UNIVERSITY OF CALIFORNIA, SAN DIEGO

Characterization of the PRE protein family in *Arabidopsis* and their interactions with Aux/IAAs

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

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by

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The thesis of Silka Jacqueline Cheng is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

I dedicate this thesis to everyone who supported and helped me both grow as an individual and as a researcher.

To my family and friends for your unwavering support and love.

To Kenny for being with me through the good times and making the harder times a little more fun.

And to Cristina, without whom I would not be the researcher I am now, for teaching and guiding me during this journey. Her enthusiasm for research will always remind me why learning is so exciting.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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ABSTRACT OF THE THESIS

Characterization of the PRE protein family in *Arabidopsis* and their interactions with Aux/IAAs

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Auxin perception and signaling requires the rapid degradation of the Aux/IAA proteins that negatively regulate ARF (Auxin response factors) transcription factors. The SCF^{TIR1} complex in the ubiquitin proteasome system (UPS) is required for Aux/IAA degradation through its role as the auxin hormone receptor and as the E3 ligase in the UPS. Any proteins that may play a role in this pathway are important for the study of cell elongation and development. KIDARI (KDR), inducible by auxin in the hypocotyl of the

model plant *A. thaliana*, is a member of the PRE (paclobutrazol resistant) family. The PRE family of atypical bHLHs do not bind to DNA directly and instead interrupt the homo-/heterodimerization of other atypical bHLHs or canonical bHLHs. Using a yeast-2-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC), KDR, PRE1, PRE2, and PRE5 are shown to interact with various Aux/IAAs. A mutation in KDR, made by a change in the conserved leucine residue in position 29 (KDRL29E), results in a loss of interaction with Aux/IAAs. Additionally, complementation of the *kdr-2* mutant phenotype shows that the short hypocotyls observed in the mutant are indeed due to a loss of KDR. The GUS reporter gene was used to characterize the expression patterns of the KDR and PRE5 promoters and proteins. The GUS reporters revealed expression in the hypocotyls, consistent with a role for these genes in promoting growth. However, it is possible that regulatory elements that facilitate spatial patterning may be absent in the transcriptional fusions of KDR and PRE5.

I:

Introduction

Plant hormones are organic substances that are present in low concentrations and usually function in locations away from their site of synthesis to coordinate the physiology and development of plants. Auxin, one of the first plant hormones to be discovered, regulates virtually every aspect of the plant life cycle including embryogenesis, vascular tissue development, root and shoot formation, and photo- and gravitropism (Abel and Theologis 1996). At the cellular level, it regulates elongation, division, and cell differentiation (Abel and Theologis 1996). Indole-3-acetic acid (IAA), the naturally abundant auxin, is conserved in all land plants and can be synthesized from tryptophan or indole (Chapman and Estelle 2009; Santner, Calderon-Villalobos, Estelle 2009). Auxin can also play a role in the pathways of other hormones such as gibberellins (GA), a phytohormone that also regulates growth and development (Santner, Calderon-Villalobos, Estelle 2009). For example, hypocotyl cell elongation due to GA can be increased with the addition of IAA (Saibo et al. 2003). The responses to this hormone greatly affect a plant as seen with the use of the synthetic auxin 2,4-dichlorophenoxyacetic acid as an herbicide to control weeds (Santner, Calderon-Villalobos, Estelle 2009).

Auxin-dependent transcriptional regulation: ARFs and AUX/IAAs

Auxin modulates the transcription of the auxin-regulated genes by the complex and dynamic interaction between two families of transcriptional regulators: the auxin reponse factors (ARFs), and the Aux/IAA nuclear proteins (Guilfoyle, Ulmasov, Hagen 1998; Tiwari et al. 2001; Ulmasov, Hagen, Guilfoyle 1999). ARFs are transcription factors that bind to specific auxin responsive DNA motifs, namely the auxin response

elements (AuxREs) (Guilfoyle and Hagen 2007) and can either activate or repress auxin responsive genes. There are 5 members of the ARF family (ARF5, ARF6, ARF7, ARF8, ARF19) that activate transcription in the model plant *Arabidopsis thaliana*. For example, ARF7 and ARF19 activate auxin response genes to regulate lateral root formation (Okushima et al. 2007; Ulmasov, Hagen, Guilfoyle 1999).15 other members may act as repressors and the function of 3 others has not yet been determined (Mockaitis and Estelle 2008; Tiwari, Hagen, Guilfoyle 2003; Ulmasov, Hagen, Guilfoyle 1999). ARFs contain 3 domains: a DNA binding domain, a middle domain that determines activation or repression of transcription, and a domain that allows for dimerization (Ulmasov, Hagen, Guilfoyle 1999). It is through this last domain, the C-terminal domain, that Aux/IAAs are capable of interacting with the ARFs (Kim, Harter, Theologis 1997; Ulmasov, Hagen, Guilfoyle 1997; Ulmasov, Hagen, Guilfoyle 1999).

