ISOLATION AND CHARACTERIZATION OF *ARABIDOPSIS* MUTANTS WITH ALTERED RESPONSE TO AUXIN (PICLORAM)

THESIS

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ISOLATION AND CHARACTERIZATION OF *ARABIDOPSIS* MUTANTS WITH ALTERED RESPONSE TO AUXIN (PICLORAM)

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF ARABIDOPSIS MUTANTS WITH ALTERED RESPONSE TO AUXIN (PICLORAM)

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The phyto-hormone auxin is vital for the plant growth and development throughout the plant's lifecycle. At the molecular level, auxin rapidly modulates the expression of auxin responsive genes by inducing degradation of a family of negative regulators known as Aux/IAA proteins. Besides the major natural auxin indoleacetic acid (IAA), there are several other natural and many synthetic auxins. Picloram and 2,4-dichlorophenoxyacetic acid (2,4-D) are two synthetic auxins that are commonly used as herbicides. Our initial results suggest that picloram may function somewhat differently

from other commonly used auxins such as IAA, 2, 4-D and 1-naphthalene acetic acid (1-NAA).

To understand the molecular mechanisms of auxin responses in plants, we isolated a range of mutants that exhibit altered response to picloram. The goal of my research was to characterize two of these mutants, *pic11* and *pic32*. The mutant *pic32* is hypersensitive to picloram but not to other commonly known auxins. *pic32* mutation was mapped to a 62 kb region that includes 17 genes in chromosome V of the *Arabidopsis* genome.

The mutant *pic11* is resistant to both picloram and 2,4-D. Compared to wild type, *pic11* exhibits many growth and development defects. It produces fewer and shorter root hairs compared to wild type and no lateral roots. Several auxins such as 2,4-D, IAA and IBA stimulate lateral root initiation in *pic11* at higher concentrations, suggesting that *pic11* mutation does not affect the ability to produce lateral roots, but may diminish sensitivity to auxin. Picloram also induces few lateral roots in *pic11*; however, all lateral roots initiate at the same point on the root just below to the hypocotyl suggesting that picloram response is severely compromised by the *pic11* mutation. While *pic11* is also defective in root hair development, it can be rescued by 1-aminocyclopropane-carboxylic acid (ACC), the immediate precursor of ethylene, suggesting that *PIC11* functions upstream of ethylene synthesis and signaling. Using map based cloning, the *pic11* mutation was identified as an allele (*iaa28*) of the *IAA28* gene that encodes an Aux/IAA protein (IAA28), a repressor of auxin induced gene transcription.

pic11/iaa28 causes a gain of function mutation in domain II of IAA28 protein. While the wild type IAA28 interacts with the auxin receptor protein AFB1 in an auxin-dependent manner, mutation of domain II interferes with this interaction, probably leading to the stabilization of the mutant *pic11/iaa28* protein. Results of this study along with previous studies by others suggest that the stabilized mutant protein may directly affect the regulation of other auxin-inducible genes that are important in growth and development.

CHAPTER I

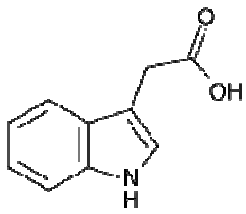
INTRODUCTION

The term auxin is derived from the Greek word “auxein” which means to grow. Auxin was the first plant hormone to be discovered. It is vital for plant growth and development throughout the plant’s lifecycle. As a critical plant hormone, it plays important roles in a number of plant activities, including embryo development, leaf formation, tropic responses to light and gravity, determination of general root and shoot architecture, organ patterning, vascular development, apical dominance, flowering, fruit development and abscission (Nemhauser *et al*., 2000; Sessions *et al*., 1997). From the very first mitotic division of the zygote, a gradient of auxin guides the patterning of the embryo into different organs of the plant. The formation of new leaves in apical meristem is initiated by the accumulation of auxin (Tames, 1988). Developing leaves deplete the surrounding cells of auxin, so that the new leaves will not form too close to them. In this way, the characteristic pattern of leaves in the plant is established (Davis, 1995). Apical dominance is maintained by auxins. It results from the downward transport of auxin produced in the apical meristem. Thus, removal of the shoot apical meristem will induce the growth of lateral buds. Further, auxin plays a major role in the abscission of leaves and fruits. Young leaves and fruits produce auxin, and so long as they produce auxin, they remain attached to the stem. When the level of auxin declines with maturity, an

abscission layer forms at the base of the petiole or fruit stalk causing the abscission of leaf or fruit (Sakamoto *et al*., 2008). Auxin also plays an important role in tropisms. In gravitrophism roots use specialized gravity sensing columella cells located in the root cap to monitor root orientation. After a gravistimulus, IAA redistributes to the lower side of the root epidermis and acts as an inhibitor of root cell elongation causing unequal cell expansion and root bending towards gravity (Bennett *et al*., 1996). Conversely, in phototropism, auxin redistributes to the side of the stem that is not exposed to light. This increases the cell elongation in the darker side of the stem bending it toward the light.

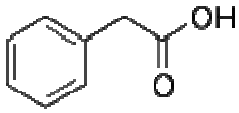
The most abundant natural auxin is indole-3-acetic acid (IAA) (Koegl *et al*., 1999). *Arabidopsis* seedlings can synthesize IAA in leaves, cotyledons and roots while young leaves have the highest biosynthetic capacity (Ljung *et al*., 2001). Most commonly plants use two biosynthetic pathways, tryptophan dependent and tryptophan independent, to synthesize IAA (Woodward and Bartel, 2005). In addition to IAA there are several other naturally occurring auxins, including 4-chloro-indoleacetic acid, phenylacetic acid (PAA) and indole-3-butyric acid (IBA) as well as many synthetic chemicals with auxinic activity such as 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), parachlorophenoxyisobutyric acid (PCIB), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram) and others (Figure 1). These synthetic chemicals exert auxin-like effects, including inhibition of root elongation and promotion of lateral root formation (Woodward and Bartel, 2005).

Natural auxins



Indole-3-acetic acid (IAA)

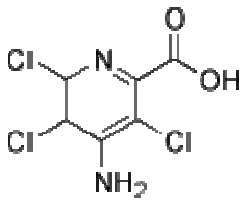
Indole-3-butyric acid (IBA)



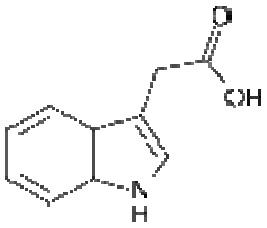
2-Phenylacetic acid (PAA)

Some synthetic auxins

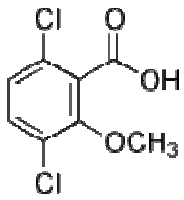
2,4-Dichlorophenoxyacetic acid (2,4-D)



4-Amino-3,5,6-trichloropicolinic acid (Picloram)



1- Naphthaleneacetic acid (1-NAA)



2-Methoxy-3,6-dichloro benzoic acid (Dicamba)

2,4,5-Trichlorophenoxy acetic acid (2,4,5-T)

Figure 1. Naturally-occurring auxins and some synthetic auxin analogs. The most abundant natural auxin is IAA. In addition there are several naturally occurring auxins and many synthetic chemicals with auxin activity. Some of these synthetic auxins have been used as herbicides for more than 50 years.

For more than 50 years synthetic auxins have been used successfully as herbicides in agriculture (Cobb, 1992; Grossmann, 2000). Major auxinic herbicide groups are chlorophenoxy acids, benzoic acids (e.g. dicamba), pyridines (e.g. picloram) and quinoline carboxylic acids (e.g. quinmerac, quinclorac). They mimic the effect of endogenous auxin compounds (Cobb, 1992; Grossmann, 2000). The early effects in sensitive dicots are characterized by growth abnormalities, such as epinasty (the downward bending of leaves), or other parts of plant and growth inhibition with intensified green leaf pigmentation within 24 hours. These phenomena are followed by chloroplast damage, chlorosis, and destruction of membrane and vascular system integrity, leading to desiccation, tissue necrosis and decay (Cobb, 1992; Grossmann, 2000). In sensitive grasses, auxinic herbicides induce the production of cyanide to a concentration that damages proper plant growth (Grossmann, 2000). Further it has been found that auxinic herbicides induce overproduction of H_2O_2 inside tissues which leads to tissue damage and cell death (Grossmann, 2000).

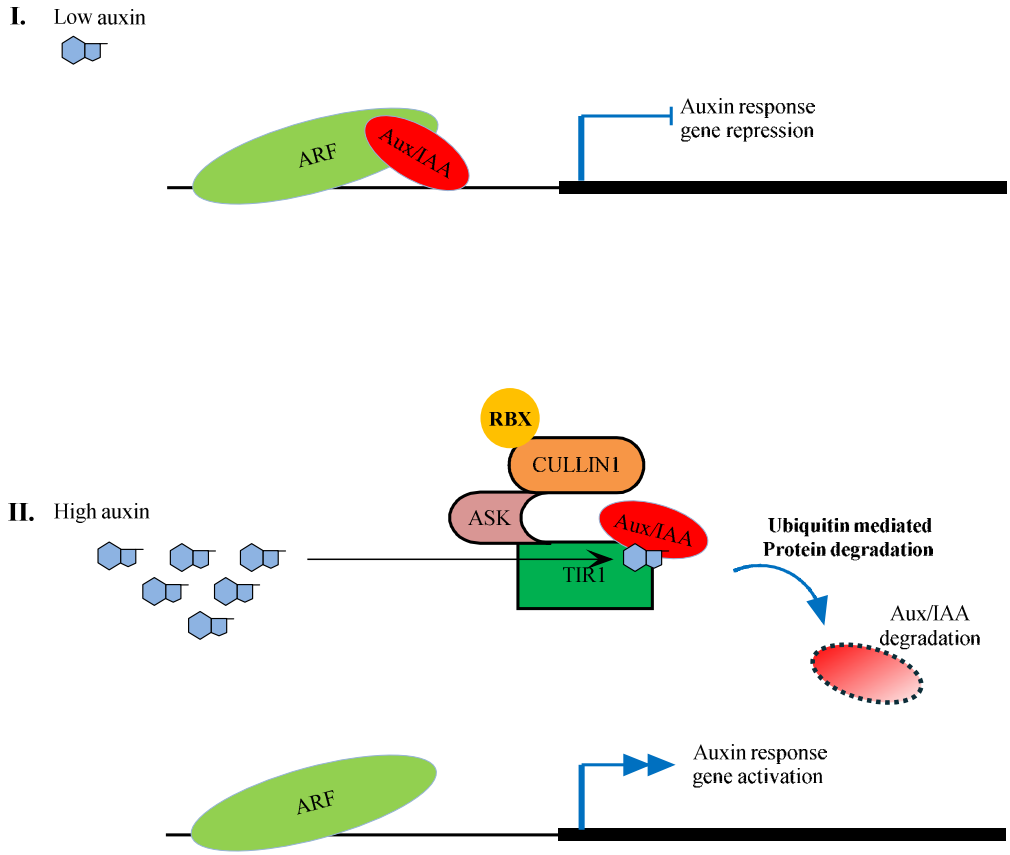
At the cellular level, auxin regulates cell division, cellular expansion and cell differentiation. Depending on the specific tissue, auxin may promote axial elongation (as in shoots), lateral expansion (as in root swelling), or isodiametric expansion (as in fruit growth). According to the acid-growth hypothesis, auxin stimulates growth of plant cells by regulating the activity of plasma membrane hydrogen adenosine triphosphatase (H^+ ATPase) which causes active transport of H^+ out of the cell. This acidification of the cell wall region activates wall-loosening proteins known as expansins that allow slippage of cellulose microfibrils weakening the cell wall. This allows the expansion of cells by turgor pressure (Senn and Goldsmith, 1988).

Auxin rapidly stimulates the transcription of many genes called primary auxin responsive genes. These include three families, *Aux/IAA* genes, *GH3* and *SAURs* (small auxin up-regulated genes) (Abel and Theologis, 1996). Molecular and genetic studies on auxin-resistant *Arabidopsis* mutants and biochemical analysis of auxin inducible genes have led to the identification of many components of the auxin signaling pathway. The current model of auxin signaling suggests that auxin rapidly modulates gene expression through the degradation of a group of repressor proteins called Aux/IAA proteins, and thereby causes expression of a network of genes resulting in proper growth and development. These repressor proteins are degraded through a highly regulated mechanism known as ubiquitin-proteasome pathway (Hellmann and Estelle, 2002) (Figure 2).

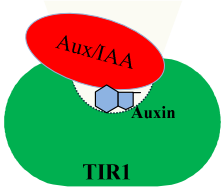
Aux/IAA proteins are short-lived, nuclear proteins. In *Arabidopsis thaliana* there are 29 *Aux/IAA* genes. Aux/IAA proteins act as negative regulators of auxin signaling by blocking the activity of a family of transcriptional factors called auxin responsive factors (ARFs). Most of Aux/IAA proteins share four conserved domains designated I to IV. Domain I of the Aux/IAA proteins is known to be involved in transcriptional repression (Tiwari *et al*., 2004), while domain II interacts with the receptor protein, TIR1 (Dharmasiri *et al*., 2005; Gray *et al*., 2001; Kepinski and Leyser, 2005). Domains III and IV are located in the C-terminal half of the protein and are involved in homodimerization with other Aux/IAA proteins, and heterodimerization with ARFs that also share domains III and IV.

There are 23 ARF genes in *Arabidopsis* (Guilfoyle and Hagen, 2001). ARFs contain an N-terminal DNA-binding domain (DBD) (Guilfoyle *et al.*, 1998b). ARFs bind to conserved DNA sequences (TGTCTC) called auxin response elements (AuxREs) in the promotor region of primary/early auxin response genes (Liscum and Reed, 2002). ARFs can act as either transciptional activators or repressors depending on the middle region of the ARF protein (Ulmasov *et al*., 1997). The ARFs with glutamine-rich middle regions function as activators whereas other ARFs with proline/serine/threonine-rich middle regions function as transcriptional repressors (Guilfoyle and Hagen, 2001). Therefore AFRs function as negative or positive regulators of transcription of Aux/IAA and other genes (Dharmasiri and Estelle, 2004).

The Aux/IAA proteins repress ARF function through heterodimerizing with domain III and IV of ARFs (Dharmasiri and Estelle, 2004; Reed, 2001). In the presence of auxin these repressor proteins are degraded through the ubiquitin proteasome pathway. First, Aux/IAA proteins are tagged with a polyubiquitin chain by a cascade of reactions involving three enzymes: ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin protein ligase (E3). The ubiquitination process starts with E1 forming a thiol-ester linkage with a ubiquitin molecule. Next, the ubiquitin molecule is transferred to E2 and finally covalently attached to the target protein with the help of an E3 ubiquitin ligase. This process is repeated until several ubiquitin molecules are attached to the target protein. Poly-ubiquitinated proteins are recognized and degraded by the 26S proteasome complex, a multiprotein complex with 20S core unit and two 19S regulatory particles (Dharmasiri and Estelle, 2004).



a. Current model of auxin signaling



b. A model of auxin regulated Aux/IAA TIR1 interaction

Figure 2. Current model of auxin signaling. (a) Aux/IAA repressor proteins degrade in the presence of high levels of auxin. Cartoon depicting some main steps in the current model of auxin signaling. The functional SCF^{TIR1} is represented in I. At low auxin concentrations the function of ARF is hindered by Aux/IAA proteins. II, at high auxin levels Aux/IAA proteins interact with E3 ligase through TIR1 and are subsequently degraded through ubiquitin proteosome pathway. (**b) A model of auxin-regulated Aux/IAA-TIR1 interaction.** A schematic diagram of auxin's function as a "molecular glue" to enhance TIR1- Aux/IAA interaction.

The specificity of the ubiquitin-proteosome pathway is determined by the E3 ligase complex. Therefore, organisms have many E3s that allow the ubiqutination of a diverse array of proteins. Currently, several groups of E3s have been identified in eukaryotes. The SCF type ligase complex is one of them and has four primary subunits; an F-box protein (that shares a conserved domain with Cyclin F) recruits the substrate, SKP1 (ASK1 in *A. thaliana*), the ring protein/RBX and CULLIN1 (CUL1). The CUL1 subunits function as a scaffold binding RBX to the SKP-F-box protein dimer (Zheng *et al*., 2002) (Figure 2a). There are more than 700 F-box proteins encoded by the *Arabidopsis thaliana* genome, suggesting that SCFs have a prominent role in cellular regulation in plants (Gagne *et al*., 2002; Kuroda *et al*., 2002).

The rapid turnover of the Aux/IAA proteins, their stabilization by proteosome inhibitors and results of some biochemical assays (e.g., pulldown assays between IAA7/IAA17 and SCF^{TIR1}) suggest that Aux/IAAs are substrates of the SCF^{TIR1} (Ramos *et al*., 2001). Genetic studies have found that domain II mutations of Aux/IAA proteins result in stabilization of Aux/IAA protein leading to gain of function mutants. The domain II has a conserved region with 13 amino acids that include --GWPPV-- which is essential for the interaction with SCF^{TIR1}. In the SCF^{TIR1} complex, TIR1 (Transporter Inhibitor Response 1) is the F-box protein that is responsible for binding with Aux/IAA proteins. TIR1 has an N-terminal F-box region and several C-terminal leucine rich repeats (Gagne *et al*., 2002; Ruegger *et al*., 1998). The SCF^{TIR1} interacts with Aux/IAA proteins in the presence of 2,4-D, 1-NAA and IAA both *in vivo* and *in vitro* (Dharmasiri *et al*., 2003; Gray *et al*., 2001). Further studies have proved that TIR1 is the receptor for IAA, 2,4-D and 1-NAA (Dharmasiri *et al*., 2005; Kepinski and Leyser, 2005).

Studies on the crystal structure of *Arabidopsis* TIR1-ASK1 complex show that a binding pocket on the top of the TIR1 leucine rich repeat domains functions in both auxin binding and substrate recruitment. Auxin binds to the bottom of the pocket that forms a promiscuous binding site that tolerates moderately different ring structures including both natural and synthetic auxins. Aux/IAA protein binds in close proximity to the auxin-binding site in the upper part of the pocket in such away that GWPPV motif is packed directly against auxin and covers the auxin binding site. Further, it has been found that of the three auxins (IAA, 1-NAA & 2,4-D) tested, IAA binds to TIR1 with the highest affinity. For this binding, both the side chain and the indole ring of IAA are important (Hayashi *et al*., 2008). Introduction of butyl or longer C-chains to the α position of IAA abolishes the auxin activity because the butyl chain is directed to the Aux/IAA binding cavity where it would prevent access of the domain II region in the binding cavity (Hayashi *et al*., 2008). The binding of auxin does not induce significant conformational changes in TIR1, but it enhances the interaction of the Aux/IAA substrate with TIR1 by occupying a cavity between substrate and receptor. Therefore, auxin mainly act as a molecular glue that effectively strengthens the binding of the substrate to the receptor (Guilfoyl, 2007; Tan *et al*., 2007).

