IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF TWO NOVEL *ARABIDOPSIS* MUTANTS THAT ARE RESISTANT TO AUXIN

THESIS

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ABSTRACT

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Auxin is a pivotal plant hormone that plays a major role in plant growth and development. While indole-3-acetic acid (IAA) is the major natural auxin found in plants, there are many synthetic chemicals with auxinic activity. 1-Naphthylacetic acid (1-NAA), 2, 4-dichlorophenoxyacetic acid (2, 4-D) and picloram are some examples of synthetic auxins. Of these, 2, 4-D and picloram are commonly used as herbicides in agriculture. While both natural and synthetic chemicals exhibit similar effects on plants, these chemicals are structurally different. Whether these structurally

different chemicals function through similar molecular mechanisms is not known. Initial work in our laboratory indicates that picloram may function differently from other commonly known auxins. To identify genes that are associated with the picloram response, we used a forward genetics approach to isolate ethyl methanesulfonate (EMS) mutagenized *Arabidopsis* mutants (pic mutants) that were resistant to picloram.

The objective of the research project was to characterize the *Arabidopsis* mutants *pic59* and *pic115* which exhibit altered response to auxin and to isolate the mutant genes by map-based cloning. Both *pic59* and *pic115* mutants are morphologically very similar to the wild type Col-0 plants with the exception of the short siliques in mutants. The primary root growth of *pic59* exhibits differential responses to different auxins. While *pic59* is highly resistant to picloram and indole butyric acid (IBA) and slightly resistant to 2, 4-D, it is sensitive to IAA. *pic115* is slightly resistant to IAA but sensitive to IBA. The primary root growth of both *pic59* and *pic115* is resistant to the plant stress hormone abscisic acid (ABA), but is sensitive to the ethylene precursor ACC and to cytokinin (kinetin). Expression of *DR5.·GFP* transgene in response to different auxins is impaired in *pic59* and *pic115* backgrounds, suggesting that auxin-dependent gene transcription is affected in both these mutants. Degradation of *HS::AXR3NT-GUS* reporter gene is accelerated in both mutant backgrounds compared to wild type. This result differs from the *AXR3NT-GUS* degradation pattern of most previously known auxin response mutants and suggests that *pic59* and *pic115* may be involved in a novel auxin regulatory mechanism.

By using a positional cloning technique, the *pic59* mutant gene was mapped to a region of fourteen genes in the south arm of chromosome V. As there are no

currently known auxin-related genes in this genetic window, *pic59* represents a newly discovered gene associated with both auxin and ABA responses in *Arabidopsis*. The *pic115* mutant gene was mapped to the north arm of chromosome II between the annotation units T23O15 and F28I8. *IBR5*, a gene encoding a dual specificity phosphatase (DSP) is located in this genetic window. Sequencing of this candidate gene revealed a single base pair change in the *IBR5* gene, creating a missense mutation in the highly conserved dual specificity active site motif of the *IBR5* gene. A *PIC115::PIC115-GUS* construct revealed that the DSP enzyme is localized to areas of cell elongation. IBR5 is known to dephosphorylate mitogen activated protein kinase12 (MPK12) in *Arabidopsis*. These results along with results from other laboratories suggest a strong connection between auxin signaling and MAP kinase pathway in plants. It is also possible that *PIC59* encodes a protein that functions as the substrate for MPK12 or a protein closely associated with the MPK12 function.

CHAPTER I

INTRODUCTION

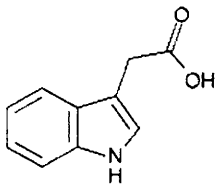
Plants grow and develop in response to many environmental and endogenous signals. The environmental and endogenous cues together with the plant's genetic makeup determine the ultimate form the plant will take. Chemicals that regulate plant growth and development are plant growth regulators collectively called plant hormones, or phytohormones (Gray, 2004). Generally, plant hormones, unlike animal hormones, are simple in structure and can function in the tissues in which they are produced as well as in distant tissues. The most common plant hormones are auxin, gibberellic acid, cytokinin, ethylene, abscisic acid, brassinosteroid, jasmonic acid, salicylic acid and systemin.

The concept of a chemical substance that could affect a plant's response to an external stimulus was first proposed by Charles Darwin in his book *The Power of Movement of Plants* in 1880. He stated "when seedlings are freely exposed to a lateral light some influence is transmitted from the upper to the lower part, causing the latter to bend" (Darwin, 1880). This fascinating observation led scientists in the early 1900's to further experiment on this "influence [that] is transmitted" which resulted in the discovery of the first plant hormone, auxin. Auxin, which means "to grow" in Greek, affects every aspect of plant growth and development. Some aspects of growth influenced by auxin given in current literature include embryogenesis, apical dominance, flower development, fruit development, root initiation, lateral root initiation, phototropism, gravitropism and vascular differentiation (Davies, 1995).

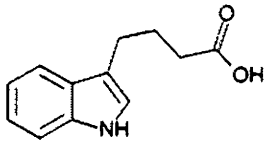
These developmental programs are primarily controlled through regulation of cell division, expansion and differentiation.

Auxinic compounds are chemically diverse but share a planar, unsaturated ring and a side chain with a carboxyl group (Jonsson, 1961). Despite efforts to develop hypotheses about the determinants of auxinic activity, how these variable chemicals elicit a common phytohormone response remains unclear (Kaethner, 1977; Farrimond *et al.,* 1978). The most abundant naturally occurring auxin in plants is indole-3-acetic acid (IAA) (Koegl, 1999). In addition to IAA there are several naturally occurring auxins. These include indole-3-butyric acid (IBA), phenyl acetic acid (PAA) and 4-chloro indole acetic acid. The use of auxin in agriculture, particularly as herbicides (Cobb, 1992; Grussmann, 2000), has led to the development of synthetic chemicals that elicit the same effects as auxins once inside the plant. Some of the auxinic herbicides are grouped as chlorophenoxy acids, benzoic acids, pyridines and quinoline carboxylic acids. Examples for synthetic plant growth regulators with auxinic activity are 1-napthalene acetic acid (NAA), 2, 4-dichlorophenoxy acetic acid (2, 4-D), picloram, dicamba, quinmerac and quinclorac (Figure 1).

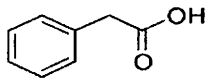
Naturally ocuuring auxins



IAA

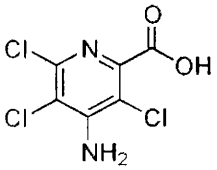


IBA

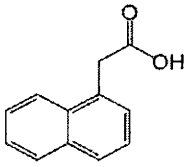


Phenylacetic acid (PAA)

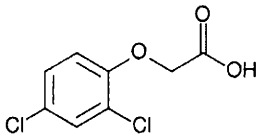
Synthtic auxins



Picloram



1-NAA



2,4-D

Figure 1. Chemical Structures of Natural and Synthetic Auxins. Auxinic compounds vary greatly in structure. IAA is the most abundant natural auxin found in plants. Most auxins are widely used as herbicides, thus some auxins are synthesized for use in industry.

Auxins are mostly synthesized in young leaves (Ljung *et al.,* 2001), and in addition they are synthesized in mature leaves, cotyledons and roots. Biosynthesis of auxins occurs in tryptophan (Trp)-dependent and Trp-independent pathways. Recent studies show that there is a previously uncharacterized auxin biosynthetic pathway that is rapidly deployed to synthesize auxin at the high levels required to initiate the multiple changes in the plant during shade avoidance (Toa *et al.,* 2008; Stepanova *et al* , 2008). Once synthesized, auxin transport is highly regulated as proper transport of auxin to the sites of its function is needed for proper plant growth. Several mutants with impaired polar auxin transport have been identified. Among the earliest identified genes is *AUX1*, which mediates influx of auxin into the cell (Marchant *et al.,* 1999). Auxin efflux is mediated by a family of proteins known as PIN proteins

(Okada *et al*, 1991). In addition, several multidrug resistance–like proteins are also known to be involved in auxin transport (Noh *et al*, 2001).

In the 1980's scientists found that at the molecular level, auxin can influence gene expression by regulating the transcription of many genes (Woodward *et al*, 2005). This led scientists to employ molecular genetic tools to elucidate the molecular mechanisms of auxin perception and signal transduction. Although significant advances have been made in this area of research, there are still many unanswered questions and unknown mechanisms. Our current understanding of the molecular mechanisms involved in auxin's action has been mainly facilitated by identification and characterization of *Arabidopsis* mutants that exhibit altered response to auxin, and this approach still remains the method of choice in elucidating novel mechanisms.

Current Model for Auxin Signaling

An early observation made was that in the presence of auxin, expression of certain genes is rapidly and specifically increased. Most of these genes belong to three well characterized gene families, *SAUR*s (Small Auxin-Up RNAs), *GH3*s (GretchenHagen3) and *Aux/IAA*s (Auxin/Indole acetic acid-binding proteins). The Aux/IAAs encode short-lived transcription repressors that regulate auxin-mediated gene expression by interacting with transcriptional factors that belong to the auxin response factor family of proteins (ARF). There are 29 Aux/IAAs and 23 ARFs in *Arabidopsis* (Overvoorde *et al.*, 2005; Okushima *et al.*, 2005) Most Aux/IAAs share four conserved domains, domain I, II, III and IV. The C-terminal domain III and IV are also found in ARFs. These domains mediate Aux/IAA-ARF heterodimerization and homodimerization. Heterodimerization between Aux/IAAs and ARFs represses transcriptional activation of auxin response genes (Tiwari *et al.*, 2003). ARFs contain

a conserved amino terminal (N-terminal) domain that facilitates the interaction with auxin-responsive elements (AuxRE) found in the promoter region of auxin-responsive genes.

The central regulator of the auxin signaling is composed of Skp1, Cullin (CUL1), an F-box protein and the small RING protein Rbx1 (RING-BOX 1). This complex of proteins is collectively referred to as SCF. SCFs are the largest family of ubiquitin ligases in plants. CUL1 acts as a scaffold, binding Skp1 at its N-terminus and Rbx1 at the C-terminus. CUL1 is modified by related to ubiquitin protein (RUB) in a dynamic process in which the SCF interacts with the COP9 signalosome (CSN), which has RUB-isopeptidase activity that cleaves the RUB modifier off of CUL1 (Figure 2). RUB conjugation requires RUB-specific E1, a heterodimer consisting of auxin resistant 1 (AXR1) and E1 C-terminal related 1 (ECR1) proteins. In addition E2 enzyme, RUB-conjugating enzyme 1 (REC1), and RBX1, which functions as a RUB E3 ligase are required for RUB conjugation. The Skp1 binds the F-box domain of the F-box protein that recruits specific proteins to the complex for ubiquitination. This complex functions in catalyzing the conjugation of ubiquitin to substrate proteins; once the substrate is ubiquitinated it is targeted to and degraded by the 26S proteasome complex. In the case of auxin signaling, SCF^{TIR1} complex acts as a positive regulator of the auxin response and functions in mediating the ubiquitination and subsequent degradation of the repressor Aux/IAA proteins.

A major discovery of the auxin signaling pathway was the identification of the auxin receptor. This question had eluded scientists for more than a century, but finally two groups independently showed that TIR1 acts as the auxin receptor (Dharmasiri *et al*, 2005; Kepinski *et al.*, 2005). Recent crystallographic studies done with the TIR1

receptor enabled scientists to elucidate the mechanism of binding between TIR1 and auxin. TIR1 contains a leucine-rich-repeat (LRR) domain that is responsible for auxin binding and Aux/IAA substrate recruitment. Auxin binds to a somewhat promiscuous site in the base of the TIR1 pocket, which has been shown to accommodate three structurally different auxins. Docked on top of auxin, the Aux/IAA protein occupies and completely encloses the rest of the hormone-binding site. Auxin is thought to function by promoting the interaction between TIR1 and Aux/IAA substrate by acting as a "molecular glue" (Tan *et al* , 2007).

The current model for auxin signaling proposes that auxin rapidly modulates gene expression through the degradation of Aux/IAA repressor proteins by interacting with the auxin receptor TIR1 in the SCF^{TIR1} protein complex. Degradation of the repressor proteins (Aux/IAAs) relieves the inhibition of ARFs. Thus, in the presence of auxin, ARFs will modulate gene transcription due to the degradation of Aux/IAA repressor proteins (Figure 2).

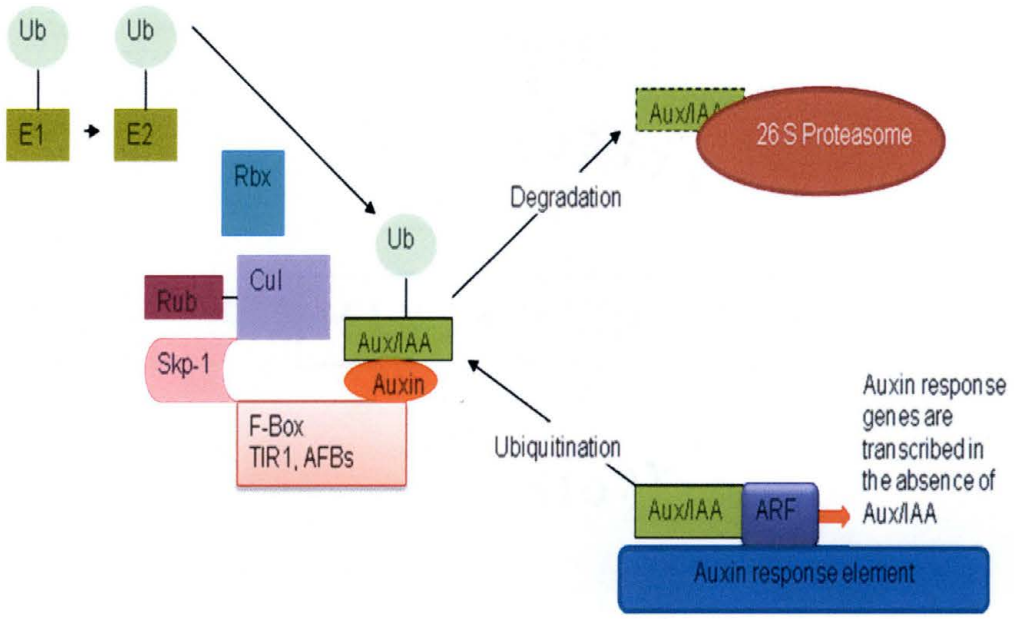


Figure 2: Current Model of Auxin Signaling. Auxin response is mediated through the SCF^{TIR1} complex. Auxin binding with TIR1 causes SCF complex-mediated ubiquitination of Aux/IAA repressor proteins, which are then targeted to and degraded by the 26S proteasome. Degradation of Aux/IAA relieves the inhibition of ARF transcription factors, which in turn activates auxin responsive genes.

Evidence of the Existence of Alternative Auxin-Signaling Mechanisms

Although most of the currently identified components fit into the model proposed for auxin signaling, some auxin-related responses cannot be explained by the current model. Components that do not fit into the current model include ABP1, repressing ARFs, SAUR proteins, MAP kinase pathway components and the indole-3-butyric acid-response (IBR5) dual-specificity phosphatase (reviewed by Quint *et al.*, 2006).

Early studies done to identify the auxin receptor focused on auxin-binding protein 1 (ABP1), a membrane bound protein found in the plasma membrane and endoplasmic reticulum. Several lines of evidence show that ABP1 is involved in auxin-mediated cell expansion although ABP1 has not been linked with auxin-related

transcriptional changes (Chen *et al.,* 2001). It was shown later that the family of F-box proteins that includes TIR1 and TIR1 homologues AFBI, AFB2 and AFB3 act as auxin receptors (Dharmasiri *et al.,* 2005a; 2005b). At the moment there is no clear role for ABP1 in auxin signaling, although homozygous *abp1* mutants are lethal (Chen *et al.,* 2001), indicating that these proteins play an essential role in plant growth and development.

A different line of evidence is that quadruple mutants created with the auxin receptor homologues (*tir1afb1afb2afb3*) are still viable and able to complete their life cycles. Auxin is essential for plant growth, so this finding suggests that auxin signaling is not completely abolished in these mutants; the ability of these quadruple mutants to complete the life cycle suggest that there may be additional receptors or signaling mechanisms other than TIR1 and its homologues that can respond to the auxin signal. On the other hand, the *afb1-1* and *afb3-1* alleles produced truncated transcripts but were not null (Dharmasiri *et al.,* 2005b). Therefore, the ability of a fraction of the quadruple mutants to complete the life cycle may be attributed to residual functionality of the truncated transcript of the two alleles.

Another well known plant response that cannot be explained by TIR1-mediated auxin signaling is rapid cell expansion in response to auxin. This response is observed within minutes of auxin application, thus possibly may not go through TIR1-related transcriptional mechanism (Masuda 1969; Chen *et al* , 2006).