The Aux/IAA family is comprised of 29 members in *A. thaliana* (Berleth, Krogan, Scarpella 2004). They are short-lived nuclear proteins that contain four conserved domains (Dharmasiri and Estelle 2002; Reed 2001; Zenser et al. 2001). Domain I is a repressor domain with an EAR (Ethylene Response Factor [ERF]-associated amphilic repression) motif and recruits the transcriptional co-repressor TOPLESS (TPL) (Long et al. 2006). TPL functions with a histone deacetylase, HDA19, to deacetylate DNA making it inaccessible for transciption (Long et al. 2006). Domain II contains a degron motif that is required for the rapid degradation of the protein in response to auxin (Ramos et al. 2001; Reed 2001). Domains III and IV are required for homodimerization with other Aux/IAAs and heterodimerization with ARFs as these two domains share high homology with the C-terminal domain of ARFs (Abel and Theologis

1996; Dharmasiri and Estelle 2002; Kim, Harter, Theologis 1997; Reed 2001; Ulmasov, Hagen, Guilfoyle 1997).

According to the current model of auxin signaling, ARF proteins permanently occupy their target DNA binding sequences. Rather than binding directly to DNA, Aux/IAAs act as transcriptional repressors by binding to ARFs and, along with TPL, repress transcription by ARFs (Calderon-Villalobos et al. 2010). At high auxin concentrations, the Aux/IAA proteins are destabilized, allowing the transcription of the auxin- responsive genes (Tiwari et al. 2001).

Mutations in the Aux/IAA domain II lead to auxin resistant phenotypes due to stabilization of the Aux/IAA and constitutive repression of ARF- target genes (Tatematsu et al. 2004; Worley et al. 2000). Consequently, plants carrying loss of function mutations in *ARF* genes are predicted to have a phenotype similar to that of Aux/IAA with mutations in domain II. BDL/IAA12 was found to inhibit MP/ARF5 and thus control initiation of root meristems (Hamann et al. 2002). A gain-of-function mutant of BDL/IAA12, due to a mutation in domain II of the protein, resulted in a failure to initiate the root meristem similar to the result of a loss-of-function mutation in MP/ARF5 (Hamann et al. 2002).

Auxin response requires the UPS system

Transcriptional and developmental responses to auxin are sensitive to the level of Aux/IAA proteins. For ARF function to occur, the Aux/IAAs must tagged with ubiquitin for degradation by the 26S proteasome (Mockaitis and Estelle 2008). Ubiquitin tagging of the Aux/IAAs requires 3 enzymes: a ubiquitin-activating enzyme (E1), a ubiqutin-

conjugating enzyme (E2), and a ubiquitin ligase (E3) (Dharmasiri and Estelle 2002; Hershko and Ciechanover 1998). The E3 ligases are more diverse than E1 and E2 enzymes as E3 ligases need to recognize their substrate proteins to tag with ubiquitin (Dharmasiri and Estelle 2002).

The SCF (Skp1-cullin-F-Box protein) class of E3 ligases is comprised of four subunits (Patton, Willems, Tyers 1998; Tyers and Willems 1999). Cullin, Rbx1, and Skp1 proteins make up the core of the SCF complex and the F-box protein binds to SKP1 (or ASK1 in plants) through its F-box domain to confer more substrate protein specificity (Calderon-Villalobos et al. 2010; Cardozo and Pagano 2004; Patton, Willems, Tyers 1998; Tyers and Willems 1999). Transport inhibitor-response1 (TIR1), a protein that encodes an F-box domain and leucine rich repeats, binds to Skp1 to make the SCF^{TIR1} complex (Dharmasiri and Estelle 2002; Patton, Willems, Tyers 1998; Ruegger et al. 1998).

TIR1 and related AFB1-5 (Auxin Signaling F-Box1-5) proteins have roles as both E3 ligases, that lead to the degradation of Aux/IAAs, and as auxin receptors (Calderon-Villalobos et al. 2010; Dharmasiri et al. 2005). TIR1/AFB's affinity for Aux/IAAs depends on the presence of auxin (Calderon-Villalobos et al. 2010). TIR1 has a pocket that binds to auxin and the Aux/IAA covers this pocket on top of auxin so that TIR1's role as part of an E3 ligase is mediated by it and Aux/IAA's role as auxin receptors (Calderon-Villalobos et al. 2010; Dharmasiri, Dharmasiri, Estelle 2005; Tan et al. 2007). When TIR1 and Aux/IAAs interact due to an increase of auxin, the SCF^{TIR1} complex tags the Aux/IAAs with ubiquitin for degradation by the 26S proteasome, which is made of the 19S and 20S subunits (Chapman and Estelle 2009; Gray et al. 1999; Gray

et al. 2001). When released from Aux/IAAs, ARF activity resumes. Regulation of Aux/IAAs through degradation is important for auxin response and the study of other proteins that interact with Aux/IAAs will help to elucidate the many aspects of auxin signaling.

