FUNCTIONS OF IAA28 IN GROWTH AND DEVELOPMENT IN ARABIDOPSIS THALIANA

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San Marcos, Texas

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FUNCTIONS OF IAA28 IN GROWTH AND DEVELOPMENT IN

ARABIDOPSIS THALIANA

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ABSTRACT

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by

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The phyto-hormone auxin plays a vital role in regulating plant growth and development throughout the plant's lifecycle. It implicates in most aspects of plant growth and development and influences overall size and shape of a plant. Auxin modulates gene transcription through the degradation of a group of transcriptional repressor proteins called Aux/IAAs. These repressor proteins interact with auxin co-receptors, TIR1/AFBs, through highly conserved domain II of Aux/IAAs in the presence of auxin, and targeted for ubquitin mediated degradation. Arabidopsis *iaa28-2* is a gain-of-function mutation in domain II of Aux/IAA28. The domain II mutation in *iaa28-2* drastically interferes with its interaction with auxin co-receptors, TIR1/AFBs in response to auxin resulting in stabilization of the mutant protein. This eventually leads to pleiotropic developmental defects such as defective lateral root development, stunted growth and reduced fertility in the mutant. *iaa28-2* mutant is severely defective in lateral

root development but produces lateral root primordia in response to high levels of 2,4-D or IBA. The mutant seedlings produce adventitious roots in response to high level of picloram, a synthetic auxin. Results of this study suggest that reduced auxin transport from hypocotyl to root and the degradation of mutant iaa28 protein through auxin co-receptor AFB1 in the hypocotyl in response to picloram may lead to induction of down-stream genes such as *LBD16* that is required for adventitious root development. Additionally, this study shows that unlike other Aux/IAA proteins, IAA28 is localized to both the nucleus and the cytoplasm and exhibit a peculiar subcellular localization pattern along the primary root. IAA28 protein is mainly localized to the cytoplasm in the basal meristem and then gradually localizes to nucleus in cell elongation and differentiation zones. Further, the data indicate that *IAA28* expression is induced by light and the IAA28 protein is modified in response to light perhaps through phytochromes. Biological significance of this localization pattern or IAA28 modification by light is currently unclear.

CHAPTER 1: INTRODUCTION

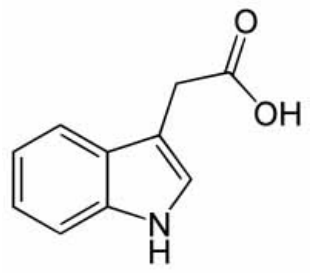
1.A. Auxin

1.A.i. Auxin effects

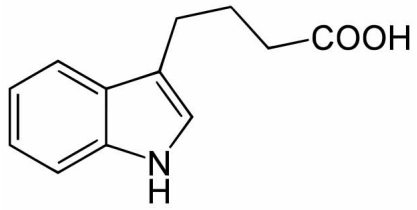
As plants are sessile they must adjust to a number of external stimuli and coordinate their growth and development accordingly. Plant growth and development is primarily controlled by the action of plant hormones. The plant hormone auxin regulates virtually every aspect of plant growth and development. Auxin regulates numerous cellular and developmental responses in plants including cell division, expansion and cell differentiation, patterning of embryo, vasculature and other tissues, lateral root initiation, gravitropism and phototropism (Liscum and Stowe-Evans 2000; Berleth and Sachs 2001; Tian *et al.* 2003). Further, recent progress on auxin biosynthesis, its metabolism and transport shows that the concentration gradient of auxin is a driving force for organogenesis and cellular patterning in plants. Collectively these observations idea that auxin is a major morphogen in plants (Perrot-Rechenmann 2010).

Many bioassays have been developed in order to isolate and identify naturally existing auxins in plants. Indole-3-acetic acid (IAA) is the most abundant natural auxin (Figure 1) (Woodward and Bartel 2005). In addition, 4-Cl-IAA, a chlorinated form of IAA also shows a high auxin activity and is found in several plants (Woodward and Bartel 2005). Indole-3-butyric acid (IBA) is identical to IAA except for two additional methyl groups and functions as an IAA precursor (Strader and Bartel 2011). Unlike other auxins, IBA efficiently induces lateral roots at very low concentrations at which the primary root elongation is minimally inhibited (Zolman *et al*. 2000). In addition to these natural auxins, there are many synthetic chemicals such as 1-naphthaleneacetic acid (1-NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), parachlorophenoxyisobutyric acid (PCIB), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 4-amino-3,5,6-trichloro-2-

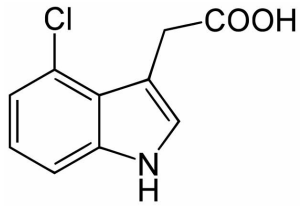
pyridinecarboxylic acid (picloram) that exert auxin-like effects (Figure1) (Woodward and Bartel 2005). At lower concentrations at the cellular site of action, auxin stimulates the growth and development, while at higher concentrations auxin inhibits growth resulting in lethality to plants. Thus, for more than 50 years, synthetic auxins have been used successfully as herbicides in agriculture (Cobb 1992; Grossmann 2000).



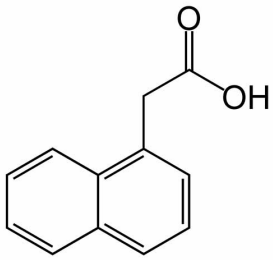
Indole-3-acetic acid



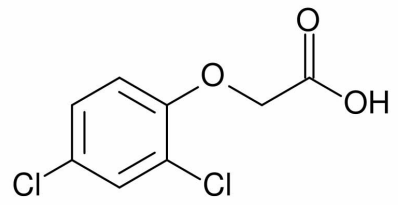
Indole-3-butyric acid



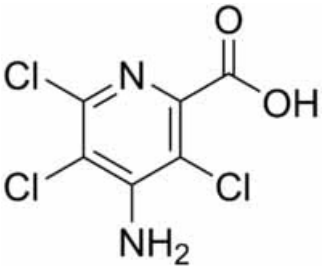
4-Chloroindole-3-acetic acid



1-Naphthaleneacetic acid



2,4-Dichlorophenoxyacetic acid



Picloram

Figure 1: **Structures of natural and some synthetic auxins**

1.A.ii. Auxin signaling

Auxin acts primarily through transcriptional regulation of auxin responsive genes. However, some responses such as induction of ion transport through the plasma membrane are too rapid to be regulated through gene transcription (Mockaitis and Estelle 2008). Auxin rapidly regulates cellular responses through a membrane protein called auxin binding protein 1(ABP1). This protein was initially isolated as a membrane protein that showed high affinity to auxin and considered as a candidate for the auxin receptor (Napier *et al.* 2002; Napier 2004). The *abp1* mutants show severe defects in cell division and cell expansion, and their growth was arrested at the globular stage of embryonic development (David *et al.* 2007).

The best characterized auxin response is the transcriptional regulation of auxin responsive genes via the actions of three major protein families called transporter inhibitor response (TIR1)/auxin signaling F-box proteins (AFB), Aux/IAA transcription repressors (Remington *et al*. 2004; Overvoorde *et al*. 2005) and auxin response factors (ARFs) (Okushima *et al.* 2005; Guilfoylea and Hagena 2007). The TIR1 F-box protein acts as an auxin receptor and directly links auxin reception to degradation of Aux/IAA proteins (Dharmasiri N. *et al.* 2005; Kepinski and Leyser 2005). Recent studies show that the high affinity of auxin binding requires proper assembly of both TIR1 and Aux/IAA as a protein complex. Therefore, TIR1 protein itself does not act as the single auxin receptor, but it functions together with Aux/IAAs as co-receptors for auxin (Calderón Villalobos *et al.* 2012) (Figure 3).

Aux/IAA proteins are short lived nuclear proteins (Abel *et al.* 1994). The known function of Aux/IAAs is to repress the transcription of auxin regulated genes. According to the current model, Aux/IAA proteins interact with ARF proteins, preventing the function of ARF transcriptional factors. In the presence of auxin, Aux/IAA proteins are poly-ubiquitinated and degraded through the proteasome pathway, allowing ARF dependent transcription *(*Tiwari *et al.* 2003). Majority of the Aux/IAA protein family members contain four conserved domains. Domain I of most Aux/IAA proteins contains an ethylene response factor associated amphiphilic response motif that is involved in transcriptional repression of other proteins, including ARFs (Tiwari *et al.* 2004).

TOPLESS (TPL) protein interacts with domain I of Aux/IAA proteins and acts as a co-repressor (H.Szemenyei *et al.* 2008). Domain II is important for the interaction with the auxin co-receptor protein TIR1 (Gray *et al.* 2001; Dharmasiri N. *et al.* 2005; Kepinski and Leyser 2005). However, recent studies have shown that sequences outside domain II are also important for the formation of auxin co-receptor complex with TIR1 protein (Calderón Villalobos *et al.* 2012). Domains III and IV are located in the C-terminal half of the protein and are involved in homodimerization with each other and heterodimerization with other Aux/IAA proteins and ARFs that also share domain III and IV (Ulmasov *et al*. 1999a; Hardtke *et al.* 2004).

During the past decades, different *Aux/IAA* mutants with altered auxin responses have been isolated and characterized. As domain II mutations in different Aux/IAA proteins inhibit the interaction with auxin receptor protein TIR1 and subsequent degradation of the repressor proteins, the mutant protein accumulates and causes pleiotropic developmental defects in plant growth and development. Thus, these domain II mutants are known as gain-of-function mutants. For example, the gain-of-function mutants in *axr2-1/iaa7, axr3-1/iaa17, shy2-2/iaa3, slr1/iaa14* and *bdl/iaa12*show dominant or semi-dominant phenotypes and each of them can recapitulate the mutant phenotype when introduced into wild type (Tian and Reed 1999; Worley *et al.* 2000). These domain II mutants of different *Aux/IAA* genes affect various tissues and developmental responses including shoot or root gravitropism, lateral root formation, shoot apical dominance, stem elongation, leaf expansion and leaf formation in the dark (Reed 2001), suggesting that different Aux/IAA proteins can regulate many of the developmental processes regulated by auxin.

In Arabidopsis there are 23 members of the ARF protein family, and each contains conserved DNA binding and dimerization domains. ARF transcriptional factors bind to the auxin responsive elements (AuxRE) in the promoter region of auxin responsive genes and subsequently either activate or repress the transcription of the particular auxin responsive gene. The middle region of ARF is responsible for transcriptional activation or repression (Hagen and Guilfoyle 2002). For example, ARFs with a glutamine (Q) rich middle region function as transcriptional activators, whereas

ARFs with proline (P)/serine (S)/ threonine (T) rich middle regions function as transcriptional repressors (Guilfoyle and Hagen 2007). Therefore, specific Aux/IAA proteins that interact with ARFs to repress their activity can act as negative or positive regulators of auxin responsive gene transcription. ARFs are involved in a broad range of functions in plant growth and development. For example, ARF5/MP functions in embryogenesis (Hardtke and Berleth 1998), ARF2, ARF7/NPH4 and ARF19 in tropism (Harper R. *et al.* 2000; Li *et al.* 2004; Okushima *et al*. 2005), ARF3/ETTIN in floral development (Sessions *et al*. 1997), and ARF2, ARF7, ARF8 and ARF19 in root and hypocotyl growth in Arabidopsis (Li *et al*. 2004; Tian *et al.* 2004; Okushima *et al.* 2005).

Protein sequence analysis shows that the auxin co-receptor TIR1 is a member of a small sub-clade with six other closely related family members, AFB1-5 and COI1 (Figure 2) (Dharmasiri N. *et al.* 2005a). Among these, TIR1 protein consists of an N-terminal F-box motif, a middle region and a C-terminal tail (Dharmasiri N. *et al.* 2005b). The middle region is a short spacer region with about 40 residues, 16 degenerate leucine rich repeats (LRRs) and a C-terminal tail of approximately 70 residues (Dharmasiri N. *et al.* 2005b). TIR1 and AFB1 co-receptors share 70% sequence similarity, whereas AFB2 and AFB3 show 84% similarity to each other. AFB4 and AFB5 share 76% similarity with each other, but their structure is distantly related to that of other members of the family by having an N-terminal overhang (Figure 2). Pull down experiments have shown that similar to TIR1, AFB1-3 proteins interact with Aux/IAA proteins in an auxin-dependent manner, strongly suggesting that they also function as auxin co-receptors (Dharmasiri N. *et al.* 2005a). These different members show different developmental and temporal expression patterns in Arabidopsis indicating complex and tissue specific responses to auxin throughout plant growth and development (Dharmasiri N. *et al.* 2005b). However, functional redundancy among these family members has hindered the understanding of the individual contribution of each gene in specific developmental processes. It has been found that quadruple mutants (*tir1,afb1, afb2, afb3*) are insensitive to exogenous auxin and exhibit seedling lethality due to the pivotal role of auxin in embryogenesis (Dharmasiri N. *et al.* 2005b). The most distant member of this group COI1 has only 30% similarity to other members. COI1 is the receptor for jasmonic acid, another plant hormone that is involved in plant defense and development (Figure 2) (Yan *et al*. 2009).

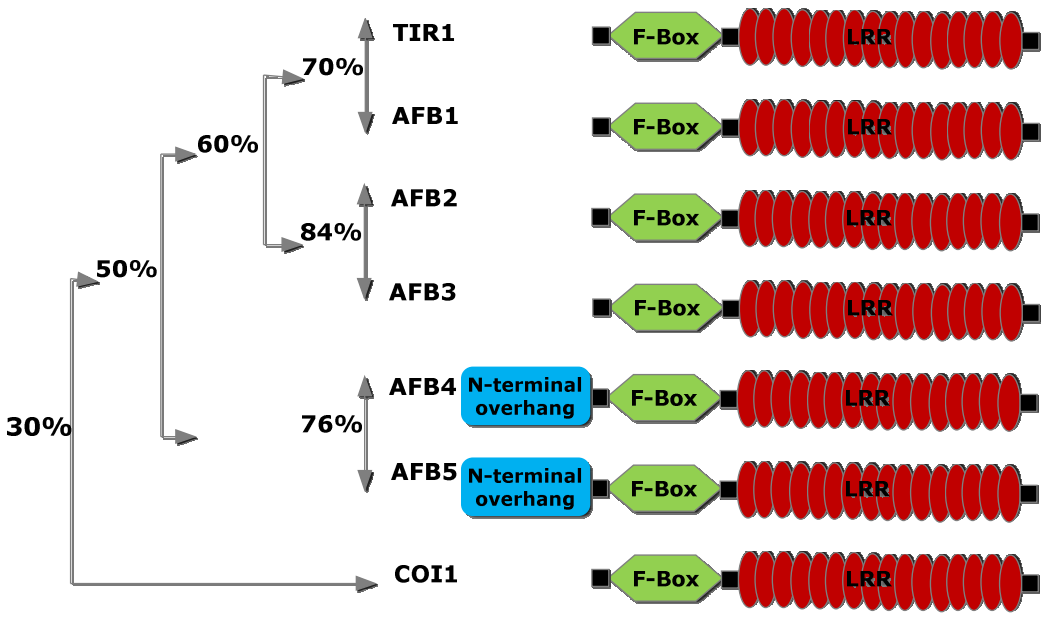


Figure 2: **Phylogenetic relationship of TIR1 and its closest relatives.** Values indicate percentage of identical amino acids (This figure is modified from Dharmasiri N. *et al.*, 2005b).

TIR1 is a component of the E3 ligase complex that is involved in the poly-ubiquitination of target Aux/IAA proteins. The E3 complex is composed of four primary subunits, ASK1, Cullin1, RBX1 and an F-box protein thus called SCF^{TIR1}. TIR1, the F-box protein, interacts with the CUL1-RBX1 sub-complex through ASK1 or ASK2 proteins in Arabidopsis (Dharmasiri N. and Estelle 2004). The *AXR6* gene encodes the Arabidopsis CULLIN1 protein (CUL1) which is required for normal embryonic and postembryonic development (Hellmann *et al*. 2003). Modification of CUL1 by RUB1 (related to ubiquitin 1) is essential for normal auxin response. RUB modification of CUL1 also involves three enzymes, E1, E2 and E3. The heterodimeric E1 enzyme is composed of AXR1 and ECR1 that activates RUB1. E2 is known as RUB conjugating enzyme (RCE), and the Arabidopsis genome contains two RCE genes, RCE1 and RCE2 (Dharmasiri N. and Estelle 2004). Recent studies show that the RBX1 functions as the E3 in RUB modification of CUL1 in Arabidopsis (Gray *et al.* 2002) (Figure 3).

Mutations in AXR1and ECR1 subunits of the RUB activating enzyme result in diverse defects in morphology (Pozo *et al.* 1998; Pozo *et al*. 2002). Similar to *axr1* and *ecr1* mutants, *rce1* mutant plants show defective growth throughout development. For example, *rce1* mutants show defective gravity responses, smaller rosette leaves, stunted inflorescence axes, shorter and rounded petioles as well as shorter siliques (Dharmasiri S. *et al.* 2003). Plants deficient in both *RCE1* and *AXR1* have a severe embryonic phenotype. These results confirm that RUB conjugation pathway is required for auxin-dependent growth and development in plants (Dharmasiri S. *et al.* 2003).

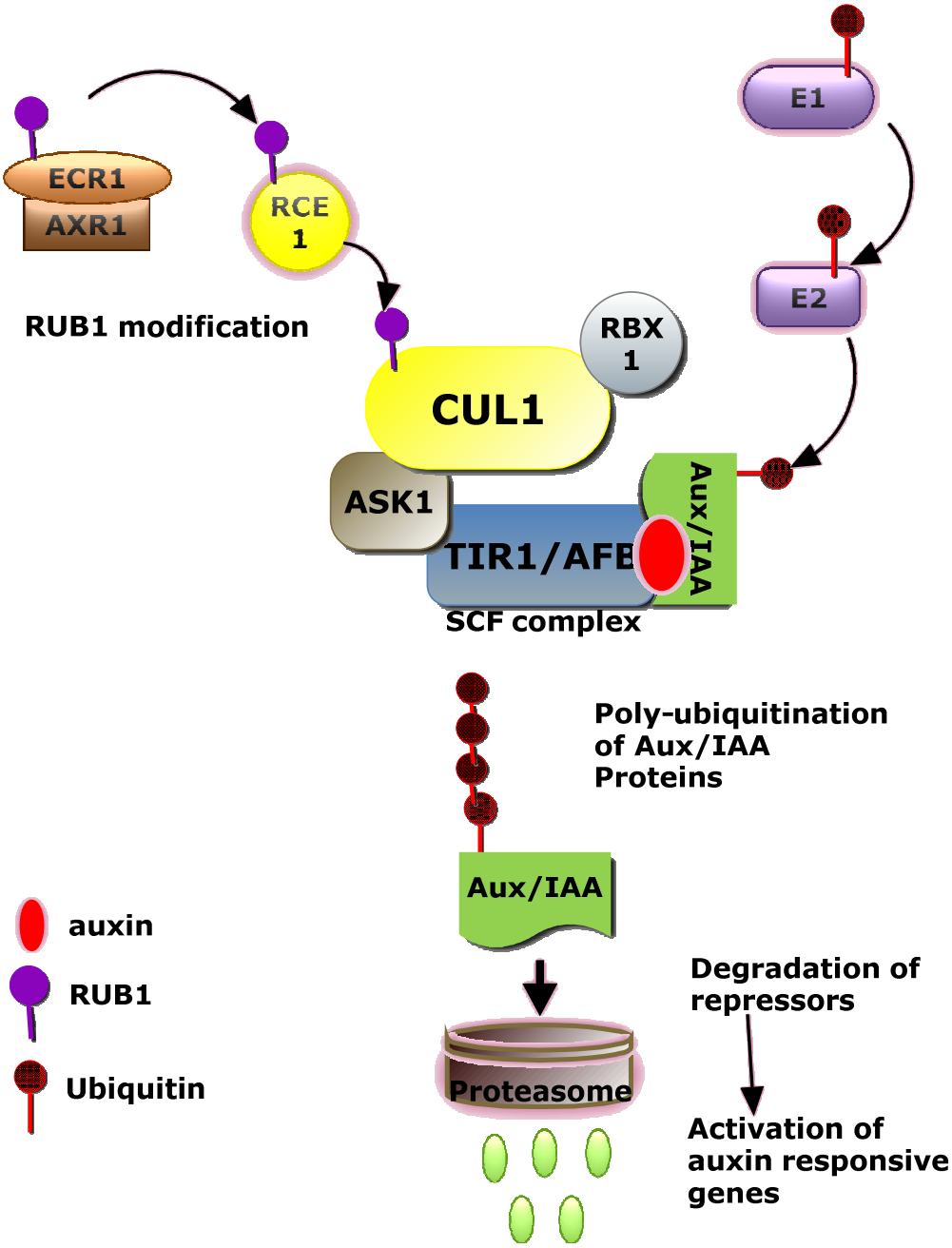


Figure 3: Different components of auxin mediated Aux/IAA protein degradation pathway

1.A.iii. Auxin biosynthesis

Many tissues including leaves, cotyledons and roots synthesize IAA. However, young leaves have the highest auxin biosynthetic capacity (Ljung *et al.* 2002). There are two major pathways to synthesize IAA; the tryptophan (Trp)-dependent and Trp-independent pathways. In the Trp-dependent pathway IAA is derived via metabolism of Trp, and in the Trp-independent pathway IAA is derived from an early indolic precursor (Woodward and Bartel 2005). The indole-3-pyruvic acid (IPA) pathway, the indole acetamide (IAM) pathway, the tryptamine pathway and indole-3-acetaldoxime (IAOx) pathways are examples of Trp-dependent IAA biosynthesis pathways. Both *yucca* and *sur1* are Arabidopsis IAA accumulating mutants. *Yucca* has a mutation in a flavin monoxygenase (FMO)-like enzyme that oxidizes tryptamine to N-hydroxyl-tryptamine in vitro (Zhao *et al.* 2001). The *sur1* mutants are defective in a C-S lyase that cleaves S-(indolylacetohydroxymoyl)-L-cystein to indole-3-thiohydroxymate, resulting in increased levels of both free IAA and IAA-conjugates (Woodward and Bartel 2005). A mutant with defective light responses, named *sav3* (*shade avoidance 3*), encodes TAA1 (an aminotransferase) that is involved in the conversion of L-tryptophan (L-Trp) to indole-3-pyruvic acid (IPA) (Tao *et al.* 2008). Another allele for TAA1 identified as *tir2* mutant (*Transporter Inhibitor Response 2*) is essential for temperature-dependent elongation of hypocotyls (Yamada *et al.* 2009).

Characterization of Arabidopsis mutants defective in the formation of tryptophan synthase shows evidence that plants can survive and synthesize IAA without Trp intermediates. Tryptophan synthase catalyzes the conversion of indole glycerol phosphate to tryptophan, which is the last step in tryptophan biosynthesis (Last *et al.* 1991). Wild type tryptophan synthase consists of two functional domains, an α-subunit and a β-subunit. Arabidopsis *trp3-1* and *trp2-1* mutants are defective Trp synthase α- and β-subunits, respectively (Last *et al.* 1991; Radwanski *et al.* 1996). However, these plants are considered as conditional tryptophan auxotrophs because both *trp3-1* and *trp2-1* require tryptophan for growth under standard illumination but not under very low light (Last *et al.* 1991; Radwanski *et al.* 1996). Thus, plants use Trp-independent pathways to synthesize the essential plant hormone auxin specifically under very low light conditions.

1.A.iv. Auxin transport

Auxin transport is a complex and highly regulated process with many different influx and efflux carriers in plants. Proper IAA transport is necessary for normal lateral root development (Reed *et al.* 1998; Bhalerao *et al.* 2002), vascular development (Mattsson *et al.* 2003), phyllotaxis (Reinhardt *et al.* 2003), embryonic axis development (Friml *et al.* 2003) and tropism (Friml *et al.* 2002). In shoots, IAA is transported basipetally and inhibits lateral shoot growth maintaining apical dominance (Thimann and Skoog 1934). However, in roots auxin is transported both acropetally and basipetally facilitating well-organized auxin distribution for proper organ formation (Scott and Wilkins 1968; Davies and Mitchell 1972). Several Arabidopsis mutants have been identified with defective polar auxin transport.

Auxin resistant mutant *aux1* encodes a transmembrane protein that mediates IAA influx into cells (Figure 4) (Bennett *et al.* 1996; Marchant *et al.* 1999). Asymmetric localization of AUX1 in the plasma membrane of different cells facilitates directional auxin transport which creates auxin gradients necessary for proper organ formation in plants (Swarup *et al.* 2001). Both IAA and its synthetic analog, 2,4-D, are AUX1 substrates, however, 1-NAA that does not require a transporter for influx and can restore the defective gravity response in *aux1* mutants (Yamamoto and Yamamoto 1998). In 2008, Swarup and co-workers showed that LAX3 (Like AUX1-3), an auxin influx transporter, is important for lateral root emergence. Similar to *aux1* mutants, *lax3* mutants showed 40% reduction in lateral root emergence. In addition, *LAX3* was mainly expressed in mature tissues adjacent to developing lateral root primordia (Swarup *et al.* 2008). They have also shown that LAX3 induces cell wall remodeling enzymes (e.g. subtilisin like protease, pectate lyase and polygalacturanose) which are involved in cell separation in order to facilitate smooth emergence of lateral root primordia through cortical cell layers (Swarup *et al.* 2008).

Several transmembrane proteins act as auxin efflux carriers. Of these, the family of PIN proteins is the best studied auxin efflux carriers in plants. The *pin-formed* (*pin1*) Arabidopsis mutant shows severe defects in shoot meristem development, forming a pin-shaped single inflorescence axis without rosette leaves or secondary branches (Okada *et*

al. 1991). Similar to AUX1, PIN1 protein also localizes asymmetrically in the plasma membrane of cells, facilitating differential auxin transport in plants (Gälweiler *et al.* 1998). Several mutant alleles of PIN2 have been identified as *ethyleneinsensitive root 1* (*eir1*), *agravitropic1* (*agr1*), and *wawy6* (*wav6*) mutants, with similar phenotypical defects (Křeček. P. *et al.* 2009). In Arabidopsis, there are eight *PIN* genes (*PIN1-PIN8*) that encode transmembrane auxin efflux carrier proteins (Figure 4A) (Woodward and Bartel 2005). However, only six PIN proteins (PIN1, PIN2, PIN3, PIN4, PIN5 and PIN7) are functionally characterized (Křeček *et al.* 2009). Recently, Barbez and co workers showed that a putative family of proteins named as "PIN-LIKES" or PILS are involved in auxin transport within the cells. PILS proteins function in auxin accumulation at the endoplasmic reticulum and then auxin availability in the nucleus for auxin signaling (Barbez *et al.* 2012). In addition to PIN proteins and PILS, multidrug resistant like (PGP/MRD) proteins are also involved in auxin transport in Arabidopsis (Woodward and Bartel 2005).

As shown in Figure 4B, PIN1, PIN7 and PIN3 are localized in the root stele, transporting auxin acropetally towards the root tip. When the flow of auxin reaches the base of the steel, PIN1, PIN4 and PIN2 proteins are involved in directing auxin towards quiescent center cells, which are involved in maintaining the surrounding meristematic cells. Subsequently, PIN2 and PIN7 redistribute auxin basipetally through outer epidermal and cortical cell layers. PIN3 and PIN7 are also involved in the programmed auxin distribution within the root columella cells (Krecek *et al.* 2009). AUX1 is also localized to the epidermal cell layers in the root tip area transporting auxin basipetally.

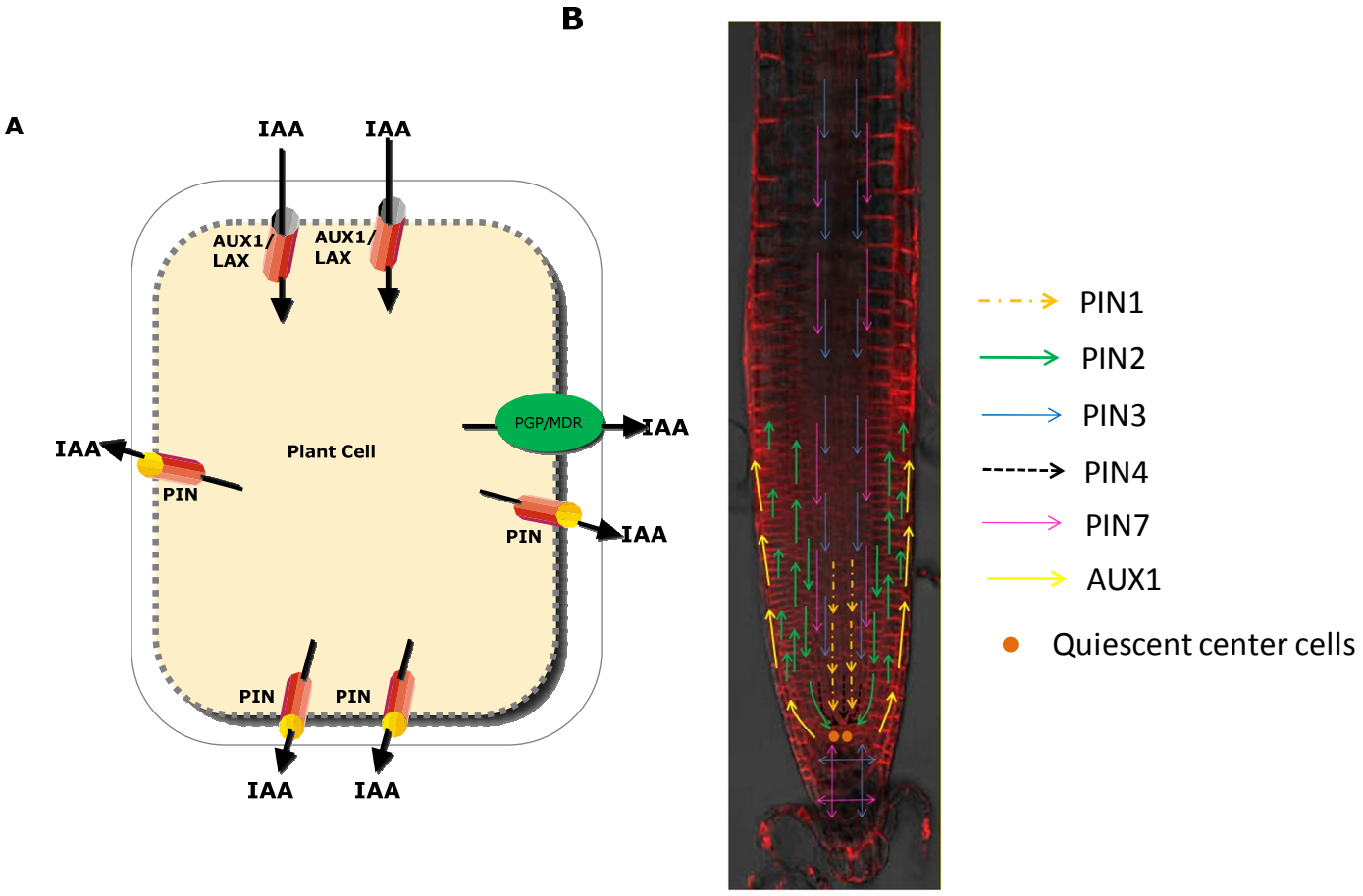


Figure 4: (A) Schematic mode of cellular trafficking of auxin through auxin transporters. The cells take up auxin via plasma membrane localized AUX1/LAX auxin influx carriers. The passage out of the cell is characterized by PIN-formed proteins (PIN1-PIN8). In addition, PGP/MDR proteins are also involved in auxin efflux. (B) PIN and AUX1 dependent auxin transport routes in Arabidopsis root tip. The arrows show the direction of auxin movement from each transport molecule. This figure is based on the data from Krecek *et al.* 2009 and Swarup *et al.* 2001.

1.A.v. Auxin homeostasis

Auxin homeostasis is the maintaining of a stable, internal environment of auxin, allowing effective plant growth and development. Auxin homeostasis results from continuous and dynamic adjustments of auxin input and output processes in cells. Common auxin input processes are auxin biosynthesis, transport (influx) and hydrolysis of IAA conjugates. Examples of auxin output mechanisms are auxin catabolism, transport (efflux), cellular compartmentalization and formation of IAA conjugates (Woodward and Bartel 2005). Existence of multiple auxin biosynthesis pathways provides a well maintained and flexible system, ensuring production and renewal of auxin at different developmental stages as well as under different environmental conditions. In addition, auxin transport plays a major role in the establishment of auxin gradients within plant tissues, which triggers differential cellular responses (Teale *et al.* 2006).

As mentioned previously, IAA conjugation is a process that higher plants use to store IAA. Generally, IAA forms conjugates with sugars, amino acids such as alanine (Ala) or leucine (Leu) or peptides (Bialek and Cohen 1986). In addition to being a form of IAA storage, these IAA-conjugates are important in auxin transport, compartmentalization, detoxification of excess IAA and protection against peroxidative degradation (Cohen and Bandurski 1982). IAA- conjugates such as IAA-glutamate (Glu) and IAA- aspartate (Asp) are not a source of free IAA, because active auxin cannot be released through the hydrolysis of these components. Therefore, these two IAA conjugates are intermediaries in a catabolic process for the degradation of IAA (Östin *et al.* 1998) (Figure 5). In conclusion, these two processes, reversible IAA-conjugation and irreversible IAA conjugation help the plant to regulate the inner auxin concentration quickly.

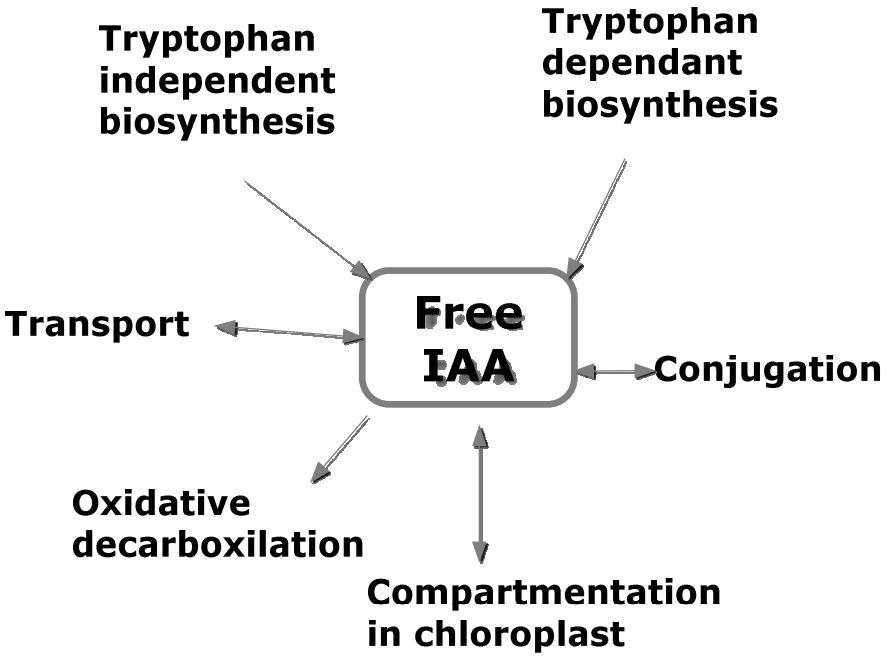


Figure 5: **Inputs and outputs to free IAA level in a plant cell**

1. A.vi. Crosstalk of auxin with other plant hormones

In addition to auxin, plants have several other hormones that are important for growth and development. For example, gibberellic acid (GA), abscisic acid (ABA), ethylene, cytokinin, brassinosteroids (BR), strigolactones (SL) and jasmonic acid (JA) are the major plant hormones that are involved in various physiological responses throughout the plant life cycle. Among these, GA, ABA, ethylene, JA and cytokinin signaling mechanisms have been well studied; however, some of the recently discovered plant hormones like SL and BR functions and signaling are not yet well characterized (Chandler 2009). In plants there are many GA-like compounds, but only active GAs regulate many physiological effects such as stimulating stem elongation, stimulating bolting, breaking seed dormancy, etc (Ross and O'Neill 2001; Chandler 2009). ABA is

known as the plant stress hormone and is involved in abscission of fruits and leaves and regulating plant responses to various abiotic stresses (Eckardt 2002; Chandler 2009). In contrast, JA is mainly involved in regulating plant responses to biotic stresses such as pathogen attack and herbivory (Stintzi *et al.* 2001). As the word cytokinin implies, this plant hormone is involved in cytokinensis (cell division) and morphogenesis (Shimizu-Sato *et al.* 2009). BR promotes cell expansion, cell elongation, vascular differentiation, and protects plants during severe chilling and drought conditions (Li *et al.* 2005). SL mainly stimulates stem branching (Dun *et al.* 2009). Some of the major functions of ethylene, a gaseous plant hormone, are fruit ripening and apical hook formation and maintenance (Yoo *et al.* 2009). Based on these broad effects of different hormones on plant growth and development, the plant cells must be able to integrate these numerous signal transduction events into a comprehensive network of signaling pathways and responses. Thus, hormones are no longer considered as response effectors in linear pathways, but are considered as members within a system of a complex interconnected web of hormone action (Chandler 2009).

Auxin signaling interacts with other plant hormone signaling pathways in order to regulate plant responses to changing environmental conditions (Chandler 2009). For example, auxin and cytokinin synergistically regulate shoot cell proliferation by controlling the expression of one of the cell cycle regulatory components, *ccD3*. However, auxin and cytokinin act antagonistically on lateral root formation (Swarup *et al.* 2002). Therefore, in tissue culture experiments, exposing the cell callus to a high auxin to cytokinin ratio induces root formation while a low ratio induces shoot formation (Skoog and Miller 1957).

Auxin and ABA act antagonistically to regulate many developmental processes in plants. For example, stomatal opening is controlled by both auxin and plant stress hormone ABA (Eckardt 2002). Under normal environmental conditions, auxin causes reduction of the turgor pressure in guard cells, which surround the stomatal pore, inducing the water movement into guard cells from surrounding cells. This will result in the opening of the stomatal pore. In contrast, ABA, under dry environmental conditions causes increase of the turgor pressure in the guard cells resulting in closure of the stomata

thus reducing water loss by transpiration (Swarup *et al.* 2002). Further, auxin and ABA are involved in root growth and seed germination. For example, auxin responsive mutants *axr2/iaa7* show ABA resistance in primary root growth while both *axr1* and *axr2* mutants show weak ABA resistant phenotype as measured by seed germination (Belin *et al.* 2009). These results show that auxin and ABA cross talk is essential in many developmental processes in plants.

Auxin and GA crosstalk is also involved in regulating many developmental processes such as stem elongation, seed germination and parthenocarpy (Sastry and Muir 1963; Ikeda *et al.* 1999; Ross and O'Neill 2001). Further, auxin regulates GA biosynthesis via the induction of several GA oxidase genes like *GA20ox* that are involved in GA biosynthesis (Jeremy *et al.* 1999).

Auxin and ethylene coordinately regulate many developmental processes in plants such as apical hook formation, root hair differentiation and elongation, root growth and hypocotyl phototropism (Masucci and Schiefelbein 1994; Lehman *et al.* 1996; Pitts *et al.* 1998; Raz and Ecker 1999) In addition, auxin induces ethylene biosynthesis by up regulating the expression of ACC synthase enzyme which acts as the rate limiting factor in ethylene biosynthesis (Abel *et al.* 1995; Abel and Theologis 1996; Bekman *et al.* 2000).

Studies with club root disease in Chinese cabbage showed evidence for the crosstalk between auxin and JA. Plants subjected to club root disease show severe hypertrophy of the roots, and this enlargement of root is related to the level of auxin. Due to club root disease the level of JA increases, resulting in the up-regulation of enzymes such as nitrilase (NIT) and myrosinase that are involved in auxin biosynthesis (Grsic *et al.* 1999). In addition, a recent study showes that, unlike JA or other JA acid conjugates, JA-Trp (tryptophan conjugates of JA) inhibits endogenous auxin responses by altering auxin homeostasis in Arabidopsis (Staswick 2009).

Arabidopsis *MAX* (*more axillary growth*) mutants show evidence for the crosstalk between auxin and SL. All *max1, max3* and *max4* mutants show increased lateral branching due to down regulation of a product in SL signaling that is required for the

inhibition of axillary bud growth (Hayward *et al.* 2009). Further, these SL mutants have high levels of free auxin, suggesting that auxin and SL levels regulate each other through a feedback mechanism to control axillary bud outgrowth (Bennett *et al.* 2006; Hayward *et al.* 2009).

1.A.vii. Auxin and light signaling

Auxin mediates many plant responses to environmental signals such as gravity, temperature stress, and light. Common examples for the auxin-regulated light responses of plants include hypocotyl elongation, shade avoidance and photomorphogenesis (Swarup *et al.* 2002). Further, auxin regulates the phototropic bending of dark grown hypocotyls. In 1996, Liscum and Briggs published their work related to Arabidopsis mutants that were unable to show phototropic bending (Liscum and Briggs 1996). The mutants were named as *nph* (*non-phototrophic hypocotyls*) and all four mutant alleles either completely lacked or had severely impaired phototrophic responses. In 1998, Stowe-Evans and co-workers explained that NPH4 is involved in the regulation of auxin-dependent differential growth in Arabidopsis (Stowe-Evans *et al.* 1998). For example, both auxin and NPH4 are involved in processes in which auxin-mediated differential growth is essential. Such processes are phototropism, gravitropism, hypocotyl curvature and apical hook maintenance (Stowe-Evans *et al.* 1998). In addition, expressions of several auxin inducible genes, *SAUR-AC1*, *IAA12*,*GH3*, *IAA4* and *IAA6* were severely impaired in *nhp4* mutant seedlings (Harper *et al.* 2000).

Auxin influences hypocotyl elongation through light signaling. For example, auxin overproducing *sur1* mutants have longer hypocotyls when grown in light but exhibit normal hypocotyls in dark compared to the wild type (Boerjan *et al.* 1995). In contrast, *iaaL* mutant that produces less auxin has shorter hypocotyls in light but normal hypocotyls in dark (Romano *et al.* 1991; Collett *et al.* 2000). Further, the auxin transport inhibitor NPA is an inhibitor of light grown hypocotyl elongation, whereas in the dark it has no significant effect on hypocotyl growth (Collett *et al.* 2000). These results clearly show that auxin plays a major role in skotomorphogenesis as well as photomorphogenesis changes in plants.

