CHARACTERIZATION OF AFB5 IN ARABIDOPSIS AUXIN SIGNALING

by

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DEDICATION

This work is dedicated to my grandfather, the late Kenneth Shivers, who taught me how to ride a horse, file my taxes, and who always believed in me.

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ABSTRACT

Auxin is a pivotal hormone that regulates nearly every aspect of plant growth and development, both genomic as well as non-genomic responses. Genomic responses are regulated through the degradation of a group of transcriptional repressors called Aux/IAA proteins. These repressors are degraded through the ubiquitin-proteasome pathway involving SCF $^{TIR1/AFBs}$ in which TIR1/AFBs function as auxin co-receptors. *TIR1* gene family in Arabidopsis consists of 6 genes, of which *AFB4* and *AFB5* are distantly related to *TIR1*. Two mutant alleles of Arabidopsis *AFB5* (*pic3* and *pic59*) were isolated through a genetic screen for picloram resistance, a synthetic auxin commonly used as an herbicide.

Both mutant alleles exhibit primary root growth resistance to picloram and indole-3-butyric acid (IBA), but not to indole-3-acetic acid (IAA) and results indicate that AFB5 may not promote Aux/IAA degradation, suggesting that it functions partially or completely differently from TIR1. *afb5* resistance to IBA in primary root elongation, lateral root proliferation, as well as altered responses to IBA-induced gene expression raises the possibility that AFB5 is involved in IBA rather than IAA signaling, however the role of IBA in auxin signaling still remains unclear. *pic3* and *pic59* display altered lateral root densities and primary root elongation, and expression of cell division reporter CyclinB::GUS is lower in *pic3* and higher in *pic59*, indicating that AFB5 regulates cell division in primary and lateral roots. *afb5* mutants also exhibit altered responses to abscisic acid (ABA) in seed germination and primary root growth and it was shown that ABA downregulates *AFB5* expression, supporting a role for AFB5 in auxin and ABA cross-talk.

Additionally, recent published data suggests that ROP GTPases regulate both auxin and ABA signaling. Two members of this family, *ROP2* and *ROP6* express

highly in actively dividing tissues and mutants show defects in lateral root development. ROP2 and ROP6 have also been shown to interact with IBR5, a phosphatase which regulates auxin signaling and displays both ABA and IBA response. Based on these data, it was hypothesized that the functions of AFB5 may be regulated through ROP GTPases. Results indicate that basal expression of *AFB5* is significantly higher in *rop2* and *rop6* and that ROP2/6 may functionally interact with AFB5 in regulating primary root elongation.

I. INTRODUCTION

Plant growth and development are orchestrated by a cache of small signaling molecules known as phytohormones. Among the known phytohormones, auxin is the only one that seems to play a role in nearly every aspect of growth and development. These morphogenic and developmental events occur via the biosynthesis, conjugation, and degradation of auxin within the closed system. In young developing seedlings, auxin is mainly synthesized in the shoot apical meristem through both tryptophan- dependent and independent pathways (Bartel, 1997). It is then transported basipetally to the root apical meristem, where it is redistributed (Reed et al., 1998; Zhao, 2011). Auxin may be in the form of free molecules, which are the most active, or it may be conjugated to various amino acids, sugars, or polypeptides, where it becomes more stable and better suited for storage (Bartel, 1997).

Although knowledge of auxin's presence in plant tissues and the general effect it has on growth dates back to the mid-1800s with Charles Darwin, it wasn't until the Cholodny-Went model, proposed in 1937, that a mechanism was first described for auxin action. In this model, auxin redistributes within tissues in response to light, elongating some cells and directing growth of the shoot toward the light source, a process known as phototropism (Went and Thimann, 1937). Over 85 years later, we now know that auxin does control phototropism, gravitropism, organogenesis, and adventitious and lateral root growth through redistribution within tissues by PIN and AUX1 transporter proteins (Estelle, 1996). At the cellular level, auxin can promote cell division and elongation, helping to form and maintain lateral and axillary meristems. Auxin also controls the differentiation of vascular tissue and lateral organs such as leaves and flowers (Mockaitis and Estelle, 2008).

In primary root development, auxin controls both cell specification during embryogenesis as well as root patterning during post-embryonic root development. During embryogenesis, auxin produced in the embryo apex is transported to the basal half of embryo to control root development by eliciting auxin-responsive gene expression (Jenik and Barton, 2005). ARF5, an auxin-responsive transcription factor, has been shown to control embryogenesis in response to auxin, and loss-of-function *arf5* has

severe defects in auxin transport and perception (Weijers et al., 2006; Möller and Weijers, 2009; Schlereth et al., 2010), resulting in rootless seedlings due to a failure to establish the hypophysis (Burleth and Jurgens, 1993; Hamann et al., 2002; Schlereth et al., 2010). In addition to its role in embryogenesis, auxin also controls and maintains the formation of the post-embryonic root through the establishment of a concentration gradient. Auxin is primarily synthesized in young leaves and is transported down the stele to the root apical meristem, where it is retained in high concentrations in a region known as the quiescent center. The high concentration of auxin in this region inhibits cell division, resulting in a cluster of inactive cells which only divide to maintain the population of stem cells around it (Aichinger et al., 2012). The establishment of this concentration gradient also controls cell division in the meristematic region, as well as the elongation and differentiation of more mature cells (Petricka et al., 2012). Secondary, or lateral, roots are formed from the pericycle region of the primary root. Auxin controls lateral root development by programming cells in the basal meristem to develop into lateral root founder cells, or initials (De Smet, 2012). Auxin initiates the formation of lateral roots by stimulating asymmetric division of the lateral root founder cells, giving rise to the first germ layer of a lateral root primordia (LRP), and then promotes the division of LRP cells as they penetrate the cortex and epidermis of the primary root (Peret et al., 2009; De Smet, 2012).

There are four known natural auxins. Indole-3-acetic acid (IAA), is the most abundant form and regulates most of the processes that are associated with auxin action. Indole-3-butyric acid (IBA) was previously believed to be only a synthetic auxin, but was shown in the 1980s and early 1990s to occur naturally in some plant species (Schneider et al., 1985; Sutter and Cohen, 1992; Ludwig-Muller and Epstein, 1991). IBA is often used in agriculture due to its ability to induce adventitious roots in plant cuttings and has been shown to be more effective than IAA at inducing lateral and adventitious roots. IBA has been the center of controversy surrounding auxin action, as some researchers suggest that its effect is solely dependent on its conversion to IAA (Epstein and Lavee 1984; Van der Krieken et al., 1997), while others assert that IBA likely has functions independent of this conversion, and that the mechanism has merely been elusive (Nordstrom et al., 1991; Ludwig-Muller, 2000; Bartel, 2000). Phenylacetic acid (PAA) is a phenyl derivative of

IAA that has been detected in several plant species including bean and maize, but not in *Arabidopsis*. PAA is thought to be associated with root-symbiont and root-pathogen interactions. 4-chloroindole-3-acetic acid (4-Cl-IAA) has been detected in legumes and some species of *Pinus*. It has, in some cases, shown to be more effective than IAA when exogenously applied, potentially due to its stability. 4-Cl-IAA is primarily involved in pericarp growth, however due to its absence in *Arabidopsis*, it has been difficult to identify other functions (Simon and Petrasek, 2011). In addition to these natural auxins, many synthetic auxins such as 2,4-D and picloram are used in agriculture as strong herbicides and in research as stable auxin analogs (Grossman, 2009; Rybel et al., 2009).

The effect of auxin on plant growth and development can be transcriptional and/or non-transcriptional. The most characterized mechanism for transcriptional IAA function is through the $SCF^{TIR1/AFB}$ pathway, in which IAA acts as a "molecular glue" between the co-receptors TIR1 and a family of proteins known as Aux/IAAs (Tan et al., 2007). In this cascade, Aux/IAAs act as repressors of ARFs which serve as auxin responsive transcription factors. In the presence of high concentrations of auxin, IAA binds to the F-box protein TIR1 allowing it to interact with Aux/IAAs (Chapman and Estelle, 2009). This interaction initiates ubiquitination of Aux/IAA, which tags it for degradation through the 26S proteasome, thus relieving repression of ARFs (Figure 1). *Arabidopsis* encodes 29 Aux/IAAs most of which contain four conserved domains, with domain II being the target for ubiquitination. There are 23 known ARFs in *Arabidopsis*, which behave as either transcriptional activators or repressors, so that degradation of Aux/IAAs may result in positive or negative regulation of gene expression (Dharmasiri and Estelle, 2004). Five members of this family contain a glutamine-rich middle region and act as transcriptional activators. The remaining ARFs which are serine-rich, serine-glycine-rich, serine-proline-rich, or serine-proline-leucine-rich have been shown to repress transcription (Leyser, 2006). In addition to the versatility of ARFs, it has also recently been shown that both ARFs and Aux/IAAs form multimeric complexes, adding another layer of complexity to what is perceived as "positive" and/or "negative" regulation of gene expression (Korasick et al., 2014).

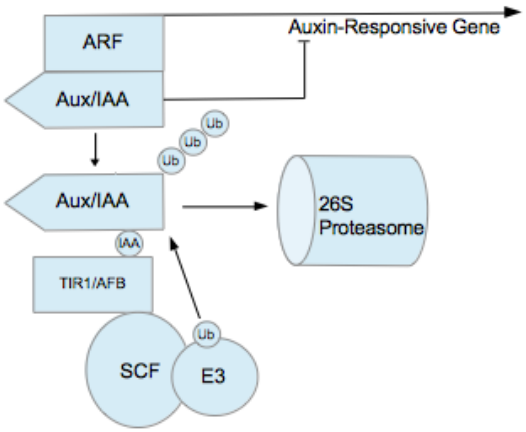


Figure 1. Hypothetical Model of SCF$^{TIR1/AFB}$ Mechanism. TIR1/AFBs initiate the ubiquitination of Aux/IAAs, tagging them for degradation, and thus relieving repression of ARF.

In addition to the well-characterized TIR1/AFB pathway, an alternative auxin signaling cascade has been proposed which includes Auxin Binding Protein 1 (ABP1). First detected in maize in 1972, ABP1 is the first putative auxin receptor to be identified. Its discovery, however, has since been marked by controversy and debate regarding its role as a receptor, primarily because its mode of action has been difficult to piece together. ABP1 is known to mediate rapid responses to exogenously applied IAA, such as cell expansion. ABP1 contains a KDEL sequence near the c-terminus, indicating that it is localized to the endoplasmic reticulum, however at this pH it shows a very low affinity for IAA. About 5% of ABP1 is localized to the apoplast, outside of the plasma membrane. Although it does not contain a transmembrane domain, ABP1 is believed to be active in the apoplast, due to it's prime location for sensing and responding to incoming auxin quickly (Shi and Yang, 2011). As ABP1 is not localized to the nucleus and responds very quickly to exogenously applied IAA, it has classically been implicated in non-transcriptional auxin responses, simply because the time frame for its mode of action is too short to be explained by gene transcription (Steffens et al., 2001; Badescu and Napier, 2006). Null *abp1* mutants are embryo-lethal (Chen et al., 2001), making it considerably more difficult to characterize its mode of action than with auxin-signaling F-box proteins. Recently, however, it has been shown that *abp1* knockdown results in the

rapid degradation of Aux/IAAs (Tromas et al., 2013), altering the general perception that its role is strictly non-transcriptional. This may be the first indication of overlap between the ABP1 and TIR1/AFB pathway and that both ABP1 and TIR1/AFB may regulate the expression of early-auxin inducible genes.

Three gene families are directly regulated by auxin: *Gretchen Hagen3* (*GH3*s), *Small auxin-upregulated RNA*s (*SAUR*s), and *Aux/IAA*s. These genes are collectively referred to as "early-auxin inducible" genes because they contain the *AuxRE* motif in their promoter region, allowing ARFs to bind (Quint and Gray, 2006). The *AuxRE* motif distinguishes genes that are direct targets of auxin regulation and those that are activated downstream of auxin signaling. *GH3*s encode three groups of enzymes which conjugate amino acids to jasmonic acid, IAA, and salicylic acid (Staswick et al., 2005; Wang et al., 2008). Because IAA is active in its "free" form, this conjugation inactivates and stabilizes IAA, allowing it to be stored or sequestered from the pool of active auxin (Kramer and Ackelsberg, 2015). *SAUR*s encode highly unstable proteins and represents the largest early-auxin responsive gene family, with 81 known members (Ren and Gray, 2015). Despite the identification of so many *SAUR* genes, this group has remained largely uncharacterized potentially due to the functional redundancy among its members (Jain et al., 2006). Auxin also induces expression of *Aux/IAA*s, forming a negative feedback loop in which Aux/IAA proteins can inhibit the expression of *Aux/IAA* genes, so that degradation of Aux/IAA proteins can initiate expression of additional Aux/IAAs, which in turn repress themselves and/or other *Aux/IAA, GH3, or SAUR* genes. This phenomenon has been modelled mathematically to show the rate of induction and degradation, demonstrating that at least one Aux/IAA, *IAA3*, does not exhibit the canonical behavior of the others (Hagen and Guilfoyle, 2002; Gray et al., 2001; Middleton et al., 2010).

Because auxin is abundant in plant tissues, it is necessary for plants to fine-tune its action. This specificity is thought to be the result of the dynamic between different Aux/IAA-ARF-TIR1/AFB combinations (Parry et al., 2009; Quint and Gray, 2006; Hayashi, 2012). In addition to TIR1, five other homologous auxin-signaling F-box (AFB) proteins are found in *Arabidopsis*. These AFBs diverged into three distinct subclades, roughly 300mya, forming "*TIR1/AFB2*" (which also includes *AFB1* and *AFB3*), "*AFB4*" (which also includes *AFB5*), and "*AFB6*" which disappeared around the time that grasses

first appeared (Parry et al., 2009). Some functional redundancy among the AFBs has been reported, however each AFB can hypothetically mediate it's own specific functions (Dharmasiri et al., 2005b). AFB1 and AFB2 share about 70% and 61% identity to TIR1, respectively, however neither can recover *tir1-1* phenotypes, indicating that despite their similarity they are not functionally redundant. In addition, AFB1 and AFB3 have little effect on Aux/IAA degradation compared to TIR1 and AFB2, suggesting that they play a lesser role in auxin signaling (Havens et al., 2012; Shimizu-Mitao and Kakimoto, 2014). While research into the function and behavior of TIR1/AFB1-3 has increased in the last ten years, less work has been done to elucidate the functions of the AFB4 clade (Parry et al., 2009).

Despite being the most distantly related homologs, AFB4 and AFB5 share about 40% identity to TIR1. What makes them considerably different, however, is their extended N-terminus of about 25 amino acids, the function of which has not yet been determined (Figure 2). A report published in 2011 suggests that AFB4 negatively regulates auxin signaling compared to TIR1 and AFB2, based on increased hypocotyl length, lateral root density, and expression of auxin-inducible genes in the mutant line

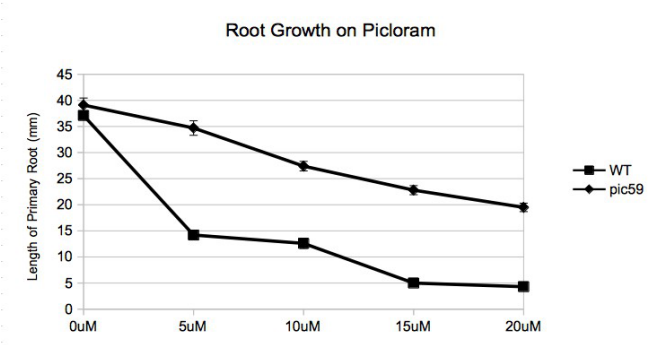
Figure 2. *AFB5* is a *TIR1* Homolog. AFB5 shares about 40% identity to TIR1, however it contains an additional 25 amino acids on the N-terminus. Both AFB5 mutants exhibit amino acid changes.