The paclobutrazol resistant family

Much of plant growth depends on auxin-induced cell expansion and division, but relatively little is known about auxin downstream genes that are controlling these processes. The *Arabidopsis* hypocotyl (or seedling stem) is a relatively simple model for studying auxin dependent growth as it grows only by cell expansion, making it possible to focus on the effects of auxin on a single growth related process.

A microarray was previously performed in the lab to elucidate auxin responsive genes in the hypocotyl (Chapman et al. 2012). 740 genes were found to be induced in the hypocotyl after a 2 hour auxin treatment. Among them were four members of the small PRE family of basic/helix-loop-helix (bHLH) genes including KIDARI (KDR) or PRE6 (Chapman et al. 2012).

bHLHs in all three eukaryotic kingdoms make up one of the largest families of transcription factors (Carretero-Paulet et al. 2010). Canonical bHLHs have a domain with many basic residues that allow for DNA binding and an HLH domain for protein dimerization (Murre et al. 1994; Toledo-Ortiz, Huq, Quail 2003). Two conserved amino acids in bHLHs are required for dimerization: leucines in position 27 of helix 1 and position 73 in helix 2 of the protein (Carretero-Paulet et al. 2010). By generating a mutation in leucine-27 (to a glutamine residue) or in leucine-73 (to a lysine residue) of

the bHLH protein PAR1, Carretero-Paulet and colleagues were able to show that PAR1's ability to homodimerize was affected (Carretero-Paulet et al. 2010). The mutation in position 27 resulted in no interactions and the mutation in position 73 resulted in reduced interactions as visualized in a yeast-2-hybrid (Y2H) assay (Carretero-Paulet et al. 2010).

Like in animals, a group of atypical bHLH proteins has also been identified in plants in which the basic domain contains fewer basic residues (Carretero-Paulet et al. 2010; Toledo-Ortiz, Huq, Quail 2003). Without the high amounts of basic residues, the atypical bHLHs, or HLH proteins, do not bind to DNA. These non-DNA binding HLHs have been shown to negatively regulate other bHLH or HLH proteins. Inhibitor of DNA binding (Id-1), a well characterized human HLH, heterodimerizes with other bHLH transcription factors, preventing them from interacting with the DNA (Hyun and Lee 2006). Id-1 has also been found to bind other proteins apart from bHLHs, as with the proteasome subunit C8 (similar to the alpha7 subunit of the plant 20S proteasome, PAG1) to destabilize HBX (Hepatitis-B virus p [HBV]- encoded protein) (Ling et al. 2008).

KDR is the sixth member of the PRE family. All members of the family lack the basic domain for DNA-binding and have been involved in different aspects of plant growth. PRE1 (from paclobutrazol resistant-1) was the first member to be described and initially found to be part of the GA signaling pathway by Lee and colleagues with their double mutant *ga2-201PRE1ox-17* plants (Lee et al. 2006). The overexpression of PRE1 in the double mutant plants rescued most of the GA-deficient phenotypes caused by the mutation in *GA2*, an enzyme in the gibberellin biosynthesis pathway (Lee et al. 2006). PRE1 localizes in the nucleus, and like KDR, PRE1 overexpressor plants have long

hypocotyls, pale green leaves, elongated petioles, and early flowering times (Hyun and Lee 2006; Lee et al. 2006).

PRE1 regulates cell elongation by interacting with IBH1, an inhibitor of transcription factors, through heterodimerization so that HBI1 and ACEs can function in cell elongation (Bai et al. 2012; Ikeda et al. 2012). KDR is thought to negatively regulate HFR1 through protein-protein interactions (Hyun and Lee 2006) allowing PIF3's function in cell elongation (Feng et al. 2008). In all of these cases, a member of the PRE family interrupts the heterodimerization between an HLH (HFR1 and IBH1) and a bHLH transcription factor (PIF3, HBI1, and ACEs) known to play a role in cell elongation.

While all PREs are involved in cell elongation processes and the function of members of the PRE family are predicted to be functionally redundant, experiments show that only PRE1 is inducible by GA (Lee et al. 2006), and only PRE1, PRE2, PRE5 and KDR are inducible by auxin in the hypocotyl (Chapman et al. 2012). This shows that although the proteins are structurally similar, they have some degree of specificity. We hope to elucidate the role these PRE proteins have in auxin signaling.

Objectives

A previously performed automated yeast-2-hybrid (Y2H) screening that used KDR as bait against a library of transcription factors showed that KDR interacts with IAA6 and IAA18. First, I will reconfirm these interactions on a smaller scale in another Y2H system. Using the Y2H assay, I will also be able to observe if KDR interacts with other Aux/IAAs or specific ones, and if other PRE proteins behave similarly. Additionally, a bimolecular fluorescence complementation (BiFC) assay in *Nicotiana*

tabacum will show the interaction of the two proteins (KDR and IAA6) in the proteins' native environment. Second, characterization of KDR and PRE5 expression during hypocotyl development and elongation will use transcriptional and translational fusions to the β-glucuronidase (GUS) reporter gene during various stages of hypocotyl growth. An emphasis on studying the hypocotyl will help with elucidating the role of the proteins in cell elongation as the main form of hypocotyl growth is due to cell elongation instead of cell division (Saibo NJ et al.).

