Dissection of the auxin response pathway using functional and chemical genetics approaches

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Abstract

Auxin plays a key role during many, if not all, developmental processes in plants. Notably, the hormone regulates the formation and emergence of lateral roots (LRs). In *Arabidopsis thaliana* the initiation of lateral root primordia (LRP) is induced by auxin and takes place deep within the parental root. Also, the emergence of the LRP through the overlying tissues is regulated by auxin. It has been shown that the gene *LAX3* is expressed in cortical and epidermal directly overlying a LRP. External auxin induces *LAX3*'s expression in all cortical and epidermal cells suggesting that it acts as the activating signal.

There are two objectives in this study: the first one is to understand how the expression of *LAX3* is regulated and the second one is to identify and characterise novel inhibitors of the induction of *LAX3*.

It has been shown that mutations in ARF7 and ARF19 or IAA14/SLR are sufficient to block *LAX3* auxin induction. Using classical genetics approaches, it is shown that ARF7 and ARF19 actually regulate *LAX3* positively and negatively, respectively. Furthermore, a canonical Auxin Response Element present in the promoter of *LAX3* is shown to negatively regulate its expression. Using transcriptomics datasets, a regulatory network is proposed and several putative candidates have been selected.

In order to obtain alternative approaches to dissect the induction of *LAX3*, a suite of 13 inhibitors (representing 8 distinct classes of compounds) have identified. The major and most promising class has been investigated and shown to interfere most probably with the E2 conjugating enzymes. A model and preliminary results with some of the other inhibitors identified are proposed.

Acknowledgments

I first would like to thank my supervisors, Malcolm Bennett and Tom Beeckman, for giving me the opportunity to carry out this PhD. It has been an extraordinary experience that has given me convictions not only in the professional part of my life but in the personal as well. Malcolm's advices while writing up simplified everything that would have been so complicated otherwise. Tom's friendliness and the great science I learned during my stay in Ghent meant that I started my PhD with an already successful project.

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Also, this thesis would not be what it is without the love Sakunkhun gave me during the last two years. We shared a fantastic time together which I hope will continue anywhere in the world.

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List of abbreviations

µM	micro Molar
2,4D	2,4-Dichlorophenoxyacetic Acid
AAAP	Auxin and Amino Acid Permeases
ABA	Abscissic Acid
ACC	1-Aminocyclopropanecarboxylic acid
Ala	Alanine (Aminoacid)
ARE	Auxin Response Element
ARF	Auxin Response Factor
At	*Arabidopsis thaliana*
Aux/IAA	Auxin/Indole-3-Acetic Acid
bp	Base Pair
BR	Brassinosteroids
CHX	Cycloheximide
CK	Citokinins
Col-0	Columbia
ddH_2O	Double distilled water
DMSO	Dimethyl Sulfoxide
DNA	Desoxyribonucleic Acid
EpiBL	Epi Brassinolide
EtOH	Ethanol
FA	Folic Acid
FC	Fold Change
GA	Gibberellins
GFP	Green Fluorescent Protein
Glu	Glutamate (Aminoacid)
GUS	Beta-glucuronidase
IAA	Indole-3-acetic acid
IVM	In Vitro Mutagenesis
JA	Jasmonic Acid
LAX3	Like AUX1 to 3
Leu	Leucine (Aminoacid)
LR	Lateral Root
LRP	Lateral Root Primordium
M	Molar
mARE	mutated Auxin Response Element
Mbp	Mega Base Pair (1,000,000 base pairs)
MeJa	Methyl Jasmonate
mM	milli Molar
mRNA	Messenger RNA
NAA	α-Naphthalene Acetic Acid
NEDD8	Neural-precursor-cell-expressed Developmentally Down-regulated 8

nM	nano Molar
NO	Nitric Oxyde
NPA	Naphthylphthalamic Acid
Nt	*Nicotiana tobacum*
PCR	Polymerase Chain Reaction
Phe	Phenylalanine (Aminoacid)
PIN	Pin-Formed
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT	Reverse Transcription
RUB	Related to Ubiquitin
SUMO	Small Ubiquitin Like Modifier
T1	Transformant 1st Generation
T2	Transformant 2nd Generation
T3	Transformant 3rd Generation
TF	Transcription Factor
Trp	Tryptophane (Aminoacid)
Ub	Ubiquitin
Ubl	Ubiquitin Like
Val	Valine (Aminoacid)
Var	Variant
WT	Wild Type
YFP	Yellow Fluorescent Protein

Chapter 1 Introduction

1.1. Arabidopsis *thaliana* as a model organism

Arabidopsis thaliana is a eudicot which belongs to the Brassicaceae family (Figure 1-1). Despite Arabidopsis having no economic value unlike other closely related crops such as broccoli or mustard, it has been used as the model plant for research on plant genetics and development for over 70 years. In 1946, Whyte called Arabidopsis the "Botanical Drosophila" in reference to the numerous wild type ecotypes identified (Whyte, 1946).

Several advantages make Arabidopsis very well suited for classical genetics studies: it has one of the smallest plant diploid genome (125 Mbp) with a simple organisation (5 chromosomes), it is a self-fertilizing plant which can be easily cross fertilized, the seed set is usually very large (>10 000 seeds/plant) and the life cycle is very short (around 2 months) (Meyerowitz, 1987; Meyerowitz, 2001). Another key advantage of Arabidopsis is the ease to transform its genome to obtain genetically engineered plants.

In the year 2000, the Arabidopsis ecotype Columbia was the first plant to have its genome fully sequenced (The Arabidopsis Genome Initiative, 2000). The last version of TAIR (The Arabidopsis Information Resource, TAIR10, 18 November 2010) concluded that 57% of the 27 416 Arabidopsis genes have a known function. Interestingly, most of Arabidopsis genes fall into multigenic families: there are only 35% of single copy genes compared to 55% in *Caenorhabditis elegans* or 72% in *Drosophila melanogaster*. Since November 2011, the sequenced genome of forty one higher plants and trees is available. Comparative genomics studies showed that many gene sequences are conserved

between highly divergent plants, such as maize (*Zea Mays*) and *Arabidopsis thaliana* (Schnable et al., 2009). In the coming decade, it will be of particular interest to see whether gene functions are also conserved.

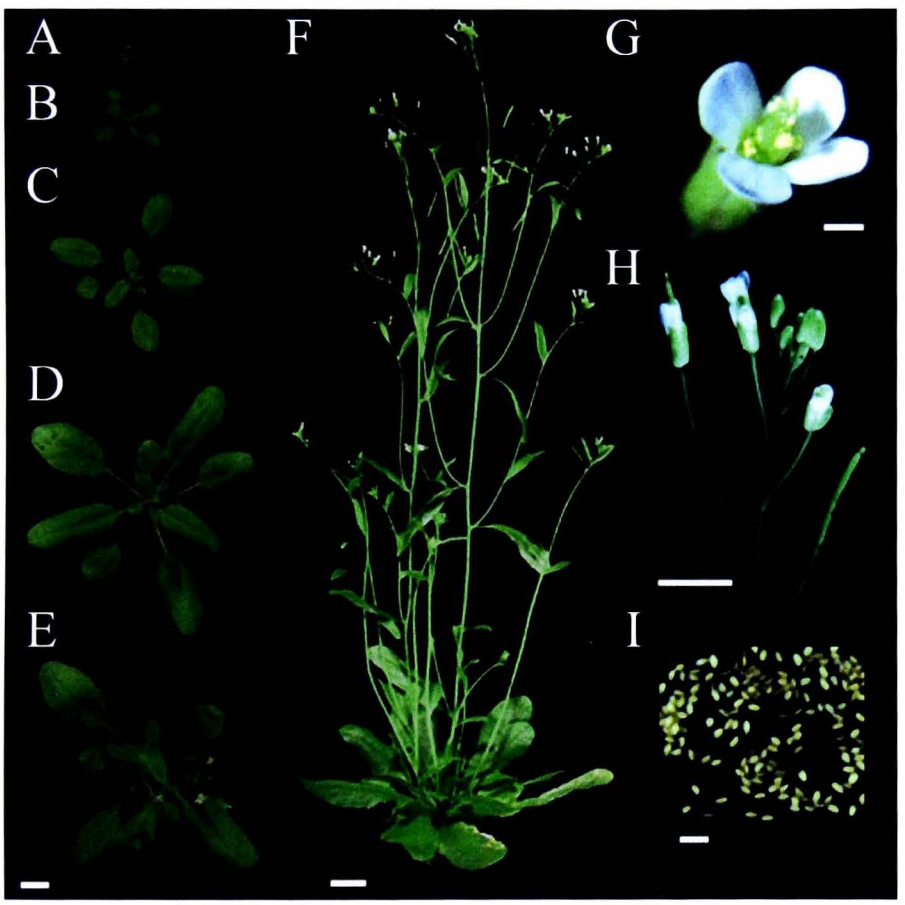

Figure 1-1 ***Arabidopsis thaliana*** **developmental stages. (A-D)** Vegetative development: **(A)** 8, **(B)** 15, **(C)** 21 and **(D)** 27 days after germination. **(E)** Transition to flowering occurs usually four weeks after germination. **(F)** Plants reach maturity usually eight weeks after germination. **(G)** Detailed view of an Arabidopsis flower showing the four floral organs: the sepals (green tissues), the petals (white tissues), the stamens (with yellow anthers at the tip) and the carpel at the centre. **(H)** Flowers are arranged in a spiral around the floral stem. On one stem, different stages of flower and fruit development development can be observed. The youngest are near the apex whereas old flowers and youg fruits are found away from the apex. Bar is 1cm except for the flower and **(I)** seeds where the bar is 1 mm.

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1.2. Plant hormones

Plant growth and development is regulated by signalling molecules such as phytohormones. Major classes of phytohormones include auxins, gibberellins, cytokinins, ethylene, brassinosteroids, abscisic acid and jasmonic acid. Each of these simple organic compounds (Figure 1-2) has specific functions in the regulation of plant growth. Auxins (IAA) strongly influences the development of both aerial and root parts. Gibberellins (GA) regulate germination, as well as shoot and root growth (Ariizumi and Steber, 2007; de Lucas et al., 2008; Feng et al., 2008; Ubeda-Tomas et al., 2008). Cytokinins (CK) regulate, in concert with auxin, cell division and differentiation at the root and shoot apex (Dello Ioio et al., 2008; To and Kieber, 2008). Ethylene regulates flower development, fruit ripening and senescence (Yang et al., 2008; Yang et al., 2010). Brassinosteroids (BR) regulate shoot elongation, male fertility and stress responses (Choe et al., 2002; Clouse et al., 1996; Li et al., 2001). Abscissic acid (ABA) and its derivatives regulate resistance to drought and germination (Leon-Kloosterziel et al., 1996; Ma et al., 2009; Park et al., 2009). Jasmonic Acid (JA) regulates defence against insects and pathogens, wound healing, and pollen fertility (Liechti et al., 2006; Xie et al., 1998).

These different classes of phytohormones transduce their signal using two different mechanisms: either protein degradation via the ubiquitination pathway (auxins, gibberellins, jasmonic acid and ethylene) or via kinases/phosphatases (cytokinins, brassinosteroids and abscissic acid). Using transcriptomic datasets, the effects of several hormones on gene expression revealed that their signalling pathways act on separate sets of genes with few common targets (Sanchez-Rodriguez et al., 2010). Interestingly, many

hormonal biosynthetic and degradation genes are found amongst differentially expressed genes (Figure 1-2). A general consensus is that auxin centralises other hormonal signals and convert it into morphological changes (Jaillais and Chory, 2010; Nemhauser et al., 2006).

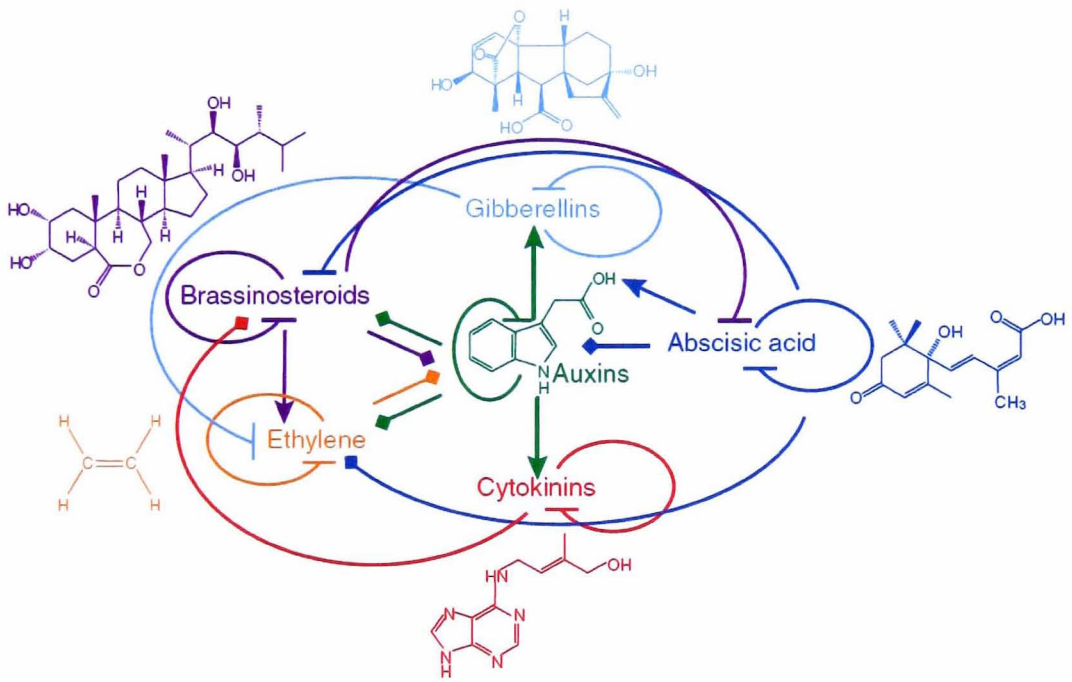

Figure 1-2 Interactions between signalling molecules in Arabidopsis. Lines with arrow head indicate a positive effect on hormone metabolism (up and/or down regulation of genes involved in biosynthesis or degradation respectively). Lines with blocked arrow head indicate a negative effect on hormone metabolism (up and/or down regulation of genes involved in degradation or biosynthesis respectively). Lines with diamond ends indicate an ambiguous effect on hormone metabolism. Reproduced from (Jaillais and Chory, 2010).

1.3. Auxin

The first description of auxin effects on plant growth was made by Charles Darwin in 1880 when studying phototropism (Darwin, 1880). Although unaware that auxin was responsible for his observations, Darwin reported that phototropism requires the coordinated action of two separate zones: one at the shoot apex which perceives light, and another one, located below the apex, where differential cell growth takes place. This observation suggested that a signal was moving from the apex to the cells below. In 1910, the term hormone was used for the first time by Hans Fitting to describe such a signal (Fitting, 1910). In 1928, Fritz Went was able to isolate the signal originally identified by Darwin, calling it auxin (from the Greek "auxein" which means "to grow") (Went, 1928). The chemical structure of Auxin was identified to be indole-3-acetic acid (IAA, Figure 1-2) by Kenneth Thimann's group in 1933 (Kögl et al., 1933). Several chemical variants, which are structurally related to auxin, were shown to have similar effects on plant growth: for example, 2,4 D (2,4-Dichlorophenoxyacetic acid) and NAA (α-Naphthalene acetic acid) are two well-known synthetic auxins.

In plants, auxin is primarily synthesised at the shoot apex and in young leaves but local synthesis occurs at the root apex and during specific developmental programs such as embryogenesis or lateral root formation (Woodward and Bartel, 2005). The hormone is then transported throughout the plant from synthesis sites to target sites (Vanneste and Friml, 2009). The transport of auxin leads to the formation of concentration gradients which are key in organogenesis programs (Benkova et al., 2003). Intracellular auxin homeostasis is regulated by several pathways that conjugate, oxidised or convert IAA (Normanly, 2010).

1.4. Auxin synthesis and metabolism

Five biosynthetic pathways leading to the formation of IAA have been identified in bacteria and plants (Normanly, 2010; Woodward and Bartel, 2005). The indole-3-pyruvic acid (IPA) and the indole-3-acetamide (IAM)) pathways have been initially characterised in bacteria but several lines of evidence suggest they function in plants as well. The triptamine (TRM) and the indole-3-acetaldoxime (IAOx) pathways have only been identified in plants. These four pathways all derive from tryptophan (TRP-dependant) but recent findings showed that there is an alternative route (TRP-independent) that uses indolic tryptophan precursors (see Figure 1-3 for details). As these 5 biosynthetic pathways are used at different levels by diverging plants it appears that there is not a single main pathway but several.

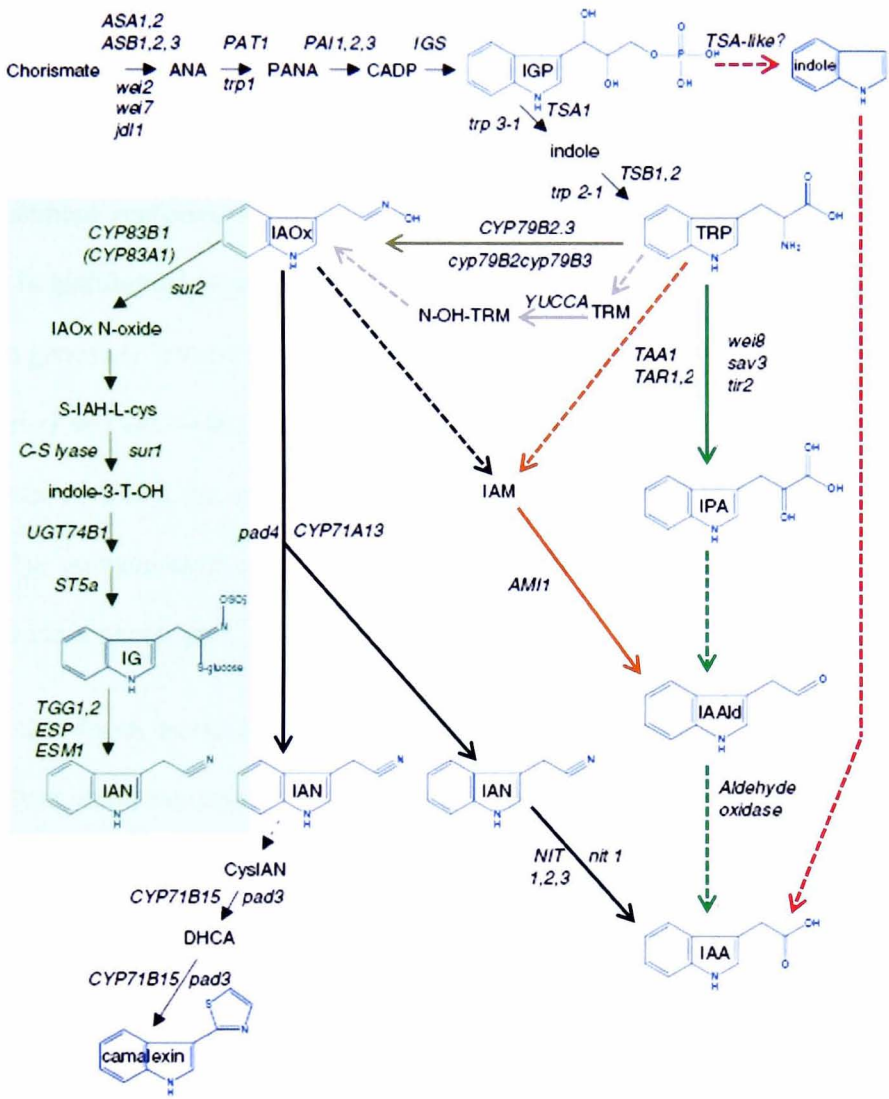

Figure 1-3 Auxin biosynthetic pathways in plants. The tryptophan dependant [IPA (green arrows), IAM (orange arrows), TAM (grey arrows) and IAOx (brown arrows)] and tryptophan independent (red arrows) pathways are shown. The glucosinolate pathway is highlighted in green. After the IAOx and TAM pathways converge, the common steps are shown with black arrows. Dashed arrows indicate that neither a gene nor an enzyme activity has been identified in Arabidopsis. Gene names are indicated in upper case italics and mutant genes are indicated in lower case italics. In the case of the Aldehyde oxidase, an enzyme activity has been proposed for the corresponding conversion, but a definitive gene assignment has not been made. (ANA) anthranilate, (PANA) 5-phosphoribosylanthranilate, (CADP) 1-(o-carboxyphenylamino)-1-deoxyribulose-5-phosphate, (IGP) indole-3-glycerol phosphate, (TRP) tryptophan, (IAM) indole-3-acetamide, (IPA) indole-3-pyruvic acid, (IAAld) indole-3-acetaldehyde, (IAOx) indole-3-acetaldoxime, (S-IAH-L-cys) S-(indolylacetohydroximoyl)-L-cysteine, (indole-3-T-OH) indole-3-thiohydroximate, (IG) indole-3-methylglucosinolate, (TRM) tryptamine, (IAN) indole-3-acetonitrile, (DHCA) dihydrocamalexic acid. Adapted from (Woodward and Bartel, 2005) and (Normanly, 2010).

A general feature of hormonal signalling pathways is a negative feedback on genes involved in biosynthesis and a positive feedback on genes involved in hormone degradation (Figure 1-2, (Jaillais and Chory; Nemhauser et al., 2006)). In Arabidopsis, auxin treatment represses strongly genes involved in the glucosinolate pathway (the pathway is highlighted in green on Figure 1-3). Therefore, it is tempting to speculate that these genes are involved in auxin biosynthesis. Nevertheless, in the loss of function mutants *sur1* and *sur2* ((Boerjan et al., 1995; Delarue et al., 1998)) seedlings accumulate more auxin. Possibly, the enzyme that converts IAOx to IAAld (CYP71A13, (Nafisi et al., 2007)) compensates *sur1* or *sur2* loss of function and results in an auxin overproduction phenotype.

On the other hand, auxin treatment strongly induces genes encoding enzyme (GH3s) that catalyse auxin conjugation with several amino acids (Figure 1-4, Asp, Glu, Phe, Val, Leu, Trp, Ala) (Normanly, 2010; Woodward and Bartel, 2005)Figure 1-4. These modifications have diverse effects: Asp and Glu conjugates appear to be markers for IAA degradation, whereas Phe, Leu and Ala conjugates appear to be used for storage purposes (Bartel and Fink, 1995; Campanella et al., 1996; Davies et al., 1999; LeClere et al., 2002; Ostin et al., 1998). IAA-Trp conjugates are auxin antagonists, rendering seedlings insensitive to IAA and 2,4D when applied exogenously (Staswick, 2009). Auxin is not only conjugated to amino acids: Walz et al. reported that an unidentified enzyme conjugates IAA with a protein in soybean (Walz et al., 2002).

The bio-active auxin (IAA) can also be converted to indole-3-butyric acid (IBA), and vice versa (Bartel et al., 2001; Zolman et al., 2008; Zolman et al., 2007). The role of IBA in Arabidopsis is unclear but exogenous application of IBA triggers auxin

responses (Chhun et al., 2003; Poupart et al., 2005; Rashotte et al., 2003). If it appears that these are partially due to the conversion of IBA to IAA, mutant analyses revealed that IBA regulates several processes, including lateral root formation, independently of IAA. Interestingly, GH3s can use IBA as a substrate as well, some of them having actually a higher affinity for IBA than IAA (Staswick et al., 2005).

IAA and IBA can both be conjugated with glucose which appears to be involved in inactivation (Jackson et al., 2001; Szerszen et al., 1994). In Arabidopsis, IAA is permanently inactivated by ring oxidation (oxIAA). IAA can also be oxidised when conjugated with amino acids or glucose which reinforces its inactivation (Barratt et al., 1999; Ostin et al., 1998). Other modifications include hydroxylation of IAA conjugated with Phe or Val, conjugation of IAA with myo-inositol and IAA methylation. The role of these modifications is mainly for storage purposes whereas methylation has an unknown effect.

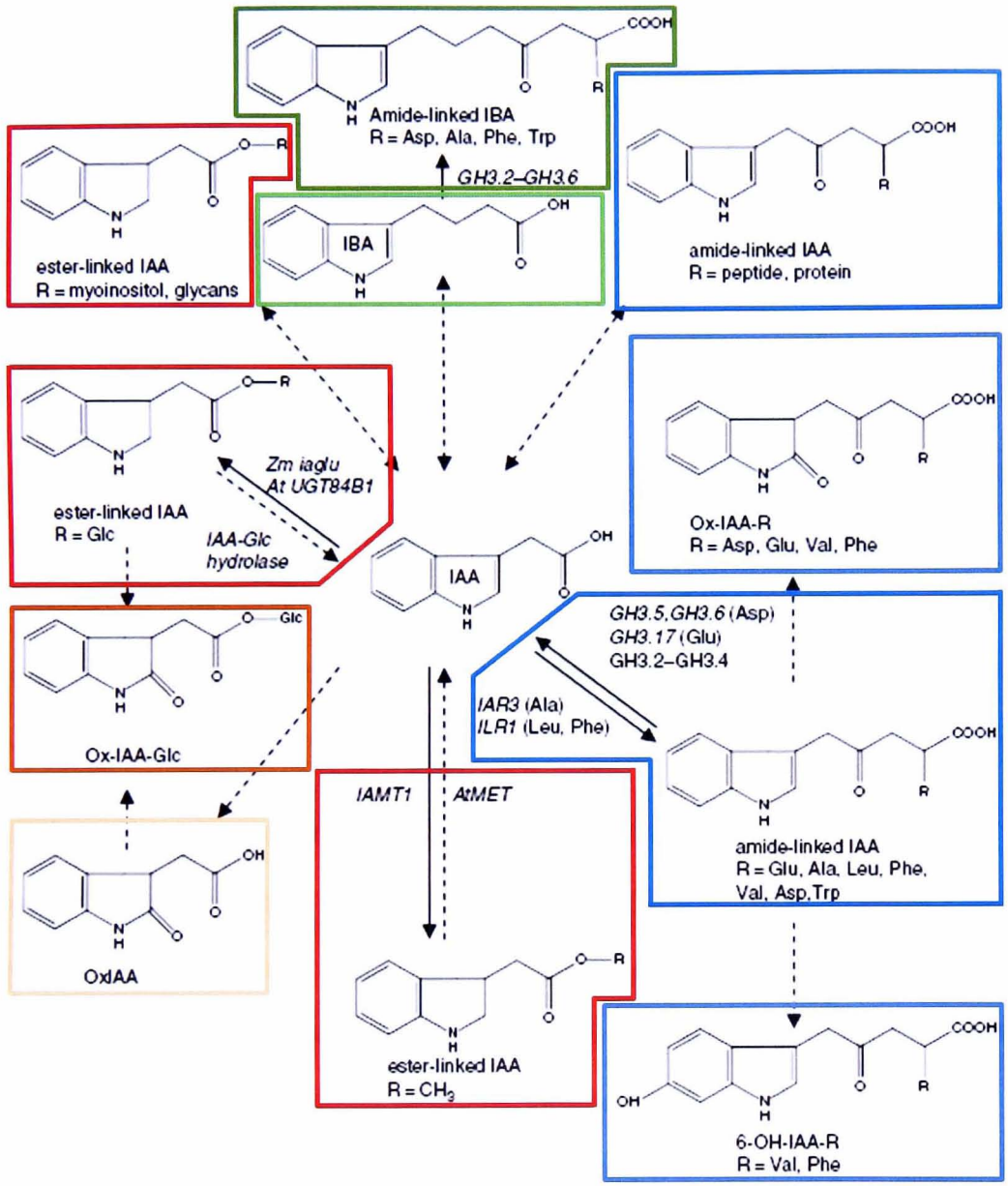

Figure 1-4 Metabolism of IAA. IAA can be converted to IBA and further conjugated via an amide linkage with several amino acids (green boxes). IAA can directly be conjugated with amino acids or proteins via an amide linkage as well (blue boxes). IAA conjugated with amino acid can be further hydroxylated or oxidised. IAA can be methylated or conjugated with sugar or myo inositol via an ester linkage (red boxes). Finally, IAA can be oxidised (orange box). Adapted from (Woodward and Bartel, 2005) and (Normanly, 2010).

1.5. Auxin transport

Two types of auxin transport have been identified in Arabidopsis (Figure 1-5). The first one is mainly gravity mediated and regulates long distance movement of auxin via the phloem vessels from source (young leaves) to sink (root apex) tissues. The second one regulates local auxin redistribution mainly via active influx and efflux carriers and to a less extent via passive diffusion (Robert and Friml, 2009).

Passive diffusion of auxin through the plasma membrane is due to its weak acidity (IAA's pKa is ±4.8) which allows protonated IAA to diffuse freely from the apoplasm (pH 5.5) into the cytoplasm (pH 7). Kramer and Bennett (2006). reported that passive diffusion of IAAH into the cell accounts only for a minor proportion (± 10%) of total auxin influx.

Active uptake is mediated by specific proteins that belong to the super family of Auxin/Amino Acid Permeases (AAAP) symporters (Kerr and Bennett, 2007). There are 4 auxin influx carriers encoded by the Arabidopsis genome termed AUX1 and LAX1 to 3 (Swarup et al., 2008). Mutations in AUX1 results in agravitropic roots with reduced lateral root density (Bennett et al., 1996). Quadruple loss of function mutants are not lethal but are severely affected in many developmental programs such as gravitropism, phototropism, lateral root initiation and emergence, root hair development and phyllotactic patterning (Bainbridge et al., 2008; Bennett et al., 1996; Jones et al., 2008; Stone et al., 2008; Swarup et al., 2008). Hence, active auxin influx is not necessary for plant growth but is required for rapid responses to environmental cues.

Efflux of auxin is regulated by two distinct families of transporters: the ABCB/PGPs and the PINs (Friml, 2010). There are 22 PGPs encoded by the Arabidopsis genome, 3 of which have been shown to be localised at the plasma membrane and to transport auxin: PGP1, PGP4 and PGP19 (Cho et al., 2007; Geisler et al., 2005; Petrasek et al., 2006). On the other hand, there are 8 PIN proteins (PIN1 to 8) encoded by the Arabidopsis genome, 5 of which (PIN1-4 and PIN7) have been shown to be localised at the plasma membrane and to actively transport auxin out of the cell (Petrasek et al., 2006; Yang and Murphy, 2009). PINs are preferentially localised on one side of the cell (polar localisation) whereas PGPs are ubiquitously localised on the plasma membrane (apolar localisation). Therefore polar auxin transport (PAT) is predominantly directed by PIN localisation. The mechanisms underlying PIN localisation, recycling and activity have been extensively studied and are summarised in several recent reviews (Friml, 2010; Grunewald and Friml, 2010; Petrasek et al., 2006; Robert and Friml, 2009). Critically, PAT leads to the formation of auxin gradient that are important to regulate, amongst many processes, lateral root formation and leaf development.

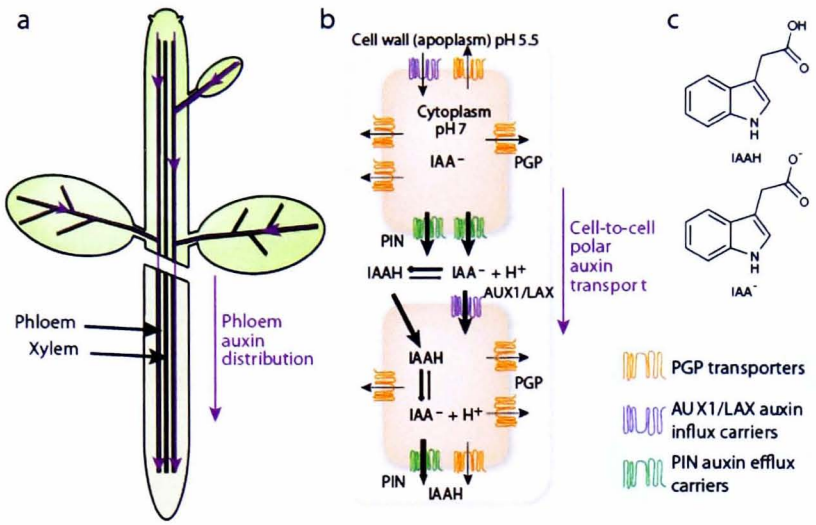

Figure 1-5 Auxin transport in Arabidopsis. (a) Long distance transport of auxin starts from the young and developing leaves down to the apex of the root. **(b)** Cell to cell transport of auxin involves different classes of influx and efflux transporters as well as passive influx. **(c)** IAAH can freely diffuse through the plasma membrane but not IAA-. Adapted from (Robert and Friml, 2009).

1.6. Auxin response

During the last decade, significant advances have been made to elucidate the molecular basis of auxin responses. It has been shown that the promoter of several classes of auxin inducible genes, such as SAURs (Small Auxin Up RNAs), GH3s and Aux/IAAs, contain repeats of a 6 nucleotide motif: GAGACA. The overall consensus sequence is GACA since there is more variability in the first and second nucleotide. Eight repeats of the 6 nucleotide motif confer auxin inducibility to a downstream reporter gene (DR5 synthetic promoter) (Abel et al., 1995; Abel et al., 1994; Abel and Theologis, 1996). The sequence GAGACA is called an ARE for Auxin Response Element (Abel et al., 1996; Ulmasov et al., 1995). The DNA binding domain of a transcription factor, ARF1 (Auxin Response Factor 1), was found to directly interact with the ARE (Ulmasov et al.,

1997a). Subsequently, 22 genes encoding ARFs were identified in the Arabidopsis genome (ARF1 to 22). Apart from few exceptions (ARF3, 13 and 17), all the ARFs share 4 conserved domains: a VP1/ABI3 like DNA binding domain at the N terminus, a middle region (MR) and two domains, called domain III and IV, at the C terminus (Hagen and Guilfoyle, 2002; Okushima et al., 2005; Tiwari et al., 2003; Ulmasov et al., 1999a) (Figure 1-6). The MR domain determines whether the ARF activates (Q-rich, ARF5-8, ARF19) or represses (S-rich, ARF1-4, ARF9-18, ARF20-22) transcription. This classification is subject to controversy since some genes are repressed or induced in *arf7* and *arf19* mutants for example, which are supposed to be two activating ARFs. Critically, ARF proteins do not regulate genes on their own but interact with themselves and with Aux/IAA proteins, a family of transcriptional repressors. These interactions are taking place through domains III and IV. Importantly, it is believed that the interaction between ARFs (whether both are activating, repressing or both) allow to fine tune gene expression depending on the genomic context of a gene which may explain some of the observations made in ARF loss of function mutants (Kim et al., 1997; Ouellet et al., 2001; Ulmasov et al., 1997b).

There are 29 Aux/IAAs encoded by the Arabidopsis genome (IAA1 to 20 and IAA26 to 34). Aux/IAAs are short living nuclear proteins that share two conserved domains (domains III and IV) with ARF (Ulmasov et al., 1999b). Besides, two additional domain are located at the N terminus of Aux/IAA proteins and are called domain I and II which repress transcription and regulate protein stability respectively (Ouellet et al., 2001; Tiwari et al., 2004; Worley et al., 2000) (Figure 1-6).

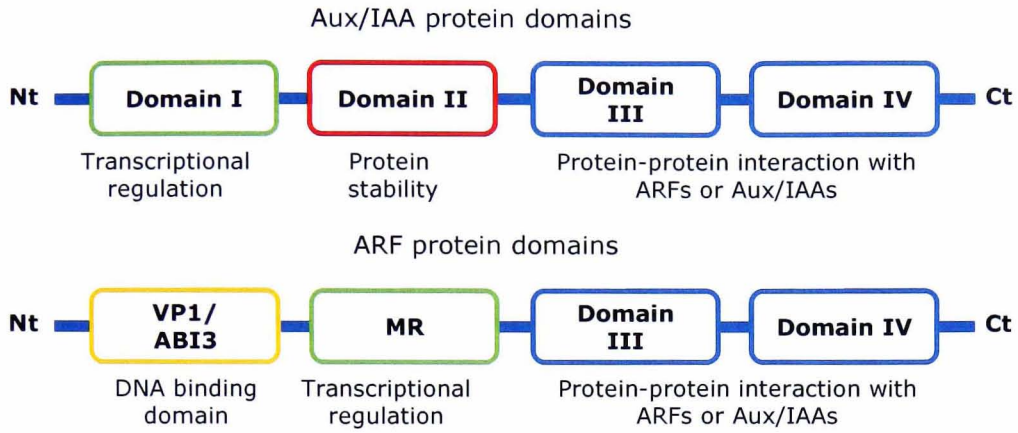

Figure 1-6 Structure of ARFs and Aux/IAA proteins. Aux/IAAs are small proteins with an average molecular weight of 24kDa (minimum: IAA31, 18kDa; maximum: IAA9, 36kDa). ARFs are much bigger proteins with an averaged molecular weight of 77kDa (minimum: ARF13, 57kDa; maximum: ARF7, 128kDa). The schematics are not at the correct scale.

Several mutants with severe auxin phenotypes were shown to bear mutations within the domain II of several Aux/IAAs: *axr5/iaa1* (Yang et al., 2004), *shy2/iaa3* (Tian and Reed, 1999), *shy1/iaa6* (Kim et al., 1996), *axr2-1/iaa7-1* (Nagpal et al., 2000), *bdl/iaa12-1* (Hamann et al., 2002), *slr-1/iaa14-1* (Fukaki et al., 2002), *axr3-1/iaa17-1* (Rouse et al., 1998), *iaa18-1* (Uehara et al., 2008), *msg2-1/iaa19-1* (Tatematsu et al., 2004) and *iaa28-1* (Rogg et al., 2001). Time course experiments revealed that these mutations extend Aux/IAAs half-lives (Gray et al., 2001; Worley et al., 2000; Zenser et al., 2001). Hence, this domain is usually referred to as a "degron". Several studies suggest that auxin regulates stability of Aux/IAA protein. Auxin treatment promotes rapid degradation of luciferase and GUS reporters fused to an Aux/IAA domain II whereas stabilising mutations reduce or block auxin effects (Dreher et al., 2006; Gray et al., 1999; Gray and Estelle, 2000; Gray et al., 2001). MG132 (a proteasome inhibitor) treatments result in an increased stability of Aux/IAA protein and a decrease in auxin

responsiveness suggesting that auxin mediates Aux/IAA degradation via the ubiquitination/proteasome pathway (Ramos et al., 2001).

Interestingly, some Aux/IAAs have a modified (IAA31) or even no domain II at all (IAA20, IAA30, IAA32 and IAA34). It has been shown that IAA31 remains slightly sensitive to auxin levels whereas IAA20, IAA30, IAA32 and IAA34 are insensitive (Dreher et al., 2006). Despite this intriguing property, no functions have been assigned to these particular Aux/IAAs.

1.7. Ubiquitination in plants: Importance of SCF complex and F-box E3s

The ubiquitination/proteasome pathway has been studied and described in great details since the identification of ubiquitin as a marker for protein degradation (Ciechanover et al., 1982; Ciechanover et al., 1984; Hershko and Ciechanover, 1986). The ubiquitin is a small peptide of 76 amino acids which is conserved in all eukaryotes and is expressed ubiquitously (Schlesinger and Goldstein, 1975; Schlesinger et al., 1975). Addition of ubiquitin to a substrate requires the concerted activity of three enzymes (Ciechanover et al., 2000; Hartmann-Petersen et al., 2003; Wilkinson, 2000). The first enzyme is an ubiquitin activating enzyme, or E1, that activates ubiquitin moieties using ATP and covalently binds it on a conserved cysteine residue (Schulman and Harper, 2009). The activated ubiquitin is then transferred to an ubiquitin conjugating enzyme, or E2, again on a conserved cysteine residue via a trans(thio)esterification reaction (Fang and Weissman, 2004). The last step consists of the transfer of the ubiquitin moiety to a target protein. The target specificity is given by an ubiquitin ligase enzyme or E3. Different

types of E3s have been identified: some of them covalently bind ubiquitin on a conserved cysteine before transferring it to the substrate (e.g. the HECT (Homologous to E6AP Carboxyl Terminus) domain like E3s) (Rotin and Kumar, 2009), whereas others bring the substrate and the E2 together but never bind the ubiquitin such as F-box and RING finger E3s) (Deshaies and Joazeiro, 2009; Stone et al., 2005) (Figure 1-7).

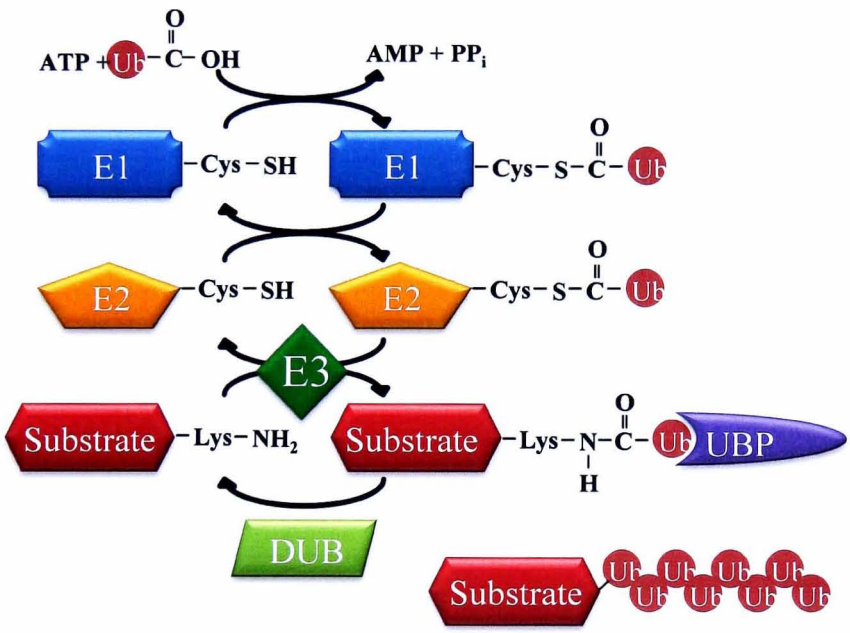

Figure 1-7 The ubiquitination proteasome pathway. 3 enzymes (E1, E2 and E3) act in concert to catalyse the addition of single or multiple ubiquitin moieties onto targeted substrate. The E1 and E2 activates and conjugates the ubiquitin whereas the E3 brings the substrate for ubiquitination. Poly ubiquitin chains are a signal for protein degradation whereas single ubiquitin is usually a signal for sub cellular localisation. DUB, Deubiquitinating enzyme, UBP, Ubiquitin Binding Protein.

In plants, the ubiquitination machinery is implicated in many if not all developmental processes including light sensing (Henriques et al., 2009), auxin signalling (Vanneste and Friml, 2009), jasmonate signalling (Gfeller et al., 2010), abiotic stress signalling (Lee and Kim, 2011), low temperature sensing (Zhou et al., 2011), and many others. Genes encoding ubiquitination machinery proteins account for 5% of all Arabidopsis genes: 2 genes encode E1s, 37 encode E2s and more than 1000 encode E3s (Bachmair et

al., 2001). The two E1s are redundant as single loss of function have none or very mild phenotypes and double mutants are embryo lethal (Goritschnig et al., 2007). Based on their amino acid content the 37 E2s can be classified into three (and more) categories (Bachmair et al., 2001; Kraft et al., 2005). Despite showing important differences, especially at the N and C terminus, few E2s have been assigned to a specific function. Only two of them, UBC1 and UBC2, were shown to have a role during the switch from vegetative to flowering phase (Xu et al., 2009). This observation suggests that E2s, like E1s, share a high degree of redundancy. On the other hand, the E3 superfamily is the largest and most complex family of proteins in Arabidopsis. Of all the sub families of E3s identified, the F-box is by far the most represented with about 700 members (Gagne et al., 2002; Lechner et al., 2006; Somers and Fujiwara, 2009).

From studies in fungi and mammals, F-box proteins act in a multimeric complex called the SCF complex, which comprises four core sub-units: a Skp1, a Cullin, a RING-like protein and an F box protein (Bai et al., 1996; Deshaies, 1999; Ho et al., 2006; Patton et al., 1998; Tyers and Jorgensen, 2000; Willems et al., 2004; Zhang et al., 1995). Genes encoding the various subunits are also known in Arabidopsis (Gray et al., 1999): there are 21 Skp1 (named ASK for *Arabidopsis* Skp1), at least 5 cullins (but only two with demonstrated activity, CUL1 and CUL2a), two RING-like protein (RBX1 and RBX1b) and almost 700 F-box proteins (del Pozo et al., 2002a; del Pozo et al., 2002b; Risseeuw et al., 2003; Shen et al., 2002; Wang et al., 2003; Xu et al., 2002).

1.8. SCF^{TIR1} regulates auxin response

Mutations in components of the SCF complex, such as ASK1 (*ask1-1*), CUL1 (*cul1-1/axr6-1*) and RBX1 (*axr1-12*), affect auxin responses in a similar way as loss of function mutations in TIR1, an E3 F-BOX protein (*tir1-1*) (Hellmann et al., 2003; Leyser et al., 1993; Quint et al., 2005; Ruegger et al., 1998; Zhao et al., 1999). Furthermore, these 4 proteins can be co-immuno-precipitated in a large complex reinforcing the idea that they form an active SCF complex, named the SCF^{TIR1} complex (Gray et al., 2001).

The active SCF complex is assembled around ASK1 which interacts with TIR1 at its C terminal and CUL1 at its N terminal (Gray et al., 1999). The role of CUL1 has been difficult to identify as loss of function alleles lead to embryo arrest (del Pozo and Estelle, 1999). The identification of a weak CUL1 allele (*cul1-6*) and a conditional CUL1 mutant (*axr6-2/eta1*) showed that the protein plays a critical role to regulate SCF activity (Moon et al., 2007; Quint et al., 2005). CUL1 is modified by a peptide, Rub (for Related to Ubiquitin), via an enzymatic cascade similar to ubiquitin. An E1, formed of two subunits (AXR1 and ECR1), activates the small Rub peptide and transfer it to an E2, RCE1 (Rub Conjugating Enzyme) (del Pozo et al., 2002b; Dharmasiri et al., 2003). The Rub moiety is then added to CUL1 via a Rub E3 ligase, RBX1 (Gray et al., 2002). Removal of the Rub moiety is catalysed by another complex, formed of several sub units and called the COP9 Signalosome (CSN). Mutations in any of these proteins lead to auxin resistant phenotypes, generally similar to *tir1-1* (Gray et al., 2002). Thus it seems that the Rub modification of CUL1 is important to regulate SCF activity. Indeed,

overexpression of RBX1 lead to an increase in the rub modified CUL1/unmodified CUL1 ratio which in turns tend to increase auxin sensitivity.

In 2005, the link between the SCF^{TIR1} complex and Aux/IAA stability was made (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Pull down experiments showed that TIR1 interact with Aux/IAA proteins via their domain II and, critically, that this interaction requires auxin. Gain of function mutations in domain II blocks this interaction which leads to Aux/IAA stabilisation. More recently, the resolution of the crystal structure of a domain II peptide in a complex with TIR1 and auxin showed that the hormone acts as molecular glue between TIR1 and the Aux/IAAs (Tan et al., 2007) and that it requires another cofactor, namely IP6 (inositol hexakisphosphate). Finally, it is only recently that poly-ubiquitinated Aux/IAAs were observed in protoplasts (dos Santos Maraschin et al., 2009) confirming that these proteins are degraded via the ubiquitination/proteasome pathway.

