UNIVERSITY OF CALIFORNIA SAN DIEGO

Analysis of the role of AFB4 and AFB5 auxin receptors during seedling development in *Arabidopsis thaliana*

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Biology

by

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The dissertation of Kathleen Marie Greenham is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chapter 1, The Introduction, consists of a review of the field of auxin biology with an emphasis on biosynthesis, conjugation, transport and signaling.

Chapter 2, in full, consists of the following manuscript published in *Current Biology* and modified for consistency:

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I was the primary researcher and author of this work. Permission to include the manuscript in this article was provided by *Current Biology* and all co-authors.

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ABSTRACT OF THE DISSERTATION

Analysis of the role of AFB4 and AFB5 auxin receptors during seedling development in *Arabidopsis thaliana*

by

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The plant hormone auxin is vital for all aspects of plant growth and development. Auxin perception is mediated by the TIR1 and the AFB1 through AFB5 family of auxin receptors. The TIR1/AFB1-3 proteins have many overlapping functions throughout plant development as shown by higher order mutant analysis. The conservation of two of the more distantly related members of the family, AFB4 and AFB5 suggests that they maintain a distinct function. The work described in this dissertation provides insight into the specialized roles of these two receptors during

Arabidopsis thaliana seedling development. Mutant analysis reveals that the AFB4 clade is the major target of the picolinate herbicides in *Arabidopsis.* AFB4 and AFB5 exhibit a unique affinity for this compound that is not shared among the other family members. Furthermore, AFB4 appears to be a negative regulator of auxin signaling in the seedling as depicted by extensive phenotypic analysis of the *afb4-2* mutant and its affect on auxin response. The discovery of the long hypocotyl phenotype in *afb4-2* led to a series of hypocotyl transcriptome experiments in *afb4-2* and *afb5-5* to uncover the downstream targets of AFB4 and the genes involved in auxin regulation of hypocotyl elongation. Results from this analysis reveal a mechanism for auxin regulated hypocotyl growth independent of the PIF4/5 pathway as well as a role for AFB4 in the circadian regulation of hypocotyl growth.

CHAPTER 1

Introduction

AUXIN

The phytohormone auxin, or indole-3-acetic acid (IAA), is essential for plant growth and development. Auxin was first described by Charles Darwin as “an influence” in the plant that he deduced from measuring oat coleoptile bending and growth after tip excision (Darwin, 1880). Following this discovery, the field of auxin biology emerged and the focus on plant hormones has become a major interest for developmental biologists. Auxin has been implicated in such processes as cell division and expansion, apical dominance, flowering, root initiation and tropic responses (Davies, 2004). Auxin is important from the onset of plant growth during embryogenesis with the establishment of apical-basal patterning (Przemeck et al., 1996; Hamann et al., 1999; Friml et al., 2003). Auxin continues to be involved in axis patterning throughout plant growth from lateral root emergence (De Smet et al., 2007; Fukaki et al., 2007) to branch formation and shoot apical meristem regulation (Cline, 1991; Okada et al., 1991; Leyser, 2003; Reinhardt et al., 2003). In addition, auxin mediates growth throughout the life cycle of the plant, from root and hypocotyl elongation in the seedling, to leaf expansion and flower development in adult plants (Evans et al., 1994; Jensen et al., 1998; Aloni et al., 2003; Cheng and Zhao, 2007).

AUXIN BIOSYNTHESIS

Given the array of processes mediated by auxin, it is not surprising that auxin levels are tightly regulated by the plant. This regulation occurs primarily at the level of biosynthesis, conjugation and transport. Although auxin has been studied since the 1880s we know very little about how it is synthesized in the plant. Early studies using isotope labeling showed that tryptophan (trp) is converted to IAA *in vivo* (Baldi et al., 1991; Bialek et al., 1992; Michalczuk et al., 1992) suggesting that a trp-

dependent IAA biosynthesis pathway exists in plants. However, analysis of IAA levels in tryptophan auxotroph mutants suggests the presence of a trp-independent biosynthetic pathway, as these mutants show greater levels of amide- and ester-linked IAA than wildtype (Wright et al., 1991; Normanly et al., 1993). The discovery of auxin overproducing mutants led to the identification of important enzymes in the trp-dependent pathways. The *superroot* (*sur1,2)* mutants, named after the large adventitious roots emerging from the hypocotyl, accumulate IAA due to a defect in C-S lyases that catalyze the conversion of indole-3-acetaldoxime (IAOx) to indolic glucosinolates. This conversion is thought to remove available IAOx for IAA synthesis since *sur2* mutants accumulate IAA (Boerjan et al., 1995; Mikkelsen et al., 2004). IAOx is produced from trp by the cytochrome P450 monooxygenases CYP79B2 and CYP79B3 (Sugawara et al., 2009).

The *yuc1D* mutant is also an auxin-overproducing mutant that was isolated in an activation-tagging screen for long hypocotyls. The phenotype is caused by overexpression of *YUCCA*, a flavin monooxygenase-like (FMO) enzyme that catalyzes the conversion of tryptamine to N-hydroxyl tryptamine that can be further converted to IAA through other intermediates (Zhao et al., 2001; Zhao, 2010). The *YUCCA* gene has 10 homologs in *Arabidopsis*, several have been characterized and shown to be important for auxin biosynthesis based on higher order mutant phenotypes (Zhao et al., 2001; Cheng et al., 2006, 2007).

Further genetic studies provided evidence for the presence of an indole-3-pyruvate (IPA) trp-dependent pathway. These studies identified the *TAA1* (Tryptophan Aminotransferase of Arabidopsis) gene that catalyzes the production of IPA from trp. Mutants for this gene (*taa1, tar1, wei8, tir2)* show a reduction in free IAA levels resulting in defects in shade avoidance, hypocotyl elongation, and

temperature and ethylene response (Stepanova et al., 2008; Tao et al., 2008; Yamada et al., 2009). The connection between the YUCCA and IPA pathways is still unclear. Recent data suggests they may be in the same pathway as similar phenotypes are observed in the *yuc1 yuc4 yuc10 yuc11* and *taa1 tar1 tar2* mutants (Zhao, 2010). However, data are still required to determine the biochemical steps in the pathway.

Although the exact pathways that synthesize IAA *de novo* are unclear, the importance of auxin biosynthesis is evident. The major sites of auxin biosynthesis are in the shoot and root tip (Zhao, 2010). However, the local accumulation of auxin by *de novo* synthesis generates important concentration gradients that are necessary for proper polarity as described in the root (Ikeda et al., 2009). The expression of biosynthesis genes in specific cell types is one mechanism used by the plant to generate these local maxima of auxin (Cheng et al., 2007) thus showing the importance of the transcriptional regulation of these genes.

AUXIN CONJUGATION

Another important mechanism for regulating auxin levels in the plant is through IAA conjugation. Amide linkage to amino acids or esterification to sugars are the two types of auxin conjugates. The ester-type conjugates are found at such low levels in plants that they are not considered a major catabolic process for IAA (Ljung et al., 2002). The amino acid conjugates are the predominant IAA catabolic pathway in plants. In *Arabidopsis,* conjugates with Ala, Leu, Asp, Phe, Glu and Glc have been measured and shown to increase in the plant following exogenous auxin treatment (Tam et al., 2000; Kowalczyk and Sandberg, 2001). These conjugates have various roles in maintaining auxin homeostasis. The IAA-Asp and IAA-Glu conjugates appear

to be involved in IAA turnover, based on metabolic labeling experiments and the absence of a phenotype following exogenous treatment with these compounds. The other conjugates are storage forms of IAA that can be hydrolyzed to produce active IAA. This is evident by the auxin related phenotypes observed following exogenous treatment with IAA-Ala, IAA-Gly, IAA-Phe, and IAA-leu (Bartel and Fink, 1995; Staswick et al., 2005).

Genetic screens in search of mutants with altered response to treatment with several IAA conjugates led to the identification of a family of amidohydrolases responsible for hydrolyzing IAA in plants (Bartel and Fink, 1995; Campanella et al., 1996; Rampey et al., 2004). Of the seven genes in the *Arabidopsis* amidohydrolase family, ILR1, ILL1, ILL2, and IAR3 have been shown to cleave IAA-amino acid conjugates *in vitro* (LeClere et al., 2002). Due to redundancy among these genes the single mutants show very weak phenotypes, but the triple *ilr1 iar3 ill2* mutant shows phenotypes consistent with a disruption in auxin distribution such as short hypocotyls and more lateral roots (Rampey et al., 2004). Still, the triple mutant is relatively healthy suggesting that IAA hydrolysis is simply one component of regulating auxin levels during seedling growth. It remains to be seen how these hydrolases are regulated in the plant and whether certain members of the family are more important under certain conditions or cell types.

The process of IAA-amino acid synthesis was largely unknown until very recently. The discovery stemmed from work on another hormone, jasmonic acid and the identification of a mutant insensitive to methyl jasmonate (MeJA), referred to as *jar1* (Staswick et al., 1992). The JAR1 gene was identified as a member of a multigene family that contained a previously identified auxin induced gene from soybean known as *GH3* (Hagen et al., 1991). Computer modeling of the structure of

JAR1 predicted a similarity to the firefly luciferase-like superfamily. Adenylation assays demonstrated that JAR1 is active on JA revealing that JAR1 is a jasmonic acid-amido synthetase (Staswick et al., 2002). Following these results, Staswick *et al.* (Staswick et al., 2002; Staswick et al., 2005) showed that six GH3 homologs of JAR1 were able to adenylate IAA and function as IAA-amido synthetases. All group II GH3 enzymes, apart from GH3.1, conjugate amino acids to IAA and have different affinities for certain amino acids (Staswick et al., 2005). These results provided a mechanism to explain the strong auxin-related phenotypes observed in the activation-tagged line *dfl1-D*, in which *GH3.6* is over-expressed leading to accumulation of IAA-Asp (Nakazawa et al., 2001; Staswick et al., 2005). The rapid induction of these group II *GH3* genes in response to auxin suggests that this is a mechanism used by the plant to remove excess auxin (Hagen and Guilfoyle, 2002; Staswick et al., 2005).

AUXIN TRANSPORT

The biosynthesis and metabolism of IAA are important to provide the plant with a source of auxin but it is equally important for the plant to be able to move auxin from one cell to another. Darwin's initial experiments describing an 'influence' in the plant suggested that an asymmetric distribution of auxin is required for the proper tropic responses, such as movement towards light (Darwin, 1880). This mode of polar auxin transport was later explained by the chemiosmotic model, developed in the 1970s and largely intact today. The model is based on the pH and electrical gradients maintained across the plasma membrane of cells. The acidic pH of the apoplast (pH 5) results in a protonated form of auxin (IAAH) that enters the cell by diffusion or through an influx-carrier. The neutral pH within the cell ionizes IAA, a form that can not diffuse across the membrane, thereby requiring basally localized

efflux-anion carriers to transport auxin down its chemical concentration gradient and out of the cell (Rubery and Sheldrake, 1974; Raven, 1975; Goldsmith, 1977).

This model was validated by the discovery of the AUXIN RESISTANCE1 (AUX1) influx-carrier and the PIN-FORMED (PIN) efflux carriers (Bennett et al., 1996; Galweiler et al., 1998; Luschnig et al., 1998). AUX1 and its relative LAX3 (Like-AUX1) were shown to transport auxin in a *Xenopus* oocyte system (Swarup et al., 2008). The *aux1* and *lax3* mutants are both deficient in lateral root development (Marchant et al., 2002; Swarup et al., 2008). AUX1 is required for the proper transport of IAA from leaf primordia to the primary root apex as well as the import of auxin to the developing lateral root primordia (Marchant et al., 2002). Auxin synthesized in lateral root primordia induces the expression of LAX3 in a distinct set of cells adjacent to the primordia. LAX3 causes the accumulation of auxin needed to promote the expression of cell wall modification enzymes required for lateral root emergence and growth (Marchant et al., 2002; Swarup et al., 2008). This auxin-dependent activation of *LAX3* expression in a specific cell-type reveals the importance of transport mediated auxin gradients during growth.

The PIN1 protein was first discovered because of the dramatic flower phenotype observed in the *pin1* (*pin-formed*) mutant that develops a pin shaped influorescence lacking floral organs. This phenotype can be mimicked by treatment with the polar auxin transport inhibitor N-(1-naphthyl)phthalamic acid (NPA) (Okada et al., 1991). The identification and characterization of the *PIN* genes revealed that these proteins exhibited polar localization within the cell consistent with the polar auxin transport model (Galweiler et al., 1998; Muller et al., 1998; Friml et al., 2002). There are 8 annotated *PIN* genes in *Arabidopsis* (*PIN1-8*) that are grouped according to the length of their central hydrophilic loop. PIN5 and PIN8 have a short hydrophilic

loop that is thought to account for their localization to the ER as opposed to the plasma membrane where the other PIN proteins are found (Krecek et al., 2009).

Extensive genetic and protein localization studies have revealed the elegant mechanism for PIN-dependent auxin distribution in the developing root. The localization of PIN proteins in the root directs the movement of basally transported auxin to the root tip where it is then directed laterally towards the elongation zone. This movement of auxin results in a concentration maximum at the root tip that is required for proper meristem size (Benkova et al., 2003; Blilou et al., 2005). The asymmetric distribution and rapid cycling of PIN proteins to the membrane provide a dynamic response mechanism to generate cell-type specific auxin gradients that are necessary during organ formation as seen during lateral root emergence and floral primordia initiation (Benkova et al., 2003; Swarup et al., 2008; Kitakura et al., 2011).

The polar distribution of the PINs relies on GNOM-dependent vesicle trafficking. GNOM, an ARF-GTPase (ARF-GEF), is membrane associated and localized to the endosome. PIN1 polar localization is disrupted in the *gnom* mutant due to loss of proper vesicle formation, a process that can be mimicked with the vesicle trafficking inhibitor brefeldin A (BFA) (Steinmann et al., 1999; Geldner et al., 2003). Rapid cycling of the PINs occurs between the plasma membrane and endosomal compartments. However, differential localization of multiple PIN proteins in a cell suggests that another regulatory mechanism determines the distribution of these proteins. One aspect of this regulation is likely to involve the phosphorylation of the PINs by the Ser/Thr kinase PINOID (PID). A current model proposes that phosphorylated PINs have a reduced affinity for the GNOM pathways and are instead recruited by a GNOM-independent trafficking pathway to the apical side of the cell (Kleine-Vehn et al., 2009).

Another family of proteins that have been found to transport auxin includes the ATP-binding cassette B/P-glycoprotein (ABCB/PGP) proteins. *Arabidopsis* ABCB1, ABCB4 and ABCB19 have been shown to function as auxin transporters in tobacco cells (Petrasek and Friml, 2009). The *abcb1* and *abcb19* single and double mutants in *Arabidopsis* show clear auxin transport related phenotypes with defects in hypocotyl elongation, shoot apical dominance and root development. The exact role of these proteins during auxin transport has yet to be determined but evidence suggests that they may be involved in interacting with certain PIN proteins to regulate their activity, as seen with ABCB19 and PIN1 (Titapiwatanakun et al., 2009).

AUXIN SIGNALING

Auxin exerts its effect on plant growth through changes in gene expression. The *Aux/IAAs, GH3*s and a family of *SMALL AUXIN UP RNA (SAUR)* genes are three classes of early auxin responsive genes (Abel and Theologis, 1996). The *Aux/IAA* genes were first described in pea because of their rapid induction within 15 minutes of auxin treatment (Walker and Key, 1982; Theologis et al., 1985). These genes encode short-lived nuclear proteins containing a putative DNA binding motif that led to the first hypothesis that they may be primary response activators or repressors of auxin signaling (Abel et al., 1994). There are 29 *Aux/IAA* genes encoded in the *Arabidopsis* genome that are grouped into 10 distinct branches in a phylogenetic tree (Overvoorde et al., 2005). Sequence alignment of these proteins reveals four conserved domains. Domains I, II and IV all contain NLS motifs (Abel et al., 1994; Abel and Theologis, 1995) whereas domain III contains a $\beta\alpha\alpha$ DNA binding domain that is required for Aux/IAA homo- and hetero-dimerization, although there is no evidence for direct DNA binding (Kim et al., 1997). Domain I contains an ERF-

associated amphiphilic repression (EAR) motif causing these proteins to act as transcriptional repressors by interacting with a family of Auxin Response Factors (ARFs) (Tiwari et al., 2004). This domain is also important for binding to the co-repressor TOPLESS (TPL) as seen for IAA12/BDL interaction (Szemenyei et al., 2008). Domains III and IV of the Aux/IAA proteins interact with similar sequences within the ARFs (Fig 1.1) (Ulmasov et al., 1997).

The ARF1 protein was originally identified in a yeast one-hybrid screen using an inverted tandem repeat of an auxin response element identified in the *GH3* promoter from soybean. The TGTCTC Auxin Response Element (AuxRE) was shown to respond to auxin when four tandem copies were fused to the β-glucuronidase (GUS) reporter gene and expressed in carrot protoplasts (Ulmasov et al., 1997). Although the TGTCTC element is found in the promoters of many auxin-responsive genes, it is not found in all; and tandem repeats of the core TGTC element, the minimum sequence required for ARF binding (Ulmasov et al., 1995), are also sufficient for auxin response (Nemhauser et al., 2004; Muller and Sheen, 2008). There are 23 ARFs encoded in the *Arabidopsis* genome that are characterized by a C-terminal dimerization domain (CTD), a B3-like DNA binding domain (DBD) and a middle region (MR) that functions as a transcriptional repressor or activator domain (Tiwari et al., 2003). The CTD region is homologous to domains III and IV of the Aux/IAAs (Reed, 2001).

Due to genetic redundancy, most of the single loss-of-function mutants of *ARF* genes have no obvious phenotypes. The most severe *ARF* mutant, *arf5/mp* or *monopteros,* is disrupted during early embryo body axis formation resulting in the complete loss of root and hypocotyl tissue (Hardtke and Berleth, 1998). The *arf3/ettin* mutant has defects in floral development with a reduction in stamen number and

anther formation and in apical-basal gynoecium patterning (Sessions et al., 1997). ARF7 and ARF8 appear to be important for hypocotyl growth based on the long hypocotyl phenotype of *arf8* (Tian et al., 2004) and the loss of proper hypocotyl gravitropism and apical hook maintenance of *arf7/nph4* (Harper et al., 2000).

Independent screens for auxin response mutants revealed several gain-of-function mutants that carry mutations in the highly conserved domain II of the Aux/IAAs causing stabilization of these nuclear repressors (Reed, 2001). These include *shy2/iaa3, shy1/iaa6, axr2/iaa7, bdl/iaa12, axr3/iaa17, msg2/iaa19* and *iaa18*; most affect multiple developmental processes throughout plant growth (Reed, 2001; Overvoorde et al., 2005). Phenotypes range from root growth defects in *shy2/*iaa3 (Tian and Reed, 1999), lack of hypocotyl gravitropism or phototropism in *msg2*/*iaa19* (Tatematsu et al., 2004), and loss of the primary root meristem during embryogenesis in *bdl/iaa12* (Hamann et al., 1999). All of these mutants carry a mutation in a 13-amino consensus sequence in domain II that is required for the degradation of these short-lived proteins (Worley et al., 2000; Ramos et al., 2001). This degradation is mediated by the TIR1/AFB auxin receptors (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b; Kepinski and Leyser, 2005).

TIR1 (transport inhibitor response 1) was identified in a genetic screen for mutants that are resistant to the auxin transport inhibitor NPA. The *tir1-1* mutant shows increased growth on NPA and reduced sensitivity to exogenous auxin (Ruegger et al., 1998). TIR1 encodes an F-box protein that interacts with ASK1, the SKP1 homolog in plants, to form the SCF complex (Gray et al., 1999). The importance of the ubiquitin-proteasome pathway in regulating hormone signaling in plants is apparent by the recent discoveries of E3 ligases required for ethylene, GA, jasmonate and auxin signaling (Santner and Estelle, 2009). Ubiquitination of a target

protein requires the coordination of three enzymes, a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin protein ligase (E3). Ubiquitin (Ub) binds to the E1 enzyme active site cysteine by a thiol-ester bond. Activated Ub is then transferred to the active site cysteine of an E2 enzyme. An E3 ligase then binds to the Ub-charged E2 and mediates the transfer of the Ub to its target substrate (Fig. 1.1) (Fang and Weissman, 2004). There are two distinct groups of E3 ligases characterized by their motifs, E3s with the ~350 homologous to E6-AP carboxyl terminus (HECT) domain and the E3s with the really interesting new gene (RING) domain. The HECT domain E3s require a thiol-ester bond with Ub before being passed to the target protein. The RING E3s do not form thiol-ester bonds but act as a bridge between the E2 and the substrate by binding to the E2-ubiquitin complex and facilitating Ub transfer (Nandi et al., 2006).

The SCF subset of E3s is made up of four subunits, SKP1 (ASK1 in plants), CULLIN (CUL1), RING-H2 finger protein RBX1 and an F-box protein. CULLIN acts as a scaffold by binding to the F-box protein at its N-terminus and RBX1 at its C-terminus. The SCF complex is regulated by the addition of an ubiquitin-like protein called RUB/Nedd8. RUB modification to CUL1 involves a similar 3-step process as ubiquitination, requiring a heterodimeric RUB-specific E1 AXR1 (AUXIN RESISTANT1) and ECR1 (E1 C-TERMINAL-RELATED), the RCE1 (RUB1 CONJUGATING ENZYME1) E2 enzyme and RBX1 (del Pozo and Estelle, 1999; del Pozo et al., 2002; Gray et al., 2002). RUB conjugation facilitates the formation of the SCF complex by preventing binding of the CULLIN-interacting protein CAND1 to CUL1 (Liu et al., 2002; Zheng et al., 2002; Goldenberg et al., 2004). Binding of CAND1 to CUL1 prevents the formation of the SCF complex by blocking the SKP1 (ASK1) binding site on CUL1. The COP9 signalosome (CSN) complex catalyzes the

removal of RUB modification allowing CAND1 to bind derubylated CUL1 (Fig. 1.1) (Goldenberg et al., 2004). The specificity of the SCF complex is provided by the F-box protein that interacts with the substrate and facilitates ubiquitination (Moon et al., 2004). In *Arabidopsis* there are more than 700 F-box proteins and represents the largest superfamily in *Arabidopsis* (Gagne et al., 2002).

The discovery that TIR1 functions as an auxin receptor finally solved the mystery of how auxin is perceived by the plant. The crystal structure of TIR1, bound to an IAA7 peptide containing the domain II degron, revealed a unique mechanism for auxin binding and SCF substrate recognition. Auxin sits in a pocket formed by the leucine rich repeat solenoid-shaped conformation of TIR1 with a binding site defined by two polar residues. Auxin acts as a 'molecular glue' by enhancing the binding affinity of TIR1 with the Aux/IAA protein (Tan et al., 2007). The *Arabidopsis* genome encodes 5 proteins related to TIR1 known as Auxin Signaling F-Box (AFB) proteins (Dharmasiri et al., 2005b). These 6 proteins can be subdivided into three clades based on amino acid similarity, TIR1/AFB1, AFB2/AFB3 and AFB4/AFB5. Phylogenetic analysis reveals that the TIR1 and AFB2 clades diverged roughly 200 million years ago (Mya) whereas the AFB4 clade diverged from the TIR1/AFB1-3 clade ~300-400 Mya (Parry et al., 2009). Previous studies showed that AFB1-3 function as auxin receptors and interact with the Aux/IAAs in an auxin dependent manner (Dharmasiri et al., 2005b). Experiments to determine the effects of auxin on root elongation and lateral root formation demonstrate that the single mutants for these genes have a mild auxin-resistant phenotype on IAA. The *tir1-1* mutant displays the strongest resistance compared to all the single mutants followed by *afb2-3* and *afb3-4*. Double, triple and quadruple mutants show a gradual decrease in auxin response as well as defects in meristem development, root growth, hypocotyl

elongation, leaf morphology and floral development. The most severely affected seedlings lack a root and have a single cotyledon (Dharmasiri et al., 2005b; Parry et al., 2009).

Based on the single mutant phenotypes it appears that the receptors have overlapping functions throughout plant development. Careful examination of higher order mutants uncovers slight differences in the contributions of these proteins during seedling growth. The *tir1-1 afb2-3 afb3-4* triple mutant shows a stronger auxin resistance in the root compared to *tir1-1 afb2-3* whereas *tir1-1 afb1-3 afb2-3* does not, suggesting that AFB3 contributes to auxin response in the root (Parry et al., 2009). This role is further supported by work from Vidal *et al.* (2010) who show that *AFB3* is the only receptor regulated by nitrate in the root and this regulation is required for lateral root growth following exposure to nitrate. The problem of redundancy and overlapping functions among the receptors, Aux/IAAs and ARFs confounds our ability to assign distinct roles to certain members.

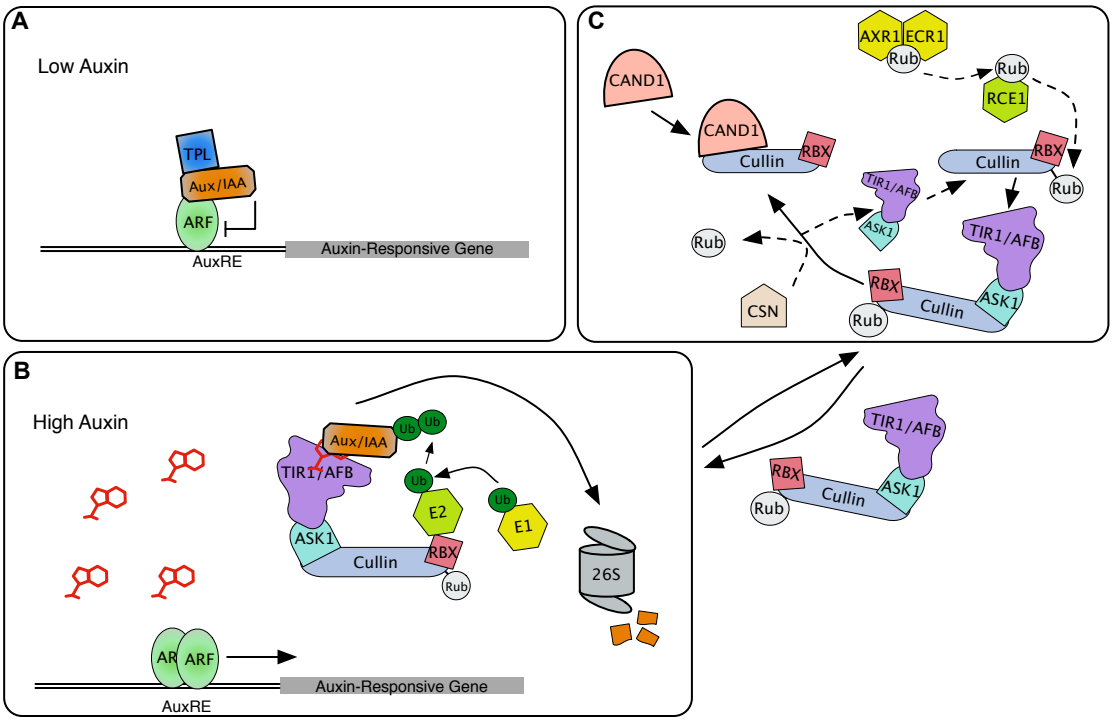

Figure 1.1 Model of auxin signaling. (A) Under low auxin conditions the Aux/IAA repressor proteins bind to and inactivate the ARF transcription factors with the help of the TPL corepressor. (B) Auxin enters the cell and mediates the interaction between TIR1/AFB and the Aux/IAA thereby releasing the repression on the ARFs allowing activation of auxin-responsive genes. The Aux/IAA proteins are poly-ubiquitinated through the $SCF^{TIR1/AFB}$ complex and degraded by the 26S proteasome. (C) The $SCF^{TIR1/AFB}$ complex is recycled via the dynamic Rub modification to Cullin. Rub modification activates the SCF complex by preventing the binding of CAND1 and allowing TIR1/AFB-ASK1 to bind to Cullin. The CSN catalyzes the removal of Rub leading to CAND1 binding and inactivation of the complex.

