CHARACTERIZATION OF THE *ARABIDOPSIS THALIANA* AUXIN F-BOX FAMILY MEMBERS AFB4 AND AFB5

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This work is dedicated to my Grandparents, Peery and Eileen Mooney, for not only their unconditional love but their simple desire for me to find and pursue the paths in life that make me happy. This work is also dedicated to my beautiful daughter, Cecilia, in the hope that I can show this purity of heart to her along with being a good mentor and guardian.

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Abstract

Sutton Mooney

CHARACTERIZATION OF THE *ARABIDOPSIS THALIANA* AUXIN F-BOX FAMILY MEMBERS AFB4 AND AFB5

Ubiquitination is a three-step pathway that links a chain of small molecules, UBQ, onto a substrate protein. UBQ serves as a signal that the tagged protein is to be targeted for degradation by the 26S proteasome. This ubiquitin-mediated protein degradation system is highly conserved throughout eukaryotic cells, including the model plant *Arabidopsis thaliana*. In *Arabidopsis* many growth and developmental process are influenced by the plant hormone auxin. Transcription factors involved in auxin response have been shown to be targets for ubiquitination and subsequent degradation. Recent work has identified a family of auxin receptors, TIR1, AFB1, AFB2 and AFB3, are key components of this process. This work is focused on the characterization of two additional members of this family AFB4 and AFB5. Using a combination of molecular biology, genetics and biochemistry I have begun the characterization of these two proteins plant development and their possible function as auxin receptors. While both proteins are clearly involved in auxin response, AFB4 has a more pleiotropic role in all aspects of the plant life cycle. This work also shows that AFB5, and AFB4 to a lesser extent, can mediate a receptor-like interaction in the presence of the synthetic auxin picloram, leaving open the question of whether or not they participate as auxin receptors in the plant to the same level as the other four family members.

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INTRODUCTION

One of the most fascinating regulatory systems to be discovered and extensively studied over the past 15 years is the ubiquitin – 26S proteasome pathway. This complex and highly regulated system is conserved across all eukaryotes and is involved in virtually all aspects of cellular growth and development from embryogenesis to the death of organisms. In the model plant system, *Arabidopsis thaliana*, groundbreaking work in the Estelle lab first linked this system to regulation of response to the phytohormone auxin through transcription factor interactions [1-3], and subsequent research has demonstrated that the pathway is involved in many other phytohormone responses [1]. Phytohormones are key signaling molecules with important roles throughout the life cycle of the plant. Auxin in particular plays an important role in all stages of plant life from embryogenesis [2] to senescence [3] as well as in responses to light [4], gravity [5] and certain stresses such as pathogens [9]. Continued research is showing that these phytohormones frequently act in overlapping processes and function in a complex network of crosstalk between one another [10-12]. The focus of this thesis is on the characterization of two members of a family of genes involved in auxin regulation via the ubiquitin/proteasome pathway.

Auxin

From Darwin's experiments in the mid-1800s on the phototropic curvature of canary grass coleoptiles to the recent identification of a family of auxin receptors, the field of auxin research has been a driving force behind the understanding of plant growth and development. Indole-3-acetic acid (IAA) is the most common form of auxin found in

plants and is derived by either tryptophan-dependent or independent pathways [6]. Several pathways for converting the amino acid tryptophan to IAA have been proposed for *Arabidopsis*, but the lack of mutants in the biosynthetic enzymes and the functional redundancy of the pathways have hampered efforts to clearly define the steps in this process. Work on one family of IAA biosynthesis genes, the YUCCAS, has led to some recent progress in understanding the importance of regulating auxin biosynthesis for organ and vasculature development [7]. YUC1 was originally identified as an auxin overexpressor [8]. This gene encodes a flavin monooxygenase that has been shown in plant pathogenic bacteria to be part of the tryptamine (TAM) pathway and is a likely branch of the trp-dependent pathway in *Arabidopsis*. There are eleven predicted YUC genes and none of the single loss-of-function mutants studied so far show obvious phenotypes. However, combinations of four of these mutants along with expression analysis have shown both redundancy and unique function. Reduction in the expression of the auxin reporter DR5-GUS in the higher order mutants indicates that the phenotypes are due to less auxin. Rescue by expression of a bacterial auxin biosynthesis gene but not exogenous auxin application indicates that the effects are linked to tissue specific auxin biosynthesis. Analysis of tryptophan biosynthesis mutants led to the discovery of a Trp-independent pathway branching off from indole-3-glycerol phosphate or indole prior to tryptophan synthesis [6]. Continued studies following metabolism of isotope labeled indole should help better define this alternate biosynthesis process. Enzymes from these pathways have been identified in the chloroplast and cytoplasm suggesting cellular trafficking of metabolites during the conversion processes rather than a localized complex for producing IAA [9].

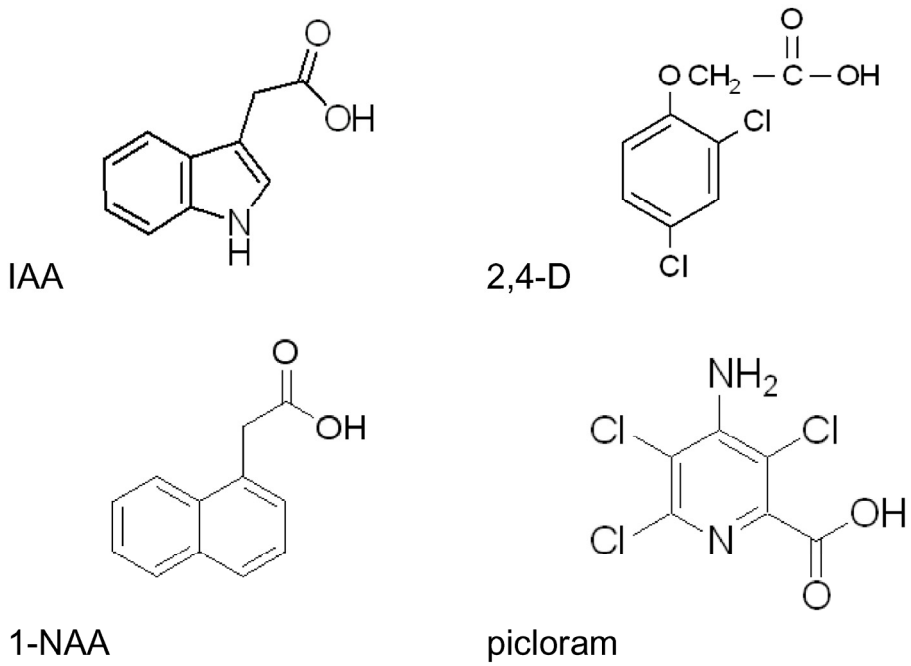


Figure 1. Structures of natural and synthetic auxins. Chemical structures of auxins commonly used in research and as herbicides.

Plants that either overproduce auxin or are given exogenous auxin display characteristic phenotypes [10]. Effects of high levels of auxin include inhibition of root elongation, increased lateral and adventitious root production, elongated hypocotyls and epinastic leaves. Chemical synthesis of IAA and other auxin derivatives has been a boon not only to plant biology research but also to the farm industry (Fig. 1). In the fields, synthetic auxins are used as herbicides and in the lab the compounds are used to study all aspects of auxin biology. Synthetic auxins have been used in screens to identify auxin resistant mutants leading to the discovery of auxin-regulated processes. Two of the most commonly used synthetic auxins are 2,4-dichlorphenoxy acetic acid (2,4-D) and naphthalene-1-acetic acid (1-NAA). Early studies in tobacco cells comparing the movement of these two auxins

compared to the natural form, IAA, yielded interesting and useful data in the study of cellular auxin transport [11]. It was shown that IAA moved efficiently into and out of the cell via influx and efflux carriers, 1-NAA passively diffused into the cell and then required efflux carriers to exit, and 2,4-D was transported into the cell by influx carriers but not efficiently transported back out by the efflux carriers (Fig. 2a).

The development of highly sensitive techniques for measuring the incorporation of deuterium from $^{2}H_2O$ solutions has made the search for the sites of auxin biosynthesis possible. Studies done with *Arabidopsis* seedlings at different stages from germination through vegetative growth, identified the highest levels of IAA in young leaves undergoing active cell division and at the onset of vascular differentiation [12]. As tissue growth switched from cell division to cell elongation IAA levels dropped by 90%. IAA levels remained low in cotyledons but in hypocotyls showed a significant increase after 12-13 days, perhaps reflecting their increased transport ability. To examine synthesis and exclude auxin transport as a source, individual organs were dissected from ten-day old seedlings and fed 30% $^{2}H_2O$ for 12 or 24 hours. Deuterium incorporated IAA was seen in all organs with the youngest leaves having the highest capacity for IAA synthesis. When intact plants were given the polar auxin transport inhibitor, NPA, to trap IAA in the cells, a two-fold increase followed by a sharp decrease of IAA levels was observed over a 24-hour period in expanding leaves but not in the youngest developing leaves. This implies a developmental stage dependent feedback regulation of auxin biosynthesis.

Even more refined mass spectrometry systems have allowed for careful dissection and

measurement of auxin levels in as little as 0.5mg of *Arabidopsis* root tissue [13]. The ability to synthesize IAA is found along the length of the entire root, but the highest capacity is at the primary and lateral root tips, which display equivalent rates. The shoot derived auxin is the main source in the young primary root and is needed for the development of early lateral roots [14]. A ten-fold increase in root-derived auxin is seen between three and ten days after germination. Measurements of 2 mm root sections of eight day old seedlings revealed that a sharp gradient was present with the highest levels at the root apex [13]. Even finer sectioning of 0.5mm at the root tip showed that the highest rates of auxin synthesis are in the root meristem. Removal of the apical part of the plant however, demonstrated that the shoot is the main auxin source and is responsible for maintaining the basipetal IAA concentration gradient in the root. The importance of polar movement of auxin down into the root for early development is seen when polar auxin transport was inhibited by NPA in four-day old seedlings. At this stage, a decrease in newly synthesized auxin in the root was measured. This effect was not seen in eight-day old seedlings. It is also postulated that phloem transport of auxin as a nonpolar route may be important in this slightly later stage of root development. Application of NPA to ten-day old plants did not block auxin transport from shoot to root supporting this idea.

Polar Auxin Transport

The directional transport of auxin has been the subject of study for many decades. Since its inception in the 1960s, the chemiosmotic hypothesis has provided a framework for thinking about this important process. This theory states that in an energy dependent manner, cells maintain a pH gradient across the membrane that favors the directional

flow of uncharged molecules into the cytoplasm. In the 1970s the chemiosmotic model of polar auxin transport was postulated, which proposed that specific carriers were involved to move auxin into and out the cell in a directional manner.

There are two ways that auxin can enter a plant cell (Fig. 2). One involves the pH gradient across the plasma membrane, which facilitates the flow of protonated IAAH, from the acidic apoplast (pH 5.5) into the neutral cytoplasm (pH 7.0). The dissociated IAA^- ion becomes trapped and cannot passively diffuse back out [15]. The second transport mechanism is through membrane-inserted carriers. For auxin to be moved through the plant in a directional manner i.e. basipetal (tip to base) or acropetal (base to tip), these carriers must be localized in the cell membrane in an asymmetric fashion. Additionally if the flow of auxin needs to be redirected to initiate new growth or in response to abiotic factors such as light or gravity, then these carriers must be efficiently redistributed in the cell. One family of influx carriers, AUX/LAX and a family of efflux carriers, the PIN proteins, have been extensively studied in *Arabidopsis*.

The *aux1* mutant was first discovered in 1980 in a screen for resistance to the synthetic auxin 2,4-D and in the mid 1990s the gene was identified as an auxin permease with three homologs in *Arabidopsis* called *LAX1-3* [16]. The rescue of the *aux1* agravitropic phenotype with 1-NAA rather than 2,4-D, and the higher incorporation of radioactive 1-NAA than 2,4-D, demonstrated that this transmembrane protein was involved in auxin uptake [24, 25]. In the lateral root cap cells AUX1 is symmetrically localized and thought

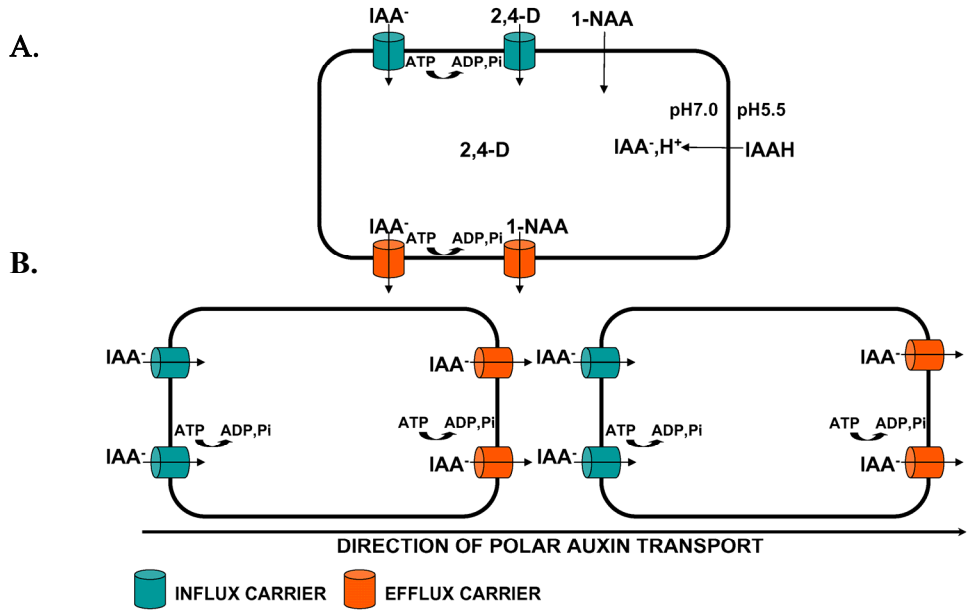


Figure 2. Auxin movement into and out of the cell. **A.** Influx carriers and diffusion mediate IAA entry into cell and efflux carriers promote transfer out of the cell. 1-NAA diffuses into the cell and is transported out by the efflux carriers. 2,4-D is a substrate for the influx carriers but a poor substrate for the efflux carriers. **B.** Asymmetrical localization of the influx and efflux carriers allows transport of auxin in a polar direction.

to permeabilize the cell to auxin influx rather than actively direct auxin transport in response to gravity. In root protophloem cells AUX1 is asymmetrically localized to the upper plasma membrane where it is proposed to unload auxin from the phloem where it is then directed into the carrier-dependent polar transport system in the root apex [17].

The polar localization of AUX1 to the membrane is dependent on AXR4 [27]. Although *axr4* plants were identified over ten years ago in a screen for auxin resistant root

elongation [18] the gene was only recently identified and cloned. This single copy, plant specific gene has one membrane-spanning domain and, through an αβ hydrolase fold, putative hydrolase activity [19]. AXR4 localizes to the endoplasmic reticulum and in *axr4* plants an accumulation of AUX1 at the ER is observed [27]. Interestingly this defect is seen only in cells were AUX1 normally displays polar localization. In other cells plasma membrane insertion of AUX1 is unaffected. Whether AXR4 activity influences AUX1 targeting directly through chaperone activity or facilitating a post-translational modification or indirectly by affecting lipid composition of vesicle trafficking components is not clear [19].

The mechanisms of cellular auxin efflux began to be uncovered with the discovery in 1991 of a mutant that resembled wild-type *Arabidopsis* grown in the presence of polar auxin transport inhibitors [20]. Named *pin1-1* for its striking phenotype, a flowerless, needle-like stem, this transmembrane protein is part of a family known as the PIN proteins. Three of the PINs (5, 6 and 8) have yet to be functionally characterized but extensive work has been done on the other five [15]. As early as the two-cell stage of embryogenesis the importance of PIN localization is seen. Apical localization of PIN7 in the lower cell directs the flow of auxin into the apical cell. When this cell grows and divides, proximal (lower) expression of PIN1 in these cells directs auxin out of the embryo. PIN7 then switches polarity and directs auxin flow to the base of the embryo thus setting the shoot-root pattern of the plant [21]. These gradients of auxin act as signals that affect the activity of transcription factors that regulate the expression of genes involved in embryo patterning and development [22]. As the plant grows, distribution of

the PIN proteins directs auxin away from the shoot apical meristematic region and into the developing tissue that acts as a sink initiating the sites of leaf formation [33].

In addition to it's role in initiation of new leaf primordia, recent work looking at leaf patterning has demonstrated that PIN1 activity is important in directing auxin to convergence points in the epidermis where the vasculature is established [23]. The most extensive work on the PIN proteins has been done in the root using fluorescent tags. This allowed the observation of their asymmetrical subcellular localization and led to recognition of their importance in enabling polar auxin transport. The PINs have both distinct and overlapping expression in root, and the functional redundancy is also seen by the increased developmental defects in the different combinations of double and triple mutants [35]. Specific apical and basal localization patterns of the PIN proteins in the different root tissue have also been observed. To begin to understand if this directionality is cell driven or sequence driven, *PIN1* was expressed behind the *PIN2* promoter and expression was compared to *PIN2* promoter driven *PIN2* in cells where *PIN1* is not normally strongly expressed [24]. Interestingly, in cortical cells PIN1 mimicked PIN2 basal localization. However in epidermal cells PIN1 was seen in the basal rather than apical cell membrane and could not rescue the *pin2* agravitropism. *PIN2* promoter driven constructs were designed which disrupted internal sequences in *PIN1,* and one was found which reversed the PIN1 polarity from basal to apical in the epidermal cells. This was sufficient to rescue the gravitropic response in *pin2* plants. It will be interesting to see if further analysis can pinpoint how specific sequences are affecting localization.

Redistribution of PIN proteins within the cell has also been seen. PIN3 is evenly distributed on the lower side of the columella cells until a gravity stimulus is applied which changes the cellular polarity. PIN3 responds to the change by not only relocating to the newly established basal side of the cells but is enhanced on the lower side of the root. The redirected flow of auxin leads to asymmetric cell elongation and organ growth which is visualized by the tropic response seen in the plant i.e. bending towards gravity or light source [15].

This ability of the PIN proteins to be redistributed in the cell underlines the importance of PIN cycling and control of its localization. Several factors have been identified that affect this process. Mutants in a Ser/Thr kinase termed PINOID (PID) displays similar phenotypes to the original *pin1* mutant. Plants overexpressing PID experience collapse of the primary root caused by auxin depletion. This could be rescued by polar auxin transport inhibitors [25] or expression in the *pin2* or *pin4* background [38]. PID overexpression resulted in a switch from basal to apical localization of PIN1, PIN2 and PIN4 and it is thought that the phosphorylation state of the proteins is the signal for membrane localization. PID transcription is auxin inducible. There is growing evidence that the PIN proteins are transported in vesicles that require the actin network and that this transport is under control of auxin. Three proteins related to vesicle trafficking and directly linked to PIN targeting have been identified. GNOM, a guanine nucleotide exchange factor for small G-proteins of the ARF class, which mediates exocytic vesicle formation, SMT1, required for sterol membrane synthesis, and SCARFACE, a vesicle trafficking regulator [15]. Additionally, plants blocked in the endosome to plasma membrane secretory

pathway trap PIN1 in intracellular compartments, and this can be reversed by the application of the actin depolymerizing drug Cytochalasin D [26]. Recent work has demonstrated the importance of auxin to the organization of the actin network [27]. Tobacco cells experiencing constitutively bundled actin filaments have increased sensitivity to the polar auxin transport inhibitor NPA and are impaired in synchronous cell division. When exposed to the polar transportable auxins IAA or 1-NAA, but not 2,4-D, the actin is debundled and cell division restored. The controversial interpretations surrounding auxin transport, likening it to animal neurotransmitter systems, add an interesting and exciting perspective to this field of plant science [28].

The importance of auxin regulation on *PIN* expression has been demonstrated both directly and through the influence of other auxin-regulated genes. The AP2 transcription factors PLT1 and PLT2 are part of an auxin-PIN feedback loop controlling patterning of root primordium [35]. Microarray data comparing *PIN* levels in wild-type and in mutants for the Aux/IAA transcription factor and SCF^{TIR1} substrate *iaa3* showed that the increase in PIN expression seen with auxin application was blocked in the *iaa3* background [29]. This, together with the stabilization of PIN2 by proteasome inhibitors [30], may correlate an aspect of PIN regulation to the auxin mediated ubiquitin-proteasome pathway.

Another 22-member family of multi-drug resistant/P-glycoproteins (MDR/PGP) has recently been identified in *Arabidopsis* of which some are discussed to function as auxin carriers. Yeast two-hybrid, coimmunoprecipitation and colocalization data, along with the stabilizing effect of PGP19 on PIN1 in the plasma membrane, imply a PGP-PIN

interaction [44, 45]. PGPs exhibit both polar and nonpolar membrane localization, actively export auxin, with one member catalyzing import, and bind tightly to the auxin transport inhibitor NPA [46-48]. Multiple mutant combinations show not only additive and synergistic effects with the PINs but also unique and separate functions for the PGPs that may encompass a non-auxin transport role [15].

Auxin-regulated transcription

There are two major families of transcription factors in *Arabidopsis* that control the activity of auxin response genes, the Aux/IAAs and the ARFs (Fig. 3). The *Aux/IAAs* were some of the first auxin responsive genes to be discovered in soybean and pea, and this group is found across many diverse plant species [31]. In *Arabidopsis* most of the 29 members [32] of this family are rapidly induced by auxin and contain four conserved domains [33]. It is through the activity of the domains that these transcription factors indirectly exert their effects on other auxin response genes. Domains III and IV contain protein binding domains which facilitate dimerization with the ARFs [34]. Domain II is important for interaction with the $SCF^{TIR/AFB}$ ubiquitin protein ligases that promote their degradation [2]. Domain I has very strong, short-range repression activity and presumably acts to repress transcriptional activation of the Aux/IAA-ARF binding partner [35]. Analysis of cis-elements of the early auxin response genes, including the Aux/IAAs, identified the Auxin Responsive Elements (AuxREs) [54, 55]. The first ARF (Auxin Response Factor) was identified in a yeast one-hybrid screen for factors that bind to an AuxRE [36]. This 23 member [32] family also carries four conserved domains, and like the Aux/IAAs, domains III and IV are involved in dimerization [36]. In most of the ARFs

domain II, or the middle region, acts as a transcriptional activator or repressor. Domain I binds the AuxRE in the DNA [37].

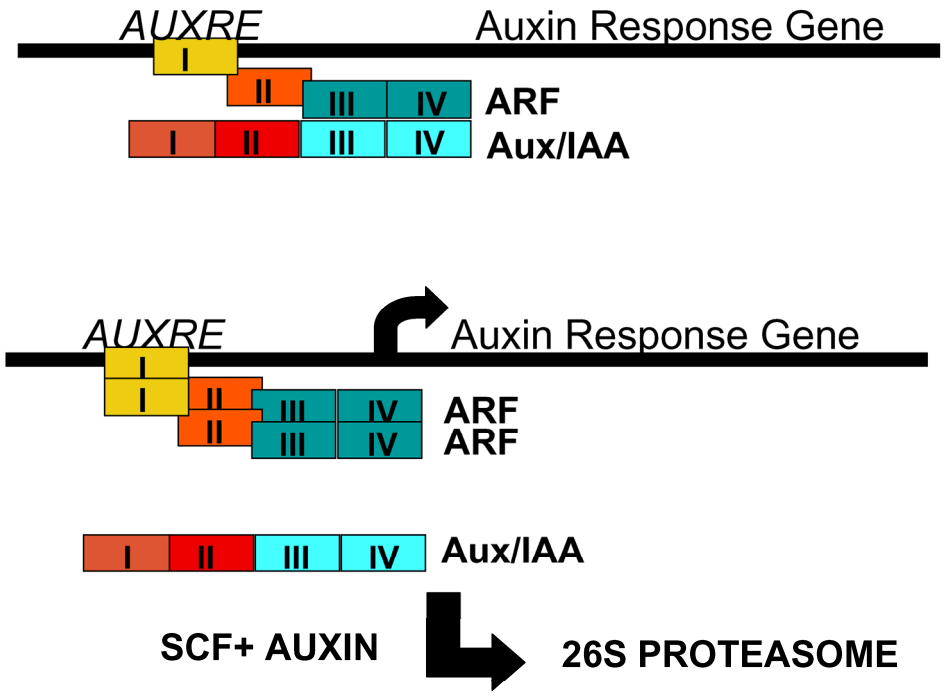


Figure 3. Transcriptional regulation of auxin response genes. Low auxin levels the Aux/IAA proteins bind to the ARF protein and inhibit transcriptional activity of the auxin response gene. High auxin levels the Aux/IAA is degraded by the 26S proteasome via the SCF and repression of transcription is released.

To determine how these transcription factors affect plant development two different types of mutants have been studied. Screens for altered auxin response have identified an interesting group of *aux/iaa* mutants that carry mutations in domain II that result in gain-

of-function. Auxin related phenotypes, such as defects in cell division and elongation, curled leaves, lack of apical dominance and auxin resistance, are seen in these mutants [38]. In addition to their important role in auxin response the influence of these genes in relation to other phytohormones such as gibberellin [39] and brassinosteroids [40] and involvement in light response [41] is also becoming apparent. Loss-of-function *aux/iaa* mutants have also been investigated. For the most part these mutants do not display any obvious changes in phenotype, probably due to genetic redundancy within this large family and/or feedback mechanisms that dampen the effects of the loss of a single protein [58, 62]. Though less dramatic, some of the loss-of-function phenotypes that have been observed are opposite to the corresponding gain-of-function mutants [38]. Surprisingly higher order mutants are largely unchanged in appearance or in gene expression profiles determined by microarray analysis [42]. The *arf* mutants are loss-of-function and show much more distinct and profound phenotypes than the *aux/iaas* [38]. In their absence expression of the AuxRE containing genes would, in most cases, be less robust and generate plants that phenotypically resemble the *aux/iaa* gain of function mutants. Overlapping phenotypes and expression patterns are also seen within this gene family [6, 63].

To date little is known about the dimerization specificity of these two families of transcription factors. However two recent studies have shed light on this question [64, 65]. Based on similar phenotypes seen in *Aux/IAA* and *ARF* mutants, and by looking at *in vivo* expression data of these genes, it is possible to speculate on possible pairings. Candidate pairs were chosen which function primarily in either embryonic root initiation

(IAA12/BDL - ARF5/MP) or auxin mediated root development (IAA3/SHY2 - ARF7/NPH and ARF19). Promoter exchange between the two gain-of-function mutant IAA genes was done to test the effectiveness of expressing the proteins in tissue where they are not normally found. Expression of either *pIAA3*:*iaa12* or *pIAA12*:*iaa3* produced less severe defects than the original mutants. This demonstrates the specificity of expression and function of the individual Aux/IAAs. Promoter exchange and gene expression was also used to analyze the importance of pairing between the families. The *pIAA12* sequence was fused to either *ARF5*, its anticipated partner, or *ARF16*, a distantly related family member, and expressed in *arf5* plants. The *pIAA12:ARF5* was much more efficient in rescuing the *arf5* embryonic defects than *pIAA12:ARF16*, again showing the importance of tissue specific expression and the effectiveness of correct Aux/IAA-ARF pairing. In the *arf7arf19* double mutant stronger *iaa3*-like root phenotypes are observed than in the singles indicating that both proteins have synergistic functions and potentially pair with IAA3. As ARF activity affects *Aux/IAA* expression it would be logical for a feedback loop to exist within the protein pairs, and in both single and double mutant *arf* plants *IAA3* mRNA was significantly reduced [43]. These experiments give a good indication that IAA3-ARF7 or ARF19 act together. This along with the promoter swapping experiments give insight into the functional pairing of the two families of auxin regulated transcription factors.

A newly developing technique called Fluorescence Cross-Correlation Spectroscopy (FCCS) quantitatively measures interaction between two proteins tagged with different fluorophores. While this technique cannot be used to visualize subcellular localization it

can compare the strength of interactions at nanometer distances. Homo- and hetero-dimerization combinations of IAA19/MSG2 and the C-terminal domains of ARF5/MP and ARF7/NPH4 were examined with FCCS in HeLa cells. In this system stronger interaction was measured between IAA19 and either ARF-CTD than for all three homodimer combinations [44]. Despite the disadvantages of the artificial system this reflects the strength of the repression of these transcription factors, which are influenced by auxin.

Recent work in the field of small RNA regulation has added another layer of complexity to the action of the *ARF* family. Together, two micro-RNAs (*miR160*, *miR167*) have five predicted *ARF* targets. Interestingly while increased auxin did not alter expression of the miRNAs, plants expressing an *miR160* resistant *ARF17* transcript showed marked changes in transcripts of several *GH3*-like early auxin response genes [45]. Analysis of *miRNA* promoter elements revealed an overrepresentation of several transcription factor binding motifs including the *AuxRE* [46]. *MiR160* and *miR167* were among the 14 *miRNAs* carrying the ARF binding site indicating that a negative feedback loop may control their expression. Additionally two other *ARFs* are targets of two trans-acting small interfering RNAs (*ta-siRNA*) from the *TAS3* locus, which are themselves targets of *miR390* [47]. The combination of the *ARFs* and the *TIR/AFBs* being targets of small RNAs suggests a careful regulation of the auxin response system.

THE UBIQUITINATION PATHWAY

Overview

Discovery and study of the ubiquitin molecule began in the 1970s and is still a very active area of research. Alone or as a chain this small protein has been shown to be

involved a variety of regulatory processes. For example monoubiquitination regulates endocytosis, protein sorting, retrovirus budding, DNA repair, histone activity and transcriptional regulation [48]. The 1980s saw the beginnings of the elucidation of the ubiquitin mediated protein degradation system [49]. This Noble prize winning work has impacted research across all fields of molecular biology and seems to play an important role in the regulation of many diverse cellular systems.

The overall process, can be simplified into three steps [50] (Fig. 4). In an ATP-dependent reaction, the C- terminal glycine of ubiquitin (Ub) forms a thiolester link to a cysteine on an ubiquitin-activating enzyme (E1). This activation step is followed by transfer to an E2 conjugating enzyme also via a cysteine, which in turn brings the Ub into the final and more complex ubiquitin ligation process involving an ubiquitin ligase (E3). The glycine 76 residue of the initial Ub peptide is attached to the E3 ligase recruited substrate at a lysl ε-amino group, and a chain of these molecules can be built up through Gly76 –Lys48 isopeptide bonds [51]. Ub carries seven lysines and alternative chain formations can also be used as signals for other cellular processes [48]. In *Arabidopsis* there are two ubiquitin activating enzyme (UBAs), and at least 37 E2 ubiquitin conjugating enzymes (UBCs) [52]. The largest group of proteins that function in ubiquitin conjugation are the E3 ligases. There are several families of E3s that I will describe below.

Single Subunit E3 Ligases

The single subunit E3s are grouped according to the prominent amino acid motif found across the classes in each family: HECT (Homology to E6-AP Carboxy Terminus), RING-H2 (Really Interesting New Gene), and U-box (UFD2-homolog domain). HECT

E3s, of which there are seven in *Arabidopsis* [53], are unique among the E3s in that they form a thiolester bond with the UBQ molecule which then facilitates its transfer and amide linkage to the substrate target [54]. U-boxes carry a modified C-terminal RING

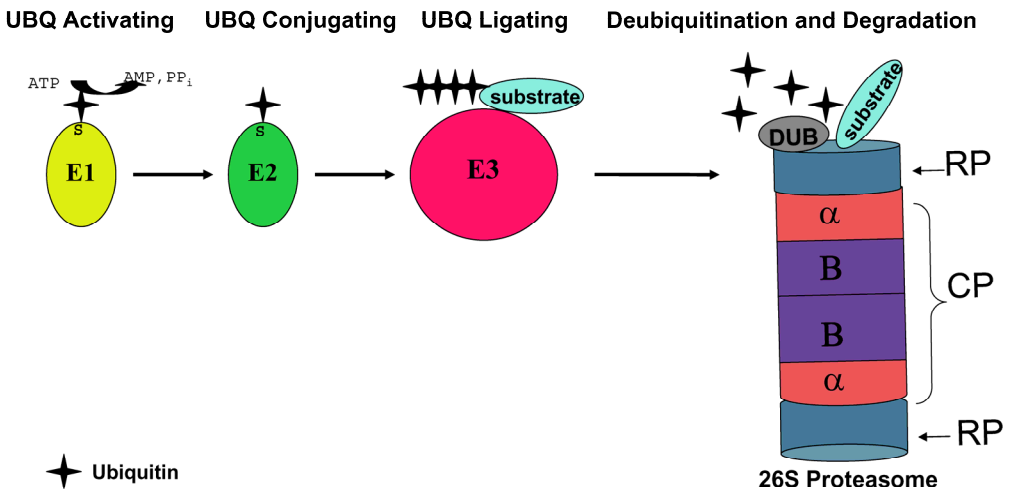


Figure 4. The Ubiquitin-26S proteasome pathway. UBQ is activated by an ATP-dependent thiol-ester bond with the E1. Upon transfer to the E2, UBQ is brought to the E3 ligase where it can be attached to the substrate. Sufficient chain formation serves as a signal for the proteasome. At the lid (RP), deubiquitinating enzymes remove the chain and the substrate is directed into the core (CP) for degradation.

finger [55] and this 70 amino acid motif has been located in 37 genes which can be further classified into 5 groups [56]. The U-box motif is also found in a newly identified class of enzymes called the E4s, which work in combination with the established ubiquitination system to facilitate extension of multi-ubiquitin chains onto substrates [57]. 469 genes have been classified as RING-type E3 members and *in vitro* assays have

demonstrated that many of these catalyze polyubiquitination [58]. Motif analysis outside the RING domain to explore potential substrate interactions has further classified these into 30 different groups [58].