The structural diversity of auxinic compounds also suggests the existence of more than one signaling mechanism for auxin signaling. Crystallographic studies have shown that IAA, 2,4-D and 1-NAA are accommodated in the same site on the TIR1 receptor (Tan *et al.,* 2007). Other than that, there is no published literature to confirm

that all auxins are able to bind with TIR1. Experiments done in our lab have shown that there are auxins that do not interact with TIR1 (S. Dharmasiri, unpublished data), suggesting that these auxins may interact with a different receptor. Whether these auxinic compounds share other molecular components that are unique is still unknown.

Aux/IAA proteins have been shown to be phosphorylated by phytochrome (Colón-Carmona *et al.,* 2000). The ability of Aux/IAA proteins to be phosphorylated is of interest. Although a previous study indicate that the auxin-induced AUX/IAA–SCF^{TIR1} interaction does not depend on phosphorylation (Dharmasiri *et al.,* 2003), there still exists the possibility of a mechanism whereby Aux/IAA proteins could be phosphorylated by MAP kinase components, leading to its rapid degradation. This proposed mechanism takes into account that the study by Dharmasiri *et al.* (2004) was not carried out on all 28 Aux/IAA proteins and that the kinase inhibitor used might not have inhibited MAP kinase. Although at the moment there is no significant evidence suggesting the existence of such a pathway, it is not possible to eliminate its existence.

Mitogen-Activated Protein Kinase and Auxin Signaling

There are data to suggest the possibility of MAP kinase components being involved in auxin signaling. The first evidence suggesting the involvement of a kinase cascade in auxin signaling was uncovered in 1994 when auxin was reported to function as an activator of MAP kinase in tobacco cells (Mizoguchi *et al* , 1994). This report was later contradicted by the finding that activation of MAP kinase was not due to the presence of auxin, but rather the acidification of the cytosol (Tena *et al* , 1998). However, in 1998 it was shown that a kinase cascade involving the tobacco

MAPKKK, NPK1, negatively regulates auxin signal transduction. The authors clearly demonstrated that NPK1 blocked the activation of the GH3 promoter and ER7 (auxin responsive element) promoter in an auxin-dependent manner but did not influence auxin-insensitive promoters (Kovtun *et al.,* 1998). In 2000 the same authors reported that the putative analog of NPK1 in *Arabidopsis,* ANP1, interfered with auxin-induced gene transcription from the GH3 promoter (Kovturn *et al.,* 2000). Mockaitis and Howell (2000) reported that MAP kinases are involved in positively regulating the auxin signal. The authors reported that auxin induced increases in protein kinase activity in *Arabidopsis* roots, suggesting that interactions among MAP kinase signaling pathways in plants influence plant response to auxin (Mockaitis and Howell, 2000).

Identification of *ibr5-1* was a significant breakthrough in identifying the components of a kinase cascade involved in auxin signaling. A forward genetic approach identified *ibr5-1* as a mutant resistant to the natural auxin indole-3-butyric acid. *IBR5* encodes a dual specificity phosphatase (Monroe-Augustus *et al.,* 2003). Recently the substrate for IBR5 was identified as a MAP kinase (MPK12) (Lee *et al* , 2008). Among the 20 different MAPKs encoded by the *Arabidopsis* genome, IBR5 interacts only with MPK12. The authors demonstrated that activated MPK12 can be dephosphorylated and inactivated by IBR5. As discussed above, several publications in the past decade provide evidence for the possibility of MAP kinase involvement in auxin response although conclusive data to suggest a complete pathway for such a signaling event are still lacking.

Taking into account that the synthetic auxin picloram may function independently of TIR1, Dharmasiri *et al.,* used a forward genetics approach to

identify molecular components that are specific to picloram. In order to identify components involved in picloram signaling, 70,000 ethyl methanesulfonate (EMS) mutagenized *Arabidopsis* seeds were screened on picloram medium and 30 putative auxin resistant mutants were identified (Dharmasiri *et al.,* unpublished data). This thesis reports the identification and characterization of two of these mutants, *pic59* and *pic115*. My hypotheses were that *pic59* and *pic115* are caused by single gene mutations and that PIC59 and PIC115 may be involved in a novel auxin signaling mechanism.

CHAPTER II

MATERIALS AND METHODS

Plant Varieties and Growth Conditions

Arabidopsis thaliana (L.) Heynh. Var. *Columbia* (Col-0) seeds obtained from the Arabidopsis Biological Resource Center (ABRC), Ohio University were used as the wild type in all experiments performed.

Seeds were surface sterilized with 40% bleach with 0.1% TritonX-100 and excessively rinsed with sterile distilled water. Seeds were plated on either *Arabidopsis thaliana* medium with 1% sucrose (ATS), pH 5.6, or where specified 0.5X Murashige and Skoog medium (MS; Murashige and Skoog, 1962; Sigma) with 1% sucrose, pH 5.6. The plates were incubated at 4°C for 24 hours and then transferred to a growth chamber at 20°C with continuous illumination (Dharmasiri *et al.*, 2003). All experiments on sterile medium were performed in the same growth chamber. All experiments with potted plants were carried out in a plant room at 22°C under continuous illumination.

Morphometric Characterization

The mutant and wild type plants were grown under similar growth conditions and the morphological characters were compared to determine phenotypical defects that are associated with the mutation.

Physiological Characterization

The Effect of Auxin

Root inhibition assays were carried out for *pic59* in picloram, 2,4-D, IAA and IBA and for *pic115* in IAA and IBA. At least 10 plants from the mutants and wild type were germinated on ATS for picloram and 2,4-D experiments or 0.5X MS medium for IAA and IBA experiments. Four-day-old seedlings were transferred on to ATS or MS (for controls) or respective media containing different auxins as described in each experiment.

The Effect of Other Phytohormones

The effect of selected plant hormones on the growth of the primary root of *pic59* and *pic115* was studied. Seedlings of Col-0 and *pic59* or *pic115* were plated on either ATS or MS (for control) or on ATS or MS containing different concentrations of abscisic acid, ethylene (ethylene precursor 1-aminocylopropane carboxylic acid) or cytokinin (kinetin) as described in the results section.

The Effect of Sugar

The effect of varying concentrations of sucrose on primary root elongation of *pic59*, *pic115* and Col-0 was studied. Four-day-old seedlings were plated on ATS medium supplemented with different concentrations of sucrose and incubated in a growth chamber under continuous illumination at 20°C for an additional 4 days.

The Effect of Temperature on Hypocotyl Growth

Previous studies have shown that *Arabidopsis* seedlings show dramatic hypocotyl elongation when grown at high temperature, and this temperature-dependent growth response is sharply reduced in some auxin response mutants (Gray *et al.*, 1998).

To determine the response of *pic59* and *pic115* mutations to high temperature, these mutants along with wild type plants were germinated and grown on ATS in

vertical orientation at 20°C and 29°C in a growth chamber under continuous illumination. The lengths of the hypocotyls were measured following 9 days of temperature treatment.

The Effect of Gravity

Col-0, *pic59* and *pic115* seeds were germinated and grown vertically on hormone free ATS medium for four days in a growth chamber at 20°C under continuous illumination, and rotated 90° to place roots in a horizontal position. The curvature of the root tip from the horizontal position was measured at 2 hour intervals for 12 hours.

Molecular and Genetic Characterization

Genetic Characterization

To determine if the mutants are dominant or recessive, *pic59* and *pic115* mutants were back-crossed in to the wild type. The F1 generation was allowed to self pollinate and the F2 generation was tested for the segregation pattern of the auxin resistant primary root phenotype on ATS medium containing either 12.5 μM picloram (for *pic59*) or 75 nM 2,4-D (for *pic115*). Sixty-four and forty F2 seedlings of *pic59* and *pic115*, respectively, were used for this experiment. The number of seedlings that were highly resistant, moderately resistant or sensitive to auxins was counted and compared with expected phenotypic ratios.

Positional Cloning of the Mutant Genes

The exact positions of the mutant genes were located through genetic mapping using genetic polymorphism between closely related *Arabidopsis* ecotypes. The availability of the entire genome sequence of *A. thaliana* ecotype Col-0 and most of the genomic sequence in *Landsberg erecta* (Ler) makes it possible to design molecular markers that show polymorphism between these two ecotypes.

To generate the mapping populations, homozygous mutant plants of *pic115* in Col-0 background was crossed in to the wild type Ler ecotype. *pic59* was crossed in to Ler ecotype - (A. Gunathilake). The resulting F1 population from each cross was self-pollinated and the F2 generation seeds were obtained. The F2 progeny was tested on auxinic media and plants homozygous for the mutation were selected. Genomic DNA was isolated from the selected plants using the sucrose preparation method (Berendzen *et al.,* 2005) and used in polymerase chain reaction (PCR) using simple sequence length polymorphic (SSLP) molecular markers.

Coarse Mapping

Sequence specific SSLP markers were used to test for linkage to the five chromosomes of *Arabidopsis thaliana* (Table 1). A ratio of 1:2:1 for Col-0: Heterozygote: Ler indicates the mutant is not located in a particular chromosome. (When a mutation is located in a particular chromosome the number of DNA samples in Col-0 background increase significantly)

Fine Mapping

Fine mapping was done to narrow down the genetic window to a region of about 40 kb. A large number of DNA samples were used to narrow down the genetic window (600 DNA samples for *pic59* and 300 DNA samples for *pic115*). Increasingly tight linkage to the mutation was tested using additional SSLP markers (Table 1).

Table 1. Oligonucleotide Primers Used for Fine Mapping of *pic59* and *pic115*			
Primer Name	Sequence	Length	Melting Temperature (^{o}C)
K17O22F	TACTCATCTACACAAGGAGCAT	22	62
K17O22R	TCAACCAATTTCGACTGCTTCT	22	62
MQL5F	TAGCCATCTACCCTTCTATTTCT	23	64
MQL5R	TTGTTCACCTGTGCTTCGCTC	21	64
MIF21	AATGTGAGGAGACTGTAACCAA	22	62
MIF21	TGGTCAACCGCACCTTTACAT	21	62
K2OJ1F	AGTGGAGGACATGTGTTGGACTT	23	68
K20J1R	TGTGTGAGGTTGTGTCTTTCAACA	24	68
K21P3F	GTGACGAGAGTTCGTTAATGGA	22	64
K21P3R	ATTACACGCCCTCACATGATTAT	23	64
K6M13F	TATCACAGTATGCCCCTGGTC	21	64
K6M13R	TCATTTTACAGTGCCACTGTAAC	23	64
K21G20F*	GCGTGTAAATGTTGTTGAGTTGA	23	64
K21G20R*	CACTAGACCACATTCACTTTTCGT	24	68
K9P8F*	CCATTCATCATTGAGGCTCTGT	22	64
K9P8R*	GGAGGCTTTTCGATTCTAATTCT	23	64
MPF21F*	GTAATGTTCGTCTCGGGAGACA	22	66
MPF21R*	CCTAAGTGTTGTCTCTCTAGCAA	23	66
K6A12F*	GGGTTTTAACTTCTCCTTCTTCTT	24	66
K6A12R*	TGGCTTGTAATATGTAGACGTAGA	24	66
MXI22-2F	GAACTCTATGGCTTCCAGTTAGAT	24	68
MX122-2R	ATTGGGTACCTGTACTCCACAG	22	66
K7B16F*	CAAAAGATGATGTTAAAGAGGTGA	24	64
K7B16R*	CTTGGAGATCCAGGACCAATCA	22	66
F19B11F	CGAGATAGACAATGTGATATTCTT	24	64
F19B11R	ACTTGAAAACCTGGCCTTTGTAT	23	64
F3C11F	AACTCGGTTACGCGAATTTAAGT	23	64
F3C11R	TTATCGCCTAGCGCTCTCTTTAA	23	66
F3L12F	CTATCTGTTATTAAGAGTCGGA	22	60
F3L12R	TGGAACTCGTGGTCGTCGATT	21	64
T16B23F	AAATATGGCTACCAAAAGTACTA	23	60
T16B23R	TCAACATTCCTTGACACGAGCCA	23	68
T23O15F	ACAGTTCGCAGACTCAGGAATA	22	64
T23O15R	TTGGTTCTGTCTGGCTCTCCAT	22	66
TI03F	ATCGTGCAACGAGCATACAAAGA	23	66
TI03R	AGTGAAGATAGCTTGGTCTGCCA	23	68
F28I8F	CGAGATAGACAATGTGATATTCTT	24	64
F28I8R	TGAATTCTATTTCTCTGTCGGCTTA	25	66
T13E11F	TCCATGCTTTCTCCGGTTTGCAA	23	68
T13E11R	CACCCAAACCATTGTTTGTATCGT	24	68

* Primers designed by N. Karunarathna

2001). The results obtained with *pic59* and *pic115* were compared with wild type Col-0 to determine whether there is an effect caused by the mutation on the stability of AXR3NT-GUS protein. (The GUS staining procedure for *pic59* was done by S. Dharmasiri)

Analysis of the Effects of Mutations on Auxin-Regulated Gene Transcription Using the *DR5::GFP* Reporter System

The expression of the green florescence protein (GFP) under the influence of *DR5*, an auxin-responsive synthetic promoter, was used to study the effects of *pic59* and *pic115* mutations on auxin-dependent gene expression. The mutant plants were crossed into Col-0 plants carrying the *DR5::GFP* reporter gene construct. The resulting F1 generation was self-pollinated to obtain F2 and then F3 plants homozygous for both the mutation and the reporter gene. The F3 seedlings grown on either ATS or ATS medium containing 12 μM picloram or 70 nM 2,4-D were observed under the confocal microscope. The expression pattern of GFP in the wild type was compared to that of the mutants to determine the effects of the mutations on GFP expression.

Image Acquisition

Images of unfixed root tips were acquired using an Olympus FV1000 (Melville, NY) and analyzed using Olympus Fluoview software. Gain and dynamic range settings were calibrated on wild type GFP expressing root tips and then kept unchanged for recording of images of the various mutant root tips. (Images were acquired by J. Koke and S. Dharmasiri)

Analysis of the Effects of the Mutations on the Expression Level of Auxin-Responsive Genes Using RT-PCR

Reverse-transcription PCR (RT-PCR) was used to determine the effect of the mutations on the level of expression of auxin-responsive genes. An early effect of auxin at the molecular level is the rapid increase in transcription of *Aux/IAA* genes. To determine whether the mutations caused an altered level of expression of auxin responsive genes, the levels of expression of selected *Aux/IAA* genes (*IAA2, IAA12*) were compared to the level of expression in Col-0.

The experiment was conducted by growing mutant seedlings along with Col-0 as the control. Four-day-old seedlings grown on ATS medium were transferred to 2,4-D, picloram and ATS medium. Following four days of incubation at 20°C, total RNA was extracted from the plant tissue. Plant tissues were dipped in liquid nitrogen and ground to fine powder. Total RNA was extracted using TriReagent (Sigma) according to the manufacturer's instructions. RNase-free DNase was used to remove any contaminating DNA from the extract. cDNA was synthesized using total RNA and Superscript reverse transcriptase (Invitrogen) following manufacturer's instructions. The amount of RNA in each preparation was standardized by the PCR amplification of ubiqutin cDNA and quantification of band intensity using ImageJ software. cDNA solutions that contained the same amount of RNA were used for the PCR amplification using gene specific primers for *IAA2* and *IAA12* (Biswas *et al*, 2007). Each PCR was repeated three times to determine consistency in results.

Examining the Spatial and Temporal Expression Pattern of *PIC115* Gene

The function of a gene is closely associated with its expression pattern. A *PIC115*:: *PIC115-GUS* recombinant translational gene construct was created to determine the spatial and temporal expression of the gene. A 2 kb size fragment from

the promoter region and the gene were amplified by using primers (Table 3) and cloned into pBluescript and then subcloned into the *pBI101* plasmid to create the recombinant gene construct. The accuracy of the gene construct was confirmed by sequencing. This construct was transformed into wild type *Arabidopsis* plants by using *Agrobacterium*-mediated transformation (Oono *et al.,* 1998; Augustus *et al.,* 2003). The T1 seeds were screened on kanamycin-containing medium and the kanamycin resistant plants were transferred to soil. T2 seeds that are homozygous for the transgene were selected for a histochemical GUS assay. Four-day-old T2 seedlings were stained with 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (X-Gluc) (0.5 mg/ml) solution as described previously (Gray *et al.,* 2001).

Table 3. Oligonucleotide Primers Used for *promoter::PIC115* Amplification

Primer Name	Sequence	Length	T_m (℃)
115-5F	GTCCAAGTATTTGAAAGCTTTGT	23	62
115-5R	CTAAGAGTCGACCATTGCAATATCA	25	68

Analysis of Genetic Interactions between Mutants

The mutants were crossed in to known mutants in the auxin signaling pathway. The F4 population that is homozygous for both mutants was used to determine the genetic interaction between the mutant gene and the known mutant genes. The known mutants used for the crosses were *afb5* (auxin receptor homolog), *sgt1b* (component of the SCF complex and a gene involved in plant disease resistance) and *tir1* (mutation in auxin receptor), to create the *pic115afb5*, *pic115sgt1b* and *pic115tir1* double mutants.