The F-box protein TIR1 is the first example of an entirely new class of receptors in which the activity is regulated by the binding of a small molecule (in this case auxin). The vast majority of the F-box proteins identified so far remain uncharacterized and their target proteins are unknown. Even for the TIR1 signaling pathway, only the involvement of three auxins IAA, 2,4-D and 1-NAA has been experimentally proved. Whether all other auxinic molecules function in the same way in the signaling mechanism is still a

mystery. If not, what are the components in the different signaling pathways? It has been found that at least some upstream components of the signaling pathway(s) may be unique for the auxinic herbicide picloram (Dharmasiri S., unpublished data). To identify new genes that are involved in picloram specific signaling, about 70,000 Ethyl methanesulfonate (EMS) mutagenized *Arabidopsis* seedlings were screened on high concentrations of picloram medium. So far, 30 putative auxin resistant *Arabidopsis* mutants have been isolated. Of which 16 are specifically resistant to picloram, while the rest is resistant to both picloram and 2,4-D, suggesting the possibility of the existence of unique and common steps in auxin signaling pathways for different auxinic chemicals.

In this thesis, I report the results of isolation and characterization of two *Arabidopsis* mutants, *pic11* and *pic32*. While *pic32* is hypersensitive to picloram, *pic11* is resistant to both picloram and 2,4-D. *pic11* shows severe defects in growth and development, including defects in lateral root development, plant height, leaf development, floral morphology and reduced fertility. *pic32* shows hypersensitivity in lateral root induction in response to picloram and also it exhibits some defects in growth and development. I hypothesize that these genetic mutations of *pic11* and *pic32* are involved in auxin responsive pathway. My results so far indicate that *pic11* is a gain of function mutation of *IAA28* which encodes the Aux/IAA protein IAA28. *pic11/iaa28* mutants exhibit both distinct and common defects relative to other *Aux/IAA* mutants in *Arabidopsis*.

CHAPTER II

MATERIALS AND METHODS

Plant varieties and growth conditions

All experiments were performed using *Arabidopsis thaliana* (L.) Heynh. Var. *Columbia* (Col-0) as the wild type. Seed varieties were obtained from Arabidopsis Biological Resource Center at the Ohio State University. Seedlings were grown in a growth chamber at 20°C with continuous illumination (Dharmasiri *et al*., 2003). All the experiments with different hormone treatments were carried out within the same growth chamber. All the experiments on soil medium were carried out in the plant room at 22°C under continuous illumination.

Isolation of mutants with altered response to picloram

Ethyl methanesulfonate (EMS) was used for mutagenesis of seed stocks of Col-0 in an effort to generate point mutations (Kim *et al*., 2006). Mutagenized M_1 seeds were grown on soil as described above. M_2 seeds were collected, germinated and grown on *Arabidopsis thaliana* growth medium with sucrose (ATS) (Lincoln *et al*., 1990) containing 10 µM picloram (Sigma Aldrich Inc. MO) under continuous illumination for 4-6 days. Since both exogenous and endogenous auxin strongly influence root morphology, inhibiting primary root elongation, increasing lateral root production and inducing adventitious roots (Zimmerman and Hitchcock, 1942), all seedlings with longer

primary roots or seedlings in which the shoot system showed healthy appearance compared to wild type in response to picloram were selected as putative picloram resistant mutants. These selected picloram-resistant mutants were directly transplanted in soil. M_3 seeds from the putative mutants were re-tested for resistance by measuring primary root elongation of seedlings grown on 10 µM picloram or 75 nM 2,4-D (Sigma Aldrich Inc. MO). All the mutants which showed resistance to these auxin concentrations were again tested on various concentrations of picloram and 2,4-D to analyze the resistance level of each mutant. For dose response experiments, 4-day-old seedlings grown on vertical plates were transferred to *Arabidopsis thaliana* medium with sucrose (ATS) medium (Lincoln *et al*., 1990) containing various concentrations of picloram or 2,4-D. The seedlings were incubated on vertically oriented plates for an additional 4 days under constant illumination. Root elongation during the 4-day period was measured by using a vernier caliper or a ruler. Root elongation of at least ten seedlings per treatment was measured . Average root length was calculated and plotted against auxin concentraions.

Morphological characterization of picloram resistant mutants

pic11 and *pic32* mutants were backcrossed to wild type Columbia (Col-0). To remove any additional mutations, F2 progeny were obtained and selected on either 10 µM picloram (for *pic32*) or 85 nM 2,4-D (for *pic11*). Seedlings with characteristics similar to the original mutant were selected for further studies.

The morphology of each mutant was compared with the same aged Col-0 seedlings. For morphometry analysis the following characteristics were observed and compared for both *pic11* and *pic32* with Col-0 in order to identify any differences.

- Height of the main inflorescence axis
- Leaf size and shape
- Apical dominance and number of inflorescences

Physiological characterization

a. Effect of temperature on mutant plants

To test whether *pic11* and *pic32* showed any defect in response to higher temperature, mutant plants along with Col-0 were grown under sterile conditions on vertically oriented petri dishes containing ATS nutrient media in an incubator at 20°C or 29°C under continuous illumination. Length of hypocotyls was measured using 9-day old seedlings. For each treatment at least 10 seedlings of Col-0 or mutants were used.

b. Effect of other phyto-hormones on mutant plants

To test effect of other hormones on *pic* mutants, each mutant was tested together with Col-0 for a selected concentration range (Table 1) for 1-Aminocyclopropane-carboxylic acid (ACC), kinetin, abscisic acid (ABA), Indole-3-butric acid (IBA) and jasmonic acid (JA) according to standard procedures (Chen *et al*., 2007; Hobbie and Estelle, 1995).

Table 1. Different concentrations of hormones used to test the effect of indicated hormones on primary root growth of *pic11* and Col-0.

Different concentrations used				
1-Aminocyclopropane-carboxylic acid (ACC)	Indole-3-butric acid	Kinetin	Abscisic acid	Jasmonic acid
0 (DMSO)	0 (ETOH)	0 (DMSO)	0 (ETOH)	0 (ETOH)
100 nM	5 μM	0.01 μM	0.01 μM	0.01 μM
500 nM	10 μM	0.1μM	0.1μM	0.1μM
1 μM	15 μM	1 μM	1 μM	1 μM
5 μM	20 μM	10 μM	10 μM	10 μM
10 μM		100 μM	100 μM	50 μM
				100 μM

Molecular and Genetic Characterization of Selected *pic* Mutants

a. Genetic characterization of the mutation

To identify whether each mutation is dominant or recessive, homozygous mutants were crossed into wild type (Col-0) plants. F1 seeds were collected, germinated on soil and allowed to self-pollinate. F2 progeny was tested for 2,4-D resistance (for *pic11*) or picloram hypersensitivity (for *pic32*), and F2 homozygous seedlings from each mutant were selected for future studies.

Positional cloning

Positional cloning is the process of identifying the genetic basis of a mutant phenotype by looking for linkage to markers whose physical location in the genome is known. As a first step in the mapping process, the each mutant was out-crossed to wild type *Landsberg erecta*, another ecotype of *Arabidopsis* which shows genetic polymorphism with Columbia ecotype. F1 plants were genotyped with a polymorphic marker to make sure F1 plants are heterozygous for the molecular marker. F2 seeds were collected from self-pollinated F1 plants, and a population of F2 seedlings was tested for auxin resistance in order to isolate the homozygous mutants.

To select a *pic32* mapping population, F2 seedlings of *pic32* x *Ler* cross were germinated on ATS medium and then transferred to medium containing15 μM picloram. Seedlings were incubated an additional 4 days, and homozygous mutants were selected based on manifesting a hypersensitive phenotype (increased number of lateral roots) on picloram medium. These mutants were then transferred to ATS medium before extracting DNA from these plants. DNA for genotype analysis was prepared from the entire seedlings separately according to the previously described procedure (Berendzen *et al*., 2005).

To select a *pic11* mapping population, *pic11* x *Ler* F2 seedlings were germinated on ATS medium, and after 4 days the seedlings were transferred on to 85 nM 2,4-D medium. Seedlings were incubated an additional 4 days before selection. Due to the semi-dominant nature of *pic11*, 3/4th of the F2 population showed resistance in primary root elongation while 1/4th was sensitive. Thus, all the sensitive seedlings were selected

and transferred on to ATS medium before extracting DNA. The entire seedling was used for DNA extraction as described previously (Berendzen *et al.*, 2005).

Coarse mapping

For coarse mapping a set of sequence specific primers made using Simple Sequence Length Polymorphism (SSLP) markers in such a way that they cover all 5 chromosomes in *A. thaliana* was used for mapping both *pic32* and *pic11*. The extracted DNA from homozygous mutants (F2 generation) of both *pic32* and *pic11* were amplified with a set of sequence specific primers made for different SSLP markers throughout the *Arabidopsis* genome (Berendzen *et al*., 2005). Next, amplified DNA was separated by agarose gel electrophoresis. In coarse mapping, the position of each mutation was identified by looking at the ratio of the parental and recombinant individuals in the F2 progeny (depending on the tightly linked marker). The two markers closest to the mutation on either side were used as flanking markers for fine mapping.

Fine mapping

Fine mapping was used to narrow down the genomic region containing the gene of interest to 40 Kb or less. More and more homozygous DNA samples were used to get heterozygotes as the size of the interval decreases. Additional SSLP markers (Table 2) were used to look for increasingly tight linkage to the mutation.

Candidate gene cloning

After narrowing down the genetic window of *pic11* mutant to about a 600 Kb region with F21J6 and F2P16 annotation units as the flankng markers on the *Arabidopsis thaliana* genome, a possible candidate gene AT5G25890 for *pic11* was identified on F18A17 annotation unit. AT5G25890 encodes an Aux/IAA protein named IAA28 (Luise *et al*., 2001). This gene was amplified by PCR using high fidelity Taq polymerase (Phusion, NEB) by using IAA28-F (5'- CACAACACAAACTTAGAAAAATGG-3') & IAA28-R (CTGATGATTTTGGCCAACCTCTC-3') with genomic DNA from the *pic11* mutant plants as well as from the Col-0 plants. DNA extractions for sequencing were done by using the CTAB method (Rogers and Benadich, 1988). Sequencing was carried out for both strands. Automated DNA sequencing was performed using AB3730 DNA analyzer by the DNA Sequencing Facility, the University of Texas at Austin. The sequence from the *pic11* seedlings was compared with the nucleotide sequence of the Col-0 in order to identify the mutation.

Molecular and genetic characterization of *pic* mutants

a. Analysis of the effect of mutations on auxin regulated protein degradation using *HS:: AXR3NT-GUS* reporter system

To determine whether Aux/IAA protein degradation is affected in *pic32* and *pic11* mutant background, both *pic32* and *pic11* alleles were crossed into plants carrying the *HS:: AXR3NT-GUS* reporter gene (Gray *et al*., 2001). This gene construct contains the N-terminal portion of *AXR3* (domains I and II) that is sufficient for nuclear localization and for targeting the protein for degradation through SCF^{TIR1} (Gray *et al*., 2001; Ramos *et al*.,

2001). Four-day-old *pic32* and *pic11* seedlings containing homozygous *HS::AXR3NT-GUS* were exposed to heat shock at 37°C for 2 h in ATS solution and then were transferred to 25°C for 45 min and either mock treated or treated with 20 μM 2,4-D. Seedlings were washed in 100 mM Na_2HPO_4, pH 7, for 20 min at 25°C. After incubation in 5-bromo-4-chloro-3-indolyl-*β*-glucuronic acid (5 mg/ml) for 4 hours at 37°C (Oono *et al*., 1998), tissues were de-stained in ethanol and observed under Nikon SMZ1500 microscope. The extent of GUS staining in both *pic32* and *pic11* backgrounds was visually compared with the Col-0 background.

b. Effect of mutation on the expression level of auxin responsive genes

To investigate the effect of *pic11* mutant on auxin regulated gene expression, the transcript levels of several selected *Aux/IAA* genes (eg. *IAA2* & *IAA28*) were visually compared with the transcript levels in wild type by using Reverse-Transcription PCR (RT-PCR).

Four day-old *pic11* and Col-0 seedlings were treated with 20 μM 2,4-D or 100 μM picloram for 1 hour. Total RNA was extracted by using Tri–reagent (Sigma) according to manufacturer's instructions. cDNA was made using oligo dT primers and reverse transcriptase enzyme. The amount of cDNA synthesized was visually estimated by amplifying ubiquitin cDNA using primers UBQ-F and UBQ-R (Table 3) in order to adjust the starting amount of cDNA in each treatment for both Col-0 and *pic11*. *IAA2* and *IAA28* cDNAs were amplified by PCR using gene specific primers (Table 3). The PCR products were separated on a 1% agarose gel (EMD Chemicals Inc. NJ) and the amount

of the amplified product was visually compared in each treatment for both Col-0 and *pic11*.

Table 2: Oligonucleotide primers used for fine mapping of both *pic11* and *pic32*.
Primers 1-8 for *pic11* fine mapping and primer 1,2 and from 9-16 for *pic32* fine mapping.

	Primer name	**Primer sequence**	**Primer length**	**Melting Temp. (^{o}C)**
1	MWD9-F	GTGCCATGTACATATACATTGAACT	25	53.2
2	MWD9-R	GATTCTTTGCAACGGGTGATTAC	24	54.6
3	F21J6-F	CACATCACCAAGATGAATCCAG	23	53.6
4	F21J6-R	ACAATCACTTGCCTCTGCCCC	21	60.1
5	F2P16-F	CCGAAACGATTTAACTAAACCAGAGA	27	55.1
6	F2P16-R	TCTTCTAGTGGTACAGTAAGTTGA	24	54.8
7	F15A18-F	GATGCCGATGATGATCCATCAAGA	25	57.5
8	F15A18-R	GCAGCTCCCTTTTTATGCCATCT	23	57.9
9	MTG13-F	GCTGCTGACGCTAGTTAAAGATGC	24	57.8
10	MTG13-R	ATACATGCACTCTTTTCTCCACTTCCA	26	55.4
11	MRG7-F	AGAGGAGATGGATATGAGCTTTC	23	53.8
12	MRG7-R	TGTTGTCTAAGTGAACTTGTTC	21	50.8
13	K16H17-F	TTGGGAGATTCTCCGAAAACGG	22	57
14	K16H17-R	CAGAATGGGATTTACTTCAAAGTGG	25	54.2
15	MWD9-2F	CCAGCTGCCAATTTTATAGGGATA	24	54.9
16	MWD9-2R	TGAAATATATAGTACTTGACCGAAG	25	50.1

Table 3. Oligonucleotide primers for RT-PCR for *pic11*.

Primer name	Primer sequence	Primer length	Melting Temp. (^{o}C)
UBQ-F	GTTGATTTTTGCTGGGAAGC	20	52.8
UBQ-R	GATCTTGGCCTTCACGTTGT	20	35.5
IAA2-F	CACCATGGCGTACGAGAAAGTCA	23	59.2
IAA2-R	CAGCTTCTCTGGATCATAAGGAAG	24	54.9
IAA28-F	AAGGAATTCTATGAACCTTAAGGAGACGGA	30	51.3
IAA28-R	TTTCTCGAGTTCATGATCTGTTCTTGAACTTCT	35	56.2

Expression of GST-IAA28 and GST-iaa28 in *E.coli*

To generate the *IAA28* expression plasmid, a 573-bp fragment containing the coding region of *IAA28* cDNA was amplified with IAA28-2F (5'- ATAAGGATCCCCAACACCAAAAACACA-3') and IAA28-2R (5'-ATCGAATTCATGATTTTGGCCAACCTC-3') primers from CD4-7 *Arabidopsis* cDNA library and cloned into the *Bam*HI-*Eco*RI site of pBluescript vector first and then into *Bam*HI-*Eco*RI site of pGE-X4T-3 vector. The recombinant vector was transformed to *E. coli* competent cells (TOP10) by using $CaCl_2$ method (Bergmans *et al*., 1981). The resulting colonies were tested for the correct insertion by using PCR with gene specific primers for *IAA28* gene (IAA28-2F and IAA28-2R). The recombinant pBluescript vector carrying the *IAA28* cDNA was sequenced with the AB3730 DNA analyzer at the DNA Sequencing Facility of the University of Texas at Austin in order to confirm the correct sequence in order to confirm the correct sequence.

Next, the *pic11/iaa28* mutation was created in pBluescript construct (*pBS-IAA28*) with sequence specific primers, IAA28-5F (5'-CTCCAGTGGTGGAATGGCCGCCGGT-3') and IAA28-5R (5'-ACCGGCGGCCATTCCACCACTGGAG-5') by site-directed mutagenesis (Stratagene), and the resulting positive colonies were confirmed for the correct mutation by using gene specific primers for mutant *iaa28*. Further, the recombinant pBS vector carrying *iaa28* was sequenced to confirm the presence of correct *iaa28* mutation. The mutant *iaa28* cDNA was sub-cloned into the *Bam*HI-*Eco*RI restriction site of the GST-fusion vector pGE-X4T-3. Finally, the recombinant vector was transformed to *E.coli* competent cells (TOP10) by using $CaCl_2$ method (Bergmans *et al*., 1981). The resulting *E.coli* colonies were tested for the correct insert by PCR using gene specific primers (Table 2).

To purify GST-IAA28 and GST-iaa28 recombinant proteins, *E. coli* (TOP10) carrying the recombinant plasmids were cultured overnight and 5 ml were inoculated into 250 ml liquid LB with carbenicillin (100 μg/ml,) and incubated at 37° C for 4 hours. Isopropyl thiogalactoside (IPTG) (Sigma Aldrich Inc. MO) was then added to a final concentration of 1 mM in order to induce protein expression, and the culture was incubated at 30° C for a further 4 hours. Bacteria were pelleted at 11.5 g for 10 minutes at 4°C (Eppendorf Centrifuge 5810R), and the pellet was re-suspended in 5 ml of phosphate-buffered saline (PBS). The cells were lysed by sonication for 30 seconds three times (Branson Sonifier 250). Protease inhibitor, phenylmethanesulfonyl fluoride (PMSF) (Sigma Aldrich Inc. MO) and Tween-20 (Sigma Aldrich Inc. MO) as the detergent were added to the extract at concentrations of 1 mM and 0.1%, respectively. Cell debris was

removed by centrifugation at 11.5 g for 10 minutes (Eppendorf Centrifuge 5810R). Glutathione-agarose beads (GST beads) (Sigma Aldrich Inc. MO) that were previously hydrated in 1X PBS were added to the supernatant and incubated at 4°C overnight with gentle agitation. Beads were recovered by centrifugation and washed at least three times each for 15 minutes with 10 ml of PBS containing 0.5% Tween-20. Washed beads were re-suspended in 250 µl of PBS containing 1 mM PMSF.

GST-pull downs and protein blots

For pull-down assays, approximately 2-3 µg of GST-IAA28 (and GST-iaa28) recombinant protein were incubated with 800 µg of crude protein extracts isolated from *Arabidopsis* seedlings carrying *CaMV-35S::AFB1-myc* transgene (Dharmasiri *et al*., 2005) separately for 1 hour at 4° C with gentle agitation. Different concentrations of 2,4-D were added directly to the reaction. At the end of incubation, glutathione beads were recovered by centrifugation and washed three times for 15 minutes with 1 ml of protein extraction buffer. Beads were resuspended in 2X Laemmli buffer, and proteins were resolved on 10% sodium dodysulfide polyacrylamide gels (SDS-PAGE). Proteins were transferred onto polyvinylidene difloride (PVDF) membrane (Pall Corporation, FL). The PVDF membrane was blocked using 5% nonfat milk in 1X Tris buffered saline containing 0.1% Tween 20 (TBST) for one hour, and washed three times with TBST. Then the blot was incubated with the primary antibody solution, α-myc (1:1000 in 1xTBST) (Cocalico Biologicals Inc. PA) for one hour on a shaker. The primary antibody bound blot was washed three times with 1X TBST. The blot was next incubated with secondary antibody (1:2000 in 1xTBST) (mouse IgG conjugated to horseradish

peroxidase) (Sigma Aldrich Inc. MO) for one hour and washed three times with TBST. Finally, the blot was developed for enhanced chemiluminescence using ECL western blotting kit (PIERCE, IL) and exposed to X-ray film according to manufacturer's instructions.