Photomorphogenesis is the light mediated developmental changes that occur in plants. For example de-etiolation of shoot and greening of leaves and stems in response to light are some of the main changes that happen when a plant is exposed to light. The Aux/IAA gain of function mutants *iaa3*/*shy2* (*suppressor of hy2 mutation*) produces leaf-like structures in dark grown seedlings unlike wild type plants (Tian and Reed 1999). This phenotype of *shy2* mutants was described as constitutive photomorphogenesis. Arabidopsis *hy2* is a phytochrome chromophore deficient mutant with elongated hypocotyl in light (Kim *et al.* 1996). Further, in 2000, Colon-Carmona and co-workers showed that recombinant proteins from Arabidopsis (SHY2/IAA3, AXR3/IAA17, IAA1 & IAA9) and pea (Ps IAA4) can be phosphorylated by oat phytochrome A in vitro (Colón-Carmona *et al.* 2000).

1.B. Aux/IAA proteins: repressors in auxin signaling

1.B.i. Diversity of Aux/IAA proteins

Plant specific Aux/IAA genes are considered as primary auxin responsive genes, due to the rapid increase in their transcript levels with auxin treatments, typically within several minutes to about 60 minutes (Abel and Theologis 1996; Hagen and Guilfoyle 2002). Additionally, *GH3* and *SAUR* gene families are also considered as primary auxin responsive genes (Abel and Theologis 1996). As previously mentioned, Arabidopsis has 29 *Aux/IAA* genes. Expression and phenotypic analysis of different *Aux/IAA* mutants show that many members of the family have redundant functions (Abel and Theologis 1996; Reed 2001; Remington *et al.* 2004; Overvoorde *et al.* 2005). However, there are some differences among group members suggesting that some *Aux/IAAs* have distinct functions during auxin signaling and may interact differently with other regulators of auxin-dependent transcription. The best example is that most of the *Aux/IAA* transcripts are auxin -induced, but the transcript level of *IAA28* decreases slightly in response to exogenous auxin application (Rogg *et al.* 2001; Karunarathna 2008).

Twenty-three of the 29 Arabidopsis Aux/IAA proteins are considered as canonical members because they all have four conserved domains including the full domain II which is essential for rapid degradation (Abel and Theologis 1995; Reed 2001; Liscum

and Reed 2002). However, the degradation rate of different Aux/IAA proteins varies significantly from each other. The half-life of IAA7 in the presence of auxin is 5 to 10 minutes whereas IAA28, which has a very similar domain II, has a half-life of 80 minutes. IAA31, which has domain II but does not have the conserved lysine in between domains I and II, has a half-life of 4 hours. These results show that the other regions in the Aux/IAA proteins may be required for recognition by the $SCF^{TIR/AFBs}$ complex (Calderon-Villalobos et al. 2010). Another five of the 29 Aux/IAA proteins of Arabidopsis, IAA20, IAA30, IAA32, IAA33 and IAA34 are considered as non-canonical members because they completely lack a domain II region. As a result they have a much longer half-life compared to the canonical members in the family (Sato and Yamamoto 2008). For example, IAA20 protein is not degraded for 12 hours and its stability is not affected by auxin treatment. IAA31 protein has only a partially conserved domain II in which the well conserved first glycine (G) has changed to aspartic acid (D) (Dreher *et al.* 2006). Similar to IAA20, in the absence of exogenous auxin IAA31 is also not degraded for 12 hours, but with auxin treatment it is degraded with a half-life of 4 hours (Sato and Yamamoto 2008).

1.B.ii. Functions of Aux/IAA proteins

Most of the loss of function mutants in different Aux/IAA proteins (with mutations in domains I, III or IV) show only mild phenotypes (Rouse *et al.* 1998; Tian and Reed 1999; Nagpal *et al.* 2000). For example, loss of function mutant *shy3/iaa3* exhibits large cotyledons and short hypocotyls, whereas loss of function mutant *axr2/iaa7* has slightly longer hypocotyls than the wild type (Tian and Reed 1999; Nagpal *et al.* 2000). These phenotypes are mild mainly due to the fact that Aux/IAA proteins function redundantly in regulating many developmental processes. However, as domain II mutations of Aux/IAA proteins stabilize the mutant protein, the mutant plants exhibit an array of developmental defects (Liscum and Reed 2002). These mutants share common as well as distinctive defects, depending on their temporal and spatial expression patterns. Therefore, these mutants can be used to understand the roles of different Aux/IAA proteins in plant growth and development. For example, the gain of function mutation in *IAA14* has no lateral roots, has few root hairs and shows abnormal gravitropic responses

in root and hypocotyls. This defective lateral root phenotype in *IAA14* is very similar to that of *arf7/arf17* double mutants. In addition, gain of functions mutation in *IAA3*, *IAA19* and *IAA28* also have reduced or no lateral root development. According to yeast two hybrid assays, ARF7 and ARF19 interact with several Aux/IAA proteins including IAA14, IAA3, IAA19 and IAA28 (Tatematsu *et al.* 2004; Fukaki *et al.* 2005). In addition, mutants of *IAA18* (*crane1* & *crane2*) show defective lateral root phenotype and IAA18 interacts with both ARF7 and ARF19 in vitro (Uehara *et al.* 2008). These results suggests that IAA3, IAA14, IAA18, IAA19 and IAA28 protein may be involved in lateral root formation, and they function cooperatively with each other in order to repress ARF7 and ARF19 (Uehara *et al.* 2008).

Arabidopsis gain of function mutation in *IAA12* affects mainly the embryogenesis process (Hamann *et al.* 1999). Mutant *iaa12* (*bodenlos/bdl*) is defective in the formation of embryonic root but not the formation of post-embryonic root. Normal Arabidopsis embryo development progresses through several stages such as 2, 4, 8, 16-cells, globular, heart, torpedo and mature embryo. However, *iaa12/bdl* mutants show defects as early as 2-cell stage at which the apical daughter cells of the zygote divide horizontally instead of vertically. In addition, basal daughter cells in the embryo, which are destined to become the hypophysis, divide abnormally and fail to generate the quiescent center of the root meristem and the central root cap in *iaa12/bdl* mutants. These initial defects will result in no primary root formation in *iaa12/bdl* mutants. In addition to this defective root formation in the embryo, some *iaa12/bdl* mutants lacked the hypocotyl as well. This lack of hypocotyls in *iaa12/bdl* mutants resembles *monopterous* (*mp/arf5*) mutants in Arabidopsis (Hamann *et al.* 1999). For example, both*iaa12/bdl* and *arf5/mp* mutations affect the orientation of the division plane of the apical daughter cells of the zygote resulting in double octant embryos with defective quiescent centers and defective lower stem cells of the root meristem (Berleth and Jurgens 1993; Hamann *et al.* 1999). Further, IAA12/BDL and ARF5/MP interact in yeast two hybrid assays. Therefore, according to these results it is obvious that IAA12 together with ARF5 is essential for proper embryogenesis in Arabidopsis (Hamann *et al.* 2002).

As mentioned above, the general function of Aux/IAA proteins is to act as repressors of ARF proteins which function as transcriptional activators or repressors. Therefore, the degradation of Aux/IAAs leads to activation or repression of ARFs, leading in turn to the transcription of auxin responsive genes. This sequence ultimately results in the regulation of many developmental and physiological responses that are essential for proper plant growth and development (Sato and Yamamoto 2008). However, some Aux/IAA proteins interact with proteins other than ARFs and Aux/IAAs. For example, IAA26 protein interacts with TMV replicase protein and localizes to the cytoplasm. This interaction is associated with disease development in Arabidopsis (Padmanabhan *et al.* 2005; Padmanabhan *et al.* 2006). In this study they used a TMV mutant defective in the IAA26 interaction was replicated and moved normally in Arabidopsis and induced milder viral disease symptoms in transformed plants compared to the wild type virus infected plants. Based on these observations, they suggested that interaction with the TMV replicase protein disrupts IAA26/PAPI localization and its function as a transcriptional regulator in auxin signaling to induce specific disease symptoms (Padmanabhan *et al.* 2005).

In 2010 Carranco and co-workers showed that Aux/IAA protein HaIAA27 (*Helianthus annuus*- Sunflower) interacts with HaHSFA9 (heat shock transcriptional factor) in young sunflower embryos using bimolecular fluorescence complementation interaction (BIFCi) technique. They also observed that IAA27 protein is stabilized in immature sunflower embryos. The stabilized IAA27 protein represses the transcriptional activation by HSFA9 protein, which regulates developmental processes such as seed longevity and embryonic desiccation tolerance. However, when a seed matures, due to its high auxin levels, the repression of HSFA9 by IAA27 will become weaker and promote the induction of HSFA9 protein. In conclusion, they suggested that both IAA27 and HSFA9 are important in regulation of the developmental processes such as seed longevity and embryonic desiccation tolerance (Carranco *et al.* 2010).

1.B.iii. Localization of Aux/IAAs

Aux/IAA proteins have a simian virus 40 (SV40)-like bipartite nuclear localization signal, and many known Aux/IAA proteins are localized to the nucleus (Abel *et al.* 1994; Ouellet *et al.* 2001; Fukaki *et al.* 2002). However, as described previously some Aux/IAA proteins such as IAA26 are localized to the cytoplasm upon interacting with TMV replicase (Padmanabhan *et al.* 2006). Padmanabhan and co-workers (2006) checked the interaction of TMV replicase protein with several other Aux/IAA proteins such as IAA27, IAA18, IAA20, IAA12, IAA28, IAA11, IAA4, IAA16 and IAA10 and found that among these only IAA27 and IAA18 interacted with the TMV replicase and localized to the cytoplasm. In contrast, the localization of non-interacting Aux/IAA proteins were unaffected by the presence of the viral protein (Padmanabhan *et al.* 2006).

Table 1: Several Aux/IAA proteins in Arabidopsis and evidence for their role in auxin-mediated development

Gene	Other names for mutants	Defects	Functions	References
IAA1	*axr5-1*	Defective root gravitropism, auxin response and defective root hair formation	*axr5-1* mutation reduces multiple auxin responses	Park *et al*. 2002; Yang *et al.* 2004; Zenser *et al.* 2001 & 2003
IAA3	*Suppressor of HY2 or Short hypocotyl2* (*shy2*)	Leaf formation in dark grown plants, defects in auxin responsive root growth, lateral root formation and timing of gravitropism	Multiple auxin responses in roots, photomorphogenesis (connects auxin and light signaling)	Tian & Reed 1999 &2003; Soh *et al.* 1999; Colon Carmona*et al*. 2000
IAA12	*Bodenlos* (*bdl*)	Defective primary root formation and hypocotyl formation, defects in auxin responsive root growth	Embryogenesis, root meristem formation & multiple auxin responses	Hamann *et al.* 1999 & 2002; Liscum and Reed 2002
IAA14	*Solitary root* (*slr*)	Reduced sensitivity to auxin, completely lacks lateral roots, defective root hair formation, defective gravity responses in both shoot and roots	Lateral root formation and multiple auxin responses	Fukaki *et al.* 2002 & 2005; Venneste *et al.* 2005
IAA17	*Auxin resistant 3* (*axr3*)	Enhanced apical dominance, reduced root elongation, increased adventitious roots, no root gravitropism	Multiple auxin responses	Dharmasiri *et al.* 2003; Leyeser *et al.* 1996; Ouellet *et al.* 2001; Overvoorde *et al.* 2005
IAA18	*Crane1*	Aberrant cotyledon placement in embryos, defective auxin transport, increased frequency of rootless seedlings, defective auxin responses	Apical patterning in embryos, multiple auxin responses	Uehara *et al*. 2008
IAA19	*Massagu 2* (*msg2*)	Auxin insensitivity, defects in hypocotyls gravitropism and phototropism, defects in maintaining apical hook, defective lateral root formation	Regulate differential growth responses of hypocotyls for different environmental conditions, lateral root formation	Tatematsu *et al*. 2004
IAA26	*Phytochrome interacting protein* (*pap1*)	Abnormal developmental phenotypes such as severe stunting, leaf epinasty those similar to viral infected plants	Connects auxin signaling with viral disease development	Padmanabhan 2005 & 2006
IAA28	*IAA-alanine resistant 28*	Abnormal shoot development, lack of lateral roots, increased adventitious root in response to high levels of picloram	Secondary root development, multiple auxin responses	Rogg *et al.* 2001; Karunarathna 2008
IAA16	N/A	ABA resistant primary root growth, reduced auxin responses, Infertile when the mutation is homozygous	Multiple auxin responses	Rinaldi *et al.* 2012

1.C. Arabidopsis as a model system

Arabidopsis thaliana is a small flowering plant that belongs to the Brassicaceae (mustard) family, which includes cultivated species such as cabbage and radish. Even though Arabidopsis does not have a major agronomic significance, it has important advantages for basic research in genetics and molecular biology due to several characteristics. Arabidopsis has a small, (125 Mb total), completely sequenced genome with extensive genetic and physical maps for all five chromosomes. It has a short life cycle of about 6 weeks from germination to mature seeds. In addition, it produces lots of seeds and is easy to transform using *Agrobacterium tumefaciens*. Arabidopsis has a large number of mutant lines and genomic resources that are available from several stock centers, such as ABRC (Arabidopsis Biological Resource Center- Ohio State University) or NASC in UK (European Arabidopsis Stock Center).

I.C.i. Root development

The plant root system is essential for growth and survival because of its role in water and nutrient uptake and in providing anchorage. In addition, to these primary functions, the plant root system is also involved in storage, phytohormone synthesis and vegetative propagation. The plant root system consists of primary roots, lateral roots and adventitious roots (Figure 6) (Osmont *et al.* 2007). The primary root is formed during embryogenesis, while LRs are derived post-embryonically from existing roots. LRs originate from the pericycle tissue layer generally adjacent to xylem pole cells in dicotyledons or phloem pole cells in monocotyledons (Casero *et al.* 1995). Adventitious roots are shoot-derived roots (Figure 6). All these roots possess epidermal root hairs that are important in increasing the absorptive surface of roots as well as for anchorage.

In angiosperms or flowering plants there are two major types of root systems: primary root systems (allorhizic) and fibrous root systems (homorhizic). Dicotyledonous, such as Arabidopsis, have primary root systems where the primary root dominates and produces many lateral roots. Adventitious roots are rare in this root system, but occasionally emerge from the hypocotyls or stems under some conditions.

Monocotyledonous such as maize and rice have fibrous root systems which consist of many adventitious roots in parallel to the primary root (Osmont *et al.* 2007).

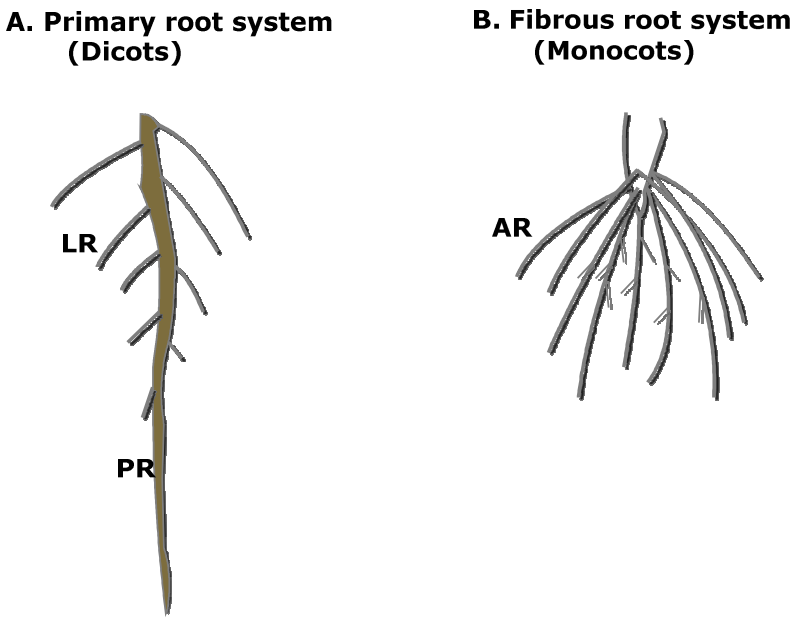


Figure 6: Different types of root systems in plants. (A) Schematic representation of a typical primary root system found in most dicotyledonous plants such as Arabidopsis, in younger and mature seedlings. Root hairs are not shown. PR (primary root), LR (lateral roots). (B) Schematic representation of a typical fibrous root system as found in most of monocotyledonous plants such as rice. AR (adventitious roots).

1.C.ii. Factors that affect root development

Root system architecture is influenced by both biotic and abiotic factors of the soil environment. RSA is highly plastic depending on the micro- and macro-environment. For example, genetically identical plants can produce significantly different root systems depending on their environment. Even though the changes of the root system architecture and their causative environmental factors have been well studied, the molecular mechanism of how changes are brought about is poorly understood. However, analysis of different mutants with defective root system architecture has strengthened the existing knowledge of root system development in plants.

Some of the abiotic factors important for root system architecture are water, nutrient availability and light conditions. The most important endogenous modulators of

root system development are different plant hormones. Auxin plays a major role in all stages of lateral root development including priming of lateral roots, initiation, emergence and growth (Figure 7) (Casimiro *et al.* 2001; Casimiro *et al.* 2003). Arabidopsis auxin overproducing mutants such *sur1* (*supperroot1*) and *rty1* (*rooty1*) have shorter primary root and increased root branching (Boerjan *et al.* 1995; King *et al.* 1995). In contrast, mutants that produce less auxin have poorly branched root systems. In addition, both acropetal and basipetal auxin transport is important in lateral root formation (Casimiro *et al* 2001). Auxin signaling mutants such as *axr1, axr6* (*cul1*), *iaa3/shy2-2* and *arf7/arf19* also show reduced lateral root numbers compared to the wild type (Péret *et al.* 2009). In conclusion, auxin signaling, transport and biosynthesis strongly influence root system architecture.

In addition to auxin, other plant homones such as cytokinin, ethylene and ABA also affect the root system development. Exogenous cytokinin application suppresses lateral root formation. For example, transgenic *Arabidopsis* plants with decreased cytokinin levels displayed increased root branching and increased primary root growth (Werner *et al.* 2003). Ethylene also affects root system architecture. Moderate concentrations of ethylene inhibit root growth and also have a role during LR emergence by promoting the breakdown of cortical cells (Ponce *et al.* 2005; Laskowski *et al.* 2006). ABA is also involved in root system development because exogenous ABA application inhibits primary and lateral root development in Arabidopsis (Beaudoin *et al.* 2000; De Smet I *et al.* 2007).

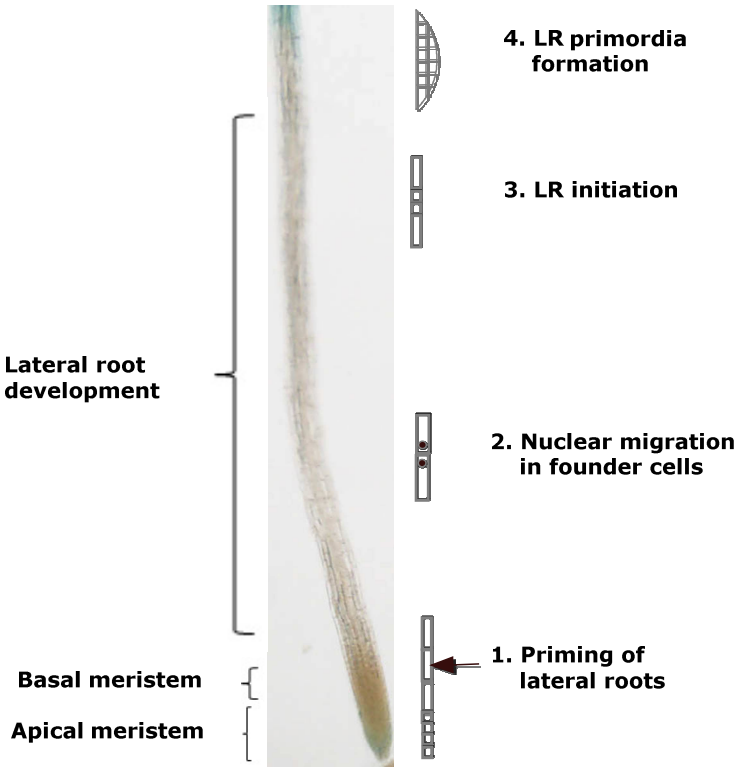


Figure 7: Developmental events during lateral root formation. (1) Pericycle cells are primed for the future lateral root initiation, (2) nuclear migration in founder cells (3) lateral root initiation (anticlinal cell divisions to produce shorter and longer cells), (4) lateral root primordium development.

1.C.iii. Mutants with defective secondary root development

As previously described, auxin plays a major role in lateral root development. Application of exogenous auxin increases the number of lateral roots whereas auxin transporter inhibitors such as 1-napthaleneacetic acid decrease the number of lateral roots (Reed *et al.* 1998; Casimiro *et al.* 2001; Marchant *et al.* 2002). Genetic studies using auxin transporter mutants show that both influx and efflux carriers are necessary for LR initiation as well as their development.

The auxin influx carrier mutant *aux1* shows highly agravitropic roots and reduced lateral root formation (Marchant *et al.* 1999). In 2002 using gas chromatography-mass spectroscopy, the same research group showed that *aux1* mutants have altered IAA distribution in young leaves (IAA source tissue) and root tissues (major IAA sink).

Therefore, the defective lateral root phenotype in *aux1* mutants is mainly due to the disruption of IAA transport from shoot to root tissues (Marchant *et al.* 2002). In addition to *AUX1, LAX3* also regulates LR emergence. In the *lax3* mutant seedlings the number of emerged LR is reduced but LR initiation is not affected. *The lax3* mutation down regulates the expression of several cell wall remodeling enzymes that are essential for the proper lateral root emergence (Swarup *et al.* 2008).

In addition, studies have shown that auxin efflux carriers are crucial for LR formation (Benkova *et al.* 2003). Multiple *pin* mutations cause dramatic defects in root patterning including LR primordium development (Benkova *et al.* 2003). The p*in1/pin4/pin7* or *pin1/pin3/pin7* triple mutants exhibit defects in lateral root primordia formation. These triple mutants produce poorly defined lateral root primordia with massive divisions of pericycle cells in response to exogenous auxin.

In addition to proper auxin transport, proper auxin signaling mediated by Aux/IAAs, ARFs and other signaling components is also required for LR initiation and development. As previously mentioned, several gain of function mutants in different Aux/IAA proteins, such as *iaa14/slr*, *iaa3/shy2*, *iaa19/msg2*, *iaa1/axr5*, *iaa28* and *iaa18*/*crane* also dramatically decrease the number of LRs (Tian and Reed 1999; Rogg *et al.* 2001; Tatematsu *et al*., 2004; Uehera *et al.* 2008; Fukaki *et al.* 2002). Similarly, *arf7arf19* double mutants have severely impaired LR initiation (Okushima *et al.* 2005; Wilmoth *et al.* 2005).

LBD16 and LBD29 are direct targets of ARF7 and ARF19. Both LBD16 and LBD29 are members of the LBD (lateral organ boundaries domain) family and are induced by auxin only if ARF7 and ARF19 are present (Shuai *et al.* 2002; Okushima *et al.* 2007). Over-expression of either LBD16 or LBD29 partially rescues the defective lateral root phenotype in *arf7arf19* mutants. Monocot plants also use a member of the LBD family in adventitious root formation. In rice a mutation in the *CRL1/ARL1* (*CROWNROOTLESS*/*ADVENTITIOUS ROOTLESS*) gene, which encodes a LBD protein homologous to Arabidopsis LBD29, causes defects in adventitious root formation and lateral root formation (Inukai *et al.* 2005; Liu *et al*. 2005). These studies in both

Arabidopsis and rice indicate that the basic mechanisms of root formation are highly conserved between dicot and monocot plants (Fukaki and Tasaka 2009).

I.D. Aim of the study

The aim of this study was to understand the functions of *IAA28* gene in plant growth and development in Arabidopsis. We have identified a mutant allele of *IAA28*, namely *pic11/iaa28-2,* from a picloram based Arabidopsis mutant screening. The mutant showed severe defects in growth and development, including defects in secondary root formation, defects in root hair development, stunted shoot growth and reduced fertility (Rogg *et al.* 2001; Karunarathna 2008).

As mentioned previously, both *iaa28-1* and *iaa28-2* showed severe defects in lateral root formation; however, higher concentrations of IBA and 2,4-D treatments were able to rescue the defective lateral root phenotype in *iaa28-2*. The mutant seedlings produce adventitious roots with picloram treatment while Col-0 (WT) produces many lateral roots along the primary root. These results show that *IAA28* plays a major role in secondary root formation in Arabidopsis. The work presented here discusses a possible mechanism to explain how IAA28 is involved in secondary root formation in Arabidopsis. Further, attempt was made to understand the expression and sub-cellular localization patterns of IAA28 in order to understand its function. Additionally, work was also focused to understand light regulation of IAA28 and to identify its interacting partners using yeast two hybrid screening.

The localization studies using *IAA28::IAA28-GUS* and *IAA28::iaa28-GUS* transgenic seedlings showed that, unlike other Aux/IAA proteins, IAA28 is localized to both nucleus and cytoplasm. This cytoplasmic localization raised the possibility that IAA28 may interact with other cytoplasmic proteins and may have multiple functions in plant growth and development. Therefore, identification of putative IAA28 interacting proteins will strengthen the current knowledge of how Aux/IAA proteins are involved in plant growth and development.

CHAPTER 2: MATERIALS AND METHODS

2.A. Plant materials and growth conditions

Arabidopsis thaliana ecotypes Columbia (Col-0) and Wassileswskija (Ws) were used in this study. Seeds were surface-sterilized with 40% bleach containing 0.08% Triton and germinated on vertically oriented, solid ATS (*Arabidopsis thaliana* medium with sucrose) nutrient medium (Lincoln *et al.* 1990) at 22^{o}C under continuous light with Sylvania cool white fluorescent bulbs. For root growth assays, four day-old seedlings were transferred onto the medium containing indicated concentrations of auxinic chemicals and allowed to grow on vertically oriented plates for four more days before measuring root lengths. Plants in soil were grown in Promix BX soil mixture in a growth chamber at 22^{o}C under continuous light with Sylvania cool white fluorescent bulbs.

2.B. Seedling phenotype assays

2.B.i. Root and hypocotyl elongation

For root growth assays, seeds were first surface sterilized as mentioned previously and stratified at 4º C for 24 hrs. The seeds were then germinated on vertically oriented ATS plates for 4 days under white light at 22º C, and then transferred to different auxin- (IAA, 2, 4-D, picloram or IBA) containing ATS medium and incubated for additional four days before measuring the root length. For hypocotyl elongation experiments, sterilized and stratified seeds were directly plated on the auxin-containing growth medium and incubated vertically for 8 days under white light, and hypocotyl length was measured using stereo microscope.

2.B.ii. Lateral and adventitious root initiation

Seeds were grown on ATS medium for 4 days and transferred onto ATS medium containing different auxins and allowed to grow for 4 more days in vertically oriented plates under white light. The total number of lateral roots and adventitious roots per seedling was counted using a stereomicroscope (Nikon SM21500). Then the number of lateral roots per 1 cm was calculated for each seedling, and the average and standard deviation were calculated. Ten to fifteen seedlings were analyzed for each genotype.

2.C. Plasmid constructs for characterization of IAA28 and iaa28-2

2.C.i. Expression of *IAA28$_{Pro}$::IAA28-GUS* and *IAA28$_{Pro}$::IAA28-GUS* in plants

To examine the expression of *IAA28* gene and localization of IAA28 protein, a translational gene construct was prepared by amplifying the 3.1 kb promoter region and the whole coding region of the IAA28 gene using the primers, 5'-ACC**AAGCTT**ACTATAAGAAACTGTGAAAT-3' and 5'-CTC**GTCGAC**TTCCTTGCCATGTTTTCTAGC-3' with introduced *Hind* III and *Sal* I sites. The *Sal* I site was introduced after the modified stop codon of *IAA28* gene in such a way that it could be in frame with the β-glucuronidase gene in pBI101. Gene sequences were amplified from wild type Col-0 and *iaa28-2* using Phusion Taq polymerase (NEB). The amplified products were cloned into the *EcoR*V site of pBluescript II SK (Stratagene) and sequenced to determine the fidelity of amplification. The gene constructs were then restricted with *Hind* III and *Sal* I and cloned into the same sites of pBI101 to generate *IAA28$_{pro}$::IAA28-GUS* and *IAA28$_{pro}$::iaa28-GUS* fusion constructs. The gene constructs were transformed into wild type Col-0 using *Agrobacterium tumefaciens* strain GV3101. Seedlings homozygous for the transgene were selected from T2 generation.

2.C.ii. Expression of *GST-IAA28* and *GST-iaa28* in an *E. coli* system

Construction details for both GST-IAA28 and GST-iaa28 in *E. coli* have been described previously (Karunarathna 2008).

2.D. Expression studies

2.D.i. GUS reporter gene expression

The following method was used for all the transgenic seedlings carrying GUS reporter gene in order to visualize the expression and localization of the particular recombinant gene. First the seedlings were fixed with the GUS fixing solution (Appendix I) (Oono *et al.* 1998) for 45 minutes at room temperature on a slowly moving shaker. Then the seedlings were washed with 100 mM Na_2HPO_4 washing solution (pH = 8.0) three times (each 5 minutes). The seedlings were stained with GUS staining buffer (Oono *et al.* 1998) containing X-Gluc (Appendix II) at 37ºC for a period of time depending on the intensity of the staining of each different transgene. The seedlings were then transferred into 50% ethanol to stop the staining and to remove chlorophyll. The expression patterns of recombinant GUS proteins were studied using a compound light microscope. Whenever necessary, the seedlings were counterstained with DAPI (1µg/ml final concentration in water) for 15 minutes to visualize nuclei. The expression of GUS transgene and DAPI (4,6-diamidino-2-phenylindole) nuclear stain were examined under compound light microscope (Olympus BH2-RFCA).

2.D.ii. Western blotting for myc tagged proteins

$IAA28_{Pro}$::IAA28-myc seedlings (kindly provided by Dr. Bonnie Bartel) were grown on ATS medium for 7 days on vertically oriented plates under the growth conditions mentioned previously. After specific treatments in each experiment, 7-8 seedlings were collected from each treatment in triplicate and frozen quickly in liquid nitrogen. Then the total protein was extracted using EZ buffers (Appendix III). The protein samples were quantified using Bradford reagents (Amresco, Solon OH) and bovine serum albumin (BSA) (B9001S BioLabs NE) as the standard. Protein samples were separated using 14% polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) (Pall Corporation, FL) membrane. Western blotting was conducted using anti-Myc (Covance) as the primary antibody and anti-mouse IgG (Invitrogen) as the secondary antibody.

Once proteins were transferred onto PVDF (Pall Corporation, FL), the membranewas blocked with 5% nonfat milk in Tris buffered saline containing 0.1% Tween 20 (TBST) for one hour on a slowly moving shaker, and then washed three times with TBST. Then the blot was incubated with primary antibody solution, anti-Myc (1:1000 in TBST) for one hour on a shaker. The primary antibody bound blot was washed three times with TBST (each 5 minutes). The blot was next incubated with secondary antibody (1:2000 in TBST) (anti-mouse IgG conjugated to horseradish peroxidase) (Sigma Aldrich Inc. MO) for one hour and washed 4 times with TBST (1^{st} wash for 15 minutes, three washes each 5 minutes). Finally, the blot was developed using PIERCE ECL western blotting substrate (an enhanced chemiluminescent substrate for detection of HRP) and exposed to X-ray film according to manufacturer's instructions.

2.E. In-vitro pull down assays

2.E.i. Protein expression, extraction and purification from *E. coli*

To purify GST-IAA28, GST-iaa28 and GST-RCE1 recombinant proteins, *E. coli* (TOP10) carrying the respective recombinant plasmids were grown overnight and 5 ml of overnight culture was inoculated into 250 ml liquid LB containing carbenicillin (100 µg/ml) and incubated at 37° C for 4 hours. Isopropyl thiogalactoside (IPTG) (Sigma Aldrich Inc. MO) was added to a final concentration of 1 mM to induce recombinant protein expression, and the culture was incubated at 30° C for a further 4 hours. Bacterial cells were pelleted at 9000 x g for 10 minutes at 4°C (Eppendorf Centrifuge 5810R), and the pellet was re-suspended in 5 ml of phosphate buffered saline (PBS, pH = 8.0). The cells were lysed by sonication for 30 seconds three times (Branson Sonifier 250). Phenylmethanesulfonyl fluoride (PMSF) (Sigma Aldrich Inc. MO) and Tween-20 (Sigma Aldrich Inc. MO) were added to the extract at concentrations of 1 mM and 0.1%, respectively. Cell debris was removed by centrifugation at 9000 x g for 10 minutes (Eppendorf Centrifuge 5810R). Glutathione-agarose beads (GST beads) (Sigma Aldrich Inc. MO) that were previously hydrated in 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.

2. E.ii. Expression and extraction of recombinant proteins from plants

Plant expressed proteins such as TIR1-myc, AFB1-myc, AFB5-myc, IAA28-myc and RCE1-myc were extracted using native extraction buffer as explained previously (Dharmasiri N. *et al.* 2003). Native extraction buffer (2.5ml) was used for 0.5-0.6g of fresh plant tissues. The tissues were ground well using a tissue grinder while adding the extraction buffer containing protease inhibitor (Complete Mini-Rochr Diagnostics, Germany), PMSF (1mM final concentration) and MG132 (10 µM final concentration). The extracts were transferred to a clean 15ml Falcon tube and kept in a rocker at 4º C for 10 minutes. The cell debris was pelleted by centrifugation at 9000xg for 10 minutes at 4°C (Eppendorf Centrifuge 5810R). The supernatant was transferred to a new tube and the level of protein quantified using Bradford reagent (Amresco Ohio). Total protein was diluted to 800 µg/ml and aliquoted into 1.5ml Eppendorf plastic tubes and stored at -80º C until further use.

2.E.iii. *In vitro* protein-protein interaction assays

Total plant proteins were prepared from *GVG::TIR1-myc*, *AFB1::AFB1-myc* and *AFB5::AFB5-myc*seedlings as mentioned previously in the section 2.E.ii. A total of 3-4 µg of purified GST-IAA28 (or GST-iaa28, GST-IAA7) proteins were added to each sample with or without auxin. The reactions were incubated for 1 hour at 4º C with gentle agitation. After 1 hour tubes were centrifuged in order to pellet glutathione bound GST-IAA28/aa28. Immobilized proteins were washed 3 times (each 5 minutes) with pull down washing buffer (Dharmasiri N. *et al.* 2003) with or without auxin. Finally protein samples were boiled in 2X LSB (Laemmli sample buffer- Appendix IV) for 6 minutes and separated using 12.5% or 14% polyacrylamide gel depending on the size of the target protein and transferred onto PVDF membrane (Pall Corporation, FL). Western blotting was carried out as explained previously (2,D.ii) in order to identify the specific myc protein used in each pull down (e.g. TIR1-myc, AFB1-myc or AFB5-myc).

2.F. Transcript studies using RT-PCR (Reverse Transcription PCR)

To investigate the effect of *iaa28-2* mutant on the expression of different auxin transporters and *LBD* genes, RT-PCR experiments were carried out as follows. Seven day-old *iaa28-2* and Col-0 seedlings were treated with liquid ATS supplemented with 20 μM 2,4-D or 100 μM picloram for 1 hour on a slowly moving shaker at room temperature. Total RNA was extracted by using TRI reagent (Sigma) according to manufacturer's instructions. Complementary DNA was synthesized using oligo dT primers and reverse transcriptase (New England Bio Labs). The amount of cDNA synthesized was visually estimated by amplifying ubiquitin cDNA using primers UBQ-F and UBQ-R (Table 2) in order to adjust the starting amount of cDNA in each treatment for both Col-0 and *iaa28-2*. The cDNA of different auxin transporters (*PINs* and *AUX1*) and *LBD* genes (*LBD16, LBD29* and *LBD18*) were amplified by PCR using gene specific primers (Table 2). 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 *iaa28-2*.

Table 2: Oligonucleotide primers used for making different constructs and for RT-PCRs

(* denotes the primers that were made by Amarah Ulghani; #, Thilanka Jayaweera; $, Dr.Yaling Song; +, common primers)

	Primer name	Primer sequence (5' to 3')	Primer length	Melting temp. (°C)
1	LBD16-F	GCTCGTGCACATCTCCGACG	20	60.9
2	LBD16-R	CATCTCATTTGTTCTCTGACTGTCT	25	54.6
3	LBD29-F	CATGACTAGTTCCAGCTCTAG	21	51.9
4	LBD29-R	GATAGAACCATACACGAGAAGGAG	24	54.3
5	LBD18-F	GGAGATAGAGTAACAACTCGTTAAT	25	52.1

Table 2 Continued…

6	LBD18-R	CAATGCGGAGATGTATCGATC	21	53.3
7	AFB1-5F	TCTCACTCTGTTTCTGATGGGTCT	24	56.9
8	AFB1-3R	ATATGACTGAAGCAGCAAGTTTACT	25	60.2
9	RCE1BamHI5F	CTGTGTGGATCCGTGTGAGTTTCTTAAG	28	60
10	RCE1SalI 3'R	GAAGAAGACAAGTCGACGCTTTG	23	58
11	IAA28-EcoRI-2F	CAAAAACAGAATCCAAACTTAGAAAAATGG	30	60
12	IAA28-BAmHI-2R	GATTTTGGCGGATCCCTCTCTATTCC	27	58
13	PIN1- F*	GGGTTGTTCATGGCGTTAAACC	22	57
14	PIN1-R *	CTT GCTGAGCTCCTACTTAAGTC	23	54.9
15	PIN2-F *	CGTGCGGAAAATCAGTAGCAGG	22	58.5
16	PIN2-R *	CAAAGTCACGTACATGCATGTGC	25	57
17	PIN3-F *	AACAATTTAGTTTGCCCGGAAAAGA	26	55.7
18	PIN3-R *	CTGAACTATAGCGACACGCAGT	22	56.8
19	PIN4-F *	TCCCACGATTCTAAGCACTG	20	54.4
20	PIN4-R *	GTGCATATTACATATCTGCTTAGC	24	51.7
21	PIN5-F *	GTTGTGGGAGAGAAGTCGTTCC	22	57.5
22	PIN5-R *	AATAAACTCCAGAGCTGCGTAGT	23	56
23	PIN6-F *	GATGCCAAATATAGTGGATTTCTC	24	51.7
24	PIN6-R *	CCGTTAATTAATCTTTCTCACAAGC	25	52.6

Table 2 Continued…

25	PIN7-F*	GGGCTCTTGTTGCTTTCAGGT	21	58
26	PIN7-R*	TACATTTTCTAGTTGCGTTCCACTA	26	54.2
27	AUX1-F*	GAAGCCACCGTTCTTTATGC	20	60
28	AUX1-R*	ACTAGCCCATCCACCGAAC	19	60
29	pACT-F*	CTATCTATTCGATGATGAAG	20	54
30	pACT-R*	ACAGTTGAAGTGAACTTGCG	22	58
31	UBQ-F*	GTTGATTTTTGCTGGGAAGC	20	52.8
32	UBQ-R*	GATCTTGGCCTTCACGTTGT	20	55.5
33	LBO2	TTGGGTGATGGTTCACGTAGTGGGCC	26	64.5
34	tir1-1F	GTGCAAGTCATGGTACGAGATCGA	23	58.6
35	tir1-1R	CTCAGGAGATTCACTGAGAGCGAA	23	58.1

2.G. Expression analysis of different genes in *iaa28-2* mutants

2.G.i. Effect of *iaa28-2* mutation on auxin transporters

Mutant *iaa28-2* was crossed to different homozygous *Arabidopsis* lines carrying *PIN1$_{Pro}$::PIN1-GFP, PIN2$_{Pro}$::PIN2-GFP* (Vieten *et al.* 2005)and *AUX1$_{Pro}$::AUX1-YFP*recombinant gene constructs (Kleine-Vehn *et al.* 2006). After several generations (F4), the plants that are homozygous for both *iaa28-2* and the transgenes auxin transporter gene were selected for expression analysis. Four day-old homozygous seedlings grown on solid ATS were transferred onto control ATS or ATS containing 85nM 2,4-D or 10 µM picloram for 18 hours, and were examined for the expression of each transgene using confocal microscope (Olympus FV1000- NSF DBI- 0821252 to Dr. Joseph R. Koke and Dr. Dana García).

2.G.ii. Expression analysis of LBD genes in *iaa28-2* background

iaa28-2 mutant plants were cross pollinated with transgenic plants carrying *LBD16*$_{Pro}$*::LBD16-GUS, LBD18*$_{Pro}$*::LBD18-GUS* and *LBD29*$_{Pro}$*::LBD29-GUS* (kindly provided by Dr. Fukaki) transgenes. Following a similar procedure explained in 2.G.i section, homozygous seeds for both *iaa28-2* and each transgene were collected and used in the experiments. Seedlings were grown on solid ATS media for 4 days and transferred onto solid ATS media containing 85nM 2,4-D, 10 µM picloram or 15 µM IBA, and the plates were kept in a growth chamber at 22ºC for 48 hours. Then the seedlings were fixed with GUS fixer (Oono *et al*. 1998) for 45 minutes with gentle agitation. The seedlings were washed with 100mM Na_2HPO_4 solution three times (5 minutes each) and incubated with GUS staining buffer for 12 hours at 37 ºC. Finally, 50% ethanol was added to each sample in order to stop further staining and to remove chlorophyll. The expression of each transgene was examined under compound light microscope (Olympus BH2-RFCA).