In addition, *pic59* and *pic115* were crossed in to each other and the *pic115pic59* double mutant was created to determine the genetic interactions, in order to find out if both mutants function in the same signaling pathway.

Data Analysis

For statistical comparison of the silique lengths and hypocotly lengths, mean and standard deviation were calculated for each sample and the results were recorded in a Microsoft Excel spreadsheet and analyzed by Student's t-test. For statistical comparison of the dose response experiments, the response to gravity and the response to sugar two-way ANOVA were done. The ANOVA was done on Microsoft Excel or software obtained from www.r-project.org/ or www.physics.csbsju.edu/stats/anova_pnp_NGROUP_form.html (ANOVA tests were done with the assistance of F. Weckerly, A. Pokaral, P. Amaradasa and N. Dharmasiri)

CHAPTER III

RESULTS

Morphometric Characterization

While homozygous mutants of *pic59* and *pic115* did not show noticeable morphological differences from wild type Col-0 throughout most of the life cycle, siliques produced by both homozygous mutants showed morphological differences compared to the wild type. Both mutants produced shorter siliques. *pic59* produced siliques that were about half the length of Col-0 siliques (Figure 3), whereas *pic115* produced siliques that were about three quarter of the length of Col-0 siliques (Figure 4).

a.

Col-0 *pic59*

b.

Col-0 *pic59*

c.

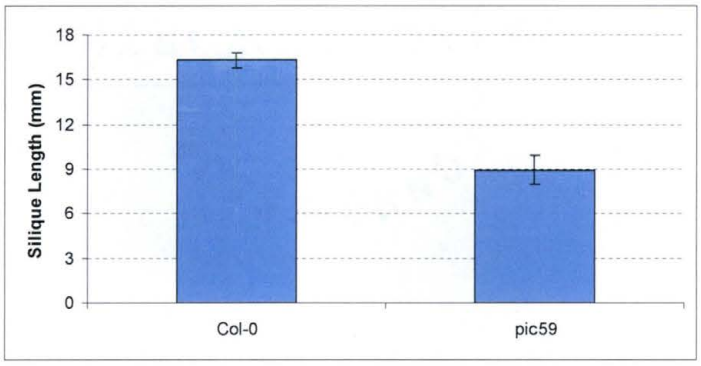


Figure 3. The Length of Siliques Produced by Col-0 and *pic59*. (a) and (b) When plants were grown under similar growth conditions, *pic59* produced siliques which were shorter in length than the siliques produced by wild type Col-0. (c) The length of siliques produced by *pic59* is significantly shorter than that of Col-0 ($P < 0.05$). Forty-two day old *pic59* and Col-0 plants were compared and analyzed. Each column shows the average silique length from 18 plants. 20 siliques from each plant were used for measurement. Error bars represent standard errors of the means.

a.

Col-0 *pic115*

b.

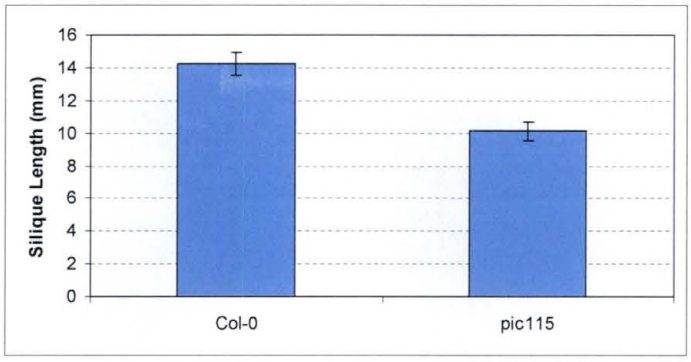
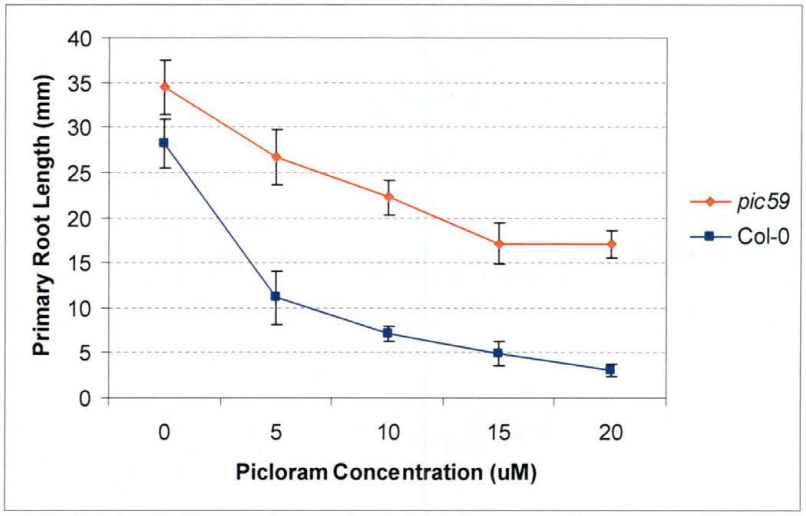


Figure 4. The Length of Siliques Produced by Col-0 and *pic115*. (a) When plants were grown under similar growth conditions, *pic115* mutant plants produced siliques that were significantly shorter in length than the siliques produced by Col-0. (b) The length of siliques produced by *pic115* is significantly shorter than that of Col-0 ($P < 0.05$). Fifty-day-old *pic115* and Col-0 plants were compared and analyzed. Each column shows the average silique length from more than 10 plants. 20 siliques from each plant were used for measurement. Error bars represent standard errors of the means.

Response of *pic59* to Auxin

Root growth assays done on *pic59* revealed that the mutant is highly resistant to picloram. The mutant plants were resistant to picloram at all concentrations tested ranging from 5 μM to 20 μM whereas the wild type plants were inhibited at all the above concentrations. The primary root growth of *pic59* was slightly resistant to 2,4-D. The mutant plants showed a resistance to 2,4-D at 50 nM and 75 nM whereas the wild type plants were significantly inhibited at these concentrations. However, both the mutant and the wild type were inhibited at the highest 2,4-D concentration (100 nM) tested. The *pic59* mutant was also resistant to IBA at 10 μM whereas the wild type was sensitive. In contrast, primary root growth of *pic59* was sensitive to IAA at all the concentrations tested (Figure 5).

a.



b.

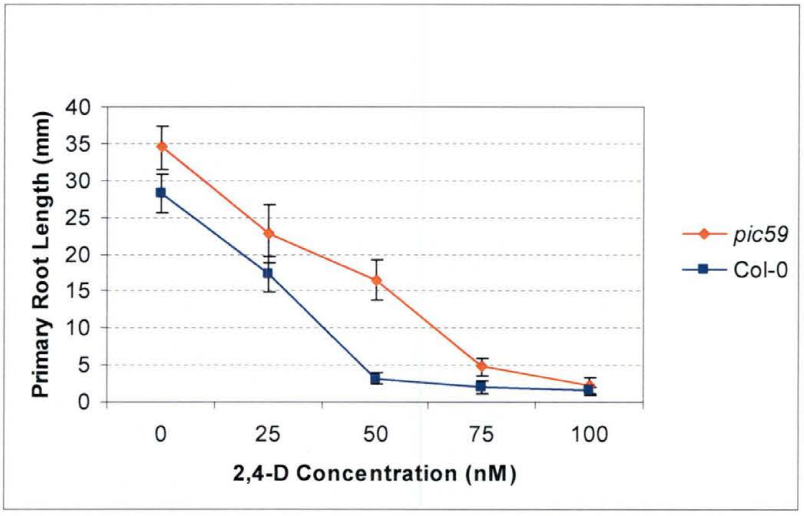
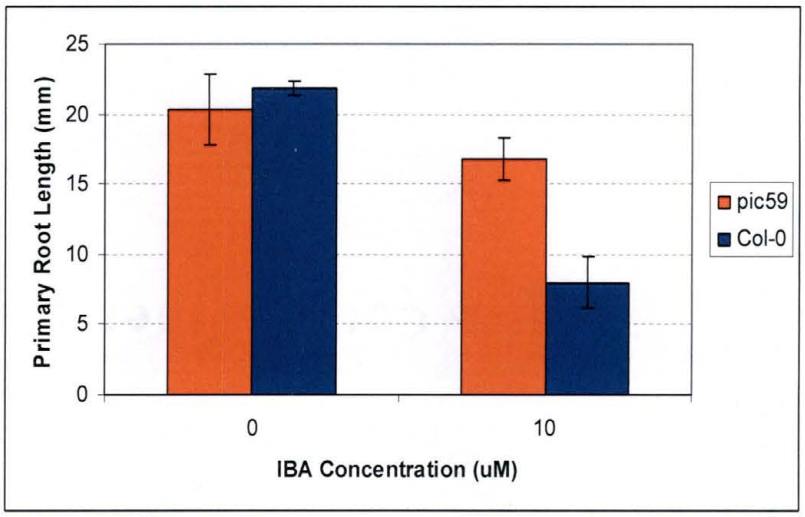


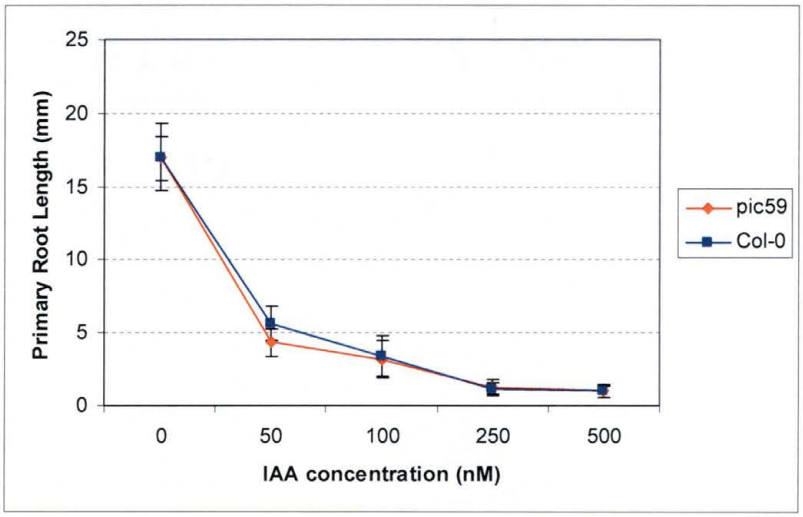
Figure 5. Primary Root Elongation of *pic59* in Response to Auxins. (a) Primary root elongation of *pic59* is significantly different than Col-0 at high concentrations of picloram; $F(1.0, 104.0) = 5.66$, $P < 0.05$. (b) Primary root elongation of *pic59* is significantly different than Col-0 at high concentrations of 2,4-D; $F(1.0, 97.0) = 5.67$, $P < 0.05$. (c) Primary root elongation of *pic59* is significantly different than Col-0 at 10 µM IBA; $F(1.0, 20.0) = 42.57$, $P < 0.05$ (d) Primary root elongation of *pic59* is not significantly different than Col-0 at high concentrations of IAA; $F(2.0, 112.0) = 0.80$, $P > 0.05$. Error bars represent standard errors of the means.

Figure 5. continued.

c.



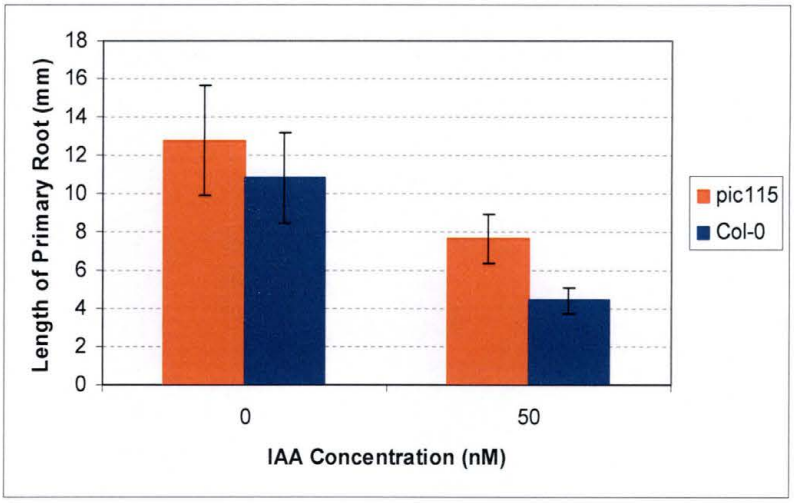
d.



Response of *pic115* to Auxin

The primary root growth of *pic115* showed a slight resistance to the most abundant naturally occurring auxins in plants, IAA. *pic115* was resistant to 50 nM IAA whereas the wild type was sensitive at the above concentration. *pic115* was also resistant to IBA to 10 μM (Figure 6). Additionally, previous studies in our laboratory have shown that primary root growth of *pic115* is resistant to synthetic auxins 2,4-D and picloram (S. Dharmasiri, unpublished data).

a.



b.

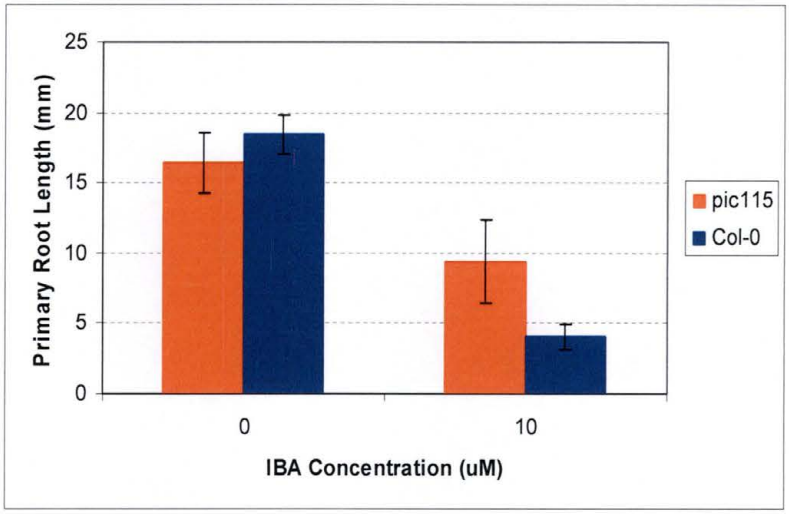


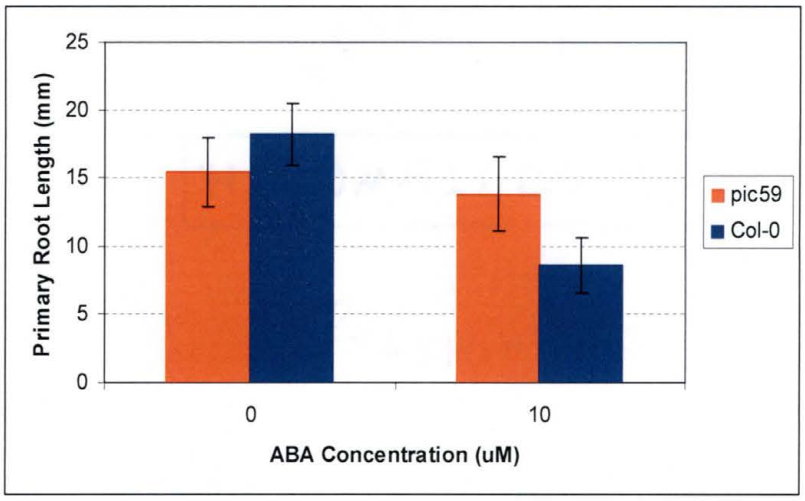
Figure 6. Primary Root Elongation of *pic115* in Response to Auxins. (a) Primary root elongation of *pic115* is significantly different than Col-0 at 50 nM IAA; $F(1.0, 38.0) = 7.66$, $P < 0.05$ (b) Primary root elongation of *pic115* is significantly different than Col-0 at 10 µm IBA; $F(3.0, 70.11) = 72.47$, $P < 0.05$. Error bars represent standard errors of the means.

Response of *pic59* and *pic115* to ABA

Cross talk between auxin and other plant hormones plays a major role in the regulation of plant development. Therefore, the same mutation may affect many other hormonal signaling pathways. To test whether this is true for *pic59* and *pic115*, these mutants were tested for ABA, ethylene and cytokinin responses as described in the methods.

Abscisic acid (ABA) is a plant hormone that mediates the plant's response to stress (Schnall *et al.,* 1992). Primary root growth of *pic59* and *pic115* was resistant to ABA at 10 μM when compared to Col-0 (Figure 7a and 7b).

a.



b.