(Greenham et al., 2011). A second paper on AFB4, however, challenges this suggestion, and shows that AFB4 mutants actually have *fewer* lateral roots than the wild type and that hypocotyl length in *afb4* is comparable to *tir1* (Hu et al., 2012). Based on both sets of published data, it is unclear what effect, if any, AFB4 has on auxin signaling. In terms of AFB5, much of the published research describes selective resistance to the auxinic herbicides picloram, dicamba, and DAS534 in mutant lines. This resistance to synthetic

auxins seems to be unique to TIR1 and AFB5 and may reveal a role for AFB5 in mediating herbicidal activity (Walsh et al., 2006; Gleason et al., 2011). In contrast, *afb5* was shown to be slightly hypersensitive to IAA, contrary to what is seen in *tir1* and *afb2* (Walsh et al., 2006). This supports the idea that AFB5 may have negative regulatory functions in the auxin signaling cascade. Although some work has been done to characterize AFB5's response to herbicides, its role in plant development and growth remains to be elucidated. Interestingly, a new report demonstrating the activity of every TIR1/AFB showed that neither AFB4 nor AFB5 contributes significantly to degradation of any Aux/IAA in the presence of IAA, further complicating the role of AFB5 in auxin signaling (Shimizu-Mitao and Kakimoto, 2014).

In a mutant screen for resistance to the synthetic auxin picloram, several alleles of *AFB5* were identified, including *pic3* and *pic59* (Figure 3). *pic3* is the more severe mutant, with two point mutations in the F-box domain, the region which binds to ASK1 in the SCF complex. These mutations confer two amino acid changes: arginine to glutamine and aspartic acid to asparagine, respectively. *pic59* contains one point mutation near the C-terminus, conferring a cysteine to tyrosine amino acid change (Figure 4). While both homozygous mutants are insensitive to primary root growth inhibition by picloram, heterozygous *pic3* shows wild type sensitivity to picloram and heterozygous *pic59* shows intermediate resistance to picloram (Data not shown). These data indicate that *pic3* is a recessive allele and *pic59* is a semi-dominant alelle. Despite it's seemingly mild mutation, *pic59* shows strong phenotypic changes compared to the wild type, indicating that this cysteine residue may be necessary to the protein's structure and/or proper function. In addition to picloram resistance, *pic3* and *pic59* both exhibit differential responses to the phytohormone abscisic acid (ABA). In the primary root, both *pic3* and *pic59* show resistance to ABA-inhibited growth, however they differ in their response to ABA-inhibited seed germination in that *pic59* is resistant while *pic3* is hypersensitive (Figure 23). Both *pic3* and *pic59* show defects in lateral root development, with *pic59* producing a higher lateral root density and *pic3* producing a lower lateral root density than wild type seedlings (Figure 10).

A.



B.

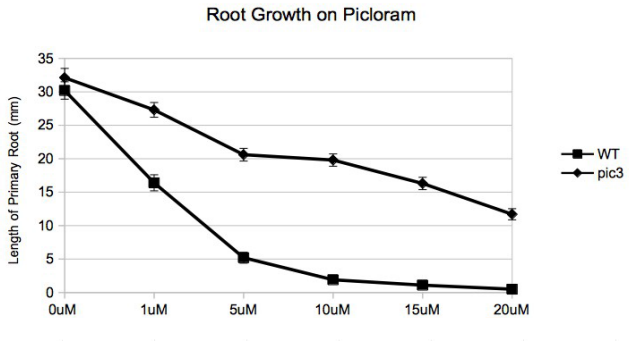


Figure 3. Primary root growth of *pic3* and *pic59* in response to picloram. Four day old seedlings were transferred to ATS media containing the indicated concentration of picloram. Primary root length was measured 4 days after transfer. *pic3* (A) and *pic59* (B) are both highly resistant to the synthetic auxin, picloram, at various concentrations. Error bars represent S.E., *n*=9. (S. Dharmasiri, Unpublished).

A.

```
AFB5     MTQDRSEMSEDDDDQQSPPLDLPSTAIADPCSSSSSPNKSRNCISNSQTFPDHVLENVLE
pic3     MTQDRSEMSEDDDDQQSPPLDLPSTAIADPCSSSSSPNKSRNCISNSQTFPDHVLENVLE
         ************************************************************

AFB5     NVLQFLDSRCDRNAASLVCKSWWRVEALTRSEVFIGNCYALSPARLTQRFKRVRSLVLKG
pic3     NVLQFLDSRCDRNAASLVCKSWWRVEALTQSEVFIGNCYALSPARLTQRFKRVRSLVLKG
         ****************************:*****************************

AFB5     KPRFADFNLMPPDWGANFAPWVSTMAQAYPCLEKVDLKRMFVTDDDLALLADSFPGFKEL
pic3     KPRFANFNLMPPDWGANFAPWVSTMAQAYPCLEKVDLKRMFVTDDDLALLADSFPGFKEL
         *****:******************************************************

AFB5     ILVCCEGFGTSGISIVANKCRKLKVLDLIESEVTDDEVDWISCFPEDVTCLESLAFDCVE
pic3     ILVCCEGFGTSGISIVANKCRKLKVLDLIESEVTDDEVDWISCFPEDVTCLESLAFDCVE
         ************************************************************

AFB5     APINFKALEGLVARSPFLKKLRLNRFVSLVELHRLLLGAPQLTSLGTGSFSHDEEPQSEQ
pic3     APINFKALEGLVARSPFLKKLRLNRFVSLVELHRLLLGAPQLTSLGTGSFSHDEEPQSEQ
         ************************************************************

AFB5     EPDYAAAFRACKSVVCLSGFRELMPEYLPAIFPVCANLTSLNFSYANISPDMFKPIILNC
pic3     EPDYAAAFRACKSVVCLSGFRELMPEYLPAIFPVCANLTSLNFSYANISPDMFKPIILNC
         ************************************************************

AFB5     HKLQVFWALDSICDEGLQAVAATCKELRELRIFPFDPREDSEGPVSELGLQAISEGCRKL
pic3     HKLQVFWALDSICDEGLQAVAATCKELRELRIFPFDPREDSEGPVSELGLQAISEGCRKL
         ************************************************************

AFB5     ESILYFCQRMTNAAVIAMSENCPELTVFRLCIMGRHRPDHVTGKPMDEGFGAIVKNCKKL
pic3     ESILYFCQRMTNAAVIAMSENCPELTVFRLCIMGRHRPDHVTGKPMDEGFGAIVKNCKKL
         ************************************************************

AFB5     TRLAVSGLLTDQAFRYMGEYGKLVRTLSVAFAGDSDMALRHVLEGCPRLQKLEIRDSPFG
pic3     TRLAVSGLLTDQAFRYMGEYGKLVRTLSVAFAGDSDMALRHVLEGCPRLQKLEIRDSPFG
         ************************************************************

AFB5     DVALRSGMHRYYNMRFVWMSACSLSKGCCKDIARAMPNLVVEVIGSDDDDDNRDYVETLY
pic3     DVALRSGMHRYYNMRFVWMSACSLSKGCCKDIARAMPNLVVEVIGSDDDDDNRDYVETLY
         ************************************************************

AFB5     MYRSLDGPRNDAPKFVTIL
pic3     MYRSLDGPRNDAPKFVTIL
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B.

```
AFB5     MTQDRSEMSEDDDDQQSPPLDLPSTAIADPCSSSSSPNKSRNCISNSQTFPDHVLENVLE
pic59    MTQDRSEMSEDDDDQQSPPLDLPSTAIADPCSSSSSPNKSRNCISNSQTFPDHVLENVLE
         ************************************************************

AFB5     NVLQFLDSRCDRNAASLVCKSWWRVEALTRSEVFIGNCYALSPARLTQRFKRVRSLVLKG
pic59    NVLQFLDSRCDRNAASLVCKSWWRVEALTRSEVFIGNCYALSPARLTQRFKRVRSLVLKG
         ************************************************************

AFB5     KPRFADFNLMPPDWGANFAPWVSTMAQAYPCLEKVDLKRMFVTDDDLALLADSFPGFKEL
pic59    KPRFADFNLMPPDWGANFAPWVSTMAQAYPCLEKVDLKRMFVTDDDLALLADSFPGFKEL
         ************************************************************

AFB5     ILVCCEGFGTSGISIVANKCRKLKVLDLIESEVTDDEVDWISCFPEDVTCLESLAFDCVE
pic59    ILVCCEGFGTSGISIVANKCRKLKVLDLIESEVTDDEVDWISCFPEDVTCLESLAFDCVE
         ************************************************************

AFB5     APINFKALEGLVARSPFLKKLRLNRFVSLVELHRLLLGAPQLTSLGTGSFSHDEEPQSEQ
pic59    APINFKALEGLVARSPFLKKLRLNRFVSLVELHRLLLGAPQLTSLGTGSFSHDEEPQSEQ
         ************************************************************

AFB5     EPDYAAAFRACKSVVCLSGFRELMPEYLPAIFPVCANLTSLNFSYANISPDMFKPIILNC
pic59    EPDYAAAFRACKSVVCLSGFRELMPEYLPAIFPVCANLTSLNFSYANISPDMFKPIILNC
         ************************************************************

AFB5     HKLQVFWALDSICDEGLQAVAATCKELRELRIFPFDPREDSEGPVSELGLQAISEGCRKL
pic59    HKLQVFWALDSICDEGLQAVAATCKELRELRIFPFDPREDSEGPVSELGLQAISEGCRKL
         ************************************************************

AFB5     ESILYFCQRMTNAAVIAMSENCPELTVFRLCIMGRHRPDHVTGKPMDEGFGAIVKNCKKL
pic59    ESILYFCQRMTNAAVIAMSENCPELTVFRLCIMGRHRPDHVTGKPMDEGFGAIVKNCKKL
         ************************************************************

AFB5     TRLAVSGLLTDQAFRYMGEYGKLVRTLSVAFAGDSDMALRHVLEGCPRLQKLEIRDSPFG
pic59    TRLAVSGLLTDQAFRYMGEYGKLVRTLSVAFAGDSDMALRHVLEGCPRLQKLEIRDSPFG
         ************************************************************

AFB5     DVALRSGMHRYYNMRFVWMSACSLSKGCCKDIARAMPNLVVEVIGSDDDDDNRDYVETLY
pic59    DVALRSGMHRYYNMRFVWMSAYSLSKGCCKDIARAMPNLVVEVIGSDDDDDNRDYVETLY
         ******************** ************************************

AFB5     MYRSLDGPRNDAPKFVTIL
pic59    MYRSLDGPRNDAPKFVTIL
         ********************
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Figure 4. Alignment of wild type AFB5 protein sequence with predicted protein sequences of *pic3* and *pic59*. (A) *pic3* mutations result in two amino acid substitutions, arginine at position 90 to glutamine and aspartic acid at position 126 to asparagine. (B) The *pic59* mutation results in single amino acid substitution, cysteine at position 562 to tyrosine (Amino acid changes are marked in red).

Cross-talk between auxin and ABA has been extensively documented and many auxin related mutants show either ABA resistance or hypersensitivity (Tiryaki and Staswick, 2002; Monroe-Augustus et al., 2003; Strader et al., 2008; Ephritikhine et al., 1999), however the mechanisms for this cross-talk are largely unknown. A variety of genes are induced by both ABA and IAA, including *Dc3* (Rock and Sun, 2005), *GH3* (Park et al., 2007), *ZmC1* (Suzuki et al., 2001), *ABI1*, *ABI2*, and *ABI3* which also function in auxin-mediated lateral root development (Brady et al., 2003). Auxin and ABA function together to promote seed dormancy (Liu et al., 2013), control embryonic axis elongation (Belin et al., 2009), and both auxin and ABA function in abiotic stress responses (Strizhov et al., 1997; Jain and Khurana, 2009; Hannah et al., 2005; Shinozaki and Yamaguchi-Shinozaki, 2000; Xiong et al., 2002; Nakashima et al., 2009), with ABA being the most intimately tied hormone in stress signal transduction.

One family of proteins, ROP GTPases, has been implicated in auxin and ABA cross-talk (Gu Y et al., 2004). ROP GTPases are considered "signaling switches" due to their ability to be converted between an active and inactive form by certain hormones (Nibau et al., 2006; Etienne-Manneville and Hall, 2002). ROP GTPases positively regulate auxin signaling by regulating Aux/IAA degradation and auxin-responsive gene expression, and expression of these GTPases is induced by auxin (Tao L-Z et al., 2002; Tao L-Z et al., 2005)). Additionally, ROP GTPases can be inactivated by ABA, placing them in a prime location for master control of this signaling cross-talk (Lemichez E et al., 2001; Zheng et al., 2002). Two members of this family, ROP2 and ROP6 display defects in auxin and ABA perception and altered lateral root densities (Lin D et al., 2012; Li H-M et al., 2001), which may be due to defects in control of cell division or expansion, as ROPs express highly in regions of actively dividing cells such as lateral root founder cells, lateral root primordia, and the tips of emerging lateral roots (Li H et al., 2001; Poraty-Gavra et al., 2013). It has been suggested that ROPs control lateral root formation by participating in an auxin signaling pathway (Li H et al., 2001).

While mutations in *TIR1* and *AFB1-3* confer stability to Aux/IAAs (Dharmasiri et al., 2005b), mutations in *AFB5* have little effect on Aux/IAA degradation (Figure 20). This is supported by a previously mentioned paper indicating that neither AFB4 nor

AFB5 could mediate degradation of Aux/IAAs in a yeast 2-hybrid system (Shimizu-Mitao and Kakimoto, 2014). This suggests that AFB5 participates in the $SCF^{TIR1/AFB}$ signaling pathway in a different capacity than TIR1. Additionally, results indicate that *afb5* shows defects in primary and lateral root growth, and that this may be due to an impaired regulation of cell division. Finally, because ROP2 and ROP6 have been shown to be regulators of auxin signaling and display similar expression patterns and phenotypes as *AFB5*, it was hypothesized that *ROP2* and *ROP6* regulate auxin signaling, at least partially, through *AFB5*.

II. MATERIALS AND METHODS

Plant varieties and growth conditions

Arabidopsis thaliana var. *Colombia* (Col-0), obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University) was used as the wild type in all experiments performed. *CycB::GUS*, *rop2*, and *rop6* were obtained from the ABRC. *tir1-1* and *HS::AXR3NT-GUS* were obtained from Dr. Mark Estelle. *pic3xHS::AXR3NT-GUS* and *pic59xHS::AXR3NT-GUS* crosses, *AFB5::AFB5-GUS* and *AFB5-myc*, *pic59-myc*, and *ΔAFB5-myc* were created by Dr. Sunethra Dharmasiri, who also mapped and identified *pic3* and *pic59*. *pic3xCycB::GUS*, *pic59xCycB::GUS*, *rop2pic3*, *rop6pic3*, *rop2pic59*, and *rop6pic59* crosses were created for this project.

Seeds were surface sterilized with 40% bleach and 0.04% Triton X-100 and rinsed several times with sterile DI water. Seeds were plated on either Arabidopsis thaliana medium with 1% sucrose (ATS) (pH 5.6), 0.6% Murashige and Skoog (MS) (Sigma-Aldrich, St. Louis, Missouri USA) with 2% sucrose (pH 5.9), or 0.5X MS with 1% sucrose (pH 5.6). Plates were incubated in 4°C for either 24 hours or 96 hours (for seed germination assays) and then transferred to either a growth chamber at 22°C with continuous light or a growth chamber at 22°C with 16 hour light/8 hour dark cycles (for lateral root growth assays). All experiments were performed in the same two growth chambers.

Treatments for reporter gene expression

For *HS::AXR3NT-GUS* assays, 4 day old seedlings were incubated in liquid ATS at 37°C for 2 hours. Seedlings were transferred to room temperature liquid ATS and kept on a shaker at room temperature for 90 minutes. Samples were taken at 0, 45, and 90 minutes and flash frozen in liquid nitrogen for quantitative β-glucuronidase assay.

