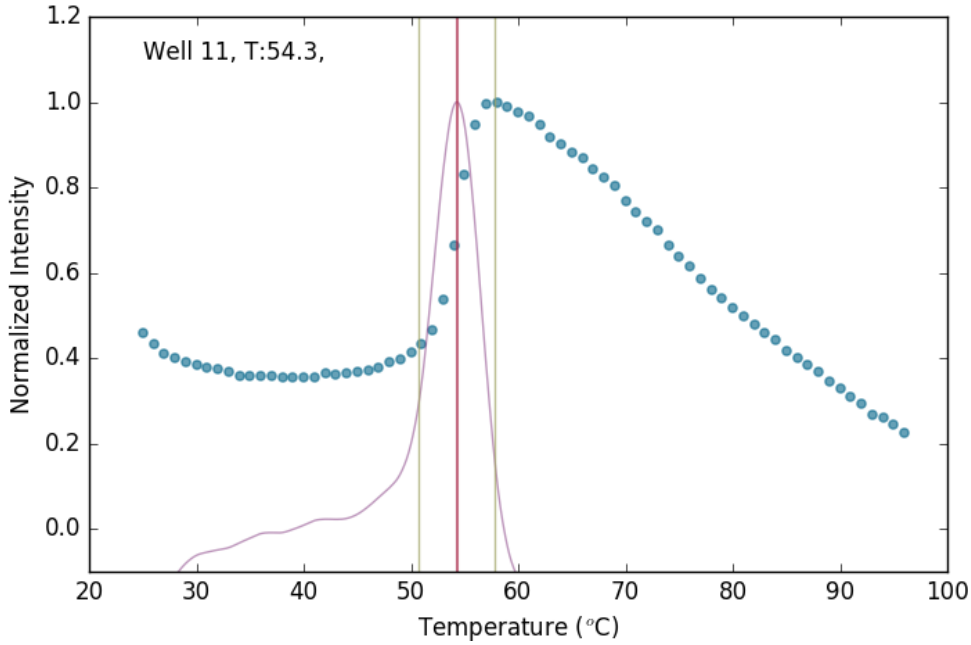


Figure 5.2: The melting point curve for TSA. The stability curve of the temperature when the hydrophobic surface is exposed and fluorescently binds to SYPRO orange dye. The Tm value is indicated by the red line while the window for Tm determination is shown by the green vertical lines.

In the experiment, ABA was used as a positive control, and the interaction was monitored by the increase of thermal shift temperature. After performing the analysis using NAMI program (see Section 2.3), ABA treatment showed a shift of between 0.6-0.8 °C, while eW5 showed a bigger shift (~0.8-1.0 °C) than ABA (Figure 5.3). This finding suggesting that eW5 binds more strongly to PYR1 as compared to ABA, especially at the concentrations of 1 µM and 100 µM.

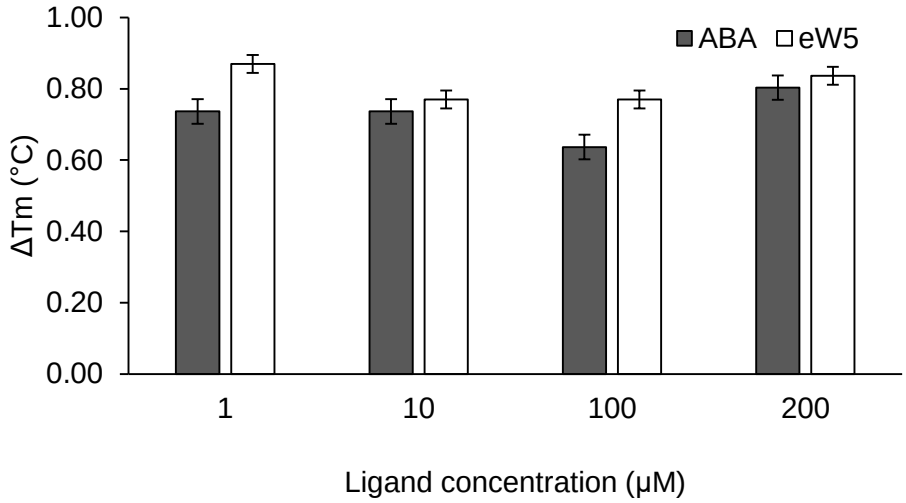


Figure 5.3: Thermal shift upon PYR1-ligand binding at four different concentrations. The experiment was performed in the presence of the indicated ligands and the resultant ΔTm value are compared to control (DMSO). The experiment was conducted in three biological replicates.

5.2.3 MicroScale Themophoresis (MST)

Since TSA can only determine whether there is a protein-ligand interaction or not, the PYR1-eW5 interaction was further investigated to determine its effect on the binding constant, K_d. To achieve this, the interaction was monitored using MicroScale Thermophoresis (MST). This technique is a powerful techniques to quantify biomolecular interactions. This technique is based on thermophoresis principle, where the particles exhibit different response to the temperature gradient. This movement depends on one of these molecular properties such as size, charge, hydration shell or conformation. Upon interaction of a ligand to the target molecule, there is an alteration in at least one of these parameter due to the changes in the thermophoretic mobility of the molecule (Entzian and Schubert, 2016). Thus, this technique allows a precise quantification of molecular events, due to its sensitivity to any change in molecular properties (Jerabek-Willemsen et al., 2014).

To ensure the homogeneity of the samples the fluorescence that covalently attached to the protein is measured without the temperature gradient. The activation

of the IR laser resulting in a decrease of fluorescent signal establishing the temperature gradient, while a reverse T-jump can be observed when the IR-laser is deactivated (Figure 5.4). Fluorescence is measured before IR-Laser heating (F_{cold}) and after a defined time of IR-Laser heating (F_{hot}). Fluorescence normalization was determined by the change in thermophoresis which is defined as F_{hot}/F_{cold}. The normalization is then plotted against ligand concentration to obtain the binding constant, K_d (Jerabek-Willemsen et al., 2014).

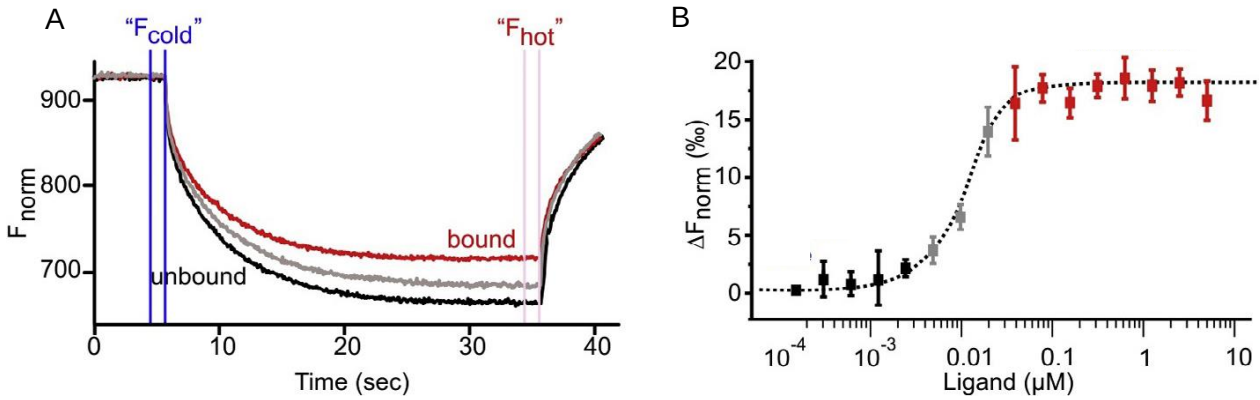


Figure 5.4: A) The MST time traces of the capillaries containing the protein and ligand solution. B) The normalized fluorescence of the MST traces plotted against the concentration of the ligands. The figure was taken from Jerabek-Willemsen et al., 2014.

With PYR1 available, the K_d of the interaction of eW5 with PYR1 was determined using a range of concentration of eW5 (12nM-200 µM). ABA was used as a positive control in this experiment and the range of ABA concentration used was 6nM-100µM. The normalized fluorescence from these analyses produced a binding curve confirming the interaction between eW5 and PYR1. Interestingly, eW5 showed an interaction with PYR1, with a K_d of 34 µM (Figure 5.5), which therefore suggests the possibility that it can act as ABA antagonist.

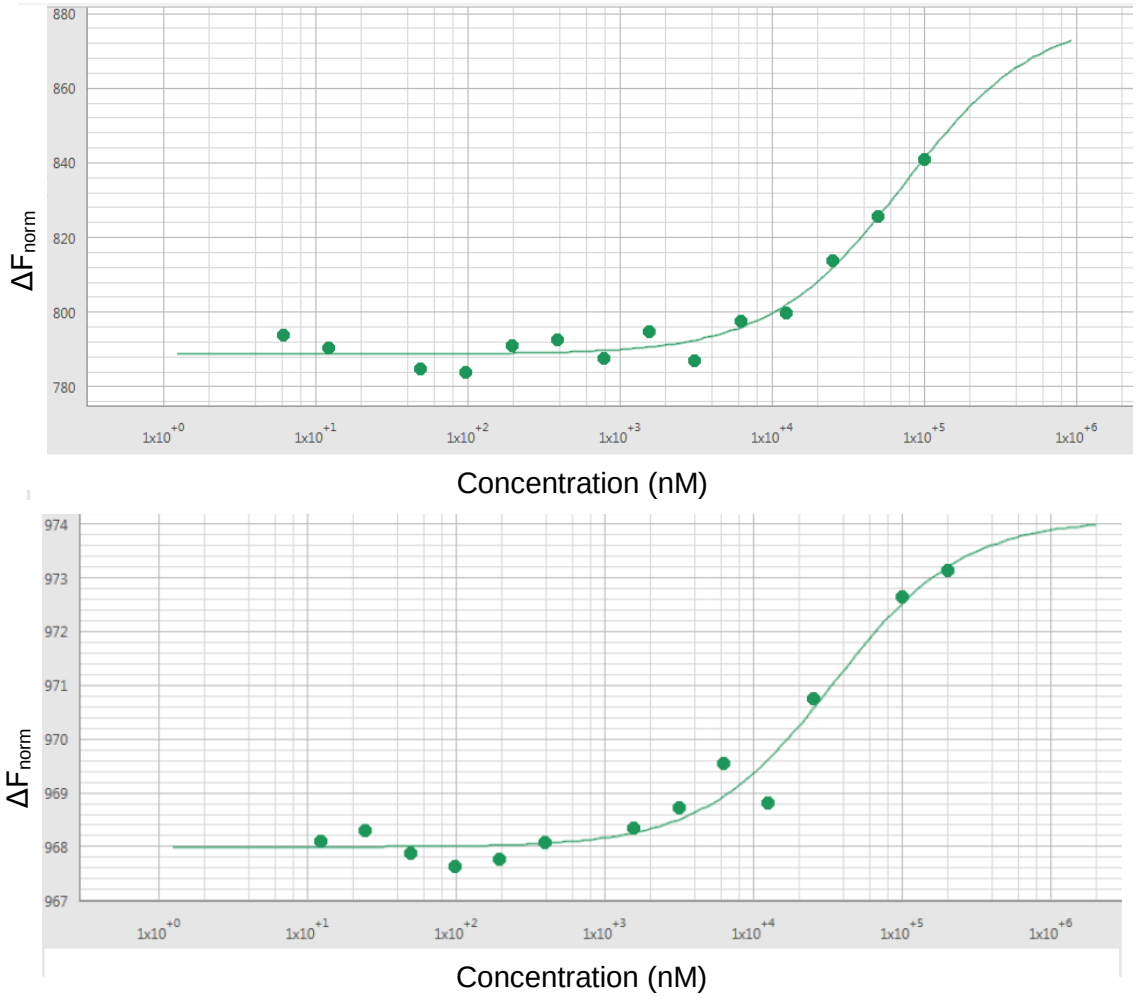


Figure 5.5: The binding curve from the normalized fluorescence plotted against concentration of the ligand A) ABA as a positive control (100µM-6nM) and B) eW5 (200µM-12nM). The curve is plotted based on 16 concentration of ligands. A K_d of 73 µM and 34 µM was determined for ABA and eW5, respectively.

5.3 Antagonistic effect of eW5 on plant physiology

The interaction between eW5 and one of the ABA receptors led to the hypothesis that eW5 might promote growth by inhibiting ABA perception. To investigate this hypothesis, the antagonistic effect of eW5 upon ABA responses was tested in relation to plant physiology, focusing on seed germination, root growth and stomatal opening. ABA is known to inhibit these three activities, hence the antagonistic effect of eW5 was tested by co-application with ABA and monitoring any reversal of ABA effects.

Seed germination is a key developmental process that has to be tightly controlled to avoid germination under unfavourable conditions (Kang et al., 2015). One of the important factors in seed germination is the balance between two hormones ABA and GA, which act antagonistically. A dynamic balance between ABA and GA controls the equilibrium between dormancy and germination (Rodriguez-Gacio et al., 2009). A higher ABA/GA ratio enables the inhibition of germination where the accumulation of ABA promotes the maintenance of seed dormancy and seed development until they are fully formed and ready to germinate. In contrast, higher GA levels remove the effect of ABA in seed dormancy and hence promoting seed germination.

In order to determine if the antagonistic effect of eW5 on ABA signalling could be observed in seed germination. In this assay, the seeds were grown on chemical-containing plates, where 1µM ABA is enough to inhibit seed germination as reported by Nishimura et al. (2007). The chemical concentration of eW5 was set at 100µM as this is the concentration that has an effect on root growth as described in Chapter 3. Seed germination was observed initially at 48 hours as this time point reflects the time that the radicle breaks dormancy and germination occurs (Koornneef and Karssen, 1994). However the observed effects were low and the analysis was repeated after 72 hours. As shown in Figure 5.6, the ABA inhibitory effect on seed germination can be observed at a low concentration of 1 µM, for both time points. The application of eW5 did not cause any difference to the control. When it is co-applied with ABA, eW5 showed a sight reversal of the ABA effect, relieving the ABA inhibition of seed germination in a small percentage of seeds.

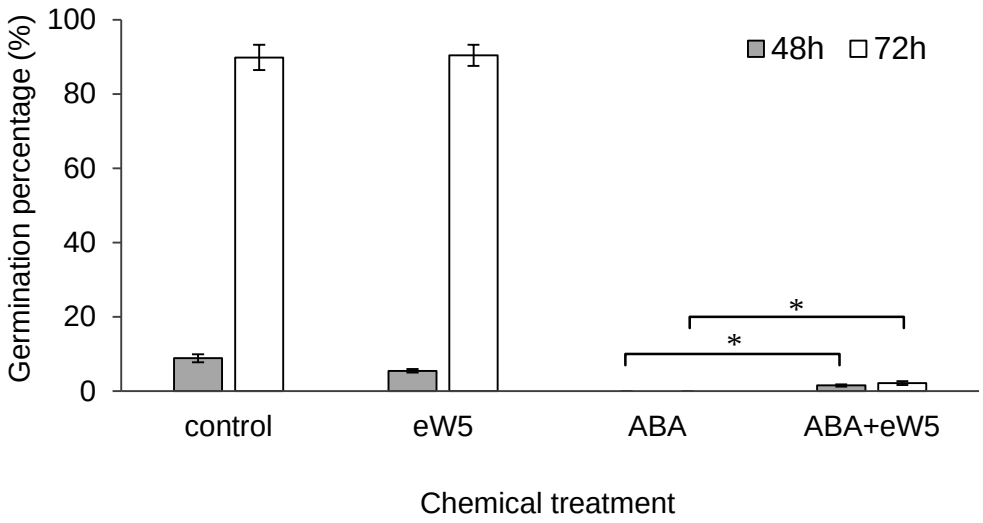


Figure 5.6: Germination percentage with different chemical treatments. The seeds were treated on chemical plates for two different times. Error bars represent standard error of three biological replicates. Asterisks indicate statistically significant differences (independent t-test, * $P<0.05$) between chemical treatment.

Having established that eW5 could antagonize ABA mediated inhibition of seed germination, the effect of eW5 on ABA regulation of root elongation was investigated through a root length assay. The root length was measured after 5 days of chemical treatment. As previously discussed, eW5 treatment increased root length while application of ABA inhibited root growth. However, eW5 could not restore any growth through its co-application with ABA in this assay (Figure 5.7). Collectively, these findings suggested that eW5 shows mild inhibition of the ABA effect on seed germination, but the inhibition effect is lost in the post germination phase.

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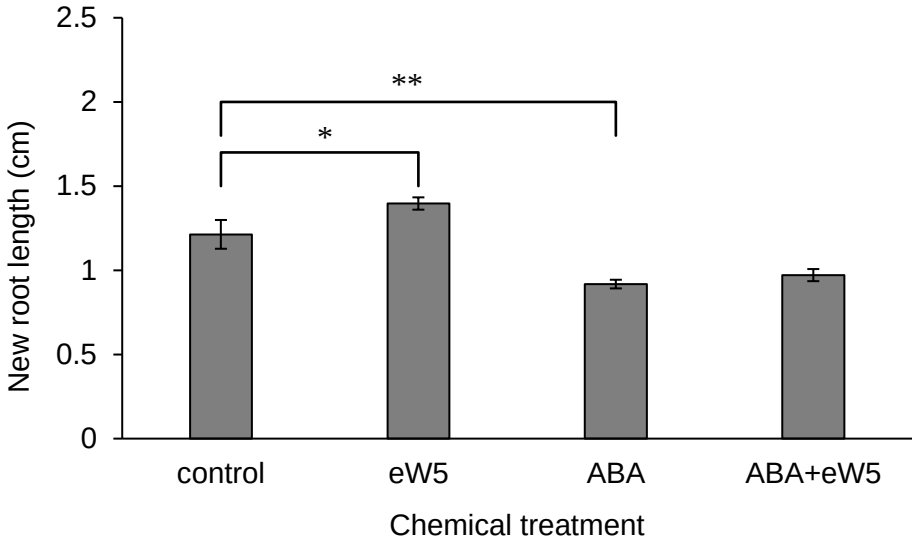


Figure 5.7: Root growth assay of eW5 and ABA competition. The seven-day old seedlings were treated with chemicals for 5 days. The concentration of ABA and eW5 used was 10μM and 100μM, respectively. The experiment was performed in three biological replicates with 18 seedlings for each replicate. Asterisks indicate statistically significant differences (independent t-test, * P<0.05, ** P<0.005) between chemical treatment.

According to Luo et al. (2014) the inhibition of root growth by ABA is mainly achieved through the promotion of ethylene biosynthesis with higher levels of ethylene inhibiting root growth. On this finding, it was hypothesized that eW5 enables higher root growth through inhibition of ethylene biosynthesis. To investigate this hypothesis, 1-aminocyclopropane-1-carboxylate (ACC), a key precursor that is involved in ethylene biosynthesis was used. Similar to ABA, the competition between eW5 and ACC was conducted on root growth assay. As shown in Figure 5.8, the treatment of the seedlings with ACC inhibits their root growth, whilst treatment with eW5 increased the root length. However, co-application of ACC and eW5 failed to reverse ACC mediated root growth inhibition, suggesting that eW5 does not affect ethylene biosynthesis.

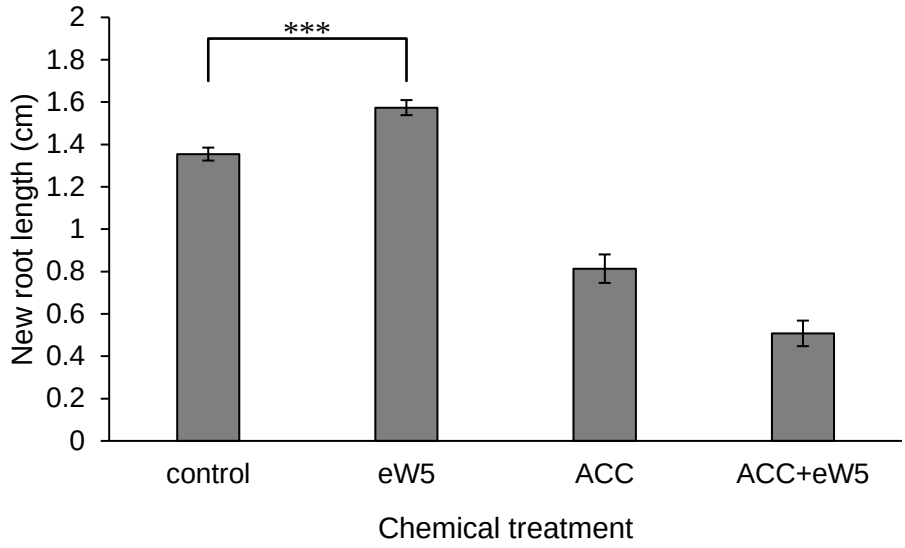


Figure 5.8: Root growth assay to investigate the competition study between eW5 and ethylene precursor, ACC. Chemical treatment was performed for 5 days using seven-day old seedlings. The experiment was conducted using at least 18 seedlings. Asterisks indicate statistically significant differences (independent t-test, *** P<0.001) between chemical treatment.

In addition to inhibiting seed germination and root growth, one of the other important functions of ABA is promoting stomatal closure and preventing stomatal opening. Stomatal pores surrounded by guard cells that are located in plant epidermis are important in controlling gas exchange in response to environmental signals. In particular increased ABA concentration leads to a closure of stomatal to prevent plants loosing too much water through transpiration, especially during periods of drought stress (Kwak et al., 2003).

Following a similar strategy as used for the seed germination and root growth assays, the antagonistic effect of eW5 on ABA on stomatal aperture was tested, by co-treating leaf epidermal layers with eW5 and ABA. The experiment was conducted by treating the epidermal layers with the chemicals for two hours before imaging them under a light microscope. As shown in Figure 5.9, treatment with ABA led to stomatal closure while eW5 treatment lead to increased stomatal aperture. A significantly bigger

stomatal aperture was observed after the co-application of eW5 and ABA, suggesting that eW5 is acting as an ABA antagonist.