II:

Materials and Methods

Yeast-2-Hybrid (Y2H)

All plasmids used to cotransform strain EGY48 were from the Estelle Laboratory stocks and from Cristina Castillejo. Cotransformation with the small-scale LiAc yeast transformation procedure (Catalog #PT3040-1, Clontech) resulted in colony growth on SD lacking uracil, tryptophan, and histidine (SD-HUW) after 2 days incubation at 30°C. 5 isolated colonies of each cotransformation were struck out to allow for propagation.

The Y2H assay used X-gal (Catalog #X4281c10, Gold biotechnology) induction plates, prepared as described (Catalog #PT3040-1, Clontech), and β-galactosidase activity on the X-gal plates shows protein-protein interaction as visualized by blue colonies. Cultures of cotransformed yeast were grown overnight at 30°C in 1 milliliter (mL) of SD-HUW; they were pelleted and resuspended in 100 microliter (μL) sterile water and 5 μL was plated on the X-gal induction plates, which were incubated at 30°C. β-galactosidase activity was checked for every 24 hours for 3 days.

Yeast Protein Extraction

Previously transformed yeast cultures were grown in 5 mL SD-HUW with 2% galactose and 1% raffinose to induce protein expression (which is repressed by glucose). The yeast were pelleted and protein was extracted in a 1:1 ratio of mass to solution using the following working solution: 95.5% YPER Reagent (Catalog #78991, Thermo Scientific), 1X protease inhibitor cocktail (Catalog #11 836 170 001, Roche) and 0.5% 0.1M PMSF (phenylmethanesulfonyl fluoride, Catalog #P7626, Sigma). The working solution is added to the pelleted yeast, vortexed, and incubated with light shaking for 30 minutes at room temperature. The solution is then pelleted and the supernatant containing

protein extracted recovered. Protein concentration was found using the Pierce BCA Protein Assay Kit (Catalog #23225, Thermo Scientific). Protein samples were diluted 1:20 in extraction buffer to make 50 μL and 1 mL of BCA working solution was added; the samples were incubated at 37°C for 30 minutes. Protein concentrations were compared to a standard curve based on bovine serum albumin (BSA) dilutions. Absorbances were read using a spectrophotometer set to the Bradford Protein Assay.

Bimolecular Fluorescence Complementation (BiFC)

The transformation of tobacco leaves for the BiFC followed a previously described with some modifications using modified versions of the binary vectors pDEST-VYCE and pDEST-VYNE (See Table 1) (Yin, Chory, Baulcombe 2001). These modified vectors are compatible for gateway cloning. Specific sequences were then inserted using LR reactions into these destination vectors to construct plasmids containing our proteins of interest and either the N- or C-terminus of the VENUS protein. *A. tumefaciens* was transformed with either the pVYCE or pVYNE plasmids and was then grown in 5 ml of Lysogeny broth (LB) media with appropriate antibiotics overnight at 30°C along with *A. tumefaciens* with the p19 plasmid (Voinnet et al. 2003). The cultures were then spun down, washed with 10 mM $MgCl_2$, resuspended in 10 mM $MgCl_2$ back to their initial volume and were left at room temperature for 1-3 hours. Before injection of tobacco leaves, the cultures were mixed in 1:1:1 ratios of a pVYCE plasmid, a pVYNE plasmid, and the p19 plasmid. Syringes were used to infiltrate the abaxial side of leaves of 4-5 week old tobacco plants. Fluorescence was visualized 3-5 days after infiltration of *Agrobacterium tumefaciens* using a confocal microscope.

Imaging using a confocal microscope

All samples were checked using the Zeiss 710 and all images were taken with the same settings.

Western Blot

Protein samples were prepared for loading into 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. 2X loading buffer was prepared with 300 μL 2-mercaptoethanol in 1 ml 2X sample buffer (0.25 M tris(hydroxymethyl)amino methane at pH6.8, 20% glycerol, 4% sodium dodecyl sulfate (Catalog #EC-874, national diagnostics), 0.1% bromophenol blue in water). Protein extracts cointaining 15-20 micrograms (μg) of total protein were added to loading buffer in a 1:1 ratio of protein to loading buffer and the mixture was heated at 90°C for 10 minutes to denature the protein. After the samples cooled to room temperature, they were loaded into 12% SDS-PAGE gels and run at 150 volts for 1.5 hours or until the dye front ran off the gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes (Catalog #RPN303D, GE Healthcare) through electroblotting at 100 volts for 1 hour or using the Thermo Scientific Pierce G2 Fast Blotter.