AFB5::AFB5-GUS seedlings were placed in milli Q water containing either 50μM, 100μM, or 200μM ABA and vacuum infiltrated for 10 minutes. Seedlings were kept in treatment on a shaker in room temperature for 1 hour and then either fixed (for histochemical staining) or flash frozen in liquid nitrogen (for quantitative β-glucuronidase assay).

Root growth assays

For primary root length, seedlings were grown for 4-10 days on ATS and the length of the primary root was measured. For lateral root density, seedlings were grown for 5-10 days and the number of lateral roots were counted and compared to the length of the primary root, or 4 day old seedlings were transferred to 0.5X MS media containing either 1μM IAA or 10μM IBA and lateral root density was measured after 4 additional days. For lateral root density in double mutants, seedlings were grown on 0.6% MS in 16 hour light/ 8 hour dark cycles for 10 days and the number of lateral roots were counted and compared to the length of the primary root. For lateral root primordia density, number of lateral root primordia compared to the length of the primary root at each cell-layer stage were counted.

To analyze primary root growth resistance, 4 day old seedlings were transferred to ATS media (or 0.5X MS for IBA) containing indicated concentrations of hormones. Primary root growth was measured after an additional 4 days of incubation in the growth chamber.

Germination assays

Seeds were surface sterilized as described above and incubated at 4°C for 96 hours and then plated on media containing either 0μM or 0.7μM ABA. Seeds were incubated at 22°C under continuous illumination. Germinated seeds were counted every 24 hours for 5 days. Seeds with an emerged radicle were considered germinated.

Histochemical staining

Histochemical staining of seedlings for GUS assays was carried out according to Jefferson et al. (1987). Treated seedlings were washed in distilled water and fixed using GUS fixer (0.3M mannitol, 10mM MES, 0.3% formaldehyde) for 30 minutes. GUS fixer was washed from seedlings twice, for 10 minutes each using GUS wash (100mM phosphate buffer pH 7.0). Seedlings were vacuum infiltrated with GUS staining buffer (0.1M 5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexylammonium, 100mM phosphate buffer, 10mM EDTA, 0.1% Triton X-100, 1mM potassium ferrocyanide, 1mM

potassium ferricyanide) and incubated in 37°C until color developed.

Quantitative β-glucuronidase assay

For quantitative β-glucuronidase assay, seedlings were flash frozen in liquid nitrogen immediately following treatment. All tissues were ground in GUS extraction buffer (100mM phosphate buffer, 10mM EDTA, 0.1% sodium lauryl sarcosine, 10μM β-mercaptoethanol) and centrifuged for 10 minutes at 13,000rpm. Supernatant was collected and the amount of total protein was quantified using Bradford assay (Bradford, 1976). 50-75μg of total protein from each sample was incubated at 37°C for 1 hour in GUS assay buffer (GUS extraction buffer containing 4-methylumbelliferyl-β-D-glucuronide hydrate). The reaction was stopped by adding samples to 0.2M sodium carbonate. Fluorescence was measured at a wavelength of 460nm using a luminometer (Turner, Sunnyvale, California USA, Model number 9200-002).

Protein extraction and western blot analysis

For pull down assays, total proteins were extracted from ~500mg of tissue using protein extraction buffer (50mM HEPES, 100mM KCl, 10% glycerol, 0.01% Tween-20) and incubated for 1 hour with GST-IAA7-bound glutathione beads and 50μM picloram. Glutathione beads were pulled down by centrifugation and boiled in 2X LSB (0.5M Tris-Cl, 0.05% SDS, 20% glycerol, and 0.003% bromophenol blue) containing 10% β-mercaptoethanol for 8 minutes. 15μL samples were separated on a 10% acrylamide SDS-PAGE gel and transferred to polyvinylidine difluoride (PVDF) membrane for western blotting.

For degradation assay, total proteins were extracted from 4 day old seedlings treated for 1 hour with 200μM ABA, 5μM MG132, 1mM cycloheximide, or combinations of treatments as indicated. Tissues were ground in E buffer (125mM Tris-Cl pH 8.8, 1% SDS, 10% glycerol, and 50mM $Na_2S_2O_5$) and centrifuged for 10 minutes at 13,000 rpm. Supernatant was collected and 5μL were boiled in 10μL of Z buffer (125mM Tris-Cl, 12% SDS, 10% glycerol, 22% β-mercaptoethanol, and 0.001% bromophenol blue) for 10 minutes. Proteins were separated on a 10% acrylamide SDS-PAGE gel and transferred to PVDF membrane for western blotting.

For all western blotting, PVDF membranes were blocked for at least 1 hour in 5% non-fat dry milk in 1X TBS with .1% Tween-20 and incubated for 1 hour in primary antibody (1:10000 anti-myc; Covance, Princeton, New Jersey USA). After washing, membranes were incubated for 1 hour in secondary antibody (1:10000 mouse IgG; Sigma-Aldrich, St. Louis, Missouri USA). Activity of secondary antibodies was detected by chemiluminescence (ECL 2 Western Blotting Substrate; Thermo Fisher Scientific, Waltham, Maryland USA).

RNA isolation and qRT-PCR

Seedlings were grown for 4 days on ATS media and treated with 1μM of either IAA or IBA for 1 hour. Tissues were flash frozen in liquid nitrogen and ground to a fine powder. Total RNA was extracted using TriReagent (38% phenol, 0.8M guanidinium thiocyanate, 0.4M ammonium thiocyanate, 0.1M sodium acetate pH 5, 5% glycerol). RNase-free DNase was used to remove any contaminating DNA. cDNA was synthesized using 3μg of total RNA and M-MuLV reverse transcriptase according to the manufacturer's instructions (NEB, Ipswitch, Maryland USA). Quantitative RT-PCR analyses were performed using SYBR Green mix (Thermo Fisher Scientific, Waltham, Maryland USA) with specific primers listed below. PCR cycling conditions for amplification were 25°C for 5 minutes, 37°C for 1 hour and 15 minutes, and then 85°C for 5 minutes. All data was normalized with respect to UBA (At1g04850). qRT-PCR was performed using Bio-Rad CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hurcules, California USA, model number 185-5200)

Image acquisition

For confocal microscopy, images of root epidermal cells were acquired using an Olympus FV1000 confocal microscope, with the assistance of Praveen Kathare, and analyzed using Olympus Fluoview software (Olympus, Shinjuku, Tokyo Japan). For light microscopy, images of seedlings were photographed using a Nikon SMZ1500 camera attached to a stereo microscope or compound microscope.

Data analysis

Data analysis was performed using VassarStats One-way ANOVA (http://vassarstats.net).

Oligonucleotide primers used in this study:

	Primer name	Primer sequence 5' to 3'	Primer length (bp)	Tm (°C)
1	pic3 F	TACCCTCTACCGCCATAGCTG	22	59.2
2	pic3 R	CAATCAGGAGGCATGAGATTGATA	25	58.4
3	pic59 F	AGGCCTGACCATGTAACAGGAA	22	58.3
4	pic59 R	CTTGCAGCATCCCTTAGACAATCGA	25	58.9
5	AFB5 Exon 3 F	ACAATCGATGCCCCTGATTCAATATGTGATG	31	59.1
6	GUS R	CGATCCAGACTGAATGCCCAC	22	59.3
7	ROP2-1F	GCGGGATCCATGGCGTCAAGGTTTATAAAG	30	62.5
8	ROP2-1R	TTTGTCGACTCACAAGAACGAGCAACG	27	63.3
9	ROP6-1F	GAGGGATCCATGAGTGCTTCAAGGTTTATC	30	60.3
10	ROP6-1R	GAGGGATCCATGAGTGCTTCAAGGTTTATC	30	60.3
11	LB02	TTGGGTGATGGTTCACGTAGTGGGCC	26	64.5
12*	qAFB5 F	TGCCAACAAGTGCAGAAAGCTG	22	58.7
13*	qAFB5 R	TCCACTTCATCATCCGTGACCTC	23	58.5
14	IAA10 F	TCGTCCAGGACTTCTATGCTTG	22	56.4
15	IAA10 R	TATTCCGATGAGCCATCCAG	20	54.1
16	IAA12 F	TATGAAGGCAGCAAGAGCG	19	55.3
17	IAA12 R	GGTTTAACCTTTTCTCGACAAG	22	51.8
18	IAA28-8F	TGGTCGGGGATGTTCCTTGGGAGA	24	63.8
19	IAA28-8R	CAACCTCTCTCTATTCCTTGCCATG	25	57.2
20	qSAUR19 F	GAAGAGATATTTGGTGCCGCTCTC	24	61.9
21	qSAUR19 R	ACTGAGCAGAGCTTGAAATGACG	23	61.8
22	SAUR53-3 Probe F	TCAGAGTTAGTTTGACTTCAAACGT	25	54.5
23	SAUR53-3 Probe R	GAACTGACAACAATCTGCACCGT	23	57.9
24	UBA F	AGTGGAGAGGCTGCAGAAGA	20	58.1
25	UBA R	CTCGGGTAGCACGAGCTTTA	20	56.7
26	Actin F	GTGGTCGCAACCGGTATTGTGTT	23	60.1
27	Actin R	CTTAGAGATCCACATCTGCTGGAA	24	56.1

***Primers designed for this project**

III. RESULTS

pic3 and *pic59* mutants exhibit altered responses to IBA, but not IAA

IAA and IBA are the two natural auxins found in Arabidopsis, and mutations in auxin-related mutants typically confer some resistance to IAA. *pic3* and *pic59*, however, display a wild type sensitivity to primary root growth inhibition by IAA. To test whether *pic3* and *pic59* exhibited altered responses to IBA instead, primary root growth on media containing IBA was measured. Both mutants exhibit significant resistance to IBA in primary root growth (Figure 5), indicating that AFB5 may play a more significant role in IBA-mediated signaling, rather than IAA signaling.

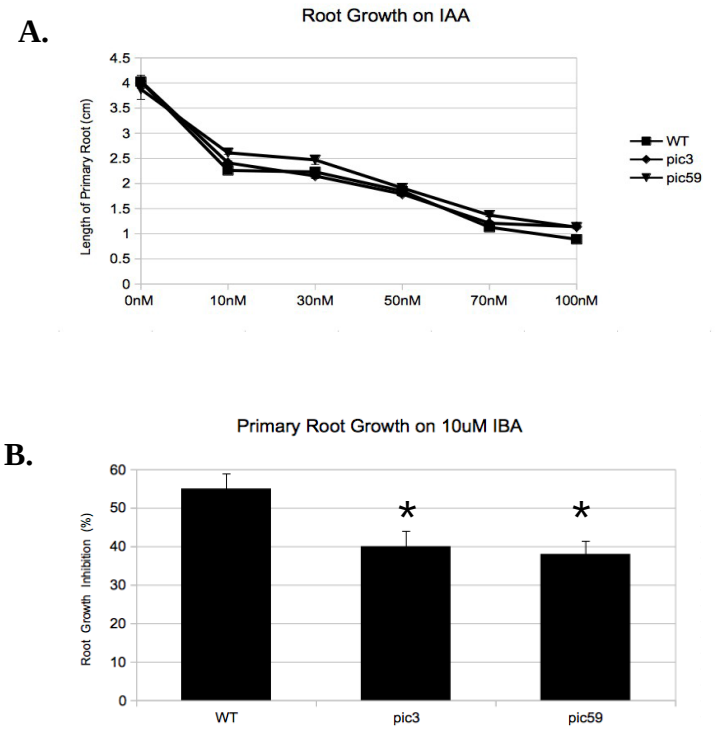


Figure 5. *AFB5* primary root growth in response to IAA and IBA. Four day old seedlings were transferred to ATS media containing the indicated concentration of IAA (A) or IBA (B). Primary root length was measured 4 days after transfer. Both *pic3* and *pic59* exhibit wild type sensitivity to IAA, but show resistance to IBA. Error bars represent S.E., *n*=12.

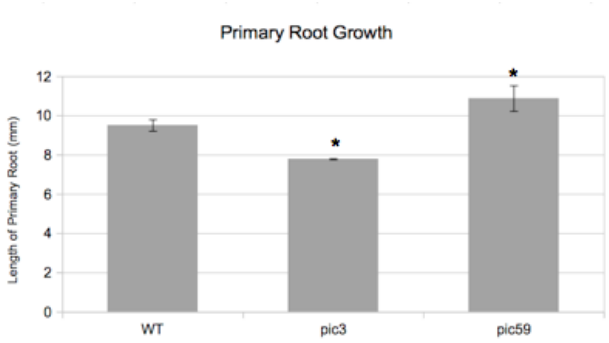
Primary root growth is altered in *afb5*

One of the key factors regulating root development is the hormone auxin. Auxin signaling mutants, such as *tir1-1* exhibit longer primary roots (Strader, et al., 2008). Therefore, the primary root lengths of *pic3* and *pic59* were measured after 4 days and 10 days of growth on ATS media. *pic3* primary roots are significantly shorter than the wild type, and *pic59* primary roots are significantly longer (Figure 6). Since this difference may simply be due to germination differences in the mutants compared to the wild type, the rate of germination was tested. Neither mutant shows differences in the rate of germination on unsupplemented media (Figure 7), indicating that this difference in primary root length is not due to early or late germination. To better understand the effect that *afb5* mutations have on primary root growth, primary root length was measured after 4-10 days of growth on unsupplemented media. Results indicate that the difference in primary root length first appears in seedlings that are about 4 days old and continues through early seedling development (Figure 8).

To test whether the difference in root length is due to defects in cell elongation, the average length of root cortical cells in *pic3* and *pic59* was compared to the wild type. No significant difference in cell length was observed between the wild type and mutants (Figure 9).

Because no difference was observed in germination rate or root cell elongation between the wild type and mutants, it was hypothesized that the difference in primary root length was due to differences in the rate of cell division. Since it is known that the cell division regulatory gene *CyclinB* is expressed highly during the mitotic phase of the cell cycle (Berckmans and Veylder, 2009; Demeulenaere and Beeckman, 2014), *pic3* and *pic59* were crossed into the *CyclinB::GUS* reporter line. GUS expression in the mutant backgrounds was compared to that of wild type (Figure 10). Intensity of GUS staining indicates that expression of *CyclinB* is slightly lower in *pic3* and higher in *pic59* compared to the wild type. Additionally, Quantification of GUS expression in the wild type, *pic3* and *pic59* background indicates that *CyclinB::GUS* expresses significantly lower in *pic3* and significantly higher in *pic59* (Figure 11).

A.



B.

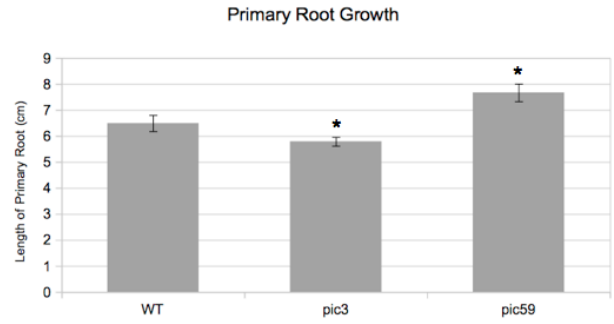


Figure 6. The growth of primary root is defective in *afb5* mutants. Seeds were germinated on ATS media and primary root length was measured after 4 days (A) and 10 days (B). *pic3* displays a shorter primary root and *pic59* displays a longer primary root than wild type seedlings. Error bars represent S.D. *n*=30

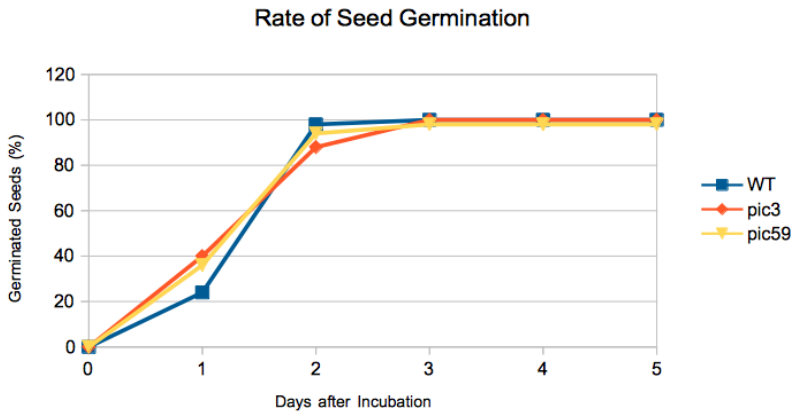


Figure 7. Rate of seed germination in *pic3* and *pic59*. Wild type, *pic3*, and *pic59* seeds were incubated in 4°C for 4 days and plated on ATS media. Germinated seeds were counted every 24 hours for 5 days. Seeds were considered germinated when the radical protruded from the seed coat. *n*=50.