Data Analysis

For each experiment at least 10 or more seedlings per sample were used for statistical analysis. Mean value and standard deviation were calculated for each sample. Data were analyzed by using student T-test.

CHAPTER III

RESULTS

Physiological and morphometric characterization of *pic32*

pic32 exhibits growth and developmental defects.

Homozygous mutants of *pic32* show morphological differences from Col-0. *pic32* plants are smaller in size compared to the wild type. This feature is clear in leaf morphology. While leaves of *pic32* are shorter and narrower compared to Col-0, they also tend to curl downward (Figure 3). Additionally, *pic32* plants lack apical dominance (results not shown). The segregation pattern of F2 populations resulting from a cross between *pic32* and wild type indicates that *pic32* mutant phenotype is inherited as 3:1 (wild type: mutant) ratio indicating that *pic32* is a recessive mutation.

pic32 is hypersensitive to picloram.

To test the effect of picloram on lateral root growth, *pic32* along with Col-0 seedlings were grown on ATS medium containing 15 μM picloram. Figure 4a & 4b illustrates the effect of picloram on lateral root development in wild-type and *pic32* seedlings. While *pic32* does not show any growth difference on ATS medium, on ATS medium containing 15 μM of picloram, *pic32* produces about 2-3 fold higher number of short lateral roots compared to Col-0 plants indicating that the mutation promotes

initiation of lateral roots while inhibiting lateral root elongation in response to picloram. Nevertheless, *pic32* response to 2,4-D was not different from Col-0 (wild type). This indicates that lateral root growth and development in *pic32* is hypersensitive to picloram, and this hypersensitivity is specific to picloram. Similar to Col-0, primary root elongation of *pic32* is sensitive to both picloram and 2,4-D (Figure 5).

a.

b.

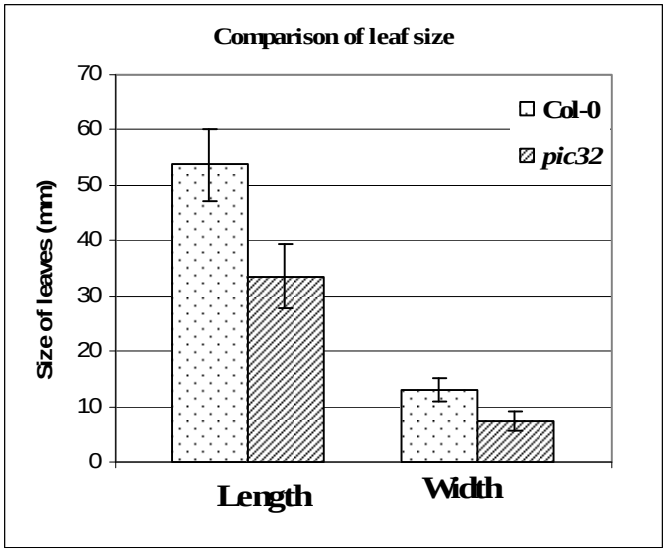
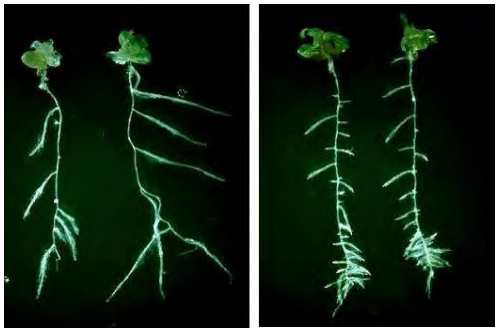


Figure 3. *pic32* exhibits defects in growth and development (a) *pic32* shows growth defects in shoot development. *pic32* shows defects in leaf morphology. It has relatively small, narrower rosette leaves with downward curved margins compared to Col-0 leaves. **(b) Comparison of leaf size of *pic32* with Col-0.** Twenty-eight day old *pic32* and wild type seedlings were compared. All the seedlings were grown under continuous light at 22°C. Each column indicates the average leaf length or width from at least 10 seedlings. Leaf length of *pic32* is shorter than that of Col-0 (Col-0 = 54 $\pm$ 6, *pic32* = 34 $\pm$ 6, $P < 0.05$) and the leaf width of *pic32* is half that of Col-0 (Col-0 = 13 $\pm$ 2, *pic32* = 7 $\pm$ 2, $P < 0.05$). Error bars indicate standard deviation of the Mean value.

a.



Col-0 *pic32*

b.

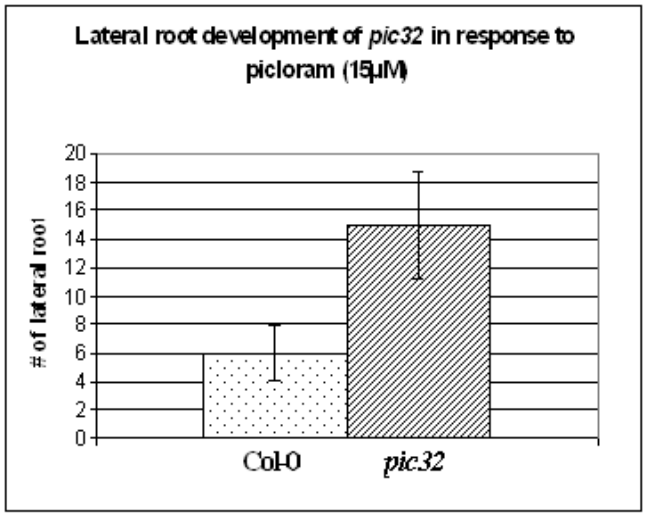
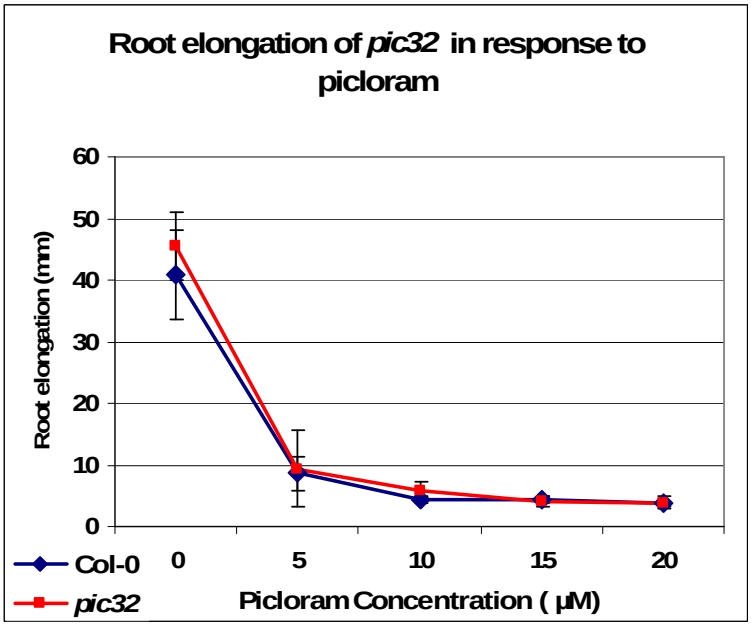


Figure 4. (a) *pic32* shows hypersensitivity in lateral root formation in response to picloram. The number of lateral roots/cm in *pic32* is approximately two-fold higher than that of Col-0 in response to 10 µM picloram. Four day-old *pic32* and Col-0 seedlings (from auxin-free ATS medium) were transferred to picloram containing ATS medium (15 µM picloram). The numbers of emerged lateral roots/cm were counted 4 days after transfer onto the medium with picloram. **(b) *pic32* produces 2.5 fold higher number of lateral roots than Col-0 on 10 µM picloram.** Each value represents the mean value of measurements on at least 10 seedlings. Error bars indicate standard deviation. $P < 0.05$.

a.



b.

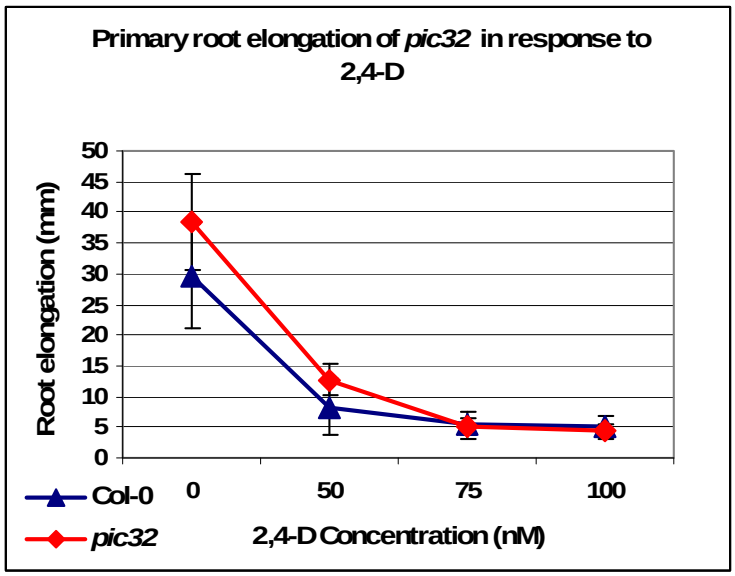


Figure 5. Primary root of *pic32* is sensitive to both picloram and 2,4-D. Four-day-old *pic32* and Col-0 seedlings were transferred from hormone-free ATS medium to ATS with different concentrations of picloram or 2,4-D. Seedlings were incubated vertically in an incubator at 20º C. Length of the primary root was measured 4 days after transfer onto the medium with auxins. **(a) Primary root elongation of *pic32* is sensitive in response to picloram. (b) Primary root of *pic32* is sensitive in response to 2,4-D**. (Each value represents the mean value of measurements on at least 10 seedlings. Error bars indicate standard deviation).

Molecular and Genetic Characterization of *pic32*

pic32 mutation was mapped to chromosome V

By using about 500 DNA samples from homozygous individuals from the *pic32* x *Ler* F2 generation, the *pic32* mutation was mapped to MWD9 BAC (Bacterial Artificial Chromosome) of chromosome V in the *Arabidopsis* genome. MWD9 BAC contains a total of 24 genes, and by using MWD9-2 forward and reverse primers the genetic window for *pic32* mutation was narrowed down to 16 genes (Table 4). Among those genes, functions of three genes are unknown while the functions of the rest of the genes have been defined. In this window, there are few possible candidate genes for *pic32*, for example, AT5G22355.1 (DC1 domain-containing protein), AT5G22400 (rac GTPase activating protein) and AT5G22380 (a transcriptional factor).

Table 4. Genes present in the genetic window of *pic32*

	Gene	Function
1	AT5G22340.2	Unknown protein
2	AT5G22355.1	DC1 domain-containing protein, UV-B light-insensitive protein
3	AT5G22360.1	Member of Synaptobrevin-like protein
4	AT5G22370.1	Encodes QQT1, required for early embryo development
5	AT5G22380.1	Transcriptional factor, identical to NAC-domain containing protein
6	AT5G22400.1	rac GTPase activating protein
7	AT5G22410.1	Identical to peroxidase 60 precursor protein
8	AT5G22420.1	Similar to acyl CoA reductase, male sterility protein
9	AT5G22430.1	Unknown protein
10	AT5G22440.2	60S ribosomal protein L10A
11	AT5G22440.1	60S ribosomal protein L10A
12	AT5G22450.1	Unknown protein
13	AT5G22460.2	Similar to esterase/lipase/thioesterase family protein
14	AT5G22460.1	Similar to esterase/lipase/thioesterase family protein
15	AT5G22470.1	Similar NAD^+ ADP^- ribosyltransferase
16	AT5G22480.1	Zinc finger (ZPR1-type) family protein

***pic11* shows abnormalities in growth and development**

The *pic11* mutation produces a characteristic shoot phenotype that includes defects in leaf, inflorescence, and flower morphology. Rosette leaves of *pic11* plants are smaller in size (Figure 6b) and tend to curl downward. The petioles are shorter than that of wild-type. Other differences between mutant and wild type plants can be seen in the inflorescences. The inflorescence of *pic11* is very small and produces only few flowers. As a result *pic11* mutants only produce about 20 seeds per plant. At maturity, the height of *pic11* plants is approximately 5- 6 cm while same aged wild type plants are about 20-25 cm in height (Figure 6a & b).

***pic11* is resistant to both picloram and 2,4-D.**

To investigate the consequences of the *pic11* mutation, several auxin responses in both wild type and *pic11* plants were examined. First, the effect of 10 µM picloram and 75 nM 2,4-D on root growth were examined. While Col-0 primary root growth was inhibited at these auxin concentrations, *pic11* showed resistant to both auxins (Figure 7).

***pic11* shows defects in lateral root development.**

The most prominent feature of *pic11* is the reduced lateral root number. *pic11* seedlings produce no lateral roots on ATS or 75 nM 2,4-D after 4 days. On picloram, Col-0 produced an average of about 16 lateral roots, while *pic11* produced about 4 lateral roots after 4 days. Application of exogenous auxin stimulates lateral root formation in *Arabidopsis* (Laskowski *et al*., 1995). Therefore, in order to determine whether auxin can rescue the lateral root phenotype of *pic11*, the seedlings were treated with 85 nM 2,4-D

or 10 μM picloram. Four days after the treatment, both auxin treatments induced the formation of many lateral roots in wild type seedlings, but in *pic11* only picloram was able to induce a few lateral roots. Picloram-induced lateral root formation in *pic11* showed a characteristic pattern compared to the wild type (Figure 8). In wild type, all the lateral roots emerge along the primary root but in *pic11* all the lateral roots arise from the same place just below to the hypocotyl region. Although, *pic11* is defective in lateral root production, at very high concentrations of 2,4-D (results not shown) and IBA (Figure 17 d), *pic11* was capable of producing lateral roots, suggesting that the *pic11* mutant does not completely lack the ability to develop lateral roots, but it is relatively insensitive to the exogenous auxins.

pic11 shows defects in root hair development

Root hairs are long, tubular extensions of root epidermal cells that help to anchor roots, interact with soil microorganism and assist in the uptake of water and nutrients (Cutler, 1978). The auxin resistant *pic11* mutant also shows defects in root hair initiation and development compared to the wild type. *pic11* produces fewer short root hairs compared to the wild-type plants on ATS medium (Figure 9a).

To examine whether the root hair phenotype of *pic11* can be rescued with auxin and/or ethylene treatments, root hair development of *pic11* seedlings were examined on 10 μM picloram, 85 nM 2,4-D and 100 nM 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate biosynthetic precursor of ethylene. Results indicate that neither picloram nor 2,4-D rescue root hair phenotype of *pic11* to the same level of Col-0 (Figure 10 b; $P < 0.05$). However, ACC treatment rescues the root hair phenotype of *pic11* to the

same level as Col-0 (Figure 10a and 10b; $P > 0.05$, between Col-0 and *pic11* in response to ACC). To find out whether root hair defective phenotype is specific for *pic11*, two other known auxin mutants, *axr5-1* and *axr2-1*, were also tested. While both *axr5-1* and *axr2-1* had fewer and shorter root hairs, auxin treatment rescued root hair phenotype in these two mutants, suggesting that root hair defect in *pic11* is specific to this mutant (Figure 9).

a.

b.

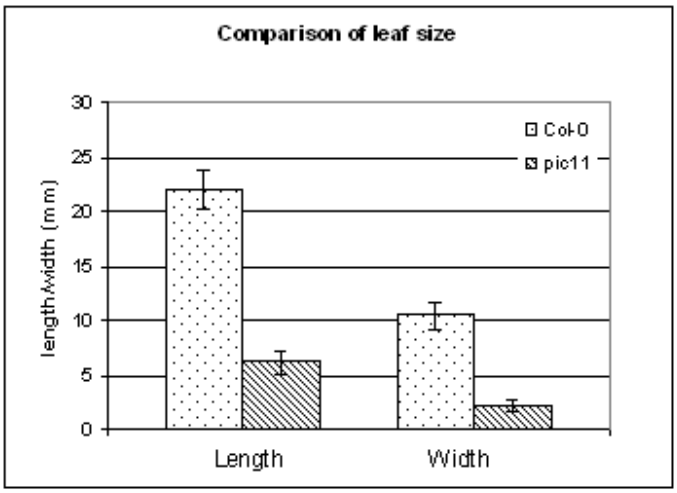


Figure 6. (a). *pic11* mutation produces a characteristic shoot phenotype that includes defects in leaf, inflorescence, and flower morphology. (b). *pic11* exhibits defects in leaf morphology. Same aged (30 days old) *pic11* and wild type seedlings were compared and analyzed. All seedlings were grown under control growth conditions. Each column indicates the average leaf length or width from at least 5 seedlings. Both leaf length and width of *pic11* have a prominent difference compared to the wild type; length of Col-0 is almost four fold higher than *pic11* (length Col-0 = 22 $\pm$ 2 and *pic11* = 6 $\pm$ 1, $P < 0.05$) the leaf width of Col-0 is about 5 fold higher than *pic11* leaf width (Col-0 = 11 $\pm$ 1, *pic11* = 2.2 $\pm$ 1, $P < 0.05$). Each value represents the mean value of measurements on at least 10 plants. Error bars indicate standard deviation.

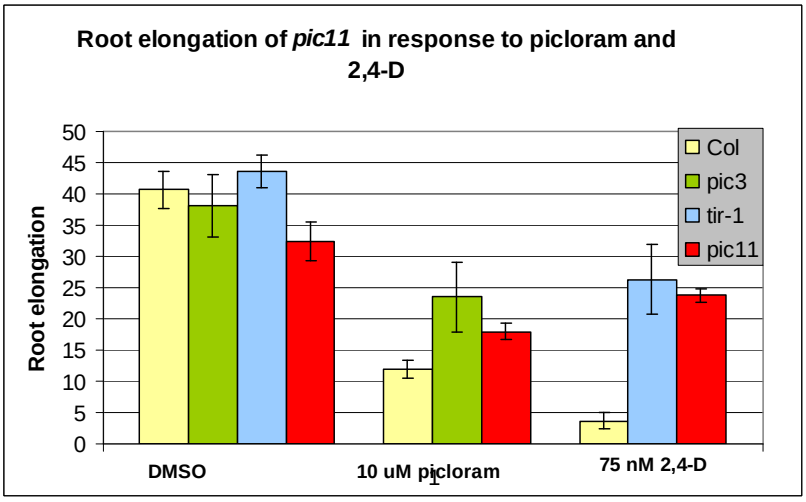


Figure 7. Primary root of *pic11* shows resistance to both 10 µM picloram and 75 nM 2,4-D. Four day-old *pic11* and Col-0 seedlings were transferred from hormone-free growth medium (ATS) to ATS with 10 µM picloram and 75 nM 2,4-D. Seedlings were incubated vertically in an incubator at 20º C. Length of primary root was measured 4 days after transfer onto the medium with auxins. *tir1* and *pic3* were used as known mutants that are resistant to 2,4-D and picloram, respectively. Each value represents the mean of measurements on at least 10 seedlings. Error bars indicate standard deviation. (Dharmasiri S. unpublished data).