Multiple subunit E3s Ligases

The multi-subunit E3 ligases now comprise five groups: ECS (Elongin C-SOCS box-CUL2), APC/C (Anaphase Promoting Complex/Cyclosome), SCF (Skp-Cullin-F-box), BRC3 (BTB-RBX1-CUL3) and DRC4 (DDB-RBX-CUL4). Of these, only the ECS has not been identified in *Arabidopsis*. All of these complexes contain a RING protein and a cullin, or in the case of the APC a cullin-type protein [80, 81] (Fig. 5).

APC/C

The APC/C is an important regulator of cell cycle progression and has been extensively studied in yeast and vertebrates but only recently in *Arabidopsis*. A 3-dimensional model at 24Å from Cryo-electron microscopy of an 11 subunit human APC has been derived and reveals a complex structure in which an outer protein wall surrounds a large inner cavity [59]. It was postulated that this cage could represent a reaction chamber where ubiquitin reactions occur, although *in vitro* reactions have shown that a single subunit, APC11, is sufficient to mediate ubiquitination [83, 84]. Single-copy homologs for most of these subunits have been identified in the *Arabidopsis* database, the exception being APC3 which is encoded by two related genes [60]. With different transcript expression patterns it was speculated that these duplicate subunits could have specific roles. Indeed while other

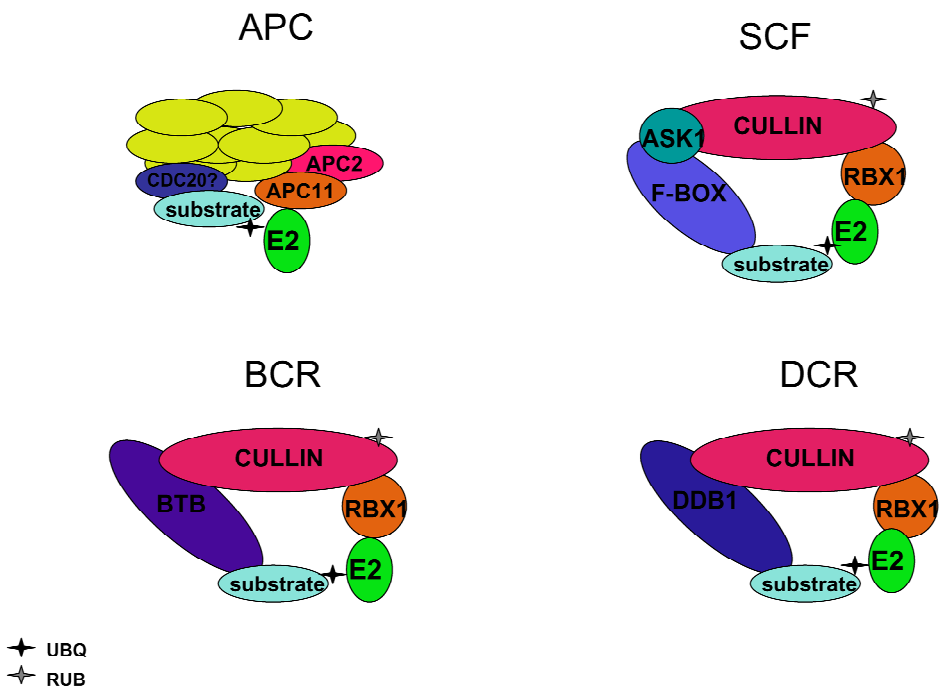


Figure 5. Multi-subunit Ubiquitin Ligases in *Arabidopsis*.

plant *APC* mutants are defective in cell cycle progression during gametophyte development and embryogenesis, one of the *APC3* homologs, *HOBBIT*, is involved in post-embryonic cell expansion and elongation [86, 87]. Interestingly, HOBBIT appears to have a role in auxin response. Mutant lines show a decrease in activity of the auxin responsive reporter DR5-GUS, and transcript levels of two auxin responsive transcription factors, *IAA17* (*AXR3*) and *IAA3* (*SHY2*), were either increased or decreased respectively [86, 87].

Two of the APC subunits have motifs found in other E3 ligase families [60]. APC2 has a cullin domain, which includes the RING-H2 protein (RBX1) binding site but not the RUB modification site. *AtAPC2* mutants have defects in female megagametogenesis [61]. The

APC11 protein has the RING-H2 domain found in many E3 ligase families, and in yeast two-hybrid studies, interaction can be demonstrated between AtAPC2 and both AtAPC11 and yeast APC11 [61]. To date, one other *Arabidopsis* APC subunit has been studied, APC6/ NOMEGA, and mutants in this subunit also have defects in female gametogenesis [62].

Cell cycle progression is regulated through sequential destruction of the APC/C substrates, cyclins being among the first identified [63]. There are two substrate recognition motifs: the nine amino acid D (destruction) box [64] and the seven amino acid KEN box [65]. Most of the ten A-type and nine B-type cyclins encoded in *Arabidopsis* have a recognizable D box [66] and fusion protein experiments have demonstrated the necessity of this motif for degradation [67]. Also associated with the APC/C are activator proteins that confer substrate specificity [68]. Nine of these WD40-repeat proteins are found in *Arabidopsis* and five of these contain a KEN box making them eligible for their own destruction via the APC/C [60].

SCF E3s

The first studies of the SCF developed from observations of cell-cycle defects in the budding yeast, *Saccharomyces cerevisiae* [69], and by the late 1990's the four core subunits of the complex had been identified and demonstrated to be active in the ubiquitin process [97-105] (Fig.6). Extensive domain analysis first demonstrated the assembly of the core complex along with the E2 [70]. The cullin subunit is the central scaffold of the SCF complex and through specific motifs at the amino- and carboxyl- ends of the protein,

serves as binding sites for the SKP1 (Suppressor of Kinetochore Protein), and the RBX (Ring-Box) subunits respectively. RBX recruits the ubiquitin carrying E2, and SKP1 binds the F-box subunit. Crystal structure has confirmed and refined the details of these interactions [107, 108]. F-box proteins are the most diverse members of the complex but all share a common N-terminal motif, the F-box, of approximately 45 amino acids [97], which is the site of binding to the SKP1 subunit. F-box swapping experiments first done in insect cell extracts identified this as the protein responsible for substrate specificity in the ubiquitin-ligase reaction [105]. The F-box motif can be accompanied by a wide range of C-terminal protein interaction domains [71]. This feature allows the SCF to be involved in the ubiquitination of a very diverse range of substrates that are involved in many different aspects of cellular functions [1].

The first F-box proteins identified in *Arabidopsis* were UFO (Unusual Floral Organs), involved in floral organ development [72], TIR1 (Transport Inhibitor Response), involved in auxin perception [111] and COI1 (Coronatine Insensitive), involved in jasmonic acid perception [73]. Subsequent analysis demonstrated that almost 700 genes, or 2.7% of the *Arabidopsis* genome, can be classified into one of 20 subfamilies of the F-box superfamily [71]. Only a small fraction of this vast number have actually been implicated in biological process and even less of these have clear substrate interactions, including UFO, the first published *Arabidopsis* F-box [1].

In *Arabidopsis,* the SKP1 subunit, which links the F-box protein to the core cullin complex, is known as ASK, for *Arabidopsis* SKP1-LIKE. While 21 members of this

family have been identified [113, 114], ASK1 and ASK2 seem to be the major participants in SCF assembly [74]. The *ask1* mutants, while viable, result in a myriad of defects involving vegetative and floral development [75] as well as hormone response [1, 116]. The *ask2* single mutant does not confer a mutant phenotype but when combined with the *ask1* mutant, results in defects in growth and development not exhibited by *ask1* alone [76].

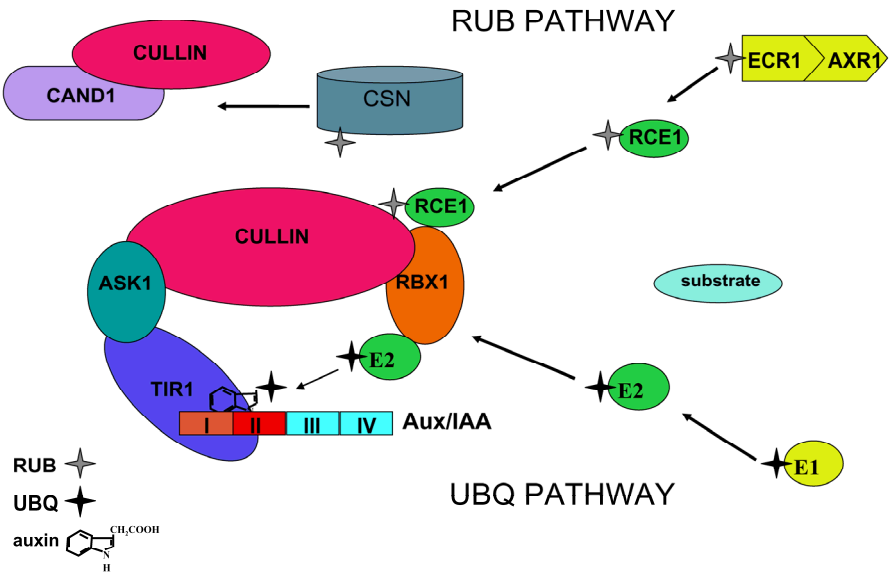


Figure 6. Model for cullin based E3 ligase activity with ASK1/F-box substrate adaptor TIR1. Cullin acts as a scaffold for ligase assembly with the substrate adaptor binding to the N-terminus of the protein and RBX1 binding at the C-terminus. RBX1 has a role in the ubiquitin and the RUB pathways. RBX1 facilitates the transfer of a RUB to the cullin and this activated form participates in the E3. The COP9 signalosome (CSN) removes the RUB and the inactivated cullin can then be bound by CAND1 until it is reactivated again. For ubiquitination RBX1 brings the ubiquitin carrying E2 into the vicinity of the adaptor

for transfer to the substrate. Auxin and substrate bound TIR1 interact with the ASK1 through the F-box motif.

Two *RBX* genes, *RBX1* and *RBX2* are present in *Arabidopsis*. *RBX1* appears to be the most important of these [118, 119]. Reduced expression of *RBX1* via dsRNA or anti-sense constructs resulted in severe defects in the plants [118, 119] and an increase in the RUB-modified form of the CUL1 protein [77].

The central scaffold of the SCF is the cullin protein. Of the 11 annotated for *Arabidopsis* [120], only five of these, *CUL1, CUL2, CUL3a, CUL3b and CUL4*, contain the 200 amino acid cullin homology (CH) domain, the Skp1/ASK1 binding domain, and the five amino acid RUB modification motif [120]. These proteins all interact with RBX1 [118, 119] [121, 122]. CUL1 is the dominant SCF cullin and null mutations in this gene are lethal in *Arabidopsis* [120]. Cullin proteins also undergo a site-specific modification by a small ubiquitin-like protein called RUB1 (Related to Ubiquitin) that is proposed to regulate activity of the cullin-containing complex [123, 124]. RUB1 (called Nedd8 in animals) is conjugated to cullins in a fashion that is similar to ubiquitin. The E1 activation enzyme for RUB1 conjugation is a heterodimer of AXR1 and ECR1 [78]. *AXR1* was originally identified in a screen for *Arabidopsis* mutants with decreased response to exogenous auxin [79]. The mutants also have defects in plant height, root gravitropism, hypocotyl elongation and fertility [80]. It was not until a few years later that it became apparent that this gene had homology to the amino half of the ubiquitin E1 [81]. AXL1 (AXR1-Like), which is 80% identical to AXR1, also functions in a redundant manner in the RUB

pathway, and while single mutants in this gene show no phenotype, *axr1axl1* double mutants are lethal [Dharmasiri and Estelle, unpublished]. Based on similar findings in the yeast system [128], and using sequence similarity, *ECR1*, the other subunit of the heterodimer, was identified [78]. RCE1 is the RUB1-conjugating enzyme [82], and through its direct interaction with RBX1 is responsible for conjugation of RUB1 to CUL1 [83]. Removal of the RUB molecule is facilitated by another complex called the CSN (COP9 Signalosome) [130], however the presence of a substrate in the SCF inhibits this process [84]. It is the RUB modified form of CUL1 that assembles the active SCF complex [85], while the unmodified form has been shown to bind to another protein called CAND1 [133, 134]. Modifications to *CUL1* that either increase or decrease CUL1-RUB levels directly affect the level of CAND1 interaction, suggesting a dynamic cycling of regulatory features that affect the assembly of the SCF complex [134, 135]. The importance of this has been underscored by the observations that in *cand1* mutants, substrates of different SCF complexes (IAA7 involved in auxin response and RGA involved in gibberellic acid response) accumulate, indicating that they are prevented from normal ubiquitination and subsequent degradation [134, 136]. Crystal structure shows extensive interaction of mammalian Cand1 with the Cul1-Roc1 scaffold of the SCF [86]. Cand1 binding at the N- terminus of Cul1 disrupts the binding site for the substrate adaptor Skp1 (ASK1 in *Arabidopsis*) and Cand1 binding at the C-terminal blocks the Nedd/RUB modification site. Dissociation of CAND1 is induced by the substrate adaptor which then allows formation of a functional SCF [84].

BCR E3s

Another cullin based class of E3s called the BCRs uses CUL3 as a scaffold. In *Arabidopsis* there are two CUL3 proteins called CUL3a and CUL3b that are 88 % identical. In *cul3a* and *cul3b* single mutants development is normal, however double mutants are embryo lethal [87]. Similar to CUL1, the C-terminal half of CUL3 undergoes RUB1 modification and interacts with RBX1 [87]. CAND1 interaction has been demonstrated for human CUL3 [88], but not yet for the *Arabidopsis* CUL3 proteins. However, given the high degree of homology and the presence of RUB1 modification it is most likely that this interaction does occur. CUL3 also interacts with the CSN to undergo removal of the attached RUB1 [89].

While CUL1 can bind to a myriad of F-box protein substrate adaptors via ASK1, in the CUL3 complex both the substrate binding and adapter proteins are combined into one protein that encompasses a large family called the BTBs (Broad-complex, Tramtrack, Bric-a-Brac). Similar to F-box proteins, the BTBs contain a core fold of 95 amino acids [90], usually in the N-terminal region, which can associate with CUL3 [87] and with other BTB containing proteins to form homo- and heterodimers [87]. A linker region separates the N-terminal and the secondary C-terminal motif, which defines the classes of BTBs. In *Arabidopsis* there are 80 BTB proteins that can be grouped into 11 different classes [140]. These proteins are involved in many different processes including phytohormone signaling, blue light signal transduction, sugar metabolism, lateral organ development and control of telomerase activity [91]. The only one with a known substrate is ETO1 (Ethylene Overproducer 1). This protein regulates the degradation of

the ACC synthase enzyme ACS5 [92].

It has recently been shown that human CUL3 dimerizes through the RUB1-like modifier NEDD8 and that this is a requirement for ubiquitination of the substrate cyclin E [93]. It will also be of interest if these CUL3 dimers are found in *Arabidopsis* and if dimerization between the different cullin proteins can be achieved.

DCR E3s

The DCR E3 ligase is the most recently investigated multi-subunit E3 ligase in *Arabidopsis*. The central scaffold of this complex is CUL4. Of the two orthologs in humans, hCUL4a is almost 50% identical to AtCUL4 making it the most highly conserved cullin across different species [121, 144]. Like CUL3 in the BCR complexes, the C-terminal half of CUL4 undergoes RUB modification and RBX1 binding, and the N-terminal carries the five helix cullin repeat [121, 144]. Unique to CUL4 is a 97 amino acid N-terminal extension outside of this conserved helical repeat which was shown to be involved in substrate adaptor binding [94].

While CUL3 has at least 80 predicted BTBs and CUL1 hundreds of SKP1/ASK1-F-box possibilities, so far only one substrate adaptor has been identified for CUL4, DDB1 (Damaged DNA-Binding1) [121, 144]. Unlike the other substrate adaptors, DDB1 does not carry the BTB/SKP1 fold used for cullin interaction [94]. DDB1 was first identified in patients with *xeroderma pigmentosum*, a genetic disorder that through defects in DNA repair causes sensitivity to UV irradiation [95]. Human DDB1 forms a heterodimer with

hDDB2 which binds damaged DNA to initiate nucleotide excision repair [96], and assembly of hDDB1-hCUL4a-hRBX1 complex which targets hDDB2 for ubiquitination in response to UV has been demonstrated [148]. The crystal structure for the 127 kD DDB1 shows three seven bladed B propellers (BPA, BPB, BPC) and a C-terminal helical domain [94]. It was proposed that a seven residue patch of invariably conserved amino acids in the BPB propeller serves as the CUL4 binding point and in vitro assays seemed to verify this [94]. Subsequent crystallization of CUL4a-DDB1-ROC1 confirmed that the BPB binds the CUL4a at the same helical folds, H2 and H5, as SKP1 binds CUL1[97]. The structure also revealed a second interaction site on the CUL4 protein at its N-terminal extension outside of the conserved five helix cullin repeat. The BPA-BPC double propeller fold has limited contact with CUL4 and is orientated towards the RING subunit. Examination of DDB1 crystallized either alone or in different associations show that while still facing the RING subunit there are distinct structural differences in the BPA-BPC structure. It is speculated that this flexibility for conformational changes along with multiple protein-protein interaction sites present in the BPA-BPC provide the opportunity for recruitment of a diverse range substrates [94]. This seems to be confirmed by the growing list of substrates which point to involvement in DNA replication, DNA damage and repair, cell cycle control, developmental patterning and epigenetic control of gene expression through histone modification [98]. Many of the hDDB1 substrates are WD-40 proteins and biochemical and modeling studies suggested that a tandem motif called a WDXR box is important for these interactions [98]. DDB1 is evolutionarily conserved across species [96] and in *Arabidopsis* there are two *DDB1* homologs that are over 90% identical, *DDB1a* and *DDB1b*, both of which can interact with CUL4 [121, 144]. Loss of *DDB1a*

shows no apparent phenotype but loss of *DDB1b* is embryo lethal [151]. The assembly of DDB2 with DDB1a in the *Arabidopsis* CUL4 complex and the presence of many substrates orthologs in the plant genome indicate that this E3 ligase is involved in similar activity as found in the mammalian cells [121, 144].

DDB1a has also been shown to associate with DET1 and COP10 in what is called the CDD complex [151, 152] and assembly of this complex with CUL4 has been demonstrated [151]. DET1 is a negative regulator of light-mediated response [99] and influences the proteasomal degradation of the circadian protein LHY [99]. COP10 encodes a small ubiquitin E2 variant that also has a connection to light signaling pathways [154]. The CUL4-CDD complex also associates with COP1, a RING E3 important in targeting several known photomorphogenesis promoting transcription factors [151]. This points to an additional function of CUL4 that is obviously unique to plants. As with CUL1, CUL4 undergoes the cycling of RUB modification that involves the CSN and CAND1 [151].

$SCF^{TIR1/AFBs}$

One of the most interesting and well-studied multi-subunit E3 ligases in *Arabidopsis* is SCF^{TIR1}. A screen of EMS mutagenized seedlings for resistance to the polar auxin transport inhibitor NPA (N-1-naphthylphthalamic acid) identified seven new genes named the *TIRs*, for *Transport Inhibitor Response* [100]. The inhibition of root elongation by NPA is caused by accumulation of auxin in the root tip, and it was postulated that resistance could be a result of either defects in auxin transport or auxin response. To

clarify this *tir1* seedlings were grown on the exogenously applied auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and indole acetic acid (IAA), which are also inhibitors of root growth. Again the mutants displayed resistance, leading to the conclusion that the defect was in auxin response rather than transport [111]. Further characterization of *tir1* mutants showed deficiencies in cell elongation responses and in lateral root formation. In addition *tir1* has a synergistic effect on auxin response and plant morphology when crossed into *axr1* mutant plants.

The *TIR1*gene encodes an F-box protein with 18 leucine rich repeats (LRRs) that associates with ASK1 and CUL1 to form SCF^{TIR1} [111]. Because *tir1* plants are deficient in auxin response, proteins involved in auxin response were likely substrates for this F-box protein. While the *Aux/IAA* transcripts are rapidly induced in the presence of auxin, the proteins have a relatively short half-life. When Aux/IAA protein levels were checked in plants treated with a proteasome inhibitor, a dramatic increase in their half-life was seen. Similar affects were seen in *tir1* plants. To test the possibility that these could be TIR1 substrates, pull-down assays with bacterially expressed GST-tagged Aux/IAA proteins and plant extracts containing myc-tagged TIR1 protein were performed. Not only was interaction detected, it was strongly enhanced with exogenous auxin. Further examination of the *Aux/IAAs* revealed that the domain II contains a highly conserved motif responsible for the TIR1 interaction [101].

Although the Aux/IAAs were known to partner with the ARFs to control auxin regulate

gene expression [36], it was not clear how the recognition of these substrates by TIR1 was accomplished. Extensive *in vitro* studies done in cell free systems demonstrated that, in contrast to previously studied SCF-substrate interactions in animals and yeast, ATP, protein phosphorylation, and proline hydroxylation were not required for TIR1-myc and GST-IAA binding [102].

Despite these studies it was still not clear how auxin promoted protein interaction, if other proteins or factors were involved or if protein modifications were required. A set of simple *in vitro* experiments revealed answers to these questions. If an enzymatically driven protein modification was occurring then this should be affected by temperature. However no difference was seen in the strength of interaction from 4^{o}C to 25^{o}C and in all cases the interaction was saturated by 20 minutes [157]. In all of these tests the one factor that was consistently required to see strong TIR1-myc and GST-IAA7 interaction was the continual presence of auxin. To look at the possibility that auxin was directly participating, radiolabeled auxin was employed in pulldown and competitive binding experiments. This pointed to the SCF^{TIR1}, but not GST-IAA7, as being the target for auxin binding [157, 158]. While TIR1 is the only recognized auxin-specific subunit of this SCF complex, to eliminate the possibility that auxin could be interacting with a tightly associated protein, tagged TIR1 was generated in either insect cell [157] or *Xenopus* embryo [103] systems. Using only two plant specific proteins, TIR1 and IAA7, pulldown assays demonstrated that auxin must be binding to TIR1 and therefore TIR1 functions as an auxin receptor. Interestingly while in *Xenopus* Skp1 was not required for the auxin induced binding,

experiments that either eliminated the F-box domain or incorporated mutations in conserved domains required for ASK1 (Skp1) binding did prevent interaction with IAA7.

The current model of auxin action is as follows [2, 157]. Under low auxin conditions Aux/IAAs bind to ARFs and repress their activity. Under high auxin conditions the Aux/IAAs becomes susceptible to ubiquitination by the SCF through its domain II interaction with the F-box/auxin receptor. Currently it remains open whether degradation of Aux/IAA proteins via the 26S proteasome requires prior disruption of Aux/IAA-ARF dimers or whether Aux/IAA proteins are degraded while still bound to ARF proteins. However, in any case release of ARF proteins from the Aux/IAA moiety most likely is the initiating step to permit ARF proteins to activate transcription of auxin-regulated genes.

Recent crystallization of TIR1 in complex with ASK1, an IAA7 domain II peptide, and three different auxins has further confirmed this model and the role of TIR1 as an auxin receptor [104]. The complex looks like a mushroom with ASK1 and the TIR1 F-box motif forming the stalk and the 18 leucine-rich repeats (LRRs) of TIR1 forming a unique horseshoe shaped solenoid cap. It is in the LRRs that auxin perception and substrate binding occurs, as well as the surprising incorporation of another small molecule, inositol hexakisphoshate ($InsP_6$). Each TIR1 LRR contains a β-strand, which comprise the concave surface of the solenoid, followed by an α-helix, which lines the convex surface. The C-terminal extension of TIR1 caps the horseshoe completing the circle. Three unusually long LRR loops in combination with the concave twist in the middle of the LRR region form the auxin pocket. IAA, the natural form of auxin found in *Arabidopsis*, and

two synthetic auxins, 1-NAA and 2,4-D, were shown to have similar contacts varying slightly according to size and hydrogen bonding capabilities, explaining the differences in affinity seen previously in biochemical studies. The mostly hydrophobic sequence of the 13 amino acid IAA7 peptide adopts a highly coiled conformation on top of the auxin molecule and fills the auxin pocket. Binding of TIR1 with the central conserved domain II motif of the peptide is through extensive hydrophobic interactions. The peptide effectively encloses the TIR1 pocket, covering the opening and coming into direct contact with the bound auxin molecule. As auxin does not induce a conformational change in the TIR1-ASK1 structure, its role seems to be one of enhancing the binding capabilities of TIR1 by extending the interaction surface with the Aux/IAA substrate. Just underneath the auxin binding residues of LRR2 is another layer of positively charged amino acids that tightly bind the $InsP_6$ cofactor. This interesting and previously unanticipated interaction appears to be critical for the structural integrity of the auxin/substrate-binding pocket. Along with addressing the impact of $InsP_6$ on TIR1 auxin receptor function, it will be interesting to examine how ubiquitination might play a role in either uncoupling the substrate from the TIR1 auxin pocket for immediate reuse by the SCF, or if this complex in part or in whole is directed to the proteasome for degradation.

While many details of *TIR1* regulation are still unknown, an examination of the effects of a pathogen induced microRNA, *miR393*, predicted to target TIR1[105], give insight into an auxin –linked stress response [9]. *Arabidopsis* treated with flg22, a peptide from bacterial flagellin, have increased level of *miR393* which results in both a decrease in *TIR1* mRNA and protein levels and the stabilization of Aux/IAA proteins. Therefore the

depletion of TIR1 by *miR393* would effectively block the auxin dependent binding and degradation of its substrates. This would lead to enhancement of transcriptional repression by Aux/IAA and their ARF dimerization partners. The downstream affect of this is plant resistance to pathogen invasion.

There are five additional genes in *Arabidopsis* that are very similar to TIR1 and these six comprise the Auxin signaling F-box protein (AFB) family**.** AFB1 is 70% identical to TIR1, while AFB2 and AFB3 are 60% and AFB4 and AFB5 50% identical to TIR1. The auxin, $InsP_6$ and Aux/IAA substrate binding sites are highly conserved across the family [104]. However there are some slight differences to note. Out of the 14 auxin contact sites in TIR1 three of these are changed in AFB4 and AFB5, one of which is defined as a key binding residue. Interestingly in AFB1 there are also three contact site changes, one that is shared with AFB4 and AFB5. COI1, the closest related non-family member only contains four out of the fourteen auxin binding sites. Of the ten $InsP_6$ binding sites four of these for AFB4 and AFB5 and two of these for AFB1 were different from TIR1. Here COI1 has five changes in binding sites. The IAA7 oligo binding sites were the most divergent across all of the family members, however of the eighteen, COI1 only carries three of the sites identical to TIR1. Work on AFB1-3 has verified that these are also auxin receptors that have overlapping and redundant function with TIR1 throughout plant development [106]. Like *tir1*, single mutant *afb1*, *afb2* and *afb3* plants have slightly auxin resistant root growth and the overall development resembles wild-type *Arabidopsis*. Combinations of doubles, triples and a quadruple mutant resulted in greater auxin resistance and in increased developmental defects as the Aux/IAA proteins are decreasingly susceptible to

degradation. The *miR393* also down-regulates *AFB2* and *AFB3* expression [9]. Presumably a single mismatch in the target sequence of *AFB1* is enough to shield it from this microRNA.

Deubiquitination and Degradation

The final step for most ubiquitinated proteins is release of the ubiquitin chain and delivery into the 26S proteasome where breakdown and recycling of the amino acids occurs (Fig. 4). Present in the nucleus and cytoplasm of eukaryotic cells, the 2Mda ATP-dependent 26S proteasome consists of a 20S core particle (CP) and a pair of regulatory particles (RP). Two peripheral rings of seven α subunits and two central rings of seven β subunits form the hollow cylinder of the CP, and the β subunits organize three chambers that house the proteolytic sites of the complex. In *Arabidopsis* there are 23 genes that can be classified as encoding the α or β CP subunits [107]. Such a high degree of functional redundancy is not seen in yeast and the homology between the *Arabidopsis* paralogs is considerable higher than those seen in human. The RP resides at either end of the CP and is divided into the Lid and the Base subcomplexes. Redundancy of the 31 RP genes in *Arabidopsis* is also seen as the structure contains a total of 17 subunits [108]. The base, which sits over the CP α ring, is six ATPase subunits (RPT1-6) and three non-ATPase subunits (RPN1, 2, 10). The Lid has eight non-ATPase subunits (RPN3, 5-9, 11, 12). [109]. The RP functions to recognize, unfold, deubiquitinate and direct substrates into the opened CP α ring where they are broken down [52]. Function of some of the RP subunits has been attributed to these processes. RPN1 can recognize Ub-like proteins and RPN11 and RPN13 have proteolytic activity probably involved in release of Ub from the substrate

[108]. Mutants in *RPN10* confer hypersensivity to abscisic acid and in RPN12a decreased sensitivity to cytokinin [109]. In humans it is thought that the inducible substitution of subunits alters specificity of the proteolytic activity perhaps giving a wider range of developmental and environmental function, and given the high number of paralogs, this has also been proposed for *Arabidopsis* [165]. This has been hinted at in the study of the two *RPN1* isoform [110]. Though they have overlapping expression patterns, these proteins are not functionally redundant during gametogenesis and embryogenesis where RPN1a is crucial.

Other proteins are loosely associated with the 26S proteasome that may enhance and regulate the RP activity. It is likely that some of these function as deubiquinating enzymes (DUBs). In *Arabidopsis* 32 potential DUBs have been identified, including the Lid subunits RPN11 and RPN13, and these fall into two major categories: ubiquitin terminal carboxy hydrolases (UCH1 and 2), and ubiquitin-specific proteases (UBPs) [167]. The DUBs remove the Ub from the substrate and process the chains into monomers. It will be interesting to see if future research identifies subunits of the 26S proteasome and DUBs that are specific to recognition of auxin response substrates and if these are regulated on a transcriptional level by auxin through the $SCF^{TIR/AFB}$ receptors.

Summary

From synthesis and transport to perception and reaction, the mechanisms that direct auxin are complex and intertwined. The volumes of data being generated on this topic, though sometimes initially difficult to interpret, are slowly bringing together the big picture of a

fascinating and crucial system in the world of plant biology. The purpose of this thesis is to begin the study of two proteins, AFB4 and AFB5, which are highly related to the TIR1/AFB auxin receptors. I will investigate their potential roles in auxin response, and in the case of AFB4 start to explore other areas of plant development that it may be affecting.

II. Characterization of the AFB4 and AFB5 proteins

Introduction

Based on sequence similarity, AFB4 and AFB5 are members of the TIR1 family of auxin response F-box proteins in *Arabidopsis*. This family comprises three additional members, AFB1, AFB2, and AFB3, that have been demonstrated to have overlapping function with TIR1 [106]. These proteins function as auxin receptors [157, 158, 161]. Auxin binding to TIR1 and the others enhanced the binding of the Aux/IAA family of transcription factors and their subsequent degradation [104]. Sequence characterization of this family has identified 18 leucine-rich repeats that are located C-terminally of the F-box motif [106]. TIR1 and AFB1 are most closely related to each other, as are AFB2 and AFB3. AFB4 and AFB5, while very similar to each other, are the least closely related to the other family members. Comparison across other plant species has shown that this family is present in other land plants and that *AFB4* and *AFB5* diverged into a separate clade from the other four *Arabidopsis* family members (M. Prigge, unpublished). TIR1/AFB1-3 has been shown to localize to the nucleus and to participate in the ASK1/CUL1 SCF complex. Transcript analysis showed expression across all tissue with *AFB2* and *AFB3* levels consistently higher than *TIR1* and *AFB1*. Promoter:GUS analysis revealed a similar expression pattern. The loss of expression of any one of these genes result in either no obvious differences from wild-type for the *afb1, afb2* and *afb3* mutants [106], or very subtle defects, like the decrease in lateral root number observed in *tir1-1* [111]. In this chapter I describe the initial characterization of *AFB4* and *AFB5* by examining the expression of the genes and how their loss-of-function affects plant development.

Results

Sequence comparison of the AFB family

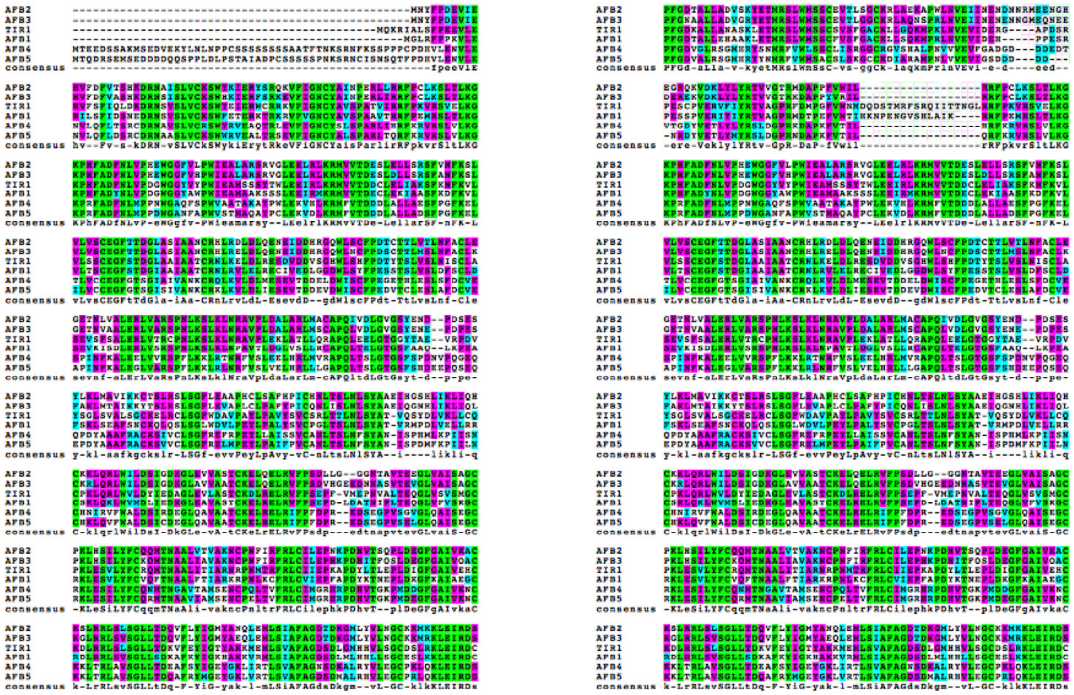


Figure 7. Amino acid sequence alignment of the *Arabidopsis* TIR1/AFB family. The alignment was done using the CLUSTAL W (1.81) multiple sequence alignment program from http://seqtool.sdsc.edu/CGI/BW.cgi. Green shaded residues are conserved in all aligned proteins. Note the N-terminal amino acid extension in AFB4 and AFB5.