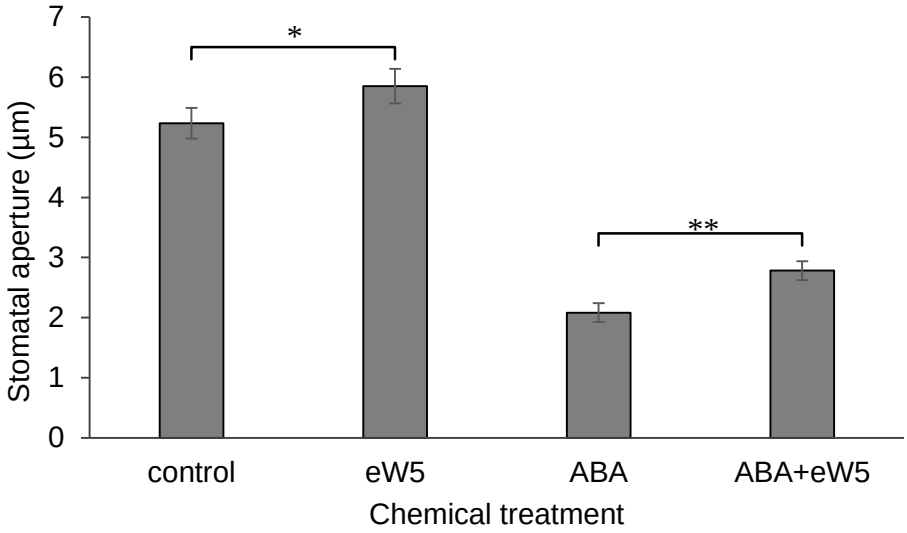


Figure 5.9: Inhibitory effect of eW5 on the size of the stomatal. The stomatal aperture was measured after two hours of chemical treatment. Error bars represent standard error. The experiment was performed in three biological replicates, with 3 peels (10 pores per peel) for each replicate. Asterisks indicate statistically significant differences (independent t-test, * $P<0.05$, ** $P<0.005$) between chemical treatment.

Taken together, these data show that eW5 has a potential to inhibit certain ABA activities in plant physiology. Specifically eW5 is acting as a weak partial ABA antagonist in seed germination and guard cell closure, but showed no inhibitory effect on ABA perception in root growth. Having observed the phenotypic effects it was important to see if they could be correlated with changes in gene expression. This is discussed in the following section.

5.4 Effect on gene expression

In the ABA signalling cascade, in the presence of ABA, active SnRK2s phosphorylate the basic leucine zipper (bZIP) transcription factors called ABFs/AREBs. These transcription factors will then bind to an ABA-responsive promoter element (ABRE) to induce ABA-responsive genes (Chan, 2012). Other than that, dehydration-responsive

element binding protein 1 (DREB1)/C-repeat binding factor (CBF) and DREB2 regulons function in ABA-independent gene expression. Other regulons such as NAC, are involved in abiotic stress-responsive gene expression (Figure 5.10) (Bansal et al., 2010; Nakashima et al., 2009). Based on the findings in the previous section, eW5 showed an antagonistic effect on ABA perception. To further explore the antagonistic effect of eW5, a study on measuring expression levels of ABA-responsive genes was then conducted. In this experiment, 10-days old seedlings were treated with eW5, with or without ABA.

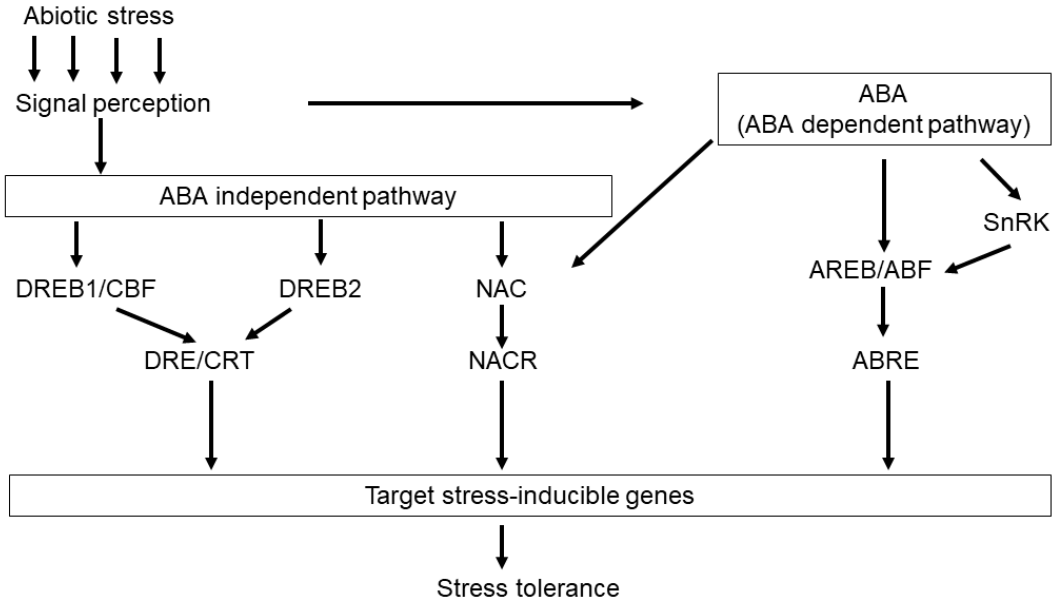


Figure 5.10: Major transcriptional regulatory network of *cis*-acting elements and transcription factors involved in abiotic stress in *Arabidopsis*. The figure was modified from Nakashima et al., 2009.

The expression levels were measured using RT-PCR at different time points, after 1, 3 and 6 hours. The expression of two genes, *ABF3* and *LTI78* was increased after ABA treatment, while the treatment of eW5 did not show any difference as compared to control. In addition, eW5 also failed to inhibit the expression of these genes when co-applied with ABA. This finding suggests that the antagonistic effect of eW5 on ABA perception cannot be observed through change in gene expression levels (Figure 5.11).

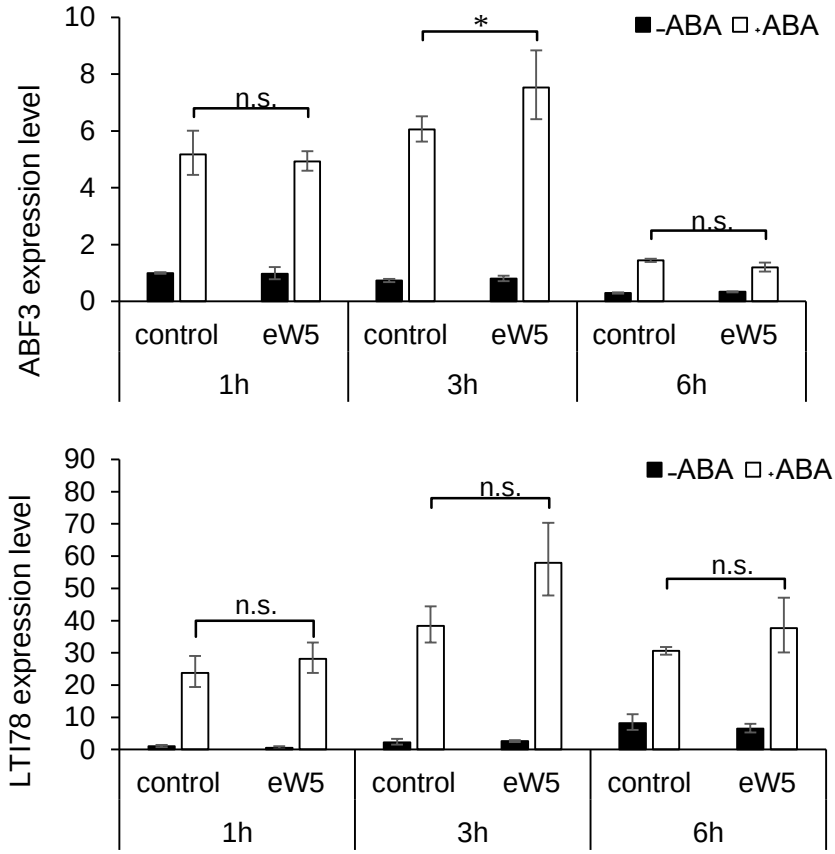


Figure 5.11: The expression level of ABA-responsive genes at different treatment time. The genes were induced by ABA hence the expression was increased after ABA treatment. Error bars represent standard error. Asterisks indicate statistically significant differences (independent t-test, * P<0.05) of eW5 as compared to ABA treatment.

Since there was no antagonistic effect of eW5 on ABA perception through ABA-responsive genes, the effect of eW5 was then tested on genes encoding components of the ABA signalling pathway. In the ABA signalling pathway, ABI1 encodes protein phosphatase 2C, and its expression is up-regulated in response to ABA treatment while the expression for ABA receptor PYL8 is decreased after ABA treatment. Similar to *ABF3* and *LTI78*, there was no inhibition by eW5 on ABA-responsiveness observed with these two genes (Figure 5.12). Collectively, these findings suggest that eW5 does not regulate ABA responses at the gene expression level.

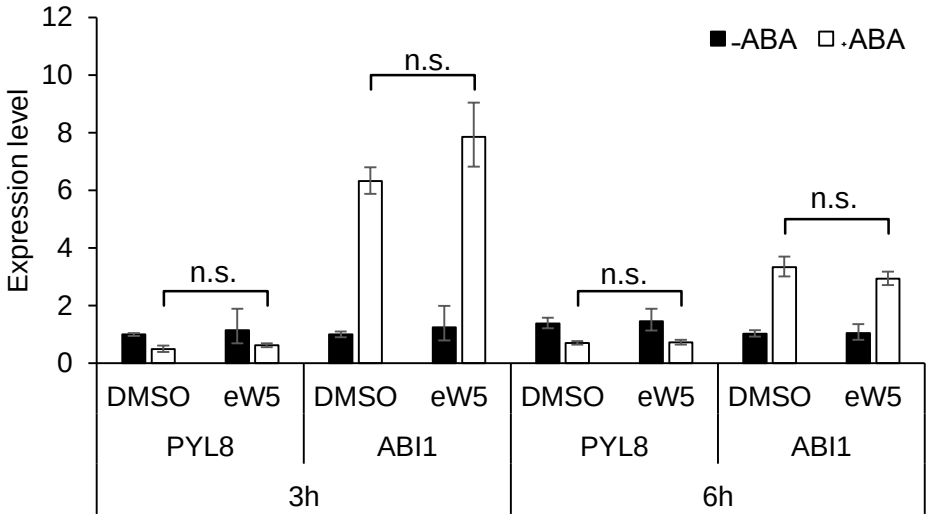


Figure 5.12: The expression levels of ABI1 and PYL8 to investigate the effect of eW5 on genes encode ABA signalling components, protein phosphatase and ABA receptor. The experiment was conducted in three replicates and the error bars represent standard error.

5.5 Phosphatase assay

As mentioned in Section 1.2, specific protein phosphatases act as negative regulators of ABA signalling. At low levels of ABA, PP2Cs interact with SnRK2 to dephosphorylate and inhibit their kinase activity and turn off ABA signalling. In contrast, at high levels of ABA, the interaction between ABA and the receptor promotes their interaction with PP2Cs and inhibits the phosphatase activity. SnRKs are then released from PP2C-SnRK2 complexes to phosphorylate downstream targets and activate ABA responses. The antagonistic effect of eW5 on ABA was therefore explored at the level of phosphatase activity, by determining whether it reverses ABA-induced PP2C inhibition through PYR1.

The assay was performed by monitoring HAB1 mediated phosphate release using a calorimetric system. The hydrolysis of p-Nitrophenyl phosphate (PNPP) to release p-nitrophenol which under the alkaline conditions of the assay exists as p-nitrophenolate, which has a strong absorption at 405 nm. In this assay, a low

concentration (1 µM) of ABA was used, since the observed antagonistic effect of eW5 on plant physiology was small (Section 4.3). As shown in Figure 5.13, in the presence of ABA, the percentage of HAB1 activity is relatively low, showing that ABA enhanced the interaction between PYR1 and HAB1, thus inhibiting phosphatase activity. The addition of increasing concentration of eW5 did not recover the activity of HAB1 even at the highest concentration used (250 µM) suggesting that eW5 is not acting as an ABA antagonist.

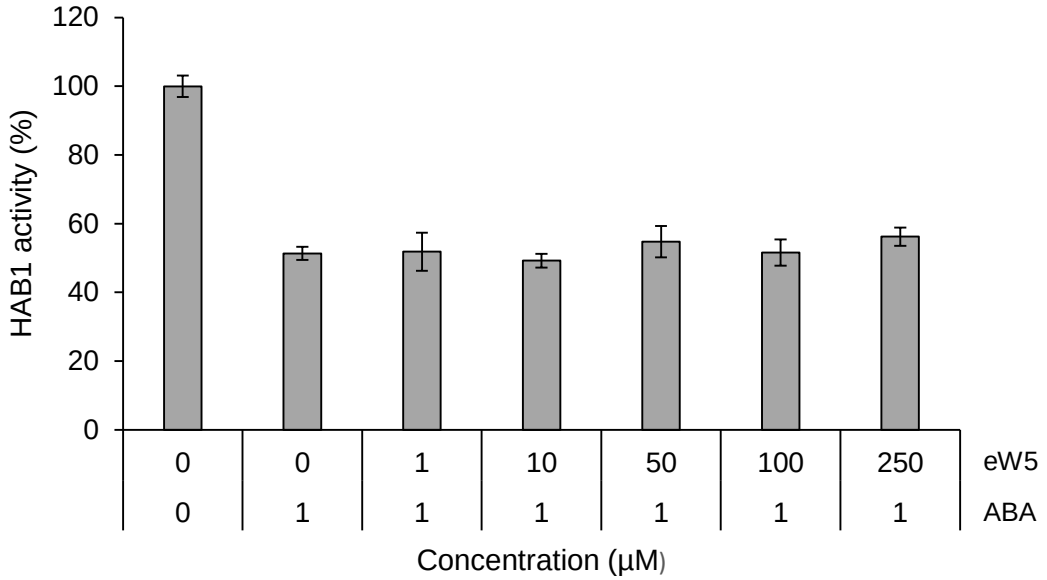


Figure 5.13: The percentage of HAB1 activity after the application of eW5 at different concentration. The concentration of ABA was fixed at 1uM since this concentration is enough to inhibit the activity of HAB1. Error bar represents standard error from three biological replicates. There is no significant different between eW5 to ABA treatment (Lane 2).

As discussed in Section 1.2, pyrabactin interacts with specific ABA receptors showing an agonistic effect through PYL1, but failed to activate PYL2 which suggests that it is acting as an antagonist instead (Melcher et al., 2010). It is therefore possible that eW5 shows a similar specificity for a selection of the PYR/PYLs receptors. Consequently, the effect of eW5 on the phosphatase activity of different receptors was explored in a similar fashion to that described above. The receptors tested in this experiment were PYL1, PYL2, PYL5 and PYL8. The latter two were chosen for their

reported roles in drought stress and root growth promotion, respectively (Santiago et al., 2009). ABA interacts with all of these receptors, hence the inhibition of HAB1 activity could be observed. However, similarly to the results with PYR1, eW5 failed to restore PP2Cs activity for any of these receptors, suggesting that eW5 does not act as an antagonist of ABA mediated phosphatase activity (Figure 5.14).

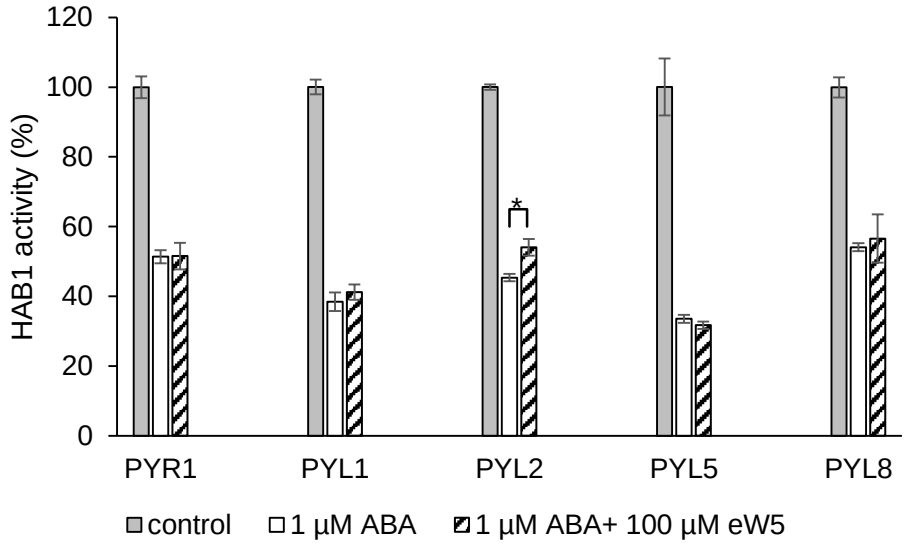


Figure 5.14: HAB1 activity measurement of different ABA receptor. The application of ABA deactivates HAB1 activity, and eW5 did not recover any the phosphatase activity for any of the receptor. The experiment was repeated twice and error bar represents standard error. Asterisks indicate statistically significant differences (independent t-test, * P<0.05) of eW5 treatment as compared to ABA.

With the failure of eW5 to restore the activity of PP2Cs through its co-application with ABA, eW5 was hypothesized to act as an agonist instead. To investigate this hypothesis, HAB1 activity was measured with ABA as a positive control. Similar to previous experiments, HAB1 activity on different receptors was measured to see if eW5 selectively interacts with the receptor. As shown in Figure 5.15, the activation of ABA-PYR1/PYLs complex interacts with PP2Cs and inhibits their activity, hence the lower activity was observed. However eW5 treatment showed no observable effect, suggesting that eW5 is not an agonist.

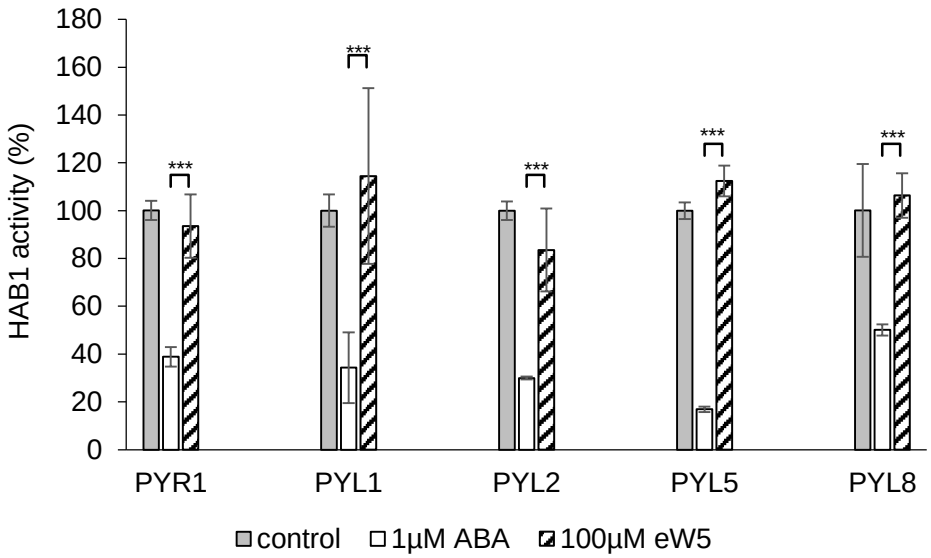


Figure 5.15: The measurement of HAB1 activity of different ABA receptors after the treatment of 100 µM eW5. ABA was used as a positive control and inhibits the activity. Asterisks indicate statistically significant differences (independent t-test, *** P<0.001) of eW5 as compared to ABA treatment.

5.6 Discussion

5.6.1 Biophysical interaction between eW5 and ABA receptor

In ABA signalling, pyrabactin is one of the agonists that has been well studied. Pyrabactin specifically interact with a subset of ABA receptors, shown by its effect on seed germination only and not in the vegetative phase (Okamoto et al., 2013). eW5 was hypothesized to repress ABA inhibition due to its root growth promotion effect. This is due to the structural similarity between eW5 and a known ABA agonist, pyrabactin. Both eW5 and pyrabactin have sulfonamide moieties and these two compounds is differentiate with the presence of bromide and pyridine group in pyrabactin. The presence of the bromide group in pyrabactin forms several Van der Waals interactions with Leu-Pro-Ala, three residues from the ligand entry gate. These interactions are important to keep the gate closed that will lead to the agonist effect of pyrabactin (Melcher et al., 2010). Therefore it could be suggested that the absence of

a bromide group might result in a different conformation of eW5-PYR interaction and hence contribute to the antagonistic effect from eW5.

Biophysical studies have been performed to determine the interaction between eW5 and PYR1. Using TSA technique, the changes of melting temperature indicates that the compound has an interaction with the protein by stabilizing the complex thus increasing the melting temperature (Pantoliano et al., 2001; Semisotnov et al., 1991; Vedadi et al., 2006). With the positive result obtained from TSA, the interaction was further observed through MST approach. By using MST approach, the binding constant can be determined and eW5 was found to bind PYR1 with a K_d of 34 µM.

5.6.2 The antagonism effect of eW5 that can be observed from plant physiology

Further observations demonstrate that some of physiological effects of ABA such as its effect on seed germination and stomatal aperture can be relieved by eW5, with small effects through its co-application with ABA. However, eW5 does not relieve all ABA physiological effects, since its co-application with ABA failed to recover the phenotype of ABA on root growth. These observations suggest that the effect of eW5 on ABA perception is different at different stages of development. This might suggest that eW5 selectively interacts with ABA receptors, therefore contributing to its relatively selective physiological effects. This is consistent with the finding from Okamoto's group who found that quinabactin activates five out of 13 ABA receptors with preferential activity on three dimeric receptors, thus acting as an ABA agonist on seed germination, vegetative growth, stomatal aperture and gene expression (Okamoto et al., 2013). Furthermore, the degree of agonistic or antagonistic effect on plant physiology depends upon their interaction with ABA receptors. According to Gonzalez-Guzman et al. (2012), different receptors contribute to different biological functions, this group discovering the contribution of PYR1, PYL1/2/4/5/8 in stomatal aperture, seed germination and root growth through multiple receptor mutants.

It was also hypothesized that eW5 might promote root growth through inhibiting ethylene biosynthesis. Root growth is influenced by meristemic activity and ethylene was found to negatively regulate root meristem size, doing so through its signalling component, ETHYLENE RESPONSE (ETR1), ETHYLENE RESPONSE SENSOR (ERS1), ETHYLENE INSENSITIVE1 (EIN1), EIN2, EIN3 and CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) (Street et al., 2015). However, no inhibition effect can be observed from a root growth assay, where eW5 did not relieve ethylene precursor, ACC treatment.