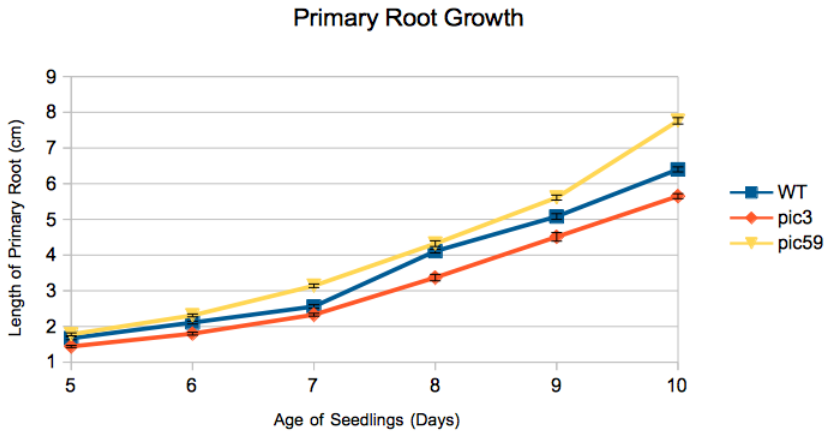


Figure 8. Primary root growth over time. Seeds were germinated on ATS media and primary root length was measured starting from the 4th until the 10th day of growth. *pic3* displays a shorter primary root and *pic59* displays a longer primary root than wild type at each stage of development. Error bars represent S.E. *n*=30.

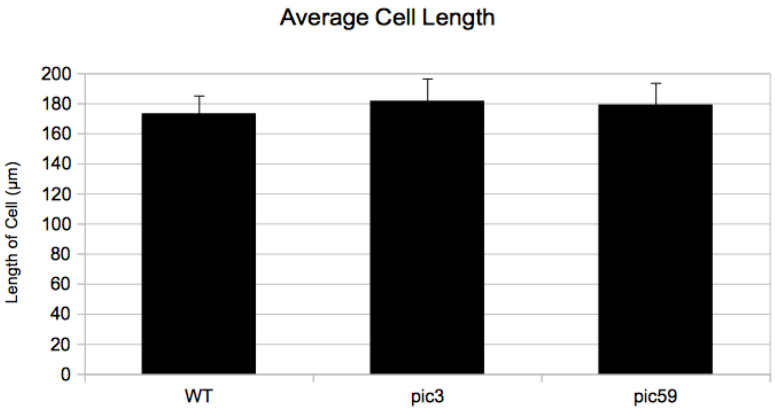


Figure 9. Average length of root cortical cells. Seedlings were grown on ATS media for 4 days and root cortical cells in the differentiation zone were imaged using confocal microscopy. Cortical cell length was measured using Olympus Fluoview software. Error bars represent S.D. *n*=100.

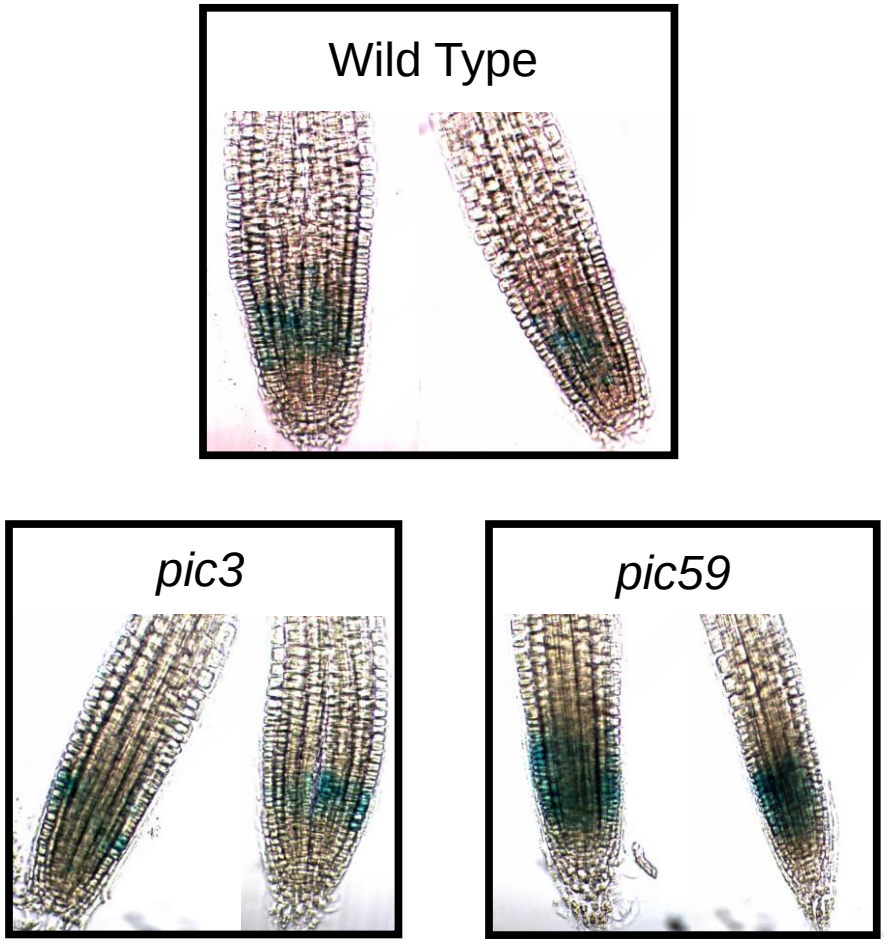


Figure 10. *CyclinB::GUS* expression in *afb5* mutants. *pic3* and *pic59* were crossed into *CyclinB::GUS* reporter line and homozygous plants were selected. Four day old seedlings were fixed and stained for GUS expression. *pic3* shows decreased GUS expression, while *pic59* shows increased GUS expression compared to GUS expression in the wild type background.

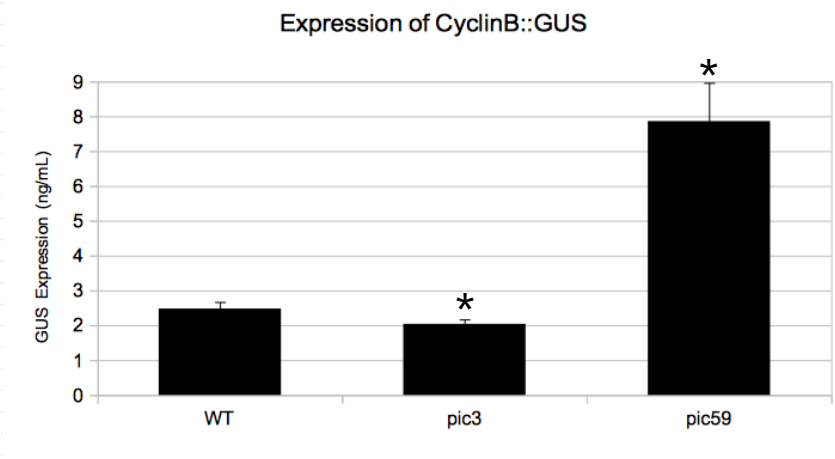


Figure 11. CyclinB::GUS expression in afb5 mutants. *pic3* and *pic59* were crossed into *CyclinB::GUS* reporter line and homozygous plants were selected. Four day old seedlings were flash frozen in liquid nitrogen. GUS expression was measured using β-glucuronidase assay. *CyclinB::GUS* expresses significantly lower in *pic3* and significantly higher in *pic59* compared to the wild type.

AFB5 may be involved in lateral root development

Auxin regulates lateral root (LR) development, and most of the auxin related mutants, including *tir1-1* show impaired LR development. In order to test LR development in *pic3* and *pic59* mutants, LR densities of 10 day old mutant seedlings were compared to that of wild type seedlings. *pic59* has a slightly higher LR density compared to the wild type, and although the LR density in *pic3* was slightly lower, the difference was not statistically significant (Figure 12). In order to determine the developmental stage at which differences in LR density become apparent, LR density was measured in seedlings grown for 5-10 days (Figure 13). Results show that the differences in LR density first appear about 6 days after germination and continue throughout seedling development.

Auxin induces LR initiation (Casimiro, et al., 2001; Himanen, et al., 2002; De Smet, et al., 2006). Since *afb5* mutants are resistant to IBA, but sensitive to IAA in primary root growth inhibition assays (Figure 4) and IBA is known to induce lateral roots more efficiently than IAA, lateral root densities were measured in *pic3* and *pic59* seedlings grown on media supplemented with IBA. Results indicate that *afb5* mutants

have a wild type sensitivity to lateral root induction by IAA, but are resistant to lateral root induction by IBA (Figure 14).

Since most of the genes that regulate LR initiation express in the LR initiation sites (primordia), it was hypothesized that *AFB5* may also express in LR primordia (LRP). To test the expression of *AFB5* in LRP, the *AFB5::AFB5-GUS* translational reporter construct was used. *AFB5::AFB5-GUS* expression was examined in 6 day old seedlings, where LRP become more prolific (Figure 15). Histochemical staining clearly shows GUS expression in developing LRP, and that this expression is apparent at all stages of LRP development, further supporting the hypothesis that *AFB5* is involved in lateral root development.

The difference in lateral root density observed in the *afb5* mutants may be attributed to an increase in lateral root initiation at the pericycle. To test this, the density of lateral root primordia was measured in seedlings from 5-7 days old and compared between the mutants and wild type (Figure 16). No significant difference in LRP density was observed between the wild type and *afb5* mutants, indicating that the difference in LR density in *afb5* mutants is not due to impaired LRP initiation. Based on this, it was hypothesized that differences in LR density were instead due to an increased and decreased rate of LR growth in *pic59* and *pic3*, respectively. To test this, the percentage of LRP that had already reached a "late stage" (3 cell files or more) were calculated for the wild type and *afb5* mutants (Figure 17). Results indicate that although there is no difference in the number of LRP in each line, LRP in *pic59* reached the later stages more rapidly than *pic3* or the wild type. This suggests that the difference in LR density between *afb5* mutants and the wild type may be due to an increased and decreased growth rate in *pic59* and *pic3*, respectively. To further show that the differences in LR density are due to differences in growth rate, the longest lateral root from each 10 day old seedling was measured. Results show that the average length of the longest lateral root is considerably shorter in *pic3* and considerably longer in *pic59* than that of the wild type (Figure 18). Based on this data, it was hypothesized that the differences in growth rate could be due to differences in cell division. *CyclinB::GUS* activity in the LRP and tips of newly emerged lateral roots in the wild type, *pic3*, and *pic59* background was observed. The level of GUS expression in *afb5* compared to wild type would indicate the relative

rate of cell division. Results indicate no significant difference in the level of GUS expression in both the LRP and LR tips in *pic3*, but a higher level in *pic59* compared to the wild type (Figure 19).

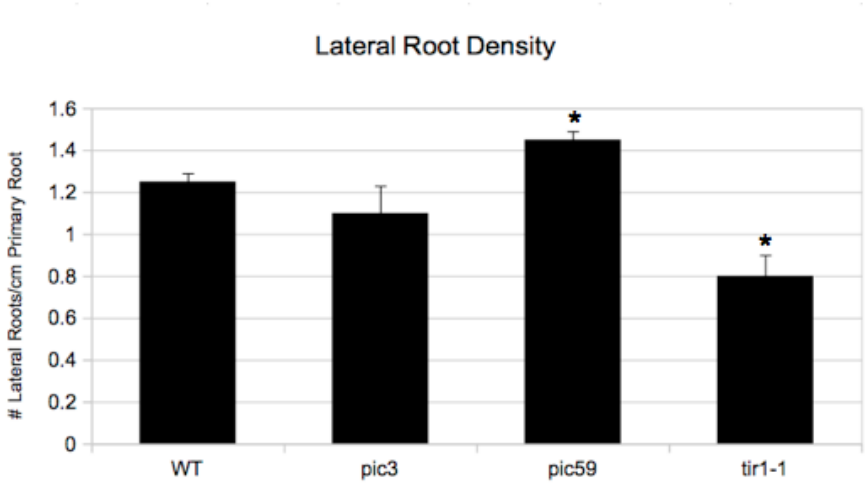


Figure 12. Lateral root density in *AFB5* mutants. Number of LR was counted and length of primary root was measured in seedlings grown for 10 days on ATS media. *pic3* shows no significant difference and *pic59* has increased lateral root density, compared to the wild-type. Error bars represent S.D., *n*=45.

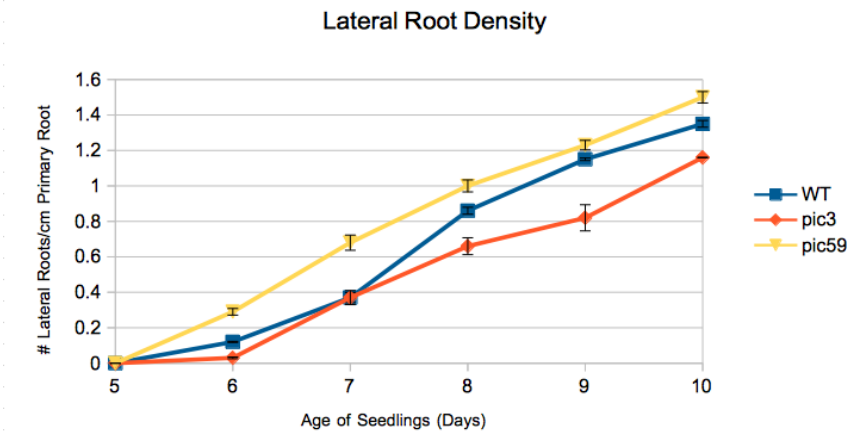


Figure 13. Lateral root density over time. Number of LR was counted and length of primary root was measured in seedlings grown for 5-10 days on ATS media. Differences in lateral root density first become apparent in 6 day old seedlings and continue throughout seedling development. Error bars represent S.E. *n*=40

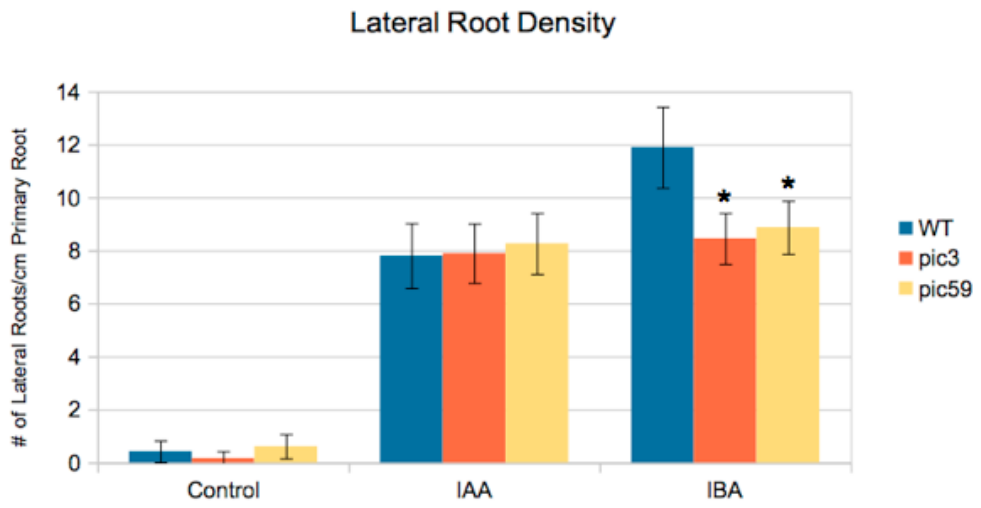


Figure 14. Density of auxin-induced lateral roots. Four day old seedlings were transferred to ATS media containing either 1μM IAA or 10μM IBA. Number of lateral roots and primary root length were measured after an additional 4 days of growth. *pic3* and *pic59* show wild type sensitivity to IAA in lateral root induction, but are insensitive to IBA in lateral root induction. Error bars represent S.D. *n*=27.