A model that integrates these observations has been proposed to explain how auxin regulates gene expressions. At low auxin concentration Aux/IAA proteins are stabilised and interact with ARF transcription factors with their domains III and IV and with another family of proteins termed TOPLESS via an EAR domain (Szemenyei et al., 2008). ARFs that bind the promoter of auxin regulated genes are thus kept in an inactive state. An increase in auxin cellular content is perceived by the auxin receptors TIR1/AFB1-3, which in turn interact with the domain II of Aux/IAAs proteins and flag them up for degradation by the 26S proteasome. The ARFs now become active and lead to changes in gene expression (Figure 1-8).

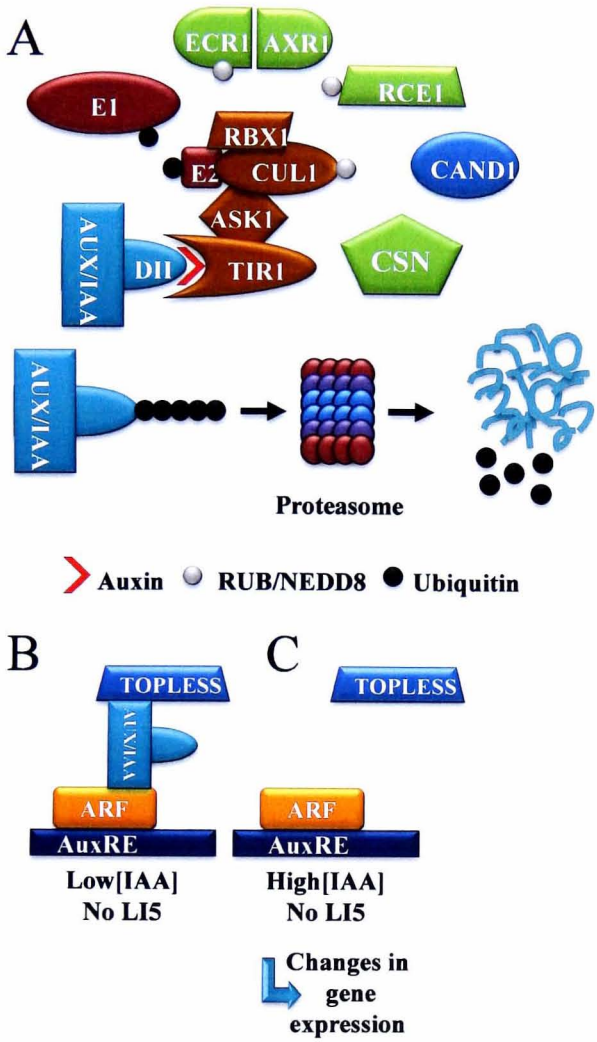

Figure 1-8 Model of the auxin response machinery. (A) Auxin acts as a molecular glue between Aux/IAAs repressors and the SCFTIR1 complex. The subsequent ubiquitination of Aux/IAAs leads to their degradation by the proteasome. The activity of the SCF complex is regulated by cycles of Neddylation (NEDD8/RUB). The Neddylation pathway is very similar to the ubiquitination pathway shown on Figure 1-7. An E1 (composed of two proteins, ECR1 and AXR1), an E2 (RCE1) and one E3 (RBX1) catalyse the addition of NEDD8 moieties on the CUL subunit. Removal of NEDD8 is catalysed by the CSN complex. Cycles of addition and removal of NEDD8 either activates or repress the complex respectively. One protein, CAND1, was shown to interact with the CUL subunit preferentially when the latter is not neddylated. **(B)** If auxin concentration is low, Aux/IAAs are stabilised and together with TOPLESS take over ARF function in regulating gene expression. **(C)** If auxin concentration is high, Aux/IAAs turnover is increased and ARFs are free to regulate the expression of downstream genes. Adapted from (Weijers and Jurgens, 2004).

1.9. Lateral root development

Auxin and its derivatives are involved in root growth and root branching, flower and fruit development, phototropism and gravitropism, as well as many other processes (Davies, 1995; Quint et al., 2005; Tanaka et al., 2006; Teale et al., 2006; Woodward and Bartel, 2005). At the cellular level auxin regulates cell division, elongation and differentiation as well as cell polarity. In the framework of my thesis, I focussed my research on the role of auxin during the formation of lateral roots (LRs).

Arabidopsis is very well suited for studies on root developments because: the Arabidopsis root has a simple organisation, made up of three layers of external tissues (epidermis, cortex and endodermis) and one layer of internal tissue (pericycle) surrounding the vascular bundle. Furthermore, the root is transparent making microscopic analysis convenient.

LR formation is a continuous process which is divided into eight stages (Malamy and Benfey, 1997) . The first stage is the initiation of a new lateral root primordium (LRP). This event takes place in the pericycle cells that are opposite to the xylem poles and is regulated by auxin (De Smet et al., 2007; De Smet et al., 2006). The hormone triggers an asymmetric cell division of two pericycle cells which give rise to a primordium composed of a pair of small and a pair of large daughter cells (De Smet et al., 2008) (Figure 1-9). The two smaller cells then form a two-layer primordium after a round of periclinal cell division (stage II). Subsequently, coordinated cell divisions lead to the formation of a dome-shaped primordium (stage VI) (Dubrovsky et al., 2001). Genes encoding auxin efflux carriers, such as PIN1, PIN2, PIN3, PIN4 and PIN7, regulate the

formation of an auxin gradient with its maximum at the apex of the primordium (Figure 1-9) (Benkova et al., 2003; Blilou et al., 2005). This gradient is required for the correct patterning of the primordium as in some PIN multiple mutants combinations primordium morphology is severely affected (Benkova et al., 2003).

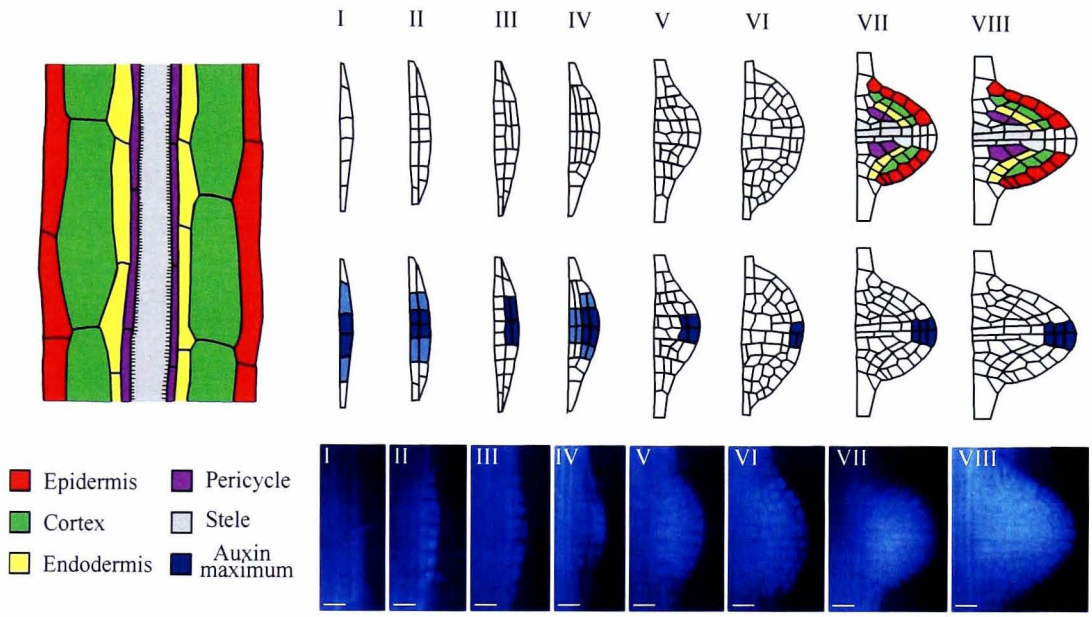

Figure 1-9 The 8 stages of LRP development. (A) Arabidopsis root structure with the pericycle (purple), layer where LRs initiates. **(B)** Primordium morphology at each stage and **(C)** location of auxin maximum (adapted from (Benkova et al., 2003)). **(D)** Picture of a LRP stained with Aniline blue (courtesy of I. Casimiro). Reproduced from (Peret et al., 2009).

In the coming chapter, the role of auxin during primordium emergence will be dissected using genetics and pharmacological approaches. Emergence is the process by which a developing LRP breaks through the three overlying layers of tissues, which are in Arabidopsis the endodermis, the cortex and the epidermis. A key gene during this process is *LAX3* (Swarup et al., 2008).

1.10. Aims and objectives

My project has three main objectives:

(1) The identification of key transcription factors involved in the *LAX3* regulatory network. The aims are to:

- Determine the precise dynamics of *LAX3* auxin induction.
- Determine which transcription factor(s) control *LAX3* auxin inducibility.
- Identify which cis active element present in the promoter of *LAX3* are involved in its regulation.

(2) The characterisation of inhibitors of *LAX3* auxin induction identified using a chemical genetics approach. The aims are to

- Characterised the effects of the positive hits identified on lateral root development.
- Select and study further the effects of a promising family of inhibitors on plant development.
- Study the relationship between structure and activity of the selected inhibitors.

(3) The identification of the target(s) of the selected family of inhibitors. The aims are to:

- Dissect the effects of this family of inhibitors on the response to auxin using transcript profiling.
- Determine the effects of the inhibitors on components of the auxin response machinery
- Generate a model of the inhibitor mode of action.

1.11. References

Abel, S., Ballas, N., Wong, L. M. and Theologis, A. (1996). DNA elements responsive to auxin. *Bioessays* **18**, 647-54.

Abel, S., Nguyen, M. D. and Theologis, A. (1995). The PS-IAA4/5-like family of early auxin-inducible mRNAs in Arabidopsis thaliana. *J Mol Biol* **251**, 533-49.

Abel, S., Oeller, P. W. and Theologis, A. (1994). Early auxin-induced genes encode short-lived nuclear proteins. *Proc Natl Acad Sci U S A* **91**, 326-30.

Abel, S. and Theologis, A. (1996). Early genes and auxin action. *Plant Physiol* **111**, 9-17.

Ariizumi, T. and Steber, C. M. (2007). Seed germination of GA-insensitive sleepy1 mutants does not require RGL2 protein disappearance in Arabidopsis. *Plant Cell* **19**, 791-804.

Bachmair, A., Novatchkova, M., Potuschak, T. and Eisenhaber, F. (2001). Ubiquitylation in plants: a post-genomic look at a post-translational modification. *Trends Plant Sci* **6**, 463-70.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W. and Elledge, S. J. (1996). SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell* **86**, 263-74.

Bainbridge, K., Guyomarc'h, S., Bayer, E., Swarup, R., Bennett, M., Mandel, T. and Kuhlemeier, C. (2008). Auxin influx carriers stabilize phyllotactic patterning. *Genes & development* **22**, 810.

Barratt, N. M., Dong, W., Gage, D. A., Magnus, V. and Town, C. D. (1999). Metabolism of exogenous auxin by Arabidopsis thaliana: Identification of the conjugate N (indol 3 ylacetyl) glutamine and initiation of a mutant screen. *Physiologia Plantarum* **105**, 207-217.

Bartel, B. and Fink, G. R. (1995). ILR1, an amidohydrolase that releases active indole-3-acetic acid from conjugates. *Science* **268**, 1745.

Bartel, B., LeClere, S., Magidin, M. and Zolman, B. K. (2001). Inputs to the active indole-3-acetic acid pool: De novo synthesis, conjugate hydrolysis, and indole-3-butyric acid -oxidation: Recent advances in auxin biology. *Journal of Plant Growth Regulation* **20**, 198-216.

Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G. and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell* **115**, 591-602.

Bennett, M. J., Marchant, A., Green, H. G., May, S. T., Ward, S. P., Millner, P. A., Walker, A. R., Schulz, B. and Feldmann, K. A. (1996). Arabidopsis AUX1 gene: a permease-like regulator of root gravitropism. *Science* **273**, 948.

Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K. and Scheres, B. (2005). The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. *Nature* **433**, 39-44.

Boerjan, W., Cervera, M. T., Delarue, M., Beeckman, T., Dewitte, W., Bellini, C., Caboche, M., Onckelen, H. V., Montagu, M. V. and Inze, D. (1995). Superroot, a recessive mutation in Arabidopsis, confers auxin overproduction. *The Plant Cell Online* **7**, 1405.

Campanella, J. J., Ludwig-Mueller, J. and Town, C. D. (1996). Isolation and characterization of mutants of Arabidopsis thaliana with increased resistance to growth inhibition by indoleacetic acid-amino acid conjugates. *Plant physiology* **112**, 735.

Chhun, T., Taketa, S., Tsurumi, S. and Ichii, M. (2003). The effects of auxin on lateral root initiation and root gravitropism in a lateral rootless mutant Lrt1 of rice (Oryza sativa L.). *Plant Growth Regulation* **39**, 161-170.

Cho, M., Lee, S. H. and Cho, H. T. (2007). P-glycoprotein4 displays auxin efflux transporter-like action in Arabidopsis root hair cells and tobacco cells. *The Plant Cell Online* **19**, 3930.

Choe, S., Schmitz, R. J., Fujioka, S., Takatsuto, S., Lee, M. O., Yoshida, S., Feldmann, K. A. and Tax, F. E. (2002). Arabidopsis brassinosteroid-insensitive dwarf12 mutants are semidominant and defective in a glycogen synthase kinase 3beta-like kinase. *Plant Physiol* **130**, 1506-15.

Ciechanover, A., Elias, S., Heller, H. and Hershko, A. (1982). "Covalent affinity" purification of ubiquitin-activating enzyme. *J Biol Chem* **257**, 2537-42.

Ciechanover, A., Finley, D. and Varshavsky, A. (1984). The ubiquitin-mediated proteolytic pathway and mechanisms of energy-dependent intracellular protein degradation. *J Cell Biochem* **24**, 27-53.

Ciechanover, A., Orian, A. and Schwartz, A. L. (2000). The ubiquitin-mediated proteolytic pathway: mode of action and clinical implications. *J Cell Biochem Suppl* **34**, 40-51.

Clouse, S. D., Langford, M. and McMorris, T. C. (1996). A brassinosteroid-insensitive mutant in Arabidopsis thaliana exhibits multiple defects in growth and development. *Plant Physiol* **111**, 671-8.

Darwin, C. (1880). The Power of Movement in Plants. *London. Murray.*

Davies, P. J. (1995). The plant hormones: Their nature, occurence and functions. The Netherlands.

Davies, R. T., Goetz, D. H., Lasswell, J., Anderson, M. N. and Bartel, B. (1999). IAR3 encodes an auxin conjugate hydrolase from Arabidopsis. *The Plant Cell Online* **11**, 365.

de Lucas, M., Daviere, J. M., Rodriguez-Falcon, M., Pontin, M., Iglesias-Pedraz, J. M., Lorrain, S., Fankhauser, C., Blazquez, M. A., Titarenko, E. and Prat, S. (2008). A molecular framework for light and gibberellin control of cell elongation. *Nature* **451**, 480-4.

De Smet, I., Tetsumura, T., De Rybel, B., Frey, N. F., Laplaze, L., Casimiro, I., Swarup, R., Naudts, M., Vanneste, S., Audenaert, D. et al. (2007). Auxin-dependent regulation of lateral root positioning in the basal meristem of Arabidopsis. *Development* **134**, 681-90.

De Smet, I., Vanneste, S., Inze, D. and Beeckman, T. (2006). Lateral root initiation or the birth of a new meristem. *Plant Mol Biol* **60**, 871-87.

De Smet, I., Vassileva, V., De Rybel, B., Levesque, M. P., Grunewald, W., Van Damme, D., Van Noorden, G., Naudts, M., Van Isterdael, G., De Clercq, R. et al. (2008). Receptor-like kinase ACR4 restricts formative cell divisions in the Arabidopsis root. *Science* **322**, 594-7.

del Pozo, J. C., Boniotti, M. B. and Gutierrez, C. (2002a). Arabidopsis E2Fc functions in cell division and is degraded by the ubiquitin-SCF(AtSKP2) pathway in response to light. *Plant Cell* **14**, 3057-71.

del Pozo, J. C., Dharmasiri, S., Hellmann, H., Walker, L., Gray, W. M. and Estelle, M. (2002b). AXR1-ECR1-dependent conjugation of RUB1 to the Arabidopsis Cullin AtCUL1 is required for auxin response. *Plant Cell* **14**, 421-33.

del Pozo, J. C. and Estelle, M. (1999). The Arabidopsis cullin AtCUL1 is modified by the ubiquitin-related protein RUB1. *Proc Natl Acad Sci U S A* **96**, 15342-7.

Delarue, M., Prinsen, E. and Va, H. (1998). Sur2 mutations of Arabidopsis thaliana define a new locus involved in the control of auxin homeostasis. *The Plant Journal* **14**, 603-611.

Dello Ioio, R., Nakamura, K., Moubayidin, L., Perilli, S., Taniguchi, M., Morita, M. T., Aoyama, T., Costantino, P. and Sabatini, S. (2008). A genetic framework for the control of cell division and differentiation in the root meristem. *Science* **322**, 1380-4.

Deshaies, R. J. (1999). SCF and Cullin/Ring H2-based ubiquitin ligases. *Annu Rev Cell Dev Biol* **15**, 435-67.

Deshaies, R. J. and Joazeiro, C. A. (2009). RING domain E3 ubiquitin ligases. *Annu Rev Biochem* **78**, 399-434.

Dharmasiri, N., Dharmasiri, S. and Estelle, M. (2005). The F-box protein TIR1 is an auxin receptor. *Nature* **435**, 441-445.

Dharmasiri, S., Dharmasiri, N., Hellmann, H. and Estelle, M. (2003). The RUB/Nedd8 conjugation pathway is required for early development in Arabidopsis. *The EMBO Journal* **22**, 1762-1770.

dos Santos Maraschin, S., Memelink, J. and Offringa, R. (2009). Auxin-induced, SCF(TIR1)-mediated poly-ubiquitination marks AUX/IAA proteins for degradation. *Plant J* **59**, 100-9.

Dreher, K. A., Brown, J., Saw, R. E. and Callis, J. (2006). The Arabidopsis Aux/IAA protein family has diversified in degradation and auxin responsiveness. *Plant Cell* **18**, 699-714.

Dubrovsky, J. G., Rost, T. L., Colon-Carmona, A. and Doerner, P. (2001). Early primordium morphogenesis during lateral root initiation in Arabidopsis thaliana. *Planta* **214**, 30-6.

Fang, S. and Weissman, A. M. (2004). A field guide to ubiquitylation. *Cell Mol Life Sci* **61**, 1546-61.

Feng, S., Martinez, C., Gusmaroli, G., Wang, Y., Zhou, J., Wang, F., Chen, L., Yu, L., Iglesias-Pedraz, J. M., Kircher, S. et al. (2008). Coordinated regulation of Arabidopsis thaliana development by light and gibberellins. *Nature* **451**, 475-9.

Fitting, H. (1910). Dei Leitung tropistischer Reize in parallelotropen Pflanzenteilen. *Jahrb. wiss. Bot.* **2**, 225-267.

Friml, J. (2010). Subcellular trafficking of PIN auxin efflux carriers in auxin transport. *European journal of cell biology* **89**, 231-235.

Fukaki, H., Tameda, S., Masuda, H. and Tasaka, M. (2002). Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY-ROOT/IAA14 gene of Arabidopsis. *Plant J* **29**, 153-68.

Gagne, J. M., Downes, B. P., Shiu, S. H., Durski, A. M. and Vierstra, R. D. (2002). The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in Arabidopsis. *Proc Natl Acad Sci U S A* **99**, 11519-24.

Geisler, M., Blakeslee, J. J., Bouchard, R., Lee, O. R., Vincenzetti, V., Bandyopadhyay, A., Titapiwatanakun, B., Peer, W. A., Bailly, A. and Richards, E. L. (2005). Cellular efflux of auxin catalyzed by the Arabidopsis MDR/PGP transporter AtPGP1. *The Plant Journal* **44**, 179-194.

Gfeller, A., Liechti, R. and Farmer, E. (2010). Arabidopsis jasmonate signaling pathway. *Science signaling* **3**, cm4.

Goritschnig, S., Zhang, Y. and Li, X. (2007). The ubiquitin pathway is required for innate immunity in Arabidopsis. *Plant J* **49**, 540-51.

Gray, W. M., del Pozo, J. C., Walker, L., Hobbie, L., Risseeuw, E., Banks, T., Crosby, W. L., Yang, M., Ma, H. and Estelle, M. (1999). Identification of an SCF ubiquitin-ligase complex required for auxin response in Arabidopsis thaliana. *Genes Dev* **13**, 1678-91.

Gray, W. M. and Estelle, I. (2000). Function of the ubiquitin-proteasome pathway in auxin response. *Trends Biochem Sci* **25**, 133-8.

Gray, W. M., Hellmann, H., Dharmasiri, S. and Estelle, M. (2002). Role of the Arabidopsis RING-H2 protein RBX1 in RUB modification and SCF function. *Plant Cell* **14**, 2137-44.

Gray, W. M., Kepinski, S., Rouse, D., Leyser, O. and Estelle, M. (2001). Auxin regulates SCF(TIR1)-dependent degradation of AUX/IAA proteins. *Nature* **414**, 271-6.

Grunewald, J. and Friml, J. (2010). The march of the PINs: developmental plasticity by dynamic polar targeting in plant cells. *The EMBO Journal* **29**, 2700-2714.

Hagen, G. and Guilfoyle, T. (2002). Auxin-responsive gene expression: genes, promoters and regulatory factors. *Plant Mol Biol* **49**, 373-85.

Hamann, T., Benkova, E., Baurle, I., Kientz, M. and Jurgens, G. (2002). The Arabidopsis BODENLOS gene encodes an auxin response protein inhibiting MONOPTEROS-mediated embryo patterning. *Genes Dev* **16**, 1610-5.

Hartmann-Petersen, R., Seeger, M. and Gordon, C. (2003). Transferring substrates to the 26S proteasome. *Trends Biochem Sci* **28**, 26-31.

Hellmann, H., Hobbie, L., Chapman, A., Dharmasiri, S., Dharmasiri, N., del Pozo, C., Reinhardt, D. and Estelle, M. (2003). Arabidopsis AXR6 encodes CUL1 implicating SCF E3 ligases in auxin regulation of embryogenesis. *Embo J* **22**, 3314-25.

Henriques, R., Jang, I. C. and Chua, N. H. (2009). Regulated proteolysis in light-related signaling pathways. *Current opinion in plant biology* **12**, 49-56.

Hershko, A. and Ciechanover, A. (1986). The ubiquitin pathway for the degradation of intracellular proteins. *Prog Nucleic Acid Res Mol Biol* **33**, 19-56, 301.

Ho, M. S., Tsai, P. I. and Chien, C. T. (2006). F-box proteins: the key to protein degradation. *J Biomed Sci* **13**, 181-91.

Jackson, R. G., Lim, E. K., Li, Y., Kowalczyk, M., Sandberg, G., Hoggett, J., Ashford, D. A. and Bowles, D. J. (2001). Identification and Biochemical Characterization of anArabidopsis Indole-3-acetic Acid Glucosyltransferase. *Journal of Biological Chemistry* **276**, 4350.

Jaillais, Y. and Chory, J. (2010). Unraveling the paradoxes of plant hormone signaling integration. *Nat Struct Mol Biol* **17**, 642-5.

Jones, A. R., Kramer, E. M., Knox, K., Swarup, R., Bennett, M. J., Lazarus, C. M., Leyser, H. M. O. and Grierson, C. S. (2008). Auxin transport through non-hair cells sustains root-hair development. *Nature cell biology* **11**, 78-84.
Kepinski, S. and Leyser, O. (2005). The Arabidopsis F-box protein TIR1 is an auxin receptor. *Nature* **435**, 446-51.
Kerr, I. D. and Bennett, M. J. (2007). New insight into the biochemical mechanisms regulating auxin transport in plants. *Biochemical Journal* **401**, 613.
Kim, B. C., Soh, M. C., Kang, B. J., Furuya, M. and Nam, H. G. (1996). Two dominant photomorphogenic mutations of Arabidopsis thaliana identified as suppressor mutations of hy2. *Plant J* **9**, 441-56.
Kim, J., Harter, K. and Theologis, A. (1997). Protein-protein interactions among the Aux/IAA proteins. *Proc Natl Acad Sci U S A* **94**, 11786-91.
Kögl, F., Haagen-Smit, A. J. and Erxleben, H. (1933). Studien über das Vorkommen von Auxinen im menschlichen und im tierischen Organismus. 7. Mitteilung über pflanzliche Wachstumsstoffe. *Hoppe-Seyler' s Zeitschrift für physiologische Chemie* **220**, 137-161.
Kraft, E., Stone, S. L., Ma, L., Su, N., Gao, Y., Lau, O. S., Deng, X. W. and Callis, J. (2005). Genome analysis and functional characterization of the E2 and RING-type E3 ligase ubiquitination enzymes of Arabidopsis. *Plant Physiol* **139**, 1597-611.
Lechner, E., Achard, P., Vansiri, A., Potuschak, T. and Genschik, P. (2006). F-box proteins everywhere. *Curr Opin Plant Biol* **9**, 631-8.
LeClere, S., Tellez, R., Rampey, R. A., Matsuda, S. and Bartel, B. (2002). Characterization of a family of IAA-amino acid conjugate hydrolases from Arabidopsis. *Journal of Biological Chemistry* **277**, 20446.
Lee, J. H. and Kim, W. T. (2011). Regulation of abiotic stress signal transduction by E3 ubiquitin ligases in Arabidopsis. *Molecules and Cells*, 1-8.
Leon-Kloosterziel, K. M., Gil, M. A., Ruijs, G. J., Jacobsen, S. E., Olszewski, N. E., Schwartz, S. H., Zeevaart, J. A. and Koornneef, M. (1996). Isolation and characterization of abscisic acid-deficient Arabidopsis mutants at two new loci. *Plant J* **10**, 655-61.
Leyser, H. M., Lincoln, C. A., Timpte, C., Lammer, D., Turner, J. and Estelle, M. (1993). Arabidopsis auxin-resistance gene AXR1 encodes a protein related to ubiquitin-activating enzyme E1. *Nature* **364**, 161-4.
Li, J., Nam, K. H., Vafeados, D. and Chory, J. (2001). BIN2, a new brassinosteroid-insensitive locus in Arabidopsis. *Plant Physiol* **127**, 14-22.
Liechti, R., Gfeller, A. and Farmer, E. E. (2006). Jasmonate signaling pathway. *Sci STKE* **2006**, cm2.
Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christmann, A. and Grill, E. (2009). Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* **324**, 1064-8.
Malamy, J. E. and Benfey, P. N. (1997). Organization and cell differentiation in lateral roots of Arabidopsis thaliana. *Development* **124**, 33-44.
Meyerowitz, E. M. (1987). Arabidopsis thaliana. *Annual Review of Genetics* **21**, 93-111.
Meyerowitz, E. M. (2001). Prehistory and history of Arabidopsis research. *Plant Physiol* **125**, 15-9.

Moon, J., Zhao, Y., Dai, X., Zhang, W., Gray, W. M., Huq, E. and Estelle, M. (2007). A new CULLIN 1 mutant has altered responses to hormones and light in Arabidopsis. *Plant Physiol* **143**, 684-96.

Nafisi, M., Goregaoker, S., Botanga, C. J., Glawischnig, E., Olsen, C. E., Halkier, B. A. and Glazebrook, J. (2007). Arabidopsis cytochrome P450 monooxygenase 71A13 catalyzes the conversion of indole-3-acetaldoxime in camalexin synthesis. *The Plant Cell Online* **19**, 2039.

Nagpal, P., Walker, L. M., Young, J. C., Sonawala, A., Timpte, C., Estelle, M. and Reed, J. W. (2000). AXR2 encodes a member of the Aux/IAA protein family. *Plant Physiol* **123**, 563-74.

Nemhauser, J. L., Hong, F. and Chory, J. (2006). Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. *Cell* **126**, 467-75.

Normanly, J. (2010). Approaching cellular and molecular resolution of auxin biosynthesis and metabolism. *Cold Spring Harbor Perspectives in Biology* **2**.

Okushima, Y., Overvoorde, P. J., Arima, K., Alonso, J. M., Chan, A., Chang, C., Ecker, J. R., Hughes, B., Lui, A., Nguyen, D. et al. (2005). Functional genomic analysis of the AUXIN RESPONSE FACTOR gene family members in Arabidopsis thaliana: unique and overlapping functions of ARF7 and ARF19. *Plant Cell* **17**, 444-63.

Ostin, A., Kowalyczk, M., Bhalerao, R. P. and Sandberg, G. (1998). Metabolism of indole-3-acetic acid in Arabidopsis. *Plant physiology* **118**, 285.

Ouellet, F., Overvoorde, P. J. and Theologis, A. (2001). IAA17/AXR3: biochemical insight into an auxin mutant phenotype. *Plant Cell* **13**, 829-41.

Park, S. Y., Fung, P., Nishimura, N., Jensen, D. R., Fujii, H., Zhao, Y., Lumba, S., Santiago, J., Rodrigues, A., Chow, T. F. et al. (2009). Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* **324**, 1068-71.

Patton, E. E., Willems, A. R. and Tyers, M. (1998). Combinatorial control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis. *Trends Genet* **14**, 236-43.

Peret, B., De Rybel, B., Casimiro, I., Benkova, E., Swarup, R., Laplaze, L., Beeckman, T. and Bennett, M. J. (2009). Arabidopsis lateral root development: an emerging story. *Trends Plant Sci* **14**, 399-408.

Petrasek, J., Mravec, J., Bouchard, R., Blakeslee, J. J., Abas, M., Seifertova, D., Wisniewska, J., Tadele, Z., Kubes, M., Covanova, M. et al. (2006). PIN proteins perform a rate-limiting function in cellular auxin efflux. *Science* **312**, 914-8.

Poupart, J., Rashotte, A. M., Muday, G. K. and Waddell, C. S. (2005). The rib1 mutant of Arabidopsis has alterations in indole-3-butyric acid transport, hypocotyl elongation, and root architecture. *Plant Physiol* **139**, 1460-71.

Quint, M., Ito, H., Zhang, W. and Gray, W. M. (2005). Characterization of a novel temperature-sensitive allele of the CUL1/AXR6 subunit of SCF ubiquitin-ligases. *Plant J* **43**, 371-83.

Ramos, J. A., Zenser, N., Leyser, O. and Callis, J. (2001). Rapid degradation of auxin/indoleacetic acid proteins requires conserved amino acids of domain II and is proteasome dependent. *Plant Cell* **13**, 2349-60.

Rashotte, A. M., Poupart, J., Waddell, C. S. and Muday, G. K. (2003). Transport of the two natural auxins, indole-3-butyric acid and indole-3-acetic acid, in Arabidopsis. *Plant physiology* **133**, 761.

Risseeuw, E. P., Daskalchuk, T. E., Banks, T. W., Liu, E., Cotelesage, J., Hellmann, H., Estelle, M., Somers, D. E. and Crosby, W. L. (2003). Protein interaction analysis of SCF ubiquitin E3 ligase subunits from Arabidopsis. *Plant J* **34**, 753-67.

Robert, H. S. and Friml, J. (2009). Auxin and other signals on the move in plants. *Nature chemical biology* **5**, 325.

Rogg, L. E., Lasswell, J. and Bartel, B. (2001). A gain-of-function mutation in IAA28 suppresses lateral root development. *Plant Cell* **13**, 465-80.

Rotin, D. and Kumar, S. (2009). Physiological functions of the HECT family of ubiquitin ligases. *Nat Rev Mol Cell Biol* **10**, 398-409.

Rouse, D., Mackay, P., Stirnberg, P., Estelle, M. and Leyser, O. (1998). Changes in auxin response from mutations in an AUX/IAA gene. *Science* **279**, 1371-3.

Ruegger, M., Dewey, E., Gray, W. M., Hobbie, L., Turner, J. and Estelle, M. (1998). The TIR1 protein of Arabidopsis functions in auxin response and is related to human SKP2 and yeast grr1p. *Genes Dev* **12**, 198-207.

Sanchez-Rodriguez, C., Rubio-Somoza, I., Sibout, R. and Persson, S. (2010). Phytohormones and the cell wall in Arabidopsis during seedling growth. *Trends Plant Sci* **15**, 291-301.

Schlesinger, D. H. and Goldstein, G. (1975). Molecular conservation of 74 amino acid sequence of ubiquitin between cattle and man. *Nature* **255**, 42304.

Schlesinger, D. H., Goldstein, G. and Niall, H. D. (1975). The complete amino acid sequence of ubiquitin, an adenylate cyclase stimulating polypeptide probably universal in living cells. *Biochemistry* **14**, 2214-8.

Schnable, P. S. Ware, D. Fulton, R. S. Stein, J. C. Wei, F. Pasternak, S. Liang, C. Zhang, J. Fulton, L. Graves, T. A. et al. (2009). The B73 maize genome: complexity, diversity, and dynamics. *Science* **326**, 1112-5.

Schulman, B. A. and Harper, J. W. (2009). Ubiquitin-like protein activation by E1 enzymes: the apex for downstream signalling pathways. *Nat Rev Mol Cell Biol* **10**, 319-31.

Shen, W. H., Parmentier, Y., Hellmann, H., Lechner, E., Dong, A., Masson, J., Granier, F., Lepiniec, L., Estelle, M. and Genschik, P. (2002). Null mutation of AtCUL1 causes arrest in early embryogenesis in Arabidopsis. *Mol Biol Cell* **13**, 1916-28.

Somers, D. E. and Fujiwara, S. (2009). Thinking outside the F-box: novel ligands for novel receptors. *Trends Plant Sci* **14**, 206-13.

Staswick, P. E. (2009). The tryptophan conjugates of jasmonic and indole-3-acetic acids are endogenous auxin inhibitors. *Plant physiology* **150**, 1310.

Staswick, P. E., Serban, B., Rowe, M., Tiryaki, I., Maldonado, M. T., Maldonado, M. C. and Suza, W. (2005). Characterization of an Arabidopsis enzyme family that conjugates amino acids to indole-3-acetic acid. *The Plant Cell Online* **17**, 616.

Stone, B. B., Stowe-Evans, E. L., Harper, R. M., Celaya, R. B., Ljung, K., Sandberg, G. and Liscum, E. (2008). Disruptions in AUX1-dependent auxin influx alter hypocotyl phototropism in Arabidopsis. *Molecular plant* **1**, 129.

Stone, S. L., Hauksdottir, H., Troy, A., Herschleb, J., Kraft, E. and Callis, J. (2005). Functional analysis of the RING-type ubiquitin ligase family of Arabidopsis. *Plant Physiol* **137**, 13-30.

Swarup, K., Benkova, E., Swarup, R., Casimiro, I., Peret, B., Yang, Y., Parry, G., Nielsen, E., De Smet, I., Vanneste, S. et al. (2008). The auxin influx carrier LAX3 promotes lateral root emergence. *Nat Cell Biol* **10**, 946-54.

Szemenyei, H., Hannon, M. and Long, J. A. (2008). TOPLESS mediates auxin-dependent transcriptional repression during Arabidopsis embryogenesis. *Science* **319**, 1384.

Szerszen, J. B., Szczyglowski, K. and Bandurski, R. S. (1994). iaglu, a gene from Zea mays involved in conjugation of growth hormone indole-3-acetic acid. *Science* **265**, 1699.

Tan, X., Calderon-Villalobos, L. I., Sharon, M., Zheng, C., Robinson, C. V., Estelle, M. and Zheng, N. (2007). Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature* **446**, 640-5.

Tanaka, H., Dhonukshe, P., Brewer, P. B. and Friml, J. (2006). Spatiotemporal asymmetric auxin distribution: a means to coordinate plant development. *Cell Mol Life Sci* **63**, 2738-54.

Tatematsu, K., Kumagai, S., Muto, H., Sato, A., Watahiki, M. K., Harper, R. M., Liscum, E. and Yamamoto, K. T. (2004). MASSUGU2 encodes Aux/IAA19, an auxin-regulated protein that functions together with the transcriptional activator NPH4/ARF7 to regulate differential growth responses of hypocotyl and formation of lateral roots in Arabidopsis thaliana. *Plant Cell* **16**, 379-93.

Teale, W. D., Paponov, I. A. and Palme, K. (2006). Auxin in action: signalling, transport and the control of plant growth and development. *Nat Rev Mol Cell Biol* **7**, 847-59.

The Arabidopsis Genome Initiative, A. G. I. (2000). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. *Nature* **408**, 796-815.

Tian, Q. and Reed, J. W. (1999). Control of auxin-regulated root development by the Arabidopsis thaliana SHY2/IAA3 gene. *Development* **126**, 711-21.

Tiwari, S. B., Hagen, G. and Guilfoyle, T. (2003). The roles of auxin response factor domains in auxin-responsive transcription. *Plant Cell* **15**, 533-43.

Tiwari, S. B., Hagen, G. and Guilfoyle, T. J. (2004). Aux/IAA proteins contain a potent transcriptional repression domain. *Plant Cell* **16**, 533-43.

To, J. P. and Kieber, J. J. (2008). Cytokinin signaling: two-components and more. *Trends Plant Sci* **13**, 85-92.

Tyers, M. and Jorgensen, P. (2000). Proteolysis and the cell cycle: with this RING I do thee destroy. *Curr Opin Genet Dev* **10**, 54-64.

Ubeda-Tomas, S., Swarup, R., Coates, J., Swarup, K., Laplaze, L., Beemster, G. T., Hedden, P., Bhalerao, R. and Bennett, M. J. (2008). Root growth in Arabidopsis requires gibberellin/DELLA signalling in the endodermis. *Nat Cell Biol* **10**, 625-8.

Uehara, T., Okushima, Y., Mimura, T., Tasaka, M. and Fukaki, H. (2008). Domain II mutations in CRANE/IAA18 suppress lateral root formation and affect shoot development in Arabidopsis thaliana. *Plant Cell Physiol* **49**, 1025-38.

Ulmasov, T., Hagen, G. and Guilfoyle, T. J. (1997a). ARF1, a transcription factor that binds to auxin response elements. *Science* **276**, 1865-8.

Ulmasov, T., Hagen, G. and Guilfoyle, T. J. (1999a). Activation and repression of transcription by auxin-response factors. *Proc Natl Acad Sci U S A* **96**, 5844-9.
Ulmasov, T., Hagen, G. and Guilfoyle, T. J. (1999b). Dimerization and DNA binding of auxin response factors. *Plant J* **19**, 309-19.
Ulmasov, T., Liu, Z. B., Hagen, G. and Guilfoyle, T. J. (1995). Composite structure of auxin response elements. *Plant Cell* **7**, 1611-23.
Ulmasov, T., Murfett, J., Hagen, G. and Guilfoyle, T. J. (1997b). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell* **9**, 1963-71.
Vanneste, S. and Friml, J. (2009). Auxin: a trigger for change in plant development. *Cell* **136**, 1005-1016.
Walz, A., Park, S., Slovin, J. P., Ludwig-Müller, J., Momonoki, Y. S. and Cohen, J. D. (2002). A gene encoding a protein modified by the phytohormone indoleacetic acid. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 1718.
Wang, X., Feng, S., Nakayama, N., Crosby, W. L., Irish, V., Deng, X. W. and Wei, N. (2003). The COP9 signalosome interacts with SCF UFO and participates in Arabidopsis flower development. *Plant Cell* **15**, 1071-82.
Weijers, D. and Jurgens, G. (2004). Funneling auxin action: specificity in signal transduction. *Curr Opin Plant Biol* **7**, 687-93.
Went, F. W. (1928). Wuchsstoffe und Wachstum. Rec. Trav. Bot. Neerl., xxv. 1. 108. 1934: On the Pea-test Method for Auxin, the Plant Growth Hormone. *Proc. K.*
Whyte, R. O. (1946). Crop production and environment: Faber.
Wilkinson, K. D. (2000). Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome. *Semin Cell Dev Biol* **11**, 141-8.
Willems, A. R., Schwab, M. and Tyers, M. (2004). A hitchhiker's guide to the cullin ubiquitin ligases: SCF and its kin. *Biochim Biophys Acta* **1695**, 133-70.
Woodward, A. W. and Bartel, B. (2005). Auxin: regulation, action, and interaction. *Ann Bot (Lond)* **95**, 707-35.
Worley, C. K., Zenser, N., Ramos, J., Rouse, D., Leyser, O., Theologis, A. and Callis, J. (2000). Degradation of Aux/IAA proteins is essential for normal auxin signalling. *Plant J* **21**, 553-62.
Xie, D. X., Feys, B. F., James, S., Nieto-Rostro, M. and Turner, J. G. (1998). COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. *Science* **280**, 1091-4.
Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W. L., Ma, H., Peng, W., Huang, D. and Xie, D. (2002). The SCF(COI1) ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. *Plant Cell* **14**, 1919-35.
Xu, L., Menard, R., Berr, A., Fuchs, J., Cognat, V., Meyer, D. and Shen, W. H. (2009). The E2 ubiquitin-conjugating enzymes, AtUBC1 and AtUBC2, play redundant roles and are involved in activation of FLC expression and repression of flowering in Arabidopsis thaliana. *Plant J* **57**, 279-88.
Yang, H. and Murphy, A. S. (2009). Functional expression and characterization of Arabidopsis ABCB, AUX 1 and PIN auxin transporters in Schizosaccharomyces pombe. *The Plant Journal* **59**, 179-191.

Yang, T. F., Gonzalez-Carranza, Z. H., Maunders, M. J. and Roberts, J. A. (2008). Ethylene and the regulation of senescence processes in transgenic Nicotiana sylvestris plants. *Ann Bot* **101**, 301-10.

Yang, X., Lee, S., So, J. H., Dharmasiri, S., Dharmasiri, N., Ge, L., Jensen, C., Hangarter, R., Hobbie, L. and Estelle, M. (2004). The IAA1 protein is encoded by AXR5 and is a substrate of SCF(TIR1). *Plant J* **40**, 772-82.

Yang, Y., Wu, Y., Pirrello, J., Regad, F., Bouzayen, M., Deng, W. and Li, Z. (2010). Silencing Sl-EBF1 and Sl-EBF2 expression causes constitutive ethylene response phenotype, accelerated plant senescence, and fruit ripening in tomato. *J Exp Bot* **61**, 697-708.

Zenser, N., Ellsmore, A., Leasure, C. and Callis, J. (2001). Auxin modulates the degradation rate of Aux/IAA proteins. *Proc Natl Acad Sci U S A* **98**, 11795-800.

Zhang, H., Kobayashi, R., Galaktionov, K. and Beach, D. (1995). p19Skp1 and p45Skp2 are essential elements of the cyclin A-CDK2 S phase kinase. *Cell* **82**, 915-25.

Zhao, D., Yang, M., Solava, J. and Ma, H. (1999). The ASK1 gene regulates development and interacts with the UFO gene to control floral organ identity in Arabidopsis. *Dev Genet* **25**, 209-23.

Zhou, M., Shen, C., Wu, L., Tang, K. and Lin, J. (2011). CBF-dependent signaling pathway: A key responder to low temperature stress in plants. *Critical Reviews in Biotechnology*, 1-8.

Zolman, B. K., Martinez, N., Millius, A., Adham, A. R. and Bartel, B. (2008). Identification and characterization of Arabidopsis indole-3-butyric acid response mutants defective in novel peroxisomal enzymes. *Genetics* **180**, 237.

Zolman, B. K., Nyberg, M. and Bartel, B. (2007). IBR3, a novel peroxisomal acyl-CoA dehydrogenase-like protein required for indole-3-butyric acid response. *Plant molecular biology* **64**, 59-72.

Chapter 2 : ARF7 and ARF19 regulate auxin inducible *LAX3* expression in an antagonistic manner

2.1. Abstract

LAX3 is an auxin inducible gene which plays a key role during lateral root emergence. Preliminary experiments reported that *LAX3* is induced by auxin in an ARF7/ARF19 dependent manner. The regulation of *LAX3* was dissected using gene expression studies, mutant analysis, promoter mining tools and promoter mutations. ARF7 and ARF19 positively and negatively regulate *LAX3* expression, respectively. The *LAX3* promoter does not appear to be a direct target for ARF7 or ARF19 as mutations in an Auxin Response Element (ARE) do not block *LAX3* auxin inducibility. Instead, an intermediate transcription factor(s) may function to induce *LAX3* expression and another one, potentially a repressing ARF, may function to repress *LAX3* expression. These two antagonistic pathways define LAX3's spatial, temporal and strength of expression. Finally, several putative transcription factors were identified that could, based on their expression patterns, directly regulate *LAX3*.

2.2. Introduction

The hormone auxin regulates many different plant developmental processes (Delker et al., 2008; Vanneste and Friml, 2009; Woodward and Bartel, 2005). Notably, the effects of auxin on root development have been described in great details using the model plant *Arabidopsis thaliana* (Overvoorde et al., 2010). Functional studies in Arabidopsis have

revealed that the hormone is involved in gravitropism, root growth, the formation of root hairs and lateral roots (Blilou et al., 2005; Casimiro et al., 2001; Dello Ioio et al., 2008; Hobbie and Estelle, 1995; Knox et al., 2003; Tian and Reed, 1999).

During lateral root formation, auxin acts at several developmental stages (Casimiro et al., 2003; Casimiro et al., 2001; Peret et al., 2009a). The hormone regulates initiation and patterning processes via its accumulation in specific cells (Benkova et al., 2003; Dubrovsky et al., 2000; Vanneste et al., 2005).

Auxin is not only redistributed within the primordium but diffuses into cell within overlying tissues. This movement of auxin was recently demonstrated to be important for primordium emergence (Swarup et al., 2008). (Swarup et al., 2008), revealed that the hormone induces the expression of a large number of genes including the auxin influx carrier *LAX3* in several cortical and epidermal cells overlying the primordium. In these cells, LAX3 activity leads to the accumulation of more auxin which is essential to activate the expression of genes involved in cell separation. A cocktail of cell wall remodelling enzymes are produced and secreted by the overlying cells which assist primordium to transit through parental root tissues. In summary, auxin is actively transported into the outer tissues where it triggers a cascade of responses which allows plants to tightly control emergence (Peret et al., 2009b).

Auxin regulates the expression of many genes depending on its concentration (Guilfoyle and Hagen, 2007; Lokerse and Weijers, 2009; Tromas and Perrot-Rechenmann, 2010). Auxin regulated genes usually possess one or several Auxin Response Elements (AREs)

within their promoter (Abel et al., 1996; Ulmasov et al., 1995). These motifs are bound by transcription factors termed Auxin Response Factors (ARFs) via a specific domain located at the N terminus (Ulmasov et al., 1997). When bound to DNA, ARF proteins can homo or hetero-dimerise via two conserved domains located at the C terminus (Ulmasov et al., 1999). The Aux/IAAs also possess these two domains which allow them to interact with the ARFs (Tiwari et al., 2003). Aux/IAAs are repressors which prevent ARFs to regulate gene expression (Tiwari et al., 2004; Tiwari et al., 2001). Aux/IAAs do this by interacting with another family of proteins termed TOPLESS (TPL), which increase their repressor activity (Szemenyei et al., 2008). Thus, Aux/IAAs are central in modulating ARF activity and, as a consequence, the expression of downstream genes.