2.G.iii. Gene expression in early lateral root development in *iaa28-2*

In *Arabidopsis thaliana*, lateral root founder cells originate from pericycle cells adjacent to xylem poles. Haseloff and coworkers screened a population of Arabidopsis GAL4-GFP enhancer trap lines and identified two lines with GAL4 expression specifically in root xylem pole pericycle cells (*J0121*) and in young lateral root primordia (*J0192*) (Laplaze *et al.* 2005). These enhancer trap lines are very useful tools as markers to visualize pericycle cells and lateral root primordial cells.

To examine the effects of *iaa28-2* mutation on the pericyle cell development, *iaa28-2* mutant was crossed to J0121 enhancer trap line. After several generations (F3), plants that are homozygous for both *iaa28-2* and *J0121* were selected and used for the expression analysis. The homozygous *iaa28-2 J0121*seedlings together with wild type *J0121* were grown on solid ATS for 6 days and transferred on to ATS media (control) or ATS containing 85 nM 2,4-D, 10 µM picloram or15 µM IBA and incubated in a growth chamber for 18 hours. The expression of J0121 in *iaa28-2* background with different auxin treatments were compared with that of wild type J0121 using confocal microscope (Olympus FV1000).

2.H. Examining the genetic interaction of *iaa28-2* with different auxin co-receptors

In order to examine the genetic interactions between different auxin co-receptor genes and *iaa28-2* mutants, *iaa28-2* mutant was crossed to *afb1*and*tir1-1* mutants. F3 seedlings that are homozygous for both mutations were selected and used for experiments. To select the plants homozygous for auxin co-receptor mutant genes (*afb1* and*tir1*), specific genotyping primers (Table 2) were used. For *afb1* mutant genotyping, gene specific 5'AFB1F primer and T-DNA specific left border primers (LB02) (Table 2) were used as previously described (Dharmasiri *et al.* 2005a). For *tir1-1* mutant identification, the gene was amplified using TIR1-1 F/R primers (Table 2) and digested with BsmA1 restriction enzyme where only the PCR product carrying the mutation is digested.

After selecting the seedlings homozygous for both mutations, plants were transferred onto soil and grown to collect seeds. The seeds were sterilized and grown on ATS medium for 4 days and transferred to control (ATS) or ATS supplemented with 10 µM picloram and allowed to grow for additional 7 days. The development of adventitious roots was examined under a stereomicroscope (Nikon SM21500).

2.I. *IAA28* and light regulated developmental responses in *Arabidopsis thaliana*

2.I.i. Effect of light on seed germination and hypocotyl development

Col-0 and *iaa28-2* seeds were surface sterilized and stratified at 4ºC as mentioned previously. Seeds were plated on solid ATS and incubated in red (λ= 660 nm, intensity = 30 µmol/m^2/s), far red (λ= 730 nm, intensity = 0.5 µmol/m^2/s), blue (λ= 475 nm, intensity = 21.3 µmol/m^2/s) and white (intensity = 42 µmol/m^2/s) light conditions for 7 days. The lengths of the hypocotyls were measured using a scale under the stereomicroscope. For seed germination assays, both Col-0 and *iaa28-2* seeds were placed on ATS medium and incubated horizontally under different light conditions (red, far red, blue and white). The percentage seed germination in both Col-0 and *iaa28-2* were calculated after 36 hrs and 48hrs of light exposure. For hypocotyl growth experiments seeds were directly

germinated under each light condition for 7 days and the length of hypocotyls was measured using stereomicroscope (Nikon SM21500).

2.I.ii. Protein expression analysis using *IAA28$_{Pro}$::IAA28-myc* and *IAA28$_{Pro}$::IAA28-GUS* and *IAA28$_{Pro}$::iaa28-GUS*

The expression of *IAA28$_{Pro}$::IAA28-myc* and *IAA28$_{Pro}$::IAA28/iaa28-GUS* were examined in both etiolated hypocotyls and roots. Surface sterilized and cold-treated seeds were germinated on vertically oriented solid ATS plates in complete darkness for 72hrs (Plates were wrapped with two layers of aluminum foil.) at 22ºC. The etiolated seedlings were exposed to different light sources (red/far red/blue or white for indicated time periods). Then for *IAA28$_{Pro}$::IAA28-myc* seedlings, shoots and roots were cut with a razor blade, collected into 1.5ml Eppendorf tubes, frozen quickly with liquid nitrogen and stored until further use. Total protein was extracted using the EZ method (Appendix I) and quantified using the Bradford method. Then, 40 µg total protein was separated on 14% polyacrylamide gel and transferred onto PVDF membrane. Western blots were carried out as described above (section 2.D.ii.) using anti-myc antibody. For *IAA28$_{Pro}$::IAA28* and *IAA28$_{Pro}$::iaa28-GUS*seedlings, after light treatments, the seedlings were fixed and stained as mentioned in section 2.D.i.

2.I.iii. Analysis of the relationship between IAA28-GUS expression and the time of the day

IAA28$_{Pro}$::iaa28-GUS seeds were germinated under dark conditions for 72 hours and kept under white light at 20 ºC. At each hour, hypocotyls (shoot) samples were collected in triplicate into 1.5ml Eppendorf tubes and quickly frozen with liquid nitrogen. Proteins were extracted with 50 µl GUS extraction buffer (Jefferson 1987) and quantified with the Bradford reagent. Equal amounts of total protein (40 µg) were added into 0.5ml of MUG assay buffer (Jefferson 1987) which had been pre-warmed to 37ºC for 30 minutes. The samples were mixed and incubated at 37ºC for 1 hour. Then 100 µl of the reaction mixture was immediately transferred to 900 µl of stop buffer (Jefferson 1987). The absorbance of each sample was measured using single tube luminometer (Modulus,

Turner Biosystems). The average was calculated for each time points and plotted as the level of GUS protein.

2.J. Identification of IAA28 interacting proteins (yeast two hybrid screening)

2.J.i. Two hybrid constructs

To isolate putative IAA28 interacting proteins, yeast two hybrid screening was carried out using GAL4 system. pAS1 and pACT1 were used as bait and prey vectors, respectively (Clontech). IAA28 cDNA was amplified from a Col-0 cDNA sample with Phusion high fidelity DNA polymerase (New England Bio Labs) using the primers ATIAA28BamHI-2F (5'…TTCGGATCCAACCACCCATATAATAAT…3') and ATIAA28SalI-2R (5'…AAAGTCGACCATCGAACTGATGATTT…3') with introduced BamHI and SalI sites. The BamHI site was created in such a way that IAA28 gene is in frame with the DNA binding domain of GAL4 transcriptional factor of pAS1 vector. The gel purified DNA product was digested with BamHI and SalI and re-purified using an Ultra clean Gel DNA extraction kit (ISE Bio Express). The digested IAA28 cDNA was then ligated to pBluescript (BamHI & SalI digested) and sequenced in order to confirm the fidelity of the sequence. The BamHI and SalI digested cDNA fragment was then ligated to the same restriction sites of pAS1. After ligation 3µl of the ligation mixture was transformed into *E. coli* (TOP10) competent cells (Bergmans *et al*. 1981). Positive transformants were selected with IAA28 gene specific primers using PCR, and also by expression analysis by western blotting using anti-HA antibody.

2.J.ii. Confirming the expression of bait vector with IAA28

The positive transformants were selected, and plasmids isolated using mini prep plasmid isolation methods (Sambrook *et al.* 2001). Plasmids were then transformed again to yeast Y190 competent cells. The yeast Y190 competent cells were prepared as described in MATCHMAKER random peptide user manual (PT3039-1). Positive transformants were selected using solid SD-Trp (Synthetic Dropout medium without Tryptophan) medium. Three positive transformants were inoculated to 5 ml liquid SD-Trp and were grown for 2 days. Yeast cells were pelleted, and total protein was extracted

(Kushnirov 2000). Then 20 µl of the extracted protein from each sample was separated on a 14% polyacrylamide gel together with a total protein sample from Y190 cells as the negative control. Proteins were transferred on to PVDF membrane (Pall Corporation-FL). The expressed IAA28-myc tag protein was detected by western blotting as described in section 2.D.ii.

To test whether IAA28 autonomously activates the reporter gene when fused to GAL4 DNA-BD, yeast Mav203 cells with IAA28 in pAS1 were grown in 25 ml of SD-Trp liquid media in shaker flasks at 30ºC for 16-18 hours at 250rpm to stationary phase (OD_{600nm}>1.5). This overnight culture was transferred to 150ml of SD-Trp liquid media and incubated for 3 hours with shaking. The cells were centrifuged at 1000x*g* for 5 minutes at room temperature, and the supernatant was discarded. The pellet was resuspended in 25ml of sterile water. The cells were centrifuged again for 5 minutes and the supernatant was removed. The pellet was resuspended in 0.75 ml of freshly prepared, sterile TE/LiAC (1X TE buffer with 1x lithium acetate/ MATCHMAKER random peptide user manual (PT3039-1)). These cells were aliquoted into 100 µl volumes and used as competent cells for cotransformation of empty vector with GAL4 DNA-AD (pACT1). Then, 100 ng of pACT plasmid DNA and 0.6ml of PEG/LiAc solution were added to these Mav203 competent cells with *pAS1-IAA28*, which were then incubated at 30ºC for 30 minutes with shaking. After 30 minutes, 70 µl of DMSO was added to the mixture, inverted gently and heat shocked in a 42 ºC water bath for 15 minutes with occasional swirling. The heat shocked cells were chilled on ice, pelleted by centrifugation and the supernatant was discarded. The pellet was resuspended in 0.5ml of TE buffer and spread on solid SD-Trp-Leu-His medium containing plates (100 µl in one plate). The plates were incubated at 30ºC for 2 days until the colonies appeared.

Several transformants (5 individual colonies) were re-inoculated into 1 ml of SD-Trp-Leu-His liquid medium and grown ~18 hours at 30ºC with shaking. A small volume of each clone was streaked separately on SD-Trp-Leu-His, SD-Trp-Leu-His + 3AT (3-amino-1,2,4-triazole, a competitive inhibitor of the yeast HIS3 protein used to inhibit background signal from yeast cells; (Fields and Song 1989). Several 3-AT concentrations (e.g. 20mM, 30mM and 40mM) were used in different plates. The plates were incubated

for 2 days at 30 ºC until the colonies appeared. Finally, the colonies were examined for the activation of LacZ gene through β-galactosidase (X-gal) assay as described in MATCHMAKER random peptide user manual (PT3039-1).

For X-gal assays, colonies were allowed to grow to 1-3mm in diameter. A sterile filter paper was placed on the surface of plates containing colonies and poked with 3 holes which provided landmarks for orienting the paper. Then the filter paper was lifted out with the colonies attached, frozen in liquid nitrogen and kept for few minutes at room temperature until it thawed completely. The filter paper was placed on another filter paper wetted with Z-buffer/X-gal solution in a Petri plate avoiding air bubbles in between filter papers. The filter papers were incubated at 30ºC and examined for the appearance of blue color. If the gene of interest was autoactive, the X-gal assay would be positive within a very short period of time (e.g. an hour).

2.J.iii. Screening of the Arabidopsis cDNA library with IAA28 in bait vector

The screening of the Arabidopsis cDNA library with in pAS1-IAA28 was carried out by Dr.Yaling Song. A single colony of pAS1-IAA28 in Mav203 cells was inoculated into 50 ml of SD-Trp liquid media and incubated at 30 ºC for overnight. The cells were collected by centrifugation at 9000 x*g* and transferred to 300 ml of liquid YPD and allowed to grow at 30ºC until the OD reached 0.1 (λ = 600nm). Cells were harvested, and competent cells were prepared as described in MATCHMAKER random peptide user manual (PT3039-1). Next, 40 µg of library DNA was transformed to the prepared Mav203 competent cells carrying pAS1-IAA28 as described previously in 2.J.ii section. Finally the cells were spread on solid SD-Trp-Leu-His media supplemented with 15 mM 3-AT and incubated at 30ºC until colonies appeared.

2.J.iv. Confirmation of putative IAA28 interacting proteins

Yeast two hybrid screening identified about 40 putative IAA28 interacting colonies. All the colonies were checked with X-Gal assay, and only the ones that produced blue color within a short period of time (less than 3 hours) were selected (by Dr. Yaling Song).

All the colonies (IAA28 interacting protein/IP-1 to IP-40) were inoculated in 1ml of liquid SD-Trp-Leu-His media and were grown at 30ºC for 24 hours. Equal volumes of each culture were spread onto SD-Trp-Leu-His + 0mM 3-AT, SD-Trp-Leu-His + 25 mM 3-AT or SD-Trp-Leu-His + 50 mM 3-AT and incubated at 30 ºC for about 48 hours. The clones that had poor growth on the media containing 25mM 3-AT and no growth on the media containing 50 mM 3-AT were considered false positives and were removed from further analysis.

2.J.v. DNA sequencing and identification of putative IAA28 interacting proteins

The positive IAA28 interacting clones that grew on higher concentrations of 3-AT were amplified with pACT-F (5'…CTATCTATTCGATGATGAAG…3', recommended by ABRC) and pACT-R primers (3'…ACAGTTGAAGTGAACTTGCG…5', recommended by ABRC) using Taq polymerase. A small amount of yeast cells from each clone was used to derive the template DNA for this PCR. The product was separated on a 2% agarose gel together with 100bp marker, and the amplified DNA product was gel purified using a DNA gel purification kit (UltraClean Gel DNA extraction kit, ISC BioExpress, CA).This DNA was used as the template for a second PCR using high fidelity Phusion Taq polymerase with pACT-F and pACT-R primers. The amplified DNA product was gel purified and ligated to pBluescript digested with EcoRV restriction enzyme. The ligated product (2 µl) was transformed into TOP10 *E. coli* competent cells using the $CaCl_2$ method. Plasmid DNA was purified from positive clones and sequenced using pACT-F primer. The resulting sequence was compared with known sequences using an online BLAST program (http://www.arabidopsis.org/Blast/index.jsp).

2.J.vi. Confirmation of interactions of some identified IAA28 interacting proteins

Among the identified putative IAA28 interacting proteins, IP4 (RUB1 conjugating enzyme 1/RCE1) was selected for further studies. IP4-transformed yeast (RCE1) were grown in 5ml of SD-Leu at 30 ºC for 24 hours and transferred to 50 ml of YPD medium and allowed to grow for about 36 hours. The cells were pelleted and plasmid DNA was isolated using the glass beads method (Amberg *et al.* 2006). The resulting DNA pellet was resuspended in 5 µl of 10mM Tris-HCl (pH-8.0), and 3 µl of

the isolated DNA was used to transform electro-competent *E. coli* (HB101). The cells were spread on solid M9 minimal medium (Sambrook *et al*. 1989) without tryptophan and incubated at 37 ºC for 36 hours until the colonies appeared. Positive transformants were reconfirmed with PCR (with pACT-F/R primers), and plasmids were isolated and transformed into *E. coli* (TOP10) competent cells. The pACT1 plasmids with each insert were isolated and transformed to Mav203 yeast cells containing pAS1-IAA28 vector. At the same time empty vector (pACT1) was also transformed into Mav203 cells with IAA28-pAS bait construct. Then, the cells were spread on SD-Trp-Leu-His without 3-AT and incubated at 30ºC for 48 hours until the colonies grow. Later five individual colonies from each transformation were picked and grown on 0.5ml of liquid SD-Trp-Leu-His medium. After 48 hours, 2µl of each culture were streaked on solid SD-Trp-Leu-His medium with different concentrations of 3-AT (0 mM, 25 mM, 50 mM and 65 mM). The plates were incubated at 30ºC for 48 hours and observed for colony growth and development. X-gal assay (MATCHMAKER random peptide user manual-PT3039-1) was conducted to confirm the interactions of selected interacting proteins and IAA28.

2.K. Microscopy

2.K.i. Compound microscopy

Seedlings with GUS reporter gene were mounted on 5 % glycerol and visualized with Olympus BH2-RFCA compound microscope. The objectives used were DPlanApo100UV (oil, NA= 1.3), DPlanApo40UV (oil, NA =1) and Olympus 40X and 10X objectives. Images were process in Adobe Photoshop.

2.K.ii. Confocal microscopy

Imaging was performed using an Olympus FV1000 laser scanning microscope and FV1000-ASW (Version 1.7) software acquired through a National Science Foundation grant (DBI-0821252) to Drs. Joseph Koke and Dana García. The emission maximum used for GFP was 525 nm. The objectives used were PlanApo 60x oil (NA= 1.4), UPlanApo 40x oil (NA= 1), UPlanSApo 20x (NA = 0.75) and XLUMPlanF1 20x water (NA = 0.95). Images were processed in Adobe Photoshop.

2.L. Statistical analysis

Using residual plots, it was determined that the particular data sets had met the assumptions of normality and homoscedasticity. Single factor analysis of variance (ANOVA) was performed when necessary. Tukey's HSD test was used to identify the significantly different means to each other. All analyses were performed using software R (http://www.r-project.org/). In addition, Student's T-test was also used when necessary.

CHAPTER 3: RESULTS

3.A. Expression and localization of *IAA28*

3.A.i. Expression of IAA28-GUS and iaa28-GUS in Arabidopsis during different developmental stages

In a previous study using RNA gel blot analysis, Rogg *et al.* (2001) reported that *IAA28* is mainly expressed in roots and inflorescence stems and at low levels in flowers, leaves and siliques. Using the transcriptional gene construct *IAA28$_{pro}$::GUS*, they further showed that GUS is mainly expressed in the root from the region of root elongation to root-hypocotyl junction with the exception at the root tip. To further understand the expression pattern of the *IAA28* gene and also the localization of the IAA28 protein, the expression patterns were studied using translational gene constructs, *IAA28$_{pro}$::IAA28-GUS* and *IAA28$_{pro}$::iaa28-2-GUS* in wild type Col-0 plants. These constructs share the same 3.1 kb 5' flanking region of *IAA28* gene similar to the previous study (Rogg *et al.* 2001). Both transgenes were strongly expressed in primary roots of developing seedlings except at the root tips (Figure 8A, B), confirming the previous results (Rogg *et al.* 2001). GUS staining was also clearly visible in root hairs but was not observed above the root-hypocotyl junction (Figure 8C). However, when an *iaa28-2* mutant version of the above transgene construct (*IAA28$_{pro}$::iaa28-2-GUS)* was expressed, GUS staining was clearly visible in the hypocotyl region, though at a lesser intensity than in the root (Figure 8K), suggesting that the *IAA28* gene is expressed in both root and hypocotyls. IAA28-GUS was highly expressed in developing lateral root primordia at very early stages, but expression gradually decreased at the lateral root tip with development (Figure 8E, F and G). In fully developed lateral roots, expression was very high in mature areas of the root except at the tip (Figure 8H, and I). When lateral roots were stained for a short period, GUS staining was mainly found in the elongated side of lateral roots (Figure 8 J), suggesting that IAA28 may play a role in cell elongation or expansion. Since the

expression of IAA28-GUS in the shoot was not detectable due to its degradation, the developmental regulation of the mutant gene was studied using *IAA28$_{pro}$::iaa28-GUS* in mature Arabidopsis transgenic plants. As shown in figure 8, GUS staining was seen in vasculature of cauline leaves and rosette leaves, inflorescence axes, pedicels, anther filaments, stigmata and the septum of the siliques (Figure 8L, M, N and O).

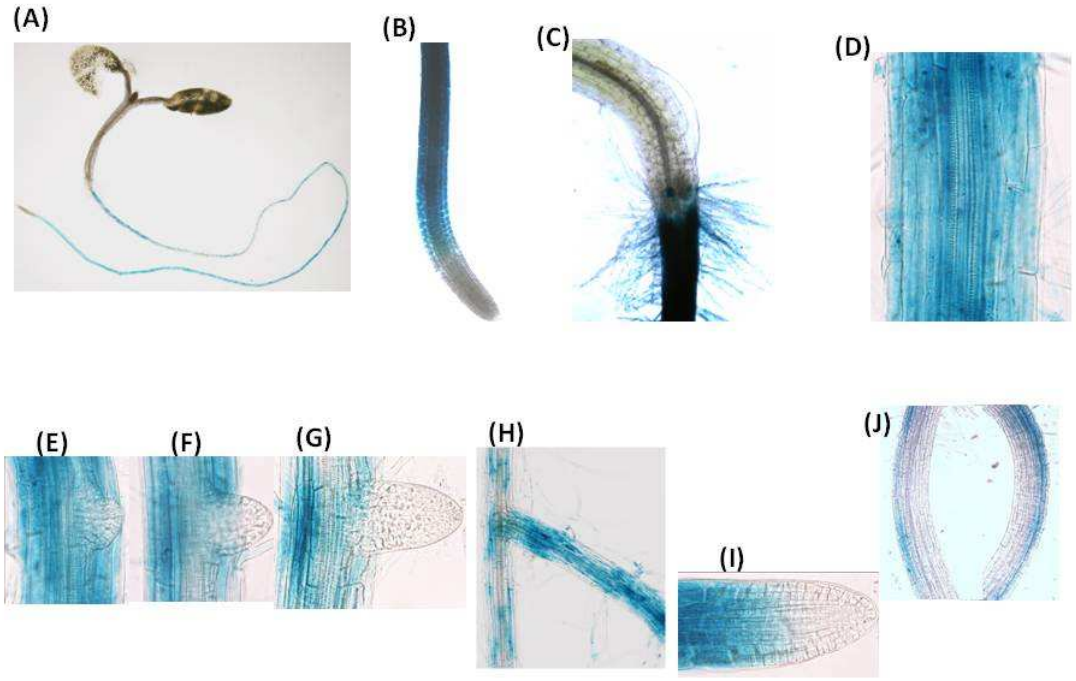
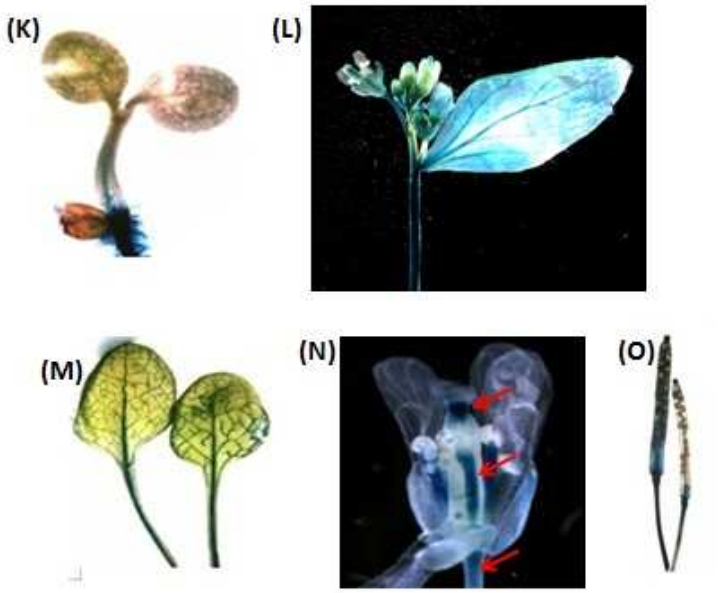


Figure 8. Expression of *IAA28* gene in Arabidopsis seedlings. Transgenic Arabidopsis seedlings homozygous for translational gene constructs, *IAA28*$_{pro}$*::IAA28-GUS* (A – J) or*IAA28*$_{pro}$*::iaa28-GUS* (K – O) containing a 3.1 kb *IAA28* 5' flanking region, were grown under continuous light at 22^{o}C and stained with 0.5 mg/ml X-Gluc. (A) GUS expression is found in the whole root except at the root tip. (B) GUS expression is visible at cell elongation and differentiation zones of the primary root tip, (C) but not in the hypocotyls. (D) GUS expression is found in the whole cytoplasm in the root cells, and (E) lateral root primordia, but (F and G) not in developing lateral root tips. (H) GUS expression in a mature lateral root and its (I) tip.(J) When roots were stained for a short time (30 min), GUS expression was mainly found at the extended side of curved lateral roots. (K) Expression of *IAA28*$_{pro}$*::iaa28-GUS* transgene in the hypocotyls, (L) cauline leaves and peduncle, (M) leaf vasculature, (N) pedicel, stamen filaments and style (marked with arrows), and (O) silique septum.

Figure 8. Continued.



3.A.ii. IAA28 localization

In order to examine the localization of IAA28 protein, transgenic seedlings carrying *IAA28$_{Pro}$::IAA28-GUS*and *IAA28$_{Pro}$::iaa28-GUS* together with *IAA7$_{Pro}$::IAA7-GUS* were fixed with GUS fixer and stained with GUS staining buffer as described in the methods section.

The results showed that IAA28-GUS is predominately localized to the cytoplasm in undifferentiated regions of the root, while IAA7 (AXR2)-GUS is localized to the nucleus (Figure 9A). To find out whether the relatively large C-terminal GUS sequence affects nuclear localization of IAA28-GUS fusion, we immunolocalized the IAA28-myc fusion protein, using the *IAA28$_{Pro}$::IAA28-myc* transgenic line. This fusion protein contains a relatively small C-terminal tag and has been described previously (Strader *et al.* 2008). When roots of wild type and *IAA28$_{Pro}$::IAA28-myc* seedlings were used for immunolocalization with anti-myc antibody and Alexa Fluor 488 conjugated to mouse IgG, IAA28-myc protein was detected in both the cytoplasm and the nucleus of *IAA28$_{Pro}$::IAA28-myc* root cells. Similarly treated cells of wild type seedlings did not cross react with the antibody (Figure 9B). This result suggests that IAA28 is localized to both the cytoplasm and the nucleus.

3.A.iii. IAA28 has a unique localization pattern in the root

To further investigate IAA28 localization IAA28-GUS expression was examined along the length of roots of *IAA28$_{pro}$::IAA28-GUS* seedlings. GUS staining appears in the basal area of cell elongation (Figure 9C and 9D), and in these cells IAA28-GUS is mainly found in the cytoplasm (Figure 9D,I and 9F). Just above this basal area, staining is still found predominantly in the cytoplasm (Figure 9G), but gradually GUS staining appears more in the nucleus in the cell differentiation zone (Figure 9D (II), (III) and 9E). Above the cell differentiation zone GUS staining is seen predominantly in the nucleus (Figure 9D (IV)). This GUS staining pattern does not depend on either duration of the staining procedure or *iaa28-2* mutation, as both IAA28-GUS and iaa28-2-GUS exhibit similar localization patterns (data not shown).

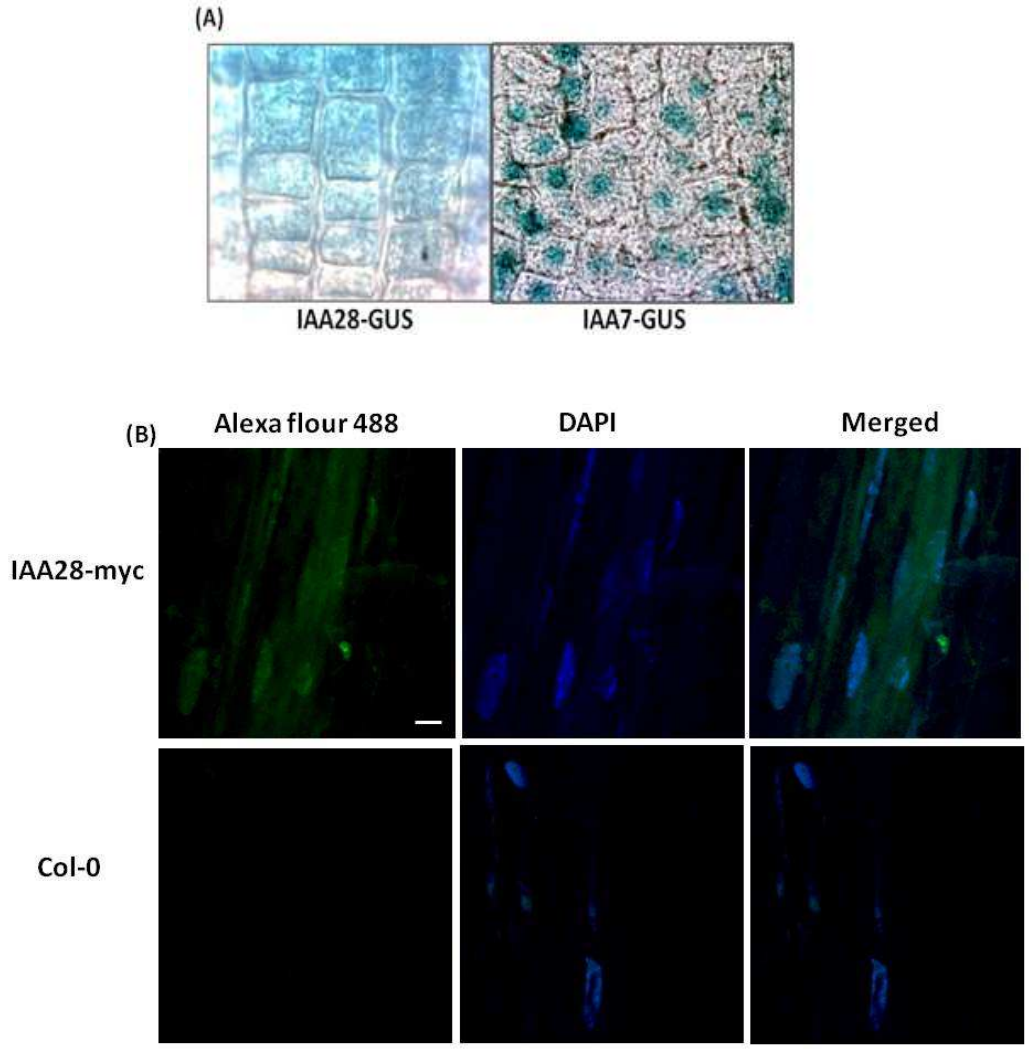
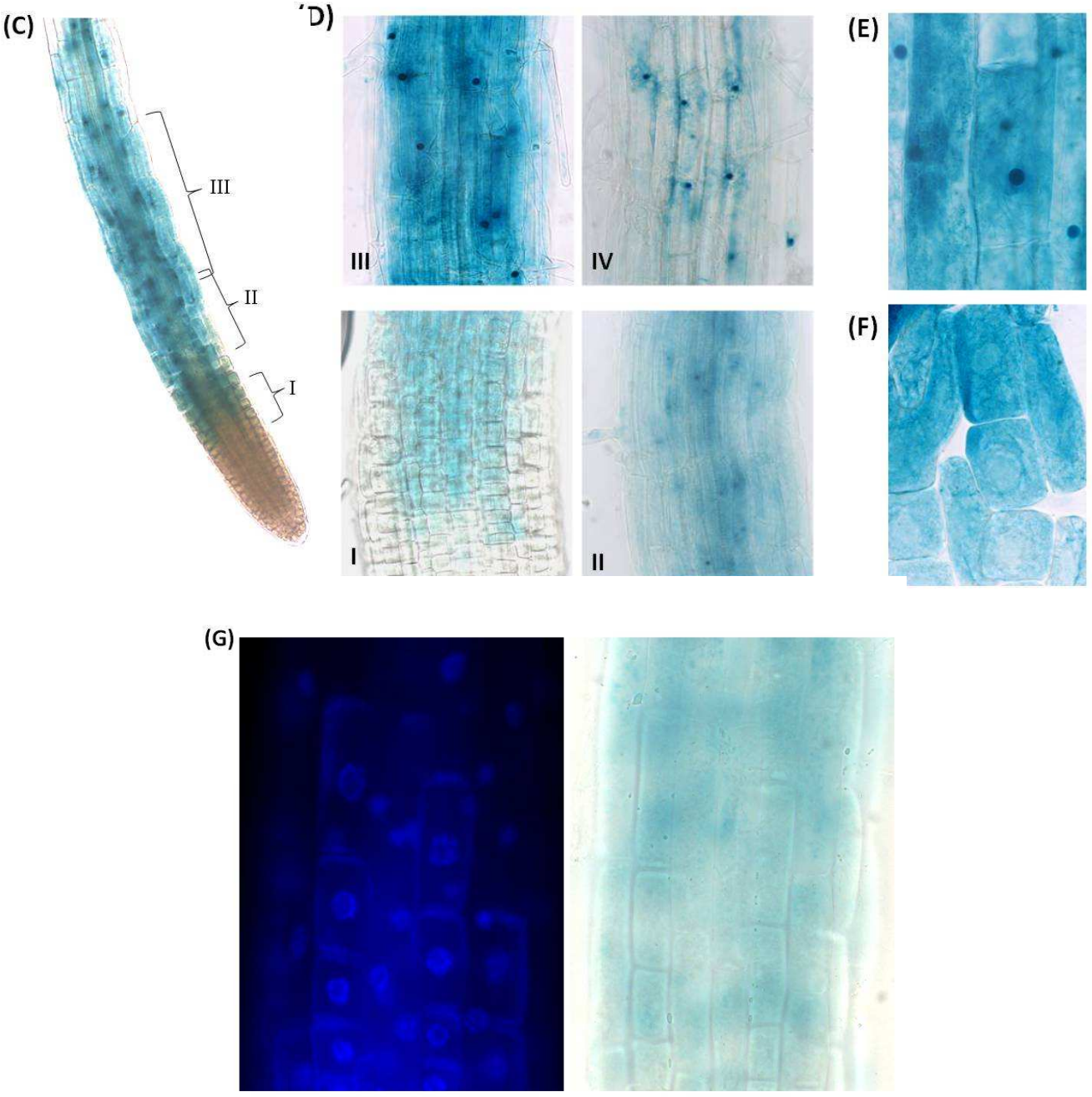


Figure 9: IAA28 is localized to both the nucleus and the cytoplasm. (A) Roots of 7 day-old transgenic homozygous seedlings of *IAA28$_{pro}$::IAA28-GUS* or *35S::AXR2(IAA7)-GUS* grown at 22^{o}C under continuous light were stained for GUS activity. (B) Confocal images of wild type (Col-0) or *IAA28$_{pro}$::IAA28-myc* (IAA28-myc).Whole mounts of roots immunostained with anti-myc primary antibody and anti-mouse IgG conjugated to AlexaFluor 488. Roots were also counter stained with DAPI (scale bar=20 μm). (C to G) The primary root of *IAA28$_{pro}$::IAA28-GUS* stained for GUS activity to show the expression and localization pattern of IAA28-GUS. (D) Localization of IAA28-GUS in different regions of the root. Cells were imaged using a light microscope (X400). Cells at the (I) basal area of cell elongation, (II) transition zone between cell elongation and cell differentiation, (III) differentiation zone and (IV) above the differentiation zone. (E) Enlarged view (X1000) of the cells in the differentiation zone, and (F) the basal area of the cell elongation zone. (G) IAA28-GUS localization in cell elongation region (right) and the same tissue counterstained with DAPI (left) (In this Figure IAA7-GUS (A-right) picture was taken by PK Kathare).

Figure 9. Continued.



3.B. IAA28 interactions with different auxin co-receptors

3.B.i. GST-IAA28 protein shows different affinities towards different auxin receptors

Several Aux/IAA proteins have been shown to interact with auxin receptor proteins TIR1, AFB1, AFB2, AFB3 (Gray *et al.* 2001; Dharmasiri N. *et al.* 2003; Dharmasiri N. *et al.* 2005; Kepinski and Leyser 2005; Parry *et al.* 2009) and AFB4 and AFB5 (Greenham *et al.* 2011). To study whether *iaa28-2* disrupts the interaction between IAA28 and auxin co-receptor F-box proteins, *in vitro* pull-down experiments were performed using bacterially expressed GST-IAA28 and GST-iaa28-2 recombinant proteins, and plant-derived TIR1-myc, AFB1-myc and AFB5-myc proteins. The pull-down results showed that GST-IAA28 protein interacted with TIR1-myc, AFB1-myc and AFB5-myc in the presence of 2,4-D or picloram in a concentration-dependent manner. However, auxin induced IAA28-TIR1 interaction is drastically diminished or abolished when *iaa28-2* mutation is introduced, indicating that the domain II mutation in IAA28 disrupts the auxin-induced interaction between mutant iaa28 and TIR1/AFB1/AFB5 (Figure 10A to E). *In vitro* pull down assays showed that GST-IAA28 interacted less with TIR1-myc protein compared to other tested Aux/IAAs, indicating IAA28-TIR1 interaction may be less efficient. In addition, auxin-induced interactions were compared between GST-IAA28 and GST-IAA7 withTIR1, AFB1 and AFB5. Results indicated that, compared to GST-IAA28, GST-IAA7 interacted more efficiently with both TIR1-myc and AFB1-myc in response to 2,4-D (Figure 10F and G). Nevertheless, both GST-IAA28 and GST-IAA7 interacted similarly with AFB5-myc in response to picloram (Figure10H), indicating that bacterially expressed GST-IAA28 and GST-IAA7 did not show qualitative differences in their general abilities to interact with F-box proteins. Thus, differential affinities of IAA28 and IAA7 toward TIR1 and AFB1 represent an actual difference between IAA28 and IAA7. These results confirmed that different Aux/IAA proteins possess different affinities towards different auxin co-receptor F-box proteins.

3.B.ii. Mutant GST-iaa28 interaction with AFB1 receptor protein in the presence of picloram

As mentioned previously, the domain II mutation in iaa28-2 drastically diminished the interaction with auxin co-receptor protein TIR1 in the presence of 2,4-D and picloram (Figure 10 A,B). Similarly, mutant GST-iaa28 does not interact with AFB5-myc in the presence of picloram (Figure 10 E). However, with AFB1 auxin co-receptor protein, the mutant GST-iaa28 interacted to some extent in a concentration-dependent manner with picloram treatments (Figure 10D), but it did not interact with the receptor in the presence of 2,4-D (Figure 10C).

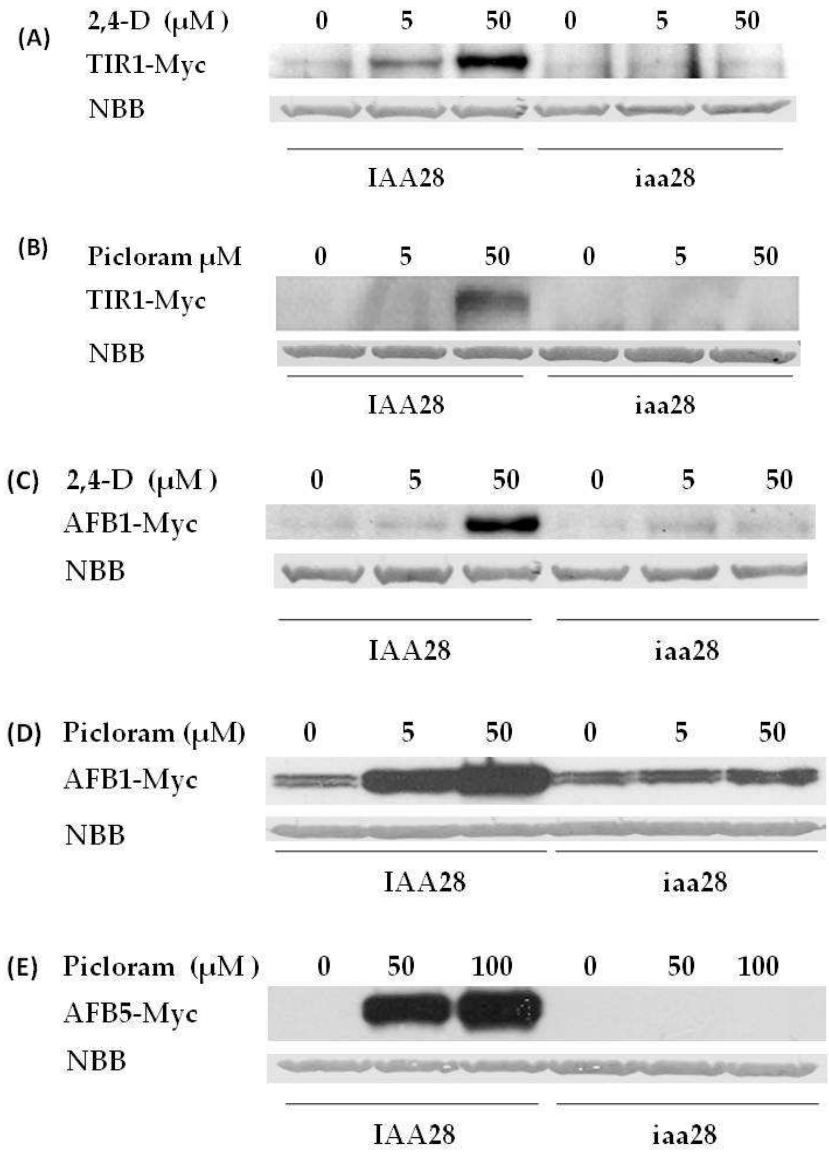
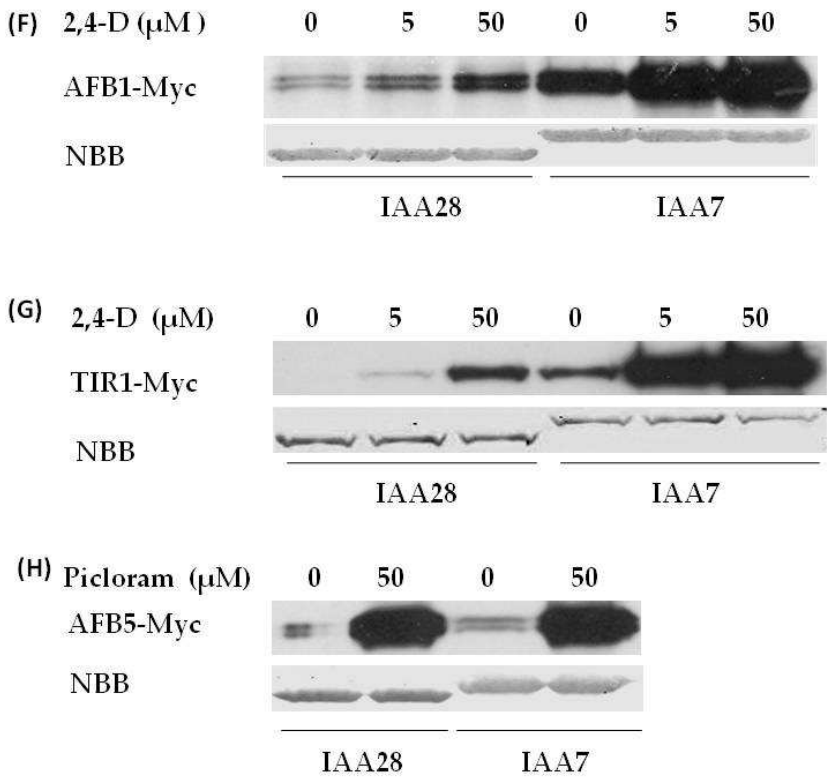


Figure 10. IAA28 interacts with TIR1, AFB1 and AFB5 proteins in an auxin-dependent manner while the *iaa28-2* mutation greatly affects this interaction. (A) Crude protein extracts from Arabidopsis seedlings expressing *TIR1-myc* (A, B), *AFB1-myc* (C, D) or *AFB5-myc* (E) were used in pull-down assays with GST-IAA28 or GST-iaa28 expressed and purified from *E. coli* in the presence of various concentrations of either 2,4-D or picloram as indicated. Auxins were directly added to the pull-down assay reaction. GST-IAA28 interacts less efficiently with both TIR1-myc (F) or AFB1-myc (G) compared to GST-IAA7 protein. (H) Both GST-IAA28 and GST-IAA7 interact with AFB5-myc with similar efficiency in response to picloram. (NBB; napthol blue black)

Figure 10. Continued.