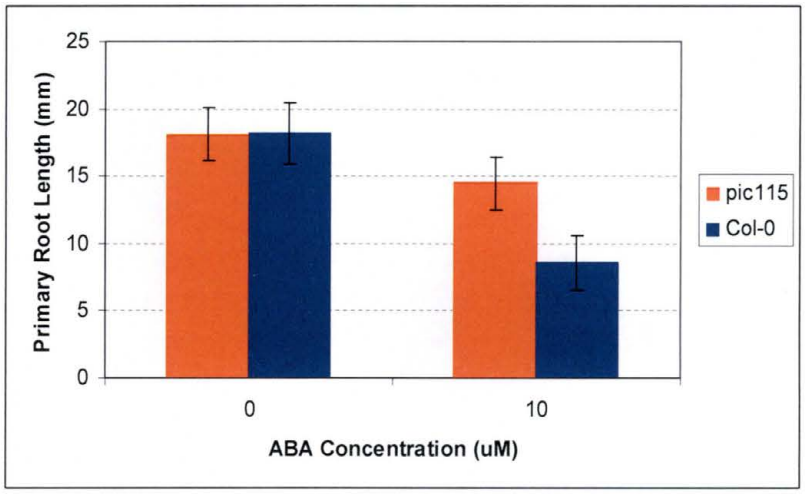
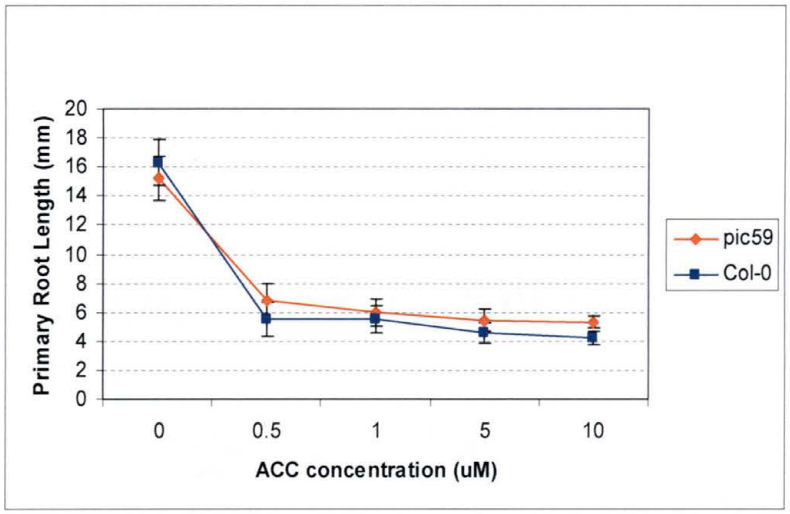


Figure 7. Primary Root Elongation of *pic59* and *pic115* in Response to ABA. (a) Primary root elongation of *pic59* is significantly different than Col-0 at 10 μM ABA; $F(1.0, 36.0) = 27.45$, $P < 0.05$ (b) Primary root elongation of *pic115* is significantly different than Col-0 at 10 μM ABA; $F(1.0, 35.0) = 20.85$, $P < 0.05$. Error bars represent standard errors of the means.

Response of *pic59* and *pic115* to ACC

Previous studies have shown that some auxin-responsive mutants exhibit cross-resistance to the plant hormone ethylene. To test whether *pic59* and *pic115* show a similar trait, these two were exposed to the precursor of ethylene synthesis, 1-aminocyclopropane-carboxylic acid (ACC). Primary root growth of both mutants were sensitive to the ethylene precursor ACC at all concentrations tested (Figure 8a and 8b).

a.



b.

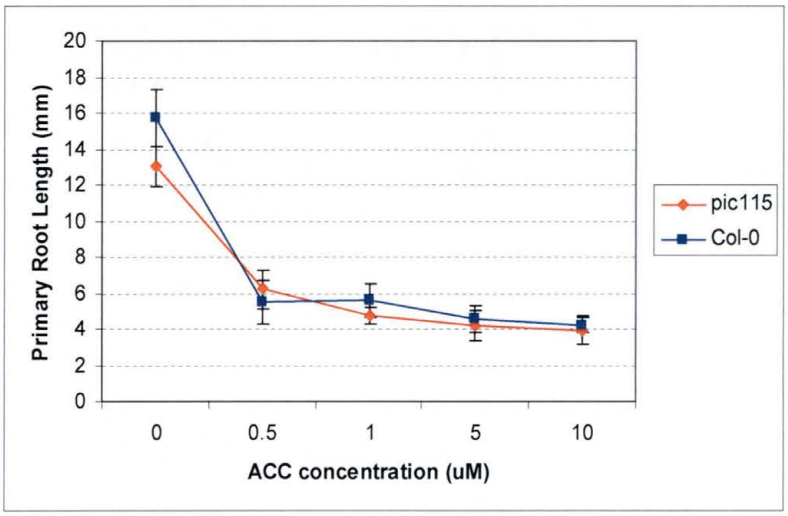
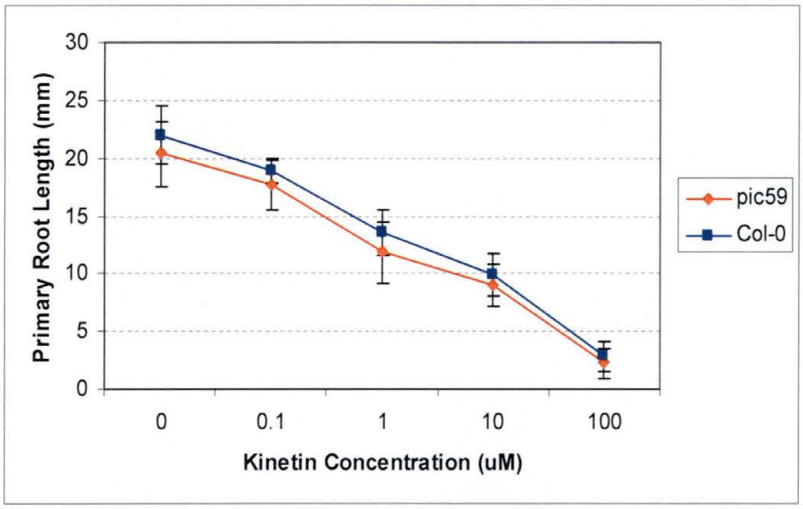


Figure 8. Primary Root Elongation of *pic59* and *pic115* in Response to ACC. (a) Primary root elongation of *pic59* is not significantly different than Col-0 at high concentrations of ACC; $F(1.0, 108.0) = 0.02$, $P > 0.05$. (b) Primary root elongation of *pic115* is not significantly different than Col-0 at high concentrations of ACC; $F(1.0, 117.0) = 0.70$, $P > 0.05$. Error bars represent standard errors of the means.

Response of pic59 and *pic115* to Kinetin

Since auxin and cytokinin are known to interact with each other to control many growth and developmental processes, both *pic59* and *pic115* were tested for cytokinin response using kinetin. Results indicate that both these mutants are sensitive to kinetin at all the concentration tested (Figure 9a and 9b).

a.



b.

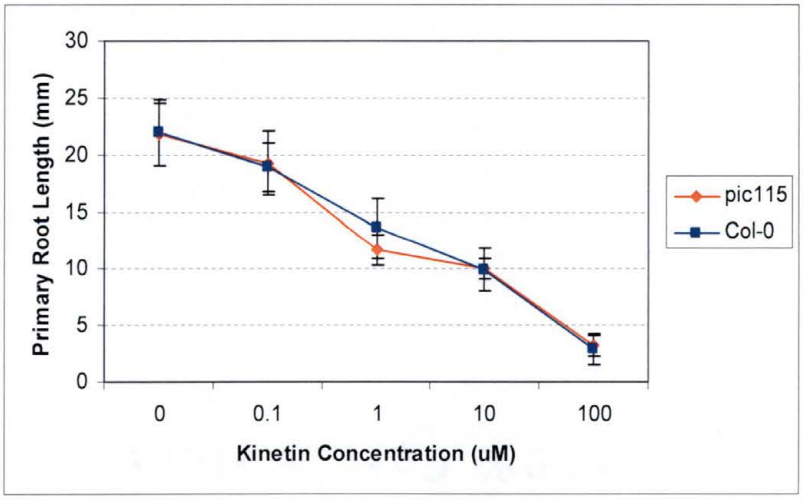
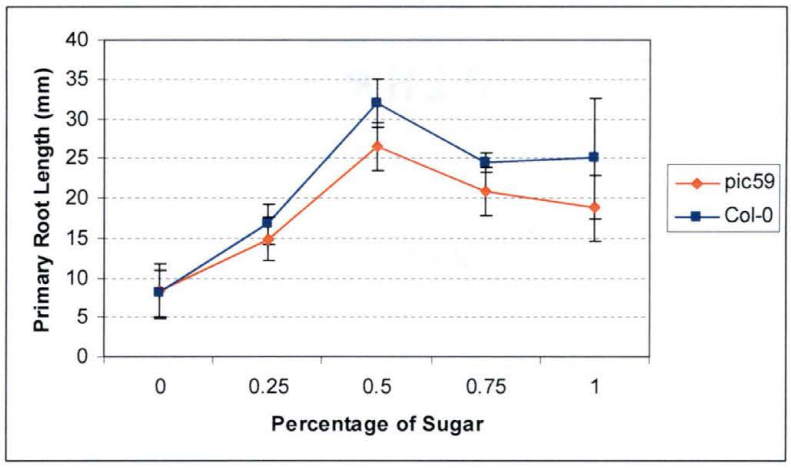


Figure 9. Primary Root Elongation of *pic59* and *pic115* in Response to Kinetin. (a) Primary root elongation of *pic59* is not significantly different than Col-0 at high concentrations of kinetin; $F(1.0, 110.0) = 0.50$, $P > 0.05$. (b) Primary root elongation of *pic115* is not significantly different than Col-0 at high concentrations of kinetin; $F(1.0, 116.0) = 0.007$, $P > 0.05$. Error bars represent standard errors of the means.

Response of *pic59* and *pic115* to Sugar

Recent literature indicates that auxin may be involved in plants' sugar-sensing mechanism (Laxmi *et al.*, 2006; Mishra *et al*, 2009). The auxin response mutants' *tir1, axr2, axr3* and *slr1* display a defect in glucose-induced changes in root length, root hair elongation and lateral root production. The objective of testing the mutants on varying concentrations of sugar was to determine if the mutations has any effect on the plants' response to sugar. Neither mutant showed an altered root growth response to sugar at any of the concentrations tested when compared to the wild type (Figure 10a and 10b).

a.



b.

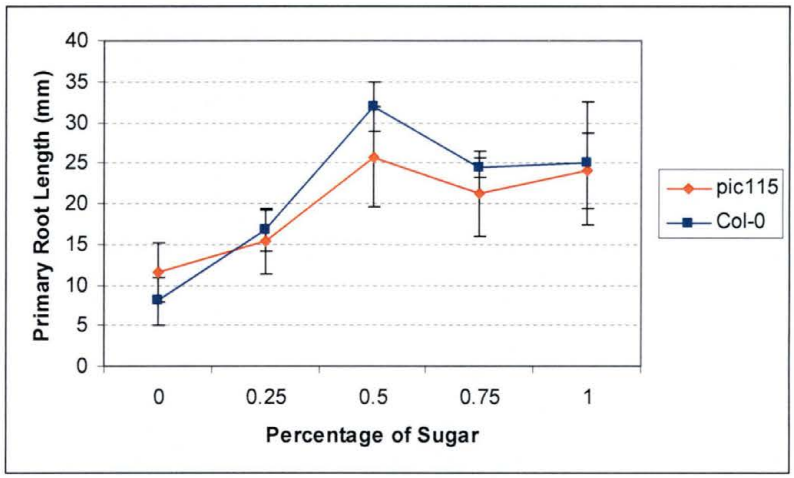


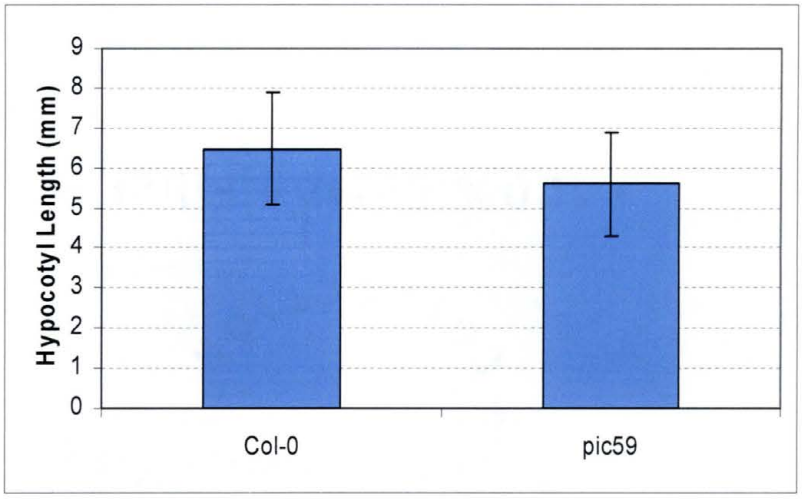
Figure 10. Response of *pic59* and *pic115* to Sugar. (a) Primary root length of *pic59* is not significantly different than Col-0 in response to sugar; F (1.0, 107.0) = 02.24, P > 0.05. (b) Primary root length of pic115 is not significantly different than Col-0 in response to sugar; F (1.0, 103.0 = 1.53, P > 0.05. Error bars represent standard errors of the means.

Response of *pic59* and *pic115* to High Temperature

Previous studies have shown that *Arabidopsis* seedlings show dramatic hypocotyl elongation when grown at high temperatures. To test whether hypocotyl growth of *pic59* and *pic115* is affected by mutations, growth responses were tested at high (29°C) temperatures under continuous illumination.

Results indicate that *pic59* and *pic115* do not show a significant difference in hypocotyl elongation compared to the wild type at high temperature (Figure 11a and 11b).

a.



b.

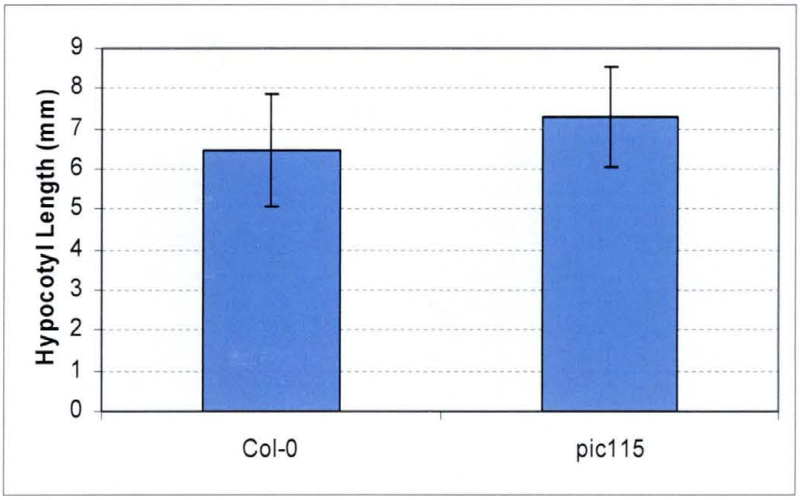
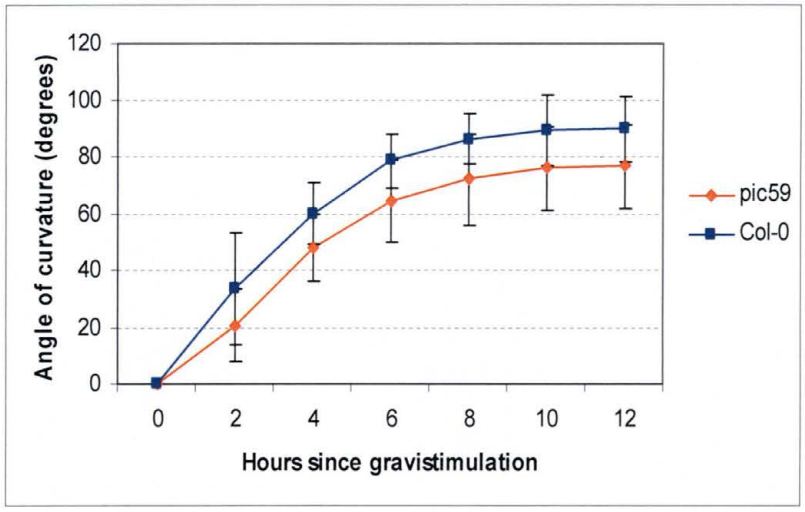


Figure 11. Hypocotyl Elongation of *pic59* and *pic115* in Response to High Temperature. (a) Hypocotyl length of *pic59* is not significantly different than Col-0 at 29°C ($P > 0.05$). (b) Hypocotyl length of *pic115* is not significantly different than Col-0 at 29°C ($P > 0.05$). Error bars represent standard errors of the means.

Response of *pic59* and *pic115* to Gravity

Gravity response in plants is regulated by auxin. Several auxin response mutants exhibit impaired gravity response (Marchant *et al* , 1999). Effect of *pic59* and *pic115* mutations on gravity response was tested as described in the methods section. Neither mutant showed a significant difference in the response to gravity compared to the wild type (Figure 12a and 12b).

a.



b.