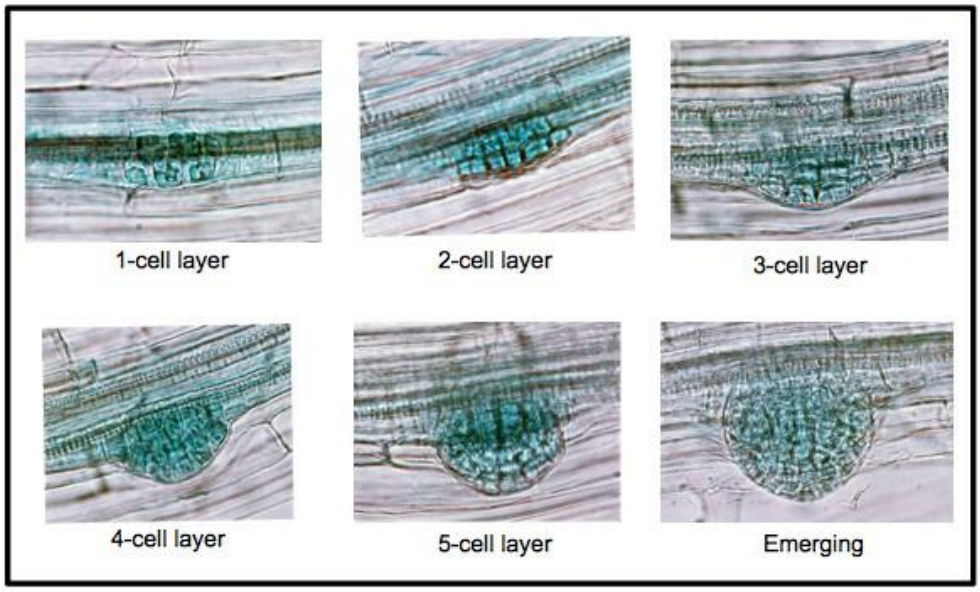


Figure 15. *AFB5::AFB5-GUS* expression in lateral root primordia. *AFB5::AFB5-GUS* expresses at all stages of lateral root development. Six day old *AFB5::AFB5-GUS* seedlings were fixed and stained for GUS and imaged.

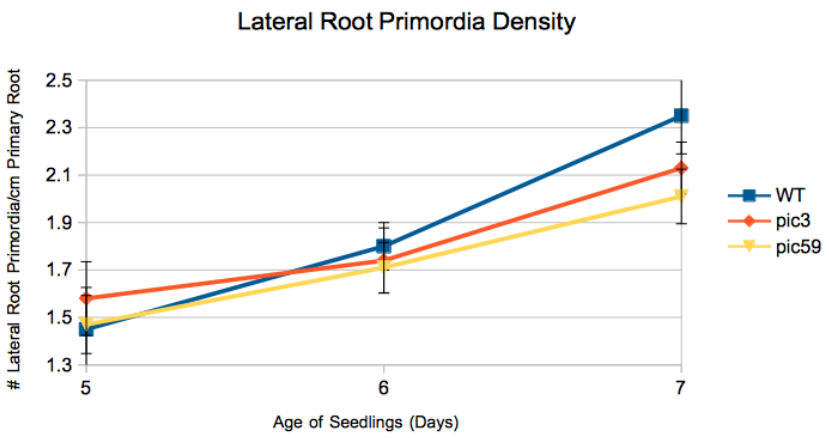


Figure 16. Density of lateral root primordia in *afb5* mutants. Number of lateral root primordia were counted and length of primary roots were measured in seedlings grown on ATS media for 5-7 days. No statistically significant difference in lateral root primordia density was observed between the wild type and *afb5* mutants. Error bars indicate S.D. *n*=15

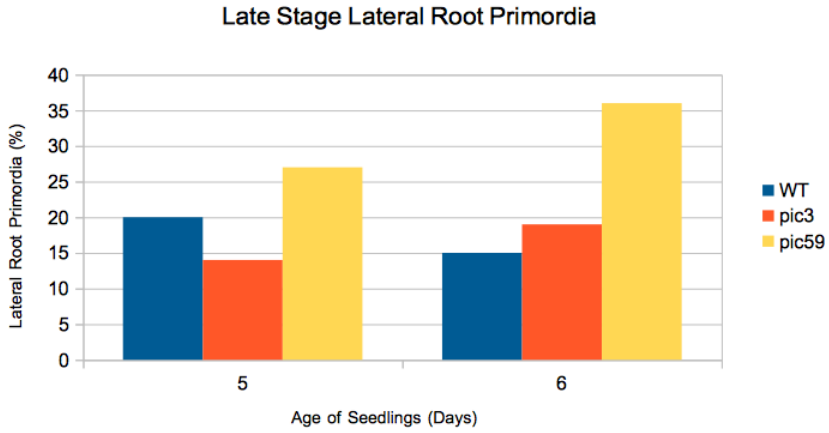


Figure 17. Percentage of late stage lateral root primordia. Number of lateral root primordia at each stage was counted in seedlings grown on ATS media for 5-6 days. Percentage of late stage lateral root primordia (3 cell files or more) was calculated. Lateral root primordia in *pic59* reach the late stage more quickly than lateral root primordia in *pic3* or the wild type.

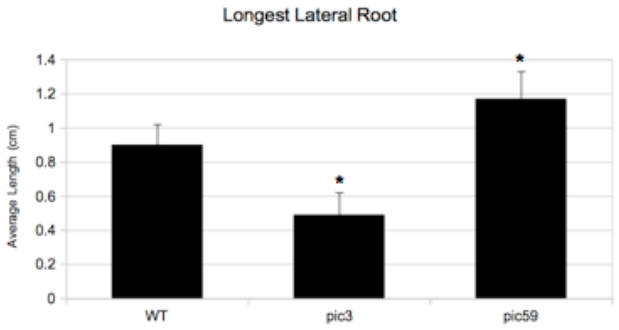


Figure 18. Average length of the longest lateral root. Seedlings were grown for 10 days on ATS media and the length of the longest lateral root from each seedling was measured. *pic3* shows significantly shorter lateral roots and *pic59* shows significantly longer lateral roots compared to the wild type. Error bars represent S.D. *n*=7.

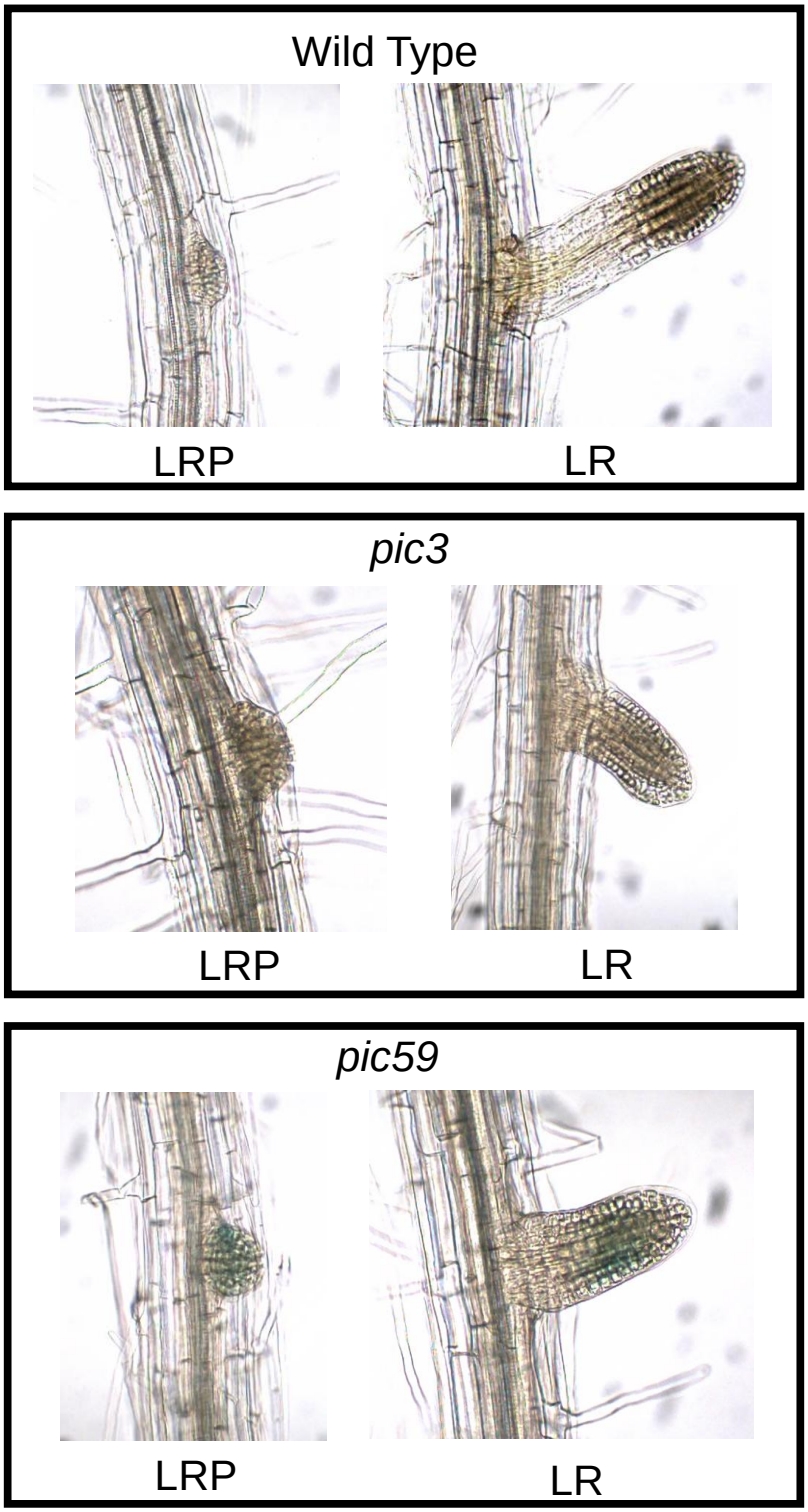


Figure 19. *CyclinB::GUS* expression in lateral root primordia and lateral root tips. Seven day old wild type, *pic3*, and *pic59* seedlings carrying *CyclinB::GUS* reporter were fixed and stained for GUS. *pic3* shows lower *CyclinB::GUS* expression and *pic59* shows higher expression, compared to the wild type.

Effect of *pic59* mutation and extended N-terminal domain of AFB5 on AFB5-Aux/IAA interaction

Members of the TIR1/AFB family are known to interact with Aux/IAAs in the presence of different auxinic compounds, the most common being IAA, 2,4-D, and 1-NAA (Kepinski and Leyser, 2005). AFB5 has been shown to interact with Aux/IAAs specifically in the presence of picloram. Because *pic3* and *pic59* are highly resistant to picloram, it was hypothesized that these mutations might affect the picloram-mediated interaction between AFB5 and Aux/IAAs.To test this, in vitro pull down assays were carried out using AFB5, PIC59, and one of the Aux/IAAs, IAA7. Here, IAA7 was expressed in bacteria as a GST-tagged protein and purified using glutathione agarose beads. Purified GST-IAA7 protein was incubated with plant-expressed AFB5-myc or PIC59-myc proteins in the presence of picloram. Results indicate that AFB5-myc but not PIC59-myc interacts with GST-IAA7 in the presence of picloram (Figure 20).

Since the extended N-terminal domain distinguishes AFB5 from TIR1/AFB1-3, it was hypothesized that this domain would be important for the picloram mediated AFB5-Aux/IAA interaction. A pull down assay using ΔAFB5-myc, which does not have the extended N-terminal domain, showed a reduced interaction with GST-IAA7 compared to AFB5-myc (Figure 20).

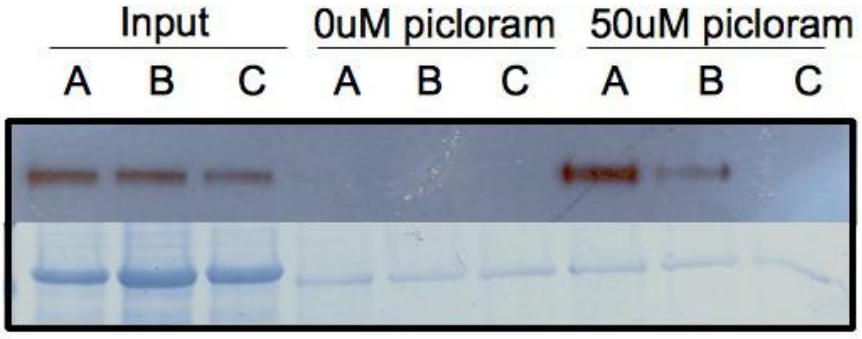


Figure 20. Mutations in *AFB5* abolish interaction between AFB5 and Aux/IAAs. Myc-tagged AFB5 (A), ΔAFB5 (B), and PIC59 (C) were incubated for 1 hour with GST-IAA7 with and without picloram. Pull-down samples were separated on SDS-PAGE gel and transferred to PVDF membrane for western blotting, using α-myc antibodies.

AFB5 may not be involved in the degradation of Aux/IAA proteins

TIR1/AFB1-3 have previously been shown to interact with Aux/IAA proteins, targeting them for degradation through the 26S proteasome. Mutations that prevent interaction between Aux/IAAs and TIR1/AFB result in accumulation of Aux/IAAs (Dharmasiri, et al., 2005b). Since PIC59 does not interact with IAA7, it was hypothesized that Aux/IAAs might be stabilized in the *pic3* and *pic59* mutant backgrounds. To test this, the *HS::AXR3NT-GUS* reporter construct was used (Gray, et al., 2001). Here, a heat-inducible promoter was used to drive the transcription of the N-terminus (domains I and II) of AXR3/IAA17, which is responsible for binding to TIR1/AFBs and subsequent degradation. The *HS::AXR3NT-GUS* reporter line was crossed into *pic3* and *pic59* (S. Dharmasiri). Rate of AXR3NT-GUS degradation was monitored following heat induction using seedlings homozygous for reporter construct and *pic3*/*pic59* mutation. Quantitative measurement of GUS activity over time showed no reasonable difference in the rate of Aux/IAA degradation between the wild type and mutants (Figure 21).