3.B.iii. Degradation of IAA28-GUS and iaa28-GUS proteins in the roots in response to different auxins

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). Previous work has shown that domain II mutations stabilize mutated Aux/IAA proteins (Reed 2001; Mockaitis and Estelle 2008). To determine how the *iaa28-2* mutation affects its own degradation in response to auxin, the degradation patterns of IAA28-GUS and iaa28-2-GUS fusion proteins were compared using 4 day-old homozygous transgenic lines of *$IAA28_{Pro}$::IAA28-GUS* and *$IAA28_{Pro}$::iaa28-2-GUS.*Five independent lines for each gene construct were observed. When transgenic seedlings were treated with auxins and stained for GUS activity, the level of GUS staining in IAA28-GUS lines was considerably lower than that of iaa28-2-GUS lines at 0 time point (data not shown), suggesting that IAA28-GUS recombinant protein may be subjected to proteasome-mediated degradation. To test whether this difference could be due to transcriptional differences of *$IAA28_{Pro}$::IAA28-GUS* and *$IAA28_{Pro}$::iaa28-2-GUS*transgenic lines, the relative *IAA28-GUS* and *iaa28-GUS*transcript levels in two, randomly selected, independent lines were measured using RT-PCR (Figure 11B). According to RT-PCR results, even though the *iaa28-GUS* transcript level is slightly lower than that of *IAA28-GUS*, the level of iaa28-GUS protein is higher than that of IAA28-GUS, suggesting that iaa28-GUS protein is less affected by proteasome-mediated degradation. Confirming this possibility, exogenous auxin treatments for 1 hour further accelerated the degradation of IAA28-GUS while iaa28-2-GUS protein was less affected, based on both histochemical staining (Figure 11A) and quantitative MUG assay (Figure 11C), indicating that the mutant iaa28 protein is stabilized.

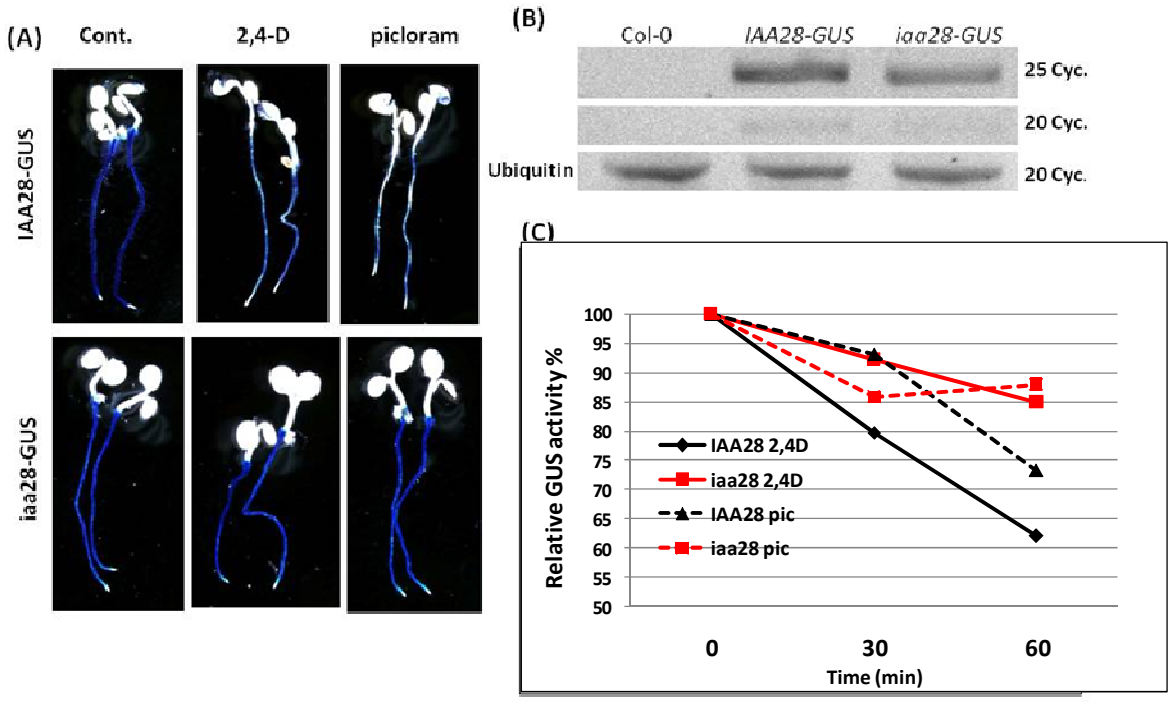


Figure 11. The *iaa28-2* mutation stabilizes mutant iaa28 protein. (A) Homozygous transgenic lines of *IAA28*$_{pro}$*::IAA28-GUS* and *IAA28*$_{pro}$*::iaa28-GUS* were treated with 20 µM 2,4-D, 50 µMpicloram or a similar volume of DMSO (control) for 1 hour and stained for GUS activity with 0.5 mg/ml X-gluc. Similar results were observed in 5 independent transgenic lines of each construct. Results are given for one independent line from each transgenic construct. (B) Relative expression of *IAA28::IAA28-GUS* and *IAA28:: iaa28-GUS* transcripts were measured by RT-PCR (done by Dr. S.Dharmasiri). (C) Quantitative GUS activity in*IAA28*$_{pro}$*::IAA28-GUS* and *IAA28*$_{pro}$*::iaa28-GUS* seedlings treated with 20 µM 2,4-D or 50 µM picloram. Data indicate mean values of three biological replicates. GUS expression values of each transgenic line at 0 time was considered as 100%. The relative GUS activity values used in graph C are shown in appendix V (MUG assay was done by T.D. Jayaweera).

3.B.iv. Effects of *iaa28-2* on auxin responsive gene expression (*DR5::GFP*)

Auxin-induced gene expression in *iaa28-2*was studied using *DR5::GFP* transgenic line. The homozygous mutant *iaa28-2* was crossed to *DR5::GFP* transgenic line and F3 seedlings that were homozygous for both the mutant and the *DR5::GFP* transgene were selected. When these seedlings were treated with 2,4-D, no difference in DR5::GFP expression was observed between wild type and mutant root tip area (Figure 13B-c,d,h and i). However, in wild type seedlings, *DR5::GFP* expression was increased in the cell elongation region in response to 2,4-D, but this enhanced *DR5::GFP* expression was absent in *iaa28-2* seedlings (Figure 13B-a,b,f and g), suggesting that auxin-induced gene expression is defective in this mutant. When wild type seedlings were treated with picloram, there was a steep increase of *DR5::GFP* expression at the root tip, especially at the lateral root cap. This enhanced GFP expression was not apparent in the *iaa28-2* mutant (Figure 13B-c, e, h and j).

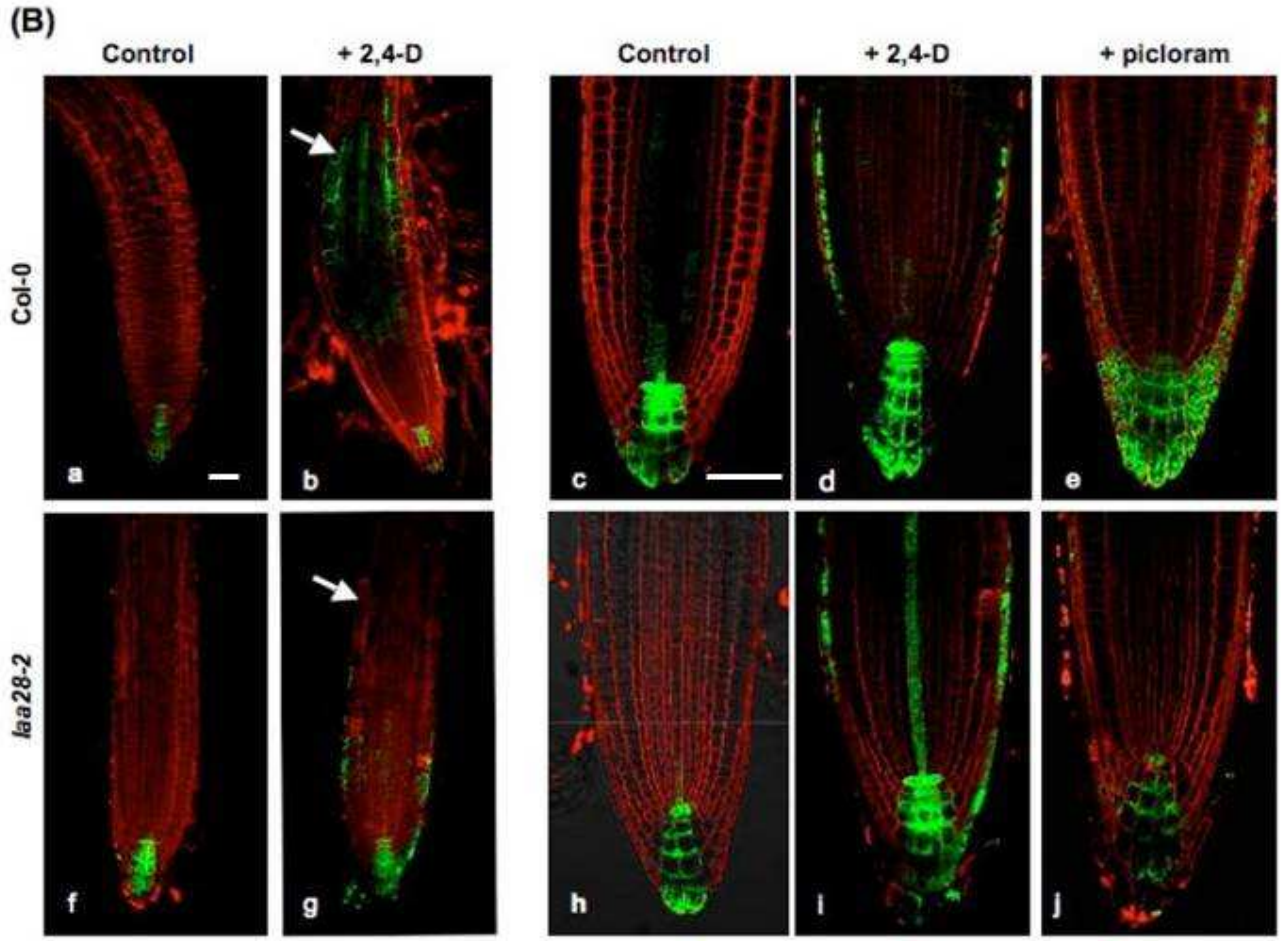


Figure 12: Expression of *DR5::GFP* is defective in *iaa28-2* mutant seedlings. Four day-old wild type (a,b,c,d,e) or *iaa28-2* (f,g,h,i,j) seedlings carrying *DR5::GFP* transgene were transferred onto ATS containing 85 nM 2,4-D (b,d,g,i) or 10 µM (e,j) picloram for 18 hours, stained with 1 µM synaptored for 10 minutes and observed under confocal microscopy. 2,4-D induces *DR5::GFP* expression in elongation zone of the primary root in wild type (b - marked with an arrow) but not in *iaa28-2* (g). While 2,4-D induces *DR5::GFP* expression in root tips of both wild type (d) and *iaa28-2* mutants (i), picloram-induced expression is only apparent in wild type (e, j).(Dr. S. Dharmasiri assisted acquiring these confocal images).

3.C. Role of IAA28 in lateral root formation

3.C. i. Mutant *iaa28-2* shows defects in lateral root formation

Application of exogenous auxin stimulates lateral root formation in Arabidopsis (Laskowski *et al.* 1995). Therefore, to determine whether exogenous auxin induces lateral root development in*iaa28-2*, four day-old seedlings were treated with 85 nM 2,4-D or 10 µM picloram. Four days after the treatment, both auxin treatments induced many lateral roots in wild type seedlings, but not in *iaa28-2* mutants. However, picloram induced a few secondary roots in *iaa28-2*with a characteristic pattern (Figure 13A, B and C). For example, *iaa28-2* seedlings developed a cluster of roots at the base of the hypocotyl region suggesting that picloram induces adventitious root formation in *iaa28-2* (Figure 13C and D) (Karunarathna 2008). To test whether this is a common feature in both mutant alleles of IAA28, the adventitious root development in *iaa28-1* mutant was also examined with picloram treatment. Similar to *iaa28-2*, picloram induced adventitious roots in *iaa28-1* (data not shown). Although *iaa28-2* is defective in auxin-induced lateral root development, several auxins such as IBA (Figure 13E and G), IAA (Figure 13F) and very high concentrations of 2,4-D (data not shown) induced lateral root primordia in *iaa28-2*, indicating that the *iaa28-2* mutant does not completely lack the ability to develop lateral roots (Karunarathna 2008).

The *CycB::GUS* marker has been used in previous studies to monitor the early cell divisions that initiate lateral root primordia (Ferreira *et al.* 1994; Smith and Fedoroff 1995). To study the characteristic pattern of secondary root induction in *iaa28-2* in response to picloram, *iaa28-2* mutants were crossed into the *CycB::GUS* transgenic line and F2 plants that were homozygous for both*iaa28-2* mutation and *CycB::GUS* transgene were identified. When both wild type *CycB::GUS* and *iaa28-2 CycB::GUS* seedlings were treated with10 µM picloram, increased GUS expression was mainly observed at the base of the hypocotyls in *iaa28-2* compared to the wild type (Figure 13H).This result corroborates the inference that secondary roots in *iaa28-2* actually arise from the base of the hypocotyl, but not from the primary root, indicating that picloram preferentially induces adventitious roots in *iaa28*.

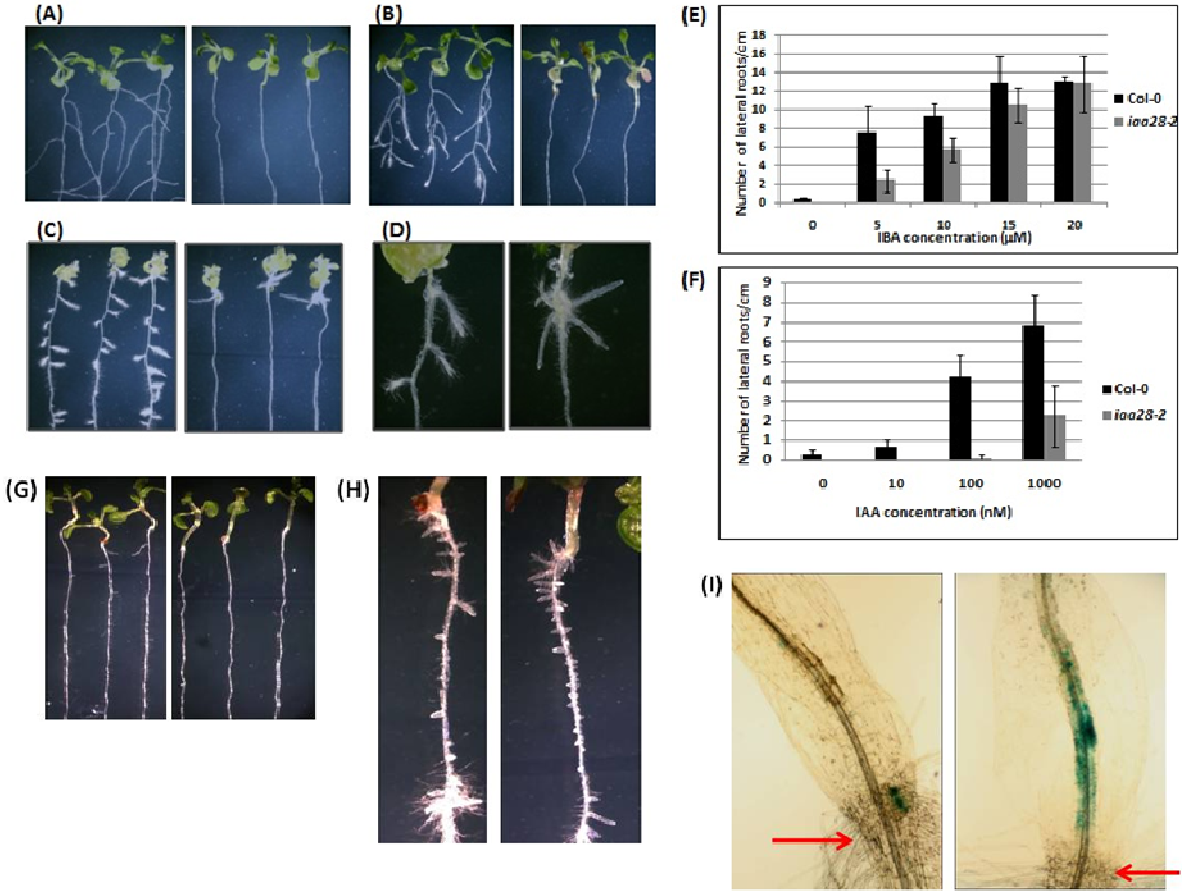


Figure 13: Auxins induce lateral roots in *iaa28-2*. (A) Wild type (left) and *iaa28-2* (right) seedlings grown on ATS medium, or ATS medium with (B) 85 nM 2,4-D or (C) 10 µM picloram. Wild type (left) develops many short lateral roots in response to 10 µM picloram while (C) *iaa28-2* produces a small number of secondary roots at the base of the hypocotyls. (D) Magnified image of secondary roots produced by wild type (left) and *iaa28-2* (right). Both IBA (E) and IAA (F) induce lateral root development in *iaa28-2*, but in the latter case only at very high concentrations. Four day-old wild type Col-0 or *iaa28-2* seedlings were transferred on to ATS media containing various concentrations of IBA or IAA as indicated. Number of lateral roots per cm of the primary root was counted 6 days after transfer on to the media with auxins. Each value represents the mean value of at least 10 seedlings. Error bars indicate standard deviation. (G) Eight day-old wild type (left) and *iaa28-2* (right) seedlings grown on ATS or (H) ATS with 10 µM IBA. (I) Expression of *CycB::GUS* in wild type (left) and *iaa28-2* (right) in response to picloram was observed using compound microscope (The *CyclinB::GUS* experiment was conducted by NK and images were acquired by P. Kathare). Seedlings were grown on ATS medium with 10 µM picloram for four days at 22^{o}C under continuous light and stained with 0.5 mg/ml X-Gluc overnight at 37^{o}C. The root-hypocotyl junction is indicated with an arrow. (In this Figure, A, B, C, D, G & H were directly taken from the MS-Thesis of Karunarathna N. 2008).

3.C.ii. Lateral root development in *iaa28-2*mutants stops at stage III

The emergence of lateral roots can be divided into 8 main stages (Peret *et al*. 2009). Lateral roots are initiated when either an individual or a pair of pericycle cells undergo several rounds of anticlinal divisions to create single layered primordia composed of up to ten small cells of equal length (stage I). Next, the cells divide periclinally, forming an inner and outer layer (stage II). Further anticlinal and periclinal divisions create a dome-shaped primordium (stage III to stage VII) that eventually emerges (stage VIII) from the parent root (Figure 14).

As mentioned previously, studies have shown that *J0121* (an Arabidopsis GAL4-GFP enhancer trap line/pericycle marker) can be used to track the divisions of the pericycle cells during different stages of lateral root development (Laplaze *et al.* 2005). To examine which stage/stages of lateral root development is affected by *iaa28-2* mutation, the mutant plants were cross-pollinated with *J0121* lines and the lines homozygous for both genes were selected as described in the methods. The expression of pericycle marker *J0121*was reduced in the differentiation and distal differentiation region of *iaa28-2*compared to wild type. With picloram treatment the expression was increased in *iaa28-2* mutants similar to that of wild type (Figure 15). Further, *iaa28-2* mutants demonstrated the capability to initiate the lateral root development through dividing the pericycle cells anticlinally and periclinally up to stage III; however, the divided cells did not undergo differentiation through stage IV to VIII showing the main defects may be in the emergence stages but not in the initiation stage (Figure 15, I to M).

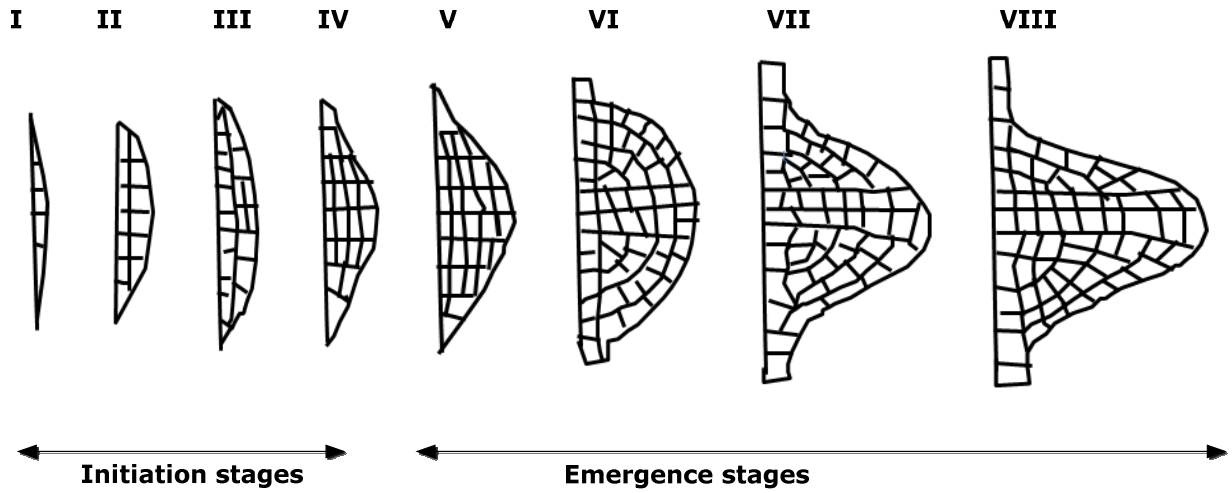


Figure 14: **Morphological changes during lateral root development.** Lateral root development can be divided into eight different stages. In these different stages programmed anticlinal and periclinal cell divisions occur while the lateral root primordia gradually enlarge and emerge through the cortical cells in the root.

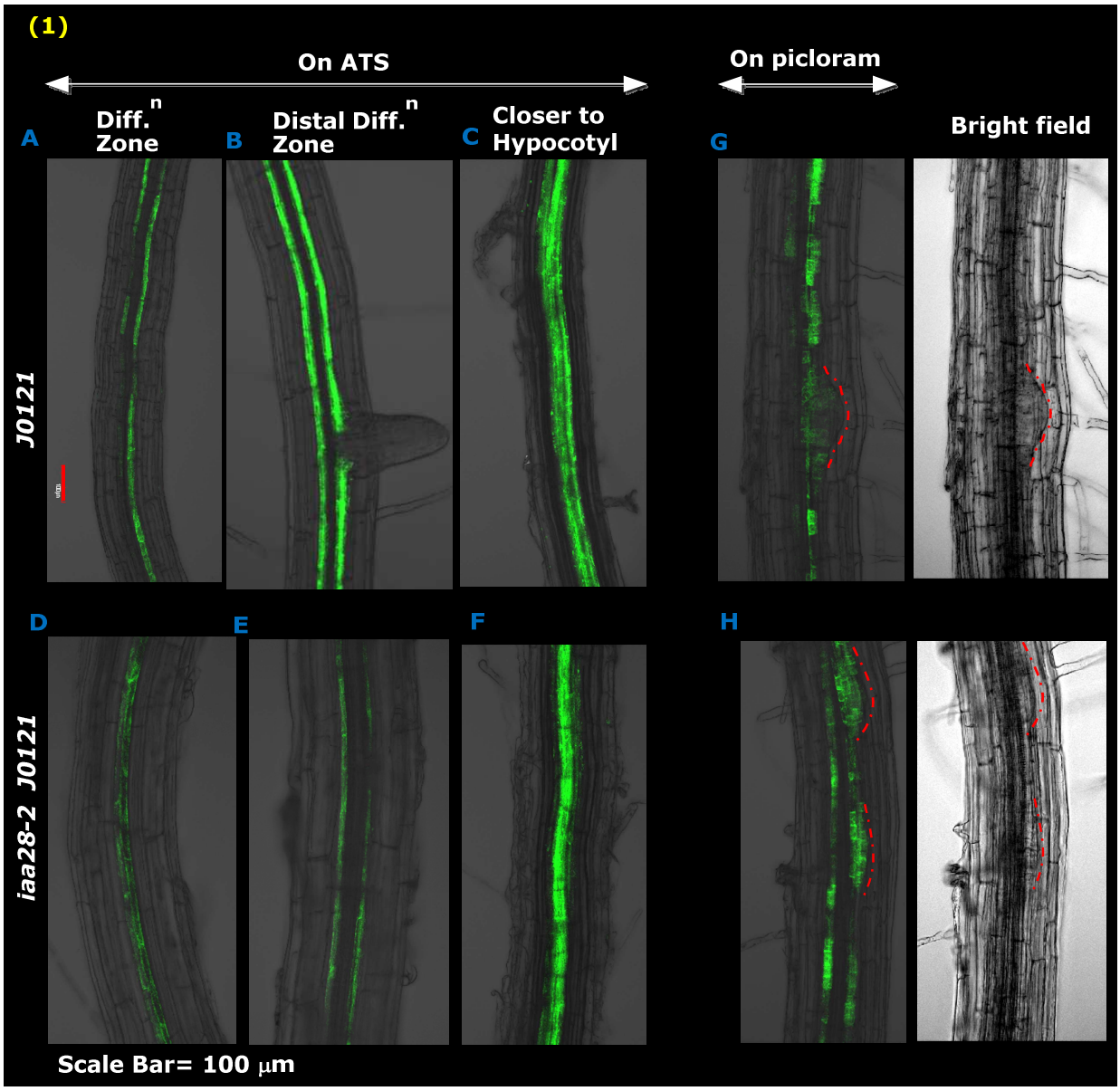
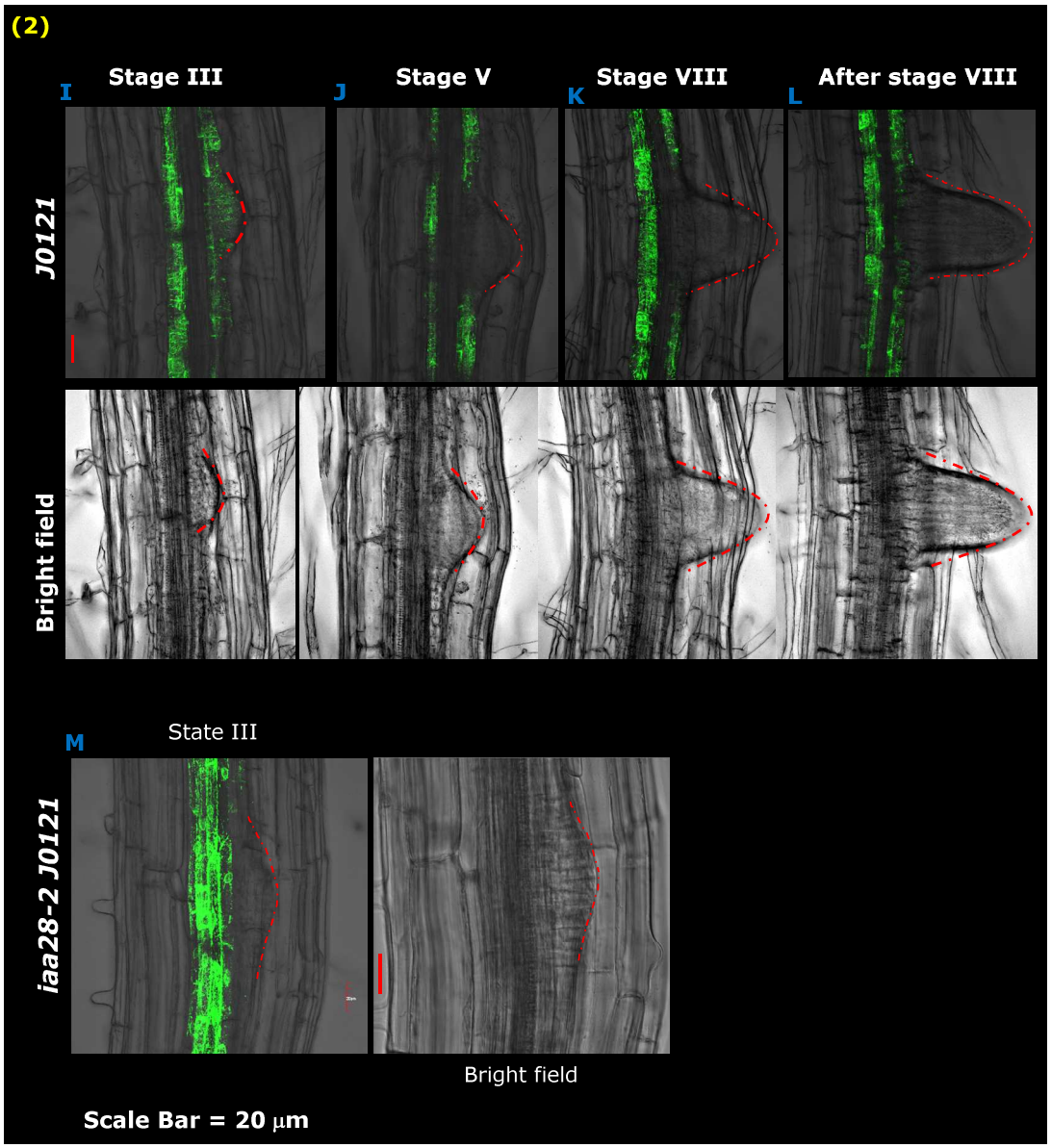


Figure 15: (1) Expression of *J0121* pericycle marker in *iaa28-2* background. (A to C) The expression of *J0121* pericycle marker in wild type roots was observed in the differentiation and distal differentiation zone as well as in the area closer to the hypocotyl. (D to F) The expression of *J0121* was lower in the differentiation zone and distal differentiation zone of *iaa28-2* mutant background compared to the wild type. (C and F) However, the expression of *J0121* is similar in both *iaa28-2* and wild type in the part of the roots closer to the hypocotyls. (G and H) The expression of *J0121* in WT and *iaa28-2* mutant plants treated with picloram was observed. In both WT and *iaa28-2* mutants, picloram induced formation of the lateral root primordia. **(2) *iaa28-2*mutants initiate lateral root development, but development stops at stage III**. (I to L) Different stages of lateral root development were observed in wild type seedlings in response to picloram treatment. (E and F) *iaa28-2* mutants exhibited up to stage III of lateral root development in response to picloram

Figure 15. Continued.



3.C.iii. Alteration of *LBD* gene expression in an *iaa28-2* background

As mentioned in the introduction, *LBD* genes, such as *LBD16, LBD29 and LBD18,* act as downstream components in lateral and adventitious root formation (Okushima *et al.* 2007; Lee *et al.* 2009). In order to examine the effect of *iaa28-2* mutation on LBD gene expression, RT-PCR experiments and expression analysis using translational gene constructs of the three *LBD* genes were conducted with different auxin treatments.

For RT-PCR experiments, seven day-old Col-0 and *iaa28-2* seedlings were treated with either 85 nM 2,4-D or10 µM picloram for one hour. RNA from seedlings was extracted and cDNA was synthesized as mentioned in methods section. The RT-PCR results showed that for all three *LBD* genes (*LBD16, 18& 29*) transcript levels were lower in an *iaa28-2* background compared to the wild type (Figure 16). However with 2,4-D and picloram treatments all the *LBD* genes were induced in the mutant background to a similar level as of Col-0.

As entire seedlings were used in the RT-PCR experiments, the results did not indicate the effects of *iaa28-2* on the spatial expression of each *LBD* gene. Therefore, the expression patterns of each *LBD* gene in *iaa28-2* background were studied using the seedlings that were homozygous for both *iaa28-2* and each of the *LBD* gene constructs (e.g. $LBD16_{Pro}$*::LBD16-GUS* and $LBD29_{Pro}$*::LBD29-GUS*).

Seedlings were first grown on ATS media and transferred to control (ATS), 10 µM picloram, 85 nM 2,4-D and 15 µM IBA for 48 hours. Seedlings were then stained with GUS staining buffer as described in methods. The results showed that the expression of *LBD16-GUS* in the hypocotyl was similar in both wild type and *iaa28-2* mutants under control conditions (Figure 17, A1 and A2). In response to auxins *LBD16-GUS* expression was increased in both wild type and iaa28-2 backgrounds to a similar level except with picloram treatment (Figure C1, C2 and D1, D2). In response to picloram, expression of *LBD16* was highly induced in *iaa28-2* background (Figure 17 C1, C2 and E).

LBD16-GUS protein was detected in the root stele mainly in the differentiation and distal differentiation region of the wild type. However, LBD16-GUS was not

detected in any part of the *iaa28-2* mutant roots, indicating that the *iaa28-2* mutation severely affects the expression of *LBD16-GUS* gene (Figure 18 A1 and A2).With picloram treatments, a clear induction of LBD16-GUS protein was detected in the root elongation region, but not in any other regions in the wild type. However, even with picloram treatments, *LBD16-GUS* expression was not detectable in the elongation zone of *iaa28-2* mutants. The expression of *LBD16-GUS* was induced in the distal differentiation region of *iaa28-2* mutant roots with picloram treatments (Figure 18 B1 and B2).

At low concentrations of 2,4-D, the level of LBD16-GUS was increased in the wild type root elongation zone, differentiation zone and distal differentiation region (Figure 18 C1 and C2). In contrast, LBD16-GUS was not detected in any region of the mutant roots with mild 2,4-D treatments (Figure 18 C2). However, with high concentrations of 2,4-D treatments (~ 5µM), the LBD16-GUS protein was induced in the mutant to a similar level as in the wild type (Figure E1 and E2).

With IBA treatments, the level of LBD16-GUS protein was increased significantly in the root differentiation region and in the distal differentiation region of root but not in the elongation region or in the tip area. Similarly, LBD16-GUS was increased in both differentiation and distal differentiation regions of *iaa28-2* mutants in response to IBA treatment (Figure 18 D1 and D2).

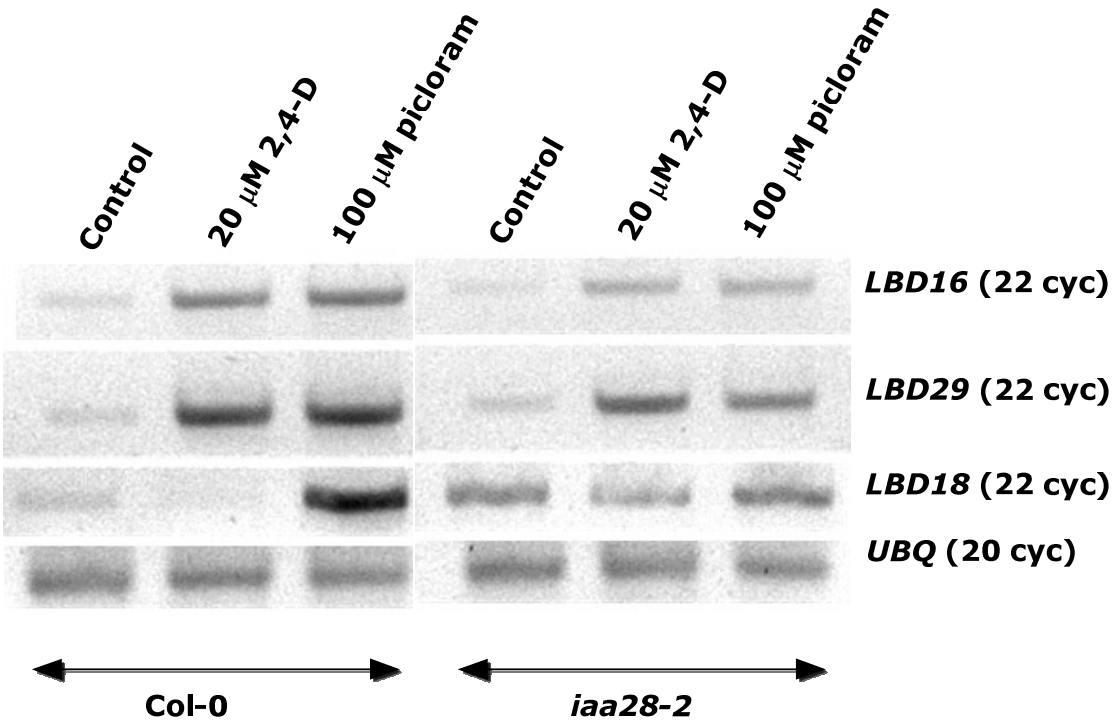


Figure 16: Transcriptional regulation of different *LBD* genes in *iaa28-2* background in response to auxin treatments. Seven day-old Col-0 and *iaa28-2* seedlings grown on ATS were treated with 20 µM 2,4-D or100 µM picloram for 1 hour, and tissues were collected and frozen quickly with liquid nitrogen. The total RNA was extracted and RT-PCR was carried out as described in methods. The transcript levels of *LBD16* and *LBD29* were low in *iaa28-2* mutant controls compared to that of Col-0. However, both 2,4-D and picloram were able to increase the transcript levels of both *LBD16* and *LBD29* in mutant background to a similar level as in Col-0. *LBD18* is constitutively expressed in *iaa28-2* mutants; however it is low in Col-0 controls. Only picloram treatment was able to induce the level of *LBD18* in Col-0 compared to control treatment.

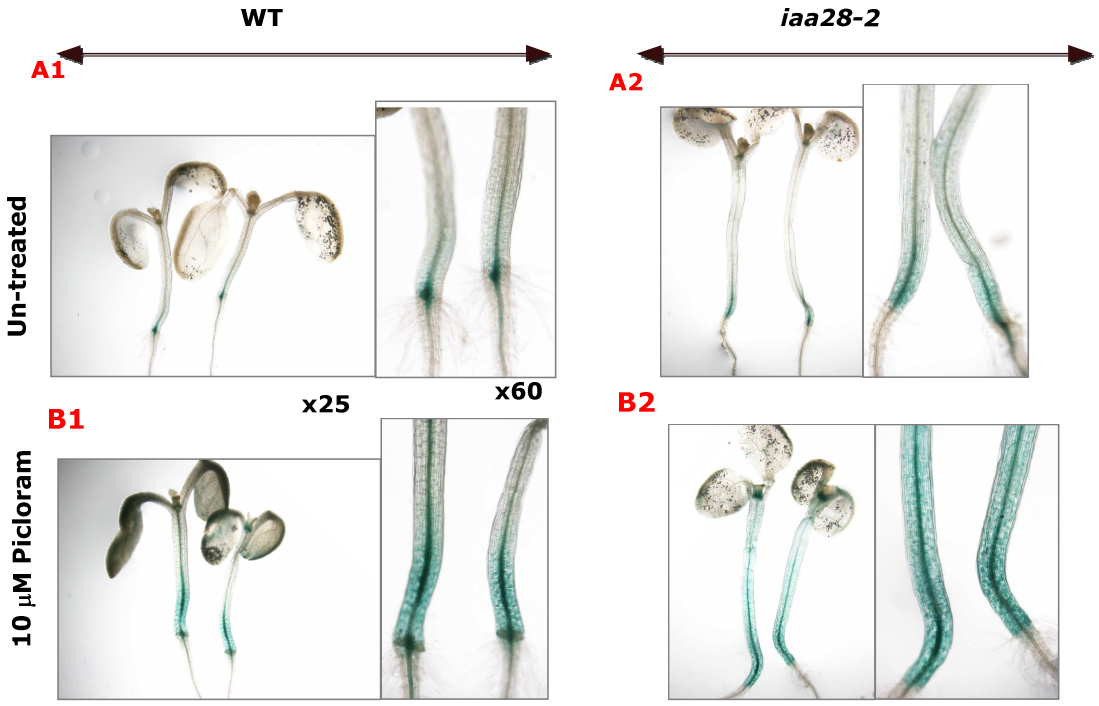
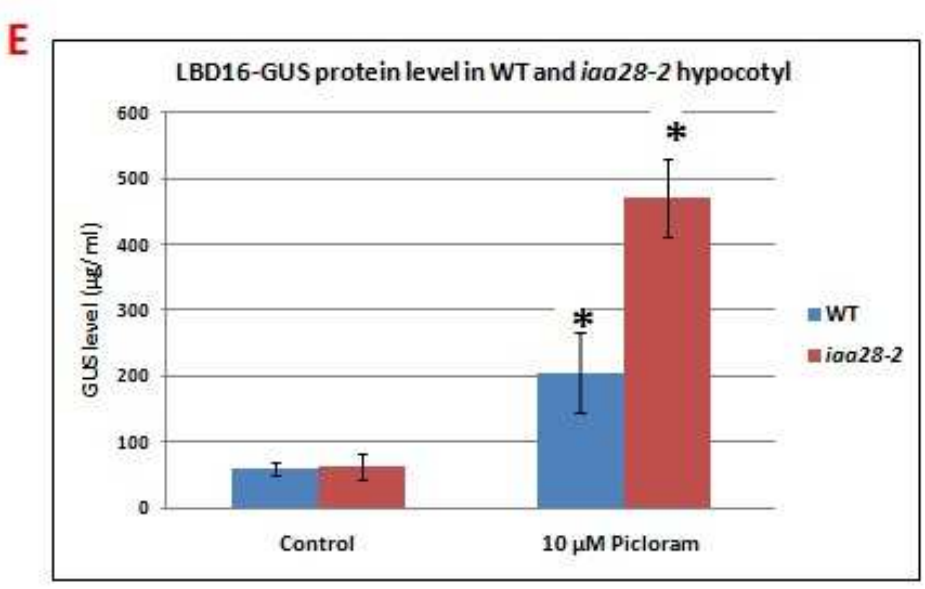


Figure 17: *LBD16$_{Pro}$::LBD16-GUS* expression in *iaa28-2* background. Four day-old seedlings of wild type or *iaa28-2* carrying *LBD16$_{Pro}$::LBD16-GUS* were transferred onto control (ATS) or ATS media containing 10 µM picloram, 85 nM 2,4-D or 15 µM IBA and incubated 48 hours. The seedlings were fixed and stained with GUS staining buffer. (A1 and A2) Both wild type and *iaa28-2* mutants had similar level of LBD16-GUS in the hypocotyls under control conditions. (B1 and B2) The level of LBD16-GUS was increased in both WT and *iaa28-2* mutant hypocotyls with picloram treatment; however, the induction was relatively higher in the *iaa28-2* background.(E) Quantitative analysis of LBD16-GUS protein levels in hypocotyl of wild type and *iaa28-2* with or without picloram. There is no difference in the level of LBD16-GUS between wild type and the mutant under control condition. With picloram, *iaa28-2* hypocotyl had a significantly higher amount of LBD16-GUS compared to the wild type ($P < 0.05$, Student's T-test). The level of LBD16-GUS increased slightly in both wild type and mutants with 2,4-D (C1, C2) and IBA (D1,D2) treatments.