At the beginning of my research project the biological roles of AFB4 and AFB5 were unknown. As mentioned in the introduction, both proteins are highly related to the other members of the AFB family and TIR1 (Fig. 8). COI1, a protein involved in jasmonic acid response [73], is the most closely related F-box protein with approximately 30% identity

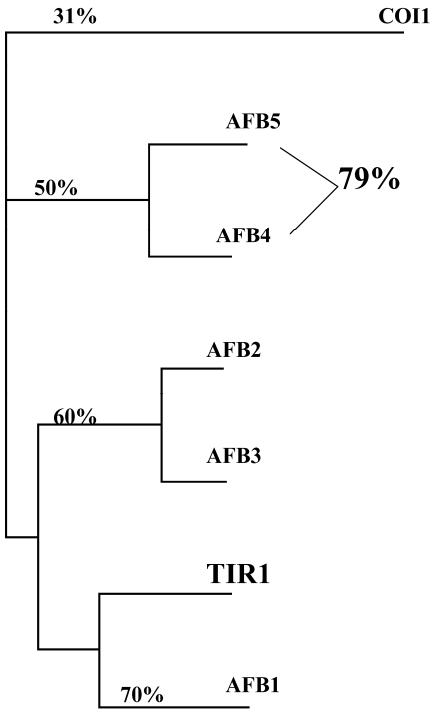


Figure 8. Phylogenic tree of the TIR1/AFB/COI family. Comparison of the percent amino acid identity by sequence alignment.

to the family members [106]. Sequence alignment of all members of the AFB/TIR1 family does not reveal major differences between the proteins, the prominent features being an F-box domain followed by the leucine-rich repeats (LRRs) (Fig. 7). However, there is an- N-terminal extension unique to AFB4 and AFB5 comprising about 50 amino acids. Although the function of the extension is unknown, it is rich in serine residues and motif analysis programs predict several phosphorylation sites.

Expression analysis of AFB4 and AFB5

To determine the pattern of *AFB4* and *AFB5* gene expression, total RNA was isolated from different tissues: flower, silique, stem, rosette, leaf, root, and two seedling stages (4 and 11 day). Amplification of *AFB4* and *AFB5* transcript by RT-PCR showed that both genes are expressed in seedlings and all tested organs (Fig. 9). *AFB4* is very weakly expressed uniformly in all tissues tested. *AFB5* is more strongly expressed, with highest levels in silique, stem, and 11-day-old seedlings, and more weakly expressed in 4-day-old seedlings

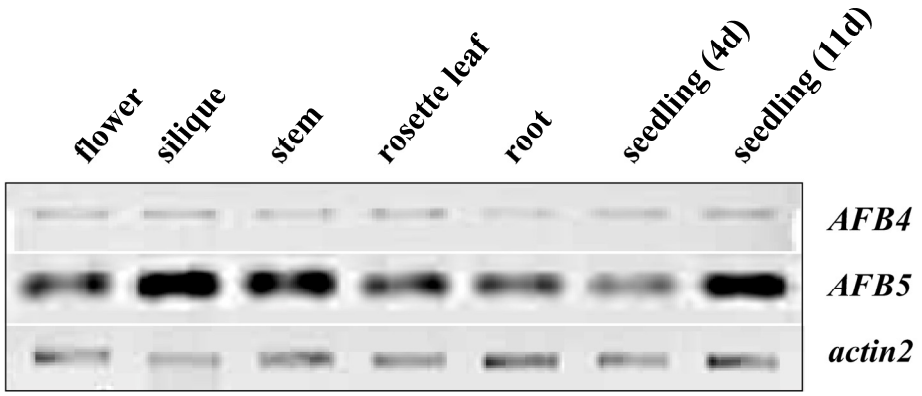


Figure 9. RT –PCR analysis revealed overlapping expression patterns of *AFB4* and *AFB5* in seedlings and different *Arabidopsis* organs. RT-PCR was done using 90ng DNA-free total RNA, with 30 cycles at 55°C annealing temperature. *Actin2* was used as loading control. 2 µl out of 25µl were loaded from the *actin2* and 6 µl from the *AFB4* and *AFB5* reactions.

To look more carefully at the expression patterns in different tissue *promoter:GUS* constructs were generated and transformed into *Col-0* wild-type plants. For the *proAFB4* construct 1047 bp and for *proAFB5,* 1044 bp upstream of the start codons were amplified from genomic DNA and cloned in front of the *GUS* reporter gene in the minibinary vector *pCB308* [111]. Between ten to fifteen transformed plants for each construct were tested for GUS staining patterns. Promoter:GUS data revealed that *AFB4* and *AFB5* are expressed early in embryogenesis (Fig. 10). *ProAFB4* and *proAFB5* driven GUS expression was detected as early as globular stage (Fig. 10A) and extended throughout embryogenesis. In addition, based on the GUS data both genes are expressed at early stages in all parts of the embryo. However, at the bent cotyledon stage, *AFB4* expression is found preferentially in the root, whereas *AFB5* is predominantly located in hypocotyl and cotyledons resembling the typical *AFB4* and *AFB5* expression patterns observed for seedlings (compare Fig. 10G and J, with Fig. 11 I and J). No expression was observed in the endosperm or seed coat.

For the *proAFB4:GUS* lines, staining in flowers increased with maturation and was mostly located in anthers, but also in carpels and the pistil (Fig. 11, B and C). GUS staining was also present in young siliques with very strong expression at the base (Fig. 11G). Leaves showed either an irregular spotty staining pattern or no staining (Fig. 11A). Interestingly, in roots the *AFB4* promoter was active in both primary and lateral roots. However staining was not observed at the root tip (Fig. 11F). Finally, in seedlings up to approximately two weeks staining was detected only in the root and never in the hypocotyl or cotyledon. However as the plants aged, staining gradually became visible in

the hypocotyl and vasculature. This pattern was consistently observed in seedlings germinated in dark or light conditions (Fig. 11D, E, F and H).

In the *proAFB5:GUS* lines staining was observed in flowers at all stages of development in anthers, carpels, pistil and petals with young siliques displaying strong staining (Fig. 11L, M, P). Leaves had a more evenly distributed GUS staining pattern with more

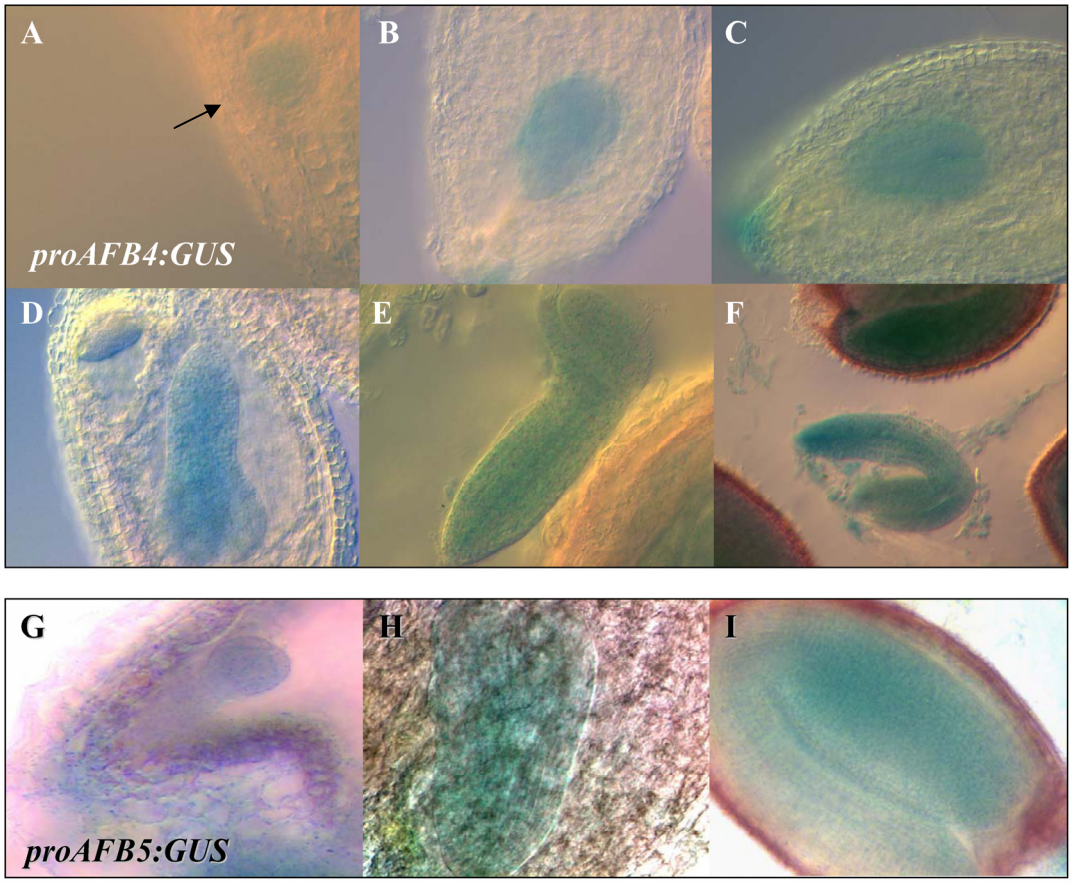


Figure 10. GUS staining in *proAFB4:GUS* **(A-F)** and *proAFB5:GUS* **(G-I)** lines. **(A,G)** globular stage; **(B)** early heart stage; **(C,H)** late heart stage; **(D, E)**, mid torpedo stage; **(F,I)** bent cotyledon stage embryo.

pronounced blue along the main vein and in the secondary veins. While there was no primary root staining, expression was seen in emerging lateral roots, which persisted in the lateral root tips of the *proAFB5:GUS* lines (Fig. 11N, O). In both light and dark grown seedling hypocotyls were positive for staining with weaker expression in the vasculature of the cotyledons (Fig. 11J, K). As the plants became older, staining increased and was visible across the whole cotyledon.

The GUS data demonstrate that, although the two *AFB* genes are expressed in the same organs as shown by RT-PCR, they have distinct expression patterns. Despite the high level of sequence similarity this suggests the possibility of specific and non-redundant function of these two proteins.

Subcellular localization of AFB4 and AFB5

Other AFB family members characterized so far are localized in the nucleus [106]. This is not surprising as they target members of the Aux/IAA transcription family for ubiquitination [101]. To assess if AFB4 and AFB5 are also found in the nucleus, *cDNAs* were translationally fused to YFP (AFB4) or GFP (AFB5) under the control of the *CaMV35S* promoter in the Gateway compatible binary vectors *pVRYFPCt* and *pVRGFP4Ct*, respectively, with fluorescent proteins located C-terminally of the AFB proteins. The resulting constructs were transformed into *Arabidopsis afb4* and *afb5* null mutants (see section III) or injected into tobacco leaves for transient expression. In both studies AFB4 and AFB5 fusion proteins were clearly located in the nucleus (Fig. 12), making them potential SCF components with transcription factor substrates.

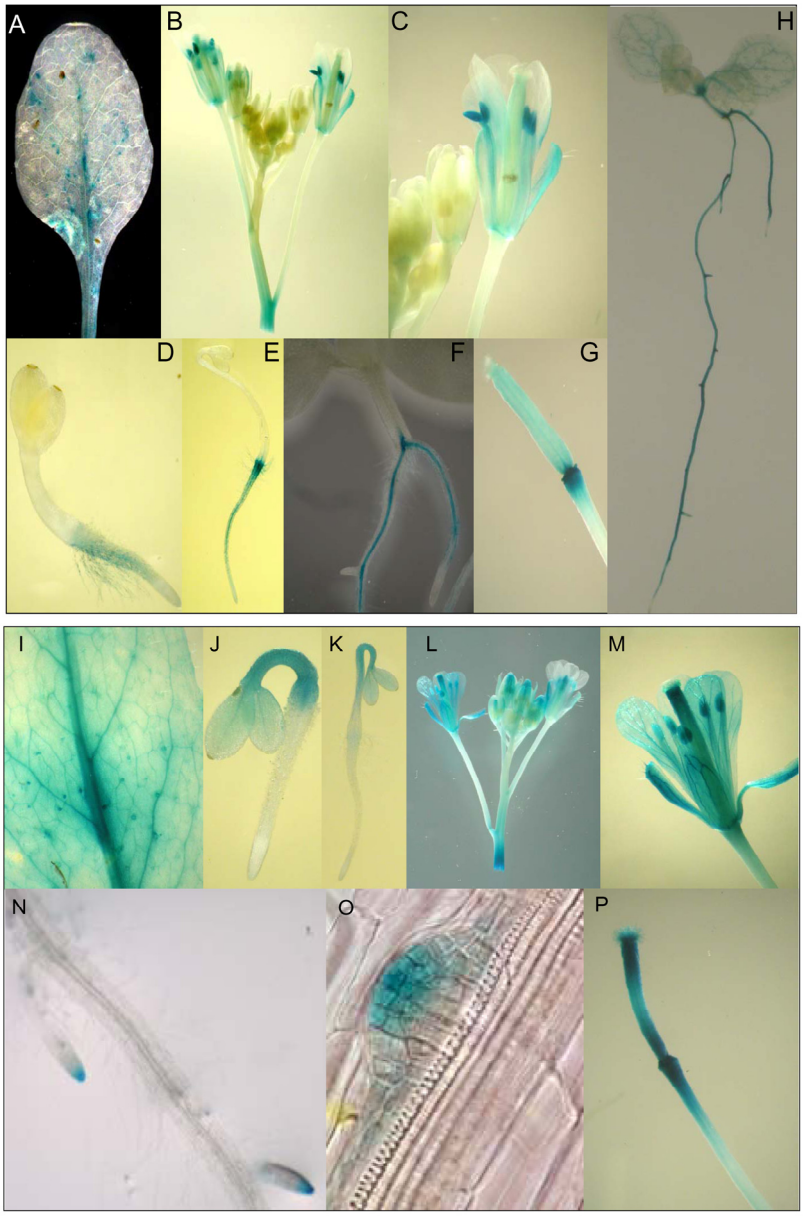


Figure 11. GUS staining in *proAFB4:GUS* (**A-H**) and *proAFB5:GUS* (**I-P**) lines. (**A**,**I**) leaf; (**B,C,L,M**) inflorescence; (**D**,**J)** seedling 1 day after germination in light; (**E**, **K)** seedling 1 day after germination in darkness; (**F**,**M,N**) primary and lateral root; (**G**, **P)** young silique; (**H**) two-week old seedling.

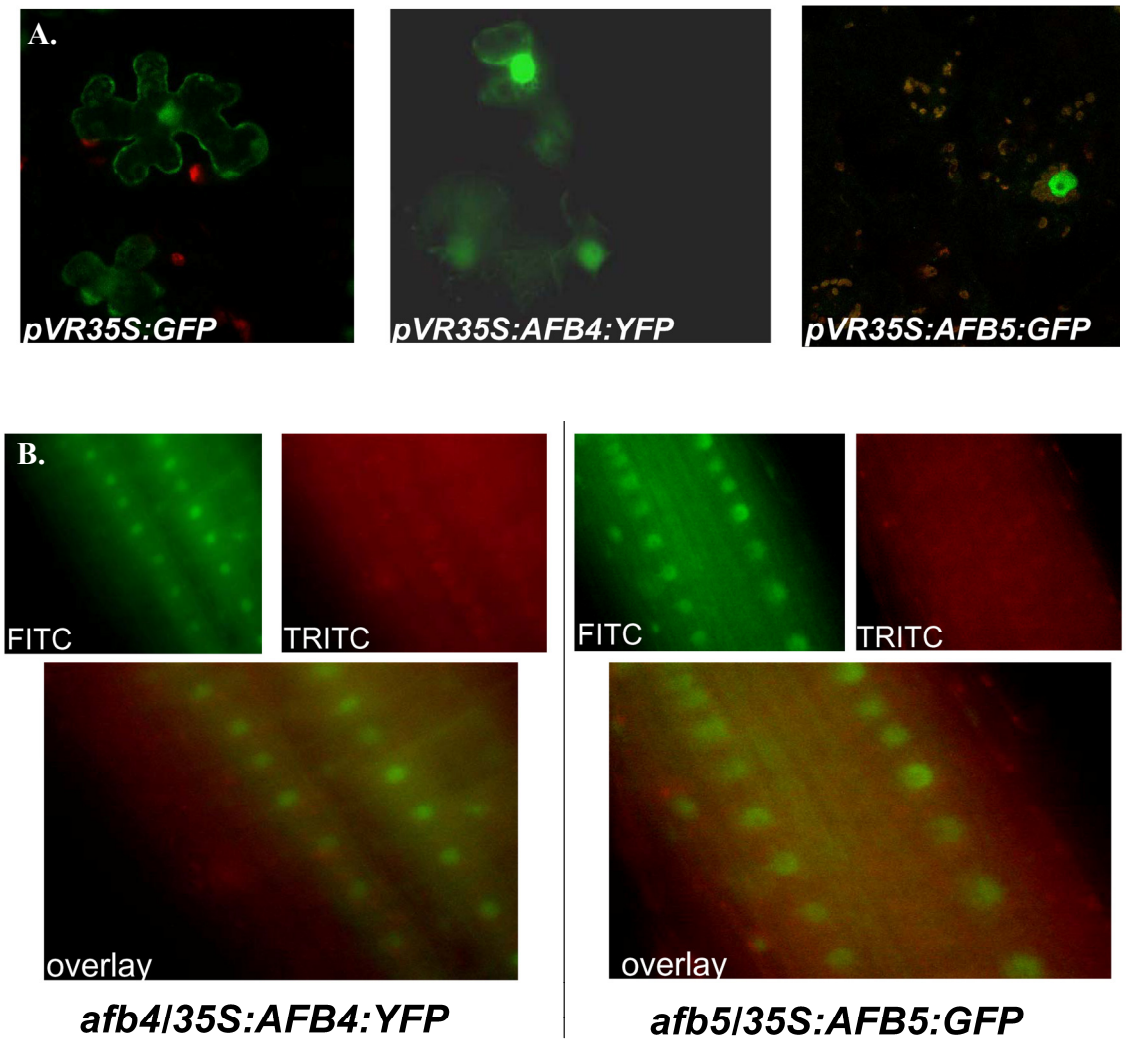


Figure 12. Subcellular localization of AFB4 and AFB5. (**A**) Localization in *Nicotiana benthamiana* by leaf infiltration of *Agrobacterium* and (**B**) One- to two-week old *Arabidopsis thaliana* root tissue expressing *pVR35S:AFB4:YFP* and *pVR35S:AFB5:GFP4Ct* were examined under a Nikon E800 microscope at 100X for fluorescence.

Identification of homozygous single and double T-DNA knockout lines

One way to understand the function of proteins in *Arabidopsis* is through reverse genetics. Fortunately, for both *AFB* genes T-DNA insertion lines were available. For *AFB4* the Gabi-Kat line GABI_068E01 [112] and for *AFB5* the SALK T-DNA insertion line SALK_110643 [113] were available. Confirmation of the insertion via PCR was followed by sequence analysis for the precise location of the T-DNA in the genomic DNA (Fig. 13A). Sequence data for the *afb4* line showed that the T-DNA is located in the second exon at nucleotide 1069 of the genomic DNA. This generated a stop codon 333 codons from the translational start. In the *afb5*line the T-DNA was inserted at the end of the first exon (gDNA nucleotide 600) generating a stop codon approximately 200 codons after the ATG. RT-PCR analysis revealed that no transcripts were detected for *afb4* or *afb5* in homozygous lines (Fig. 13B). This demonstrated that the insertions fully disrupted gene expression and that the corresponding mutant lines are knockouts. The corresponding *afb4* and *afb5* mutants were crossed into each other and homozygous, stable *afb4 afb5* double knockouts were successfully identified in the F2 generation (Fig. 13B).

Phenotypes of *afb4 and afb5* mutant lines

Single *afb4* and *afb5* mutant plants along with the *afb4 afb5* double mutant line underwent a detailed phenotypic characterization. Most striking was the observation that the *afb4* mutant was strongly affected in many aspects of plant development, whereas the

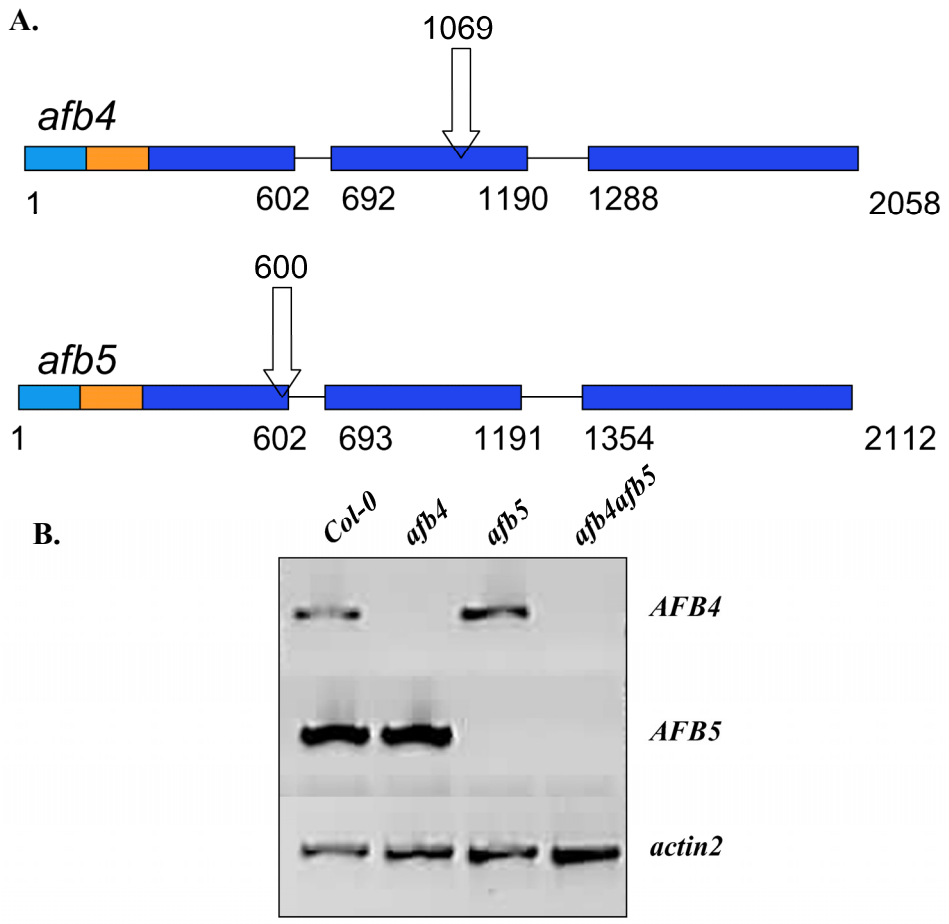


Figure 13. T-DNA insertions in *AFB4* and *AFB5* and effect on expression. **A.** T-DNA insertion into *AFB4* and *AFB5* is represented by an arrow. The precise location of the T-DNA was confirmed by sequencing from the left-T-DNA border into the surrounding genomic DNA. Numbers represent genomic sequence. **B.** RT-PCR analyses demonstrated

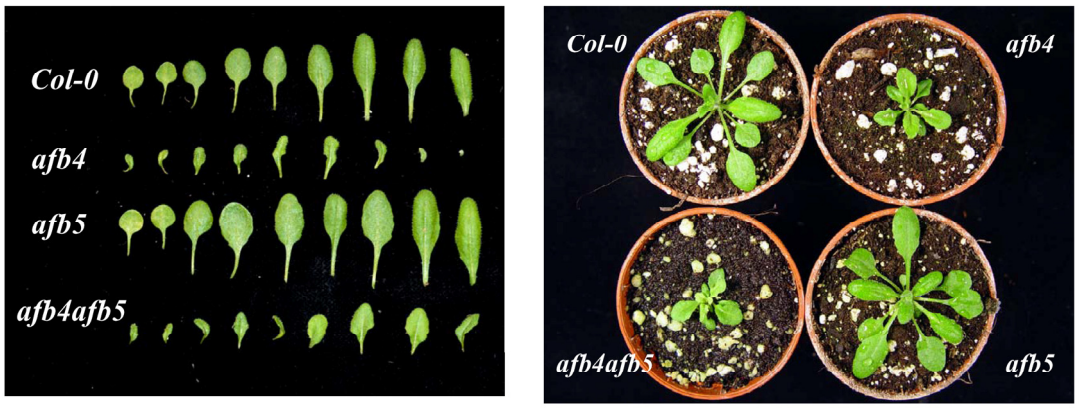
loss-of *AFB4* and *AFB5* in respective single and double *afb4 afb5* mutants. RT PCR was done using 90ng of total RNA, 30 cycles, 5 µl out of a 25 µl reaction were loaded.

afb5 mutant was mostly indistinguishable from *Col-0* wild-type. The double *afb4 afb5* mutant was generally very similar to the single *afb4* mutant.

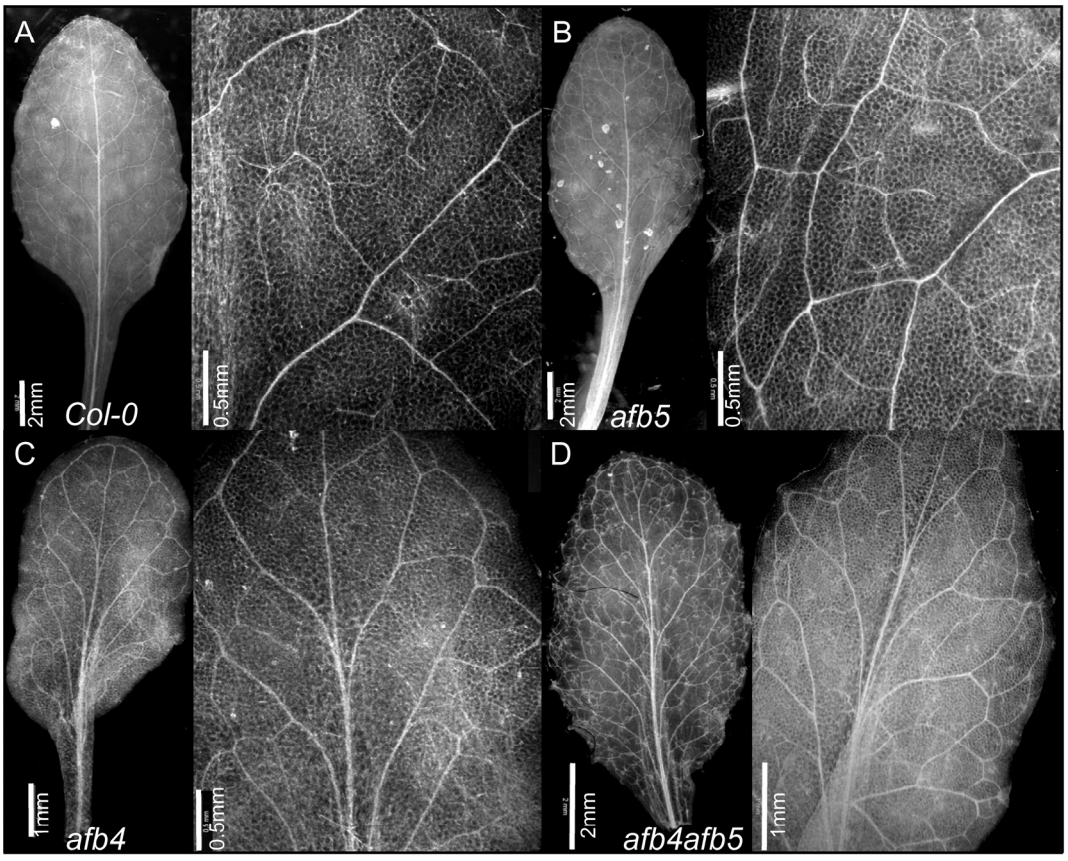
A variety of phenotype changes were clearly observed in *afb4* and double mutant plants. Both lines developed small and often distorted rosette leaves and produced an overall smaller rosette (Fig. 14A). Whereas 20-day-old soil grown wild-type plants developed rosettes with an average diameter of 4 cm, rosettes of *afb4* mutants had a maximum diameter of around 1.5 cm (Fig 17). Because of the leaf-curling phenotype, differentiated rosette leaves were cleared and examined under the microscope to see if vascular development was affected (Fig.14B). No obvious aberrations in major veins were observed. Upon closer examination of the leaf tissue under the light microscope a striking difference in the cell size between wild-type and *afb4* became apparent (Fig. 14C). The smaller cells in the *afb4* leaves clearly account for its dramatically reduced size in relation to *Col-0*.

To verify that the phenotypes observed in the *afb4* background resulted from the absence of functional AFB4, complementation studies were done with constructs carrying tagged versions of the cDNA driven by the strong, constitutive *CaMV35S* promoter. Plants were rescued to wild-type growth as seen in Figure 15.

A.


Col-0
afb4
afb5
afb4afb5
Col-0
afb4
afb4afb5
afb5

B.


A
2mm
Col-0
0.5mm
B
2mm
afb5
0.5mm
C
1mm
afb4
0.5mm
D
2mm
afb4afb5
1mm

C.

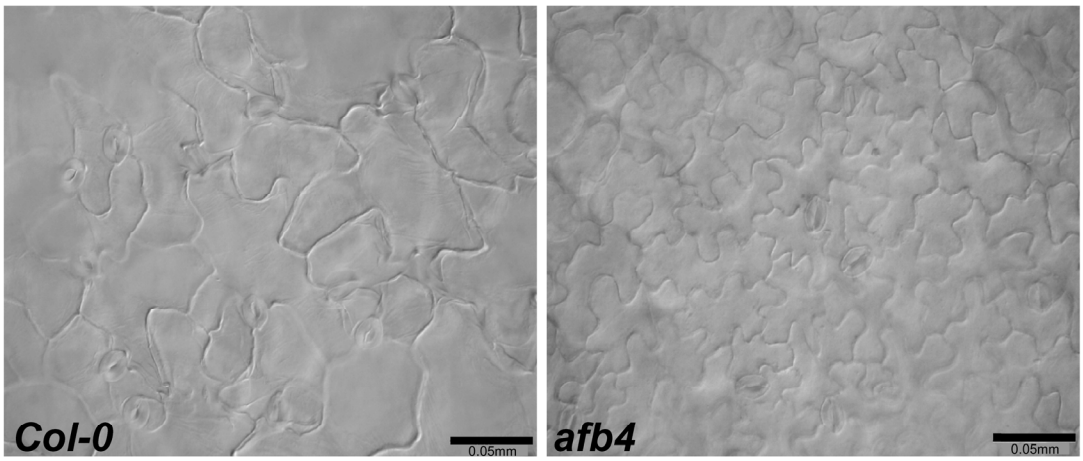


Figure 14. (**A**) Rosette size and leaf comparison at 20 days. (**B**) Differentiated rosette leaves, cleared for observation and (**C**) Light microscopy of leaf cells comparing *Col-0* and the *afb4* mutant, bar represents 0.05mm.

An area of development that seems to be affected in both *afb4* and *afb5* mutant backgrounds is the height of the inflorescence. When measured under long-day growth conditions there was a noticeable reduction in comparison to wild-type, which was augmented in the double mutant plants (Fig. 16).

Interestingly *afb4*/+ plants have a phenotype intermediate between homozygous *afb4* and wild-type plants as seen by the rosette diameter and plant height (Fig. 17). RT-PCR analysis of *AFB4* expression in wild-type and *afb4*/+ lines did not show a clearly reduced expression in the heterozygous mutant but it appears to be likely that the weaker growth defects in *afb4*/+ plants are due to a dose dependent effect.