SYSTEMS BIOLOGY AND AUXIN

The presence of large gene families with overlapping functions in *Arabidopsis* poses a difficult challenge for biologists seeking to understand what genes are involved in a physiological process. The field of systems biology is gaining increasing recognition in large part due to recent advances in high-throughput technologies such as NextGeneration sequencing. A systems approach to address a biological question queries whole-genome datasets to uncover pathways or groups of genes involved in a cellular response. Microarray technology was one of the first methods used to measure global changes in transcript levels (Hoheisel, 2006). Several studies have been performed using microarray technology to define the auxin responsive transcriptome in *Arabidopsis* and to identify genes of interest. Expression profiling by Sawa *et al.* (2002) performed in whole seedlings demonstrated that following a short 15 min treatment with IAA, 29 genes were upregulated including an unknown homeobox gene *HAT2*. Further characterization of this gene revealed a role in auxin mediated root and shoot development (Sawa et al., 2002). Tissue specific microarrays performed in root tissue following auxin treatment have lead to the identification of a large set of auxin responsive genes involved in a wide range of processes including cell wall modification, signal transduction, vascular transport and transcription (Laskowski et al., 2006). Detailed mapping of the temporal and spatial expression patterns of genes during root development using fluorescent cell sorting coupled with microarray analysis has been used to identify the transcriptional response occurring in a cell-type specific manner, for example during the formation of lateral roots (Birnbaum et al., 2003; Brady et al., 2007; Moreno-Risueno et al., 2010).

Another advantage of microarray technology is the ability to do comparative analysis between treatments, tissues or developmental time periods. Comparative

analysis between hormone treatments have uncovered hormone specific transcriptional targets for auxin, giberrellin, jasmonic acid, ethylene and brassinosteroids, as well as overlapping targets, highlighting the complexity of hormone crosstalk during plant growth (Nemhauser et al., 2004; Nemhauser et al., 2006; Stepanova et al., 2007). The availability of these microarray studies to the scientific community enable researchers to analyze gene families or their particular genes of interest across a wide set of auxin treatment experiments in order to examine the contribution of individual genes under various conditions (Paponov et al., 2008).

The complexity of auxin biology and the coordinated network of biosynthesis, transport and signaling components has motivated the use of computational modeling to generate a dynamic system that integrates all of these processes. The formation of leaf and flower primordia from the shoot apical meristem occurs with extreme precision to create phyllotactic patterns. Auxin is essential for this process and many hypotheses exist to explain how auxin transport occurs to generate this precise patterning. Computer models are being used to address the mechanism of phyllotaxy by predicting the localization and movement of PIN1 during this process to generate testable hypotheses for the mechanism of auxin distribution (Jonsson et al., 2006; Merks et al., 2007; Bayer et al., 2009). The model by Bayer *et al.* (2009) is based on two mechanisms for PIN1 polarization, the 'up-the-gradient', or towards the auxin maximum, and 'with-the-flux', or towards transient zones of increased auxin levels created by flux. An apical polarization at the tip of the midvein was predicted by this model and experimentally confirmed, demonstrating the advantages of using mathematical models to form testable hypotheses (Bayer et al., 2009).

An important challenge for the future is to apply what we know from studies in *Arabidopsis* to crop plants. High-throughput sequencing technologies such as RNA-seq provide a platform for pursuing studies in species without sequenced genomes. This technology can be used for transcript profiling, gene discovery, identifying splice forms and small RNAs and for developing molecular markers for QTL mapping (Wang et al., 2010; Yang et al., 2011). An RNA-seq experiment on stem tissue between two alfalfa genotypes resulted in the identification of 10,826 single nucleotide polymorphisms (SNPs), a subset of which are located in genes related to auxin (Yang et al., 2011). This demonstrates the success of this technology when applied to non-model organisms.

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CHAPTER 2

The AFB4 Auxin Receptor is a Negative Regulator of Auxin Signaling in Seedlings

REPRODUCTION OF THE MANUSCRIPT

The AFB4 auxin receptor is a negative regulator of auxin signaling in seedlings

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SUMMARY

The plant hormone auxin is perceived by a family of F-box proteins called the TIR1/AFBs. Phylogenetic studies reveal that these proteins fall into four clades in flowering plants called TIR1, AFB2, AFB4, and AFB6 (Parry et al., 2009). Genetic studies indicate that members of the TIR1 and AFB2 groups act as positive regulators of auxin signaling (Dharmasiri et al., 2005; Parry et al., 2009). In this report we demonstrate a unique role for the AFB4 clade. Both AFB4 and AFB5 function as auxin receptors based on *in vitro* assays. However, unlike other members of the family, loss of AFB4 results in a range of growth defects that are consistent with auxin hypersensitivity including increased hypocotyl and petiole elongation, and increased numbers of lateral roots. Indeed, qRT-PCR experiments show that *afb4-2* is hypersensitive to IAA in the hypocotyl indicating that AFB4 is a negative regulator of auxin response. Further, we show that AFB4 has a particularly important role in the response of seedlings to elevated temperature. Finally, we provide evidence that the AFB4 clade is the major target of the picloram family of auxinic herbicides. These results reveal a previously unknown aspect of auxin receptor function.

RESULTS AND DISCUSSION

An extensive phylogenetic analysis revealed that the AFB4/AFB5 clade diverged from the TIR1/AFB1-3 clade ~300-400 million years ago whereas the AFB2/AFB3 clade diverged from TIR1/AFB1 ~200 million years ago (Parry et al., 2009). Genetic and biochemical studies have demonstrated that members of the TIR1 and AFB2 clades are positive regulators of auxin response and differ in their relative contributions to seedling development (Parry et al., 2009). However, the phylogenetically distinct AFB4 group comprised of AFB4 (At4g24390) and AFB5 (At5g49980) have not been characterized in detail. Since these proteins have only 50% identity to the other TIR1/AFB proteins, it is likely that they have evolved distinct functions. To explore this possibility we performed a series of experiments focusing on the role of AFB4 and AFB5 during seedling development.

The AFB4 and AFB5 proteins are auxin receptors

Our first objective was to determine if AFB4 and AFB5 are subunits of SCF complexes. Transgenic lines expressing c-myc-tagged versions of AFB4 and AFB5 under the control of the *AFB5* promoter were generated for co-immunoprecipitation experiments. AFB4-myc and AFB5-myc were immunoprecipitated from plant extracts with the anti-myc antibody coupled to agarose beads. After washing, the samples were resolved by SDS-PAGE, blotted, and probed with antibodies to the Arabidopsis SKP1-related protein ASK1 (Gray et al., 1999). A line expressing TIR-myc was included for comparison (Gray et al., 1999). Consistent with their similarity to the TIR1 and AFB1-3 proteins both AFB4 and 5 interact with ASK1 and presumably form an SCF complex (Fig 2.1A).

To determine whether AFB4 and AFB5 also exhibit the characteristics of auxin receptors, we performed pull-down experiments with the Aux/IAA protein IAA3. Equivalent amounts of total protein extract from AFB4-myc and AFB5-myc plants were incubated with GST-IAA3 bound beads in the presence or absence of 50μM IAA. Both AFB4 and AFB5 interact with IAA3 in an auxin dependent manner demonstrating that these proteins probably function as auxin receptors (Fig 2.1B).

AFB4 and AFB5 are the major targets of the picolinate class of auxinic herbicides

The synthetic auxin picloram (4-amino-3,5,6-trichloropicolinic acid) has been well studied for its auxinic herbicidal properties on a variety of plant species (Hamaker et al., 1963; Scott and Morris, 1970; Chang and Foy, 1983). To identify genes required for herbicide response, Walsh and colleagues (Walsh et al., 2006) screened EMS-mutagenized *Arabidopsis* seedlings to identify mutants that were specifically resistant to picolinate auxins (Walsh et al., 2006). One of the genes identified in this screen was *AFB5*. Further characterization revealed that the *afb5* mutants were highly resistant to picloram but sensitive to 2,4-D (2,4-dichlorophenoxyacetic acid), a synthetic auxin from the aryloxyacetate class (Walsh et al., 2006). To further explore this specificity, we obtained a T-DNA insertion allele of *AFB5* referred to as *afb5-5*. This allele has an insertion in exon 2 that results in the loss of full-length *AFB5* mRNA (Fig 2.S1A, B). In addition six *afb4* mutants were recovered from the *Arabidopsis* TILLING project, 2 of which were characterized in more detail (Till et al., 2003). The position and nature of the amino acid substitutions is shown in Fig 2.S1C. The root growth response of several of these mutants to picloram was determined and compared to Col-0 and *tir1-1*. Consistent with Walsh *et al.* (Walsh et al., 2006), *afb5-5* seedlings were resistant to picloram-mediated root

inhibition. Similarly, *afb4-2* and *afb4-3* were picloram-resistant while *tir1-1* exhibited wild-type sensitivity (Fig 2.2A). Further, picloram resistance was enhanced in both double mutant combinations. In contrast, neither *afb4-2* nor *afb5-5* displayed significant resistance to either IAA or 2.4-D (Fig 2.2B).

Picloram is known to promote hypocotyl elongation (Savaldi-Goldstein et al., 2008). To examine whether AFB4 and AFB5 contribute to this response, seedlings were grown for 4 days under short day photoperiods before being transferred to fresh plates containing picloram. As expected based on previous studies picloram stimulates elongation of Col-0 hypocotyls (Fig 2.2C)(Savaldi-Goldstein et al., 2008). In contrast, the *afb4-2* mutant is slightly picloram-resistant while *afb5-5* and *afb4-2 afb5-5* are almost completely resistant to picloram. Picloram sensitivity was restored in *afb5-5* by introducing the AFB5-myc construct described in Figure 2.1. Similarly, we showed that an *AFB4:AFB4-GUS* transgene restores picloram sensitivity to the *afb4-2* mutant (fig 2.S1D,E). These results demonstrate that the picloram dependent hypocotyl elongation is primarily AFB4/5-dependent.

To determine if picloram selectivity is expressed at the biochemical level, pull-down assays were carried out as before but with the addition of 50μM picloram. Both AFB4 and AFB5 interacted with IAA3 in a picloram dependent manner whereas TIR1 did not (Fig 2.2D) suggesting a unique specificity of the AFB4 clade for picloram. To compare the interaction of AFB4 and AFB5 with picloram with that of other auxins, we also did side by side pulldown experiments (Fig 2.2E). The results indicate that both proteins also respond to IAA, 2,4-D and 1-NAA. Interestingly, picloram was only slightly more effective than 2,4-D in promoting the interaction between AFB4 and 5 and GST-IAA7 while IAA appears to be most effective in promoting the interaction. However, it is important to note that these pulldown assays

are not quantitative. Our results indicate that members of the AFB4 clade have a high affinity for IAA but also exhibit structural differences compared to the other TIR1/AFB proteins that allow them to respond to picloram.

Taken together, these data indicate that members of the AFB4 clade are the major targets of the picolinate herbicides in Arabidopsis. This finding is particularly important because of the broad use of picloram in agriculture. Identifying the genes that contribute to picloram sensitivity will provide the basis for the development of picloram resistant crops.

AFB4 is a negative regulator of auxin-dependent processes

To assess the role of AFB4 and AFB5 in the developing seedling we examined several auxin-dependent growth processes in the mutants, including petiole and hypocotyl elongation and lateral root formation. For comparison, we also examined the *tir1-1 afb2-3* double mutant, which is known to be deficient in hypocotyl elongation and lateral root formation (Dharmasiri et al., 2005). This was confirmed in our experiments (Fig 2.3A-D). Surprisingly the *afb4-2* single and *afb4-2 afb5-5* double mutants had the opposite effect on these processes. The *afb4-2* mutant had elongated petioles (Fig 2.3A) and a longer hypocotyl (Fig 2.3B) than wild-type seedlings. The *afb5-5* single mutant was like wild type but enhanced the *afb4-2* hypocotyl phenotype in the double mutant suggesting that AFB5 has a similar, but lesser role in these processes. Importantly, the increase in length of the *afb4-2* hypocotyl was not due to a prolonged growth phase. Rather, the growth rate of mutant seedlings was increased relative to wild type, particularly early in development (Fig 2.S2A). In addition, the *afb4-2* mutant had shorter roots and produced more lateral roots/primary root length than wild-type seedlings (Fig 2.3D, E). At this point, it

is not clear whether increased lateral root density is a direct effect of the mutation or related to the shorter primary root. In addition, it is important to note that we have not determined the origin of these roots in detail, and it is possible that *AFB4* has a role in anchor or adventitious root production. A recent study has demonstrated an important role for auxin in the development of these roots (Lucas et al., 2011). To confirm that these defects are due to the loss of AFB4, the *AFB4:AFB4-GUS* transgene, was crossed into the *afb4-2* mutant. We find that the transgene substantially restores the mutant to wild-type levels with respect to hypocotyl length, petiole length, and root growth (Fig 2.S2B-G). These results indicate that AFB4 is a negative regulator of petiole and hypocotyl elongation as well as lateral root formation. It is interesting to note that the growth phenotype of the *afb4-2* mutant is stronger than any of the other single mutants in the *TIR1/AFB* family.

The opposite phenotype of the *tir1-1 afb2-3* and *afb4-2 afb5-5* mutants is striking. To understand the relationship between the AFB4 clade and the other receptors, the *afb4-2* mutant was introduced into the *tir1-1 afb2-3* background. The *tir1-1 afb2-3 afb4-2* triple mutant exhibited an *afb4-2*-like phenotype with longer petioles and hypocotyl than *tir1-1 afb2-3* and wild type (Fig 2.3A, B). It is evident that, in the case of these tissues, *afb4-2* is epistatic to *tir1-1* and *afb2-3* suggesting that AFB4 function does not depend on the other members of the TIR1/AFB family. In the case of growth, the situation is more complex. The triple mutant did not exhibit an increase in lateral root density compared to *tir1-1 afb2-3* suggesting that *afb4-2* is not epistatic in this tissue (Fig 2.3C). However, *afb4-2* does appear to be epistatic with respect to primary root elongation. Further experiments are required to understand the relationship between TIR1/AFB proteins in the roots.

The *PIF4* and *PIF5* genes encode related basic helix-loop-helix proteins that function in a variety of growth processes. They are positive regulators of hypocotyl growth and are regulated by the GA, light and clock pathways (Fujimori et al., 2004; Duek and Fankhauser, 2005; Nozue et al., 2007; de Lucas et al., 2008; Lorrain et al., 2009). To determine if the effect of *afb4-2* on the hypocotyl is dependent on PIF4 and PIF5, we introduced *afb4-2* into the *pif4-101 pif5-1* double mutant. We found that the *pif4-101 pif5-1* double mutant has a shorter hypocotyl at 22°C under short day conditions but that *afb4-2* acts to partially suppress this phenotype (Fig 2.3E). This result suggests that AFB4 functions at least partially independently of PIF4 and PIF5.

Expression of the AFB4 and AFB5 genes

To determine whether differences in expression pattern between *AFB4* and *AFB5* can account for the difference in mutant phenotype we measured transcript levels for each of the *TIR1/AFB* genes in tissue collected from 4-day-old seedlings by quantitative RT-PCR. The results in Fig 2.3F indicate that the level of *AFB4* transcript is extremely low compared to other members of the family in all three tissues examined. In contrast *AFB5* transcript levels are relatively high, particularly in the hypocotyl (Fig 2.3F). *AFB4* transcript levels are similar in the root, hypocotyl, and cotyledon, whereas the other members of the *TIR1/AFB* family exhibit different levels of expression in cotyledons, hypocotyls and roots.

To confirm these results we also generated transgenic lines in which the *AFB4* and *AFB5* cDNA was fused to *GUS* and placed under the control of the *AFB4* and *AFB5* promoters respectively. Seedlings were stained after 4 days under short day growth conditions. In the case of the *AFB4:AFB4-GUS* lines, staining was only visible at the root-shoot junction and in the root tip (Fig 2.S2C) consistent with the low levels

of the transcript. In contrast, in *AFB5:AFB5-GUS* lines, fairly uniform staining was observed in all tissues at a much higher level (Fig 2.S2C). These results indicate that the greater role of *AFB4* in seedling development compared to *AFB5,* is not related to expression level.

We obtained similar results in a recent study of TIR1 and AFB1 (Parry et al., 2009). In this case, *AFB1* is expressed at a much higher level than *TIR1* but has a lesser role in root development. We show that the interaction between AFB1 and Aux/IAA proteins is weaker than that of TIR1 explaining why TIR1 has a greater contribution to auxin response. Thus the difference between AFB4 and AFB5 may be related to differences in the biochemical activity of the two proteins. Further studies will be required to determine if this is the case.

AFB4 is a negative regulator of auxin response in the hypocotyl

All of the defects observed in *afb4-2* seedlings can be simulated in wild-type seedlings by treatment with auxin. In addition, *afb4-2* seedlings have a similar phenotype to that of the *yucca-2D* mutant (Zhao et al., 2001). This mutant over-expresses a flavin monooxygenase-like enzyme involved in auxin biosynthesis, resulting in increased IAA levels. Like *afb4-2*, *yucca-2D* seedlings have a long hypocotyl in the light but a shorter hypocotyl in the dark (Fig 2.S3). To determine if *afb4-2* seedlings also have high levels of IAA we measured free IAA levels in excised 4-day-old *afb4-2* and Col-0 hypocotyls and in a separate experiment, cotyledon and hypocotyl combined. In both experiments, IAA levels were similar in both genotypes, indicating that increased IAA levels are not responsible for enhanced hypocotyl elongation (Fig 2.4A). To determine if IAA response is disrupted in *afb4-2* plants, transcript levels of a selection of auxin response marker genes were examined in

cotyledon, hypocotyl and root tissue collected from short day grown *afb4-2* and wild type seedlings after a 2hr treatment with and without 1μM IAA. Tissue was dissected from 4-day-old seedlings when hypocotyls are in their maximum stage of growth under our conditions (Fig 2.S2A). The early auxin responsive genes were selected based on microarray data from Nemhauser *et al.* (Nemhauser et al., 2006). The level of each transcript in the untreated samples is similar in wild type and *afb4-2* tissue. However, the response to auxin was greater in the mutant compared to wild type indicating that *afb4-2* is hypersensitive to IAA in the hypocotyl (Fig 2.4B). In contrast we did not observe hypersensitivity in either the root or cotyledons of *afb4-2* plants (data not shown).

To determine if *afb4-2* is hypersensitive with respect to hypocotyl elongation, we treated wild-type and mutant seedlings with increasing concentrations of IAA (Fig 2.4C). The wild-type response curve was bell shaped, typical of many auxin growth responses. As observed previously, the mutant had longer hypocotyls in the absence of IAA. The response curve was also slightly bell shaped, but in this case, the maximum response occurred at a lower IAA concentration consistent with auxin hypersensitivity (Fig 2.4C).

AFB4 is required for temperature induced hypocotyl elongation

Previous studies have shown that hypocotyls of seedlings grown at 29°C accumulate free IAA and as a result elongate more than seedlings at 20°C (Gray et al., 1998). To determine if AFB4 regulates this response, seedlings were grown for 4 days at 22°C and shifted to 29°C. After two days at 29°C hypocotyls were measured and the length expressed as a percentage of growth at 22°C. As expected, wild-type hypocotyls were over twice as long at the higher temperature (Fig 2.4D). The *afb5-5*

mutation had no effect on the response while the *tir1-1 afb2-3* mutants were modestly resistant to increased temperature. In contrast, the effect of higher temperature on the *afb4-2* mutant was strongly reduced compared to wild type (Fig 2.4D). This aspect of the *afb4-2* phenotype was also rescued by the *AFB4:AFB4-GUS* transgene (Fig. 2.S2G).

CONCLUSIONS

In this report we demonstrate that the AFB4 clade of auxin receptors have a novel function in auxin signaling and seedling development. We show that AFB4 and AFB5 are required for picloram response and appear to be the major targets of this class of herbicide. Paradoxically, AFB4 is a negative regulator of IAA response in the developing seedling. At this point the biochemical basis for these effects is not clear. However auxin action is highly regulated with multiple feedback systems affecting every level of the auxin network (Leyser, 2003). It is possible that AFB4 has a particularly important role in mediating a negative regulatory loop. If this were the case, loss of AFB4 would result in increased auxin response. At the biochemical level, the unusual activity of AFB4 may be related to interaction with specific Aux/IAA and ARF proteins. Further detailed studies of the role of AFB4/5 in different aspects of the network will be required to resolve this issue. In addition, biochemical and structural studies will be required to determine the basis of picloram action and the specific role of the AFB4 clade in this response.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions and treatments

Arabidopsis thaliana mutants and transgenic lines used in this study were all in the Columbia (Col-0) ecotype. The SALK T-DNA insertion lines *afb5-5* (SALK_110643) and *afb2-3* (SALK_137151) were obtained from the Arabidopsis Biological Resource center at The Ohio State University. The *afb4-2* mutant was backcrossed to Col-0 twice and was genotyped for the loss of the erecta lesion that is present in TILLING mutants. Seeds were surface sterilized for 2 min in 70% (v/v) ethanol followed by 10 min in 30% commercial bleach and plated on medium containing ½ X MS media, 1% sucrose, 1% agar and stratified for 2-4 days at 4°C.

Growth Assays

All root assays were completed under constant light and hypocotyl assays were performed under short day photoperiods (8:16) at 80 μmol m^{-2} sec^{-1}, unless otherwise stated. For auxin inhibited root growth assays, 5-day-old seedlings were transferred onto fresh MS media ± auxin for 3 additional days after which root length was measured. Hypocotyl assays were performed similarly except the seedlings were transferred at day 4 for a 2-day treatment unless otherwise stated. Emerged lateral roots were counted on 10-day-old long day grown seedlings using a Nikon SMZ1500 dissecting scope. Petiole length was measured on 7-day-old short day grown seedlings. All measurements were performed using a Nikon SMZ1500 dissecting scope and ImageJ software (http://rsbweb.nih.gov/ij/index.html).

Generation of Transgenic Lines

The TIR1-myc line was generated as previously described (Gray et al., 1999). The AFB4 and AFB5 c-myc lines were generated using a 2-kb 5' upstream region of the *AFB5* gene with the AFB4 and AFB5 cDNA. The *AFB5* promoter was used for expressing AFB4 due to the low activity of the *AFB4* promoter. These constructs were transformed into the *afb5-5* mutant background. Transformants were assayed for their ability to restore picloram sensitivity in the hypocotyl (Fig S1). The GUS fusions were generated with the 2-kb promoter region of each gene ahead of the GUS cDNA for the translational fusions and the *AFB4* and *AFB5* cDNA fused to GUS in the transcriptional fusions.

Protein Expression and Pulldown experiments

For pulldown assays, GST-IAA3 and GST-IAA7 were recombinantly expressed in *E.coli* and purified with glutathione agarose beads (SIGMA) using standard methods. Seedlings expressing c-myc-tagged AFB4, AFB5 and TIR1 were grown for 8 days in liquid MS medium. TIR1-myc expression was induced by treatment with 30μM Dex for 24hrs. The ASK1-antibody was generated as previously described (Gray et al., 1999). For the various auxin comparisons (Fig 1D) seedlings were incubated for 2hrs in 50μM of the compounds or an equivalent volume of DMSO prior to harvest. For all other pulldown experiments samples were incubated with auxin for 45min following harvest. Tissue was harvested by grinding to a powder in liquid nitrogen and vortexed vigorously in extraction buffer (50mM Tris pH7.5, 150mM NaCl, 10% glycerol, 0.1% NP-40, complete protease inhibitor (Roche), 50μM MG-132). Cellular debris was removed by centrifugation and total protein concentration was determined by Bradford assay. Each pulldown reaction included 1mg total

protein extract and equal volumes of GST-IAA protein bound to agarose beads for each sample in a 500μl total volume. The pulldown reactions were incubated at 4°C for 45min with rocking and transferred to a Micro Bio-Spin Chromatography Column (Biorad). Samples were washed 3 times in 1 ml extraction buffer without protease inhibitors or MG-132 in the presence or absence of auxin. Samples were eluted using reduced glutathione (Sigma) and separated on SDS-PAGE and stained with Ponceau (0.1% (w/v) Ponceau S in 5%(v/v) acetic acid) for loading control. AFB/TIR1-myc proteins were detected by immunoblotting with anti-c-myc-Peroxidase antibody (Roche). Proteins were visualized using the ECL Plus Western Blotting Detection System (Amersham).

GUS Staining Assays

Seedlings were fixed in 90% acetone for 20 min followed by incubation in GUS staining solution (50mM sodium phosphate buffer pH 7, 0.1% Triton X-100, 10mM EDTA and 0.5mg/ml 5-bromo-4-chloro-3-indolyl-β-glucuronic acid in N,N-dimethylformamide) overnight at 37°C. Seedlings were cleared in 70% (v/v) ethanol and imaged using a Nikon SMZ1500 dissecting scope.

RNA extraction and quantitative PCR

Hypocotyl, cotyledon and root tissue frozen in liquid N_2 and ground using a mortar and pestle was used for RNA extractions using the INVITROGEN PureLink RNA minikit. RNA yield was quantified using the Thermo Scientific NanoDrop 2000. For quantitative RT-PCR, 1μg RNA, pre-treated with DNase using the DNA-free Kit (Ambion) according to manufacturer's instructions, was used for generating cDNA with SuperScript III First-Strand Synthesis (Invitrogen) and 20-mer oligo(dT) primers.

Quantitative RT-PCR was performed using SyBR green and the primers listed in the table below. Primer pairs were evaluated for specificity and efficiency using three serial dilutions of cDNA using the CFX96^{TM} Real-Time PCR Detection System (Biorad). Data was normalized to the reference primer PP2AA3 (Czechowski et al., 2005b) according to the $\Delta\Delta$Ct method. All other primers were designed using QuantPrime (Arvidsson et al., 2008b). Two biological replicates were performed, each replicate containing roughly 700 individual seedlings that were dissected into cotyledon, hypocotyl and root samples.

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Chapter 2, in full, consists of the manuscript “The AFB4 Auxin Receptor Is a Negative Regulator of Auxin Signaling in Seedlings” as published in Current Biology (2011) *21*, 1-6 and modified for consistency with the other chapters and with permission from all co-authors.

I was the primary researcher of the manuscript and performed the experiments described in figures 1B, 2A-D, 3A-F, 4B, D, 6, 7.