Figure 17. Continued.



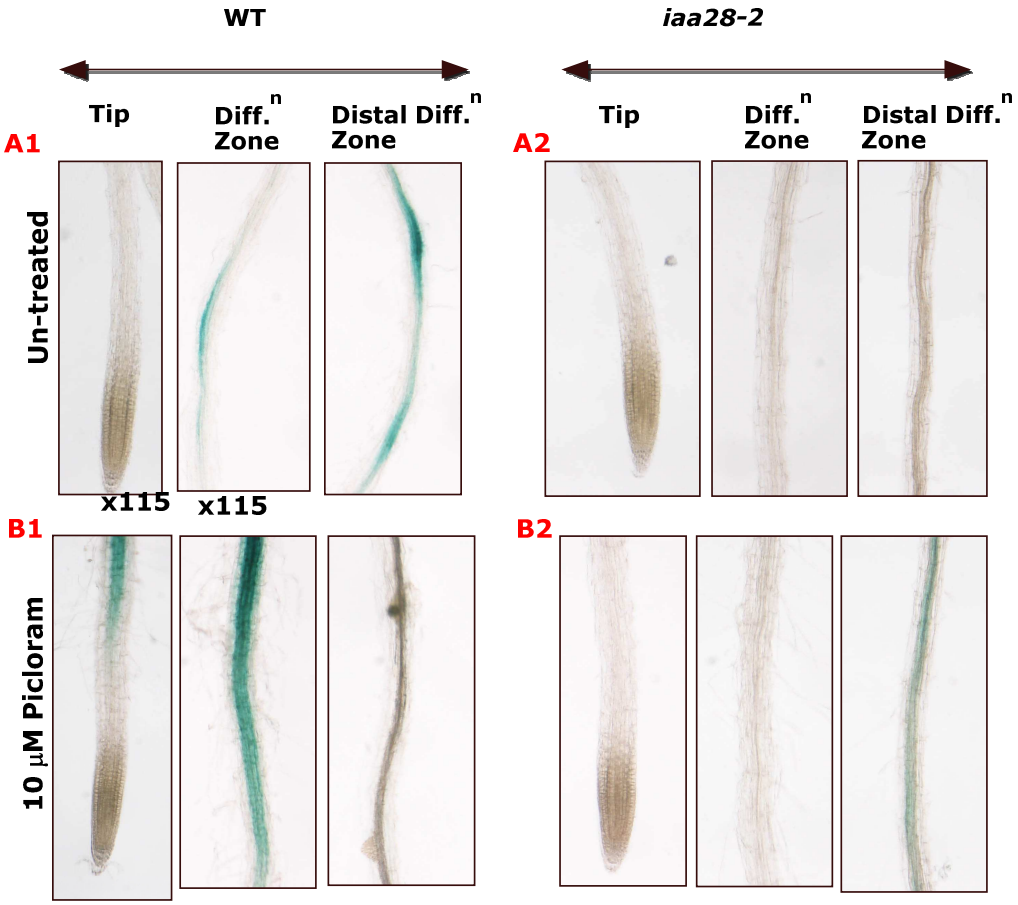
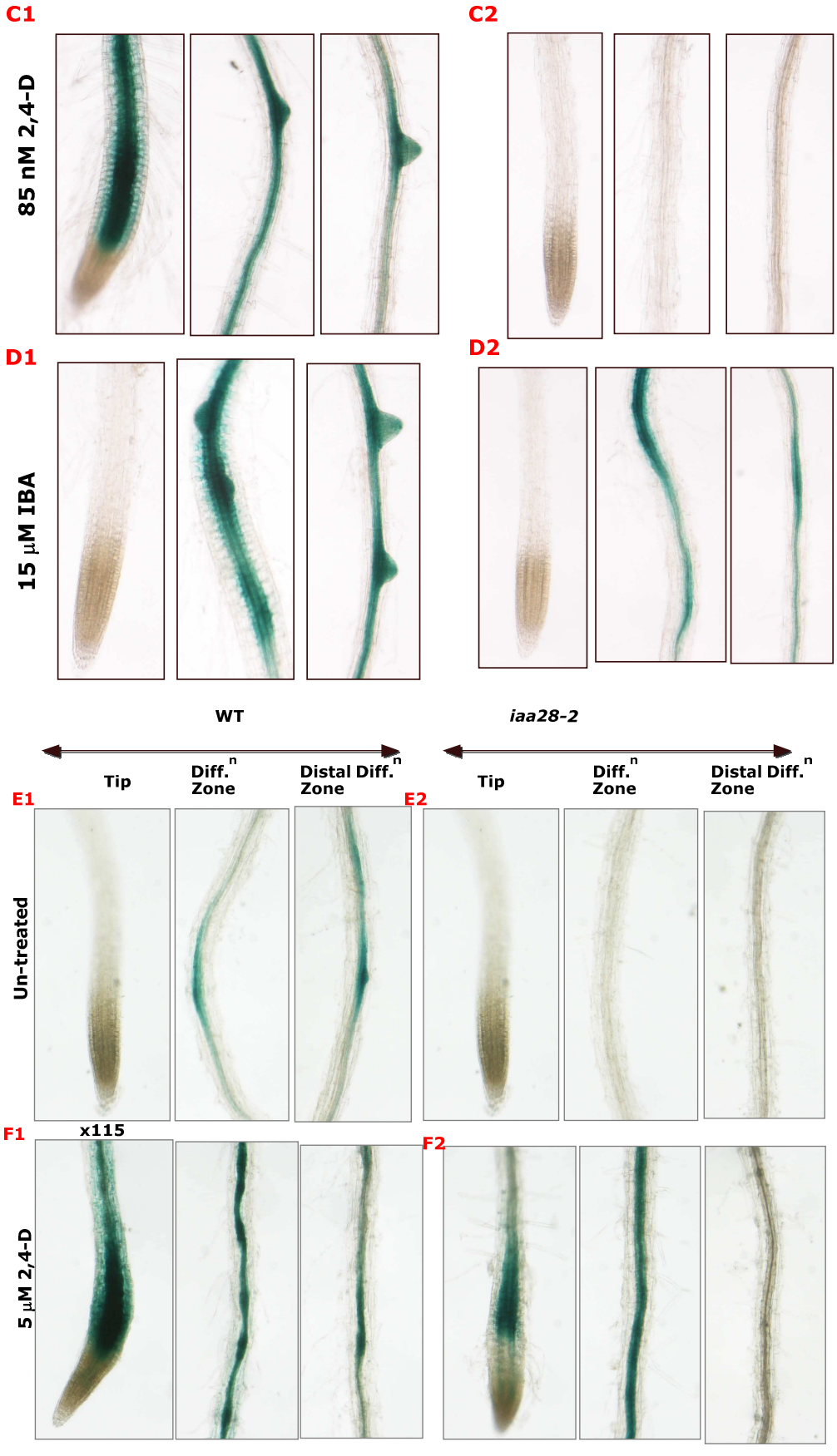


Figure 18: *LBD16-GUS* expression in wild type and *iaa28-2* mutant roots with different auxin treatments. Four-day old seedlings of wild type and *iaa28-2* carrying *LBD16*$_{Pro}$*::LBD16-GUS* were transferred onto ATS media containing 10 µM picloram, 85 nM 2,4-D or 15 µM IBA and incubated for 2 days (A to D) or 1 day (E to F). The seedlings were fixed and stained with GUS staining buffer. (A1 and A2). *LBD16-GUS* was mainly expressed in differentiation and distal differentiation zone of wild type seedlings; however, LBD16-GUS was not detected in any region of the mutant roots. (B1 and B2) With picloram treatments LBD16-GUS protein level was increased in the cell differentiation zone of wild type, but in mutants the protein was increased slightly in the distal differentiation region of the root. (C1 and C2) With 2,4-D treatments, the level of LBD16-GUS was increased throughout the wild type root except in the root meristem area, but this induction was completely absent in the *iaa28-2* mutant background. (D1 and D2)With IBA treatment, LBD16-GUS level was increased in the differentiation and distal differentiation region of both wild type and mutant seedlings; however, the level of LBD16-GUS was less in the mutant roots compared to the level in wild type. (E and F) LBD16-GUS expression increases similarly in mutant roots to wild type with 5µM 2,4-D treatments (Magnification for all the images is 115X).

Figure 18. Continued.



Homozygous lines for both *iaa28-2* mutation and *LBD29$_{Pro}$::LBD29-GUS* transgene were constructed as described in the methods. The expression of *LBD29-GUS* in *iaa28-2* mutant background was compared with the wild type in the presence of different auxin treatments in a similar way that was explained previously for *LBD16$_{Pro}$::LBD16-GUS*.

LBD29-GUS protein was not detected in either wild type or *iaa28-2* mutant hypocotyls under control growth medium (Figure 19 A1, A2). However, with both picloram and 2,4-D treatments, the level of LBD29-GUS protein increased slightly in the wild type hypocotyls but not in the mutant hypocotyl (Figure 19, B1,B2, C1, C2). The expression of LBD29-GUS was not detected in *iaa28-2* hypocotyl with any of the auxin treatments tested (Figure 19 A2, B2, C2 and D2). LBD29-GUS expression was induced in wild type hypocotyls in response to IBA but to a lesser extent than 2,4-D or picloram (Figure 19 D1).

In the wild type, LBD29-GUS protein was expressed in the stele of distal differentiation region but was absent in both differentiation and the root tip area. LBD29-GUS was not detected in any part of the *iaa28-2* mutant roots. With picloram and IBA treatments the induction of *LBD29-GUS* was observed in differentiation and distal differentiation region of wild type roots; however, in *iaa28-2* mutants the induction was only observed in the distal differentiation region (Figure 20 A1, A2, B1, B2, D1 and D2). In wild type seedlings, 2,4-D treatment increased the level of LBD29-GUS throughout the root except in the apical meristem (Figure 20, C1, C2). Similarly in the mutant background, the level of LBD29-GUS was increased throughout the root.

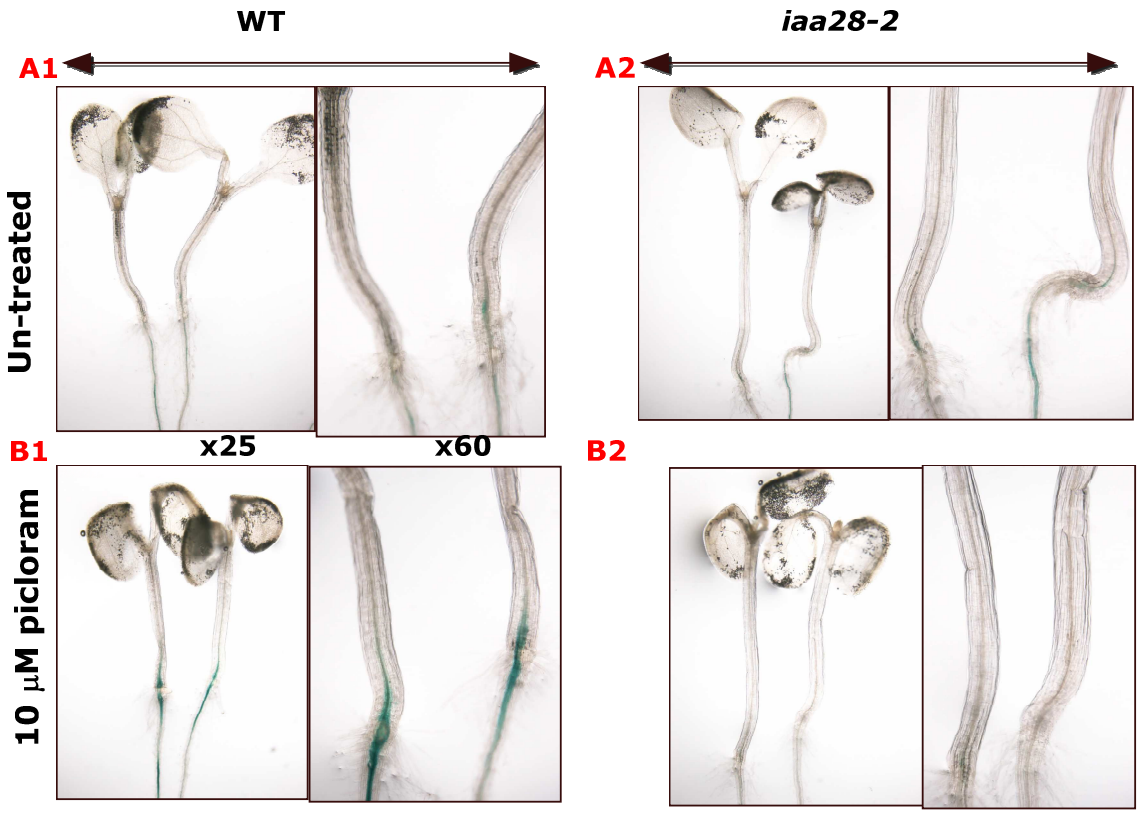
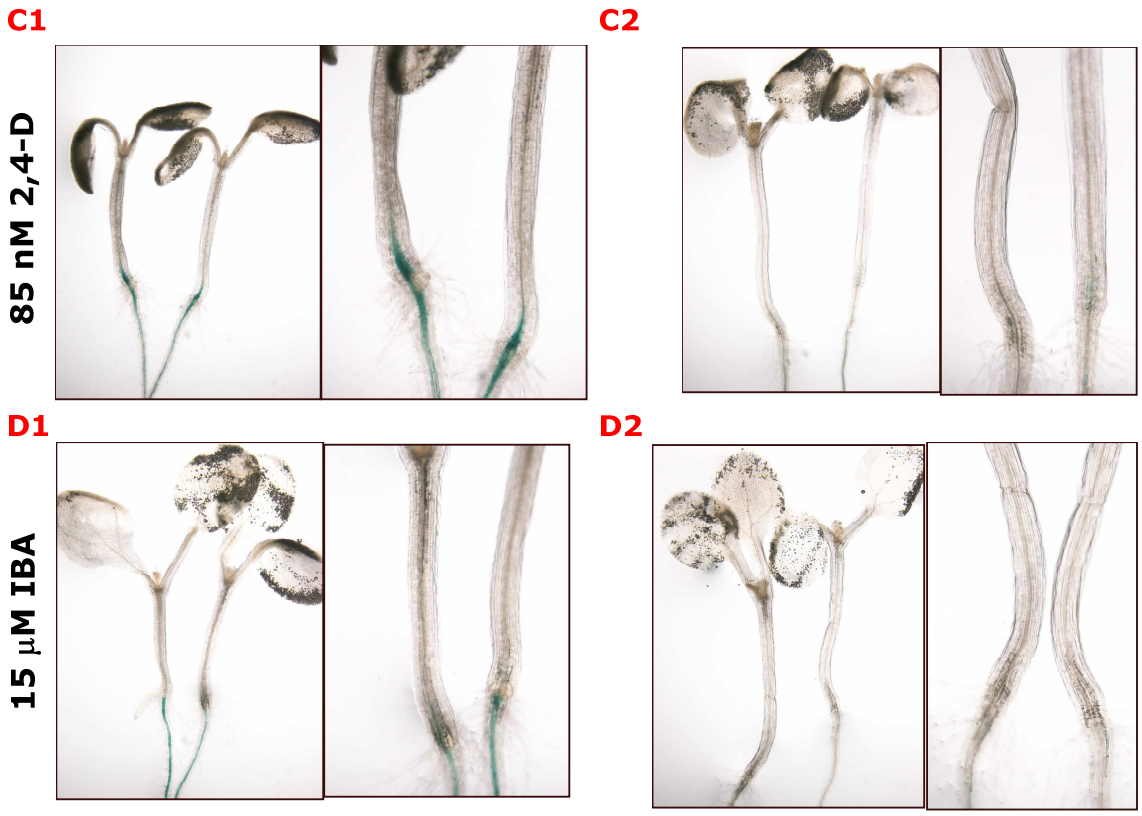


Figure 19: Expression of *LBD29$_{Pro}$::LBD29-GUS* in wild type and *iaa28-2* mutant hypocotyls with different auxin treatments. Four-day old seedlings of wild type and *iaa28-2* mutants with*LBD29$_{Pro}$::LBD29-GUS* were transferred to control (ATS) or to solid ATS media containing 10 µM picloram, 85 nM 2,4-D or 15 µM IBA media and allowed to grow 2 more days. The seedlings were fixed and stained with GUS staining buffer. (A1 and A2) LBD29-GUS protein was not detected in either wild type or *iaa28-2* mutant hypocotyls under control conditions. (B1, B2, C1 and C2). In response to both picloram and 2,4-D, the level of LBD29-GUS was increased slightly in wild type hypocotyls but not in the mutants. (D1 and D2) In response to IBA treatments wild type hypocotyls showed slight expression of LBD29-GUS protein, while no protein was detected in *iaa28-2* hypocotyl.

Figure 19. Continued.



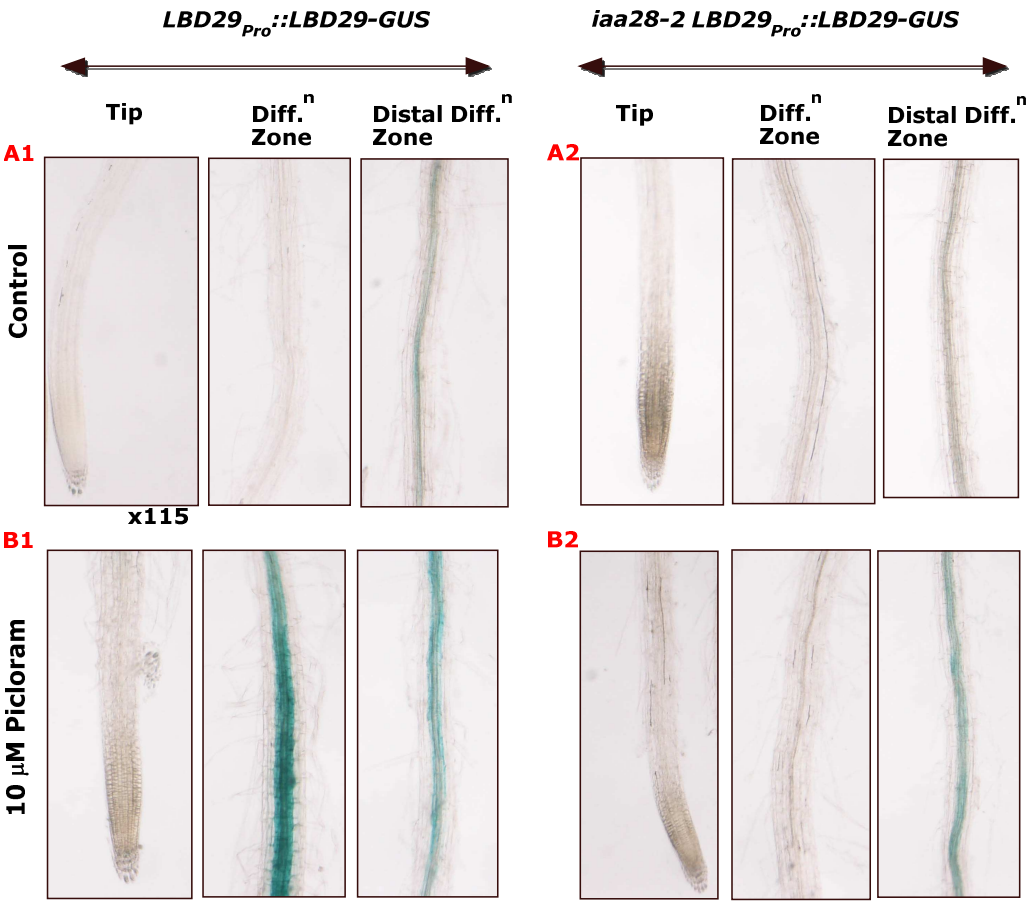
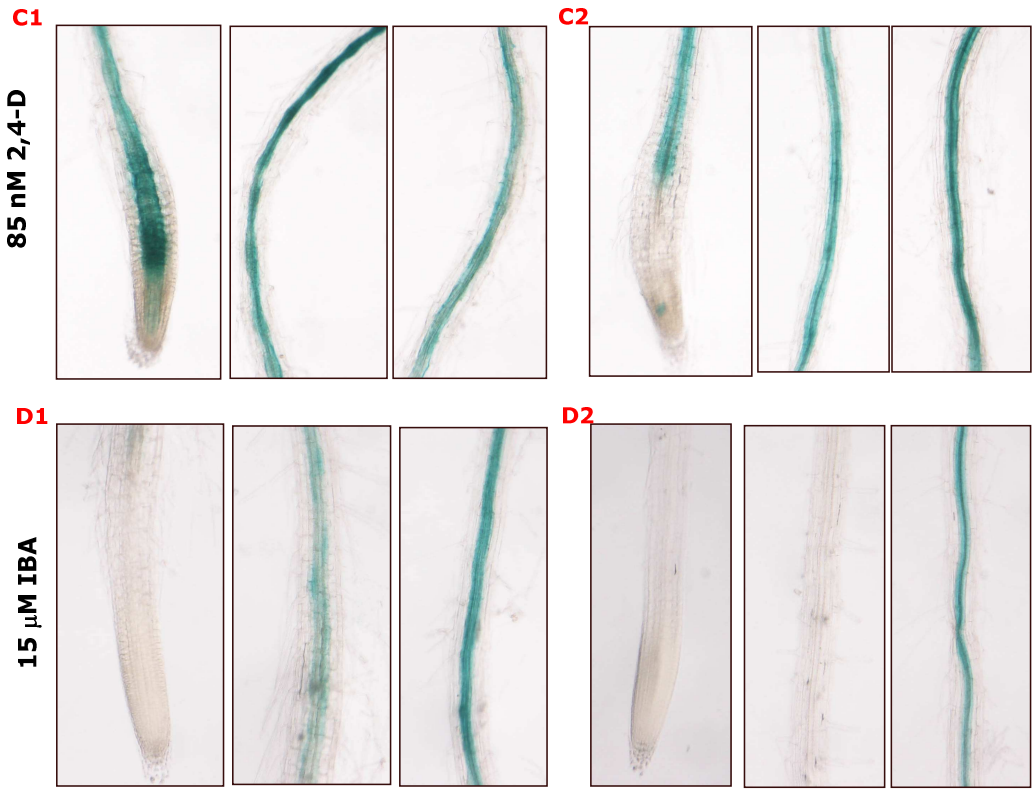


Figure 20: *LBD29$_{Pro}$::LBD29-GUS* expression in roots with different auxin treatments. Four-day old wild type and *iaa28-2* mutants with *LBD29$_{Pro}$::LBD29-GUS* were transferred to control (ATS) or solid ATS media containing 10 µM picloram, 85 nM 2,4-D or 15 µM IBA and allowed to grow 2 more days. The seedlings were then fixed and stained with GUS staining buffer. (A1 and A2) Under control conditions, LBD29-GUS was detected only in the distal differentiation region of wild type root, but not in any part of the mutant roots. (B1 and B2) In response to picloram, the expression of LBD29-GUS increased in the differentiation and maturation region of wild type, but induction was only found in the maturation region of *iaa28-2* roots. (C1 and C2) LBD29-GUS protein level was increased throughout the wild type roots except in the root meristem with 2,4-D treatment. Similarly, *iaa28-2* mutant roots had increased levels of LBD29-GUS in the root differentiation region and distal differentiation region of root except the cell elongation zone. (D1 and D2) IBA treatment increased the LBD29-GUS protein level in the differentiation and distal differentiation region of the wild type roots but only increased LBD29-GUS protein level in the maturation region of *iaa28-2* mutants.

Figure 20. Continued.



3.D. Role of IAA28 in adventitious root formation in *iaa28-2* mutants

3.D.i. Mutant *iaa28-2* produces adventitious roots in response to picloram

As explained previously in 3.C.i. section, mutant *iaa28-2*seedlings produce adventitious roots in response to picloram while wild type seedlings produce many lateral roots along the primary root (Figure 13 C & D). As shown in Figure 13 I, the expression of cell cycle marker *CyclinB::GUS* was studied in *iaa28-2* background with picloram treatment, and the results showed that the expression of *CyclinB::GUS* was higher in *iaa28-2* mutants compared to the wild type, indicating higher cell division rates in *iaa28-2*hypocotyl in response to picloram (Figure 13 I).

3.D.ii. Mutant iaa28-GUS protein degradation in the hypocotyl in response to picloram

As *iaa28-2* mutants produce adventitious roots in response to picloram, and the mutant GST-iaa28 protein interacts with AFB1-myc auxin co-receptor in the presence of picloram, the degradation of iaa28 protein in the hypocotyls was examined using *IAA28$_{Pro}$::iaa28-GUS*. Interestingly, the mutant protein was rapidly degraded in the *iaa28-2* mutant hypocotyls in response to picloram compared to the root (Figure 21).

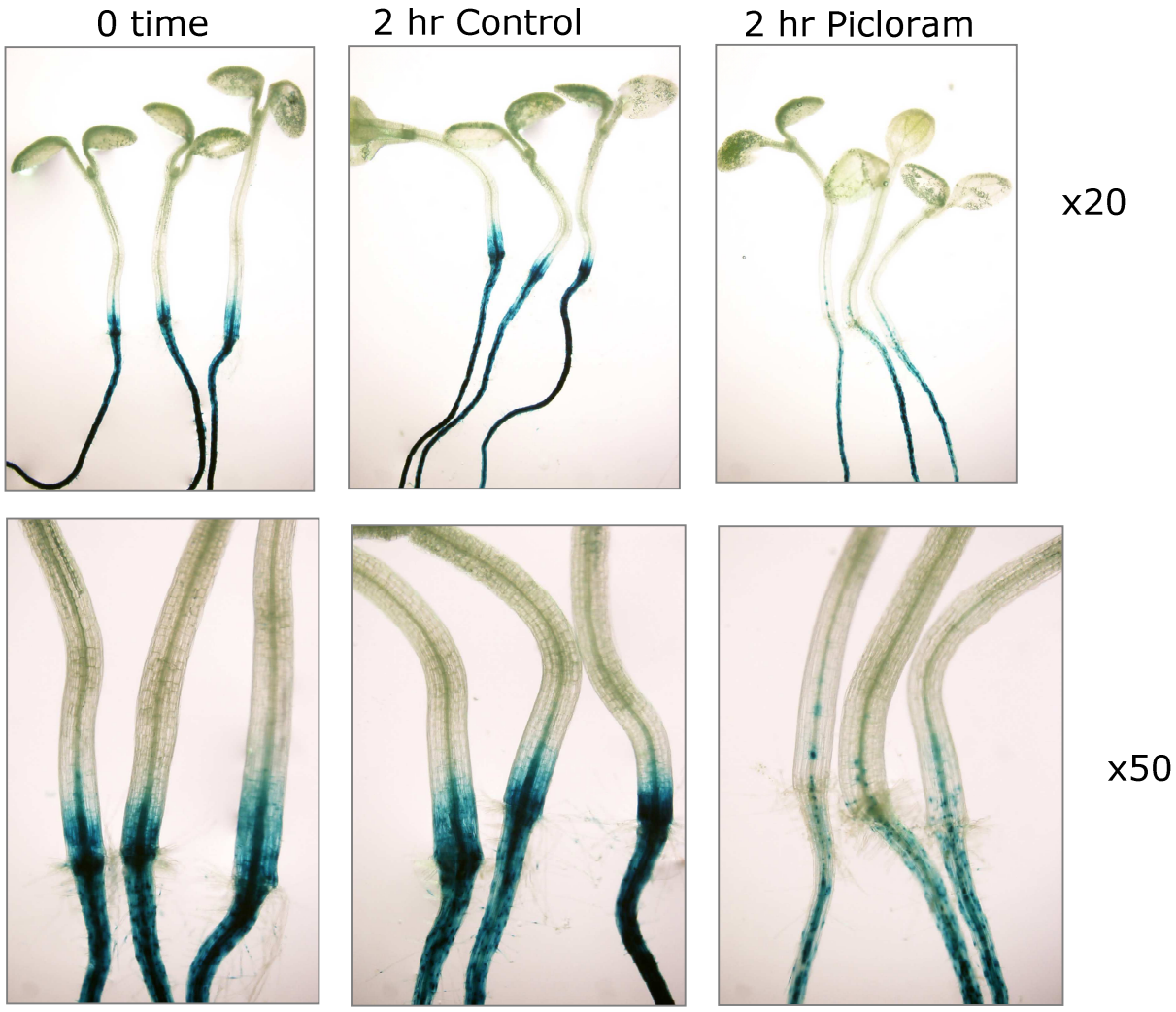


Figure 21: iaa28-GUS recombinant protein was degraded in response to picloram. *IAA28$_{pro}$::iaa28-GUS* seeds were germinated in dark for two days and then transferred to light for another 3 days before the experiment. The seedlings were incubated with liquid ATS containing 100 µM picloram or similar volume of DMSO (control) for 2 hrs. Seedlings were then fixed and stained for GUS activity. Seedlings for 0 time point were directly fixed and stained for GUS activity.

3.D.iii. AFB1-GUS protein level increases in the wild type hypocotyls in response to picloram

As described previously in figure 10D, in the presence of picloram, even the mutant GST-iaa28 showed concentration dependent interactions with AFB1 auxin co-receptor protein. More supporting evidence for this was described in figure 21 showing that, in the presence of picloram, the mutant iaa28-GUS degrades in the hypocotyls region of Arabidopsis. As F-box proteins are important as co-receptors of auxin for Aux/IAA protein degradation, the expression of closely related F-box proteins that act as auxin co-receptors was studied. Here, the expression patterns of *AFB1* together with *AFB2*, *AFB3*, *AFB5* and *TIR1* were studied using translational gene constructs with the *GUS* reporter gene. In this experiment, four-day old $AFB1_{Pro}$*::AFB1-GUS*, $AFB2_{Pro}$*::AFB2-GUS*, $AFB3_{Pro}$*::AFB3-GUS*, $AFB5_{Pro}$*::AFB5-GUS* and $TIR1_{Pro}$*::TIR1-GUS* were transferred to liquid ATS containing 100 µM picloram and incubated with gentle shaking for 2 hours. Then, tissues were fixed with GUS fixer and stained with GUS staining buffer as described previously.

The results showed that, only *AFB1*, *AFB3* and *AFB5* were expressed to some extent in hypocotyls under control conditions (Figure 22, A, C, D). *AFB1* was slightly expressed in the base of hypocotyl, whereas both *AFB3* and *AFB5* were expressed in the shoot apical meristem. Neither *AFB2* nor *TIR1* were expressed in the hypocotyl tissues (Figure 22 B, E). The level of AFB1-GUS increased throughout the hypocotyl with picloram treatment (Figure 22 A, F). AFB5-GUS level was increased slightly in the cotyledons with picloram treatment but not in the hypocotyl region (Figure 22 D, I). However, the level of AFB2-GUS did not show any difference in response to picloram treatment (Figure 22 B, G). Neither AFB2-GUS nor TIR1-GUS showed any detectable levels of protein in hypocotyls with or without picloram treatment (Figure 22 B, G, E, and J).

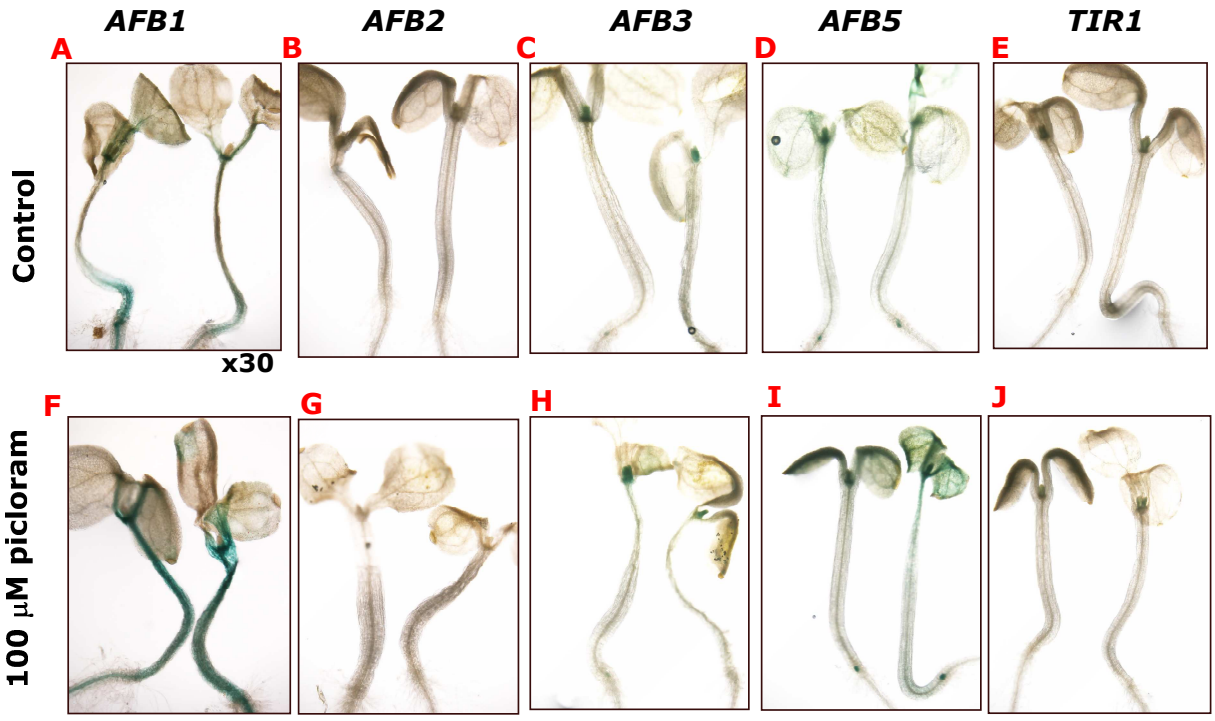


Figure 22: Expression of different auxin co-receptors in hypocotyl tissues in response to picloram treatment. Four-day old seedlings carrying translational fusion constructs with *GUS* reporter gene of *AFB1*, *AFB2*, *AFB3*, *AFB5* and *TIR1* were treated with 100 µM picloram for 2 hours and stained with GUS staining buffer. (A, C and D) *AFB1*, *3* and*AFB5* were expressed slightly in the hypocotyls under control conditions. (F) The level of AFB1-GUS was increased throughout the hypocotyl with picloram treatment. (H and I) Neither *AFB3* nor *AFB5* were induced in the hypocotyl with picloram treatment. (B, G, E and J) Neither *AFB2* nor*TIR1* were expressed in the hypocotyl tissues with or without picloram treatment.

The effects of other auxins such as 2,4-D and IBA on the *AFB1-GUS* expression were also studied. Four-day old *AFB1$_{Pro}$::AFB1-GUS* seedlings were treated with 100 µM picloram, 20 µM 2,4-D and 50 µM IBA for 2 hours,and then tissues were fixed and stained with GUS staining buffer. The results show that, similar to picloram, both 2,4-D and IBA were able to induce AFB1-GUS expression in hypocotyls (Figure 23). However, the expression of AFB1-GUS in roots shows different patterns in response to each auxin. For example, the level of AFB1-GUS is reduced in the root tip area and differentiation region as well as in the distal differentiation region in response to picloram. AFB1-GUS expression was only found in the top of the root that is closer to the hypocotyls in response to picloram. With both 2,4-D and IBA, the level of AFB1-GUS protein was lesser compared to un-treated roots, however, it was higher that picloram treated roots. AFB1-GUS was expressed throughout the root except in the root elongation region in response to both 2, 4-D and IBA (Figure 23).

To test whether picloram-induced AFB1-GUS expression in the hypocotyl is due to transcriptional activation or posttranslational regulation, a similar experiment was conducted using transcriptional construct of *AFB1* with *GUS* reporter gene (*AFB1::GUS*). Here, four-day old *AFB1::GUS* seedlings were treated with 100 µM picloram, 20 µM 2,4-D and 50 µM IBA for 2 hours, and then tissues were fixed and stained with GUS staining buffer. The results showed that the transcript level of *AFB1::GUS* was not affected by picloram treatment in the hypocotyl tissues (Figure 24, A, B). Only 2,4-D and IBA treatments were able to induce the *AFB1::GUS* transcript levels slightly in hypocotyls (Figure 24, A, C, D). These results indicate that picloram probably regulates AFB1 induction in the hypocotyls at the posttranslational level.

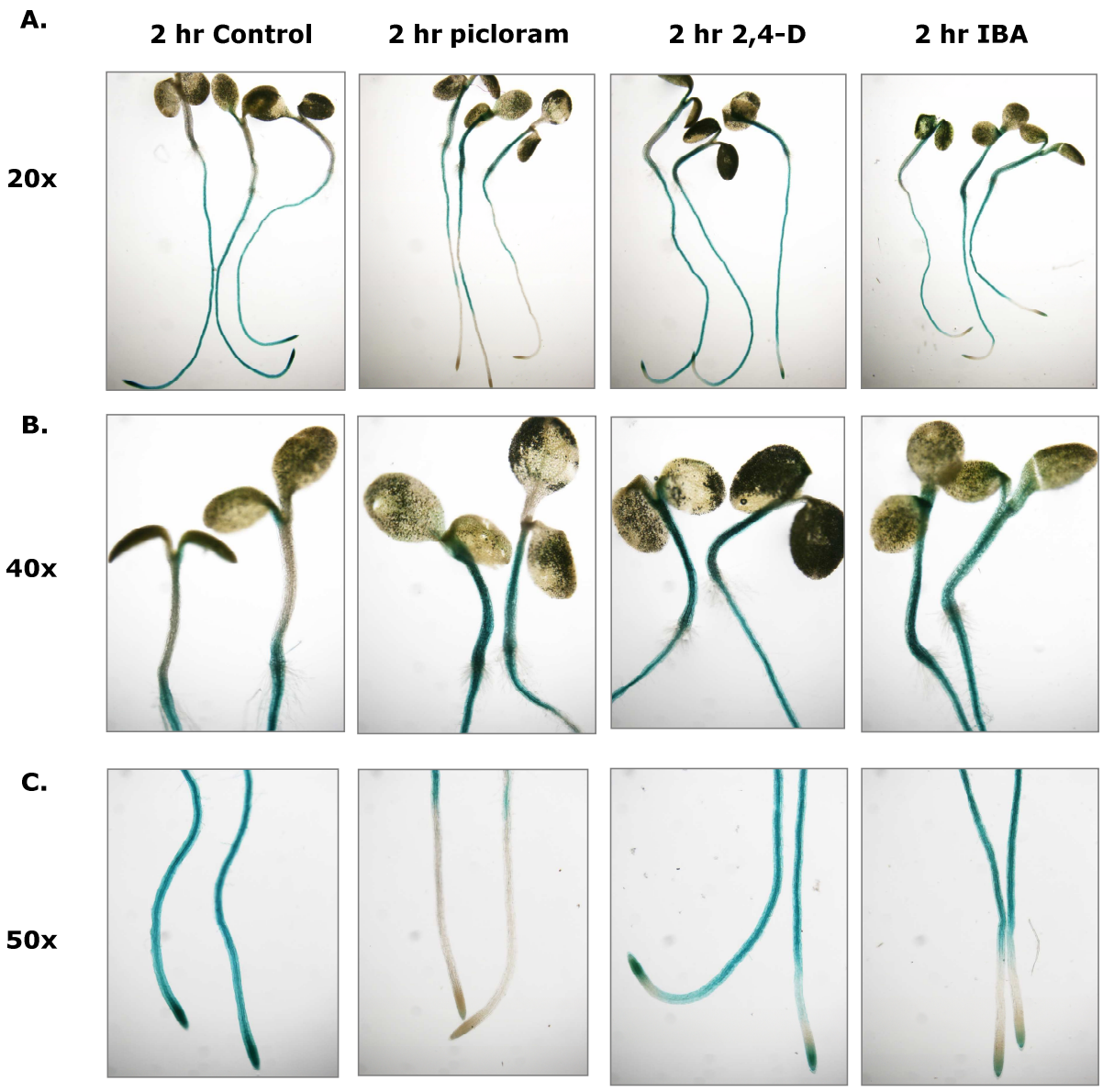


Figure 23: The expression of *AFB1$_{Pro}$::AFB1-GUS* with different auxin treatments. Four-day old seedlings were treated with 100 µM picloram, 20 µM 2,4-D or 50 µM IBA for 2 hours and stained with GUS staining buffer. (Panel A)The expression patterns of AFB1-GUS in whole seedling were examined in the presence of different auxin treatments.(Panel B) The expression levels of AFB1-GUS in hypocotyls with different auxins. (Panel C) The level of AFB1-GUS in root tips with different auxins. With picloram treatment, the level of AFB1-GUS was higher in the hypocotyls but lower in the root tip area and differentiation region of the root. With both 2,4-D and IBA, the level of AFB1-GUS increased in the hypocotyls. In roots the level of AFB1-GUS was less affected with 2,4-D or IBA compared to picloram.