5.6.3 The failure of eW5 to show its antagonistic effect from *in-vitro* assay

The ABA signalling is activated upon the repression of PP2Cs inhibition of SnRK2 kinase protein. As a negative regulator of ABA signalling, the inhibition activity of PP2Cs is crucial, and can be achieved through its interaction with ABA-PYR/PYLs complex (Cutler et al., 2010). From an *in vitro* assay, it was demonstrated that eW5 does not seem to act as an ABA antagonist. This is due to the failure of eW5 to activate the receptors to restore PP2Cs activity after the application of ABA. Even at a low concentration of ABA (1 µM), higher concentrations of eW5 (250 µM) still could not restore PP2Cs activity. This might be due to the failure of eW5-PYR1 complex to induce the conformational changes that lead to the binding interface for PP2Cs (Melcher et al., 2010). This is supported by the finding from (Park et al., 2009), who reported that ABA-PYR1 complex enhanced the interaction with PP2Cs due to the ability of ABA-PYR1 complex to induce the conformational changes.

The conformational changes in ABA perception involves an open/close-latch-gate conformation due to the interaction between the receptor and chemical (ABA or its agonist or antagonist). Open gate conformation leads to the inactivation of ABA signalling due to an inability to provide the interface for PP2Cs interaction. In contrast, close-latch-gate conformation will show an agonist effect, where the lock in the binding

pocket provides the interface for the interaction with protein phosphatase (Melcher et al., 2010). Consistent with this finding, it is suggested that the interaction between eW5 and PYR1 might lead to an open-latch-gate conformation. Therefore, although the eW5-PYR1 complex can be formed, the complex fails to enhance the interaction between the complex and protein phosphatase, hence does not contribute to any biological function.

Moreover, the finding that eW5 is not an ABA antagonist is supported by the gene expression experiments, where there was no inhibition of ABA-responsive genes through eW5 co-application with ABA.

5.6.4 Conclusion

The hypothesis that eW5 might act as an ABA antagonist derived due to a structural similarity between eW5 and pyrabactin. This hypothesis was supported by the interaction between eW5 and PYR1 through TSA and MST studies. With a weak interaction between eW5 and PYR1, it was observed that eW5 has a small reversal effect in physiological effect. However, the failure of eW5-PYR1 to induce the interaction with PP2Cs protein, HAB1, which can be observed from phosphatase assay suggests that eW5 does not act as an ABA antagonist. Therefore with no antagonistic effect of eW5 that can be monitored *in vitro*, it is possible that eW5 regulates the GA signalling pathway hence repressing ABA activity as we can see in seed germination and guard cell assays. Moreover, it is widely studied that the plant growth and development is influenced by the antagonistic effect between GA and ABA. The possible regulation of eW5 in GA signalling pathway is discussed in the following chapter.

CHAPTER 6

Stimulation of eW5 in GA signalling pathway

6.1 Introduction

Gibberellic acid (GA) belongs to a family of tetracyclic diterpenoid plant hormones and have an important role in plant growth and development. GA controls a lot of developmental processes throughout the plant life cycle such as stimulating seed germination, and stimulates stem elongation and leaf expansion through cell expansion and cell division (Alabadı et al., 2008; Feng et al., 2008) (Koornneef and van der Veen, 1980; Yamauchi et al., 2004; Seo et al., 2009). GA also triggers transitions from meristem to shoot growth, juvenile to adult leaf stage, transition from vegetative to reproductive growth (Gupta and Chakrabarty, 2013). GA was also found to regulate stomatal aperture, discovered from a study conducted by Goring et al. (1990) on *Vicia faba*.

As discussed in Section 1.2, GA stimulates plant growth by targeting the growth repressor, DELLA protein, for destruction by proteasomal degradation thus overcoming DELLA restraint of growth (Harberd et al., 1998; Sasaki et al., 2003). The complex between GA and its receptor, GID1, initiates the interaction with DELLA protein triggering binding with SCF^{SLY1} before undergoing proteasomal degradation (Murase et al., 2008; Dill et al., 2004). Furthermore, GA signalling is often controlled by changes in the enzymes encoding *GA20ox*, *GA3ox* and *GA2ox* in response to environmental or developmental stimuli (Hedden and Phillips, 2000).

This chapter describes the possible modes of action of eW5 in regulating the GA signalling pathway. This was hypothesised due to the growth promoting effect of eW5 that can be seen through phenotypic assays as described in Chapter 3. Aims of the research described in this chapter include:

- Investigating if eW5 application can promote DELLA degradation.
- Determining the target of eW5 on core components of the GA signalling pathway using different genetic backgrounds.
- Investigating the possible effect of eW5 on GA biosynthesis and GA sensitivity.

6.2 GA signalling pathway and DELLA degradation

As described in Section 1.2.2, DELLA proteins are the negative regulators of plant growth, and as such are key players in regulating GA responses (Yoshida et al., 2014; Hauvermale et al., 2012) controlling gene transcription through interaction with specific transcription factor targets (Zentella et al., 2007; Daviere et al., 2008; Yoshida et al., 2014). For example, DELLAs interact with the *Arabidopsis* nuclear transcription factor, PHYTOCHROME INTERACTING FACTORs (PIFs) and inhibit PIF-induced hypocotyl length by blocking the transcription of PIFs target genes (Feng et al., 2008; de Lucas et al., 2008). DELLA proteins belong to the GRAS family transcriptional regulator that have a number of important role such as in light and hormone signalling (de Lucas et al., 2008).

The function of DELLA proteins is regulated by the change in their stability. High levels of bioactive GAs lead to their destruction (Hussain and Peng, 2003). This degradation is initiated by the binding of gibberellic acid (GA) to its receptor, GID1. The GA-GID1 complex is then able to interact with the N-terminal sequence of the DELLA (known as the DELLA domain) enabling ubiquitination by SCF^{SLY} E3 ligase and subsequent degradation by the 26S proteosome (Dill et al., 2001; Dill et al., 2004) (Figure 6.1). The downstream effect of DELLA degradation is seen in enhanced root growth (Ubeda-Tomas et al., 2008), seed germination and plant cell elongation. Parallel with the observed promotion of root growth by eW5 as described in Section 3.3, leads to the hypothesis that eW5 might promote root growth through the same

mechanism. To investigate this hypothesis, the stability of DELLA proteins upon treatment with eW5 was investigated.

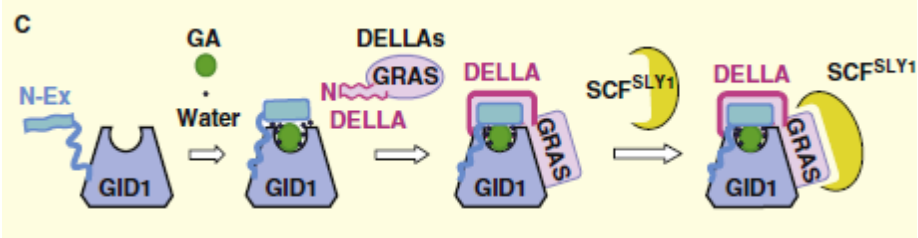


Figure 6.1: The mechanism of DELLA degradation process which induced by GA. The conformational changes has been induced by the GA-GID1 complex and therefore enhanced the interaction with SCFSLY1 before undergo proteosamal degradation. The figure was taken from Sun et al., 2010.

In order to monitor this a method for monitoring DELLA levels was required. A RGA-GFP fusion that enables the monitoring of DELLA proteins degradation through loss of fluorescence has been demonstrated by Silverstone et al. (2001). Building on this precedent, a RGA-GFP transgenic line was utilised in order to establish the effect of eW5 on DELLA stability. *Arabidopsis thaliana* seedlings expressing RGA-GFP were treated with eW5 with GA as a positive control. While paclobutrazol (PAC), an established inhibitor of GA biosynthesis was used as a negative control. Roots were then imaged after 2 and 24 hours using confocal microscopy (Figure 6.2). The application of eW5 led to a reduced fluorescence that indicates loss of DELLA proteins. The eW5 response on DELLA degradation was as fast as GA, as the response could be observed from the treatment after 2 hours. Application of PAC, 48 hours prior to measurement stabilized DELLA protein due to the low level of GA (Silverstone et al., 2001). Together, these results suggested that eW5 promotes root growth by targeting DELLA protein degradation.

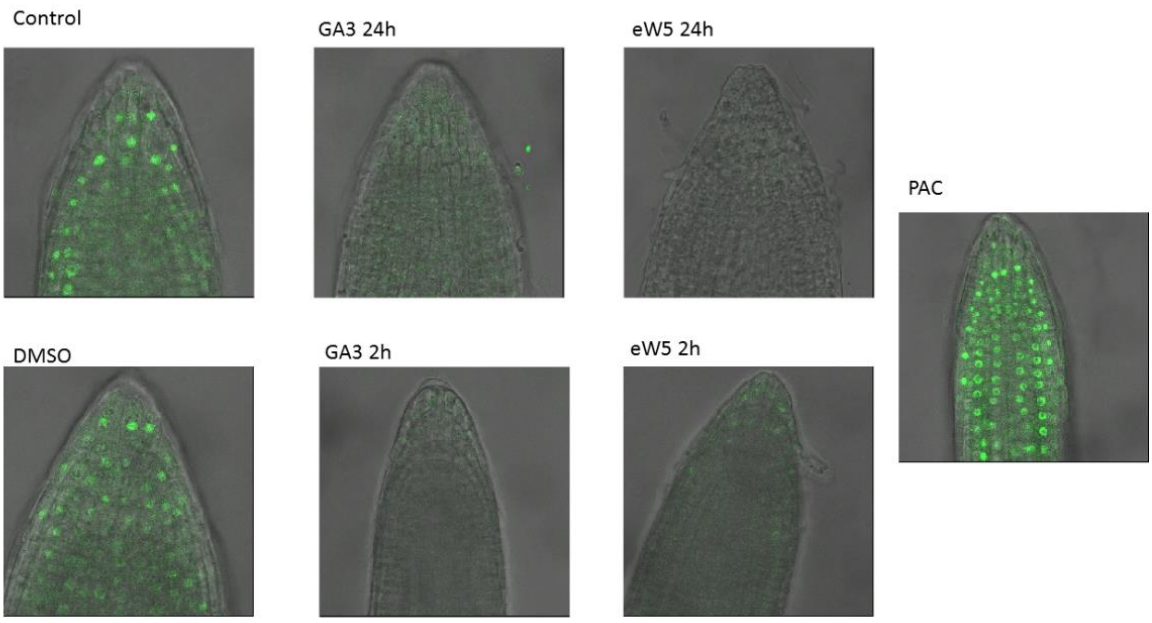


Figure 6.2: Effect of eW5 on the fluorescence level in the roots of a transgenic *Arabidopsis* line expressing RGA-GFP. The concentration for GA_3, eW5 and PAC was 100 µM. The seedlings were treated with GA and eW5 at different time point, 2hr and 24hr, while the treatment of PAC was for 48hr.

To further explore the effect of eW5 on DELLA degradation a study of hypocotyl growth was then undertaken. It is well established that DELLA can inhibit the binding of PIFs to their target promoter leading to a reduction in hypocotyl growth (Castillon et al., 2007; de Lucas et al., 2008; Feng et al., 2008). Significantly the process is affected by light levels with high light promoting plant photomorphogenesis as characterized by a shorter hypocotyl. This is due to PIF degradation, inhibition of GA accumulation and increased level of DELLA protein (Sun, 2010). In contrast, the seedling hypocotyls are tall and fully expanded when grown in the dark, which has been shown to be due to a lower level of DELLA protein (Alabadi et al., 2004).

As hypocotyl length is related to DELLA levels, the effect of eW5 on hypocotyl elongation was investigated. Hypocotyl growth assays were performed by growing the seedlings on the chemical plate for three days with reduced light intensity. The reduced light intensity will avoid the higher accumulation of DELLA protein and inhibition of PIF-mediated light control of hypocotyl elongation. As before, GA and PAC were used as

positive and negative control, respectively. Consistent with this hypothesis eW5 and GA led to significantly longer hypocotyl while PAC inhibits hypocotyl growth (Figure 6.3). This result suggests that eW5 promotes the hypocotyl growth through DELLA degradation.

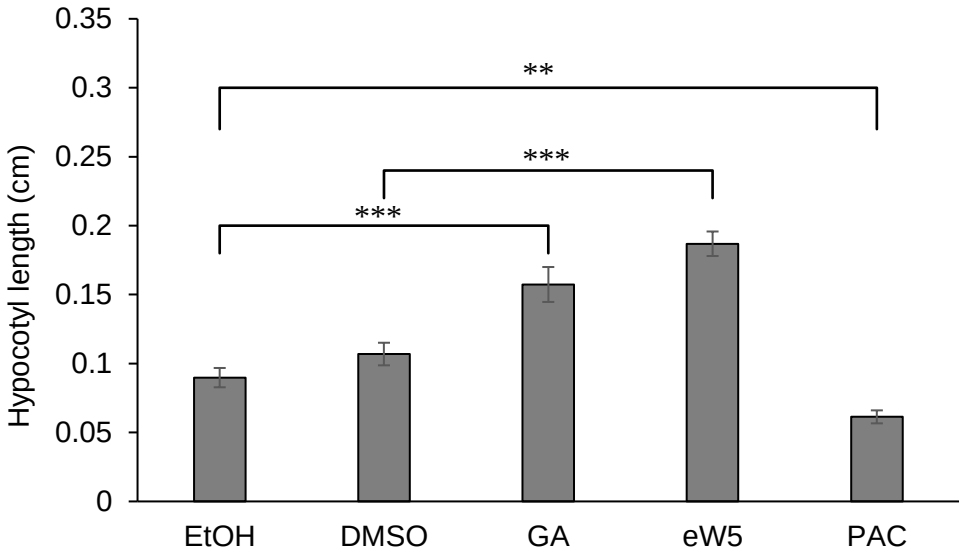


Figure 6.3: Hypocotyl length of *Arabidopsis* after eW5 treatment. GA and PAC represent positive and negative control, respectively. Ethanol was used as a control for GA and PAC while the control for eW5 was DMSO. Error bars represent standard error from 30 seedlings. Asterisks indicate statistically significant differences (independent t-test, ** $P<0.005$, *** $P<0.001$) between chemical treatment.

The finding strongly supported the idea that eW5 effect upon growth was DELLA-dependent. To test this hypothesis genetically, a mutant line that lacks all DELLA (GAI, RGA, RGL1, RGL2 and RGL3) function was then used. Due to the loss of all DELLA function, this mutant displays longer root and hypocotyl as compared to wild type. No promotion of either root or hypocotyl growth was observed after eW5 treatment on this mutant, suggesting that eW5 requires DELLA for its effect on root and hypocotyl elongation (Figure 6.4).

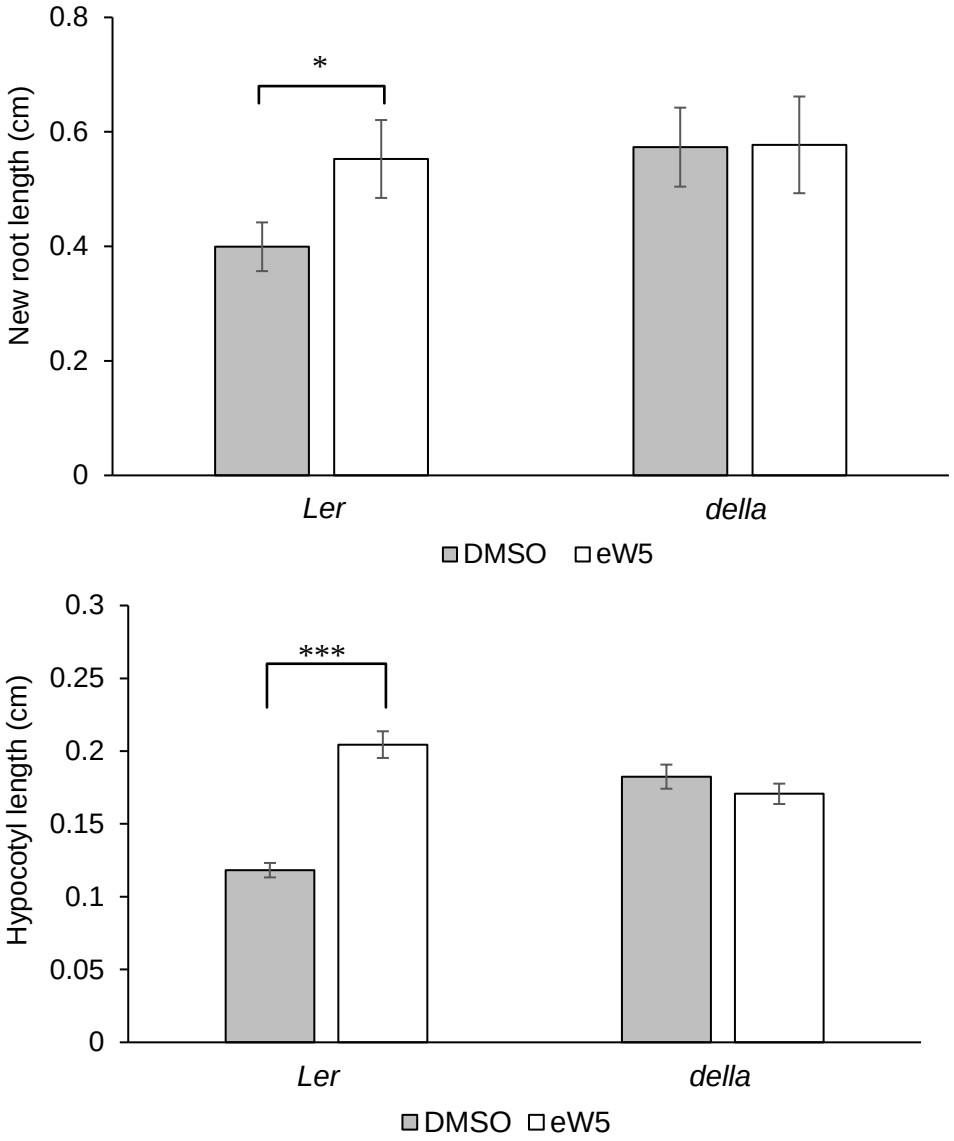


Figure 6.4: Bar chart of (A) root growth and (B) hypocotyl growth assay for *della* quintuple mutant. This mutant lacks of all DELLA function, hence it was used to investigate the DELLA-dependency of eW5. Error bars represent standard error, based on three biological replicates. Asterisks indicate statistically significant differences (independent t-test, * $P<0.05$, *** $P<0.001$) between chemical treatment.

As the literature reports that GA has a role in promoting stomatal aperture in *Vicia faba*, its effect was thus investigated in *Arabidopsis*. Due to the similarity in effect of eW5 and GA in root growth and DELLA protein degradation, an investigation on the effect of eW5 on stomatal aperture was also undertaken. These guard cell experiments were conducted using the same procedure described in the previous chapter. To reduce the size of the stomatal apertures in the control condition, these experiments were conducted in a minimal source of light. The stomatal aperture were larger than

control upon addition of GA, suggesting that GA induces stomatal opening in *Arabidopsis* as it does in *Vicia faba*. The treatment of the epidermal peels with eW5 showed that eW5 opens stomata less than GA, supporting the idea that eW5 triggers the same mechanism as GA (Figure 6.5).

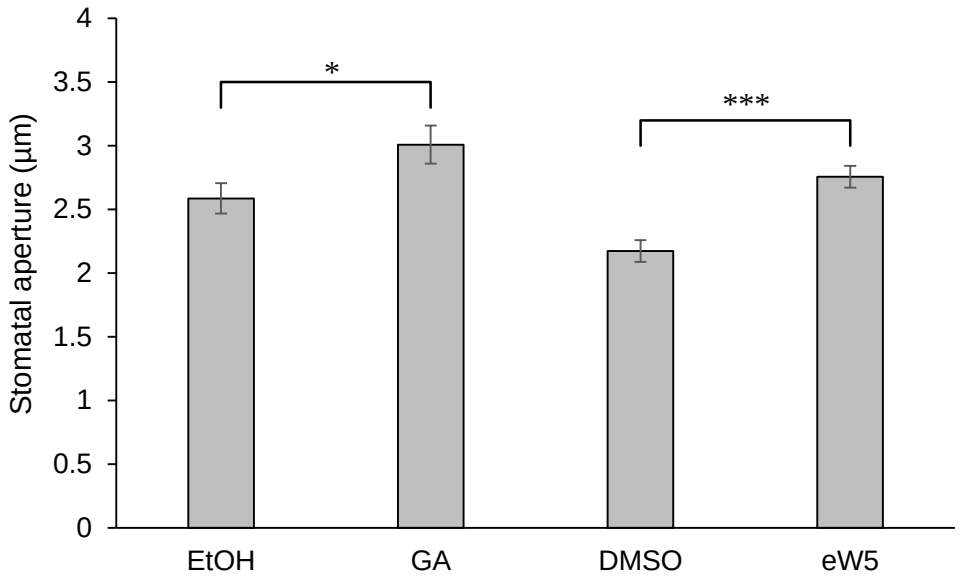


Figure 6.5: The stomatal aperture after GA and eW5 treatment. Ethanol and DMSO was used a control for GA and eW5, respectively. The concentration of both GA and eW5 was 100µM. Asterisks indicate statistically significant differences (independent t-test, * P<0.05, *** P<0.001) between chemical treatment.

Due to the promoting effect of GA on stomatal aperture, the assay was conducted using a *della* quintuple mutant to determine if the stomatal aperture promotion is through DELLA-dependent mechanism. In the absence of DELLA, the stomatal aperture was bigger than the control, suggesting that DELLA protein might (in the wild type state) induce stomatal closure. No promotion of stomatal aperture opening was observed in the *della* quintuple mutant after GA application, indicating that GA promotion of stomatal guard cell opening is DELLA-dependent. Furthermore, due to the stomatal opening promotion shown by eW5 previously, it was hypothesized that the regulation of eW5 in stomatal guard cell was through the same mechanism as root and hypocotyl growth ie. DELLA-dependent. Using the same genetic backgrounds, the pore sizes of the epidermal layers were measured in order to

investigate this hypothesis. Similar to growth promotion in root and hypocotyl that requires DELLA protein, eW5 required DELLA proteins to control stomatal aperture, with no promotion observed upon eW5 treatment of the *della* quintuple mutant (Figure 6.6).