Protein transfer was confirmed using Ponceau Staining (0.1% Ponceau (Catalog #P-3504, Sigma) and 5% acetic acid). The membrane is first washed in 1X TBS-T (tris buffered saline with Tween 20 (Catalog #786-517, G-Biosciences)) and then stained in Ponceau solution for 10 minutes. The membrane is rinsed in 1X TBS-T 5 times or until protein bands are visualized.

A Coomassie Brilliant Blue stain (50% methanol, 10% acetic acid, 39.75% water, 0.25% Coomassie Brilliant Blue (Catalog #161-0400, BioRad)) was also used to visualize equal protein loading between samples in an SDS-PAGE gel.

The membrane is incubated in blocking buffer (1X TBS-T with 5% milk powder) for 1 hour at room temperature or at 4°C if overnight. After blocking the membrane to ensure no non-specific binding of the antibody, the primary antibody is added.

If probing for an HA-tagged protein, an α-HA-horse radish peroxidase antibody (Catalog #3F10, Sigma) was used in a 1:1000 ratio of α-HA-HRP to blocking buffer; the membrane was incubated in this solution at room temperature for 2 hours with rocking. After incubation with the primary antibody, the membrane is rinsed 5 times with 1X TBS-T and chemiluminescent detection is used to detect the protein of interest, as the horseradish perioxidase will cleave the chemiluminescent agent to give off light. 500 μL of the ECL Prime Western Blotting Detection Reagent (Catalog #RPN2232, GE Healthcare) is used for each membrane and the light given off is captured through film. The time given for exposure of the film depends on the strength of the luminescence.

If probing for a LexA-tagged protein, an α-LexA-Rabbit antibody (Catalog #06-719, Millipore) was used in a 1:1000 ratio of antibody to blocking buffer; the membrane was incubated at 4°C overnight with rocking. After rinsing out the primary antibody 5 times with 1X TBS-T, the secondary antibody, goat α-rabbit-POD (Catalog #A6154, Sigma) was added at a ratio of 1:5000 of antibody to blocking buffer. The membrane was incubated for 1.5 hours in the secondary antibody at room temperature with rocking. After incubation, the membrane was rinsed 5 times with 1X TBS-T and imaged with the ECL Prime Western Blotting Detection Reagent.

Growth of *Arabidopsis thaliana*

Murashige and Skoog (MS) media was used to grow *Arabidopsis thaliana* seedlings. The media was prepared with 0.22% Murashige and Skoog powder (Catalog

#30630059-3, plantmedia), 1% sucrose, 0.05% MES hydrate (Catalog #M8250, Sigma), and the pH was adjusted to 5.70 using KOH. If plates were used, 0.8% agar was added before autoclaving. Seeds were plated on agar in sterile hoods and grown in light (long day or short day) chambers.

Adult plants were grown on soil at 24°C in greenhouse conditions.

Protein extraction from *Arabidopsis thaliana* adult leaves

Extraction of protein from adult plants used the EZ protein extract as described (Martinez-Garcia, Monte, Quail 1999). As some components of the extraction buffer are incompatible with the Pierce BCA Protein Assay Kit, another assay was used to quantify the protein concentrations in each sample. The DC Protein Assay Kit I (Catalog #500-0112, Bio-Rad) was used instead.

Creating Transgenic Lines of *Arabidopsis thaliana* (ecotype- Columbia)

Plasmids containing appropriate constructs are transformed into *A. tumefaciens* and a 500 mL culture is prepared in Lysogeny broth (LB) media with an appropriate antibiotic. This 500 mL culture is grown overnight and pelleted for 20 minutes at room temperature and 5500 g. The supernatant is removed and the pellet is air-dried. The cells are then resuspended in 5% sucrose and 0.05% Silwett-L77 Surfactant (Catalog #505869, GE). Approximately 2 week old (or bolting) *Arabidopsis thaliana* plants are then dipped into the solution containing *A. tumefaciens* and allowed to lay flat for 2 days before being returned to the growth room.

DNA extraction from *Arabidopsis* leaves

1 leaf was harvested and added to a 1.5 ml microcentrifuge tube containing 2 BB beads. The tube was frozen in liquid N2, the leaves were shredded by vortex, and 400 μL

of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS in H2O) was added to the tube. The tube was centrifuged at 12400rpm for 5 minutes and the supernatent containing DNA was removed to a new tube. 300 μL isopropanol is added to the supernatent, the tube was vortexed and centrifuged at 12400rpm for 5 minutes to pellet DNA. 70% EtOH was added, the tube was centrifuged at 12400rpm for 2 minutes and the supernatent was removed. The DNA pellet was air dried and resuspended in 50 μL of sterile H_2O. The DNA was stored at -20 deg C.