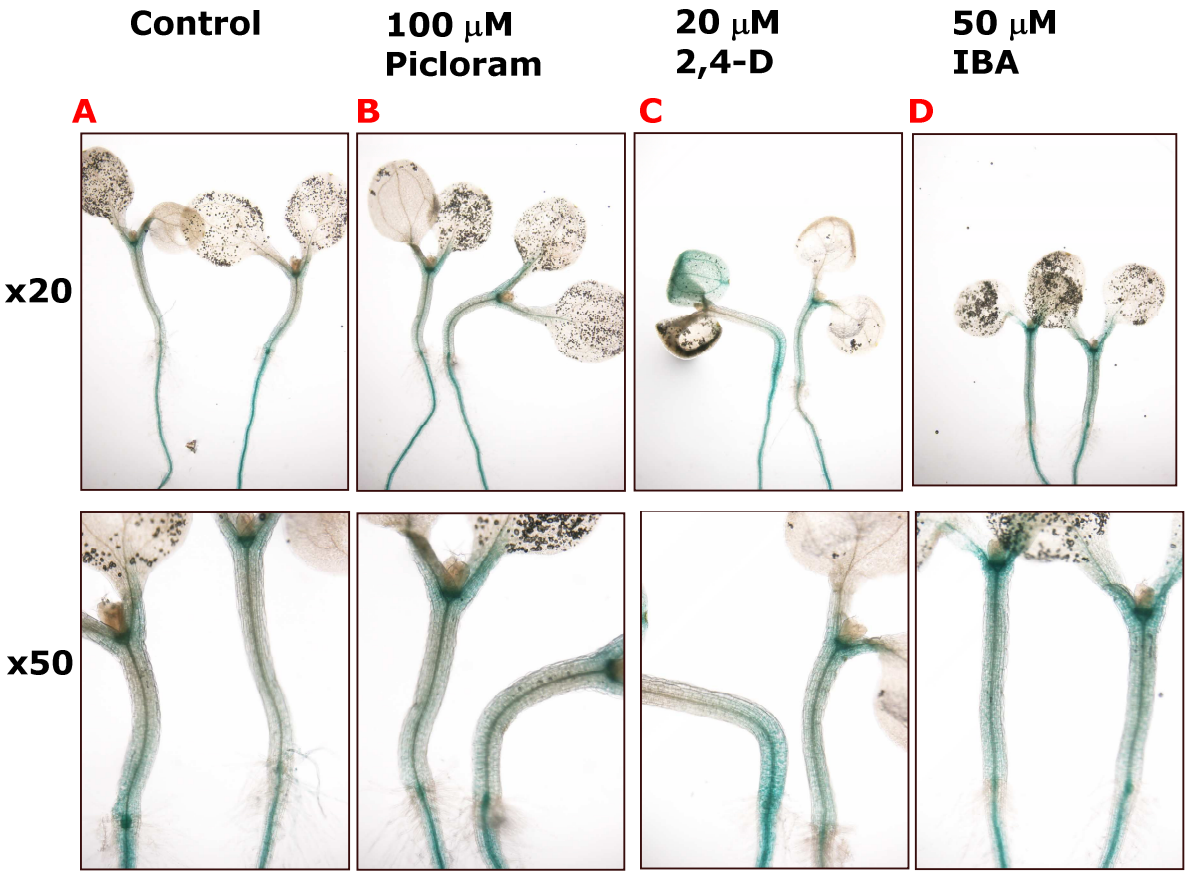


Figure 24: Effects of different auxins on the transcript levels of *AFB1*. Four-day old *AFB1::GUS* seedlings were treated with 100 µM picloram, 20 µM2,4-D or 50 µM IBA for 2 hours and stained with GUS staining buffer. (A and B)There is no difference in the level of *AFB1::GUS* transcript level in the hypocotyls with or without picloram.(C and D) Both 2,4-D and IBA increased the *AFB1::GUS* transcript level slightly in hypocotyl tissues.

3.E. Auxin transport is altered in *iaa28-2* mutants

3.E.i. Expression of some transporter genes is altered in *iaa28-2*

Studies have shown that, auxin transport plays an essential role in both adventitious and lateral root formation. More importantly programmed auxin distribution within root cells is important for lateral root initiation, emergence and growth (Casimiro *et al.* 2001; Casimiro *et al.* 2003). As *iaa28-2* mutants show severe defects in lateral and adventitious root formation, one factor for this defective lateral root may be altered auxin transport in *iaa28-2* mutant. Therefore, transcript levels of different auxin transporters (*PIN*s and *AUX1*) were examined under control and auxin treatments using RT-PCR technique. In addition, expression pattern variations were studied using different reporter gene constructs of PIN and AUX1 protein. RT- PCR results showed that transcript levels of *PIN2* and *PIN3* were slightly lower in *iaa28-2* control treatment compared to that of the wild type (Figure 25). However, the transcript levels of *PIN1*, *PIN4*, *PIN5*, *PIN6* and *PIN7* in *iaa28-2* control samples were similar to those of wild type. All *PIN1*, *PIN2* and *PIN3* transcript levels in mutants increased in both wild type and the mutants with both picloram and 2,4-D treatments (Figure 25).

To examine the effects of *iaa28-2* mutation on the spatial expression of auxin influx and efflux carriers, translational gene constructs of *PIN1*, *PIN2* and *AUX1* were used. Here *iaa28-2* mutant plants were crossed to $PIN1_{Pro}$*::PIN1-GFP*, $PIN2_{Pro}$*::PIN2-GFP* and $AUX1_{Pro}$*::AUX1-YFP*. Then the plants homozygous for both *iaa28-2*and the transgene were selected as described in methods.

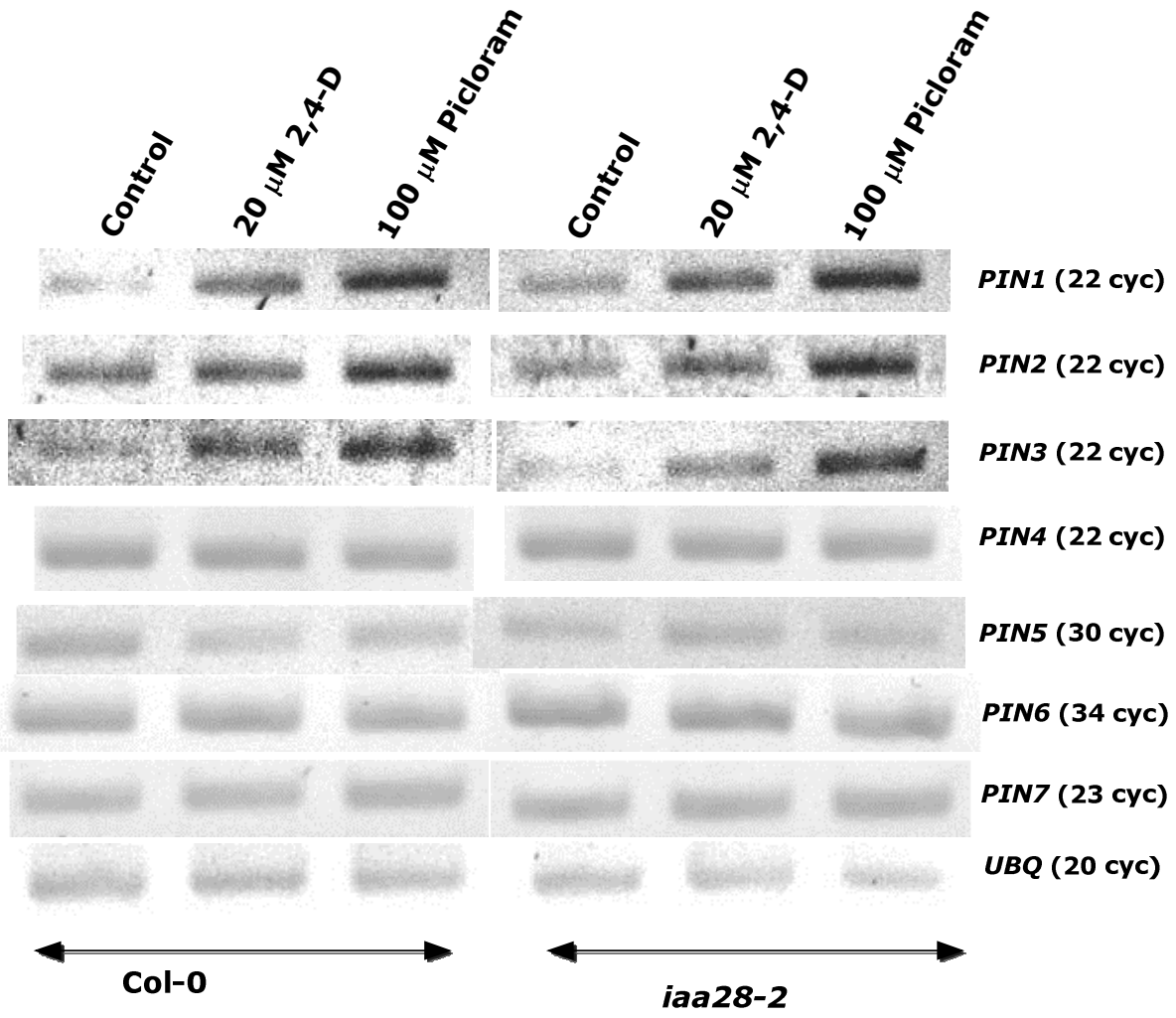


Figure 25: RT-PCR results showing the expression of different auxin transporters in *iaa28-2* background. Seven-day old Col-0 and *iaa28-2* seedlings were treated with 20 µM 2,4-D or100 µM picloram for 1 hour, and tissues were frozen with liquid nitrogen. Total RNA was extracted and cDNA was synthesized as described in methods. The transcript levels of *PIN2* and *PIN3*were slightly less in an *iaa28-2* control compared to that of wild type. The level of *PIN1*was slightly higher in the mutant background compared to wild type. All three PINs (*PIN1*,*PIN2* and *PIN3*) were increased with 2,4-D and picloram treatment in both Col-0 and *iaa28-2* background. *PIN4*, *PIN5*, *PIN6* and *PIN7* didn't show any difference in both wild type and *iaa28-2* background under any conditions used (e.g. control, 2,4-D or picloram). Different numbers of cycles were used in PCR for each gene tested and similar pattern was observed in response to auxin treatments.

3.E.ii. Expression analysis of auxin efflux carrier *PIN1$_{Pro}$::PIN1-GFP* in *iaa28-2* background

To study the effects of *iaa28-2* mutation on the *PIN1-GFP* expression, *iaa28-2* mutants with *PIN1$_{Pro}$::PIN1-GFP*were generated as described previously. *PIN1* is mainly expressed in the root tip area. The level and pattern of *PIN1-GFP* expression in *iaa28-2* were very similar to that in wild type. Neither 2,4-D nor picloram had effects on the expression of *PIN1-GFP* in either the wild type or mutants (Figure 26).

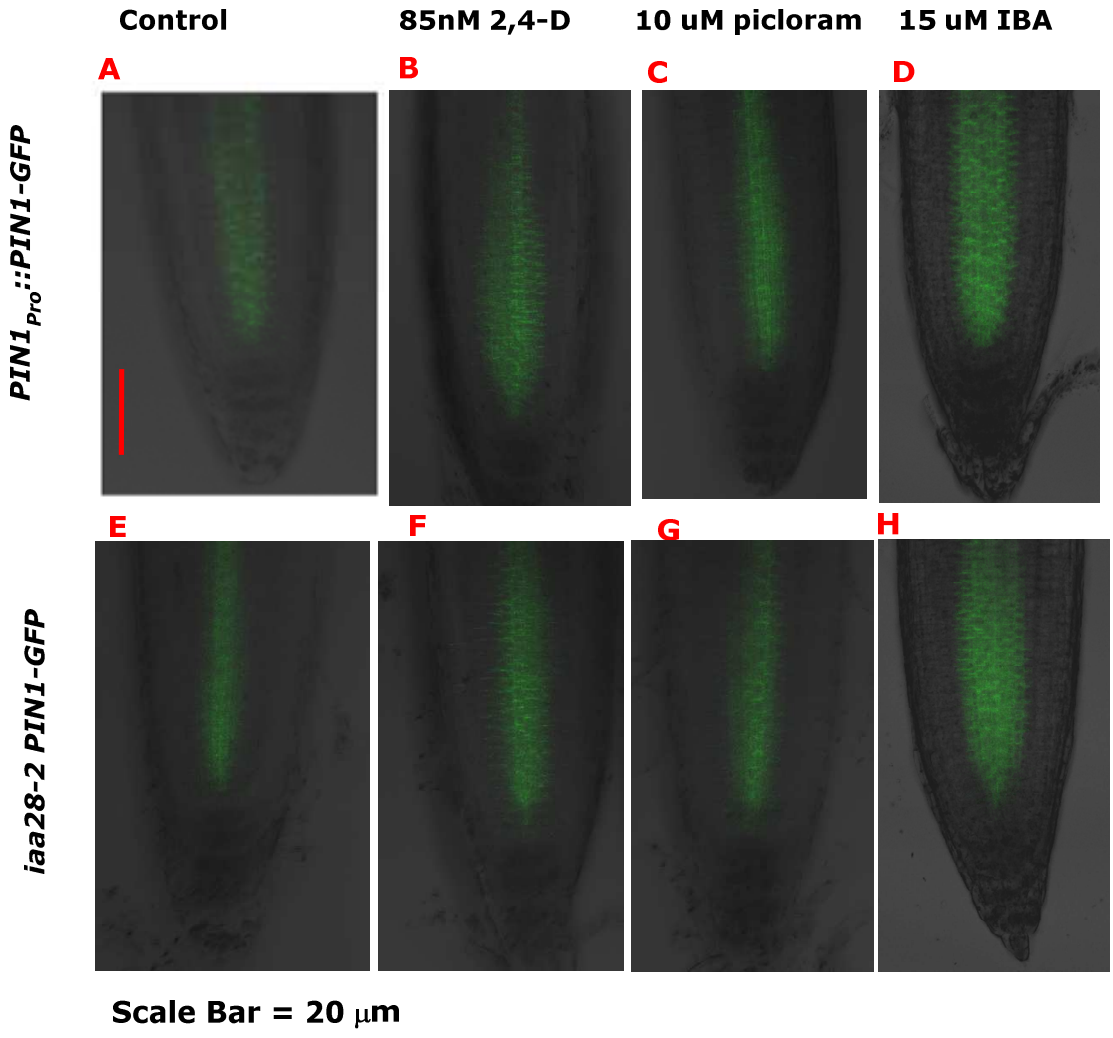


Figure 26: Effects of *iaa28-2* mutation on *PIN1-GFP* expression. (A and E) Both *iaa28-2* and wild type had similar levels of *PIN1-GFP* expression. (B, C, D and F, G, H) Treatments with 2,4-D, picloram or IBA did not affect expression of *PIN1-GFP* in wild type or *iaa28-2* mutants. (These figures are combined images of all the Z stacks taken. The scanning was performed with 3 µm section thickness).

3.E.iii. Expression of *PIN2$_{Pro}$::PIN2-GFP* in *iaa28-2* mutants

In the stele, auxin is transported acropetally towards the root tip. In the tip region PIN2 efflux carrier is specifically involved in auxin distribution from the stele into cortical and epidermal cell layers and re-distribution of auxin towards the elongation zone. Plants homozygous for both *iaa28-2* and *PIN2$_{Pro}$::PIN2-GFP* were used to examine the effects of different auxin treatments on *iaa28-2* on *PIN2-GFP* expression. In the mutant, the level of PIN2-GFP protein was considerably lower in the root tip area compared to the wild type (Figure 27 A and D). With 2,4-D the level of PIN2-GFP in *iaa28-2* was increased to the same level as 2,4-D-treated wild types. Although picloram enhanced the PIN2-GFP expression in wild type, it did not have any effect on *iaa28-2*.

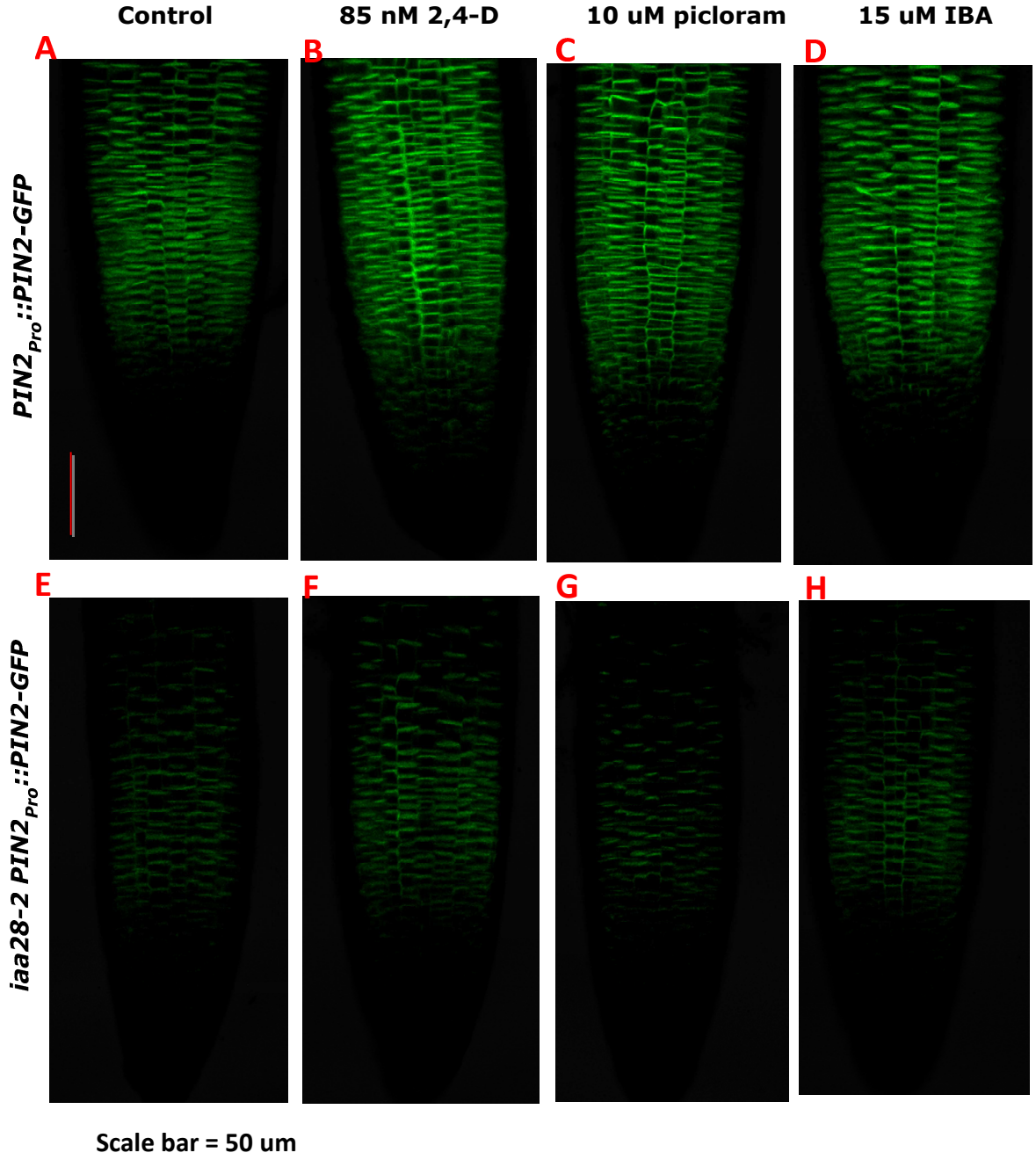


Figure 27: The expression of *PIN2-GFP* in the *iaa28-2* mutant background. Four-day old wild type and *iaa28-2* carrying *$PIN2_{Pro}$::PIN2-GFP* were transferred onto control (ATS) or solid ATS with 10 µM picloram or 85 nM 2,4-D and incubated for 18 hour before obtaining images with the confocal microscope. (A and E) The mutant had low levels of *PIN2-GFP* expression in the root tip compared to wild type; (B and F) however, with 2,4-D treatment the levels of PIN2-GFP was increased in the mutant but to a lesser extent compared to the wild type.(C and G) With picloram treatment, the level of PIN2-GFP was increased in the wild type but not in *iaa28-2* mutant roots.(D and H) PIN2-GFP was slightly increased in *iaa28-2* mutants with IBA treatments. (These figures are combined images of all the Z stacks taken. The scanning was performed with 3 µm section thickness).

3.E.iv. Mutant *iaa28-2* alters the expression and localization of auxin influx carrier, *AUX1$_{Pro}$::AUX1-YFP*

To examine the effect of *iaa28-2* mutation on the expression and localization of *AUX1* auxin influx carrier, seedlings homozygous for both *iaa28-2* mutation and *AUX1$_{Pro}$::AUX1-YFP* transgene were treated with different auxins. Under control conditions *AUX1-YFP* protein was detected in the root tip area in both wild type and mutant plants. However, the level of expression was lower in the mutant compared to the wild type (Figure 28). Even though the expression was low in mutants, the AUX1-YFP protein showed similar localization as wild type. With 2,4-D and picloram treatments, the *AUX1-YFP* expression was increased to a similar level in both mutant seedlings and in wild type seedlings (Figure 28). Interestingly, in wild type, *AUX1-YFP* expression was increased in the cell differentiation region with 2,4-D treatment. This induction was not apparent in the mutants (Figure 29 A1 and A2). Picloram treatment did not induce AUX1-YFP expression in the cell differentiation zone of wild type or mutants, similar to the effect explained previously with 2,4D (data not shown).

In *iaa28-2* hypocotyls, the level of AUX1-YFP expression was lower than that in the wild type but showed similar localization pattern as wild type facilitating proper basipetal auxin transport (data not shown). With picloram treatments, the level of AUX1-YFP was slightly increased in *iaa28-2* mutants in the shoots and also localized properly (data not shown). However, *iaa28-2* mutation disrupts the localization of AUX1-YFP protein in the root just below the hypocotyls with picloram treatments. In wild type, the AUX1-YFP influx carrier was localized properly in the entire root including the area just below the hypocotyls (Figure 29 B1, B2, C1, C2 and C3). The effect of other auxins such as 2,4-D and IBA on the *AUX1-YFP* expression in *iaa28-2* mutants was also studied following the same experimental procedure as described for picloram. *AUX1-YFP* expression was increased in *iaa28-2* background to a similar level as in the wild type with 2,4-D (Figure 29, D1 and D2). IBA treatments were also able to induce the *AUX1-YFP* expression in *iaa28-2* mutants; however the level of protein was relatively lower than that in wild type (Figure 29, E1 and E2). According to these results, AUX1-YFP

localization is disrupted in *iaa28-2* mutants compared to that of wild type in the presence of picloram but not with either 2,4-D or IBA.

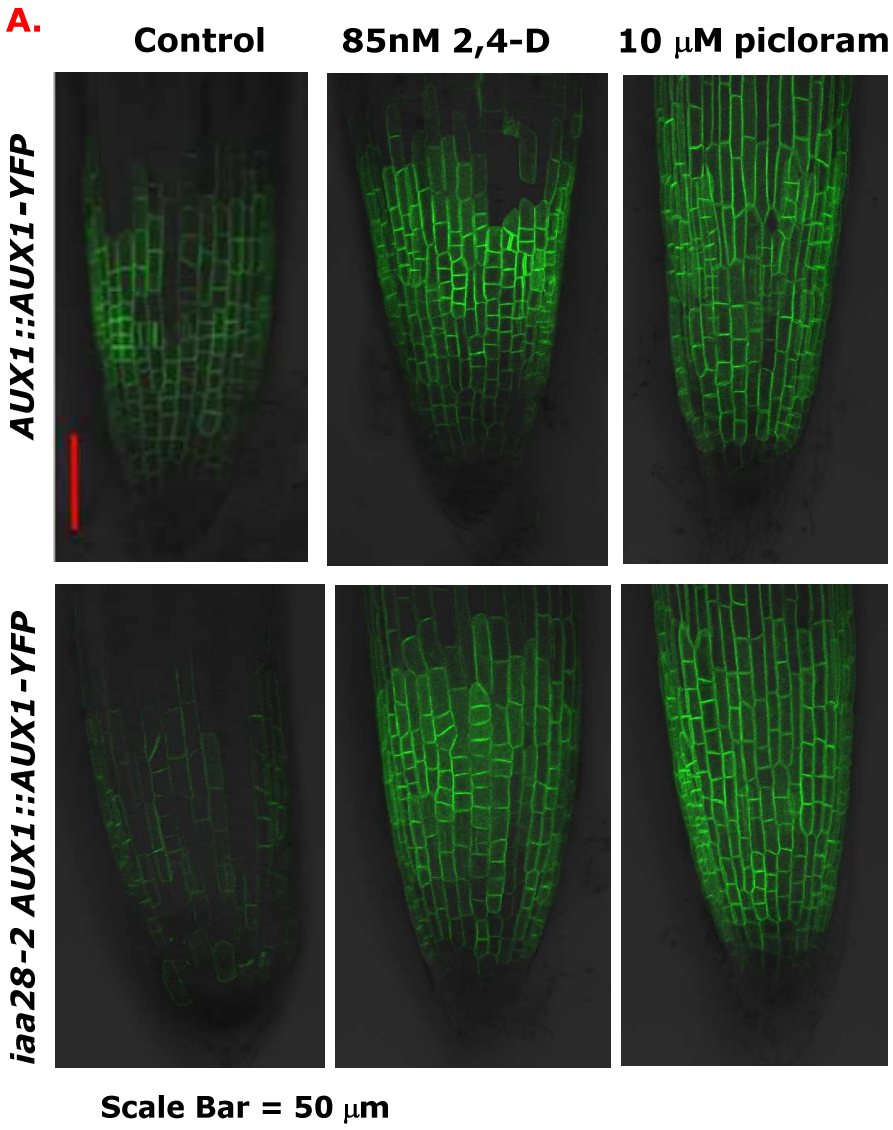


Figure 28: Comparison of *AUX1*$_{Pro}$*::AUX1-YFP* expression in *iaa28-2* root tip area. (A) The level of AUX1-YFP was lower in the mutant root tip area compared to that of wild type. However, with both 2,4-D and picloram treatments the level of AUX1-YFP increased to the same extent as in wild type. (These figures are combined images of all the Z stacks taken. The scanning was performed with 3 µm section thickness).

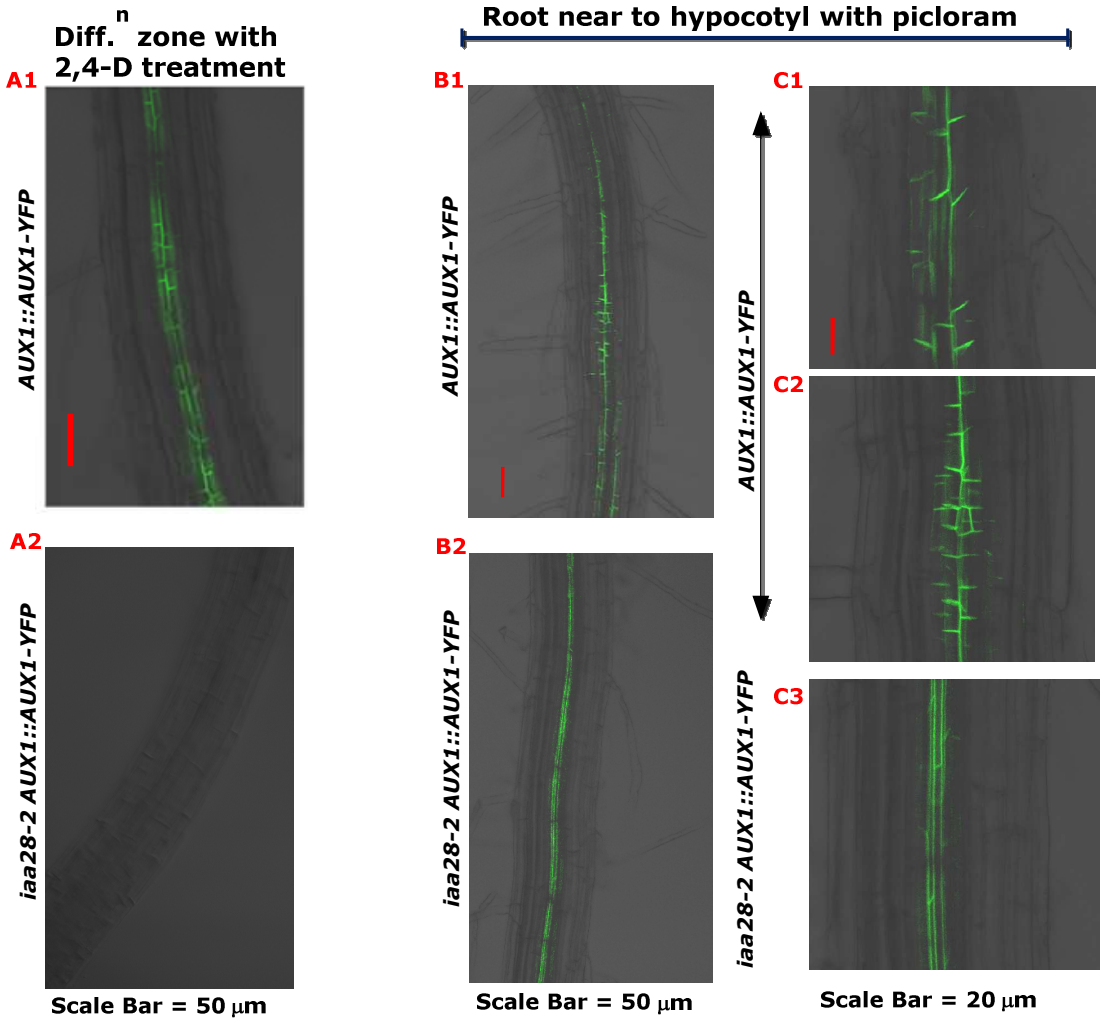
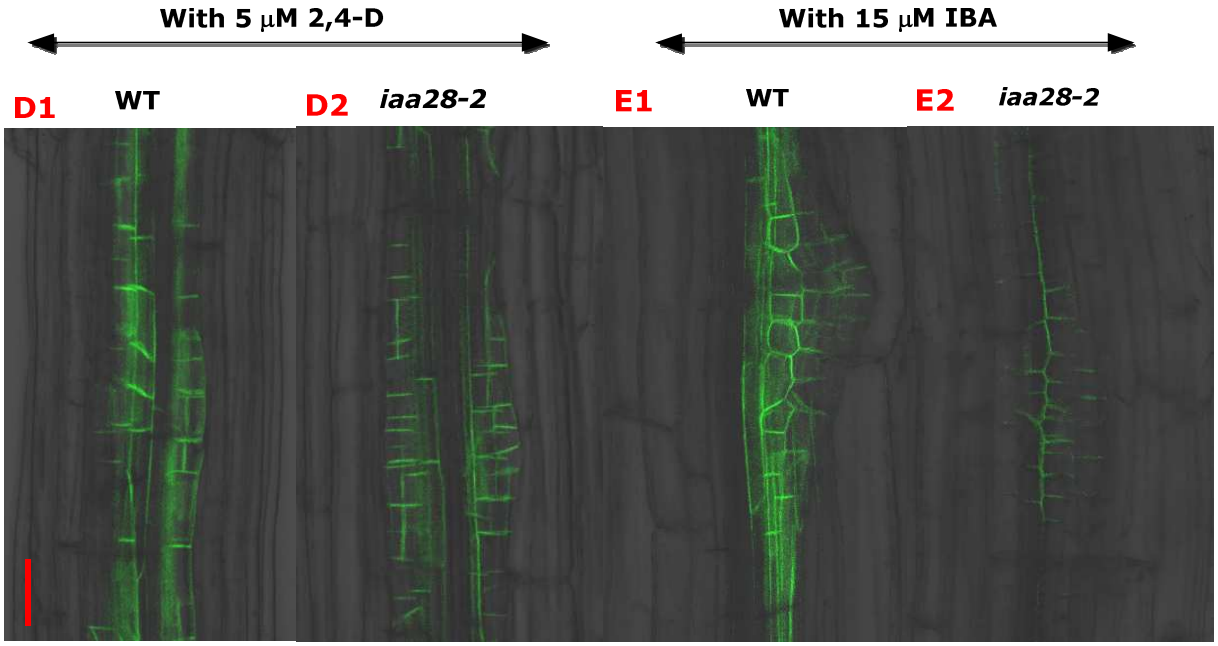


Figure 29: Effect of *iaa28-2* mutation on *AUX1$_{Pro}$::AUX1-YFP* expression and localization. Four-day old wild type and *iaa28-2* mutant seedlings carrying *AUX1$_{Pro}$::AUX1-YFP* were transferred to control (ATS), 10 µM picloram, 5 µM 2,4-D or 15 µM IBA for 18 hours, and the expression and localization of AUX1-YFP was examined using a confocal microscope. (A1 and A2) Expression of AUX1-YFP in the root differentiation zone in the presence of 2,4-D. AUX1-YFP expression was induced in wild type root differentiation region but not in *iaa28-2* mutants. (B1 and B2) Expression of AUX1-YFP in the root below the hypocotyls in both wild type and mutants in response to picloram was observed. In wild type AUX1-YFP was localized properly. However, in *iaa28-2* mutants, AUX1-YFP protein localization was disrupted in the roots just below the hypocotyls, presumably causing defective auxin transport from shoot to root. (C1 and C2) Enlarged images of B1 that show proper subcellular localization of AUX1-YFP in wild type root in response to picloram. (C3) Enlarged image of B2, which shows the defective AUX1-YFP localization in *iaa28-2* mutant in response to picloram. (D1, D2, E1 and E2) Unlike picloram, both 2,4-D and IBA did not affect the localization of AUX1-YFP in the mutant roots compared to that of wild type.

Figure 29. Continued.

***AUX1*$_{Pro}$*::AUX1-YFP* expression in *iaa28-2* background with 2,4-D and IBA treatments**



Scale Bar = 20 μm

3.F. Role of IAA28 in light signaling

3.F.i. Mutant *iaa28-2* shows altered growth in response to different light sources

To examine the effect of *iaa28-2*mutation on light responses, seed germination assays and hypocotyl growth assays were conducted using *iaa28-2* seeds and Col-0 seeds. Seeds were sterilized and stratified at 4º C for 3 days and transferred to different light sources (red, FR and Blue) in a dark growth chamber at 20 ºC. For white light treatments, the regular growth chamber at 20 ºC was used. Seed germination was observed after 36 hours and 48 hours under each light condition. For hypocotyl growth experiments, seeds were incubated for 7 days under different light conditions. Seed germination experiments showed that the mutant*iaa28-2* had a higher percent seed germination under red light compared to the wild type (Figure 30A) after 36 hours in red light. However, longer exposure of red light showed similar percent seed germination in both wild type and the mutants (Figure 30B).

Studies have shown that far red light inhibits hypocotyl growth, whereas red light induces hypocotyl elongation (Kang and Ni 2006). Under red light, the increase of hypocotyl length in *iaa28-2* was significantly higher than that of Col-0. Under blue light, the increased length of hypocotyls was significantly higher in *iaa28-2* background compared to wild type (Figure 31). These results show that IAA28 may be involved in light-regulated growth and developmental events in plants.

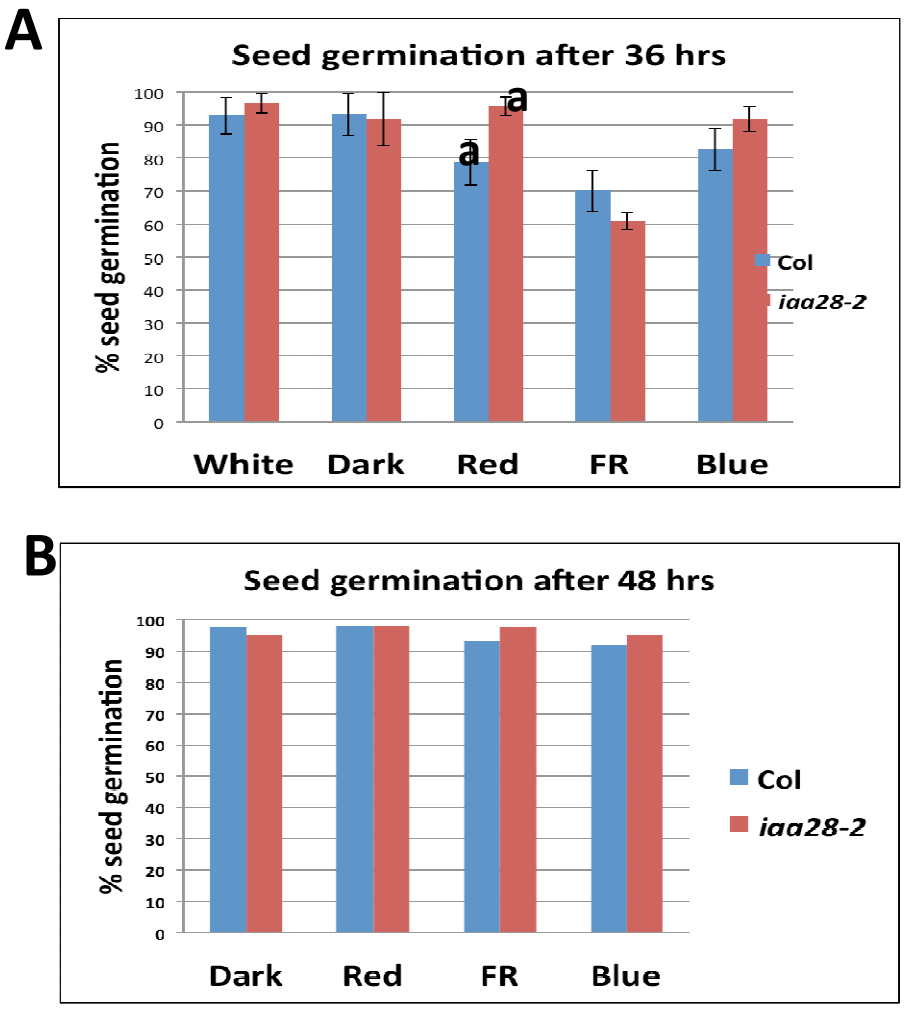


Figure 30: Effect of different light sources on *iaa28-2* growth and development. (A) Seed germination after 36 hours. Mutant *iaa28-2* showed significantly more seed germination under red light compared to Col-0. (P<<<<0.05, single factor ANOVA and Tukey's HSD) (B) But after 48 hours both Col-0 and *iaa28-2* showed similar seed germination under different light sources. The responses connected by "a" are significantly different (P<<<<0.05, Single factor ANOVA).

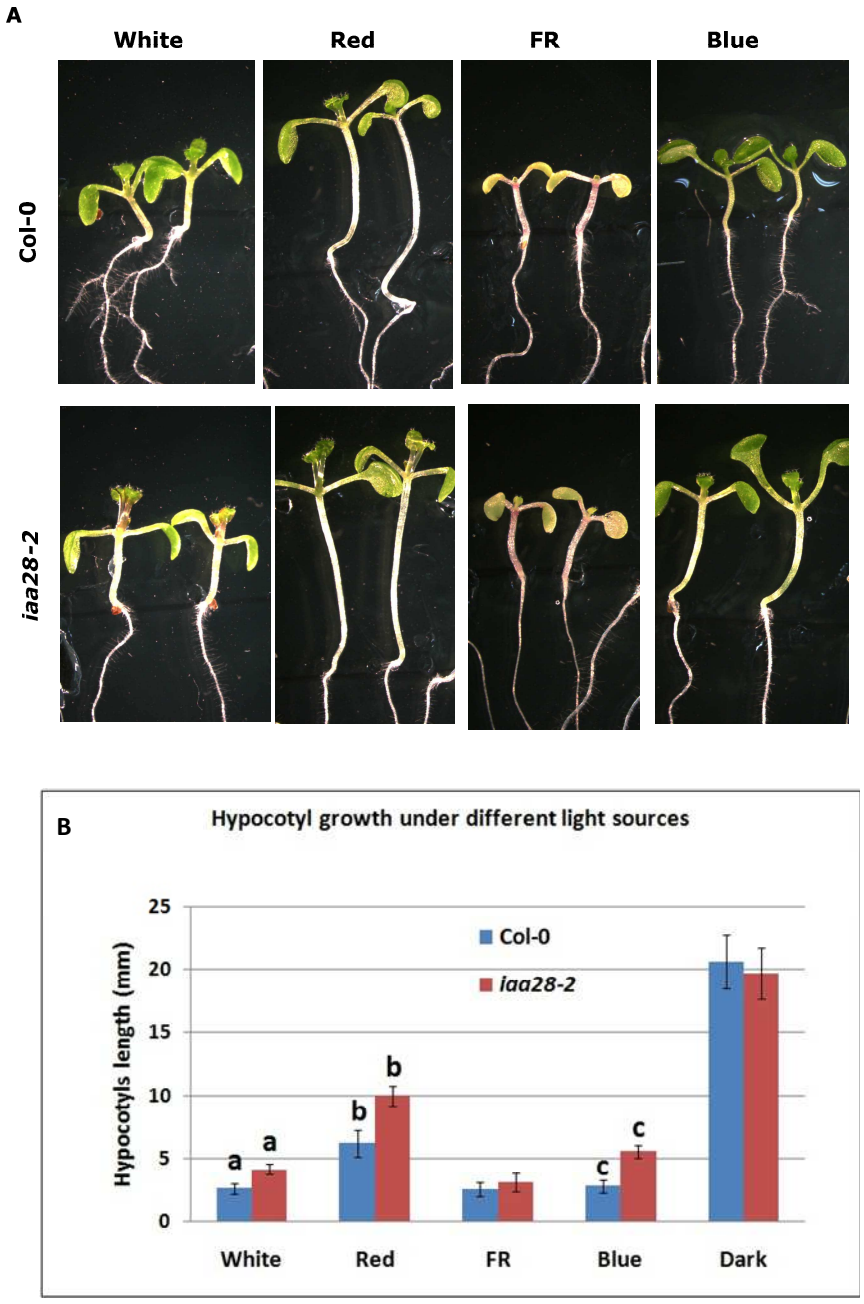


Figure 31: Effects of different light sources on hypocotyl elongation. Col-0 and *iaa28-2* seeds were grown under different light sources for 7 days, and the hypocotyls' lengths were measured using stereomicroscope. (A) Both red and blue light induced the hypocotyls' length to increase in wild type as well as in mutants while FR light inhibited growth. (B) The red light-induced hypocotyl elongation was significantly higher in *iaa28-2* compared to wild type (P<<<0.05, Single factor ANOVA). Inhibition of hypocotyl growth by FR light was similar in both *iaa28-2* and Col-0, while induction of hypocotyls length by blue light was significantly higher in *iaa28-2* mutants (P<<<0.05, Single factor ANOVA). The dark induction of hypocotyl length also significantly lower in mutants compared to wild type (P<<<0.05, Single factor ANOVA).The responses connected by the same letter are significantly different (P<<<<0.05, Single factor ANOVA and Tukey's HSD).