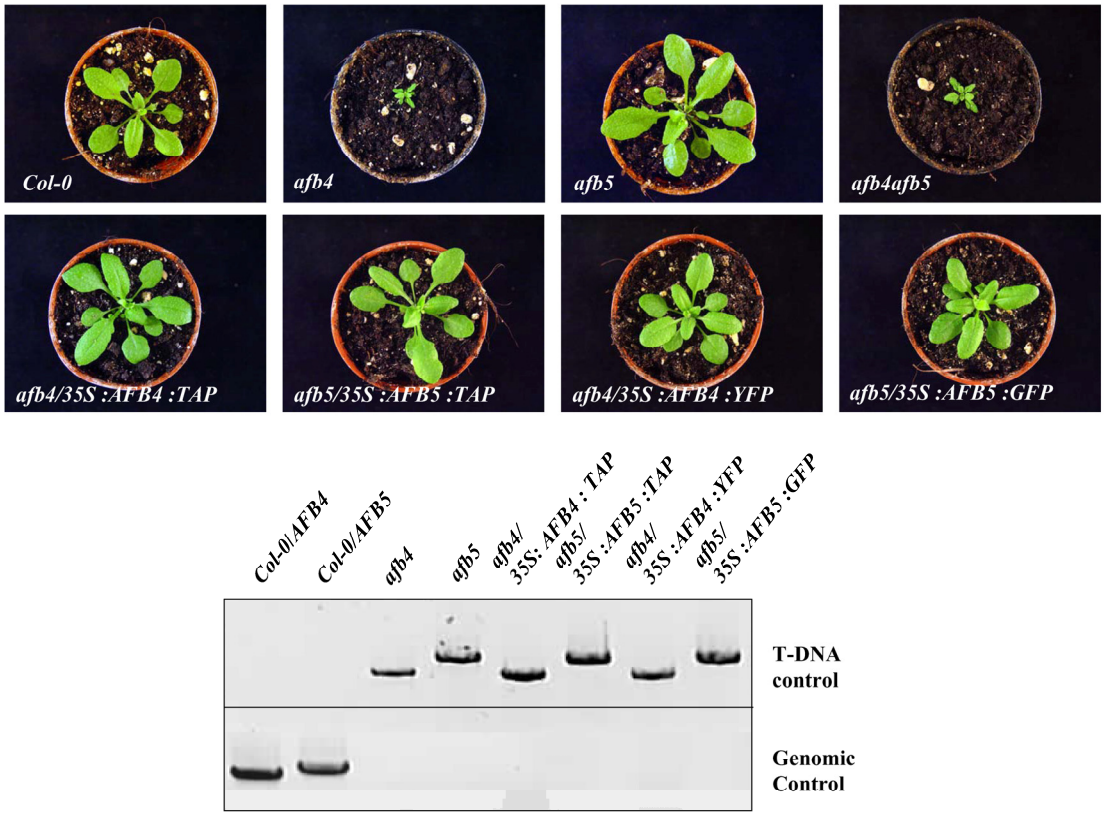
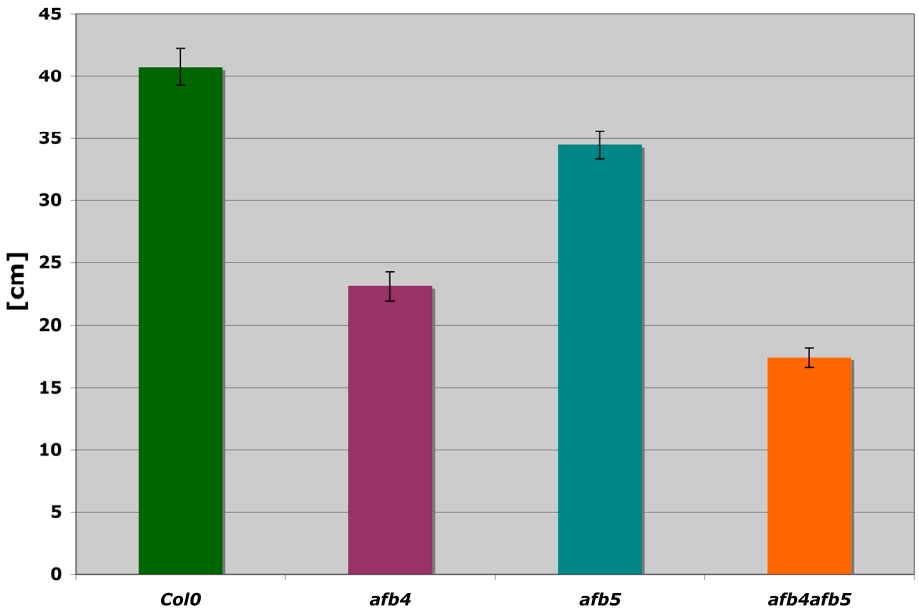


Figure 15. Complementation by overexpressing *AFB4* and *AFB5*. RT-PCR verified the presence of the T-DNA insertion in the mutant genomes.

A.



B.

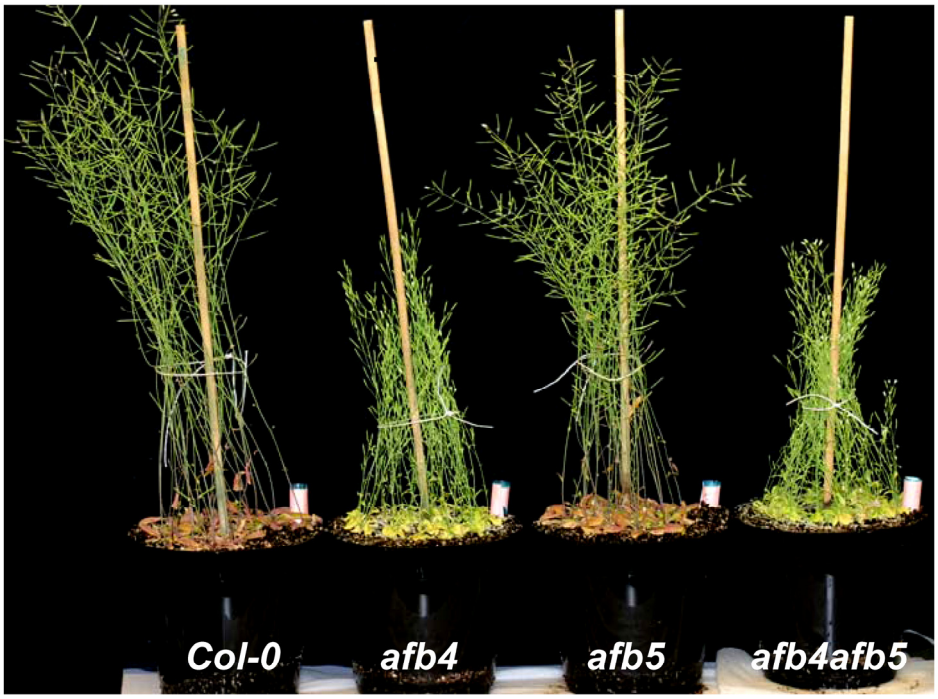


Figure 16. A. Average plant height grown under constant light conditions. Error bars indicate SE. **B**. Plants grown under constant light.

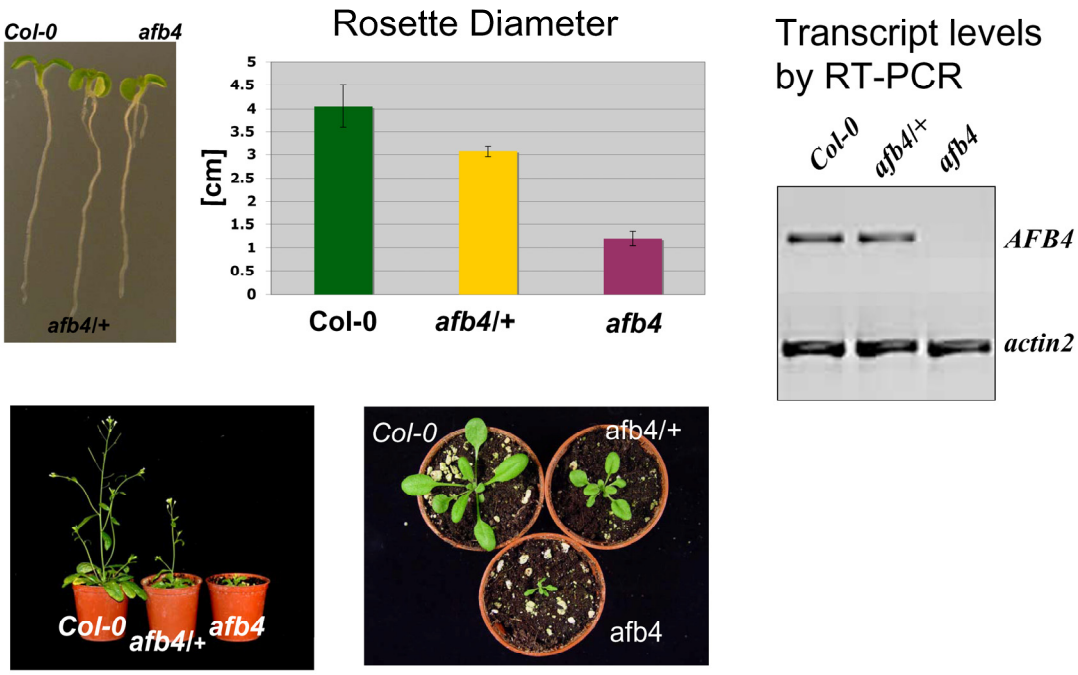


Figure 17. Heterozygous *afb4* plants display an intermediate phenotype, although RT-PCR did not show a noticeable reduction of *AFB4* transcript in heterozygous plants compared to wild-type. Error bars indicate SE.

A difference in flowering time was also observed under long-day growing conditions (Fig. 18). While *afb5* mutants flowered slightly earlier than *Col-0*, *afb4* and the *afb4 afb5* mutants flowered significantly later. An increase in rosette leaf number at flowering time is characteristic for mutants with late-flowering phenotypes [114]. There was only a difference of one to two leaves at flowering time on the mutants compared to wild-type, which would indicate that this could be more of an overall growth delay rather than a late-flowering time.

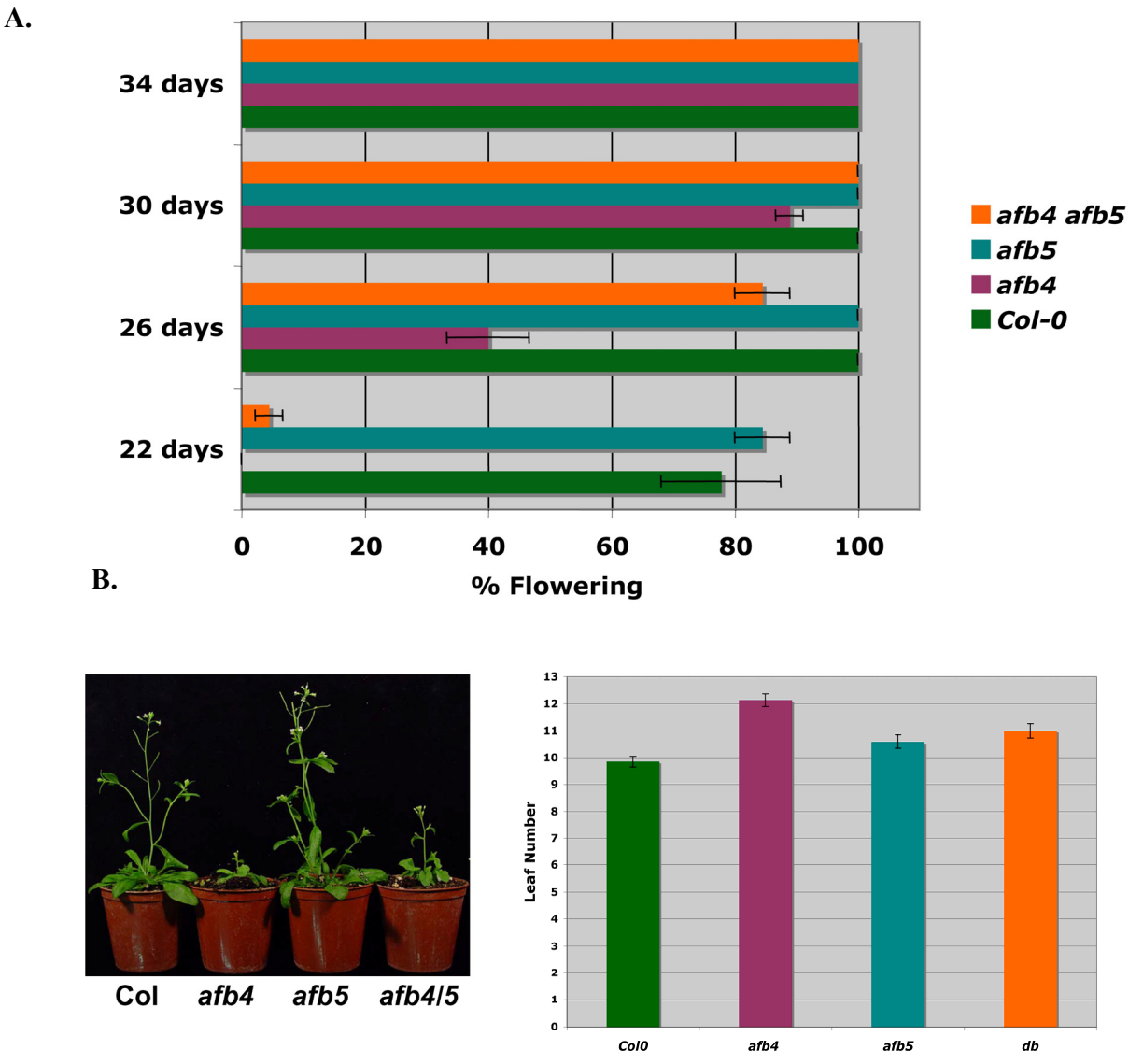
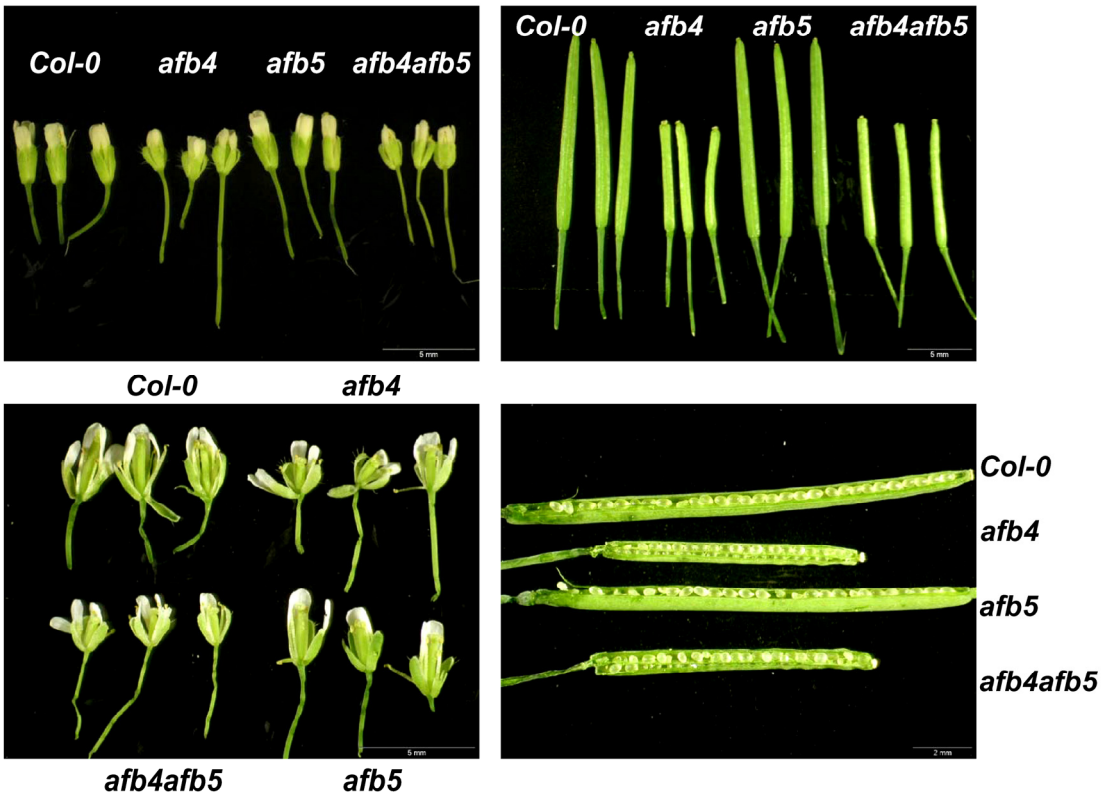


Figure 18. Comparison of (**A**) flowering-time and (**B**) leaf number under long-day growth conditions. Error bars represent SE.

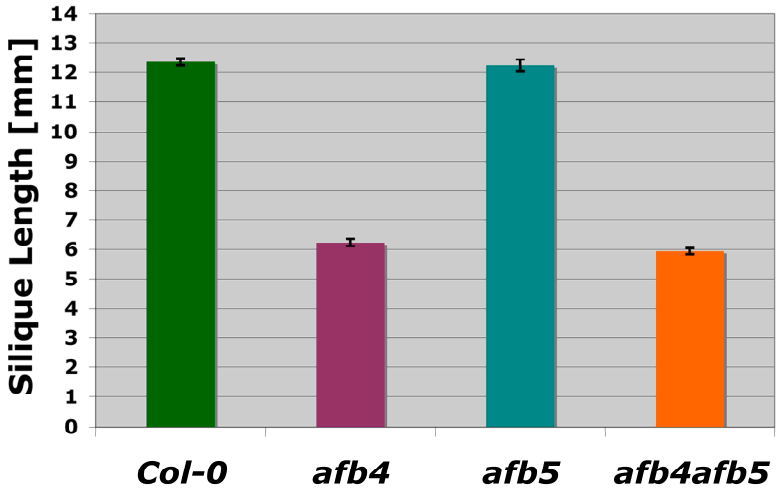
A closer look at the inflorescences from wild-type and mutant lines also revealed developmental differences. *afb4* and *afb4 afb5* plants had slightly smaller flowers and smaller siliques than wild-type (Fig. 19). Measurement of silique length showed that siliques from *Col-0* and *afb5* plants were on average 12.2 mm long, whereas *afb4* and *afb4 afb5* siliques have an average length of approximately 6mm (Fig. 19), which represents a 50% reduction compared to wild-type. As expected the shorter siliques contained fewer seed, but the significant reduction in size and number, however, was not connected with reduced viability. Opening of the siliques revealed that seeds were not aborted (Fig. 19), and harvested seeds germinated normally under laboratory conditions.

Although seed viability was not reduced in the *afb4* background the smaller siliques contained slightly smaller seeds (Fig. 21B). *Col-0* and *afb5* seeds had an average size of 0.14 mm^2. In comparison, the average size of both *afb4* and *afb4 afb5* seeds was 0.12 mm^2 (Fig. 21A). Characteristic for seeds from *Arabidopsis* and other angiosperms is the rapid development of a transparent gel-like coating around the seed after contact with water. This material, called mucilage, is synthesized in the testa during seed development and exists in a dehydrated form before contact with water. The mucilage consists of acidic polysaccharides, mainly unbranched rhamnogalacturonan, and can be stained with the dye Ruthenium Red [172]. Noticeably, staining of wild-type and *afb4* mutant seeds revealed a strong reduction of mucilage coating in the mutant, compared to the wild-type (Fig. 21C). This reduction might provide one explanation for the reduced seed size of *afb4* as it indicates that the production or storage of mucilage is impaired in *afb4* seeds.

A.


Col-0
afb4
afb5
afb4afb5
Col-0
afb4
afb5
afb4afb5
Col-0
afb4
afb4afb5
afb5
Col-0
afb4
afb5
afb4afb5

B.


Silique Length [mm]
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Col-0
afb4
afb5
afb4afb5

C.

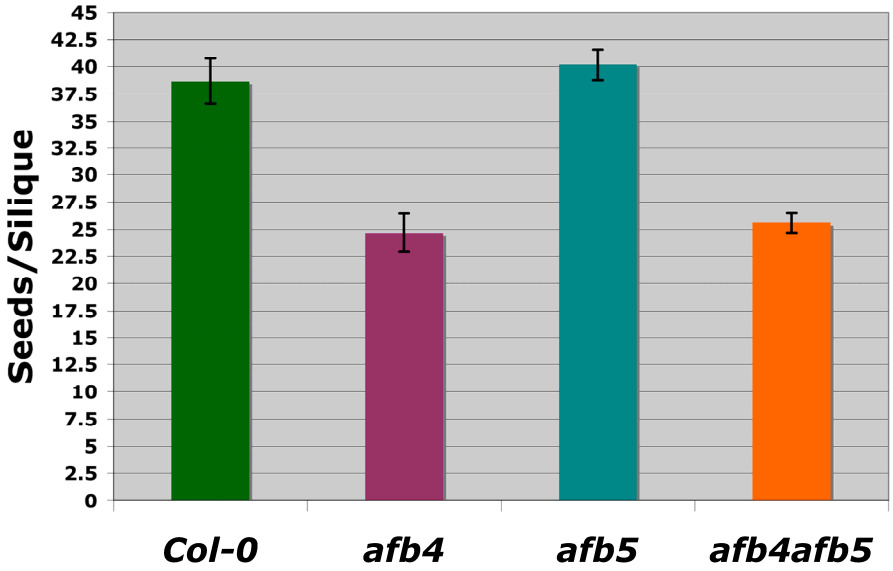


Figure 19. (**A**) Appearance of flowers and siliques in mutant plants. *afb4* and *afb4 afb5* plants have slightly smaller flowers and significantly smaller siliques. Smaller silique length (**B**) correlated with fewer seeds (**C**). Error bars represent SE.

This seed coat phenotype is associated with *glabrous* mutants, which have defects in trichome development [173, 174]. Examination of trichomes on the mutant seedlings did not reveal any obvious defects (Fig. 20).

Figure 20. The *afb4* mutant plants are able to develop trichomes

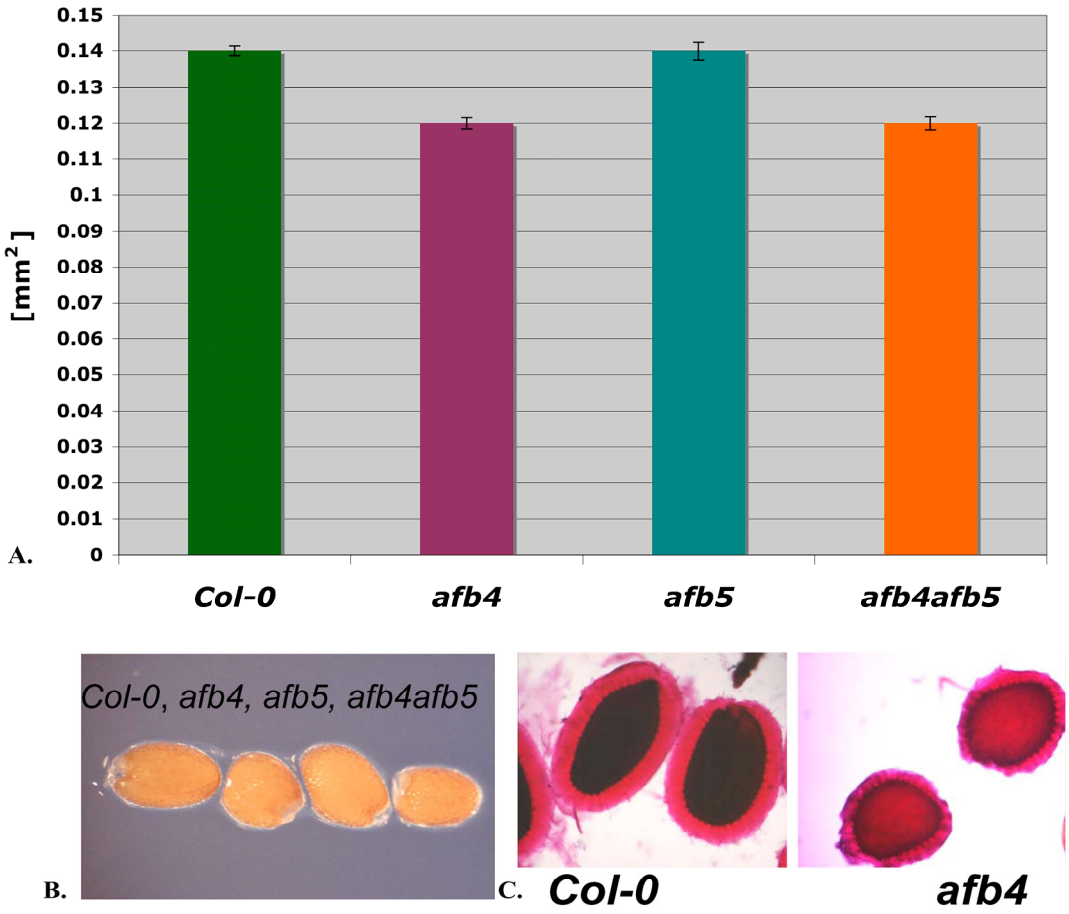


Figure 21. Comparison of seed size and seed coat. **(A)** Quantification of wild-type and *afb* mutant seed surface. Error bars represent SE. **(B)** Light microscopy of representative seeds demonstrates smaller size of *afb4* and *afb4 afb5* seeds. **(C)** Pectin staining with ruthenium red staining demonstrates well-developed mucilage for *Col-0* and strongly reduced mucilage in case of *afb4*.

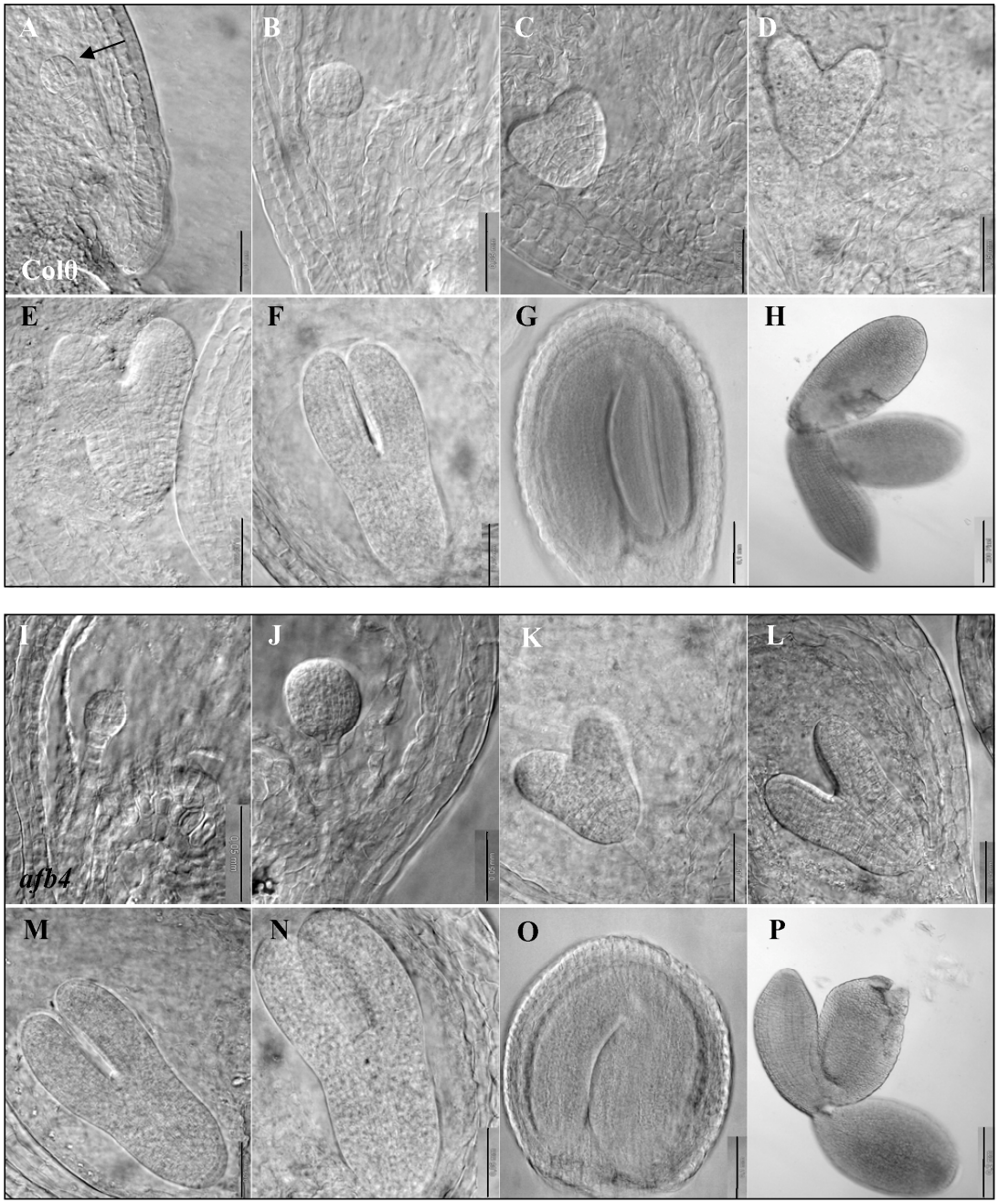


Figure 22. Normal embryogenesis is seen in *afb4* mutants. (**A-H**) *Col-0*; (**I-P**) *afb4*, (**A, B, I, J**) globular stage, bar is 0.05mm; (**C, D, K, L**) heart stage, bar is 0.05mm; (**E, F, M, N**) torpedo stage, bar is 0.05mm; (**G, O**) bent cotyledon stage, bar is 0.1mm; (**H, P**) emerged, bar is 0.2mm.

Expression of the *AFB4* gene was detected in all stages of embryogenesis (Fig. 10), To see if the anomalous seed development had any obvious affects on embryo development, embryos at different developmental stages (globular to bent embryo stage) were examined in wildtype and mutant lines (Fig. 22). Embryos were cleared and examined using DIC microscopy. No pronounced defects were observed. However, at the bent cotyledon stage, *afb4* embryos appear to be slightly smaller than *Col-0* embryos (Fig. 17G, H and O, P).

Many aspects of the aberrant phenotype, including the reduced leaf size and curling, were detected in young seedlings. Another early difference was reduced height of the hypocotyl in the *afb4* background (Fig. 23). This defect was observed under all growth conditions including darkness. Microscopic examination of hypocotyls revealed a shorter, more square-like cell structure in the *afb4* background compared to the longer more rectangular structure found in wild-type plants (Fig. 24). As with the smaller cells in the leaves, this reduction could account for the difference in length seen between *afb4* and *Col-0* hypocotyls.

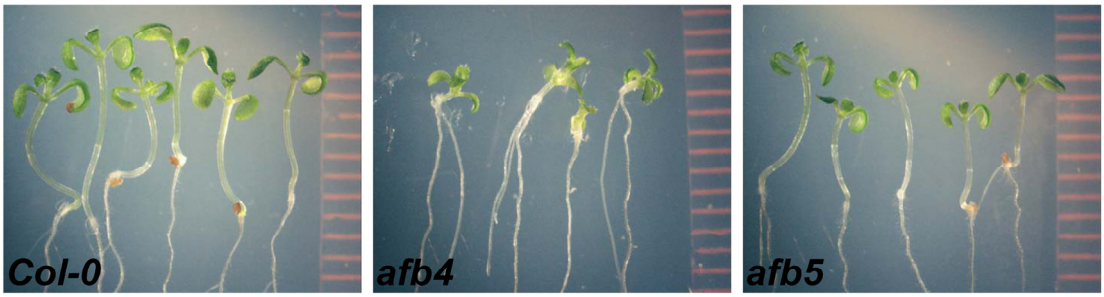


Figure 23. Seedlings grown on ATS eight days at 22^{o}C under yellow filter. *afb4* plants have significantly shorter hypocotyls than wild-type and *afb5* plants.

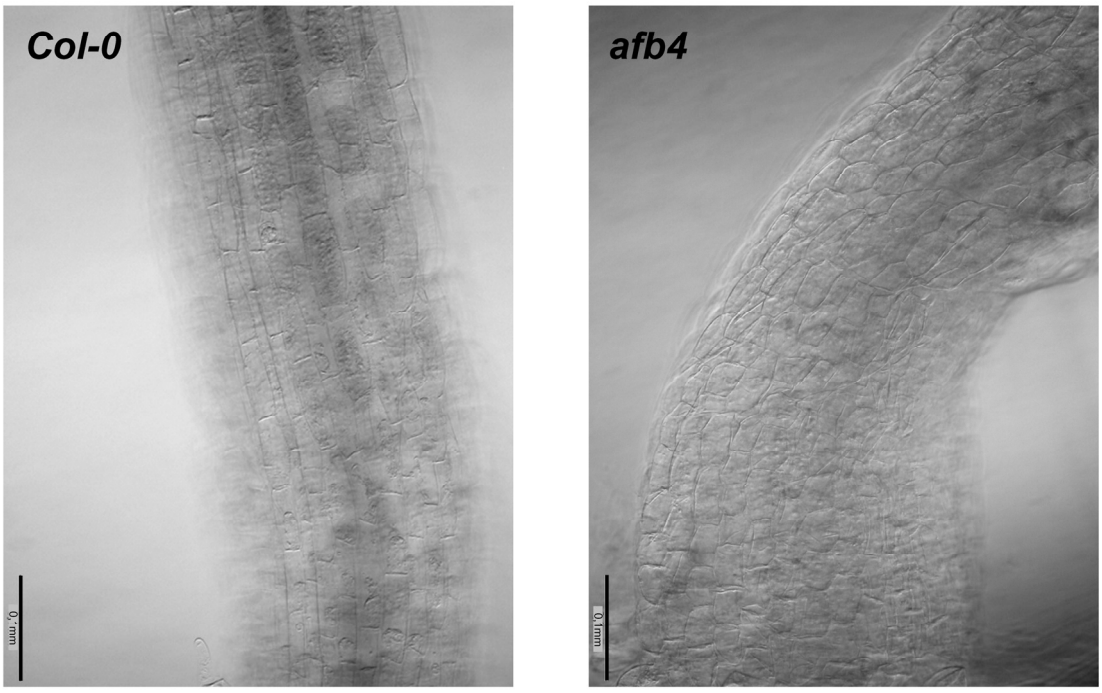


Figure 24. There is a significant difference in the hypocotyl cell size and shape of *afb4* mutants compared to wild-type. Bar represents 0.1mm.

afb4 and the *afb4 afb5* mutants developed yellow, bleached rosette leaves at early seedling stages when grown under constant white light in sterile culture, indicating reduced chlorophyll content (Fig. 25A). Quantification of chlorophyll showed that the *afb4* mutant had nearly 40% less chlorophyll A and B in comparison to wild-type, and the double mutant had a 60% decrease (Fig. 25B). The much stronger reduction in the double mutant was surprising, since chlorophyll content in the *afb5* mutant was around 20% increased in comparison to wild-type and one might have anticipated an intermediate phenotype for the double mutant. A similar situation existed for carotenoids (Fig. 25C). Again the double mutant had the strongest reduction in comparison to wild-type (60%), whereas *afb5* plants had on average up to 25% more carotenoids.