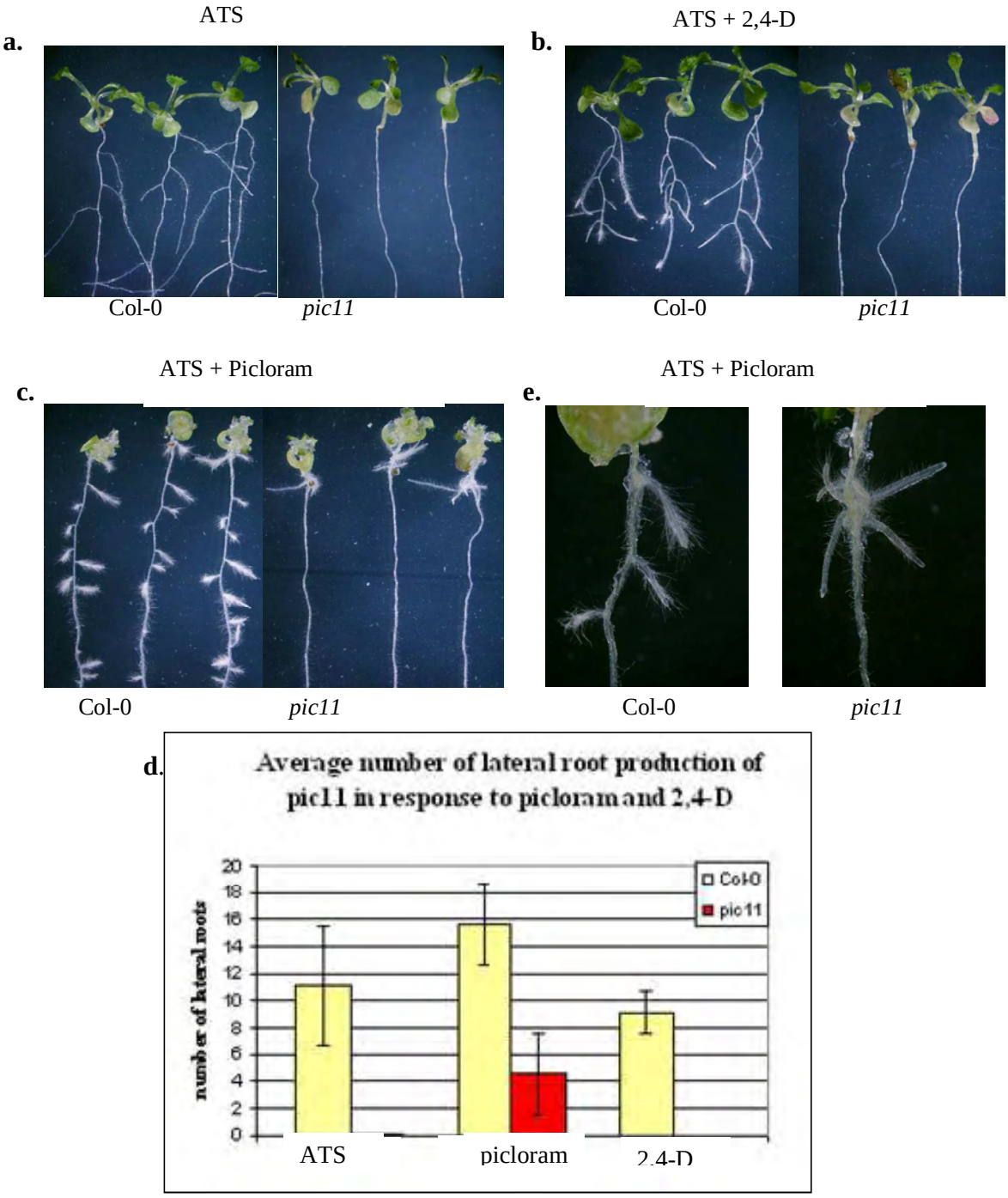


Figure 8. ***pic11*** **shows defects in lateral root initiation and development. (a) on ATS, (b) on 2,4-D, (c) on picloram medium, (d) on picloram** ***pic11*** **produces only few number of lateral roots compared to Col-0**. *pic11* produces average about 4-5 lateral roots per plant while Col-0 produces about 16 lateral roots per plant on picloram medium. Each value represents the mean value of measurements on at least 10 seedlings. Error bars indicate standard deviation. **(e) Development of lateral roots in response to picloram shows a characteristic pattern compared to the wild type** (Dharmasiri S. unpublished data).

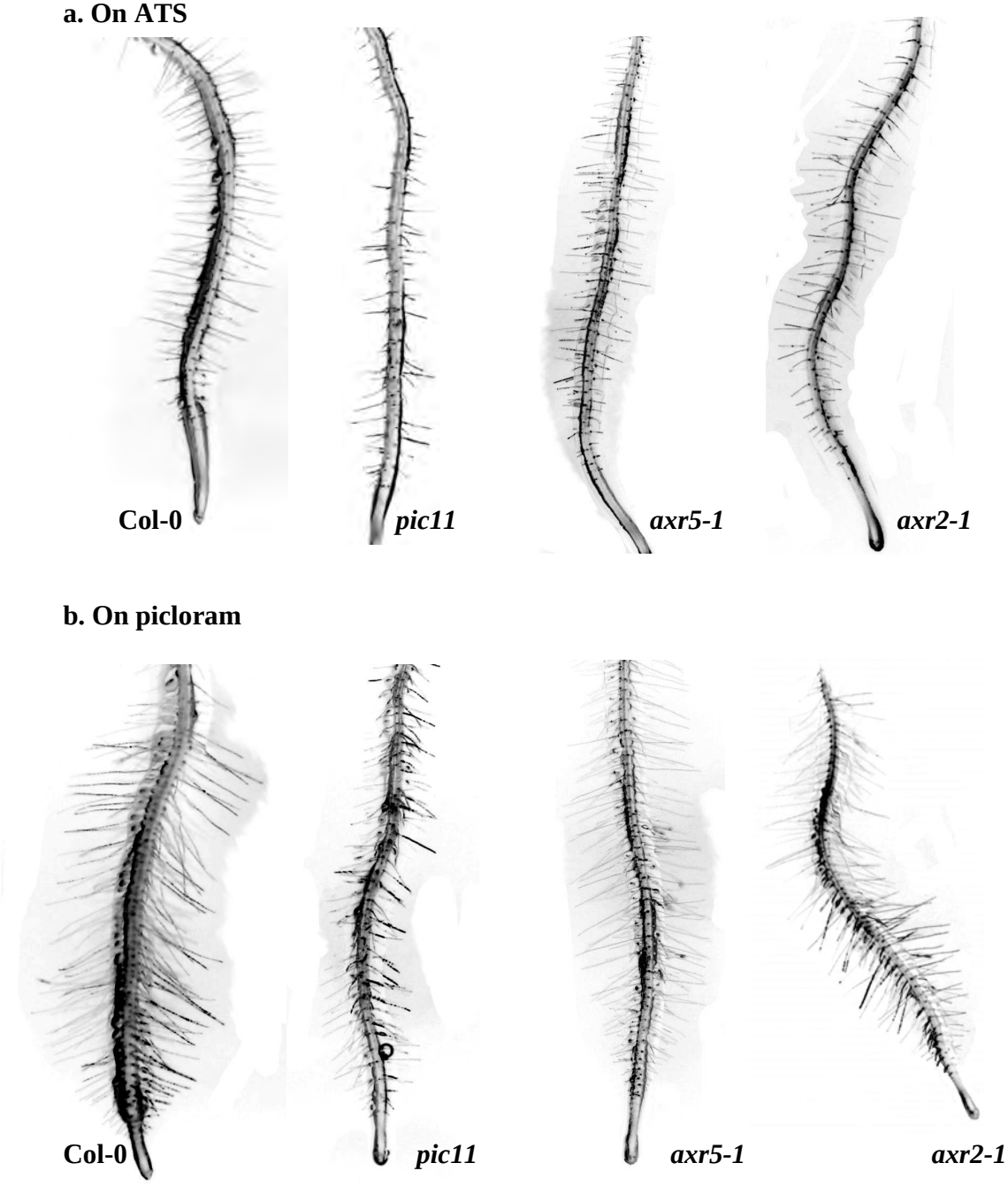
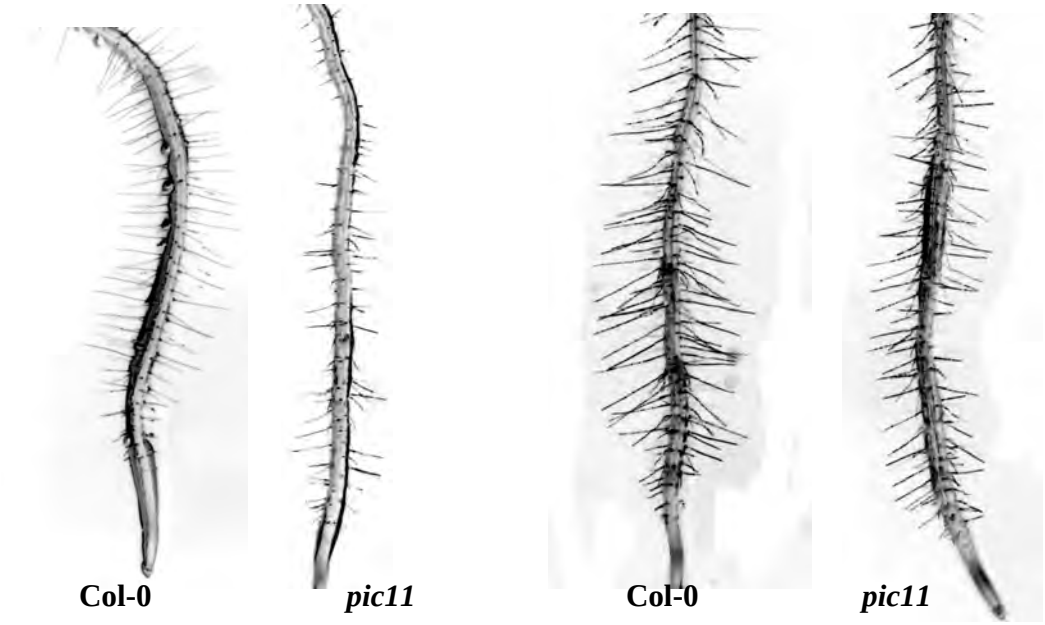


Figure 9. *pic11* shows defects in root hair development compared to the wild type. Four days old *pic11* seedlings together with Col-0, *axr2-1* and *axr5-1* were transferred onto 10 μM picloram. *pic11* produces fewer and shorter root hairs compared to the wild type. While picloram treatment rescues *axr2-1* and *axr5-1* root hair phenotype, it has less effect on *pic11*.

With ACC treatment

a.



b.

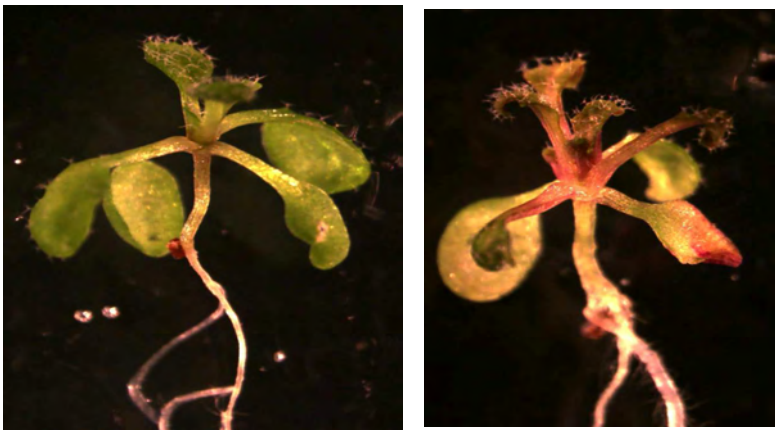
	Average number of root hairs per 5mm along the primary root		
	ATS	**10 µM picloram**	**50 nM ACC**
Col	94± 6	116 ± 15	111 ± 7
pic11	68 ± 14	74 ± 13	113 ± 11
P value	P < 0.05	P < 0.05	P >0.05

Figure 10. (a) ACC can rescue the root hair phenotype of *pic11*. Four day-old *pic11* seedlings together with Col-0 were transferred onto 50 nM ACC. After 4 days incubation, *pic11* produces higher and longer root hairs similar to Col-0 in response to ACC treatment indicating ACC can rescue the root hair phenotype of *pic11*. **(b) The average number of root hairs of both Col-0 and *pic11* on ATS and ATS supplemented with picloram or ACC**. The results indicate that ACC can rescue the root hair phenotype of *pic11*. Each value represents the mean value of measurements on at least 10 seedlings. Error bars indicate standard deviation.

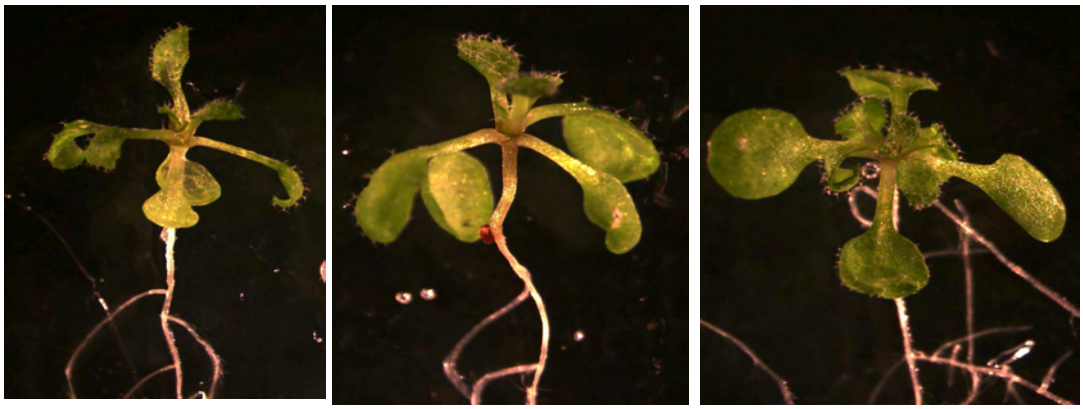
pic11 accumulates higher level of anthocyanin compared to wild-type

pic11 appeared to produce more anthocyanin in leaf petiole area compared to the wild type on ATS medium, and with the application of exogenous 2,4-D the level of anthocyanin increases not only in petiole areas but also in younger leaves. In contrast, some other known auxin mutants *tir1-1, axr2-1* and *axr5-1* do not accumulate anthocyanin in response to auxin (Figure 11), suggesting the possibility that *pic11* is involved in regulating anthocyanin metabolism in *Arabidopsis*.

Shoot phenotype in response to 2,4-D (100 nM)



Col-0 *pic11*



tir1 *axr2-1* *axr5-1*

Figure 11. *pic11* appeared to produce more anthocyanin near leaf petioles compared to the wild type. Four day-old *pic11* seedlings together with Col-0, *tir1*, *axr2-1* and *axr5-1* were transferred onto 100 nM 2,4-D. Five to six days after transferring onto 2,4-D anthocyanin accumulation was evident in *pic11,* but not in other auxin-treated mutants.

pic11 shows defects in hypocotyl elongation

Hypocotyl elongation in *Arabidopsis* is controlled by both environmental conditions and hormones (Gendreau *et al*.,1997). Some known auxin responsive mutants show defects in hypocotyl elongation compared to the wild type. Thus, auxin resistant *pic11* seedlings were examined to identify any defects in hypocotyl growth. Interestingly *pic11* produces longer hypocotyls compared to the wild type on ATS medium (Figure 12). Increased levels of auxin will cause hyocotyl elongation in *Arabidopsis* (Gray *et al*., 1998). To examine whether *pic11* shows a similar behavior in response to exogenous auxin, both 4 day-old Col-0 and *pic11* seedlings were transferred on to 10 µM picloram and 85 nM 2,4-D. Seedlings were incubated four more days and elongation of the hypocotyls was measured . The Col-0 hypocotyls were elongated in response to picloram treatment while the growth of *pic11* hypocotyls did not show any significant difference to any auxin treatment (Figure 12).

pic11 shows defects in temperature-induced hypocotyl elongation

When *Arabidopsis* seedlings grown in the light at high temperature (29° C), they exhibit dramatic hypocotyl elongation compared with seedlings grown at 20° C (Gray *et al*., 1998). To examine the effect of *pic11* mutation on temperature-induced hypocotyl elongation, Col-0 and *pic11* seedlings were grown at 29° C. Unlike the wild type *pic11* mutant seedlings do not exhibit temperature-induced hypocotyl elongation. Hypocotyls of Col-0 elongated almost two-fold higher than that of *pic11* in response to high temperature (Figure 13).

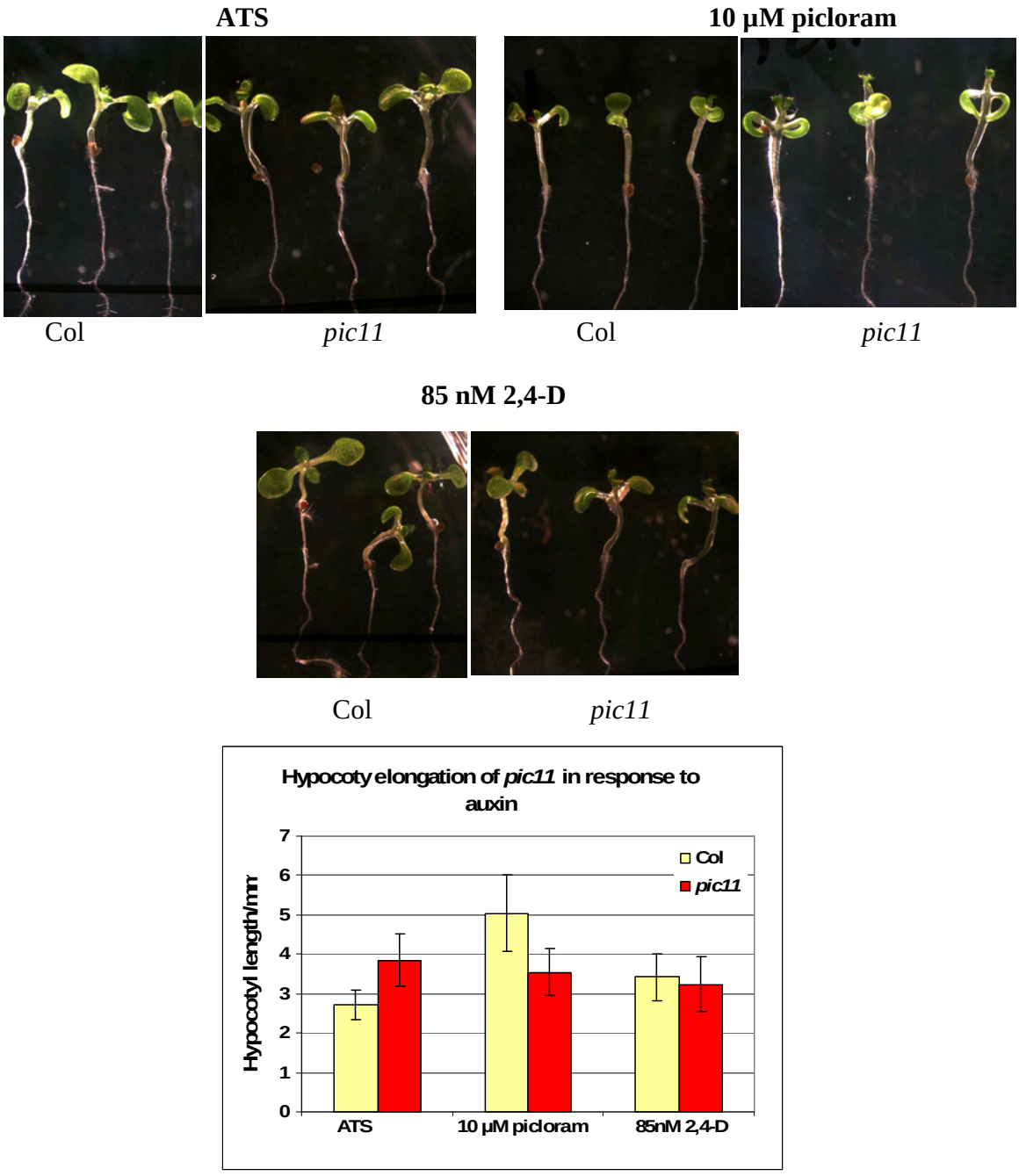
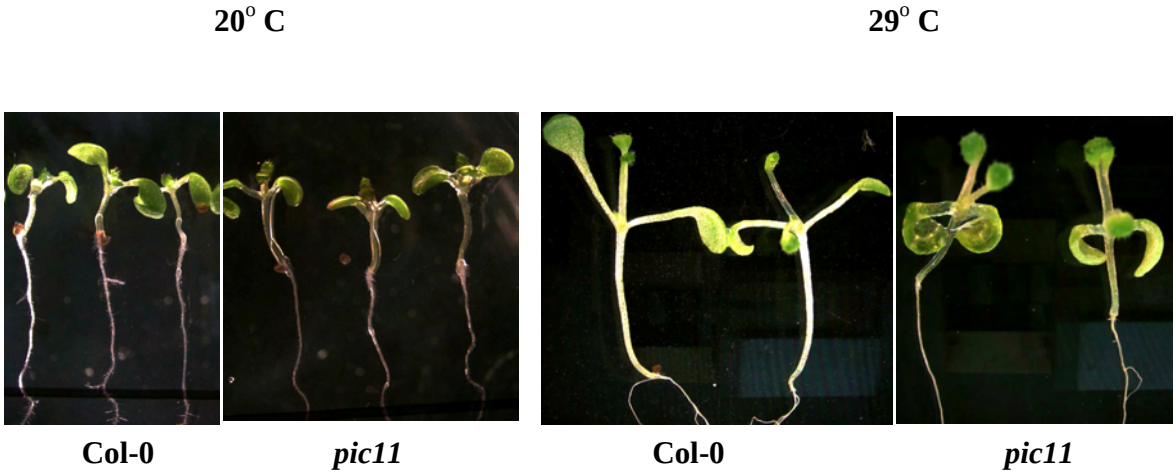