Table 2.1 Primer sequences for qRT-PCR

Annotation	AGI	5'->3' Primer Sequences (F/R)
TIR1	AT3G62980	ATCGCTGCCACTTGCAGGAATC TGGCCACTAACGTCGTCAACATC
AFB1	AT4G03190	GCTACTGTCCGAATGCCTGATCTTG GCCTTGTTCCGTCAGAGGTATGTTG
AFB2	AT3G26810	GCCGCTAATTGCAGGCATCTTC AGTCGTGCAAGTGTCTGGGAAAC
AFB3	AT1G12820	AGGTTGAAGCGGATGGTTGTAACAG GCAAGTCCAGCTCACGAAGATGC
AFB4	AT4G24390	TGCTCAAGCCCATCATAAGCAAC TCGAGTCAAGAGCCCAGAAGACTC
AFB5	AT5G49980	TGCCAACAAGTGCAGAAAGCTG TCCACTTCATCATCCGTGACCTC
ACS8	AT4G37770	TGGCCTTCCTTCCTTCAAGAATGC TGAGAGTCTCGTTAGCCGGAGTAG
IAA5	AT1G15580	TCCGCTCTGCAAATTCTGTTCG ACGATCCAAGGAACATTTCCCAAG
IAA19	AT3G15540	GGTGACAACTGCGAATACGTTACCA CCCGGTAGCATCCGATCTTTTCA
SAUR15	AT4G38850	TTGAGGAGTTTCTTGGGTGCTAAG GCCATGAATCCTCTTGGTGTCG
GH3.1	AT2G14960	CGATCGTCGCCAGCTTCTTTAC CCCGGCACATACAAATTCATTACG
GH3.3	AT2G23170	ACCGGAGATTCAACGTATTGCC CAGAGCTTGTGAGGAACTCTGTG
HAT2	AT5G47370	AACGTCGAGGAAGAAGCTCAGG AGCTTCTGTTTGGGATTGAGAGTG

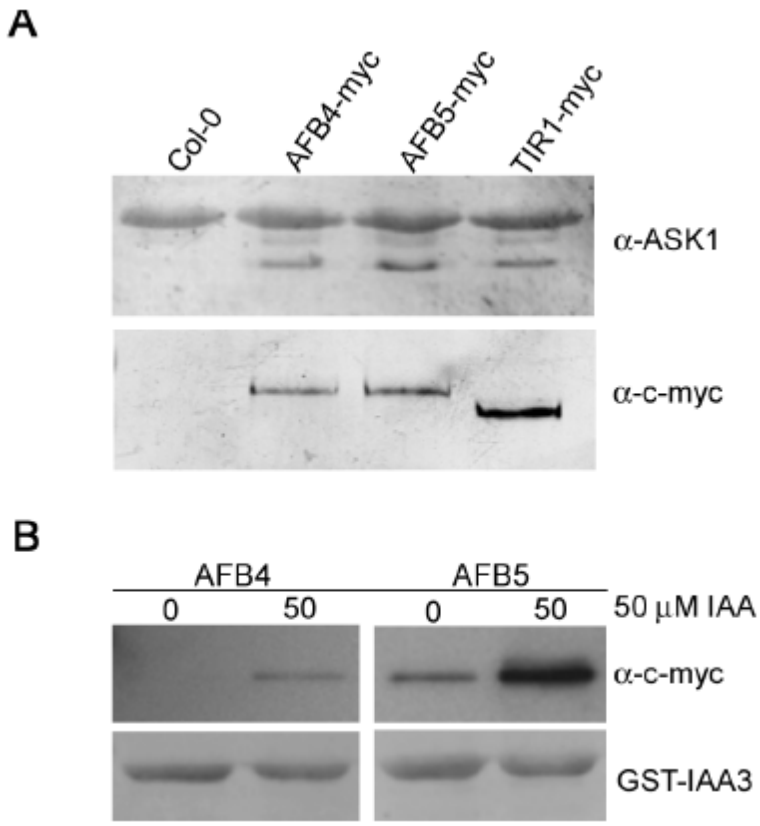


Figure 2.1 AFB4 and AFB5 interact with ASK1 and interact with the Aux/IAAs in an auxin dependent manor revealing their role as auxin receptors.

(A-B) Pull-down experiments were carried out using crude plant extracts prepared from [*tir1-1*] GVG-TIR1-*myc*, [*afb5-5*] *AFB5*-AFB5-*myc* and [*afb5-5*] *AFB5*-AFB4-*myc* seedlings and recombinant GST-IAA3. (A) TIR1-myc, AFB4-myc and AFB5-myc was immunoprecipitated with the anti-myc antibody coupled to agarose beads and ASK1 was detected with an anti-ASK1 antibody. (B) GST-IAA3 was immunoprecipitated with glutathione agarose beads, AFB4-myc and AFB5-myc protein was detected with the anti-c-myc-Peroxidase antibody. Pull-down reactions were incubated for 45min in the presence or absence of 50μM IAA.

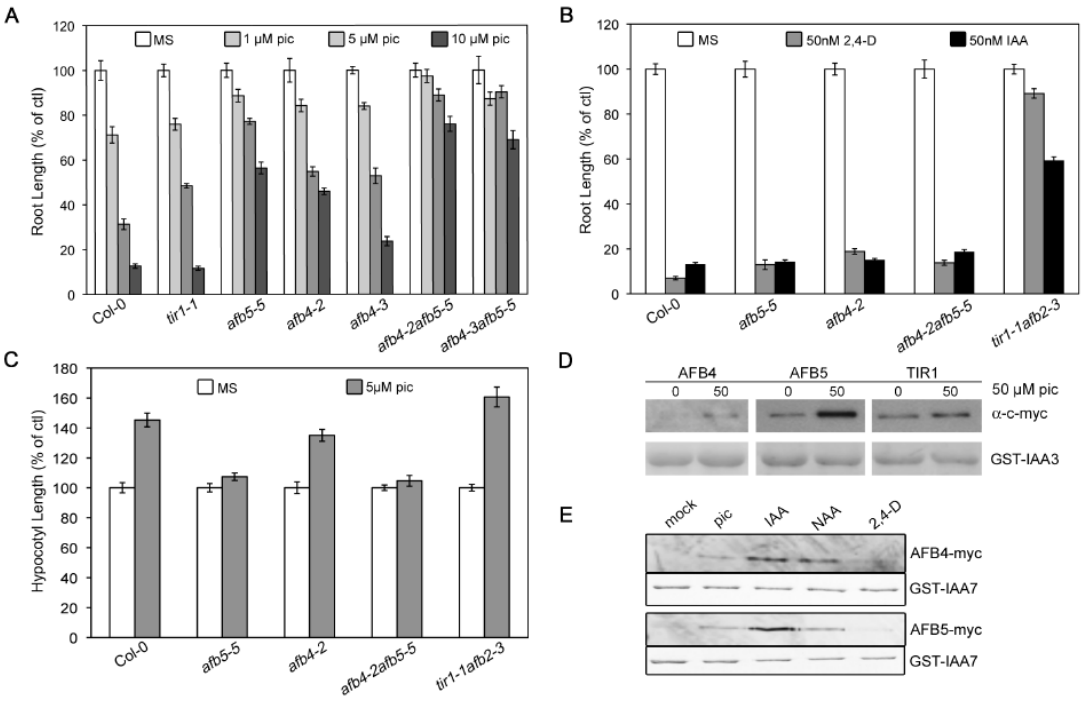


Figure 2.2 AFB4 and AFB5 are required for the picloram response. (A-B) Five-day-old LD grown WT and *tir1/afb* mutant seedlings grown on MS media were transferred to media containing 0, 1, 5 and 10μM picloram (A), 50nM 2,4-D or IAA (B) for an additional 3 days. (C) Four-day-old WT and *tir1/afb* grown seedlings were transferred to 5μM picloram for an additional 2 days. Root and hypocotyl length was expressed as a percent elongation based on the no auxin control growth. Error bars = SE. (D-E) Pull-down reactions were carried out as in figure 1 with 50μM of the indicated auxin.

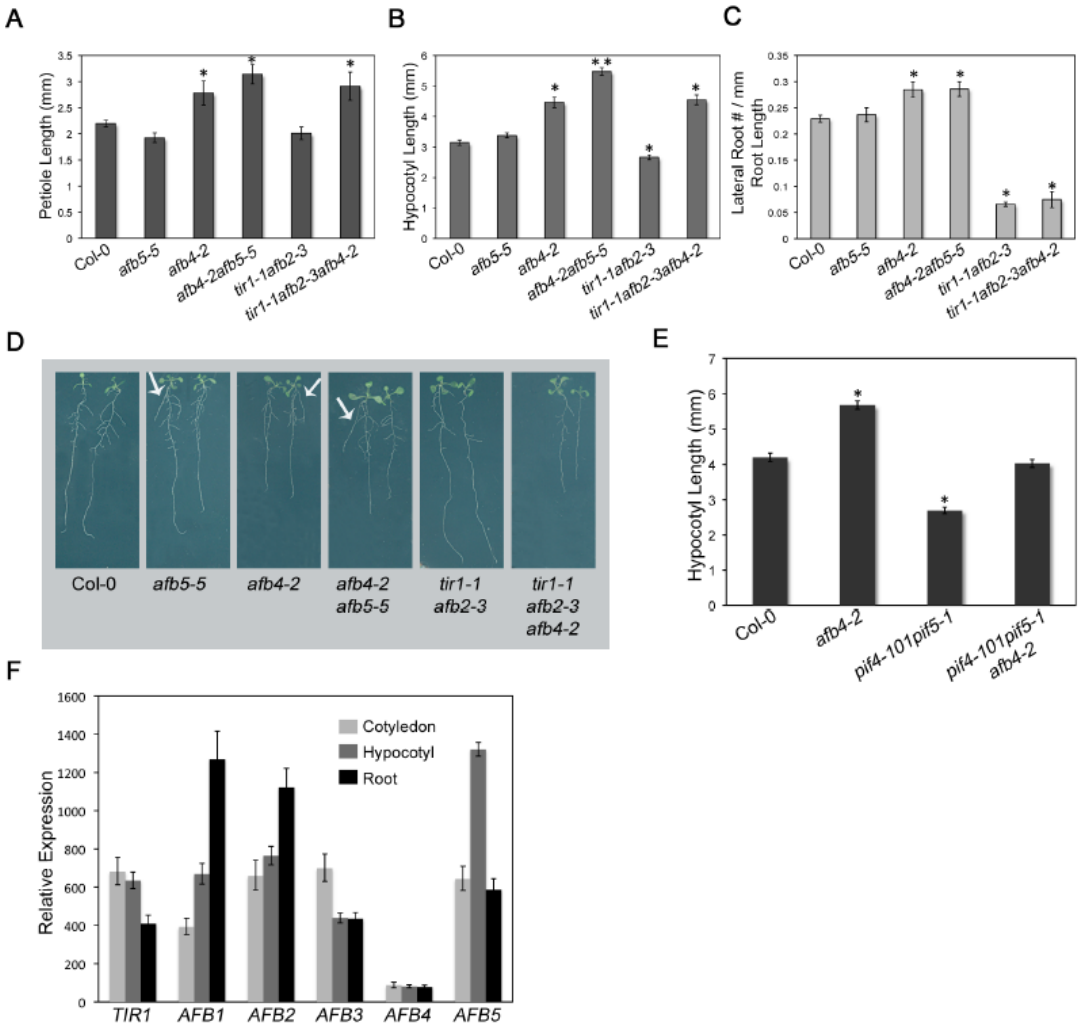


Figure 2.3 The *afb4-2* mutant shows stronger auxin related phenotypes than *afb5-5* and has much lower expression levels. Petiole (A) and hypocotyl (B) lengths of 6d old WT and *tir1/afb* mutant seedlings. Lateral root number (C) of 10d old WT and *tir1/afb* mutant seedlings grown under LD photoperiods. Images (D) of 7d old WT and *tir1/afb* mutant seedlings grown under LD photoperiods. Arrows point to lateral roots emerging from root-shoot junction. (E) Hypocotyl lengths of four-day-old WT and mutant seedlings grown at 22°C. Error bars represent SE. * represents significant difference compared to wildtype and ** represents significant difference compared to each other and wildtype (student's t-test $p<0.05$). (F) qRT-PCR of *TIR1, AFB4* and *AFB5* in hypocotyl tissue from 4d old WT seedlings.

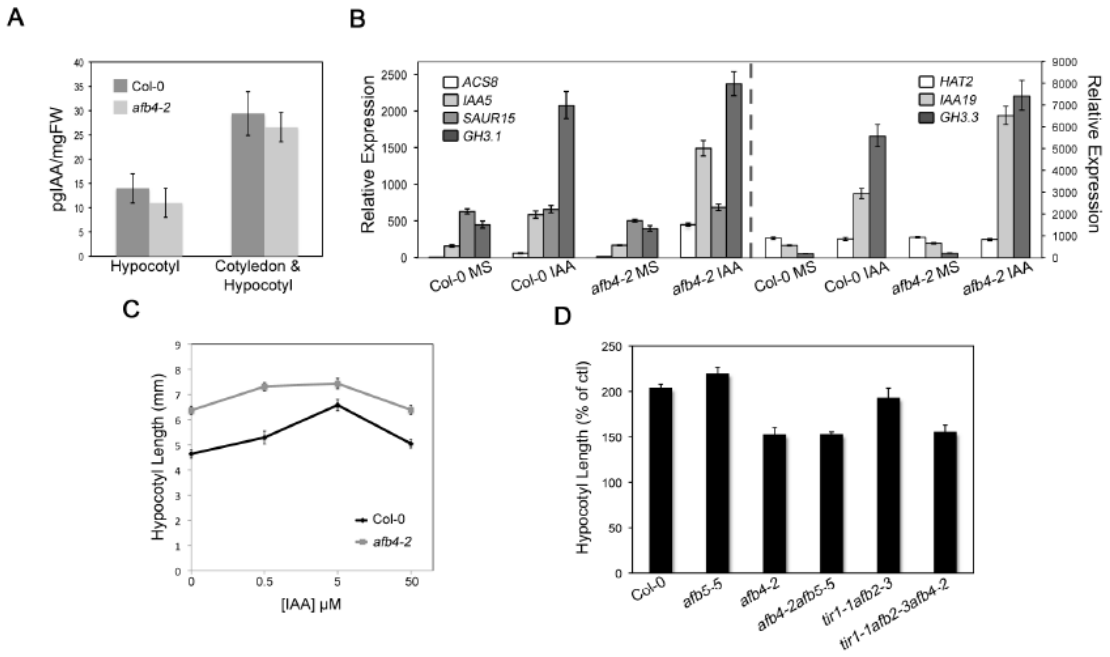


Figure 2.4 The *afb4-2* mutant is hypersensitive to endogenous IAA. (A) IAA measurements from 4-day-old dissected hypocotyls and cotyledon and hypocotyls combined. Error bars = SD. (B) qRT-PCR of IAA marker genes in hypocotyl tissue from 4-day-old WT and *afb4-2* mutant seedlings following a 2hr treatment with 1μM IAA. Expression values are normalized to the PP2AA3 reference gene (Czechowski et al., 2005a). (C) IAA dose response curve for wildtype and *afb4-2*. Seedlings grown for 4 days under a short day photoperiod were transferred to the indicated IAA concentration for 3 days. (D) Four-day-old WT and mutant seedlings were transferred to 29°C for 2 days. Control plates were maintained at 22°C. Hypocotyl length is expressed as a percent elongation based on the control plates. Error bars represent SE.

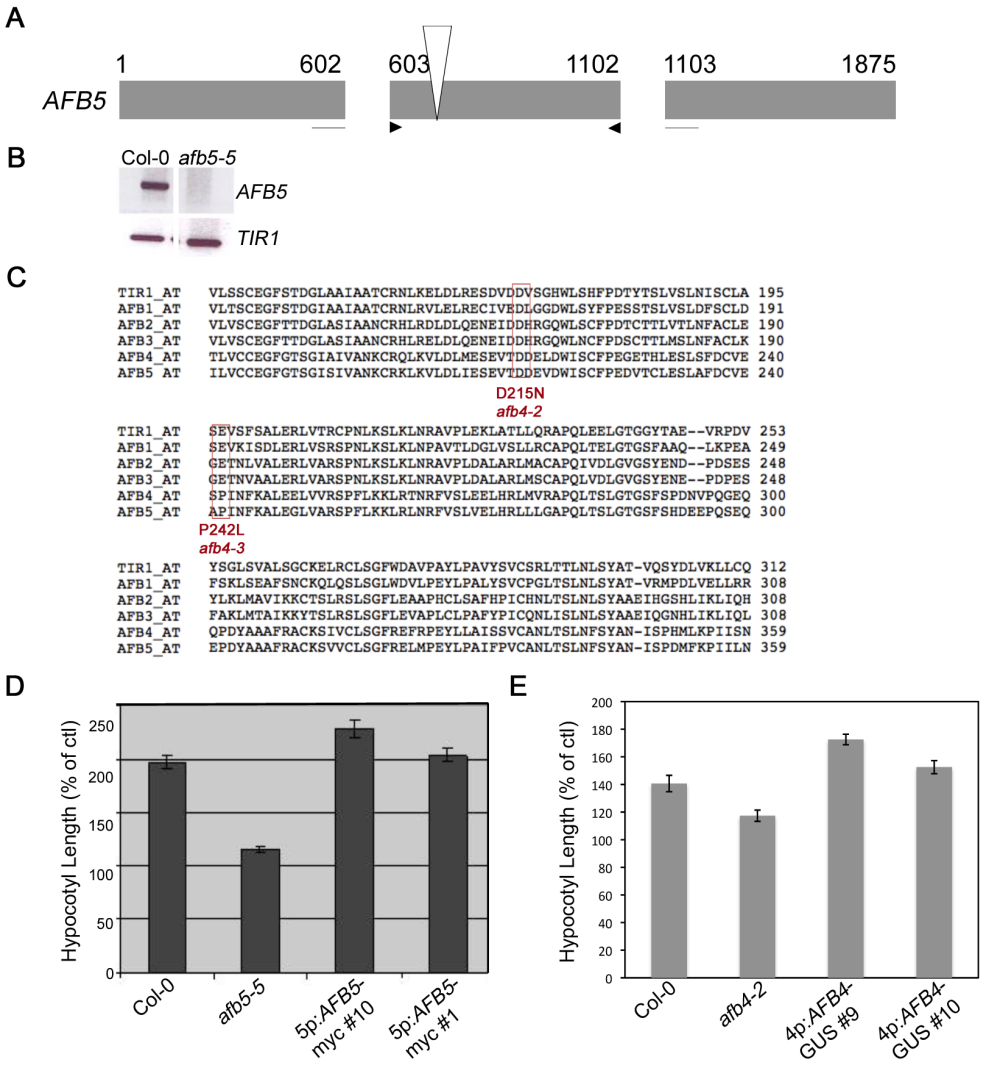
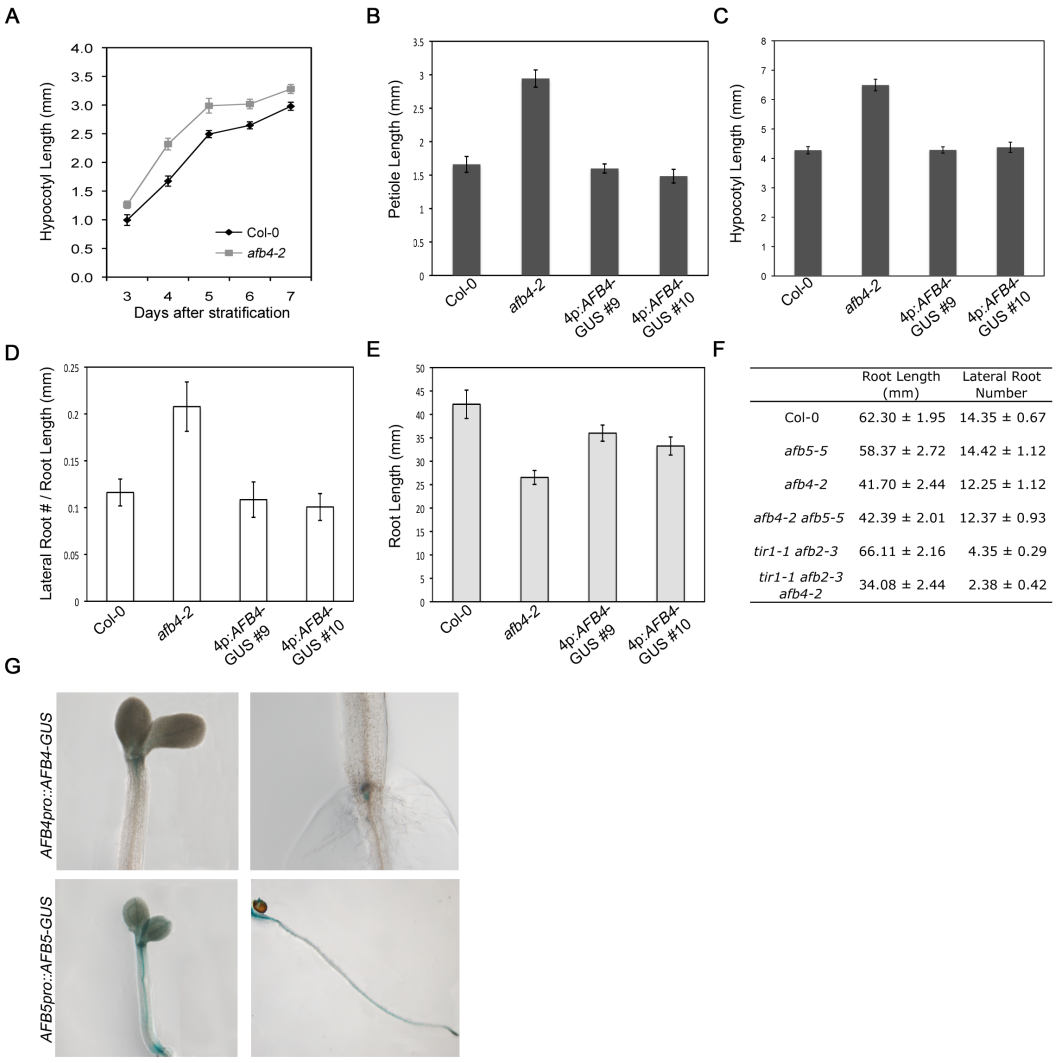


Figure 2.S1 The mutant alleles for *AFB4* and *AFB5.* (A) Diagram of the *AFB5* gene and T-DNA insertion site. Arrows indicate primer position for RT PCR. (B) RT PCR demonstrating loss of AFB5 transcript in *afb5-5*. Reactions with TIR1 primers were used as a control for afb5-5 cDNA integrity. (C) Alignment of TIR1/AFB proteins demonstrating *afb4* tilling line point mutation sites and their conservation among the family members. (D) Hypocotyl elongation assay on 5μM picloram demonstrating complementation of *afb5-5* with AFB5-myc expressed from the *AFB5* promoter. Error bars = SE. (E) Hypocotyl elongation assay on picloram demonstrating complementation of *afb4-2* following a cross with a line carrying the *AFB4:AFB4-GUS* construct. Seedlings were grown for 4 days and transferred to 5μM picloram for 2 days. Error bars = SE.



	Root Length (mm)	Lateral Root Number
Col-0	62.30 ± 1.95	14.35 ± 0.67
afb5-5	58.37 ± 2.72	14.42 ± 1.12
afb4-2	41.70 ± 2.44	12.25 ± 1.12
afb4-2 afb5-5	42.39 ± 2.01	12.37 ± 0.93
tir1-1 afb2-3	66.11 ± 2.16	4.35 ± 0.29
tir1-1 afb2-3 afb4-2	34.08 ± 2.44	2.38 ± 0.42

Figure 2.S2 The *afb4-2* mutation accounts for several auxin related defects in the developing seedling. (A) Hypocotyl growth assay with wild type and *afb4-2* seedlings beginning 3 days after stratification under short day photoperiods at 22°C. Hypocotyl length was measured for the next four days. Error bars = SE. (B-E) Complementation of *afb4-2* phenotypes with the line described in Figure S1E. Petiole (B) and hypocotyl length (C) of 6 day old seedlings. Lateral root number (D) and primary root length (E) of 10 day old seedlings grown under long day photoperiods. Error bars = SE. (F) Primary root length and lateral root number measurements used to calculate the lateral root #/mm root length shown in figure 3C. Mean length and lateral root number is represented with standard error. (G) *AFB4:AFB4-GUS* and *AFB5:AFB5-GUS* activity in 4 day old short day grown wild type seedlings.

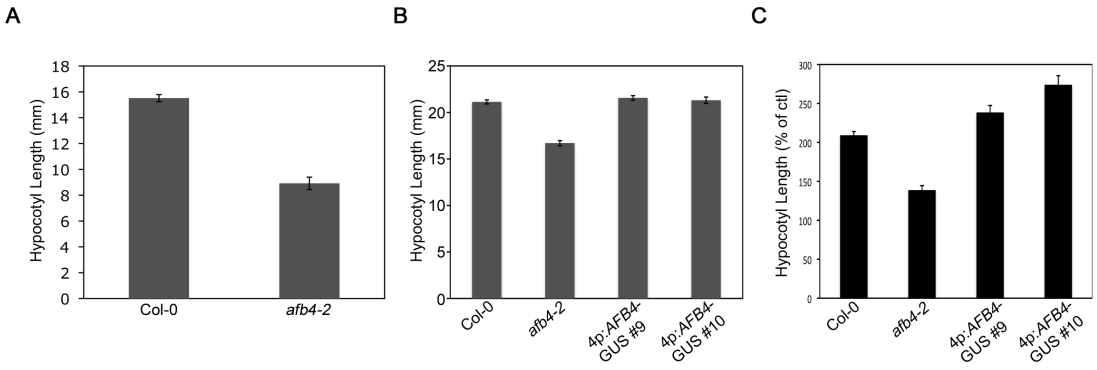


Figure 2.S3 Dark grown hypocotyl growth of *afb4-2.* (A) Hypocotyl length of wild type and *afb4-2* seedlings following 7 days in the dark. Seedlings were stratified for 2 days and subjected to 1hr of light before being shifted to constant dark conditions. Error bars = SE. (B) Complementation of the dark grown hypocotyl phenotype of *afb4-2* using the line described in Figure S1E. (C) Complementation of the 29°C induced hypocotyl elongation resistance of *afb4-2* using the line described in Figure S1E. Seedlings were grown for 4 days at 22°C and shifted to 29°C for an additional 2 days. Elongation is expressed as a percentage of the 22°C control.

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CHAPTER 3

Hypocotyl Transcriptome Reveals Auxin Regulation of Growth-Promoting Genes Through GA-Dependent and –Independent Pathways

REPRODUCTION OF THE MANUSCRIPT

Hypocotyl Transcriptome Reveals Auxin Regulation of Growth-Promoting Genes Through GA-Dependent and -Independent Pathways

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ABSTRACT

Many processes critical to plant growth and development are regulated by the hormone auxin. Auxin responses are initiated in part through activation of a transcriptional response mediated by the TIR1/AFB family of F-box protein auxin receptors as well as the AUX/IAA and ARF families of transcriptional regulators. To explore the role of auxin signaling pathways in the regulation of cell expansion and plant growth, we selected the Arabidopsis hypocotyl as a model system. By analyzing the behavior of a series of mutants, we show that auxin-mediated hypocotyl elongation is dependent upon the TIR1/AFB family of auxin receptors and degradation of AUX/IAA repressors. We present evidence from microarray- and

qPCR-based transcriptional profiling of elongating hypocotyls that gibberellin biosynthesis, cell wall reorganization and biogenesis, and other growth-associated processes are activated by auxin. We further show that GA biosynthesis and signaling are partially required for the cell expansion response to auxin and that the overall transcriptional auxin output preceding hypocotyl elongation consists of PIF-dependent and –independent genes whose expression correlates with growth. We propose that auxin acts independently from and interdependently with PIF and GA pathways to regulate expression of growth-associated genes in cell expansion.

AUTHOR SUMMARY

In growth and development, the hormone auxin is associated with a variety of specific processes such as cell identity determination, organ initiation, and tropism. In recent years a molecular mechanism for auxin action has emerged in which hormone-receptor interactions directly regulate the turnover of a family of transcriptional repressors and thereby control expression of auxin-responsive genes. We are using hypocotyls of Arabidopsis seedlings as a model to explore functions of the auxin-regulated transcriptome in cell expansion and plant growth. Here, we characterize the hypocotyl elongation response to auxin and identify auxin receptors and transcriptional regulators required for this growth response. We profile the transcriptome in elongating hypocotyls and present evidence that auxin promotes cell expansion by regulating expression of genes associated with growth. We further show that this regulatory effect occurs in part through gibberellin and in part through pathways independent of this second hormone. With these results we identify factors required for auxin perception, signal transduction, and transcriptional output in a specific auxin-mediated growth response. We also establish the hypocotyl as a model

system for further studies of hormone signaling in cell expansion, and elucidate striking parallels between the auxin-regulated and the growth-associated transcriptome.