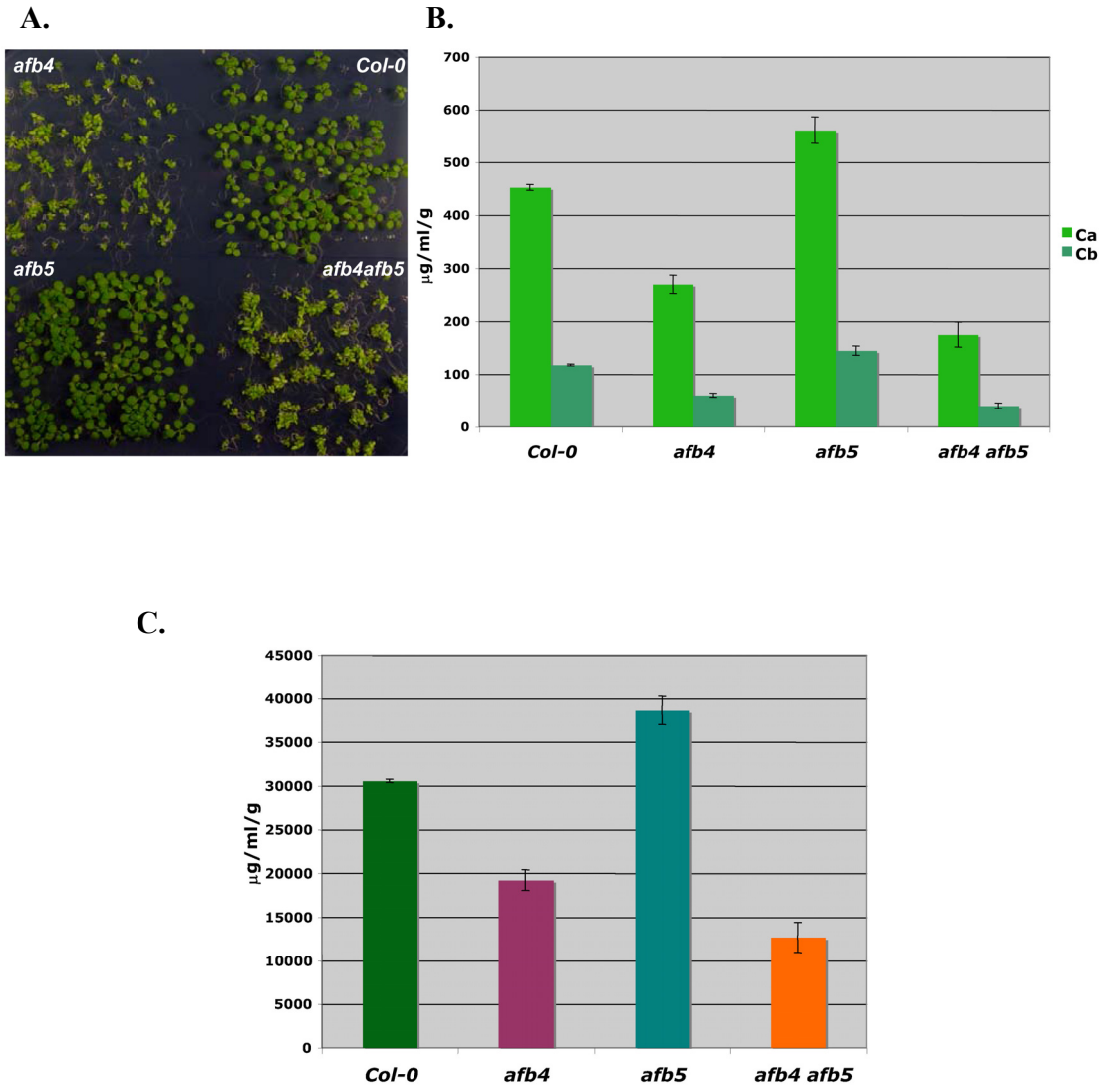


Figure 25. Reduction in pigment levels of *afb4* seedlings. **A.** Seedlings on ATS. **B.** Chlorophyll measurements. **C.** Carotenoid measurements. Error bars represent SE.

One possible approach to further explore the changes in pigment levels is to investigate the sensitivity of the *afb* mutants to protochlorophyllide in bleaching assays. The idea behind these assays is that when grown in darkness seedlings accumulate protochlorophyllide. Upon irradiation, protochlorophyllide absorbs light energy and can dissipate reactive oxygen species causing cell damage. This is prevented by photo

activated protochlorophyllide oxidoreductase, which rapidly converts protochlorophyllide to chlorophyllide as a critical step in chlorophyll biosynthesis [175, 176]. Consequently, an increase in protochlorophyllide, reduced oxidoreductase activities, or lower levels of reactive oxygen quenching pigments like carotenoids, can be fatal to plants. In any of these cases one might expect bleaching of dark-cultivated mutants after irradiation. Seeds were exposed to white light for one hour, grown in darkness for four days, transferred back to white light and compared after two days for green or bleached leaves. As a positive control for increased sensitivity *pif1* (phytochrome A interacting factor) was used. The *PIF1* gene participates in red light signal transduction and the mutant over-accumulates protochlorophyllide [115, 116], making it hypersensitive to the bleaching assay. As shown in Fig. 26, *afb4* and the *afb4 afb5* double mutant are even more sensitive to the bleaching conditions than *pif1* seedlings, while *afb5* seedlings were not hypersensitive. The increased sensitivity of the *afb4* mutant might be due to reduced levels of protecting carotenoids, suggesting that proto-chlorophyllide levels are similar to wild-type. Further investigation, such as assays to measure chlorophyll conversion upon exposure to light, would need to be performed to definitively confirm whether or not the chlorophyll biosynthesis pathway is indeed impaired in these mutants.

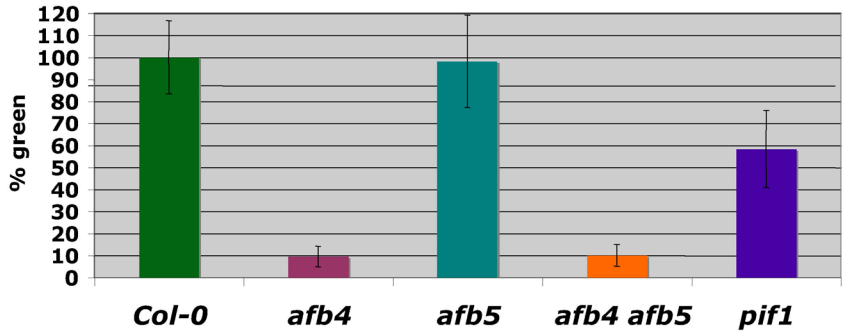


Figure 26. Bleaching assays. The *afb4* and the *afb4 afb5* double mutants showed greater sensitivity than *pif1*, a mutant that has been shown to accumulate protochlorophyllide in bleaching assays. Error bars represent SE.

It has been shown that growing plants under yellow-filtered light not only reduces degradation of nutrients in plant culture media but also slows the degradation of free IAA in media [117]. In general growth under yellow-filtered light may be less stressful to the plants than the commonly used white fluorescent light. Because the *afb4* plants showed sensitivity to white light conditions in the growth chambers, most experiments, including root analysis, were performed under yellow-filtered light.

A comparison of wild-type and mutant primary root growth on ATS at 21^{o}C under yellow filters did not reveal any major changes (Fig. 27A). However, *afb4*, *afb5*, and the double mutant each developed fewer lateral roots than wild-type. While 12-day-old *Col-0* seedlings had an average of nearly two lateral roots per cm of the primary root, *afb5* seedlings had 1.5/cm while *afb4* and *afb4 afb5* seedlings had approximately 1 lateral root/cm (Fig. 27B). In contrast, *afb4* and *afb4 afb5* plants developed increased numbers

of adventitious roots at the hypocotyl (Fig. 27C). This difference was not observed in *afb5* seedlings. These findings indicate that AFB4 and AFB5 are involved in lateral root development and demonstrate an additional function for AFB4 in adventitious root development.

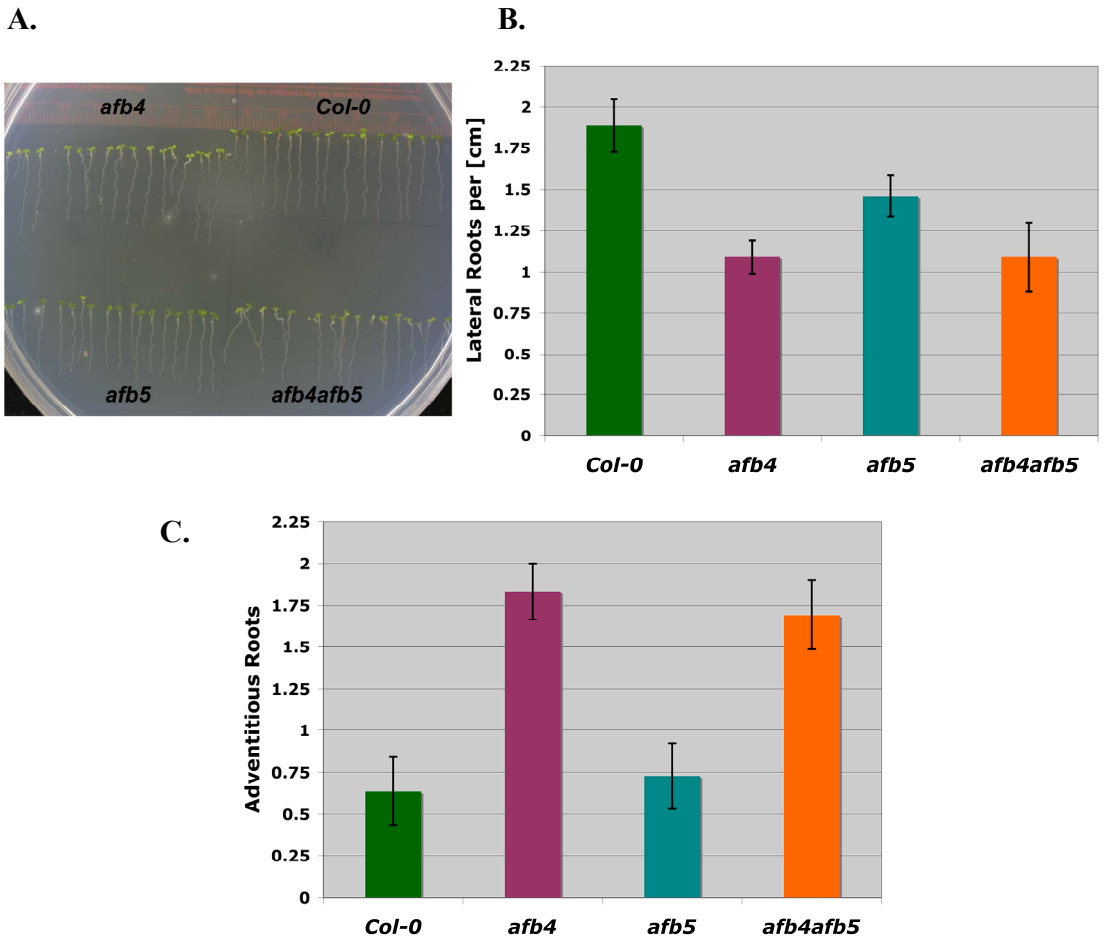


Figure 27. Root development. **A.** Primary root growth of five-day-old mutant seedlings was similar to *Col-0.* **B.** The mutants have decreased lateral root development compared to *Col-0* at 12 days. **C.** *afb4* and *afb4 afb5* seedlings have increased adventitious root production compared to *Col-0* and *afb5* seedlings at 12 days. Error bars represent SE.

The mutants carrying either the *35S:AFB4:TAP* or the *35S:AFB5:TAP* constructs (Fig. 15) were examined for complementation of the root developmental defects. Ectopic expression of AFB4:TAP fully restored lateral root development back to wild-type levels in *afb4* plants. Interestingly, *afb5* plants over-expressing AFB5:TAP developed more lateral roots than wild-type (Fig. 28A). This indicates complementation of the mutant and functionality of the tagged protein; however, it also indicates that the amount of AFB5 in the plant is critical for correct lateral root development. Surprisingly, over-expression of AFB4:TAP did not reduce adventitious root growth in *afb4 35S:AFB4:TAP* plants and *afb5 35S:AFB5:TAP* lines showed significant increase in adventitious root growth (Fig. 28B). This was unexpected in both cases and might be due to special hypersensitivity of the hypocotyl towards constitutive expression of both *AFB4* and *AFB5* genes.

Auxin response is crucial for gravitropic responses, and promoter-GUS data showed both AFB4 and AFB5 to be expressed in root tissues (Fig.11). Adventitious, lateral and primary roots of seedlings between two to three week-old were examined for gravity response after two days of stimulus. No obvious defects were seen in the different root types of the mutants (Fig.29)

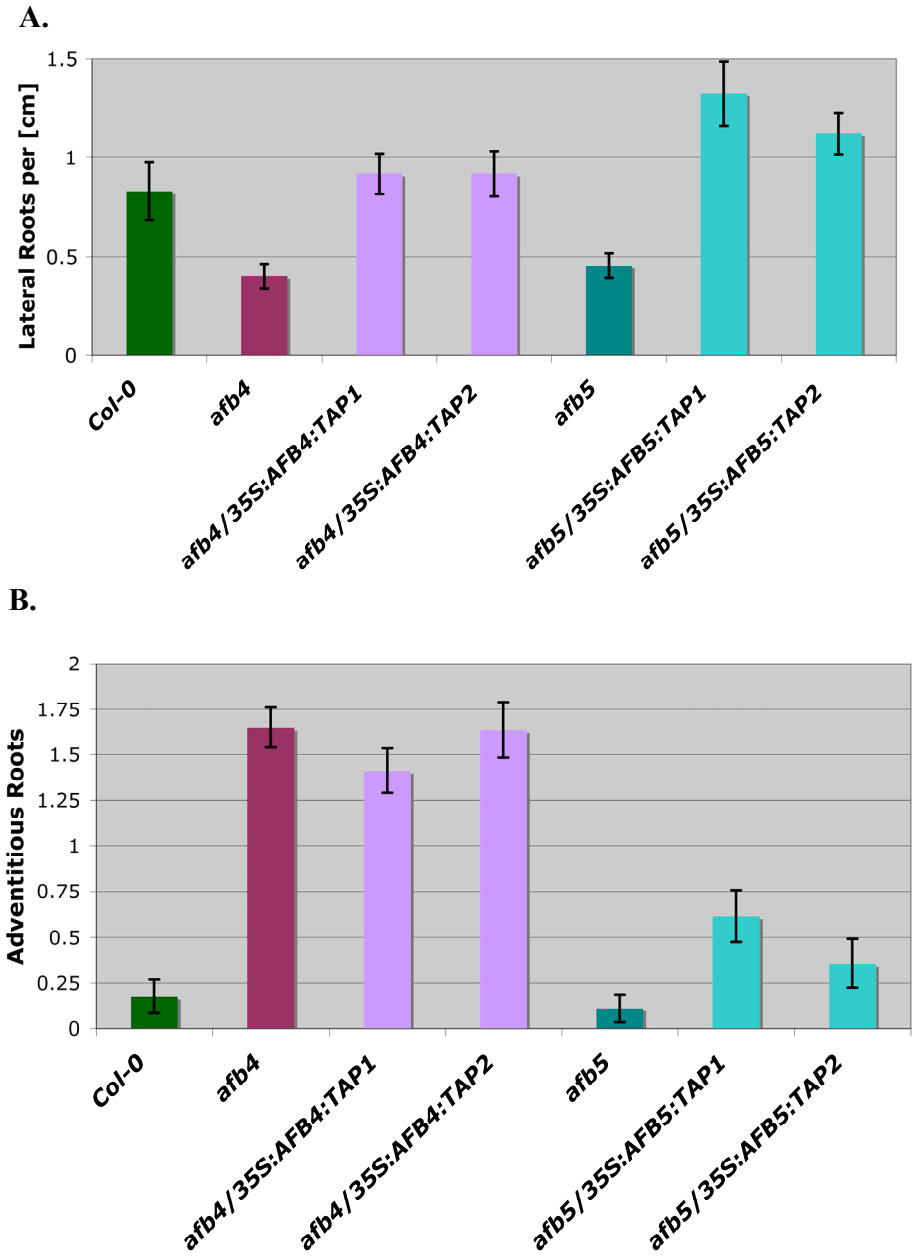


Figure 28. Analysis of root development in mutant lines over-expressing *AFB4* and *AFB5*. **A.** Lateral root development was restored to at least wild-type levels. **B.** Adventitious root growth was not rescued in *afb4* seedlings and was slightly promoted in *afb5* seedlings. Error bars represent SE.

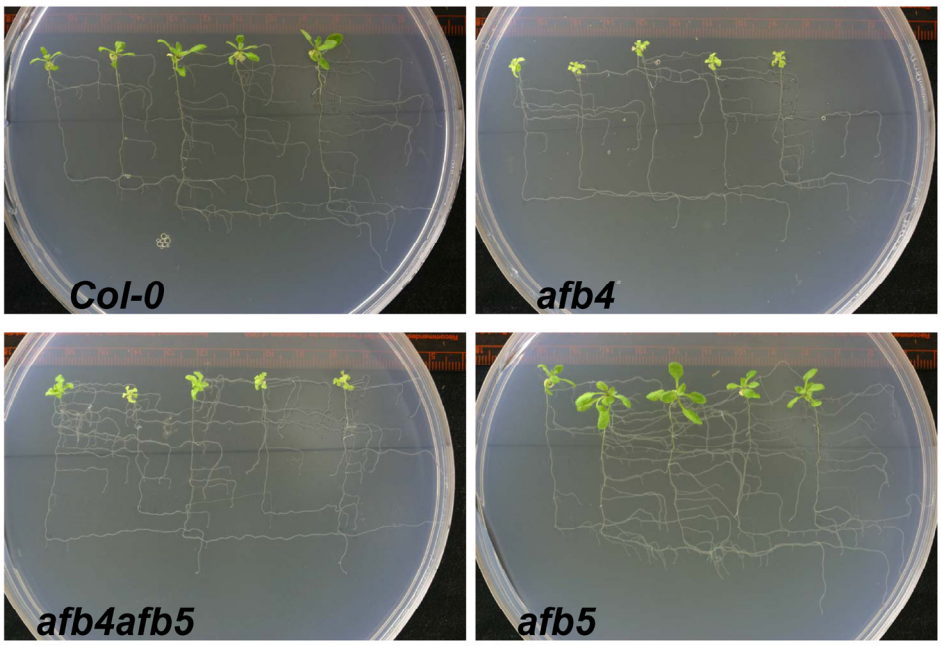


Figure 29. Long-term gravitropic response is not delayed in the mutant backgrounds

DISCUSSION

Sequence of AFB4 and AFB5:

The most striking difference between AFB4 and AFB5 compared to the other family members is the N-terminal extension of around 50 amino acids. Most prominent within this stretch is a repetition of serine residues (AFB4$^{22\text{-}34}$, PPCSSSSSSSSAA; AFB5$^{30\text{-}39}$, PCSSSSSPNK). Computational analysis using the ELM program, http://elm.eu.org, of these first 50 amino acids predict phosphorylation sites for several different kinases including cyclin-dependent protein kinases (CDK) and casein Kinase I (CKI). This leads to the question of whether or not this serine-rich N-terminal extension plays a special role in the function of AFB4 and AFB5. Gel shift assays utilizing specific antibodies to probe

for changes in the different AFB bands appearing on Western blots could provide a possible indication for phosphorylation events. Expression of N-terminal deleted versions of the proteins under control of the native promoters to look at complementation and expression patterns might also shed light on the importance of this sequence. An N-terminal extension was also noted for two other leucine-rich F-box proteins, EBF1 and EBF2 [179, 180]. These were reported to participate in an SCF that regulates the ethylene response transcription factor, EIN3. Although there was no sequence similarity such as the serine-rich area, ELM also predicts some phosphorylation motifs on the *EBF* sequence, though not nearly as extensive as for *AFB4* and *AFB5*.

Expression of *AFB4* and *AFB5*:

RT-PCR and analyses of *promoter:GUS* lines were done to look at transcript levels and expression patterns of *AFB4* and *AFB5*. RT-PCR showed expression in all tissues that were tested with *AFB5* RNA levels significantly higher than *AFB4*. This is similar to what is seen for the other family members with *AFB2* and *AFB3* displaying much higher RNA levels than *TIR1* and *AFB1* [106]. In addition, the *promoter:GUS* lines indicate that *AFB5* expression is higher than that of *AFB4*. For the most part, expression of *AFB5* is similar to the other family members except in the primary root. Here *AFB5* was detected only in the emerging lateral root and in the developing lateral root tips. While *AFB4* is expressed in the primary root and lateral roots, interestingly, expression was not seen in the root tips. These experiments utilized only the promoter region of the genes, so if there are transcriptional or translational controls or post-translational protein modifications that affect protein function, this may not be reflected in this data. Recent information about

miRNA regulation of *TIR1*, *AFB2* and *AFB3* highlight this possibility [9]. While *AFB4* and *AFB5* have not yet been identified as having sites for any miRNA regulation, they do have the unique N-terminal region containing sites that could be phosphorylated. It would be interesting and important to examine plants that express AFB4: and AFB5:GUS fusion proteins under the control of their natural promoters. Here expression and localization of the GUS protein is also under the control of potential regulatory steps post-transcriptionally affecting AFB4 or AFB5. Not surprisingly AFB4 and AFB5, like the others [106], were nuclear localized making them possible candidates to also be auxin receptors of SCF ligases for the Aux/IAA transcription factors.

Phenotype of *afb4* and *afb5* mutant plants:

T-DNA insertion knockout lines of *AFB4* and *AFB5* were examined to determine their roles in plant development. Because these proteins are around 80% identical at the amino acid level it was very surprising that such a difference was seen between the *afb4* and *afb5* phenotypes. *afb5* plants, like the other family members, were generally similar to the wild-type in appearance. *afb4* plants however were dramatically different. Also because of the similarity between AFB4 and AFB5, the double mutant line was generated. The double mutant, except for a few exceptions, generally resembled the *afb4* plants.

The most dramatic feature of *afb4* plants is their overall decrease in size compared to wild-type. *afb4* and *afb4 afb5* rosettes are less than half the size of wild-type rosettes. The small rosette leaves are curled. This is common among other auxin response mutants such

as many of the gain-of-function mutations in *Aux/IAA* genes, and triple mutant combinations of the other *TIR1/AFB* family members [58, 127, 161].

Recent work on leaf patterning has shown that repression of the AS1 Myb transcription factor promotes leaf formation. AS1 is a class I KNOX transcription factor, which positively affects meristem activity. This is in concert with the auxin gradients established by PIN1 [118]. Expression of the *KNOX* gene *BP* results in misformed leaves. In both the *as1* and *pin1* backgrounds *BP* transcript is increased. This effect was also observed in the *axr1* background and in the *axr3* background. *axr3* results in a gain-of function mutant in a IAA17 which inhibits its substrate capacity with the $SCF^{TIR1/AFB}$ [101]. Given the pronounced defects in *afb4* leaf development and formation it would be interesting to see whether expression or activity of any of these proteins is aberrant in the mutant.

Closer examination of the rosette leaves of the *afb4* mutant showed that the cells are smaller than in wild-type, which would clearly be a contributing factor to the smaller leaf size. Global profiling of the 29 *Aux/IAAs* showed that each gene is widely expressed with distinct profiles for tissue and levels of expression [42]. Also each tissue expresses large sets of the family. Further underlying their redundancy, analysis of triple loss-of-function mutants did not show a change in auxin regulated gene expression. However, the gain-of-function mutant *axr3-1* (*IAA17*) profile displayed many transcriptional changes with 114 auxin-regulated genes affected. Transcription of many other genes not directly related to

auxin signal transduction were affected including genes involved in cell wall biosynthesis and cell elongation.

Another interesting observation in the *afb4* mutant leaves was the paler green color in the early stages of seedling growth. Leaves contain a number of different pigments including chlorophylls and carotenoids [119]. Measurements of these pigments revealed that this mutant contains half the level compared to wild-type. Recent work on a family of early light-induced genes, *ELIPs*, has shown that these genes are induced under stress and that overexpression leads to pale green leaves with a decrease in photosynthetic pigments [120].

Also strong link between GA biosynthesis and pigment biosynthesis has been established through a shared enzymatic reaction. In plastids the committed step in GA biosynthesis is catalyzed by ent-copalyl diphoshate synthase (CPS). The substrate of CPS is also a direct precursor of carotenoids and of the phytol side chain of chlorophyll [121]. Another link with GA metabolism has been shown through PIF1, a transcription factor which regulates GA metabolism [122]. *pif1* mutant seedlings displayed a similar response to the *afb4* mutant seedlings in bleaching assays for photooxidative stress recovery and PIF1 has been shown to be degraded by the 26S proteasome [116]. While no direct links between auxin and pigment biosynthesis have been shown so far, it could be possible that AFB4 targets a protein that affects this pathway, or that the absence of AFB4 leads to a stress response that results in the induction of genes such, as the *ELIP* family, that are then affecting pigment biosynthesis.

The reduced cell size that was seen in the leaves was also evident in the hypocotyls of the *afb4* seedlings. Shorter hypocotyls were seen in both light and dark, a defect that has also been documented for many of the gain-of-function *Aux/IAA* mutants such as *axr2-1* (*IAA7*) [123] and *shy2-1* (*IAA3*) [124], clearly demonstrating a function of auxin and members of the Aux/IAA family in cell elongation. Given a possible role of AFB4 in targeting Aux/IAA proteins for proteasomal degradation it appears to be plausible that the observed shortened hypocotyl of *afb4* is caused by stabilization of Aux/IAA proteins.

Another striking feature of the *afb4* hypocotyl is the increase in adventitious roots. Increased adventitious roots are seen in the auxin overproducers *sur1* and *sur2* [188, 189]. However unlike any of the TIR1/AFB family members, these plants have generally increased root development. Work done on *ago1*, a mutant in the RNA-silencing complex, has linked adventitious root development to ARF17 [190]. *ago1* mutants accumulate miRNA targets, which include members of the *ARF* family. Decreased levels of free auxin were measured in *ago1*, and this mutant is resistant to auxin mediated hypocotyl elongation, and it has decreased adventitious root formation. Levels of *ARF17* transcript were increased by seven-fold and *ARF10* by two-fold in the hypocotyl of this mutant. No change was measured for *ARF6, 8* or *16*. Down-regulation of the early auxin-response *GH3* genes, as measured by *GH3:uidA* GUS staining, was also seen in the *ago1* background. *GH3* genes encode for auxin conjugating enzymes and three of these have been correlated to adventitious root number. In an *ARF17* overexpressing line not only were all three significantly repressed, there was also decreased adventitious root development.

Primary root development in the *afb4* and *afb5* seedlings is similar to wild-type, but in both backgrounds a reduction in lateral root development was observed. The importance of the TIR/AFB family in root development is dramatically seen in the *tir1 afb1 afb2 afb3* mutant [106]. Root development was severely compromised and ranged from no discernable root to highly disorganized root meristem, even if root development appeared relatively normal. Promoter:GUS staining was not seen in the primary root tips of *afb4* and *afb5* seedlings, and Lugol staining of the singles or the double showed no defects in the root meristems. Recent work on lateral root patterning has shown the importance of auxin signaling and perception in lateral root positioning, initiation and development [125]. Lateral root development is initiated in the root pericycle [126] which is triggered by auxin signaling and response in the adjacent protoxylem cells.

Lateral roots form at regular spaced interval alternating between 'left' and 'right' sides off of the primary root. This corresponds to the root wave or bend in response to gravity as the primary root grows. In mutants of the auxin influx carrier, *aux1*, gravitropic growth and response is disrupted, the root bends to the right and lateral root distribution is favored on the left or outer side of the coil [193]. Closer examination of the role of auxin revealed an oscillation of auxin response by *DR5::GUS* with the strongest response in the protoxylem cells adjacent to the pericycle cells where the new lateral root is initiated. This periodicity from auxin signal through perception and subsequent lateral root initiation took about twenty hours and followed the pattern of the cell cycle marker *CYCB1;1::GUS* expression for lateral root formation. To look at auxin response, the gain-of-function IAA17 mutant *axr3-1* was selectively expressed (UAS:*axr3-1*) in these

xylem pole cells. This mutant has been previously reported to have reduced lateral roots [127]. The *UAS:axr3-1* disrupted all stages of lateral root development and very few made it past the first stages of initiations. Another gain-of-function mutant, *iaa14*, leads to no lateral root formation. This gene is expressed in the pericycle where initiation occurs, but not in the cells where auxin response and priming occur. Dex-inducible promoter driven expression of *iaa14* inhibited lateral root initiation. Interestingly this inhibition was reversible when roots were transferred off of Dex in all but the very proximal region of the primary root. These experiments highlight the importance of auxin response by the *Aux/IAA* genes for lateral root priming, initiation, and development. Regulation of these proteins by the TIR/AFBs including AFB4 and AFB5 is crucial to proper root development as evidenced by the inhibition in their absence and in the case of AFB4 the increased production of adventitious roots.

Flowers in the *afb4* mutant are normal, just smaller than wild-type. Similarly to leaf development, polar transport of auxin plays a role in flower initiation and differentiation [128] and antibodies against IAA show that young flowers accumulate high concentration of auxin pools which may be released later in development. *DR5:GUS* analysis has identified high levels of free auxin production and accumulation in anthers and stigma. Following fertilization auxin levels increase and are then available for embryo development [129]. Examination of embryos in the mutants showed no major developmental problems, although the *afb4* embryos seemed slightly smaller than wild-type in the final stages of development.

The smaller flowers of *afb4* resulted in smaller siliques, which corresponded to fewer seeds. Seed size and shape were quite different in the mutant. The paler *afb4* seeds had reduced seed coat mucilage. This defect has been seen not only in plants mutant for enzymes involved in biosynthesis and extrusion of mucilage, but also in several transcription factors including *APETALA2*, *TRANSPARENT TESTA GLABRA1* and *GLABRA2* [172, 174]. The transcripts of these genes are up-regulated in developing siliques and expression of one of the mucilage biosynthesis genes is severely decreased in siliques of the transcription factor mutants. Interestingly though, none of these mutants had the smaller, misformed seeds seen in *afb4*, and in fact *ap2* mutants produce seeds with increased mass [197]. Germination and viability can be a problem for seeds that have storage or metabolism defects if the media is not supplemented with sucrose [130]. There were no problems with viability of the mutant seeds, and they could germinate and grow on media with or without sucrose. This seed phenotype was also observed for the *tir1 afb1 afb2 afb3* quadruple mutant (Hong Ren pers.comm.). Closer examination of this interesting and unusual phenotype would need to be done to get a clearer idea of how this could be related to these mutants and to auxin response.

An antagonistic effect of AB4 and AFB5 function was seen in flowering time. While *afb4* was delayed in flowering, the *afb5* mutant showed a modestly early flowering time point in comparison to wild-type. Consequently, the *afb4 afb5* double mutant had an intermediate flowering time point: earlier than the *afb4* single mutant but still clearly later than the *afb5* mutant. The delayed flowering time for *afb4* is interesting as it brings into question whether flowering time is directly affected by AFB4 activity, or whether the late

flowering time point is simply a result of the overall reduced growth and aberrant development of the mutant. However, to provide a better understanding of the aberrant flowering phenotype a more detailed set of studies needs to be performed for both mutants. Growth conditions for long day and constant light for these experiments should be standardized for less variation and short day measurements need to be taken. It would also be necessary to investigate in greater detail whether expression of classical genes involved in flowering control, like for *constans* or *leafy* [131] are aberrant in one of the *afb* mutants.

Summary

The observed mutant phenotypes clearly demonstrated that AFB4 is very important for various aspects in plant development. AFB5, although more strongly expressed than AFB4, appears not to be as crucial as AFB4 for most aspects of plant development.

III. AFB4 and AFB5 Function in Auxin Signal Transduction

Introduction

The characterization of AFB4 and AFB5 point strongly to a possible role in auxin response that, in many respects, parallels the other family members. Although the phenotypes seen for *afb4* mutants are quite dramatic, many of these have also been observed in the higher order *tir1/afb* mutants previously examined [106]. This chapter will examine auxin response through classic auxin resistance assays and begin investigating the auxin regulatory pathways that AFB4 and AFB5 would be expected to participate in.

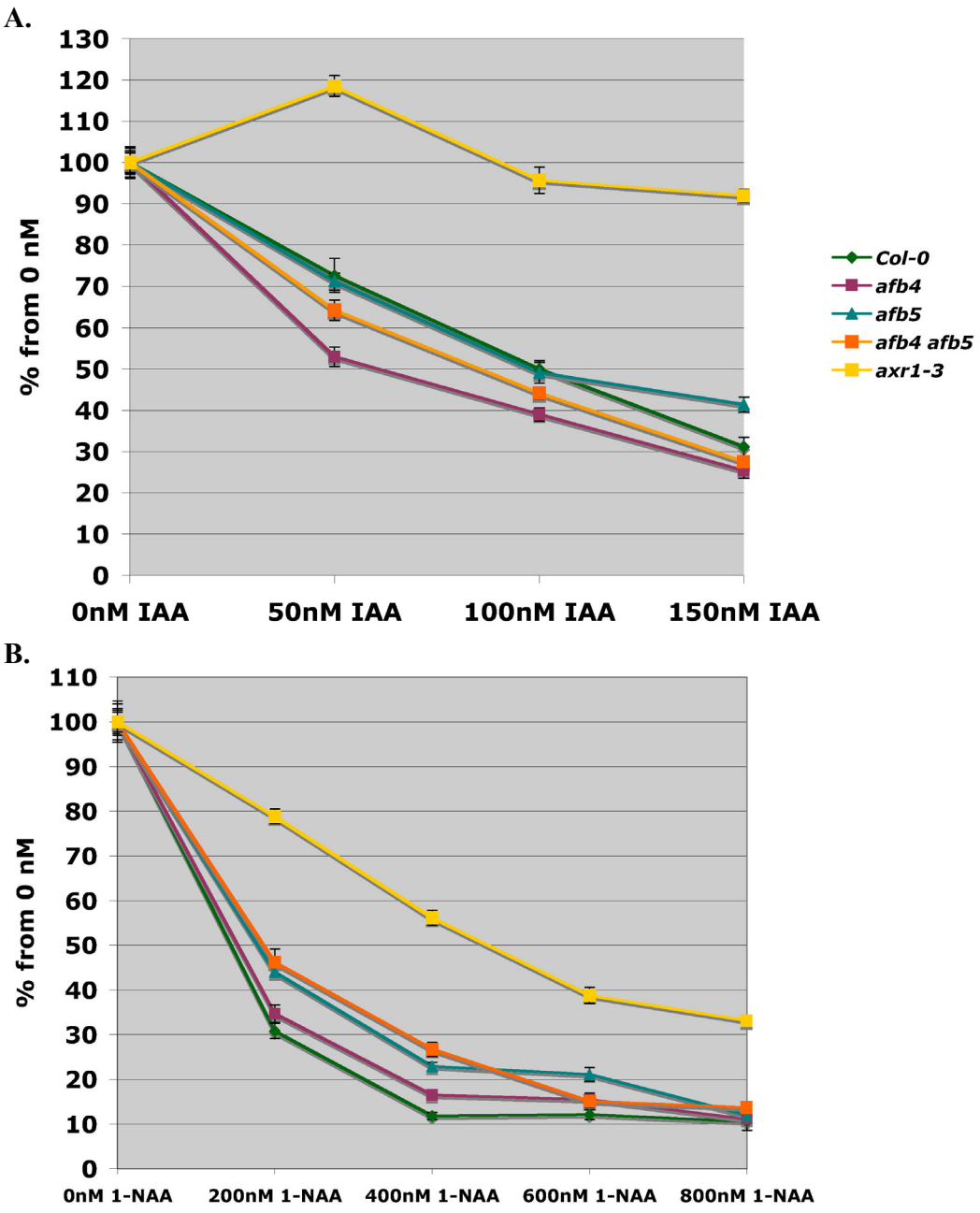
Results

Auxin response in roots of *afb4* and *afb5* mutants

Due to the close similarity between AFB4, AFB5, and TIR1, it likely that both proteins are involved in auxin response. One approach to assess this is to investigate the sensitivity of plants to auxin in root elongation growth assays [132]. In these assays primary root growth is measured in relation to the concentration of auxin in the growth medium.