Figure 12. Hypocotyl elongation of *pic11* compared to Col-0. *pic11* has longer hypocotyls on ATS compared to the wild type ($P < 0.05$). Col-0 hypocotyls elongate with the application of exogenous picloram ($P < 0.05$) while *pic11* seedlings show no significant change in hypocotyl length in response to picloram ($P > 0.05$). Neither *pic11* nor Col-0 appeared to respond to 2,4-D treatments ($P > 0.05$). Each value represents the mean value of measurements on at least 10 seedlings. Error bars indicate standard deviation.



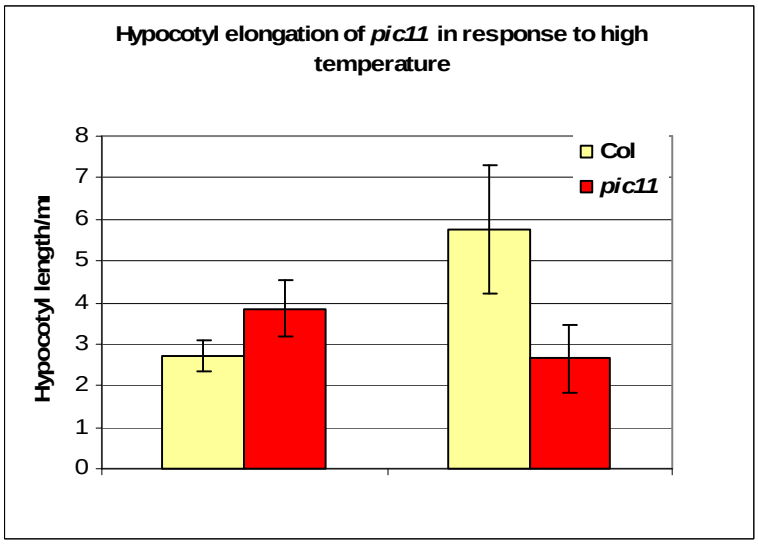


Figure 13. *pic11* shows defects in temperature-induced hypocotyl elongation. High temperature (29°C) causes hypocotyl elongation of Col-0 seedlings (Gray *et al.*, 1998), while it does not show any effect on hypocotyl elongation of *pic11*. Each value represents the mean value of measurements on at least 10 seedlings. Error bars indicate standard deviation.

Effect of other plant hormones on *pic11*

pic11 is slightly resistant to 1-aminocyclopropane-carboxylic acid (ACC)

The growth response of auxin resistant *pic11* mutant was examined by using different concentrations of ACC, the immediate precursor of the plant hormone ethylene. In this experiment 4 day-old seedlings of both Col-0 and *pic11* were transferred onto ACC-containing ATS medium and allowed to grow. After 4 days primary root elongation of seedlings in each treatment was measured (Figure 14). The results indicate that primary root elongation of *pic11* shows slight resistance to ACC at 1 μM concentration compared to the wild type, but become sensitive at concentrations above that level ($P > 0.05$).

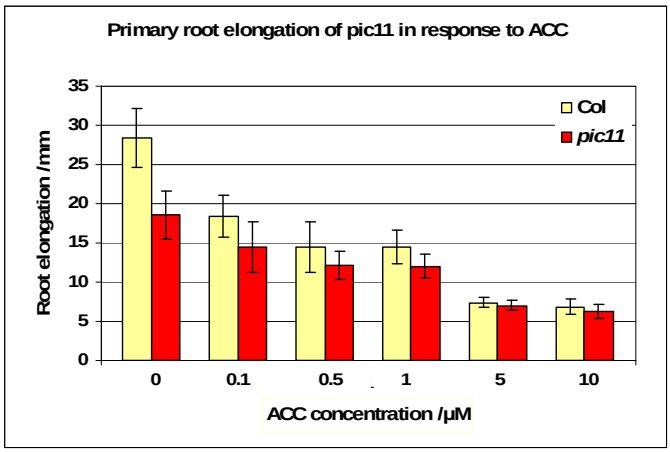
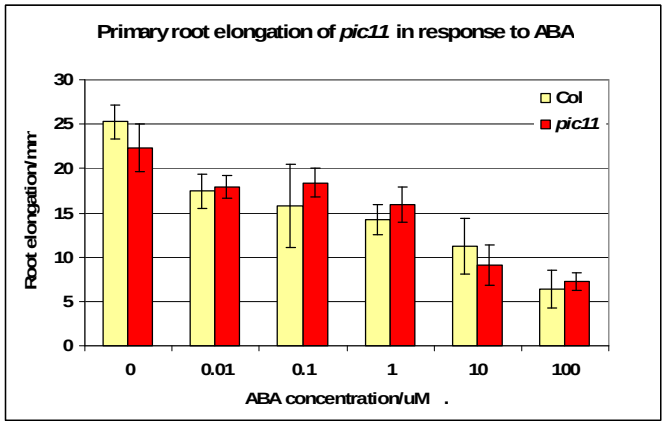


Figure 14. Primary root elongation of *pic11* shows slight resistance to ACC at lower concentrations. Primary root elongation of *pic11* shows slight resistance to ACC at lower concentrations as 1 µM level ($P < 0.05$) compared to Col-0, but becomes sensitive at higher concentrations of ACC ($P > 0.05$). Four-day-old *pic11* and Col-0 seedlings were transferred from hormone-free growth medium (ATS) to ATS with different concentrations of ACC and kept vertically in an incubator at 20° C. Length of primary root was measured 4 days after transfer onto the medium with different plant hormones. Each value represents the mean value of measurements on at least 10 seedlings. Error bars indicate standard deviation.

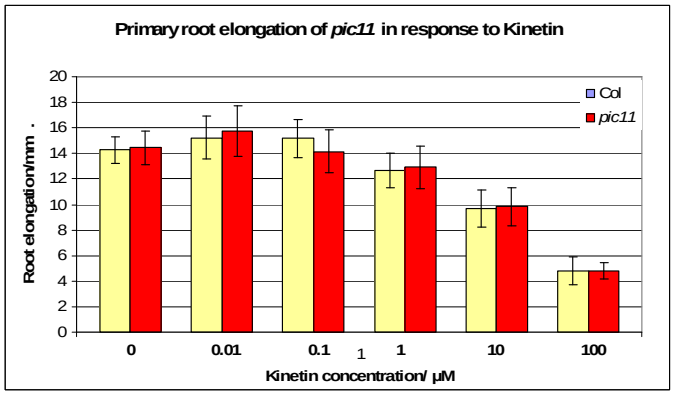
pic11 is not defective in other plant hormone responses

To find out whether *pic11* exhibit any defects in responding to other plant hormones, dose experiments were carried out using range of concentrations of abscisic acid (ABA) (Figure 15 a), kinetin (Figure 15 b), and jasmonic acid (JA) (Figure 15 c). Results indicate that similar to Col-0, primary root growth of *pic11* is sensitive all three hormones indicating that *pic11* exhibit normal responses to these hormones. Additionally, both Col-0 and *pic11* appeared to accumulate anthocyanin in response to jasmonic acid (Figure 16), further confirming that jasmonic acid response in *pic11* has not been compromised by the mutation.

a.



b.



c.

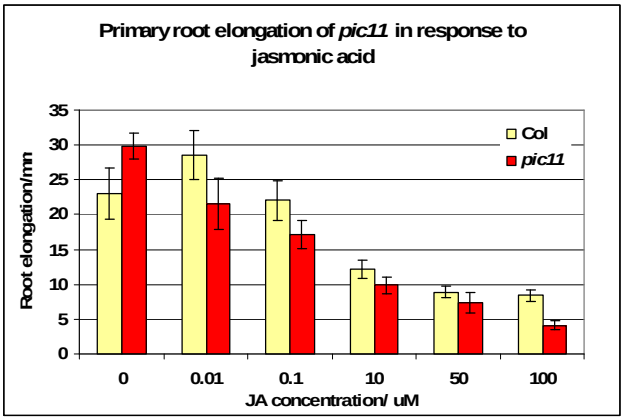
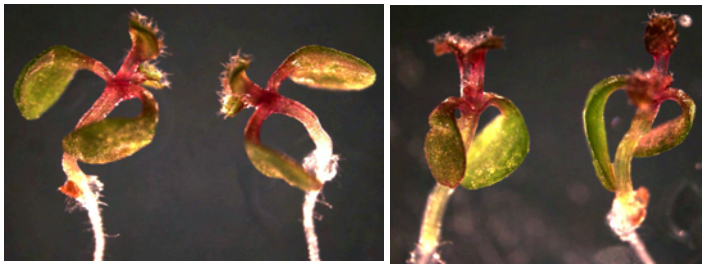


Figure 15. ***pic11*** **primary root elongation is sensitive to ABA (a), kinetin (b) and jasmonic acid (c).** Four-day-old *pic11* and Col-0 seedlings were transferred from hormone-free growth medium (ATS) to ATS with different concentrations of ABA, kinetin and jasmonic acid. Seedlings were kept vertically in an incubator at 20º C. Length of primary root was measured 4 days after transfer onto the medium with different plant hormones. Each value represents the mean value of measurements on at least 10 seedlings. Error bars indicate standard deviation.

b. ATS

Col-0 ***pic11***

c. 50 µM Jasmonic acid



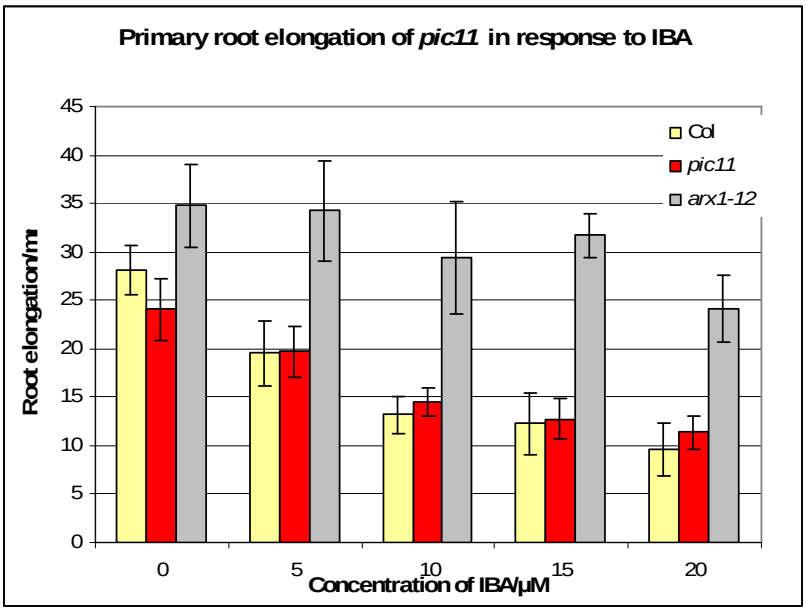
Col-0 ***pic11***

Figure 16. JA induces anthocyanin production in both *pic11* and Col-0.

***pic11* is sensitive to Indole-3-butric acid (IBA)**

The effect of IBA on the primary root elongation of *pic11* was tested over concentrations ranging from 0 to 20 µM. . As illustrated in figure 17a, the primary root elongation is sensitive to IBA at all the concentrations tested.. However, IBA rescues the lateral root phenotype of *pic11* at higher concentrations (Figure 17b). Lateral root initiation points of *pic11* also showed a pattern similar to the wild type (Figure 17c).

a.



b.

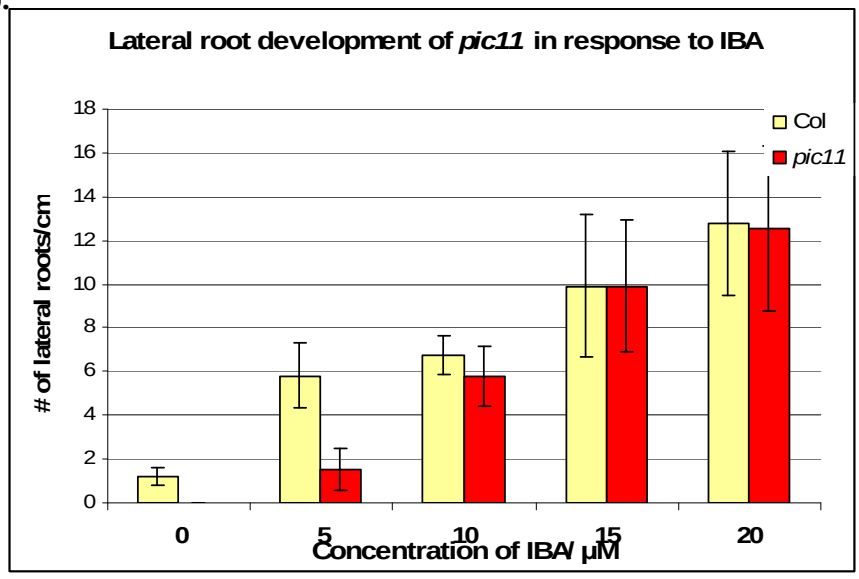
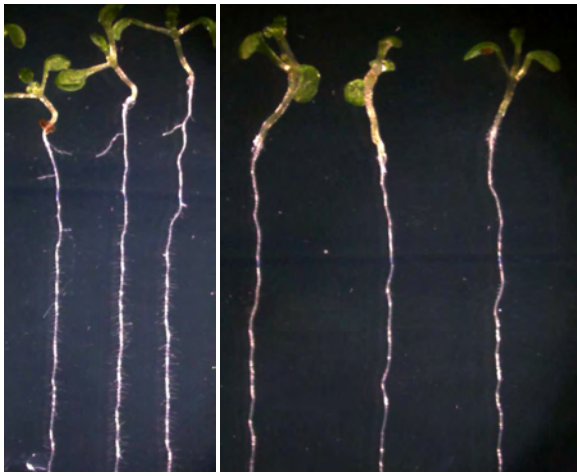


Figure 17. Primary root growth of *pic11* is sensitive to IBA. However IBA rescues the lateral root phenotype of *pic11*. **(a) Primary root elongation of *pic11* is sensitive to IBA. (b) *pic11* produces lateral roots similar to Col-0 in response to IBA. (c) *pic11* does not produce any lateral roots on ATS while Col-0 produce average of 2 lateral roots per cm on the primary root. (d) Higher concentrations of IBA rescues lateral root phenotype of *pic11*.** At higher concentrations of IBA (20 µM) *pic11* produces average of 12 lateral roots/cm along the primary root similar to the wild type.

Figure 17. Continued.

c. ATS



Col-0 *pic11*

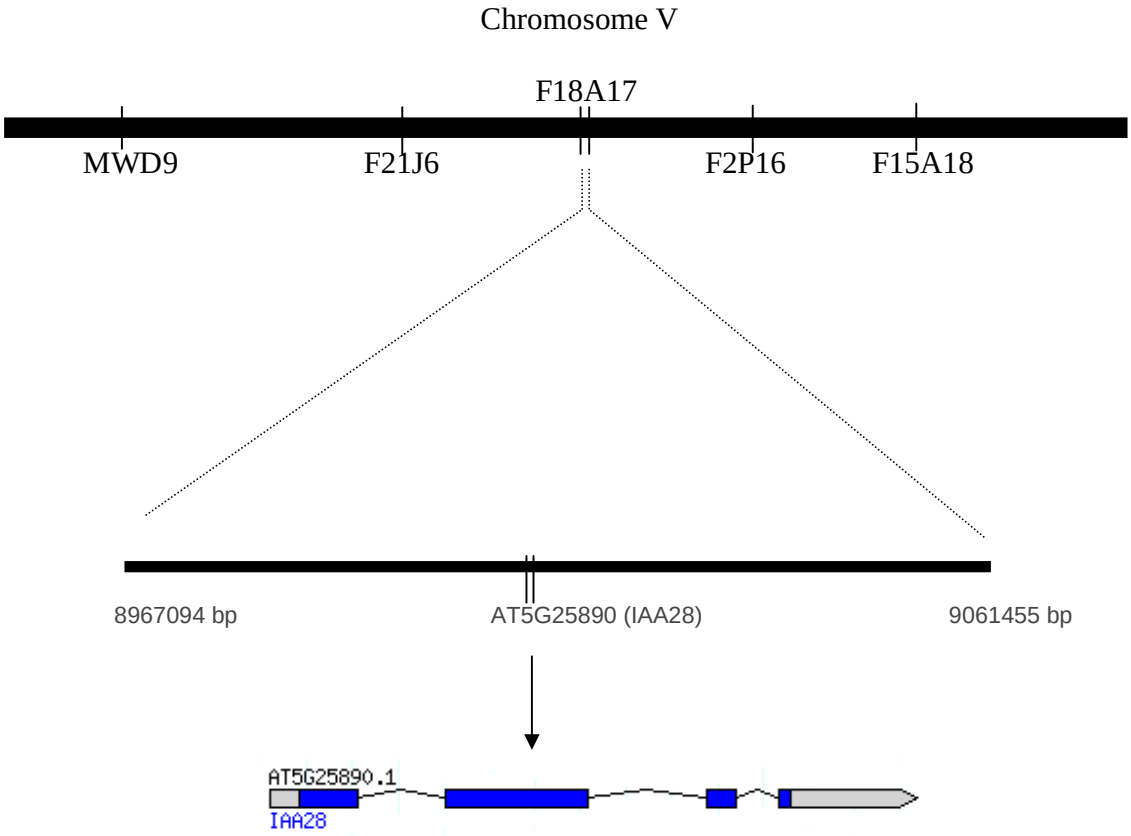
d. 20 µM IBA

Col-0 *pic11*

Molecular and Genetic Characterization of selected *pic11* mutant

pic11 mutation was identified as an allele of *IAA28*

Using about 300 homozygous DNA samples from the F2 progeny of *pic11* x *Ler*, the *pic11* mutation was mapped on to the chromosome V between F21J6 and F2P16 annotation units on the *Arabidopis* genome. This genetic window includes 8 BACs (Bacterial Artificial Chromosome) and the total size of the genetic window was about 735 Kb (Figure 18). Even though this is a wide genetic window, one of the most possible candidate gene for *pic11* was identified in F18A17 BAC (Figure 18). This candidate gene, AT5G25890 (IAA28) encodes an Aux/IAA protein. Aux/IAAs act as negative regulators in auxin signaling. Using gene specific primers (IAA28 -1F & IAA28-1R) (Table 3) AT5G25890gene was amplified from both wild type and *pic11* genomic DNA samples. Sequence analysis of AT5G25890 gene from Col-0 and *pic11* revealed a single nucleotide change in *pic11* background (Figure 19). The nucleotide substitution was G408A of the *IAA28* gene. This single nucleotide change has led to a missense mutation, changing an amino acid from glycine (G) to glutamic acid (E) (Figure 20).