INTRODUCTION

The hormone auxin (indole-3-acetic acid [IAA] and structurally similar compounds) plays a diverse role in plant growth and development including, but not limited to, embryogenesis, cell division and expansion, root initiation, tropic responses, apical dominance, flowering, and fruit and seed development (Davies, 2004). A major challenge in the field of auxin biology is to understand how a small molecule can specify such distinct changes in morphogenesis and growth throughout the life cycle of a plant. Current models suggest that auxin levels are highly regulated through changes in auxin biosynthesis, conjugation and storage, degradation, and polar transport. Auxin level is then interpreted by the auxin perception machinery resulting in tissue- and cell type-specific changes in gene expression (Chapman and Estelle, 2009; Leyser, 2010; Tromas and Perrot-Rechenmann, 2010).

Auxin regulation of transcription involves a large family (23 in Arabidopsis) of DNA-binding transcription factors called the AUXIN RESPONSE FACTORs (ARF) (Hagen and Guilfoyle, 2002; Overvoorde et al., 2005). ARFs bind to promoters of auxin-responsive genes at *cis*-elements referred to as auxin response elements (*AuxREs*) (Ulmasov et al., 1999; Tiwari et al., 2003). A TGTCTC sequence motif first identified in the auxin-responsive *GH3* promoter from soybean was shown to recruit multiple members of the *Arabidopsis* ARF family, with TGTC being absolutely required for ARF-DNA binding (Ulmasov et al., 1995). However, the TGTCTC element is not found in all auxin-responsive promoters. In some cases tandem

repeats of the TGTC portion of the *AuxRE* are sufficient for auxin induction (Nemhauser et al., 2004; Muller and Sheen, 2008). ARF proteins are characterized by a B3-like DNA binding domain, a middle region associated with transcriptional repression or activation, and a C-terminal domain (CTD) involved in homo- and hetero-dimerization (Ulmasov et al., 1999; Tiwari et al., 2003; Chapman and Estelle, 2009). The CTD region is similar to the C-terminal domains III and IV of the Aux/IAA transcriptional regulators (Reed, 2001).

The Aux/IAAs are a 29 member family of small nuclear proteins in *Arabidopsis* that are involved in repressing auxin-regulated transcription (Berleth et al., 2004). Aux/IAA proteins contain four conserved domains (I-IV), of which domains I, II and IV contain nuclear localization motifs. Domain III contains a sequence that is related to the βαα DNA binding domain that is required for Aux/IAA homo- and hetero-dimerization. However, there is currently no evidence that Aux/IAA proteins bind DNA directly (Abel et al., 1994; Kim et al., 1997). Rather, Aux/IAAs are recruited to promoters through interactions with ARF proteins that are mediated by domains III and IV of the two proteins. Domain II of Aux/IAAs is highly conserved and contains a degron motif that is important for degradation by the SCF^{TIR1} E3 ubiquitin ligase complex (Ramos et al., 2001; Reed, 2001). Mutations in this degron result in stabilization of the protein and reduced auxin response, causing various defects in growth and development (Ramos et al., 2001; Overvoorde et al., 2005; Sato and Yamamoto, 2008).

Functional redundancies within the ARF and Aux/IAA gene families make assigning specific roles of each protein a challenge. However, genetic studies have revealed ARF and Aux/IAA combinations that are essential for certain processes. BDL/IAA12 and MP/ARF5 specify apical-basal polarity during embryogenesis

(Hamann et al., 2002), SLR/IAA14 and NPH4/ARF7 are required for lateral root initiation, and MSG2/IAA19 and NPH4/ARF7 are involved in tropic hypocotyl growth (Okushima et al., 2005). ARF2, ARF8, and ARF19 are involved in root and hypocotyl growth and development, although Aux/IAA partners in these processes are not clear (Li et al., 2004; Tian et al., 2004; Okushima et al., 2007). Recently, the apical-basal polarity determinant TOPLESS (TPL) was shown to act as a transcriptional co-repressor with IAA12/BDL to repress ARF5/MP transcriptional activity (Szemenyei et al., 2008). It has yet to be seen whether all the Aux/IAAs interact with TPL to repress the auxin response in specific developmental pathways.

Auxin exerts changes in gene expression by interacting with the TIR1/AFB family of auxin receptors. These proteins are the F-box protein subunits of SCF (Skp1/Cullin/F-box) complexes that target the Aux/IAAs for proteasome-mediated degradation (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b; Kepinski and Leyser, 2005). The *Arabidopsis* genome encodes 5 proteins related to TIR1, Auxin Signaling F-Box (AFB) proteins AFB1, 2, 3, 4 and 5. Previous work has shown that, like TIR1, AFB1-5 function as auxin receptors that interact with Aux/IAA repressors in an auxin-dependent manner (Dharmasiri et al., 2005b; Greenham et al., 2011). Mutant analysis reveals overlapping functions of TIR1/AFB1-3. The most severely affected *tir1 afb1 afb2 afb3* quadruple mutants arrest shortly after germination (Dharmasiri et al., 2005b). The AFB4 clade of receptors, including AFB4 and AFB5, display a unique affinity for the synthetic auxin picloram. The *afb5-5* single mutant shows almost complete resistance to picloram-induced hypocotyl growth (Greenham et al., 2011).

In order to develop successful models for auxin regulation of growth and development, it will be important to identify the gene targets of the TIR1/AFB

pathway(s) and understand their function in cell growth. Several studies of auxin-responsive transcriptomes have identified large numbers of candidate auxin targets. The results of supporting genetic studies ascribe developmental roles to a small number of these (De Rybel et al., 2010). A potential barrier to identification of distinct auxin pathways from such studies lies in the complexity of the tissue sampled for the experiment. Auxin mediates distinct responses in different tissue types, for example inhibiting primary root elongation while stimulating lateral root initiation and outgrowth (Scott, 1972). Therefore, auxin-responsive transcriptomes in entire plants are too complex to facilitate separation of distinct developmental pathways.

In this study we focus on the role of auxin signaling in cell expansion. We chose the hypocotyl, which grows entirely by cell expansion, as a model tissue for this study (Gendreau et al., 1997). The hypocotyl elongates in plants overexpressing auxin biosynthetic genes (Zhao et al., 2002) and in response to high temperature (Gray et al., 1998), due to elevated auxin levels. Hypocotyl elongation is tightly regulated and many signaling pathways overlap to regulate uniform, as well as directional, hypocotyl cell expansion. Light is a major repressor of hypocotyl growth and as a consequence, mutations in the phytochrome light receptors result in seedlings with long hypocotyl phenotypes (Shin et al., 2009). Light-activated forms of the phytochromes interact with members of the phytochrome-interacting factor (PIF) family of bHLH transcription factors, signaling rapid phyA- and phyB-mediated degradation of PIF,3,4 and 5 in the light (Bauer et al., 2004; Al-Sady et al., 2008; Lorrain et al., 2008). PIFs have also recently been shown to function in GA signaling (de Lucas et al., 2008). The PIFs appear to be the major positive regulators of hypocotyl growth, as they are required for growth responses to time of day, direction of light source, nutrients, high temperature and other stimuli (Kunihiro et al., 2011;

Nozue et al., 2007; Lucyshyn and Wigge, 2009; Koini et al., 2009)). PIF mRNA and protein levels are controlled by the circadian clock, light, and GA signaling, such that PIF activities and hypocotyl growth are repressed during the day (Nozue et al., 2007; Niwa et al., 2009; de Lucas et al., 2008). Within the PIF family, several *PIF* and *PIF-LIKE (PIL)* genes are implicated in germination and early seedling growth (Duek and Fankhauser, 2005). PIF4 and PIF5 seem to be particularly important for hypocotyl growth as expression of these factors is circadian regulated and correlates with hypocotyl growth (Nozue et al., 2007; Niwa et al., 2009). In addition the *pif4pif5* double mutant has a short hypocotyl phenotype (Lorrain et al., 2008).

Here we outline the auxin signaling pathways required for auxin-responsive hypocotyl elongation and present a series of hypocotyl transcriptome experiments designed to explore the mechanism by which auxin induces cell expansion. We demonstrate the benefits of using hypocotyl tissue compared to whole seedlings for microarray experiments to address the role of auxin in regulation of cell expansion. Our findings indicate that auxin-induced hypocotyl elongation is associated with regulation of a suite of growth-associated genes and involves GA biosynthesis. However we also show that auxin works in part through pathways independent from GA and PIF activities.

RESULTS AND DISCUSSION

Auxin promotes elongation of Arabidopsis hypocotyls

To explore the function of auxin in plant growth, we selected the *Arabidopsis* hypocotyl and developed a robust assay for auxin response in this system. Seedlings were grown at 22°C for 5 days in various day-night cycles, exposed to auxin or other compounds, and measured after different treatment times. Under these

conditions, auxin promoted hypocotyl growth in continuous light (LL), long days (LD), or short days (SD) (Fig 3.1A). The synthetic auxin picloram elicited a growth response similar to that observed with IAA, consistent with previous reports (Fig 3.1B) (Walsh et al., 2006; Greenham et al., 2011). Elongation was clearly visible following 24 hours of auxin exposure and continued for at least 72 hours (Fig 3.1B). Unless otherwise stated, LD conditions were used for additional experiments designed to characterize auxin-responsive hypocotyl growth (Fig 3.1C).

Auxin-mediated hypocotyl elongation requires transcriptional auxin signaling

To confirm that the auxin-dependent elongation response requires activation of transcriptional auxin signaling pathways, we measured the response in a series of *Aux/IAA* gain-of-function mutants in which auxin-regulated transcription is repressed (Tatematsu et al., 2004; Fukaki et al., 2002; Timpte et al., 1994; Yang et al., 2004; Wilson et al., 1990; Tian and Reed, 1999). In *slr-1/iaa14, axr2-1/iaa7, axr5-1/iaa1* and *shy2-2/iaa3,* the auxin response was reduced compared to wild-type plants (Fig 3.1D). Interestingly, the response of *msg2-1/iaa19* seedlings was similar to wild type, even though this mutant is deficient in differential growth in the hypocotyl. This suggests that different auxin signaling pathways have specific roles in hypocotyl growth. This has been shown for apical-basal polarity determination (Hamann et al., 2002) and lateral root initiation (Okushima et al., 2007).

We explored the possibility of functional specialization among the TIR1/AFB auxin receptors in hypocotyl elongation by analyzing the phenotypes of various *tir1/afb* mutants. We observed slight auxin resistance or hypersensitivity in single *tir1/afb* receptor mutants (Fig 3.2A,B) with the exception of *afb5-1* and *afb4-2 afb5-5,* which are highly resistant to picloram (Greenham et al., 2011). The basis for auxin

hypersensitivity in *afb1-3* and *afb3-4* mutants is unclear, however, double mutant combinations among *afb1-3, afb2-3,* and *afb3-4* eliminated this hypersensitivity (Fig 3.2C), suggesting that increased growth response may be due to enhanced activity of other TIR1/AFB family members that is lost in the higher order mutants. Future analysis of the expression patterns of the receptors in the single and double mutant backgrounds will be necessary to address this hypersensitivity.

Double and triple mutants carrying *tir1-1* each displayed increased auxin resistance when compared to the *tir1-1* mutant (Fig 3.2D). The triple mutant *tir1-1 afb2-3 afb3-4* displays an incompletely penetrant phenotype in which a significant percentage of individuals fail to develop basal structures such as roots and hypocotyls (Dharmasiri et al., 2005b). In *tir1-1 afb2-3 afb3-4* individuals with developed basal structures, hypocotyls were shorter than those of wild-type plants and displayed the highest degree of resistance to IAA-mediated elongation of all *tir1/afb* receptor mutants (Fig 3.2D). The reliance of the elongation response on the TIR1/AFB auxin receptors and degradation of Aux/IAA proteins confirms that auxin mediated growth requires transcriptional auxin signaling pathways. Importantly, in our conditions the hypocotyl growth response of wild type seedlings forms a bell-shaped curve, which differentiates our growth conditions from those in which auxin treatment or constitutive auxin signaling inhibits hypocotyl elongation, such as growth in the dark, in yellow light, or at elevated temperatures (Collett et al., 2000; Harper et al., 2000; Tian et al., 2004).

Identification of auxin-responsive cell expansion-associated genes in elongating hypocotyls

Based on our findings that auxin-mediated hypocotyl elongation requires transcriptional auxin signaling, we hypothesized that elongation is preceded by changes in expression of a suite of auxin-responsive genes. To identify such genes, we profiled auxin-responsive transcription in hypocotyls in a series of microarray experiments. We incorporated several parameters into our microarray design to maximize the likelihood of identifying auxin-regulated genes associated with anisotropic cell expansion. To enrich our dataset for cell expansion genes that may not be uncovered in whole seedling experiments, we sampled hypocotyl tissue dissected from auxin- or control-treated whole seedlings. To minimize time-of-day and circadian effects and avoid mis-identification of auxin-responsive genes, we treated experimental and control seedlings at the same time of day and limited the dissection time to 30 minutes. To maximize the amplitude of the transcriptional auxin response, we treated seedlings two hours after subjective dawn, when hypocotyl growth is minimal in the absence of exogenous auxin (Dowson-Day and Millar, 1999). Finally, we used the synthetic auxin picloram and included the *afb5-5* mutant in our microarray design, as this mutant is picloram-resistant but does not otherwise show overt phenotypes (Walsh et al., 2006; Greenham et al., 2011). We theorized that cell expansion-associated genes differentially expressed in wild-type hypocotyls elongating in response to picloram might not be responsive in *afb5-5* hypocotyls, which fail to elongate in response to picloram.

For microarray experiment “a”, we sampled hypocotyls from wild-type plants treated for 30 minutes or 2 hours with picloram or a solvent-only control. For experiment “b”, we sampled hypocotyls from wild-type or *afb5-5* mutant plants treated

for 2 hours with picloram or a solvent-only control (Fig 3.3A). Following auxin or control treatment of seedlings, hypocotyls were individually dissected and frozen for subsequent RNA isolation.

To identify genes differentially expressed among the treatments, we used a moderated linear model (Smyth, 2004) and an FDR cutoff of <0.05 to filter data from each microarray experiment. From this initial analysis we identified 65 genes differentially expressed following the 30-minute auxin treatment, and 3544 (experiment "a") or 804 (experiment "b") genes differentially expressed following a 2-hour auxin treatment (Fig 3.3A). Consistent with the picloram-resistant phenotype of *afb5-5*, no differential expression was detected in *afb5-5* following picloram treatment using the analysis method described. Interestingly, we were also unable to identify genes differentially expressed between wild-type and *afb5-5* untreated samples (Fig 3.3A). So far, picloram perception and regulation of picloram-responsive transcription is the only known function of the AFB5 auxin receptor. The identification of additional functions for AFB5 will require alternative experimental approaches. Analysis of genes differentially expressed following 30 minutes of picloram treatment indicated that *SAUR* genes, *AUX/IAA* genes, *GH3* genes and others shown elsewhere to be early auxin-responsive (Paponov et al., 2008) were induced by picloram and were the predominant genes to be regulated at this time-point. For additional insight into gene expression associated with auxin response, we focused on data from the 2-hour time-point samples.

Comparison of gene lists from the 2-hour auxin treatment in experiments "a" and "b" identified 267 genes differentially expressed in both experiments. This modest overlap may be due to experimental variables such as differences in RNA extraction methods and microarray hybridization parameters, or perhaps more importantly, to

'lab-effects' such as those previously shown to serve as a source of variability among microarray experiments performed on the same platform at different laboratories (Vert et al., 2005)(see Methods). To increase the validity and statistical strength of the comparison we used the RankProd package in R that accepts pre-processed data generated from different laboratories and platforms (Hong et al., 2006). This package is an extension of the rank product method that implements a non-parametric statistic to compare the expression-based rankings of genes across samples (Breitling et al., 2004). From this analysis, we identified 1193 genes differentially expressed between control and 2 hour auxin-treated samples; 740 of these genes are induced, and 453 are repressed by picloram. The mean expression levels of these two gene sets in microarray "b" are not affected by picloram treatment of *afb5-5* mutant plants (Fig 3.3B), suggesting that these are indeed downstream targets of picloram-stimulated transcriptional auxin signaling. The failure of *afb5-5* to regulate this set of genes or to elongate in response to picloram is consistent with a model in which these genes are targets of auxin signaling and are involved in the elongation response. We focused on this set of 1193 candidate auxin-responsive cell expansion-associated genes for all additional experiments.

Picloram and IAA regulate a common set of target genes

The synthetic auxin picloram induces a hypocotyl elongation response similar to that observed with IAA, suggesting that the downstream targets of picloram- and IAA-stimulated auxin signaling are common between these two auxin pathways. To confirm this, we performed a comprehensive comparison between our auxin-responsive gene set and publicly available microarray data. Our first comparison was done using the MASTA package available from the BAR website

(http://bar.utoronto.ca/welcome.htm) that probes differentially expressed genes against a database of 600 contrasts obtained from publicly available microarray datasets. Of the 740 genes upregulated by picloram in our dataset, 219 were identified as auxin-upregulated in IAA treatment arrays; of 453 genes downregulated by picloram in our dataset, 121 genes were identified as auxin-downregulated in IAA arrays (data not shown). These overlaps are statistically significant (p.value <0.001) and confirm that picloram affects known IAA-responsive genes. We also performed independent comparisons with the Nemhauser *et al.* (Nemhauser et al., 2006) and Stepanova *et al*. (Stepanova et al., 2007) auxin treatment datasets (see Methods for details of comparison). In both cases, more than 50% of the IAA-induced genes were induced by picloram in our experiments (Fig 3.4A). The Stepanova *et al.* (Stepanova et al., 2007) dataset was obtained from experiments using root tissue suggesting that many of the genes involved in hypocotyl growth are common to root tissue. We would expect these genes to be specifically involved in cell elongation during root growth.

To further validate the effects of picloram on auxin-responsive genes, we confirmed that a set of auxin "marker" genes, proposed to serve as hallmarks of auxin activity (Nemhauser et al., 2006), were identified as picloram-responsive in our microarray data analysis. Overall, expression of the marker genes was responsive to picloram in wild-type hypocotyls, but not in hypocotyls from *afb5-5* mutant plants (Fig 3.S1A). We further validated the picloram response of several of these genes, *GH3.3, GH3.5, HAT2, IAA5, IAA19 and SAUR15*, by quantitative RT-PCR using wild-type and *afb5-5* hypocotyls. Expression of each gene was induced in wild-type hypocotyls by picloram treatment, and induction was dependent upon AFB5 (Fig 3.4B). This indicates that picloram and IAA regulate an overlapping set of target genes, although the picloram signal is uniquely transduced by AFB5.

Finally, we analyzed our picloram-responsive gene set for association with auxin Gene Ontology terms and overrepresentation of *AuxRE*-containing promoter elements. GO terms associated with auxin response and hormone signaling are enriched in the annotations of our auxin-responsive gene set, and we identified several overrepresented *AuxRE*-containing promoter elements in the promoter gene set (Fig 3.S1B). From these results we conclude that picloram regulates the same downstream transcriptional targets as IAA, and therefore promotes hypocotyl elongation through the same transcriptional pathways as IAA. For the remaining experiments, we used picloram and IAA interchangeably or in parallel, and we did not observe qualitative differences in responses to these two auxins.

A profile of the transcriptional auxin response preceding hypocotyl elongation

Further examination of GO terms associated with our auxin-responsive gene set revealed overrepresentation of genes involved in cell wall maintenance, cell expansion, growth and hormone signaling (Fig 3.5A, Fig 3.S2). Enriched GO terms associated with the auxin-induced gene set included cell wall metabolism and gibberellin biosynthesis. Terms associated with the auxin-repressed gene set included carbohydrate metabolism and plastoquinone assembly (Fig 3.5A). Representation of these GO processes in our auxin-responsive gene set is consistent with a role for auxin in transcriptional activation of cell expansion-associated genes. Cell expansion in the hypocotyl, as well as in other growing plant tissues, is gated by the circadian clock and shows non-uniform patterns across a 24-hour period (Michael et al., 2008; Dowson-Day and Millar, 1999; Nozue et al., 2007). This is likely due in part to circadian patterns of expression of many genes involved in auxin signaling, biosynthesis and transport, and varying sensitivity to auxin at different times of day

(Covington and Harmer, 2007). We theorized that genes we found to be auxin-responsive in elongating hypocotyls might follow circadian expression patterns. To determine whether circadian-regulated genes are overrepresented in our auxin-responsive gene set, we generated a gene subset consisting of the top 400 auxin-induced genes according to statistical significance, and analyzed this subset using the Phaser tool (http://phaser.cgrb.oregonstate.edu/)(Michael et al., 2008). We observed significant enrichment of genes showing peak expression during phases 0-2 and 22-23, during which hypocotyl growth is active (Fig 3.5B) (Michael et al., 2008). We further explored our auxin-induced gene set for additional determinants of expression profile by analyzing the corresponding promoter set for overrepresented regulatory elements. Interestingly, the predicted MYC/MYB binding site 'CACATG' was the most highly overrepresented element identified in this analysis (data not shown). The 'CACATG' element was previously identified as the Hormone Up at Dawn (HUD) element enriched in promoters of genes responsive to phytohormones and showing peak expression levels during periods of growth (Michael et al., 2008). Together, these findings suggest that auxin promotes hypocotyl growth by regulating expression of cell expansion-associated genes whose expression levels are controlled by the circadian clock. This is consistent with auxin gating by the clock to maintain the diurnal pattern of hypocotyl elongation under normal growth conditions (Covington and Harmer, 2007).

Auxin-mediated hypocotyl elongation requires GA signaling

Among the genes we identified in our microarray analysis, we noted that *GA20OX1*, *GA20OX2*, *GA2OX8*, and *GA3OX1*, GA oxidases that either promote (*GA3OX* and *GA20OX* families) or diminish (*GA2OX* family) accumulation of bioactive

gibberellins, were present. This was particularly interesting to us due to the established role of gibberellins in cell expansion and plant growth (Davies, 2004), the role of the PIFs in hypocotyl elongation (de Lucas et al., 2008) and GA signal transduction (de Lucas et al., 2008), and the identification of GA oxidases as primary targets of auxin signaling (Frigerio et al., 2006). We therefore selected GA oxidases as representatives of our auxin-responsive gene set and further explored the role of GA in auxin-responsive growth. To address the role of GA biosynthesis in auxin response, we tested the effect of adding paclobutrazol, a GA biosynthesis inhibitor, to hypocotyl elongation assays. Paclobutrazol inhibited the effects of exogenous auxin in our system, as co-treatment with paclobutrazol attenuated, but did not abolish, the hypocotyl elongation promoted by picloram (Fig 3.6A) or IAA (Fig 3.6B). This suggests that active GA biosynthesis is required for optimal hypocotyl auxin response. We asked whether auxin regulates GA signaling by testing the effects of exogenous auxin on stability of the DELLA protein RGA, a repressor of GA signaling (Dill et al., 2001). Treatment of seedlings expressing RGA-GFP with IAA or GA resulted in loss of RGA protein from hypocotyl cells within 2 hours (Fig 3.6C). This auxin effect was abolished by co-treatment with paclobutrazol (Fig 3.6C). While it is possible that the observed loss of RGA protein in auxin-treated seedlings is due to an effect of auxin on transcription of *RGA*, we think this is unlikely, as we did not identify *RGA* as an auxin-downregulated gene in our microarray experiments (although we did identify *RGA-LIKE1* (AT1G66350) and *RGA-LIKE3* (AT5G17490) as auxin-upregulated genes). A more likely possibility is that auxin regulation of GA levels results in degradation of RGA-GFP protein in the seedlings.

We further explored the requirement for GA biosynthesis and signaling in auxin response by examining the behavior of various GA mutants in the hypocotyl

elongation assay. Plants compromised in endogenous GA levels due to mutations in *GA20OX1* and *GA20OX2* showed partial auxin resistance (Fig 3.6D). Plants deficient in GA perception (*gid1a, gid1b, gid1c*) or signaling (*gai* and *pif4pif5*) were also partially resistant to auxin treatment (Fig 3.6D). These data indicate that GA synthesis and signaling are required for optimal auxin response. A quintuple mutant for five DELLA protein repressors of GA signaling (*della5*) has a long hypocotyl phenotype due to constitutive activation of GA signaling. Interestingly, this phenotype was partially suppressed by treatment with the auxin transport inhibitor NPA (Fig 3.S3A), suggesting that the *della5* phenotype is partly due to the elongation-promoting effects of auxin in the hypocotyl. Elongation in response to auxin treatment was additive to the elongation response to GA, even at high exogenous GA concentrations (Fig 3.S3B). However, the GA response required endogenous auxin as it was partially inhibited by co-treatment with the auxin transport inhibitor NPA (Fig 3.S3C). These data suggest that auxin and GA act interdependently in hypocotyl cell expansion.

We did not observe complete auxin resistance in any of the GA mutants, in fact the response of the *rga-∆17* mutant was similar to the response in wild-type plants. This indicates that plants deficient in GA signaling can respond to auxin, albeit at a reduced level, and suggests that while GA contributes to the auxin response, auxin promotes elongation in part through a pathway independent of GA. In hormone treatment assays, paclobutrazol did not completely abolish the auxin effect (Fig 3.6B), again suggesting that the elongation-promoting effects of auxin are not limited to regulation of GA pathways. We propose that auxin promotes hypocotyl growth in part through GA and in part through an unknown independent pathway(s). The short hypocotyl phenotypes of several gain-of-function Aux/IAA mutants are only partially restored by treatment with GA (Fig 3.S3D), which indicates that constitutive

repression of auxin signaling in the Aux/IAA mutants serves to repress not only the auxin-GA crosstalk but also unknown auxin-regulated growth pathways. A mechanism by which auxin can induce hypocotyl growth independently of GA synthesis may be important for rapid growth responses.

It is important to note that while the results of our paclobutrazol experiments are consistent with a model in which auxin stimulates synthesis of GA, which then contributes to the elongation response, this may be an oversimplification. GA levels are under negative feedback regulation in which expression of GA biosynthesis genes is repressed as GA levels increase (Hedden and Kamiya, 1997). This feedback loop is perturbed in GA signaling mutants such as *gai-1* such that active GA accumulates to higher levels than found in wild-type plants (Talon et al., 1990). The effect of exogenous auxin on such mutants may be difficult to predict, as high levels of exogenous auxin lost efficacy for stimulating hypocotyl elongation as GA concentration increased (Fig 3.S3B). Indeed, we observed that the *gai* and *gid 1a* +/+ mutants elongated less on exogenous auxin than their untreated controls (Fig 3.6D). It is clear from the results of our experiments, however, that auxin and GA pathways interplay in the control of hypocotyl elongation during the day.

Auxin promotes cell expansion independent of time of day in part through regulation of PIF-independent pathways

As previously mentioned, several signaling pathways are important for controlling hypocotyl growth, including light signaling and the circadian clock, as well as hormone signaling (Nozue et al., 2007; de Lucas et al., 2008; Niwa et al., 2009). Many of the growth-associated downstream genes in these pathways are regulated by PIF transcription factors (Nozue et al., 2007; de Lucas et al., 2008; Niwa et al.,

2009), recently shown to be required for activation of transcription downstream of GA signaling (Nozue et al., 2007; de Lucas et al., 2008; Niwa et al., 2009). PIF4 and PIF5 are two members of the PIF family that are circadian regulated and for which expression level is correlated with hypocotyl growth (Nozue et al., 2007; Niwa et al., 2009; Nozue et al., 2011). A recent study by Nozue *et al.* (Nozue et al., 2011) suggests that PIF5 is a modulator of auxin signaling and that PIF4 and PIF5 regulate auxin sensitivity to control hypocotyl growth.