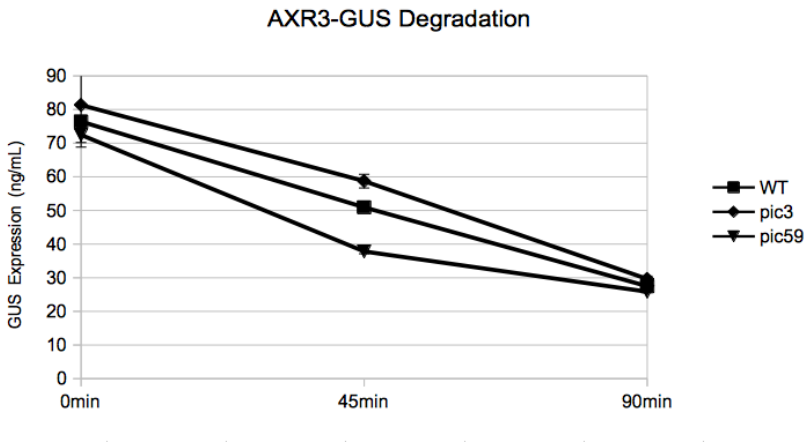


Figure 21. Degradation of AXR3NT-GUS in *afb5*. Four day old seedlings were heat shocked for 2 hours at 37°C. Heat shocked seedlings were transferred to ATS media at room temperature and samples were collected at the indicated times. GUS expression was measured using β-glucuronidase assay. Mutations in *AFB5* have little effect on Aux/IAA degradation. Error bars represent S.D., *n*=3.

Primary auxin-responsive gene expression in *afb5* mutants

Mutations in TIR1/AFB1-3 affect the expression of primary auxin-responsive genes (Dharmasiri, et al., 2005b). *afb5* mutants, therefore, may also show altered expression of primary auxin responsive genes. Based on the resistance of *AFB5* mutants to IBA but not IAA, it was hypothesized that AFB5 may regulate IBA-modulated gene expression. To test this, total RNA was isolated from seedlings treated with either IAA or IBA and cDNA was synthesized. Expression of primary auxin-responsive genes were analyzed by qRT-PCR. (Figures 22 and 23).

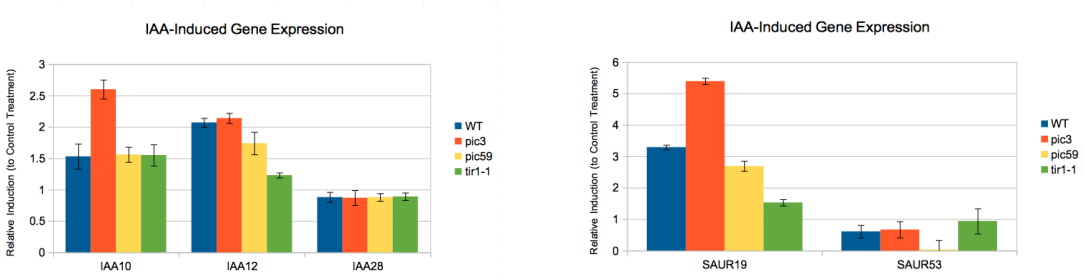


Figure 22. IAA-induced gene expression in *afb5*. Total RNA was extracted from 4 day old seedlings treated with 1µM IAA for 1 hour and cDNA was synthesized. Expression levels of IAA10, IAA12, IAA28, SAUR19, and SAUR53 was examined using qRT-PCR. Error bars represent S.D. *n*=3.

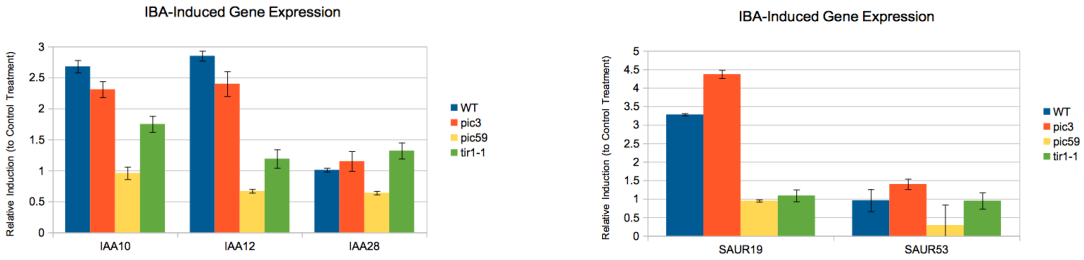
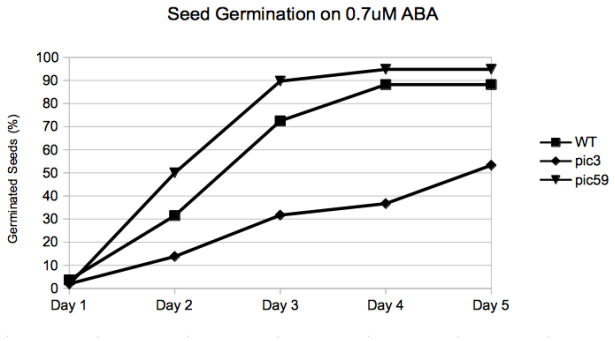


Figure 23. IBA-induced gene expression in *afb5*. Total RNA was extracted from 4 day old seedlings treated with 10µM IBA for 1 hour and cDNA was synthesized. Expression levels of IAA10, IAA12, IAA28, SAUR19, and SAUR53 was examined using qRT-PCR. Error bars represent S.D. *n*=3.

AFB5 is involved in auxin-ABA cross-talk

Mutants, such as *pic30* and *ibr5-4* were identified, along with *pic3* and *pic59*, in the screening for picloram resistance (Jayaweera, et al. 2014; Kathare, unpublished). *pic30*, *ibr5-4*, as well as *ibr5-1*, which was isolated through an IBA screening, show an altered sensitivity to abscisic acid (ABA) (Jayaweera, et al. 2014; Kathare, unpublished; Monroe-Augustus, et al. 2003). In fact, many auxin-related mutants show altered responses to ABA (Tiryaki and Staswick, 2002; Monroe-Augustus et al., 2003; Strader et al., 2008; Ephritikhine et al., 1999). Therefore, it was hypothesized that *pic3* and *pic59* would also show an altered sensitivity to ABA. Supporting the hypothesis, both mutants exhibit resistance to ABA-inhibited primary root growth compared to the wild type. Moreover, while *pic59* was resistant, *pic3* showed increased sensitivity to ABA-inhibited seed germination compared to the wild type (Figure 24).

A.



B.

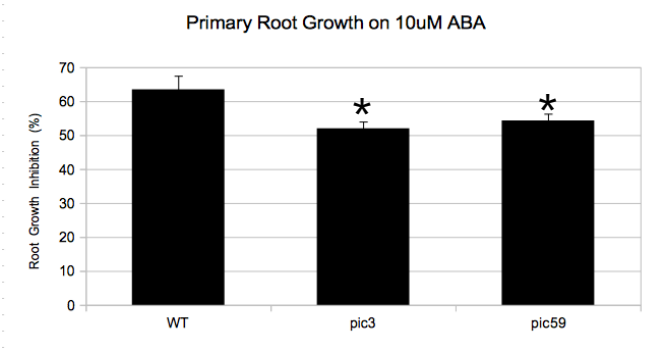


Figure 24. *afb5* displays a defective response to abscisic acid. (A) Wild type, *pic3*, and *pic59* seeds were incubated at 4°C for 4 days and plated on ATS media supplemented with 0.7µM ABA. Germinated seeds were counted every 24 hours for 5 days. Seeds were considered germinated when the radical protruded from the seed coat. *pic59* is resistant to ABA-inhibited seed germination, while *pic3* is highly sensitive. (B) Four day old seedlings were transferred to ATS media containing 10µM ABA. Primary root length was measured 4 days after transfer. Both *pic3* and *pic59* are resistant to ABA-inhibited primary root elongation. Error bars represent S.E., n=12.

ABA downregulates the expression of *AFB5*

Expression of many genes involved in ABA responses, including *IBR5* and *PIC30*, are regulated by ABA, and ABA has been shown to upregulate *TIR1* expression (Jayaweera, et al. 2014; Kathare, unpublished; Jayaweera, unpublished). Since *afb5* mutants also show defective ABA responses, the effect of ABA on *AFB5* expression was examined using the *AFB5::AFB5-GUS* translational construct as well as qRT-PCR. Histochemical and quantitative analysis shows a reduction in GUS expression levels after ABA treatment, and that this reduction was concentration dependent (Figure 25). Since the reduced AFB5-GUS expression may be due to protein degradation, seedlings were treated with the 26S proteasome inhibitor, MG132. Interestingly, MG132 does not

recover AFB5-GUS expression downregulated by ABA (Figure 26). To further confirm that the ABA-downregulated AFB5 expression is not due to protein degradation, the *35S::AFB5-myc* construct was used. A similar level of AFB5-myc was observed in the control, ABA, and combined ABA/MG132 treatments. When treated with cycloheximide to prevent further protein synthesis, the level of AFB5-myc decreased, suggesting is general degradation. However, when the seedlings were treated with ABA and cycloheximide, there was no further decrease in AFB5-myc levels when compared to cycloheximide treatment alone, suggesting that ABA does not induce AFB5 degradation (Figure 27). These results strongly suggest that ABA might transcriptionally downregulate *AFB5*. To further confirm that possibility, RT-PCR was carried out using on the *AFB5-GUS* transgene using ABA treated *AFB5::AFB5-GUS* seedlings. Results showed reduced expression of the transgene (Figure 28). In order to show that this same transcriptional downregulation occurs in the endogenous *AFB5*, qRT-PCR was performed on cDNA synthesized from wild type seedlings treated with ABA. Reduced expression of *AFB5* was observed in ABA-treated wild type seedlings, confirming the transcriptional downregulation of *AFB5* by ABA.

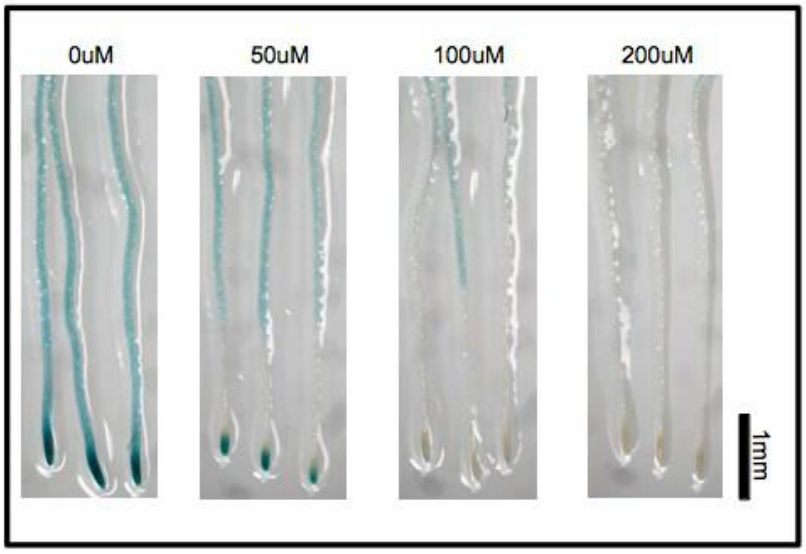


Figure 25. ABA downregulates *AFB5::AFB5-GUS* in a concentration-dependent manner. Four day old *AFB5::AFB5-GUS* seedlings were treated with increasing concentrations of ABA for 1 hour. Fixed seedlings were stained for GUS and imaged. Expression of AFB5-GUS reduces with increasing ABA concentrations.

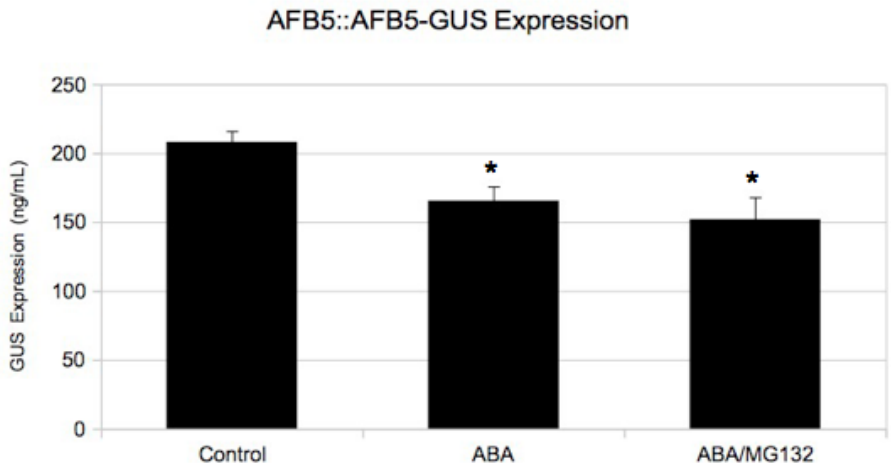


Figure 26. ABA transcriptionally downregulates *AFB5* expression. Four day old *AFB5::AFB5-GUS* seedlings treated with ABA (50µM) or ABA and MG132 (5µM) for 1 hour. Seedlings were frozen in liquid nitrogen and processed for a quantitative GUS assay. *AFB5::AFB5-GUS* expression is significantly reduced by ABA treatment and is not recovered by treatment with the 26S proteasome inhibitor, MG132. Error bars represent S.D., *n*=3.

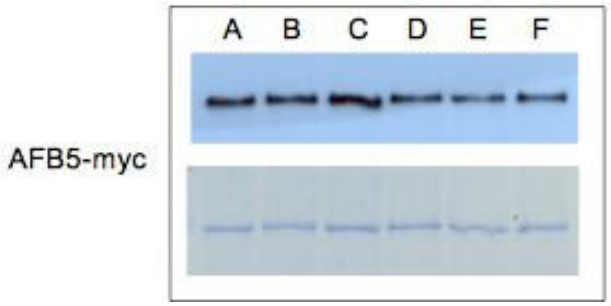


Figure 27. ABA does not promote the degradation of AFB5. Four day old *35S::AFB5-myc* seedlings were treated with mock (A),200µM ABA (B), ABA/5µM MG132 (C), 1mM cycloheximide (D), ABA/Cycloheximide (E), or ABA/MG132/Cycloheximide (F) for 1 hour. Total proteins were isolated using a denaturing buffer and separated by SDS-PAGE. AFB5-myc was detected by western blotting using anti-myc antibody.

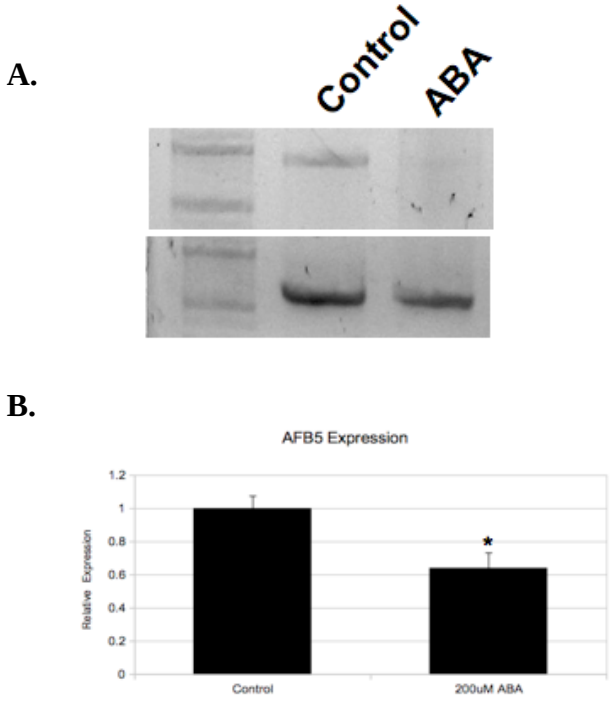


Figure 28. RT-PCR analysis of *AFB5* expression in response to ABA. Total RNA was isolated and cDNA was synthesized from 4 day old *AFB5::AFB5-GUS* (A) or wild type (B) seedlings treated with 200uM ABA. Expression of *AFB5-GUS* (A) and endogenous *AFB5* (B) were analyzed by RT-PCR and qRT-PCR, respectively. ABA treatment significantly reduces the transcription of *AFB5-GUS* as well as endogenous *AFB5*. Expression of AFB5 in panel (A) is compared to the expression of actin in the lower bands. Error bars represent S.D., n=3.