(From Munich Information Center for Protein Sequence)

Figure 18. **Positional cloning of *pic11* and diagrammatic representation of *IAA28* gene.** *pic11/iaa28* mutation was initially mapped between MWD9 and F15A18 BACs on chromosome V. Additional markers, F21J6 and F2P16 were made in order to narrow down *pic11/iaa28* mutant location to 13 BACs (1100 kb) region. The AT5G25890 candidate gene was found in F18A17 BAC. Sequencing of AT5G25890 from *pic11* background revealed that G to A transition that changes conserved glycine to glutamic acid in the conserved region of IAA28 domain II. Schematic diagram of AT5G25890 gene. Blue color thick regions indicate the position of 4 domains of IAA28 protein. BAC stands for Bacterial Artificial Chromosome.

Figure 19. Alignment of nucleotide sequences for *IAA28* gene from both Col-0 (query) and *pic11* (subject). In *pic11* background, the 408th nucleotide of the *IAA28* gene (Col-0) has changed from guanine (G) to adenine (A).

Figure 19. Continued.

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Query  573  AGCTTTCACATGCCGTTGACCAACTCTTCTCTAAGAAAGATTCGTGGGATCTAAACAGAC  632
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  495  AGCTTTCACATGCCGTTGACCAACTCTTCTCTAAGAAAGATTCGTGGGATCTAAACAGAC  554

Query  633  AATACACTTTGGTCTACGAAGACACTGAAGGAGATAAAGTTCTGGTCGGGGATGTTCCTT  692
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  555  AATACACTTTGGTCTACGAAGACACTGAAGGAGATAAAGTTCTGGTCGGGGATGTTCCTT  614

Query  693  GGGAGTAAGTTTTAAATCAAACAAAACTAATTCCATTTTTTTTTCTGGAACTGAATATGA  752
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  615  GGGAGTAAGTTTTAAATCAAACAAAACTAATTCCATTTTTTTTTCTGGAACTGAATATGA  674

Query  753  ACTTACTTTATCAAAAATTCATTTTATATCTATCTACTTTTGTTTTTGTCGAATTTAACA  812
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  675  ACTTACTTTATCAAAAATTCATTTTATATCTATCTACTTTTGTTTTTGTCGAATTTAACA  732