Auxin directly regulates levels of Aux/IAA proteins via its co-receptor TIR1/AFB1-5 (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Tan et al., 2007). The TIR1/AFB genes encode F-BOX ubiquitin E3 ligases which are component of an $SCF^{TIR1/AFB}$ complex. Auxin acts as a molecular glue between TIR1/AFBs and the Aux/IAAs, greatly promoting the strength and stability of their interaction. As a result of TIR1/AFBs activity, Aux/IAAs are poly-ubiquitinated and subsequently degraded by the proteasome (dos Santos Maraschin et al., 2009). An increase in auxin concentration will therefore reduce Aux/IAA levels and release the ARFs to activate target genes (Tiwari et al., 2003).

23 ARFs are encoded by the Arabidopsis genome, which are roughly separated into two functional groups: the putative activating ARFs (ARF5-8 and ARF19) and the putative repressing ARFs (all the others) (Okushima et al., 2005; Tiwari et al., 2003; Wilmoth et al., 2005). Six of them have been shown to regulate primary root and lateral root development (ARF3, ARF7, ARF10, ARF16, ARF17 and ARF19) (Mallory et al., 2005; Okushima et al., 2005; Wang et al., 2005; Yoon et al., 2010). Two of them, ARF7 and ARF19, appear to be most important for root development as *arf7arf19* double mutants exhibit agravitropic roots with no emerged lateral roots 14 days after germination (Fukaki et al., 2006; Okushima et al., 2005; Wilmoth et al., 2005). These phenotypes have not been observed to that extent in any other single or combinations of ARF mutants. ARF7 and ARF19 have been shown to have largely overlapping role, regulating most of the auxin responsive genes in the Arabidopsis root, (Okushima et al., 2005; Swarup et al., 2008).

29 Aux/IAAs are encoded by the Arabidopsis genome, of which seven have been shown to regulate primary root and lateral root development (IAA1, IAA3, IAA14, IAA18, IAA19 and IAA28) (Fukaki et al., 2002; Overvoorde et al., 2005; Rogg et al., 2001; Tatematsu et al., 2004; Tian and Reed, 1999; Uehara et al., 2008; Yang et al., 2004). The role of IAA14 has been particularly well described during lateral root formation (Fukaki et al., 2005; Fukaki et al., 2006; Okushima et al., 2005). The gain of function mutant *iaa14/slr-1* has a severe root phenotype quite similar to *arf7arf19* double mutants. This observation correlates with similar deleterious effects of the *slr-1* mutation on gene expression (Vanneste et al., 2005).

Primary responsive genes are generally rapidly induced or repressed due to the very fast turnover of Aux/IAAs proteins in the presence of auxin (Dreher et al., 2006; Ramos et al., 2001; Tiwari et al., 2001; Worley et al., 2000; Zenser et al., 2003). These genes do not require *de novo* protein synthesis as they are induced in the presence of auxin and cycloheximide (CHX), a potent translation inhibitor (Koshiba et al., 1995). Despite these recent advances, regulation of secondary (or late) auxin responsive genes, which are not direct targets of an ARF-Aux/IAA pair and are not induced in presence of auxin and CHX, have proved more elusive (Baumann et al., 1999; Inukai et al., 2005; Lee and Kim, 2010; Lee et al., 2009; Okushima et al., 2007; Shin et al., 2007; Xie et al., 2000). Whilst several families of transcription factors have been identified as responsive to auxin (LBDs (Lateral organ Boundaries Domain), HB (Homeo Box), DOF (DOF domain)), very few downstream targets genes or developmental processes have been defined to date.

In this chapter, I dissect the transcriptional regulation of *LAX3* by auxin. *LAX3* is shown to be a late auxin responsive gene that requires the *de novo* synthesis of primary auxin responsive genes. Using promoter mining tools, a selection of cis acting regulatory elements were identified within the *LAX3* promoter which were functionally characterised employing promoter deletions and point mutations experiments. These results suggest two ARF pathways, one positive and one negative, which regulates *LAX3*. Finally, transcriptomics datasets were used to identify putative transcription factors that function as signalling intermediates between ARF7 and LAX3.

2.3. Material and methods

2.3.1. Materials

2.3.1.1. Chemicals

Indole-3-Acetic Acid (IAA) and α-Naphthalene acetic acid (NAA) were purchased from Sigma, Cycloheximide (CHX) from VWR International Ltd. IAA and CHX were dissolved in 100% EtOH, NAA was dissolved in DMSO. Plant seeds were sown on ½ MS medium (Murashige and Skoog, 1962) (Sigma) (2.17g salts/L), at pH5.7 solidified with 1% bacto-agar (Appleton Woods).

2.3.1.2. Seeds

The *arf7arf19* double mutant (in col-0 background) was kindly provided by Dr. Hidehiro Fukaki (University of Kobe, Japan).

2.3.1.3. Primers used for LAX3 promoter deletions and point mutations.

Primer name	Sequence (5'->3')
Lx3-R2	ttctaagtaattccctgcgacc
(KPN I)-Lx3-22	(CCGGTACC)tttctaagaaattagtgggtta
(KPN I)-Lx3-23	(CCGGTACC)aatatgttttattcattgtttc
(KPN I)-Lx3-24	(CCGGTACC)atataattaacaatctcaaacc
Lx3-25	tttctaagaaattagtgggttaaataaagc
Lx3-26	agtctccttttagccccatgcttttacaatgg

2.3.1.4. Primers used for RT qPCR

Primer name	Sequence (5'->3')
qCTRL1-F	agtggagaggctgcagaaga
qCTRL1-R	ctcgggtagcacgagcttta
qGH3.1-F	aacttatgccgaccattaaagaa
qGH3.1-R	tctagacccggcacatacaa
qLAX3-F	tcaccattgcttcactccttc
qLAX3-R	aagcaccattgtggttggac
qARF19-F	caccgatcacgaaaacgata
qARF19-R	tgttctgcacgcagttcac

2.3.2. Methods

2.3.2.1. Seed sterilisation and seedling growth

Seeds were surface sterilized for 5 minutes in 50% bleach, 0.1% triton X-100 then washed three times with sterile ddH_2O. Seeds were stratified at 4° for 2 days to synchronise germination. 5 days after germination, 100 seedlings were transferred onto solid agar plates containing the indicated chemicals (IAA, NAA or CHX) for the indicated length of time.

2.3.2.2. Transformation vectors and construction of transgenic plants

To create point mutations, the *LAX3* promoter was cloned from pENTR11-LAX3-YFP (Swarup et al., 2008) into pBluescript KM+ (Invitrogen) using unique KpnI and SpeI restriction sites. The plasmid was PCR amplified using primers Lx3-25 and Lx3-26, which were designed to modify a single nucleotide within the auxin response element (GAGACA to GAGACT). PCR amplification was carried out using Pfx proofreading DNA Polymerase. Purified PCR products were digested with dpnI, treated with T4

Polynucleotide Kinase (NEB) and ligated with T4 Ligase (NEB). Point mutated promoters were cloned back into pENTR11-LAX3-YFP and sequenced to check no other mutations were created during the PCR.

For PCR generated promoter deletions, a combination of primers Lx3-R2 and (KpnI)-Lx3-22 for Δ2, (KpnI)-Lx3-23 for Δ4 or (KpnI)-Lx3-24 for Δ5 were usd. PCR amplification was carried out using Pfx proofreading DNA Polymerase. Purified PCR products were digested with dpnI and cloned into pENTR11-LAX3-YFP using KpnI and SpeI restrictions sites.

For deletions generated using restriction enzymes, KpnI and MunI for Δ1 or only BamH1 for Δ3 were used. The correct band was gel purified, if necessary 3' overhang were filled using T4 DNA Polymerase (NEB), and both fragments ligated using T4 Ligase.

Constructs were then cloned in the binary pGWB7 vector using the Gateway LR reaction (Invitrogen). This vector was transformed into *Agrobacterium tumefaciens* strain C58 and finally transformed into *Arabidopsis thaliana aux1 lax3* double mutants. At the T1, at least 20 lines were selected for each construct. 3 lines segregating ¾ for Kan^R at the T2 were kept to get homozygous T3s. All the lines used in this analysis have been selected following these criteria except for two lines, IVM1-1#2-1 and #6-3 which were 100% homozygous at the T2 (more than one insert). All enzymes were purchased from NEB or Invitrogen and were used following manufacturer recommendations.

2.3.2.3. Histology and histochemistry

GUS activity was revealed by incubating seedlings in a phosphate buffer (500mM, pH 7) containing 0.5 mM potassium ferricyanide ($K_3[Fe(CN)_6]$), 0.5 mM potassium ferrocyanide ($K_4[Fe(CN)_6]$), 1 mM ethylenediaminotetraacetic acid (EDTA pH 8), 0.5 % (v/v) Triton X-100 and 1 mM X-bromo-4-chloro-3-indolyl ß-D-galactopyranoside (Sigma) (X-Glc) at 37° for 1 hour. X-Glc was initially dissolved in 100% dimethylformamide (DMF). After GUS staining, seedlings were cleared for at least 12 hours in 260% chloral hydrate in 33 % glycerol (all chemicals were purchased from Fischer Scientific and Sigma).

2.3.2.4. Confocal microscopy

Confocal microscopy was performed using a Leica SP2 confocal laser scanning microscope (Leica Microsystems). Cell walls were stained using propidium iodide (10 µg/ml) (Sigma). Scanning settings were optimised and kept unchanged throughout the experiment. Fluorescence was quantified using Leica SP2 Image Analysis software and figures created using Adobe Photoshop (version 7.01; Adobe Systems) and Microsoft PowerPoint 2007 (Microsoft Corporation, Redmond, USA).

2.3.2.5. RNA extraction and RT qPCR

RNA was extracted from plant tissues using Trizol Reagent (Invitrogen) and cleaned up using the RNeasy kit (Qiagen). Poly(dT) cDNA was prepared from 0.5 µg total RNA with Superscript II reverse transcriptase (Invitrogen) and analyzed on a LightCycler 480 apparatus (Roche Diagnostics) with the Quantace SYBRGREEN mix (Quantace)

according to the manufacturer's instructions. Targets were quantified with specific primer pairs designed with the Universal Probe Library Assay Design Center (Roche Applied Science). All individual reactions were done in quadruplicate and data were analyzed with Microsoft Excel 2007 (Microsoft Corporation, Redmond, USA). Expression levels were normalized to At1G04850 (CTRL1, thanks to Tara Holman for its sequence).

2.3.2.6. Promoter mining tools

Database name	Website
PlantCare (Rombauts et al., 1999)	http://bioinformatics.psb.ugent.be/webtools/plantcare/html/
PLACE (Higo et al., 1998)	http://www.dna.affrc.go.jp/PLACE/signalscan.html
AtCis (Athena) (Davuluri et al., 2003)	http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl

Table 2-1 Web-based cis-acting regulatory elements mining tools

2.3.2.7. Picture analysis

Root lengh was measured using ImageJ (ImageJ 1.40g).

2.3.2.8. Phylogenetic trees and heat maps

Gene families were obtained from the Plant Transcription Factor Database (http://plntfdb.bio.uni-potsdam.de/v3.0/, (Perez-Rodriguez et al., 2009)). Protein sequences were retrieved from TAIR (http://www.arabidopsis.org/, (Swarbreck et al., 2007)), aligned using clustal X2 (Larkin et al., 2007) and the phylogenetic trees were drawn using Dendroscope (Huson et al., 2007). Heat maps were generated using Microsoft Excel 2007 (Microsoft Corporation, Redmond, USA). The number shows the fold change of the indicated gene in

the cases where there is a significant change and the expression is above background. If the gene is absent from the microarray chip used (ATH1) the cell is filled in black.

There are two distinct heat maps: one for the four Okushima datasets (WT, *arf7*, *arf19* and *arf7arf19* treated with IAA) and one for the NAA treated WT roots. The heat maps consist of a gradient of red (highest value) to blue (lowest value) either for each gene separately (Okushima datasets) or either for all the genes (NAA dataset). For the Okushima heat map, when a gene changes its expression in only genotype it appears in red even if it is down-regulated. The heat map for the last dataset (NAA) is set on red for the gene with the highest induction fold and blue for the gene with the highest repression fold. If the gene is present on the lateral root emergence dataset (LRED), the last box appears in white. FC: Fold Change.

2.3.2.9. Microarray experiment set up

Col-0 Arabidopsis seeds were sown on plates containing NPA (10 μM). 5 days after germination, seedlings were transferred onto plates containing NAA at 10μM for 6 hours and RNA was extracted as described before. Hybridization of the RNAs on the Affymetrix ATH1 chips was performed at NASC (Nottingham Arabidopsis Center).

2.3.2.10. Microarrays data analysis

Data were normalised from .cel files using the RMA protocol within R/Bioconductor (Gentleman et al., 2004; Team, 2007). Further analyses were performed using Excel 2007 (Microsoft Corporation, Redmond, USA). Differentially regulated loci had a fold change greater than 1.5 and a Benjamini and Hochberg False Discovery Rate of 0.05 (or

5%) (Benjamini and Hochberg, 1995). Data is the average of 3 biological replicates per treatment (NAA on Col-0) or of 2 biological replicates per treatment (IAA with Col-0/*arf7*/*arf19*).

2.4. Results

2.4.1. *LAX3* is not a primary auxin responsive gene

To determine the kinetics of *LAX3* mRNA induction by auxin, WT seedlings were treated with exogenous auxin (1 μM IAA) then sampled at different time points. The expression levels of *LAX3* and of two primary auxin responsive genes, *GH3.1* and *ARF19* were then quantified by RT qPCR. Figure 2-1 (A) reports that *LAX3* starts to be induced after 180 minutes and its expression peaks after 720 minutes of treatment. In contrast, *GH3.1* and *ARF19* are induced within 15 and 30 minutes of auxin treatment respectively (inset in Figure 2-1 A) whilst their expression peaks after 300 minutes. The two hours delay prior to *LAX3* induction suggests that it is a late auxin responsive gene, which requires the *de novo* translation of a transcription factor in order to be activated. To test that, WT plants were treated with IAA (1μM) and with or without CHX (50 μM) for different lengths of time and the mRNA levels of the same genes were determined by RT qPCR. Figure 2-1 (B) shows that in presence of IAA and CHX, *LAX3* mRNA is not induced, even after 40 hours, whereas *GH3.1* and *ARF19* are still induced. Taken together, the results show that *LAX3* is a secondary auxin responsive that requires *de novo* translation of a transcription factor(s) to be induced. In this chapter, this unknown transcription factor is referred to as "Transcription Factor X" or TFX.

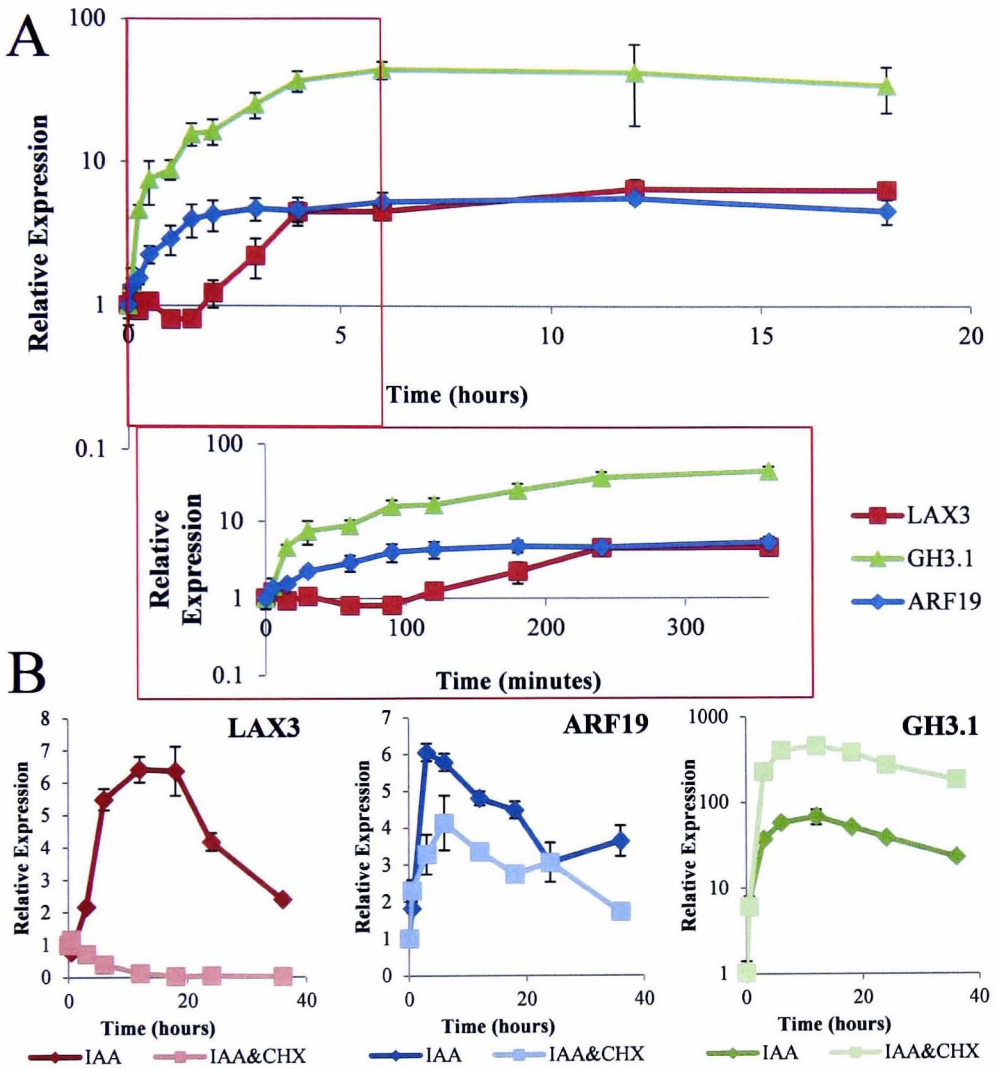

Figure 2-1 *LAX3* is not a primary auxin responsive gene. (A) Dynamics of *LAX3*, *GH3.1* and *ARF19* induction by auxin (up to 18 hours). Inset shows early time points (0, 5, 15, 30 and 60 minutes). **(B)** Dynamics of *LAX3*, *GH3.1* and *ARF19* induction by auxin with or without CHX. Error bars on all the charts show the SD of the mean of four technical replicates.

2.4.2. Bioinformatics analysis of the *LAX3* promoter sequence

To provide insights into which gene family TFX may belong to, promoter mining tools were used to look for auxin responsive motifs within the 2kb promoter of *LAX3* (Table 2-1). Using three different databases, 11 non-overlapping cis-acting elements, which correspond to 7 different motifs due to multiple occurrences of site 1 (3 times), site 2 (2 times) and site 7 (2 times), were identified (Table 2-2). All of these motifs were previously shown to be important for the auxin responsiveness of a downstream gene. If available, the transcription factor associated with the binding site is indicated on Table 2-2. Notably, there is an ARF binding site (ARE, motif 5) and two Dof domain transcription factor binding sites in tandem (motif 7).

Motif # and Reference	Name	Sequence	Distance	TF associated
1 (Sakai et al., 1996)	Auxin RR	GGTCMAT	-1520, -371	*Unknown*
2 (Xu et al., 1997)	CATATG	CATATG	-1475, -727	*Unknown*
3 (Benfey and Chua, 1990)	ASF1	TGACG	-1349	NtTGA1a, bZIP TF
4 (Pastuglia et al., 1997)	TGA-element	AACGAC	-1303	*Unknown*
5 (Ulmasov et al., 1997)	ARF binding site	TGTCTC	-936	ARF TF
6 (Bai et al., 2005)	Auxin RR	AAGGAC	-581	*Unkown*
7 (Baumann et al., 1999)	*Nt*BBF1	ACTTTA	-426, -419	NtBBF1 DOF TF

Table 2-2 The *LAX3* promoter contains 7 auxin responsive motifs. Details of the promoter mining tools used can be found on Table 2-1. Name: Name of motif. Distance: Distance to ATG. TF: Transcription Factor. Nt: *Nicotiana tobacum*.

2.4.3. Antagonistic regulation of *LAX3* by ARF7 and ARF19

ARF7 and ARF19 are the two main activating ARFs expressed in the root (Okushima et al., 2005). They have been shown to regulate *LAX3* but their individual roles have not been determined (Swarup et al., 2008). ARF19 could be TFX for the following reasons: First, *ARF19* is a primary auxin responsive gene (Figure 2-1). Secondly, the gene is expressed in cells overlying a primordium just where *LAX3* is expressed. Thirdly the *LAX3* promoter contains an ARF binding site (Table 2-2). We therefore hypothesised that *ARF19*, which is induced by auxin in an ARF7 dependent manner, binds LAX3's ARE and activates its expression. If this is the case, then we expect *LAX3* to be auxin insensitive in both *arf7* and *arf19* single mutants.

To test this model, the double *arf7arf19* mutant was crossed with two reporter lines, LAX3::GUS and LAX3:LAX3-YFP and the progenies of all the possible genotypes were selected. Figure 2-2 (A and B) shows the expression pattern of the GUS and YFP reporters. In both cases the induction of reporters is affected in *arf7* and *arf7arf19* but not in *arf19* mutants. Hence, ARF19 cannot function as the transcription factor intermediate between ARF7 and LAX3.

To quantify the effects of the mutations, WT, *arf7*, *arf19* and *arf7arf19* seedlings were treated with IAA (1μM) for different time points and the induction of *LAX3* mRNA monitored by RT qPCR.

Figure 2-2 (C) shows that only ARF7 is required to induce *LAX3*, though the level of *LAX3* mRNA did slightly increase after 24 hours of treatment, probably due to the

activity of other activating ARFs. Interestingly, the *arf19* mutation had an opposite effect to *arf7* as *LAX3* was induced to higher levels by auxin. These results suggest that ARF7 but not ARF19 is required for *LAX3* auxin induction and that ARF7 and ARF19 act antagonistically to regulate *LAX3* auxin induction. Taking into account the fact that ARF19 but not ARF7 are auxin inducible then none of them could be transcription factor X (TFX has to be auxin inducible and has to block LAX3 auxin induction).

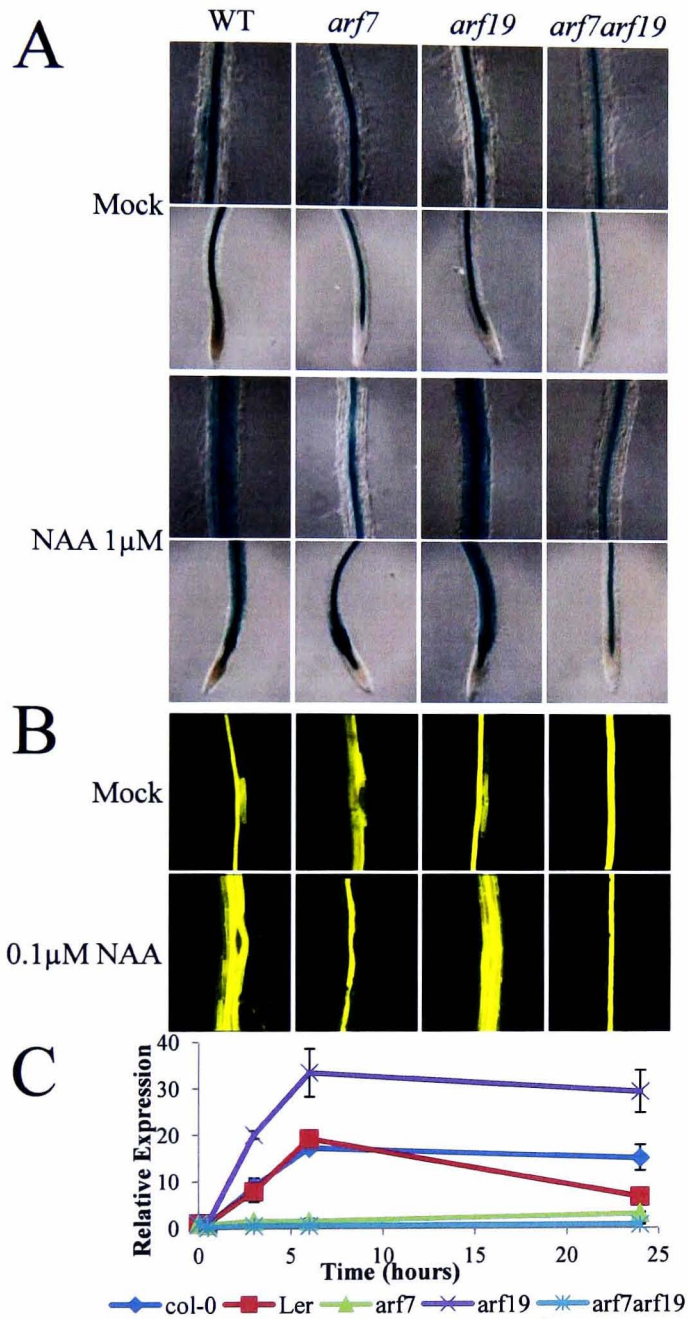

Figure 2-2 ARF7 is required for induction of *LAX3* by auxin. (A) Expression pattern of the LAX3::GUS reporter in WT, *arf7, arf19* and *arf7arf19* mutants in the presence or absence of auxin. **(B)** Expression pattern of the LAX3::LAX3-YFP reporter in WT, *arf7, arf19* and *arf7arf19* mutants in presence or absence of auxin.**(C)** Dynamics of *LAX3* induction by auxin visualised by RT qPCR in col-0, Ler, *arf7*, *arf19*, and *arf7arf19* mutants. Errors bars show the SD of the mean of four technical replicates.

2.4.4. *LAX3* is negatively regulated via its promoter's Auxin Response Element (ARE)

To test whether the ARE identified in the promoter of *LAX3* has any effects, a point mutation was introduced into its sequence which was changed from GAGACA to GAGACT (mARE). Such mutation has previously been shown to abolish the auxin sensitivity of a downstream reporter as well as ARF transcription factor binding (Ulmasov et al., 1997). Sequencing revealed that two different mARE variants had been generated (Figure 2-3 A). Mutated promoters containing each mARE were cloned upstream of a LAX3-YFP transgene and the constructs transformed into a *lax3aux1* double mutant background. The double mutant was used rather than the single *lax3* or *aux1* mutant since its phenotype is much more severe (no emerged lateral root two weeks after germination) compared to single *lax3* or *aux1* mutants (Figure 2-3 B and (Swarup et al., 2008)). The lateral root phenotype was used to assay whether the mutated promoter can drive the expression of the *LAX3YFP* transgene and rescue the *lax3aux1* lateral root phenotype. Lines segregating 3/1 for the two constructs were selected. Following these criteria we obtained two independent homozygous lines for IVM1 and IVM2. An additional line for IVM1 (line 3) was selected but did not segregate 3/1 at the T2 (probably because of multiple insertions).

In a control experiment, *lax3aux1* double mutants were transformed with a construct containing an unmodified *LAX3* promoter driving the *LAX3-YFP* transgene (LAX3YFP on Figure 2-3 B). This construct completely rescued the lateral root defects of *lax3aux1* mutants demonstrating that the LAX3-YFP fusion protein used is functional and that the

2kb *LAX3* upstream sequence is sufficient to drive *LAX3* expression. The same double mutants transformed with either of the transgene driven by the mutated promoters also complemented the lateral root phenotype (Figure 2-3 B). This result suggests that the ARE is not necessary for proper *LAX3* expression. Interestingly, few lines had a higher density of lateral roots.

The effects of the mutation on the induction by auxin of the LAX3-YFP reporter were then analysed using confocal microscopy. Figure 2-3 (C) shows that the mutated promoter is still sensitive to auxin. Interestingly, the fluorescence appeared stronger when the LAX3-YFP transgene is driven by the mutated promoter. When the intensity of the fluorescence was quantified in the two strips of cortical cells the YFP signal was consistently higher (up to two fold increase) in several seedlings of three independent lines as shown on Figure 2-3 (C) compared to a representative independent WT line. Taken together the results suggest that the ARE is not necessary for *LAX3* expression or auxin induction but that it has a negative impact on *LAX3* auxin induction.

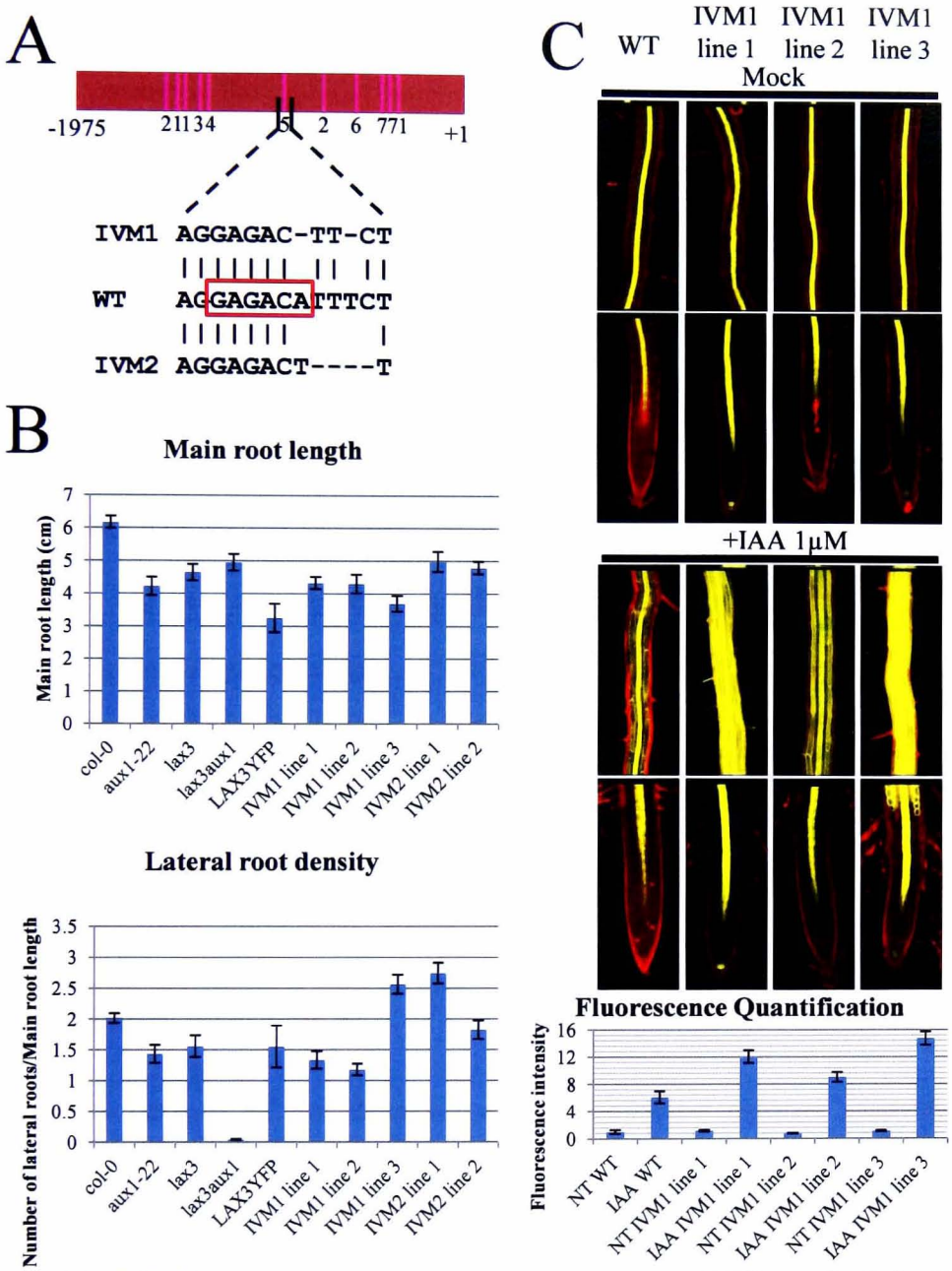

Figure 2-3 LAX3's promoter ARE is not necessary for *LAX3* auxin induction. (A) Schematic representation of *LAX3* promoter, the different binding sites identified and the mutations generated in the ARE. Numbers correspond to the sites on Table 2-2. **(B)** Main root length and lateral root density of WT, *aux1*, *lax3*, *lax3aux1* and *lax3aux1* plants transformed with the WT (LAX3YFP) or the modified *LAX3* promoter driving the LAX3YFP fusion protein. Error bars show the SE of the mean (n≥8). **(C)** Induction of the LAX3YFP fusion protein by auxin and quantification of the fluorescence in cortical cells. Error bars show the SD of the average of at least 4 strips of cortical cells.

2.4.5. *LAX3* promoter deletions reveal several regulatory regions

The ARE present in the *LAX3* promoter does not appear to be essential for LAX3 auxin induction. To assess the role of the other motifs identified, a promoter deletion approach was employed. The *LAX3* promoter was truncated at four different position (Figure 2-4 A), fused to the LAX3YFP reporter and the constructs transformed into *lax3 aux1* double mutants for the same reason as explained before. At least three independent lines segregating 3/1 at the T2 generation were obtained for each construct.

Promoter deletion lines were scored for complementation of the *lax3aux1* lateral root phenotype. Ten days after germination, the lateral root density was compared with an intact promoter. We observed that all Δ1 and Δ2 lines fully complemented the mutant phenotype (Figure 2-4 B). This result demonstrates that the 826 base pairs sequence upstream of the *LAX3* gene is sufficient to drive expression and regulate lateral root emergence. On the other hand, it has been observed that all Δ3 lines only partially complemented the root phenotype whereas no complementation was observed for every Δ4 lines analysed (Figure 2-4 B).

Quantitative analysis of the induction of the LAX3YFP reporter following auxin treatment (as describe in section 2.4.4) yielded similar results. No changes in LAX3YFP intensity were observed in either Δ1 or Δ2 lines whereas there was a reduction of fluorescence in Δ3 lines and barely any detectable fluorescence in Δ4 lines (the results using a representative line for each promoter deletion construct is shown on Figure 2-4 C).

These results suggest that *LAX3* is positively regulated by at least two different portions of its promoter: first, a domain between -826 and -570 regulates the intensity of *LAX3* induction, as the reporter can still be induced in the Δ3 line but at reduced levels, and second a domain between -570 and -363 regulates *LAX3* induction as the reporter is not induced in the Δ4 line 24 hours after auxin treatment.

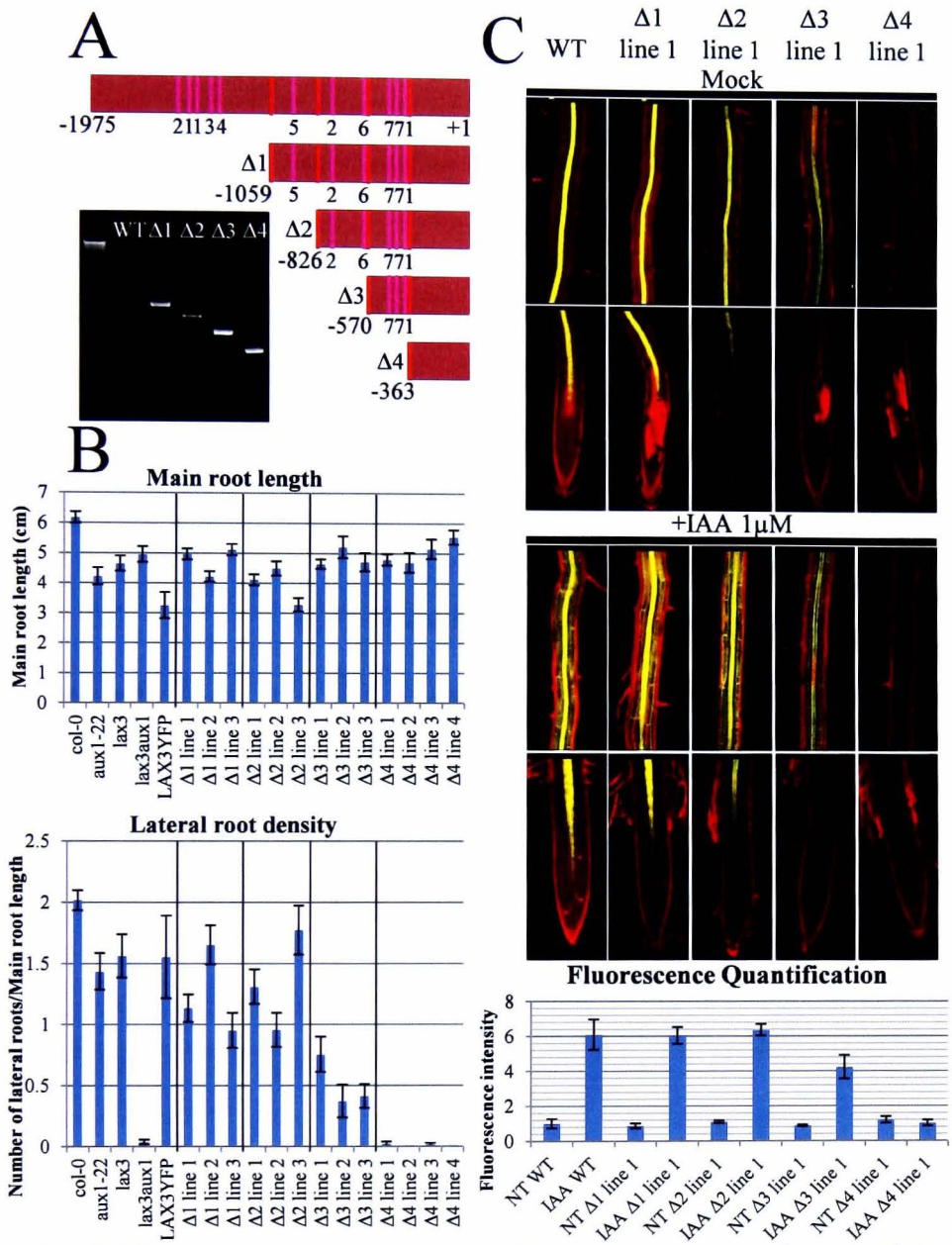

Figure 2-4 *LAX3*'s promoter sequence contains several regions that regulate *LAX3* expression and induction. (A) Schematic representation of *LAX3* promoter, the different binding sites identified and the position of the deletions. Numbers correspond to the sites on Table 2-2. **(B)** Main root length and lateral root density of WT, *aux1*, *lax3*, *lax3aux1* and *lax3aux1* plants transformed with the WT (LAX3YFP) or the truncated LAX3 promoter driving a functional LAX3YFP fusion protein. Error bars show the SE of the mean (n≥8). **(C)** Induction of the LAX3YFP fusion protein by auxin and quantification of the fluorescence in cortical cells. Error bars show the SD of the average fluorescence intensity of at least 4 strips of cortical cells (2 plants).

2.4.6. Transcript profiling to determine the *LAX3* regulatory network

Based on the data presented so far, the network analysis suggests that *LAX3* auxin induction has two edges: an activating edge, which is mediated by ARF7 and a repressive edge that is mediated by ARF19. On Figure 2-5 a regulatory network for *LAX3* is presented. As ARF19 is thought to be an activator (Okushima et al., 2005), another transcription factor, termed TFY for Transcription Factor Y, has been added. It is supposed to be a repressor, that is ARF7 and ARF19 dependent and which is a secondary auxin responsive gene.

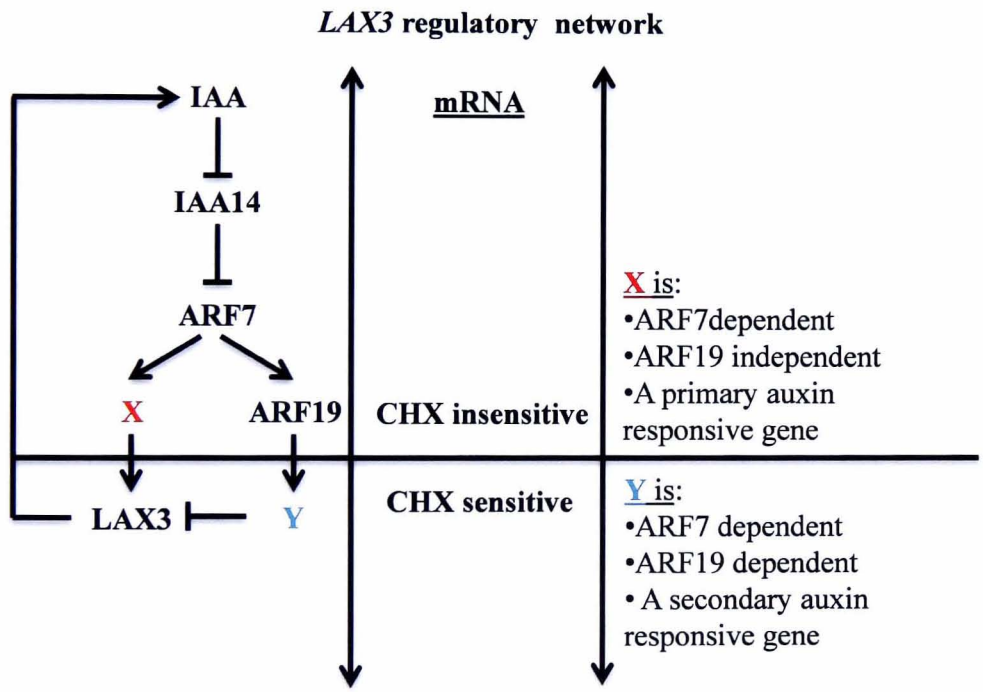

Figure 2-5 The *LAX3* regulatory network. Expected properties of transcription factors X and Y are indicated on the right side of the figure.

To obtain a list of putative candidates for transcription factors X (TFX) and Y (TFY) several transcriptomics datasets were analysed. First, genes differentially expressed after 2 hours of auxin treatments (IAA, 1 μM) in whole seedlings of Col-0, *arf7*, *arf19* and *arf7arf19* mutants were identified using published datasets (Okushima et al., 2005). Secondly, genes differentially expressed after 6 hours of auxin treatment (NAA 10μM) in the mature part of Col-0 roots were identified using a dataset generated in our laboratory (see chapter 4 for detail). The main use of the latter is to filter candidate genes from the former datasets to restrict it to root specific genes.

Based on the model of the *LAX3* regulatory network (Figure 2-5) the following filters were applied: (1) TFX is differentially expressed in *col-0* and *arf19* mutants but not in *arf7* or *arf7arf19* mutants. (2) Y is differentially expressed in col-0 but not in *arf19*, *arf7* or *arf7arf19* mutants. (3) X and Y are differentially expressed in the root dataset.

An initial list of 152 transcription factors differentially expressed in the Okushima datasets was reduced to 43 candidate genes for TFX and 25 for TFY. Because the root dataset contains differentially expressed genes after 6 hours of auxin treatment it won't include rapidly induced genes. If the third filter is not applied, there are 78 candidate genes for X and 51 for Y. The list of genes obtained when taking into account the root dataset is the "top priority" list and the one without is the "medium priority" list (Figure 2-6 and Figure 2-7).

Candidate genes for TFX, group 1						
ATG	Description	col-0	arf7	arf19	arf7arf19	NAA
AT1G05710	ethylene	1.93		1.70		2.08
AT1G15580	IAA5	21.57	4.90	21.27	1.62	16.09
AT1G33420	PHD	1.83		1.58		6.23
AT1G53170	ERF8	1.56		1.55		2.38
AT1G71030	MYBL2	1.56		1.59		-2.45
AT2G34140	Dof	2.69		2.05		4.47
AT2G43060	TF	1.75		1.63		1.58
AT2G47260	WRKY23	2.62		2.41		16.59
AT3G16500	PAP1	1.89		1.88		3.42
AT3G55730	MYB109	1.89		1.60		1.56
AT4G09460	MYB6	1.63		1.54		4.66
AT4G14550	IAA14	2.21		2.36		8.33
AT4G28640	IAA11	4.80	2.15	2.64		27.62
AT4G29190	CCCH	2.07		2.00		2.26
AT4G30080	ARF16	2.84		3.32		4.08
AT4G32280	IAA29	23.79	5.16	25.04		85.48
AT5G26930	GATA	6.39	3.04	4.25		114.59
AT5G47370	HAT2	8.01	2.18	8.28		10.98
AT5G48150	PAT1	1.87		1.87		-1.52
AT5G53290	CRF3	2.95		2.39		38.13
AT5G58620	CCCH	1.98		2.01		2.18
AT5G65320	bHLH	4.58		4.96		11.09
AT5G65640	BHLH093	2.00		1.71		2.96
AT5G65670	IAA9	1.71		2.29		3.10

Candidate genes for TFX, group 3						
ATG	Description	col-0	arf7	arf19	arf7arf19	NAA
AT1G01010	ANAC001	1.89		1.60		
AT1G09530	PIF3	1.80		1.98		
AT1G18400	BEE1	1.83		2.95		
AT1G18570	MYB51	1.74		1.79		
AT1G21910	AP2	1.95		2.64		
AT1G25560	AP2	2.71		2.36		
AT1G34670	MYB93	3.68		2.83		
AT1G44830	AP2	2.93		3.55		
AT1G79700	TF	1.79		1.74		
AT1G80840	WRKY40	3.33		2.67		
AT2G01200	IAA32	3.68		2.76		
AT2G43140	TF	1.50		1.63		
AT3G01970	WRKY45	3.33		2.08		
AT3G23050	IAA7	1.75		1.86		
AT3G23240	ERF1	2.71		2.04		
AT3G25710	bHLH	1.55		1.99		
AT3G60530	GATA	2.53		2.19		
AT4G00050	UNE10	1.92		1.84		
AT4G23810	WRKY53	1.98		1.94		
AT4G25410	bHLH	2.32		2.14		
AT5G15160	bHLH	2.30		2.32		
AT5G25190	ethylene	4.67	1.77	5.23		
AT5G28300	TF	2.08		2.05		
AT5G44260	CCCH	5.98		4.82		
AT5G60200	Dof	1.64		1.70		
AT5G67060	HEC1	5.83		6.44		

Candidate genes for TFX, group 2						
ATG	Description	col-0	arf7	arf19	arf7arf19	NAA
AT1G13300	myb	-1.73		-2.18		-5.44
AT1G72360	ethylene	-1.84		-1.61		-2.19
AT1G74840	myb	-2.46		-2.28		-5.19
AT1G76890	GT2	-1.80		-1.77		-5.17
AT1G77920	bZIP	-1.69		-1.67		-1.82
AT2G23340	AP2	-1.61		-1.83		-3.30
AT2G28550	TOE1	-1.62		-1.59		-2.29
AT2G44940	AP2	-1.92		-1.56		-4.56
AT3G46130	MYB111	-3.12		-2.57		-25.99
AT5G05790	myb	-2.01		-1.69		-4.14
AT5G07580	TF	-2.13		-1.81		-6.08
AT5G15830	BZIP3	-2.55		-2.48		-13.08
AT5G25810	TNY	-2.70		-2.49		-6.59
AT5G44190	GLK2	-1.89		-1.89		-2.16
AT5G57150	bHLH	-2.00		-1.79		-9.61
AT5G59780	MYB59	-1.87		-1.73		-4.35
AT5G60890	MYB34	-5.59	-1.61	-4.65	-1.51	-8.03
AT5G61420	MYB28	-3.37		-3.01		-16.07
AT5G65210	TGA1	-2.06		-1.89		-1.75

Candidate genes for TFX, group 4						
ATG	Description	col-0	arf7	arf19	arf7arf19	NAA
AT1G77200	AP2	-1.58		-1.55		
AT2G23760	BLH4	-1.56		-1.82		
AT4G32980	ATH1	-1.71		-1.56		
AT5G07690	MYB29	-2.05		-2.17		
AT5G11060	KNAT4	-1.71		-1.59		
AT5G14340	MYB40	-2.05		-1.75		
AT5G46690	BHLH071	-2.12		-2.05		
AT5G49330	MYB111	-1.63		-1.57		
AT5G56860	GNC	-1.64		-1.66		

LAX3						
ATG	Description	col-0	arf7	arf19	arf7arf19	NAA
AT1G77690	LAX3	2.40		2.54		3.20

Figure 2-6 Putative candidates for TFX are differentially expressed after two hours of auxin treatment in col-0 and *arf19* but not in *arf7* and *arf7 arf19* mutants. Some genes are still induced in *arf7* mutants but the induction fold is at least two times reduced compared to the WT control. Group 1 and 2 contain genes differentially expressed in the root after 6 hours of auxin treatment whereas genes in group 3 and 4 were not expressed or differentially expressed in the root

dataset. Group 1 and 3 contain induced genes, group 2 and 4 repressed genes. Values are fold changes compared to untreated controls. Values highlighted in red indicate the highest fold change and in green the lowest. Values in between are shown in a gradient between red and green.