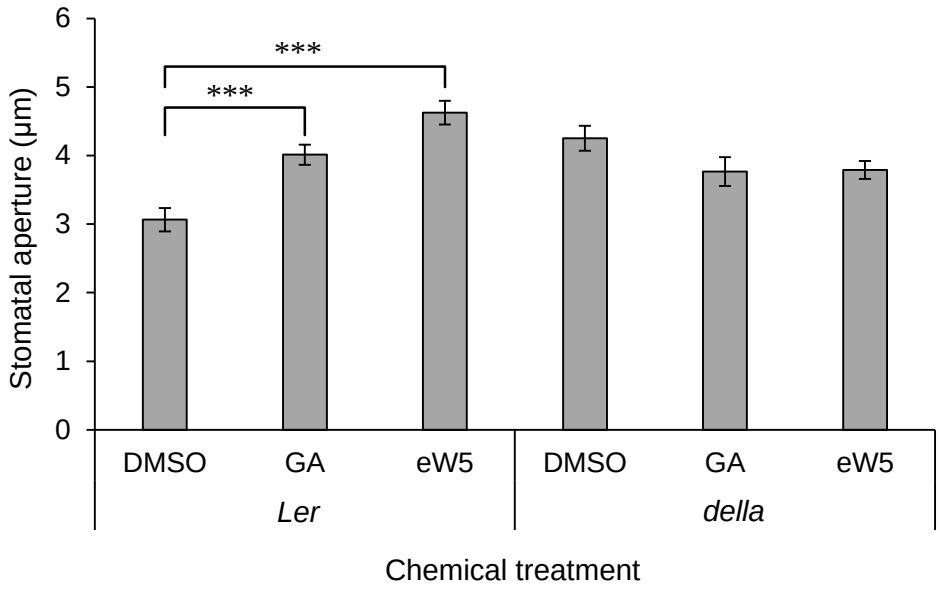


Figure 6.6: Stomatal aperture on *della* quintuple mutants after their chemical treatment. The concentration used for GA and eW5 was 100µM. The experiment was conducted in three replicates and the error bars represent standard errors. Asterisks indicate statistically significant differences (independent t-test, *** P<0.001) between chemical treatment.

In summary, the root growth and hypocotyl assays suggested that activity of eW5 is DELLA-dependent with the mechanism for eW5 promotion of growth is through DELLA degradation as demonstrated by genetics and microscopy. In addition to this, both GA and eW5 have a role in inducing the opening of stomatal aperture, as shown through genetic study. This suggests that eW5 and GA may have a related mode of action. Therefore it is important to understand the mode of action of eW5 in promoting growth. The exploration of the signalling pathway upon eW5 application is discussed in the following section.

6.3 Identification of eW5 target

Based on the finding that eW5 promotes growth through DELLA proteins degradation, it was proposed that eW5 could be regulating GA signalling pathway. Therefore, to investigate the effect of eW5 on this pathway, the hypocotyl and root growth assays were performed on the specific genetic background for each of the core component in the pathway; GA-deficient, GA receptor and F-box mutants.

Since eW5 has the same effect as GA, it was hypothesized to mimic GA function. Therefore, the first genetic background that has been tested was GA-deficient mutant, *ga1-5*. This mutant contains low levels of bioactive GA and has a dwarfed phenotype. Reduction of GA levels in the *ga1-5* mutant causes an increase in DELLA protein, hence cause growth inhibition (Fridborg et al., 1999). When plants were grown in a reduced light intensity condition, *ga1-5* mutants had shorter hypocotyls than wild type, confirming that GA is important in hypocotyl elongation in the dark. The treatment of the seedlings with eW5 could not recover the hypocotyl elongation (Figure 6.7), therefore suggesting that eW5 is not acting like GA and it needs endogenous GA to obtain its effect on growth promotion.

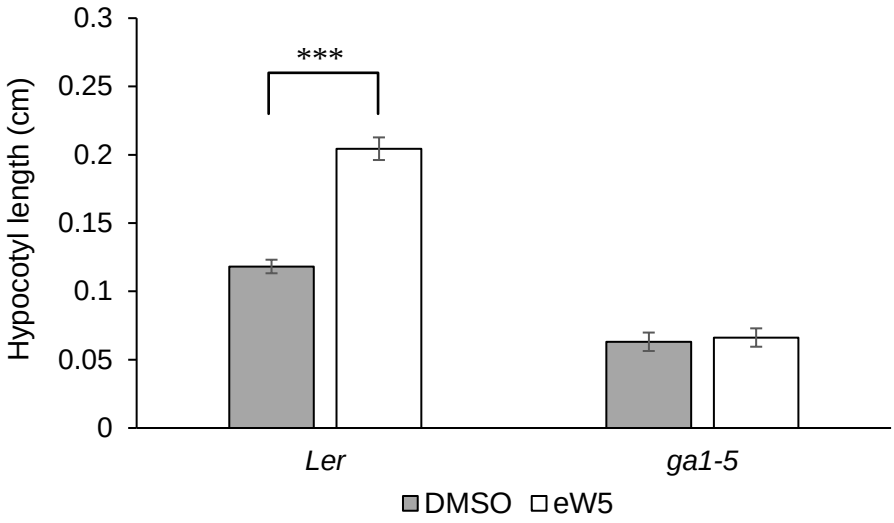


Figure 6.7: Hypocotyl length of GA biosynthesis mutant, *ga1-5* after eW5 treatment for three days. The concentration used for this assay was 100 μM. Error bars represent standard error. Asterisks indicate statistically significant differences (independent t-test, *** P<0.001) between chemical treatment.

Due to the promotion of stomatal aperture after eW5 application, the pore sizes was measured using different genotypes. The application of GA on *ga1-5* mutants restore GA accumulation and promoted stomatal aperture. Unlike in the hypocotyl growth assay, eW5 was found to have a similar effect as GA in guard cell assays. The application of eW5 was able to restore the stomatal aperture of *ga1-5* mutants, suggesting that eW5 can mimic GA function specifically in promoting stomatal aperture (Figure 6.8).

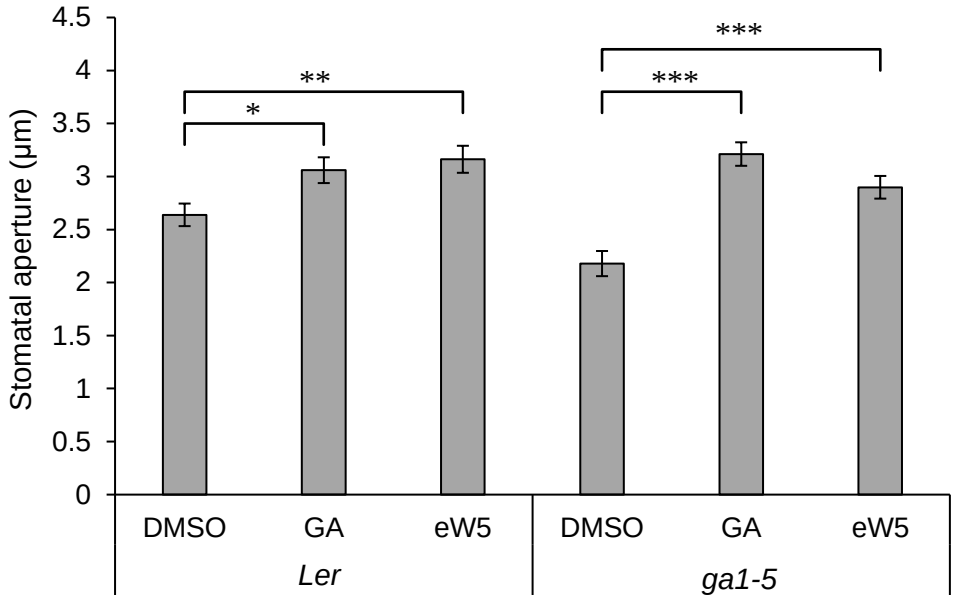


Figure 6.8: The stomatal aperture of *ga1-5* mutants after the application of GA and eW5 at 100µM concentration. The assay was conducted in a minimum source of light and performed in three replicates. Error bars represent standard errors. Asterisks indicate statistically significant differences (independent t-test, * $P<0.05$, ** $P<0.005$, *** $P<0.001$) between chemical treatment.

The observation on the effect of eW5 on GA signalling pathway was continued with the receptor mutants. In *Arabidopsis*, there are three GA receptors named *GID1a, GID1b* and *GID1c*. These three receptors showed functional redundancy as there was no phenotype observed in *gid1* single mutants in stem elongation. However, a defect can be observed in flower development in single mutant, suggesting that the single mutant can show a phenotype under specific development (Griffiths et al., 2006). For stem elongation, severe dwarfed phenotype can be observed from triple receptor

mutant, *gid1a1b1c* and one of double mutants, *gid1a1c*. The phenotype of these mutants cannot be rescued by the application of GA. Therefore, if eW5 affect upstream of the GA signalling pathway, no difference can be observed after eW5 application. In this study, only root length of *gid1a* mutant was increased after eW5 treatment for *gid1a* single mutants, while there was no difference for *gid1b* and *gid1c* (Figure 6.9A). In *gid1* double mutants, eW5 did not affect the root growth of *gid1a1b* and *gid1a1c*, since no difference could be observed between the mutants and wild type seedlings. Application of eW5 increase the root length for *gid1b1c*, as shown in Figure 6.9B. This double mutant phenotype data suggested that the single mutant should have a phenotype, however it did not show in this experiment. Therefore, it would be suggested to perform genomic PCR to check the mutant genotypes.

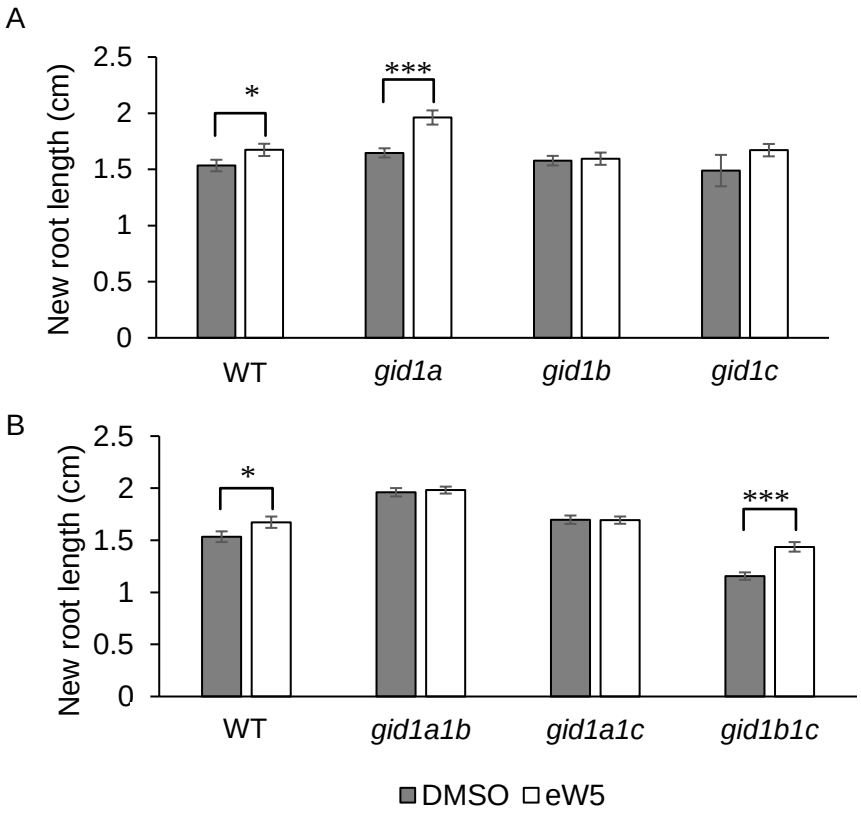


Figure 6.9: Root growth assay of GA receptor single (A) and double (B) mutants with and without eW5 treatment. The assay was conducted using seven day old seedlings and treated for five days. Error bars represent standard error for 18 seedlings. Asterisks indicate statistically significant differences (independent t-test, * $P<0.05$, *** $P<0.001$) between chemical treatment.

The effect of eW5 on GA receptor mutants was also investigated on hypocotyl growth. The growth of *Arabidopsis* is obviously dwarfed for *gid1a1c* mutants, which cannot be observed in the other double *gid1* mutants, *gid1a1b* and *gid1b1c*. As shown in Figure 6.10, eW5 treatment increased the hypocotyl length in wild type seedlings, however the effect was lost in the mutants. This finding therefore leads to two suggestions; eW5 is acting upstream of the pathway by regulating the biosynthesis or that eW5 is involved in GA sensitivity, and therefore it needs the receptor to show its growth promotion effect.

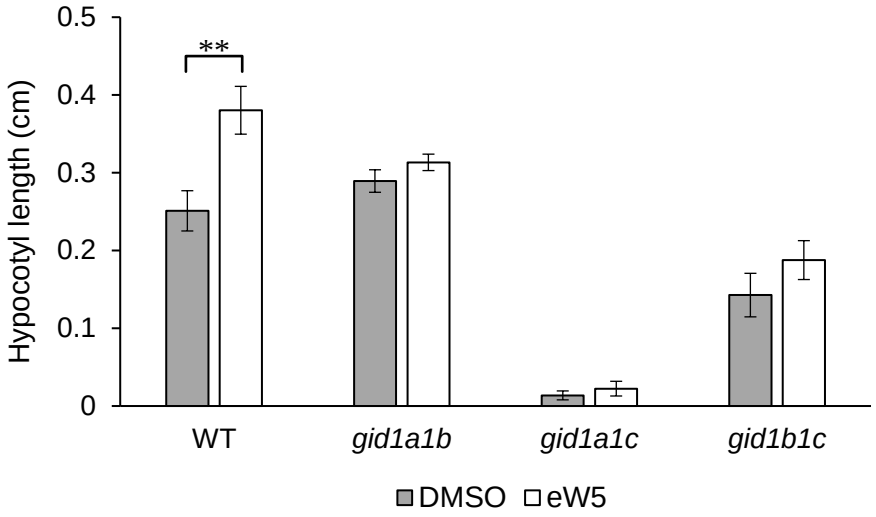


Figure 6.10: The graph of hypocotyl length for GA receptor double mutants. The assay was performed using at least 30 seedlings per treatment. Asterisks indicate statistically significant differences (independent t-test, ** P<0.005) between chemical treatment.

Apart from GA and its receptor, one of the important component in GA signalling pathway is F-box protein. In *Arabidopsis*, SCF^{SLY1} is homologous to F-box protein and has been identified to have the interaction with DELLA protein before inducing proteosamal degradation. This protein is a positive regulator of the pathway and the mutation of this protein will cause an accumulation of DELLA protein, hence it can be characterized by a dwarfed phenotype (Dill et al., 2004; Fu, 2002). To investigate whether eW5 can show its effect in the absence of F-box protein, the effect of eW5 was monitored from root growth assay. As shown in Figure 6.11, eW5 still could promote growth in *sly1-10* mutants, suggesting that DELLA degradation does

not require F-box protein in the presence of eW5. This is consistent with the study performed by Ariizumi et al. (2008) where they discovered a SLY1-independent mechanism for GA signalling that can still function without DELLA degradation.

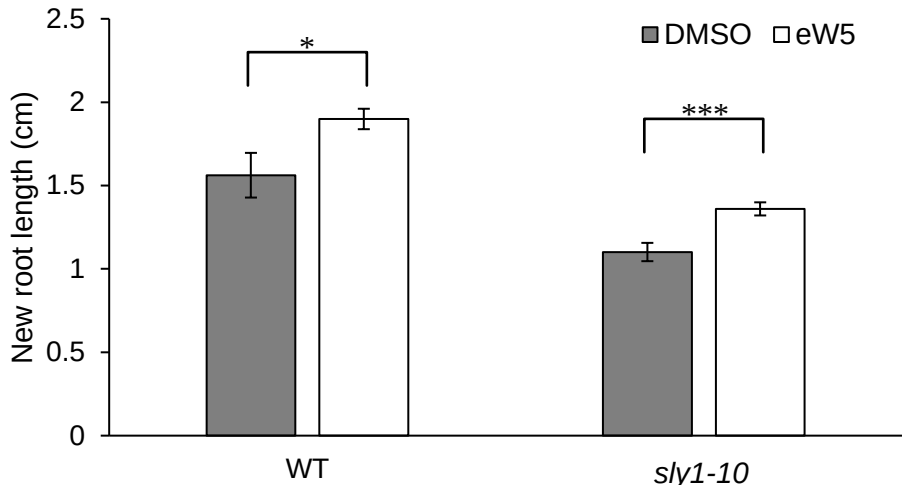


Figure 6.11: The root growth assay for *sly1-10* mutants as compared to wild type after eW5 treatment. The experiment was performed on 7-day old seedlings with 5 days of chemical treatment. The data is represents three biological replicates with error bars represent standard error. Asterisks indicate statistically significant differences (independent t-test, * $P<0.05$, *** $P<0.001$) between chemical treatment.

Taken together, with the loss of eW5 effect on the mutants, it was suggested that eW5 regulates upstream of the pathway. Therefore, to investigate this hypothesis, the analysis on the GA biosynthesis and sensitivity was further explored, as discussed in the following section.

6.4 Effect of eW5 on GA biosynthesis and GA sensitivity

In *Arabidopsis*, GA biosynthetic enzymes are encoded by a family of five genes: *AtGA20ox1-AtGA20ox5*. This gene family was chosen to investigate the involvement of eW5 on the GA biosynthesis pathway, by monitoring the expression levels using real-time quantitative PCR. The expression of these genes was tested at several time points after eW5 treatment; 1h, 3h and 6h. The application of eW5 however did not affect the expression levels of the GA metabolism enzymes, *GA20ox1, GA20ox2* and

GA20ox3, as there was no difference in the expression level as compared to control (DMSO), as shown in Figure 6.12. These findings suggest that eW5 does not regulate GA biosynthesis pathway.

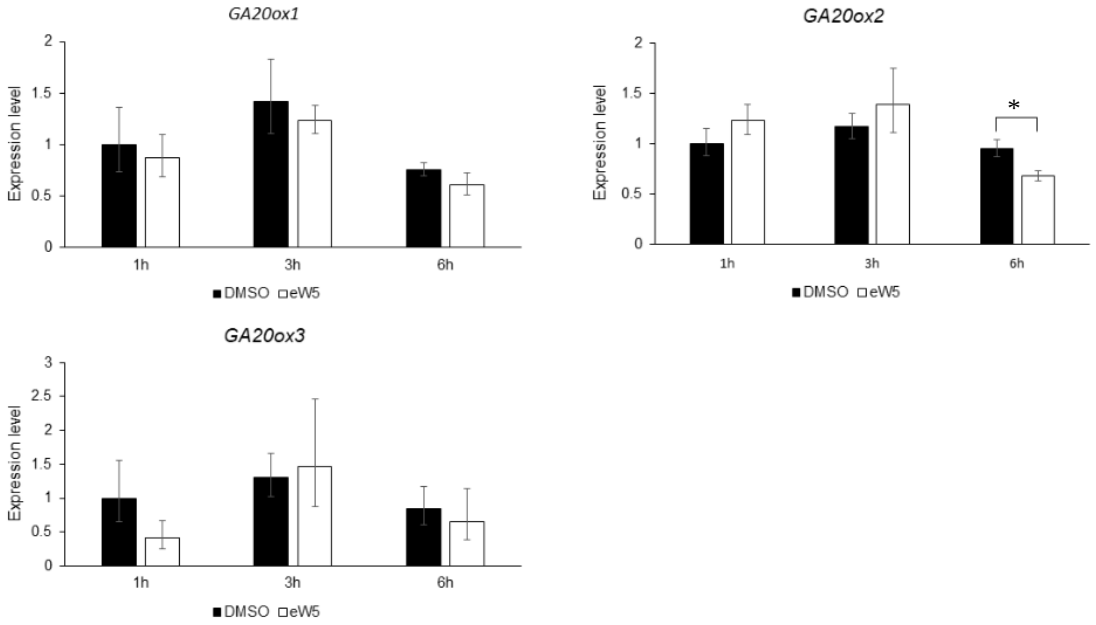


Figure 6.12: The expression level of GA metabolism genes at 1,3 and 6 hours. *GA20ox1*, *GA20ox2* and *GA20ox3* are GA biosynthesis genes that will increase GA production. High levels of GA down-regulate the transcript level of these enzyme for its homeostasis. Asterisks indicate statistically significant differences (independent t-test, * P<0.05) between chemical treatment.

To further explore the effect of eW5 on the GA biosynthesis pathway, *GA20ox3* gene expression in a GA deficient mutant was measured. The experiment was conducted to investigate whether eW5 can regulate the expression of GA biosynthetic enzymes when the content of GA is low. As expected, a high level of *GA20ox3* expression was observed in the control and the application of GA activated the feedback loop, which can be seen by a decrease expression of *GA20ox3*. The greatest effect was observed after an hour treatment. With eW5 treatment, the expression is higher for both 30 mins and one hour time points (Figure 6.13), indicating that there was no feedback loop regulation triggered by eW5, suggesting strongly that eW5 does not increase GA levels.