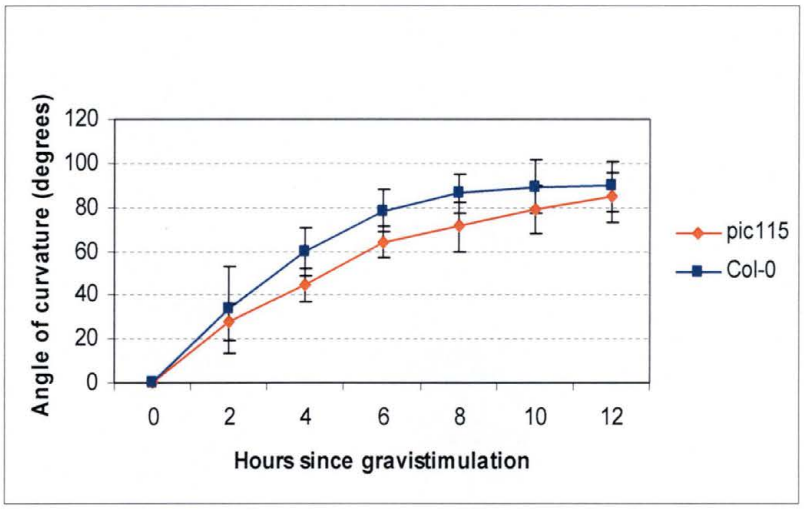


Figure 12. Response of *pic59* and *pic115* to Gravity. (a) The angle f curvature of *pic59* is not significantly different than Col-0; F (1.0, 131.0) = 0.92, P > 0.05. (b) The angle of curvature of *pic115* is not significantly different than Col-0; F (1.0, 136.0) = 0.45, P > 0.05. Error bars represent standard errors of the means.

pic59 is a Semi-Dominant Mutant

Segregation pattern of auxin resistance of F2 seedlings resulting from a cross between wild type Col-0 and *pic59* was tested on 12 μM picloram medium using a root growth assay. The results obtained indicate that ¼ of total seedlings were highly resistant to picloram, $^{2}/_{4}$ of the seedlings were moderately resistant and ¼ of the seedlings were sensitive to picloram. These results indicate that *pic59* is a semi-dominant mutation (Table 4 and Figure 13).

Table 4. Segregation Pattern of Auxin Resistance in *pic59*			
Total Number of Plants	Number of Plants with High Resistance	Number of Plants with Moderate Resistance	Number of Sensitive Plants
64	16	32	16

Figure 13. Segregation Pattern of Auxin Resistance in *pic59*. One forth of total seedlings were highly resistant (*pic59/pic59*), 2/4 of the seedlings were moderately resistant (pic59/+) and ¼ of the seedlings were sensitive (+/+).

pic115 is a Recessive Mutant

The segregation pattern of auxin resistant root growth of F2 seedlings of the cross between wild type Col-0 and *pic115* was tested on ATS media containing 75 nM 2,4-D. The results showed that approximately three-fourths of the total seedlings were sensitive to 2,4-D and one-forth of the seedlings were resistant to 2,4-D, indicating that the *pic115* is a recessive mutation (Table 5 and Figure 14).

Table 5. Segregation Pattern of Auxin Resistance in *pic115*

Total Number of Plants	Number of Resistant Plants	Number of Sensitive Plants
28	6	22

Figure 14. Segregation Pattern of Auxin Resistance in *pic115*. One fourth of the total seedlings were resistant (*pic115/pic115*) and three fourths of the seedlings were sensitive (*pic115*/+ or +/+).

Mapping *pic59*

Using around 600 DNA samples from homozygous individuals of *pic59* x Ler F2 generation, the *pic59* mutation was mapped to MPF21 BAC (bacterial artificial chromosome) in the south arm of chromosome V. The location of the mutant was narrowed down to a region of 14 genes in MPF21. This region contains no known genes associated with auxin signaling. Therefore, *pic59* will reveal a newly discovered gene associated with auxin signaling. Most of the genes in the genetic window are of unknown functions but contain conserved domains that may help to find the possible function of PIC59. Currently, these genes are being cloned and sequenced to find the exact mutation.

Mapping *pic115*

The locus for the *pic115* mutation was narrowed down to a region between BACs T23O15 and F28I8 representing *Arabidopsis* chromosome II. This genetic window contained T23O15, T1O3 and F28I8 with an approximate total size of 100 kb. The most likely candidate gene for *pic115* in this genetic window was AT2G04550 (indole-butyric acid resistance5 [*IBR5*]) in T1O3, which encodes a dual specificity phosphatase. This gene was cloned and sequenced. Comparison of sequence data with the wild type gene sequence revealed a single base pair change in the second exon of *pic115*. The nucleotide substitution was a G727A in the IBR5 gene. The single base pair change resulted in a missense mutation in the amino acid sequence, replacing the non-polar, neutral glycine (G) at position 132 with a polar, acidic glutamate (E) residue (Figure 15, 16 and 17).

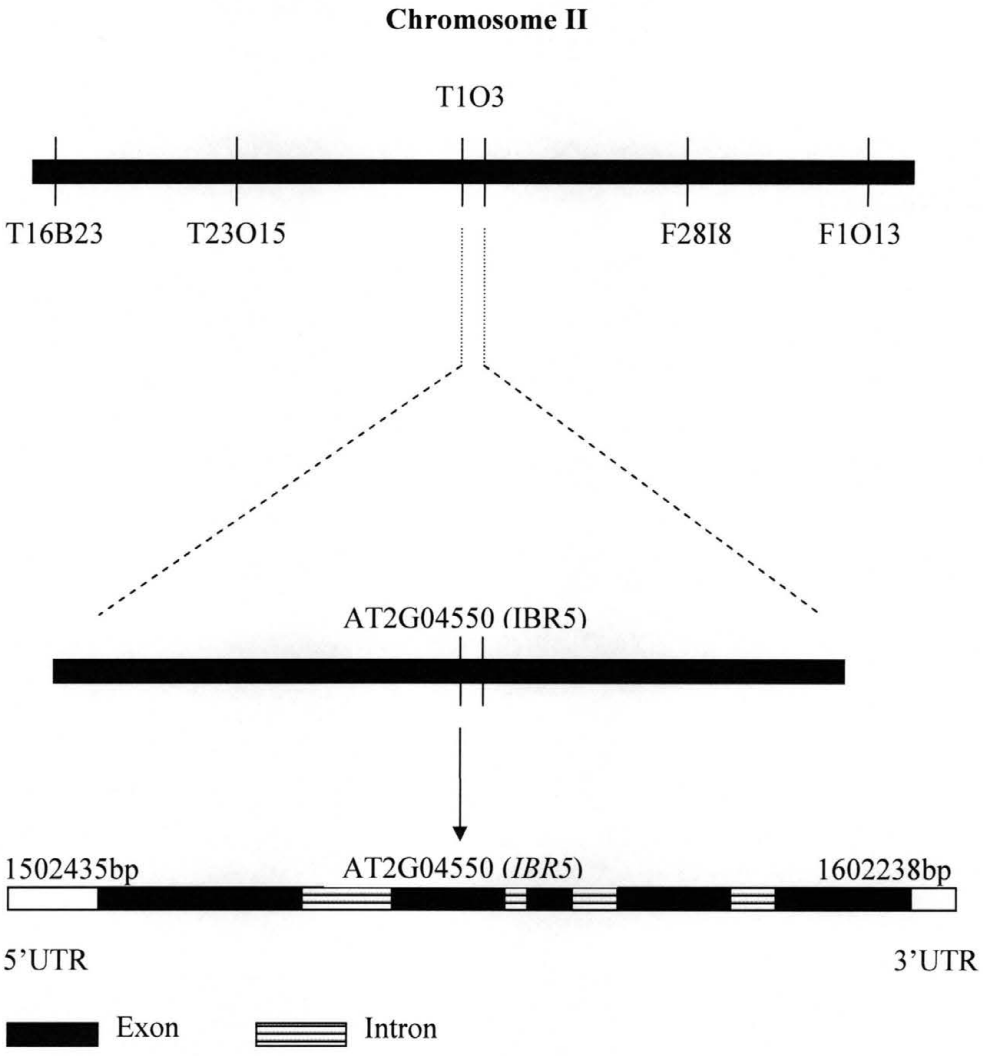


Figure 15. Positional Cloning of *pic115* and Diagrammatic Representation of *IBR5*. *pic115* mutation was mapped between the BACs T23O15 and F28I8 on chromosome II. The most likely candidate gene in the genetic window was in the BAC T1O3. Sequencing of AT2G04550 from *pic115* background revealed a G to A nucleotide change resulting the glycine (G) to glutamate (E) substitution in PIC115 protein.

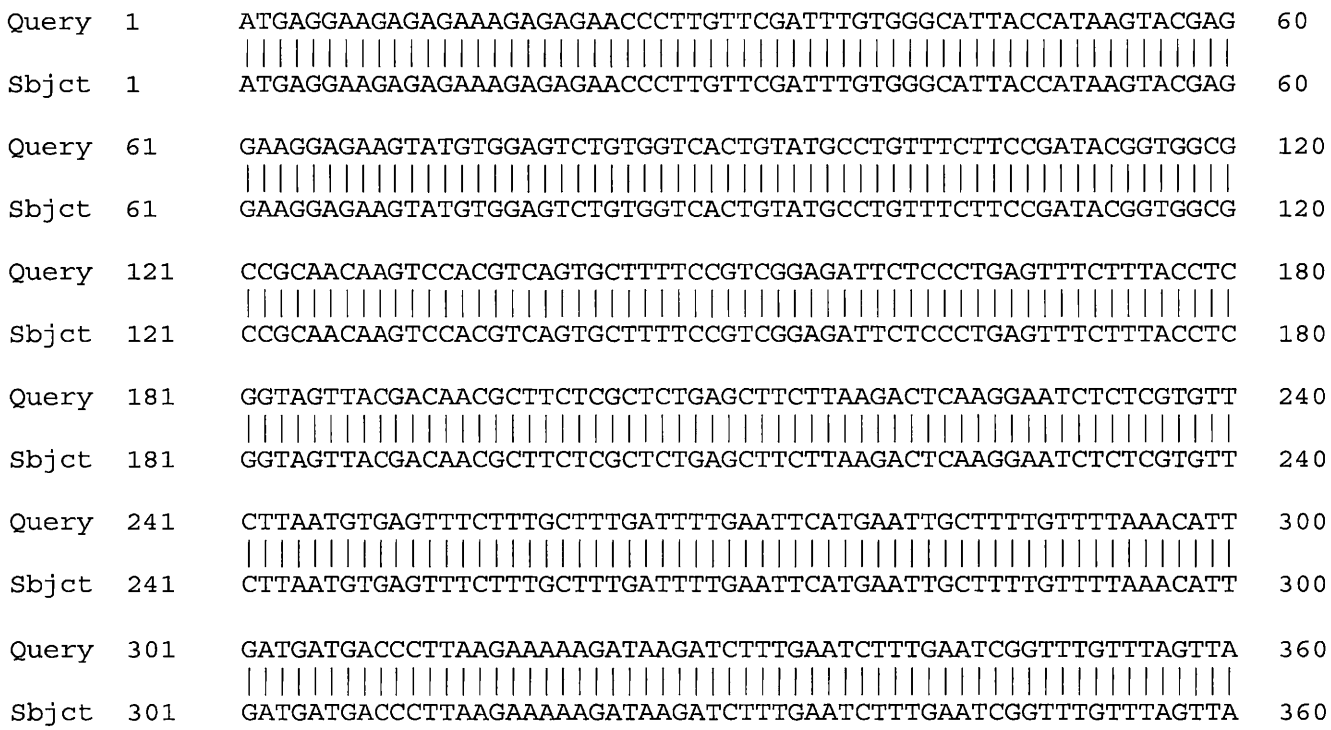


Figure 16. Alignment of the Nucleotide Sequence of *IBR5* Gene from the Wild Type Background (Query) and *pic115* Background (Subject). In *pic115* background, the 727 nucleotide (highlighted in red) of the *IBR5* gene (Col-0) has changed from guanine (G) to adenine (A).

Figure 16 continued.

```
Query  361    GGGTTTTGCCTTGTAGATGAATCTTTCTTATACTTATACGTTTTGTTGACTTATGGTTTC  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361    GGGTTTTGCCTTGTAGATGAATCTTTCTTATACTTATACGTTTTGTTGACTTATGGTTTC  420

Query  421    TGTTAAAGTTCCTGTTATGGTAATAGAGAGGAATGTGTTGTTTACTATTTGTTGGTATTG  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421    TGTTAAAGTTCCTGTTATGGTAATAGAGAGGAATGTGTTGTTTACTATTTGTTGGTATTG  480

Query  481    ACTCTGTTTTTGTTACTCTGTTTCAGACGGTTCCTATGTGCCAGAATCTCTACAGGAATT  540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481    ACTCTGTTTTTGTTACTCTGTTTCAGACGGTTCCTATGTGCCAGAATCTCTACAGGAATT  540

Query  541    CATTCACTTATCATGGTCTTGATAATGAAAAAGTTTTACAGTTTGATGATGCTATTAAGT  600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541    CATTCACTTATCATGGTCTTGATAATGAAAAAGTTTTACAGTTTGATGATGCTATTAAGT  600

Query  601    TCTTAGGTATGTTGATTCTATCTCAAGTTTCTAGATTTCATCTTTATGATTTTTTCTTGA  660
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601    TCTTAGGTATGTTGATTCTATCTCAAGTTTCTAGATTTCATCTTTATGATTTTTTCTTGA  660

Query  661    CGGTCTTGCTTTGTGTAGACCAATGTGAGAAGGACAAGGCACGTGTTCTTGTGCATTGCA  720
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661    CGGTCTTGCTTTGTGTAGACCAATGTGAGAAGGACAAGGCACGTGTTCTTGTGCATTGCA  720

Query  721    TGTCTGGGAAAAGTAGGTGAGTACATTTATCATTCTCTTACTCTCATCCTAAACTTGCGG  780
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721    TGTCTGAGAAAAGTAGGTGAGTACATTTATCATTCTCTTACTCTCATCCTAAACTTGCGG  780

Query  781    CTGCTTTCTTATGATTACCATTAATTATTTAGACTAATAGAGTGATATGTTTTGTGTTTC  840
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Sbjct  781    CTGCTTTCTTATGATTACCATTAATTATTTAGACTAATAGAGTGATATGTTTTGTGTTTC  840
```

Figure 16 continued.

```
Query  841    GATTACATGACAGATCACCAGCGGTTGTTGTAGCGTACTTGATGAAACGAAAAGGGTGGA  900
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841    GATTACATGACAGATCACCAGCGGTTGTTGTAGCGTACTTGATGAAACGAAAAGGGTGGA  900

Query  901    GACTCGCTGAGAGTCATCAGTGGGTTAAACAACGGAGACCAAGCACTGACATAAGTCCAG  960
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901    GACTCGCTGAGAGTCATCAGTGGGTTAAACAACGGAGACCAAGCACTGACATAAGTCCAG  960

Query  961    GCAAGTACTTAGACTTTGGATTAGAAACTTACTTATTGTTCTTGAAACTGTTACTCATTG  1020
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  961    GCAAGTACTTAGACTTTGGATTAGAAACTTACTTATTGTTCTTGAAACTGTTACTCATTG  1020

Query  1021   CTTTTTCTTTGTACACAGAGTTTTACCAACAACTGCAGGAGTTTGAGCAGGGGATATTCG  1080
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1021   CTTTTTCTTTGTACACAGAGTTTTACCAACAACTGCAGGAGTTTGAGCAGGGGATATTCG  1080

Query  1081   GATCTGAGATGATGTCGGCGATGAATATTAATGATGCTCCAACGTTTGGGTTTGGTTTCC  1140
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1081   GATCTGAGATGATGTCGGCGATGAATATTAATGATGCTCCAACGTTTGGGTTTGGTTTCC  1140

Query  1141   CTAAGATTGATAATCAAGCACAAGCTCCTGTGTTCAACAATGCTCCTACTTCTTCTATAT  1200
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1141   CTAAGATTGATAATCAAGCACAAGCTCCTGTGTTCAACAATGCTCCTACTTCTTCTATAT  1200

Query  1201   TTTCATCTCCTGCTTCAAGTATCCCTCCTCAGGAGTTCACTTTTGGAGCAACTCCACCAA  1260
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1201   TTTCATCTCCTGCTTCAAGTATCCCTCCTCAGGAGTTCACTTTTGGAGCAACTCCACCAA  1260

Query  1261   AGCCAACAACTGGTGGTGATATTGCAATGGATGGCTCTTAG
              |||||||||||||||||||||||||||||||||||||||
Sbjct  1261   AGCCAACAACTGGTGGTGATATTGCAATGGATGGCTCTTAG
```

```
Query  1      MRKRERENPCSICGHYHKYEEGEVCGVCGHCMPVSSDTVAPQQVHVSAFPSEILPEFLYL    60
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Query  61     GSYDNASRSELLKTQGISRVLNTVPMCQNLYRNSFTYHGLDNEKVLQFDDAIKFLDQCEK    120
Sbjct  61     GSYDNASRSELLKTQGISRVLNTVPMCQNLYRNSFTYHGLDNEKVLQFDDAIKFLDQCEK    120

Query  121    DKARVLVHCMSGKSRSPAVVVAYLMKRKGWRLAESHQWVKQRRPSTDISPEFYQQLQEFE    180
Sbjct  121    DKARVLVHCMSEKSRSPAVVVAYLMKRKGWRLAESHQWVKQRRPSTDISPEFYQQLQEFE    180

Query  181    QGIFGSEMMSAMNINDAPTFGFGFPKIDNQAQAPVFNNAPTSSIFSSPASSIPPQEFTFG    240
Sbjct  181    QGIFGSEMMSAMNINDAPTFGFGFPKIDNQAQAPVFNNAPTSSIFSSPASSIPPQEFTFG    240

Query  241    ATPPKPTTGGDIAMDGS  257
Sbjct  241    ATPPKPTTGGDIAMDGS  257
```

Figure 17. Alignment of Amino Acid Sequence of IBR5 Protein from Wild Type Background (Query) and *pic115* Background (Subject). In *pic115* background, the glycine (G) at position 132 is converted to glutamate (E) (highlighted in red) due to a missense mutation.

pic115 Mutation is in the Active Site of the DSP

The position of the mutation in *pic115* is significant. The mutation is located in the highly conserved active site of the phosphatase loop of the dual specificity phosphatase (DSP) enzyme. This active site $VxVHCx_2GxSRSx_5AYLM$ motif is conserved in almost all known eukaryotic DSPs (Monroe-Augustus *et al*, 2003). The glycine (G) that is changed in the *pic115* background is conserved in almost all of the eukaryotic DSPs (Figure 18). In *pic115* glycine, which is an uncharged amino acid with a non-polar side chain, is replaced by a glutamate (E), which is a negatively charged amino acid.