Query  813  AATATTTTTAGTATGAGT  830
            ||||||||||||||||||
Sbjct  733  AATATTTTTAGTATGAGT  749
```

pic11 mutation (*iaa28*) is a gain of function mutant

In *pic11*, the point mutation described above leads to a substitution of the first glycine (G) of the conserved amino acid region of domain II (GWPPV) to glutamic acid (E). Similar mutations in the domain II of several Aux/IAA proteins have resulted in gain of function mutations (see the discussion) suggesting that iaa28 protein may be stabilized due to the mutation. Thus, *pic11*/*iaa28* mutation was identified as another gain of function mutation in Aux/IAA proteins (Figure 21).

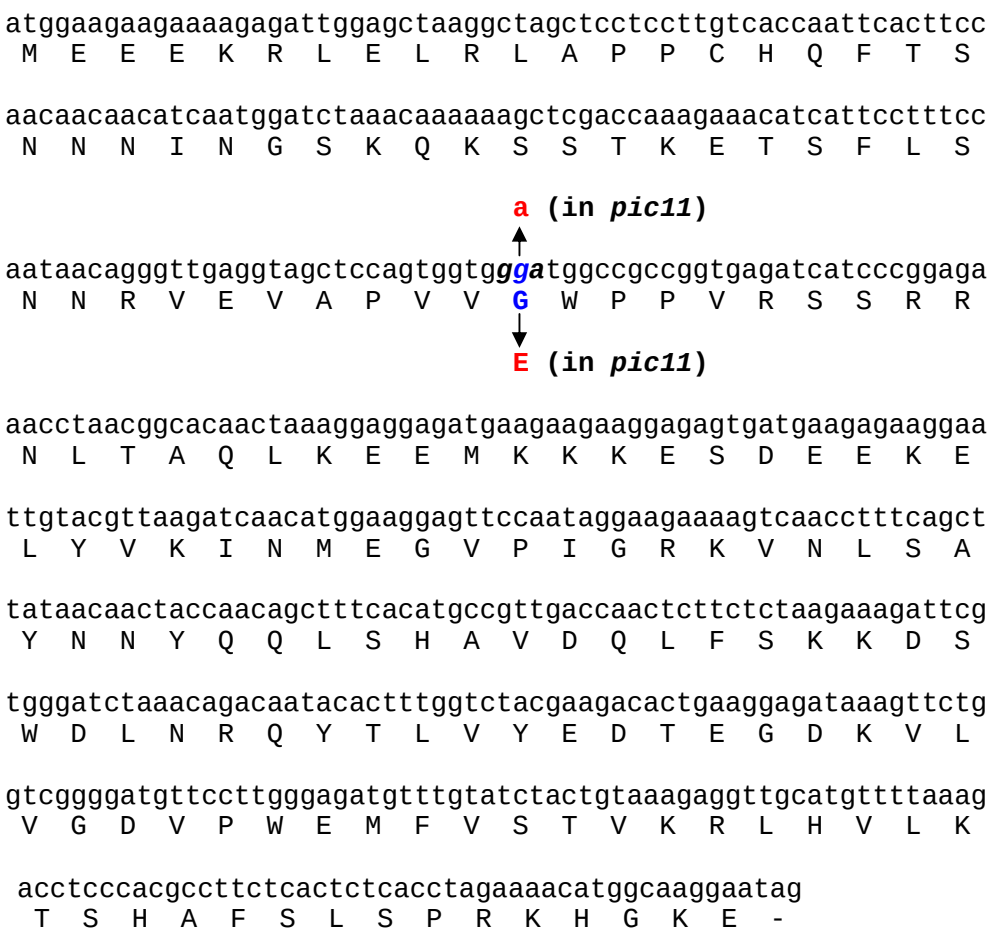

Figure 20. Point mutation in *pic11* has lead to a missense mutation changing amino acid from Glycine (G) to Glutamic acid (E).

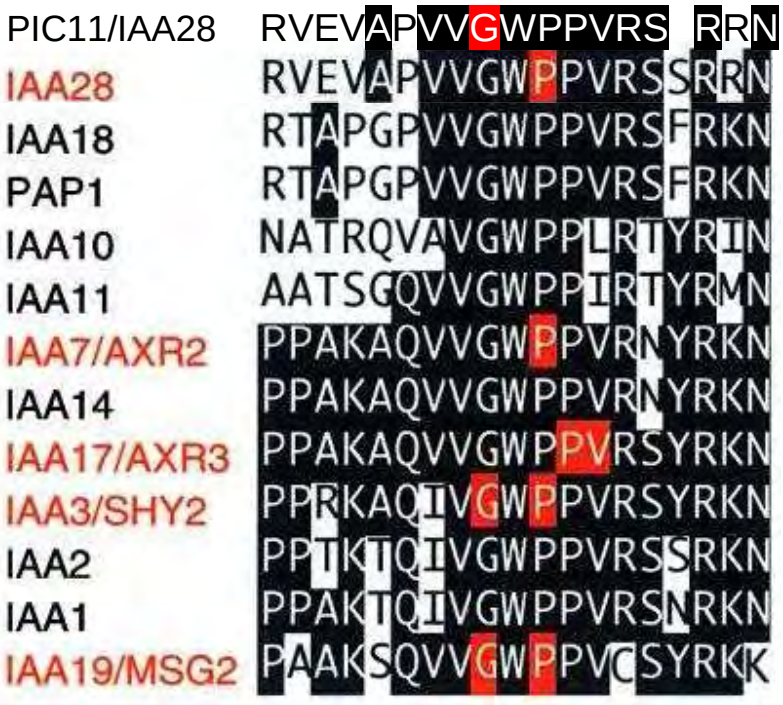

Figure 21. All reported Aux/IAA gain of function mutants have mutations in the conserved domain II. Domain II of pic11/IAA28 protein is aligned with the other Aux/IAA proteins (adapted from Luise *et al*., 2001) and the domain II of several other *Arabidopsis* Aux/IAA proteins, including those for which gain of function mutations have been reported. Red colored protein names indicate known gain of function mutants in different Aux/IAA proteins in *Arabidopsis*. Reported mutated residues from each protein are highlighted in red. The black colored proteins indicate some other Aux/IAA proteins in order to show the domain II conserved region. Black shaded amino acids are the conserved amino acid in different Aux/IAA proteins.

Expression of auxin induced genes in *pic11/IAA28*

Aux/IAA genes are one of the primary auxin responsive genes (Abel and Theologis, 1996). The expression of the most of Aux/IAA genes induce with the treatment of exogenous auxin within less than one hour period. To observe the expression of different *IAA*s in *pic11/iaa28* background compared to the wild type, expression of IAA2 and *IAA28* were examined in untreated *pic11* and Col-0 seedlings as well as seedlings treated for one hour with 20 μM 2,4-D or 100 μM picloram. In Col-0 seedlings, mRNA levels of *IAA2* gene increased with both 2,4-D and picloram treatment while no expression was found in untreated seedlings. Further, mRNA levels of IAA2 in Col-0 background showed higher increases with picloram treatment compared to the 2,4-D treatment. In a *pic11/iaa28* background the mRNA levels of IAA2 showed no difference with auxin treatments compared to the control sample. In contrast to *IAA2* transcript that is strongly induced by auxin it was found that *IAA28* mRNA levels actually decreased slightly in Col-0 background in response to both auxin treatments; this decrease was especially prominent with picloram. But in *pic11/iaa28* background, mutant *iaa28* gene induced with both auxin treatments compared to the control, and the highest induction was observed with picloram treated samples (Figure 22). While most other *Aux/IAA* genes are known to be induced by auxin, this study shows *IAA28* gene expression is repressed by auxin, suggesting that *IAA28* shows a unique expression pattern compared to other *Aux/IAA* genes.

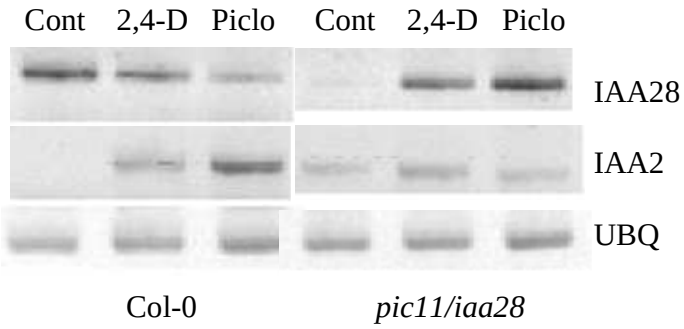

Figure 22. RT-PCR results showing the expression of *IAA2* and *IAA28* genes in both Col-0 and *pic11* background with and without auxin treatments. Total RNA was extracted from four days old seedlings that were treated with 100 μM picloram or 20 μM 2,4-D. cDNA was synthesized with oligo dT primers and the transcript levels of both IAA2 and IAA28 was checked in each treatment for both Col-0 and *pic11/iaa28* by regular PCR. The amplified products were separated on 1% agarose and the resulted bands were visually compared with each other. *IAA2* transcript levels were increased in Col-0 with auxin treatment while such difference was absent in *pic11/iaa28* background. In contrast *IAA28* transcript levels were decreased with auxin treatments in Col-0 background while it gradually increased in *pic11/iaa28* with auxin treatments.

GST-IAA28 interacts with AFB1-myc protein in an auxin-dependent manner

GST-IAA28 and GST-iaa28 proteins were purified from *E. coli*. Both GST-IAA28 and GST-iaa28 purified protein extracts had equal amounts of protein (Figure 23 a.). For pull-down experiments, GST-IAA28 or GST-iaa28 was incubated with crude plant extracts from *Arabidopsis* plants carrying *35S::AFB1-myc* transgene. 2,4-D was added to the reaction mixture and incubated at 4°C for 30 minutes. Proteins that interacted with GST recombinant protein were separated on SDS-PAGE and immunoblotted with anti-myc antibody. GST-IAA28 interacted with AFB1-myc effectively in an auxin dependant manner, but the *pic11/iaa28* mutation affected this interaction (Figure 23b).

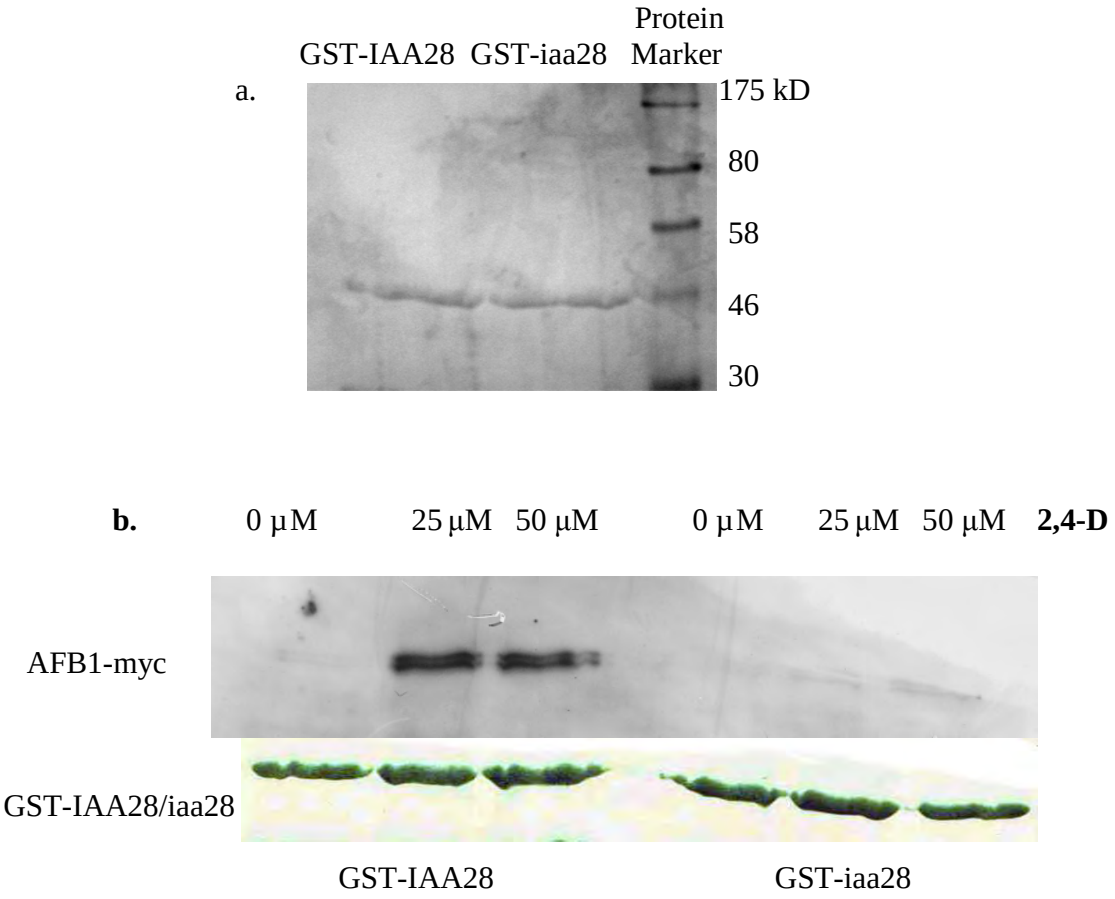

Figure 23. Pull-down results showing the interaction of GST-IAA28 with TIR1-myc protein in an auxin-dependent manner. (a) Purified recombinant proteins of GST-IAA28 and GST-iaa28. Recombinant proteins were expressed in *E.coli* and purified using glutathione agarose. **(b) Wild- type IAA28 protein interacts with AFB1-myc protein in an auxin-dependent manner**. The *pic11/iaa28* mutation prevents the interaction between the mutant protein and AFB1-myc. Pull-down assays were carried out using bacterially expressed GST-IAA28 or GST-iaa28 with plant derived AFB1-myc protein in the presence of auxin.

AXR3-GUS recombinant protein is stabilized in *pic11/iaa28* background

Many of the Aux/IAA proteins have a very short half-life and are rapidly degraded in response to auxin (Worley *et al*., 2000; Zenser *et al*., 2003). To test whether the *pic11* mutation affects the degradation of Aux/IAA proteins, the degradation of the recombinant protein AXR3-GUS was tested in the mutant background. Expression of AXR3-GUS is under the control of a heat shock promoter. Plants carrying this transgene construct (*HS::AXR3-GUS*) were crossed to *pic11* and homozygous plants for *pic11* and the transgene were used to monitor the degradation of AXR3-GUS in response to auxin. AXR3-GUS protein is degraded in Col-0 background after 45 minutes with 20 μM 2,4-D treatment, while in the *pic11/iaa28* mutant background the protein is stabilized (Figure 24).

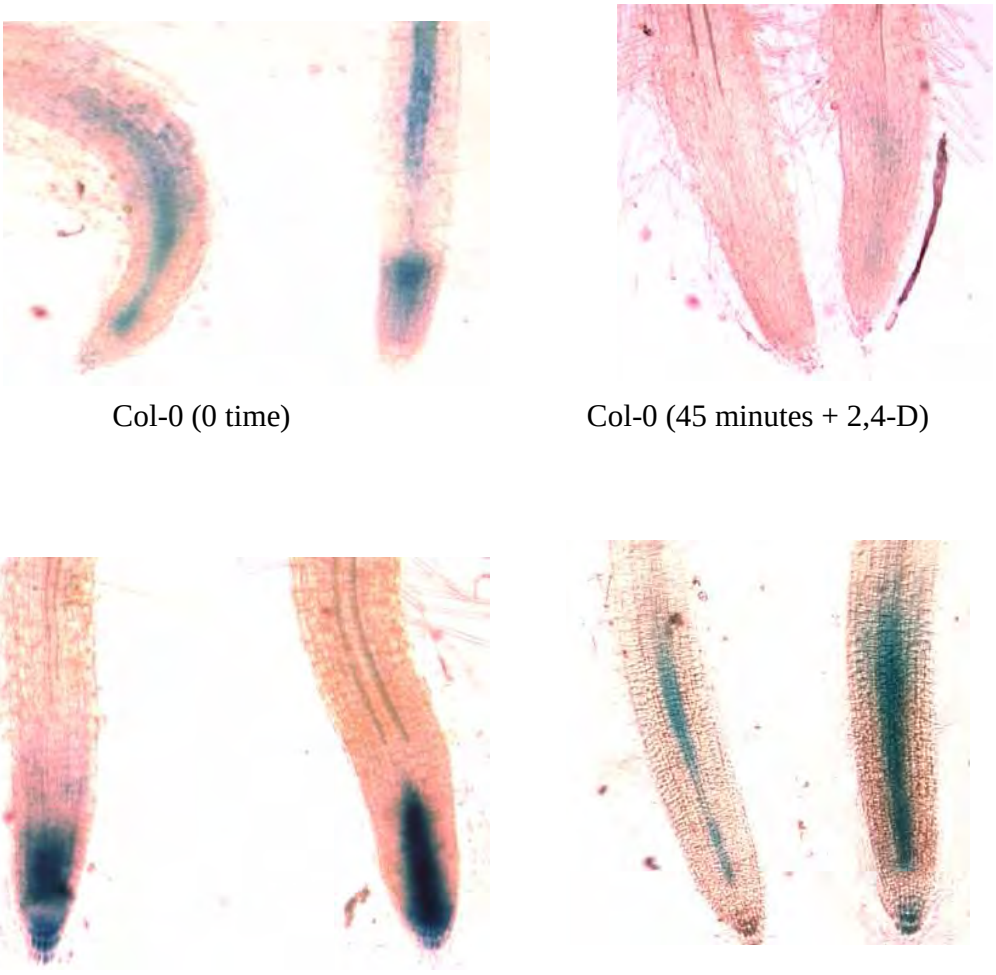

Figure 24. AXR3-GUS recombinant protein is stabilized in *pic11/iaa28* background. In Col-0 background AXR3-GUS protein is degraded in response to auxin treatment while in *pic11/iaa28* background AXR3-GUS protein degradation is significantly inhibited resulting the stabilization of AXR3-GUS protein.

CHAPTER IV

DISCUSSION

Auxin is a plant hormone that regulates many aspects of plant growth and development. In this study a chemical genetic approach was used to identify components in auxin response in *Arabidopsis* using the synthetic auxin picloram, a widely used herbicide. The use of different synthetic auxinic compounds like picloram is valuable in dissecting auxin response pathway/s in plants. A genetic screen was employed to identify mutants that were resistant to picloram in an attempt to isolate picloram targets or downstream components. After screening about 70,000 EMS mutagenized *Arabidopsis* seedlings, we isolated 30 putative mutant lines. *pic11* and *pic32* represent two of those mutants with altered response to auxins.

The *pic32* mutation was mapped to a 62 Kb region in chromosome V

Lateral root development in *pic32* is hypersensitive to higher concentrations of picloram while the primary root elongation is sensitive to both picloram and 2,4-D. Additionally, *pic32* shows some defects in growth and development especially the leaves of *pic32* that are relatively small with margins that tend to curl downward. *pic32* is inherited as a recessive mutation and is mapped to the MWD9 annotation unit of chromosome V. Using closely linked SSLP markers, the genetic window was further

narrowed down to a region with 16 genes. Among those 16 genes, there are 3 unknown genes and few possible candidate genes for *pic32*, such as AT5G22380, a transcriptional factor identical to the NAC-domain containing protein, and AT5G22355, a DC1 domain-containing protein, UV-B light-insensitive protein. In order to identify the *pic32* mutation, these selected candidate genes should be sequenced from the *pic32* and Col-0 backgrounds and sequences should be compared.

***pic11* is a gain of function mutation in *IAA28,* a gene that encodes an Aux/IAA protein**

pic11 mutation was mapped to F18A17 annotation unit on chromosome V north arm of *Arabidopsis* genome. The mutation in *pic11* background was identified as a single nucleotide change in one of the Aux/IAA proteins named IAA28 in *Arabidopsis thaliana*. Aux/IAA proteins are small nuclear proteins (Abel *et al*., 1994). Many members of this protein family show increased levels of mRNA within 5 to 20 minutes after exogenous auxin application, and this mRNA induction is specific for all biologically active auxins (Guilfoyl *et al.*, 1998). There are at least 29 Aux/IAA genes in the *Arabidopsis* genome. Most of the Aux/IAA proteins share four conserved domains. Domain I and II are unique to Aux/IAA proteins whereas domain III and IV are shared with the C-terminal domain of ARF proteins (Hagen and Guilfoyl, 2002; Liscum and Reed, 2002). Domain I of the Aux/IAAs contains an ethylene response factor (ERF)-associated amphiphilic repression motif (Tiwari *et al*., 2004), which is known to be involved in transcriptional repression (Ohta *et al*., 2001). Domain III and IV are located in the C-terminal half of the protein and are involved in homo- and hetero-dimerization with other Aux/IAA proteins, and

hetero-dimerization with ARFs (Dharmasiri and Estelle, 2004). Domain II of Aux/IAA proteins is important for the interaction with TIR1 auxin receptor in the SCF^{TIR1} complex, and for subsequent degradation of Aux/IAA proteins through ubiquitin-proteosome pathway. Using transient assays, it has been found that a 13 amino acid region of domain II is sufficient for the interaction with TIR1 and subsequent degradation of a recombinant protein (Ramos *et al*., 2001). Further, this 13 amino acids stretch includes a highly conserved amino acid sequence of GWPPV. Single amino acid substitutions in these conserved amino acids result in a 6 to 20-fold increase in accumulation of the mutated protein (Ramos *et al.*, 2001).

In *pic11*, the nucleotide change 408^{G-A} results in change of the **G**WPPV conserved region to **E**WPPV. Pull-down data shows that bacterially expressed recombinant GST-IAA28 interacts with plant derived Auxin Binding F-box 1 (AFB1) protein (which acts as an auxin receptor similar to TIR1) in an auxin-dependent manner. However, if the *pic11* mutation is introduced to GST-IAA28, the mutant protein does not interact efficiently with AFB1. This result suggests that mutant protein may be stabilized in *pic11* background. The increased level of mutant iaa28 protein may inhibit the activity of the ARF proteins by preventing them from activating auxin-induced genes, leading to pleiotropic defects in growth and development of *pic11* mutants. Similar observations were made with other known Aux/IAA mutants in *Arabidopsis thaliana*. For example, gain of function mutations in several Aux/IAA genes including *shy2*/*iaa2* (Tian and Reed, 1999), *shy1*/*iaa6* (Kim *et al.*, 1996; Reed, 2001), *axr2/iaa7* (Nagpal *et al*., 2000; Timpte *et al*., 1994), *bdl/iaa12* (Hamann *et al.*, 1999), solitary root *slr*/*iaa14* (Fukaki *et al*., 2002), *axr3*/*iaa17* (Leyser *et al*., 1996; Rouse *et al*., 1998) and *msg2*/ *iaa19*

(Tatematsu *et al.*, 2004) have pleiotropic effects on plant growth. All of the above mutations are caused by a single amino acid change in domain II of the corresponding Aux/IAA proteins. Biochemical analyses have revealed that these domain II mutants stabilize the levels of the corresponding Aux/IAA protein. For example mutant axr3-1/iaa17 protein has a seven-fold increased half-life compared with the wild type version of the protein (Ouellet *et al*., 2001). Similarly, the shy2-1/iaa3 protein accumulated in *shy2-2/iaa3* mutant plants (Colon-Carmona *et al*., 2000). These observations from other studies along with the *pic11* phenotype and lack of in vitro protein interaction suggest that mutant iaa28 protein may be stabilized in *pic11*.

Expression of auxin-induced genes in *pic11/IAA28*

Transcription of most *Aux/IAA* genes is induced by treatment with exogenous auxin within less than one hour (Abel and Theologis, 1996). For instance, increased steady-state mRNA levels were observed for all the *Aux/IAA* genes from *IAA1* to *IAA14* except for *IAA7* and *IAA8* with 20 μM 2,4-D treatment (Abel *et al.*, 1995). Further, accumulation of *Aux/IAA* mRNAs ranged from a two-fold increase for *IAA9* to a 25-fold increase for *IAA5* (Abel *et al*., 1995). Previous studies have shown that auxin-induced transcriptional activation of many *Aux/IAA* genes is affected in many auxin related mutants (Leyser and Pickett, 1996). Results of this study show that both picloram and 2,4-D increase the abundance of *IAA2* mRNA in wild type plants. However, neither auxin has any effect on *IAA2* mRNA abundance in *pic11/iaa28* plants, suggesting a defect in auxin-induced gene transcription in *pic11/iaa28*. Most importantly, IAA2 transcript is not detectable in Col-0 in the absence of auxin, but is readily detectable in

pic11/iaa28 even in the absence of exogenous auxin, suggesting that *IAA28* may be involved in down regulating the *IAA2* gene expression in wild type background and that expression of *IAA2* (and perhaps other *Aux/IAAs*) in *pic11/iaa28* background is misregulated, leading to pleiotropic growth and developmental defects. It may be interesting to see whether the expression of other *Aux/IAA* genes is also misregulated in the mutant background.

In contrast to *IAA2*, the transcript level of *IAA28* was decreased slightly in the wild type background in response to both auxin treatments, especially with picloram. But in *pic11/iaa28* background, expression of the mutant *iaa28* gene was induced with both auxin treatments compared to the control, and the highest induction was observed with the picloram-treated sample. These results suggest that the regulation of *IAA28* gene expression follows unique pattern compared to other known *Aux/IAA* genes that are induced by auxins.

***PIC11*/*IAA28* and other *AUX/IAA* genes may have overlapping and distinct roles in growth and development**

Auxin regulates many aspects of plant growth and development including lateral root induction, root hair development, primary root elongation, apical dominance, stem elongation, gravitropic responses and hypocotyl elongation (Davis, 1995).

Gain of function mutations in different *AUX/IAA* genes cause distinct as well as similar adult phenotypes. *pic11/iaa28* mutants showed reduced apical dominance, reduced length of inflorescence axis, and relatively smaller leaves, resulting in short small plants compared to the wild type. Similarly several other Aux/IAA mutants such as

shy2/iaa3, *axr2-1/iaa7*, *bdl/iaa12*, *axr3/iaa17* had bushier plants due to reduced apical dominance, shorter inflorescence axes and stems with relatively smaller leaves compared to the wild type (Hamann *et al.*, 1999; Leyser *et al.*, 1996; Negpal *et al.*, 2000; Tian and Reed, 1999; Timpte *et al.*, 1994). In *axr3/iaa17* the smaller leaves were curled with epinastic petioles and had higher levels of anthocyanin production compared to the wild type. Similarly higher anthocyanin production was observed in *pic11/iaa28* plants.

Not all gain of function mutants of *Aux/IAA* genes exhibit similar defects in growth and development. For example, *Arabidopsis msg2/iaa19* plants had similar size and rosette leaf morphology to the wild type (Tatematsu *et al*., 2004). On the other hand *msg2/iaa19* displays neither gravitropic nor phototropic defects (Tatematsu *et al*., 2004). Similarly, in this study *pic11*/*iaa28* showed no defects in either shoot or root gravitropic responses (data not shown). In contrast, several mutants such as *axr3/iaa17*, *axr2-1/iaa7*, *slr1/iaa14*, that share shoot/root developmental defects with *pic11*/iaa28, showed reduced shoot and root gravitropic responses (Timpte *et al*., 1994; Negpal *et al.*, 2000).

Role of PIC11/IAA28 in hypocotyl elongation

When grown in the light at higher temperatures (29^{o}C), *Arabidopsis* seedlings exhibit a dramatic increase in hypocotyl elongation, and this temperature induced-growth is sharply reduced in auxin signaling or transport mutants. Moreover, temperature induced hypocotyl elongation is associated with increased levels of IAA in the hypocotyl, suggesting that high temperature induces the synthesis or transport (Gray *et al*., 1998). *pic11/iaa28* has elongated hypocotyl compared to the wild type under normal growth temperature (22^{o}C) (Figure 12). However, at high temperature (29^{o}C), wild type

hypocotyls elongated to a greater extent than *pic11* hypocotyls, indicating that temperature-induced auxin metabolism, transport or signaling processes have been affected due to the mutation (Figure 13). Interestingly, *shy2/iaa3* had short hypocotyls compared to wild type at normal temperature (Tian and Reed, 1999), suggesting these two Aux/IAA proteins, SHY2/IAA3 and PIC11/IAA28, may have opposing regulatory functions in hypocotyl elongation.

Role of PIC11/IAA28 in lateral root development

Most of the known *Aux/IAA* mutants show defects in normal lateral root initiation and development. For example, in *shy2/iaa3* produce many longer lateral roots compared to wild type (Tian and Reed, 1999); conversely, in both *slr1/iaa14* and *msg2/iaa19*, lateral root development is strongly inhibited (Fukaki *et al*., 2002; Tatematsu *et al*., 2004). Similar to *slr1/iaa14* and *msg2/iaa19*, lateral root development in *pic11/iaa28* is severely defective. While 8-10 day-old light grown wild type seedlings produce many lateral roots, similarly aged *pic11/iaa28* seedlings produce no lateral roots. Previous studies have shown that application of exogenous auxin induces lateral root formation in *Arabidopsis* (Laskowski *et al*., 1995). Nevertheless in this study 85 nM 2,4-D had no effect on lateral root formation in *pic11/iaa28*. Interestingly, a higher concentration of picloram induced a few lateral roots in *pic11/iaa28* with a characteristic pattern. Picloram induced 4-5 lateral roots that emerged from the same point just below the hypocotyl region, suggesting that though picloram shares auxinic activities with other known auxins, it may also have a distinct mechanism of action. To find out whether *pic11/iaa28* is incapable of producing lateral roots, mutant seedlings were treated with

very high levels of auxin. When mutant seedlings were treated with 5 μM 2,4-D, many lateral roots were induced (data not shown). Moreover, *pic11/iaa28* showed similar sensitivity to wild type to another natural auxin named indole-3-butyric acid (IBA). In *pic11/iaa28,* IBA treatment inhibits primary root elongation while promoting the development of many lateral roots along the primary roots similar to the wild type. It has been found that even though plants have slightly less IBA than IAA (Ludwig-Muller and Epstein, 1994; Ludwig-Muller *et al*., 1993), IBA is often more effective than IAA in inducing root initiation (Hartmann *et al*., 1990). According to the results, it can be suggested that *IAA28* is a negative regulator of lateral root initiation and development, and the *pic11/iaa28* is not defective in initiation or development of lateral roots, but defective in sensitivity to auxin. In an earlier study, it has been found that other gain of function mutants of other Aux/IAA genes such as *shy2/iaa3* (Tian and Reed, 1999), *axr5/iaa1* (Yang *et al*, 2004) and *iaa14* (Fukaki *et al*., 2002) also inhibit lateral root development in *Arabidopsis* suggesting that all these mutants may act as negative regulators of lateral root development.