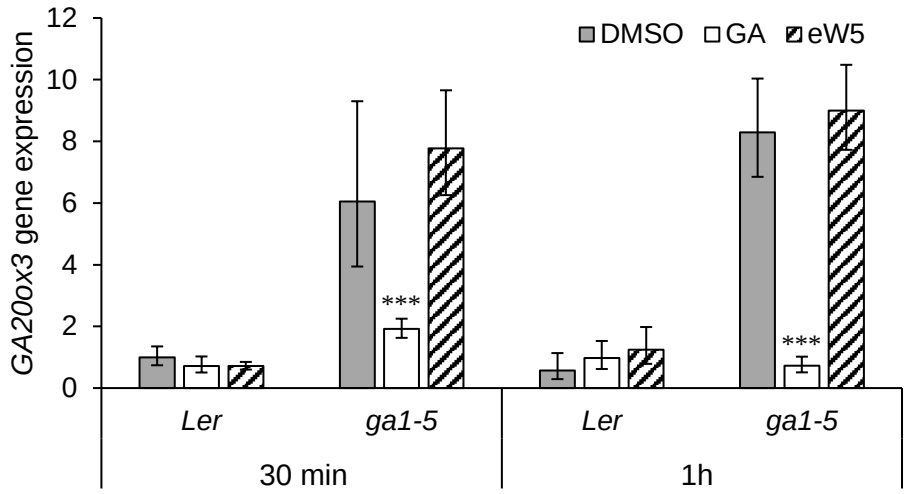


Figure 6.13: *GA20ox3* expression level in *ga1-5* mutants after the application of GA and eW5 at two time points. Error bars represent standard errors from three biological replicates. Asterisks indicate statistically significant differences (independent t-test, *** P<0.001) between chemical treatment and the control, DMSO.

Since there is no changes in metabolic enzymes encoding *GA20ox1-GA20ox3* after eW5 treatment, it shows that eW5 did not affect these genes. Based on the finding that eW5 needs GA to promote growth, its growth promotion effect might be due to the increased GA biosynthesis, hence its effect upon GA production was then investigated. As a result, to investigate if eW5 has an effect on GA production, GA measurement was performed using HPLC-MS. Metabolites extraction was performed on 2 weeks old seedlings as described in details in Chapter 2. In this experiment, the content of GA_3 was measured at different time points, from 10 minutes to 2 hours of eW5 treatment. This time point was chosen to see the effect of eW5 on GA production at the early treatment. 2 hours treatment was chosen as the longest time according to the finding on DELLA degradation, where eW5 can degrade DELLA protein in this period of time. As shown in Figure 6.14, there is no difference in GA production for every time points of eW5 treatment. Furthermore, the level of GA for each time point of eW5 treatment was significantly lower than control, suggesting that eW5 affects GA production by reducing its production.

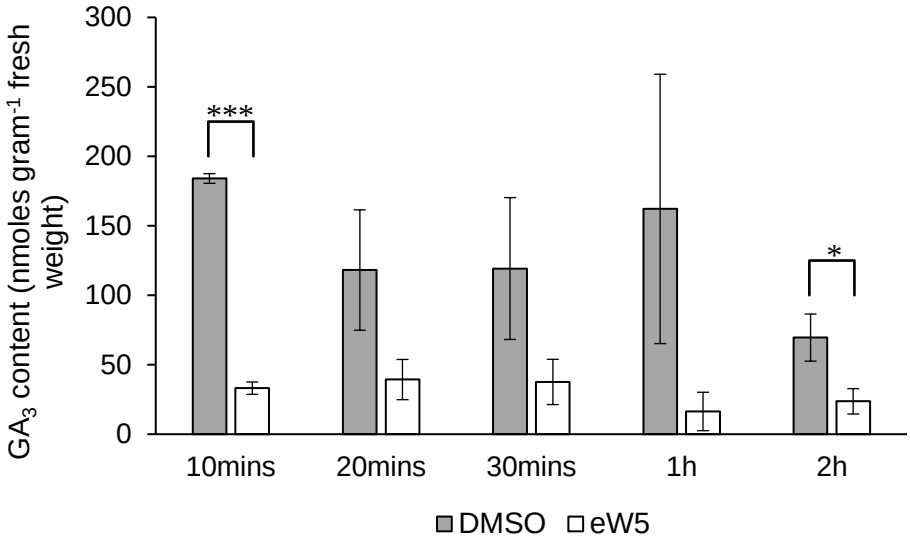


Figure 6.14: GA_3 content after eW5 treatment as compared to its control at different time points. The concentration of eW5 used was 100µM. The experiment was performed in three biological replicates. Asterisks indicate statistically significant differences (independent t-test, * $P<0.05$, *** $P<0.001$) between eW5 and its control.

To confirm that eW5 does not increase GA production, a phenotypic analysis of *GA20ox1, GA20ox2* and *GA20ox1/2* mutants was then performed. These mutants have a dwarfed phenotype due to their low levels of GA, and it is known that this phenotype can be recovered through GA application (Rieu et al., 2008). eW5 did not recover the phenotype of these mutants, indicating that eW5 does not increase the GA biosynthesis and production (Figure 6.15).

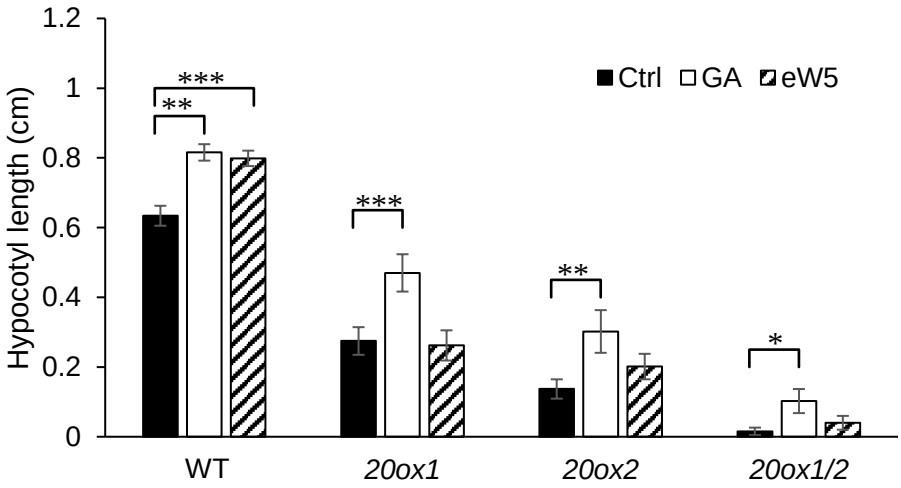


Figure 6.15: The effect of eW5 on hypocotyl growth of ga20ox single and double mutants. The production of GA is low in these mutants therefore GA was used as a positive control. The experiment was conducted in three biological replicates with error bars represent standard error. Asterisks indicate statistically significant differences (independent t-test, * P<0.05, ** P<0.005, *** P<0.001) between chemical treatment.

With no promotion effect observed in root growth assay for both GA-deficient and GA receptors mutant, and the finding that eW5 decreased GA biosynthesis, it was hypothesized that eW5 might promote growth by enhancing the sensitivity to GA. To investigate this hypothesis, eW5 was co-applied with GA to see if this treatment can give synergy effect on hypocotyl growth. To perform this experiment, lower concentration of GA was used as there was no difference between in hypocotyl growth at different concentration of GA. GA can increase the hypocotyl growth at the concentration as low as 1µM, and the hypocotyl grow longer as the concentration of GA increase. Interestingly, the co-application of eW5 increase more of the hypocotyl growth, suggesting that eW5 have an additive effect on GA application (Figure 6.16).

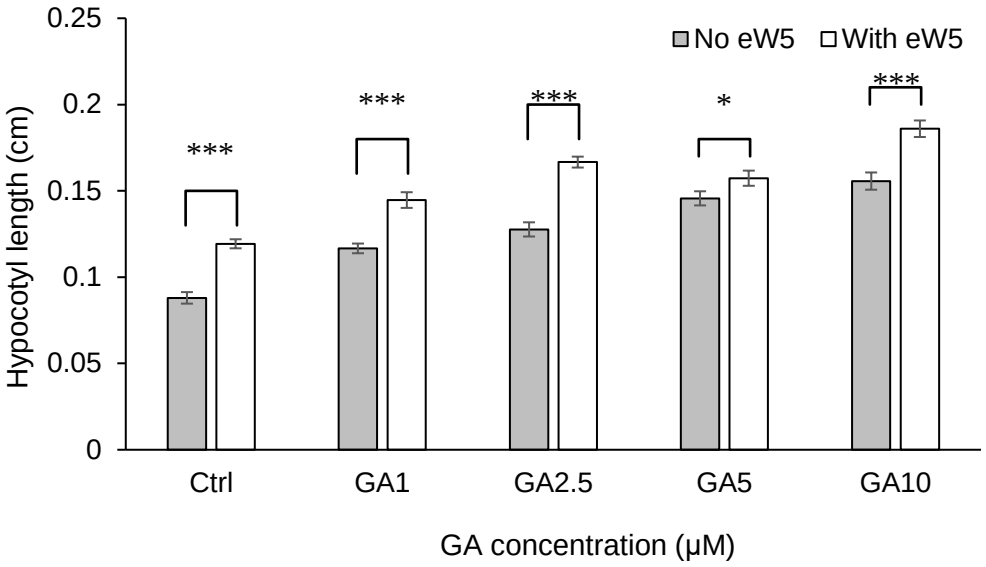


Figure 6.16: The hypocotyl growth of GA treatment with and without the addition of eW5. eW5 was added to investigate the sensitivity of GA. Lower concentration of GA was used as 1 μM is enough to promote hypocotyl elongation. The assay was performed with at least 30 seedlings. Error bars represents standard error. Asterisks indicate statistically significant differences (independent t-test, * P<0.05, *** P<0.001) between chemical treatment.

Collectively, through genetic study, it was ascertained that eW5 needs endogenous GA in order to perform its growth promoting effect: hypocotyl growth of GA deficient mutant, *ga1-5*, was not promoted by eW5. The effect of eW5 on growth was investigated through its stimulation on expression of genes encoding GA biosynthesis enzymes. However, eW5 did not stimulate expression of those genes, suggesting that eW5 does not affect the growth at the gene expression level. eW5 was found not to increase the GA production either, which can be seen in biochemistry analysis. However, the possible mechanism of eW5 in regulating growth is through enhancing GA sensitivity.

6.5 Discussion

6.5.1 GA (and eW5) on DELLA protein degradation

GA is one of the plant hormones that is responsible for regulating plant growth and development. High levels of GA promotes root and hypocotyl growth, elongate stem

and promotes cell division (Sun, 2010). As a key negative regulator of the GA signalling pathway, the degradation of DELLA proteins is considered as a major event during GA-mediated growth (Qin et al., 2014). It is well known that DELLAs integrate endogenous and environmental cues in the regulation of plant growth (Achard et al., 2006; Fu and Harberd, 2003). The GA signalling pathway is activated when GA represses the DELLA restraint by promoting the destruction of DELLAs in the proteasome (Dill et al., 2004; Silverstone et al., 2001). The growth promoting effect of eW5 is likely due to the activation of the GA signalling pathway deduced from its application that mediates DELLA protein degradation after 2 hours. This finding is supported by its promoting effect observed in hypocotyl growth which indicates the accumulation of free PIF4 proteins, resulting from DELLA protein degradation (de Lucas et al., 2008).

GA signalling modulates growth responses to darkness through DELLA proteins. Light and GAs antagonistically regulate hypocotyl elongation in plants. Hypocotyl growth assays were conducted in a reduced light intensity condition due to the inhibitory effect of light on growth by the activation of phytochromes and photoreceptors (Achard et al., 2007). In the light, where plants undergo photomorphogenesis, the transcription factor that is involved in cell elongation, PIF4, is degraded by the light receptor, phyB hence cell elongation is inhibited. In addition, GA metabolic gene transcript levels have been shown to change rapidly in response to changes in the light environment. The expression *GA20ox1* and *GA3ox1* expression is inhibited whereas light increases *GA2ox1* expression in de-etiolated seedlings (Zhao et al., 2007). The lower expression of *GA20ox* and *GA3ox* correlates with transient reduction in GA level (Rieu et al., 2008). At low levels of GA, DELLA proteins accumulate, bind to PIF4 and block the transcriptional activity of this protein. DELLA proteins accumulate in light-grown seedlings causing short hypocotyls hence they are

a major component affecting growth regulation during photomorphogenesis (de Lucas et al., 2008).

On the other hands, GA was found to regulate stomatal aperture in *Arabidopsis*. This is proven by the guard cell assay on GA-biosynthesis mutants, where the stomatal aperture was smaller as compared to wild type in controls. The application of GA restored activity (opening), suggesting that GA is important in stomatal aperture. The promotion of stomatal aperture after GA application is supported by the study conducted by Goring et al. (1990) where a similar observation was made in *Vicia faba*. Consistent with the finding that eW5 regulates root and hypocotyl growth through GA signalling, the promoting effect of stomatal aperture can be observed upon its application. Goring et al. (1990) suggests that the promotion of stomatal aperture is due to the accumulation of sugar and potassium. Therefore it could be suggested that eW5 might increase the accumulation of sugar and potassium that enables the promotion of stomatal aperture. Interestingly, the activity of GA in regulating stomatal aperture is through DELLA proteins as can be observed by the fact that *della* quintuple mutants show bigger stomatal apertures as compared to wild type and no changes are observed in these mutants after GA application. However, the mechanism for this process is still unknown.

6.5.2 The investigation on eW5 target on the pathway

GA destabilizes DELLA proteins through its promotion of ubiquitination and proteasomal degradation. The interaction between GA and its receptor (GID1) induces the conformational changes of GID1 and promotes its interaction with DELLA protein (Sun, 2011; Murase et al., 2008). This stable complex then enables the interaction with SCF components before undergoing ubiquitination and proteasomal degradation (Fu, 2002; Dill et al., 2004). As eW5 promotes growth with a similar mechanism to GA; by targeting DELLA degradation, supported by the finding that eW5 growth promotion effect is DELLA dependent, the effect of eW5 on the GA signalling pathway was

investigated at the point of each of the core components of this pathway. This investigation was performed using specific genetic backgrounds.

Although eW5 has the same effect as GA, namely degrading DELLA proteins, it was found not to mimic GA function directly as demonstrated by the analysis of GA-deficient mutants: no recovery of the phenotype was observed after the application of eW5. Due to this effect, together with its effect that lost in the receptor mutants, eW5 was suggested to act upstream of the pathway, possibly through regulating GA biosynthesis.

6.5.3 The effect of eW5 on GA biosynthesis

As mentioned in the previous section, genetic evidence suggests that eW5 affecting GA biosynthetic pathway. As GA biosynthesis is important to regulating the concentration of GA in cells, GA production upon eW5 treatment was measured. Unexpectedly, GA levels were lower after eW5 treatment, as compared to control after only 10 minutes of eW5 treatment. 10 minutes was chosen as the shortest time because it reflects the time required for GA to degrade DELLA proteins as performed by Zentella et al. (2007). This observation suggests that eW5 might regulate GA production in less than 10 minutes. A number of studies have revealed that GA homeostasis is achieved by a feedback mechanism (Sun, 2010). To maintain its homeostasis, enhanced GA deactivation is important which can be achieved by *GA2ox* that can convert active GAs to inactive forms (Sun, 2008). Therefore it could be suggested that the high accumulation of bioactive GA in addition of eW5 has been converted to inactive forms in a short period of time. This could be tested by measuring the inactive forms of GA such as GA8 or GA29 using the same biochemical approach as mentioned in Section 6.4.

The failure of eW5 to produce more GA was supported by the finding in measuring the transcript level of genes encoding GA biosynthesis genes, *GA20ox3*.

In the GA biosynthesis pathway, there are several enzymes that are responsible for GA metabolism and catabolism. Addition of chemical (GA or GA biosynthesis inhibitor) or genetic mutation causes changes in GA signalling activity, which will affect the expression for some of these GA biosynthesis genes (Olszewski et al., 2002; Sun and Gubler, 2004; Sun, 2008). Under GA-deficient conditions or in mutants that have reduced GA signalling, transcripts level of GA biosynthetic genes, encoding *GA20ox* and *GA3ox* are up-regulated, whilst the expression of the GA catabolic genes encoding *GA2ox* is downregulated (O'Neill et al., 2010). In contrast, the transcript levels of *GA20ox* and *GA3ox* have been shown to be under negative feedback regulation by GA treatment, while *GA2ox* expression level is increased upon GA application (Hedden and Phillips, 2000). This is supported by a study conducted by Thomas et al., 1999 where they discovered that the expression of *GA2ox* is up-regulated after the application of GA to the flower buds of the *Arabidopsis ga1-2* mutants. This finding contrasts with the down-regulation of *GA20ox* expression levels by GA activity (Thomas et al., 1999; Schomburg et al., 2003). Thus, the level of GA is likely to be maintained by modulating both their synthesis and catabolism. Bioactive GA homeostasis is maintained by the feedback regulation of such genes involved in GA metabolism and for that, an active GA response pathway is necessary (Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000). Since these genes are important in the GA biosynthesis pathway, their transcript levels were measured upon eW5 application. eW5 had no effect on these genes suggesting that eW5 does not regulate GA biosynthesis through the feedback mechanism described above.

Several GA biosynthetic genes have been shown to be downregulated by GA, constituting a negative feedback loop of GA on its own biosynthesis. The concentration of bioactive GA is decreased with the reduction of *GA20ox1* or/and *GA20ox2*, with double mutants of these genes producing lower levels of GAs than single mutants or wild type (Rieu et al., 2008). The finding that eW5 did not affect GA biosynthesis was

confirmed through the hypocotyl growth assay in *ga20ox* mutants. The roles of these mutants were identified previously by Rieu et al. (2008), where a *ga20ox2* mutant showed reduced hypocotyl growth, indicating that this gene is required for cell elongation. A *ga20ox1/2* mutants has a low levels of GA and a dwarfed phenotype which can be recovered through GA application. However, there was no effect observed from eW5 treatment, confirming that eW5 is not affecting the GA biosynthesis pathway. This result is consistent with the *GA20ox3* gene expression pattern observed in *ga1-5* mutants, where there was no *GA20ox3* expression difference observed between eW5 treatment and control, whilst its expression was reduced after GA application. As mentioned above, the expression of *GA20ox3* is reduced when there are high levels of endogenous GA therefore strongly suggesting that eW5 does not regulate GA biosynthesis and produce GA.

6.5.4 The enhancement of GA sensitivity by eW5

Since eW5 does not seem to regulate GA biosynthesis, it was hypothesized that it might enhance sensitivity to GA. In order to investigate whether eW5 enhanced GA sensitivity, a hypocotyl growth assay was performed at a low concentration of GA. This condition was chosen to minimize the accumulation of GA and hence the synergistic effect could be observed. 1 µM GA was able to promote hypocotyl growth and the co-application with eW5 further increased hypocotyl growth, suggesting that eW5 might increase the sensitivity to GA. However, rather than synergistic affect, the additive effect of GA and eW5 were found in hypocotyl growth, where the co-application of eW5 with GA increased the length of the hypocotyl. The co-application of eW5 with GA showed a similar increment of hypocotyl length for every concentration. This result was supported by the treatment of eW5 on *gid1* double mutants, where the treatment of eW5 did not affect the growth. This could be due to eW5 losing the synergistic effect due to the absence of the receptors themselves. In a recent finding, GA receptor RING E3 ubiquitin ligase (GARU) has been identified as a protein that reduces GA sensitivity

and acts as a negative regulator of GA signalling (Nemoto et al., 2017). This protein ubiquitinates GID1 hence leading to the destabilization of GID1 and higher accumulation of DELLA protein. In the context of this finding, it is therefore possible that eW5 inhibits GARU to affect the sensitivity of GA before induces DELLA degradation. Thus, the binding activity between eW5 and GARU is one of the experiment that can be performed in order to explore this suggestion.

There was a difference between the effect of eW5 on hypocotyl and root growth in *gid1* double receptor mutants. The measurements showed that there was no growth increase for hypocotyl in any of the double mutants, while for root growth assay, one of the double mutants, *gid1b1c,* had a longer root after eW5 treatment. These results suggests that eW5 effect is specifically on the GA pathway. This is due to the effect of GA that is more specific in hypocotyl growth. Hypocotyl growth is influenced by degradation of DELLA protein and GA is needed to degrade them so that the transcription factor, PIF4 can be released to function. GA is known to promote hypocotyl growth and is strictly required for hypocotyl elongation in dark-grown seedlings (Saibo et al., 2003).

6.5.5 Conclusion

Taken together, strong genetic evidence indicates eW5 is targeting DELLA protein degradation to show its growth promotion effect. Further investigation using genetic analysis shows that eW5 does not act as GA. However, eW5 was found to have an effect on GA production, where it decreases GA content in a short period of time. This suggests that eW5 might have an effect on GA feedback loop which can be confirmed through the identification of the GA inactive forms. Further to this, it was found that eW5 might be acting through increasing GA sensitivity, which was suggested by the additive effect observed between eW5 and GA. This can be supported by the biophysical experiment between eW5 and GA receptors such as Thermal Shift Assay and Microscale Thermophoresis.

CHAPTER 7

eW5 Analogues

7.1 Introduction

Small molecule analogues are important tools to discover and manipulate plant signalling pathways (Toth and van der Hoorn, 2010). To achieve this, the modifications of the compounds using structure activity relationships (SAR) is normally done, due to the approach that can evoke a target biological effect in the organism. Structural variants may have functional groups modified or removed. This allow a study on how certain structural changes can affect biological activity (Capdeville et al., 2002).

In ABA signalling, upon binding ABA PYR1 undergoes conformational changes inducing a closed conformation which in turn creates the interaction surface that enables binding with PP2C and thus downstream ABA-mediated responses. Takeuchi et al. (2014) by generating a series of ABA analogues was able to demonstrate that these small molecules regulators of the ABA signalling pathway were highly structurally-specific. The analogues that have a longer alkyl chain interfered with the PYR-PP2C interaction hence blocked activity and deactivated ABA signalling (Figure 7.1) (Takeuchi et al., 2014).