Four different auxins were used including the naturally occurring auxin IAA, and three synthetic compounds, 1-NAA (1-naphtaleneacetic acid), 2,4-D (2,4-dichlorphenoxyacetic acid), and picloram (4-amino-3, 5, 6-trichloropicolinic acid). 1-NAA is a lipophilic auxin that can passively diffuse through membranes. IAA, 2,4-D and picloram can diffuse through the membrane but are also actively transported into the cell [133]. 2,4-D has also been reported to be a substrate of the auxin influx carrier but not the efflux protein [134,

135]. Picloram, like 2,4-D, is a commercially available auxin-like herbicide in use since the 1960s [134, 136].



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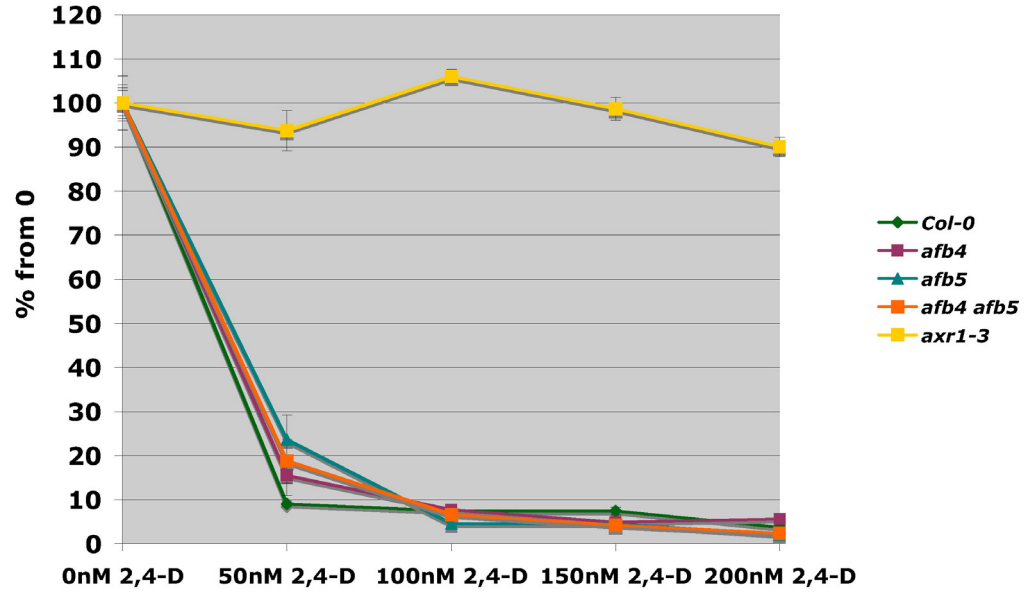

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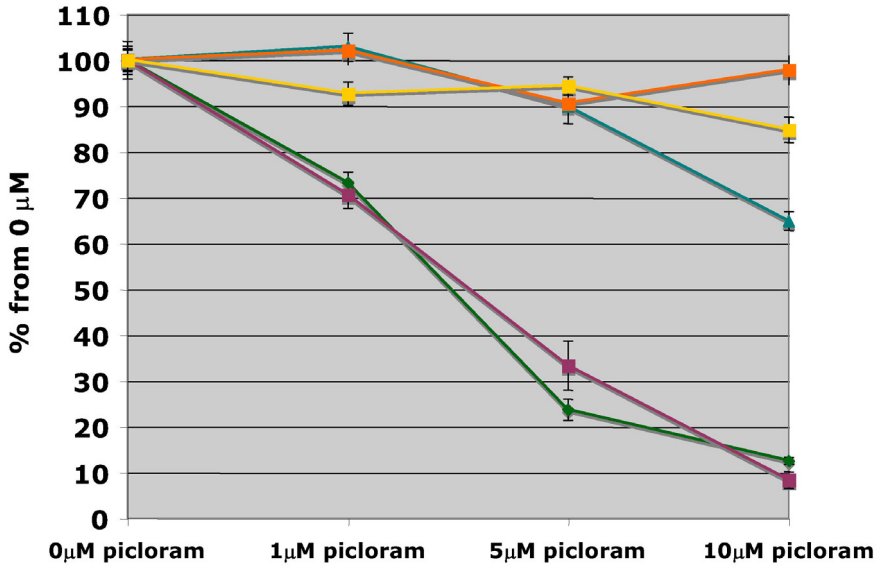


Figure 30. Root elongation assays with the naturally occurring auxin IAA, and the three synthetic auxins 1-NAA, 2,4-D, and picloram. Root growth is expressed relative to unsupplemented medium. Error bars represent SE.

Besides *Col-0* wild-type and the different *afb* mutants, the auxin resistant *axr1-3* mutant was also used as a control for reduced auxin sensitivity [80]. While *axr1-3* showed clear resistance at all concentrations, primary root growth of wild-type and all *afb* mutants was strongly inhibited on 2,4-D, IAA and 1-NAA (Fig. 30). Picloram was tested in a range of 0 to 10 μM and had varying effects on the different *afb* mutants. The *afb4* primary root behaved similarly to wild-type, but *afb5* and the double mutant were highly resistant to picloram with no obvious reduction in primary root growth. Only at very high concentrations of 10 μM was *afb5* root growth inhibited. Surprisingly growth of the double mutant was not inhibited at this concentration and appeared to be even more picloram resistant than *axr1-3* roots.

The impact of picloram on lateral and adventitious root development was also investigated (Fig. 31). Like other auxins, the compound increased the number of lateral roots that developed in wild-type plants. Interestingly *afb4* plants were more sensitive to picloram with an approximately five-fold increase in lateral root development compared to the just over three-fold observed in wild-type at 10μM. In contrast, the *afb5* mutant response was very weak, with hardly any change detectable for lateral root development even at the highest concentrations of picloram. In keeping with its *afb5*-like response to picloram on primary root elongation, the *afb4 afb5* double mutant was also insensitive towards picloram with respect to lateral root development. Picloram also induced adventitious root development in wild-type plants at 5 μM and 10 μM, with the strongest effect at 5 μM. Similar to the effect on

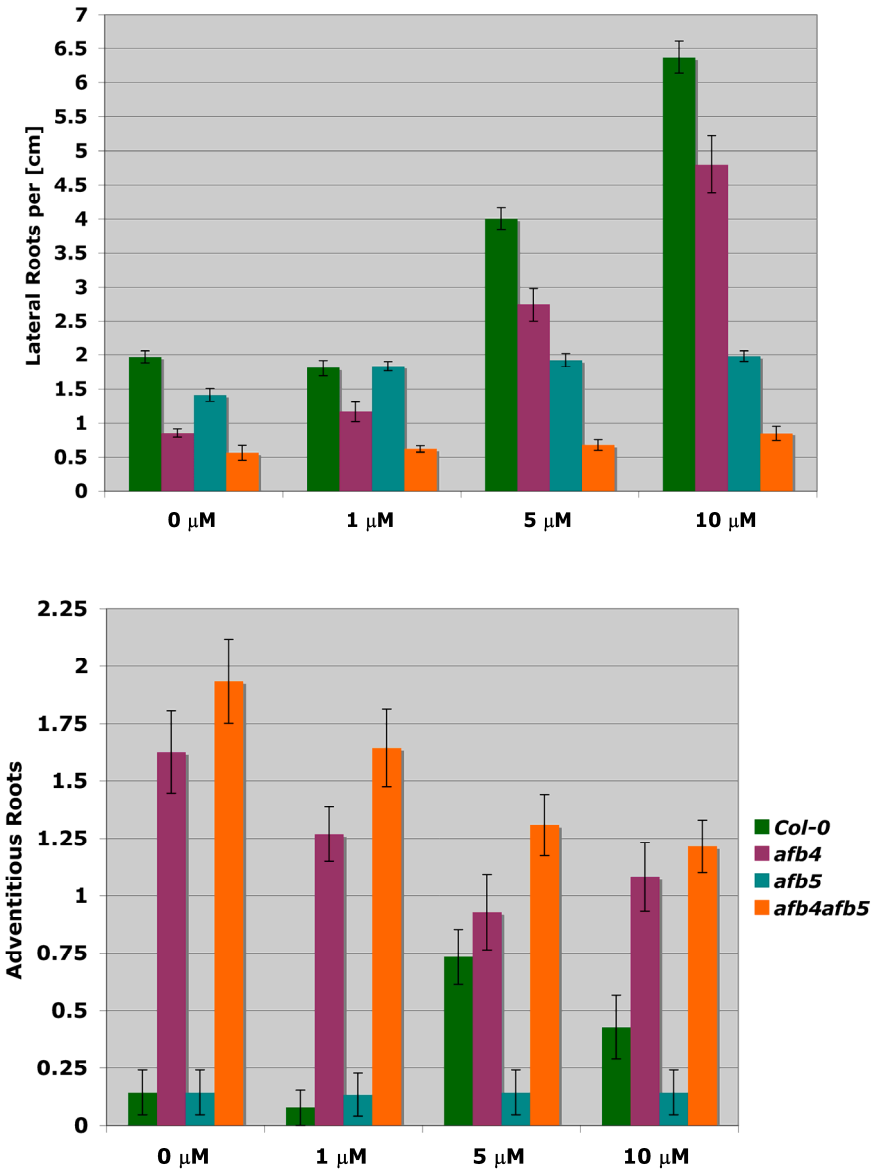


Figure 31. Effects of picloram on root development of *Col-0* and *afb* mutants at 12 days.

lateral roots, picloram did not enhance adventitious root development in *afb5*. This finding indicates a general resistance to picloram in *afb5* plants. Surprisingly, increased picloram application caused a slight reduction in the adventitious roots of the *afb4* which *afb5* was not able to overcome in the double mutant. This demonstrates not only the hypersensitivity in *afb4* but also highlights the different functions of these two genes.

Auxin response in hypocotyls of *afb4* and *afb5* mutants

Dramatic effects of picloram on the hypocotyls of mutant plants were also observed. To determine if this was compound specific, hypocotyls were also examined in the presence of 2,4-D. On 200nM 2,4-D, *Col-0*, *afb5* and *axr1-3* plants were not affected, while *afb4* plants and the double mutants were affected by picloram (Fig. 32 middle row). On 10 μM picloram *Col-0* and *afb4* plants were dramatically affected, forming callus-like growths on the hypocotyls and rosettes accompanied by yellowing and death of the early leaves (Fig. 32, bottom row). The double mutant, while not as severely affected, showed some callus-like growth along with curling, yellowing, and narrowing of the leaves. Mutant *afb5* and *axr1-3* had some curling and narrowing of the leaves and *axr1-3* had slight growth at the hypocotyl base (Fig. 32, third and last columns). The *afb5* hypocotyl continued to exhibit robust growth under this high concentration of picloram. The sensitivity of *afb4* and the double could account for the picloram effect seen on adventitious roots (Fig. 31).

The sensitivity of roots and hypocotyls was restored to *Col-0* levels in *afb5 35S:AFB5:TAP* plants (Fig. 33), again demonstrating functionality of AFB5:TAP and confirming that the observed picloram-induced phenotypes of *afb5* plants were in fact caused by loss-of AFB5.

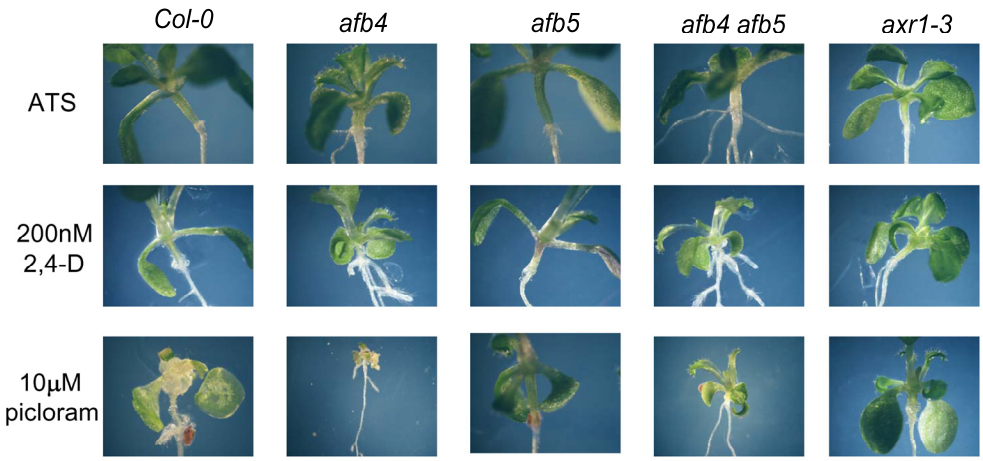


Figure 32. The effect of auxin on hypocotyl development in wild-type, *axr1-3*, and *afb* mutants. Plants were cultured for 10 days on ATS under constant light with yellow filters before transfer to auxin for 2 days of additional growth under the same conditions.

AFB4 and *AFB5* transcriptional response to auxin

Although there was no observable change in expression levels of either *AFB4* in the *afb5* background or of *AFB5* in the *afb4* background compared to wild-type (Fig. 9), it is still possible that picloram induces expression of *AFB4* and *AFB5*. This can affect mutant sensitivities towards picloram: for example, if *AFB5* is inducible but not *AFB4* then perhaps upon picloram treatment increased *AFB5* expression could account for sensitivity of *afb4* and reciprocally the resistance of the *afb5* mutant. In this context it is also important to confirm that picloram is activating auxin-responsive pathways comparable to IAA or 2,4-D. To answer these questions, *AFB4* and *AFB5 promoter:GUS* lines were analyzed for auxin response and compared with the *GUS* expression of plants carrying the auxin responsive *DR5* promoter (*proDR5:GUS*) [137]. Incubation of *proAFB4:*,

proAFB5:, and *proDR5:GUS* lines with 2,4-D (10 µM) or picloram (10 µM) showed that neither of the *proAFB:GUS* plants responded to the treatment (Fig. 34). In contrast, *proDR5:GUS* plants showed equally strong induction of *GUS* expression after 1h treatment for both 2,4-D and picloram. These findings strongly suggest that transcription of *AFB4* and *AFB5* is not auxin regulated. It further shows that picloram is regulating the *DR5* promoter in a manner similar to 2,4-D, thus activating auxin responsive pathways.

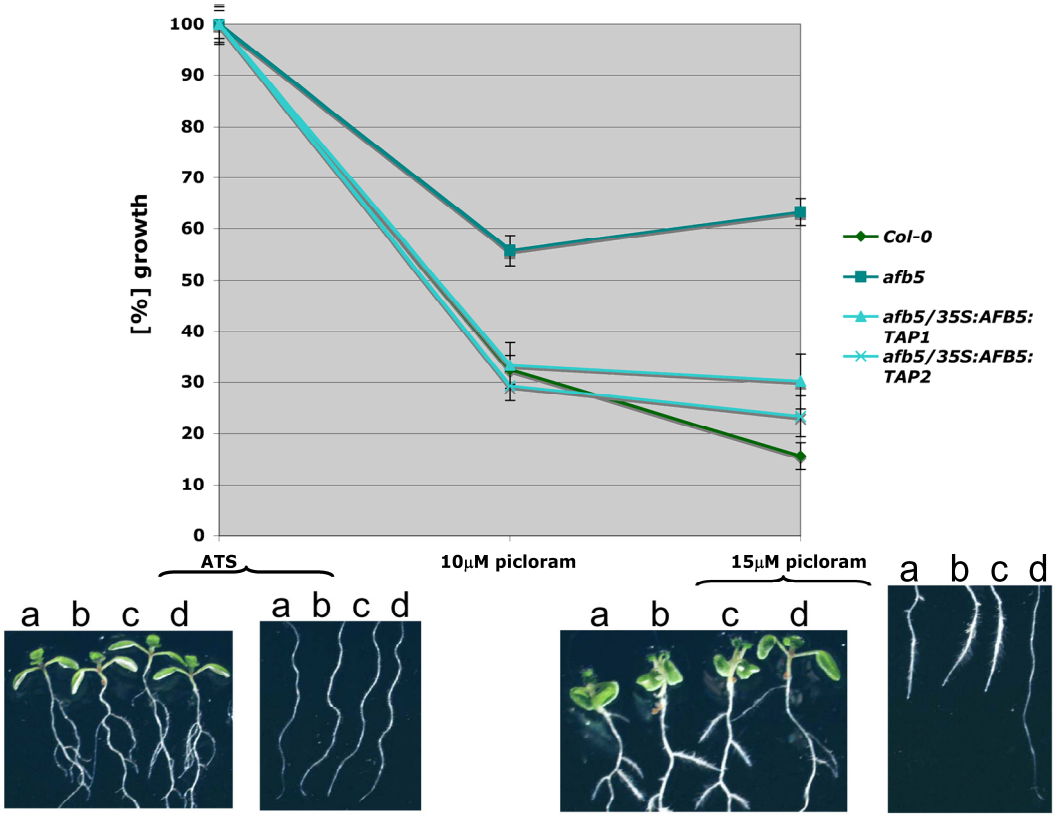


Figure 33. Complementation of *afb5* by ectopic expression of AFB5:TAP in the mutant background. *Col-0*, *afb5*, and two independent transgenic lines were investigated for picloram sensitivity. The graph shows restoration of sensitivity in root elongation assays when the transgene is expressed. Likewise, the apical affects of picloram can be seen in the pictures. (a. *Col-0*, b. *afb5 35S:AFB5:TAP1*, c. *afb5 35S:AFB:5TAP2*, d. *afb5*).

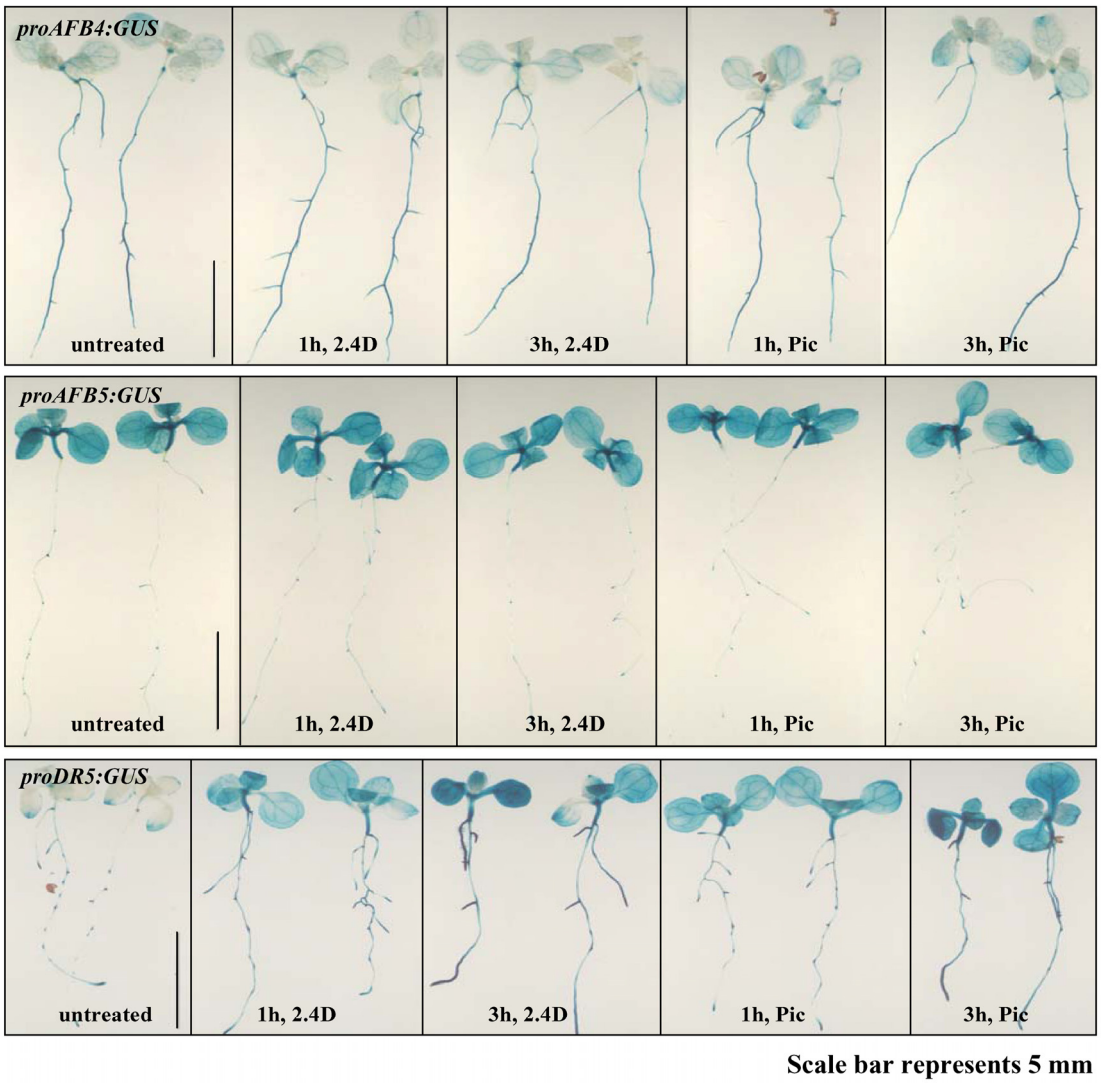


Figure 34. *AFB4* and *AFB5 promoter:GUS* plants do not respond to 2,4-D or picloram treatment with enhanced GUS expression. Plants were treated for 1h and 3h with 10μM 2,4-D or 10μM picloram before overnight incubation in GUS staining buffer.

To further corroborate results from *promoter:GUS* experiments, 2.4-D and picloram dependent expression of *AFB4* and *AFB5* was directly monitored. Eight day old seedlings

grown in liquid ATS medium were treated for 1h with either 10μM 2,4-D or 10μM picloram as done for the *promoter:GUS* analysis. Expression of the two *AFB* genes in treated plants was analyzed by RT-PCR and compared with untreated samples. As shown in Figure 35 no change in expression was detected for either of the two *AFB* genes, confirming the results from *promoter:GUS* analysis, and making it unlikely that the picloram dependent phenotypes of *afb* null mutants are connected with picloram induced or aberrant *AFB4* or *AFB5* expression.

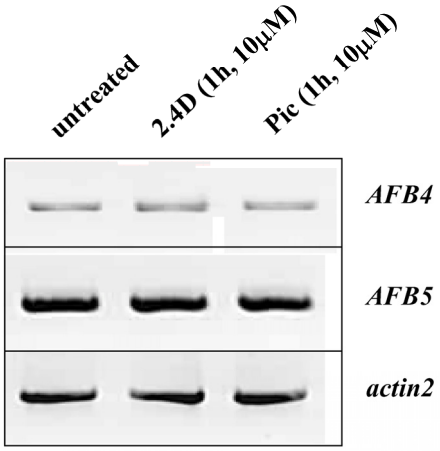


Figure 35. *AFB4* and *AFB5* expression is not inducible by 2,4-D or picloram as measured by RT-PCR. 8-day old seedlings treated for one hour on 10μM 2,4-D or 10μM picloram were compared to untreated. No change with auxin treatment was observed.

Auxin responsive promoter activity in *afb4* and *afb5* mutants

Alternatively loss of *AFB4* or *AFB5* function could cause defects in picloram uptake, distribution or perception, and may be a possible explanation for the strong picloram resistance of *afb5*. Again the *DR5* promoter provided a valuable tool to examine this

question. The *proDR5rev:GFP* transgene was crossed into *afb4* and *afb5* to analyze auxin regulated gene expression (Fig. 36). Interestingly, roots in the *afb4* background had less fluorescence than the *Col-0* and *afb5* background even before application of auxin (Fig: 37).

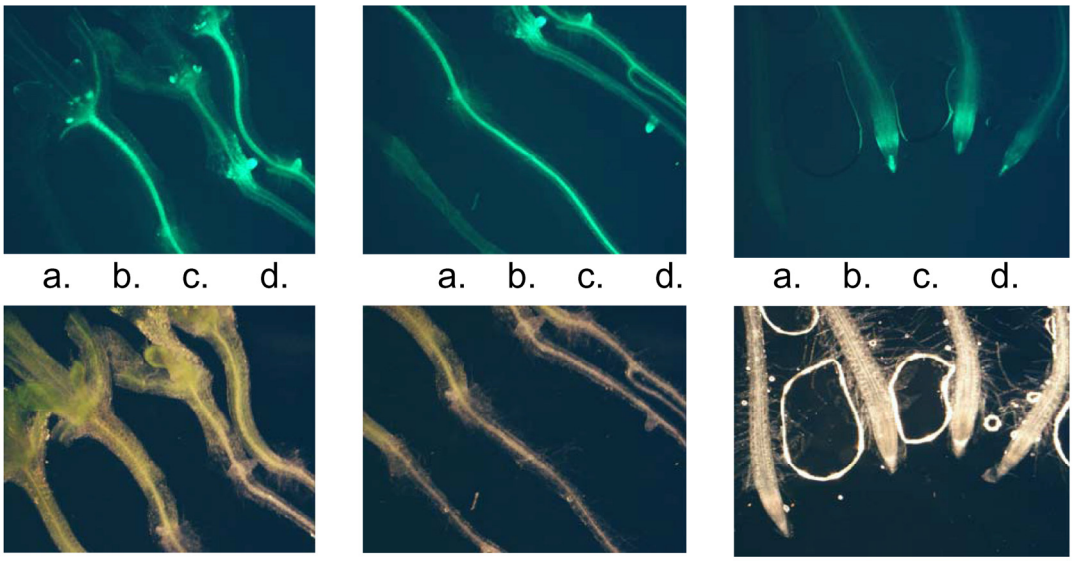


Figure 36. Fluorescence in hypocotyl, roots, and primary root tip on 2,4-D. From left to right are a. *Col-0*, b. *Col-0 proDR5rev:GFP,* c. *afb4 proDR5rev:GFP,* d. *afb5 proDR5rev:GFP*.

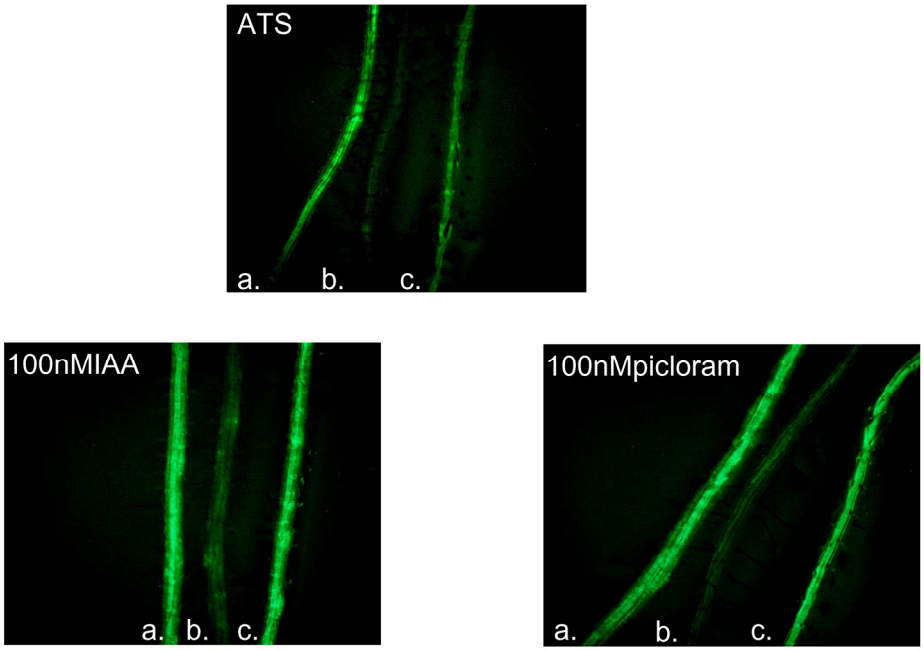


Figure 37. *DR5rev::GFP* expression in *afb4*, but not *afb5*, is reduced in the primary root. This correlates with the *proAFB4:GUS* expression that is strongly present in the primary root. a. *Col-0 proDR5rev::GFP,* b. *afb4 proDR5rev::GFP,* c. *afb5 proDR5rev::GFP*.

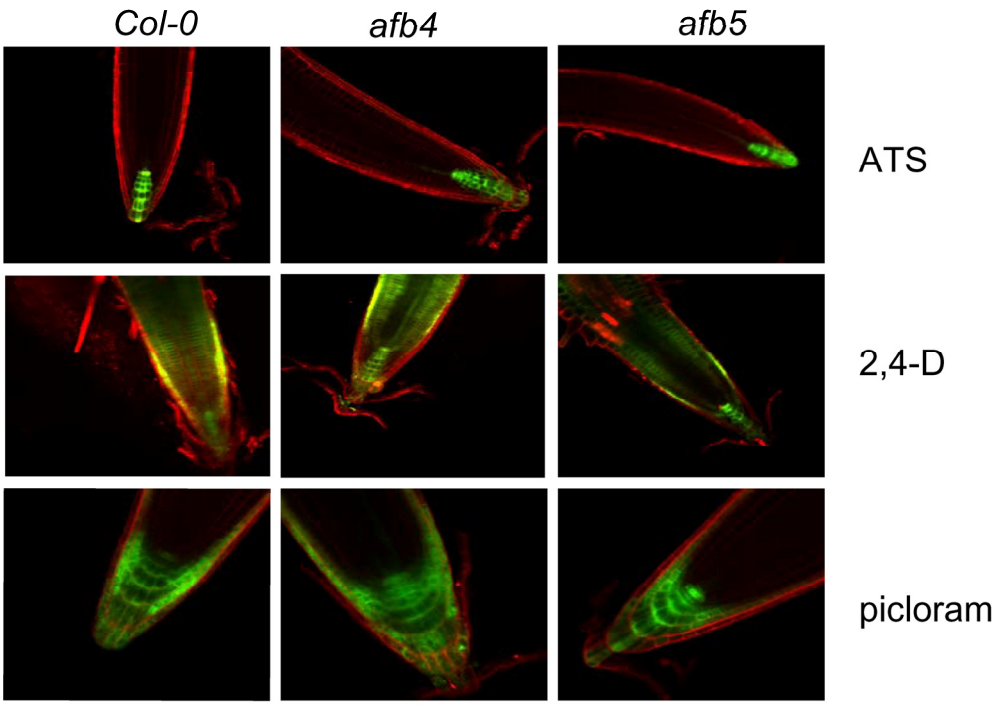


Figure 38. Confocal images of *DR5rev::GFP* showed decreased expression in the primary root in *afb4* mutants, but is not affected in the root tip in either the *afb4* or *afb5* mutants.

To compare auxin response in the *afb* mutants, plants were treated with 100nM IAA or picloram. Both auxins enhanced GFP expression in *proDR5rev:GFP* and *afb5 proDR5rev:GFP* plants along the primary root but this was not observed in *afb4 proDR5rev:GFP* (Fig. 37). This was surprising since *afb5* but not *afb4* mutants exhibited picloram resistance in root elongation assays, and in addition *afb4* was hypersensitive in root elongation assays towards exogenously supply of IAA at 50nM and 100nM concentrations (compare Fig. 30).

In contrast to *DR5* expression along the root, no major differences were observed in the root tip (Fig. 38). 2,4-D and picloram-induced GFP expression was similar in level and pattern in wild-type and mutant backgrounds.

Auxin induced *IAA5* expression in *afb4* and *afb5* mutants

The known substrates for the TIR1/AFB family are the auxin regulated *Aux/IAA* transcription factors [101, 103, 106, 138]. To further test if AFB4 and AFB5 are involved in auxin induced gene expression, transcript levels of *IAA5* were examined in wild-type, *afb4*, *afb5*, and the double mutant after treatment for 1h with either 10μM 2,4-D or picloram (Fig. 39). 2,4-D and picloram were equally competent to induce expression of *IAA5* in wild-type, indicating that the two compounds activated similar regulatory pathways. A similar response was observed in *afb4* plants. Interestingly, in both the single *afb5* and the *afb4 afb5* double mutant, 2,4-D induction of *IAA5* was normal, but the addition of picloram did not result in increased *IAA5* expression (Fig. 39). These findings are novel and important for understanding the TIR1/AFB family. The results provide a plausible explanation for why *afb5* but not *afb4* is resistant against picloram; in the absence of AFB5, picloram cannot induce the normal auxin response. It also demonstrates the different biochemical properties of AFB4 and AFB5 towards picloram.