There are several possible mechanisms by which transcriptional auxin signaling may promote growth either by feeding into a PIF-mediated pathway or acting independently. First, auxin might promote PIF activity by inducing *PIF* transcription during the day; second, auxin might indirectly promote PIF activity by stimulating GA synthesis consequently degrading the DELLA repressors of the PIFs; third, auxin might act independently of the PIFs and regulate transcription of PIF targets during the day; last, auxin might act independently of the PIFs and regulate PIF-independent growth genes. We addressed the first possibility by analyzing our microarray data. We did not detect a transcriptional response to auxin for the *PIF4/5* genes, suggesting that auxin either enhances residual PIF activity that may be present during the day, or acts in parallel to promote elongation independently of these proteins.

We asked whether PIF4/5 are required for the initial growth response to a pulse of auxin using a time course elongation assay done during the day. A 2-hour auxin treatment led to an increase in hypocotyl length in wild-type seedlings within 2 hours (Fig 3.7). The response of *pif4pif5* mutant seedlings was indistinguishable from that of wild type, suggesting that this initial growth response does not require PIF4/5 protein. This result is not surprising given that PIF4 and PIF5 are rapidly degraded

by a phyb-dependent mechanism and transcriptionally inhibited by the DELLAs during the day, and so are unlikely to be required for daytime growth (de Lucas et al., 2008).

To address whether the transcriptional targets of auxin signaling are also PIF targets, we performed an extensive comparison of our auxin-responsive cell expansion data set with existing growth-related microarray datasets. Nozue *et al.* (Nozue et al., 2011) describe a series of global expression analyses in the *pif4pif5* mutant to classify sets of "growth" and "stationary" phase genes that are PIF4/5-dependent or -independent. Using the resulting gene lists as well as datasets obtained using various light conditions in wild type and a *pif1 pif3 pif4 pif5 PIF* quadruple mutant (*pifq*) (Leivar et al., 2009), we compared our gene lists to the growth-regulated genes identified in these selected arrays. For a description of the arrays selected and the method of comparison see Methods and Table 3.1. We compared our auxin-induced and auxin-repressed gene lists to each array dataset and identified 490 auxin-induced genes and 270 auxin-repressed genes also presented in these growth datasets. We converted these results into a matrix in which each row represents an auxin-responsive gene from our list, and each column represents a microarray condition. We then used hierarchical clustering to generate maps of each matrix. We divided each map into 'growth' and 'stationary' sections to reflect the conditions with which regulation of each gene is associated, as described by Nozue et al. (Fig 3.8A). We also included a column of genes associated with cell wall reorganization, 'CW' (Jamet et al., 2009).

A pattern that emerges from our matrix maps is that many picloram-induced genes are co-regulated by conditions where growth is occurring. We found that 46% of our auxin-induced genes are induced in wild type 2-day-old seedlings grown in the

dark when compared to light-grown seedlings (Fig 3.8A column 1), and 21% are repressed by a 2-day red light treatment that inhibits hypocotyl elongation (Fig 3.8A column 3). Similarly, the overlap between stationary phase genes and auxin-repressed genes is greater (the sum of values in columns 9-15 is 348 for 269 genes) than between stationary phase genes and auxin-induced genes (the sum of values in columns 9-15 is 191 for 490 genes) (Fig 3.8A, left and right maps, columns 11,12,14,15). Therefore, our auxin-induced gene list consists at least in part of genes that are associated with growth, such as *ARGOS (*AT3G59900) and *ARGOS-LIKE* (AT2G44080) (Hu et al., 2003; Hu et al., 2006), *LONGIFOLIA1* (AT5G15580) and *LNG2* (AT3G02170) (Lee et al., 2006) and several *EXPANSIN* and *EXPANSIN-LIKE* genes (Cosgrove et al., 2002; Lee et al., 2003; Li et al., 2003).

The matrix maps highlight a significant overlap between PIF-regulated genes and auxin targets in elongating hypocotyls (Fig 3.8A columns 3-6). This is consistent with previous results from Nozue et al. that show that auxin-regulated genes are overrepresented among genes differentially expressed between *pif4pif5* double mutant and wild type plants. Not surprisingly, genes in this category include genes associated with GA pathways including gibberellin biosynthesis genes *GA3OX1* and *GA2OX8*, the GA repressor *RGL1*, *PIF3-LIKE2* (AT3G62090) and SOMNUS (AT1G03790), a germination gene downstream of *PIL5* (AT2G20180). Of the 81 genes defined by Nozue et al. as upregulated by growth and PIF4- or PIF5-dependent, 38 are also classified in that study as auxin regulated. Of these 38, 35 are in our auxin-induced list. Our auxin-induced list also includes an additional 17 PIF4/5-dependent genes not classified by Nozue *et al.* as auxin-responsive (Nozue et al., 2011).

These findings raise the question of whether auxin regulates PIF target genes through induction of GA biosynthesis and consequent PIF activation, through a GA-independent PIF process, or through a PIF-independent mechanism. We predicted that a set of PIF4/5-dependent growth-associated genes might be auxin-regulated in the absence of PIF activity, since the hypocotyl growth response to the transient auxin treatment during the day did not require PIF4/5 (Fig 3.7). We tested the response of a subset of growth-associated genes, including *SAUR23* (AT5G18060), *IAA2* (AT3G23030) and *ARGOS,* to auxin using qRT-PCR. We found that each of these three genes was induced by a 2hr IAA treatment in *pif4pif5* double mutant seedlings (Fig 3.8B). This suggests that these genes are directly regulated by auxin. This has been confirmed for *IAA2*, which is rapidly induced by auxin in the presence of cyclohexamide (Abel et al., 1995). As the regulation of these three genes in growth-promoting conditions is PIF-dependent (Nozue et al., 2007; Niwa et al., 2009; Nozue et al., 2011), our results support a growth model in which a number of important cell expansion dependent genes are common targets of multiple growth-promoting pathways.

Finally, our analysis also revealed overlap between auxin-responsive genes and growth-upregulated genes that are PIF4/5-independent. More than 200 of our auxin-induced genes are in this category. While this group predictably includes auxin transport (*e.g. PINOID-BINDING PROTEIN1*, AT5G54490; *TOUCH3*, AT2G41100 (Benjamins et al., 2003) and signaling factors (*IAA7*, AT3G23050; *IAA5*, AT1G15580), genes in the GA pathway (*GAI,* AT1G14920; *GA20OX2,* AT5G51810), ethylene pathway (*ETHYLENE RESPONSE 2*, AT3G23150; *ETHYLENE RESPONSE SENSOR 1*, AT2G40940; *ERS2*, AT1G04310), and brassinosteroid pathway (*BES1/BZR1 HOMOLOG 2* (AT4G36780; *BRASSINAZOLE-RESISTANT 1*,

AT1G75080) are also present. Additionally, several genes with roles in cell wall metabolism are present, including *XYLOGLUCAN ENDOTRANSGLUCOSYLASE /HYDROLASE 16* (AT3G23730), *XTH17* (AT1G65310) and *XTH8* (AT1G11545), *CELLULOSE SYNTHASE-LIKE D3* (AT3G03050), and *CELLULOSE SYNTHASE-INTERACTIVE PROTEIN 1* (AT2G22125). Together, these genes are candidate direct auxin targets involved in growth and in cross talk with other signaling pathways.

Auxin regulates additional candidate cell expansion genes

In our auxin-responsive gene list, 81 genes we identified as auxin-induced and 70 genes we identified as auxin-repressed are interrogated by the NimbleChip but not by the Affymetrix ATH1 chip (ftp://ftp.arabidopsis.org/home/tair/Microarrays /Affymetrix/). Several genes among these have predicted functions in cell expansion, including *BREVIS RADIX* (AT1G31880), which promotes leaf, root and shoot growth (Beuchat et al., 2010), *KIDARI* (AT1G26945), which promotes shoot elongation downstream of GA (Hyun and Lee, 2006), and *PAR2* (AT3G58850), a transcription factor induced during the shade avoidance response (Roig-Villanova et al., 2007). Due to a lack of available microarray data, we did not further explore the expression profiles or functions of these genes. However, we confirmed auxin-responsiveness of *PAR2* as well as of *CTR1* (AT5G03730) and *BRIL* (AT1G55610) (new candidate growth genes that are not represented on the ATH1 chip) in seedlings using qRT-PCR (Figure 3.S5B).

Concluding remarks

Our hypocotyl sampling approach enabled us to detect auxin-responsive growth-associated genes that have not been detected in whole seedling arrays. It is

possible that the large number of genes in our auxin-responsive lists that were not found in the MASTA analysis or the comparison with the Nemhauser *et al.* (Nemhauser et al., 2006) and Stepanova *et al.* (Stepanova et al., 2007) datasets represent genes that are auxin-responsive in a specific spatio-temporal pattern that is masked in experimental designs using diverse tissue homogenates. Results from this study emphasize the value of tissue-specific analyses when addressing a particular developmental question. We have uncovered a large set of auxin-regulated genes that are expressed in elongating hypocotyls, including several GA biosynthesis enzymes. Our results suggest that auxin regulates GA biosynthesis to release DELLA-dependent growth repression (de Lucas et al., 2008). Genetic analyses confirmed the importance of auxin-GA cross-talk for a complete hypocotyl growth response, a process that has also been reported in pea (Ross et al., 2000). However, we also demonstrated that regulation of GA is not the only mechanism for auxin-stimulated hypocotyl growth and an independent pathway is required for optimal response. Interestingly, auxin-GA interplay is also involved in tropic hypocotyl growth, although in these processes GA is required to attenuate growth through repression of auxin signaling (Gallego-Bartolome et al., 2011). It will be important for a complete understanding of hormone-regulated growth to assign downstream growth genes to specific hormone pathways or identify mechanisms and conditions in which these genes are downstream of multiple signaling pathways, as we have proposed for *IAA2*, *ARGOS*, and other genes.

Under normal growth conditions, the circadian clock maintains diurnal hypocotyl growth by gating auxin response primarily through PIF4 and PIF5 (Nozue et al., 2007; Koini et al., 2009; Niwa et al., 2009). However, various stress conditions cause plants to stimulate rapid changes in growth during the day in order to survive.

For example, rapid flooding causes changes in hormone levels within 1h of submergence. Studies in *Rumex palustris* have revealed the importance of ethylene, IAA and GA in stimulating stem elongation following submergence due to rapid flooding (Voesenek et al., 2003). Our hypocotyl transcriptome analysis was performed under conditions that mimic a rapid increase in auxin levels during the day leading to a hypocotyl elongation response. We have identified many cell elongation genes that are known to be growth-associated but have not been previously described as auxin-responsive. A subset of these genes is described as being PIF4/5 dependent and we would expect this regulation to be active during normal hypocotyl growth conditions during the night when the PIFs are present (Nozue et al., 2007; Niwa et al., 2009). However, our results suggest that auxin activates these genes in the absence of PIF4/5 suggesting that auxin promotes hypocotyl growth by an independent pathway during the day. Among our auxin-responsive gene list are genes involved in cell wall biogenesis and secretory pathways known to be important for cell elongation. Using the hypocotyl tissue-specific approach and the NimbleChip, we have also uncovered additional hypocotyl growth genes that may also be important for other cell expansion dependent processes such as petiole growth. With this study we propose a mechanism by which the plant can regulate a growth-associated transcriptome to stimulate rapid hypocotyl elongation during the day, independent of PIF4 and PIF5.

MATERIALS & METHODS

Plant Material

Arabidopsis thaliana mutants and transgenic lines used in this study were all in the *Columbia* (*Col-0*) ecotype, with the exception of *shy2-2* (*Landsberg erecta*).

Mutants *msg2-1* (Watahiki and Yamamoto, 1997)*, slr-1* (Fukaki et al., 2002)*, shy2-2* (Reed et al., 1998), *pif4-101/pif5* (Lorrain et al., 2008), and *della5* (*rga-t2 rgl3-1 gai-t6 rgl2-1 rgl1-1*) (Cao et al., 2005) were described previously. The *rga-∆17* line was generated by transforming Col-0 with the *rga-∆17* construct as described in Dill et al., 2001 (Dill et al., 2001). *tir1-1, afb1-3, afb2-3, afb3-4, afb4-2, afb5-5* and higher-order combinations among these mutants were described previously (Parry et al., 2009; Greenham et al., 2011). *RGA::GFP-RGA* (CS16360) and *gai-1* (CS63) were obtained from the Arabidopsis Biological Resource Center at The Ohio State University. Additional mutants were generous gifts from the following: *ga20ox1ox2* mutant (Rieu et al., 2008) was from Peter Hedden; *gid1* mutants (Griffiths et al., 2006) were from Claus Schwechheimer. For hormone treatment assays and RNA isolations, seeds were plated on ½ x Murashige-Skoog medium containing 1% sucrose and 1% agar, and stratified 2-4 days in the dark at 4°C.

Hypocotyl Growth Assays and Imaging

Seedlings were grown under long day photoperiods (16h light/ 8h dark) at 23ºC unless otherwise indicated, with white light intensity of ~80µmol/m^2/s. For treatment assays and RNA isolations, 5-day-old seedlings were transferred to plates containing the chemical being tested or the solvent control (DMSO was used for picloram; ethanol was used for GA_3, cyclohexamide, IAA and NPA) for an additional 48 hours unless otherwise stated. Hypocotyl images were taken using a Nikon SMZ1500 dissecting scope and all measurements were done using ImageJ software. Data shown represent an average of at least 10 seedlings per treatment; error bars represent standard error. GFP fluorescence in pRGA:RGA-GFP was visualized using a Nikon SMZ1500 dissecting scope.

Transcriptome Experiments

Microarray “a”.

Stratified seeds were plated on medium overlaid with sterilized nylon mesh (110 micron pore size; www.smallparts.com). Two hours after chamber lights came on, mesh rafts containing 5-day-old seedlings were transferred to medium containing 5 μM picloram or an equivalent volume of DMSO for 30 min or two hours. Hypocotyls were dissected over a 30-minute period and frozen in liquid nitrogen. Tissue samples were collected over several days and pooled into biological replicates containing at least 400 hypocotyls. RNA extractions were done using Trizol reagent (Sigma) followed by additional phenol extraction and ethanol precipitation steps. mRNA was amplified using the MessageAmp II aRNA Amplification kit (Ambion) and the manufacturer’s protocol. Labeled cDNA was prepared from aRNA using the Superscript ds cDNA synthesis kit (Invitrogen), Cy3- and Cy5-labeled random nonomers (TriLink) and Klenow fragment (Promega). Samples representing three biological replicates were selected for hybridization to the 4-plex NimbleGen chip at the Center for Genomics and Bioinformatics at Indiana University. Experiment ‘a’ was hybridized to the NimbleGen 4-plex chip using dual-color labeling.

Microarray “b”.

Seedlings were grown and treated as described for experiment ‘a’; however, roughly 700 hypocotyls were included in each biological replicate to avoid RNA amplification. RNA extractions were performed using the Invitrogen PureLink RNA mini Kit. Three biological replicates were sampled and used for cDNA synthesis and hybridization to the 12-plex NimbleGen chip according to manufacturer’s instructions. Experiment ‘b’ was hybridized to the NimbleGen 12-plex chip using single-color

labeling. Microarray 'b' was carried out at the GeneChip™ Microarray Core facility at the University of California San Diego.

Transcriptome Analysis

All microarray analysis was done using R (R Development Core Team (2011), http://www.R-project.org/) and Bioconductor (Gentleman et al., 2004). Microarray 'a' was annotated based on the TAIR8 version and 'b' was annotated based on TAIR10. Raw xys data files were used to build a pdInfoPackage in pdInfoBuilder and used in oligo for RMA normalization. Normalized data for array 'a' and 'b' were analyzed independently using a linear model method (Smyth, 2004) performed in the LIMMA package in R. Differentially expressed genes were chosen based on an Empirical Bayes method and an FDR of less than 0.05. The comparative analysis of processed data from 'a' and 'b' was done using the RankProd package in R that accounts for multiple sites of origin when comparing expression data (Hong et al., 2006). Genes or splice forms that were not present on both chips were removed from the analysis. Upregulated and downregulated gene lists from RankProd were used for the comparisons described below. Microarray data and additional details are available from the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/) (Accession number provided upon publication).

Array Comparisons

The MASTA package available from the BAR website (www.bar.utoronto.ca) was used to compare RankProd-generated lists with the 7 IAA wild-type treatment arrays included in the MASTA package. The IAA root treatment data from Stepanova *et al.* (20) was downloaded from the Gene Expression Omnibus (GEO) database

(www.ncbi.nlm.nih.gov/geo/). CEL files were RMA normalized using the Affymetrix package and input into RankProd. Differentially expressed genes with an FDR less than 0.05 were selected and compared to the picloram-responsive gene lists. For the Nemhauser *et al.* (Nemhauser et al., 2006) comparisons the genes defined by the authors as auxin-responsive were used.

Microarrays selected for the growth gene comparisons are listed in Table 3.1. CEL files were not available for all of the arrays selected; picloram-responsive genes identified in this study were compared with genes defined as differentially expressed according to the publication associated with the data in GEO. Matrices were generated with the picloram-induced and –repressed genes in which each row represents an auxin-responsive gene and each column represents a treatment condition from the array being compared. Genes were assigned a value of 1 if defined as differentially expressed in the associated publication, or a value of 0 if absent from the data set. The resulting matrix was used to generate a hierarchical clustered based map in R. Columns were manually arranged based on conditions where growth is occurring (growth phase) or inhibited (stationary phase). The middle column in each map (cw) includes genes that were defined by Jamet *et al.* (Jamet et al., 2009) as being involved in cell wall biogenesis or secretory pathways likely important for cell wall expansion.

Quantitative RT-PCR

RNA samples collected from hypocotyl and whole seedling tissue were obtained from tissue frozen in liquid N2 using the INVITROGEN PureLink RNA minikit. RNA yield and quality was quantified using the Thermo Scientific NanoDrop 2000. Equal amounts of RNA were pre-treated with DNase using the DNA-free Kit

(Ambion) according to manufacturer's instructions and used to generate cDNA with SuperScript III First-Strand Synthesis (Invitrogen) with 20-mer oligo(dT) primers. Quantitative RT-PCR was done with SyBR green and the primers listed in Table S8. Primer pairs were evaluated for specificity and efficiency using three serial dilutions of cDNA using the CFX96^{TM} Real-Time PCR Detection System (Biorad). Data were normalized to the reference gene PP2AA3 (Czechowski et al., 2005) according to the ΔΔCt method (Pfaffl, 2001). Primers were designed using QuantPrime (Arvidsson et al., 2008). Experiments with hypocotyl or seedling tissue were done with two biological replicates and three technical replicates.

ACKNOWLEDGEMENTS

Chapter 3, in full, consists of the manuscript "Hypocotyl transcriptome reveals auxin regulation of growth-promoting genes through GA-dependent and –independent pathways" as it was submitted to PLoS Genetics. Modified for consistency with the other chapters and with permission from all co-authors. I was an equal principal researcher with Dr. Chapman on this manuscript. I contributed to experiments described in figures 3, 4, 5b, 7, and 11. Colleen Doherty assisted with microarray analysis. Mon-Ray Shao, Amanda Budiman, Tatiana Manchenkov and Britta Baynes assisted with plant propagation and provided technical assistance. Jason Reed provided helpful comments on early drafts of the manuscript.

Table 3.1 Microarray datasets selected for growth gene comparisons.

**pifq* – quadruple *pif1 pif3 pif4 pif5* mutant; Rc – 7.5 µmol/m^2/s of red light

**upG PIF4/5 – Genes up in growth phase and PIF4/5 dependent; upG – Growth phase genes PIF4/5 independent; upS PIF4/5 – Genes up in stationary phase and PIF4/5 dependent; upS – stationary phase genes PIF4/5 independent

Publication	**GEO #**	**Conditions**			
*Leivar *et al.* 2009	GSE17159	WT **vs** *pifq* 2d-Dark	WT **vs** *pifq* 2d Drk + 1h Rc	WT 2d Rc **vs** WT 2d Drk	WT seed **vs** WT 2d Drk
**Nozue *et al.* 2011	GSE21684	upG PIF4/5	upG	upS PIF4/5	upS
Ma *et al.* 2005	GSE14648	6d old L grown hypocotyls	6d old Drk grown hypocotyls		

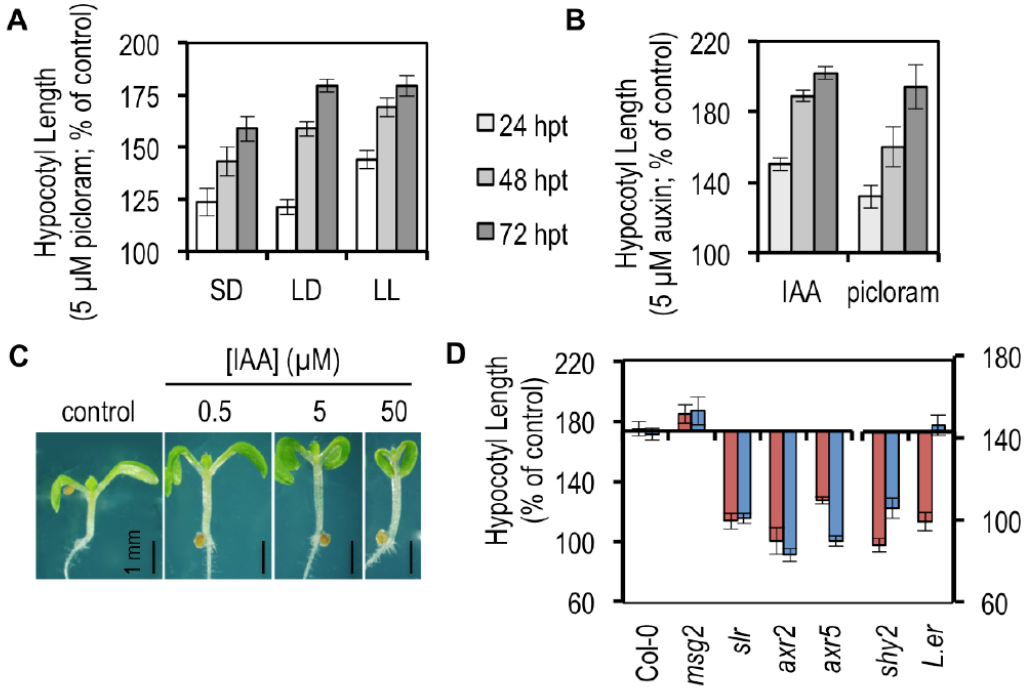

Figure 3.1 Auxin promotes hypocotyl elongation in light-grown seedlings. (A) Auxin promotes hypocotyl elongation in a range of day-length conditions. Average hypocotyl length of wild-type seedlings grown in short days (SD; 8/16), long days (LD; 16/8) or constant light (LL) and treated with 5 µM picloram was determined following 24, 48, or 72 hours of auxin treatment. Hypocotyl length on auxin is shown as a percentage of length on control medium. (B) IAA and picloram promote hypocotyl elongation. Average hypocotyl length of wild-type seedlings grown in long days and treated with 5 µM IAA or picloram was determined following 24, 48, or 72 hours of auxin treatment. Hypocotyl length on auxin is shown as in (a). (C) Auxin response in seedlings increases with auxin concentration. Images of aerial portions of individual 7 day-old seedlings were captured following 48 hours of picloram treatment at the indicated concentrations. (D) Hypocotyl auxin response requires auxin signaling. Average hypocotyl length of wild-type or *aux/iaa* mutant seedlings treated with 5 µM picloram (red bars) or IAA (blue bars) was measured following 48 hours of auxin treatment. Hypocotyl length on auxin relative to the untreated control is shown as in (a).

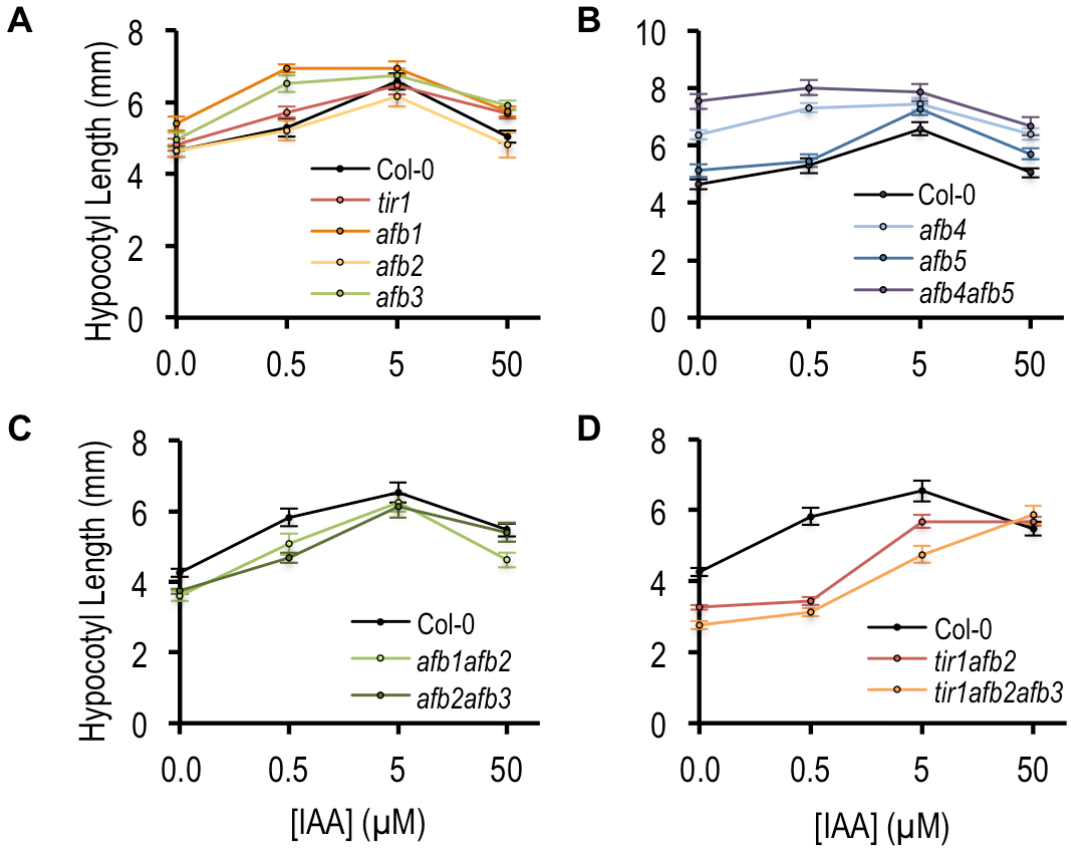

Figure 3.2 Hypocotyl auxin response requires TIR1/AFB auxin receptors. (A-D) Hypocotyl length of wild-type or *tir1/afb* single or multiple mutant seedlings grown in short days and treated with IAA at the indicated concentrations was measured following 48 hours of auxin treatment.