Candidate genes for TFY, group 1						
ATG	Description	col-0	*arf7*	*arf19*	*arf7arf19*	NAA
AT1G19220	ARF19	2.21				6.61
AT1G25280	TLP10	1.67				1.86
AT1G28370	ERF11	6.23	3.32	2.96		1.89
AT1G52880	NAM	1.86				3.62
AT1G57560	MYB50	1.65				-1.70
AT1G68840	RAV2	1.59				-1.94
AT1G74660	MIF1	2.44				3.14
AT2G25900	ATCTH	1.68				-4.02
AT3G55980	CCCH	1.80				-1.51
AT3G60630	SCL	1.76				1.98
AT4G05100	MYB74	1.79				-2.60
AT4G23750	CRF2	1.59				57.54
AT5G13330	RAP2.6L	2.11				2.13
AT5G18560	PUCHI	3.25	1.75			66.34
AT5G47230	ERF5	1.83				-1.53
AT5G60450	ARF4	1.53				7.84

Candidate genes for TFY, group 3						
ATG	Description	col-0	*arf7*	*arf19*	*arf7arf19*	NAA
AT1G01720	ATAF1	1.75				
AT1G29160	Dof	1.60				
AT1G50420	SCL3	1.66				
AT1G74650	MYB31	2.30				
AT3G24500	MBF1C	1.59				
AT3G49530	ANAC062	1.56				
AT5G04340	C2H2	2.11				
AT5G43170	AZF3	1.56				
AT5G65310	ATHB5	1.75				

Candidate genes for TFY, group 2						
ATG	Description	col-0	*arf7*	*arf19*	*arf7arf19*	NAA
AT1G19700	BEL10	-1.73				-3.12
AT1G76880	TF	-1.56				-1.60
AT2G16400	BLH7	-1.52				-2.40
AT3G13040	myb	-1.61				-2.00
AT3G51910	HSFA7A	-1.63				-2.59
AT4G17880	bHLH	-1.85				-1.87
AT5G25160	ZFP3	-1.84				-4.38
AT5G25830	GATA	-2.44				-3.99
AT5G61590	AP2	-1.75				-4.66

LAX3						
ATG	Description	col-0	*arf7*	*arf19*	*arf7arf19*	NAA
AT1G77690	LAX3	2.40		2.54		3.20

Candidate genes for TFY, group 4						
ATG	Description	col-0	*arf7*	*arf19*	*arf7arf19*	NAA
AT1G12860	bHLH	-1.50				
AT1G16060	TF	-1.63				
AT1G69810	WRKY36	-1.51				
AT2G25000	WRKY60	-1.53				
AT2G31070	TCP10	-1.52				
AT2G39250	SNZ	-1.51				
AT2G45190	AFO	-1.50				
AT3G28910	MYB30	-1.61				
AT3G49930	C2H2	-1.65				
AT3G54990	SMZ	-1.51				
AT3G57800	bHLH	-1.56				
AT3G58710	WRKY69	-1.71				
AT4G00480	MYC1	-1.60				
AT5G15130	WRKY72	-1.65				
AT5G42630	ATS	-1.64				
AT5G54630	TF	-1.51				
AT5G67450	AZF1	-1.51				

Figure 2-7 Putative candidates for TFY are differentially expressed after two hours of auxin treatment in col-0 but not in *arf19*, *arf7* and *arf7 arf19* mutants. Some genes are still differentially expressed in *arf7* or *arf19* mutants but the fold change is at least two times reduced compared to the WT control. Group 1 and 2 contain genes differentially expressed in the root after 6 hours of auxin treatment whereas genes in group 3 and 4 were not expressed or differentially expressed in the root. Group 1 and 3 contain induced genes, group 2 and 4 repressed genes. Values are fold changes compared to untreated controls. Values highlighted in red indicate the highest fold change and in green the lowest. Values in between are shown in a gradient between red and green.

2.5. Discussion

LAX3 is a secondary auxin responsive gene, which is induced three hours after auxin treatment. The delay in induction is likely to be due to the requirement to transcribe and translate an intermediate transcription factor as treatments with auxin and CHX failed to induce *LAX3*. Mutant analysis revealed that ARF7 is the only ARF required for *LAX3* activation and more surprisingly that ARF19 negatively regulates *LAX3*. It is hypothesised that ARF7 induces a transcription factor (TF X) that induces LAX3 and that ARF19 induces a repressor (TF Y) that attenuates the induction of LAX3 by auxin.

It has been shown in previous studies that a transcription factor, NAC1, which positively regulate auxin signalling, is down regulated by two pathways (ubiquitination/proteasome via SINAT5 (Xie et al., 2002) and micro RNA with miRNA164 (Guo et al., 2005)) so to attenuate the auxin signal during lateral root formation. Unfortunately, in the microarray datasets and in the RT qPCR analysed in this chapter work, NAC1 is not induced by auxin and therefore was not retained as a putative candidate for TFX or TFY.

Analysis of the *LAX3* promoter revealed the presence of 11 auxin responsive motifs with notably one ARE. Mutations in the ARE revealed that it functions negatively to regulate *LAX3*. This suggests that a repressive ARF binds this site. On the other hand, a promoter deletion approach showed that several regions positively regulate *LAX3*. Notably, two Dof domain transcription factor binding sites are located in what appears to be a key region (Table 2-2 and Figure 2-4). In contrast to the increased induction of the LAX3YFP reporter when the ARE is mutated (Figure 2-3), Δ2 lines do not show the same observation despite the fact that they do not include the ARE which is counter

intuitive. The most likely explanation is that in the deletion constructs all the sequences upstream of the ARE are removed and therefore the genomic context of the ARE is lost including enhancer sequences.

The results are summarised in a model of the *LAX3* regulatory network in Figure 2-5.

The analysis of several transcriptomics datasets yielded lists of transcription factors that could potentially regulate *LAX3* (Figure 2-6 and Figure 2-7). This approach is comprehensive but has several issues: First, Gene Ontologies (GO) annotations are not always accurate (for example, LBD transcription factors are not annotated as transcription factors). Secondly, the biological function of the selected genes is not taken into consideration (functions in root formation and/or auxin response). Thirdly, homologous genes are not included (the proteins involved in the auxin response pathway usually belong to multi gene families). To overcome these issues, a thorough analysis of several transcription factor families was done based on published data showing their role in the auxin response pathway (Chapman and Estelle, 2009; Paponov et al., 2008). Hence, the analysis included the following gene families: DOF domain, LOB/LBD/ASL (Lateral Organ Boundaries-Domain/Asymmetric Leaves Like), HB (HOMEOBOX, including the related ZF-HD, Zinc Finger Homeodomain) and the ARFs.

Several lines of evidence support a role for Dof transcription factors in the auxin response pathway. First, NtBBF1, a tobacco Dof domain protein, was shown to bind on the promoter of a late auxin responsive gene, rolB (Baumann et al., 1999). A single mutation in its binding site blocked the auxin induction of the gene suggesting that rolB is a direct target of NtBBF1. Nevertheless, the authors reported that NtBBF1 levels are

not regulated by auxin; hence another primary auxin responsive gene must regulate rolB together with NtBBF1. In other cases, it has been shown that some Dof proteins are auxin inducible. For example, in Arabidopsis, the auxin response factor MONOPTEROS/ARF5 directly binds the promoter of AtDOF5.3 and induces its expression (Schlereth et al., 2010). Transcriptomic datasets have revealed several other auxin inducible Dof proteins (this study).

Several LOB proteins were identified as downstream targets of ARF7 and ARF19 in Arabidopsis (Lee and Kim, 2010; Okushima et al., 2007). Overexpression of LBD16, 18 or 29 partially rescued the lateral root defects of *arf7arf19* double mutants. Interestingly, an orthologous gene of LBD16 and 29 in rice, termed CRL1, encodes a transcription factor involved in the formation of crown and lateral roots (Inukai et al., 2001; Inukai et al., 2005). CRL1, like its Arabidopsis counterpart, is a primary auxin responsive gene which is directly regulated by OsARF1. Other LBD genes from rice (ARL1) and maize (RTCS) have been shown to be auxin inducible and to regulate root architecture (Liu et al., 2005; Taramino et al., 2007). Many LOB genes are regulated by auxin and ARFs in Arabidopsis roots as well (Okushima et al., 2007).

In the case of the HB family of transcription factors, one member has been shown to be rapidly induced by auxin (HAT2 within 15 minutes) (Sawa et al., 2002). Overexpression of HAT2 leads to opposite phenotypes in the shoot and in the root. Whereas shoot phenotypes resemble auxin overproducing mutants (long hypocotyls, epinastic cotyledons, long petioles, and small leaves) the root phenotypes resemble auxin insensitive mutants (reduction in lateral root number, reduced sensitivity of root growth

to auxin). Hence, HAT2 may function as a repressor of the auxin response pathway in the Arabidopsis root. Many other members of the HB family were shown to be up or down-regulated by auxin (Baima et al., 1995; Donner et al., 2010; Plesch et al., 1997; Son et al., 2004) with diverse role during development (notably vascular differentiation). Finally, members of the ARF family of transcription factors are obvious candidates for TFY since mutation in the ARF binding site leads to an increased induction of *LAX3*. Interestingly, several negative ARFs fit with the properties of TFY and are shown on Figure 2-8. ARF19 is amongst those candidates except that it is not a negative ARF. Further studies will be required to assess the role of ARF19 on LAX3 expression, using protoplasts systems for example. Nevertheless, as these experiments are done in different tissues using plant material that has been protoplasted, a complimentary approach will be to swap ARF7 and ARF19 domains I and II and analyse the effects on LAX3 expression.

To summarize the analysis, Figure 2-8 shows members of these 5 genes families which could be, based on their expression values, TFX or TFY. To take into account redundancy, closely related genes are shown even if they don't change their expression in presence of auxin. In conclusion, a list of 45 top priority targets for TFX and 27 for TFY will be further analysed to identify TFX and TFY.

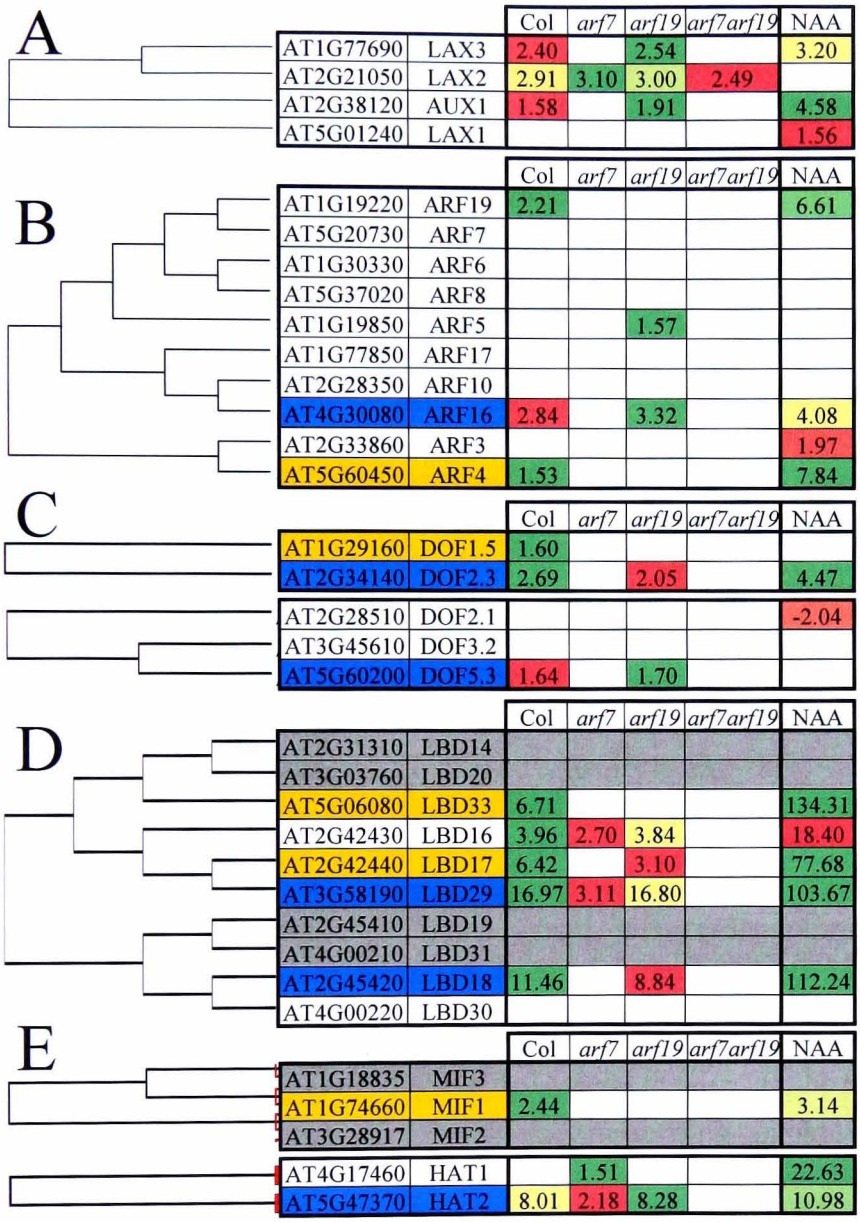

Figure 2-8 Phylogenetic trees of candidate genes for TFX and TFY. Differentially expressed genes of several families in the Okushima (col, *arf7*, *arf19* and *arf7 arf19*) and the root (NAA) datasets along with their closely related homologs are displayed. **(A)** AUX/LAX, **(B)** ARF, **(C)** Dof domain, **(D)** LOB domain and **(E)** HB/HD zinc finger. Gene numbers and names highlighted in blue show candidate genes for TFX, in yellow for TFY and in dark grey genes that are not represented on the ATH1 chip. Values are fold changes compared to untreated controls. Values highlighted in red indicate the highest fold change and in green the lowest. Values in between are shown in a gradient between red and green.. Differentially expressed genes have an adjusted p-Value < 0.05 and a fluorescence intensity above 50.

2.6. References

Abel, S., Ballas, N., Wong, L. M. and Theologis, A. (1996). DNA elements responsive to auxin. *Bioessays* **18**, 647-54.

Bai, F., Watson, J. C., Walling, J., Weeden, N., Santner, A. A. and DeMason, D. A. (2005). Molecular characterization and expression of PsPK2, a PINOID-like gene from pea (Pisum sativum) *Plant Sci* **168**, 1281-1291

Baima, S., Nobili, F., Sessa, G., Lucchetti, S., Ruberti, I. and Morelli, G. (1995). The expression of the Athb-8 homeobox gene is restricted to provascular cells in Arabidopsis thaliana. *Development* **121**, 4171.

Baumann, K., De Paolis, A., Costantino, P. and Gualberti, G. (1999). The DNA binding site of the Dof protein NtBBF1 is essential for tissue-specific and auxin-regulated expression of the rolB oncogene in plants. *Plant Cell* **11**, 323-34.

Benfey, P. N. and Chua, N. H. (1990). The Cauliflower Mosaic Virus 35S Promoter: Combinatorial Regulation of Transcription in Plants. *Science* **250**, 959-966.

Benjamini, Y. and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)* **57**, 289-300.

Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G. and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell* **115**, 591-602.

Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K. and Scheres, B. (2005). The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. *Nature* **433**, 39-44.

Casimiro, I., Beeckman, T., Graham, N., Bhalerao, R., Zhang, H., Casero, P., Sandberg, G. and Bennett, M. J. (2003). Dissecting Arabidopsis lateral root development. *Trends Plant Sci* **8**, 165-71.

Casimiro, I., Marchant, A., Bhalerao, R. P., Beeckman, T., Dhooge, S., Swarup, R., Graham, N., Inze, D., Sandberg, G., Casero, P. J. et al. (2001). Auxin transport promotes Arabidopsis lateral root initiation. *Plant Cell* **13**, 843-52.

Chapman, E. J. and Estelle, M. (2009). Mechanism of auxin-regulated gene expression in plants. *Annual Review of Genetics* **43**, 265-285.
Davuluri, R. V., Sun, H., Palaniswamy, S. K., Matthews, N., Molina, C., Kurtz, M. and Grotewold, E. (2003). AGRIS: Arabidopsis gene regulatory information server, an information resource of Arabidopsis cis-regulatory elements and transcription factors. *BMC bioinformatics* **4**, 25.
Delker, C., Raschke, A. and Quint, M. (2008). Auxin dynamics: the dazzling complexity of a small molecule's message. *Planta* **227**, 929-941.
Dello Ioio, R., Nakamura, K., Moubayidin, L., Perilli, S., Taniguchi, M., Morita, M. T., Aoyama, T., Costantino, P. and Sabatini, S. (2008). A genetic framework for the control of cell division and differentiation in the root meristem. *Science* **322**, 1380-4.
Dharmasiri, N., Dharmasiri, S. and Estelle, M. (2005). The F-box protein TIR1 is an auxin receptor. *Nature* **435**, 441-445.
Donner, T. J., Sherr, I. and Scarpella, E. (2010). Auxin signal transduction in Arabidopsis vein formation. *Plant signaling & behavior* **5**.
dos Santos Maraschin, S., Memelink, J. and Offringa, R. (2009). Auxin-induced, SCF(TIR1)-mediated poly-ubiquitination marks AUX/IAA proteins for degradation. *Plant J* **59**, 100-9.
Dreher, K. A., Brown, J., Saw, R. E. and Callis, J. (2006). The Arabidopsis Aux/IAA protein family has diversified in degradation and auxin responsiveness. *Plant Cell* **18**, 699-714.
Dubrovsky, J. G., Doerner, P. W., Colon-Carmona, A. and Rost, T. L. (2000). Pericycle cell proliferation and lateral root initiation in Arabidopsis. *Plant Physiol* **124**, 1648-57.
Fukaki, H., Nakao, Y., Okushima, Y., Theologis, A. and Tasaka, M. (2005). Tissue-specific expression of stabilized SOLITARY-ROOT/IAA14 alters lateral root development in Arabidopsis. *Plant J* **44**, 382-95.
Fukaki, H., Tameda, S., Masuda, H. and Tasaka, M. (2002). Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY-ROOT/IAA14 gene of Arabidopsis. *Plant J* **29**, 153-68.
Fukaki, H., Taniguchi, N. and Tasaka, M. (2006). PICKLE is required for SOLITARY-ROOT/IAA14-mediated repression of ARF7 and ARF19 activity during Arabidopsis lateral root initiation. *Plant J* **48**, 380-9.
Gentleman, R., Carey, V., Bates, D., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y. and Gentry, J. (2004). Bioconductor:

open software development for computational biology and bioinformatics. *Genome biology* **5**, R80.
Guilfoyle, T. J. and Hagen, G. (2007). Auxin response factors. *Curr Opin Plant Biol* **10**, 453-60.
Guo, H. S., Xie, Q., Fei, J. F. and Chua, N. H. (2005). MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for Arabidopsis lateral root development. *The Plant Cell Online* **17**, 1376.
Higo, K., Ugawa, Y., Iwamoto, M. and Higo, H. (1998). PLACE: a database of plant cis-acting regulatory DNA elements. *Nucleic Acids Res* **26**, 358-9.
Hobbie, L. and Estelle, M. (1995). The axr4 auxin-resistant mutants of Arabidopsis thaliana define a gene important for root gravitropism and lateral root initiation. *Plant J* **7**, 211-20.
Huson, D. H., Richter, D. C., Rausch, C., Dezulian, T., Franz, M. and Rupp, R. (2007). Dendroscope: An interactive viewer for large phylogenetic trees. *BMC bioinformatics* **8**, 460.
Inukai, Y., Miwa, M., Nagato, Y., Kitano, H. and Yamauchi, A. (2001). Characterization of rice mutants deficient in the formation of crown roots. *Breeding Science* **51**, 123-129.
Inukai, Y., Sakamoto, T., Ueguchi-Tanaka, M., Shibata, Y., Gomi, K., Umemura, I., Hasegawa, Y., Ashikari, M., Kitano, H. and Matsuoka, M. (2005). Crown rootless1, which is essential for crown root formation in rice, is a target of an AUXIN RESPONSE FACTOR in auxin signaling. *Plant Cell* **17**, 1387-96.
Kepinski, S. and Leyser, O. (2005). The Arabidopsis F-box protein TIR1 is an auxin receptor. *Nature* **435**, 446-51.
Knox, K., Grierson, C. S. and Leyser, O. (2003). AXR3 and SHY2 interact to regulate root hair development. *Development* **130**, 5769.
Koshiba, T., Ballas, N., Wong, L. M. and Theologis, A. (1995). Transcriptional regulation of PS-IAA4/5 and PS-IAA6 early gene expression by indoleacetic acid and protein synthesis inhibitors in pea (Pisum sativum). *Journal of molecular biology* **253**, 396-413.
Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A. and Lopez, R. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947.

Lee, H. W. and Kim, J. (2010). Ectopic Expression of LBD18/ASL20 Results in Arrest of Plant Growth and Development with Repression of AINTEGUMENTA and PLETHORA Genes. *Journal of Plant Biology*, 1-8.
Lee, H. W., Kim, N. Y., Lee, D. J. and Kim, J. (2009). LBD18/ASL20 regulates lateral root formation in combination with LBD16/ASL18 downstream of ARF7 and ARF19 in Arabidopsis. *Plant physiology* **151**, 1377.
Liu, H., Wang, S., Yu, X., Yu, J., He, X., Zhang, S., Shou, H. and Wu, P. (2005). ARL1, a LOB-domain protein required for adventitious root formation in rice. *Plant J* **43**, 47-56.
Lokerse, A. S. and Weijers, D. (2009). Auxin enters the matrix. Assembly of response machineries for specific outputs. *Current opinion in plant biology* **12**, 520-526.
Mallory, A. C., Bartel, D. P. and Bartel, B. (2005). MicroRNA-directed regulation of Arabidopsis AUXIN RESPONSE FACTOR17 is essential for proper development and modulates expression of early auxin response genes. *Plant Cell* **17**, 1360-75.
Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. plant* **15**, 473-97.
Okushima, Y., Fukaki, H., Onoda, M., Theologis, A. and Tasaka, M. (2007). ARF7 and ARF19 regulate lateral root formation via direct activation of LBD/ASL genes in Arabidopsis. *The Plant Cell Online* **19**, 118.
Okushima, Y., Overvoorde, P. J., Arima, K., Alonso, J. M., Chan, A., Chang, C., Ecker, J. R., Hughes, B., Lui, A., Nguyen, D. et al. (2005). Functional genomic analysis of the AUXIN RESPONSE FACTOR gene family members in Arabidopsis thaliana: unique and overlapping functions of ARF7 and ARF19. *Plant Cell* **17**, 444-63.
Overvoorde, P., Fukaki, H. and Beeckman, T. (2010). Auxin Control of Root Development. *Cold Spring Harbor Perspectives in Biology* **2**.
Overvoorde, P. J., Okushima, Y., Alonso, J. M., Chan, A., Chang, C., Ecker, J. R., Hughes, B., Liu, A., Onodera, C., Quach, H. et al. (2005). Functional genomic analysis of the AUXIN/INDOLE-3-ACETIC ACID gene family members in Arabidopsis thaliana. *Plant Cell* **17**, 3282-300.
Paponov, I. A., Paponov, M., Teale, W., Menges, M., Chakrabortee, S., Murray, J. A. H. and Palme, K. (2008). Comprehensive transcriptome analysis of auxin responses in Arabidopsis. *Molecular plant*.

Pastuglia, M., Roby, D., Dumas, C. and Cock, J. M. (1997). Rapid induction by wounding and bacterial infection of an S gene family receptor-like kinase gene in Brassica oleracea. *Plant Cell* **9**, 49-60.

Peret, B., De Rybel, B., Casimiro, I., Benkova, E., Swarup, R., Laplaze, L., Beeckman, T. and Bennett, M. J. (2009a). Arabidopsis lateral root development: an emerging story. *Trends Plant Sci* **14**, 399-408.

Peret, B., Larrieu, A. and Bennett, M. J. (2009b). Lateral root emergence: a difficult birth. *J Exp Bot.*

Perez-Rodriguez, P., Riano-Pachon, D. M., Correa, L. G. G., Rensing, S. A., Kersten, B. and Mueller-Roeber, B. (2009). PlnTFDB: updated content and new features of the plant transcription factor database. *Nucleic Acids Research.*

Plesch, G., Störmann, K., Torres, J. T., Walden, R. and Somssich, I. (1997). Developmental and auxin-induced expression of the Arabidopsis prha homeobox gene. *The Plant Journal* **12**, 635-647.

Ramos, J. A., Zenser, N., Leyser, O. and Callis, J. (2001). Rapid degradation of auxin/indoleacetic acid proteins requires conserved amino acids of domain II and is proteasome dependent. *Plant Cell* **13**, 2349-60.

Rogg, L. E., Lasswell, J. and Bartel, B. (2001). A gain-of-function mutation in IAA28 suppresses lateral root development. *Plant Cell* **13**, 465-80.

Rombauts, S., Dehais, P., Van Montagu, M. and Rouze, P. (1999). PlantCARE, a plant cis-acting regulatory element database. *Nucleic Acids Res* **27**, 295-6.

Sakai, T., Takahashi, Y. and Nagata, T. (1996). Analysis of the promoter of the auxin-inducible gene, parC, of tobacco. *Plant Cell Physiol* **37**, 906-13.

Sawa, S., Ohgishi, M., Goda, H., Higuchi, K., Shimada, Y., Yoshida, S. and Koshiba, T. (2002). The HAT2 gene, a member of the HD Zip gene family, isolated as an auxin inducible gene by DNA microarray screening, affects auxin response in Arabidopsis. *The Plant Journal* **32**, 1011-1022.

Schlereth, A., Möller, B., Liu, W., Kientz, M., Flipse, J., Rademacher, E. H., Schmid, M., Jürgens, G. and Weijers, D. (2010). MONOPTEROS controls embryonic root initiation by regulating a mobile transcription factor. *Nature* **464**, 913-916.

Shin, R., Burch, A. Y., Huppert, K. A., Tiwari, S. B., Murphy, A. S., Guilfoyle, T. J. and Schachtman, D. P. (2007). The Arabidopsis

transcription factor MYB77 modulates auxin signal transduction. *The Plant Cell Online* **19**, 2440.

Son, O., Cho, H. Y., Kim, M. R., Lee, H., Lee, M. S., Song, E., Park, J. H., Nam, K. H., Chun, J. Y. and Kim, H. J. (2004). Induction of a homeodomain-leucine zipper gene by auxin is inhibited by cytokinin in Arabidopsis roots. *Biochemical and biophysical research communications* **326**, 203-209.

Swarbreck, D., Wilks, C., Lamesch, P., Berardini, T. Z., Garcia-Hernandez, M., Foerster, H., Li, D., Meyer, T., Muller, R. and Ploetz, L. (2007). The Arabidopsis Information Resource (TAIR): gene structure and function annotation. *Nucleic Acids Research.*

Swarup, K., Benkova, E., Swarup, R., Casimiro, I., Peret, B., Yang, Y., Parry, G., Nielsen, E., De Smet, I., Vanneste, S. et al. (2008). The auxin influx carrier LAX3 promotes lateral root emergence. *Nat Cell Biol* **10**, 946-54.

Szemenyei, H., Hannon, M. and Long, J. A. (2008). TOPLESS mediates auxin-dependent transcriptional repression during Arabidopsis embryogenesis. *Science* **319**, 1384.

Tan, X., Calderon-Villalobos, L. I., Sharon, M., Zheng, C., Robinson, C. V., Estelle, M. and Zheng, N. (2007). Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature* **446**, 640-5.

Taramino, G., Sauer, M., Stauffer, J. L., Jr., Multani, D., Niu, X., Sakai, H. and Hochholdinger, F. (2007). The maize (Zea mays L.) RTCS gene encodes a LOB domain protein that is a key regulator of embryonic seminal and post-embryonic shoot-borne root initiation. *Plant J* **50**, 649-59.

Tatematsu, K., Kumagai, S., Muto, H., Sato, A., Watahiki, M. K., Harper, R. M., Liscum, E. and Yamamoto, K. T. (2004). MASSUGU2 encodes Aux/IAA19, an auxin-regulated protein that functions together with the transcriptional activator NPH4/ARF7 to regulate differential growth responses of hypocotyl and formation of lateral roots in Arabidopsis thaliana. *Plant Cell* **16**, 379-93.

Team, R. D. C. (2007). R: A language and environment for statistical computing. *Vienna, Austria: R Foundation for Statistical Computing.*

Tian, Q. and Reed, J. W. (1999). Control of auxin-regulated root development by the Arabidopsis thaliana SHY2/IAA3 gene. *Development* **126**, 711-21.

Tiwari, S. B., Hagen, G. and Guilfoyle, T. (2003). The roles of auxin response factor domains in auxin-responsive transcription. *Plant Cell* **15**, 533-43.
Tiwari, S. B., Hagen, G. and Guilfoyle, T. J. (2004). Aux/IAA proteins contain a potent transcriptional repression domain. *Plant Cell* **16**, 533-43.
Tiwari, S. B., Wang, X. J., Hagen, G. and Guilfoyle, T. J. (2001). AUX/IAA proteins are active repressors, and their stability and activity are modulated by auxin. *Plant Cell* **13**, 2809-22.
Tromas, A. and Perrot-Rechenmann, C. (2010). Recent progress in auxin biology. *Comptes Rendus Biologies*.
Uehara, T., Okushima, Y., Mimura, T., Tasaka, M. and Fukaki, H. (2008). Domain II mutations in CRANE/IAA18 suppress lateral root formation and affect shoot development in Arabidopsis thaliana. *Plant Cell Physiol* **49**, 1025-38.
Ulmasov, T., Hagen, G. and Guilfoyle, T. J. (1997). ARF1, a transcription factor that binds to auxin response elements. *Science* **276**, 1865-8.
Ulmasov, T., Hagen, G. and Guilfoyle, T. J. (1999). Dimerization and DNA binding of auxin response factors. *Plant J* **19**, 309-19.
Ulmasov, T., Liu, Z. B., Hagen, G. and Guilfoyle, T. J. (1995). Composite structure of auxin response elements. *Plant Cell* **7**, 1611-23.
Vanneste, S., De Rybel, B., Beemster, G. T., Ljung, K., De Smet, I., Van Isterdael, G., Naudts, M., Iida, R., Gruissem, W., Tasaka, M. et al. (2005). Cell cycle progression in the pericycle is not sufficient for SOLITARY ROOT/IAA14-mediated lateral root initiation in Arabidopsis thaliana. *Plant Cell* **17**, 3035-50.
Vanneste, S. and Friml, J. (2009). Auxin: a trigger for change in plant development. *Cell* **136**, 1005-1016.
Wang, J. W., Wang, L. J., Mao, Y. B., Cai, W. J., Xue, H. W. and Chen, X. Y. (2005). Control of root cap formation by microRNA-targeted auxin response factors in Arabidopsis. *The Plant Cell Online* **17**, 2204.
Wilmoth, J. C., Wang, S., Tiwari, S. B., Joshi, A. D., Hagen, G., Guilfoyle, T. J., Alonso, J. M., Ecker, J. R. and Reed, J. W. (2005). NPH4/ARF7 and ARF19 promote leaf expansion and auxin-induced lateral root formation. *Plant J* **43**, 118-30.
Woodward, A. W. and Bartel, B. (2005). Auxin: regulation, action, and interaction. *Ann Bot (Lond)* **95**, 707-35.

Worley, C. K., Zenser, N., Ramos, J., Rouse, D., Leyser, O., Theologis, A. and Callis, J. (2000). Degradation of Aux/IAA proteins is essential for normal auxin signalling. *Plant J* **21**, 553-62.

Xie, Q., Frugis, G., Colgan, D. and Chua, N. H. (2000). Arabidopsis NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. *Genes Dev* **14**, 3024-36.

Xie, Q., Guo, H. S., Dallman, G., Fang, S., Weissman, A. M. and Chua, N. H. (2002). SINAT5 promotes ubiquitin-related degradation of NAC1 to attenuate auxin signals. *Nature* **419**, 167-170.

Xu, N., Hagen, G. and Guilfoyle, T. (1997). Multiple auxin response modules in the soybean SAUR 15A promoter. *Plant Sci* **126**, 193-201

Yang, X., Lee, S., So, J. H., Dharmasiri, S., Dharmasiri, N., Ge, L., Jensen, C., Hangarter, R., Hobbie, L. and Estelle, M. (2004). The IAA1 protein is encoded by AXR5 and is a substrate of SCF(TIR1). *Plant J* **40**, 772-82.

Yoon, E. K., Yang, J. H. and Lee, W. S. (2010). Auxin and Abscisic Acid Responses of Auxin Response Factor 3 in Arabidopsis Lateral Root Development. *Journal of Plant Biology* **53**, 150-154.

Zenser, N., Dreher, K. A., Edwards, S. R. and Callis, J. (2003). Acceleration of Aux/IAA proteolysis is specific for auxin and independent of AXR1. *Plant J* **35**, 285-94.

Chapter 3 Identification of novel classes of auxin response inhibitors employing a chemical genetics approach

3.1. Abstract

Auxin regulates lateral root emergence via the activation of the auxin influx carrier *LAX3* in cortical and epidermal cells overlying lateral root primordia. The pathway that leads to *LAX3* activation is complex and is likely to involve multiple transcription factors, of which several remain to be identified as it has been discussed in the previous chapter (Transcription Factor X and Transcription Factor Y). To overcome common issues associated with classical genetic approaches, such as redundancy or embryonic lethality, a chemical genetics screen was conducted to identify inhibitors of *LAX3* auxin induction. Phenotypic analysis of the positive hits showed that all affect root development (root growth or lateral root formation or both). A family of structurally related sulfonamides represented the largest class and was shown to disrupt primordia emergence. The results presented in this chapter provide important background information prior to target identification of these inhibitors.

3.2. Introduction

With the advent of Arabidopsis as a model organism, plant geneticists had the opportunity to easily carry out large scale forward genetics screens in order to identify key genes required during plant development (Meyerowitz, 2001). Similarly, reverse genetics has been greatly simplified with the availability of large libraries of T-DNA insertion lines (Alonso et al., 2003; Kuromori et al., 2004; Parinov and Sevugan, 1999;

Rosso et al., 2003; Sessions et al., 2002) and alternative approaches, such as RNAi or overexpression lines (Holtorf et al., 1995; Mello and Conte, 2004). Nevertheless, these approaches are often hampered by genetic redundancy and embryonic lethality (McCourt and Desveaux, 2010; Tóth and van der Hoorn, 2010).

In a chemical genetics approach, instead of modifying DNA sequences as in classical genetics, small organic molecules are used to directly affect protein activity (reviewed in (Stockwell, 2000; Stockwell, 2004). Chemical genetics screens use large libraries of compounds (>10 000) to test their effect on a given pathway. These screens are usually carried out either on a cell line or in whole organism (for example *Caenorhabditis elegans* and *Arabidopsis thaliana*). Screens carried out in Arabidopsis led to the identification of several important biological targets and compounds. These include the ABA receptor (Park et al., 2009), auxin response activators or inhibitors (reviewed in (De Rybel et al., 2009a), auxin transport inhibitors (De Rybel et al., 2009a), GSK3 kinases inhibitors (De Rybel et al., 2009b), cellulose synthase inhibitors (DeBolt et al., 2007), and others. In many of these discoveries, the chemical genetics approach helped overcoming either genetic redundancy or lethality: on one hand a compound can affect several proteins of the same family by targeting a conserved motif and on the other hand a compound can be applied at any time points to avoid interference with other cellular and/or developmental processes.

The importance of knocking down several proteins in one go is highlighted by the fact that 65% of all Arabidopsis genes are in families containing at least 2 members (The Arabidopsis Genome Initiative, 2000). This issue can be exemplified with the auxin response machinery. Auxin binds one of its 6 nuclear receptors (TIR1/AFB1-5) which

triggers the degradation of most of the 29 Aux/IAAs repressors and releases one or several of the 23 ARFs transcription factors to change gene expression (Okushima et al., 2005; Overvoorde et al., 2005; Parry et al., 2009). Loss of function mutations in single receptors, Aux/IAA or ARF very often do not have a clear phenotype due to the high degree of redundancy amongst each gene family. Therefore, multiple loss of function mutants or gain of function mutants had to be obtained in order to shed a light on how auxin regulates gene expression.

Auxin is a key signal during multiple developmental programs (Woodward and Bartel, 2005). In the root, this hormone triggers the genetic reprogramming of xylem pole pericycle cells to generate new lateral roots (Overvoorde et al., 2010). All the way from the priming of the pericycle cells to the emergence of the lateral root primordia, auxin regulates a large repertoire of genes that are involved in cell division, cell differentiation and cell elongation (Swarup et al., 2008; Vanneste et al., 2005). Components of the auxin response machinery involved in lateral root development include at least 6 ARFs and 6 Aux/IAAs (Fukaki et al., 2002; Mallory et al., 2005; Okushima et al., 2005; Rogg et al., 2001; Tatematsu et al., 2004; Tian and Reed, 1999; Uehara et al., 2008; Wang et al., 2005; Yang et al., 2004; Yoon et al., 2010). Two ARFs, ARF7 and 19, play a key role during lateral root formation at all stages (initiation, patterning and emergence respectively). Unfortunately, this complicates the analysis of their function during later stages since there are few (in single *arf7* mutants) or no (in double *arf7arf19* mutants) initiated primordia 10 days after germination (Okushima et al., 2005; Shin et al., 2007).

Previous studies carried out in our laboratory focussed on characterising the regulation and function of an auxin influx carrier, *LAX3*, during lateral root emergence (Swarup et

al., 2008). This gene is induced by auxin via ARF7 in several cells that directly overlay a lateral root primordium. The LAX3 protein is located at the plasma membrane where it increases the auxin influx in the overlying cells. A complex network of genes, which depends on the auxin content of the cell, triggers the activation of cell wall remodelling genes that facilitate emergence. In order to dissect the genetic program involved during lateral root emergence, a chemical genetic screen was conducted to identify chemicals that can inhibit the auxin induction of *LAX3*. Several positive hits were identified and their role during lateral root formation and root growth was analysed and a subset of promising compounds was further characterised.

3.3. Material and Methods

3.3.1. Material

3.3.1.1. Chemicals

Indole-3-Acetic Acid (IAA), α-Naphthalene acetic acid (NAA), *N*-1-Naphthylphthalamic Acid (NPA), Sulfameter and Sulfadiazine were purchased from Sigma. IAA was dissolved in 100 % EtOH, NAA, NPA; Sulfameter and Sulfadiazine were dissolved in DMSO. Plant growth medium consist of ½ strength MS salts (Murashige and Skoog, 1962) (2.17 g MS salts/L (Sigma)) at pH 5.7 which was either liquid (for the chemical genetics screen) or solidified (for characterisation of the positive hits) with 1 % bacto-agar (Appleton Woods).

3.3.1.2. LAX3 inhibitors

All *LAX3* inhibitors were purchased from ChemBridge (ChemBridge, UK) as well as LI5 derivatives VarA to E (Chembridge catalogue number for LI4 is 5343444, LI5 5344621, LI7 5349647, LI8 5350185, LI9 5350587, VarA 5352384, VarB 5350341, VarC 5346720, VarD 5356458, VarE 5358024). LI5 and its derivatives AW1 to AW12 were produced in house at the University of Nottingham, School of Chemistry, by Neil J Oldham and Anna Westacott.

3.3.1.3. RT qPCR primers

Primer name	Sequence (5'->3')
qCTRL1-F	agtggagaggctgcagaaga
qCTRL1-R	ctcgggtagcacgagcttta
qLAX3-F	tcaccattgcttcactccttc
qLAX3-R	aagcaccattgtggttggac

3.3.2. Methods

3.3.2.1. Seed sterilisation

Seeds were surface sterilized for 5 minutes in 50 % bleach, 0.1 % triton X-100 then washed three times with sterile ddH_2O. Seeds were stratified at 4° for 2 days to synchronise germination.

3.3.2.2. Chemical genetics screen

A commercial library consisting of 10,000 compounds (DiverSet™, ChemBridge, UK) was screened to identify inhibitors of *LAX3* auxin induction. 2 to 3 seeds of an *Arabidopsis thaliana* transgenic line expressing a pLAX3::GUS reporter were sown in the wells of a 96-well filter plates (Multiscreen HTS MSBVS1210; Millipore) containing liquid ½ MS medium with NPA at 10 µM. Plates were incubated under continuous light (110 $\mu E.m^{-2}.s^{-1}$), continuous shaking (50 rpm/min) and at constant temperature (22°C). 5 days after germination, the medium was replaced with fresh medium containing a compound from the library at a final concentration of 50 µM (final DMSO concentration is 0.25 %). 24 hours after medium replacement, NAA was added in each well to a final concentration of 10 µM. 24 hours after auxin induction, GUS staining was performed.

3.3.2.3. Histology and histochemistry

GUS activity was revealed by incubating seedlings in a phosphate buffer (500mM, pH 7) containing 0.5 mM potassium ferricyanide ($K_3[Fe(CN)_6]$), 0.5 mM potassium ferrocyanide ($K_4[Fe(CN)_6]$), 1 mM ethylenediaminotetraacetic acid (EDTA pH 8), 0.5 % (v/v) Triton X-100 and 1 mM X-bromo-4-chloro-3-indolyl ß-D-galactopyranoside

(Sigma) (X-Glc) at 37° for 1 hour. X-Glc was initially dissolved in 100% dimethylformamide (DMF). After GUS staining, seedlings were cleared for at least 12 hours in 260% chloral hydrate in 33 % glycerol (all chemicals were purchased from Fischer Scientific and Sigma).

3.3.2.4. Picture analysis

Roots and hypocotyles length were measured using ImageJ (ImageJ 1.40g).

3.3.2.5. Statistical analysis

Statistical analysis was performed using Minitab version 15 (Minitab Inc, UK).

3.3.2.6. Cell wall remodelling enzymes assays

Pectin methyl sterase activity was measured using the pH stat method. 1 g of citrus pectin was incubated in a 6mM NaCl solution at pH 8 with or without LAX3 inhibitors. After addition of the enzyme (Pectin methyl esterase, Sigma) the pH was maintained at 8 by an automatic titrator using 5 mM NaOH. The volume added was monitored during time to determine enzymatic activity (Hagerman and Austin, 1986).

Polygalacturonase activity was measured using the cyanoacetamide method. Polygalacturonase enzyme (Sigma) was added to the assay mix (50 mM sodium acetate, 150 mM NaCl, 0.5 g polygalacturonic acid (Sigma) pH 4) with or without inhibitors and incubated at 37°C for 30 minutes. Enzymatic activity was revealed following addition of cyanoacetamide (1 % final) and sodium tetraborate (100 mM final) and incubation at 100°C for 10 minutes. Absorbance at 276 nm was measured and used to determine enzymatic activity using a standard curve (Gross, 1982).

3.3.2.7. RNA extraction and RT qPCR

RNA was extracted from plant tissues using Trizol Reagent (Invitrogen) and cleaned up using the RNeasy kit (Qiagen). Poly(dT) cDNA was prepared from 0.5 µg total RNA with Superscript II reverse transcriptase (Invitrogen) and analyzed on a LightCycler 480 apparatus (Roche Diagnostics) with the Quantace SYBRGREEN mix (Quantace) according to the manufacturer's instructions. Targets were quantified with specific primer pairs designed with the Universal Probe Library Assay Design Center (Roche Applied Science). All individual reactions were done in quadruplicate and data were analyzed with Microsoft Excel 2007 (Microsoft Corporation, Redmond, USA). Expression levels were normalized to At1G04850 (CTRL1, thanks to Tara Holman for primer sequence).

3.4. Results

3.4.1. Chemical genetics screen to identify *LAX3* auxin induction inhibitors

A library of 10,000 molecules was screened to identify inhibitors of the induction by auxin of the lateral root emergence marker p*LAX3*::GUS. Given the large number of molecules tested, a robust, reproducible and high throughput assay using a p*LAX3*::GUS reporter was designed (Figure 3-1 A). The reporter is normally expressed in the vascular tissues and in cortical and epidermal cells overlying a lateral root primordium (Figure 3-1 B, Untreated). Auxin treatment strongly induces its expression in all cortical and epidermal cells of the root (Figure 3-1 B, NAA). Chemicals that blocked auxin induction of LAX3::GUS were selected for further analysis.

Following these criteria, 13 compounds were identified and confirmed as inhibitors of *LAX3* auxin induction. Based on the pattern of GUS expression the positive hits were classified into two categories. The first category consists of 12 molecules which blocked the induction of the reporter in the outer tissues but did not or only slightly affected the vascular expression. Compounds from this category were named LI1 to 12 (for LAX3 Inhibitors). The second category consists of one molecule that blocked the expression of the reporter in all tissues. The compound from this category was named LT1 (LAX3 Total inhibitor) (Figure 3-1 (B)).

To simplify the phenotypical analysis, the compounds were grouped together based on their structure: LI2 and 3, which share a common structure, are in the first group, LI4, 5, 7, 8 and 9, which also share a common structure, are in the second group and finally, as all the remaining compounds are structurally distinct, they compose a third group.