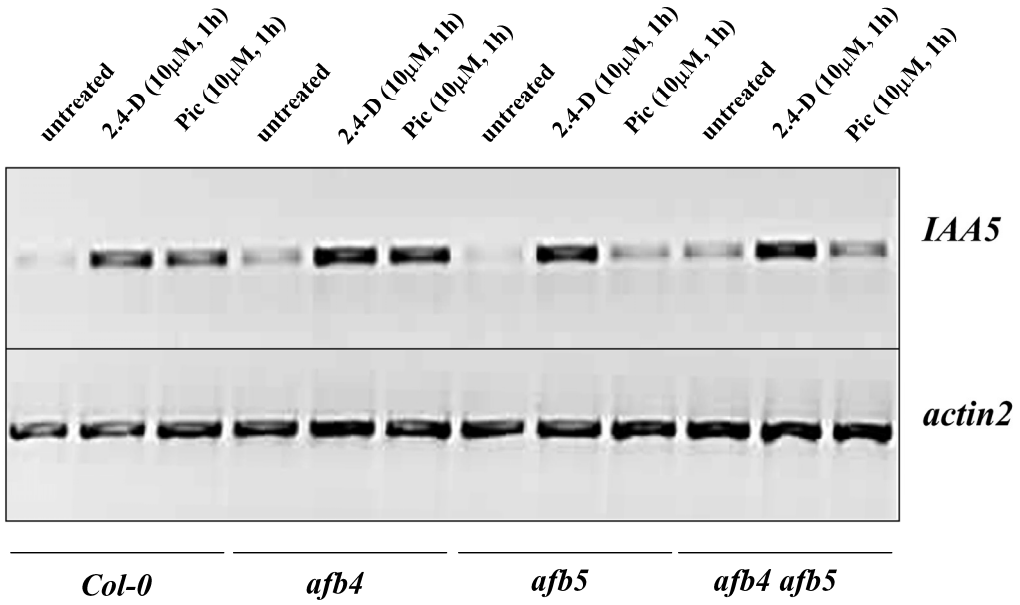


Figure 39. RT-PCR analysis shows that transcript levels of *IAA5* are not induced in the *afb5* background after picloram treatment. Seedlings were treated for 1h with 10μM 2,4-D or picloram respectively, before RNA was isolated.

AFB4 and AFB5 interaction with IAA7

One of the major breakthroughs in the last years for the understanding of auxin signal transduction was the finding that the SCF subunits TIR1, and AFB1, 2, and 3, interact with Aux/IAA proteins when they bind auxin [101, 106, 138]. As both AFB4 and AFB5 are F-box proteins with high similarity to the four other TIR1/AFB family members it is likely that they can also interact with Aux/IAA proteins to initiate their degradation. In addition, one would anticipate that auxin enhances this interaction as for the other members of the TIR1/AFB family.

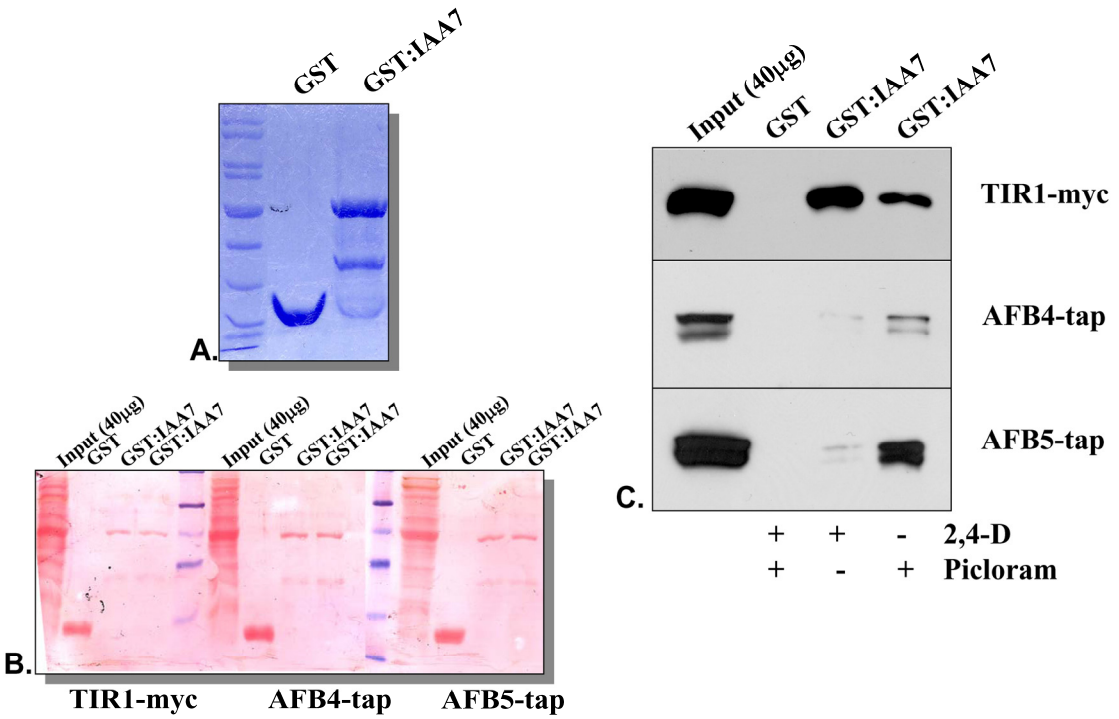


Figure 40. Pulldowns of *in planta* expressed myc- or tap-tagged AFB4, AFB5, and TIR1 proteins. **(A)** Coomassie stained gel with GST, and GST:IAA7 inputs. **(B)** Ponceau S staining of membrane used for immunodetection. **(C)** Pulldown results for AFB4-, AFB5-, TIR1-myc by GST:IAA7 fusion protein in the presence of 50µM 2,4-D or picloram.

To determine if AFB4 and AFB5 also interact with Aux/IAAs in an auxin-dependent manner, pull down assays were performed with a GST:IAA7 fusion protein, expressed in and purified from *E. coli*, and *in planta* expressed myc, or tap-tagged TIR1, AFB4 and AFB5 proteins. Pull down experiments were done as described earlier [138] in the continuous presence of either 50µM 2,4-D or picloram, respectively. Recovered proteins

were subject to SDS-PAGE and Western-blotting using a specific, monoclonal myc antibody (Fig. 40). As expected, TIR1-myc was recovered with GST-IAA7 but not with GST alone. Also significantly more TIR1-myc protein was recovered in the presence of 2,4-D than in the presence of picloram. In comparison, very little AFB4-myc protein pulled-down GST:IAA7 in the presence of 2,4-D. However, in the presence of picloram more AFB4-myc protein was detected on the Western-blot, though still less than was detected with TIR1-myc. In the case of AFB5-Myc, 2,4-D had a very small effect on the interaction between GST:IAA7, while picloram substantially increased the effect.

Discussion

Perception and Response

Many proteins that are part of the auxin perception and response network in *Arabidopsis* were identified through screens for auxin-resistant mutants. Mutants of *TIR1, AFB1, AFB 2,* and *AFB 3* exhibited resistance when tested on IAA, 2,4-D and NAA. Combinations of double, triple and quadruple mutants enhanced this effect. This made it quite surprising when *afb4*, *afb5* and even the *afb4 afb5* mutants did not follow this pattern. One synthetic auxin that had been tried but which had not given the same resistance response on the *tir1, afb1, afb2* and *afb3* mutants was picloram (Dharmasiri pers.comm.).

Part of a family of synthetic auxins called the picolinates, picloram has been a long-standing herbicide produced by DOW chemicals. Combined with 2,4-D in a mixture known as Agent White, picloram was part of the second most actively used herbicide (after Agent Orange) to exfoliate the jungles of Vietnam. 2,4-D is classified with the

aryloxyacetates while IAA and 1-NAA are arylacetates. Experiments at DOW to look for mutants that were resistant to a novel picolinate compound identified *afb5* [139]. Interestingly and in agreement with the previous work in the lab, *tir1* did not show the robust resistance to this compound or to picloram. Likewise, they also demonstrated that *afb5* did not show the *tir1*-like resistance to 2,4-D. When *afb4*, *afb5* and *afb4 afb5* were examined for root elongation on picloram, I also saw strong resistance with *afb5*, however *afb4* was as sensitive as wild-type. Because *afb4* showed wild-type like picloram sensitivity it was surprising that *afb4 afb5* seedlings showed an enhanced picloram resistance higher than the single *afb5* mutant. This was also reflected in the production of lateral roots. The high levels of picloram that stimulated lateral root growth in wild-type and *afb4* did not do so in *afb5* plants. This again appeared to be enhanced in the double mutant. Interestingly, a decrease in the number of adventitious roots in *afb4* and *afb4 afb5* plants was observed upon picloram treatment. The detrimental effects of picloram on the hypocotyl of wild-type and *afb4* were quite dramatic. Curiously the robust resistance of *afb5* was not reflected in *afb4 afb5* to the same extent as seen in the root and may account for the decrease in adventitious roots. The developmental defects observed in the *afb4* hypocotyl may be contributing to the picloram susceptibility seen in *afb4 afb5*. While biochemical experiments and structural data do show that there is a preference for the natural auxin IAA [156, 159], it is interesting that such a dramatic difference is seen in the recognition of the auxin compounds demonstrated by *afb5*. In these experiments, it appears that the other family members, that would presumably still be present and active in the *afb5* mutant, have an extremely limited ability to respond to the picolinate class of synthetic auxin. It is equally interesting that in the absence of the

very similar AFB4 protein, the plants have no resistance, but when coupled with the removal of AFB5, loss of AFB4 results increased resistance to picloram in the root. Perhaps further assessment of *afb4* plants on lower concentrations of picloram would uncover a slight resistance that could explain this additive effect. Additionally, more rigorous analysis of transcript levels in the mutants to check for feedback regulation among the family member could be explored.

To verify that plants are responding to picloram as an auxin compound, activity of the auxin responsive element DR5 was assessed. GUS driven expression in response to picloram was similar to 2,4-D across the seedling. As for the other *TIR/AFB* family members, auxin does not appear to regulate expression of these genes.

In the triple mutant *tir1 afb2 afb3,* auxin response in the root tip as measured by *DR5rev::GFP* expression was strongly reduced. Auxin response measured by the induction of transcript of the auxin response gene *IAA1* and *IAA5* was also greatly reduced in this background. Presumably the single mutants were not so drastically affected, and it is the cumulative loss of these redundant genes that yielded such dramatic responses. In *afb5* mutant plants the *DR5rev::GFP* responded normally whereas *DR5rev::GFP* expression was decreased down the length of the primary root of *afb4* mutants, but not in the root tip. Reduced GFP fluorescence in the *afb4* mutant was evident prior to auxin application. This was surprising because the *afb4* mutant had wild-type sensitivity to auxin applications. Additionally one would expect that the other family members should still be present and active to mediate auxin response. However, currently the precise reason for reduced *DR5rev::GFP* expression remains elusive. While GFP is

an excellent marker for cellular expression with strong magnification, fluorescence in the whole tissue is strong. It might be beneficial to examine auxin response in a more quantitative approach and with other markers of auxin reporter driven expression such as GUS. A strong fluorescence was also seen at the hypocotyl –root junction, which also had strong *AFB4prom:GUS* expression. Because this fluorescence could be due to the excess tissue from the adventitious roots, GUS analysis here might clarify if there is an abnormal accumulation of auxin at this point. This also could indicate that auxin is not being transported down the root accounting for the reduced *DR5rev::GFP* expression. Alternatively, a reduced GUS expression could reflect decreased auxin response in the mutant.

Auxin response measured by the induction of the early auxin response gene *IAA5* was not impaired in *afb4* when subjected to either 2,4-D or picloram. It would be interesting if *afb4* in combination with *tir1*, *afb1, afb2 or afb3* had an impact on auxin regulated IAA feedback. In *afb5*, and similarly for *afb4 afb5*, however the induction by picloram was inhibited. This further proves that AFB5 is unique in its strong response to picloram and is the main regulator of the picloram dependent expression of at least *IAA5.*

Auxin Mediate Interaction Between AFB4 and AFB5 and IAA7

Auxin dependent interaction between the F-box protein and the Aux/IAA substrate can be measured by pulldown assays. Previous work has used this assay to determine that TIR1 and AFB1, 2, and 3 function as auxin receptors to mediate substrate recognition. Pulldowns with AFB5 and a potential substrate, IAA7, again show that this F-box protein

has a uniquely strong response to picloram. In the presence of picloram TIR1-myc did also interact with GST:IAA7 but binding of IAA7 was much stronger when samples were treated with 2,4-D. Interestingly in this experiment, AFB4 showed, like AFB5, a stronger interaction with GST:IAA7 when picloram was present rather than 2,4-D, and this could account for the increased root elongation resistance seen in the *afb4afb5* double mutant.

The receptor interaction region of TIR1 binds domain II of the Aux/IAAs and this domain is highly conserved within the Aux/IAA family. Thus, it is possible that all Aux/IAA proteins could be targets of the TIR1/AFB family. However, although one might expect some specific assembly with TIR1/AFB proteins, it is clear that assembly between members of the two families is restricted to their expression in the same cell. However binding affinities have not been quantitatively tested, and there could be preferences of the TIR1/AFB family to assemble with Aux/IAA proteins based on differences in folding of the substrate. IAA7 could therefore be a bad substrate for AFB4 explaining the observed weak interaction, and it might be that AFB4 displays a much higher affinity for other members of the Aux/IAA family. Currently it is open whether AFB4 is specialized for certain members of the Aux/IAA family. Other GST:IAA proteins could be tested in pulldown assays to look for stronger interactions. Another indicator of interaction and proteasome-mediated degradation that has been frequently used is the stabilization of IAA17 as measured by the levels of expression of the *HS:AXR3NT-GUS* transgene. When tested in the *tir1-1* [101] and the *afb1-1*, *afb2-1*, *afb3-1* single and *tir1-1 afb2-1 afb3-1* triple mutant backgrounds [106] the degradation of AXR3NT-GUS was impaired. This transgene could likewise be crossed into *afb4* plants and measured for changes in GUS expression. Although a lack of change would also

imply that the other family members are active and efficiently mediating IAA17 degradation in the absence of AFB4.

In comparison to AFB1, 2, and 3, which are 70% and 60% identical to TIR1, AFB4 and AFB5 are only 50 % identical to the other AFB members and TIR1. This lower degree of identity can be taken as an argument that the evolutionary divergence of *AFB4* and *AFB5* accounts for what seems to be an inability of these two proteins to respond to the auxins like the other family members do. However based on the TIR1 crystal structure the amino acids used for auxin receptor activity, $InsP_6$ binding and substrate peptide interaction are mostly conserved in these two proteins [104]. However maybe these few changes, including one of the sites defined as a key auxin binding point are enough to alter AFB4 and AFB5 so that they do not bind IAA and 2,4-D in a TIR1 comparable manner, but instead have a preference for picloram or even some other compounds that are found naturally in the plant. It would be interesting to see if site-directed mutagenesis to this key residue and other conserved amino acids alter the affinity for AFB4 and AFB5 to both auxin and picloram. Perhaps there are also other modifications to either AFB4 or AFB5 that would alter their specificity for auxin binding which could be examined. As one of the main differences between these two proteins and the other family member is the N-terminal extension, deletion of this may also create a protein that has a more TIR1-like affinity for auxin. Additionally if the *afb4* phenotype can be complemented by overexpression of the other family members or perhaps other closely related proteins in the same clade from other species, this would argue that it could be functioning as an auxin receptor if it can be replaced by one. Further examination with transgenes utilizing

native promoters or promoter exchanges among the family members may also clarify the effectiveness of complementation studies.

Summary

Although the phenotypes of the *afb4* mutant plants indicate that this protein could be involved in auxin response, the data at this point does not clarify what this role may be. The DR5-GFP expression does strongly indicate a problem in auxin response in the absence of AFB4. However there is not yet enough data to verify that this protein functions in a similar manner as the other family members. AFB5 on the other hand, clearly responds to the synthetic auxin picloram in a strong and unique way compared with all of the other family members. Additionally the reduced interaction in the presence of 2,4-D compared to TIR1 and the sensitivity of the *afb5* mutant towards the other auxins tested in the root elongation assays indicate that perhaps it plays a minor role as a receptor in comparison to TIR1, AFB1, AFB2 and AFB3.

IV. Function of AFB4 in Response to Light

Introduction

The previous chapter demonstrated that AFB5 can function strongly and AFB4 weakly as receptors for the synthetic auxin picloram. Auxin perception, as measured by an auxin responsive reporter, was clearly affected in the *afb4* mutant background, and coupled with the phenotypic characterization of this mutant, implies that AFB4 functions in auxin response. This final chapter examines the possibility that AFB4 may be acting either directly or through cross-talk between auxin and other pathways, to have a much broader impact on plant development.

Results

Growth of *afb4* and *afb5* mutants under different light conditions

One of the critical processes in the plant life cycle is germination of the seed. As both *AFB4* and *AFB5* are expressed in the embryo and in early seedlings, it was of interest to study the role of these genes during early development. No obvious differences in germination rates under white light or in the dark were observed in *afb4, afb5,* or *afb4 afb5* mutants compared to *Col-0*. However, distinct post-germination differences between wild-type and *afb4* plants were observed in the dark. Normally seedlings that germinate in the dark have a characteristic de-etiolated phenotype which includes enhanced hypocotyl elongation, formation of an apical hook, and closed cotyledons [140]. To investigate *afb4* and *afb5* development in the dark, wild-type and mutant lines were plated on ATS medium, exposed to one hour of white light to synchronize germination, and grown in complete darkness for 5 days. As shown in Fig. 41, *afb5* seedlings behaved like wild-type under these conditions. Interestingly *afb4* and *afb4afb5* seedlings lacked

the apical hook and had open cotyledons. The hypocotyls in the *afb4* mutant background maintained their characteristic reduction in length compared to wild-type. Open cotyledons and the lack of an apical hook is often associated with defects in ethylene production or perception [140]. When wild-type seeds are germinated and grown in the presence of additionally supplied ethylene they display what is known as the triple response. This includes reduced hypocotyl elongation, enhanced apical hook formation and inhibition of primary root growth [140]. To investigate whether the *afb* mutants respond to ethylene normally, seedlings were germinated in the dark in the presence of the ethylene precursor ACC. The ethylene mutants *ctr1* (*constitutive triple response 1*, [141]), and *hls1* (*hookless1*, [142]) were used as controls. *ctr1* seedlings display the triple response even without an exogenous supply of ethylene and *hls1* seedlings do not form a hook even in the presence of applied ACC. When seeds were germinated and grown on medium containing 10 μM ACC, both the wild-type and the *afb* mutants displayed the triple response. In *afb4* seedlings, apical hook formation and the inhibition of cotyledon expansion were reduced compared to the wild-type and *afb5* plants (Fig. 41). However, the fact that both mutants did respond suggests that AFB4 and AFB5 are probably not directly involved in ethylene biosynthesis or signal transduction.

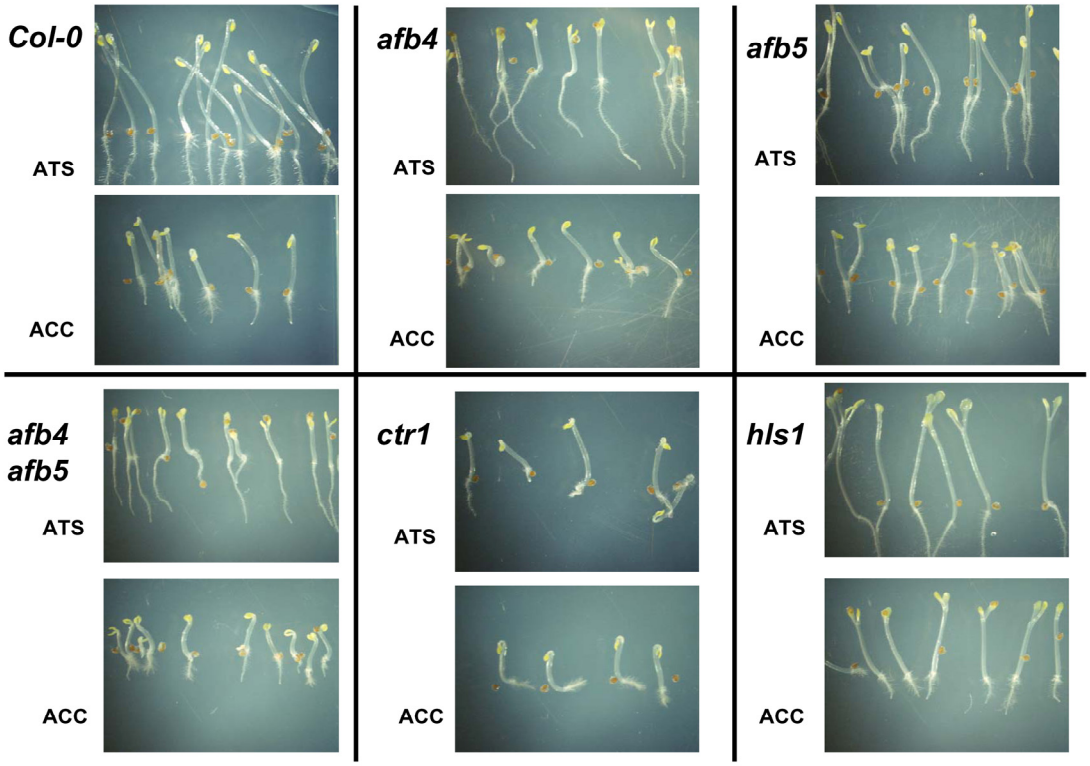


Figure 41. Dark grown *Col-0*, *afb*, *ctr1,* and *hls1* mutants. Seedlings were grown in the dark for 5 days on ATS +/- 10 μM ACC. *afb4* mutants lack apical hook and closed cotyledons on ATS and have a modest response to ethylene.

To verify that the dark grown phenotype is a result of the *afb4* mutation, germination of the *afb4 35S:AFB4TAP* line was also examined (Fig. 42). Expression of AFB4 restored the wild-type-like triple response in dark grown seedling confirming that AFB4 has an important role in seedling development in the dark.

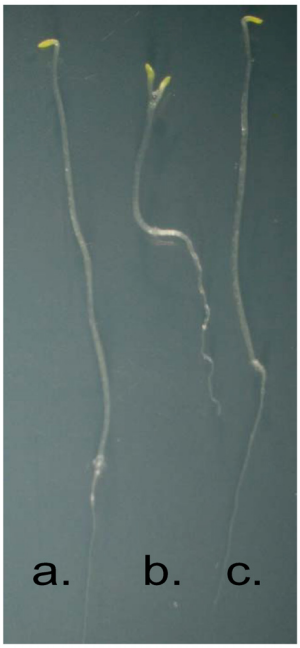


Figure 42. Ectopic expression of AFB4 complements the *afb4* mutant phenotype in dark grown seedlings. a. *Col-0*, b. *afb4*, c. *afb4 35S:AFB4:TAP1*

An alternative explanation for the aberrant skotomorphogenesis is the involvement of AFB4 in light signaling pathways mediated by the photoreceptors phytochromes, cryptochromes, and phototropins [143]. These proteins are important for germination, shade avoidance, hypocotyl elongation and other developmental processes [144]. Phytochromes are required for detection of red (Rc; 600-700nm) to far-red (FRc; 700-800nm) light. Phototropins and cryptochromes are key mediators of blue (400-700nm) and UV-A light signal transduction. Thus, plants that are deficient in phytochromes or cryptochromes are blind to the specific wavelengths of light and when germinated in Rc, FRc or blue light conditions, develop much longer hypocotyls than wild-type. Defects in one of the photoreceptor classes can be monitored by exposing germinating plants to a

defined light wavelength. However, the shorter hypocotyls of dark grown *afb4* seedlings suggested that a light-responsive pathway is already activated and that the mutant may be hypersensitive to light. Consequently, increasing fluence levels of Rc, FRc and blue light, were applied to germinating wild-type and *afb4* seedlings. The *pif1* mutant was used as a control. These seedlings have been shown to have reduced hypocotyl elongation under red and far-red growth conditions [116]. As shown in Fig. 43 increasing light levels resulted in increased inhibition of hypocotyl growth. Because *afb4* hypocotyls are shorter than wild-type in the dark the percent inhibition was measured in relation to dark grown seedlings. In far-red and blue light the overall elongation inhibition trends of the mutants are similar to wild-type, but in red light *afb4* hypocotyls had a more dramatic response demonstrated by the initial steepness of the slope of the elongation inhibition curve. While the slope levels off more quickly, most likely due to the constraints of the originally shorter hypocotyls, the inhibitory effects of red light are more pronounced on *afb4* plants. One important point to note here is that normally dark grown seedlings should exhibit longer hypocotyls than all of the light conditions, however in these experiments this was not the case. While all of the samples were treated simultaneously for each data point, one explanation for this discrepancy could be a difference in temperature between the dark chamber and the light chambers. If the dark chamber was slightly cooler then the seedlings would have grown slower resulting in this unexpected reduction.

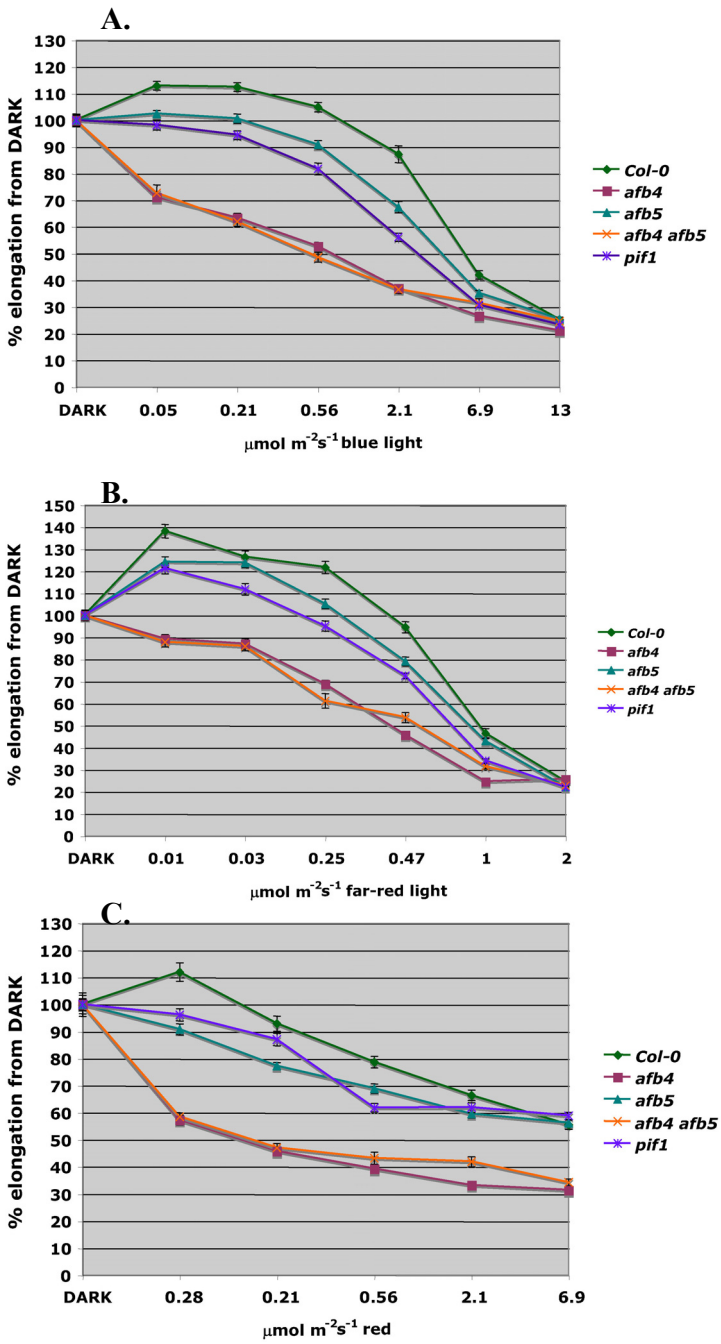


Figure 43. Hypocotyl elongation under different light conditions. **A.** blue light; **B.** far-red light, **C.** red light.

PhyB-independent germination of *afb4* and *afb5* mutant seeds

A third approach to examine whether AFB4 and AFB5 are involved in light responsive pathways, is to monitor germination rates under red light (phyB-mediated) or far-red light (phyA-mediated) conditions. A red-light pulse induces germination by activating PhyB and subsequent processes, whereas a far-red light inhibits germination most likely by preventing translocation of PhyB into the nucleus [145]. When wild-type seeds are exposed to a pulse of far-red light and then placed in the dark they are unable to germinate. When the *afb* mutants were tested for germination after an far-red light pulse almost all *afb4* and *afb4afb5* seeds were able to overcome this inhibition whereas nearly all *afb5* seeds remained dormant.

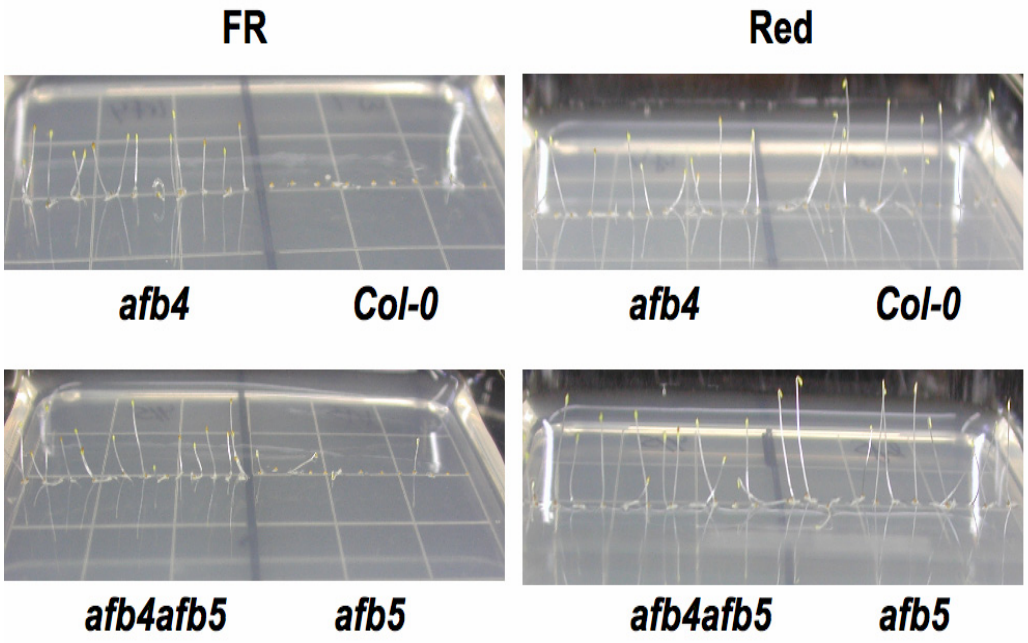


Figure 44. *afb4* seeds overcome germination inhibition of far-red light. **A.** 15 minute far-red light pulse 3 μmol $m^{-2}s^{-1}$. **B.** 15 minute red light pulse 20 μmol $m^{-2}s^{-1}$.

To corroborate that AFB4 is required for normal far-red light response, *afb4 35S:AFB4:TAP* lines were tested for complementation of the mutant phenotype. As shown in Fig. 45, ectopic expression of AFB4:TAP in *afb4* seeds resulted again in far-red light dependent inhibition of seed germination, clearly demonstrating that AFB4 is indeed a critical player in this process.

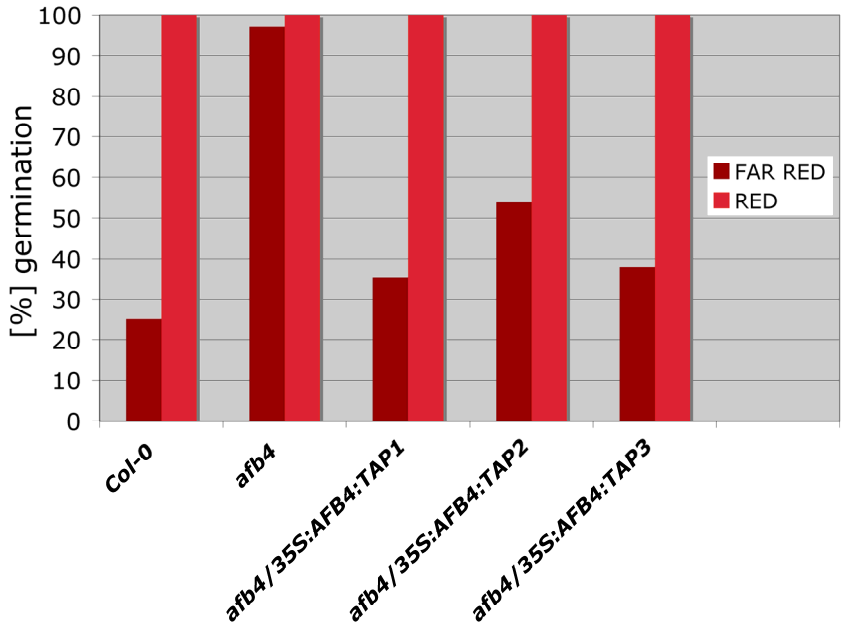


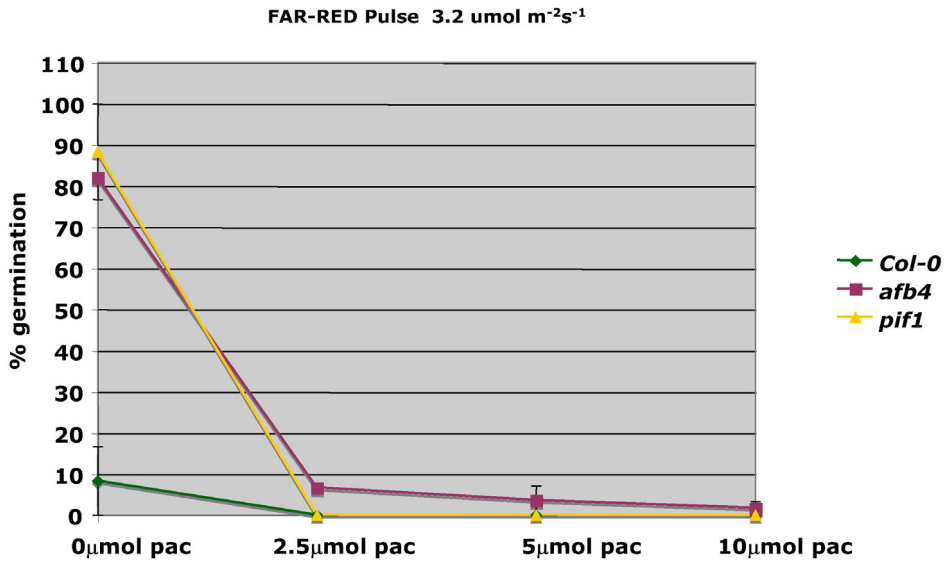
Figure 45. Ectopic expression of *AFB4* complements the *afb4* mutant phenotype of germination after a 15 minute 3 μmol $m^{-2}s^{-1}$ far-red pulse.