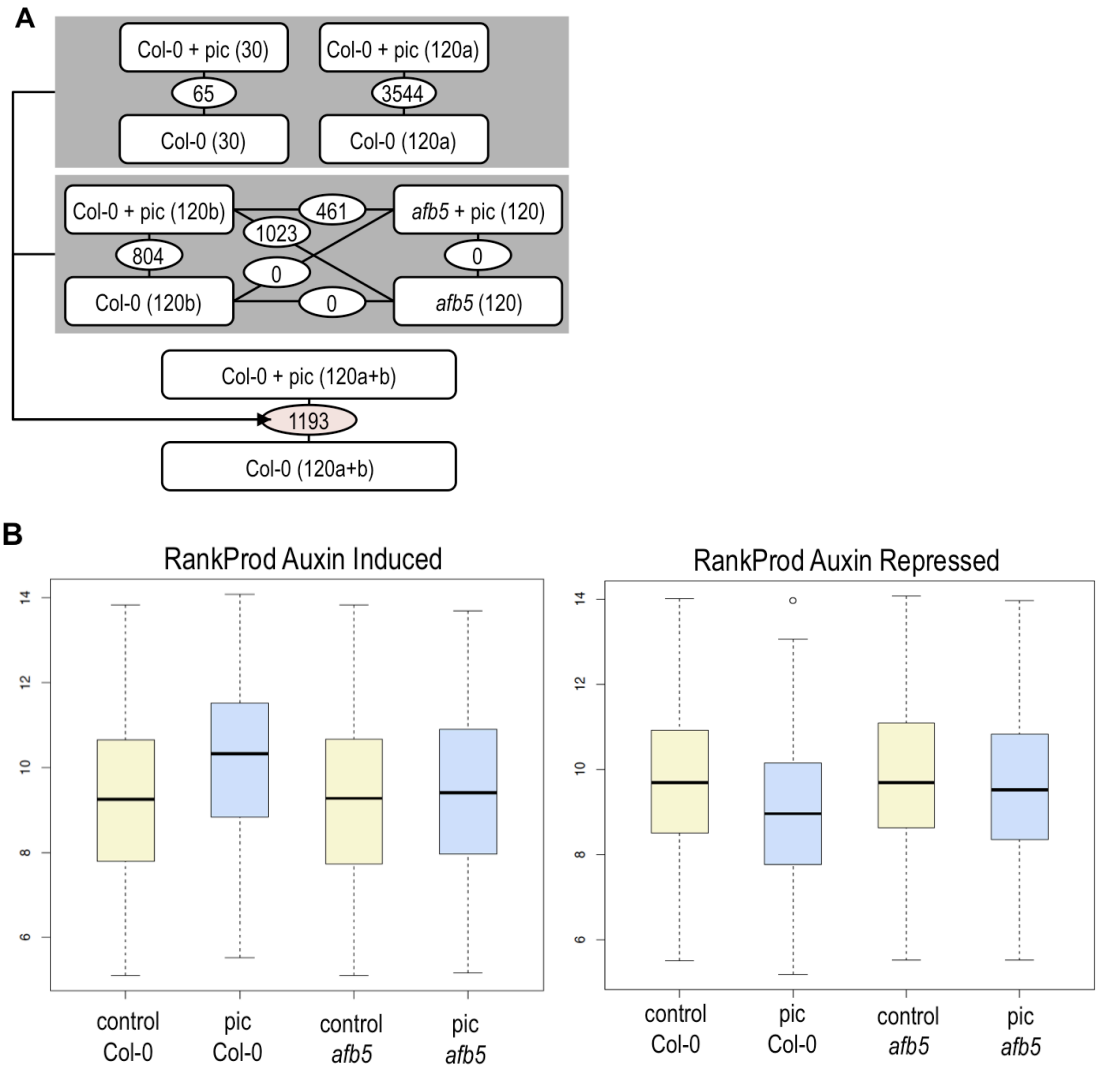

Figure 3.3 The *afb5-5* mutant fails to respond to picloram. (A) Differential gene expression between hypocotyl samples of solvent-treated wild-type (Col-0) or *afb5-5* seedlings (*afb5*) or seedlings treated with picloram (+ pic) for 30 minutes (30) or 120 minutes (120), as determined by analysis of microarray data. The number of genes differentially expressed between samples is shown in lines connecting each sample pair. Data from microarray experiments (a) and (b) were combined for identification of 1193 picloram-responsive genes. (B) Average expression value of 740 auxin-induced or 453 auxin-repressed genes. Differentially expressed genes identified using the RankProd package were selected and average expression values for microarray 'b' are shown.

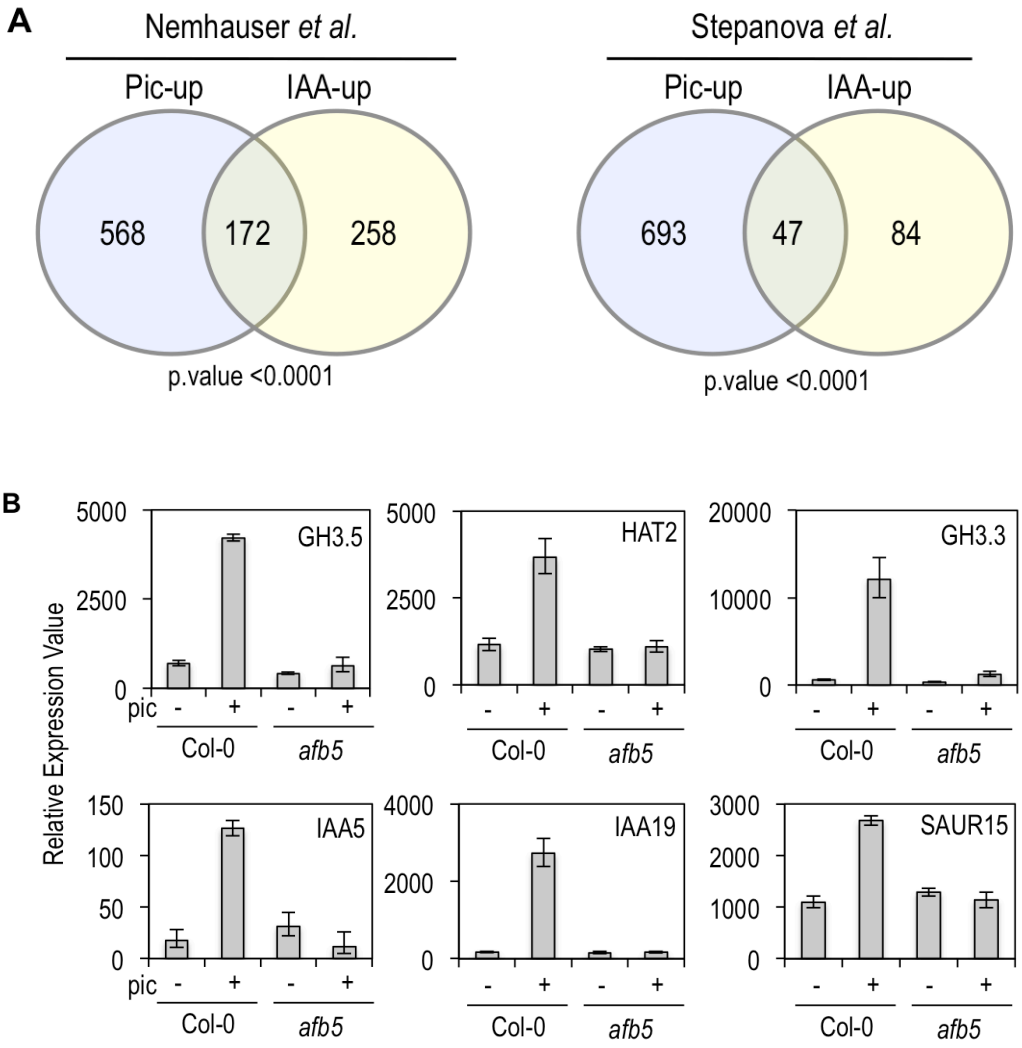

Figure 3.4 Picloram and IAA regulate common target genes. (A) Microarray analysis of picloram-regulated genes in hypocotyls and IAA-regulated genes in seedlings or roots identified common target genes. Venn diagrams of auxin-upregulated genes in IAA-treated materials (IAA-up) and hypocotyls of picloram-treated seedlings (Pic-up) are shown. Numbers of genes identified in common are shown in the overlap sections of each diagram. (B) IAA marker genes are regulated by picloram in hypocotyls of picloram-treated seedlings. Wild-type (Col-0) or *afb5-5* mutant seedlings were treated with picloram or a solvent control for 2 hours and used for hypocotyl dissection and RNA isolation. Expression value of each gene shown, relative to a control gene, was determined by RT-qPCR.

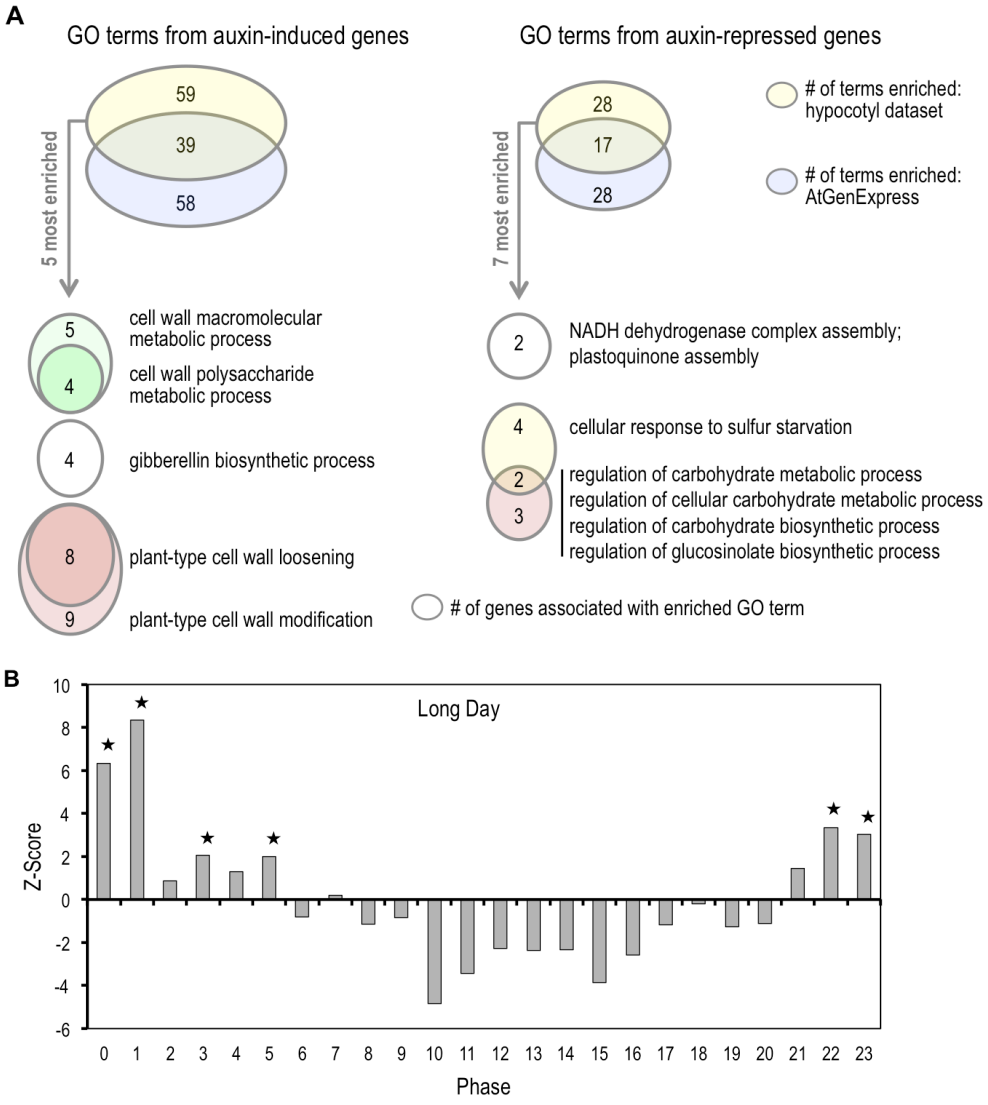

Figure 3.5 Auxin regulates a suite of growth-associated genes preceding hypocotyl elongation. (A) Venn diagrams indicating the number of enriched GO terms in the auxin-induced or -repressed hypocotyl datasets or IAA datasets from the AtGenExpress project (Nemhauser et al., 2006) are shown. The top-ranked GO terms unique to the hypocotyl dataset are shown in the lower set of Venn diagrams. (B) The top 400 statistically significant picloram induced genes were analyzed using the Phaser tool (http://phaser.cgrb.oregonstate.edu/). Bars represent z-scores for the enrichment of cycling genes within our gene list compared to all the genes shown to cycle under long day conditions at a given phase of the day. Phase 0 signifies the start of the day. Asterisks indicate significant enrichment with a $p<0.05$.

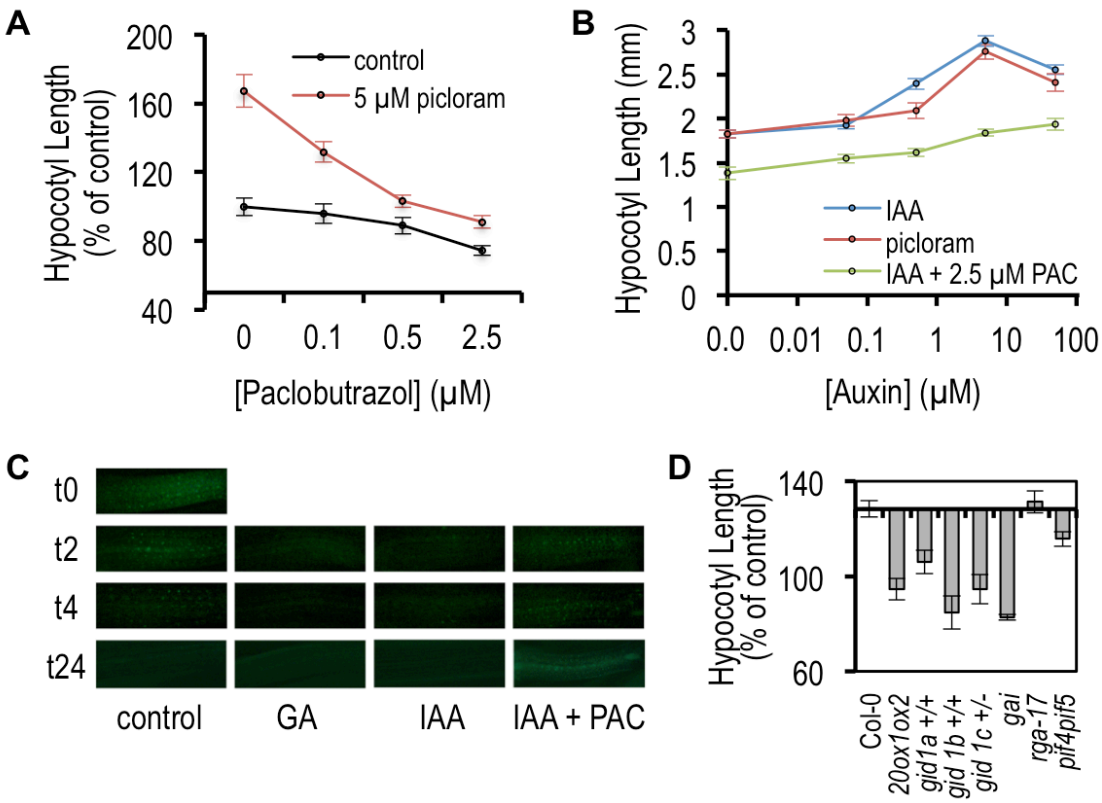

Figure 3.6 Gibberellin biosynthesis and signaling is required for hypocotyl auxin response. (A) Hypocotyl length of wild-type seedlings treated with paclobutrazol at the indicated concentrations (black line) or paclobutrazol plus 5 µM picloram (red line) was measured following 48 hours of treatment. (B) Hypocotyl length of wild-type seedlings treated with IAA (blue line) or picloram (red line) at the indicated concentrations or IAA and 2.5 µM paclobutrazol (green line) was measured following 48 hours of treatment. (C) Abundance of RGA-GFP protein in hypocotyl tissues of 4 day-old plants treated for 2, 4, or 24 hours with 50 µM GA_3, 5 µM IAA or 5 µM IAA + 2.5 µM paclobutrazol was analyzed by epifluorescence microscopy. (D) length of wild-type seedlings (Col-0) or indicated GA mutants treated with 5 µM picloram was measured following 48 hours of auxin treatment. Hypocotyl length on auxin is shown as a percentage of length on control medium. For *rga-Δ17*, the hypocotyl length was measured using a mixture of hemizygous and homozygous seedlings because the *rga-Δ17* homozygotes are sterile. *gid1* double mutants are named as in Griffiths *et al.,* 2006 (Griffiths et al., 2006) and carry one (+/-) or two (+/+) functional copies of only *gid1a*, *gid1b*, or *gid1c.*

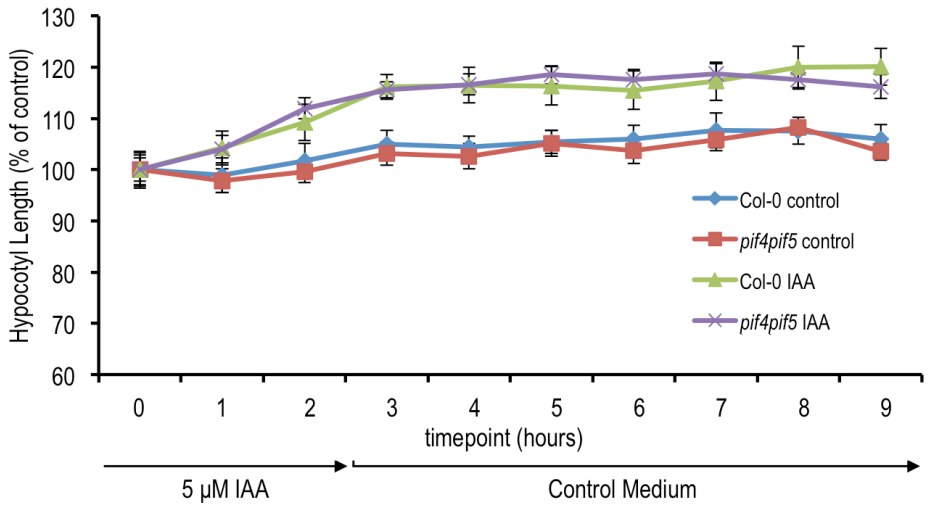

Figure 3.7 Auxin promotes hypocotyl elongation during the day independent of PIF4/5. Average hypocotyl length of wild-type (Col-0) or *pif4pif5* mutant seedlings treated with 5 µM IAA for two hours was measured each hour for 7 hours. Hypocotyl length at each timepoint is shown as a percentage of length at time 0.

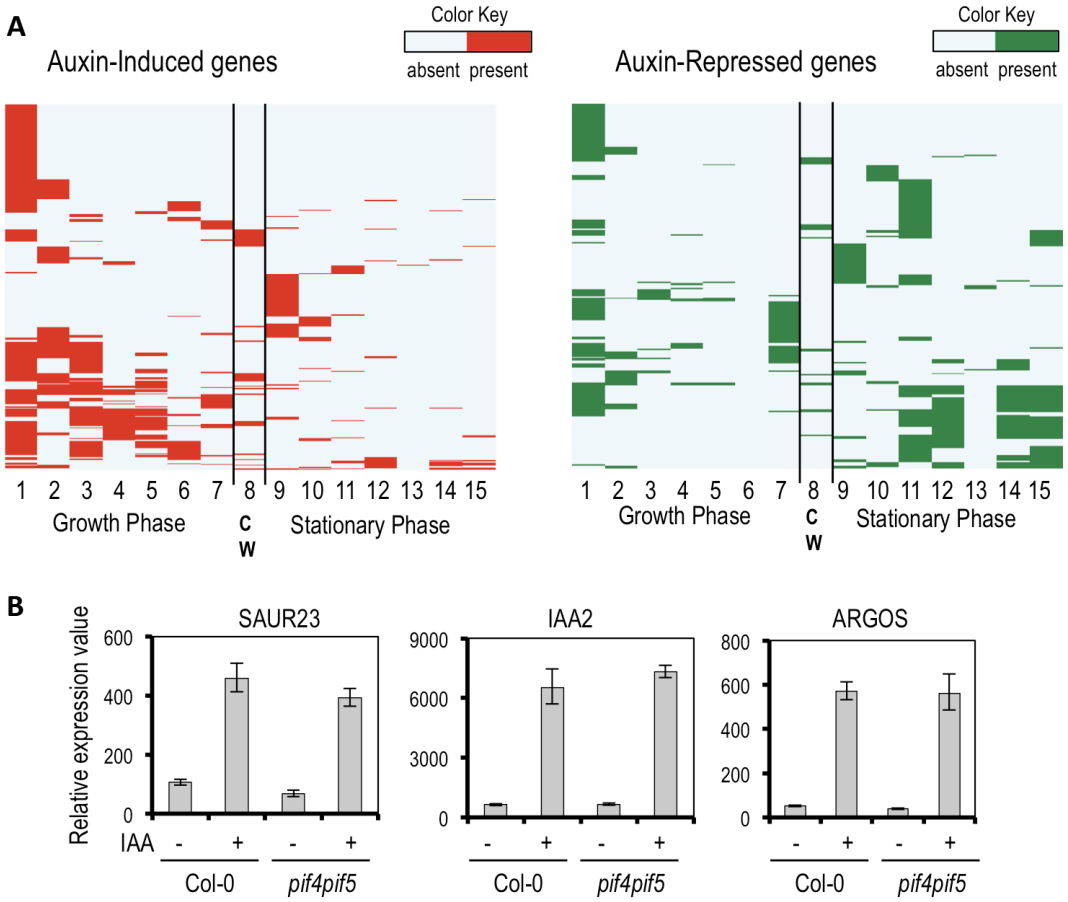

Figure 3.8 Auxin promotes hypocotyl elongation through PIF-dependent and –independent pathways. (A) Picloram-induced genes are also induced in the dark (column 1, up in WTD) and during growth (2, upG; 6, upG PIF4/5), and repressed by light (3, down in WTRc; 7, DL) and in the pifq mutant (4, down in pifqR1; 5, down in pifqD). Picloram-repressed genes are also repressed in the dark (column 9, down in WTD) and upregulated by light (10, upL; 15 up in WTRc), during stationary phase (11, upS; 13 Nozue upS PIF4/5), and in the pifq mutant (12, up in pifqR1; 14, up in pifqD) (see Methods and Table S6 for complete description of array conditions shown). CW indicates genes associated with cell wall metabolism (column 8). (B) Wild-type (Col-0) or *pif4pif5* mutant 5-day-old seedlings were treated with 5 μM IAA or a solvent control for 2 hours and used for RNA isolation. Expression value of each gene shown, relative to a control gene, was determined by qRT-PCR.

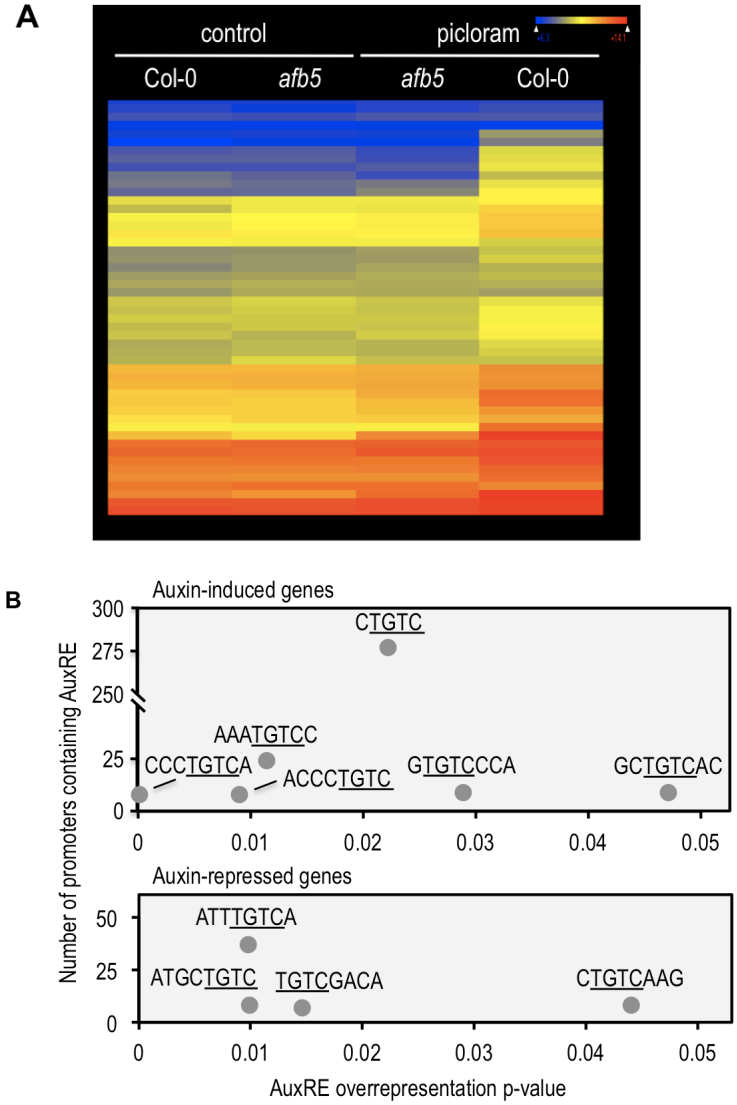

Figure 3.S1 Picloram and IAA share transcriptional targets. (A) Auxin marker genes are picloram-responsive in hypocotyls from wild-type, but not *afb5-5* mutant, seedlings. Hierarchical clustering result of IAA marker gene expression in hypocotyls of picloram-treated or control wild-type (Col-0) or *afb5-5* (*afb5)* seedlings, as determined by analysis of microarray data using ArrayStar, is shown. (B) Auxin response elements are overrepresented in picloram-responsive promoters. Statistical significance of overrepresentation of each AuxRE-containing sequence element (p-value) is plotted on the x-axis; the number of promoters containing the element is plotted on the y-axis. Overrepresented sequences were identified using ELEMENT (Mockler et al., 2007).

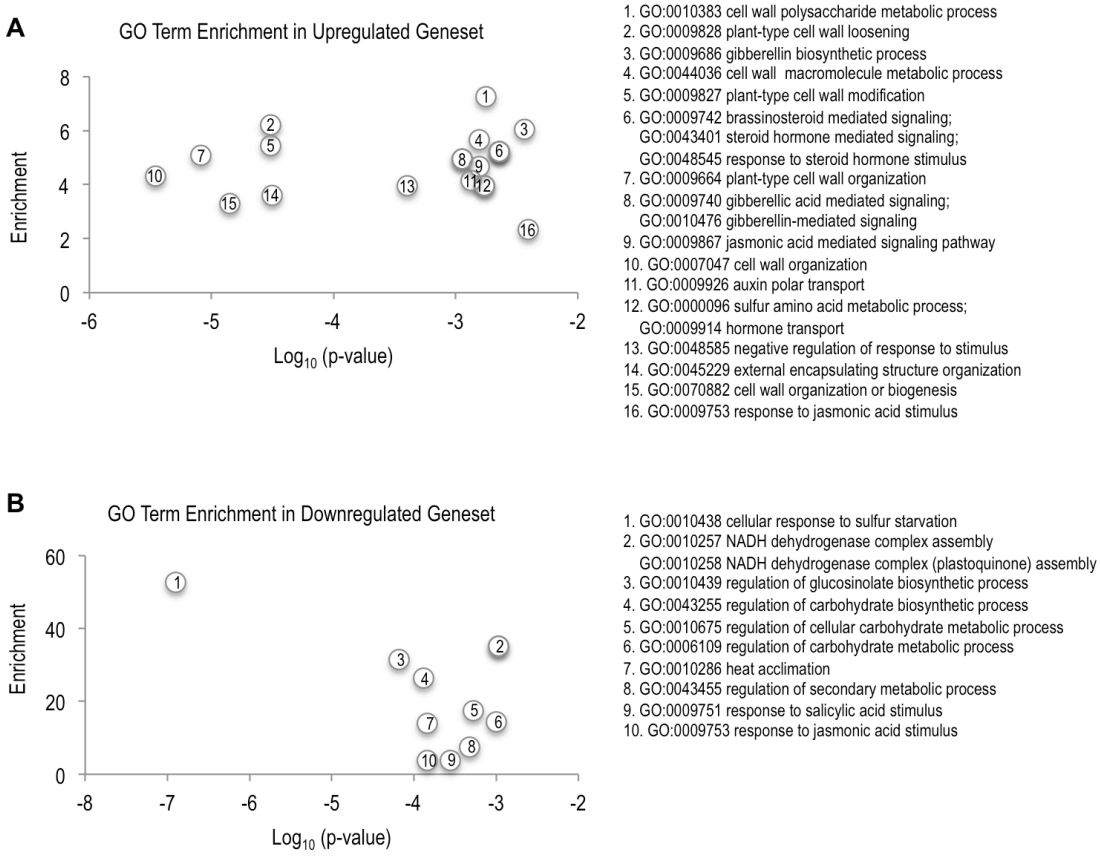

Figure 3.S2 GO terms newly associated with auxin-responsive transcription. Overrepresented GO terms and enrichment scores were identified using GOMiner (Zeeberg et al., 2003). Only GO terms not overrepresented in the AtGenExpress datasets (Nemhauser et al., 2006) are shown.