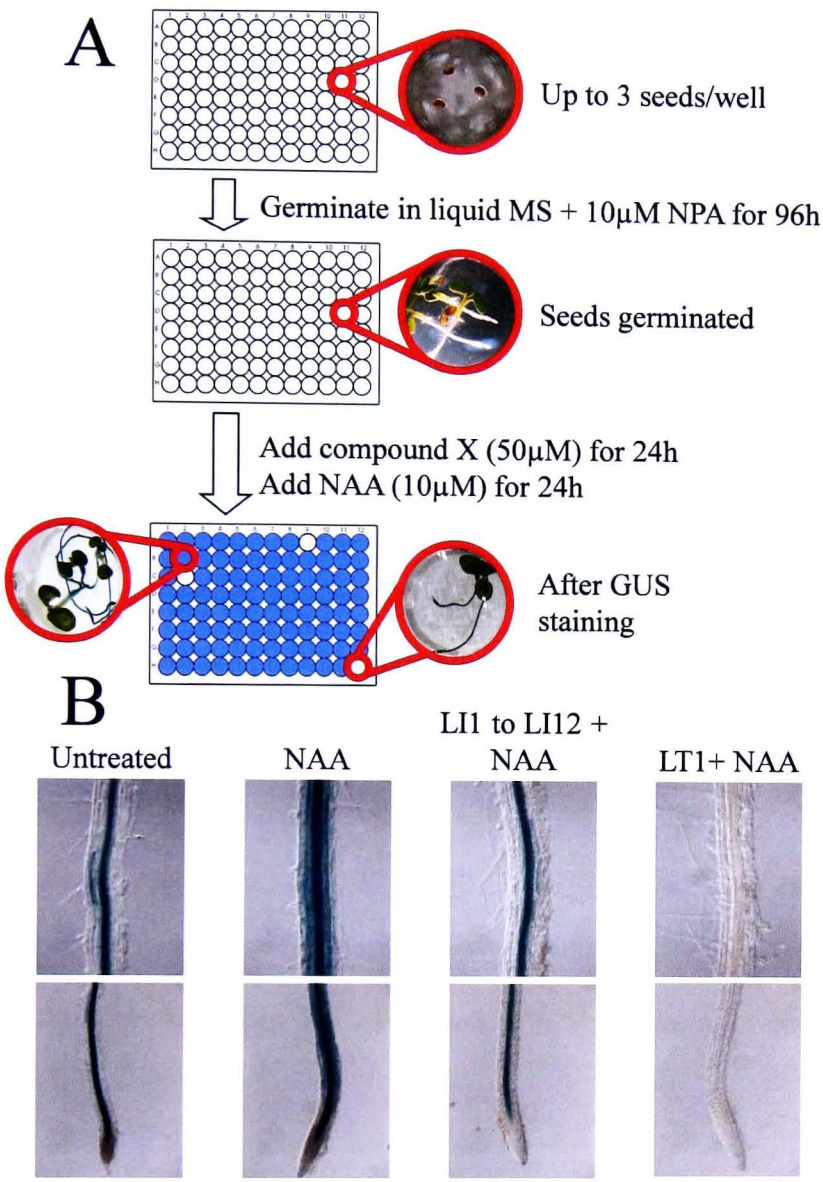

Figure 3-1 Identification of inhibitors of *LAX3* auxin induction. (A) Procedure used during the chemical genetics screen. The screen was carried out at the Compound Screening Platform at the VIB (University of Ghent) by Bert de Rybel, Long Nguyen and Dominique Audenaert). Original figure from Bert de Rybel. **(B)** Phenotype of LAX3::GUS seedlings treated with a mock solution, auxin (NAA, 10µM), with inhibitors LI1 to 12 (50µM) and auxin (NAA, 10µM) (representative image of an LI5+NAA treated seedling, results are similar with other compounds) or with inhibitor LT1 (50µM) and auxin (NAA, 10µM).

3.4.2. Effects of *LAX3* inhibitors on lateral root development and root growth (dose response curves)

To assess the effects of *LAX3* inhibitors on lateral root development and root growth, 5-day-old Col-0 seedlings were transferred onto plates containing increasing concentrations of the inhibitors for 7 days before the number of lateral roots and the main root length were measured. Figure 3-2 A shows that compounds of group 1 have effects at varying doses on root development. LI2 affects root growth at a concentration around 30 times lower than LI3 (0.1 μM and 3 μM respectively), but, on the other hand, LI3 affects lateral root density at a concentration ±3 times lower than LI2 (3 μM and 10 μM respectively). Conversely, the compounds of group 2 affect root growth and lateral root formation at 5μM, with LI5, 8 and 9 being the most active ones. Finally, compounds of group 3 have very different effects but since they all have different structures it is not suprising. LI1, 10, 11 and 12 have little effects on lateral root formation but affect root growth at concentrations of 10μM (LI1) and 30 μM (LI10, 11 and 12). On the other hand, LI6 and LT1 affect both processes at 0.3 μM and 10 μM respectively.

To determine at which developmental stage the chemicals affect lateral root formation (initiation, emergence or both), 5 day-old Col-0 seedlings were germinated on NPA (to prevent lateral root initiation) and then treated with the inhibitors for 7 days before the number of emerged and non emerged lateral root primordia were counted. Figure 3-2 (B) shows that 7 out of the 13 inhibitors affect primordia emergence (LI4, 5, 7, 8, 9, 10 and LT1), which include all compounds from group 2 and two from group 3. Notably, compounds of group 2 also affect initiation, as the density of emerged and non emerged

primordia is reduced compared to the untreated control. The other compounds from group 1 and 3 that did affect lateral root density in the previous bioassay had no effect on emergence, thus they mainly appear to affect initiation.

As the number of roots analysed was initially limited (for practical reasons) the statistical comparison of the data did not yield any significant difference (Tukey multiple comparison test, $p < 0.05$). Thus, the experiment was repeated but this time characterising only three inhibitors in greater depth (LI4, 5 and LT1). Figure 3-3 shows that the three treatments affected lateral root emergence significantly (Tukey multiple comparison test, $p < 0.01$).

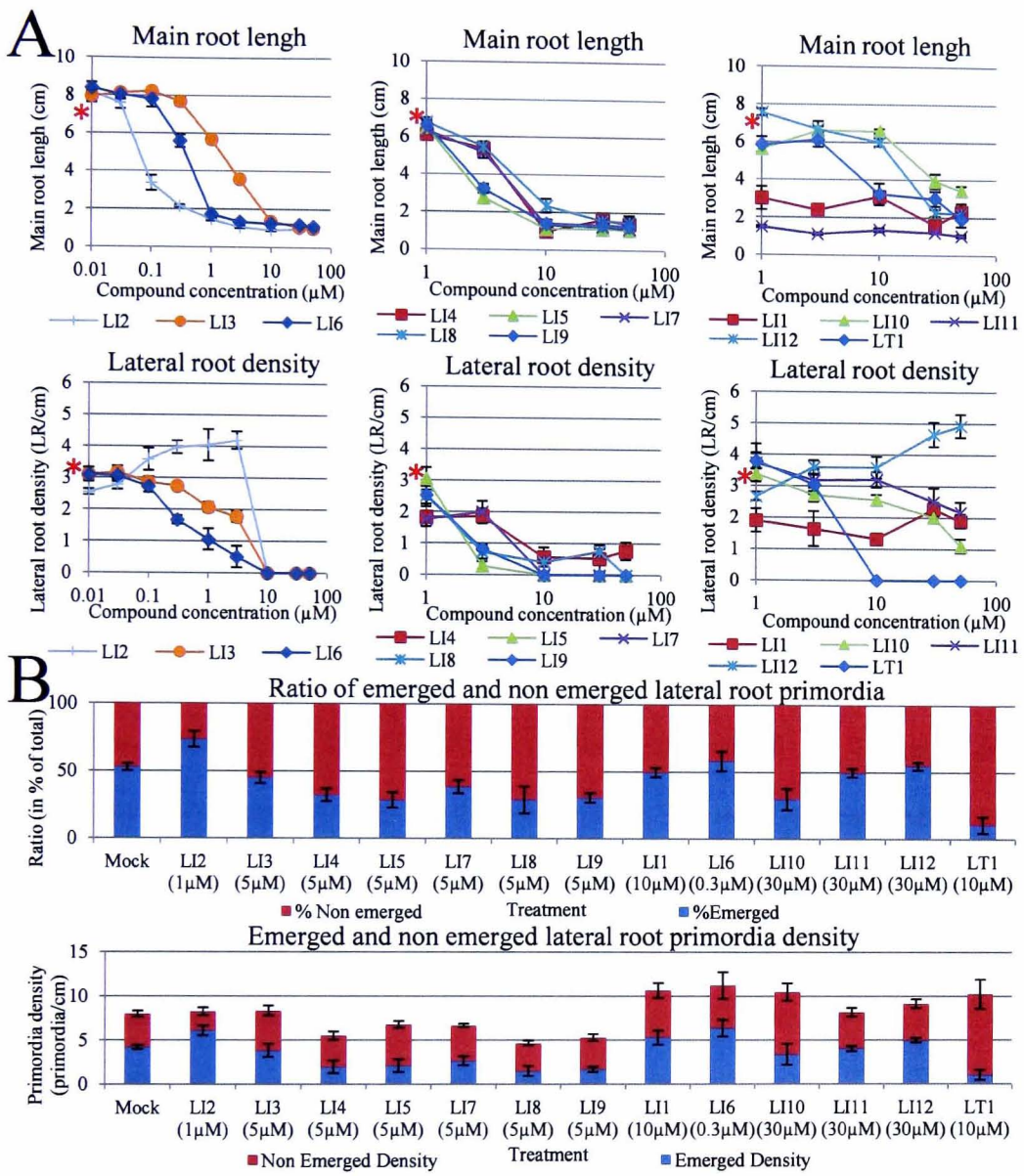

Figure 3-2 Effects of *LAX3* inhibitors on lateral root formation and root growth. (A) Main root length and lateral root density of 5-day-old Col-0 seedlings treated with the indicated inhibitors for 7 days. Error bars indicate the standard error (n≥8). The red star on each chart indicates the main root length or lateral root density of a mock treated control. The x-axis is in $\log_{10}$ scale. **(B)** Ratio of emerged and non emerged lateral root primordia of col-0 seedlings germinated on NPA and transferred on plates containing the inhibitors at the indicated concentration for 7 days. Below is shown the density of all primordia (emerged + non emerged). Error bars indicate the standard error (n=4).

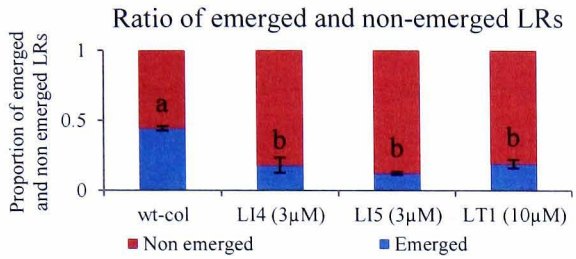

Figure 3-3 Effects of LI4, LI5 and LT1 on lateral root emergence. Ratio of emerged and non emerged lateral root primordia of Col-0 seedlings germinated on plates containing the inhibitors at the indicated concentration for 10 days. Error bars indicate the standard error (n=5). A different letter indicates a statistically significant difference (Tukey multiple comparison test, $p \leq 0.01$).

3.4.3. Effects of NAA on seedlings treated with the inhibitors

To determine whether NAA can rescue the lateral root phenotype caused by some inhibitors, 5-day-old Col-0 seedlings were treated with the compounds and several concentrations of NAA for 5 days after which the number of lateral roots and the main root length were measured. Figure 3-4 shows that seedlings treated with inhibitors from group 1 can be rescued by NAA but not those of group 2. LI4 and 7 appear to respond at concentration of 0.1 and 1µM. Nevertheless, this is probably due to their reduced potency compared to other group 2 inhibitors. Finally, out of the six group 3 inhibitors, three are rescued by NAA (LI1, LI11 and to a less extent LI6). Interestingly, LI10 and LT1, which affect emergence, are not rescued, as well as LI12.

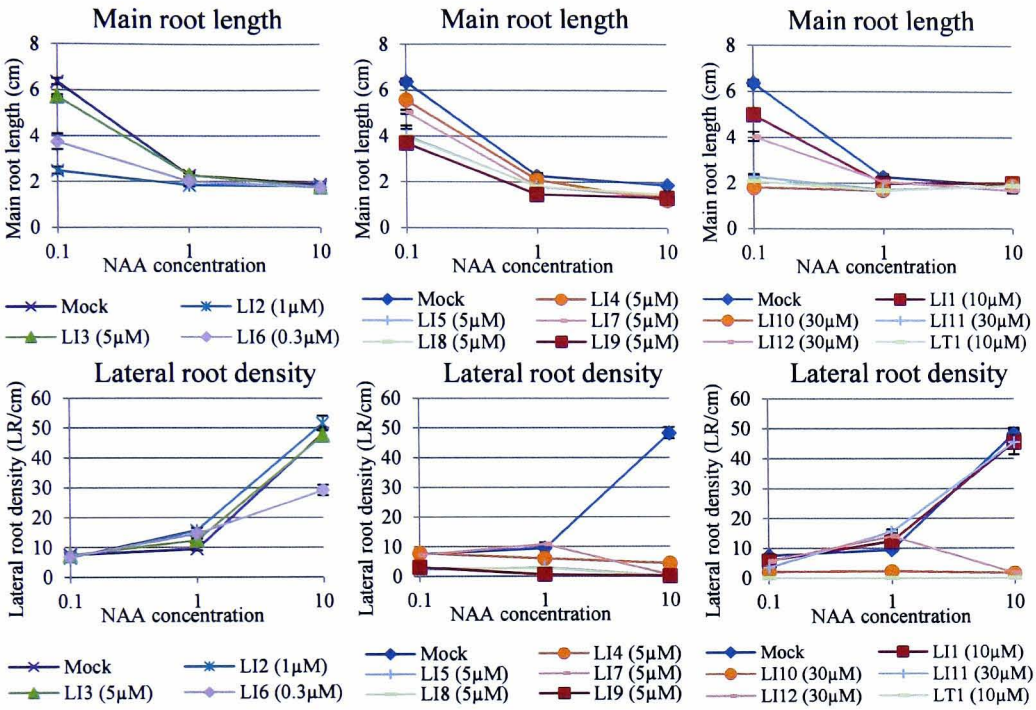

Figure 3-4 Effects of NAA on seedlings treated with the inhibitors. Quantification of main root length and lateral root density of seedlings treated with the inhibitors. Error bars indicate the standard error (n≥8). The x-axis is in $\log_{10}$ scale.

3.4.4. Phenotype of plants treated with LI5

Out of 13 inhibitors initially identified, 7 affect lateral root initiation, emergence and root growth and render plants insensitive to NAA as well. Out of these 7 compounds, 5 share a common structure (group 2): LI4, 5, 7, 8 and 9 are all benzene sulfonamides which differ only by the position of chlorine atoms on the two benzene rings, and, in one case, by the substitution of chlorine by fluorine (Figure 3-5 A). For the following reasons we decided to focus our work on this family. First, they are active at a relatively low concentration (5µM). Second, they affect lateral root emergence which is our developmental programme of interest. Third, these five compounds allow to have an idea of the structure/function relationship of this family of compounds. Finally benzene sulfonamides are relatively easy to synthesise in the laboratory. Therefore, we have

made a more detailed phenotypic analysis of a representative member of this family, LI5, which in several bio-assays was the most active compound (Figure 3-5 B and C).

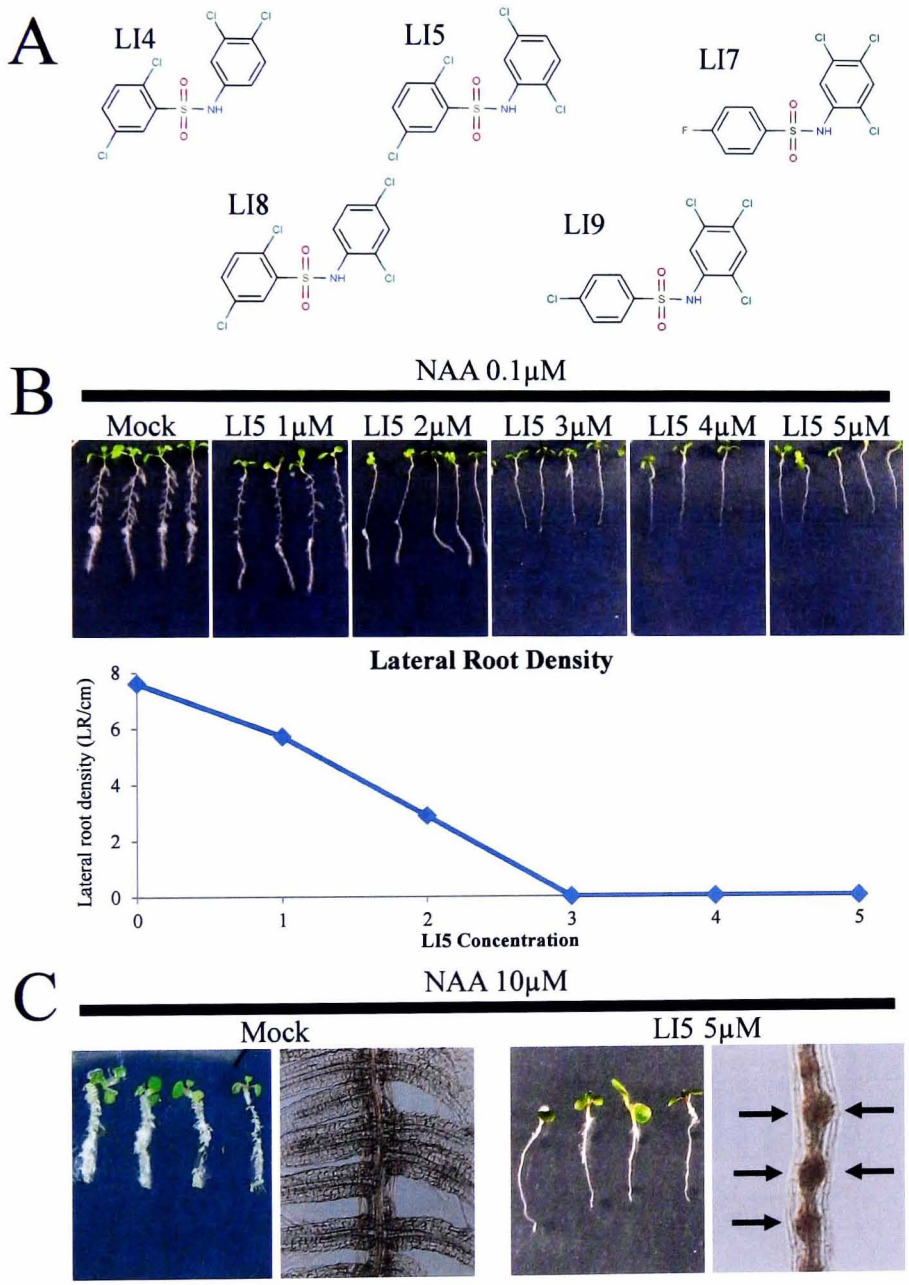

Figure 3-5 Effects of LI5 on lateral root formation. (A) Structures of the 5 sulfonamides identified during the chemical genetics screen. **(B)** Effects of different concentrations of LI5 in presence of 0.1 μM NAA on the lateral root density. Error bars indicate the standard error (n≥8). **(C)** When plants are treated with high amounts of auxin, many non emerged primordia can be observed along the main root (arrows) while in the mock treated control they all emerged from the main root within 2 days (picture taken after 5 days of treatments).

3.4.5. LI5 and related sulfonamides do not affect cell wall remodelling enzymes

During lateral root emergence, Swarup et al. (2008) showed that genes encoding different types of cell wall remodelling enzymes, such as polygalacturonases and pectin methyl esterase, are specifically induced in overlying tissues(Swarup et al., 2008). Because of the lateral root emergence phenotype observed in plants treated with 10μM NAA and LI5 (Figure 3-5 C), we hypothesised that this could be due to a defect in cell wall remodelling as the primordia remain within the primary root. To determine if these cell wall remodelling enzymes were affected by LI5 and/or other inhibitors from group 2, several *in vitro* enzymatic assays were used. Figure 3-6 shows that the activity of a pectin methyl esterase and a polygalacturonase is not affected by the inhibitors.

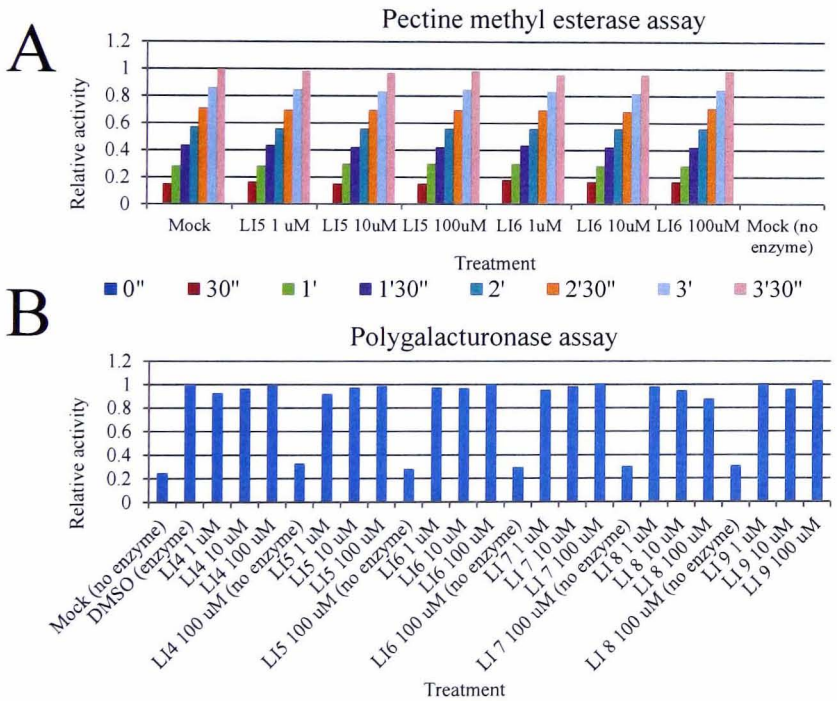

Figure 3-6 Group 2 inhibitors do not affect pectin methyl esterase or polygalacturonase activity. (A) Relative activity of a pectin methyl esterase during time in presence of the indicated inhibitors. **(B)** Relative activity of a polygalacturonase in presence of the indicated inhibitors.

3.4.6. Group 2 sulfonamides do not inhibit folate synthesis

Historically, sulfonamides were identified as the first class of compounds to have antimicrobial properties (the "sulfa drugs") (Domagk, 1935). These compounds were shown to inhibit the activity of the enzyme dihydropteroate synthetase (DHPS), which is involved in folate biosynthesis, as they compete with one of its substrate (*para*-aminobenzoic acid) (Wise and Abou-Donia, 1975). The folate biosynthesis pathway is largely conserved between plants and bacteria (Hanson and Gregory Iii, 2002). The Arabidopsis genome encodes two DHPS genes, which are as well sensitive to sulfa drugs. Arabidopsis plants treated with sulfa drugs, such as sulfameter or sulfadiazine (Figure 3-8 A), have severely impaired hypocotyl and root growth which can be conveniently quantified when seeds are germinated in the dark (Figure 3-8 B). Importantly, the phenotype can be chemically rescued by adding exogenous folic acid (FA). Despite being structurally different (Figure 3-8 A), treatments with the sulfonamide identified in this study also affect hypocotyl elongation (Figure 3-7). This is a property unique to group 2 inhibitors. To determine whether group 2 inhibitors affect plant growth by blocking folate synthesis, we have germinated Col-0 seeds in the dark in presence of the inhibitors and with or without exogenous FA. Figure 3-8 B shows that FA does not rescue LI5 treated plant whereas it rescues seedlings treated with sulfa drugs. This result suggests that group 2 inhibitors do not affect plant growth by inhibiting folate synthesis.

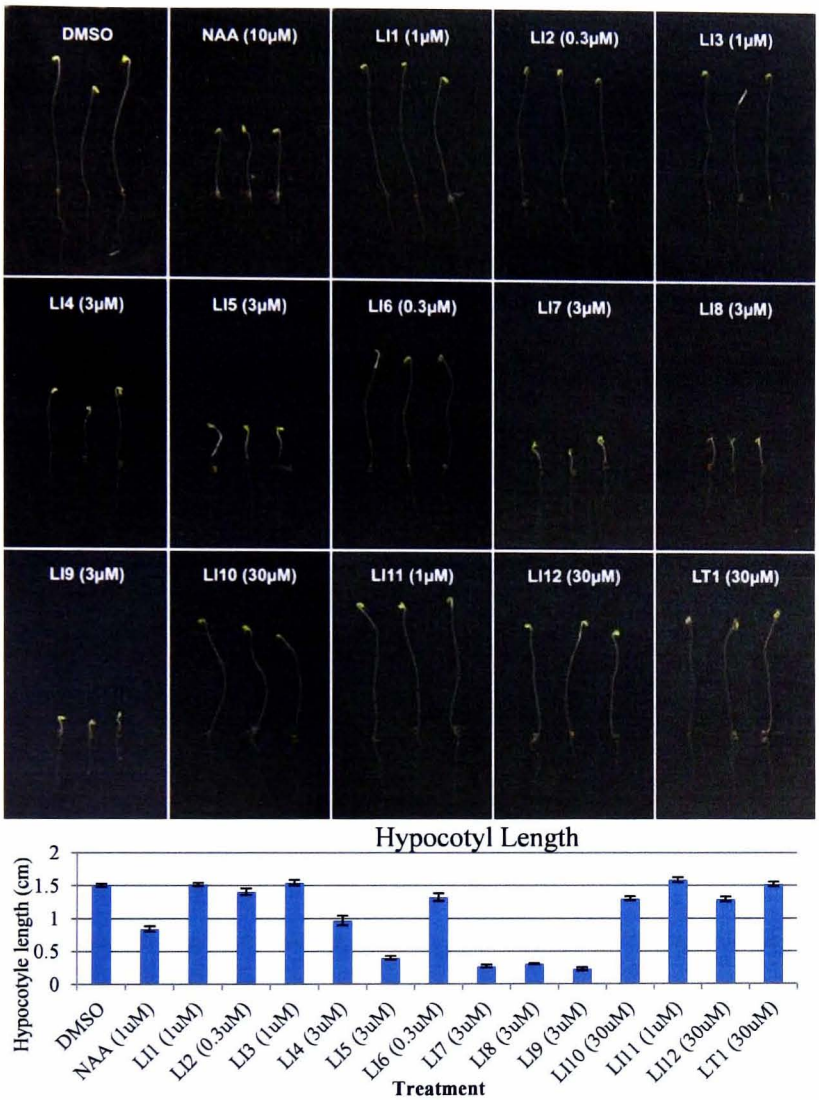

Figure 3-7 Group 2 inhibitors block hypocotyl elongation in dark grown seedlings. Error bars indicate the standard error ($n \geq 10$).

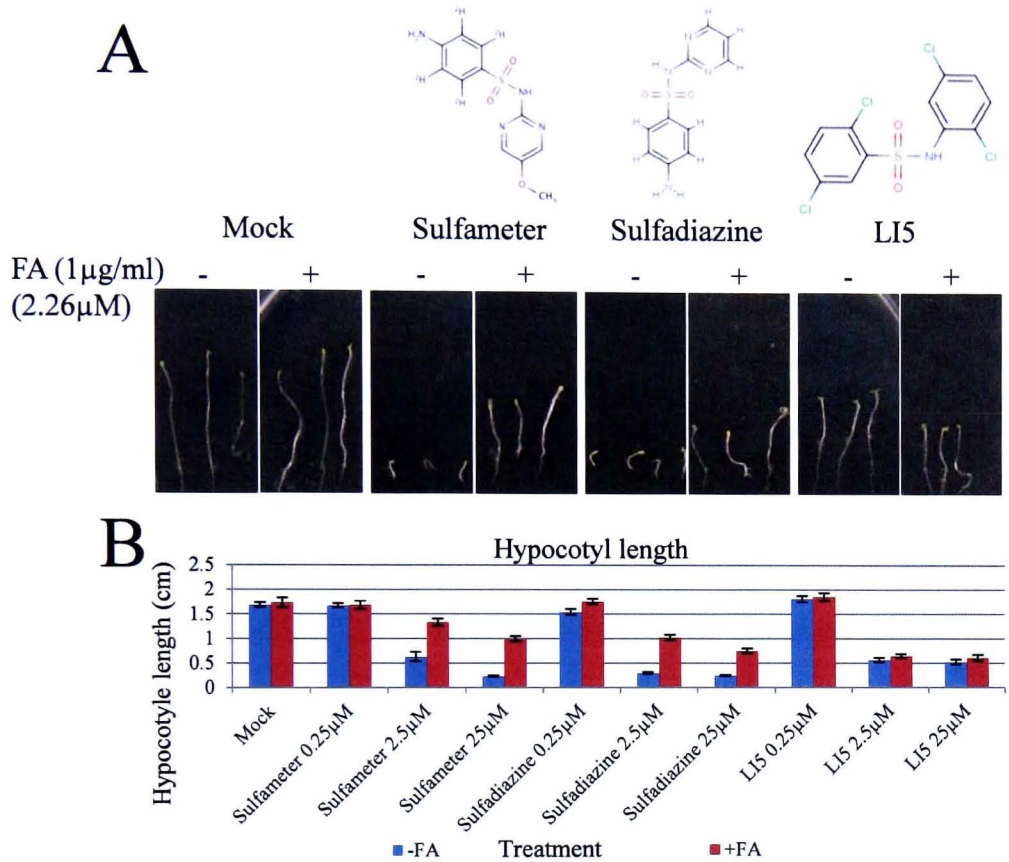

Figure 3-8 Inhibition of hypocotyl elongation by LI5 cannot be rescued with exogenous FA. **(A)** Structures of two sulfa drugs (Sulfameter and Sulfadiazine) and LI5. Below is a picture of seedlings which have been germinated in the dark on the indicated compounds (at 25 µM) in presence or absence of FA (folic acid). **(B)** Quantification of hypocotyl length of seedlings germinated in the dark on the indicated compounds in presence or absence of FA. Error bars indicate the standard error (n≥10).

3.4.7. Analysis of biological activities of benzene sulfonamide structural variants

In order to study the relationship between the structure and the biological activity of group 2 inhibitors, a suite of chemical variants was generated using LI5 as a template and their biological activity was determined using the following three bio assays:

1) **Lateral root density:** 5 day-old Col-0 seedlings were transferred onto plates containing the variants at 0.5, 5 or 50 μM ("AW" series) or at 0.1, 1 and 10 μM ("Var" series) for 7 days before measuring the emerged lateral root density

2) **Auxin (NAA) induced lateral root formation:** 5 day-old Col-0 seedlings were transferred onto plates containing the variants at 5 or 50 μM ("AW" series) or at 5 μM ("Var" series) and NAA at 1 μM for five days before measuring the emerged lateral root density.

3) **Hypocotyl elongation of seeds germinated in the dark:** Col-0 seeds were germinated on plates containing the variants at 0.5, 5 or 50 μM ("AW" series) or at 0.1, 1 and 10 μM ("Var" series) in the dark for 5 days before the hypocotyl length was measured.

To simplify the interpretation, the compounds were grouped in 4 batches depending on the changes made to the structure.

The first batch of compounds consists of LI5 variants LI4, 7, 8, 9, AW5, 7 and VarA (Figure 3-9 A). All these compounds are very similar to LI5 as only minor changes are made to the position and number of chlorine groups. Figure 3-9 B shows that all the variants are almost as active as LI5 except for LI4 and AW7, which have a reduced activity in the three bioassays. Nevertheless, they are both active at higher concentrations.

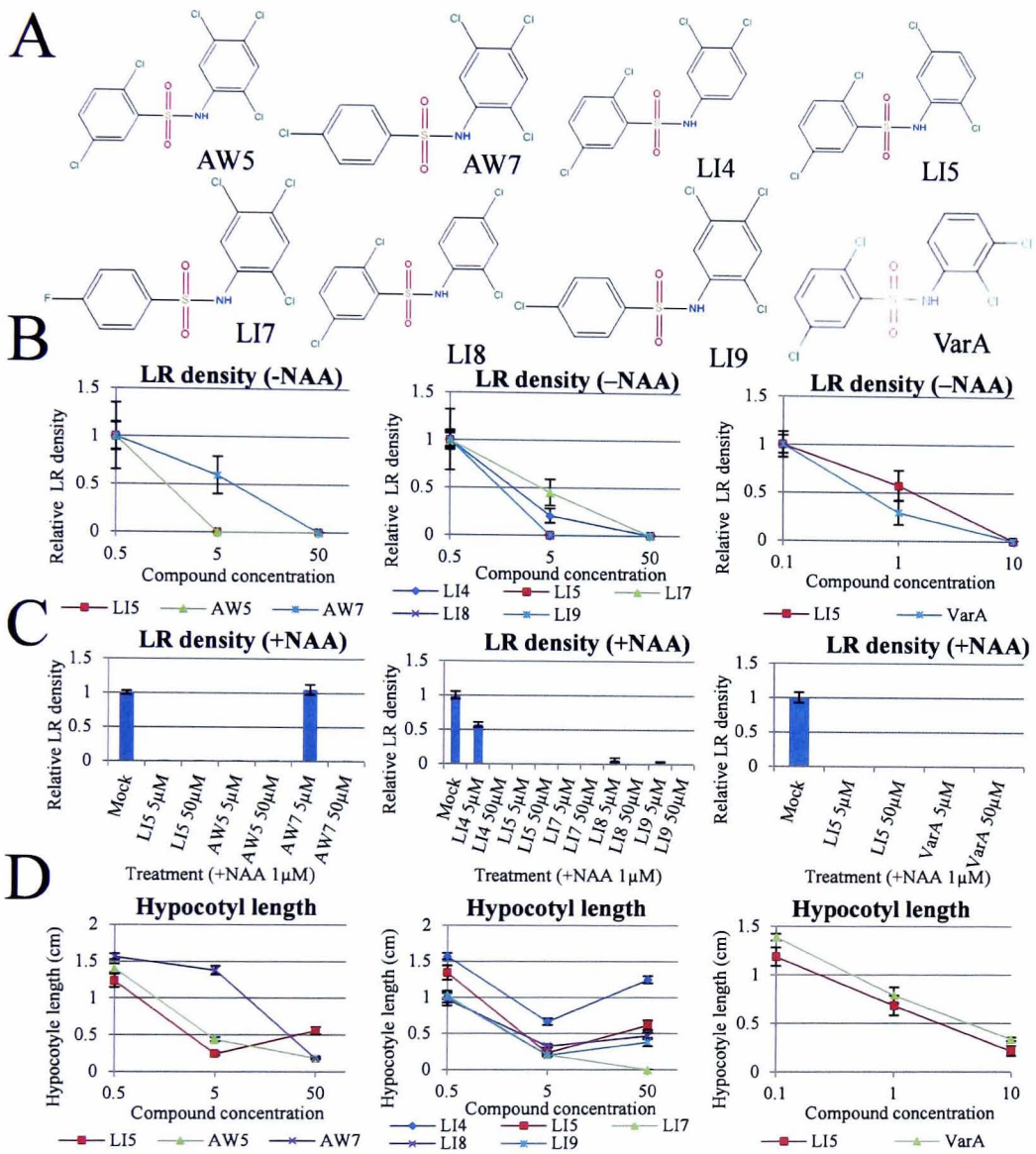

Figure 3-9 LI5 structural variants (A) Structures of LI5 and its variants LI4, LI7, LI8, LI9, AW5, AW7 and VarA. **(B)** Effects of the variants on lateral root density without auxin. The x axis is in $\log_{10}$ scale. **(C)** Effects of the variant on lateral root density with auxin. **(D)** Effects of the variants on hypocotyl elongation in the dark. The x axis is in $\log_{10}$ scale. In all experiments the error bars show the SE (n≥8). Lateral root density in **(B)** and **(C)** is relative to Mock + NAA control (set at 1).

The second batch of compounds consists of LI5 variants AW1, 2, 3, 4 and 9 (Figure 3-10 A). These compounds were designed to determine (1) the role of the chlorination on both benzene rings (AW1-3), (2) the role of the sulfonamide functional group (AW4)

and (3) the feasibility of adding a linker to LI5 (AW9). Figure 3-10 B shows that only the chlorination on the aniline ring can be removed with a reduced loss of activity as AW1, but not AW2 or 3, is active. Nevertheless, removal of the aniline ring results in a complete loss of activity (AW9). Hence, it appears that the sulfonamide group plays a crucial role as AW4 (sulfoxylate) is completely inactive.

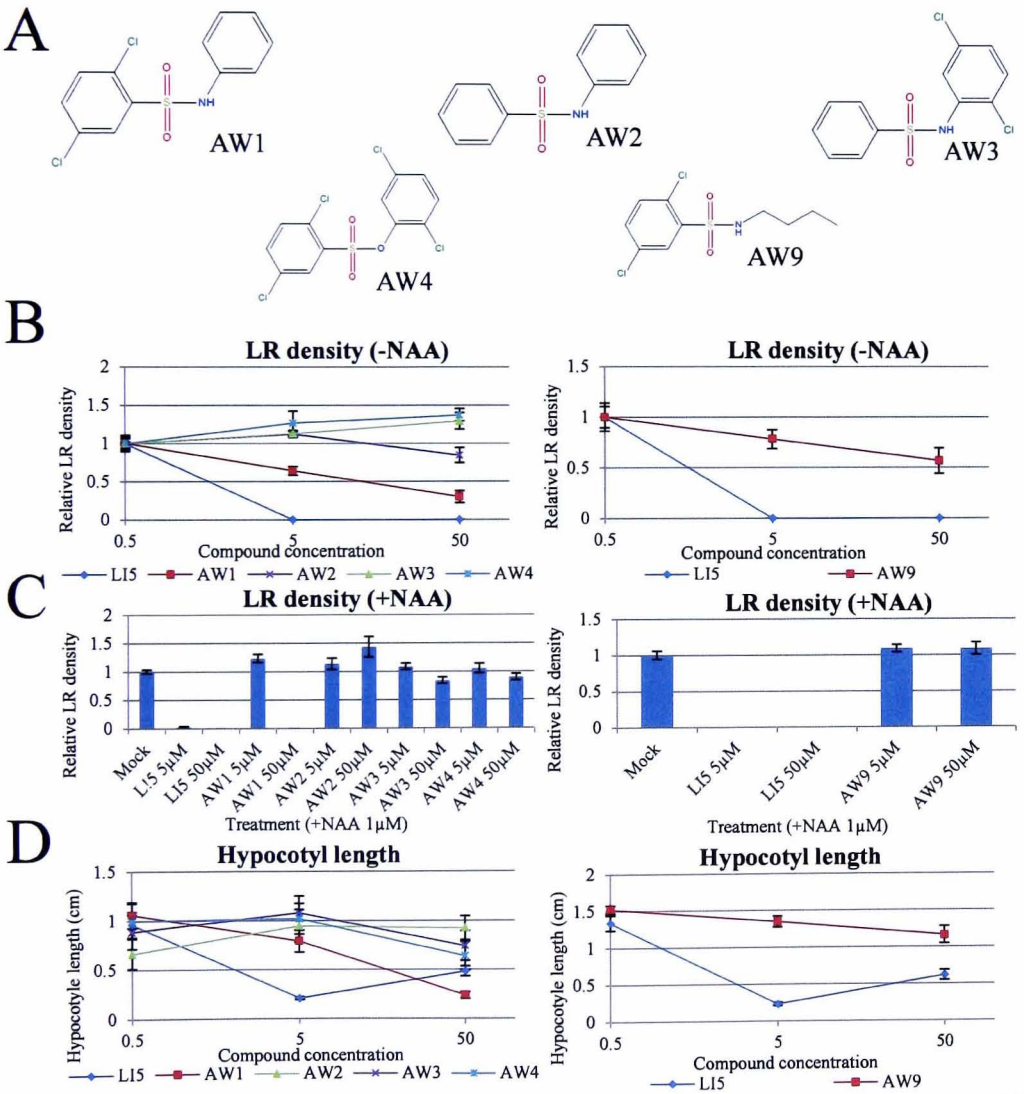

Figure 3-10 LI5 structural variants (A) Structures of LI5 and its variants AW1, AW2, AW3, AW4 and AW9. **(B)** Effects of the variants on lateral root density without auxin. The x axis is in $\log_{10}$ scale. **(C)** Effects of the variant on lateral root density with auxin. **(D)** Effects of the variants on hypocotyl elongation in the dark. The x axis is in $\log_{10}$ scale. In all experiments the error bars show the SE ($n \geq 8$). Lateral root density in **(B)** and **(C)** is relative to Mock + NAA control (set at 1).

The third batch of compounds consists of LI5 variants VarB, C, D and E (Figure 3-11 A). These compounds were designed to determine to what extent the aniline could be modified by substituting chlorines with either iodine or methyl groups. Figure 3-11 C shows that iodine substitutions remain active but their activity strongly depends on its position (VarB and D). In contrast, exchanging chlorines with methyl groups leads to a strong reduction in activity (VarC and E).

The fourth and last batch of compound consists of LI5 variants AW6, 10, 11 and 12 (Figure 3-11 B). These compounds were designed to determine the effects of the position of the chlorine (ortho, AW10; meta, AW11; para, AW6) on the benzene sulfonic acid ring. Figure 3-11 D shows that all substitutions lead to an active compound, though the para subsitution seems to be slightly more active. AW12 biological activity is similar to AW11 despite an extra methyl group in the para position. This addition only reduces very slightly the activity of AW11.

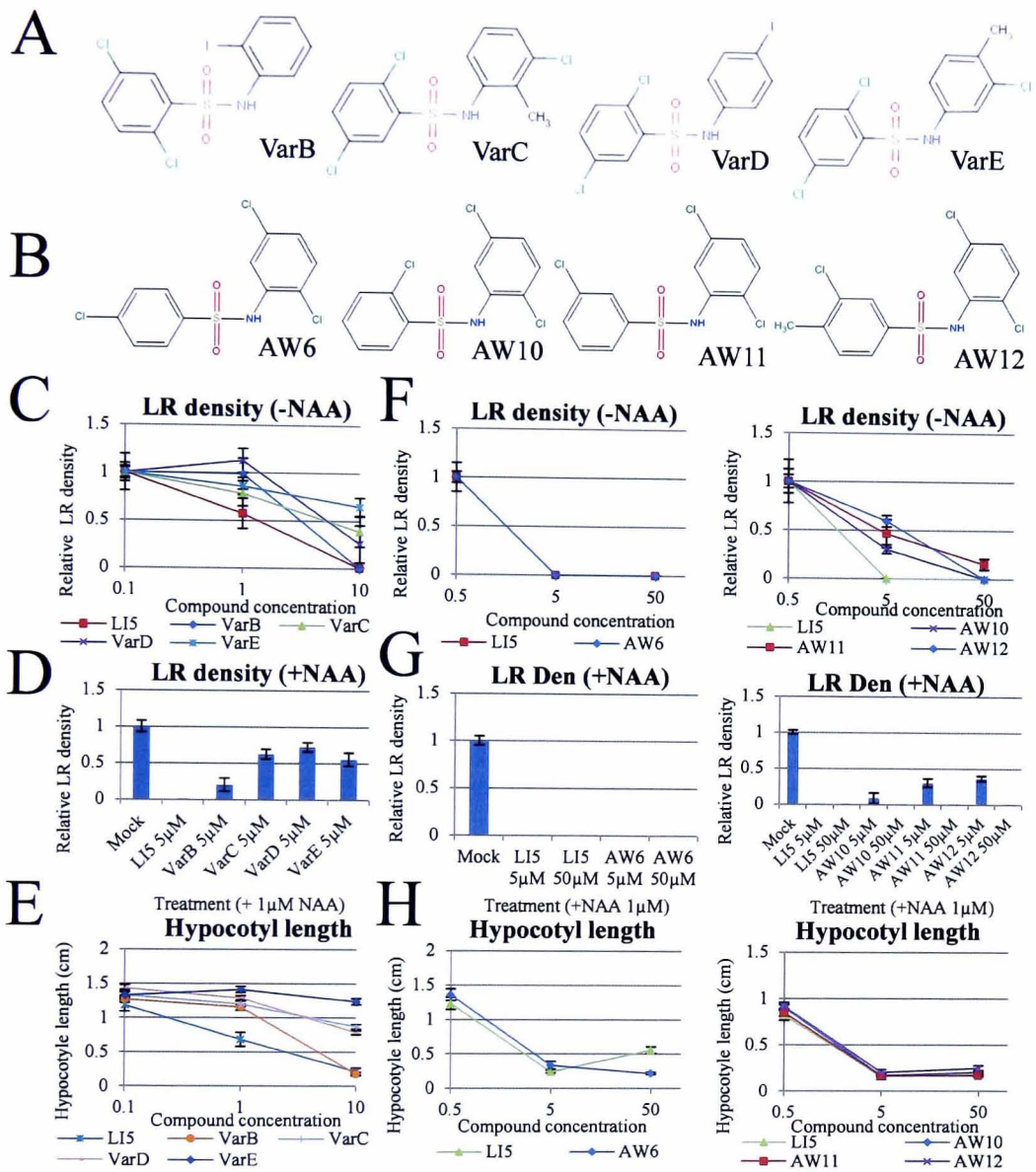

Figure 3-11 LI5 structural variants (A) Structures of LI5 and its variants VarB, VarC, VarD and VarE. **(B)** Effects of the variants on lateral root density without auxin. The x axis is in $\log_{10}$ scale. **(C)** Effects of the variant on lateral root density with auxin. **(D)** Effects of the variants on hypocotyl elongation in the dark. The x axis is in $\log_{10}$ scale. In all experiments the error bars show the SE ($n \geq 8$). **(E)** Structures of LI5 and its variants AW6, AW10, AW11 and AW12. **(F)** Effects of the variants on lateral root density without auxin. The x axis is in $\log_{10}$ scale. **(G)** Effects of the variant on lateral root density with auxin. **(H)** Effects of the variants on hypocotyl elongation in the dark. The x axis is in $\log_{10}$ scale. In all experiments the error bars show the SE ($n \geq 8$).

On Figure 3-12 the variants are classified in four categories depending on their biological activity. We analysed further several variants from each category by looking at their effects on the auxin induction of *LAX3* mRNA. 5 day old Col-0 seedlings were treated for 6 hour with or without auxin (1 μM NAA) and with or without variants at 5μM before gene expression was analysed by RT qPCR. Figure 3-13 shows that the results are in agreement with the data from the bio assays: AW2 is completely inactive, AW1 and AW12 have only minor effects, AW10, AW11 and LI4 are more active and, finally, AW5, LI5 and LI8 almost completely abolish *LAX3* induction.