Genotyping using the Polymerase Chain Reaction (PCR)

After extraction of DNA from *Arabidopsis,* the sequence of interest was amplified using PCR. 5 μL of GoTaq (Catalog #M791B, Promega), 4 μL of extracted DNA, 0.5 μL of a 10 μM forward primer, and 0.5 μL of a 10 μM reverse primer were mixed and the PCR was run with an elongation time appropriate to the length of the sequence of interest (where the Taq DNA polymerase works at 1000 basepairs per minute).

Histochemical localization of GUS reporter activity

Tissue was placed in enough GUS staining solution (50 mM sodium phosphate at pH7.0, 0.1% Triton X-100 (Catalog #T9284, Sigma), 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 0.5 mg/ml X-Gluc (Catalog #G1281C5, Gold biotechnology) and H_20) to cover the tissue, and vacuum infiltrated for 20 minutes (for seedlings). After, the plate was incubated in the dark at 37°C overnight. The tissue was cleared with 70% ethanol overnight and pictures were taken the next day.

Measurement of hypocotyl lengths of *Arabidopsis* seedlings

When seedlings were at various stages of growth, the hypocotyl lengths were measured for analysis purposes. Seedlings grown on plates were imaged using a dissecting microscope and hypocotyl lengths were determined using ImageJ (NIH).

Table 1. List of plasmids/vectors used in this thesis. pB42AD and pGILDA vectors were used in the Y2H assays. pDEST-VYNE and pDEST-VYCE were used in the BiFC assays.

Vector Name	**Purpose**	**Selection Marker**	**Marker for Western Blotting**
pB42AD	Contains the activating domain of the GAL1 promoter	Growth on media lacking tryptophan	HA-tagged
pGILDA	Contains the binding domain of the GAL1 promoter	Growth on media lacking histidine	LexA-tagged
pDEST-VYNE	Contains the N-terminus of the fluorescent protein VENUS	Kanamycin and hygromycin resistance	c-myc-tagged
pDEST-VYCE	Contains the C-terminus of the fluorescent protein VENUS	Kanamycin resistance	HA-tagged

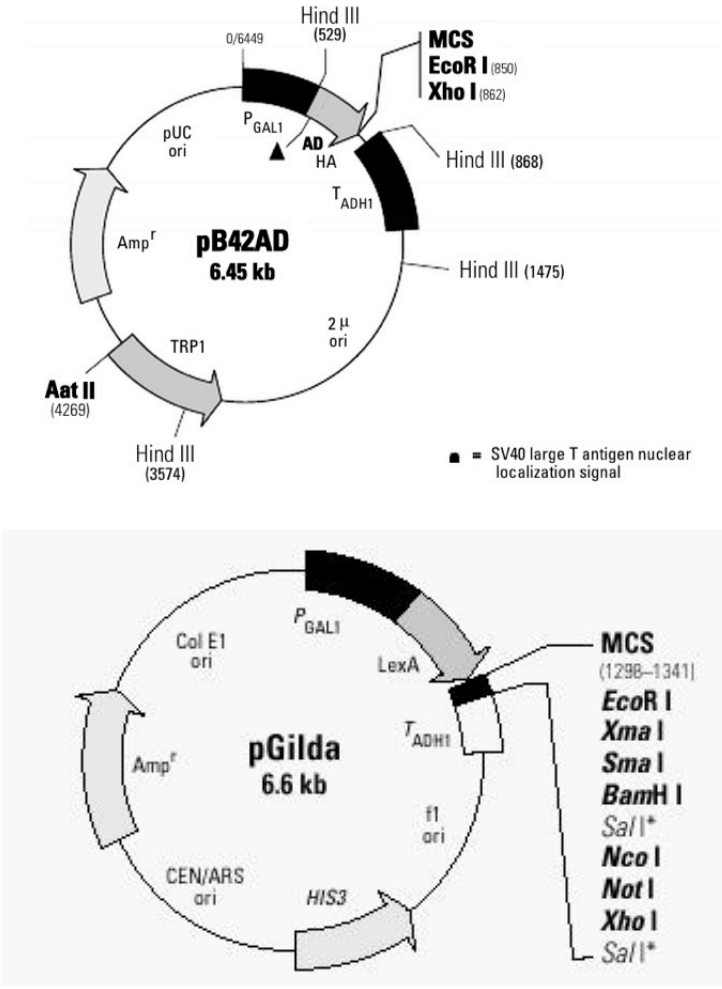

Figure 1. Vector maps of pGILDA and pB42AD. pGILDA encodes for the binding domain of LexA, driven by the GAL1 promoter, and the HIS3 gene that allows for growth on medium lacking histidine. pB42AD encodes for the activating domain of LexA, also driven by the GAL1 promoter, and the TRP1 gene allows for growth on medium lacking tryptophan. Modified versions of these two vectors that allow gateway cloning were made in the lab. When yeast strain EGY48 is cotranformed with the vectors, growth can occur on medium lacking uracil, histidine, and tryptophan. When grown on medium with galactose (instead of glucose), the GAL1 promoter is induced to express the LexA binding domain and activating domains along with any coding sequences inserted into the vectors for use in a Y2H.