3.F.ii. IAA28 expression in shoots and roots under different light conditions

To examine the effect of different light sources on *IAA28* expression, *$IAA28_{Pro}$::iaa28-GUS* and *$IAA28_{Pro}$::IAA28-Myc* transgenic lines were used. Expression of iaa28-GUS is altered under different light conditions (Figure 32). The protein level of iaa28-GUS in shoots was relatively lower in all red, FR and blue light treatments compared to white light treatment (Figure 32).

A similar experiment was carried out using *$IAA28_{Pro}$::IAA28-Myc* transgenic seedlings to see the effect of different light on the wild type IAA28 protein.*$IAA28_{Pro}$::IAA28-myc* seedlings grown in the dark for 72 hours were exposed to different light sources for 4 hours, and total protein was collected from tissues and used for western blotting with anti-myc antibody. The results showed that there was a qualitative difference in the expression of IAA28-myc in plants grown under different light regimens. For instance, IAA28-myc protein isolated from plants grown under red and FR light conditions appeared as two closely separated bands. In contrast, IAA28-myc protein isolated from plants grown under blue and white light treatments appeared as a single band (Figure 33 A). Further, a time course experiment using plants transformed with *$IAA28_{Pro}$::IAA28-myc* and grown under different light showed gradual changes in the IAA28-myc protein isoforms under white, red and blue light conditions (Figure 33B). In all three light conditions, no IAA28-myc protein was detected in hypocotyls at time 0 (Figure 33 B). Under white light, the lower IAA28-myc band appeared starting from 5 minutes and the level increased gradually up to the1 hour time point. At one hour, the formation of upper IAA28-myc band was detected, but to a lesser extent compared to the lower one. After an additional hour, the protein level of upper IAA28-myc band gradually increased while that of lower IAA28-myc isoform gradually decreased (Figure 33 B, top). Red light treatment also showed a similar expression pattern with time; however, under blue light only one isoform of IAA28-myc (upper band) was observed throughout the experiment, starting from the 1 hour time point (Figure 33B).

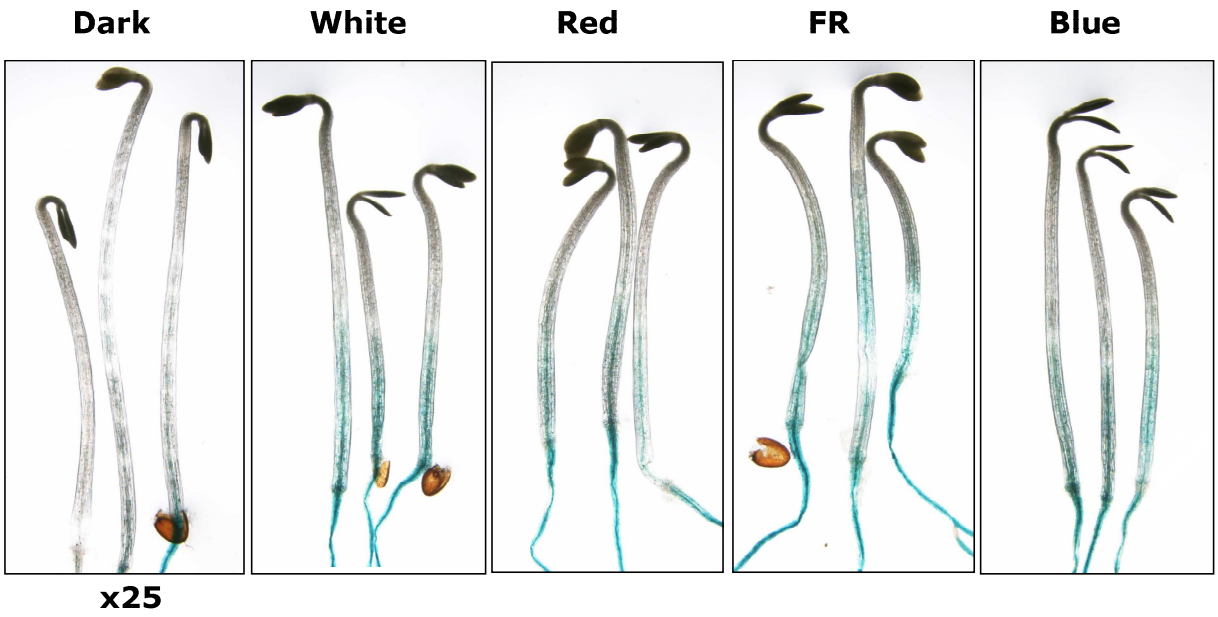


Figure 32: Expression of *IAA28$_{Pro}$::iaa28-GUS* under different light conditions. *IAA28$_{Pro}$::iaa28-GUS* seedlings were grown in dark for 72 hrs and exposed to white, red, FR and blue light for 4 hours. The tissues were fixed and stained with GUS staining buffer.iaa28-GUSwas not detected in dark grown hypocotyls but was induced with white light and showed altered expression under different conditions.

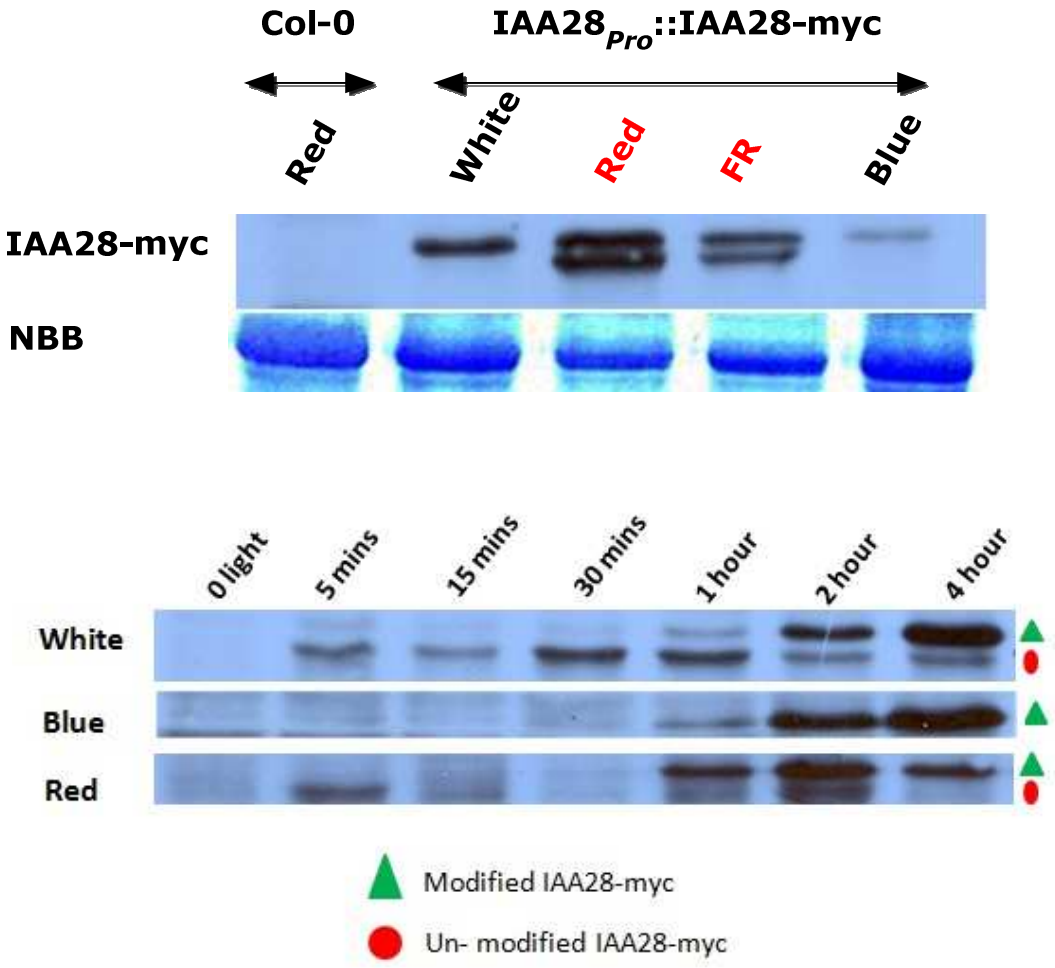


Figure 33: Expression of *IAA28$_{Pro}$::IAA28-myc* under different light conditions. (A) *IAA28-myc* protein appeared as two isoforms under red and FR light conditions. (B) Gradual formation of two isoforms of IAA28-myc was shown at different time points under white, blue and red light sources. (NBB; Napthol blue black).

3.F.iii. IAA28 protein degrades under dark conditions

To examine the effect of dark on IAA28-myc protein level, experiments were conducted using *IAA28$_{Pro}$::IAA28-GUS* under different periods of darkness. The level of IAA28-GUS protein in the root was reduced gradually with dark treatments (Figure 34). However, the *IAA28$_{Pro}$::IAA28-GUS*seedlings that were kept in light continuously did not show any difference in the level of protein in roots.

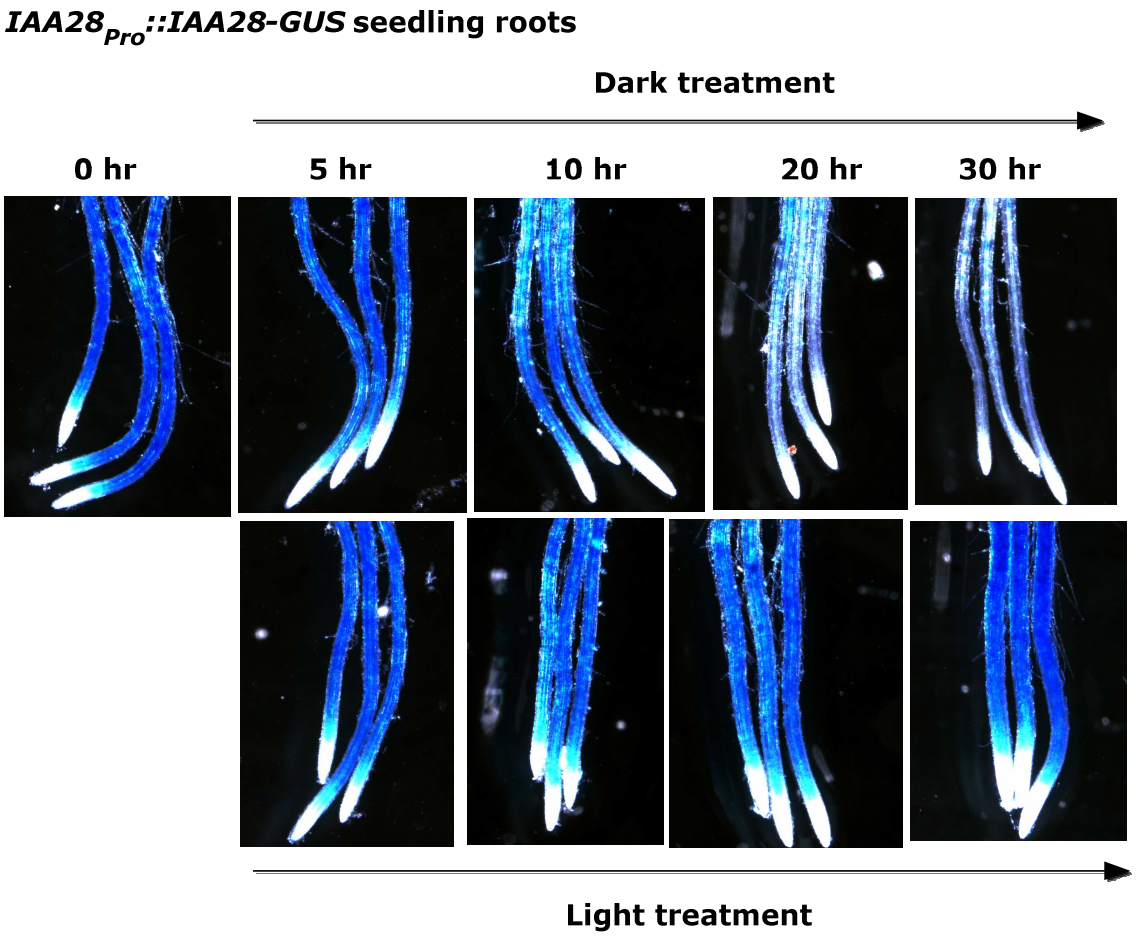


Figure 34: IAA28-GUS protein level was decreased under dark conditions. (A) IAA28-GUS protein level in root was decreased gradually under dark conditions, while there was no difference in light grown seedling roots.

Similar experiments were conducted using *IAA28$_{Pro}$::IAA28-myc* seedlings. In this experiment, seedlings were first grown in light and transferred to dark for 20 hours. Proteins were extracted and a western blot was carried out to examine the level of IAA28-myc protein. The results with IAA28-myc construct corroborated the results described previously with IAA28-GUS constructs, indicating that the level of IAA28 protein decreases under dark conditions (Figure 35). In the same experiment, MG132, a proteasome inhibitor, and cyclohexamide (CYX), a protein translational inhibitor, were used to determine whether this decrease of IAA28-myc protein was due to protein degradation. In the dark, the level of IAA28-myc protein was less compared to the light control samples; however, with added MG132, the level of protein was stabilized in the dark. Addition of CYX aggravated the drop in protein levels in the dark (Figure 35). These results indicate that dark conditions increase the rate of degradation of IAA28-myc protein through the proteasome-mediated pathway.

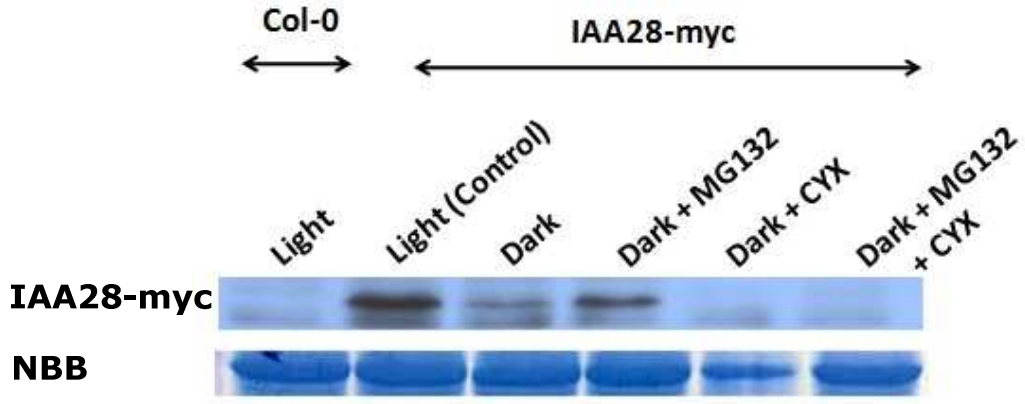


Figure 35: IAA28-myc degrades faster under dark conditions. *IAA28$_{Pro}$::IAA28-myc* seeds were grown in light and transferred to dark for 20 hrs with added MG132 or CYX. IAA28-myc degrades in the dark compared to the light control. Addition of MG132 stabilizes the protein under dark conditions. (NBB, Napthol blue black).

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3.F.iv. Light-induced expression of IAA28

To examine the effect of light on IAA28 expression, *IAA28$_{Pro}$::IAA28-myc* seedlings were grown under dark for 72 hours and exposed to white light for different time intervals. At each time points, shoot tissues, roots and entire seedlings were used, and westerns blots were carried out as described in methods.

Dark grown shoots have very low or no IAA28-myc protein, but in roots there was a detectable amount of IAA28-myc protein (Figure 36, A and B). The levels of IAA28-myc were induced drastically in both shoots and roots upon exposure to white light for 4 hours. With longer exposure to white light, shoots showed a slight fluctuation of IAA28-myc levels while roots had stable levels of IAA28-myc protein (Figure 36, A and B). White light-induced IAA28-myc expression was considerably higher in shoots compared to that of roots (Figure 36 D).

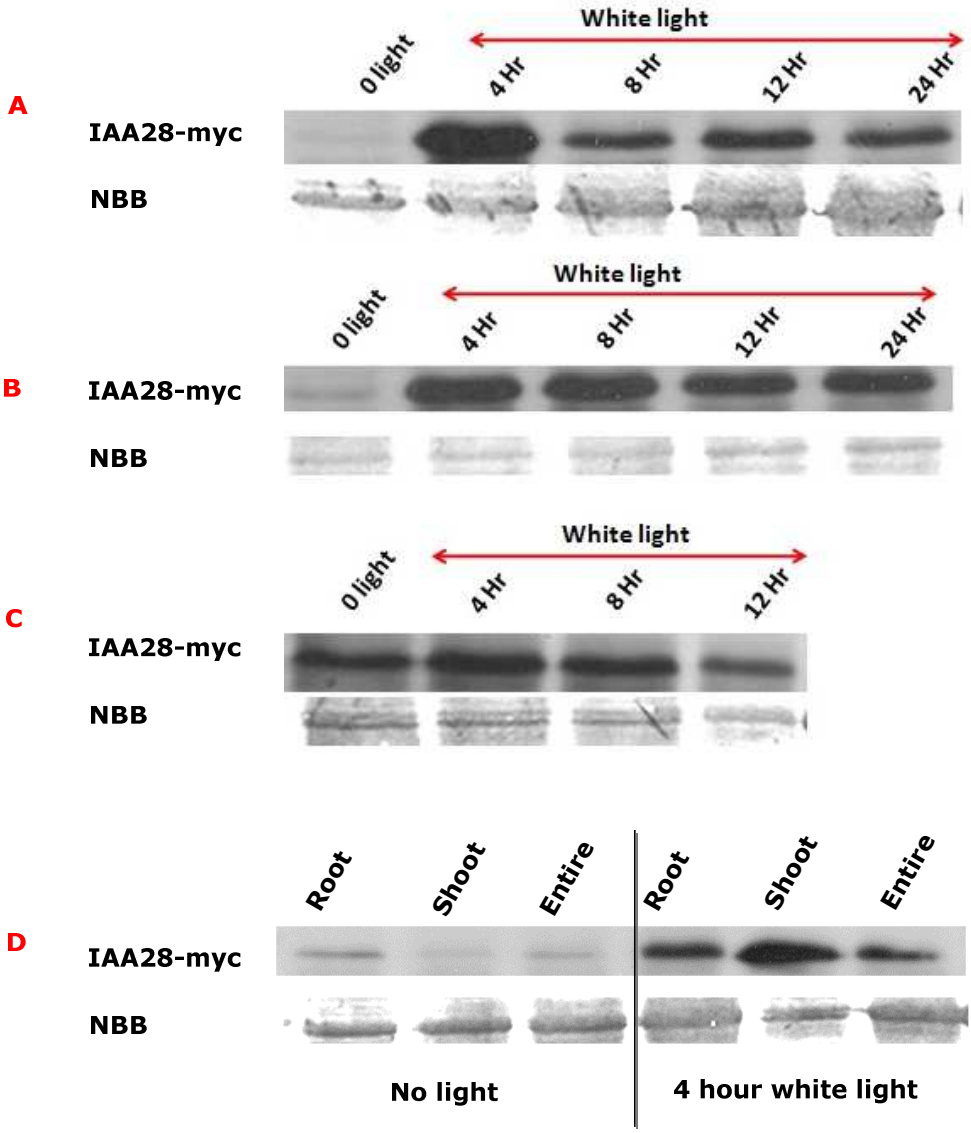


Figure 36: Effect of light on the regulation of IAA28-myc protein in dark-grown seedlings. All the *IAA28*$_{Pro}$*::IAA28-myc* seedlings were grown under dark conditions for 72 hours and exposed to white light. Samples were collected at different time points. (A) Only shoots, (B) Only roots, (C).Entire seedlings. (A and B) IAA28-myc protein was induced rapidly in both shoots and roots in response to white light. However, after 4 hours the levels of IAA28-myc dropped in shoots while roots had steady level of IAA28-myc protein. (C) Light induced induction of IAA28-myc protein was also evident when entire seedlings were used for protein extraction. (D) Induction of IAA28-myc protein in response to white light was relatively higher in shoots compared to that of roots.

3.F.v. The level of iaa28-GUS protein fluctuates in shoots in response to white light

As previous result showed a relationship between IAA28 and light signaling, further experiments were carried out to test whether IAA28 expression is regulated by circadian rhythm. For this *IAA28$_{Pro:}$:iaa28-GUS* construct was used where there is minimum protein degradation. For histological experiment, seeds were germinated in dark for 72 hours and then transferred to white light and samples were collected in every 4 hour time points and the tissues were fixed and stained for the GUS activity. The expression of iaa28-GUS was examined with a stereo-microscope. For quantitative analysis of iaa28-GUS, MUG assay was used as described in the methods section. *IAA28$_{Pro:}$:iaa28-GUS*seeds were germinated in dark for 72 hours and then transferred to white light. Shoot tissues were collected at every hour for 48 hours. At each hour, three sets of hypocotyl samples were collected and finally the average protein levels were plotted in a bar graph (Figure 37).

Both histochemical and quantitative experiment results showed that iaa28-GUS expression fluctuated during the day (Figure 37 and 38). In histochemical experiments, at the 0time point, the level of iaa28-GUS was very low or negligible and gradually increased with time until the12 hour point. The level of iaa28-GUS then slowly decreased and again increased slightly (Figure 37). The quantitative analysis also showed a similar pattern. At time zero, the level of iaa28-GUS activity was very low and gradually started to increase, producing a maximum at the 5 hr time point (11am). Then it started to decrease and again increased with time (Figure 38).

The expression of *IAA28$_{Pro}$::iaa28-GUS* in hypocotyls at different time points under white light

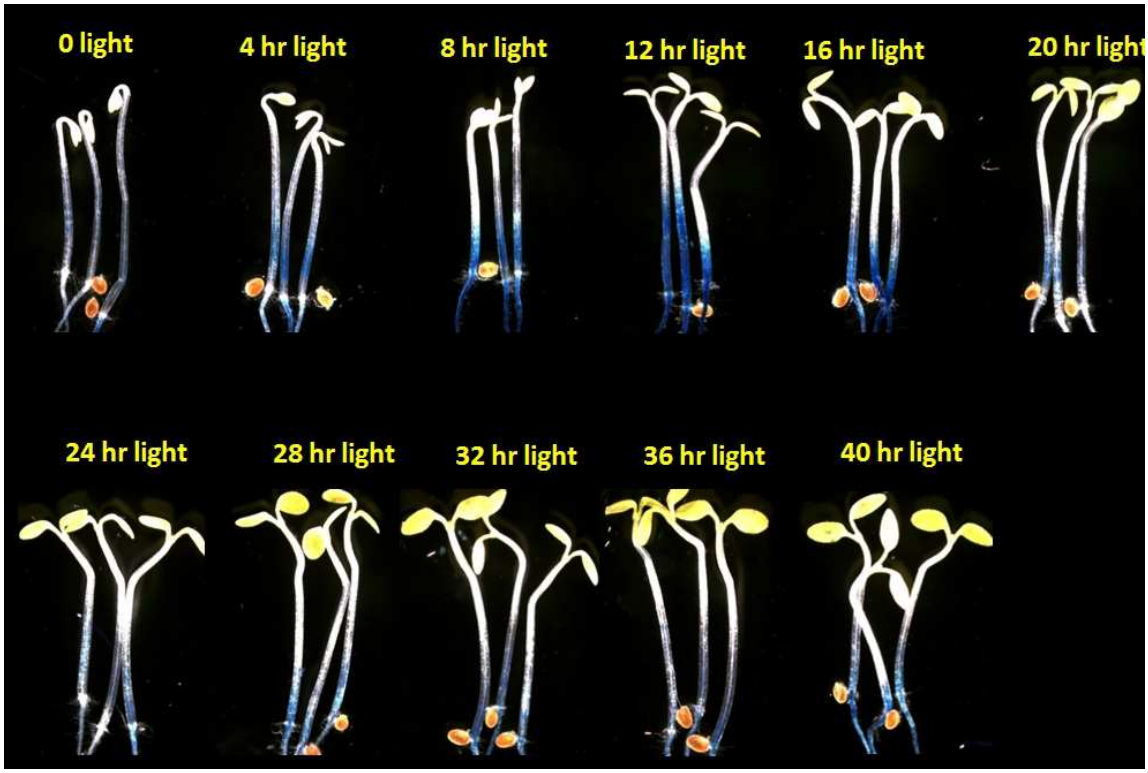


Figure 37: The expression of iaa28-GUS in shoots after different time of white light exposure. *IAA28$_{Pro}$::iaa28-GUS* seedlings were grown in dark for 72 hours and exposed to white light and collected samples at every 4 hours. The tissues were fixed and stained with GUS staining buffer. iaa28-GUS fusion protein was very low at 0 time and gradually increased till 12 hours and started to decrease and had a very low level of protein at 24 hours. Then again iaa28-GUS protein started to increase slightly in the shoots.

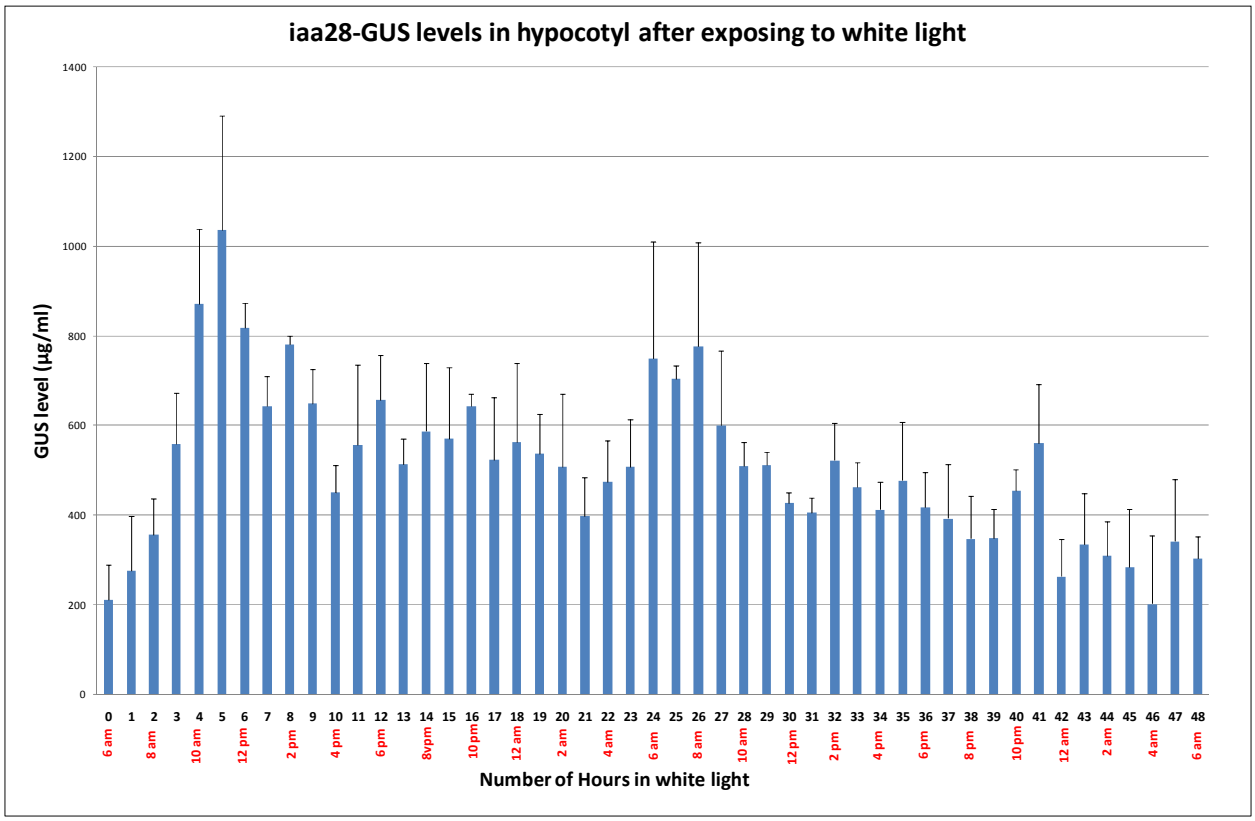


Figure 38: *IAA28$_{Pro}$::iaa28-GUS* expression in shoot tissues under white light condition (MUG assay). *IAA28$_{Pro}$::iaa28-GUS* seedlings were grown in dark for 72 hours and exposed to white light, and samples collected every hour. Shoot samples were collected in triplicate and frozen in liquid nitrogen. Proteins were extracted, and MUG assay was conducted to quantify the level of iaa28-GUS in shoots as described in methods. The level of iaa28-GUS protein fluctuated in shoots at different times after exposure to white light. At time 0, the lowest level of iaa28-GUS protein was observed, and the level increased gradually with time. The highest level of iaa28-GUS was observed at the 5 hour time point which was 11:00 am.

3.G. Identification of IAA28 interacting proteins

3.G.i. IAA28 shows wide range of different interactions in yeast two hybrid assay

The cytoplasmic localization of IAA28 suggests that it may interact with one or more cytoplasmic proteins and may have multiple functions in plant growth and development. In order to identify the putative IAA28 interacting proteins, yeast two hybrid experiments were conducted as described in methods. Here, the initial identification of first 40 putative IAA28 interacting proteins will be discussed. These 40 clones were inoculated to triple drop-out solid medium (SD-Trp-Leu-His) with different concentrations of 3-AT (0 mM, 25 mM and 50 mM). Out of these 40 clones, 8 clones grew poorly on 25 mM 3-AT containing medium and did not grow completely on 50 mM 3-AT supplemented medium. Therefore, these 8 clones were removed from further screening as false positives.

Table 3: Identified putative IAA28 interacting proteins

IAA28 Interacting protein number	Gene number/name	Functions
IP3	AT2G04550 (IBR5)	Dual specificity phosphatase
IP9	AT2G04550 (IBR5)	Dual specificity phosphatase
IP4	AT4G36800 (RCE1)	RUB conjugating enzyme
IP7	AT4G36800 (RCE1)	RUB conjugating enzyme
IP15	AT1G75590 (SAUR)	Primary auxin responsive genes
IP23	AT1G75590 (SAUR)	Primary auxin responsive genes
IP25	AT5G09590 (HS70)	Heat shock protein 70
IP40	AT2G32090	Lactoylglutathione lyase/glyoxalase family protein
IP11	AT3G23050 (IAA7)	Aux/IAA protein, repressor of auxin inducible genes

CHAPTER 4: DISCUSSION

4.A. *iaa28-2* mutation stabilizes iaa28 protein

Most of the Aux/IAA proteins share four conserved domains, domains I to IV. Domain I contains an ethylene response factor (ERF)-associated, amphiphilic, repression motif, which is known to be involved in transcriptional repression (Ohta *et al.* 2001; Tiwari *et al.* 2004). Domain II of Aux/IAA proteins is involved in the interaction with TIR1/AFB auxin co-receptors in the $SCF^{TIR1/AFBs}$ complexes and in subsequent degradation of Aux/IAA proteins through ubiquitin-proteasome pathway (Calderon-Villalobos *et al.* 2010). 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). This 13 amino acid stretch includes a highly conserved amino acid sequence of GWPPV. Single amino acid substitutions in this conserved region result in a 6 to 20-fold increase in accumulation of the mutated protein (Ramos *et al.* 2001).

In *iaa28-2*, the nucleotide change 408^{G-A} results in change of the conserved peptide sequence, **G**WPPV in domain II to **E**WPPV (Karunarathna 2008). Interestingly, this mutation causes both semi-dominant and recessive phenotypes in mutant plants. While auxin-resistant root elongation exhibits semi-dominant behavior, stunted shoot phenotype inherits as a recessive character. Similar results have also been reported for *iaa28-1* (Rogg *et al.* 2001). Pull-down data show that the bacterially expressed GST-IAA28 recombinant protein interacts with plant-derived TIR1-myc, AFB1-myc and AFB5-myc proteins in an auxin-dependent manner (Figure 10, A, B, C). However, the *iaa28-2* mutation severely interferes with this interaction, suggesting that mutant protein may be stabilized in *iaa28-2* background. The increased level of mutant iaa28 protein may inhibit the activity of ARF proteins by preventing them from modulating auxin-responsive genes, leading to pleiotropic defects in growth and development of the *iaa28-2* mutant. Similar observations have been made with other known *Aux/IAA* domain II mutants in Arabidopsis. 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* (Timpte *et al.* 1994; Nagpal *et al.* 2000), *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. Biochemical analyses have revealed that these domain II mutations stabilize the corresponding Aux/IAA proteins. For example, the mutant axr3-1/iaa17protein shows a seven-fold increase in half-life compared to the wild type version of the protein (Ouellet *et al.* 2001). Similarly, the shy2-1/iaa3 protein accumulates in *shy2-2/iaa3* mutant plants (Colón-Carmona *et al.* 2000). Expression analysis data show that while the IAA28-GUS fusion protein is degraded in response to auxin treatments, the iaa28-GUS fusion protein is less affected by auxin. These *in vivo* GUS fusion protein degradation data and *in vitro* pull down results suggest that the mutant iaa28 protein is stabilized in *iaa28-2,* resulting in the gain-of-function phenotype due to enhanced repressor activity.

4.B. Compare to IAA7, IAA28 interacts less efficiently with auxin co-receptor proteins TIR1 and AFB1

Aux/IAA proteins are degraded through the ubiquitin-proteasome pathway in response to high auxin levels. This process requires E3 ubiquitin ligases involving TIR1/AFBs. Aux/IAAs and TIR1/AFBs function as co-receptors of auxin in this process (Calderon-Villalobos *et al.* 2010; Calderón Villalobos *et al.* 2012). Previous work indicates that several Aux/IAA proteins interact with F-box proteins, TIR1 (Gray *et al.* 2001; Dharmasiri N. *et al.* 2003; Tian *et al.* 2003; Dharmasiri N. *et al.* 2005; Kepinski and Leyser 2005) and other TIR1 related F-box proteins, AFB1, AFB2, AFB3 (Dharmasiri N. *et al.* 2005b; Parry *et al.* 2009), AFB4 and AFB5 (Greenham *et al.* 2011). The presence of 29 Aux/IAA proteins and six auxin receptor F-box proteins in the Arabidopsis genome raises the possibility that different Aux/IAAs may have different affinities toward individual F-box proteins. Using an *in vitro* pull-down assay, Parry et al. (2009) showed that different F-box proteins bind to the same Aux/IAA protein with different affinities. Recently, Calderon Villalobos and coworkers showed that different Aux/IAAs have different affinities towards different TIR1/AFBs. They also showed that IAA28 interacts less efficiently with TIR1 compared to other canonical Aux/IAA

proteins such as IAA7 and IAA14 (Calderón Villalobos *et al.* 2012). In this dissertation, it has been shown using pull down experiments that compared to IAA7, IAA28 interacts less efficiently with both TIR1 and AFB1 confirming that different Aux/IAA proteins may have different affinities toward the same F-box protein(Figure 10 F,G). Since both GST-IAA28 and GST-IAA7 interact similarly with AFB5-myc in response to picloram (Figure 10 H), lower affinity of IAA28 toward TIR1/AFB1 is not due to altered properties of bacterially expressed GST fusion proteins, and may represent an actual difference between IAA28 and IAA7. It is also interesting to note that these binding data are in agreement with previously published data on IAA7 and IAA28 degradation. For example, Dreher *et al*. (2006) reported that while the half-life of IAA7 is about 5 to 10 min in the presence of auxin, the half-life of IAA28 is 80 min under same conditions. Thus, it is possible that the lower affinity of IAA28 toward TIR1/AFB1 (and possibly other AFBs) reduces the rate of its degradation. Collectively, these findings suggest that individual Aux/IAA proteins may have different affinities toward different auxin-signaling F-box proteins, and peptide sequences other than domain II of Aux/IAA proteins and the nature of auxin molecule may be important in determining the specificities of Aux/IAA-auxin-F-box protein interactions.

4.C. IAA28 shows a peculiar pattern of sub-cellular localization

Most known Aux/IAA proteins are localized to the nucleus (Ouellet *et al.* 2001; Fukaki *et al.* 2002; Song *et al.* 2009), and this is in agreement with the presence of bipartite and SV40-type nuclear localization signals (NLS) in Aux/IAA proteins (Abel and Theologis 1995; Thakur *et al.* 2005). Interestingly, this dissertation study indicates that recombinant IAA28-GUS and IAA28-myc proteins are localized to both the cytoplasm and the nucleus. Experiments with *IAA28-GUS* indicate that IAA28 is expressed at the base of the cell elongation region of the root and extends toward the root/hypocotyl junction. While IAA28-GUS recombinant protein is predominantly found in the cytoplasm of the cells in the base of the cell elongation region, recombinant protein is predominantly found in the nucleus of the cells at the mature region of the root (Figure 9 C, D, E, F, and G). However, there is still a possibility that C-terminal tags may affect nuclear localization of IAA28, but IAA7-GUS was clearly localized to the nucleus under

same conditions (Figure 9A), and IAA28-GUS was localized to the nucleus in mature root cells (Figure 9, C, D, E, F, G). Previous studies show that C-terminal tags of GUS and GFP do not interfere with nuclear localization of other Aux/IAA proteins (Abel and Theologis 1995; Ouellet *et al.* 2001; Fukaki *et al.* 2002).

Recent studies have shown that nuclear localization of some Aux/IAA proteins is affected when they interact with other proteins. For example, tobacco mosaic virus (TMV) replicase protein interacts with PAP1/IAA26, IAA18 and IAA27 and interferes with nuclear localization of these Aux/IAA proteins but does not interfere with nuclear localization of Aux/IAA proteins that do not interact with TMV replicase (Padmanabhan *et al.* 2005; Padmanabhan *et al.* 2006). These findings suggest that proteins interacting in the cytoplasm may specifically alter the localization of certain Aux/IAA proteins. Interestingly, the IAA26-P108L domain II mutation does not alter the interaction between TMV replicase and the mutant IAA26-P108L protein or the localization pattern of IAA26 (Padmanabhan *et al.* 2006). Similarly, both IAA28-GUS and iaa28-GUS proteins are localized in the same manner suggesting that domain II mutation does not affect IAA28 localization pattern. Thus, it is possible that despite the presence of nuclear localization signals, IAA28 is localized to the cytoplasm through an interaction with a cytoplasmic protein. It should also be noted that IAA28 interacts less efficiently with TIR1and AFB1 (Figure 10F and G) compared to IAA7 (Calderón Villalobos *et al.* 2012). Previous work has shown that IAA28 degrades at a slower rate compared to IAA7 (Dreher *et al.* 2006). Thus, it could be argued that slower degradation along with higher expression of IAA28 results in its cytoplasmic accumulation. However, present results with IAA28 as well as previous work (Rogg *et al.* 2001; De Rybel *et al.* 2010) on IAA28 expression do not support this idea, because high expression of *IAA28*does not show a positive correlation with the cytoplasmic localization pattern. Expression analyses with $IAA28_{Pro}$*::IAA28-GUS* seedlings along with previous studies (Rogg *et al.* 2001; De Rybel *et al.* 2010) indicate that IAA28 is expressed at a lower level in the basal area of the cell elongation zone where IAA28-GUS is predominantly found in the cytoplasm, while IAA28-GUS expression is higher in the cell differentiation zone where the protein is localized to both the nucleus and the cytoplasm. In mature root cells, although IAA28 expression is comparatively low, IAA28-GUS is predominately localized to the nucleus.

Recent work shows that IAA28 function is necessary for GATA23-dependent specification of lateral root founder cells (De Rybel *et al.* 2010). Expression analyses with both wild type and mutant constructs (*$IAA28_{Pro}$::IAA28-GUS* and *$IAA28_{Pro}$::iaa28-GUS)* showed that domain II mutation in *iaa28-2* does not interfere with the unique pattern of IAA28 localization (data not shown). Whether this pattern of IAA28 localization is involved in *GATA23*-dependent lateral root founder cell specification or any other biological function is not yet clear. However, it is interesting to note that *GATA23::GUS* is expressed just above the basal meristem region (De Rybel *et al.* 2010), which coincides with the region where IAA28-GUS protein first appears to be localized to the nucleus. Thus, nuclear localization of IAA28 may induce the transcription of *GATA23*, which is involved in lateral root priming process. De Rybel and co-workers also found that the expression level of GATA23 is significantly reduced in *iaa28-1* mutants relative to wild type plants. In addition, the expression of GATA23 in the lateral root founder cells is correlated with auxin signaling maxima in the basal meristem. In this experiment, they have examined the activity of *DR5::GUS* (an indicator of auxin levels) and *pGATA23::GUS* in the basal meristem every 5 hours from seed germination until 50 hours after germination. The results showed oscillating *DR5::GUS* maxima in the protoxylem cells of the basal meristem with a period of about 15 hours with the first peak at the 10 hour point. Simultaneously, they observed the expression of *pGATA23::GUS*, as patches along the basal meristem starting from 18 hours. Interestingly, a new patch of pGATA23::GUS appeared exactly after 10 hours of DR5::GUS maximum. Based on these results it appears that auxin maxima in the basal meristem are essential for the *GATA23* expression (De Rybel *et al.* 2010). However, this paper did not explain the reason why the *GATA23* expression is defective in *iaa28-1* background, resulting in defective lateral root phenotype.