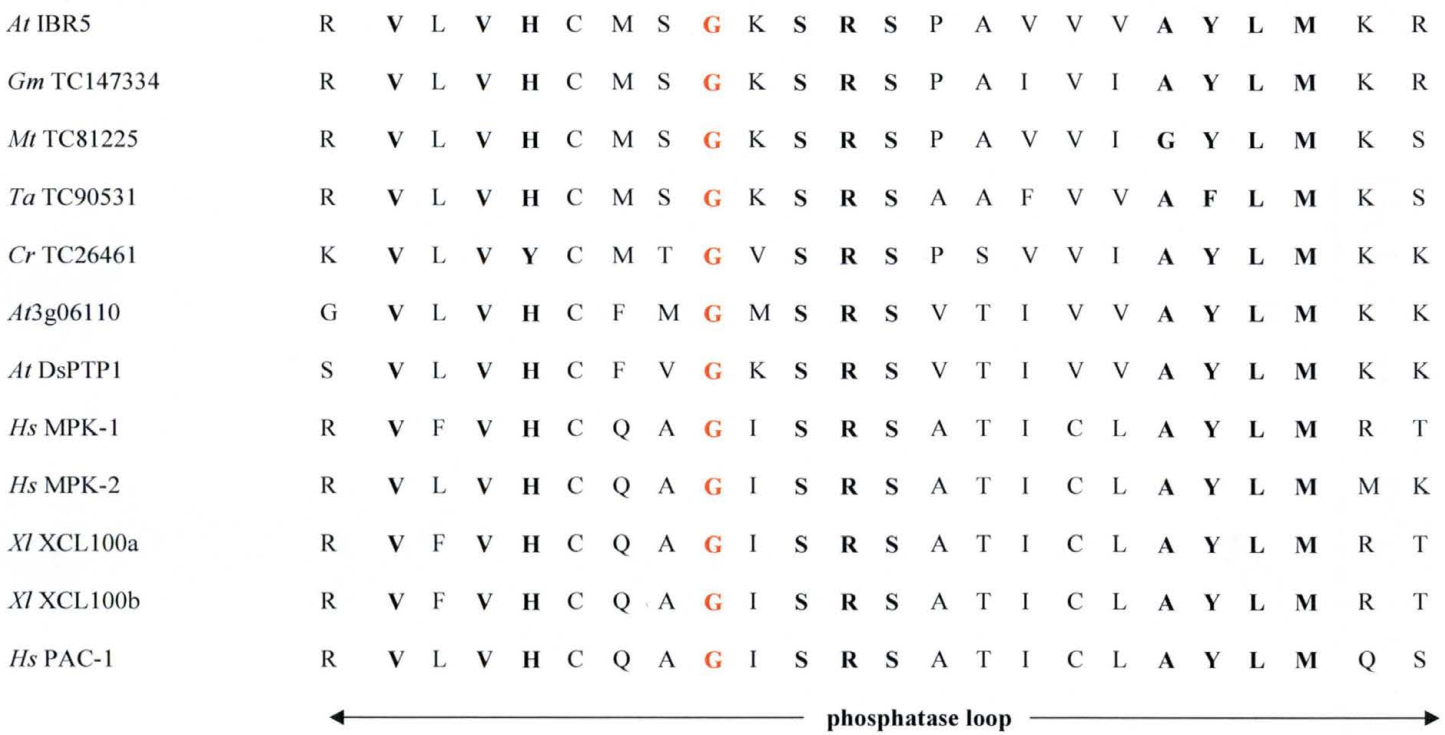


Figure 18. The DSP Active Site Motif VxVHCx$_2$GxSRSx$_5$AYLM in the Phosphatase Loop is Conserved Throughout Eukaryotes. The glycine (G) (highlighted in red) is replaced by a glutamate (E) in *pic115* mutants. [*Arabidopsis thaliana* (*At*), soybean (*Gm*), *Medicago truncatula* (*Mt*), wheat (*Ta*), *Chlamydomonas reinhardtii* (*Cr*), human (*Hs*), *Xenopus laevis* (*Xl*)].

Stability of the AXR3-GUS Recombinant Protein in *pic59* and *pic115* Background

Aux/IAA proteins have a very short life cycle and are rapidly degraded in the presence of auxin (Worley *et al.,* 2000; Zenser *et al.,* 2003). In most of the currently known auxin response mutants, the Aux/IAA repressor proteins are stabilized. The stability of Aux/IAA proteins in *pic59* and *pic115* backgrounds was studied using the AXR3-GUS recombinant protein. Seedlings that are homozygous for both *pic59* and *HS AXR3-GUS* or *pic115* and *HS:AXR3-GUS* were selected and used for studying the stability of AXR3-GUS recombinant protein. When compared to the wild type, the recombinant protein was less abundant in both *pic59* and *pic115* backgrounds (Figure 19 and 20). This indicates that AXR3-GUS protein is rapidly degraded in these mutant backgrounds.

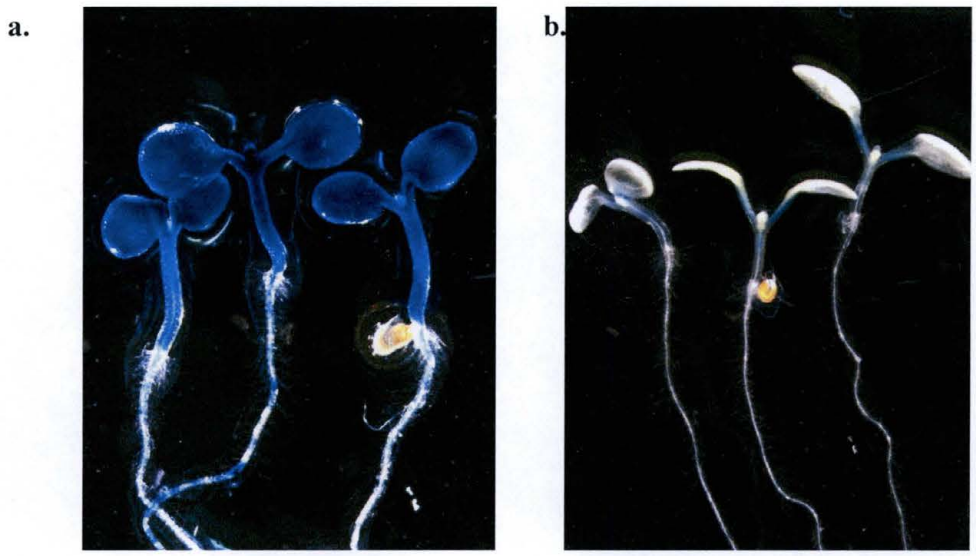


Figure 19. Stability of AXR3-GUS Recombinant Protein in *pic59*. (a) Stability of AXR3-GUS recombinant protein in Col-0 plants is shown by GUS staining immediately after a two hour heat shock. (b) Stability of AXR3-GUS recombinant protein in *pic59* plants is shown by GUS staining immediately after a two hour heat shock. The low stability of AXR3-GUS in *pic59* background compared to wild type is indicated by the lower color intensity. (The GUS staining was done by S. Dharmasiri)

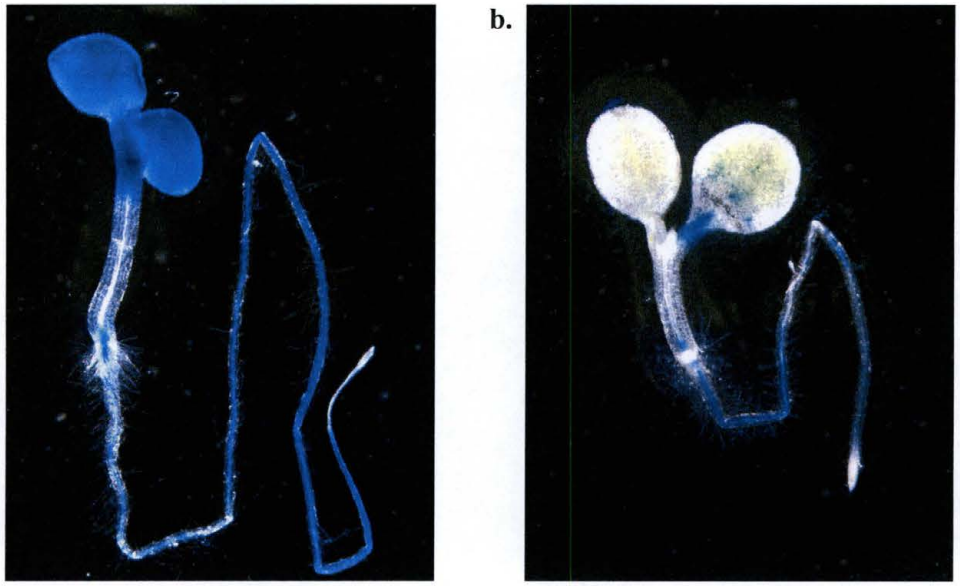


Figure 20. Stability of AXR3-GUS Recombinant Protein in *pic115*. (a) Stability of AXR3-GUS recombinant protein in Col-0 plants shown by GUS staining immediately following a two hour heat shock. (b) Stability of AXR3-GUS recombinant protein in *pic115* plants is shown by GUS staining immediately following a two hour heat shock. The low stability of AXR3-GUS in *pic115* background compared to wild type is indicated by the lower color intensity.

Auxin-Dependent Gene Transcription in *pic59* and *pic115* Background

Auxin-dependent gene transcription was examined using the *DR5::GFP* reporter system. *DR5* is a synthetic auxin promoter that is induced in the presence of auxin. When F3 seedlings homozygous for *pic59* and *DR5::GFP* or *pic115* and *DR5::GFP* were treated with auxins such as picloram or 2,4-D, auxin-induced GFP expression levels were lower in both *pic59* and *pic115* backgrounds compared to those of the wild type Col-0 background. These results indicate that auxin-dependent gene transcription is impaired in *pic59* (Figure 21) and *pic115* mutants (Figure 22).

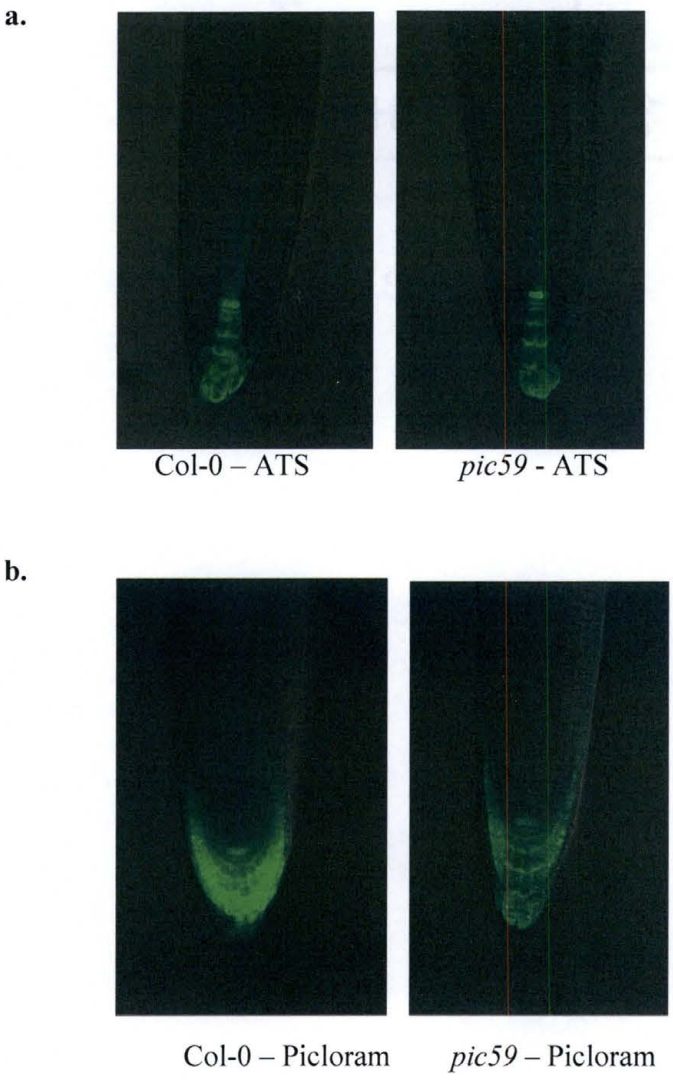


Figure 21. GFP Expression in *pic59*. Confocal images of root tips of (a) Col-0 and *pic59* seedlings on ATS medium (b) Col-0 and *pic59* on ATS containing 12 μM picloram. When grown on picloram medium, visual observations indicate a higher expression of GFP in Col-0 background compared to *pic59* background. (Confocal images were taken by S. Dharmasiri)

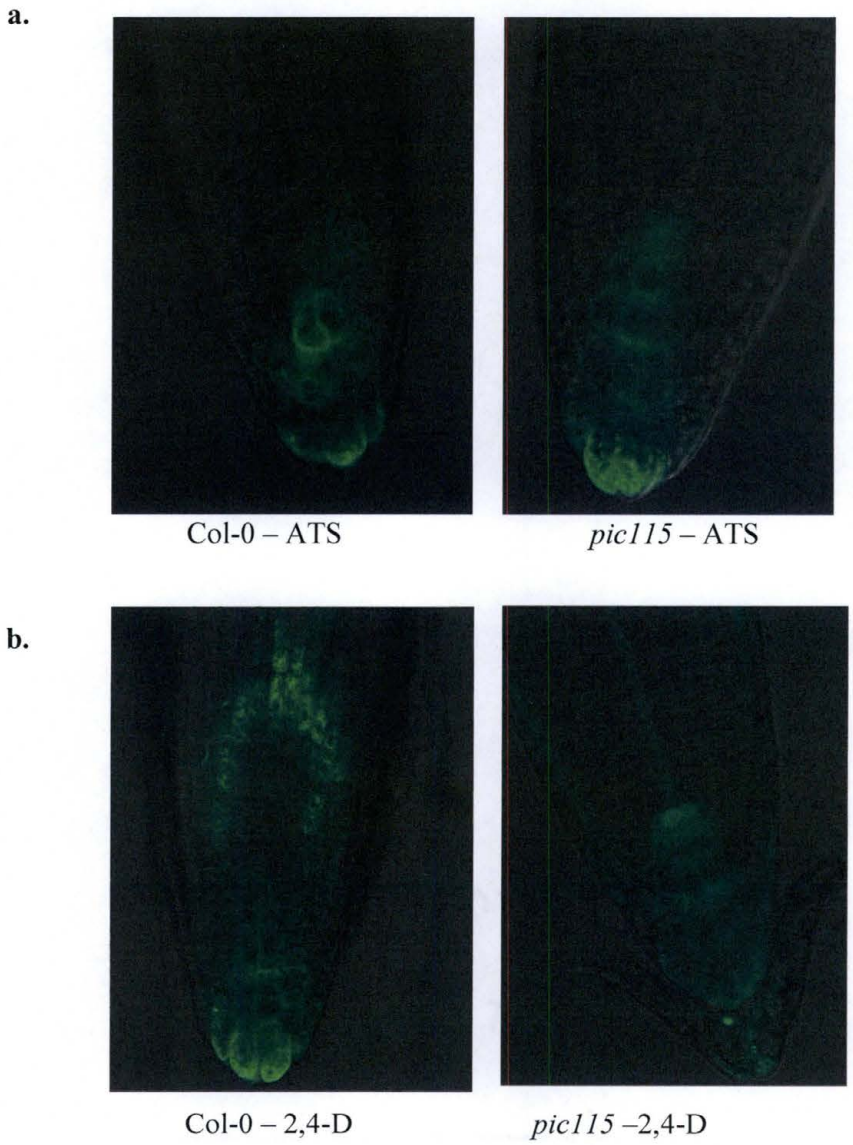


Figure 22. GFP Expression in *pic115*. Confocal images of root tips of (a) Col-0 and *pic115* seedlings on ATS medium (b) Col-0 and *pic115* on ATS containing 70 nM 2,4-D. When grown on 2,4-D medium, visual observations indicate a higher expression of GFP in Col-0 background compared to *pic115* background. (Confocal images were taken by J. Koke)

Expression of Auxin Induced Aux/IAA Genes in *pic115*

In both Col-0 and *pic115* background the mRNA level of *IAA2* increased in the presence of picloram. The mRNA level was low in the untreated sample and in the presence of 2,4-D in both Col-0 and *pic115* background.