III:

Results

Interactions of PRE family with Aux/IAAs in Y2H Assays

Preliminary results from a large scale automated Y2H library screening of various transcription factors with KDR as bait in a GAL4-based system performed in the lab found that both IAA6 and IAA18 interact with KDR. To further confirm those interactions, the MATCHMAKER LexA Two-Hybrid System was used. Using this system will reconfirm the results seen in the screening on a smaller scale with additional replicates and also verify the positive interactions by using a different two-hybrid system.

The MATCHMAKER LexA Two-Hybrid System was used to visualize potential interactions between members of the PRE family (PRE1, PRE2, PRE5, and KDR) and various Aux/IAAs belonging to the different clades. The EGY48 yeast strain carries the p8op-*lacZ* reporter in its genome (Catalog #PT3040-1, Clontech), the DNA-binding domain (DNA-BD) is provided by the prokaryotic LexA protein, and the activation domain (AD) is an acidic *E. coli* peptide that activates the transcription in yeast. The DNA-BD binds to a specific promoter sequence (LexA promoters, p8op) and the AD directs the RNA polymerase II complex to transcribe the *lacZ* gene downstream of the DNA binding site (Catalog #PT3040-1, Clontech). An interaction between a target protein fused to the DNA-BD and another protein fused to the AD creates a novel transcription factor with binding affinities for LexA promoters. When *lacZ* transcription is activated, the cells produce β-galactosidase, whose activity can be monitored using the substrate X-gal in the media, making the protein-protein interaction phenotypically detectable by blue colonies. If the two hybrid proteins do not interact with each other, the reporter genes will not be transcribed.

Modified versions based on pB42AD and pGILDA vectors were used to generate fusions of the AD and DNA-BD to genes encoding proteins that potentially interact with each other (See Figure 1 for plasmid maps). The PRE1, PRE2, PRE5, KDR, or KDRL29E (a mutant KDR) coding sequence is fused to the DNA-BD while Aux/IAA members are fused to the AD. KDRL29E is the KDR protein with a glutamine residue in position 29 instead of a leucine. By introducing this mutation in KDR, the protein is no longer able to interact with other proteins. Position 29 in KDR is a the conserved leucine residue required for interaction with other bHLHs (Toledo-Ortiz, Huq, Quail 2003). Without this residue, there should not be any protein-protein interactions. Using these constructs, the interaction or lack thereof between the PRE proteins and Aux/IAAs can be visualized.

As negative controls, the DNA-BD was cotransformed with all of the ADs fused to Aux/IAAs. Additionally, the AD was cotransformed with all the DNA-BD fused to PRE proteins. These controls are used to ensure that any phenotypes seen from the colonies is due to protein-protein interaction and not due to autonomous activation of LacZ by the fused proteins with either the DNA-BD or AD. There was no interaction seen from these cotransformed controls until day 2 or 3 of incubation. DNA-BD is fused to IAA7 and cotransformed with AD fused to ARF7 as a positive control. These two proteins are known to interact and blue colonies were observed after the first 24 hour incubation at 30°C.

There was strong interaction between KDR and seven of the 10 Aux/IAAs (IAA5, IAA6, IAA8, IAA18, IAA19, IAA28, and IAA31) and weaker interaction with

IAA1 and IAA12 (Figure 2). There was no interaction of KDR with IAA7 (Figure 2). In contrast, KDRL29E did not interact with any of the Aux/IAAs (Figure 2).

As for other members of the PRE family, only PRE1, PRE2, and PRE5 were cotransformed with Aux/IAAs because these were the other members that were inducible by auxin in the hypocotyl (Chapman et al. 2012). Interactions of PRE1, PRE2, and PRE5 with the Aux/IAAs on day 1 (not shown) are amplified by day 2 as seen with the more intense blue colonies. PRE1, PRE2, and PRE5 showed strong interaction with IAA5, IAA6, IAA28, and IAA31 (Figure 3). Weaker interactions can be seen with other IAAs but these color variations may or may not reflect the strength of the protein-protein interaction as this specific assay is not quantitative (Figure 3). Despite this, PRE1 seemed to interact more with IAA18 and IAA19 than PRE2 and PRE5 did. By Day 2 (Figure 3), the 3 PRE family members interacted with all IAAs used as compared to the negative controls. Note that the negative control for IAA28 was not included in the assay for PRE1, PRE2, and PRE5 but all other controls acted as expected and there was no autoactivation by AD-IAA28 seen in the previous Y2H (Figure 2).