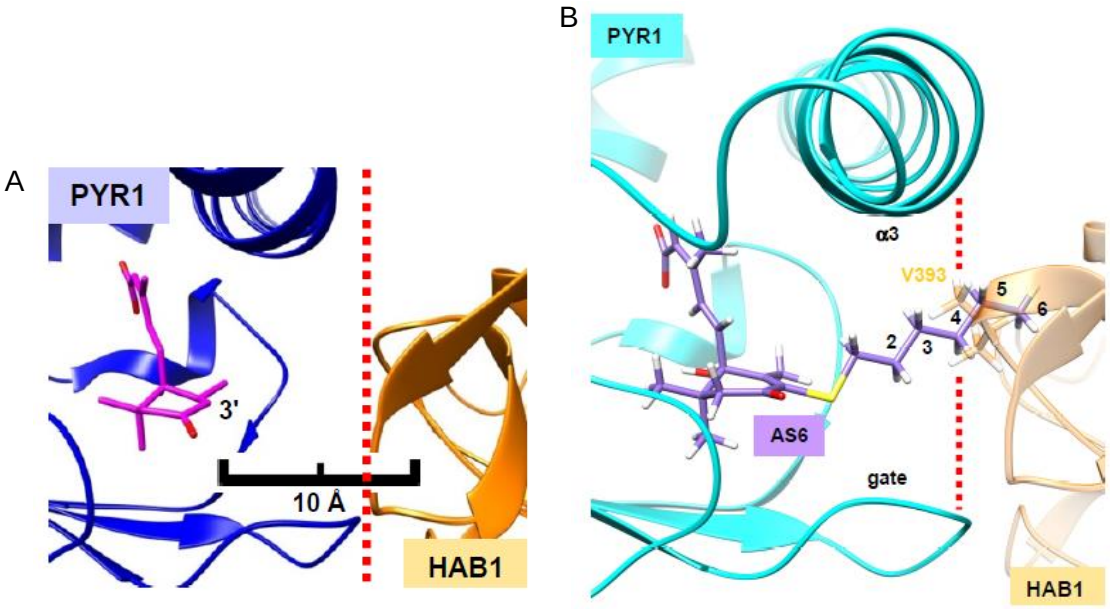


Figure 7.1: Modelling structure of the interaction between PYR1 and A) ABA, and B) Takeuchi's representative ABA analogue. A longer hexyl chain interrupt the interaction between PYR-PP2C complex. The figures were adapted from Takeuchi et al., 2014.

It was thus proposed that eW5 function might be modulated in a similar manner. Using the same approach, the objectives in this chapter include:

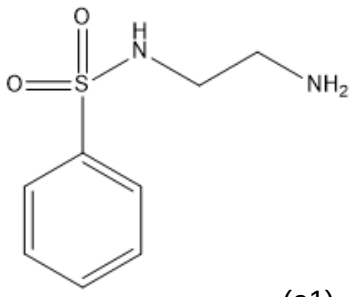
- Synthesis of small analogues with structures related to eW5.
- Investigation of the effect of these analogues on plant physiology.

7.2 The small analogues of eW5

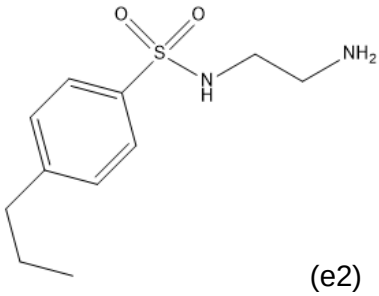
As mentioned in Chapter 5 and Chapter 6, eW5 has a positive effect in root and hypocotyl growth, and stomatal opening. The promotion of root and hypocotyl growth is influenced by the cell elongation regulated by numbers of proteins such as PHYTOCHROME INTERACTION FACTOR (PIF4) (de Lucas et al., 2008). On the other hand, stomatal opening is tightly regulated by Ca^{2+}-dependent protein kinases (CDPK) (Schroeder et al., 2001). Having established that different developmental processes are regulated by different proteins, it was suggested that the positive effect

of eW5 on root and hypocotyl growth and stomatal opening might be due to an interaction with different, but specific proteins for each response. To explore this idea, eW5 was modified with relatively simple alterations, targeted to the naphthalene and ethylenediamine groups. The analogues were synthesized to explore if the naphthalene group plays a major role in growth promotion. Therefore, e1 and e3 were synthesized to explore the effect of replacing the naphthalene ring with a simple benzoic structure, while e2 and e4 were synthesized to investigate the effect of the corresponding compounds with the addition of the alkyl group at the para position. To further study the effect of the amine group in eW5, the addition of methyl chain at the primary amine (e5) and secondary amine (e6) were synthesized. The series of the analogues produced is shown in Table 7.1. In a similar fashion to that described for the production of analogues in Chapter 3, the synthesis was achieved by reacting the corresponding sulfonyl chloride with ethylene diamine, followed by Boc-protection and deprotection to aid compound purification.

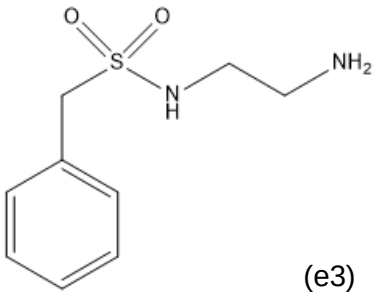
Table 7.1: The analogues of eW5 produced by modifying the functional group to identify the side that is responsible for the growth promotion effect.



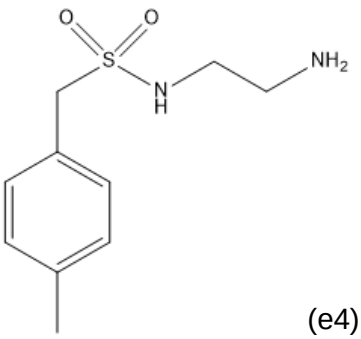
(e1)



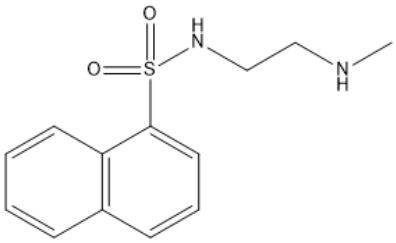
(e2)



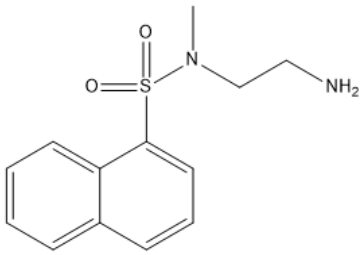
(e3)



(e4)



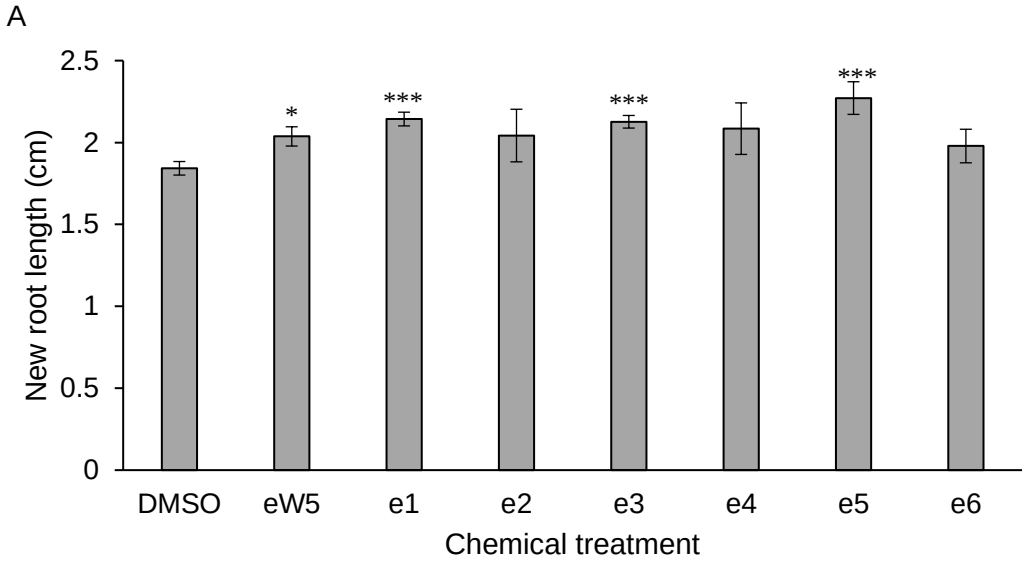
(e5)



(e6)

7.3 The effect of eW5 analogues on plant physiology

With this set of analogues available the next plan was to investigate the effect on root and hypocotyl growth, and stomatal aperture. These three experiments were conducted with eW5 as a control. In order to allow a direct comparison with eW5, the concentration used in these assays was 100 µM. The root growth assay was performed by treating the 7-day old seedlings on chemical plates for 5 days. Measurement of root growth was performed using ImageJ software. The root length for each compound is shown in Figure 7.2A, whilst the relative percentage of root growth is presented in Figure 7.2B. All of the compounds tested promoted root growth with most of them showing a higher activity than eW5, suggesting that any modification of the naphthalene or ethylene diamine group does not change the growth promotion effect of eW5.



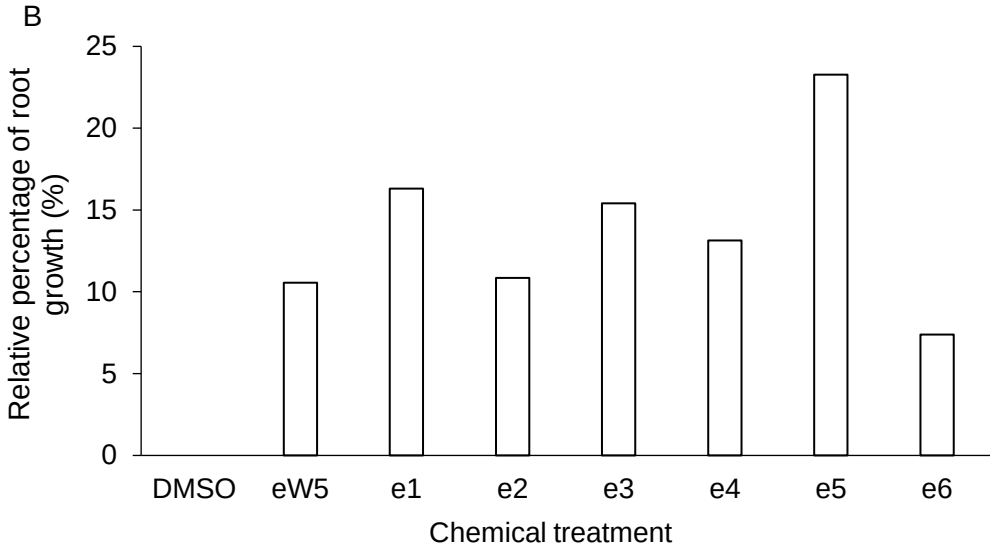


Figure 7.2: Root growth assay for eW5 analogues with eW5 as a positive control. The data shown is based on three biological replicates. A) New root growth (in cm) after treatment with the chemicals at 100µM concentration. B) The relative percentage of growth as compared to control (DMSO). Asterisks indicate statistically significant differences (independent t-test, * $P<0.05$, *** $P<0.001$) between chemical treatment and the control, DMSO.

Subsequently the compounds were tested for effects on hypocotyl growth assay. The same procedure as described in Chapter 6 was performed, whereby the seeds were grown in reduced light intensity. Similar to the root growth assay, it was found that all of the analogues showed a different degree of promotion of hypocotyl growth (Figure 7.3A). Among them, only e3 and e4 showed lower hypocotyl growth promotion with quantitatively less than eW5, which suggests that an aryl sulfonamide is important in promoting hypocotyl growth (Figure 7.3B).

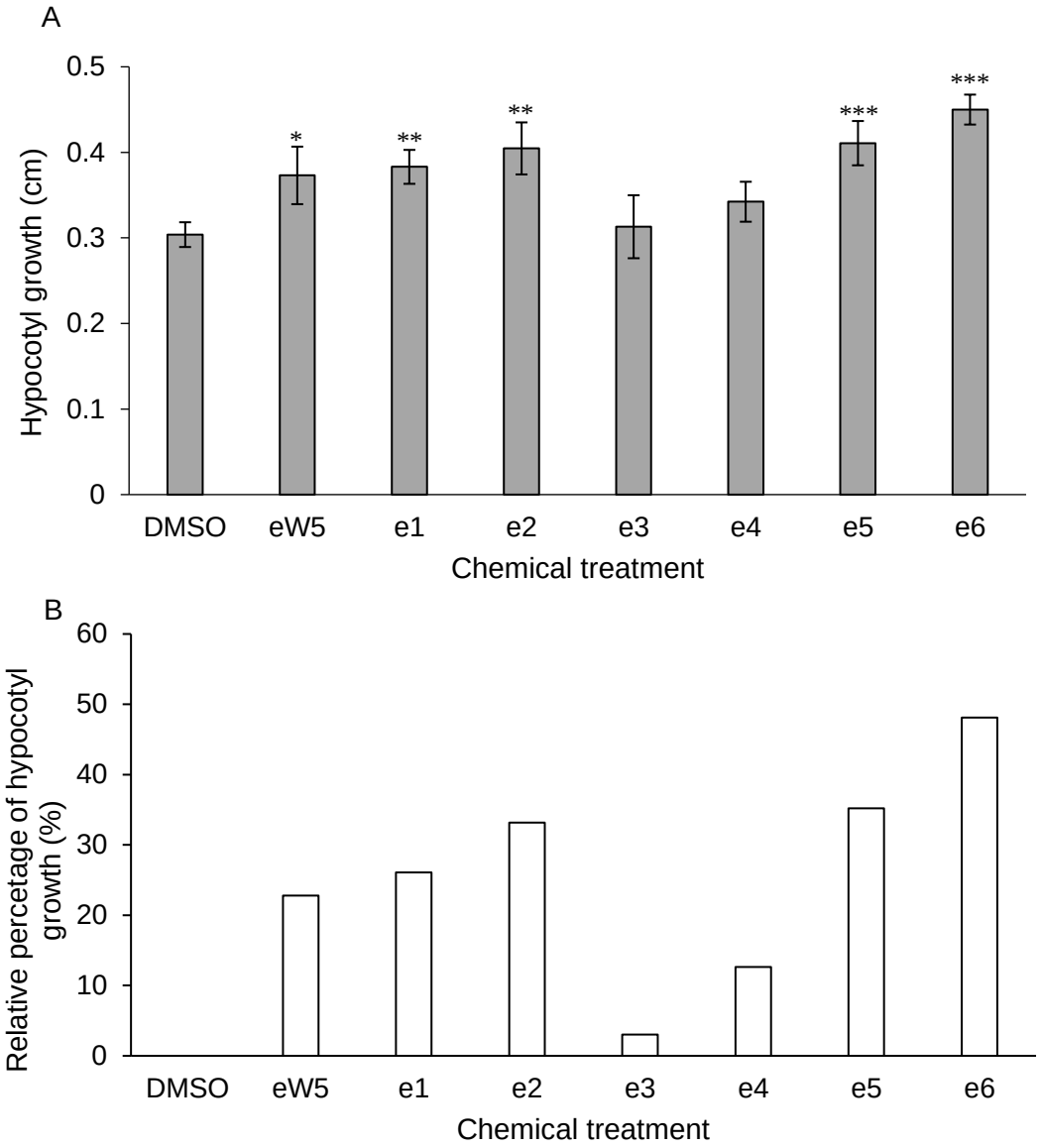
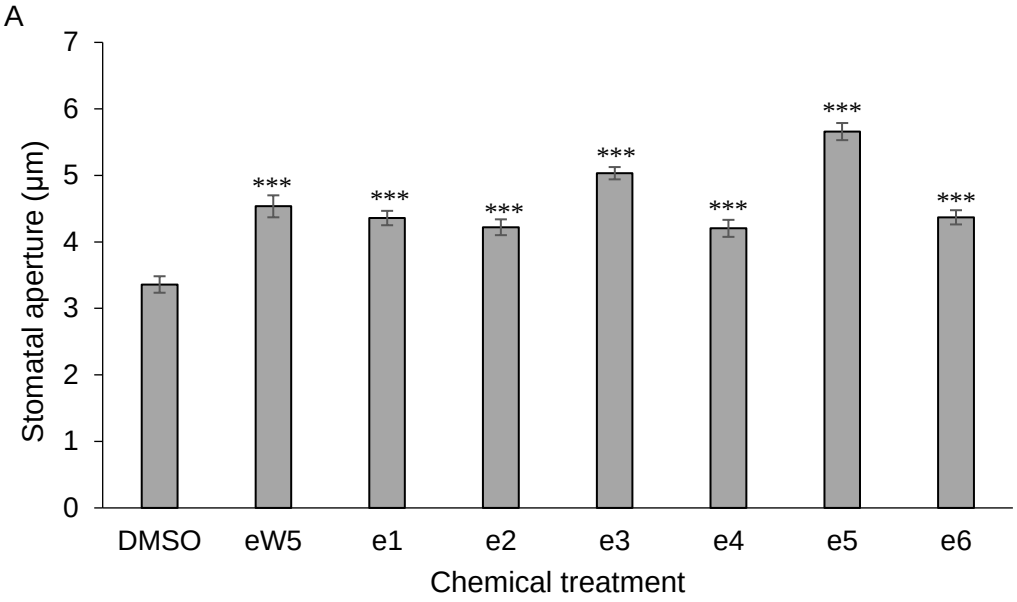


Figure 7.3: Hypocotyl growth assay for eW5 analogues with eW5 as a positive control. A) New hypocotyl growth (in cm) after treatment with the chemicals at 100µM concentration. The error bars represent standard error. B) The relative percentage of growth as compared to control (DMSO). The graph was based on three biological replicates. Asterisks indicate statistically significant differences (independent t-test, * P<0.05, ** P<0.005, *** P<0.001) between chemical treatment and DMSO (as control).

Whilst all of the compounds showed a positive growth enhancement, there is a different promotion activity in root and hypocotyl growth regulated by the position of sulfonamide as observed for the e3 and e4 treatment specifically. These two compounds showed higher activity than eW5 in root growth, but failed to show the corresponding effect in hypocotyl growth. Therefore, it is suggested that the

compounds interact differently with the protein regulating root and hypocotyl growth. The addition of methyl group at the end of the ethylenediamine chain (e5) had the biggest effect upon root and hypocotyl growth, suggesting that the presence of this particular group is important for a quantitatively greater activity.

As well as an effect of root growth, eW5 also has an effect on stomatal aperture where it has a potential to promote stomatal aperture. Therefore eW5 analogues were tested using a guard cell assay. The experiment was performed as described in Chapter 5 using eW5 as a positive control. Interestingly, all of the analogues promote stomatal aperture with different quantitative levels as compared to control, with e3 and e5 displaying a larger effect than eW5 (Figure 7.4).



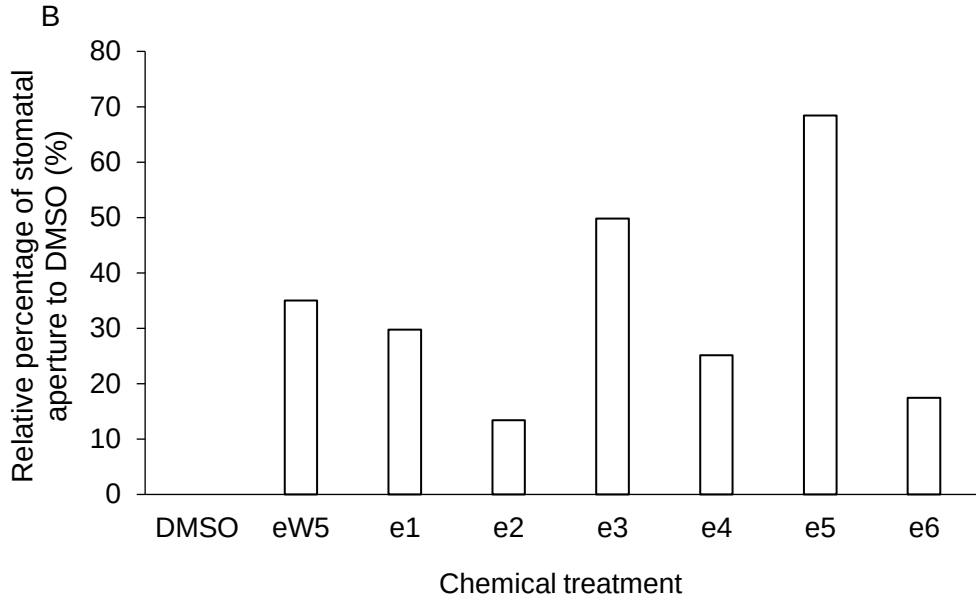


Figure 7.4: Stomatal aperture assay for eW5 analogues with eW5 as a positive control. The data shown is based on three biological replicates. A) The stomatal aperture after treatment with the chemicals at 100µM concentration. B) The relative percentage of aperture as compared to control (DMSO). Asterisks indicate statistically significant differences (independent t-test, *** P<0.001) between DMSO and chemical treatment.

Collectively, the chemical modifications of eW5 lead to different degrees of affecting its growth promotion effect. Further to this, the compounds do not show the same effect for every developmental process, suggesting that they have different specific targets in regulating different aspects of plant growth. Screening of the analogues resulted in the identification of e5 as the compound with a promising effect in both root and hypocotyl growth, and stomatal opening. In addition, the difference in response from the application of e3 and e4 in root and hypocotyl assays suggests that the position of the sulfonamide is important in regulating hypocotyl growth but not root growth.

7.4 Discussion

7.4.1 The modification of the compound and their biological activity

Generally, structure activity relationships (SAR) gives the information about the active site of the compound that is responsible for their bioactivity. Different effects observed in guard cell, root and hypocotyl growth assays after the treatment of different eW5

analogues suggest that the small alteration of the compound can indeed affect its biological function. This is due to the presence of a functional group that can interact with specific protein(s) and affect their protein function.

The modification of eW5 by adding a methyl group at the end of the chain (e5) improved it activity, with it being the most active in promoting root and hypocotyl growth, and stomatal aperture. Interestingly, there is only one difference between e5 and A3 (from Chapter 3) at the end of the diamine moiety, where there is methyl and propyl chain for e5 and A3, respectively (Figure 7.5). However, this difference gave different effects of the compounds, where with alkyl addition, root growth is promoted, while with a longer alkyl chain the effect is the opposite. The longer alkyl chain might therefore block the activity for root growth therefore this compound acts as an antagonist for root growth. This might be due to the different interaction of the compounds with their target proteins and the presence of a longer alkyl chain might change the conformation hence leading to the lower activity of the compound. The longer alkyl chain may tend to interfere with the interaction between proteins hence acting as an antagonist. Hayashi et al. (2008a) has discovered that the access of Aux/IAA to the TRANSPORT INHIBITOR RESPONSE 1 (TIR) auxin-binding pocket is blocked after the application of compounds with butyl and longer alkyl chains. Aux/IAA is a repressor and its interaction with auxin-TIR is important to activate the pathway downstream. Similarly, in order to activate the ABA signalling pathway, the interaction between ABA-PYLs receptor enhances the interaction with PP2Cs therefore PP2Cs inhibition on SnRKs can be released. ABA analogues have been designed by adding different alkyl chains at the 3’ position of the ring with a longer alkyl chain which interferes with the interaction due to steric hindrance (Takeuchi et al., 2014).