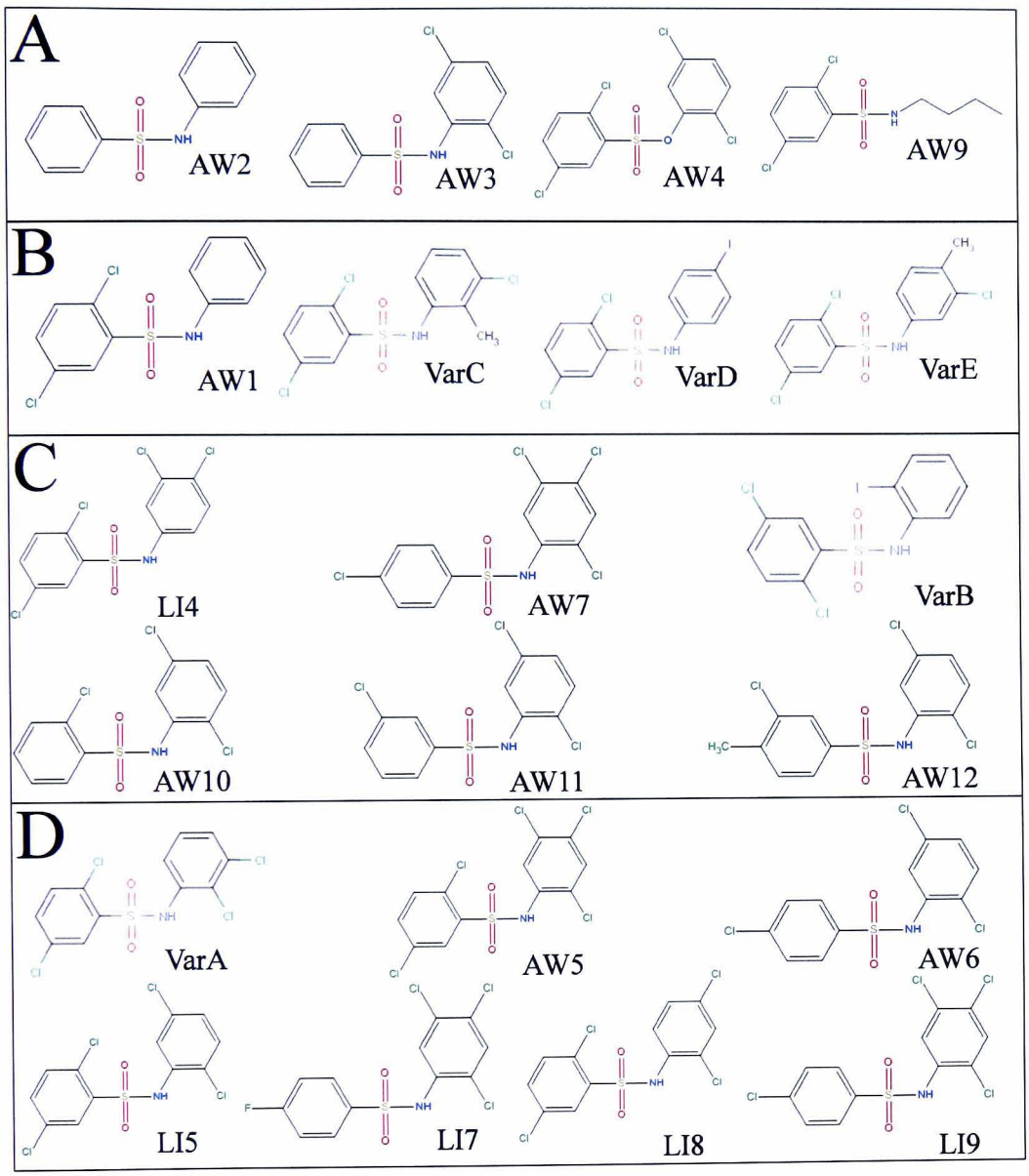

Figure 3-12 Classification of the variants depending on their activity in the three bio assays at a concentration of 5µM. In **(A)** are shown the inactive variants, in **(B)** the low active ones, in **(C)** the medium active ones and in **(D)** the most active ones.

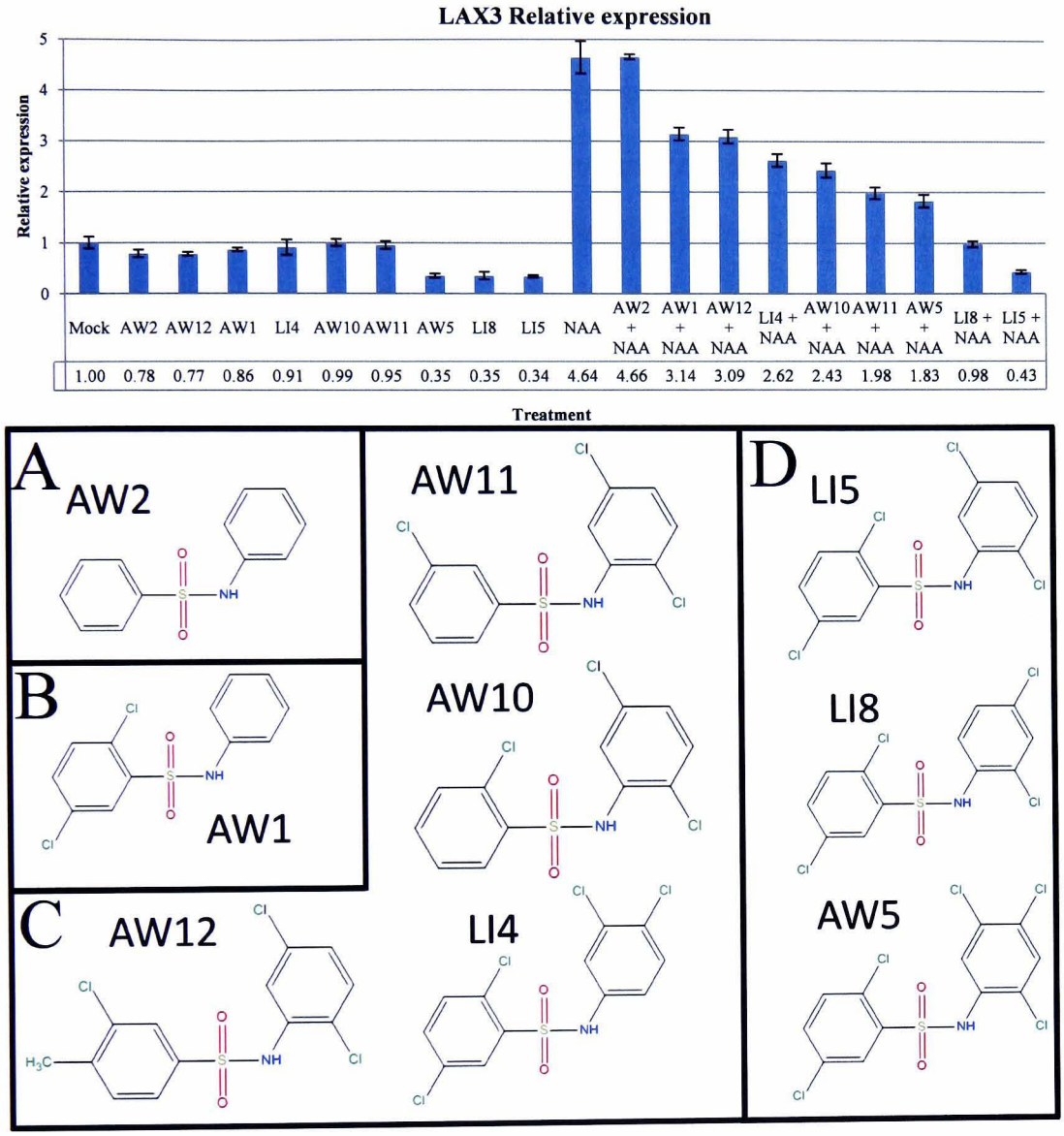

Figure 3-13 Relative induction of *LAX3* mRNA by auxin in the presence of the variants from the 4 categories defined in Figure 3-12.

3.5. Discussion

Auxin redistribution between lateral root primordia and overlying tissues triggers the expression of the auxin influx carrier *LAX3* (Swarup et al., 2008). However, *LAX3* is a late auxin responsive gene (chapter 2) necessitating the involvement of an unknown transcription factor(s) intermediate. To aid our characterisation of *LAX3* expression, a chemical genetics screen was conducted to identify inhibitors of its auxin induction. In this chapter, the effects of 13 *LAX3* inhibitors on root growth and lateral root formation was analysed and a promising family of compounds was characterised.

The primary focus of this chapter was to identify compounds affecting emergence. 5 of the 13 compounds identified to disrupt *LAX3* auxin induction (termed LI4, 5, 7, 8, 9, 10 and LT1) also blocked emergence. Treatments with LI10 and LT1 affect lateral root initiation very slightly (or not at all) but strongly blocked emergence. Also, these two compounds render the root insensitive to NAA. At concentrations over 10μM, main root growth is severely reduced but the formation and elongation of adventitious root –or of emerged lateral roots- is not affected. These two compounds may target similar processes in the primary root (rather than lateral root) and are therefore outside the scope of this study.

The remaining 5 compounds have been extensively studied (group 2 inhibitors). They are likely to all target the same protein(s) as they share a common structure (a sulfonamide functional group that links two benzene rings) and induce similar effects on lateral root development. These effects correlate with an inhibition of the induction by auxin of *LAX3* mRNA. Hence, the effects of the inhibitors on root architecture are most probably due to their effects on gene expression.

In previous studies, several inhibitors of auxin signalling have been identified (De Rybel et al., 2009a). A common feature of active auxins (either natural forms or synthetic agonists) is to bind several related F-BOX E3 ligases (termed TIR1 and AFB1-5) to promote their interaction with Aux/IAA repressors (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Tan et al., 2007). The TIR1-auxin-Aux/IAA complex leads to the ubiquitination and degradation of Aux/IAAs by the proteasome (dos Santos Maraschin et al., 2009). Some structural variants of auxin still have the ability to bind TIR1 and/or AFB1-5 but will not promote the degradation of Aux/IAA proteins. Such compounds are known as "anti-auxins" (or auxin antagonists). The structural requirements for a compound to bind TIR1 (and potentially AFB1-5) and to promote the degradation of Aux/IAAs have been dissected in a recent paper (Hayashi et al., 2008). PCIB (or p-Chlorophenoxyisobutyric Acid), a commonly used auxin inhibitor is believed to be an anti-auxin, though its interactions with TIR1 has not been clearly demonstrated (Oono et al., 2003).

Apart from anti-auxins, other auxin response inhibitors have been described such as Yokonolide B (Hayashi et al., 2003), Terfestatin A (Yamazoe et al., 2005), Toyocamycin (Hayashi et al., 2009) or Compound A, B, C or D (Armstrong et al., 2004; Sungur et al., 2007). Because these compounds are structurally very different from auxin, it is unlikely that they act as anti-auxin. All of these inhibitors, apart from compound D, have been shown to stabilise Aux/IAA proteins (it has also not been shown that Compound D does not stabilise Aux/IAA) but their target is currently unknown.

The compounds described in this chapter are also structurally very different to auxin and most likely do not compete with its binding to TIR1. To dissect how these compounds, and especially the one from group 2, affect the auxin induction of *LAX3* several assays can be used to monitor where they act in the pathway. First, the assumption that these compounds are not anti auxin is simply based on their structure. What makes an anti-auxin is the capacity of a compound to interact with TIR1 and the related receptors in a competitive manner with auxin. To address this point, pull downs experiments that test the auxin dependant interaction of TIR1 and Aux/IAAs can give a direct answer (Gray et al., 2001). Secondly, do the compounds affect auxin responses in all the tissues? Do they affect only early responsive gene, only late responsive genes or both? Using RT qPCR and GUS reporter lines it will be possible to address this at a gene specific level. Using microarrays will give an overview at the genomic level. A key aspect of auxin responses is Aux/IAA stability: are these compounds stabilising Aux/IAA? To determine that, reporter genes fused to relevant Aux/IAA can be used. These experiments will help to determine where the compounds act in the auxin response pathway and more precisely in the LAX3 regulatory network as shown on Figure 3-14.

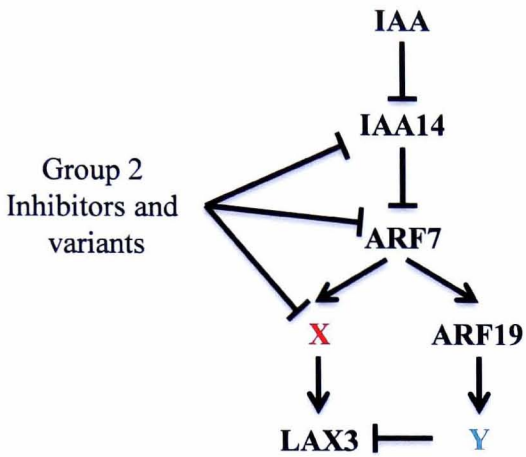

Figure 3-14 Model for group 2 inhibitors effects on *LAX3* auxin induction. Group 2 inhibitors affect lateral root emergence by blocking the induction by auxin of *LAX3*. Three mode of action are proposed: LI5 stabilise the Aux/IAA protein IAA14,

3.6. References

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P. and Cheuk, R. (2003). Genome-wide insertional mutagenesis of Arabidopsis thaliana. *Science* **301**, 653.

Armstrong, J. I., Yuan, S., Dale, J. M., Tanner, V. N. and Theologis, A. (2004). Identification of inhibitors of auxin transcriptional activation by means of chemical genetics in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 14978.

De Rybel, B., Audenaert, D., Beeckman, T. and Kepinski, S. (2009a). The past, present, and future of chemical biology in auxin research. *ACS Chemical Biology* **4**, 987-998.

De Rybel, B., Audenaert, D., Vert, G., Rozhon, W., Mayerhofer, J., Peelman, F., Coutuer, S., Denayer, T., Jansen, L. and Nguyen, L. (2009b). Chemical inhibition of a subset of Arabidopsis thaliana GSK3-like kinases activates brassinosteroid signaling. *Chemistry & biology* **16**, 594-604.

DeBolt, S., Gutierrez, R., Ehrhardt, D. W., Melo, C. V., Ross, L., Cutler, S. R., Somerville, C. and Bonetta, D. (2007). Morlin, an inhibitor of cortical microtubule dynamics and cellulose synthase movement. *Proceedings of the National Academy of Sciences* **104**, 5854.

Dharmasiri, N., Dharmasiri, S. and Estelle, M. (2005). The F-box protein TIR1 is an auxin receptor. *Nature* **435**, 441-445.

Domagk, G. (1935). A report on the chemotherapy of bacterial infections. *Deut Med Woch* **61**, 250.

dos Santos Maraschin, S., Memelink, J. and Offringa, R. (2009). Auxin-induced, SCF(TIR1)-mediated poly-ubiquitination marks AUX/IAA proteins for degradation. *Plant J* **59**, 100-9.

Fukaki, H., Tameda, S., Masuda, H. and Tasaka, M. (2002). Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY-ROOT/IAA14 gene of Arabidopsis. *Plant J* **29**, 153-68.

Gray, W. M., Kepinski, S., Rouse, D., Leyser, O. and Estelle, M. (2001). Auxin regulates SCF(TIR1)-dependent degradation of AUX/IAA proteins. *Nature* **414**, 271-6.

Gross, K. C. (1982). A rapid and sensitive spectrophotometric method for assaying polygalacturonase using 2-cyanoacetamide *HortScience* **17**, 933-934.

Hagerman, A. E. and Austin, P. J. (1986). Continuous spectrophotometric assay for plant pectin methyl esterase. *Journal of agricultural and food chemistry* **34**, 440-444.
Hanson, A. D. and Gregory Iii, J. F. (2002). Synthesis and turnover of folates in plants. *Current opinion in plant biology* **5**, 244-249.
Hayashi, K., Jones, A. M., Ogino, K., Yamazoe, A., Oono, Y., Inoguchi, M., Kondo, H. and Nozaki, H. (2003). Yokonolide B, a novel inhibitor of auxin action, blocks degradation of AUX/IAA factors. *Journal of Biological Chemistry* **278**, 23797.
Hayashi, K., Kamio, S., Oono, Y., Townsend, L. B. and Nozaki, H. (2009). Toyocamycin specifically inhibits auxin signaling mediated by SCFTIR1 pathway. *Phytochemistry* **70**, 190-197.
Hayashi, K., Tan, X., Zheng, N., Hatate, T., Kimura, Y., Kepinski, S. and Nozaki, H. (2008). Small-molecule agonists and antagonists of F-box protein–substrate interactions in auxin perception and signaling. *Proceedings of the National Academy of Sciences* **105**, 5632.
Holtorf, S., Apel, K. and Bohlmann, H. (1995). Comparison of different constitutive and inducible promoters for the overexpression of transgenes in Arabidopsis thaliana. *Plant molecular biology* **29**, 637-646.
Kepinski, S. and Leyser, O. (2005). The Arabidopsis F-box protein TIR1 is an auxin receptor. *Nature* **435**, 446-51.
Kuromori, T., Hirayama, T., Kiyosue, Y., Takabe, H., Mizukado, S., Sakurai, T., Akiyama, K., Kamiya, A., Ito, T. and Shinozaki, K. (2004). A collection of 11 800 single copy Ds transposon insertion lines in Arabidopsis. *The Plant Journal* **37**, 897-905.
Mallory, A. C., Bartel, D. P. and Bartel, B. (2005). MicroRNA-directed regulation of Arabidopsis AUXIN RESPONSE FACTOR17 is essential for proper development and modulates expression of early auxin response genes. *Plant Cell* **17**, 1360-75.
McCourt, P. and Desveaux, D. (2010). Plant chemical genetics. *New Phytologist* **185**, 15-26.
Mello, C. C. and Conte, D. (2004). Revealing the world of RNA interference. *Nature* **431**, 338-342.
Meyerowitz, E. M. (2001). Prehistory and history of Arabidopsis research. *Plant Physiol* **125**, 15-9.
Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. plant* **15**, 473-97.
Okushima, Y., Overvoorde, P. J., Arima, K., Alonso, J. M., Chan, A., Chang, C., Ecker, J. R., Hughes, B., Lui, A., Nguyen, D. et al. (2005). Functional genomic analysis of the AUXIN RESPONSE FACTOR gene

family members in Arabidopsis thaliana: unique and overlapping functions of ARF7 and ARF19. *Plant Cell* **17**, 444-63.
Oono, Y., Ooura, C., Rahman, A., Aspuria, E. T., Hayashi, K., Tanaka, A. and Uchimiya, H. (2003). p-Chlorophenoxyisobutyric acid impairs auxin response in Arabidopsis root. *Plant physiology* **133**, 1135.
Overvoorde, P., Fukaki, H. and Beeckman, T. (2010). Auxin Control of Root Development. *Cold Spring Harbor Perspectives in Biology* **2**.
Overvoorde, P. J., Okushima, Y., Alonso, J. M., Chan, A., Chang, C., Ecker, J. R., Hughes, B., Liu, A., Onodera, C., Quach, H. et al. (2005). Functional genomic analysis of the AUXIN/INDOLE-3-ACETIC ACID gene family members in Arabidopsis thaliana. *Plant Cell* **17**, 3282-300.
Parinov, S. and Sevugan, M. (1999). Analysis of flanking sequences from dissociation insertion lines: a database for reverse genetics in Arabidopsis. *The Plant Cell Online* **11**, 2263.
Park, S. Y., Fung, P., Nishimura, N., Jensen, D. R., Fujii, H., Zhao, Y., Lumba, S., Santiago, J., Rodrigues, A., Chow, T. F. et al. (2009). Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* **324**, 1068-71.
Parry, G., Calderon-Villalobos, L. I., Prigge, M., Peret, B., Dharmasiri, S., Itoh, H., Lechner, E., Gray, W. M., Bennett, M. and Estelle, M. (2009). Complex regulation of the TIR1/AFB family of auxin receptors. *Proceedings of the National Academy of Sciences* **106**, 22540.
Rogg, L. E., Lasswell, J. and Bartel, B. (2001). A gain-of-function mutation in IAA28 suppresses lateral root development. *Plant Cell* **13**, 465-80.
Rosso, M. G., Li, Y., Strizhov, N., Reiss, B., Dekker, K. and Weisshaar, B. (2003). An Arabidopsis thaliana T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. *Plant molecular biology* **53**, 247-259.
Sessions, A., Burke, E., Presting, G., Aux, G., McElver, J., Patton, D., Dietrich, B., Ho, P., Bacwaden, J. and Ko, C. (2002). A high-throughput Arabidopsis reverse genetics system. *The Plant Cell Online* **14**, 2985.
Shin, R., Burch, A. Y., Huppert, K. A., Tiwari, S. B., Murphy, A. S., Guilfoyle, T. J. and Schachtman, D. P. (2007). The Arabidopsis transcription factor MYB77 modulates auxin signal transduction. *The Plant Cell Online* **19**, 2440.
Stockwell, B. R. (2000). Chemical genetics: ligand-based discovery of gene function. *Nature Reviews Genetics* **1**, 116-125.
Stockwell, B. R. (2004). Exploring biology with small organic molecules. *Nature* **432**, 846-854.

Sungur, C., Miller, S., Bergholz, J., Hoye, R. C., Brisbois, R. G. and Overvoorde, P. (2007). The small molecule 2-furylacrylic acid inhibits auxin-mediated responses in Arabidopsis thaliana. *Plant and Cell Physiology* **48**, 1693.

Swarup, K., Benkova, E., Swarup, R., Casimiro, I., Peret, B., Yang, Y., Parry, G., Nielsen, E., De Smet, I., Vanneste, S. et al. (2008). The auxin influx carrier LAX3 promotes lateral root emergence. *Nat Cell Biol* **10**, 946-54.

Tan, X., Calderon-Villalobos, L. I., Sharon, M., Zheng, C., Robinson, C. V., Estelle, M. and Zheng, N. (2007). Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature* **446**, 640-5.

Tatematsu, K., Kumagai, S., Muto, H., Sato, A., Watahiki, M. K., Harper, R. M., Liscum, E. and Yamamoto, K. T. (2004). MASSUGU2 encodes Aux/IAA19, an auxin-regulated protein that functions together with the transcriptional activator NPH4/ARF7 to regulate differential growth responses of hypocotyl and formation of lateral roots in Arabidopsis thaliana. *Plant Cell* **16**, 379-93.

The Arabidopsis Genome Initiative, A. G. I. (2000). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. *Nature* **408**, 796-815.

Tian, Q. and Reed, J. W. (1999). Control of auxin-regulated root development by the Arabidopsis thaliana SHY2/IAA3 gene. *Development* **126**, 711-21.

Tóth, R. and van der Hoorn, R. A. L. (2010). Emerging principles in plant chemical genetics. *Trends in plant science* **15**, 81-88.

Uehara, T., Okushima, Y., Mimura, T., Tasaka, M. and Fukaki, H. (2008). Domain II mutations in CRANE/IAA18 suppress lateral root formation and affect shoot development in Arabidopsis thaliana. *Plant Cell Physiol* **49**, 1025-38.

Vanneste, S., De Rybel, B., Beemster, G. T., Ljung, K., De Smet, I., Van Isterdael, G., Naudts, M., Iida, R., Gruissem, W., Tasaka, M. et al. (2005). Cell cycle progression in the pericycle is not sufficient for SOLITARY ROOT/IAA14-mediated lateral root initiation in Arabidopsis thaliana. *Plant Cell* **17**, 3035-50.

Wang, J. W., Wang, L. J., Mao, Y. B., Cai, W. J., Xue, H. W. and Chen, X. Y. (2005). Control of root cap formation by microRNA-targeted auxin response factors in Arabidopsis. *The Plant Cell Online* **17**, 2204.

Wise, E. M. and Abou-Donia, M. M. (1975). Sulfonamide resistance mechanism in Escherichia coli: R plasmids can determine sulfonamide-

resistant dihydropteroate synthases. *Proceedings of the National Academy of Sciences of the United States of America* **72**, 2621.

Woodward, A. W. and Bartel, B. (2005). Auxin: regulation, action, and interaction. *Ann Bot (Lond)* **95**, 707-35.

Yamazoe, A., Hayashi, K., Kepinski, S., Leyser, O. and Nozaki, H. (2005). Characterization of terfestatin A, a new specific inhibitor for auxin signaling. *Plant Physiol* **139**, 779-89.

Yang, X., Lee, S., So, J. H., Dharmasiri, S., Dharmasiri, N., Ge, L., Jensen, C., Hangarter, R., Hobbie, L. and Estelle, M. (2004). The IAA1 protein is encoded by AXR5 and is a substrate of SCF(TIR1). *Plant J* **40**, 772-82.

Yoon, E. K., Yang, J. H. and Lee, W. S. (2010). Auxin and Abscisic Acid Responses of Auxin Response Factor 3 in Arabidopsis Lateral Root Development. *Journal of Plant Biology* **53**, 150-154.

Chapter 4 Identification of a novel class of auxin response inhibitors using a chemical biology approach

4.1. Abstract

In the previous chapter a novel class of chemicals identified in a screen for inhibitors of the induction of the reporter gene LAX3 has been studied and structurally characterised. Using different approaches, the mode of action of this family of inhibitors in plants is now being dissected. It is shown that these inhibitors block the induction of many auxin responsive genes most likely by stabilising the Aux/IAA family of repressors. Importantly they don't act as anti-auxins, which is consistent with their structure very different from IAA. Using various methods, it is also shown that the inhibitors block the response to hormonal pathways dependent on the SCF complex, such as gibberellins or jasmonic acid, but not kinase dependent pathways, such as brassinosteroids or abscissic acid. Finally, some data suggest that this SCF specific effects could be due to a reduction of CUL1 neddylation.

4.2. Introduction

The plant hormone auxin (IAA) regulates a wide variety of developmental processes (Woodward and Bartel, 2005). These are mainly mediated via a nuclear localised machinery that triggers the transcriptional reprogramming of target cells (Vanneste and Friml, 2009). The transduction pathway leads to the rapid degradation of Aux/IAA repressor proteins via the ubiquitin/proteasome pathway (Chapman and Estelle, 2009). The accelerated Aux/IAA turnover relieves ARFs transcription factors to induce or

repress gene expression (Lokerse and Weijers, 2009; Tiwari et al., 2003; Tiwari et al., 2001; Ulmasov et al., 1999; Ulmasov et al., 1997).

IAA induces the degradation of Aux/IAAs by promoting their interaction with the auxin receptor TIR1 (TRANSPORT INHIBITOR RESPONSE 1) (or the related AFB1-3) (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Crystallographic studies revealed that IAA sits in a pocket within TIR1, greatly increasing its affinity with a domain of Aux/IAA proteins called "Domain II" (Tan et al., 2007). TIR1 is an F-BOX protein that is a sub-unit of an SCF complex (Gray et al., 2001; Kepinski and Leyser, 2004; Ruegger et al., 1998). The other sub-units that form the active SCF^{TIR1} complex in Arabidopsis have been identified and are termed ASK1 or ASK2 (ARABIDOPSIS SKP1-LIKE), CUL1 (CULLIN1) and RBX1 (RING BOX1) (del Pozo and Estelle, 1999; Gray et al., 1999; Gray et al., 2002; Hellmann et al., 2003; Moon et al., 2007; Quint et al., 2005; Shen et al., 2002; Zhao et al., 1999).

Several signalling pathways in plants rely on the ubiquitination machinery. Notably, several members of the F-BOX protein family, which provide substrate specificity to the SCF E3 ubiquitin ligase complex, are involved in hormonal signal transduction pathways:

- Ethylene (EBF1 and 2) (Guo and Ecker, 2003; Potuschak et al., 2003)
- Auxin (TIR1 and AFB1-5) (Dharmasiri et al., 2005; Kepinski and Leyser, 2005)
- Gibberellins (SLY1) (Dill et al., 2004; McGinnis et al., 2003)
- Jasmonate (COI1) (Thines et al., 2007; Xie et al., 1998)

- and potentially strigolactones (MAX2) (Gomez-Roldan et al., 2008; Stirnberg et al., 2002; Umehara et al., 2008), (Gagne et al., 2002; Ho et al., 2006; Willems et al., 2004).

In eukaryotes, the activity of the SCF complex is regulated by cycles of addition and removal of NEDD8/RUB on the CULLIN subunit (NEDD8 stands for Neural-precursor-cell-expressed Developmentally Down-regulated 8 and RUB for Related to Ubiquitin) (Kamitani et al., 1997; Kirkin and Dikic, 2007; Rabut and Peter, 2008). NEDD8 is a polypeptide that is highly similar to ubiquitin. The enzymatic reaction that leads to the addition of a NEDD8 moiety (a process known as Neddylation) is reminiscent of the ubiquitination machinery but with notable structural differences (Huang et al., 2009; Huang et al., 2007; Huang et al., 2004; Huang et al., 2005; Walden et al., 2003).In plants, a heterodimer functions as a NEDD8 E1 activating enzyme (the two proteins are termed AXR1 and ECR1 in Arabidopsis) (del Pozo et al., 2002; del Pozo and Estelle, 1999; del Pozo et al., 1998; Leyser et al., 1993). Subsequently, a NEDD8 E2 conjugating enzyme, termed RCE1, together with a NEDD8 E3 ligase, termed RBX1, add a NEDD8 moiety to the CUL1 subunit (del Pozo et al., 2002; Dharmasiri et al., 2003; Gray et al., 2002; Willems et al., 2004). When modified, the CULLIN protein is able to interact with the SCF complex (via SKP1, which is termed ASK1/2 in Arabidopsis) (Chuang et al., 2004; Feng et al., 2004; Schwechheimer et al., 2001; Zhang et al., 2008). Removal of the NEDD8 subunit by the COP9 signalosome complex (also known as CSN complex) triggers the dissociation of CUL1 from the SCF complex and its subsequent interaction with CAND1 (CULLIN ASSOCIATED and NEDD8-DISSOCIATED1) (Lyapina et al., 2001; Schwechheimer et al., 2001).

The importance of the Neddylation pathway in the response to auxin is highlighted by mutant phenotypes: loss of function of any sub-units of the CSN complex is embryo lethal and reduced expression of *CSN5*, the catalytic sub unit of the complex, leads to stabilisation of Aux/IAA proteins (Cope and Deshaies, 2003; Cope et al., 2002; Lyapina et al., 2001; Schwechheimer and Isono, 2010; Schwechheimer et al., 2001). Also, loss of function in the NEDD8 E1, E2 or E3 (*axr1*, *ecr1* and *rbx1)* result in auxin resistant phenotypes (del Pozo et al., 2002; del Pozo and Estelle, 1999; Dharmasiri et al., 2003; Gray et al., 2002). Taken together, it appears that cycles of addition and removal of NEDD8 on CUL1 are required to maintain the SCF complex active (Gray et al., 2002; Schwechheimer et al., 2001).

In the previous chapter, five related sulfonamides that inhibit the induction by auxin of *LAX3* have been identified using a high throughput chemical genetics screen. In this chapter, it is shown that this class of inhibitors affects many auxin regulated genes probably because they stabilise Aux/IAA proteins. It is further shown that they do not target TIR1 but affect CUL1 Neddylation. As a consequence, several hormonal transduction pathways that require an active SCF complex are affected. On the other hand, SCF independent pathways, such as brassinosteroids or abscissic acid, are not or only slightly affected.

4.3. Material and Methods

4.3.1. Materials

4.3.1.1. Chemicals

Cycloheximide (CHX) was purchased from VWR International Ltd and was dissolved in 100% EtOH. The *LAX3* inhibitor LI5 was synthesised in house at the University of Nottingham, School of Chemistry, by Neil J. Oldham and Anna Westacott. All *LAX3* inhibitors were dissolved in 100% DMSO to a stock concentration of 20 mM.

4.3.1.2. Hormones

Indole-3-acetic acid (IAA), α-naphthalene acetic acid (NAA), gibberellic acid (GA4), methyl jasmonate (MeJa), epibrasinolide (EpiBL), 1-Aminocyclopropanecarboxylic acid (ACC, ethylene precursor) and (±)-Abscisic acid (ABA) were purchased from Sigma. IAA, GA4 and BL were dissolved in 100% EtOH, NAA and MeJa were dissolved in DMSO, ACC was dissolved in ddH_2O and ABA in 100% methanol. All compounds were dissolved to a stock concentration of 50 or 100 mM.

4.3.1.3. Arabidopsis Mutants

Arabidopsis seeds for the lines p35S::JAZ-GUS (Thines et al., 2007) and p35S::DII(IAA28)-VENUS (Vernoux et al., 2011) were kindly provided by Pr. John Browse (Washington State University) and Dr Teva Vernoux (ENS Lyon). The transgenic lines used were initially described in the following publications: pHS::AXR3NT-GUS and pHS::GUS (Gray et al., 2001), pRGA::RGA-GFP (Silverstone

et al., 2001), pDR5::GUS (Ulmasov et al., 1997), pIAA19::GUS (Tatematsu et al., 2004) and *prt6-1* (Garzón et al., 2007).

4.3.1.4. Primers for qPCR

Primer name	Sequence (5'->3')
qCTRL1-F	agtggagaggctgcagaaga
qCTRL1-R	ctcgggtagcacgagcttta
qIAA3F	caaagatggtgattggatgct
qIAA3R	tgatccttagtctcttgcacgta
qIAA14F	caaagatggtgactggatgc
qIAA14R	gcatgactcgacaaacatcg
qPG-R	catcgatggacgaggatca
qPG-F	cctcaaagctgttggtttgg
qCYCB11-F	ttccattgcagacgaaaaga
qCYCB11-R	tgatggactgaacattatcatcg
qLAX3-F	tcaccattgcttcactccttc
qLAX3-R	aagcaccattgtggttggac
qARF19-F	caccgatcacgaaaacgata
qARF19-R	tgttctgcacgcagttcac
qERF1-F	cttcccttcaacgagaacga
qERF1-R	gtttgttgcgtggactgct
qEDF1-F	gtggcggttccgttacagt
qEDF1-R	ccagcccttggtcaacac
qEDF2-F	cacgaaagctaccatcttcaaa
qEDF2-R	atctgagctccccatcttcc
qEDF3-F	acggtaacggaaaagagacg
qEDF3-R	ccgttttaaaccccgtca
qEDF4-F	gcatctttcacgcctcgta
qEDF4-R	gcaaataaagaaaacgaaaatgg
qEBF2-F	ctggaatcttcagatttagtggtg
qEBF2-R	cttacgcgctgggtaatataca
qRD29B-F	gaagagtctccacaatcacttgg
qRD29B-R	caactcacttccaccggaat
qRAB18-F	ggcttgggaggaatgctt
qRAB18-R	ttgatcttttgtgttattcccttct
qRD22-F	agggctgtttccactgagg
qRD22-R	caccacagatttatcgtcagaca
qKIN2-F	ggcaaagctgaggagaagag
qKIN2-R	actgccgcatccgatatact
qBR6OX2-F	caatagtctcaatggacgcagagt
qBR6OX2-R	aaccgcagctatgttgcatg
qBR6OX1-F	tggccaatctttggcgaa
qBR6OX1-R	tcccgtatcggagtctttggt
qBAS1-F	ttggcttcataccgtttggc
qBAS1-R	ttacagcgagtgtcaatttggc
qDWF4-F	gtgatctcagccgtacatttgga
qDWF4-R	cacgtcgaaaaactaccacttcct

4.3.2. Methods

4.3.2.1. Seed sterilisation and seedling growth

Seeds were surface sterilized for 5 minutes in 50% bleach, 0.1% triton X-100 then washed three times with sterile ddH_2O. Seeds were stratified at 4° for 2 days to synchronise germination. Seeds were sown on ½ MS medium (Murashige and Skoog, 1962) (Sigma) (2.17g salts/L), at pH5.7 solidified with 1% bacto-agar (Appleton Woods).

4.3.2.2. Histology and histochemistry

GUS activity is revealed by incubating seedlings in a phosphate buffer (500mM, pH 7) containing 0.5 mM potassium ferricyanide ($K_3[Fe(CN)_6]$), 0.5 mM potassium ferrocyanide ($K_4[Fe(CN)_6]$), 1 mM ethylenediaminotetraacetic acid (EDTA pH 8), 0.5% (v/v) Triton X-100 and 1 mM X-bromo-4-chloro-3-indolyl ß-D-galactopyranoside (Sigma) (X-Glc) at 37° for 1 hour. X-Glc is initially dissolved in 100% dimethylformamide (DMF) to reach a final DMF concentration of 0.5%. After GUS staining, seedlings are cleared for at least 12 hours in 260% chloral hydrate in 33% glycerol (all chemicals purchased from Fischer Scientific and Sigma) before observation on microscope.

4.3.2.3. Confocal microscopy

Confocal microscopy was performed using a Leica SP5 confocal laser scanning microscope (Leica Microsystems) and a Nikkon EZ-C1 confocal laser scanning microscope. Scanning settings used for one experiment were optimised and kept unchanged throughout the experiment.

4.3.2.4. Image analysis

Root length was measured using ImageJ (ImageJ 1.40g) and confocal scanning microscope as well as light microscope images were processed using Fiji (Fiji Is Just ImageJ, ImageJ 1.44b) and figures assembled using Adobe Photoshop (version 7.01; Adobe Systems) and Microsoft PowerPoint 2010 (Microsoft Corporation, Redmond, USA).

4.3.2.5. RNA extraction and RT qPCR

RNA was extracted from plant tissues using Trizol Reagent (Invitrogen) and cleaned up using the RNeasy kit (Qiagen). Poly(dT) cDNA was prepared from 0.5 µg total RNA with Superscript II Reverse transcriptase (Invitrogen) and analyzed on a LightCycler 480 apparatus (Roche Diagnostics) with the Quantace SYBRGREEN mix (Quantace) according to the manufacturer's instructions. Targets were quantified with specific primer pairs designed using the Universal Probe Library Assay Design Center (Roche Applied Science). All individual reactions were done in quadruplicate and data were analyzed with Microsoft Excel 2010 (Microsoft Corporation, Redmond, USA). Expression levels were normalized to At1G04850 (CTRL1, thanks to Tara Holman for primer sequences).

4.3.2.6. TIR1 pull downs

Immunoprecipitations were performed as previously described (Gray et al., 1999). For GST-AXR3 pull-down assays, 4 mg of purified fusion protein was added to 2.5 mg of crude Arabidopsis protein extract prepared from 7-day-old seedlings. Extracts were prepared by homogenizing seedlings in Buffer C (Gray et al., 1999) supplemented with

1mM dithiothreitol, 10 mM MG132, 10mM b-glycerolphosphate, 1mM NaF and 1mM orthovanadate. The resulting homogenate was cleared by microcentrifugation for 15min. Following addition of the glutathione±agarose-bound GST fusion protein, extracts were incubated at 4 8C with gentle agitation for 3 h. Glutathione beads were collected by brief centrifugation, washed three times in the above buffer, resuspended in SDS-PAGE sample buffer and subjected to SDS-PAGE electrophoresis and immunoblotting.

4.3.2.7. CUL1 Neddylation

Total proteins were extracted (as described in (Criqui et al., 2000) from 2-week-old *Arabidopsis* seedling of wild-type Col-0 genotype treated for 24 hours with or without NAA and with or without LI5. CUL1 was detected with a specific CUL1 antibody using western blot.

4.3.2.8. Hormone responses

To probe the response to various hormones, the following procedures were followed. Seeds of the GUS reporters (IAA19p::GUS, DR5p::GUS, 35S::JAZ1-GUS and HS::AXR3 NT-GUS) were germinated on ½ MS agar plates and transferred to liquid ½ MS media for treatments. Various times of incubation (IAA19p::GUS, 2 hours, DR5p::GUS: 20 minutes, 35S::JAZ1-GUS, overnight and HS::AXR3 NT-GUS, 2 hours) were used to obtain optimum GUS staining. For live imaging of fluorescent reporters (RGAp::RGA-GFP and 35S::DII(28)-VENUS) seedlings were transferred on glass bottom Petri dishes and a block of agar containing the chemicals for treatments was gently and carefully laid down on the seedlings. Images were collected on a Leica SP5 confocal scanning microscope as described in 4.3.2.

4.3.2.9. Microarray experiment set up

Col-0 Arabidopsis seeds were sown on plates containing NPA (10μM). 5 days after germination, seedlings were transferred on plates containing DMSO (mock treatment), LI5 at 5μM, NAA at 10μM or LI5 and NAA at 5 and 10μM respectively, for 6 hours. RNA was extracted as described in the RNA extraction and RT qPCR section. Hybridization of the RNAs on the Affymetrix ATH1 chips was performed at the microarray facility at NASC (Nottingham Arabidopsis Stock Center).

4.3.2.10. Microarray quality controls

M vs A plots, chip images, distribution of probe intensities before and after normalisation, Normalised Unscaled Standard Error (NUSE), Relative Log Expression (RLE), PCA analysis, array to array correlation, RNA degradation curves as well as Affymetrics recommended quality controls were performed within R/Bioconductor (Gentleman et al., 2004; Team, 2007).

4.3.2.11. Microarrays data analysis

Data were normalised from .cel files using the RMA protocol (background correction: rma, normalisation: quantiles, PM correction: pmonly, expression: median polish) within R/Bioconductor (Gentleman et al., 2004; Team, 2007) and a recent CDF file (http://brainarray.mbni.med.umich.edu/, version 12). Lists of differentially expressed gene were obtained by fitting a linear model using the limma package in R/Bioconductor. Further analyses were performed using Excel 2010 (Microsoft Corporation, Redmond, USA). Differentially regulated loci had a fold change greater than 2, a Benjamini and Hochberg False Discovery Rate of 0.05 (or 5%) and an

expression value above 6.64 (log2) (Benjamini and Hochberg, 1995). Data is the average of 3 biological replicates per treatment. Enrichment in GO terms was determined using TAIR and BAR websites. Heat maps were generated using MeV (Multi Experiment Viewer, part of TM4 Software Suite). To determine the extent of LI5 effects on NAA regulated genes, the following parameters were used:

- If a gene is up regulated by NAA and in presence of LI5 and NAA its up-regulation is more than 2 fold increased, the induction of the gene is considered to be **additive**
- If a gene is up-regulated by NAA and in presence of LI5 and NAA its up-regulation is less than 2 fold different compared to NAA on its own, the induction of the gene is considered to be **unaffected or slightly affected**.
- If a gene is up-regulated by NAA and in presence of LI5 and NAA its up-regulation is more than 2 fold decreased compared to NAA on its own, the induction of the gene is considered to be **affected**.
- If a gene is up regulated by NAA and in presence of LI5 and NAA the gene is not differentially regulated the induction of the gene is considered to be **completely affected**.
- If a gene is up regulated by NAA and in presence of LI5 and NAA the gene is down-regulated the induction of the gene is considered to be **opposite**.

These rules are similarly applied to down-regulated genes.

4.4. Results

4.4.1. LI5 affects early auxin response in all root tissues

To determine whether LI5 affects only secondary auxin responsive genes (such as *LAX3*) or primary responsive genes as well (such as *IAA19* (Tatematsu et al., 2004) or the synthetic reporter DR5 (Ulmasov et al., 1997)), 5-day-old seedlings expressing IAA19::GUS or DR5::GUS reporters were treated with IAA at 1μM and with various concentrations of LI5 then sampled at different time point and stained.

Figure 4-1 shows that LI5 affects the induction of both IAA19 and DR5 GUS reporters in presence of IAA. This result suggests that LI5 blocks not only a secondary auxin responsive gene (LAX3) but also primary auxin responsive genes (at least IAA19 and the synthetic DR5 reporter). Interestingly, DR5::GUS staining is still visible in the columella cells at the root tip of seedlings treated with IAA and LI5 at 5 or 10μM for 24 hours. To rule out the possibility that LI5 affects auxin uptake from the medium, a protein synthesis inhibitor (cycloheximide, CHX) was used to determine how long the GUS enzyme is stable at the root tip. Figure 4-1 shows that it is stable for at least 24 hours but not 72 hours. This result suggest that LI5 blocks auxin responses most likely by acting at the transcriptional level.

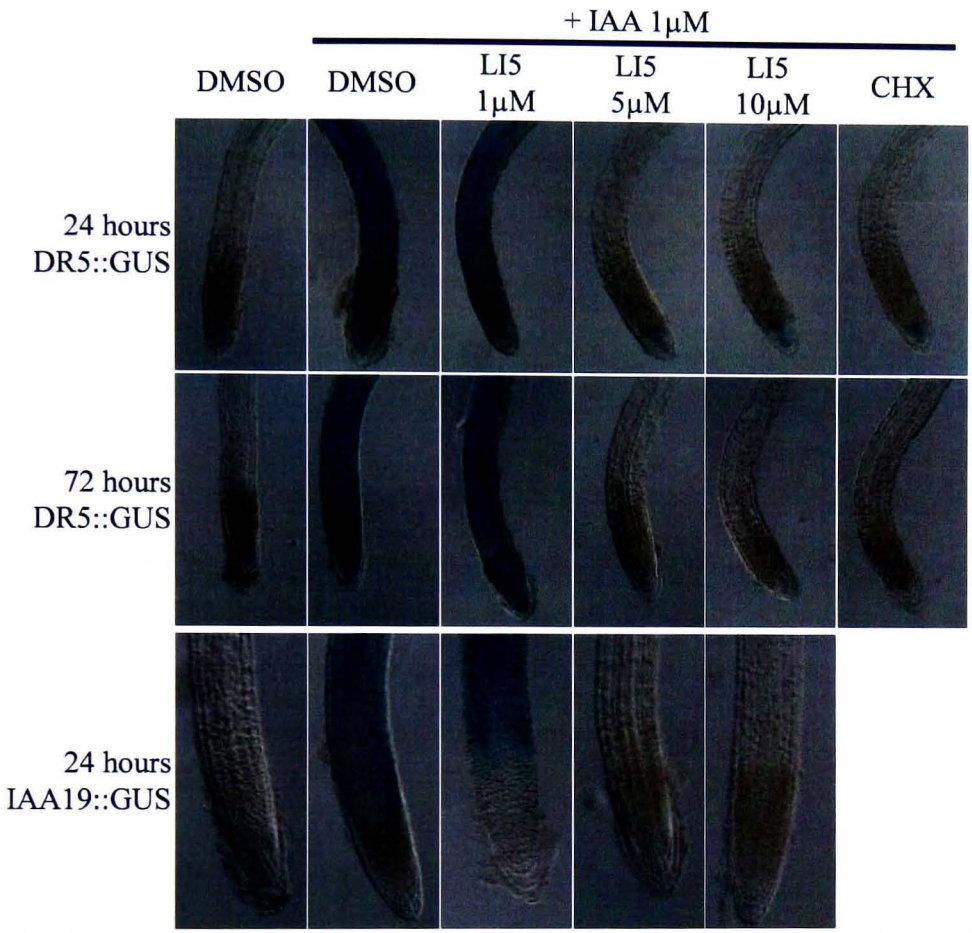

Figure 4-1 Effects of LI5 concentration on the induction of two auxin responsive markers. Cycloheximide (CHX) is used as a control to show that LI5 does not block the flux of IAA from the medium into the root.

To confirm these observations, 5-day-old col-0 seedlings were treated with NAA (10μM) and with or without LI5 at various concentrations then sampled at different time points to analyse gene expression by RT qPCR. First, the dynamics of auxin induction of *LAX3* were analysed. Figure 4-2 (A) shows that *LAX3* is not induced by NAA in presence of LI5at 5 or 10 uM (but it does at 1uM) even after 10 hours of treatments. Therefore, LI5 does not delay the response to auxin but completely blocks it. Secondly, the induction fold of auxin responsive genes specific for different root tissues, such as *CYCB1;1* (pericycle), *IAA3* (endodermis), *IAA14* (pericycle, cortex and epidermis), *PG*

(cortex and epidermis), *LAX3* (vasculature, cortex and epidermis) and *ARF19* (all root tissues) were measured. Figure 4-2 (B) shows that LI5 blocks the induction by auxin of all the gene analysed in all tissues of the mature part of the root.