ABA enhances the degradation of unstable PIC59 protein

Degradation experiments showed that AFB5 is subjected to normal degradation. Similarly, TIR1 has also been shown to degrade through the 26S proteasome pathway (Yu, et al., 2015). Based on *pic59* mutant's semi-dominant nature-which may be due to a change in the stability of the mutant protein-and defective ABA responses, it was hypothesized that ABA may affect the stability of PIC59 protein. To test this, a degradation experiment like the one described previously with AFB5-myc, was carried out using the 35S::PIC59-myc construct. Cycloheximide drastically reduces the expression of PIC59-myc, suggesting the instability of the mutant protein. More importantly, ABA only enhances the degradation of PIC59-myc and not AFB5-myc. ABA-induced degradation of PIC59-myc can be recovered by MG132 treatment,

indicating that the protein is degraded through the 26S proteasome pathway (Figure 29).

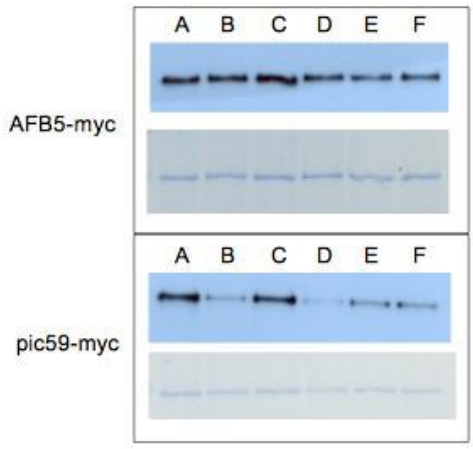


Figure 29. Relative Stability of pic59 protein compared to wild type AFB5. Four day old 3*5S::AFB5-myc* or *35S::PIC59-myc* seedlings were treated with mock (A), 200µM ABA (B), ABA/5µM MG132 (C), 1mM cycloheximide (D), ABA/cycloheximide (E), or ABA/MG132/cycloheximide (F) for 1 hour. Total proteins were isolated using a denaturing buffer and separated by SDS-PAGE. Myc-tagged proteins were detected by western blotting using anti-myc antibody.

AFB5 activity may be regulated by ROP GTPases

ROP GTPases have recently been identified as players in auxin as well as ABA signaling. Since ROP GTPases are predicted to regulate both auxin and ABA signaling, and similarly to *AFB5*, they express highly in regions of actively dividing cells such as LRP and primary root tips (Li H et al., 2001; Poraty-Gavra et al., 2013), it was hypothesized that AFB5 activity may be regulated by ROP GTPases. Two members of this family, *ROP2* and *ROP6*, have previously been shown to regulate lateral root development (Lin D et al., 2012; Li H-M et al., 2001). Additionally, data from our own lab indicates that these ROP GTPases interact with IBR5 (Lopez, unpublished), another regulator of auxin signaling which was identified in the same mutant screening for picloram and shows an altered response to ABA. This phenotypic overlap provides a foundation for a possible link between ROP GTPase and $SCF^{TIR1/AFB}$ signaling through AFB5. Based on these phenotypic and expression pattern overlaps, it was hypothesized that ROP GTPases and AFB5 are functionally connected. In order to test this, the expression of *AFB5* was first checked in the *rop2* and *rop6* mutant backgrounds using qRT-PCR. Results indicate that *AFB5* expression is significantly higher in the *rop2* and

rop6 background, supporting the hypothesis that ROP2 and ROP6 regulate AFB5 activity, and suggesting that ROP2 and ROP6 function to downregulate *AFB5* (Figure 30).

Like *AFB5*, both *ROP2* and *ROP6* have been shown to regulate lateral root development. Based on this it was hypothesized that these ROP GTPases regulate this development through *AFB5*. In order to check the functional interaction between ROP2/6 and AFB5 in LR development, *rop2* and *rop6* were crossed into *pic3* and *pic59* to produce double mutants and homozygous mutants were selected by picloram resistance and genotyping. Double mutants were grown under long day conditions for 10 days and lateral root density was compared between the wild type, single, and double mutants. The experiment, however, failed to produce any discernible results, due to unknown reasons.

Both ROP2 and ROP6 are known to regulate cell division, and *AFB5* as well as *ROP2/6* express in overlapping regions of the primary root. Moreover, ROP2 and ROP6 directly or indirectly regulate *AFB5* expression. It was therefore hypothesized that AFB5 may functionally interact with ROP2 and/or ROP6 to regulate primary root growth. To test this hypothesis, the primary root length of double mutants was compared to the wild type and single mutants (Figures 31 and 32). Results indicated that *rop2pic3 and rop6pic3* double mutants show an intermediate phenotype. Both *rop2pic59* and *rop6pic59* double mutants, however, show significantly shorter primary roots than either single mutants and the wild type.

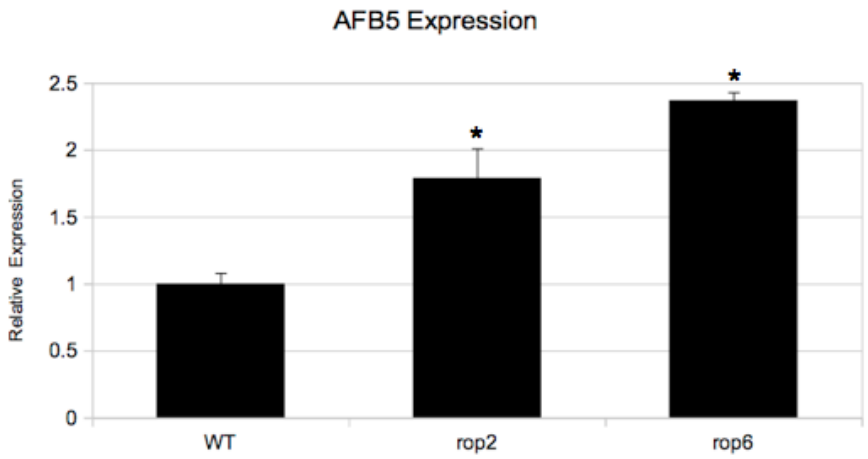


Figure 30. *AFB5* expression in the *rop2* and *rop6* mutant backgrounds. Total RNA was isolated from 4 day old *rop2*, *rop6*, and wild type seedlings and cDNA was synthesized. QRT-PCR analysis shows that *AFB5* expression is significantly higher in the *rop2* and *rop6* mutant background. Error bars represent S.D. *n*=3.

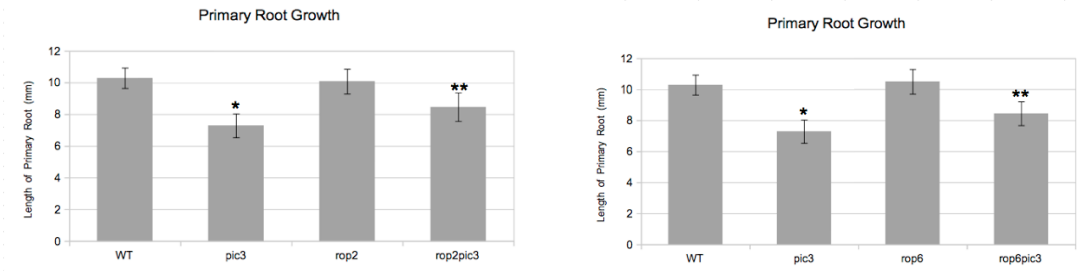


Figure 31. Primary root growth of *pic3* and *rop2*/*rop6* double mutants. Seedlings were grown for 4 days on ATS media and imaged. Primary root length was measured using ImageJ. *pic3* shows a significantly shorter primary root, and *rop2* and *rop6* show no difference from the wild type. Double mutants display an intermediate phenotype. A significant difference from the wild type is represented with (*) and a significant difference from the wild type and single mutants is represented as (**) (P<0.001). Error bars represent S.D. *n*=27.

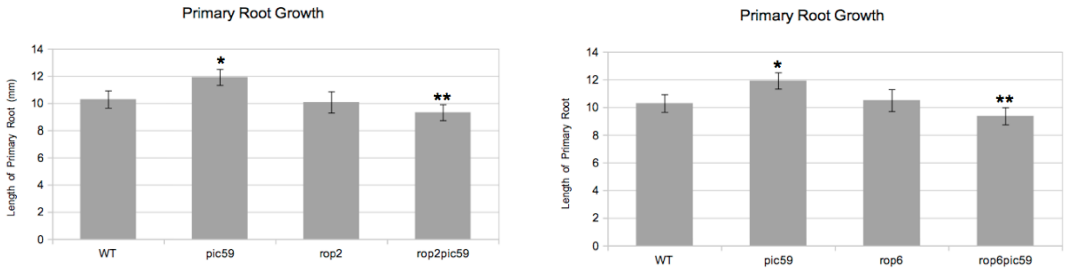


Figure 32. Primary root growth in *pic59* and *rop2*/*rop6* double mutants. Seedlings were grown for 4 days on ATS media and imaged. Primary root length was measured using ImageJ. *pic59* shows a significantly longer primary root, and *rop2* and *rop6* show no difference from the wild type. Double mutants display a shorter primary root than either single mutants or the wild type. A significant difference from the wild type is represented with (*) and a significant difference from the wild type and single mutants is represented as (**) ($P<0.001$). Error bars represent S.D. $n=27$.

IV. DISCUSSION

Auxin, as one of the most pivotal plant hormones, programs plant growth and development at the cellular, tissue, and organ levels. These developmental programs are often modulated by functional interactions between environmental cues and other phytohormones, such as abscisic acid and cytokinin. After nearly 80 years of research into the synthesis, transport, and perception of auxin within the cell, we are now beginning to understand this hormone's complex molecular mechanisms. At the cellular level, auxin induces both genomic and non-genomic responses. The most understood genomic responses are primarily through the $SCF^{TIR1/AFB}$ complex, in which TIR1/AFB functions as auxin co-receptors. Through this pathway, auxin mediates the interactions between TIR1/AFBs and a group of repressor proteins known as Aux/IAAs, targeting Aux/IAAs for degradation, and relieving their repression of auxin responsive transcription factors (ARFs). Because auxin is responsible for a vast number of developmental programs, the effect of auxin must be tightly regulated. It is believed that this regulation is partially achieved through the variety of possible combinations of auxin-signaling F-box proteins, Aux/IAAs, and ARFs. In addition to this, many other hormones are capable of modulating auxin responses, in what is referred to as hormonal cross-talk. Cytokinin, for example, acts antagonistically to auxin in lateral root development and in the establishment of the apical meristem by altering auxin transport, and thus disrupting the effect of auxin's concentration gradient (Su, et al., 2011; Bishopp, et al., 2011). It has also been shown that ethylene and auxin regulate the biosynthesis of each other and as a result of this, ethylene can impact root cell elongation by upregulating auxin biosynthesis (Swarup et al., 2007; Stepanova et al., 2007). Some of the most well documented accounts of hormonal cross-talk are between auxin and the stress-associated hormone, abscisic acid (ABA), and many auxin mutants are defective in ABA perception and response (Tiryaki and Staswick, 2002; Monroe-Augustus et al., 2003; Strader et al., 2008; Ephritikhine et al., 1999).

Despite the strides that have been made in auxin research, many gaps exist in our knowledge of the regulatory mechanisms of auxin-programmed development. Thus, to identify novel genes involved in auxin transport and perception, a forward genetic screen

was performed using picloram, a synthetic auxin often used as herbicide. In this screening, multiple alleles of auxin-signaling F-box protein 5 (*AFB5*) were uncovered, including *pic3* and *pic59*. Because little work has been done previously to characterize the molecular action and developmental function of AFB5, this study was primarily focused on characterizing the functional role of AFB5 in auxin signaling and in plant growth and development.

pic3 and *pic59* mutations introduce amino acid changes

Genetic mapping and sequencing identified two point mutations in *pic3* conferring an arginine to glutamine amino acid change at position 90 and an aspartic acid to asparagine at position 126 (S. Dharmasiri, unpublished). The SCF complex consists of an F-box protein and ASK1 bound to the scaffolding protein, Cullin1. Since both mutations in *pic3* are likely to be within the F-box region, the domain responsible for interaction with the SCF complex, *pic3* may have a lower or abolished interaction with ASK1, the adaptor protein which links the F-box to the complex. *pic59* contains a single base pair substitution, resulting in a cysteine to tyrosine amino acid change at position 562. Because this mutation is in the leucine-rich repeat domain, the region which interacts with Aux/IAAs, it is possible that PIC59 protein still interacts with ASK1, but has a reduced interaction with Aux/IAA proteins. Because the nature of the mutations are essentially different between *pic3* and *pic59*, it was not surprising that these mutants often exhibited phenotypes opposite to each other, such as lateral root density and primary root length.

Picloram is known to promote interactions between TIR1/AFB and Aux/IAA proteins similarly to other auxins, however its ability to promote TIR1-Aux/IAA interactions is weaker than that of IAA (Calderon Villalobos et al., 2012). On the contrary, picloram is more efficient than IAA at promoting AFB5-Aux/IAA interactions, and it is believed that AFB4 and/or AFB5 are its major targets. (Greenham et al., 2011). Since *pic59* was identified in a screening for picloram, a PIC59-myc construct was used to examine the effect of the mutation on Aux/IAA interaction in the presence of picloram. Only AFB5-myc, but not PIC59-myc interacted with IAA7 in the presence of picloram. This indicates that the mutation in *pic59's* LRR-domain has a severe effect on the

protein's ability to interact with Aux/IAAs. AFB5-myc, even when treated with the translation inhibitor, cycloheximide, showed reasonable stability. PIC59-myc, however, degraded rapidly when treated with cycloheximide, indicating that the mutation in *pic59* also renders the protein highly unstable. This effect could result in significantly lower endogenous levels of the protein, essentially making *pic59* a *knockdown* mutant.

Since AFB5 contains an extended N-terminus compared to TIR1, the interaction with GST-IAA7 in the presence of picloram was tested with ΔAFB5-myc, which has a deleted N-terminus. ΔAFB5-myc showed a significantly reduced interaction compared to that of AFB5-myc. It is possible that this N-terminal extension serves a role in AFB5's picloram affinity, but it is more likely that this deletion results in a partially misfolded protein.

AFB5 may not function in Aux/IAA degradation

Since the known primary role of TIR1/AFBs is to mediate Aux/IAA degradation, and mutations in these F-box proteins result in stabilization of Aux/IAAs, it was hypothesized that mutations in *AFB5* would also stabilize Aux/IAAs. This hypothesis was tested using the heat shock-inducible reporter line AXR3NT-GUS, and results show that mutations in *AFB5* have little to no effect on Aux/IAA degradation. This data is supported by a recent finding that neither AFB4 nor AFB5 could mediate degradation of any Aux/IAA (Shimizu-Mitao and Kakimoto, 2014). These data together suggest that AFB5 regulates auxin responses independently of Aux/IAA degradation and that the molecular mechanism of AFB5 may be partially or completely different from TIR1.

pic3 and *pic59* display selective resistance to auxin

Because many auxin-related mutants show resistance to IAA-inhibited primary root growth, and because *pic3* and *pic59* show significant resistance to the synthetic auxin picloram, it was hypothesized that *pic3* and *pic59* might also be resistant to IAA. Results, however, show that both mutants have a wild type sensitivity to IAA in both primary root elongation and lateral root induction, suggesting that AFB5 has little to no role in IAA-mediated signaling. On the contrary, both *pic3* and *pic59* were significantly resistant to another natural auxin found in Arabidopsis, IBA, in both primary root

elongation and lateral root induction.

Since auxin is primarily known to induce expression of early auxin-responsive genes, and that mutations in TIR1/AFB1-3 tend to impair this response, it was hypothesized that *afb5* might also be impaired in auxin-induced gene expression. Highly auxin-inducible genes *IAA10*, *IAA12*, and *SAUR19* as well as two genes that are not auxin-inducible, *IAA28* and *SAUR53* (Rogg et al., 2001; Kathare, unpublished) were used to test the above hypothesis. Induction of *IAA10*, *IAA12*, and *SAUR19* by IAA, but not *IAA28* or *SAUR53* in wild type indicated the accuracy of the assay. Results showed a slight impairment in IAA-induced gene expression in *pic59*, however nothing to the extent of that seen in *tir1-1*. *pic3*, however, showed even more of an increase in gene expression than the wild type, suggesting that the nature of the mutations in *pic3* enhance auxin-induced gene expression.