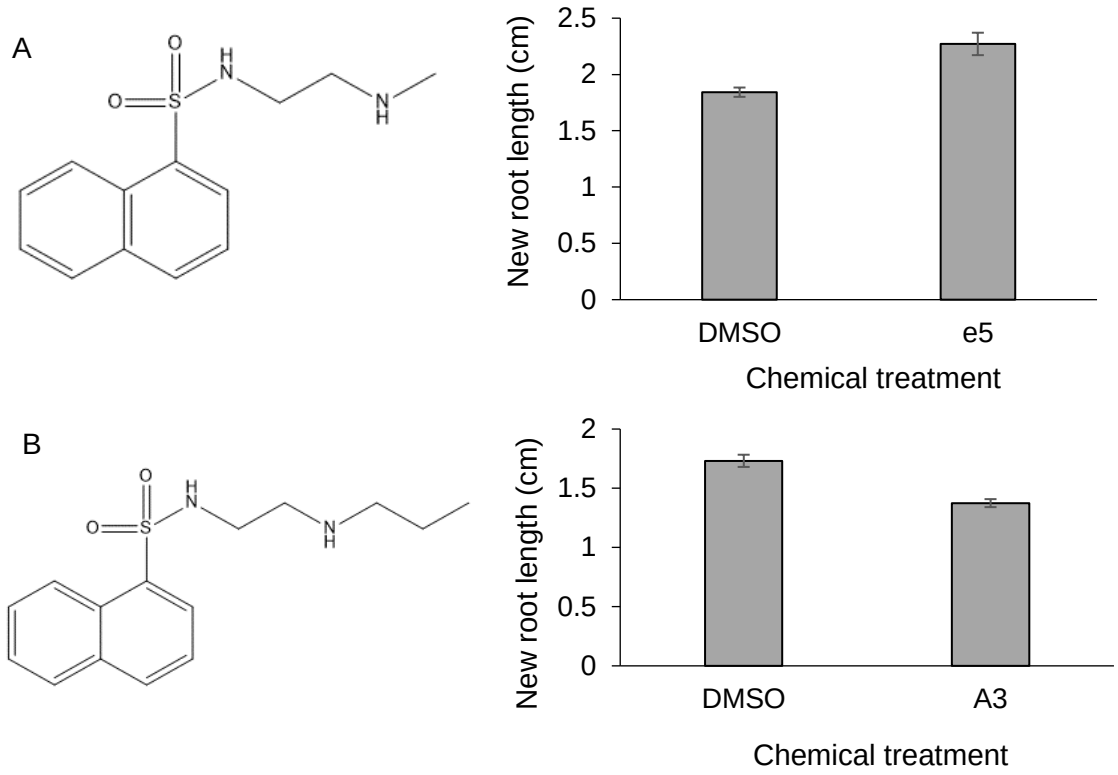


Figure 7.5: The difference between A) e5 and B) A3 (from Chapter 3) and their effect on root growth. The only difference for these compounds is the length of the alkyl group at the end of the amine chain but the activity for these compounds are totally opposite.

7.4.2 Molecular mechanism of the compounds

To understand the molecular mechanism of the protein, modelling and X-ray crystallography could be attempted, where the key interaction of the signalling can be determined. From there, the analogues could then be designed through a structure-based approach. For example, in the ABA signalling pathway, X-ray crystallography revealed that ABA binding induces the conformational change of the PYLs and brings about the “gate-closed” conformation, which will create an interaction surface that enables the interaction with the active site of the PP2Cs. Takeuchi et al. (2014) designed a series of ABA analogues and produced a series of 3’-alkylsulfanyl ABA. This series allow the discovery that a longer alkyl chain interrupt and failed to induce the close-gate conformation hence acts as an antagonist. These findings revealed that structure-based analogue design together with crystallography is important to understand the molecular mechanism of the protein and to further explore the activity

of the compound/ analogue (Choi et al., 2014). In this study, it is suggested that the position of sulfonamide is important for hypocotyl growth promotion. Therefore, the modification of the analogues by retaining this site is important, and the compounds can be used to identify all target proteins that interact with the compound, which is normally achieved through affinity based purification protein (ABPP) (Tashiro and Imoto, 2012).

Based on the finding that eW5 both stimulates the GA signalling pathway by enhancing GA sensitivity (Chapter 6) and interacts with an ABA receptor, it is possible that eW5 analogues described in this chapter operate through the same mechanisms. The comparison between the effect of eW5 analogues on root and hypocotyl growth and stomatal aperture suggest that the compound that has an effect in promoting growth does not necessarily promote stomatal aperture in the same degree as well. For example, e2 promotes hypocotyl growth with a higher percentage than eW5, however in guard cell assays, this compound did not show the same effect. This observation suggests that the analogues might stimulate the GA signalling pathway and interact with different ABA receptor/s. The sextuple *pyr/pyl* mutant, *pyr1/pyl1/2/4/5/8* shows strongly reduced sensitivity to ABA-mediated inhibition of growth as well as stomatal aperture (Gonzalez-Guzman et al., 2012), which suggest that these receptors (PYR1/PYL1/2/4/5/8) are important in both root growth and stomatal aperture. Therefore different binding affinities to different receptors would potentially determine the activity of the compounds in these developmental processes.

7.4.3 Conclusion

With the modification of eW5 and the analysis on plant physiology, it was found that all of the compounds promote growth with a different degree of promotion. The difference in promotional effect might be due to the binding specificity of the compounds with different protein targets. Therefore the further step like affinity based

protein purification should be taken in order to identify the target proteins of eW5 followed by biophysical interaction techniques to understand the molecular mechanism of the compounds in regulating plant growth and development.

CHAPTER 8

Discussion

8.1 Implication of the work

It is well known that plant growth and development is regulated by small molecules called phytohormones. Phytohormone perception is a complex process involving a cross talk between each hormones to modulate the specific developmental changes (Rigal et al., 2014). Due to its complexity, hence it is a challenge to understand the hormonal signalling pathway. Therefore, a more versatile approach, chemical genetics, always be used to achieve the understanding. This is due to the advantage of this approach that can reduce gene redundancy problem (Mccourt and Desveaux, 2010). The research work presented in this thesis was aimed at exploring the specificity of particular protein targets, as well as understand the mode of action of synthesised small molecules in plant biological system. This chapter will briefly conclude the results of this thesis and provide some suggestions that can be taken for future research.

8.2 Chemically induced gene expression

Plants are constantly exposed to their environment and develop the mechanism for their adaptation. The understanding of their ability to respond to their environment has advanced through studies controlling for genotypic variation (Pigliucci, 2001; van Kleunen and Fischer, 2005; Valladares et al., 2007). These studies typically contribute to the phenotypic variation, which is revealing the mechanisms of heritable epigenetic effects. These epigenetic effect include DNA methylation, histone modification, microRNA and small interfering RNA. These mechanisms lead to phenotypic changes

due to the changes in gene expression, without variation in genotype (Finnegan, 2002; Jablonka and Raz, 2009).

Environmental signals, as well as the application of small molecules such as phytohormones contributes to epigenetic modification by regulating the levels of histone acetylation (Yamamuro et al., 2016; Chinnusamy et al., 2008; Chinnusamy and Zhu, 2009). For example, HDA6 is induced by jasmonic acid and ethylene and mediate histone deacetylation in order to adapt to the environment, including biotic and abiotic stress (Zhou et al., 2005a). Further to this, it is also responsible in regulating transcriptional gene silencing and RNA-directed DNA methylation (RdDM) (Probst et al., 2004; Aufsatz et al., 2002).

The work presented in Chapter 4 was aimed at investigating the effect of eW5 on DNA methylation that might be correlated to epigenetic changes. The most common epigenetic mechanism is DNA methylation, one of the factor that regulates gene expression by blocking the promoters at which activating transcription factor binds, and thus inhibits their activity (Hudson et al., 2011; Suzuki and Bird, 2008). DNA methylation can be observed from restriction enzyme digestion experiment that differentiate the methylated DNA from unmethylated DNA (Figure 4.2). However, this result did not determine if eW5 regulates epigenetic changed, but this could be further tested by other experiment such as bisulfite sequencing. Moreover, together with transcriptomic analysis, the upregulated and downregulated genes upon the application of the eW5 could determine which genes are responsible for the specific phenotype. With a detail analysis such as undirected analysis, the pathway that is regulated by eW5 can be discovered.

8.3 eW5 regulation in plant hormonal signalling

ABA is one of the plant hormones that has a major role in regulating plant adaptation during stress. It is important in seed dormancy and germination, root development, stomatal movement and adaptive stress responses (Nambara and Marion-Poll, 2005; Cutler et al., 2010). Higher level of ABA cause a growth defects which suggests that ABA is important in regulating plant development (Barrero et al., 2005; Fujii and Zhu, 2009; Fan et al., 2009; Nambara and Marion-Poll, 2005).

As discussed in Chapter 1, the ABA response occurs through the activation of SnRK2 protein kinases following the repression of PP2C inhibition upon the interaction with ABA-PYR/PYLs. Structural biology has revealed that ABA-PYR/PYLs interaction induced the closed-gate conformation that enables the complex to bind to PP2Cs (Melcher et al., 2010). Therefore, the agonism and antagonism effect of the small molecules can be predicted from this interaction. Pyrabactin successfully forms the closed gate conformation with PYR1, but showing its antagonistic effect in PYL2, where the interaction does not change to this conformational arrangement. The work performed in Chapter 5 was aimed at discovering the potential of eW5 as an ABA antagonist due to the structural similarity between eW5 and pyrabactin. However, the failure of eW5 to recover PP2C activity indicates that it has no interaction with PP2Cs proteins therefore does not inhibit ABA perception (Figure 5.14). It could be suggested that the interaction of eW5 and PYR1 in this study does not induce PYR1 conformational change therefore cannot create the binding surface to bind PP2Cs. This is due to a weak interaction between eW5 and PYR1. Moreover, it could be suggested that the agonism or antagonism effect of the compound is important, which can be identified from the structural biology.

The finding is consistent with the physiological effect of eW5 which displays a weak effect to ABA responses; in seed germination (Figure 5.6) and in stomatal

aperture (Figure 5.9). It is well known that GA and ABA always antagonize each other activity to regulate many physiological processes including seed germination and plant growth, which is discussed in Chapter 1. The promotion of eW5 of these physiology effects suggests that eW5 might regulate the GA signalling pathway and in this way it can reduce the ABA effect (or increase GA:ABA ratio), instead of acting as ABA antagonist.

GA regulates plant growth by promoting the degradation of the plant repressor, DELLA protein (Lee et al., 2002a; Peng et al., 1997). This process involves GA, its receptor, GID1, DELLA protein itself, F-box protein, SLY1, where all of these core components have to be interacting with each other in order to promote such mechanism (Alabadi et al., 2004). The work performed in Chapter 6 was aimed at determining the role of eW5 in the GA signalling pathway, due to the similarity of its effect upon application to the GA response; DELLA degradation, promoting seed germination, hypocotyl and root growth, as well as promoting stomatal aperture (Silverstone et al., 2001; Goring et al., 1990; Griffiths et al., 2006; de Lucas et al., 2008).

With the similarity between eW5 and GA, it raises the hypothesis that it is mimicking GA function. However, through hypocotyl growth assays using a GA-deficient mutant, no recovery of hypocotyl growth was observed which suggests that it is not simply mimicking GA function, and it needs endogenous GA to show its growth promoting effect (Figure 6.7).

Higher levels of GA degrade DELLA proteins therefore GA-responsive genes are activated and the growth promotion effect can be observed. GA level is mainly regulated by expression of genes *GA20ox* and *GA3ox* encoding metabolic enzymes, as well as *GA2ox* encoding a catabolic enzyme. These three gene families play an important role in maintaining the level of GAs. The level of expression of GA metabolic

genes is decreased in the presence of GA and in contrast, the absence or low level of GA increases *GA2ox* expression. Other than monitoring the expression level, the level of GA can be observed through the metabolite identification by MS as performed by Forcat et al. (2008). However, the growth promotion effect induced by eW5 is not through affecting GA level, as concluded from the low levels of GA detected after its application (Figure 6.14). With no growth promotion in the mutants of each component of the signalling and GA biosynthesis, the promotion of hypocotyl can be observed however with the co-application of eW5 and GA (Figure 6.14). Therefore, although eW5 decreased the production of GA, the other possible mechanism on how eW5 regulates GA signalling pathway is through increasing GA sensitivity. Further to this, eW5 might regulate GA biosynthesis shorter than 10 minutes, before regulating the GA feedback loop.

A new finding from guard cell assays in this study suggests that the correlation between GA and ABA might be through DELLA protein. GA was found to promote stomatal aperture, and this activity was proposed to act through DELLA protein as with *della* quintuple mutant, the stomatal aperture was bigger than wild type and there was no change in stomatal aperture after GA application (Figure 6.6). The potential mechanism of this phenomenon is that the stomatal aperture regulated by DELLA protein, whereby their degradation plays a major role in promoting stomatal aperture. Interestingly, it is well-studied that stomatal aperture is controlled by ABA level, in order to control water loss during transpiration, especially during drought stress. It is therefore suggested that when there is a low level of DELLA proteins, stomatal aperture is promoted, whilst higher levels of DELLA protein induce stomatal closure (Achard et al., 2006). Genetic evidence of the antagonistic effect between GA and ABA have also been found where the ABA biosynthetic mutant *aba2* shows an increase levels of GA and, conversely, the biosynthetic mutants accumulates higher level of ABA (Seo et al., 2006; Oh et al., 2007).

8.4 eW5 analogues and their effects on plant physiology

Structural biology is an important approach in chemical genetic studies. Normally this approach has been used in order to generate the analogues of hormones and determine the agonistic or antagonistism activity of these analogues. Small chemical analogues represent a powerful tool to discover, visualize and manipulate plant signalling pathway (Toth and van der Hoorn, 2010; Blackwell and Zhao, 2003). Here, structural resolutions of small chemical hormone binding site to corresponding receptors enables the rational design of analogues that interfere with endogenous hormone signalling.

An excellent example for such a structural biology approach has been demonstrated by Takeuchi et al. (2014) and Hayashi et al. (2008a) for the ABA and auxin signalling pathways, respectively. In their studies, they investigated the key interaction between the hormone and its receptor before generating analogues and identifying the compound that had an antagonistic effect to these particular pathway. The antagonistic effect of the compounds is derived from their interaction with the receptor that was resolved by X-ray crystallography and phenotypic analyses.

This study has been focused on the growth promoting effect due to the application of eW5. Further analogues of eW5 were synthesized (Table 7.1) and similar phenotypic investigations were performed, especially investigating the GA signalling pathway, to determine if the analogues can still retain the original eW5 effect. The generation of eW5 analogues described in Chapter 7 was aimed at investigating the specificity of the compounds on protein target. The specificity of the compound was shown by e3 and e4, where these compounds showed a bigger promotion effect as compared to eW5 in root growth but not in hypocotyl growth (Figure 7.4). Overall, combining structural biology with genetic studies is the obvious next step in chemical

genetics, as this would allow the complete understanding on the molecular mechanism of certain signalling pathways.

8.5 Future work and suggestion

This section will provide some suggestions for future work that could be performed in order to understand more of the effect of eW5 in promoting growth. Further to this, more exploration of eW5 on ABA and GA signalling pathway will be discussed.

8.5.1 The changes of gene expression and transcript level

Plants adaptation to the environment requires epigenetic modifications that lead to changes in gene expression and transcript level which contribute to the phenotypic changes in plants (Sahu et al., 2013; Golldack et al., 2011). Work performed in Chapter 4 suggested that eW5 has a potential in regulating epigenetic modifications. Therefore, to study further the involvement of eW5 in epigenetic regulation could provide a number of answers regarding the changes in gene expression. One of the most widely studied is through bisulfite profiling (Wreczycka et al., 2017). This technique would provide a series of data that indicate specifically which genes have been subject to epigenetic modification. Treatment of DNA with bisulfite converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected. Therefore, DNA that has been treated with bisulfite retains only methylated cytosines (Frommer et al., 1992; Li and Tollefsbol, 2011). After bisulfite modification, DNA sequencing need to be performed for the comparison with the control (Li and Tollefsbol, 2011). Together with transcriptomic analysis, an understanding regarding the genes affected can be enhanced, thus provide us the set of genes that are responsible for phenotype of interest.

8.5.2 The regulation of hormone signalling pathways by eW5

The mechanism by which eW5 causes a similar effect to GA was suggested to be through altered GA sensitivity, demonstrated by a synergistic effect in hypocotyl elongation (Chapter 6). Nemoto et al. (2017) discovered that GA ring ubiquitin E3 (GARU) was involved in GA sensitivity and DELLA phosphorylation. DELLA phosphorylation promotes their direct interaction with the SLY1 subunit of SCF^{SLY1} E3 ubiquitin ligase that promotes ubiquitination and 26S proteasomal degradation (Dill et al., 2004). GARU has been found to ubiquitinate GID1A and destabilize it. However, GARU-dependent GID1a ubiquitination decreased in the presence of DELLA protein, suggesting that GARU cannot mediate the ubiquitination of GID1a in GID1a-GA-DELLA complex. As a result, it can be hypothesized that eW5 might bind to GARU and abolish its effect, thus the promotion effect can be observed due to the degradation of DELLA protein. To achieve this, the biophysical interaction between eW5 and this protein is important to be investigated. Furthermore, due to the finding that suggest that eW5 might regulate GA sensitivity, it would be interesting to investigate whether eW5 has an interaction with the GID1 receptors themselves. This interaction can be monitored through either thermal shift assay (TSA) or MicroScale Thermophoresis (MST) technique. This finding can enhance our understanding on the mode of action of eW5 in GA signalling pathway.

Furthermore, the application of eW5 to understand hormonal cross talk can be investigated, regarding to a small ABA antagonistic effect that can be observed in Section 5.3. As mentioned earlier, plant physiology is regulated by the antagonistic mechanism between GA and ABA. Therefore, to discover the basis of this correlation could lead to an understanding of the hormone cross talk that is known as a complex system. The finding that GA (and eW5) promotes stomatal aperture could be a starting point to take this investigation further, with the hypothesis that PYR/PYL2 and DELLA protein might be correlate to each other. Some experiments that could be conducted

include measuring the stomatal aperture of *della* quintuple mutants after ABA treatment.

8.5.3 Exploration of eW5 in other hormone signalling pathways

The growth promotion of roots and hypocotyls is often regulated by GA and brassinosteroids (BRs). Interestingly, these two hormones have the similar responses in plant physiology and it has been discovered from full genome expression analysis that BRs- and GA- responsive genes are highly interconnected (Nemhauser et al., 2006). It has been discovered that BRs regulate GA biosynthesis. In BRs mutant, the expression of GA metabolism genes, *GA20ox* and *GA3ox* are reduced, which results in severe dwarfed phenotype (Tong et al., 2014; Unterholzner et al., 2015). BRs induce GA biosynthesis to stimulate the degradation of DELLA proteins, which release their inhibition on BES1/BZR1 transcription that required for plant growth and development (Unterholzner et al., 2015; Ross and Quittendan, 2016; Bai et al., 2012; Gallego-Bartolome et al., 2012). BRs regulate cell division, cell elongation, and seedling development and root growth. Due to the similarity in their action, it would be interesting to investigate the possible regulation of eW5 in BRs signalling pathway, or the cross talk between GA-BRs.

8.5.4 Ultimate objective: Identifying the target protein(s) of eW5

As discussed in Chapter 1, the ultimate objective for chemical genetics is the identification of specific identity of the proteins. The identification of the protein in question can be performed using a biochemical approach, by the addition of a linker to the small molecule without losing its activity (Taunton et al., 1996). To retain the activity is the most challenging aspect, therefore the application of structure-activity relationship (SAR) is important. SAR studies involved the modification of the functional groups of the small molecule to determine the important site for the bioactivity. The nonessential sites are then used as a site for the attachment to an affinity before the

immobilization (Lomenick et al., 2010). The bound protein are separated by performing SDS-PAGE and the identification of protein is conducted by MS analysis (Kawatani and Osada, 2014).

Following this, the target identification of eW5 could be performed based on its analogues as discussed in Chapter 7. With the growth promotion observed from the application of the analogues, the modification could be performed on any of the compounds in this series. However, e2 could be a good candidate to attach a linker such as biotin due to its ability to retain eW5 activity with a longer chain on its benzyl ring.

The more recent techniques in protein identification is drugs affinity responsive target stability (DARTS). Unlike an affinity approach, this technique provides a straightforward technique to identify small molecules target proteins, by using a native small molecule. The basic strategy for DARTS is that the stabilization of the protein target upon ligand binding, thereby reducing protease sensitivity of the target protein (Lomenick et al., 2009; Rodriguez-Furlan et al., 2017). In this technique, the protein extract are incubated with the small molecule and treated with proteolytic enzymes, where bound proteins are protected from proteolysis, while non-target protein are digested away (Lomenick et al., 2009; Rodriguez-Furlan et al., 2017). With this advantage, the protein target of eW5 can be identified without any modification of the compound, which increase the confidence that the activity can be retained.

8.6 Conclusion

In conclusion, the work performed in this thesis describes the use of a chemical genetics approach to understand the mechanisms of hormonal regulation of plant development. In particular, the exploration of small molecules has uncovered a potential mechanism in regulating plant hormonal signalling pathways. Using the

suggested approaches to identify the protein target(s) will be an important advance to enhance our knowledge on the mechanism of action of this small compound.