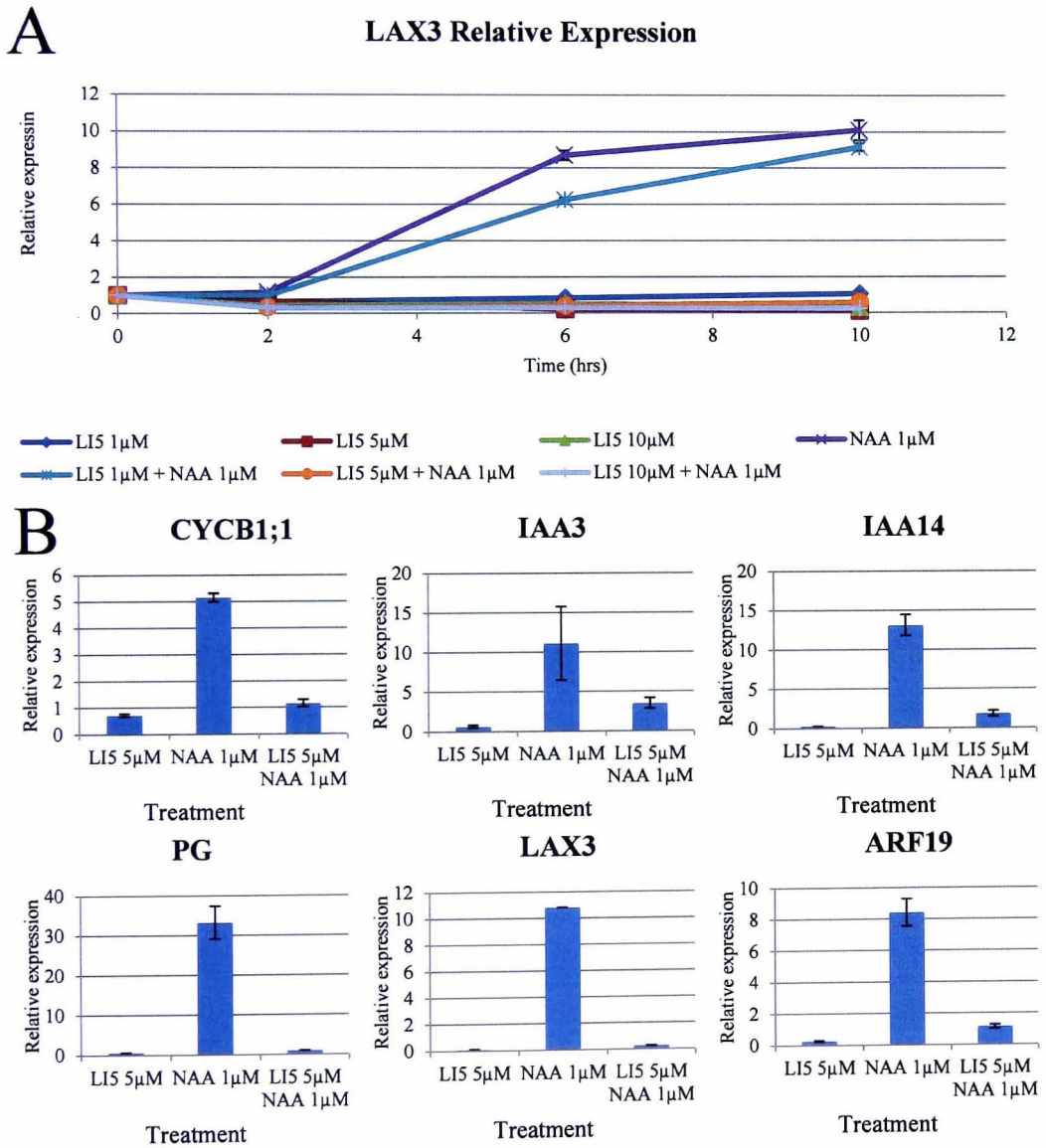

Figure 4-2 LI5 does not delay auxin response and affect all tissues. (A) Induction of *LAX3* is affected by LI5 at 5µM and is not delayed. **(B)** Several auxin responsive genes expressed in specific tissues are not induced in presence of NAA and LI5. Error bars on all the charts show the SD of the mean of four technical replicates.

4.4.2. Transcriptomics analysis

4.4.2.1. Experimental set-up and quality controls.

To have an overview of the genomic effects of LI5 on the response to auxin, we performed a transcriptomic analysis on the mature part of the root of wild type (col-0) seedlings treated for 6 hours with or without auxin (NAA at 10μM) and with or without LI5 at 5μM. We generated 3 biological replicates for each treatment, resulting in 12 samples in total. Several quality control checks were done (details are in material and methods) and based on these it is concluded that the quality of the dataset is very high. Figure 4-3 and Figure 4-4 show the results of some of these QC.

The procedures used for probe sets corrections and normalisation, identification of differentially expressed genes and microarray data mining are in the material and methods. Analysis of differentially expressed genes was done by looking at significantly enriched Gene Ontologies (GO) in sets of genes induced or repressed in the different treatments using TAIR (The Arabidopsis Information Resource) and BAR (The Bio-Array Resource for Plant Biology) websites. Details of the method use for the analysis are described in Material and Methods

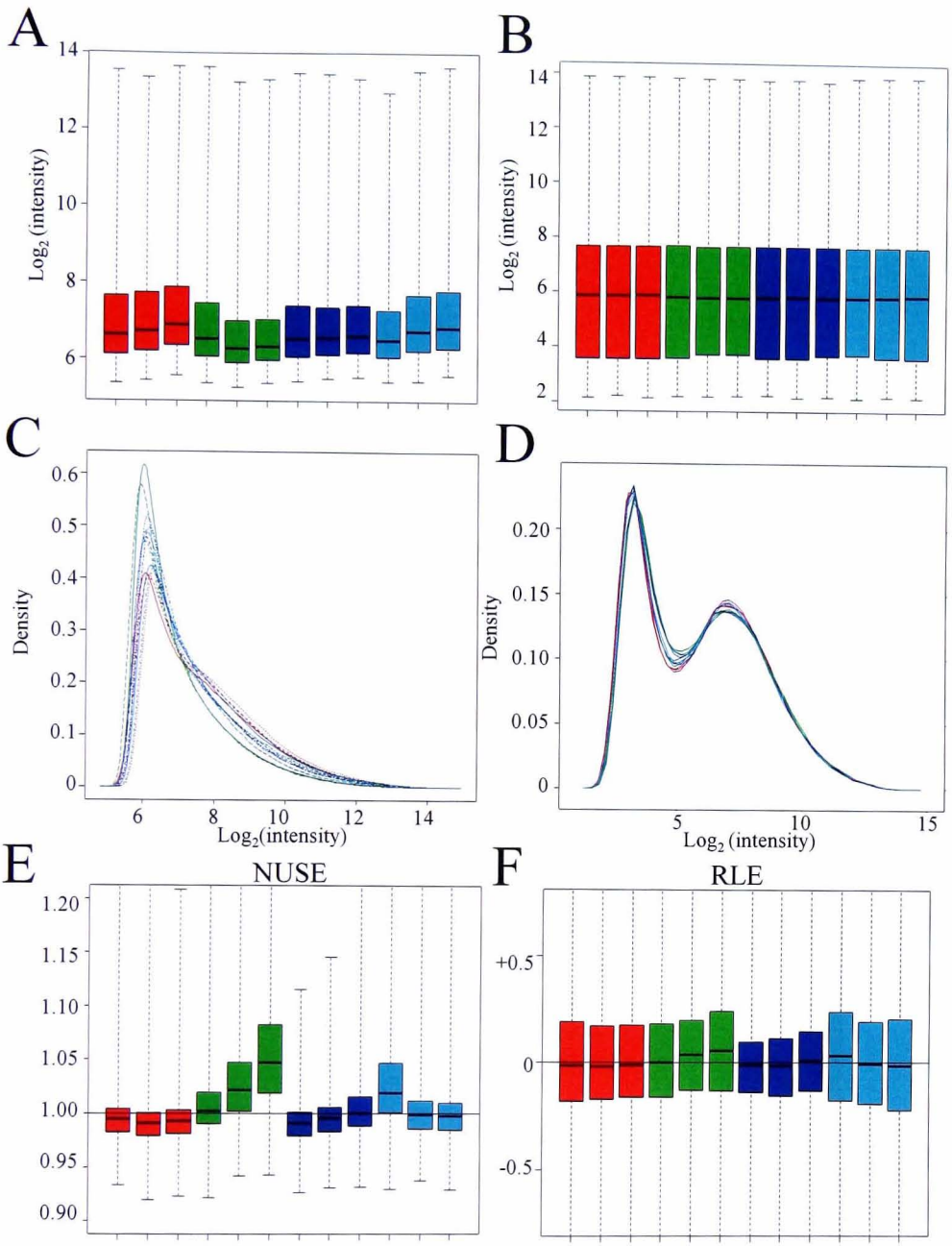

Figure 4-3 Quality controls (1) of the arrays analysed. (A) and **(B)** Boxplots of the probe intensities (log_2) on the arrays before **(A)** and after **(B)** normalisation and background corrections. **(C)** and **(D)** Distribution of the probe intensities (log_2) before **(C)** and after **(D)** normalisation and background corrections. In **(D)** the two peaks represent the distribution of the non-expressed probe sets (below background, first peak) and the distribution of the expressed probe sets (above background, second peak). **(E)** NUSE (Normalised Unscaled Standard Errors) and **(F)** RLE (Relative Log Expression) charts of the arrays analysed. In **(E)**, it is expected that the normalised standard errors of the arrays centre around 1 and have an equivalent spread. It appears that the third green array (LI5 replicate 3) is different from the others. Low quality arrays typically centre above 1.1 and, therefore, despite this array showing signs of low quality, it remains acceptable. On each chart, the 3 replicates of each treatment are shown in red (mock), green (LI5), dark blue (LI5 and NAA) and light blue (NAA).

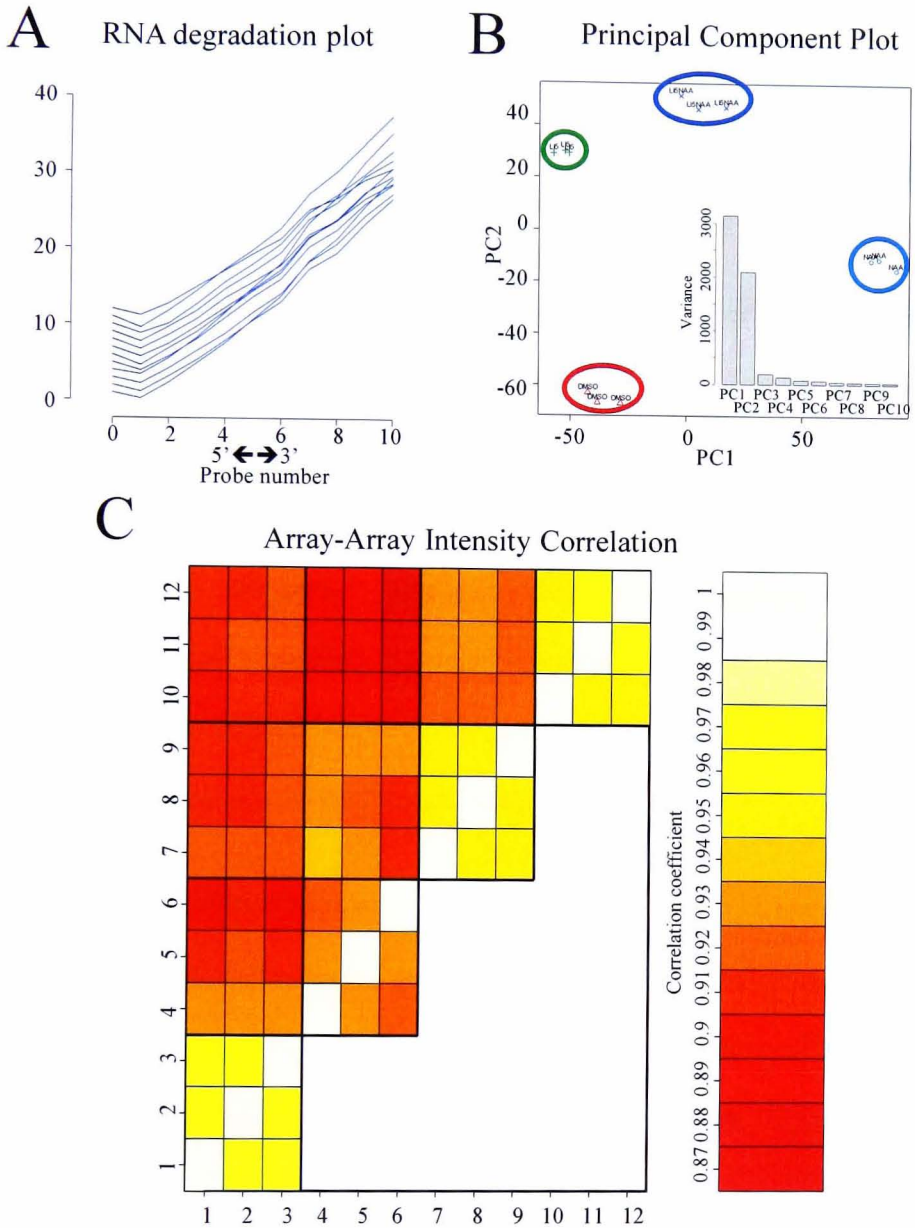

Figure 4-4 Quality controls (2) of the arrays analysed. (A) The RNA degradation curves show no obvious signs of RNA degradation in the samples. **(B)** Principal component analysis (PCA) of the two components that explain most of the variance (as shown on the scree plot). The PCA analysis shows that component 1 explains most of the variation due to NAA whereas component 2 explains most of the variation due to LI5. **(C)** Array to array correlation analysis shows that arrays obtained from similar treatments (1-3, mock; 4-6, LI5; 7-9, LI5 and NAA; 10-12, NAA) have a high correlation coefficient. On the other hand, arrays obtained from two very different treatment (LI5 (4-6) and NAA (9-12) have a low correlation coefficient.

4.4.2.2. Effects of LI5

At the phenotypic level, treatment with LI5 at 5μM severely affects plant growth and lateral root formation (see Chapter 3). Seedlings grown on LI5 have shorter roots with no lateral roots and the cotyledons show signs of anthocyanin accumulation (red colour).

At the genomic level, treatment with LI5 at 5μM induces 716 genes and represses 734 genes in the root. GOs of induced genes fall into four main categories: responses to heat, to organic substances, to abiotic stimulus and to ABA. The top 10 induced genes include two multi drug efflux transporter and two heat shock proteins. GOs of repressed genes fall into three main categories: hydrogen peroxide metabolism, glucosinolate biosynthesis and cell wall formation. The top 10 repressed genes include three disease resistance proteins, two peroxidases and one sulfotransferase. Figure 4-5 shows GOs overrepresented in genes induced and repressed by LI5.

4.4.2.3. Effects of NAA

Treatment with NAA at 10μM triggers many developmental and morphological changes in the Arabidopsis root: formation of root hairs, initiation and emergence of lateral roots, arrest of root growth and many others.

At the genomic level, treatment with NAA at 10μM induces 1475 genes and represses 1174 genes in the root. Most of these genes have been reported before as auxin responsive. Notably, the GOs of induced genes fall into three main categories: lateral root formation, ribosome biogenesis and cell cycle. The top 10 induced genes include three LOB domain proteins, one Aux/IAA and one GH3. GOs of repressed genes fall into three main categories: glucosinolate biosynthesis, photosynthesis and response to

reactive oxygen species. The top 20 repressed genes include one peroxidase, one glucosinolate biosynthesis gene, one aquaporin and two proteases. Figure shows GOs overrepresented in genes induced and repressed by NAA. Figure 4-5 shows GOs overrepresented in genes induced and repressed by NAA. Figure 4-6 and Figure 4-7 compares the effects of LI5 on genes up and down regulated by NAA respectively.

4.4.2.4. Effects of LI5 and NAA

Treatment with LI5 at 5μM and NAA at 10μM blocks all the developmental and morphological changes induced by NAA, apart from root growth which is already severely reduced by LI5 on its own (Chapter 3). Seedlings are very similar to the one treated with LI5 only.

At the genomic level, treatment with LI5 at 5μM and NAA at 10μM induces 1146 genes and represses 1059 genes. GOs of induced genes fall into the same main categories as LI5 and also to a less extent NAA: responses to heat, organic substances, abiotic stimulus, ABA, auxin and lateral root formation. The top 10 induced genes include one LOB domain protein, one GH3, two multidrug efflux transporters and two proteins involved in the degradation of ROS (Reactive Oxygen Species). GOS of repressed genes fall into the same main categories as LI5 and, to a less extent, NAA as well: glucosinolate biosynthesis, hydrogen peroxide metabolism, response to reactive oxygen species and photosynthesis. The top 10 repressed genes include two peroxidases. Figure 4-5 shows GOs overrepresented in genes induced and repressed by LI5 and NAA

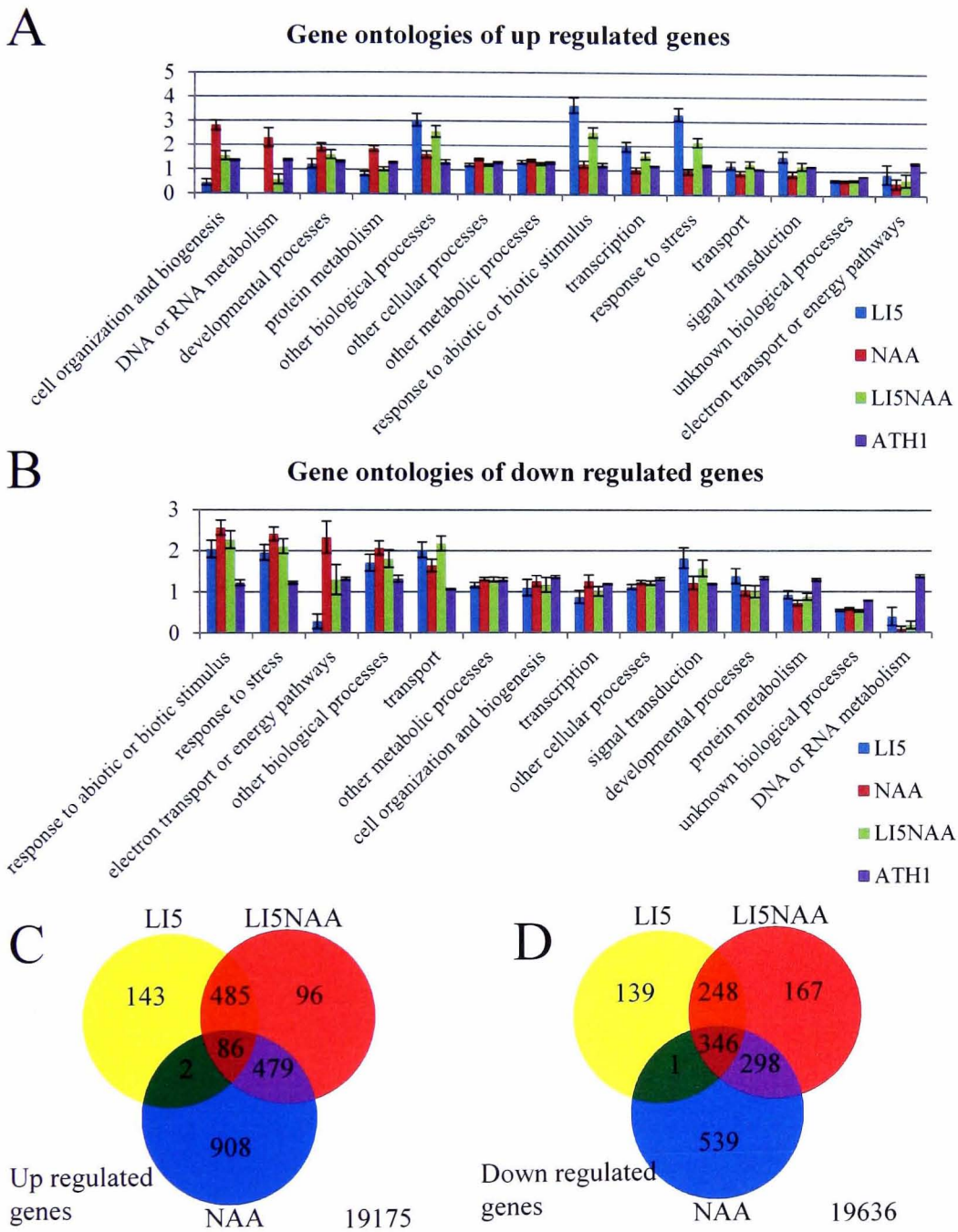

Figure 4-5 LI5 and NAA affects separate sets of genes. (A and B) Over and under representation of gene ontologies (GO) of genes up **(A)** and down **(B)** regulated in the different treatments. GO terms are ordered with the most overrepresented in the NAA treatment on the left and the most underrepresented in the NAA treatment on the right. The mean and standard deviation for 100 bootstraps of the datasets were used to estimate the relevance of the over and under representation of the GO terms. **(C and D)** Venn diagram of genes up **(C)** and down **(D)** regulated in the different treatments.

4.4.2.5. To what extent LI5 affects auxin responsive genes?

A detailed comparison of the effects of LI5 on auxin regulated genes has been carried out. Figure 4-6 and Figure 4-7 show that LI5 affects 75% of auxin induced genes and 52% of auxin repressed genes. Interestingly, amongst the auxin regulated genes which are not affected by LI5 some are actually more induced or more repressed (genes classified as "additive" on Figure 4-6 and Figure 4-7). Interestingly, 67% of these genes have at least one Auxin Response Element (ARE) in their promoter (this proportion is of 44% on the ATH1 chip). Importantly, these genes are up or down regulated by LI5 on its own as well which explains the increased expression in presence of both LI5 and NAA. The microarray data show that LI5 blocks the response to auxin almost completely and induces stress responsive genes, which are also induced by NAA on its own. Therefore, it appears that the inhibitor targets a general and conserved mechanism of the auxin response machinery.

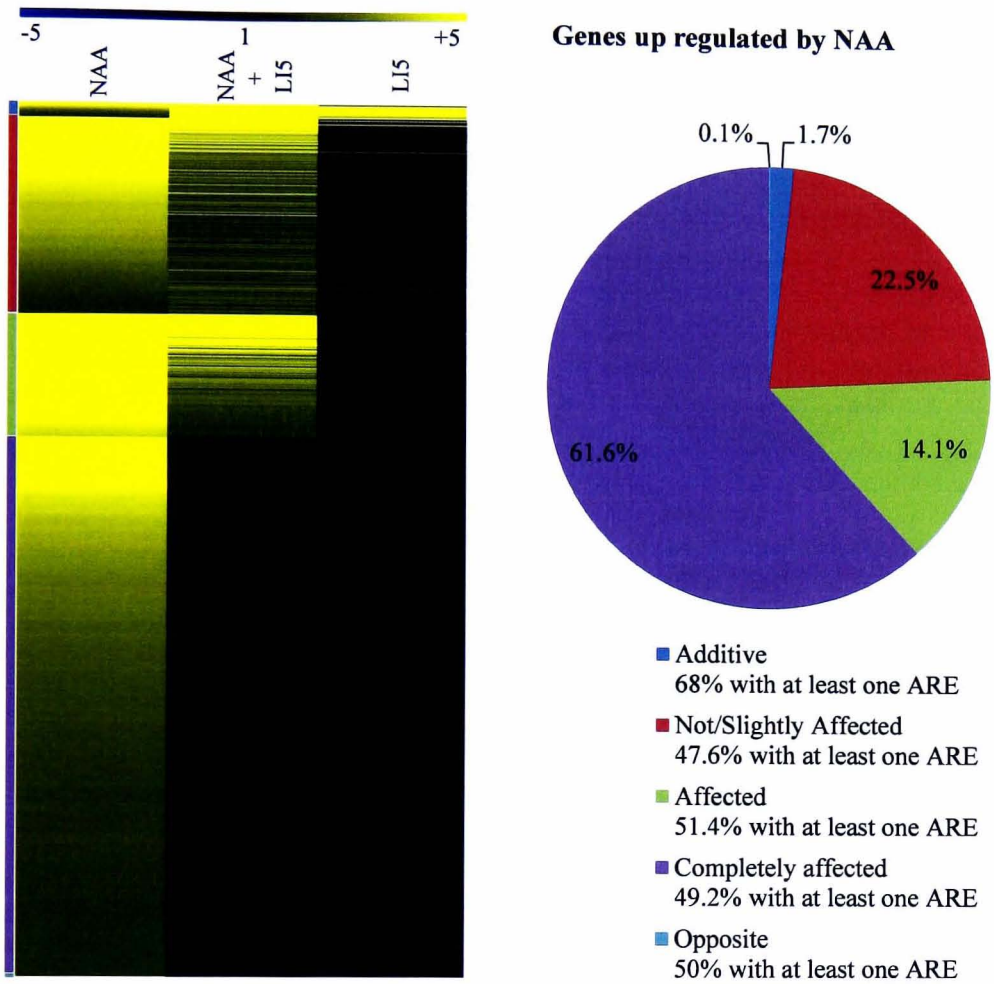

Figure 4-6 LI5 affects 75% of genes up regulated by NAA. The heat map shows the 1475 genes up regulated in presence of NAA. The genes are clustered depending on how LI5 affects their induction. The coloured strips on the left of the heat map indicate the clusters which are detailed on the pie chart. Details on how genes were categorised are in the materials and methods. ARE: Auxin Response Elements. On average, 44% of all the genes on the ATH1 chip have at least one ARE.

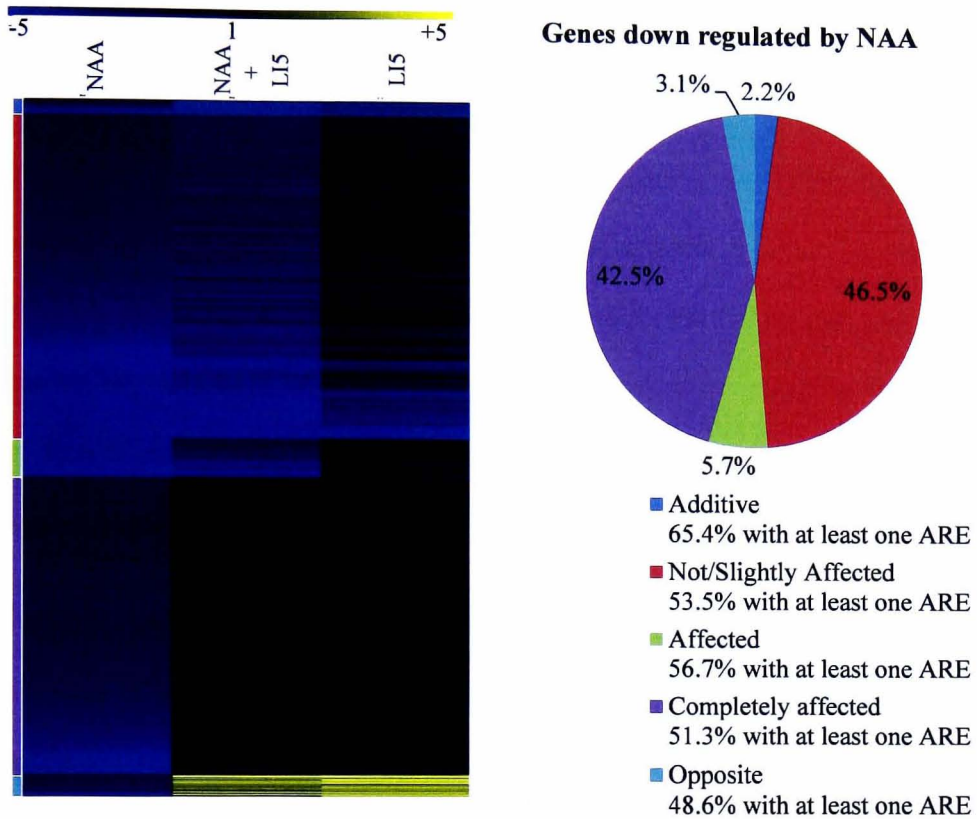

Figure 4-7 LI5 affects 50% of genes down regulated by NAA. The heat map shows the 1174 genes down regulated in presence of NAA. The genes are clustered depending on how LI5 affects their induction. The coloured strips on the left of the heat map indicate the clusters which are detailed on the pie chart. Details on how genes were categorised are in the materials and methods. ARE: Auxin Response Elements. On average, 44% of all the genes on the ATH1 chip have at least one ARE.

4.4.3. LI5 stabilises Aux/IAA proteins

Most of the auxin response inhibitors identified previously stabilise Aux/IAA proteins (Armstrong et al., 2004; Hayashi et al., 2003; Hayashi et al., 2009; Oono et al., 2003; Yamazoe et al., 2005). To test if LI5 inhibits the response to auxin because it stabilises Aux/IAA proteins, two different reporters have been used. The first one uses a fusion between the domain II of AXR3 (IAA17) (Gray et al., 2001) and the GUS enzyme and the second one uses a fusion between the domain II of IAA28 and Venus, a yellow fluorescent protein (Vernoux et al., Submitted).

The AXR3NT-GUS transgene is under the regulation of a temperature inducible promoter which is inactive at the restrictive temperature of 21° and active at the permissive temperature of 37°. Aux/IAA stability is measured by performing a GUS staining of the seedlings 30 minutes after induction of the reporter and subsequent treatment with or without auxin and with or without LI5. Figure 4-8 A shows that LI5 reduces the NAA or IAA induced degradation of the GUS reporter.

The Aux/IAA28-Venus fusion is constitutively expressed (35S promoter) and targeted to the nucleus. Aux/IAA stability is measured by following the total fluorescence of seedlings in presence of auxin and/or LI5. Figure 4-8 B shows the quantification of fluorescence. It seems that there is a delay before LI5 stabilises the Aux/IAA reporter, which could be explained by a slower uptake of the drug compared to NAA or to the higher potency of NAA compared to LI5 towards its target protein.

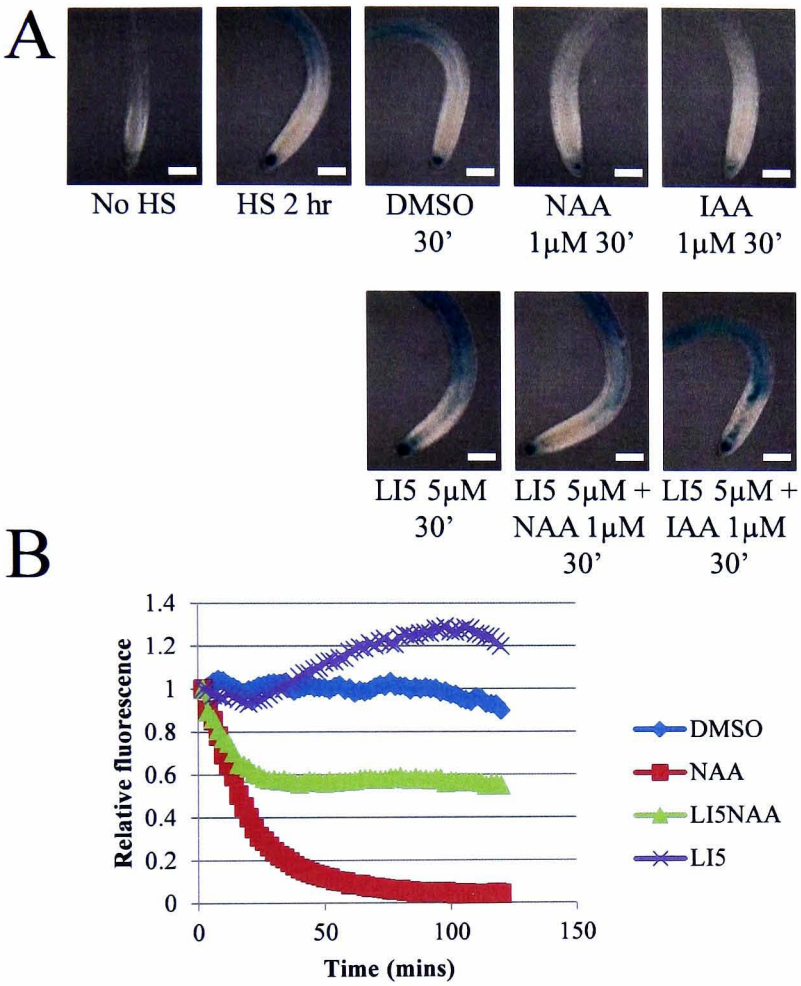

Figure 4-8 LI5 stabilises Aux/IAA proteins. (A) Representative pictures of GUS stained AXR3NT-GUS seedlings after treatments with or without auxin (IAA or NAA) and with or without LI5 for 30 minutesat 21 degrees C just after a 2 hours heat shock at 37 degrees C. No HS: No Heat Shock (GUS staining before heat shock). HS: Heat Shock (GUS staining just after 2 hours of heat shock at 37 degrees C). **(B)** Quantification of DII Aux/IAA28-Venus fluorescence in seedlings treated with or without NAA and with or without LI5.

4.4.4. LI5 affects Cullin Neddylation

Based on the current model of the auxin response pathway, there are several ways to explain Aux/IAA stabilisation. LI5 could act as an anti auxin and block the interaction of TIR1 with the domain II of Aux/IAA proteins, LI5 could also block the ubiquitination of Aux/IAA proteins by disturbing the activity of the SCF^{TIR1} complex or LI5 could affect the activity of the proteasome.

To determine if LI5 acts as an anti-auxin, pull down experiments were carried using GST tagged AXR3 N terminal peptides (that include its domain II) which were immobilised on sepharose beads and incubated with a protein extract from transgenic Arabidopsis seedlings expressing TIR1-myc (Gray et al., 2001). Figure 4-9 shows that LI5 does not disrupt their auxin dependant interaction (experiment performed by Dr Stefan Kepinsky). Therefore, it appears that LI5 does not act as an anti-auxin.

To determine if LI5 affects CUL1 neddylation wild type seedlings treated with auxin and with or without LI5 for 24 hours and protein extracts were analysed by western blot (del Pozo and Estelle, 1999). Cullin neddylation is required to promote the assembly of the SCF complex and, *in vivo,* both forms (modified and unmodified) can be observed in untreated WT seedlings (Figure 4-9). Auxin treatments did not change the ratio of modified to unmodified CUL1 but treatments with auxin and LI5 seemed to reduce the amount of modified CUL1 (Figure 4-9) (experiment performed by Pr Pascal Genschik and Dr. Esther Lechner). Hence it appears that the inhibitor may affect the neddylation of the cullin subunit of the SCF complex.

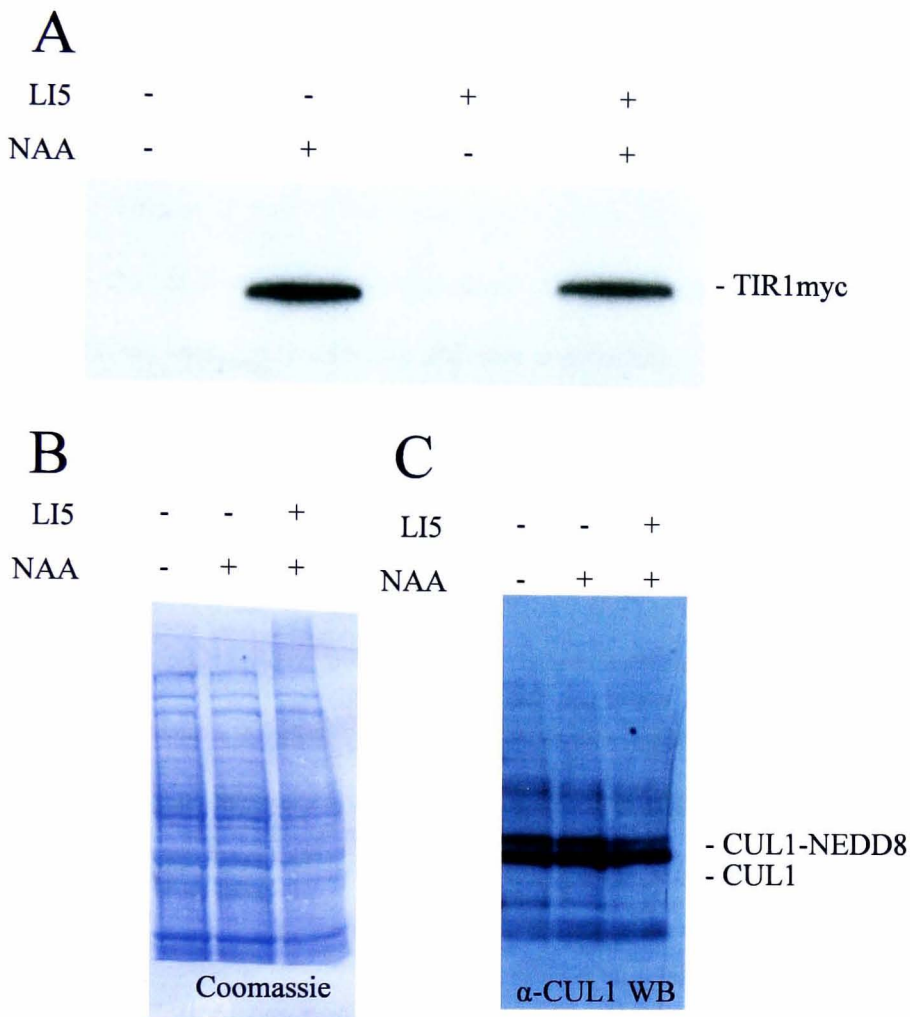

Figure 4-9 LI5 affects CULLIN1 neddylation *in vivo* but not Aux/IAA TIR1 auxin dependant interaction *in vitro*. **(A)** LI5 does not affect the auxin dependant interaction between TIR1 and the N terminus of AXR3 (Aux/IAA17) *in vitro*. LI5 concentration is 25µM and NAA concentration is 10µM (courtesy of Dr. Stefan Kepinsky). **(B and C)** LI5 reduces the proportion of neddylated CUL compared to unmodified CUL1 *in vivo* (courtesy of Pr. Pascal Genschik and Esther Lechner).

4.4.5. LI5 affects hormonal pathways that require a functional SCF complex

The results obtained suggest that LI5 affects cullin neddylation. Most likely, this is going to have an impact on the response to other hormones that are dependent on the SCF complex. To test if that is the case, the response to several hormones that are dependent on the SCF complex (gibberellins (GA), jasmonates (JA) and ethylene (Figure 4-10)) have been analysed using different approaches:

GA functions by promoting the degradation of DELLA repressor proteins (Dill et al., 2004; McGinnis et al., 2003) (Figure 4-10). *In vivo*, GA responses can be monitored using a fusion between a DELLA (RGA) and a green fluorescent protein (GFP) (Silverstone et al., 2001). Live imaging using a confocal microscope shows that the RGA-GFP fusion protein is destabilised after addition of exogenous GA. Treatment with LI5 and GA results in the stabilisation of the reporter suggesting that LI5 blocks the response to GA (Figure 4-11, A).

JA functions by promoting the degradation of JAZ (jasmonate ZIM domain proteins) repressor proteins (Thines et al., 2007) (Figure 4-10). *In vivo*, JA responses can be monitored using a fusion between a JAZ (JAZ1) and the GUS reporter gene. This transgene is expressed in transgenic plants via a 35S promoter and treatment with MeJa promotes the rapid degradation of the fusion protein. Treatment with LI5 and MeJa results in the stabilisation of the JAZ1-GUS fusion protein which suggests that LI5 blocks JA responses (Figure 4-11, B).

In the absence of ethylene, the transcription factors EIN3 and EIN3-LIKE (EILs) are constitutively degraded by the $SCF^{EBF1/2}$ complex (Figure 4-10) (Guo and Ecker, 2003;

Potuschak et al., 2003). Ethylene functions by inactivating the EBF1/2 proteins. As LI5 inactivates the SCF complex it is expected that LI5 mimics ethylene treatments. To test that, 5 day old col-0 seedlings were treated with or without ACC (1-Aminocyclopropane-1-carboxylic acid, ethylene precursor) and LI5 then sampled at different time points and gene expression analysed by RT qPCR. The expression levels of several ethylene responsive genes (ERF1, EDF1-4 ((Stepanova and Alonso, 2009))) were then quantified by RT qPCR. The result suggests a mild but reproducible effect of LI5 on ethylene responsive genes (Figure 4-11, C).

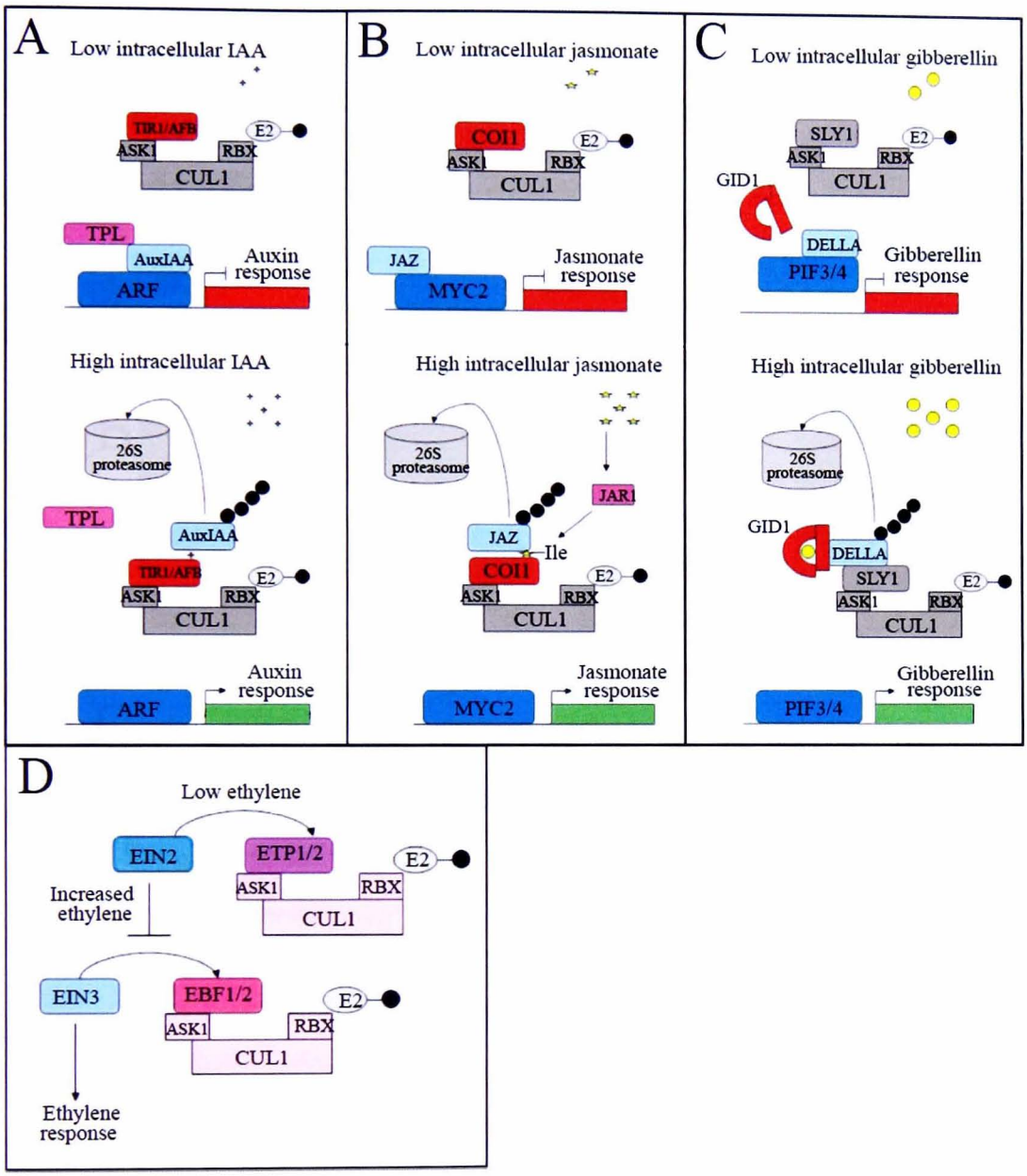

Figure 4-10 Hormone signal transduction pathways dependant on the SCF complex. (A) The auxin signal transduction pathway (as described in Chapter 1). The small stars represent IAA. **(B)** The jasmonic acid signal transduction pathway. An increase in JA concentration is perceived by the JA receptor COI1, which triggers the degradation of JAZ repressors and releases MYC2 to regulate downstream genes. The stars represent JA. **(C)** The gibberellin signal transduction pathway. An increase in GA concentration is perceived by the GA receptors GID1a-c, which triggers the degradation of DELLA repressors and releases PIFs transcription factors to regulate downstream genes. The circles represent GA. **(D)** The ethylene signal transduction pathway. An increase in ethylene concentration is perceived by the ethylene receptor CTR1 which triggers the stabilisation of the transcriptional activator EIN3 via EIN2. Adapted from (Santner and Estelle, 2009).

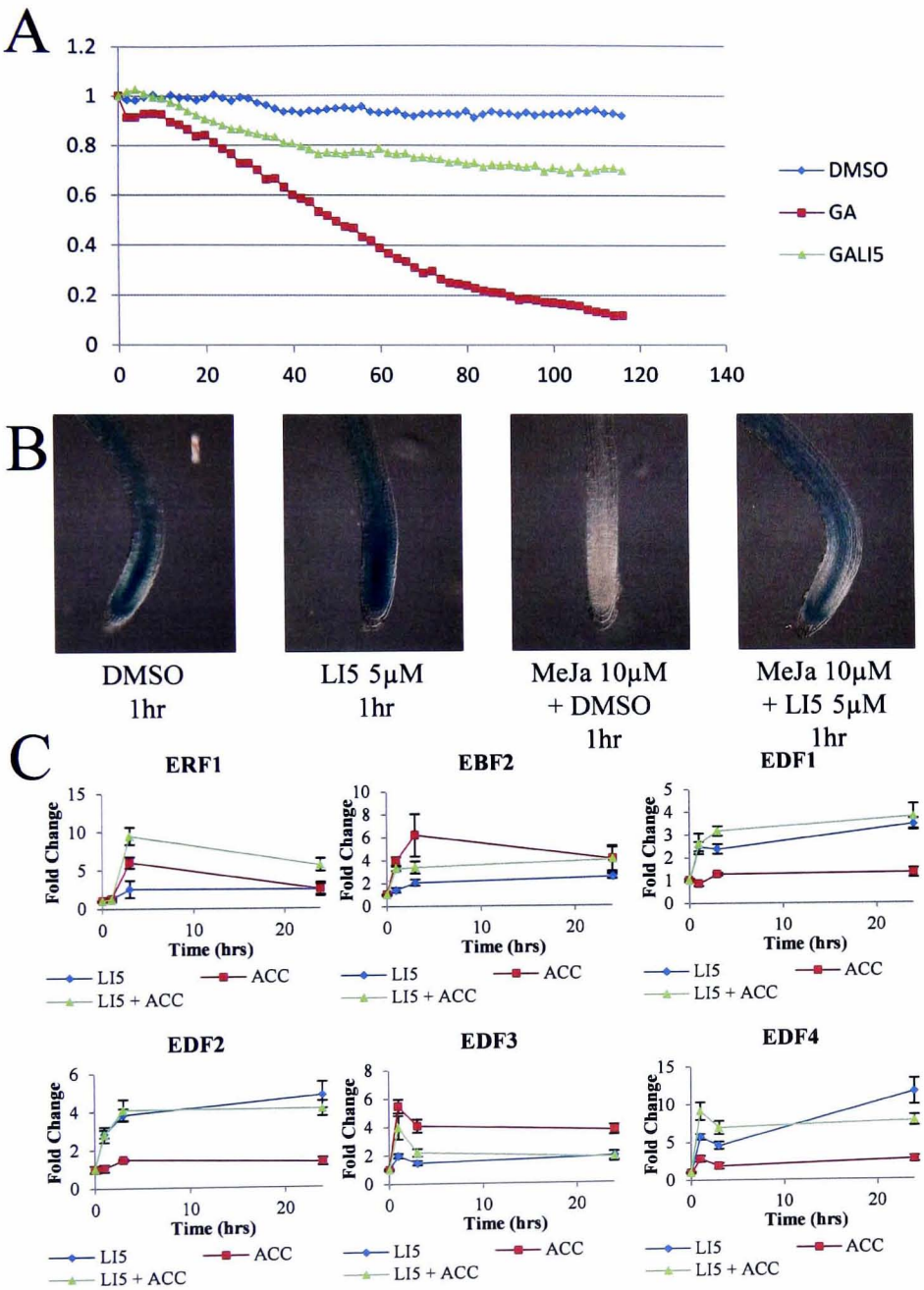

Figure 4-11 LI5 affects SCF dependant hormonal pathways. (A) GA responses are monitored at the protein level using the fluorescent reporter RGA::RGA-GFP. The chart shows the quantification of total GFP fluorescence over two hours following the indicated treatments. Data acquisition and analysis was performed as described in Material and Methods. **(B)** JA responses are monitored at the protein level using a 35S::JAZ-GUS reporter. Data acquisition and analysis was performed as described in Material and Methods. **(C)** Ethylene responses are monitored after treating seedlings with ACC, LI5 or ACC and LI5 for 1 hour, 3 hours and 24 hours. The amount of mRNA of typical ethylene responsive genes is determined using RT qPCR. Error bars on the RT qPCR charts show the SD of the mean of four technical replicates. GA3 concentration is 10µM, LI5 is 5 µM, JA is 10 µM and ACC is 10 µM.