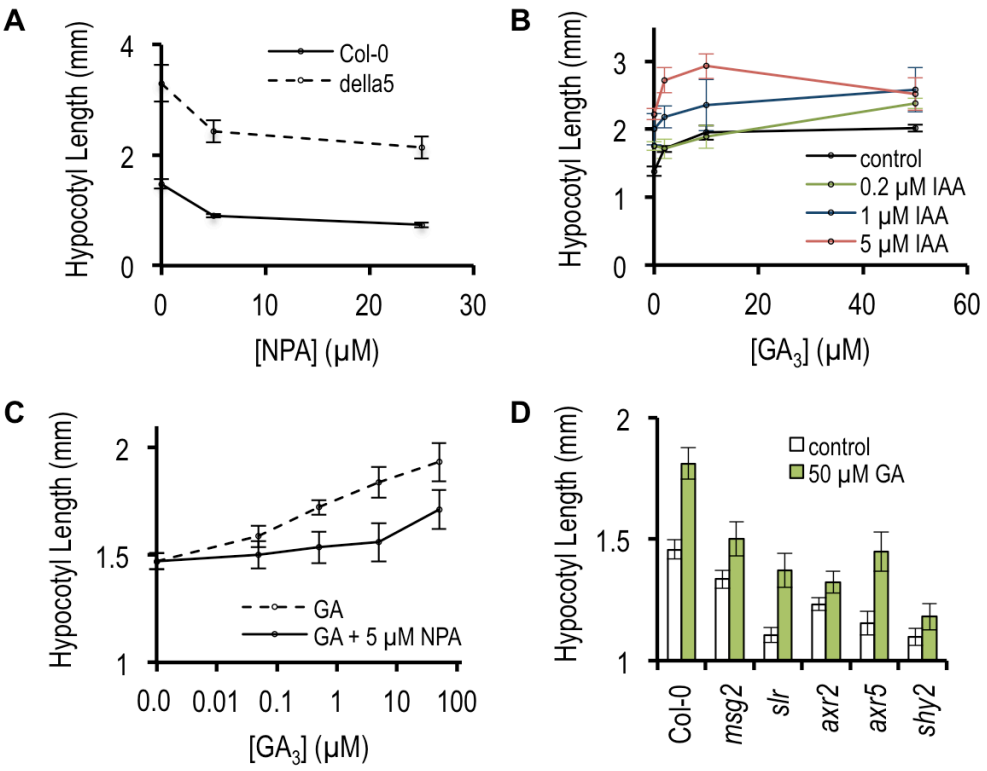

Figure 3.S3 GA and auxin act independently and interdependently to regulate hypocotyl elongation. (A) The *della5* mutant phenotype is dependent upon auxin transport. Average hypocotyl length of wild type seedlings (Col-0; solid line) or a pentuple DELLA mutant (*rga-t2 rgl3-1 gai-t6 rgl2-1 rgl1-1*; dotted line) germinated and grown in long days on medium supplemented with NPA at the indicated concentrations is shown. (B) Auxin and GA are additive in promoting hypocotyl elongation. Average hypocotyl length of wild type seedlings (Col-0) grown in long days and treated with IAA and GA_3 at the indicated concentrations is shown. (C) Hypocotyl growth response to GA requires auxin transport. Average hypocotyl length of wild type seedlings grown in long days and treated with GA_3 and NPA at the indicated concentrations in shown. (D) Auxin signaling mutants are partially restored by treatment with GA_3. Average hypocotyl length of wild-type seedlings or the indicated mutants grown in long days and treated with 50 µM GA_3 was determined following 48 hours of treatment.

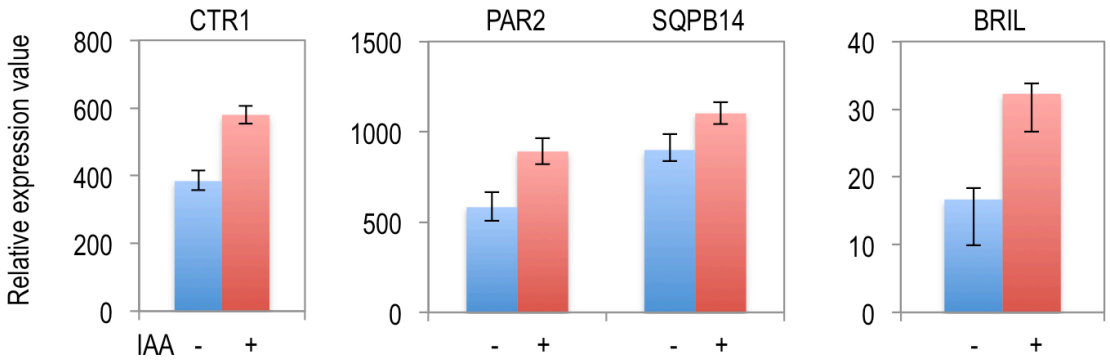

Figure 3.S4 PIF4/5-independent genes are regulated by auxin in seedlings. Wild-type seedlings were treated with IAA or a solvent control for 2 hours and used for RNA isolation. Expression value of each gene shown, relative to a control gene, was determined by RT-qPCR.

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CHAPTER 4

AFB4 and the Circadian Clock

ABSTRACT

To gain insight into the role of AFB4 during seedling development we focused on the hypocotyl and addressed the basis for the long hypocotyl phenotype of *afb4-2*. A series of microarray experiments were performed on wild type and *afb4-2* hypocotyls sampled from seedlings grown at 22°C and 29°C, a condition that results in a long hypocotyl in wild-type seedlings. The results from the analysis reveal a significant overlap between genes differentially expressed in *afb4-2* at 22°C compared to wild type at 29°C. This suggests that a similar set of genes is contributing to the long hypocotyl phenotype observed in both conditions. Further analysis reveals significant enrichment of circadian regulated genes in both datasets. A connection with the clock is confirmed by the short period phenotype of the *afb4-2* mutant suggesting that AFB4 is contributing to the circadian regulation of hypocotyl growth. These results provide the first evidence suggesting that auxin acts as an input into the circadian clock in *Arabidopsis.*

INTRODUCTION

The circadian clock is a molecular oscillator that controls many essential cellular processes in diverse species from cyanobacteria to humans (McClung and Gutiérrez, 2010). In plants, this regulation ranges from photosynthesis, stress responses, metabolic pathways such as the TCA cycle (Fukushima et al., 2009), hormone signaling and growth (Nozue et al., 2007; Michael et al., 2008). The circadian clock is comprised of a series of interlocked negative feedback loops with two morning expressed MYB transcription factors, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) together with an evening expressed pseudo-response regulator (PRR), TIMING OF CAB

EXPRESSION 1 (TOC1), making the central loop. *CCA1* and *LHY* repress the expression of *TOC1*, through an EVENING ELEMENT (EE) motif in the promoter of *TOC1.* *CCA1* and *LHY* activate three other PRRs (PRR5, 7 and 9), which in turn repress *CCA1* and *LHY* expression, forming an interlocked 'morning loop'. *CCA1* and *LHY* are activated by TOC1, which is recruited to their promoters by an unknown mechanism. TOC1 is also a component of the 'evening loop' by repressing the positive regulator of TOC1, GIGANTEA (GI) as well as a predicted unknown factor referred to as 'Y' (McClung and Gutiérrez, 2010; Pruneda-Paz and Kay, 2010).

An important characteristic of the circadian clock is its ability to maintain a ~24 hr rhythm over a range of temperatures, a process known as temperature compensation. The exact mechanism for this effect is not clear although several important components have been identified. The *gi-11* mutant containing a mutation in the *GI* gene has a short period phenotype following a shift to temperatures above or below 17°C revealing a temperature-dependent circadian phenotype. The *lhy* loss-of-function mutant is affected in period length at high temperatures while the *cca1* mutant is more affected at low temperatures suggesting differences in how these transcription factors function at different temperatures (Gould et al., 2006). Further insight into the mechanism for temperature compensation came from the observation that the *prr7 prr9* mutant has an overcompensation phenotype at temperatures above 12°C, characterized by a long period. This phenotype is completely dependent on *CCA1* and *LHY* since *prr7 prr9* mutants expressing amiRNAs against both *CCA1* and *LHY* are indistinguishable from *amiR-CCA1-LHY* lines in a wild type background. This suggests that the controlled regulation of *CCA1* and *LHY* by PRR7 and PRR9 is required for temperature compensation (Salomé et al., 2010).

At 29°C, *Arabidopsis* seedlings accumulate IAA leading to an increase in hypocotyl length (Gray et al., 1998). This response requires the bHLH transcription factor PHYTOCHROME INTERACTING FACTOR 4 (PIF4), as *pif4-1* mutants fail to elongate in response to high temperature (Koini et al., 2009). PIF4 and PIF5 are both involved in promoting hypocotyl growth, evident by the short hypocotyl phenotype of *pif4-1 pif5-101* (Nozue et al., 2007; Lorrain et al., 2008; Niwa et al., 2009). To control the timing of hypocotyl growth, the light and clock pathways regulate PIF4 and PIF5 expression and protein accumulation. The photoreceptor PHYB directly interacts with PIF4 and PIF5 to induce their degradation and repress hypocotyl growth during the day (Khanna et al., 2004; Lorrain et al., 2008). The circadian clock regulates the expression of *PIF4* and *PIF5* in a photoperiod specific manner. Under short day (SD) conditions *PIF4* and *PIF5* expression is activated at the end of the night to promote hypocotyl growth from subjective dusk to dawn (Nozue et al., 2007; Niwa et al., 2009). The direct binding of an ELF4-ELF3-LUX protein complex to PIF4 and PIF5 promoters controls this end of night activation. The *elf3, elf4* and l*ux* mutants all exhibit defects in hypocotyl growth under diurnal conditions due in part to arrhythmic circadian oscillations. *ELF3* and *ELF4* are nuclear proteins with no known functional domains whereas *LUX* is a myb-domain-containing SHAQYF-type GARP transcription factor. This complex represses the expression of *PIF4* and *PIF5* in the early evening, a mechanism that connects the circadian clock to the diurnal control of hypocotyl growth (Nusinow et al., 2011)

All hormone-signaling pathways show some connection with the circadian clock in *Arabidopsis*. Mutants involved in ethylene (ACC), cytokinin (CK) and abscisic acid (ABA) signaling and synthesis show various clock related defects including alteration in period length. Through exogenous treatment assays it was suggested

that brassinosteroid (BR) and ABA affect the periodicity of the clock whereas CK has a greater effect on phase (Hanano et al., 2006). Given that roughly a third of the genes expressed in *Arabidopsis* are circadian regulated, it is not surprising that a large portion are involved in hormone signaling. Covington *et al.* (2008) showed that of the genes that respond to auxin (IAA), BR, CK, ACC, gibberellin (GA), methyl jasmonate (MJ), salicylic acid (SA) or ABA treatment within 0.5 - 4 hr there is significant enrichment of circadian-regulated genes. A collection of genes implicated in biosynthesis, catabolism, perception or signaling for ABA, ACC, BR, CK, GA, and IAA were examined for their time of day expression patterns and found to peak during the hypocotyl growth phase (Michael et al., 2008).

Genes involved in auxin biosynthesis, conjugation, transport, signaling and response are circadian regulated. Taking into account the fold-change of auxin induced genes more than half of high-induced genes are circadian regulated. The circadian control of auxin response is apparent by the time-of-day change in the plants sensitivity to hypocotyl elongation by auxin treatment (Covington and Harmer, 2007). It is clear that the circadian clock is involved in gating auxin response in order to maintain coordinated growth. This regulation likely involves PIF4 and PIF5. Many auxin responsive genes are also regulated by PIF4 and PIF5 and the altered sensitivity of the *pif4-1 pif5-101* mutant to auxin suggests that they are involved in regulating auxin sensitivity in the plant (Nozue et al., 2011). There is no evidence to suggest that auxin has any major effect on the clock itself. Although it remains to be seen whether certain stress or environmental conditions that require rapid growth responses independent of the normal circadian control may rely on auxin-dependent modifications to circadian pathways.

RESULTS AND DISCUSSION

Identification of downstream targets of AFB4 in elongating hypocotyls

The AFB4 auxin receptor plays a unique role during hypocotyl growth as determined by the long hypocotyl phenotype observed in the *afb4-2* mutant. Following transfer to 29°C, *afb4-2* hypocotyls fail to elongate to the extent of wild-type seedlings suggesting that the response is saturated due to a hypersensitivity to auxin as seen by exogenous auxin treatment (Greenham et al., 2011). To identify downstream targets of AFB4 in the hypocotyl as well as genes important for the high temperature-dependent hypocotyl elongation response, a series of microarray experiments were performed. Seedlings were grown at 22°C and 29°C under SD photoperiods (8L:16D), a condition that promotes hypocotyl growth. Hypocotyls were dissected from 4-day-old seedlings when the hypocotyls are in their maximum growth stage under our conditions (Greenham et al., 2011). To minimize time-of-day effects, dissection time was limited to 30 min per sample and carried out over a 2 hr period at the same time of day.

An initial analysis of the data using a moderated linear model approach (Smyth, 2004) with an FDR cutoff of 0.05 resulted in 1013 differentially expressed genes between wild type hypocotyls grown at 29°C compared to 22°C. Of these genes, 577 are induced by high temperature and 436 are repressed. Surprisingly, using this analysis there no significant differences between wild type and *afb4-2* hypocotyls at 22°C were observed. This may be due to technical issues, such as variability among the replicates. To determine if this is the case, we performed an analysis of the data using the rank product method that implements a non-parametric statistic to compare the expression-based rankings of genes across samples and can improve the ability to detect differential expression with high replicate variation

(Breitling et al., 2004; Hong et al., 2006). Using the RankProd package in R (Hong et al., 2006) we identified 156 genes that are differentially expressed between wild type and *afb4-2* hypocotyls at 22°C. To ensure these genes are in fact significantly different between these two samples a t-test was performed between the averaged replicates for wild type and *afb4-2*. Using a p-value cutoff of 0.05 we found 96 genes that are upregulated in *afb4-2* and 26 that are downregulated compared to wild type at 22°C (Fig 4.1A).

Included in the list of genes upregulated in *afb4-2* are genes involved in stress response (*TRIACYLGLYCEROL BIOSYNTHESIS DEFECT 1*, AT2G19450; *DARK INDUCIBLE 11*, AT3G49620; *HEAT SHOCK PROTEIN 90-1*, AT5G52640), cell organization and biogenesis (*CASPARIAN STRIP MEMBRANE DOMAIN PROTEIN* (*CASP*) *1,2&3*, AT2G36100, AT3G11550, AT2G27370; *MICROTUBULE-ASSOCIATED PROTEIN 65-4* (*MAP65-4*), AT3G60840) and response to abiotic or biotic stimulus (*DARK INDUCIBLE 2* (*DIN2*), AT3G49620; *AZELAIC ACID INDUCED 1*, AT4G12470; *FLOWERING LOCUS C*, AT5G10140; *TONOPLAST INTRINSIC PROTEIN* (*TIP*) *2;3*, AT5G47350). Several of these genes are known to be involved in hypocotyl growth or associated with cell wall modifying processes including: *GA2OX7*, which is involved in GA biosynthesis (Schomburg et al., 2003); *FAR1*, which is involved in phyA signaling (Hudson et al., 1999); and *TIP2;3* and *PIP2;4* (AT5G60660), aquaporins that transports water across membranes, an important process for cell elongation (Boursiac et al., 2005; Eisenbarth and Weig, 2005).

Identification of temperature responsive genes in Arabidopsis hypocotyls

Given that *afb4-2* appears hypersensitive to endogenous IAA (Greenham et al., 2011) we hypothesized that the genes misregulated in *afb4-2* might overlap with

the genes responding to increased IAA at 29°C. To be able to compare both datasets we analyzed the wild type 22°C and 29°C data using the RankProd package. Following the same methodology we identified 388 genes upregulated by 29°C and 325 genes downregulated (Fig 4.1B). Although these lists are smaller compared to the previous method, 75% of the genes identified using RankProd were also identified with the linear model approach. The upregulated genes in common between both analysis methods cover a range of biological processes. Genes involved in abiotic stress response such as members of the *CYTOCHROME P450* family, including *CYP705A5* and *CYP708A2* that catalyze steps in triterpene synthesis and *CYP710A2* that catalyzes the formation of brassicasterol (Ohnishi et al., 2009); genes responding to heat stress including several *HEAT SHOCK PROTEINS* (Wahid et al., 2007); and several *Peroxidase* genes that respond to oxidative stress are also present. Additionally, several genes associated with growth, such as *EXPANSINS* (Cosgrove et al., 2002), *ELF4* (AT2G40080) (Nusinow et al., 2011), *TINY* (AT5G25810) (Wilson et al., 1996), and *TAA1* (AT1G70560) (Stepanova et al., 2008; Tao et al., 2008; Yamada et al., 2009) are present.

A GO enrichment analysis of the RankProd generated lists reveals a number of significantly enriched categories we would expect following a prolonged heat treatment (Table 4.1A, B), including response to heat, response to reactive oxygen species, and cell wall and glucosinolate biosynthesis processes. The GO enrichment for the downregulated genes revealed a number of terms related to chloroplast components and photosynthesis, in particular photosystem II and oxidation-reduction pathways. It has been previously shown that high temperature stress causes reduced photosynthesis due to a reduction in chlorophyll biosynthesis and chloroplast production (Kumar Tewari and Charan Tripathy, 1998; Dutta et al., 2009).

Chloroplasts located in the hypocotyl are necessary for phototropic bending (Jin et al., 2001); the GO enrichment results suggest that they are also responding to heat stress similarly to the chloroplasts found in leaf tissue.

Hypocotyl elongation in afb4-2 and at elevated temperature includes a common set of genes

To determine whether a similar set of genes are contributing to the long hypocotyl phenotypes observed in *afb4-2* at 22°C and wild type at 29°C, we compared the gene lists identified by RankProd. We found a significant number of genes in both the upregulated and downregulated lists (p-value < 0.001). In fact, close to half of the genes upregulated in *afb4-2* at 22°C were also upregulated by high temperature and the same was true for the downregulated set (Fig 4.1C). Many of the upregulated genes in the overlap list are associated with lipid and cell wall modification and water channel activity such as *TIP2;3* (AT5G47450), *PIP2;4* (AT5G60660) and *CASP1*, *2* & *3*. This is consistent with a role for these genes in cell elongation during hypocotyl growth. These results suggest that a significant number of genes that are targets of AFB4 are required for the high temperature induced auxin response in wild type hypocotyls. This is consistent with the hypersensitivity of *afb4-2* to high temperature as seen by delayed growth of adult plants (Fig 4.2) and reduced hypocotyl elongation (Greenham et al., 2011). Among the upregulated overlap list we noticed the *PSEUDO-RESPONSE REGULATOR 3 (PRR3)* gene that is related to the circadian clock genes *PRR5, 7, 9* and *TOC1*. The exact role of PRR3 is not clear but it interacts with TOC1 both in yeast and plants, suggesting that it may be involved in stabilizing TOC1 by preventing the interaction with ZEITLUPE (ZTL), an F-box protein that targets TOC1 for degradation (Para et al., 2007).

Loss of AFB4 results in a circadian clock defect

Due to the role of the circadian clock in gating hypocotyl growth we decided to look for other circadian regulated genes in our overlap lists. Using the Phaser tool (http://phaser.cgrb.oregonstate.edu/) (Michael et al., 2008) we looked for enrichment of cycling genes compared to all the genes in the genome that cycle at a given phase of the day. Both the up and downregulated lists showed significant enrichment for genes that cycle under constant light and dark conditions (Fig 4.3). The enrichment of circadian regulated genes in the microarray datasets suggested that the long hypocotyl phenotype of *afb4-2* might be a consequence of a clock defect since the samples for the microarray analysis were performed at the same time of day. To test this hypothesis *afb4-2* was crossed into a line expressing the *LHY* promoter fused to the *luciferase (luc)* reporter gene. Under constant white light the period length of *LHY:Luc* was roughly an hour shorter in *afb4-2* compared to wild type. To determine whether this is specific to *LHY* or a general defect in the clock, we performed a leaf movement assay. In wild type seedlings the cotyledons rise and fall in a 24 hr rhythmic pattern that can be quantified to determine the period of the clock (Dowson-Day and Millar, 1999). The results from this assay confirmed a 23 hr-shortened period in *afb4-2* (Fig 4.4). A short phenotype was not observed in the *tir1-1 afb2-3* double mutant suggesting that the clock defect is specific to *afb4-2*. Several circadian clock mutants that affect period length also show altered hypocotyl growth such as *lhy-1*, *gi-100* and *elf3-1* (Schaffer et al., 1998; Dowson-Day and Millar, 1999; Huq et al., 2000).

The work described in this study provides evidence to suggest a role for auxin as an input into the circadian clock. If auxin is involved in the circadian regulation of hypocotyl growth it is possible that this may be an important component of the

temperature compensation mechanism since an increase in IAA levels is observed at elevated temperature (Gray et al., 1998). If AFB4 is integrating the auxin signaling pathway with circadian regulation of hypocotyl growth it is not surprising to see significant overlap between the genes differentially expressed in *afb4-2* at 22°C and responding to 29°C in wild type. Future work will address the mechanism for this regulation through more detailed analysis of the expression patterns of various clock reporters in *afb4-2* as well as other auxin-signaling mutants. Of course, we cannot rule out the possibility that the short period defect in *afb4-2* is an indirect effect. If the clock is signaling through AFB4 to inhibit hypocotyl growth then it is possible that in the *afb4-2* mutant a consequence of this loss in inhibition may lead to a change in metabolite levels or other factors that feedback to the clock and cause the change in period.

MATERIALS & METHODS

Plant Material

Arabidopsis wild type and all mutants were in the Columbia (Col-0) ecotype. The receptor mutants have been previously described: *tir1-1* (Ruegger et al., 1998), *afb2-3* (Savaldi-Goldstein et al., 2008), *afb4-2* and *afb5-5* (Greenham et al., 2011). For RNA isolations, seeds were plated on 0.5 X Murashige-Skoog medium containing 1% sucrose and 0.8% agar and stratified for 2-4 days in the dark at 4°C. Following stratification plates were moved to a SD photoperiod (8L:16D) at a fluence rate of 80μmol/m^2/s, unless otherwise stated.

Transcriptome Experiments

Following stratification, wild type and *afb4-2* seeds were plated on medium overlaid with sterilized nylon mesh (110 micron pore size; www.smallparts.com) and grown either at 22°C or 29°C under SD conditions. Hypocotyls were dissected on day 4 starting when the chamber lights came on. Each sample set (ex. Col 22°C) was dissected over a 30 min period and frozen in liquid nitrogen. The total dissection time for one day was 2 hrs. Each biological replicate contains tissue pooled over several dissection days and contains ~700 hypocotyls. RNA extractions were performed using the Invitrogen PureLink RNA mini kit. Three biological replicates were sampled and used for cDNA synthesis and hybridization to the 12-plex NimbleGen chip according to manufactures instructions. The cDNA synthesis, labeling and hybridization were done at the GeneChip™ Microarray Core facility at UC San Diego.

Transcriptome Analysis

Microarray analysis was performed using R (R Development Core Team (2011), http://www.R-project.org/) and Bioconductor (Gentleman et al., 2004). TAIR10 was used for annotation. Annotation packages were built with pdInfoBuilder using raw data files (.xys) along with a NimbleGen microarray design file (.ndf). Data was RMA normalized in oligo using this annotation package. Initial analysis was performed using a moderated linear model (Smyth, 2004) available in the LIMMA package in R. Differentially expressed genes were defined based on an Empirical Bayes method and an FDR cutoff of less than 0.05. The RankProd analysis was performed using the package available in R (Hong et al., 2006) and an FDR cutoff of less than 0.05. To confirm the differential expression of these genes a student t-test

was performed on the average expression values of the replicates between samples and genes that did not have a p.value less than 0.05 were discarded. GO enrichment analysis was performed using DAVID with all the genes included on the NimbleGen chip used as the background for the analysis (Huang da et al., 2009a, b).

Bioluminescence Assays

Seedlings were grown under white light with a fluence rate of 60 μmol/m^2/s under the given temperature and day length condition described. Bioluminescence rhythms were analyzed as previously described (Somers et al., 1998). Period lengths were determined using a fast fourier transform nonlinear least square (FFT-NLLS) fit method as described (Plautz et al., 1997; Zhong et al., 1997).

Leaf Movement Assays

Seeds were surface-sterilized and plated on MS medium with 2% sucrose and stratified for 3-4 days in the dark at 4°C. Seedlings were grown for 5 days under a 12L:12D photoperiod and then a cube of medium was cut out surrounding the seedling and transferred to a 24-well plate with Whatmann paper on the back to provide contrast. The plates were shifted to continuous white light and a Panasonic CCTV camera (model WV-BP120, Matsushita Communications Industrial, Laguna, Philippines) was used to image the leaf movement every 20 min over 7 days. The analysis was performed using Kujamorph software as described (Millar et al., 1995b; Millar et al., 1995a) and using fast Fourier-transform-nonlinear least squares (Plautz et al., 1997; Zhong et al., 1997) and BRASS (http://millar.bio.ed.ac.uk/PEBrown/BRASS/BrassPage.Htm).