These results further confirm that AFB5 behaves differently from TIR1/AFB1-3 in inducing primary auxin-responsive genes. Since *pic3* might not interact with the SCF complex, high expression of genes in *pic3* might be due to the increased availability of SCF complexes to TIR1/AFB1-3 in this mutant background. On the other hand, PIC59 may sequester the SCF complex without rendering Aux/IAA degradation, hence the availability of SCF complexes to TIR1/AFB1-3 may be less in the *pic59* background. However, due to the normal degradation of AXR3-GUS in the *pic3* and *pic59* backgrounds and the relative instability of PIC59 protein, this seems unlikely. More research into the structural changes as a result of the *pic3* and *pic59* mutations may provide more clarity into how these mutant proteins affect overall SCF activity, if at all. Furthermore, these results suggest that primary auxin-responsive genes may be induced by a novel mechanism alternative to Aux/IAA degradation, and that AFB5 could play a role in this.

Although it has not previously been shown that IBA induces primary auxin-responsive gene expression, it was hypothesized that, based on *pic3* and *pic59*'s selective resistance to IBA-if IBA induces primary auxin-responsive gene expression-both mutants might show defects in IBA-induced gene expression. IBA induces tested primary auxin-responsive gene expression. In fact, IBA was more efficient than IAA at inducing gene expression, however this could be due to the higher concentration of IBA that was used.

Results indicate that both mutants are impaired in IBA-induced gene expression, but the most dramatic difference was seen in *pic59*. Since IBA is converted to IAA in the plant, it is possible that the observed gene induction is due to IAA rather than IBA. However, since pic3 and pic59 did not respond in the same manner to IBA as IAA, it is more likely that AFB5 is involved in IBA signaling rather than IAA.

Several other mutants identified in the picloram screen are also IBA resistant, suggesting that IBA may be part of a novel signaling pathway and, as an auxin receptor, AFB5 might play a central role in this cascade. Although the status of IAA-independent IBA signaling is controversial, alternatives have been proposed to explain the effects of IBA on growth. One such alternative is that IBA may modulate IAA responses, resulting in certain developmental programs which are IBA-dependent (Ludwig-Muller et al., 2005; van der Krieken et al., 1993, 1994).

AFB5 regulates cell division

No obvious phenotypic differences can be observed between *afb5* and the wild type. Upon closer inspection, however, it was revealed that both mutants showed small, albeit consistent, differences in primary root growth and lateral root density, and that this difference is apparent throughout early seedling development. In both cases, these differences are observed as early as 4 days after germination, suggesting that a developmental event regulated by AFB5 may occur at this stage, and the differences persist as the seedling develops, possibly because the roots cannot recover from this initial stunting or acceleration of growth. Because *pic3* displays a shorter primary root and *pic59* displays a longer primary root compared to the wild type, it was hypothesized that these differences could be due to either defects in the rate of germination, cell elongation, or cell division. Neither mutant shows a significant difference in the rate of germination or the length of the root cortical cells, indicating that the difference may be due to cell division instead. This was tested using the *CyclinB::GUS* cell division marker. *pic3* shows a slight, albeit significant, decrease in *CyclinB::GUS* expression in the primary root tip, suggesting a lower rate of cell division in this mutant background. In addition to this, *pic59* shows a significant increase in *CyclinB::GUS* expression, indicating a higher rate of cell division. Therefore, the differences observed in primary

root length may be due to impaired cell division in *afb5* mutants. This result also suggests that AFB5 is involved in regulation of cell division.

AFB5 may regulate lateral root growth

Auxin regulates lateral root development by coordinating several different processes. Initially, auxin programs cells in the basal meristem to eventually develop into lateral root founder cells, specific cells along the pericycle which give rise to lateral roots. Auxin also helps to initiate the asymmetric division of these lateral root founder cells to give rise to the first germ layer of lateral root primordia, and then promotes the division of these lateral root primordia cells as they penetrate the cortex and epidermis of the primary root (Peret et al., 2009). According to *AFB5::AFB5-GUS* expression, AFB5 expresses highly in lateral root primordia, during all stages of development, indicating that AFB5 may function in lateral root development. Because both *pic3* and *pic59* showed defects in lateral root density, it was initially hypothesized that these defects arose from the initiation of lateral roots. No significant difference, however, was observed in the density of lateral root primordia compared to the wild type, suggesting that both mutants function normally in lateral root initiation. When the percentage of lateral root primordia which had reached a "late" stage (3 cell layers or more) was examined in *pic3*, *pic59,* and the wild type, it was observed that a much lower percentage of *pic3* LRP and a much higher percentage of *pic59* LRP had reached a late stage, compared to the wild type. Thus, it was hypothesized that the differences observed in lateral root density were due to differences in the rate of growth of LRP and LR. The average length of the longest lateral root was significantly shorter in *pic3* and significantly longer in *pic59,* indicating that this difference may indeed be due to growth rate. Using the *CyclinB::GUS* lines described previously, GUS expression was observed in the *pic3*, *pic59,* and wild type LRP and newly emerged LR. No GUS expression was detected in *pic3* LRP and a lower expression was observed in the emerged lateral roots compared to the wild type, indicating a lower rate of cell division in *pic3* lateral roots. On the contrary, a much higher level of GUS expression was observed in the LRP and LR of *pic59,* indicating a higher rate of cell division compared to the wild type. Based on this data, the hypothesis that the differences in LR density of *pic3* and *pic59* is due to an

decrease and increase in cell division, respectively, was supported.

AFB5 expression is regulated by ABA

Since auxin and ABA are known to engage in hormonal cross-talk, and because many auxin mutants show defects in their response to ABA, ABA response in *pic3* and *pic59* was examined. Both mutants are resistant to ABA in primary root growth, however *pic3* is hypersensitive and *pic59* is resistant to ABA-inhibited seed germination. In accordance with the above results, ABA downregulated AFB5 expression indicated by the *AFB5::AFB5-GUS* reporter. The inability of proteasome inhibitor, MG132, to recover the downregulation of AFB5, as well as lower transcript levels of *AFB5-GUS* in ABA-treated seedlings, suggested that ABA regulates AFB5 at the transcriptional level. Additionally, qRT-PCR analysis of AFB5 transcripts in wild type seedlings after ABA treatment also showed a significant reduction, confirming the above results. Moreover, AFB5-myc protein, which was expressed under the control of the CaMV 35S promoter was not downregulated by ABA. Altogether, this indicates that ABA transcriptionally downregulates *AFB5*, and as the AFB5 promoter region contains putative ABA-responsive elements, this regulation may occur via the binding of one or more ABA-regulated transcription factors. Interestingly, PIC59-myc showed rapid degradation following ABA treatment, and this degradation was recovered by treatment with MG132, indicating that although ABA transcriptionally regulates native *AFB5*, it is also capable of promoting degradation of PIC59 protein. Since the wild type protein is not subjected to ABA-promoted degradation, it is more likely that degradation of PIC59 is an indirect effect and further experiments are required to confirm this possibility. Contrary to what was expected, simultaneous treatment with ABA and cycloheximide did not result in further degradation, but seemed to partially recover the expression. This could be because cycloheximide inhibits the translation of another protein necessary for this degradation.

AFB5 activity may be regulated by ROP GTPases

ROP2 and ROP6 are members of the Rho of plants (ROP) GTPases, a family of small GTPases which function as signaling switches ((Nibau et al., 2006; Etienne-Manneville and Hall, 2002). Both *ROP2* and *ROP6* are induced by auxin, and both have

been shown to be inactivated by ABA, placing them in the center of hormonal cross-talk (Tao L-Z et al., 2002; Tao L-Z et al., 2005). Additionally, both have been shown to interact with IBR5 (Lopez, unpublished), a regulator of auxin signaling which also shows altered response to IBA. *ROP2* and *ROP6* are known to regulate lateral root development and express in regions of actively dividing cells ((Lin D et al., 2012; Li H-M et al., 2001; Poraty-Gavra et al., 2013). Based on this expression and phenotypic similarity, and the fact that both AFB5 and ROP2/ROP6 function in Auxin and ABA cross-talk, it was hypothesized that AFB5 may functionally interact with ROP2 and/or ROP6. Basal expression of *AFB5* is significantly higher in both *rop2* and *rop6*, suggesting that these GTPases function to downregulate *AFB5*. *rop2* and *rop6* did not display any obvious phenotype. This may be due to their functional redundancy, however when combined with *afb5*, their root lengths did change. Although *rop2pic3* and r*op6pic3* double mutants only displayed an intermediate primary root length, *rop2pic59* and *rop6pic59* double mutants showed a significantly shorter primary root when compared to corresponding single mutants and wild type. Similar to most other phenotypes, *pic3* and *pic59* show opposite functional interactions with *rop2* and *rop6*. Since *afb5* expression in the *rop2/6* background is higher than wild type, it can be assumed that expression of the *afb5* mutant proteins are also higher in the double mutant background. Therefore, increased expression of *pic59* may negatively regulate primary root growth. Multiple attempts were made to examine lateral root density in the *afb5* and *rop2*/*rop6* double mutants, however no discernible results were produced. Since this was the first time these growth conditions and media composition (0.6% MS, 2% sucrose, pH5.9) were used, more fine tuning will be needed in order to carry out this experiment successfully.

Conclusions and further directions

In this study, *pic3* and *pic59* were shown to have a selective resistance to picloram and IBA, but wild type sensitivity to IAA. Additionally, it appears that AFB5 does not significantly mediate Aux/IAA degradation, however, both *pic3* and *pic59* still show defects in IAA and IBA-induced gene expression. This raises the possibility that AFB5 regulates auxin signaling independently of Aux/IAA degradation, perhaps via an IBA-dependent mechanism. Because several mutants identified in the picloram screening also

show resistance to IBA, it is likely that these proteins participate in a common signaling pathway, however further research is required to establish this link. It was very recently shown that TIR1 naturally degrades and that mutations in *tir1* which prevent its binding to the SCF complex render the protein stable (Yu et al., 2015). This may be the result of TIR1 degrading along with the Aux/IAA protein that it is bound to. Since AFB5 shows only a small amount of degradation compared to TIR1, it is possible that AFB5 does not bind with Aux/IAAs, but rather occupies SCF complexes for the purpose of inhibiting Aux/IAA degradation. Mutations in *pic3* are likely to inhibit the protein from binding to ASK1, allowing other F-box proteins to occupy SCF complexes, and thus resulting in an increase in auxin response. It would be of value in the future to test the interaction between *pic3* and ASK1.

In addition to exploring the implications of the *pic3* mutations, a better understanding of the *pic59* mutation might provide additional insight into the molecular action of AFB5. Since a single amino acid substitution in the LRR region of AFB5 is enough to confer resistance to several compounds, as well as affect the stability of the protein, it is likely that this amino acid residue is of importance to the protein's structure and/or function. Cysteine residues are capable of undergoing several types of post-translational modifications (PTM), such as S-nitrosylation or sulfhydration, and are capable of forming disulfide bonds with other Cys residues. It is possible that changing this cysteine residue results in structural changes to the protein, effectively causing a misfolding in the tertiary structure. Since misfolded proteins are often recycled via the 26S proteasome, this may explain why *pic59*'s mutation renders the protein unstable. By the same token, introducing a tyrosine residue, which is also a target for PTM, may introduce unnatural PTMs to AFB5, again resulting in a misfolded or functionally impaired protein. Future work should explore the possible PTM of AFB5, and Cys562 seems to be a good place to start.

Both AFB4 and AFB5 contain an extended N-terminus, the function of which has not been elucidated. Phylogenetic analysis (Parry, et al, 2009) suggests that it is possible that the ancestral AFB also contained this extension, and that the domain could have been *lost* in the TIR1/AFB2 lineage, a more probable scenario than AFB4 and AFB5 *adopting* additional amino acids. This could indicate that the extension present in AFB4 and AFB5

is vestigial and serves no additional function. However, if this domain is indeed functional, it may be valuable to employ a genome-wide protein interaction study, such as a yeast two-hybrid assay on AFB5 and ΔAFB5 to find interacting proteins and to identify any proteins which interact with AFB5, but not ΔAFB5. Aside from this N-terminal extension, another major structural difference is present between TIR1 and AFB5. In the auxin binding pocket of TIR1, two amino acid residues are necessary for binding to IAA, Arginine403 and Serine438 (Tan et al., 2007). In AFB5, only the arginine residue is present, with an alanine in place of the serine. The auxin binding pocket of TIR1 has been shown to have the ability to bind a variety of auxinic compounds, such as 2,4-D, picloram, and 1-NAA (Tan et al., 2007). Thus, this structural difference in AFB5 may confer a lower affinity for IAA without affecting the binding of other auxinic molecules. This could be one explanation for why AFB5 binds tightly to picloram and weakly to IAA. In the future, changing this alanine residue in AFB5 to a serine may help to reveal if this difference in the auxin binding pocket plays a role in AFB5's specific activity.

In this study, it was revealed that AFB5 participates in auxin and ABA cross-talk and that *AFB5* is transcriptionally downregulated by ABA. Since *afb5* shows no obvious phenotypic differences from the wild type, exploring the functional relevance of this hormonal regulation may help to uncover AFB5's role in plant growth and development. In addition to ABA, NaCl was also shown to downregulate *AFB5* (data not shown). However, concomitant treatment with NDGA, an ABA biosynthesis inhibitor, did not recover *AFB5::AFB5-GUS* expression, indicating that the effect NaCl has on AFB5 is ABA-independent. Since NaCl and ABA are both known to induce the production of reactive oxygen species (ROS), what is seen may still be a common effect between NaCl and ABA, albeit independently of each other. Additional research into the role of ROS in AFB5 signaling may help to reveal more functions for this F-box protein.

One of the findings of this study was that AFB5 regulates the rate of cell division in roots, resulting in a primary root and lateral root phenotype. *ROP2* and *ROP6*, two small GTPases known to mediate auxin and ABA cross-talk have also been shown to express highly in actively dividing tissues, and both *rop2* and *rop6* exhibit altered lateral root densities. It was therefore hypothesized that AFB5 may have a functional interaction with ROP2 and/or ROP6. Neither *rop2* nor *rop6*, however, display a phenotypic

difference from the wild type when grown under continuous illumination, suggesting that the function of these proteins is at least partially light-regulated. Both *rop2* and *rop6* showed a functional interaction with *pic59* in primary root growth, begging the question of whether AFB5 activity is also light-regulated. It is possible that the absence of phenotypic difference observed in *afb5* is due to a lack of light regulation rather than a lack of function. In future work on AFB5, it will be important to explore this possibility.

Picloram, a synthetic auxin commonly used as an herbicide, selectively targets broadleaf (dicot) plants. Because of this, the use of picloram is limited to grass crops, such as corn and small grains. Because *afb5* shows no obvious defects in growth and development, and both seedlings and adult plants are resistant to the application of picloram, AFB5 may prove to be a useful target in engineering picloram resistance in dicot crops. This could expand the use of picloram to a variety of crop plants. Additionally, AFB5 has been shown to function in lateral root growth, which is one important factor in determining drought tolerance (Gowda et al., 2011). Research indicates that protection against dehydration may result in an increase in lateral root growth (Ludwig-Muller et al., 2005). In this study, AFB5 was shown to be regulated by ABA, a hormone best known for it's role in abiotic stress tolerance. AFB5 also seems to participate in IBA signaling and previous work indicates that IBA synthesis is increased under drought stress (Ludwig-Muller et al., 1995). Based on the data presented here, future work on AFB5 might focus on it's possible role in drought-stress tolerance.

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