In both Col-0 and *pic115*, *IAA12* mRNA was not detected in either samples derived from untreated plants or plants grown in the presence of 2,4-D. In the Col-0 background, there was a slight increase in *IAA12* in the presence of picloram. This increase of *IAA12* in the presence of picloram was not observed in the *pic115* background (Figure 23).

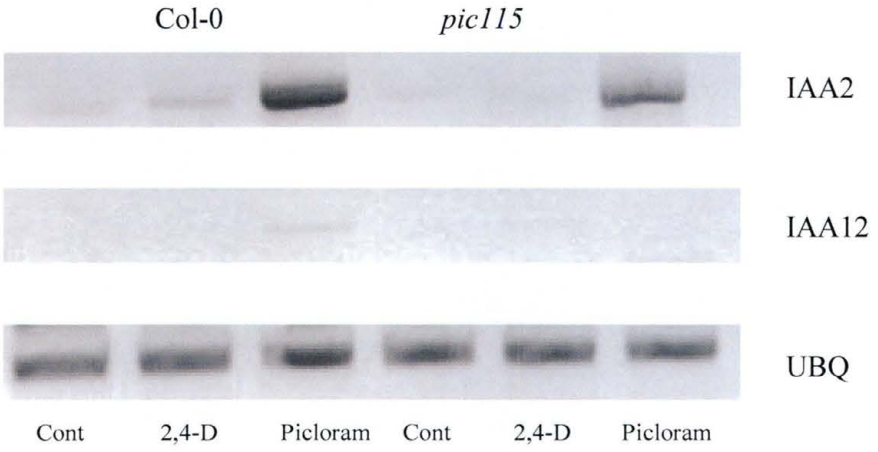


Figure 23. Auxin Induced Expression of Selected IAA Genes in *pic115*. RT-PCR results show the expression of *IAA2* and *IAA12* genes in Col-0 and *pic115* background with and without auxin treatment. Ubiquitin was used to standardize the amount of mRNA in all the samples.

Expression Pattern of *PIC115*

PIC115::*PIC115-GUS* gene construct revealed that the IBR5 DSP enzyme encoded by the *PIC115* gene expression is restricted to specific areas during the seedling stage. In the root the protein was found in the region a few millimeters above the root tip and in the junctions where secondary roots branch off. At these junctions the protein was located specifically in the primary root; the secondary roots did not contain the PIC115-GUS protein. In the shoot, the protein was localized to a region just below the shoot apex. The protein was also localized to the junction between the root and shoot (Figure 24).

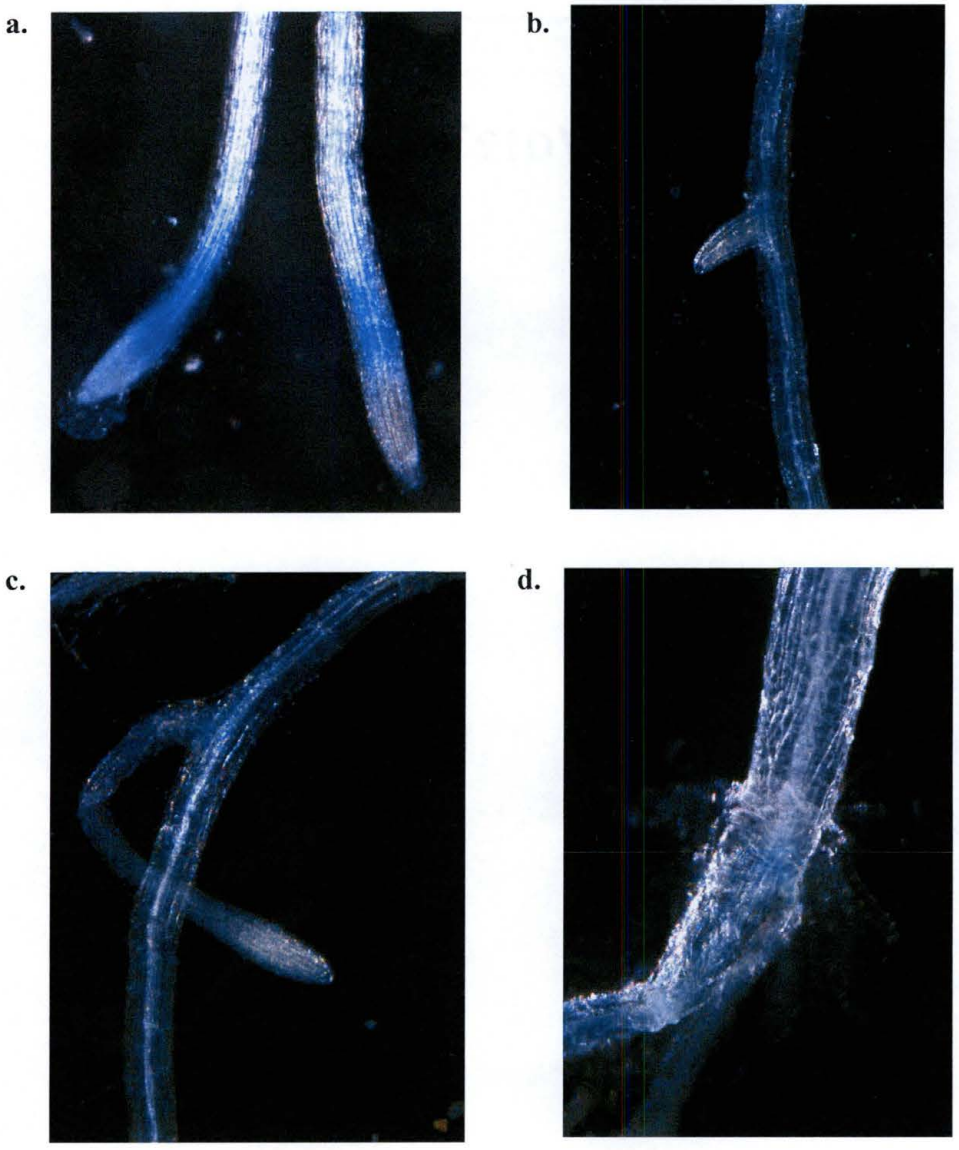


Figure 24. Expression of *PIC115-GUS* Recombinant Gene. (a) In the root tip PIC115-GUS is localized to a region a few millimeters above the root tip. (b) and (c) PIC115-GUS is located in the root junctions where secondary roots branch off. In these areas the protein is localized only in the primary root; no protein is observed in the secondary root. (d) PIC115-GUS is located in the area that connects the shoot and root. (e) In the shoot the protein was localized just below the shoot apex and throught the leaves.

Figure 24. continued.

e.

Genetic Interactions between Mutants

The homozygous double mutants created for *pic115 afb5*, *pic115 sgt1b* and *pic115 pic59* were analyzed to determine the genetic interactions between the mutants. *pic115 afb5* double mutant had a very severe shoot phenotype. The double mutants were very small in size compared to wild type. Also it had small leaves, produced very small siliques and less than five seeds per silique. *pic115 sgt1b* double mutant although less severe than *pic115 afb5*, was also shorter than the wild type and had smaller leaves and siliques. The *pic115 pic59* double mutants were the same size as the parents and wild type but had smaller siliques and lacked apical dominance (Figure 25). In addition when grown on 12 μM picloram the primary root length of *pic115 pic59* double mutants were longer than either of the individual parent mutant (Figure 26).

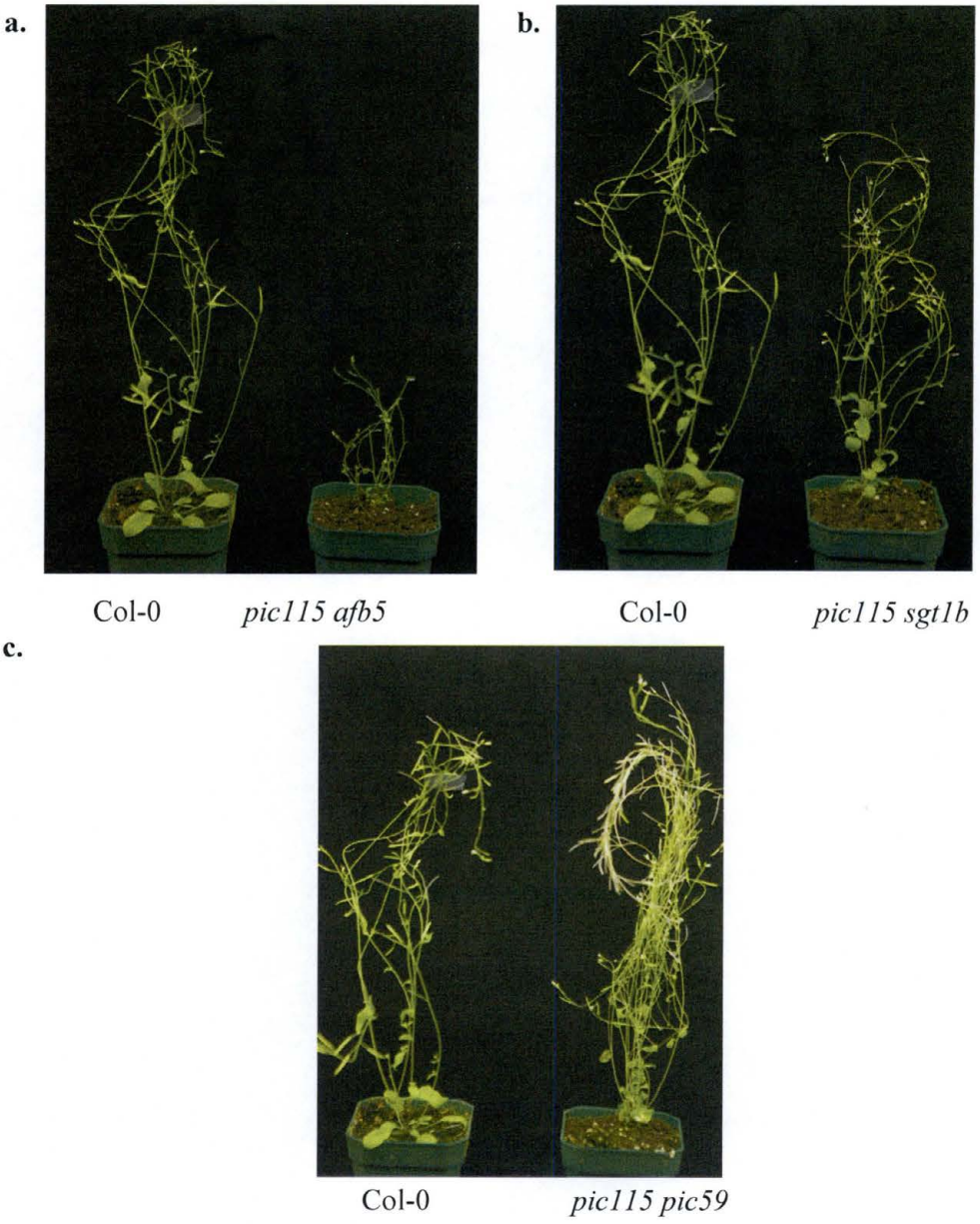


Figure 25. Phenotype of Double Mutants. (a) *pic115afb5* double mutant shows severe defects compared to the wild type. (b) *pic115sgt1b* double mutant shows defects compared to the wild type (c) *pic115pic59* double mutant shows defects compared to wild type.

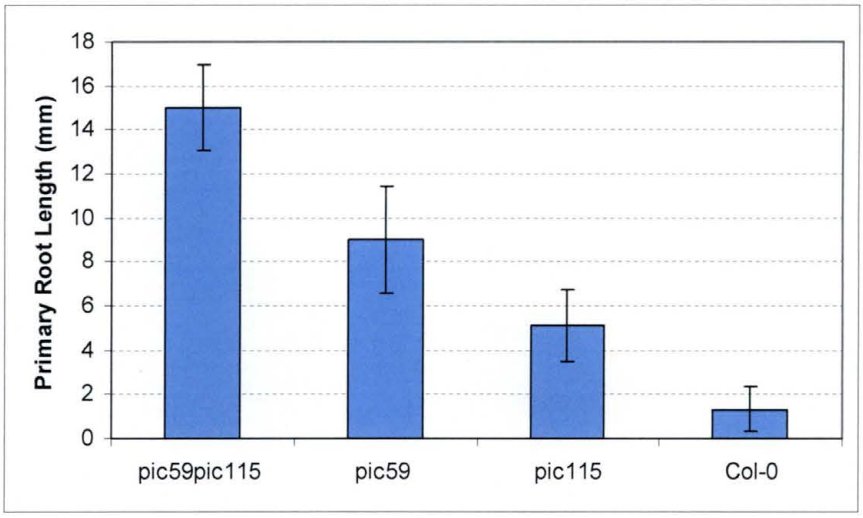


Figure 26. Primary Root Length of *pic115 pic59*. When grown on 12 μM picloram the primary root length of *pic115 pic59* was significantly higher than either of the individual parent *pic59* ($P < 0.05$) or *pic115* ($P < 0.05$) or the wild type Col-0 ($P < 0.05$). Error bars represent standard errors of the means ($N > 10$).

CHAPTER IV

DISCUSSION

Auxin is a pivotal plant hormone that regulates almost all growth and developmental processes of a plant. Many components involved in auxin response pathways in plants, including auxin synthesis, transport and signaling, have been identified; nevertheless, our knowledge of the molecular mechanisms involved in auxin signaling is far from complete. To identify novel genes involved in auxin response in *Arabidopsis*, this study utilized a chemical genetic approach using the synthetic auxin picloram to screen auxin resistant mutants. Previous studies in our laboratory indicate that picloram may function in a pathway different from many other known auxins which act through the TIR1-dependent pathway (Dharmasiri *et al*, unpublished data). By screening approximately 70,000 EMS mutagenized *Arabidopsis* seedlings, 30 putative auxin-resistant mutant lines were identified (Dharmasiri *et al.*, unpublished data). *pic59* and *pic115* represent two of these mutant lines with altered response to picloram. As these mutants are resistant to picloram and the *tir1* mutant is sensitive to picloram, it was hypothesized that these mutants may be involved in plant auxin response but in TIR1-independent manner.

Auxin Resistance of *pic59* and *pic115*

Similar to many other known auxin response *Arabidopsis* mutants, results in this study show that *pic59* and *pic115* mutants are resistant to synthetic auxins, such

as 2,4-D and picloram. The primary root growth of *pic59* and *pic115* are also resistant to the naturally occurring auxin IBA. Primary root growth of *pic115* is also resistant IAA, *pic59* is sensitive in this response. While both *pic59* and *pic115* mutants are resistant to picloram and 2,4-D, *pic115* is more resistant to 2,4-D but is less resistant to picloram (Dharmasiri S, unpublished data); in contrast *pic59* is more resistant to picloram but is less resistant to 2,4-D. Additionally, both these mutants are also defective in normal ABA responses. These results suggest that both these mutants may be involved in a signaling network with overlapping pathways that has both distinct and common signaling components. In support of this argument, another picloram resistant mutant *pic30* is only resistant to picloram but not to any other auxin (Dharmasiri S, unpublished data). However, it is also possible that *PIC59* and *PIC115* may function in the same pathway as discussed below.