***afb4* seed germination on paclobutrazol**

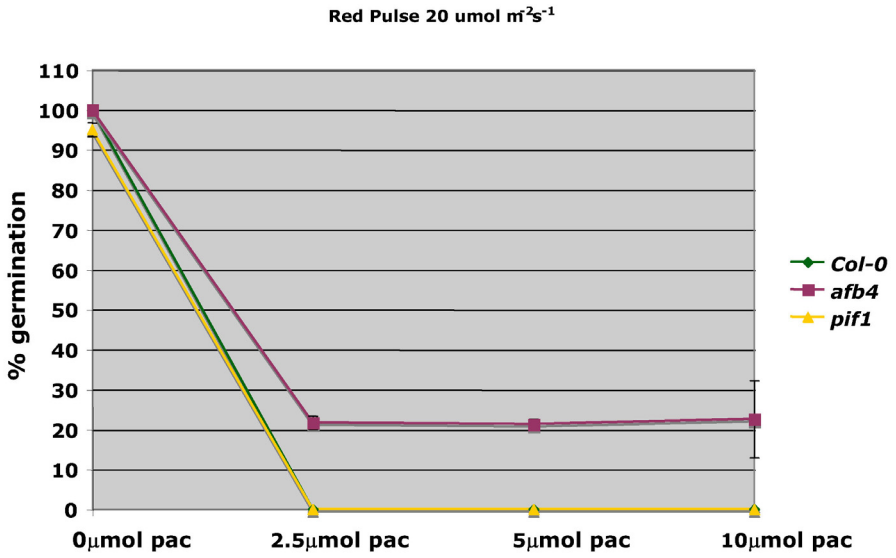
Gibberellic acid plays a key role in stimulating the germination process, and seeds unable to produce GA fail to germinate [146]. It has been demonstrated that PhyB is responsible for promoting GA biosynthesis in the seed, although PhyA also has a role [147, 148]. To

test if there is a connection between the far-red light insensitivity of *afb4* and GA biosynthesis, seed germination of *Col-0*, *pif1* mutants, and *afb4* mutants was analyzed in the presence of increasing concentrations of the GA biosynthetic inhibitor paclobutrazol [148]. Even at a concentration of 2.5µM paclobutrazol germination of *Col-0* and *pif1* seeds were completely inhibited in dark, red and far-red conditions (Fig.46). In white light, 5 µM paclobutrazol inhibited the germination of *Col-0* and *pif1* seeds while *afb4* seeds were only slightly affected. In all cases, however, the *afb4* germination rate was significantly higher than the wild-type and *pif1* mutant controls. The results clearly indicate that *afb4* mutants are resistant to paclobutrazol, but it remains unclear how this behavior is connected to gibberellin biosynthesis or phytochrome dependent regulation of germination.

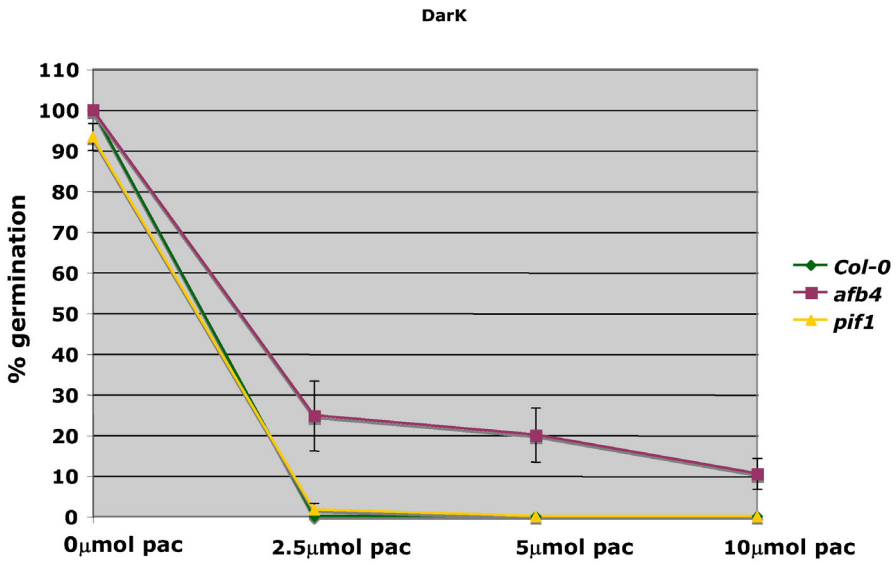
A.



B.


Red Pulse 20 umol m-2s-1
% germination
110
100
90
80
70
60
50
40
30
20
10
0
0µmol pac
2.5µmol pac
5µmol pac
10µmol pac
Col-0
afb4
pif1

C.


DarK
% germination
110
100
90
80
70
60
50
40
30
20
10
0
0µmol pac
2.5µmol pac
5µmol pac
10µmol pac
Col-0
afb4
pif1

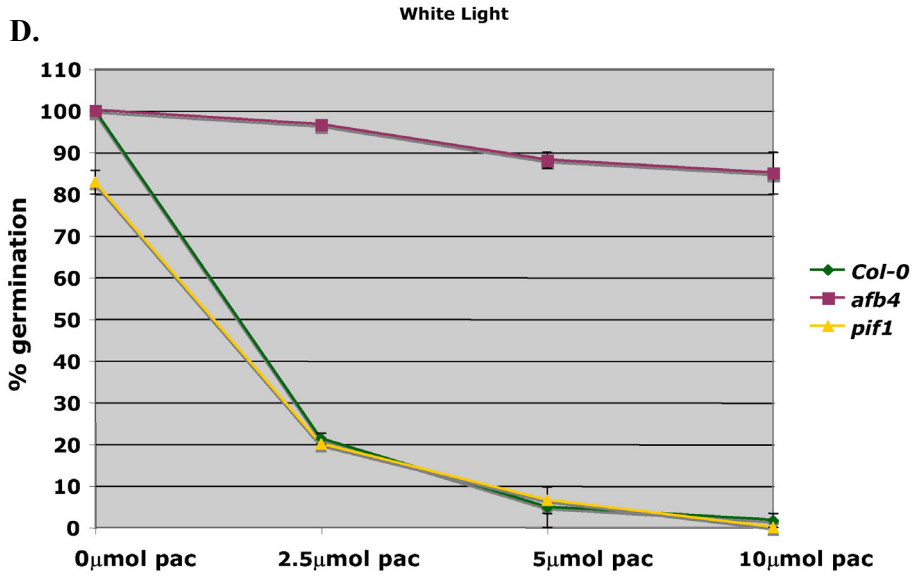


Figure 46. *afb4* seed germination with the GA biosynthesis inhibitor paclobutrazol. A. far-red light. B. red light. C. dark. D. white light

Discussion

AFB4 and the light response

It is interesting to observe that the dark-grown *afb4* mutant plants are affected in skotomorphogenesis. The *afb4* mutants actually weakly resemble the *cop*, *constitutive photomorphogenesis*, mutants that are affected in repression of photomorphogenesis [149]. The development of shorter hypocotyls and open cotyledons under these conditions also resembled the gain-of-function *aux/iaa* mutants *axr2, axr3*, and *shy2* [186, 194, 216]. Earlier studies have demonstrated that *cop* mutants also play critical roles in the ubiquitin conjugation and RUB deconjugation pathways and are therefore important regulators within the ubiquitin proteasome pathway [130, 154]. One major player is the CSN, or COP9 signalosome. This multi-subunit complex resembles the lid

of the 19S subunit of the 26S proteasome, and one of its functions is to remove the RUB protein from cullins [150]. Consequently the CSN complex physically interacts with subunits of SCF^{TIR1} and *csn* mutants have decreased auxin response and are impaired in Aux/IAA degradation in a *tir1* comparable manner [130]. Also several Aux/IAAs proteins have been connected to light dependent regulatory pathways by being targets of PHYA and PHYB dependent phosphorylation [187, 218]. Currently it is not clear what precisely the effect of this phosphorylation event on Aux/IAA protein activity is. Hypothetically a decrease in one of the phytochromes may prevent the phosphorylation and subsequent degradation of the Aux/IAA proteins, while an increase could lead to more rapid degradation and release of the repression of these transcription factors. Two of the Aux/IAAs, SHY1/IAA6 and SHY2/IAA3, were identified in screens for mutants deficient in phytochrome red/far-red activity [219, 220]. *shy2* gain-of-function mutants have a decreased response to red-light induced hypocotyl elongation [151] which also seems to be the case for *afb4* mutants. This finding that loss-of AFB4 affects photomorphogenesis might point to a novel link between light and auxin response.

AFB4 and germination

Light is a key regulator of germination [11. Whereas red light is a promoter of germination, treatment of seeds with far red light causes inhibition of germination. At the molecular level, red light response is mediated by phytochrome B, which moves, in its then active form, into the nucleus to interact with transcription factors, including PIF1/PIL5. As a result of this interaction, PIF1/PIL5 is degraded by the 26S proteasome [176, 177, 214]. PIF1/PIL5 is a negative regulator of GA biosynthesis and germination, and thus, upon degradation of PIF1, GA biosynthetic genes are activated and germination

is promoted. *afb4* seeds are also able to germinate in a PHYB independent manner. This strongly indicates that AFB4, like PIF1/PIL5, is a negative regulator of germination. If AFB4 is participating as a substrate adaptor in an SCF complex, then one can predict that this protein targets an activator of phyB dependent germination for degradation. Hence, in the absence of AFB4 if the activator cannot not be degraded then germination is not blocked. Similar examples have been described earlier for other F-box proteins. For example, in *Arabidopsis* the EBFs (ethylene binding factors) act as negative regulators of ethylene signaling by promoting the degradation of EIN3, a transcriptional regulator and positive mediator of ethylene signaling [221]. While it is unclear what the targets of AFB4 may be, it has been shown that the Aux/IAA binding partners, the ARFs, can act as both activators and repressors of transcriptional regulation [37]. Another striking behavior of *afb4* mutants was increased resistance to the GA biosynthesis inhibitor paclobutrazol. This chemical inhibits the monooxygenases involved in the oxidation of ent-kaurene to ent-kaurentioc acid in the second stage of GA biosynthesis [152]. If seeds are placed on paclobutrazol containing growth medium, germination under all light conditions is impaired. In contrast to *afb4*, *pif1* seeds were consistently more sensitive to paclobutrazol. For example, at concentrations of 2.5 μM no germination was detectable for both wild-type and *pif1* seeds. In contrast, *afb4* seeds germinated at a statistically significantly higher level at this concentration independently of the tested light conditions. Assuming that the altered seed coat of the *afb4* mutants does not restrict passage of this chemical into the seed, the reduced paclobutrazol sensitivity of *afb4* could indicate two different situations. First, in *afb4* seeds *de novo* GA biosynthesis is not as critical as in wild-type to promote germination. This would mean that AFB4 is active

downstream of the GA signal and negatively controls germination in a GA-independent manner. Alternatively, AFB4 function might be to keep GA biosynthesis low and loss-of AFB4 would therefore result in increased GA levels. Here, it is conceivable that higher amounts of paclobutrazol are necessary for depletion of existing GA pools. However, independently of both scenarios, the findings that *afb4* mutants are far red light insensitive, and to a much greater extent than *pif1*, paclobutrazol resistant, indicates that AFB4 represent a novel regulator in germination that might act even in a phytochrome independent manner.

One would expect that increased GA in the embryo would result in early germination. However there appear not to be obvious differences in germination rates between *afb4* and wild-type seeds. There could however be slight differences that a more thorough germination assay may reveal.

If AFB4 is regulating GA biosynthesis, then this could be examined by looking at transcripts of the GA biosynthesis genes in *afb4* mutants. Another explanation could again be that AFB4 is acting downstream of GA biosynthesis. If GA regulated transcription results in germination, but AFB4 is repressing an activator of germination, then in the absence of AFB4 this activator is not repressed and seeds can germinate. Recent work has shown a connection between auxin signaling and regulation of GA metabolic genes [39]. When seedlings are treated with auxin, transcript levels of several GA metabolic genes are up-regulated. This up-regulation in the presence of auxin could be blocked by the addition of the proteasome inhibitor MG132. Because transcription of

the *Aux/IAA* genes is known to be up-regulated by auxin and the corresponding proteins are degraded in response to auxin, GA metabolic expression was examined in the gain-of-function mutants, *axr2*, *axr3*, and *shy2*, along with a *tir1* mutant. While there were some minor changes in the basal levels of gene expression in these mutants the more dramatic differences were detected upon application of exogenous auxin. Response to endogenous auxin was also tested using the auxin over-producing *yucca* mutants. Many of these same transcriptional changes were also seen in these mutants. Additionally application of paclobutrazol to the *yucca* mutants inhibited hypocotyl elongation, further linking auxin response to GA metabolism.

Abscisic acid (ABA) has been shown to inhibit germination in an antagonistic effect to GA [153]. PIF1/PIL5 has also been implicated as an activator of ABA biosynthesis [122]. Another possibility for the *afb4* germination phenotype could be that the seeds contain a lower level of ABA or that AFB4 functions downstream of ABA metabolism. Investigation of this alternative would again be similar to the connection of AFB4 and GA.

Summary

While this data is quite preliminary there seems to be evidence that AFB4 may be participating in both light regulated pathways and gibberillic response pathways. Future work to not only determine this, but also to further understand the crosstalk between the different pathways will undoubtedly be exciting and enlightening for the study of plant development.

Conclusions

At the beginning of this work the expectation was that AFB4 and AFB5 would function as auxin receptors and even be redundant to the other four members of the TIR1/AFB family. Through phenotype analysis, auxin perception and response assays, and interaction studies, the data presented here has shown that while some overlapping roles may exist, these two proteins have diverged into their own niche from the TIR1/AFBs. Of the two, AFB5 fits most closely into the family with a similar phenotype, expression pattern and strong ability to interact with an Aux/IAA substrate protein in the presence of an auxin-like compound. However, while the TIR1/AFBs work preferentially with the natural auxin IAA and almost as well with the two synthetic auxins, 2,4-D and 1-NAA [1, 2], AFB4 and AFB5 interact preferentially with the synthetic auxin picloram. The most likely reason for this discrepancy is the difference in the auxin binding sites [104]. In this highly conserved binding pocket three of the fourteen sites, one of which was defined as a key binding residue, are different in both AFB4 and AFB5 from TIR1. Given that picloram is not naturally occurring in the plant, like IAA, this leads to the question of why AFB5 utilizes this compound so efficiently.

According to evolutionary analysis, *AFB4* and *AFB5* diverged into a separate clade from the other family members (M. Prigge, pers. comm.). The introduction of an auxin-like compound that can utilize the mutated binding pocket indicates that at one time these two proteins were probably able to function as TIR1-like auxin receptors. Hypothetically there could be another plant generated compound with which AFB5 interacts that is functioning like picloram. Another possibility is that a pathogen is producing picloram-

like substances and AFB5 activity helps to protect the plant from invasion. It is interesting that while they share the same amino acid exchanges in not only the auxin binding sites, the substrate binding sites, and the $InsP_6$ binding sites, AFB4 is much less effective in utilizing picloram to promote substrate binding. Additionally *afb4* mutant plants are equally as sensitive to picloram as to the other auxins. However the *afb4 afb5* double mutant plants displayed an increased resistance compared to the *afb5* single mutant plants. This could be explained by the ability of AFB4 to function weakly as a picloram receptor. The preferential binding of AFB4 and AFB5 to picloram could be compared to ABP1 (Auxin Binding Protein 1) which preferentially binds 1-NAA [154]. It has been suggested that this might define a separate auxin signaling pathway from the IAA response pathway resulting in similar downstream effects [155].

Although this work did not show AFB4 to be a strong auxin receptor candidate, plants mutated in this gene had a remarkable display of phenotypes compared to mutants in the other *TIR1/AFB* family members. While many of the *afb4* mutant phenotypes resemble other auxin response mutants, some are more consistent with the cross-talk between GA biosynthesis and light response. In particular there was a reduction of pigments in the young seedlings [121] and PHYB-independent germination under far-red light [122]. Whether or not there is a direct downstream effect due to AFB4 substrates not getting degraded in the mutant is open. Recent work has begun to indicate that there is a complex network between the different hormone response pathways [5, 6], and the work with AFB4 opens up the exciting possibility that there is a new level of cross-talk between these pathways and auxin response.

Materials and Methods

Plant Methods

Plant Genotyping

The following established plant lines were used:

axr1-3 [81]; *GVG::TIR1-myc[tir1-1]* [132]; *DR5rev::GFP* [156] ; *hls1* [142]; *ctr1-1* [157]; *pif1-2* [115, 116]; DR5::GUS [158]

Homozygous lines for *afb4* and *afb5* were established by:

GABI-Kat Line 068E01 At4g24390 (*afb4*) T-DNA insertion with sulfadiazine resistance

Screening for insert: GabiLB 5'-atattgaccatcatactcattgc-3'

AFB4 5'-atgacagaagaagatagc-3'

AFB4 5'-tcataaaattgttacaaa-3'

Salk Line 110643 At5g49980 (*afb5*) T-DNA insertion with kanamycin resistance

Screening for insert: LB1 5'-gccttttcagaaatggataaatagccttgcttcc-3'

AFB5 5'-ctataaaatcgtgacgaa-3'

AFB5 5'-atgacacaagatcgctca-3'

Plant Cultivation and Seedling Growth

All plants used for this study were in the *Arabidopsis thaliana* Columbia (*Col-0*) background along with the tobacco species *Nicotiana benthamiana.*

Unless otherwise stated, *Arabidopsis* seeds were surface sterilized with 30% bleach solution containing 0.1% Triton for 15 minutes on a mixing rotor, washed four times in sterile de-ionized water and stratified in the final wash at 4°C in the dark for at least twelve hours. Seeds were plated on ATS media [79] supplemented with 1% sucrose and

1% agar and grown vertically either in growth chambers with constant white lights directly above the trays at 21°C or in a growth room under white light covered with yellow saran filter purchased from Hobby Lobby.

Soil grown plants were either started from non-sterilized seeds or transferred as seedlings from agar plates into pots with Scots Metro Mix soil and watered to saturation with a solution of 1:10,000 Adept (active ingredient diflubenzuron)(Crompton Uniroyal Chemical) diluted in tap water for the control of fungus gnat larvae. Plants were bottom watered with tap water containing fertilizer (1:4 dilution of Peters Professional 20-20-20, Scotts) as needed. Growth of plants was in a room with 24-hour white light at 22°C. Plants used for flowering time were soil grown and transferred into individual pots at two weeks at the Institute for Biology/Applied Genetics, Freie Universität Berlin. Long day growth was in the greenhouse and constant light growth was in a Rumed Rubarth Appate GmbH chamber with white lights along the sides.

Cloning and Plant Transformation

Promoter regions of *AFB4* and *AFB5* were PCR amplified from Col-0 gDNA using the following oligos:

AFB4(*Xba*I):5'-gctctagagagcaaagcttttaatta-3'

AFB4(*Spe*I):5'-gcactagtaagaaagactctgacttt-3'

AFB5(*Xba*I):5'-gggctcgagtctagattttggaggcttttcgattctaat-3'

AFB5(*Bam*HI):5'-gggggatccaaagagagaggctttggattcaga-3'

Restriction sites generated are in parenthesis and were used for directional cloning into the binary vector pCB308 with kanamycin resistance in bacteria and basta resistance in

plants [159]. Sequencing oligo for correct insertion of promoter sequence in front of uidA (GUS): 5'-cgatccagactgaatgcccaca-3'

A cDNA clone of *AFB4* was ordered from TAIR (Order# 15012119197) which was generated from RT-PCR in ecotype Columbia, inserted into the vector pUNI51 (kan^R) and maintained in the host strain *E.coli* PIR1. AFB5 was amplified from a cDNA library generated from auxin treated Columbia seedlings by Bill Gray in the Estelle lab using the following oligos:

AFB5(*Bgl*II): 5'-gggagatctatgacacaagatcgctcagaaatg-3'

AFB5(*Bam*HI): 5'-gccggatccctataaaatcgtgacgaactttgg-3'

Restriction sites generated are in parenthesis and were used for cloning into the vector pBluescript SK+, GenBank # 52325 (amp^R) and maintained in host strain *E. coli* DH5α. These cDNA clones were amplified by PCR for insertion into the Gateway Entry Vector pENTR-D (Invitrogen) with the following oligos:

*AFB4*F:5'-caccatgtcagaggatgttgagaaatatctcaac-3'

*AFB4*R:5'-taaaattgttacaaactttggagcatcctt

*AFB5*F:5'-caccatgacacaagatcgctcagaaatg

*AFB5*R:5'-taaaatcgtgacgaactttggtgcatcatt

Directional TOPO cloning into the following binary expression vectors with spectinomicin resistance in bacteria and gentamycin resistance in plants from Vicente Rubio [160] was performed using the previously described Entry constructs. To look at cellular localization, *35S-AFB:GFP or YFP* constructs were made using the Gateway compatible vectors *pVR GFP4 Ct* and *pVR YFP Ct*. For complementation and protein

interaction experiments, *35S-AFB:TAP* constructs were made with *pYLTAPCt*. Verification of cloning by sequencing was done with the following oligos:

AFB4(503R):5'-gccaaatcatcatccgtaacaaaca-3'

AFB4(1501F):5'-gcgtttgctgggaacagtga-3'

AFB5(315R):5'-cctcgccggagaaagagcgtaacagt-3'

AFB5(1441F):5'-actcgccttgcagtgtcggg-3'

Binary vectors were electroporated into *Agrobacterium*, strain GV3101, and selected on standard Luria Broth agar plates with antibiotics. Positive colonies were confirmed by PCR and used for plant transformation by the floral dip method [161]. T1 seeds were selected on ATS plates with antibiotics to select vector and T-DNA mutant lines when *afb4* and *afb5* plants were used. Positive transformants were transferred to soil and further screened by PCR and expression analysis.

To ensure that the transgenes were being expressed in the mutant lines, plants were screened for T-DNA insertions as described above and for disruption in the gDNA by amplification across the UTR region of either AFB4 orAFB5 using the following oligos:

AFB4(UTRF):5'- gagagagtccaaacaccaagaccagc-3'

AFB4 5'-tcataaaattgttacaaa-3'

AFB5 5'-atgacacaagatcgctca-3'

AFB5(UTRR):5'-gaagttacattatagtttgctgacaaatgac-3'

Experimental Methods

Root growth assays

For root elongation assays seeds were bleach sterilized, stratified and plated as described above. After five days vertical growth under yellow filter in 24-hour light at 22°C,

seedlings with approximately the same size root length were transferred to plates containing ATS or ATS supplemented with hormone. The length of the root at time of transfer was indicated by a mark on the plate and digital pictures were taken after 3 days. The root growth measurements were made using Image J freeware (v.1.32) (http://rsb.info.nih.gov/ij/index.html).

Lateral and adventitious roots were counted under Nikon dissecting microscope after 12 days of vertical growth under yellow filter in 24-hour light at 22°C.

Root Gravitropism

Seedlings were grown vertically on ATS plates under yellow filter in 24-hour light at 22°C for two weeks to insure adventitious and lateral root production. Plates were turned at 90^{o} and after two days turned again at 90^{o}. After an additional two days pictures were taken to illustrate that all of the roots were responsive to the gravity stimulus.

Hypocotyl Growth Assays

To determine the effect of light fluence on hypocotyl elongation, *Arabidopsis* seeds were surface sterilized and sown on Murashige-Skoog (MS) growth medium containing 0.9% agar as described by [162]. After stratification at 4°C for four days, plated seeds were exposed to one hour white light and placed horizontally in the different light conditions at 21°C. Red, Far Red and Blue light treatments were performed in growth chambers equipped with light emitting diodes (LEDs) (Model E30LED, Perceival Scientific, Madison, WI). Light fluence rates were measured using a spectroradiometer (Model EPP2000, StellarNet Inc.,Tampa, FL).

After 3 days of growth seedlings were flattened onto the agar and digital images were taken for growth measurements using Image J freeware (v.1.32) (http://rsb.info.nih.gov/ij/index.html).

Germination Assays

For germination assays, seeds were sterilized and plated on MS (as described above) or MS supplemented with paclobutrazol. Plating and administration of the indicated light treatments was performed in less than one hour. Plates were then wrapped in aluminum foil and dark grown at 21°C for five days and scored for germination. Emergence of seedling was used as the minimum to be counted.

RT-PCR

RNA was extracted using the Qiagen RNAeasy kit as per manufacturer's instructions. Samples were eluted twice in 30ul DEPC-treated water and stored at -20°C (less than 2 months) or at -80°C (up to a year). RT-PCR was done with 90ng DNA-free total RNA using the Qiagen OneStep RT-PCR kit as per manufacturer's instructions, with 30 cycles at 55°C annealing temperature. Amplicification of cDNA to analyze transcript levels and control for gDNA contamination was done with the following oligos:

*ACTIN2*F: 5'-ggctgaggctgatgatattc-3'

*ACTIN2*R: 5'-tctgtgaacgattcctggac-3'

AFB4(1F): 5'-atgacagaagaagatagc-3'

AFB4(999R): 5'-gttaagagaggtgagattagcacaaactga-3'

AFB5(1F): 5'-atgacacaagatcgctca-3'

AFB5(655R): 5’-ccacttcatcatccgtgacctcaga-3’

*IAA5*F: 5’-atggcgaatgagagtaataatc-3’

*IAA5*R: 5’-caaaattgatatagcatccg-3’

GUS Histochemical Staining

For GUS staining of whole tissue, fresh samples were washed once with 100mM Na_2PO_4 and vacuum infiltrated with the following staining buffer [163]: 100mM Na_2HPO_4(pH7), 0.5mM potassium ferricyanide, 0.5mM potassium ferrocyanide, 10mm EDTA, 0.1% Triton X-100. 5mg X-Gluc was dissolved in 50ul N,N DMF and added into 5-10mls staining buffer. Incubation at 37°C overnight was followed by destaining in 50% ETOH for one hour and 75% ETOH until cleared tissue was mounted in 30% glycerol and visualized under light microscope.

For GUS staining of embryos, protocol from [164] was followed. Siliques were opened and permeabilized in 90% acetone at -20°C for 30 minutes, rinsed with phosphate buffer and vacuum infiltrated with staining buffer minus detergent for ten minutes. 1mg/ml X-Gluc was added and samples were incubated at 37°C overnight. Staining solution was removed with 75% ETOH:25% acetic acid and samples were cleared in ETOH series of 75%, 50%, 25% followed by a dH_2O rinse to remove ETOH. Siliques were dipped in chlorohydrate solution (8g Chloral hydrate, 3ml dH_2O, 1ml glycerol), ovules were scrapped out, mounted in chlorohydate solution and cleared at 37°C for at least 30 minutes for Neumarski visualization under light microscope.

Tissue Clearing

To examine rosette vasculature and cell structure tissues of corresponding 20-day old plants were cleared with 0.24N HCL, 20% methanol for 15 minutes at 57°C, followed by 7% NaOH, 60% EtOH for 15 minutes at room temperature and then 5% EtOH, 25% glycerol for microscopy.

To examine embryos siliques at different developmental stages were opened and ovules were removed and directly mounted into chlorohydrate solution for clearing and microscopy.

To examine hypocotyl cells one-week old light grown seedlings were immersed in chlorohydrate solution, rinsed in dH_2O and mounted in 30% glycerol for microscopy.

Visualization of GFP and YFP expression

To see cellular localization of fluorescent tagged AFB4 and AFB5 in *Nicotiana benthamiana* [165], a 5ml overnight of Agrobacteria carrying the constructs of interest was used to inoculate 50ml cultures which were grown to late log phase at 30°C and resuspended to 0.5 at OD_{600} in 10mM MgCl, 10mM MES, 150uM acetosyringone. Cells were incubated in solution at room temperature for two hours and then used for infiltration by syringe into young tobacco leaves. After two days thin epidermal peels at the infected areas were taken for Nikon E800 light microscopic analysis.

To see cellular localization of fluorescent tagged AFB4 and AFB5 in *Arabidopsis thaliana*, plants transformed with the constructs of interests were grown on ATS plates for no more than two weeks and root tips of seedlings were removed and mounted for Nikon E800 light microscopic analysis.

To see expression of DR5:GFP, seedling were grown on ATS plates for one week and transferred to ATS plates supplemented with auxin for overnight exposure. Whole seedlings were examined on a Nikon dissecting scope or mounted root tips were examined by confocal microscopy.

Visualization of Seed Coat Mucilage

0.01% ruthenium red was used to stain seed coat mucilage [166, 167]. Dry seeds were shaken in ruthenium red solution for ten minutes and observed under a Nikon dissecting scope.

Pulldown Assays

To test interaction between TIR1-Myc, AFB4-Tap and AFB5-Tap with GST-IAA7 basic protocols from [101, 102] were followed. Bacterially expressed GST-IAA7 was collected on glutathione beads, checked on SDS-PAGE by Coomassie staining for integrity and concentration and approximately 3-4 μg were used for each interaction sample. TIR-Myc, AFB4-Tap, AFB5-Tap were expressed in *Arabidopsis* seedlings which were homogenized in extraction buffer containing the following: 150mM NaCl, 0.1M Tris-HCl pH7.5, 0.5% NP-40 (Igepal CA-630) for stock kept at 4°C and before use proteinase inhibitors were added: 10mM β-glycerolphosphate, 1mM NaF, 1mM PMSF in ETOH, 1mM DTT. Total protein was measured by Bradford Assay and 3mg of extract used for each sample. Incubation of plant extract with beads was in the presence of 50uM auxin for four hours rocking gently at 4°C followed by three washes with buffer containing the same auxin application. Samples were loaded on 10% SDS-PAGE, subjected to Western

transfer and visualized by Ponceau S to control for even loading and transfer. Anti-myc antibody was used for immunodetection.

Pigment Measurements

For pigment measurements according to [119], approximately 0.03g of 13-day old leaf tissue from seedling grown on ATS plate under white light was homogenized in 100% acetone. Supernatant was adjusted to 80% acetone for spectrophotometer reading and calculations with the following formulas were done: chlorophyll a=12.25*A663.2 - 2.79*A646.8; chlorophyll b=21.50*A646.8 - 5.10*A663.2; carotenoids=1000*A470 - 1.82*Ca - 104.96*Cb. μg/ml concentrations were normalized according to fresh weight of tissue used for each sample.

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1991: spring semester undergraduate assistant in lab of Dr. James Mahaffey, NC State University Genetics Dept.

This lab studies homeobox genes in *Drosophila*; I was learning basic molecular techniques to assist graduate students

1991: summer internship at NIEHS in lab of Dr. Janssen, Research Triangle Park, NC

Worked with lab technician studying effects of retinoic acid on rat tissue culture lines

1991-1992: Part-time undergrad assistant in lab of Dr. Scott Chilton, NC State University, Biology Dept.

Assisted postdoc in purification of phytochemicals from fungi and performed general lab duties

1992: Graduated North Carolina State University, BS Biology and Biochemistry

1992-1993: Clinician at cytogenetics lab, GeneCare, Chapel Hill, NC

Main duties included tissue culture of amniotic fluid samples and chromosomal analysis

1993: Technician through temporary agency at Orgenon-Tecknica, Durham, NC

Assisted in testing growth conditions for large scale tuberculosis screening method being designed to market to hospitals

1994-1995: Lab technician at Duke University Medical Center for Dr. Tom Mitchell, Microbiology Dept., Durham, NC

Performed general lab upkeep, assisted postdocs in testing rats for infectious responses to *Cryptococcus neoformans* and PCR and sequencing for genotyping *Candida albicans* for phylogenetic studies. Also performed fungal resistance tests on patient samples from Duke Hospital to determine best fungicide and dose for doctors to use to treat infections.

1995-1997: Lab technician at UNC-Chapel Hill University for Dr. Steve Matson, Biology Dept., Chapel Hill, NC

Performed general lab upkeep duties, assisted postdoc in characterization of bacterial and yeast helicases undertaking genetic, molecular and biochemical assays.

1997: began Graduate school at University of Texas in Austin

1998: Started thesis project in the lab of Dr. Ian Molineux, Microbiology Dept. UT

Main topic was trying to determine the DNA entry mechanism from lambda phage into *E.coli* bacteria host by modifying a T7phage transcription entry assay and measuring rates of DNA transfer under different conditions to see if this 1-2 minute process could be slowed down.

My arrangements in this lab did not work out and the project has not been continued.

2001: Started thesis project in the lab of Dr. Mark Estelle, currently at Indiana University Biology Dept. Bloomington, Indiana

Title of thesis project – CHARACTERIZATION OF THE *ARABIDOPSIS THALIANA* AUXIN F-BOX FAMILY MEMBERS AFB4 AND AFB5

Expected completion in 2007