The work in this dissertation shows that the *iaa28-2* mutation severely affects expression of auxin transporters. In brief the expression of *PIN2,* an auxin efflux carrier, which is involved in redistributing auxin basipetally through cortical and epidermal cells in the root tip area showed reduced expression in *iaa28-2* mutant roots. In addition, the expression of the auxin influx carrier *AUX1*was also severely affected in *iaa28-2* mutants roots. AUX1 plays a major role in redistribution of auxin basipetally in roots. Thus, it is

possible that, defective auxin transport in *iaa28-2* mutant roots affects the formation of auxin maxima in the basal meristem which is essential for the GATA23 expression.

4.D. IAA28 affects the auxin transport in Arabidopsis

In roots, auxin is transported acropetally through the central cylinder toward the root tip and columella cells, and then redistributed into the basipetal stream through the lateral root cap and the epidermis (Muller *et al*. 1998; Friml *et al*. 2003). Studies have shown that auxin transport plays a major role in controlling the gravity response (Muller *et al*. 1998; Marchant *etal*. 2002). Further it is also known that both gravity responses and lateral root initiation are highly correlated with each other. For example, the root waving induced by rhythmic gravistimulation promotes an alternating left to right lateral root positioning on the convex side of the bent root (De Smet *et al*. 2007). PIN proteins (auxin efflux carriers) and AUX1 (auxin influx carriers) are essential for this programmed distribution of auxin in roots that leads to bending towards gravity and lateral root initiation (Benkova and Bielach 2010; Marchant *et al*. 2002).

In general, PIN proteins function as auxin transporters (efflux carriers) at the plasma membrane for intercellular transport or at the endoplasmic reticulum membrane for intracellular regulation of auxin homeostasis (Krecek *et al*. 2009). The direction of the auxin flow is mainly determined by the polarization of PIN proteins in the membrane. This highly regulated polarization of PIN proteins in cell membranes is the key element in the formation of the auxin gradients and auxin maxima that underlie many developmental processes such as embryonic apical–basal polarity, root patterning, organogenesis and organ positioning (Krecek *et al*. 2009).

RT-PCR results showed that *iaa28-2* mutation affects the transcript level of some *PIN* genes. For example the transcript level of *PIN2* and *PIN3* are slightly lower in the mutant compared to the wild type in control condition. However, the transcript level of *PIN1* is slightly higher in the mutant compared to that of the wild type. *PIN4*, *PIN5*, *PIN6* and *PIN7* transcript levels do not show any difference in the *iaa28-2* mutants compared to the wild type. With both 2,4-D and picloram treatment the transcript levels of *PIN1*, *PIN2* and *PIN3* were increased in both wild type and *iaa28-2* mutants (Figure 25). Even

though the transcript levels of *PIN* proteins was similarly induced with both 2,4-D and picloram, effects of these two auxins on *iaa28-2* secondary root development had a significant difference from each other (Figure 13 B,C). For example, as described previously, lower concentrations of 2,4-D were unable to induce any lateral roots in *iaa28-2*; however, higher concentrations of 2,4-D were able to induce the formation of lateral root primordia along the primary root of *iaa28-2* mutants. The picloram treatments induced the formation of adventitious roots in mutants while wild type produced many lateral roots along the primary root (Figure 13). As the entire seedlings were collected in RT-PCR experiments in order to extract RNA, the differences in the localization and expression pattern of each auxin transporter was not discerned.

Thus, $PIN1_{Pro}$*::PIN1-GFP* and $PIN2_{Pro}$*::PIN2-GFP* translational construct was used to study the effect of *iaa28-2* on efflux movement of auxin. PIN1 protein is an auxin efflux carrier with 22 putative transmembrane segments (Krecek *et al.* 2009). As shown in Figure 26, the expression level of PIN1-GFP is very similar to that of in wild type under control and auxin treatments, showing that there is no effect of *iaa28-2* mutation on the *PIN1*expression. This is well supported by the fact that *PIN1* expression is restricted only to the mid cells in the root meristem area where IAA28-GUS is not expressed. As shown in Figure 9C, IAA28-GUS expression starts from the basal meristem region (only localized to the cytoplasm) and extends to the cell elongation (both cytoplasmic and nuclear localization) and maturation region (only nuclear localization). Therefore, the gain-of-function mutation in *iaa28-2* has no effect on the *PIN1* expression in the root meristem. Another possibility is that *PIN1* gene may not be regulated by *IAA28* at all in the root.

PIN2 is mainly localized in the cortical and epidermal cell layers starting from the cell elongation region (Krecek *et al.* 2009). Under control conditions the level of *PIN2-GFP* expression is considerably lower in *iaa28-2* mutant background compared to the wild type (Figure 27). PIN2 is localized to the membranes of cortical and epidermal cells in the root meristamatic and elongation region (Muller *et al*. 1998). The results in this work show that the level of *PIN2* expression was affected by *iaa28-2* mutation; specifically the level of PIN2 in the *iaa28-2* mutant root tips was considerably lower

compared to that in the wild type under control conditions (Figure 27). Even though 2,4-D treatment increased the PIN2 expression in the *iaa28-2* mutants to the same level as in wild type, picloram treatment could not induce the PIN2 expression in the mutants to the same level as in wild type (Figure 27). Based on these results, under control conditions defective expression of PIN2 in *iaa28-2* mutant roots may prevent auxin from being redistributed properly in the root tip area which is essential for lateral root priming and lateral root initiation. Therefore, the mutant seedlings show no lateral root development. However, the mutants do produce lateral root primordium like structures with high concentrations of 2,4-D treatments (data not shown). This observation can be explained by the fact that PIN2 expression increases in the mutant background with 2,4-D treatments, facilitating the redistribution of auxin in *iaa28-2* mutant roots, allowing lateral root initiation. As explained in the results section, *iaa28-2* mutants do not produce any lateral roots in response to picloram, but do produce adventitious roots from the base of hypocotyls. This observation may be due to expression of PIN2 in the mutants treated with picloram. As described earlier picloram induction of PIN2 expression is much lower in mutant roots compared to wild type, which may affect the redistribution of auxin that is essential for lateral root formation.

In addition, $AUX1_{Pro}$*::AUX1-YFP* was used to examine the effect of *iaa28-2* mutation on the expression and localization of auxin influx carriers in *iaa28-2* mutant background. Mutations in the auxin influx carrier *AUX1* lead to modified root architecture, resulting in 50% reduction in lateral root number (Hobbie and Estelle 1995). This is mainly due to the disruption in AUX1-mediated transport between the IAA source and sink tissues. In brief, AUX1 promotes IAA accumulation in the root apex, influencing the rate of initiation of lateral root primordial. Later, *AUX1* regulates lateral root emergence through IAA transport from leaves to developing lateral root primordia (Marchant *et al*. 2002; Bhalerao *et al*. 2002).

The results in this work show that the level of AUX1-YFP protein was considerably lower in *iaa28-2* mutant root tips compared to wild type but was induced to a similar extent with both 2, 4-D and picloram treatments (Figure 28). AUX1-YFP was induced in the cell differentiation region of wild type roots in response to 2,4-D, but this induction was completely defective in *iaa28-2* mutants (Figure 29). According to this

result it is possible that at lower concentrations of 2,4-D (85nM 2,4-D, the concentration used in the experiment explained in Figure 29), due to the defective AUX1-YFP localization in the cell differentiation zone of *iaa28-2*, the mutant may not have proper auxin re-distribution in the root in order to initiate lateral roots. With high 2, 4-D treatments (e.g., 5 µM 2,4-D, the concentration that induce the formation of lateral root primordial in the mutant background) the AUX1-YFP may be induced properly even in the cell differentiation region, resulting the formation of lateral root primordia.

In order to examine whether there is any connection between auxin transport and the formation of adventitious roots in response to picloram, auxin transport was studied in *iaa28-2* using translational gene construct of different auxin carriers such as *PIN1*, *PIN2* and *AUX1*. Neither *PIN1* nor *PIN2* were detected in hypocotyls or in the mature primary roots of wild type seedlings. In wild type, $AUX1_{Pro}$*::AUX1-YFP* was expressed in hypocotyls and in the mature regions of the root just below the hypocotyls (Figure 29). Even though the expression level of AUX1-YFP is lower in the *iaa28-2* mutant hypocotyls compared to wild type; it showed a similar localization as in the wild type (data not shown). With picloram treatment, the expression of AUX1-YFP increased in both wild type and *iaa28-2* mutant hypocotyls. However, the level of AUX1-YFP was still lower in the mutant background compared to the wild type but localized similarly to that of wild type (Figure 29). According to these results *iaa28-2* mutants are able to transport auxin properly from the cotyledon or young leaves towards the base of the hypocotyls. However, the mature root of *iaa28-2* mutants, just below the root-hypocotyl junction, showed a defective AUX1-YFP localization compared to wild type in response to picloram (Figure 29). Briefly, AUX1-YFP was localized to transverse membranes of each cell in the wild type, facilitating proper basipetal auxin movement, but in mutants AUX1-YFP was localized throughout the cell membrane presumably resulting in defective basipetal auxin transport. Thus, auxin transport from hypocotyls to the base of the root may be inhibited or blocked due to the mis-localization of AUX1-YFP in the mutant root. This transport defect may lead to the accumulation of auxin in the base of hypocotyls in the mutant in response to picloram, leading to the formation of adventitious roots. A mechanism to explain how picloram induces adventitious roots in *iaa28-2* in response to picloram will be discussed in detail in the next section.

4.E. Role of IAA28 in secondary root development

4.E.i. IAA28 and lateral root formation

Lateral roots originate from a subset of pericycle cells called pericycle founder cells, which are adjacent to proto-xylem pole cells (Casimiro *et al*. 2003). To examine the activity of the pericycle in *iaa28-2*, homozygous seedlings for both *iaa28-2* and *J0121* (a GAL4 enhancer trap line specific for pericycle) (Laplaze *et al.* 2005) were examined using confocal microscopy with and without auxin treatments. Expression of the pericycle marker (J0121) was lower in the *iaa28-2* roots compared to the wild type (Figure 15 A to F), however, with picloram the expression of *J0121-GFP* increased in *iaa28-2* mutants to a level similar to that in the wild type (Figure 15 G and H). Interestingly with picloram treatment, the *iaa28-2* mutants were even able to initiate the programmed cell division in pericycle cells and to produce the early cell stages of lateral root primordia (stages I, II & III) (Figure 16 E and F). These results indicate that the defect in *iaa28-2* lateral root formation is not at the initial programmed cell division but may be in emergence of lateral root primordia. This hypothesis is well supported from the results with LBD gene analysis in *iaa28-2* background discussed below.

As mentioned in the introduction, both *ARF7* and *ARF19* are involved in lateral root development. Even though the single mutants of each transcription factor showed little effects *arf7* and *arf19* double mutants showed severe defects in growth and development. For example, double mutants were defective in lateral and adventitious root formation and had reduced leaf cell expansion (Wilmoth *et al.* 2005). These results suggest that both *ARF7* and *ARF19* are positive regulators of lateral root development. In 2007, Okushima and co-workers found that *ARF7* and *ARF19* directly regulate other downstream genes such as *LATERAL ORGAN BOUNDARIES-DOMAIN16/ASYMMETRIC LEAVES2-LIKE18* (*LBD16/ASL18*), *LBD29/ASL16* (Okushima *et al.* 2007) and *LBD18/ASL20*(Lee *et al.* 2009). The defective lateral root phenotype in *arf7 arf19* double mutants was rescued through over-expression of both *LBD16* and *LBD29* in Arabidopsis. These data suggest that *ARF7* and *ARF19* regulate lateral root formation via direct activation of *LBD/ASL* genes in Arabidopsis (Okushima *et al.* 2007). In addition, studies have shown that, *SLR1/IAA14, MSG2/IAA19* and *AXR5/IAA1* and *IAA28* interact with *ARF7* and *ARF19* in yeast system (Tatematsu *et al*.

2004; Fukaki *et al*. 2005; Weijers *et al*. 2005; De Rybel *et al*. 2010). Therefore, it is possible that these Aux/IAA proteins may interact with ARF7 and ARF19 to regulate the downstream genes *LBD16, LBD18* and *LBD29*that positively regulate lateral root development.

Expression analysis of *LBD* genes using RT-PCR and histological assays (Figure 17-21) showed that both transcript and protein levels of *LBD16* and *LBD29* were lower in *iaa28-2* mutants compared to those in wild type. The transcript levels of all *LBD* genes (*LBD16, LBD29* and *LBD18*) increased in response to both 2,4-D and picloram treatments in *iaa28-2* background similar to that in wild type (Figure 16). However, even though transcript levels of all the *LBD* genes tested increased in response to both picloram and 2,4-D, these two auxins showed different effects on the secondary root formation in *iaa28-2* mutant background (section 3.C.i). Therefore, expression analysis of *LBD16* and *LBD29* in *iaa28-2* background was done by using $LBD16_{Pro}$*::LBD16-GUS* and $LBD29_{Pro}$*::LBD29-GUS* transgenic lines.

The expression analysis data showed that LBD16-GUS was mainly detectable in wild type hypocotyls, differentiation regions and distal differentiation regions of root but not in the root tip or the cell elongation region (Figure 18). Previous studies have shown that even though *LBD16* is strongly expressed in vascular tissues in the mature region of roots, the auxin-mediated induction is only observed in the elongation zone (Okushima *et al*. 2007). However, with picloram, the induction of LBD16-GUS was mainly observed in the differentiation region of the wild type (Figure 18 A) which was not observed in *iaa28-2* mutants. Picloram was only able to induce the LBD16-GUS expression in the distal differentiation region of *iaa28-2* seedlings. According to these results, it is obvious that *iaa28-2* mutation interferes with the expression of *LBD16*, resulting in defective lateral root formation under control conditions. As picloram treatments were unable to rescue the expression of LBD16-GUS in mutants similar to wild type, the mutants were unable to develop lateral roots in response to picloram.

Similar results were also obtained for LBD16-GUS expression in the mutants with low concentrations of 2,4-D treatments. In wild type, the LBD16-GUS protein level was highly induced throughout the root except for the root meristem with 85 nM 2, 4-D treatments. However, with this 2,4-D treatment, no LBD16-GUS was observed in the

mutants (Figure 18 C). In contrast, in the presence of high concentrations of 2,4-D (5 µM) both *iaa28-2* mutants and wild type roots showed a similar induction pattern of LBD16-GUS (data not shown). This defective LBD16-GUS expression well explains the defective lateral root phenotype of *iaa28-2* in response to 2,4-D. At lower concentration, 2,4-D was unable to induce any lateral roots in the mutants; however, higher concentrations of 2,4-D were able to induce the formation of many lateral root primordia along the primary root of both *iaa28-2* mutants and the wild type.

The expression of $LBD16_{Pro}$::*LBD16-GUS* with IBA was also studied. As shown in figure 18 D, IBA was able to induce LBD16-GUS in *iaa28-2* mutants in a similar pattern as in wild type, but to a lesser extent. Activation of LBD16-GUS in the mutant roots in response to IBA completely rescues the defective lateral root phenotype in *iaa28-2* (Figure 13 H).

In addition to *LBD16-GUS*, the expression of *LBD29-GUS* was also studied in *iaa28-2* in order to understand the effects of *iaa28-2* mutation on *LBD29*-mediated lateral root development. According to Okushima *et al.* 2007, auxin-mediated induction of *LBD29* was observed in the steles of mature regions in primary and lateral roots. In this experiment they used only 1-NAA as the auxin. However, the work presented here showed that induction profiles of LBD29 (and LBD16) are different with different auxins, resulting in slightly different outcomes in relation to lateral root formation. For instance, translational fusion construct of LBD29-GUS was detected only in the maturation region of the wild type roots under control conditions. With picloram treatment, the level of LBD29-GUS increased in both differentiation and distal differentiation regions of the wild type; however, in *iaa28-2* mutants *LBD29-GUS* was expressed only in the distal differentiation region (Figure 20 A and B).

In wild type seedlings treated with 2, 4-D, $LBD29_{Pro}$::*LBD29-GUS* was expressed along the primary root, including the cell elongation region but not the root meristem. Mutants also showed an induction pattern of LBD29-GUS similar to wild type, but did not show the induction in the cell elongation region or the start of cell differentiation region (Figure 20 C). It is interesting to note that even though LBD29-GUS was induced in *iaa28-2* with a partial similarity to the wild type with 2,4-D (85 nM) treatment, this 2,4-D concentration was unable to induce any lateral roots in the mutants.

Further, expression analysis of *DR5::GFP* (Figure 12 B and G) and *AUX1$_{Pro}$::AUX1-YFP* (Figure 30) in *iaa28-2* background with 2,4-D showed a defective expression of both *DR5::GFP* and *AUX1$_{Pro}$::AUX1-YFP* in the mutant root differentiation zone. Thus, it is possible that both auxin responsive gene transcription and auxin transport is defective in *iaa28-2* mutants resulting in defective lateral root phenotype.

With IBA treatments, LBD29-GUS increased in the cell differentiation and distal differentiation region, but not in the elongation region of the wild type. However, in mutants LBD29-GUS was only detected in the distal differentiation region of the root with IBA treatment (Figure 20D).

In a summary, *iaa28-2* mutation affects the expression of both *LBD16* and *LBD29* transcription factors. Under control conditions both LBD16-GUS and LBD29-GUS were not detectable in the root system. These results show that the *iaa28-2* mutation severely affects the expression of downstream targets of lateral root formation like *LBD16* and *LBD29,* leading to defective lateral root development. Due to the domain II mutation of *iaa28-2* protein, the mutant *iaa28-2* protein may accumulate in the cells, resulting in inhibition of the transcriptional activation of downstream *LBD16* and *LBD29* genes through *ARF7* and *ARF19* transcriptional factors. Due to the lack of LBD gene expression under control conditions, the mutant seedlings do not initiate or develop lateral roots.

4.E.ii Role of IAA28 in adventitious root formation

Interestingly, a high concentration of picloram induces a few secondary roots in *iaa28-2* with a characteristic pattern (Figure 13 D and I). These secondary roots emerge from the same point at the base of the hypocotyl region. This result was confirmed by studying the *CycB::GUS* expression in *iaa28-2* in response to picloram. In the presence of picloram, GUS staining appears in the lower hypocotyl region of *iaa28-2*, indicating that picloram induces adventitious roots in *iaa28-2*. The reason picloram especially induces adventitious roots in *iaa28* is not clear. However, pull down data indicate that mutant iaa28 protein still interacts with AFB1 in response to picloram, although less efficiently compared to the wild type IAA28 protein (Figure 10D). In addition, despite the domain II mutation, iaa28-GUS recombinant protein was still degraded in the

hypocotyl with picloram treatment (Figure 21). This finding can be further supported from the results obtained with *AFB1-GUS* expression in the hypocotyls. The results show that the protein level of AFB1-GUS was drastically increased in hypocotyls in response to picloram treatment (Figure 22 and 23). Thus, this high level of AFB1 auxin co-receptor protein may further accelerate the degradation of mutant iaa28-2 protein in the hypocotyls in response to picloram. Therefore, this AFB1 mediated mutant iaa28-2 protein degradation may de-repress the activity of ARF7 and ARF19. This de-repression will induce the expression of downstream genes such as LBD genes (LBD16 and LBD29), resulting in adventitious root formation. This observation was well supported from the results obtained with the expression analysis of $LBD16_{Pro}$*::LBD16-GUS* in an *iaa28-2* mutant background. The results showed that the level of LBD16-GUS was increased significantly in *iaa28-2* hypocotyls compared to the wild type hypocotyl in response to picloram (Figure 17 B and E). However, neither 2,4-D nor IBA were able to induce LBD16-GUS in the mutant hypocotyls as picloram could. This picloram-mediated induction of LBD16-GUS in *iaa28-2* hypocotyls may induce the formation of adventitious roots. Surprisingly the expression of $LBD29_{Pro}$*::LBD29-GUS* did not show any induction in the mutant hypocotyls with picloram treatment. In wild type there was a slight induction of LBD29-GUS in the hypocotyls in response to picloram compared to the hypocotyls in the control seedlings. Based on these results, it can be proposed that both AFB1 and LBD16 are involved in the formation of adventitious roots in *iaa28-2* mutants in response to picloram.

Based on these results, the next question raised was if the mutant iaa28-2 protein can be degraded through AFB1 in the mutants in response to picloram, why are the mutant seedlings incapable of producing lateral roots similarly to the wild type? This observation can be explained in several different ways. For example, GUS staining data indicate that iaa28-GUS protein accumulates in roots to a much higher level than in hypocotyls. Therefore, it is possible that high iaa28 levels in the primary root prevent lateral root induction, but the degradation of iaa28 in hypocotyls in response to picloram may induce adventitious roots in *iaa28*. This conjecture can be further supported by the induction profile of AFB1-GUS in the primary root in response to picloram. As shown in Figure 23 A and C, even though the level of AFB1 was increased in the hypocotyls with

picloram, in roots the level of AFB1-GUS was drastically reduced especially in the root tip, cell elongation region, cell differentiation region as well as in some parts of the maturation region. However, under control conditions as well as with both 2,4-D and IBA treatment, *AFB1$_{Pro}$::AFB1-GUS* was expressed throughout the primary root. Therefore, it is possible that this low level of AFB1-GUS protein in the primary root in response to picloram may retard the degradation of mutant iaa28-2 protein in the primary root, inhibiting the formation of lateral roots as in the wild type. Even though IAA28 expression is high in the primary roots of wild type, efficient basal level degradation of IAA28 in response to picloram may induce lateral roots thereby preventing adventitious root induction in wild type seedlings. Another possibility is that *iaa28-2* mutation may interfere with auxin transport in the primary root leading to the accumulation of auxin at the base of the hypocotyls resulting in picloram-induced adventitious root development in *iaa28-2*. In addition, as described previously, auxin transport is also defective in iaa28-2 mutations. Specifically, auxin influx carrier AUX1-YFP showed defects in the localization in the mutant roots just below the hypocotyls in response to picloram. However, AUX1-YFP was localized properly in the same region of wild type root in response to picloram. Thus, this picloram-mediated, defective auxin transport from hypocotyls to roots in the mutants leads to the formation of adventitious roots. Unlike picloram, both 2,4-D and IBA did not affect the localization of AUX1-YFP in mutant roots.

4.F. Role of IAA28 in light signaling

Light influences the auxin response in many different ways, including modulation of auxin levels, transport and auxin responsiveness. Light induces auxin biosynthesis in young leaves, and due to this, dark grown seedlings are largely devoid of auxin (Bhalerao *et al.* 2002). Many studies have shown that light signaling and auxin responses are interconnected to each other and recently, Laxmi and co-workers published that light signaling directly regulates the function of auxin efflux carriers such as *PIN1*, *PIN2* and *PIN7* (Laxmi *et al*. 2008). In this paper, they found that in seedlings growing in dark, the plasma membrane localization of PIN2 is largely reduced and it is more localized to vacuolar compartments. A similar effect was also observed when plants were kept under

far red or red light continuously. In the same study they found that dark-mediated vacuolar compartmentalization is very much reduced in the presence of the protease inhibitor MG132 and also in the *cop1* (*Constitutive photomorphogenic1*) mutant. COP9 is an E3 ubiquitin ligase involved in 26S proteasome-mediated protein degradation (Laxmi *et al*. 2008). In the dark, COP1 accumulates in the nucleus and stimulates the degradation of transcription factors such as HY5, HYH (HY5 homology) and HFR. As a consequence the expression of light regulated genes is suppressed. In contrast, in light COP1 is degraded and its degradation is permits expression of light-regulated genes, promoting photomorphogenesis (Laxmi *et al*. 2008).

The data discussed in the section 3F in this dissertation support the idea that *IAA28* links light to auxin signaling. For example under white light the mutant plants produce a longer hypocotyl compared to the wild type. In addition *iaa28-2* mutants show altered responses to growth under different light conditions. As mentioned previously, continuous red light induces the elongation of hypocotyl through the action of *PhyB* (shade avoidance syndrome) (Quail 1995). In contrast continuous far red illumination inhibits hypocotyl elongation through *PhyA* action (shade-survival mechanism) (Quail 1995). The results in this work showed that *iaa28-2* mutants had significantly longer hypocotyls under red light compared to those in Col-0 grown under the same conditions. In addition, the percent seed germination of *iaa28-2* seeds was higher in red light compared to that of wild type. In conclusion, these results show that some of the developmental processes of *iaa28-2* are altered under red light.

Interestingly, expression analysis of IAA28-myc in shoots under different light conditions showed that IAA28-myc may be modified under red and far red light conditions. According to Figure 34, IAA28-myc appeared as two closely separated bands, indicating that one might be a modified version (top) and the other is an un-modified form (bottom). It is worth noting that the shoot tissues used in this experiment were collected after 4 hours in different light treatments. However, IAA28-myc exists as a single band under both blue and white light conditions, and this band was paralleled the top band observed from plants incubated under both red and far red light, indicating that IAA28-myc exists in its modified form under white and blue light. Time course

experiments showed that the formation of the modified form of IAA28-myc occurs gradually (Figure 34). In white light and red light initially IAA28-myc appeared as un-modified form, and at the 1 hour time point both forms were detectable. Later the modified form became dominant. However, under blue light only the modified form of IAA28-myc was observed.

In 2000, Colon-Carmona and co-workers showed that some of the Aux/IAA proteins such as IAA3, IAA17, IAA1, IAA9 and Ps-IAA4 are phosphorylated by recombinant oat phytochrome A in vitro. However, they did not identify the exact amino acid residues that are phosphorylated. In addition, pull-down experiments showed that IAA3 interacts with Arabidopsis PhyB invitro (Tian *et al.* 2002). These findings raise the possibility that IAA28 may be phosphorylated by phytochromes. The sequence analysis of IAA28 with IAA1, IAA3 and IAA17 showed putative phosphorylatable sites; however, further studies are required to confirm this hypothesis.

Further, this work has shown that both IAA28-GUS and IAA28-myc degrades in roots faster under dark conditions and is induced rapidly in shoots when exposed to light conditions. In 2007, Salisbury and co-workers published that both *IAA1* and *IAA3* are expressed at higher levels in *PhyB* mutant shoots (Salisbury *et al.* 2007). They proposed that both *IAA1* and *IAA3* are regulated through *PhyA*- and *PhyB*-mediated light signaling through the transcriptional activation of *HY5*. HY5 protein has been shown to regulate transcription of light-regulated genes though binding to a specific sequence in the target promoter sequence named the G-box sequence (CCACGTG) (Ang *et al*. 1998). This G-box sequence was found within the promoter regions of *SLR/IAA14*, *AXR2/IAA7* and *IAA28* (Cluis *et al.* 2004; Salisbury *et al.* 2007). Interestingly, the transcript levels of these 3 *Aux/IAA* genes, including *IAA28,* were found to be reduced in *hy5* mutant background, supporting the idea that HY5 may be regulating the transcription of these *Aux/IAA* genes (Cluis *et al.* 2004). According to the current model, under dark conditions the level of COP1 (an E3 ligase) is high in the nucleus where it interacts with HY5 and other transcriptional factors such as CIP1. This interaction prevents transcriptional activation by HY5 and CIP1. However, under light conditions COP1 degrades and moves back to the cytoplasm, creating a low level in the nucleus. Lack of COP1 in the nucleus

under light conditions releases HY5 and other transcriptional factors, activating expression of other light-regulated genes (Wang *et al.* 2005). These findings together with data presented here work strengthen the idea that IAA28 may be involved in phytochrome-mediated light signaling in plants.

4.G. IAA28 shows a wide range of different interactions in yeast two hybrid assay

A yeast two hybrid screening was conducted to identify putative IAA28 interacting proteins. Forty putative IAA28 interacting proteins were chosen for further analysis. Here a total of nine clones were sequenced for identification. As shown in Table 3, the candidates included a wide variety of proteins involved in different functions in plant growth and development. Two proteins out of these nine were IBR5, a dual specificity phosphatase that is involved in dephosphorylating MAPK12 (Augustus *et al*. 2003; Lee *et al.* 2008). *ibr5* is an Arabidopsis indole-3-butyric acid response mutant, and it also is less responsive to indole-3-acetic acid and the plant stress hormone abscisic acid. Studies have shown that IBR5 is a phosphatase that modulates phytohormone signal transduction and support a link between auxin and ABA signaling pathways (Augustus *et al*. 2003).

IP4 interacting protein was identified as RCE1 (RUB conjugating enzyme 1) a protein that is involved in RUB modification of CUL1 protein, which is a member of E3 ligase complex in Arabidopsis. Studies have shown that RUB modification is important for SCF function and RUB modification of CUL1 is required for normal function of SCF^{TIR1}. RCE1 can directly interact with the RING protein RBX1, and it is present in a stable complex of SCF^{TIR1} (Dharmasiri N. *et al*. 2003). IP11 was identified as IAA7 another Aux/IAA protein in which the gain-of-function mutation causes severe agravitropic responses in roots and shoots, short hypocotyls and auxin-resistant root growth (Nagpal *et al*. 2000). IP15 and IP23 were identified as At1g19840 (SAUR-like protein) which also act as a primary auxin responsive genes. IP25 was identified as At5g09590, a heat shock cognate protein (HSC-70) which is involved in responding to heat, salt stress in Arabidopsis. IP40 was identified as At2g32090, lactoylglutathione lyase/glyoxalase1 family protein mentioned above. It is also involved in responding to biotic and abiotic stresses in plants. However, more studies will be needed to confirm

these interactions and to understand what the biological significance of these interactions in plant growth and development are?

4.H. Hypothetical model to explain functions of IAA28 in secondary root formation in Arabidopsis

As discussed above, IAA28 shows a unique expression pattern along the primary root. IAA28-GUS is localized only to the cytoplasm in the root area just below the basal meristem. In some regions IAA28 is found in both nucleus and cytoplasm, and in the mature regions of the root, IAA28 is localized in the nucleus. The nuclear localization of IAA28 was first observed in the basal meristem region where *GATA23*-dependent lateral root priming occurs. In 2010, De Rybel and co-workers showed that *GATA23* expression is highly correlated to the auxin maxima in the basal meristem and the expression level of *GATA23* is severely reduced in *iaa28-1* background. This work shows that the *iaa28-2* mutation affects auxin transport, specifically *PIN2*- and *AUX1*-dependent auxin redistribution in the root tip area. This defective auxin redistribution may lead to defective *GATA23* expression in *iaa28-2* mutants, resulting in defective lateral root formation. In addition, *LBD16* and *LBD29* genes, which act as downstream targets of lateral root formation, show defective expression in *iaa28-2* mutants. Similarly, the expression of pericycle marker, *J0121* is lower in *iaa28-2* background; however, the results show that with picloram, *iaa28-2* mutants are able to initiate lateral roots but are unable to form fully developed lateral roots. Further, high concentrations of 2,4-D and IBA were also able to induce lateral roots. This observation is also well supported by the fact that *LBD16* and *LBD29* expression was induced similarly to the wild type in *iaa28-2* mutants in response to high concentrations of 2,4-D and IBA.

Studies were conducted to understand the mechanism of how picloram induces the formation of adventitious roots in *iaa28-2* background. Interestingly, the localization of AUX1-YFP was disrupted in *iaa28-2* in response to picloram. This disruption may prevent auxin transport from shoot to roots in the mutant background. In addition, the expression of *AFB1* auxin co-receptor is induced in hypocotyls in response to picloram. As mutant iaa28-2 interacts with AFB1 in the presence of picloram, this induction of AFB1 in the hypocotyls accelerates the degradation of mutant *iaa28* protein in hypocotyls. This degradation may eventually lead to transcription of *ARFs* such as *ARF7*

and *ARF19* that are involved in lateral root formation. The transcription of *ARFs* will eventually induce their downstream targets, such as *LBD* genes involved in secondary root formation. Supporting this hypothesis, the level of *LBD16* is highly induced in mutant hypocotyls compared to that of wild type in response to picloram. Finally as a combination of all these events, mutant *iaa28-2* forms adventitious roots in response to picloram. In conclusion this work had identified that *IAA28* and *AFB1* together regulate adventitious root formation in Arabidopsis through the activation of downstream genes such as *LBD16* (Figure 39).

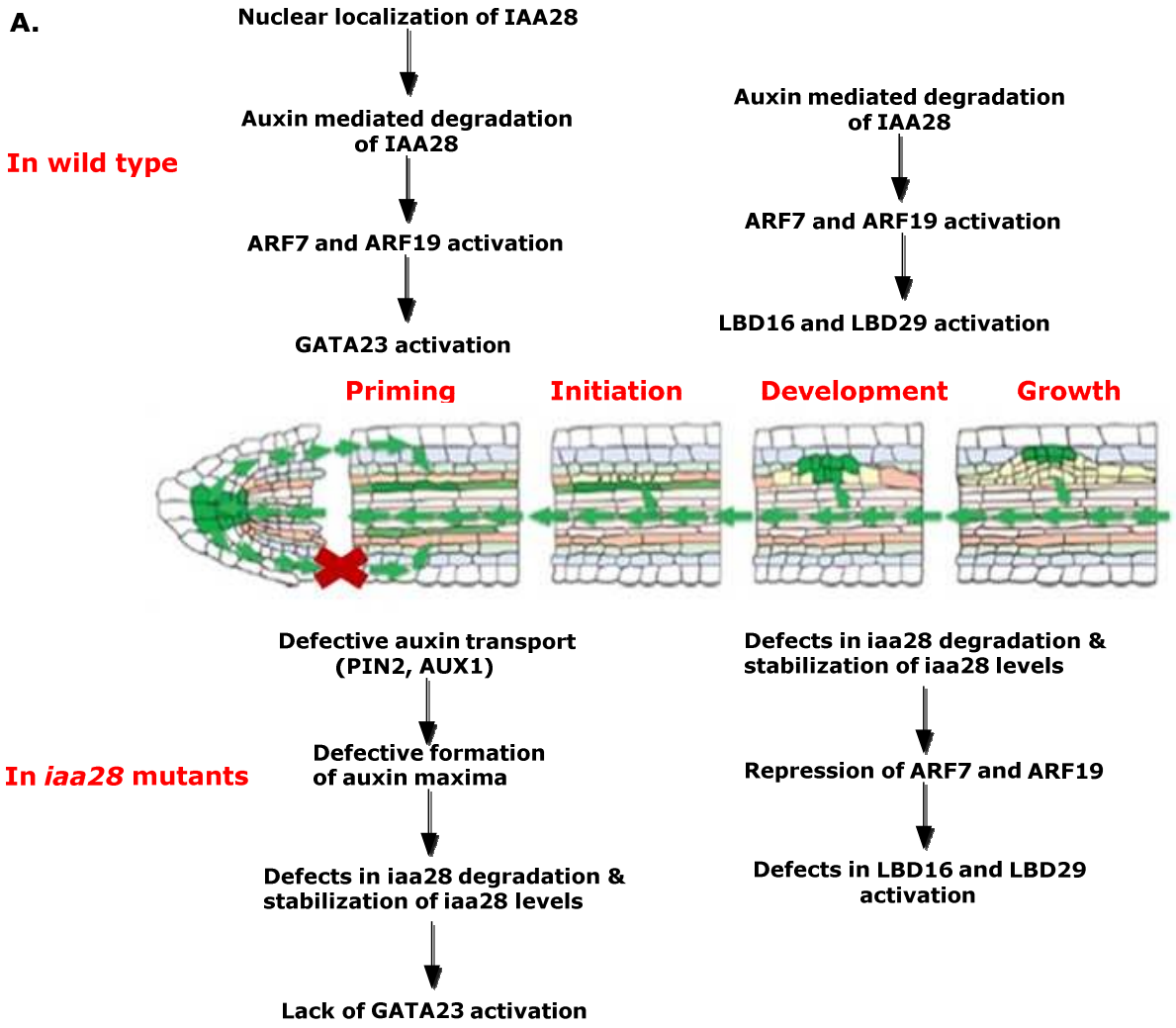
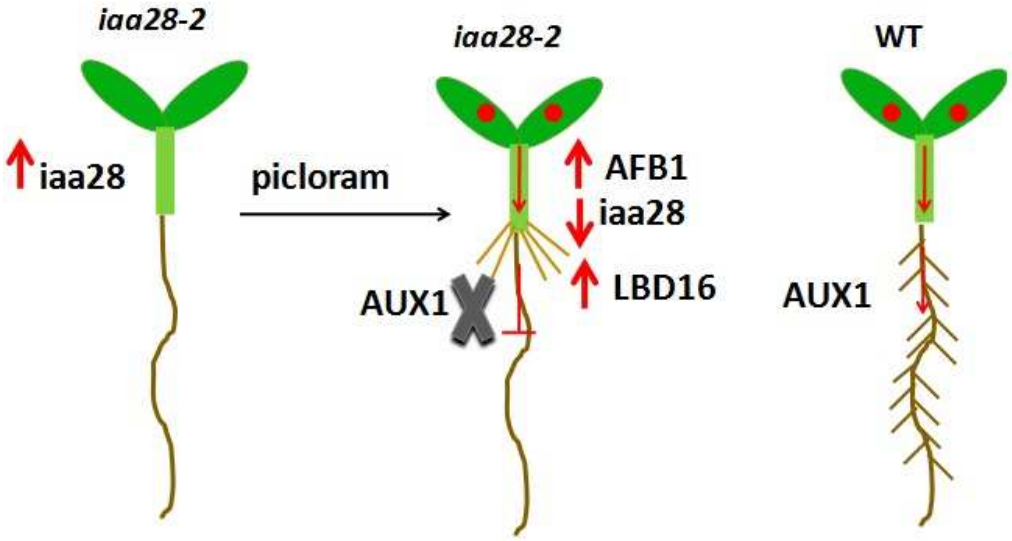


Figure 39: Schematic representation of a model indicating the roles of IAA28 in secondary root formation. (A) Roles of *IAA28* in lateral root formation. IAA28 is localized in the nucleus in the basal meristem and is involved in the activation of GATA23-dependent priming of lateral roots. Creation of auxin maxima through programmed auxin transport is essential for the priming process. The *iaa28-2* mutation severely affects auxin transport in the root tip, specifically*PIN2*- and *AUX1*-dependent auxin transport involved in redistributing auxin in the root tip area. These defects in auxin transport may affect the creation of auxin maxima in the basal meristem. Thus, mutant iaa28 protein is stabilized in the system, resulting in lack of GATA23-dependent lateral root priming. Defective lateral root priming causes defective lateral root phenotype in *iaa28-2* mutants. (B) Role of IAA28 in adventitious root formation. Mutant *iaa28-2* hypocotyls have stabilized iaa28 protein. The level of AFB1 increases in the hypocotyls in response to picloram. AFB1 degrades mutant iaa28 protein in hypocotyls in the presence of picloram. This degradation may activate the transcription of ARF7 and ARF19 in an *iaa28-2* mutant background, resulting in the induction of downstream targets of lateral root formation such as LBD16. Additionally, in *iaa28-2* mutants AUX1-dependent auxin transport from shoot to root is affected compared to that of wild type. These events together induce the formation of adventitious roots in *iaa28-2* mutant background. (The root structure in A section was modified from (Dastidar *et al.* 2012).

Figure 39. Continued.

B



Appendix I

GUS fixing solution (Oono *et al.* 1998)

For 10 ml

Formaldehyde	= 81 µl (0.3%)
Mannitol	= 6 ml from 0.5M (0.3 M)
100 mM MES	= 1 ml

Appendix II

GUS staining solution (Oono *et al.* 1998)

For 10 ml

Na_2HPO_4	= 2 ml from 0.5M (100 mM final)
Potassium ferricyanide	= 50 µl from 100 mM (0.5mM final)
Potassium ferrocyanide	= 50 µl from 100 mM (0.5mM final)
EDTA	= 200 µl from 0.5 M (10 mM final)
Triton	= 10 µl from 20% (0.1% final)
X-gluc	= 5-7 mg
Water	= up to 10 ml

Appendix III

EZ- Protein extraction for western blots (protocol modified in Dharmasiri Lab)

Buffer E

125 mM tris-HCl (pH = 8.8)

1% SDS (w/v)

50% glycerol (v/v)

50mM $Na_2S_2O_5$)

Buffer Z

125 mM tris-HCl (pH = 6.8)

12% SDS (w/v)

10% glycerol (v/v)

22% β- mercaptoethanol (v/v)

0.001% Bromophenol blue (w/v)

1. Grind 10 mg leaf sample in a 50 µl of buffer E till the tissue is homogenous and transfer on to ice. When all samples are prepared keep all samples at room temperature for 5 minutes to solubilize SDS. Centrifuge at 13000g for 10 minutes and take the supernatant.
2. Add $1/10^{th}$ volume of buffer Z and boil the samples for 6 minutes immediately before loading on to the SDS-PAGE.

Appendix IV

2X Laemmli sample buffer (2X LSB) for 9 ml (protocol modified in Dharmasiri Lab)

0.5 M Tris-HCl (Ph= 6.8)	= 5 ml
SDS	= 0.4 g
Glycerol	= 2 ml
Milli Q water	= 2 ml
Bromophenol blue	= 0.3 mg

1. Heat to about 40 ºC to dissolve.

2. Store at -20 ºC as 0.9 ml aliquots. Add BME (β- mercaptoethanol) to a final concentration of 10%.

Appendix V

Figure 11 C. Quantitative GUS activity in *IAA28$_{Pro}$::IAA28-GUS* and *IAA28$_{Pro}$-iaa28-GUS* seedlings treated with 20 µM 2,4-D or 50 µM picloram

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