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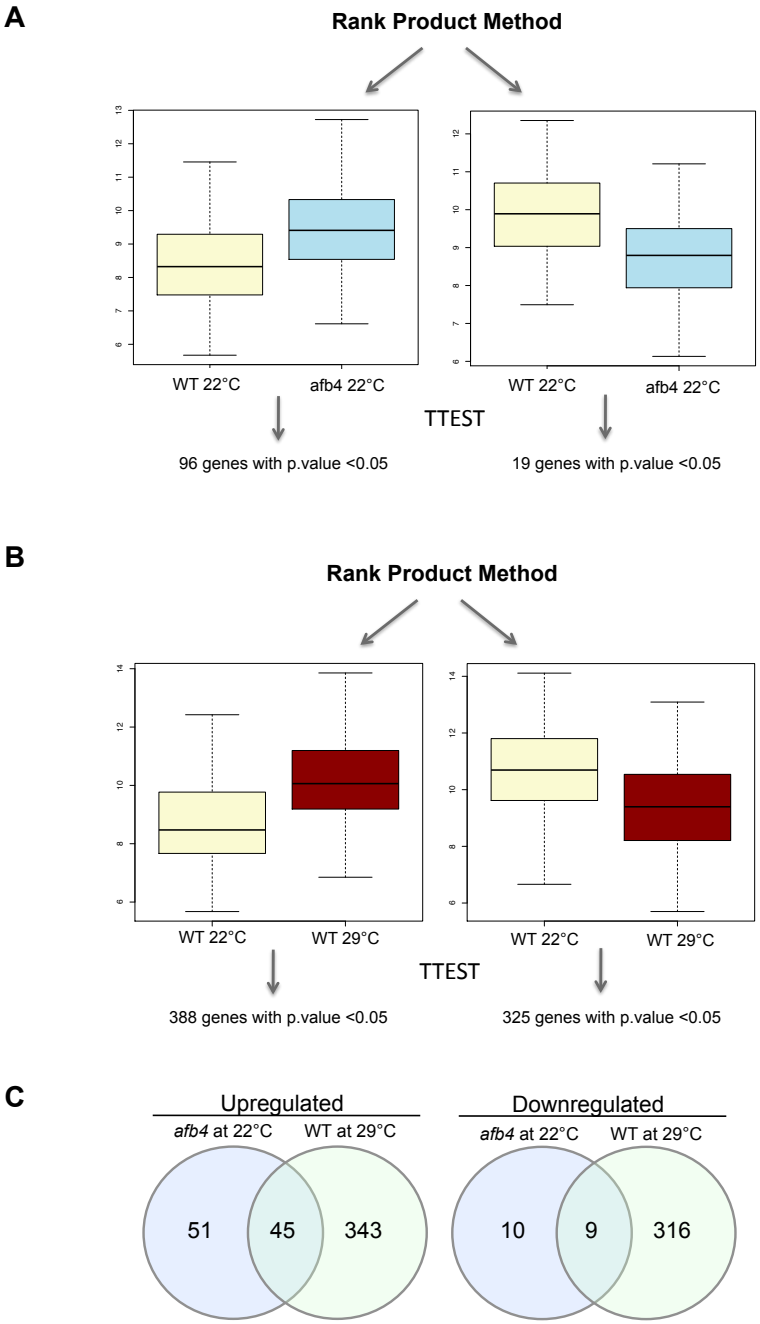

Figure 4.1 AFB4 regulates a set of genes that are temperature responsive in the hypocotyl. Average expression values for genes identified as differentially expressed in *afb4-2* at 22°C (a) and WT at 29°C (b) using the RankProd package, genes were further filtered by a student's t-test using a 0.05 cutoff. (c) Venn diagrams showing the overlap between the significantly up and downregulated genes from (a) and (b).

Table 4.1 Genes responding to 29°C show enrichment of temperature related GO categories. GO enrichment of the genes identified by RankProd as significantly upregulated (a) and downregulated (b) by 29°C in wild type using the DAVID functional annotation tool with a Benjamini p.value cutoff of 0.05 (http://david.abcc.ncifcrf.gov/) (Huang da et al., 2009a, b).

A

Category	Term	PValue	Benjamini
BP	GO:0019748~secondary metabolic process	5.28E-11	3.31E-08
BP	GO:0006979~response to oxidative stress	1.57E-09	4.92E-07
BP	GO:0042542~response to hydrogen peroxide	4.02E-07	8.39E-05
BP	GO:0009408~response to heat	9.87E-07	1.54E-04
BP	GO:0000302~response to reactive oxygen species	2.81E-06	3.51E-04
BP	GO:0009644~response to high light intensity	6.36E-06	5.69E-04
BP	GO:0016143~S-glycoside metabolic process	6.36E-06	5.69E-04
BP	GO:0019757~glycosinolate metabolic process	6.36E-06	5.69E-04
BP	GO:0019760~glucosinolate metabolic process	6.36E-06	5.69E-04
BP	GO:0019758~glycosinolate biosynthetic process	6.07E-06	6.33E-04
BP	GO:0019761~glucosinolate biosynthetic process	6.07E-06	6.33E-04
BP	GO:0016144~S-glycoside biosynthetic process	6.07E-06	6.33E-04
BP	GO:0009642~response to light intensity	2.37E-05	0.001646
BP	GO:0010035~response to inorganic substance	2.32E-05	0.001810
BP	GO:0009266~response to temperature stimulus	6.74E-05	0.003830
BP	GO:0044272~sulfur compound biosynthetic process	6.19E-05	0.003865
BP	GO:0009407~toxin catabolic process	9.50E-05	0.004946
BP	GO:0009404~toxin metabolic process	9.50E-05	0.004946
CC	GO:0030312~external encapsulating structure	2.58E-04	0.014573
BP	GO:0016137~glycoside metabolic process	4.34E-04	0.020673
BP	GO:0016138~glycoside biosynthetic process	5.02E-04	0.020753
BP	GO:0006790~sulfur metabolic process	4.94E-04	0.021865
CC	GO:0005618~cell wall	2.08E-04	0.023458
MF	GO:0004364~glutathione transferase activity	9.28E-05	0.030152
BP	GO:0080003~thalianol metabolic process	7.85E-04	0.030243

Table 4.1 Continued.

B

Category	Term	PValue	Benjamini
CC	GO:0044434~chloroplast part	1.07E-25	3.49E-24
CC	GO:0044436~thylakoid part	9.46E-26	4.13E-24
CC	GO:0031984~organelle subcompartment	8.48E-26	5.55E-24
CC	GO:0009534~chloroplast thylakoid	6.79E-26	8.90E-24
CC	GO:0031976~plastid thylakoid	6.79E-26	8.90E-24
CC	GO:0044435~plastid part	5.31E-25	1.39E-23
CC	GO:0009579~thylakoid	1.11E-24	2.43E-23
CC	GO:0042651~thylakoid membrane	1.01E-23	1.90E-22
CC	GO:0034357~photosynthetic membrane	1.79E-23	2.93E-22
CC	GO:0055035~plastid thylakoid membrane	2.39E-23	3.48E-22
CC	GO:0009535~chloroplast thylakoid membrane	2.39E-23	3.48E-22
BP	GO:0015979~photosynthesis	9.05E-20	5.17E-17
CC	GO:0009526~plastid envelope	1.63E-13	2.14E-12
CC	GO:0009941~chloroplast envelope	3.11E-13	3.70E-12
CC	GO:0009507~chloroplast	5.94E-12	6.48E-11
CC	GO:0031090~organelle membrane	1.02E-11	1.03E-10
CC	GO:0009536~plastid	1.96E-11	1.84E-10
CC	GO:0009521~photosystem	9.27E-11	8.10E-10
BP	GO:0019684~photosynthesis, light reaction	3.94E-12	1.13E-09
CC	GO:0009543~chloroplast thylakoid lumen	5.48E-10	4.49E-09
CC	GO:0031978~plastid thylakoid lumen	5.48E-10	4.49E-09
CC	GO:0009532~plastid stroma	1.58E-09	1.22E-08
CC	GO:0009570~chloroplast stroma	2.80E-09	2.04E-08
CC	GO:0031977~thylakoid lumen	3.50E-09	2.41E-08
CC	GO:0031967~organelle envelope	4.88E-09	3.20E-08
CC	GO:0031975~envelope	5.95E-09	3.71E-08
BP	GO:0009063~cellular amino acid catabolic process	1.90E-08	3.61E-06
CC	GO:0009523~photosystem II	6.09E-07	3.62E-06
BP	GO:0009310~amine catabolic process	3.50E-08	5.00E-06
CC	GO:0010287~plastoglobule	1.54E-06	8.79E-06
BP	GO:0006091~generation of precursor metabolites and energy	1.26E-07	1.44E-05
MF	GO:0016168~chlorophyll binding	6.24E-08	2.23E-05
BP	GO:0009416~response to light stimulus	2.37E-07	2.26E-05
CC	GO:0048046~apoplast	5.54E-06	3.03E-05
BP	GO:0009765~photosynthesis, light harvesting	4.65E-07	3.32E-05
BP	GO:0009314~response to radiation	4.39E-07	3.58E-05
BP	GO:0046395~carboxylic acid catabolic process	5.77E-07	3.66E-05
BP	GO:0016054~organic acid catabolic process	5.77E-07	3.66E-05
CC	GO:0030076~light-harvesting complex	1.99E-05	1.04E-04
BP	GO:0016051~carbohydrate biosynthetic process	7.99E-06	4.56E-04
CC	GO:0030095~chloroplast photosystem II	9.16E-05	4.61E-04
CC	GO:0009522~photosystem I	2.51E-04	0.001218
BP	GO:0019253~reductive pentose-phosphate cycle	3.57E-05	0.001851
BP	GO:0019685~photosynthesis, dark reaction	4.94E-05	0.002346
BP	GO:0009628~response to abiotic stimulus	7.70E-05	0.003375
BP	GO:0015977~carbon utilization by fixation of carbon dioxide	1.44E-04	0.005847
BP	GO:0009637~response to blue light	1.97E-04	0.007466
BP	GO:0010114~response to red light	2.18E-04	0.007738
CC	GO:0009654~oxygen evolving complex	0.001800	0.008394
BP	GO:0009639~response to red or far red light	3.14E-04	0.010483
BP	GO:0055114~oxidation reduction	3.38E-04	0.010679
MF	GO:0008453~alanine-glyoxylate transaminase activity	6.59E-05	0.011721
BP	GO:0009853~photorespiration	4.03E-04	0.012032
BP	GO:0010218~response to far red light	5.62E-04	0.015924
BP	GO:0006073~cellular glucan metabolic process	6.82E-04	0.018372
BP	GO:0016052~carbohydrate catabolic process	0.001293	0.033030
MF	GO:0046906~tetrapyrrole binding	3.05E-04	0.035732
CC	GO:0005576~extracellular region	0.008477	0.037727

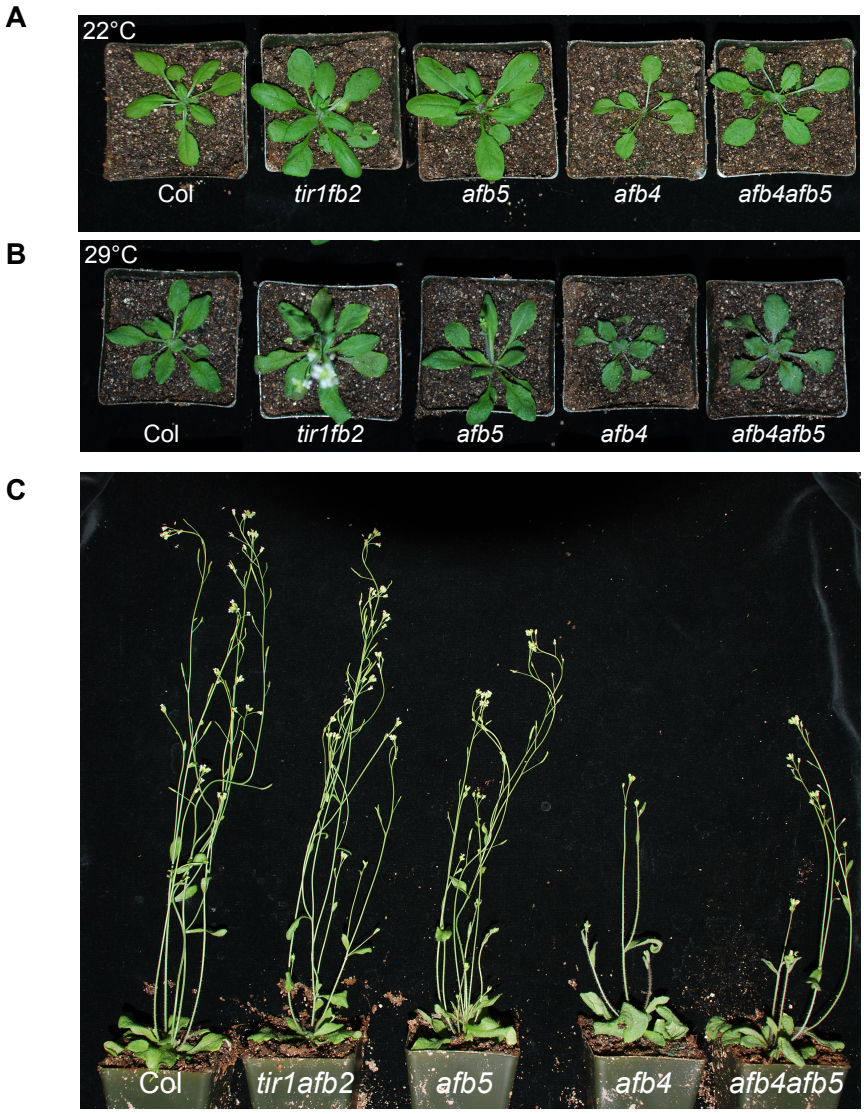

Figure 4.2 *afb4-2* adult plants are hypersensitive to 29°C. Wild type and mutant 10 day old seedlings were transferred to soil and grown under constant light at 22°C (a) or 29°C (b & c). Plants were imaged prior to bolting (a & b) and following bolting of *afb4-2* at 29°C (c).

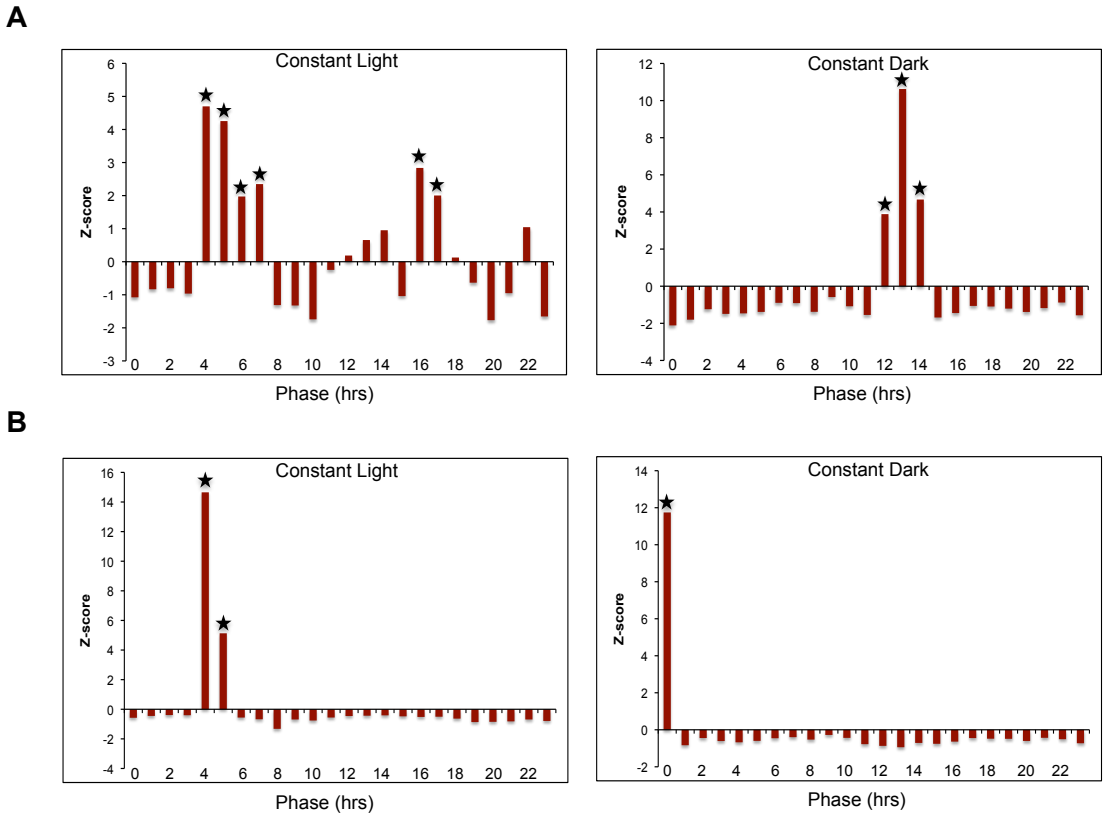

Figure 4.3 Genes differentially expressed in long hypocotyl conditions are enriched for circadian regulated genes. Genes upregulated (a) and downregulated (b) in common between *afb4-2* at 22°C and wild type at 29°C were input into the Phaser analysis tool and compared against cycling genes under constant light and dark conditions (http://phaser.cgrb.oregonstate.edu/) (Michael et al., 2008). Bars represent the z-scores for the enrichment of cycling genes within our list compared to all the genes shown to cycle under constant light or dark conditions at a given phase of the day. Phase 0 represents the start of the day. Asterisks indicate significant enrichment with a $p<0.05$.

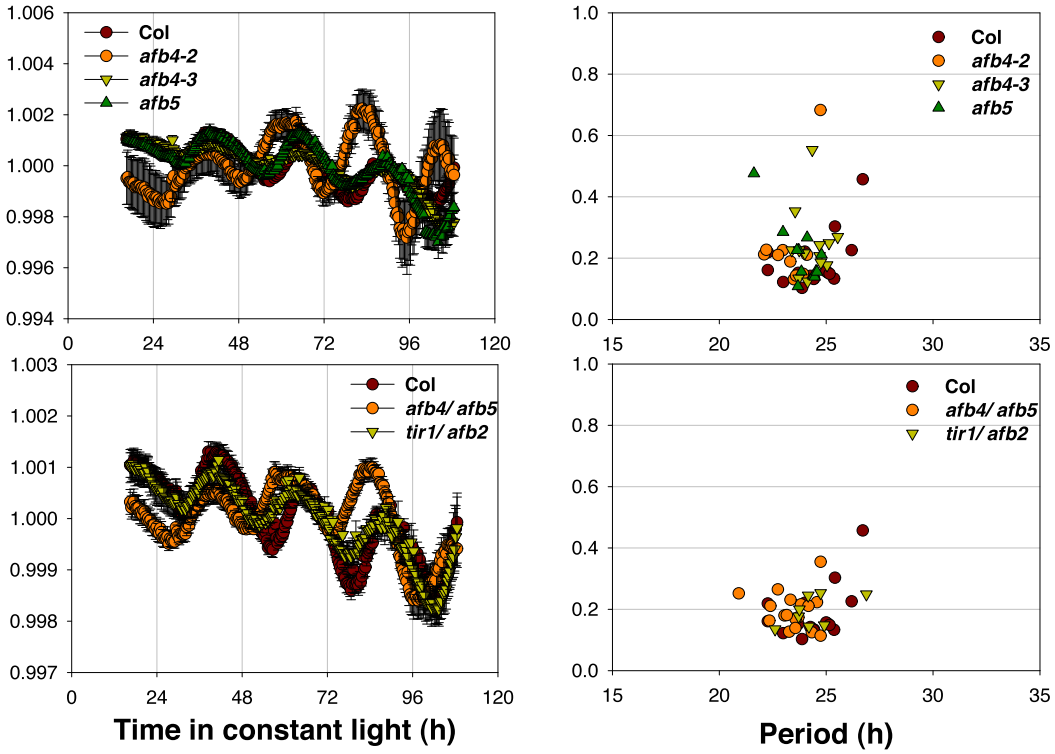

Figure 4.4 The *afb4-2* mutant has a short period phenotype. Cotyledon movement assays for Col-0, *afb4-2, afb4-3, afb5-5, afb4-2 afb5-5, tir1-1 afb2-3*. Seedlings were entrained in 12L:12D conditions for 5 days and then transferred to 24-well plates and shifted to constant light. Cotyledon movement was imaged for 7 days.

Table 4.2 The *afb4-2* mutant has a unique ~23 hr period phenotype. Quantification of the leaf movement assay shown in Fig. 4.4. Data was analyzed by fast Fourier transform-nonlinear least squares (Plautz et al., 1997; Zhong et al., 1997).

		Col	*afb4-2*	*afb4-3*	*afb5*	*afb4/afb5*	*tir1/afb2*
Number		16	10	13	12	17	8
Period	*Mean=*	24.44	23.32	24.36	23.86	23.43	24.37
	SEM=	0.32	0.26	0.19	0.25	0.24	0.44
RAE	*Mean=*	0.19	0.24	0.24	0.21	0.20	0.19
	SEM=	0.02	0.05	0.03	0.03	0.01	0.02
	T Test=		0.011	0.825	0.160	0.017	0.890

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CHAPTER 5

Conclusions and Future Directions

The AFB4 clade of auxin receptors are the targets of picolinate herbicides

Auxinic herbicides have been widely used in agriculture for over 50 years because of their selectivity for broadleaf weeds. Included among this group of herbicides is the synthetic auxin picloram of the pyridine class. Understanding how these herbicides act in the plant is crucial for identifying the cause of herbicide resistance in weeds, a growing problem over the past two decades (Zheng and Hall, 2001). Chapter two outlines work performed to characterize picloram resistance in *Arabidopsis* based on an *afb5* allele that was first identified in a screen for picloram resistant mutants (Walsh et al., 2006). Characterization of the *afb4-2* and *afb5-5* alleles revealed that these receptor mutants show a selective resistance to picloram whereas the other receptor mutants do not. This selectivity was validated through biochemical analysis demonstrating that AFB4 and AFB5 are able to interact with the Aux/IAA repressor proteins in a picloram dependent manner. This affinity for picloram is unique to AFB4 and AFB5 and suggests that the structure of the auxin-binding pocket in these proteins differs compared to TIR1/AFB1-3 in such a way that allows the binding of picloram. These results suggest that the AFB4 clade is the major target of picloram in plants and raises a number of important questions about the structural differences of AFB4 and AFB5 compared to the other family members. It is unknown whether the ~42 amino acid N-terminal extension of AFB4/5 contributes to their function, although there is only ~30% sequence identity between them. Further biochemical studies are needed to elucidate the functional differences of these proteins compared to TIR1/AFB1-AFB3 in terms of their interaction with the SCF complex and their Aux/IAA targets.

AFB4 is a negative regulator of auxin signaling in developing seedlings

Along with the unique resistance to picloram, the *afb4-2* mutant showed a number of other distinctive phenotypes. These phenotypes include short primary roots, an increase in lateral root production and long hypocotyl and petioles. These are opposite to the phenotypes observed in the *tir1-1 afb2-3* double mutant that has long primary roots, few lateral roots and a short hypocotyl, consistent with reduced auxin response. In addition, AFB4 appears to be epistatic to TIR1/AFB1-3 in terms of hypocotyl, petiole and primary root growth as seen by the *afb4-2*-like phenotypes in the *tir1-1 afb2-3 afb4-2* triple mutant. This data suggests a novel function for AFB4 as a negative regulator of auxin signaling. The phenotypes observed in *afb4-2* are similar to those of auxin overproducing mutants, such as the *yucca* overexpressor *yuc-1D* (Zhao et al., 2001). IAA measurements in *afb4-2* did not show any differences in auxin levels in hypocotyls or cotyledons compared to wild type suggesting that AFB4 is not regulating auxin biosynthesis, conjugation or transport.

Interestingly, several auxin marker genes showed an increase in transcript level by qRT-PCR following a 2hr IAA treatment in *afb4-2* hypocotyl tissue compared to wild type. This result, along with the observation that *afb4-2* hypocotyl growth is inhibited at lower concentrations of IAA than wild type, suggests that *afb4-2* is hypersensitive to endogenous levels of auxin. This is consistent with a role for AFB4 as a negative regulator of auxin signaling. The presence of another negative feedback loop in auxin signaling is not surprising given the multitude of regulatory loops occurring at the level of auxin biosynthesis, conjugation and transport (Leyser, 2010). This inherent network topology of auxin signaling emphasizes the need for new systems biology approaches to map these interconnected regulatory modules. With this in mind, we simplified our question and chose to focus on the process of

auxin-dependent cell elongation in the hypocotyl, a tissue that grows entirely by cell expansion (Gendreau et al., 1997).

Picloram regulates IAA responsive genes in the hypocotyl

Although simple in structure, hypocotyl growth relies on the coordination of light, clock and hormone signaling pathways (Nozue et al., 2007; Michael et al., 2008; Niwa et al., 2009). This complex regulation limits the ability to use classical genetics to uncover important components of the auxin-dependent pathway due to extensive crosstalk with these parallel pathways. The work in chapter three describes a hypocotyl transcriptomics approach to uncover genes involved in auxin-mediated cell expansion. The strong resistance of the *afb5-5* mutant to picloram induced hypocotyl growth provided a good control for isolating genes required for the growth response following picloram treatment. This analysis resulted in the identification of a large set of picloram-responsive hypocotyl growth-dependent genes. Comparisons with IAA treatment microarrays revealed that picloram acts through the same transcriptional network as IAA to promote hypocotyl growth. The lack of response in the *afb5-5* mutant confirmed that the picloram promotion of hypocotyl growth is mediated almost exclusively by AFB5. This result supports the hypothesis that AFB4 is unique in its role as a negative regulator and although it plays a minor role as a positive regulator in response to picloram, AFB5 is the primary picloram receptor.

Auxin regulates growth dependent genes in the hypocotyl in part independently of PIF4/5

A subset of the genes identified as auxin-responsive in the hypocotyl were previously shown to be PIF4/5 dependent (Nozue et al., 2011). Our experiment was

designed to limit the detection of auxin-independent growth promoting genes by treating seedlings during the day when growth is typically inhibited by the light and GA signaling pathways through inhibition of PIF4/5 (Dowson-Day and Millar, 1999; de Lucas et al., 2008). A time course experiment following the same experimental design with the *pif4-1 pif5-101* double mutant resulted in a similar increase in hypocotyl growth following IAA treatment compared to wild type. A selection of PIF4/5-dependent genes were shown to be induced in *pif4-1 pif5-101* following IAA treatment by qRT-PCR suggesting that the initial hypocotyl growth response to auxin during the day is not dependent on PIF4/5. This could explain a mechanism for the plant to respond to rapid changes in the environment, such as flooding, with stem elongation through activation of auxin signaling.

Although a number of genes identified in this analysis are targets of the auxin-signaling pathway it remains unclear which Aux/IAA-ARF combinations are mediating this response. Further transcriptome experiments in the various *iaa* and *arf* mutants would provide valuable insight into the complex regulation of hypocotyl growth. The long hypocotyl phenotype of the *arf8* mutant and the recent study demonstrating a role for ARF8 during petal growth suggests an important role for this clade in regulating cell expansion and division (Tian et al., 2004; Varaud et al., 2011). Identifying the targets and interactors of the ARFs remains a central challenge. A recent study by Vernoux *et al.* (2011) used a yeast-two-hybrid approach to test all the ARF/Aux/IAA combinations in order to create an ARF-Aux/IAA interaction network. Information about the ARF-Aux/IAA network, ARF and TIR1/AFB expression data, auxin-responsive genes and Aux/IAA degradation was inputted into a mathematical model to predict the timing and spatial requirements for patterning the shoot apex (Vernoux et al., 2011). As more high-throughput data becomes available

mathematical models will be important tools for integrating the information into a more dynamic network view.

AFB4 is important for the circadian regulation of hypocotyl growth

The discovery of the short period phenotype in the *afb4-2* mutant suggests a role for auxin as an input into the clock, a process that has been discounted in previous studies (Hanano et al., 2006; Covington and Harmer, 2007). The exact mechanism for this process is unknown but appears to be specific to AFB4. The significant overlap between genes regulated by AFB4 at 22°C and temperature responsive genes suggests that AFB4 might be important for an auxin-dependent role in temperature compensation or entrainment by the circadian clock. Work is underway to test this hypothesis. Given the importance of auxin for plant growth and the coordination of these developmental processes by the circadian clock, it is not surprising that their signaling pathways are intertwined. It is important for future experimental designs to consider the involvement of the clock and to take into account the environmental conditions that affect plant growth including seasonal changes and daily temperature fluctuations. These factors will become increasingly more relevant as knowledge from *Arabidopsis* is translated to crop plants in order to improve productivity in the future. A better understanding of the molecular pathways in the context of the natural environment will improve our ability to apply knowledge from *Arabidopsis* to other plant species.

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