Role of PIC11/IAA28 in root hair development

Another striking feature of *pic11/iaa28* is the defects in the development of root hairs. *pic11/iaa28* produces fewer and shorter root hairs than the wild type. Similarly, *slr1/iaa14* and *axr3/iaa17* mutants also show defects in root hair formation (Fukaki *et al*., 2002: Leyser *et al*., 1996; Rouse *et al*., 1998). Both auxin and ethylene are known to be involved in root hair formation in plants (Reed *et al*., 1993; Okada and Shimura, 1994; Pitts *et al*., 1998). To examine whether the application of auxin or ethylene can rescue the

root hair phenotype of *pic11/iaa28*, 4-day old seedlings were transferred onto different media containing 85 nM 2,4-D, 10 μM picloram or 100 nM ACC, the immediate precursor of ethylene. After incubation for an additional 4 days, both auxin treatments induced root hair formation in wild type seedlings but not in *pic11/iaa28* seedlings. In contrast ACC treatment was able to recover the root hair phenotype of *pic11IAA28* to a greater extent though not to the same level as wild type. These results indicate that the *pic11/iaa28* mutant retains the ability to differentiate root hairs in response to ethylene. The *GLABRA2* (*GL2*) gene, which is required for cell specification of non-hair cell identity in the epidermis, is expressed in the non-hair cell layer (Masucci and Schiefelbein, 1996). It has been proposed that auxin and ethylene act downstream of *GL2* in promoting root hair formation, because mutations affecting hormone sensitivity to auxin and/or ethylene do not alter the expression of *GL2* gene (Masucci and Schiefelbein, 1996). For example, the *axr2/iaa7* mutant resistant to both auxin and ethylene has reduced root hair formation, but has a normal expression pattern of the *GL2* $_{promoter}$*::GUS* reporter in the non-hair cell file, indicating that the *axr2/iaa7* acts downstream of *GL2* (Masucci and Schiefelbein, 1996). Therefore, our observation that the *pic11/iaa28* can develop normal root hairs after ACC treatment along with previous studies strongly suggests that *PIC11/IAA28* acts upstream of ethylene synthesis/signaling and downstream of *GL2* in root hair development of *Arabidopsis*. However, independent pathways of ethylene, GL2 and auxin that converge to regulate root hair development cannot be ruled out. Comparisons of growth defects of *pic11/iaa28* with other known gain of function mutants in *Arabidopsis thaliana* reveal that *pic11/iaa28* has both unique and common defects compared to other domain II mutants of *AUX/IAA* gene family. Therefore, these

overlapping yet distinct root and shoot phenotypes of gain of function mutants suggest that *AUX/IAA* genes play only partially redundant roles in several auxin-mediated responses.

Conclusions and future directions

Identification of mutants in auxin signaling pathway leads to better understanding of the mechanisms involved in auxin response. In this study, two mutants *pic11* and *pic32* with altered response to auxin were characterized in detail. *pic32* exhibits hypersensitivity to exogenous picloram and shows a characteristic shoot phenotype with smaller, curled leaves and reduced apical dominance. While the *pic32* gene has not yet been identified, the mapping efforts identified a genetic window with 17 genes in the south arm of the chromosome V. Future efforts will focus on sequencing of candidate genes in this window and confirmation of the mutant gene.

pic11 is a semi-dominant gain of function mutation that exhibits resistance to both picloram and 2,4-D. Mutant plants show severe growth and developmental defects, suggesting the importance of this gene in plant development. Through map based cloning the mutant gene was identified as an allele of *IAA28*, a member of the *Aux/IAA* gene family. Aux/IAA proteins function as repressors of auxin-induced gene expression. In response to auxin, Aux/IAA proteins interact with the auxin receptors (TIR1, AFB1, AFB2 and AFB3) and then are degraded through the unbiquitin-proteasome pathway. Domain II of Aux/IAA proteins is known to be involved in the interaction with auxin receptors in the presence of auxin.

In *pic11*, the conserved amino acid residues GWPPV of domain II of *IAA28* gene has been changed to EWPPV due to a single nucleotide change. This domain II mutation prevents the interaction of mutant iaa28 protein with the receptor protein AFB1 (and perhaps with other receptor proteins such as TIR1, AFB2 and AFB3) and probably stabilizes the mutant protein. The increased mutant protein level may inhibit the activity of the ARF proteins by preventing the activation of auxin-induced genes that are necessary for growth and development.

This conjecture is well supported by the results of pull down experiments that were carried out using recombinant GST-IAA28 or GST-iaa28 with AFB1-myc protein. The results of these assays indicate that GST-IAA28 interacts with AFB1-myc in an auxin-dependant manner, while the mutant protein, GST-iaa28, does not interact efficiently with AFB1-myc protein.

In the suggested model (Figure 25), it is proposed that this stabilized mutant protein may directly affect the regulation of other genes that are related to growth and development. According to the RT-PCR results, one of the *Aux/IAA* genes tested, *IAA2,* is misregulated in the *pic11* background. The *pic11/iaa28* gene itself is also mis-regulated. Thus, in future work, it would be interesting to see whether other Aux/IAA genes are also misregulated in *pic11* background. As Aux/IAAs are transcriptional repressors, they should regulate many downstream genes that are essential for growth and development. Therefore, it may be interesting to find out what downstream targets are regulated by *IAA28*. Microarray analysis and/or proteomic analysis of *pic11/iaa28* and Col-0 may help to identify downstream targets.

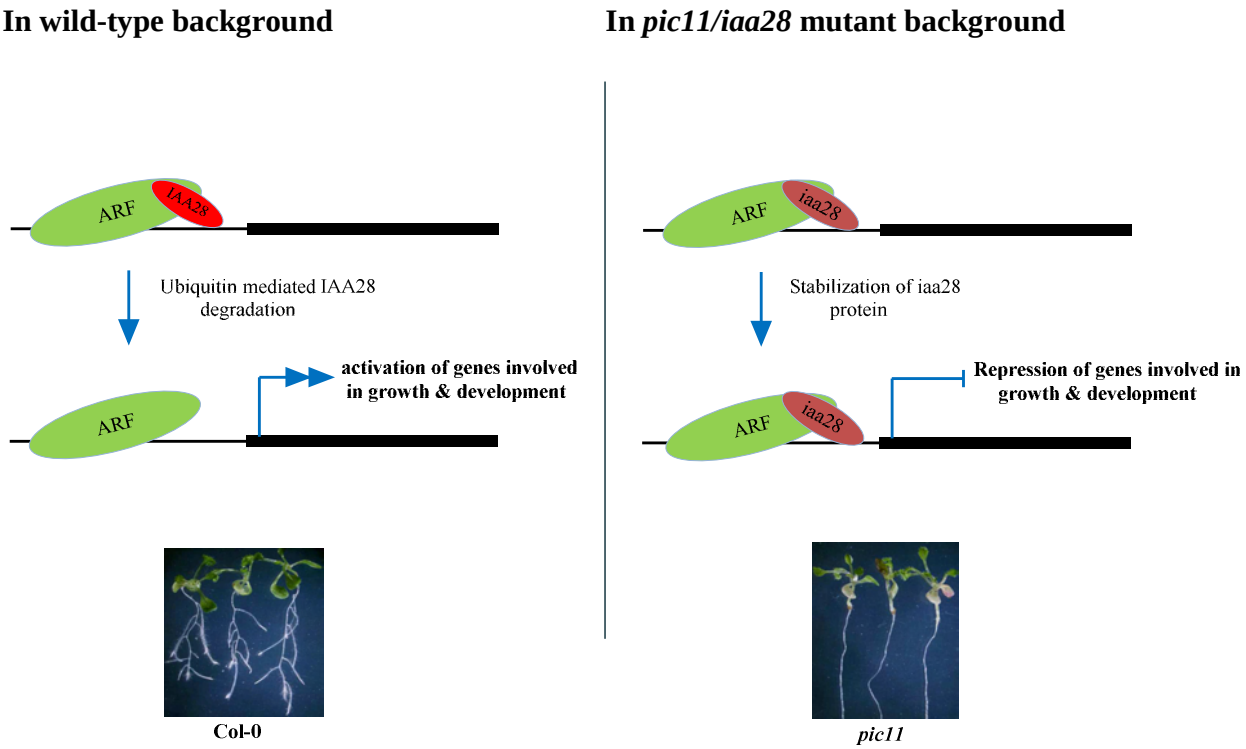

Figure 25. Proposed model for *IAA28* mediated growth and development in *Arabidopsis thaliana*.

REFERENCES

Abel S., and Theologis A. 1996. "Early genes and auxin action." *Plant Physiology*,111: 9–17.

Abel S., Oeller P.W., and Theologis A. 1994. "Early auxin-induced genes encode short-lived nuclear proteins." *Proceedings of the National Academy of Science of the USA*, 91:326–330.

Abel, S., Nguyen M.D., and Theologis A. 1995. "The PS-IAA4/5 like family of early auxin- inducible mRNAs in *Arabidopsis thaliana*." *Molecular Biology*, 251:533-549.

Bennett M.J., Marchant A., Green H.G., May S.T., Ward S.P., Millner P.A., Walker A.R., Schulz B., and Feldmann K.A. 1996. "*Arabidopsis* AUX1 gene: a permease-like regulator of root gravitropism." *Science*. 273:948-950.

Berendzen K., Searle I., Ravenscroft D., Koncz c., Batschauer A., Coupland G., Somssich I.E., and Ulker B. 2005. "A rapid and versatile combined DNA/RNA extraction protocol and its application to the analysis of a novel DNA marker set polymorphic between *Arabidopsis thaliana* ecotypes Col-0 and *Landsberg erecta*." *Plant Methods*. 1:1-4.

Bergmans H.E.N., Die I.M.V., and Hoekstra W.P.M. 1981. "Transformation in *Escherichia coli*: Stages in the Process." *Journal of Bacteriology*, 146(2):564-570.

Chen Q., Dai L., Xiao S., Wang Y., Liu X., and Wang L. 2007. "The COI1 and DFR Genes are Essential for Regulation of Jasmonate-Induced Anthocyanin Accumulation in *Arabidopsis*." *Integrative Plant Biology*, 49(9):1370-1377.

Cobb A. 1992. "Auxin type herbicides." *Herbicides and Plant physiology*, Chapman & Hall. London. 82-106.

Colon-Carmona A., Chen D.L., Yeh K.C., and Abel S. 2000. "Aux/IAA proteins are phosphorylated by phytochrome in vitro." *Plant Physiology*, 124:1728-1738.

Cutler E.J. 1978. "The epidermis". *Plant Anatomy*. Clowers & Sons. London. 94-106.

Davis P.J. 1995. "Plant hormones physiology, biochemistry, and molecular biology." (Dordrecht,the Netherlands: Kluwer Academic Publishers).

Dharmasiri N., and Estelle M. 2004. "Auxin signaling and regulated protein degradation." *Trends in Plant Science*, 9(6):302-308.

Dharmasiri N., Dharmasiri S., and Estelle M. 2005. "The F-box protein TIR1 is an auxin receptor." *Nature*, 435(26):441-445.

Dharmasiri N., Dharmasiri S., Jones A.M., and Estelle M. 2003. "Auxin action in a cell-free system." *Current Biology*, 13: 1418–1422.

Dharmasiri N., Dharmasiri S., Weijers D., Lechner E., Yamada M., Hobbie L., Ehrismann J.S., Jürgens G., and Estelle M. 2005. "Plant Development is regulated by a family of Auxin Receptor F Box Proteins." *Development Cell*, 9:109-119.

Fukaki H., Tameda S., Masuda H., and Tasaka M., 2002. "Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY-ROOT/IAA14 gene of *Arabidopsis*." *The Plant Journal*, 29:153-168.

Gagne J.M., Downes B.P., Shiu S.H., Durski A.M., and Vierstra R.D. 2002. "The F-box subunit of the SCF E3 complex is encoded by a diverse super family of genes in *Arabidopsis*." *Proc Natl Acad Sci USA*, 99:11519-11524.

Gendreau E., Traas J., Desnos T., Grandjean O., Caboche M., and Hofte H. 1997. "Cellular basis of hypocotyls growth in *Arabidopsis thaliana*." *Plant Physiology*, 114:295-305.

Gray W.M., Ostin A., Sandberg G., Romano C.P., and Estelle M. 1998. "High temperature promotes auxin mediated hypercotyl elongation in *Arabidopsis*." *Proc. Natl. Acad. Sci.*, 95:7197-7202.

Gray W.M., Kepinski S., Rouse D., Leyser O., and Estelle M. 2001. "Auxin regulates SCF^{TIR1}- dependant degradation of AUX/IAA proteins." *Nature*, 414:271-276

Grossmann K. 2000a. "The mode of action of quinclorac: a case study of a new auxin-type herbicide." *Herbicides and their mechanism of action*, Sheffield Academic Press, Sheffield. 181–214.

Guilfoyle T. 2007. "Sticking with auxin." *Nature*, 446:621-622.

Guilfoyle T., and Hagen G. 2001."Auxin response factors." *J. Plant Growth Regulation*, 10:281-291.

Guilfoyle T., Hagen G., Ulmasov T., and Murfett J. 1998. "How does auxin turn on genes." *Plant Physiology*, 118:341-347.

Guilfoyle T.J., Ulmasov T., and Hagen G. 1998b. "The ARF family of transcription factors and their role in plant hormone responsive transcription." *Cell Mol. Life Sci*. 54:619-627.

Hagen G., and Guilfoyle T. 2002. "Auxin responsive gene expression: genes, promoters and regulatory factors." *Plant Molecular Biology*, 49:373-385.

Hamann T., Mayer U., Jurgens G. 1999. "The auxin-insensitive bodenlos mutation affects primary root formation and apical-basal patterning in the *Arabidopsis* embryo." *Development*, 126:1387-1395.

Hartmann H.T., Kester D.E., and Davis F.T. 1990. "Plant propagation; Principles and practices." (Prentce-Hall) 99-245.

Hayashi K., Tan X., Zheng N., Hatate T., Kimura Y., Kepinski S., and Nozaki H. 2008. "Small molecule agonists and antagonists of F-box protein- substrate interactions in auxin perception and signaling." *Proc. Natl. Acad. Sci. USA*, 105:5632-5637.

Hellmann H., and Estelle M. 2002. "Plant development: regulation by protein degradation." *Science*, 297:793-797.

Hobbie L., and Estelle M. 1995. "The axr4 auxin-resistant mutants of *Arabidopsis thaliana* define a gene important for root gravitropsim and lateral root initiation." *The Plant Journal*, 7(2):211-220.

Kepinski S., and Leyser O. 2005. “The *Arabidopsis* F-box protein TRI1 is an auxin receptor.” *Nature*, 435:446-451

Kim B.C., Soh M.S., Kang B.J., Furuya M., and Nam H.G. 1996. “Two dominant photomorphogenic mutations of *Arabidopsis thaliana* identified as suppressor mutations of hy2.” *Plant J*, 9:441-456.

Kim Y.S., Schumaker K.S., and Zhu J.K. 2006. "EMS mutagenesis of *Arabidopsis*." *Methods in Molecular Biology*, 323:101-103.

Koegl M., Schlenker S., Ulrich H.D., Jentsch T.U., and Jentsch S. 1999. “A novel ubiquitination factor, E4 is involved in multiubiquitin chain assembly.” *Cell*, 96:635-644.

Kuroda H., Takahashi N., Shimada H., Seki M., Shinozaki K., and Matsu M. 2002. “Classification and expression analysis of *Arabidopsis* F-box containing protein genes.” *Plant Cell Physiology*. 43:1073-1085.

Laskowski M.J., Williams M.E., Nusbaum H.C., and Sussex I.M. 1995. "Formation of lateral root meristems is a two-stage process." *Development*, 121:3303–3310.

Leyser H.M.O., Pickett F.B., Dharmasiri S., and Estelle M. 1996. "Mutations in the AXR3 gene of *Arabidopsis* result in altered auxin response including ectopic expression from the SAUR-AC1 promotor." *Plant Journal*, 10(3):403-413.

Lincoln C., Britton J.H., and Estelle M. 1990. “Growth and development of the axr1 mutants of *Arabidopsis*.” *Plant Cell*, 2:1071-1080.

Liscum E., and Reed J.W. 2002. “Genetics of Aux/IAA and ARF action in plant growth and development”. *Plant Mol.Biology* 49:387-400.

Ljung K., Bhalerao R.P., and Sandberg G. 2001. "Sites and homeostatic control of auxin biosynthesis in *Arabidopsis* during vegetative growth." *Plant Journal*, 28:465–474.

Ludwig-Muller J., Saas S., Sutter E.G., Wonder M., and Epstein E. 1993. "Indole-3-butric acid in *Arabidopsis thaliana*. I. Identification and quantification." *Plant Growth Regul*, 13:179-187.

Ludwid-Muller J., and Epstein E. 1994. “Indole-3-butyric acid in *Arabidopsis thaliana*, in vitro biosynthesis.” *Plant Growth Regulation*, 14: 7-14.

Luise E., Lasswell R.J., and Bartel B. 2001. “A gain of function mutation in IAA28 suppresses lateral root development.” *Plant Cell*, 13:465-480.

Munn-Bosch S., Weiler S., Alegre E.W., Maller L., Dachting M., and Falk P. 2007. “Tacopherol may influence cellular signaling by modulation jasmonic acid levels in plants”. 225:681-691.

Musucci J.D., and Schiefelbein J.W. 1996. "Hormones act downstream of TTG and GL2 to promoteroot hair initiation through an auxin and ethylene- associated process." *Plant Cell*, 8:1505-1517.

Nagpal P., Walker L.M., Young J.C., Sonawala A.,Timpte C., Estelle M., and Reed J. 2000. "AXR2 Encodes a Member of the Aux/IAA Protein Family 1." *Plant Physiology*, 123:563–573.

Nemhauser J.L., Feldman L.F., and Zambryski P.C. 2000. "Auxin and ETTIN in *Arabidopsis* gynoecium morphogenesis." *Development*, 127:3877-3888.

Ohta M., Matsui K., Hiratsu K., Shinshi H., and Ohme-Takagi M. 2001. “Repression domains of class II ERF transcriptional repression.” *Plant Cell*. 13:1959-1968.

Okada K., and Shimura Y. 1994. “Modulation of root growth by physical stimuli.” *Arabidopsis* (ed. E.M. Meyerowitz and C.R. Somerville). Cold Spring Harbor. NY. Cold Spring Habor Laboratory Press. 665-648

Oono Y., Chen Q.G., Overvoorde P.J., Kohler C., and Theologis A. 1998. “age mutants of *Arabidopsis* exhibits altered auxin regulated gene expression.” *Plant Cell*. 10:1649-1662.

Ouellet F., Overvoorde P.J., and Theologis A. 2001. "IAA17/AXR3: Biochemical insight into an auxin mutant phenotype." *The Plant Cell*, 13:829-841.

Pitts R.J., Cernac A., and Estelle M. 1998. "Auxin and ethylene promotes root hair elongation in *Arabidopsis*." *Plant Journal*, 16:553-560.

Ramos J.A., Zenser N., Leyser O., and Callis J. 2001. "Rapid Degradation of Auxin/Indoleacetic Acid Proteins Requires Conserved Amino Acids of Domain II and is Proteasome Dependent." *The Plant Cell*, 13:2349–2360.

Reed J.W. 2001. "Roles and activities of Aux/IAA proteins in *Arabidopsis*." *Trends in Plant Science*, 6:420-425.

Reed J.W., Nagpal P., Poole D.S., Furuya M., and Chory J. 1993. "Mutations in the gene for the red/ far red light receptor phytochrome B alter cell elongation and physiological response throughout *Arabidopsis* development." *Plant Cell*, 5:147-157.

Rogers O.S., and Bendich A.J. 1988. "Extraction of DNA from plant tissues". *Plant Molecular Biology Manual*, A6:1-10.

Rouse D., Mackay P., Stirnberg P., Estelle M., and Leyser O. 1998. "Changes in auxin response from mutations in an AUX/IAA Gene." *Science*, 279:1371-1373.

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

Sakamoto M., Munemura I., Tomita R., and Kobayashi K. 2008. "Involvement of hydrogen peroxide in leaf abscission signaling, revealed by analysis with an in vitro abscission system in *Capsicum* plants". *Plant J*, 56:13-27.

Senn A.P., and Goldsmith M.H.M. 1988. "Regulation of electrogenic proton pumping by auxin and fusicoccin as related to the growth of *Avena* coleoptiles." *Plant Physiology*, 88:131-138.

Sessions A., Neuhauser J.L., McColl A., Roe J.L., Feldmann K.A., and Zambryski P.C. 1997. "ETTIN patterns the *Arabidopsis* floral meristem and reproductive organs." *Development*, 124:4481- 4491.

Tames I. 1988. "Hormonal regulation of apical dominance. In Plant hormones and their roles in Plant growth and development." (Dordrecht: Kluwer Academic Publishers). 393-410.

Tan X., Irina L.A., Villalobos C., Sharon M., Zheng C., Robinson C.V., Estelle M., and Zheng N. 2007. "Mechanism of auxin perception by the TIR1 ubiquitin ligase." *Nature*, 446:640-645.

Tatematsu K., Kumagai S., Muto H., Sato A., Watahiki M.K., Harper R.M., Liscum E., and Yamamoto K.T. 2004. "MASSUGU2 encodes Aux/IAA19 an auxin-regulated protein that functions together with the transcriptional activator NPH4/ARF7 to regulate differential growth responses of hypocotyl and formation of lateral roots in *Arabidopsis thaliana*." *The Plant Cell*, 16:379–393.

Tian Q., and Reed J.W. 1999. "Control of auxin-regulated root development by the *Arabidopsis thaliana* SHY2/IAA3 gene." *Development*, 126:711-721.

Timpte C., Wilson A.K., and Estelle M. 1994. “The axr2-1 mutation of *Arabidopsis thaliana* is a gain of function mutation that disrupts an early step in auxin response.” *Genetics*, 138:1239-1249.

Tiwari S.B., Hagen G., and Guilfoyle T.J. 2004. "Aux/IAA proteins contain a potent transcriptional repression domain." *The Plant Cell*, 16:533–543.

Ulmasov T., Murfett J., Hagen G., and Guilfoyle T.J. 1997. "Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response eleents." *Plant Cell*, 9:1963–1971.

Woodward A.W., and Bartel B. 2005. "Auxin: regulation, action, and interaction." *Annals of Botany*, 95:707-735.

Worley C.K., Zenser N., Ramos J., Rouse D., Leyser O., Theologis A., and Callis J. 2000. "Degradation of Aux/IAA proteins is essential for normal auxin signalling." *Plant Journal*, 21:553–562.

Yang X., Lee X., So J., Dharmasiri S., Dharmasiri N., Ge L., Jensen C., Hangarter R., Hobbie L., and Estelle M. 2004. “The IAA1 protein is encoded by AXR5 and is a substrate of SCF^{TIR1}.” *The Plant Journal*. 40:772-782.

Zenser N., Dreher K.A., Edwards S.R., and Callis J. 2003. “Acceleration of Aux/IAA proteolysis is specific for auxin and independent of AXR1”. *Plant J*, 35:285-294.

Zheng J., Yang X., Harrell J.M., Ryzhikov S., Shim E.H., Lykke A.K., Wei N., Sun H., Kobayashi R., and Zhang H. 2002. "CAND1 binds to unneddylated CUL1 and regulates the formation of SCF ubiquitin E3 ligase complex." *Molecular Cell*, 10:1519–1526.

Zimmerman P.W., and Hitchcock A.E. 1942. "Substituted phenoxy and benzoic acid growth substances and the relation of structure to physiological activity." *Contributions of the Boyce Thompson Institute*.