Table 6. Resistance Pattern of Mutants Based on Primary Root Growth

Mutant	Auxin			
	2,4-D	picloram	IBA	IAA
pic59	+	+++	++	-
pic115	+++	+	+	++

+ signifies resistance; - signifies sensitivity

Both these mutants are also defective in normal ABA responses, suggesting that both *PIC59* and *PIC115* genes may be involved in both auxin and ABA signaling networks that share common components. However, the two mutants used in this study did not show any defect in cytokinin or ethylene response suggesting *pic115* or *pic59* may not be involved these hormonal responses.

PIC59, a Novel Gene Associated with Auxin Responses

pic59 is a mutant that shows resistance to the synthetic auxins picloram and 2,4-D and the naturally occurring auxin IBA. In addition *pic59* is resistant to the plant stress hormone ABA. *pic59* is inherited as a semi-dominant mutation. The mutation

was mapped to a narrow genetic window of 14 genes in the south arm of chromosome V. This genetic window contains no previously identified genes associated with auxin signaling; therefore, the identity of *pic59* will reveal a newly described gene involved in auxin signaling. According to the Genbank annotations most of the genes in the genetic window encode proteins with unknown functions. Nevertheless, a closer look at the predicted amino acid sequences of these genes indicate the presence of conserved domains such as WD40 repeats, AP2 domains, zf-DHHC, PMEI, DUF393 and NADB-Rossmann (http://www.ncbi.nlm.nih.gov/). While candidate genes in this window are being sequenced, the presence of these domains will help to predict the possible function of PIC59 in plant auxin and ABA response.

pic115 Encodes a DSP

pic115 was located to the north arm of chromosome II in the annotation unit T1O3. The mutation was identified as an allele *of ibr5*. *IBR5* encodes a 257-amino acid protein that contains the highly conserved dual-specificity phosphatase active site motif $VxVCHx_2GXSRSx_5AYLM$ suggesting that IBR5 is a dual specificity phosphatase (putative Map Kinase Phosphatase - MKP). It is among the five identified MKPs in the *Arabidopsis* genome (Monroe-Augustus *et al.,* 2003). The mutant *ibr5-1* was first identified using a forward genetic approach to isolate genes that are defective in IBA response in *Arabidopsis* (Monroe-Augustus *et al.,* 2003). Dual specificity phosphatases are involved in dephosphorylation of phosphorylated MAP kinases (Mishra *et al.,* 2006). Therefore, this finding indicates that MAP kinase signaling is associated with auxin signaling. Previous attempts to find the substrate for IBR5 were not successful (Monroe-Augustus *et al.,* 2003). However, in a recent study using yeast-two hybrid system, Lee *et al.,* (2008) identified *Arabidopsis* MPK12

as the substrate for IBR5. Moreover, this study showed that homozygous *mpk12* knock-out lines were lethal, and suppression of MPK12 expression by RNAi led to auxin hypersensitivity, suggesting that MPK12 negatively regulates the plant's sensitivity to auxin (Lee *et al.,* 2008). This inference is further supported by the positive regulation of auxin signaling by IBR5 (Strader *et al.,* 2008). The *ibr5-1* mutation resulted from a premature stop codon at position 42 that results in a null mutant (Monroe-Augustus *et al* , 2003). In *pic115*, the single base pair change of $132^{G\text{-}A}$ results in a substitution in the highly conserved DSP active site domain $VxVCHx_2GXSRSx_5AYLM$. This missense mutation results in replacement of glycine (G) with glutamate (E). *pic115* is the first identified allele of *IBR5* with a change in the DSP active site of the enzyme. This amino acid substitution may either completely abolish or reduce the activity of the enzyme, leading to inhibition of the auxin response that results in *pic115* becoming resistant to the auxins. This conjecture is supported by the finding that auxin-induced Aux/IAA mRNA expression is reduced in the *pic115* mutant as is induction of *DR5::GFP* expression. Nevertheless, whether these altered auxin responses are caused by the reduced stability of the mutant PIC115 is not known at this time.

While previously described *ibr5-1* allele shows more severe shoot phenotype, *pic115* is morphologically similar to wild type plants except for silique length. Since *ibr5-1* appears to be a null allele, these observations suggest that despite the catalytic site mutation, the mutant protein in *pic115* may retain some enzymatic activity. Alternatively the PIC115 protein may have functions other than DSP activity which are still intact despite the mutation. It is also possible that morphological differences

we see in these two different alleles of IBR5 are due to different growth conditions or practices used in two laboratories.

PIC115 Expression in *Arabidopsis*

The function of a gene is closely related toits expression pattern. The *PIC115::PIC115-GUS* translational gene construct created to examine the spatial and temporal expression pattern of *PIC115* revealed that *PIC115* is expressed highly in cotyledons, at the base of young leaves, in the expansion region of the primary root and at the sites of lateral root emergence. All these tissues are associated with cell expansion. Thus, it is possible that PIC115/IBR5 may have a function in cell expansion, especially during the early seedling development. Experiments are underway to assess the developmental regulation of *PIC115/IBR5*. A previous study shows that IBR5 is expressed at the very end of the primary root tip in the cell division zone (Monroe-Augustus *et al.,* 2003) however we observed IBR5 expression in cell expansion zone. This discrepancy may be due to the fact that the previous study used a transcriptional reporter construct whereas this study used a translational reporter construct. The presence of additional gene sequences may be necessary to regulate the exact expression pattern.

pic59 and *ibr5/pic115* in Auxin Signaling

It has been proposed that IBR5 promotes auxin response through a novel mechanism that is distinct from TIR1-mediated repressor degradation (Strader *et al* , 2008). Experimental evidence showed that the Aux/IAA repressor reporter protein AXR3NT-GUS was not stabilized in an *ibr5* background. In several identified auxin response mutants, Aux/IAA proteins are stabilized. These include *tir1* (Gray *et al.,* 2001), *axr-1*(Gray *et al.,* 2001), *ecr1* (Woodward *et al* , 2007), *tir1afb2afb3* triple

mutant (Dharmasiri *et al.,* 2005), *cul1* (Quint *et al.,* 2005), *eta2/cand1* (Chuang *et al.,* 2004), *eta3/sgt1b* (Gray *et al.,* 2003) and *aar1* (Rahman *et al* , 2006). These results are expected as a mutation in the SCF complex or related molecular components will affect the degradation of Aux/IAA proteins. In an *ibr5* background AXR3NT-GUS is rapidly degraded. This rapid degradation in AXR3NT-GUS was also observed in the *tir1ibr5* double mutant, suggesting that *ibr5* suppressed AXR3NT::GUS accumulation in *tir1* background. In addition the authors showed that one of the Aux/IAA repressors, IAA28, is less abundant in *ibr5* background compared to the wild type. Using these experimental evidences it was postulated that IBR5 regulates auxin response in a TIR1 independent pathway (Strader *et al.,* 2008).

The data obtained through this research revealed that an allele of *ibr5*, *pic115* also shows a rapid degradation of AXR3NT-GUS proteins. This indicates that both alleles of *ibr5*, despite the difference in the location of the mutation, promote destabilization of Aux/IAA proteins.

Interestingly, *pic59* shows a similar degradation pattern as *ibr5/pic115*. The AXR3NT-GUS was rapidly degraded in *pic59* background. This suggests that both mutants are affecting the degradation of Aux/IAA proteins in a similar manner. Also this degradation pattern differs from the pattern seen in most published auxin response mutants. These data suggest that *pic59* is an unidentified mutant that maybe involved in the same or overlapping signaling pathway as *ibr5/pic115*. Therefore, *pic59* may reveal a novel gene associated with auxin signaling mechanism involving MAP kinase components.

The data obtained through the *pic115 pic59* double mutants also suggest that both mutants may be components of the same or overlapping signaling pathway.

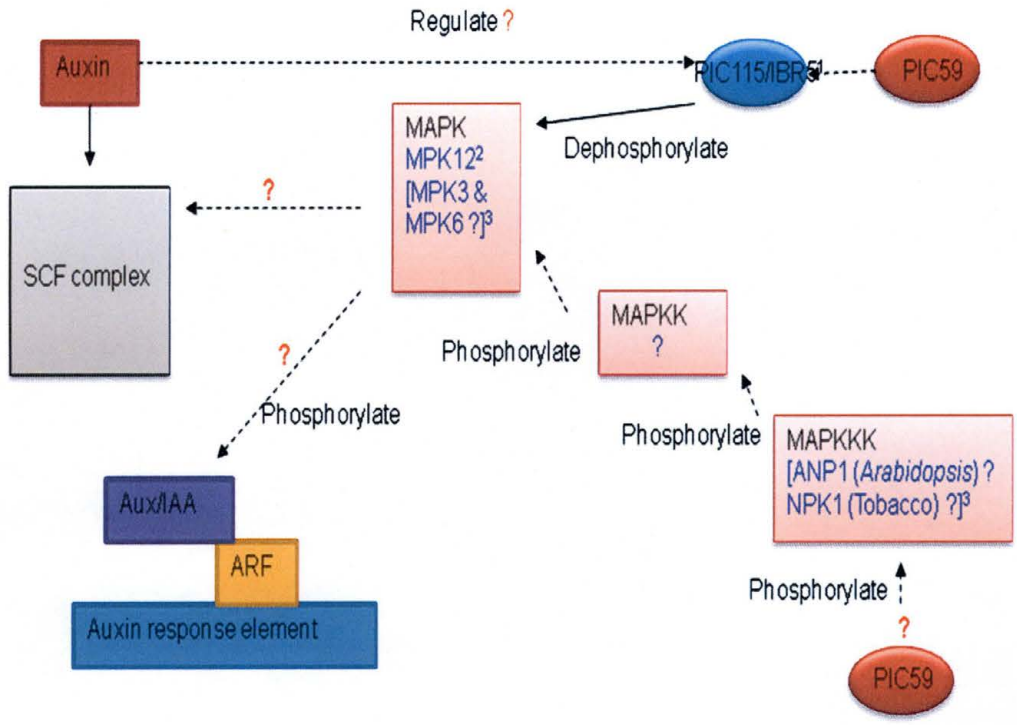
While *pic115 pic59* double mutant exhibits relatively minor enhancement of defects in shoot system, level of auxin resistant root growth shows an additive effect. It is possible that these two genes may have different functional relationships in root and shoot systems. In addition *pic115 afb5* and *pic115 sgt1b* both show more severe defects in shoot system than their respective single mutants suggesting synergistic effects. While these observations suggests the possibility that these genes may function in the same or overlapping pathways; further analysis of these double mutants and biochemical analyses will be necessary for an unambiguous conclusion.

PIC59 and *PIC115* – Reveal a Novel Auxin Signaling Mechanism

IBR5/PIC115, a DSP enzyme, may be involved in a MAP kinase signaling pathway in auxin signaling. This hypothesis is further supported by the recent observation that MPK12 functions as the substrate for IBR5 (Lee *et al*, 2008). The high similar physiological and molecular characteristics of *pic59* and *pic115* and their genetic interactions suggest that both these genes may function in the same or closely related signaling pathways. The involvement of a MAP kinase signaling pathway in auxin response has long been proposed but still suffers from a lack conclusive evidence for the major components and signaling mechanisms.

MAPK signaling is proposed to involve a sequential phosphorylation cascade of three consecutively acting protein kinases with MAPKs being the last component. MAPK is activated by the phosphorylation of threonine and/or tyrosine residues by MAP kinase kinase (MAPKK), which in turn is activated by phosphorylation on two serine or threonine residues by MAP kinase kinase kinase (MAPKKKs) (Nakagami *et al.*, 2005). The MAPKK and MAPKKK involved in auxin signaling are yet to be identified. In addition interaction of MPK12 with the currently known components of

the auxin response pathway such as the SCF complex or Aux/IAA proteins is yet to be demonstrated. Interestingly, TIR1 and other AFBs contain putative phosphorylatable sequences (N. Karunarathna., unpublished data). Additionally, some Aux/IAA proteins are known to be phosphorylated (Colón-Carmona *et al.*, 2000). Whether the MAP kinase pathway is involved in phosphorylating these proteins is currently not known. Taking all the current evidence together it is possible to present a hypothetical model on the mechanism of auxin signaling through the MAP kinase pathway. It is important to note that this model is based on the very few facts known to date, and therefore is tentative (Figure 27).



Interaction has been published

Hypothetical interaction

Figure 27: Schematic Diagram of a Possible Pathway for Auxin Signaling through a Mitogen-Activated Protein Kinase Cascade. Monroe-Augustus *et al.,* (2003) and the work presented in this thesis identified IBR5 as a DSP involved in auxin signaling. MPK12 was identified as the substrate of IBR5 (Lee *et al.,* 2008). ANP, NPK1, MPK3 and MPK6 have been demonstrated to be involved in auxin signaling (Kovtun *et al.,* 1998a; 2000b). Most of the other components and the signaling mechanism remain unidentified to date. The current research identified PIC59 as a possible previously unidentified component of this mechanism. Although the position of PIC59 in this pathway was not identified, PIC59 may be a component of the mechanism that activates MAPKKK or on the other hand deactivates IBR5. This prediction can be made based on the degradation pattern of AXR3-GUS, where the recombinant protein is rapidly degraded in the mutant background. Strader *et al.,* (2008) showed that in the null mutant *ibr5-1* the degradation of AXR3-GUS occurs more rapidly than in the wild type. Since *pic59* is a gain of function mutation, based on the semi dominant nature of the mutant, it is possible that PIC59 functions in the mechanism that deactivates IBR5. Alternatively it is also possible that PIC59 may be involved in activating MAPKKK, which would also result in a rapid degradation of the recombinant protein in the mutant background.

CONCLUSIONS AND FUTURE DIRECTIONS

In this study, *pic59* and *pic115*, two mutants with altered response to picloram, were characterized. My hypotheses that *pic59* and *pic115* are caused by single gene mutations and that PIC59 and PIC115 may be involved in a novel auxin signaling mechanism were supported by the data obtained. *pic115* was identified as an allele of *ibr5-1*, with the mutation G727A. IBR5 is known to dephosphorylate Mitogen Activated Protein kinase 12 (MPK12) in *Arabidopsis*. These results along with results from other laboratories suggest a strong connection between auxin signaling and a MAP kinase pathway in plants. *pic59* is a mutant with altered response to some synthetic and natural auxins as well as ABA, a plant hormone associated with stress response. While *pic59* has not yet been identified, the segregation pattern of F2 seeds obtained from the cross between *pic59* and Col-0 suggest that *pic59* is a single gene mutation. As there are no genes associated with auxin signaling in the genetic window to which *pic59* has been mapped, once identified *pic59* will reveal a novel gene associated with auxin signaling. The data obtained with the degradation of AXR3-GUS suggests that PIC59 may function in the same signaling mechanism as IBR5/PIC115.

This research gives evidence that links auxin signaling with MAP kinase pathway, and there are many future directions for research. Identification of the remaining components of this pathway in order to build a testable model will be of great importance. Suppressor mutants have been created from *ibr5* (Strader *et al.*, 2008). The authors have 42 confirmed mutants, which when identified will reveal novel components of this signaling pathway. In addition, our laboratory has created mutant lines selected on picloram. The screen reported herein identified *pic115*, an allele of *ibr5*. In addition our lab has isolated several mutants that maybe involved in

the MAP kinase pathway (Dharmasiri, unpublished data). Identification of these mutants, some of which were not included in the genetic windows proposed by Strader *et al.,* (2008), will further help in completing the pathway. The *pic59* mutation affects an as yet uncharacterized gene associated with auxin signaling. As the current data suggest that PIC59 and IBR5/PIC115 function in the same or closely related signaling pathways, once the gene is identified it will be crucial to find the function of the protein produced by *pic59* with respect to auxin signaling.

The immediate targets of MPK12 are unknown and have to be identified. It is possible that MPK12 phosphorylates a target in the SCF complex or directly acts on Aux/IAA repressor proteins. Interactions among these molecular components should be studied with biochemical and molecular genetic approaches. Evidence so far suggests that there is a conserved mitogen activated protein kinase cascade involved in auxin signaling. This pathway strictly would contain highly conserved MAPK, MAPKK and MAPKKK. Researchers have so far been able to identify the MAPK in this cascade as MPK12 (Lee *et al.,* 2008) and possibly the MAPKKK, NPK1 (Tobacco) ANP1 (*Arabidopsis*) (Kovtun *et al.,* 1998, 2000). Adding to the complexity, although IBR5 is shown to specifically dephosphorylate MPK12, MPK3 and MPK6 have also been suggested to be involved with auxin signaling (Hirt 2000). Further, the mechanism by which the MAPKKK is activated in order to begin the phosphorylation cascade is unknown. It would be of great interest to find out if the kinase cascade is activated in response to auxin and/or other stress factors. In addition the cues that activate IBR5 and that dephosphorylate MAP kinases should be identified. Whether auxin regulates *IBR5* gene transcription is another avenue that should be studied. Currently, there are no data on the mechanism regulating *IBR5* genes although there is a high likelihood that auxin may be involved.

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