4.4.6. Effects of LI5 on hormonal pathways that do not require a functional SCF complex

4.4.6.1. Abscissic Acid

LI5 consistently affects pathways that utilises the SCF complex in their signalling cascade. The abscissic acid signal transduction pathway utilises protein kinases and phosphatases to modify target proteins activity (mainly ABI3 and ABI5). There is no report of a role of a cullin-associated complex, though several RING E3s have been shown to modulate ABA responses by regulating ABI3 and ABI5 protein levels (Bu et al., 2009; Raghavendra et al., 2010; Santner and Estelle, 2010; Stone et al., 2006; Zhang et al., 2005; Zhang et al., 2007).

To test whether LI5 has an effect on ABA signalling, 5 day old Col-0 seedlings were treated with or without ABA and with or without LI5 then sampled at different time points and gene expression analysed by RT qPCR. Figure 4-13 shows that LI5 on its own triggers an ABA response (Figure 4-13). This is consistent with the microarray results which showed that "ABA responsive gene" is one of the top statistically over represented term in the set of genes differentially regulated by LI5 on its own (Figure 4-5). Treatments with LI5 and ABA had a mild effect on genes induced by ABA (around 10 fold reduction). Hence, it appears that LI5 blocks partially ABA response possibly because it affects the degradation of ABI3 and 5 (Bu et al., 2009; Raghavendra et al., 2010; Santner and Estelle, 2010; Stone et al., 2006; Zhang et al., 2005; Zhang et al., 2007).

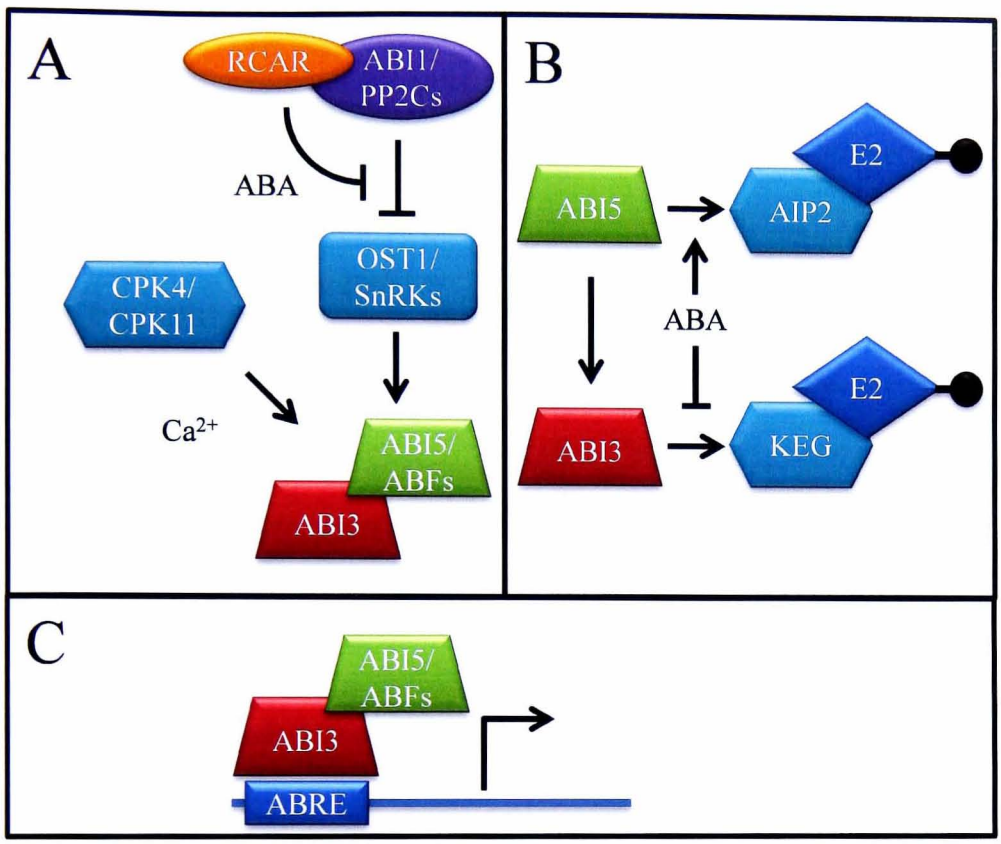

Figure 4-12 ABA signal transduction pathways. (A) ABA signals via a protein kinase cascade that lead to ABI3 and 5 activation. Ca^{2+} also activates these two transcription factors but via different protein kinases. **(B)** ABA triggers the degradation of ABI5 via the AIP2 RING E3 ligase and ABA stabilises ABI3 by inhibiting its degradation by KEG (Keep On Going) E3 ligase. (C) ABI3 and ABI5 interact and regulate genes by binding directly their promoter on Abscissic acid Response Element (ABRE). Adapted from (Raghavendra et al., 2010; Santner and Estelle, 2009).

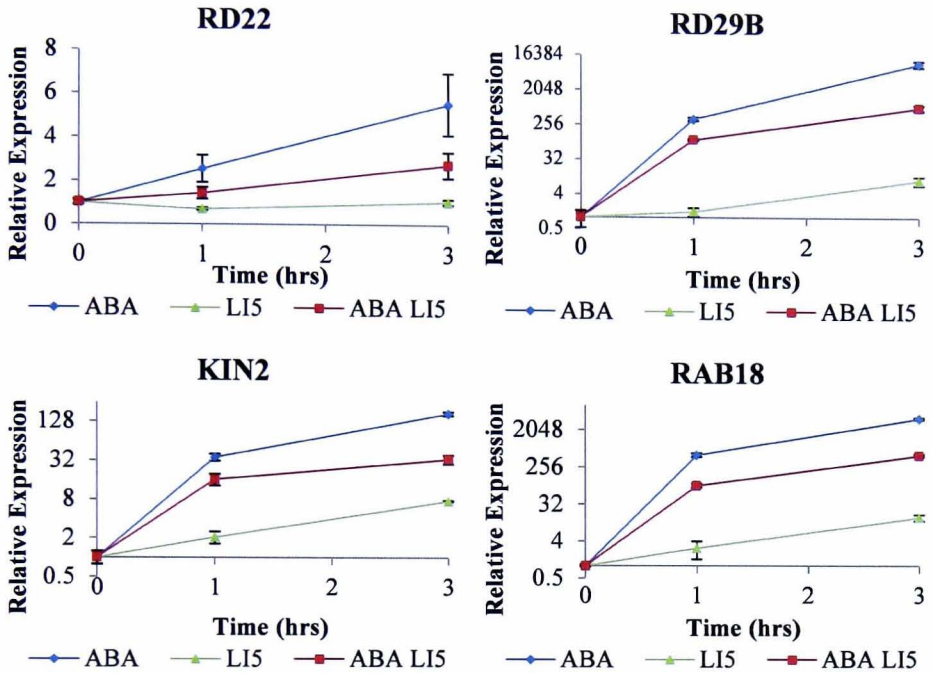

Figure 4-13 LI5 affects slightly ABA responsive genes. ABA responses are monitored after treating seedlings with ABA, LI5 or ABA and LI5 for 1 hour and 3 hours. The amount of mRNA of typical ABA responsive genes is determined using RT qPCR. Error bars on all the charts show the SD of the mean of four technical replicates. Concentration of LI5 is 5μM and ABA is 10μM.

4.4.6.2. Brassinosteroids

Brassinosteroids function by activating a cell-surface receptor kinase (BRI1) which triggers a phosphorylation and dephosphorylation cascade that leads to transcriptional reprogramming (He et al., 2000). A negative regulator of BR signalling BIN2 (BRASSINOSTEROID INSENSITIVE 2)) was shown to be degraded by epi-brassinolide treatments in a proteasome dependant manner but the E3 ligase involved has not been identified. (He et al., 2002; Peng et al., 2008).

To test whether LI5 has an effect on brassinosteroid signalling, 5 day old Col-0 seedlings were treated with or without epibrassinolide (EpiBL, 1 μM) and with or without LI5 and then sampled at different time points. Figure 4-15 shows that LI5 does not affect brassinosteroid responsive genes.

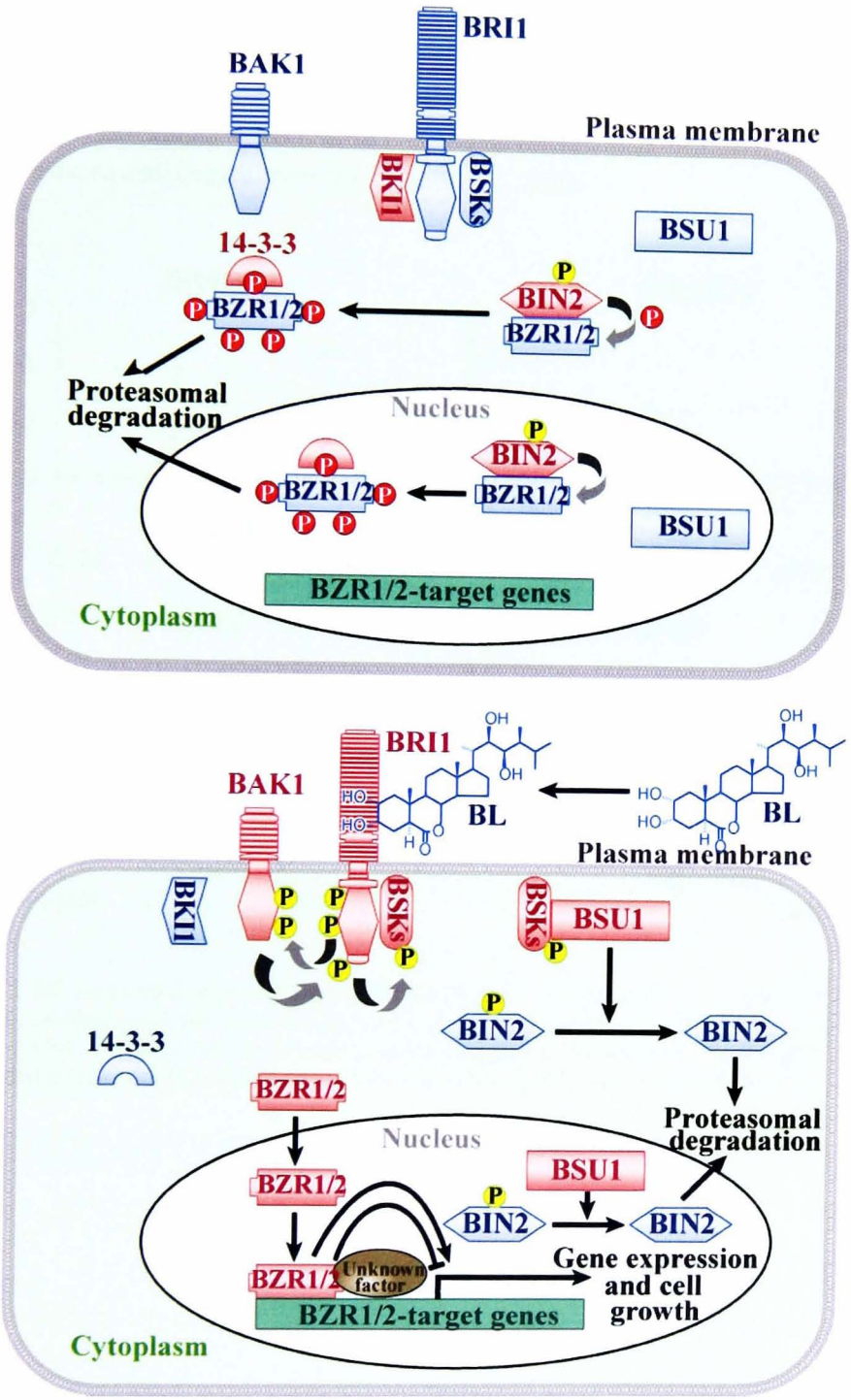

Figure 4-14 The brassinosteroid signal transduction pathway. The signalling components in active and inactive status are shown in pink and blue, respectively. Phosphorylation confers positive effects (*yellow circles*) and negative effects (*red circles*). In the absence of BR (top panel), BKI1 is inactive and, consequently, BSU1 is inactive. BIN2 constitutively phosphorylates BZR1 and BZR2/BES1, leading to nuclear export and proteasomal degradation

of BZR1 and BZR2/BES1. In the presence of BR (lower panel), BR binds to BRI1, which induces association with BAK1 and disassociation of BKI1. Sequential transphosphorylation activates BSKs and BSU1. Activated BSU1 inhibits BIN2 through dephosphorylation of the phospho-tyrosine residue of BIN2, which allows accumulation of unphosphorylated BZR1 and BZR2/BES1 in the nucleus. Active BZR1 and BZR2/BES1 bind the promoter of BR-target genes. Reproduced and adapted from (Kim and Wang, 2010).

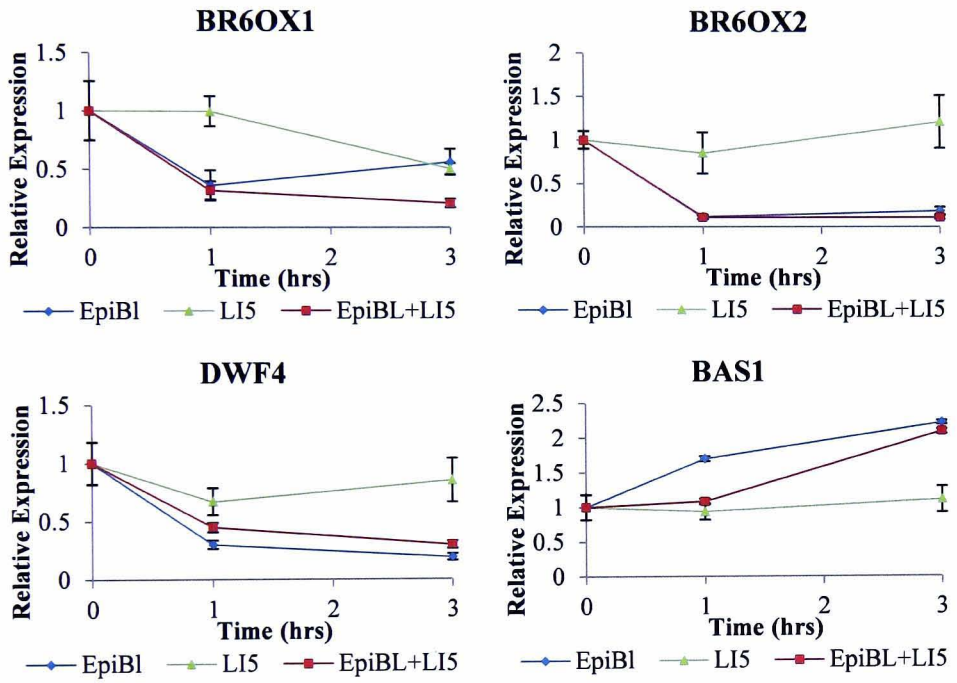

Figure 4-15 BL responsive genes are not affected by LI5. Brassinosteroids responses are monitored after treating seedlings with Epi Brassinolide, LI5 or Epi Brassinolide and LI5 for 1 hour and 3 hours. The amount of mRNA of typical brassinosteroids responsive genes is determined using RT qPCR. Error bars on all the charts show the SD of the mean of four technical replicates.LI5 concentration is 5μM, EpiBL 1μM.

4.5. Discussion

In this chapter, the mode of action of a class of sulfonamides that were identified in a screen for inhibitors of the induction by auxin of the gene *LAX3* (Chapter 3) has been dissected. It has been shown that LI5, the most active member of this class of compounds, stabilises a family of repressor proteins of the auxin signalling pathway, the Aux/IAAs (Figure 4-8). Using *in vivo* assays it has been shown that this stabilisation could be a consequence of a reduction in CUL1 neddylation (Figure 4-9). The addition of a NEDD8/RUB moiety (neddylation) on cullins has been described in animals and plants (Kamitani et al., 1997; Kirkin and Dikic, 2007; Rabut and Peter, 2008).. The pathway is very similar to ubiquitination as it requires the concerted action of three enzymes: an E1 (or activating enzyme), which in Arabidopsis is a heterodimer (AXR1 and ECR1), an E2 (or conjugating enzyme) (RCE1) and an E3 (or ligase enzyme) (RBX1) (Huang et al., 2009; Huang et al., 2007; Huang et al., 2004; Huang et al., 2005; Walden et al., 2003).. Any of these three enzymes could be targeted by LI5 as mutations in any of them result in reduced auxin responsiveness (del Pozo et al., 2002; del Pozo and Estelle, 1999; Dharmasiri et al., 2003; Gray et al., 2002). Preliminary result suggests that ubiquitin E1 enzyme is not affected by the compound but there are no evidences that it does not affect the NEDD8 E1.

By affecting CUL1 neddylation, LI5 could trigger the dissociation of the SCF complex. Un-neddylated CUL1 interacts with CAND1 whereas neddylated CUL1 interact with ASK1 and other sub-units of the SCF complex (Chuang et al., 2004; Feng et al., 2004; Schwechheimer et al., 2001; Zhang et al., 2008). Removal of NEDD8 on cullins is necessary since loss of function mutations in the catalytic sub-unit which catalyses

NEDD8 removal leads to embryo lethal phenotype (Lyapina et al., 2001; Schwechheimer et al., 2001). Consistent with this idea, many SCF dependant pathways have been shown to be affected (Figure 4-10 and Figure 4-11). In the case of GA and JA, there is a clear stabilisation of the repressor proteins (DELLA for GA, JAZ1 for JA) in presence of LI5 (Dill et al., 2004; McGinnis et al., 2003; Thines et al., 2007; Xie et al., 1998). In the case of ethylene, responses are more complex to analyse. The transduction pathway relies primarily on the stabilisation of an activator (EIN3) rather than the degradation of a repressor(Guo and Ecker, 2003; Potuschak et al., 2003). Nevertheless, another protein, EIN2, has to be degraded by the proteasome to allow EIN3 stabilisation. As one would expect, LI5 does not block ethylene responses but enhances it. Treatments with LI5 on its own only slightly induce ethylene responses which could be due to the negative effects of EIN2 stabilisation.

Aux/IAA stabilisation correlates well with the marked effects of LI5 on the auxin response transcriptome (Figure 4-5, Figure 4-6 and Figure 4-7). Importantly, it appears that LI5 does not affect the entire auxin responsive gene transcriptome to the same extent. Overall, 75% of the auxin induced and 50% of the auxin repressed genes are affected. In most cases, the genes that are not affected are also differentially expressed in presence of LI5 on its own. In particular, the gene IAA30 is induced by auxin (15 fold after 6 hours) and by LI5 (2 fold after 6 hours). In presence of both compounds the gene is induce more than 40 fold after 6 hours. IAA30 is not the only gene where treatments with LI5 and NAA have synergic effects: MYB39 (transcription factor), GA2OX6 (GA catabolism) or CYP707A1 (ABA catabolism) are other examples of LI5 and auxin common targets.

Interestingly, hormonal pathways such as abscisic acid and brassinosteroids are not significantly affected by LI5 (Figure 4-13 and Figure 4-15). This is consistent with these pathways shown to be kinase (rather than SCF) dependent (He et al., 2000; Peng et al., 2008; Raghavendra et al., 2010). In the case of ABA, the 4 genes tested all responded to ABA and LI5 which is consistent with LI5 triggering ABA responses. Nevertheless, treatments with both compound lead to a small reduction compared to ABA.

In plants and animals, the cullin family of proteins is associated with several RING E3 ligases (called CRLs for cullin-RING ubiquitin ligases) complexes such as the SCF but also the cyclosome (APC/C or Anaphase Promoting Complex) the CUL3-BTB, the CUL4-DDB1 and probably many more (Thomann et al., 2005) (Barford, 2011; Biedermann and Hellmann, 2010; Hua and Vierstra, 2011; Jia and Sun, 2011; Zimmerman et al., 2010). In each case, the CULLIN subunit is consistently modified by a NEDD8 moiety. It will be of interest to determine if these complexes are also affected by looking at other CULLINS modifications.

The results presented in this chapter are summarised in the model on Figure 4-16. The family of benzene sulfonamides identified have been shown to affect the responses to several hormones regulated by an SCF complex. Further biochemical characterisation of SCF complex activity in presence of LI5 may help determining if the complex is still active and will allow to narrow down potential targets.

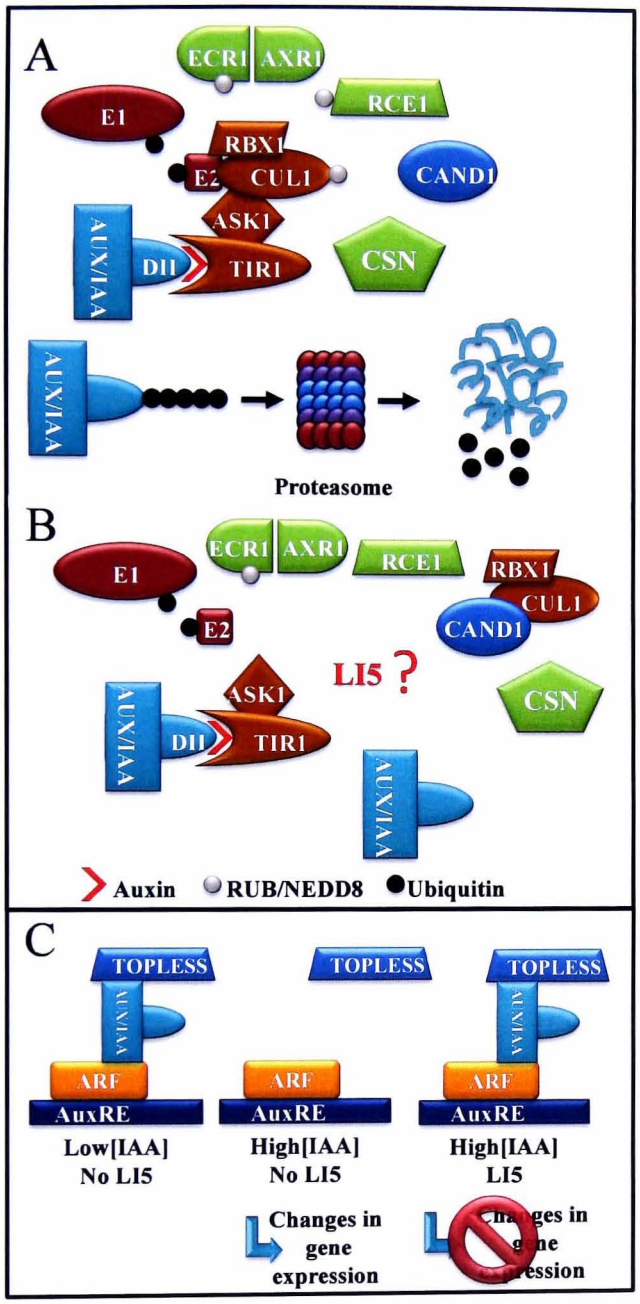

Figure 4-16 Model of LI5 action on the auxin response pathway. (A) In presence of auxin, SCF^{TIR1} interacts with Aux/IAA repressors and promote their degradation. Cycles of addition and removal of RUB/NEDD8 maintain the SCF complex active. RUB/NEDD8 is added to the CUL1 sub unit of the SCF complex by an E1 (heterodimer AXR1/ECR1), an E2 (RCE1) and an E3 (RBX1) and is removed by the CSN complex. Unmodified CUL1 interacts with CAND1 which promotes SCF dissociation. Addition of ubiquitin to the Aux/IAA is catalysed by an ubiquitin E1, an ubiquitin E2 and by the FBOX E3 TIR1. **(B)** In presence of LI5, the CUL1 sub-unit seems to not be modified by RUB/NEDD8 which triggers SCF dissociation. Consequently, Aux/IAAs are stabilised. **(C)** As a result of Aux/IAA stabilisation, auxin responsive genes are not induced or repressed.

4.6. References

Armstrong, J. I., Yuan, S., Dale, J. M., Tanner, V. N. and Theologis, A. (2004). Identification of inhibitors of auxin transcriptional activation by means of chemical genetics in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 14978.

Barford, D. (2011). Structure, function and mechanism of the anaphase promoting complex (APC/C). *Quarterly Reviews of Biophysics* **44**, 153-190.

Benjamini, Y. and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)* **57**, 289-300.

Biedermann, S. and Hellmann, H. (2010). WD40 and CUL4-based E3 ligases: lubricating all aspects of life. *Trends in plant science*.

Bu, Q., Li, H., Zhao, Q., Jiang, H., Zhai, Q., Zhang, J., Wu, X., Sun, J., Xie, Q. and Wang, D. (2009). The Arabidopsis RING finger E3 ligase RHA2a is a novel positive regulator of abscisic acid signaling during seed germination and early seedling development. *Plant physiology* **150**, 463.

Chapman, E. J. and Estelle, M. (2009). Mechanism of auxin-regulated gene expression in plants. *Annual Review of Genetics* **43**, 265-285.

Chuang, H. W., Zhang, W. and Gray, W. M. (2004). Arabidopsis ETA2, an apparent ortholog of the human cullin-interacting protein CAND1, is required for auxin responses mediated by the SCF(TIR1) ubiquitin ligase. *Plant Cell* **16**, 1883-97.

Cope, G. A. and Deshaies, R. J. (2003). COP9 Signalosome:: A Multifunctional Regulator of SCF and Other Cullin-Based Ubiquitin Ligases. *Cell* **114**, 663-671.

Cope, G. A., Suh, G. S. B., Aravind, L., Schwarz, S. E., Zipursky, S. L., Koonin, E. V. and Deshaies, R. J. (2002). Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. *Science* **298**, 608.

Criqui, M. C., Parmentier, Y., Derevier, A., Shen, W. H., Dong, A. and Genschik, P. (2000). Cell cycle-dependent proteolysis and ectopic overexpression of cyclin B1 in tobacco BY2 cells. *The Plant Journal* **24**, 763-773.

del Pozo, J. C., Dharmasiri, S., Hellmann, H., Walker, L., Gray, W. M. and Estelle, M. (2002). AXR1-ECR1-dependent conjugation of RUB1 to the Arabidopsis Cullin AtCUL1 is required for auxin response. *Plant Cell* **14**, 421-33.

del Pozo, J. C. and Estelle, M. (1999). The Arabidopsis cullin AtCUL1 is modified by the ubiquitin-related protein RUB1. *Proc Natl Acad Sci U S A* **96**, 15342-7.

del Pozo, J. C., Timpte, C., Tan, S., Callis, J. and Estelle, M. (1998). The ubiquitin-related protein RUB1 and auxin response in Arabidopsis. *Science* **280**, 1760-3.

Dharmasiri, N., Dharmasiri, S. and Estelle, M. (2005). The F-box protein TIR1 is an auxin receptor. *Nature* **435**, 441-445.

Dharmasiri, S., Dharmasiri, N., Hellmann, H. and Estelle, M. (2003). The RUB/Nedd8 conjugation pathway is required for early development in Arabidopsis. *The EMBO Journal* **22**, 1762-1770.

Dill, A., Thomas, S. G., Hu, J., Steber, C. M. and Sun, T. (2004). The Arabidopsis F-box protein SLEEPY1 targets gibberellin signaling repressors for gibberellin-induced degradation. *The Plant Cell Online* **16**, 1392.

Feng, S., Shen, Y., Sullivan, J. A., Rubio, V., Xiong, Y., Sun, T. and Deng, X. W. (2004). Arabidopsis CAND1, an unmodified CUL1-interacting protein, is involved in multiple developmental pathways controlled by ubiquitin/proteasome-mediated protein degradation. *The Plant Cell Online* **16**, 1870.

Gagne, J. M., Downes, B. P., Shiu, S. H., Durski, A. M. and Vierstra, R. D. (2002). The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in Arabidopsis. *Proc Natl Acad Sci U S A* **99**, 11519-24.

Garzón, M., Eifler, K., Faust, A., Scheel, H., Hofmann, K., Koncz, C., Yephremov, A. and Bachmair, A. (2007). PRT6/At5g02310 encodes an Arabidopsis ubiquitin ligase of the N-end rule pathway with arginine specificity and is not the CER3 locus. *FEBS letters* **581**, 3189-3196.

Gentleman, R., Carey, V., Bates, D., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y. and Gentry, J. (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome biology* **5**, R80.

Gomez-Roldan, V., Fermas, S., Brewer, P. B., Puech-Pagès, V., Dun, E. A., Pillot, J. P., Letisse, F., Matusova, R., Danoun, S. and Portais, J. C. (2008). Strigolactone inhibition of shoot branching. *Nature* **455**, 189-194.

Gray, W. M., del Pozo, J. C., Walker, L., Hobbie, L., Risseeuw, E., Banks, T., Crosby, W. L., Yang, M., Ma, H. and Estelle, M. (1999). Identification of an SCF ubiquitin-ligase complex required for auxin response in Arabidopsis thaliana. *Genes Dev* **13**, 1678-91.

Gray, W. M., Hellmann, H., Dharmasiri, S. and Estelle, M. (2002). Role of the Arabidopsis RING-H2 protein RBX1 in RUB modification and SCF function. *Plant Cell* **14**, 2137-44.

Gray, W. M., Kepinski, S., Rouse, D., Leyser, O. and Estelle, M. (2001). Auxin regulates SCF(TIR1)-dependent degradation of AUX/IAA proteins. *Nature* **414**, 271-6.

Guo, H. and Ecker, J. R. (2003). Plant responses to ethylene gas are mediated by SCFEBF1/EBF2-dependent proteolysis of EIN3 transcription factor. *Cell* **115**, 667-677.

Hayashi, K., Jones, A. M., Ogino, K., Yamazoe, A., Oono, Y., Inoguchi, M., Kondo, H. and Nozaki, H. (2003). Yokonolide B, a novel inhibitor of auxin action, blocks degradation of AUX/IAA factors. *Journal of Biological Chemistry* **278**, 23797.

Hayashi, K., Kamio, S., Oono, Y., Townsend, L. B. and Nozaki, H. (2009). Toyocamycin specifically inhibits auxin signaling mediated by SCFTIR1 pathway. *Phytochemistry* **70**, 190-197.

He, J. X., Gendron, J. M., Yang, Y., Li, J. and Wang, Z. Y. (2002). The GSK3-like kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the brassinosteroid signaling pathway in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 10185.

He, Z., Wang, Z. Y., Li, J., Zhu, Q., Lamb, C., Ronald, P. and Chory, J. (2000). Perception of brassinosteroids by the extracellular domain of the receptor kinase BRI1. *Science* **288**, 2360.

Hellmann, H., Hobbie, L., Chapman, A., Dharmasiri, S., Dharmasiri, N., del Pozo, C., Reinhardt, D. and Estelle, M. (2003). Arabidopsis AXR6 encodes CUL1 implicating SCF E3 ligases in auxin regulation of embryogenesis. *Embo J* **22**, 3314-25.

Ho, M. S., Tsai, P. I. and Chien, C. T. (2006). F-box proteins: the key to protein degradation. *J Biomed Sci* **13**, 181-91.

Hua, Z. and Vierstra, R. (2011). The Cullin-RING Ubiquitin-Protein Ligases. *Annual Review of Plant Biology*.

Huang, D. T., Ayrault, O., Hunt, H. W., Taherbhoy, A. M., Duda, D. M., Scott, D. C., Borg, L. A., Neale, G., Murray, P. J. and Roussel, M. F. (2009). E2-RING expansion of the NEDD8 cascade confers specificity to cullin modification. *Molecular cell* **33**, 483-495.

Huang, D. T., Hunt, H. W., Zhuang, M., Ohi, M. D., Holton, J. M. and Schulman, B. A. (2007). Basis for a ubiquitin-like protein thioester switch toggling E1–E2 affinity. *Nature* **445**, 394-398.

Huang, D. T., Miller, D. W., Mathew, R., Cassell, R., Holton, J. M., Roussel, M. F. and Schulman, B. A. (2004). A unique E1-E2 interaction required for optimal conjugation of the ubiquitin-like protein NEDD8. *Nature structural & molecular biology* **11**, 927-935.

Huang, D. T., Paydar, A., Zhuang, M., Waddell, M. B., Holton, J. M. and Schulman, B. A. (2005). Structural basis for recruitment of Ubc12 by an E2 binding domain in NEDD8's E1. *Molecular cell* **17**, 341-350.

Jia, L. and Sun, Y. (2011). SCF E3 Ubiquitin Ligases as Anticancer Targets. *Current Cancer Drug Targets* **11**, 347-356.

Kamitani, T., Kito, K., Nguyen, H. P. and Yeh, E. T. H. (1997). Characterization of NEDD8, a developmentally down-regulated ubiquitin-like protein. *Journal of Biological Chemistry* **272**, 28557.

Kepinski, S. and Leyser, O. (2004). Auxin-induced SCFTIR1-Aux/IAA interaction involves stable modification of the SCFTIR1 complex. *Proc Natl Acad Sci U S A* **101**, 12381-6.

Kepinski, S. and Leyser, O. (2005). The Arabidopsis F-box protein TIR1 is an auxin receptor. *Nature* **435**, 446-51.

Kim, T. W. and Wang, Z. Y. (2010). Brassinosteroid signal transduction from receptor kinases to transcription factors. *Plant Biology* **61**, 681.

Kirkin, V. and Dikic, I. (2007). Role of ubiquitin-and Ubl-binding proteins in cell signaling. *Current opinion in cell biology* **19**, 199-205.

Leyser, H. M., Lincoln, C. A., Timpte, C., Lammer, D., Turner, J. and Estelle, M. (1993). Arabidopsis auxin-resistance gene AXR1 encodes a protein related to ubiquitin-activating enzyme E1. *Nature* **364**, 161-4.

Lokerse, A. S. and Weijers, D. (2009). Auxin enters the matrix. Assembly of response machineries for specific outputs. *Current opinion in plant biology* **12**, 520-526.

Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D. A., Wei, N. and Deshaies, R. J. (2001). Promotion of NEDD8-CUL1 conjugate cleavage by COP9 signalosome. *Science* **292**, 1382.

McGinnis, K. M., Thomas, S. G., Soule, J. D., Strader, L. C., Zale, J. M., Sun, T. and Steber, C. M. (2003). The Arabidopsis SLEEPY1 gene encodes a putative F-box subunit of an SCF E3 ubiquitin ligase. *The Plant Cell Online* **15**, 1120.

Moon, J., Zhao, Y., Dai, X., Zhang, W., Gray, W. M., Huq, E. and Estelle, M. (2007). A new CULLIN 1 mutant has altered responses to hormones and light in Arabidopsis. *Plant Physiol* **143**, 684-96.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. plant* **15**, 473-97.

Oono, Y., Ooura, C., Rahman, A., Aspuria, E. T., Hayashi, K., Tanaka, A. and Uchimiya, H. (2003). p-Chlorophenoxyisobutyric acid impairs auxin response in Arabidopsis root. *Plant physiology* **133**, 1135.

Peng, P., Yan, Z., Zhu, Y. and Li, J. (2008). Regulation of the Arabidopsis GSK3-like kinase BRASSINOSTEROID-INSENSITIVE 2 through proteasome-mediated protein degradation. *Molecular plant* **1**, 338.

Potuschak, T., Lechner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, C. and Genschik, P. (2003). EIN3-Dependent Regulation of Plant Ethylene Hormone Signaling by Two Arabidopsis F Box Proteins:: EBF1 and EBF2. *Cell* **115**, 679-689.

Quint, M., Ito, H., Zhang, W. and Gray, W. M. (2005). Characterization of a novel temperature-sensitive allele of the CUL1/AXR6 subunit of SCF ubiquitin-ligases. *Plant J* **43**, 371-83.

Rabut, G. and Peter, M. (2008). Function and regulation of protein neddylation.'Protein modifications: beyond the usual suspects' review series. *EMBO reports* **9**, 969-976.

Raghavendra, A. S., Gonugunta, V. K., Christmann, A. and Grill, E. (2010). ABA perception and signalling. *Trends in plant science.*

Ruegger, M., Dewey, E., Gray, W. M., Hobbie, L., Turner, J. and Estelle, M. (1998). The TIR1 protein of Arabidopsis functions in auxin response and is related to human SKP2 and yeast grr1p. *Genes Dev* **12**, 198-207.

Santner, A. and Estelle, M. (2009). Recent advances and emerging trends in plant hormone signalling. *Nature* **459**, 1071-1078.

Santner, A. and Estelle, M. (2010). The ubiquitin-proteasome system regulates plant hormone signaling. *The Plant Journal* **61**, 1029-1040.

Schwechheimer, C. and Isono, E. (2010). The COP9 signalosome and its role in plant development. *European journal of cell biology* **89**, 157-162.

Schwechheimer, C., Serino, G., Callis, J., Crosby, W. L., Lyapina, S., Deshaies, R. J., Gray, W. M., Estelle, M. and Deng, X. W. (2001). Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCFTIRI in mediating auxin response. *Science* **292**, 1379-82.

Shen, W. H., Parmentier, Y., Hellmann, H., Lechner, E., Dong, A., Masson, J., Granier, F., Lepiniec, L., Estelle, M. and Genschik, P. (2002). Null mutation of AtCUL1 causes arrest in early embryogenesis in Arabidopsis. *Mol Biol Cell* **13**, 1916-28.

Silverstone, A. L., Jung, H. S., Dill, A., Kawaide, H., Kamiya, Y. and Sun, T. (2001). Repressing a repressor: gibberellin-induced rapid reduction of the RGA protein in Arabidopsis. *The Plant Cell Online* **13**, 1555.

Stepanova, A. N. and Alonso, J. M. (2009). Ethylene signaling and response: where different regulatory modules meet. *Current opinion in plant biology* **12**, 548-555.

Stirnberg, P., van De Sande, K. and Leyser, O. (2002). MAX1and MAX2 control shoot lateral branching in Arabidopsis. *Development* **129**, 1131-1141.

Stone, S. L., Williams, L. A., Farmer, L. M., Vierstra, R. D. and Callis, J. (2006). KEEP ON GOING, a RING E3 ligase essential for Arabidopsis growth and development, is involved in abscisic acid signaling. *The Plant Cell Online* **18**, 3415.

Tan, X., Calderon-Villalobos, L. I., Sharon, M., Zheng, C., Robinson, C. V., Estelle, M. and Zheng, N. (2007). Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature* **446**, 640-5.

Tatematsu, K., Kumagai, S., Muto, H., Sato, A., Watahiki, M. K., Harper, R. M., Liscum, E. and Yamamoto, K. T. (2004). MASSUGU2 encodes Aux/IAA19, an auxin-regulated protein that functions together with the transcriptional activator NPH4/ARF7 to regulate differential growth responses of hypocotyl and formation of lateral roots in Arabidopsis thaliana. *Plant Cell* **16**, 379-93.

Team, R. D. C. (2007). R: A language and environment for statistical computing. *Vienna, Austria: R Foundation for Statistical Computing*.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S. Y. and Howe, G. A. (2007). JAZ repressor proteins are targets of the SCFCOI1 complex during jasmonate signalling. *Nature* **448**, 661-665.

Thomann, A., Dieterle, M. and Genschik, P. (2005). Plant CULLIN-based E3s: Phytohormones come first. *FEBS letters* **579**, 3239-3245.

Tiwari, S. B., Hagen, G. and Guilfoyle, T. (2003). The roles of auxin response factor domains in auxin-responsive transcription. *Plant Cell* **15**, 533-43.

Tiwari, S. B., Wang, X. J., Hagen, G. and Guilfoyle, T. J. (2001). AUX/IAA proteins are active repressors, and their stability and activity are modulated by auxin. *Plant Cell* **13**, 2809-22.

Ulmasov, T., Hagen, G. and Guilfoyle, T. J. (1999). Activation and repression of transcription by auxin-response factors. *Proc Natl Acad Sci U S A* **96**, 5844-9.

Ulmasov, T., Murfett, J., Hagen, G. and Guilfoyle, T. J. (1997). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell* **9**, 1963-71.

Umehara, M., Hanada, A., Yoshida, S., Akiyama, K., Arite, T., Takeda-Kamiya, N., Magome, H., Kamiya, Y., Shirasu, K. and Yoneyama, K. (2008). Inhibition of shoot branching by new terpenoid plant hormones. *Nature* **455**, 195-200.

Vanneste, S. and Friml, J. (2009). Auxin: a trigger for change in plant development. *Cell* **136**, 1005-1016.

Vernoux, T., Band, L. R., Brunoud, G., Wells, D. M., Larrieu, A., Middleton, A. M., Sun, J., French, A., Sato, E. M., Wilson, M. H. et al. (2011). Root gravitropism is regulated by a transient lateral auxin gradient dependent on angle. *Nature* **Under review at Nature**.

Walden, H., Podgorski, M. S., Huang, D. T., Miller, D. W., Howard, R. J., Minor Jr, D. L., Holton, J. M. and Schulman, B. A. (2003). The structure of the APPBP1-UBA3-NEDD8-ATP complex reveals the basis for selective ubiquitin-like protein activation by an E1. *Molecular cell* **12**, 1427-1437.

Willems, A. R., Schwab, M. and Tyers, M. (2004). A hitchhiker's guide to the cullin ubiquitin ligases: SCF and its kin. *Biochim Biophys Acta* **1695**, 133-70.

Woodward, A. W. and Bartel, B. (2005). Auxin: regulation, action, and interaction. *Ann Bot (Lond)* **95**, 707-35.

Xie, D. X., Feys, B. F., James, S., Nieto-Rostro, M. and Turner, J. G. (1998). COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. *Science* **280**, 1091-4.

Yamazoe, A., Hayashi, K., Kepinski, S., Leyser, O. and Nozaki, H. (2005). Characterization of terfestatin A, a new specific inhibitor for auxin signaling. *Plant Physiol* **139**, 779-89.

Zhang, W., Ito, H., Quint, M., Huang, H., Noel, L. D. and Gray, W. M. (2008). Genetic analysis of CAND1-CUL1 interactions in Arabidopsis supports a role for CAND1-mediated cycling of the SCFTIR1 complex. *Proc Natl Acad Sci U S A* **105**, 8470-5.

Zhang, X., Garreton, V. and Chua, N. H. (2005). The AIP2 E3 ligase acts as a novel negative regulator of ABA signaling by promoting ABI3 degradation. *Genes & development* **19**, 1532.

Zhang, Y., Yang, C., Li, Y., Zheng, N., Chen, H., Zhao, Q., Gao, T., Guo, H. and Xie, Q. (2007). SDIR1 is a RING finger E3 ligase that positively regulates stress-responsive abscisic acid signaling in Arabidopsis. *The Plant Cell Online* **19**, 1912.

Zhao, D., Yang, M., Solava, J. and Ma, H. (1999). The ASK1 gene regulates development and interacts with the UFO gene to control floral organ identity in Arabidopsis. *Dev Genet* **25**, 209-23.

Zimmerman, E. S., Schulman, B. A. and Zheng, N. (2010). Structural assembly of cullin-RING ubiquitin ligase complexes. *Current opinion in structural biology.*

Chapter 5 Summary and concluding remarks

In this thesis, pharmacological and genetic approaches have been employed to give new insight into mechanisms of auxin regulated genes.

5.1. Summary

(1) A new regulatory network for *LAX3*:

- *LAX3* is a secondary auxin responsive gene that appears to be positively regulated by an ARF7 dependent transcription factor (TF X) and negatively regulated by an ARF19 dependent transcription factor (TF Y).
- The promoter of *LAX3* has an ARE that negatively regulate its auxin induction and a 200 base pairs domain that positively regulate its expression and auxin induction
- Using transcript profiling of *arf7* and *arf19* mutants, 78 and 51 putative candidates have been identified for TF X and TF Y respectively.

(2) The characterisation of new inhibitors of *LAX3* auxin induction:

- 13 new inhibitors have been identified that affect *LAX3* auxin induction.
- 5 of the 13 inhibitors, which belong to the sulfonamide class of compounds, share a common structure and affect lateral root emergence.
- Studies on the structure/function relationship of the sulfonamides showed that minor changes to the structure dramatically affect the activity of the compound.

(3) Identification of the mode of action of the sulfonamides:

- The inhibitors affect very broadly auxin responses and trigger stress responses.
- The inhibitors stabilise Aux/IAA repressors, maybe by affecting CUL1 neddylation.
- The inhibitors affect hormonal response pathways depending on the SCF complex

5.2. Concluding remarks

The wide range of developmental processes regulated by auxin is fascinating when considering the simplicity of its chemical structure. A partial explanation to this extraordinary potential resides in the large number of combinations possible between the components of the auxin response pathway, which is shown on Figure 1-8. The results presented in this thesis challenges some of these concepts and should be further investigated:

- Positive ARFs (such as ARF19) can have negative effect on gene expression. The current model, which suggests that a combination of positive and negative ARFs fine tune the level of gene expression, is not sufficient to explain this observation. Further studies on the downstream targets of ARF19 will have to be investigated in order to determine how they regulate the expression of *LAX3* and potentially many other genes.
- The regulation of secondary auxin responsive genes is lacking clear genetic and physiological evidences. Several member of various families of transcription factors, which are primary auxin responsive genes, have been identified and further studies on loss function mutants will shed a light on their function
- New groups of auxin response inhibitors have been identified and characterised. Further studies of their effects on auxin signalling, for example by looking at their effects on Aux/IAA reporters will uncover if they affect early or late responses.

- The large number of processes regulated by the SCF complex in plants is illustrated by the large numbers of F-BOX proteins (over 700 in Arabidopsis).

  The sulfonamides studied in Chapter 3 and 4 seem to affect SCF activity by affecting the modification of a sub-unit of the complex (CUL1). Further characterisation of the mode of action of the compound and the identification of its target(s) may provide new insights into processes regulated by an SCF complex.