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APPENDIX A

Oligonucleotides

A.1 Primers used for Rapid Amplification Polymorphic DNA

ATC33:	CGG TAG CCG C
ATC51:	ATG TGG CGA C
ATC71	GTC GTT CGG G
OPB11	GTA GAC CCG T
OPB12	CCT TGA CGC A
OPB14	TCC GCT CTG G
OPB17	AGG GAA CGA G
OPB18	GGA GGG TGT T
RAPDa	GGT GCG GGA A
RAPDb	GTT TCG CTC C
RAPDc	GTA GAC CCG T
RAPDd	AAC GCG CAA C
RAPDe	CCC GTC AGC A

A.2 Primers used for qPCR using Applied BioSystems

GA20ox1F	GCC GCT TCT TTG ATA TGC CT
GA20ox1R	TTC CAT GGA AGC TTG GTG GA
GA20ox2F	CAT TCC AGA GCT CAA CGT CC
GA20ox2R	GAG GAA GAA GCC GTG TTT GG
GA20ox3F	TCG TGG ACA ACA AAT GGC A
GA20ox3R	TGA AGG TGT CGC CTA TGT TCA C
GA2ox7F	CGA AGA ATA GTT ACC GTT GGG GAA ACC
GA2ox7R	ACG ATT GTT CTG AGG TTG TTG CGA TC
ABF3F	CAG AAA TTG CGC AAC TCA AA
ABF3R	CAG AGG CTC CAG AAG CTG AT

LTI78F	GCACCCAGAAGAAGTTGAACA
LTI78R	TCATGCTCATTGCTTTGTCC
ABI1F	TGA GAT GGC AAG GAA GCG GAT TCT
ABI1R	GGC TTC AAA TCA ACC ACC ACC ACA
PYL8F	TGT GGT GAA AGG AAA CAT GG
PYL8R	CTC AGT GCT TCT AGT TGC TGG T

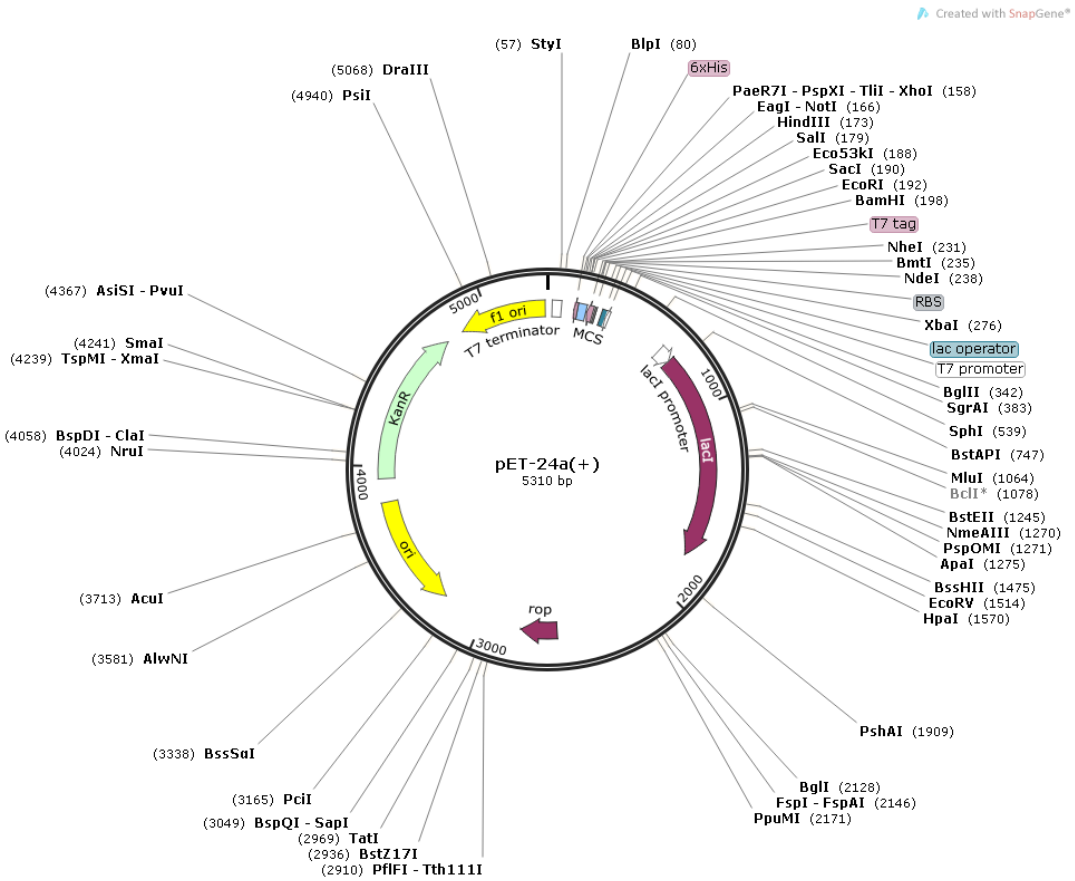
A.3 Primers used for gene cloning

PYR1 F	CGCGCATATGCCTTCGGAGTTAACACC
PYR1 R	CGCGCTCGAGTCACGTCACCTGAGAACCACTTC

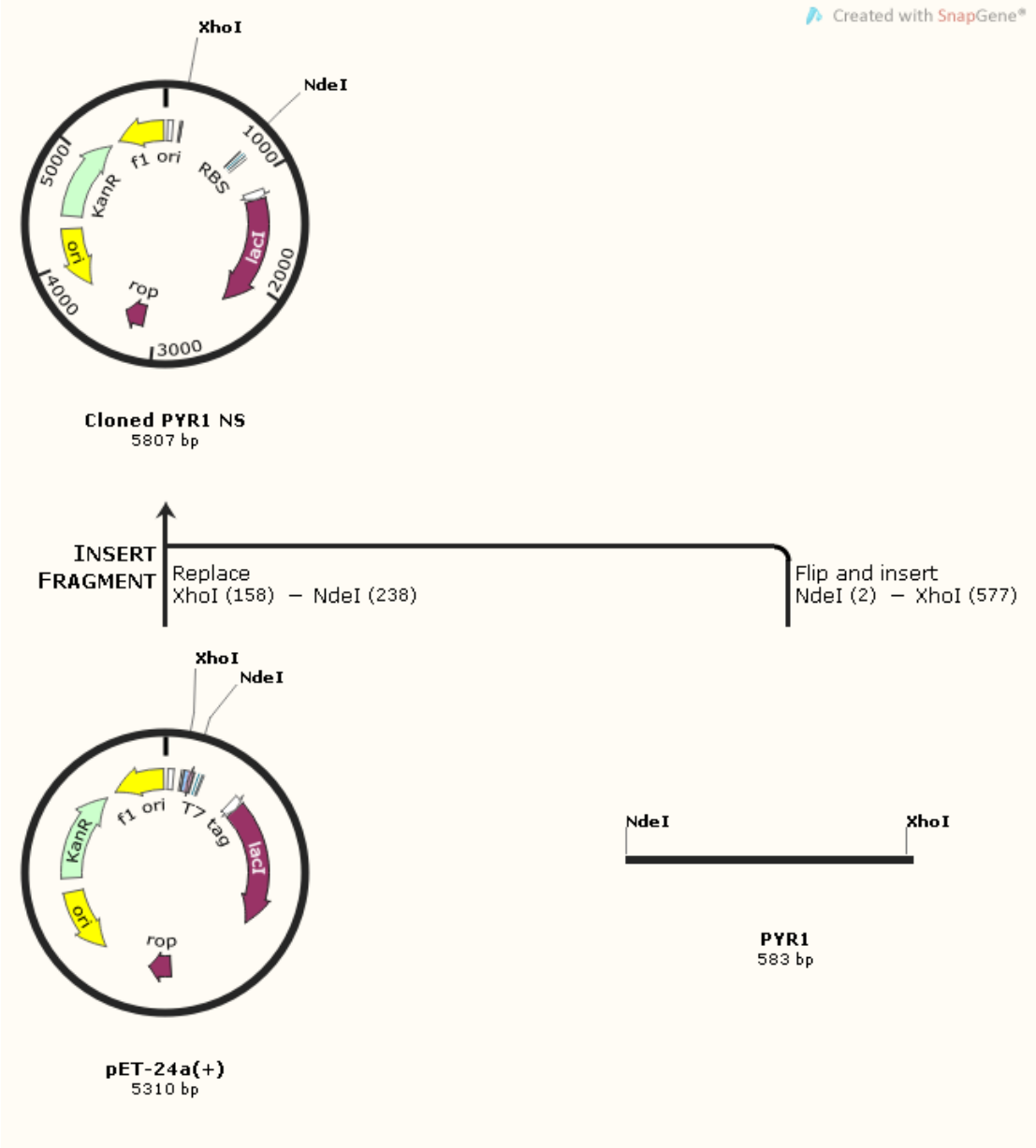
APPENDIX B

Protein identification

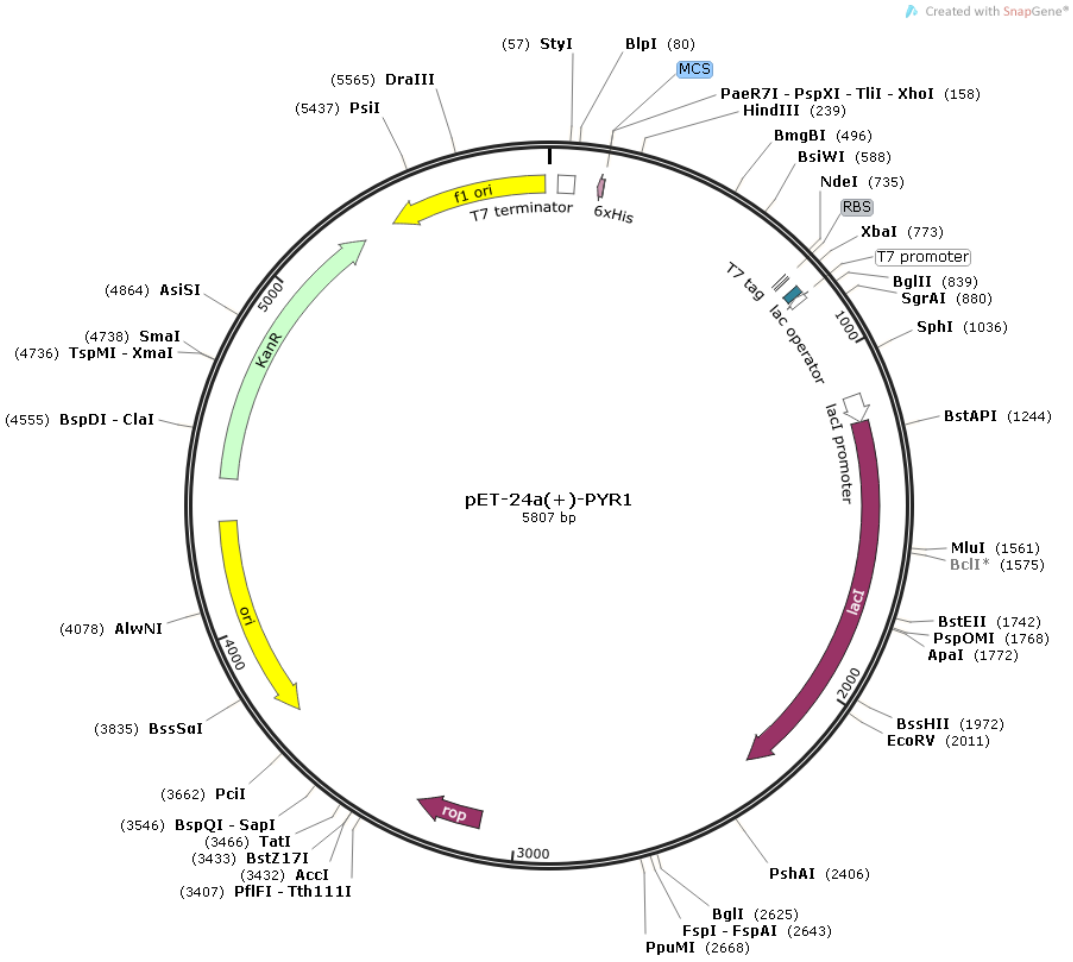
B.1 Vector map of pET24a



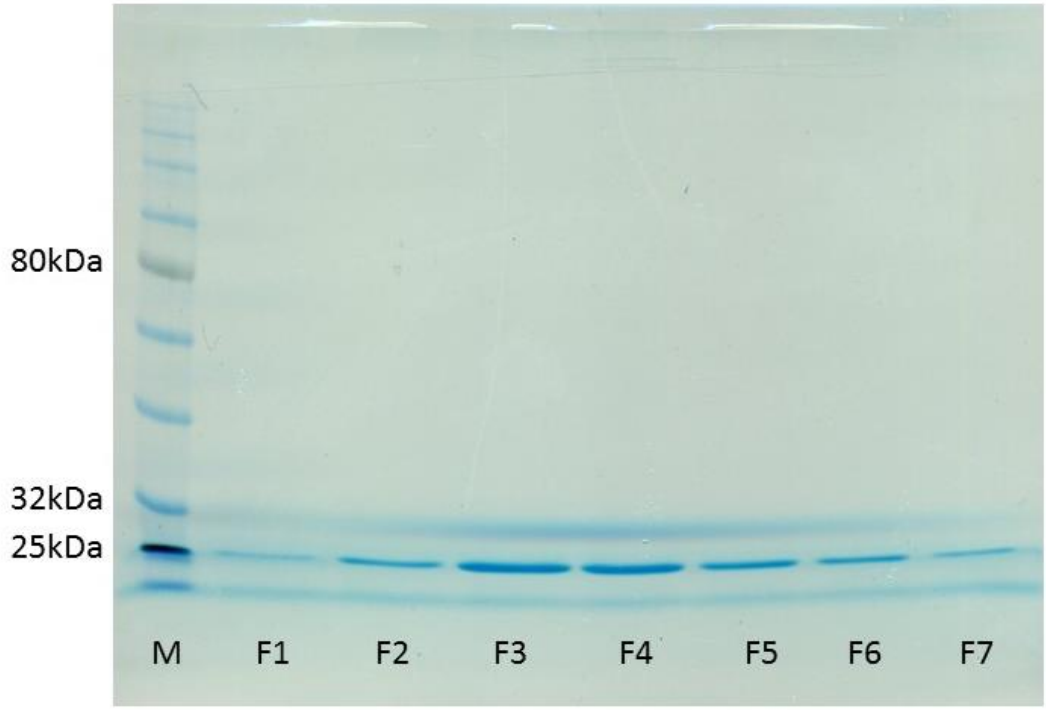
B.2 Cloning product



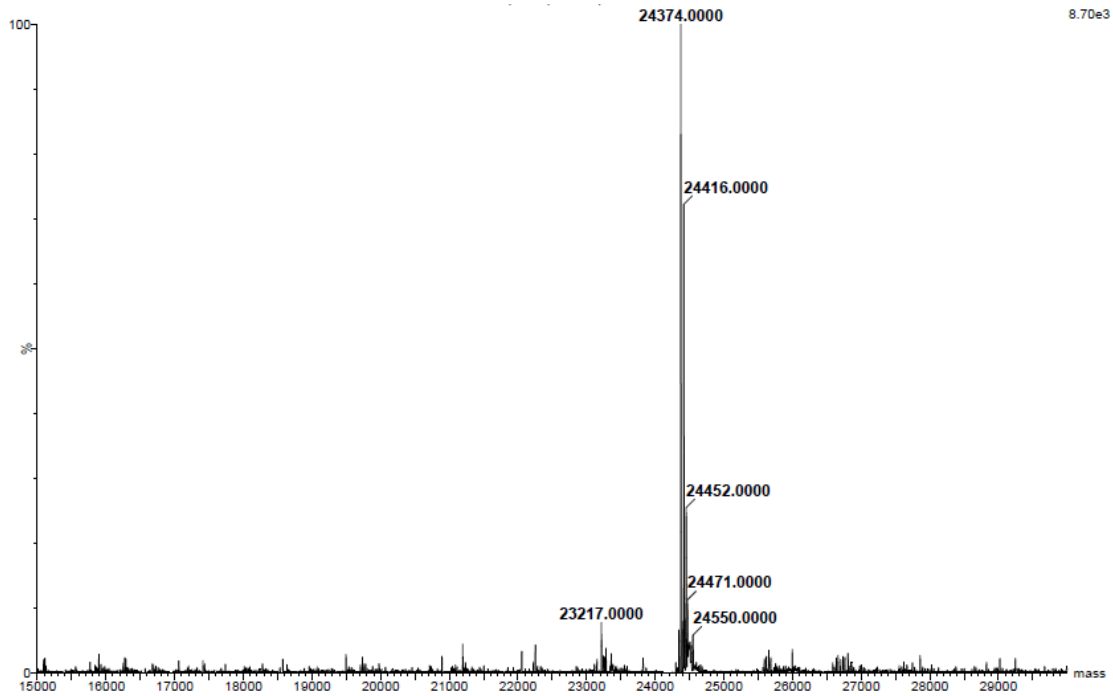
B.3 PYR1 construct map



B.4 Coomassie staining for PYR1 protein



B.5 Mass identification of PYR1 protein



APPENDIX C

RNA-seq analysis

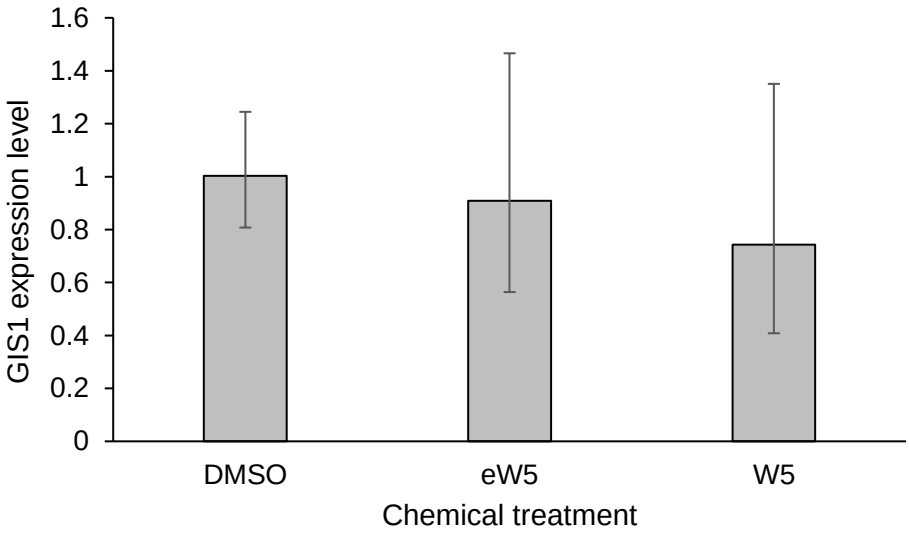
C.1 Raw counts per sample of RNA-seq data (the file can be found on the enclosed CD-ROM)

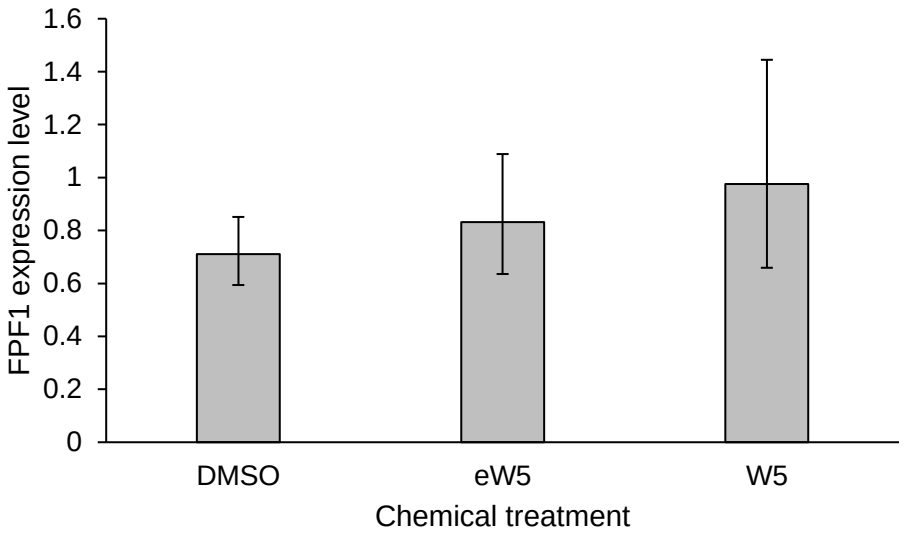
C.2 Gene list for pairwise comparison (eW5 vs DMSO) (the file can be found on the enclosed CD-ROM)

C.3 Gene list for pairwise comparison (W5 vs DMSO) (the file can be found on the enclosed CD-ROM)

C.4 Gene list for pairwise comparison (W5 vs eW5) (the file can be found on the enclosed CD-ROM)

C.5 qRT-PCR from the gene listed on the hormone signalling




1.6
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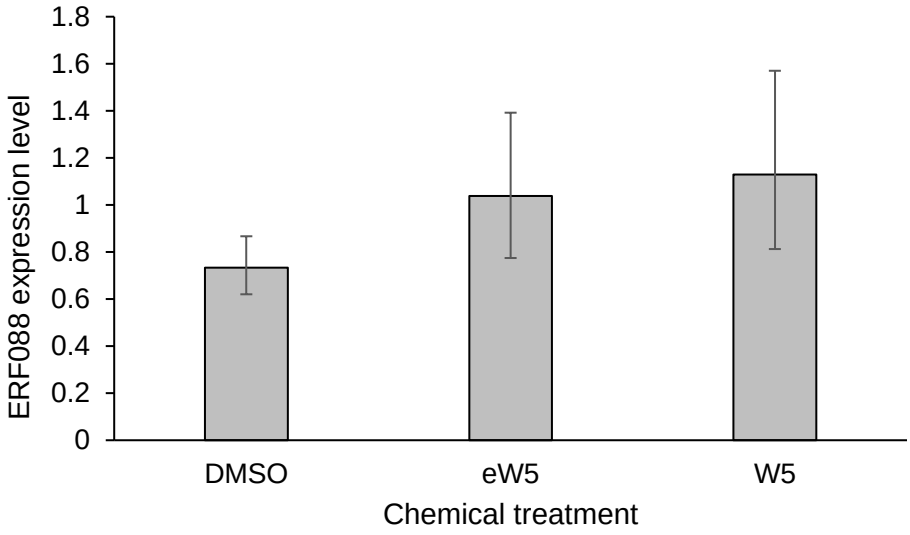
1.2
1
0.8
0.6
0.4
0.2
0
FPF1 expression level
DMSO
eW5
W5
Chemical treatment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1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ERF088 expression level
DMSO
eW5
W5
Chemical treatment