While any interactions observed confirm presence of the proteins of interest, western blots were run to determine whether or not the differences in interaction strength or lack of interactions was due to protein expression levels. Yeast were grown in SD-HUW with galactose and raffinose sugars instead of glucose. Galactose would induce the GAL1 promoter to drive expression of our fused proteins and raffinose was used to promote the growth of the yeast in the case that galactose was not enough. Protein extracts from this yeast were then used in western blots. Presence of all AD fused proteins was confirmed (Figure 4) by using an α-HA antibody. To ensure that the DNA-

BD fused proteins were present, an α-LexA antibody was used (Figure 5). The western blots confirm that all proteins were present and that any lack of interactions observed was because the proteins don't interact.

Bimolecular fluorescence complementation

The detection of a specific protein-protein interaction in a heterologous system, as it is in yeast, is not necessarily indicative of the proteins' behavior in its native environment. By using BiFC in *Nicotaiana tabacum* we are able to analyze our proteins of interest *in planta* and thus confirm results previously seen in the Y2H. While using GFP and its variants (VENUS in this case) is beneficial because of its ability to be appropriately expressed in almost every cell type and no reagent needs to be added to visualize it, there are a few drawbacks applicable to the experiments performed (as reviewed in (Bhat, Lahaye, Panstruga 2006)). The full length GFP requires several hours to fold and its fragmented form in the assay may take even longer (Bhat, Lahaye, Panstruga 2006). Due to this characteristic of the fluorophore, be it GFP or VENUS, and the short half lives of Aux/IAAs, the Aux/IAA used in the BiFC must be stabilized. As KDR does not interact with the domain II of Aux/IAAs, a mutation in this domain is possible for stabilization as it will no longer be able to be tagged with ubiquitin for degradation hence the IAA6 with a mutation in domain II (IAA6mII).

Additionaly, many peptides under control of the CaMV 35S promoter, as is the case with all proteins used, were shown to autofluoresce without an interacting partner (Bhat, Lahaye, Panstruga 2006). Modified versions of both the experimental protein (KDR) and the positive control (ARF7) that cannot interact with IAA6mII were

constructed: KDRL29E and ARF7Δ, respectively. These two negative controls would ensure that any fluorescence visualized between KDR with IAA6mII and ARF7 with IAA6mII are indeed due to protein-protein interactions and not due to any fluorescence caused by the constitutive expression of the protein (Bhat, Lahaye, Panstruga 2006). In these experiments, two binary GATEWAY vectors, pDEST-VYNE(R)^{GW} and pDEST-VYCE(R)^{GW} were used and they encode for the N-terminus and C-terminus of VENUS, respectively (Gehl et al. 2009). VENUS is a brighter version of the yellow fluorescence protein (YFP). When fused to proteins that are known to interact, the two previously physically separated fragments of VENUS come together and a yellow fluorescence is visualized with a confocal microscope.

Using LR reactions to insert my proteins of interest into the vectors allowed me to reconfirm their interaction as seen in the Y2H. The coding sequences (CDS) of KDR, KDRL29E, ARF7, and ARF7Δ were inserted into pDEST-VYNE(R)^{GW} and therefore would be expressed along with the N-terminus of VENUS. The CDS of IAA6mII was inserted into pDEST-VYCE(R)^{GW} and expressed with the C-terminus of VENUS. The vector constructs containing fragments of VENUS and my proteins of interest were then transformed into *A. tumefaciens* which were then used to transiently infiltrate *N. tabacum* leaves.

Fluorescence was viewed in leaves that were infiltrated by KDR and IAA6mII, and ARF7 with IAA6mII (Figure 6). The fluorescence visualized confirmed the interactions seen in the Y2H (Figure 2) as well as showed that the fluorescence localized in the nucleus. Additionally, negative controls worked appropriately as leaves infiltrated

with ARF7Δ and IAA6mII or KDRL29E and IAA6mII did not exhibit any fluorescence, especially not in the nucleus, and mimicked the lack of fluorescence in wild type leaves.

KDR overexpression in *Arabidopsis thaliana*

In order to study KDR in *A. thaliana*, a line had been previously produced to overexpress KDR and it had a longer phenotype than wild type, consistent with published data (Hyun and Lee 2006). As KDRL29E showed no interaction in the Y2H or BiFC, it is a good candidate as a negative control or comparison for any phenotypes seen in KDR in future experiments. It's hypothesized that if the interaction between KDR and Aux/IAAs is important for the long hypocotyl phenotype seen in the KDR overexpressor, then an overexpression of KDRL29E should not exhibit this phenotype. A KDRL29E overexpressor in *A. thaliana* was created by *A. tumefaciens* dipping, T1 selection based on GFP expression in the seed, PCR genotyping, and protein analysis in a western blot. Results of the western blot (Figure 7) using an α-HA antibody show that 5 lines (9,10,12,17,19) of the newly dipped plants do express KDRL29E. Line 5 did not show any protein most likely due to a smaller amount of total protein loaded as shown in the Coomassie blue stain below the blot. Also as positive controls, two lines of the KDR overexpressor showed KDR expression whereas the wild type negative control had no HA-tagged protein.

Analysis of PRE5 and KDR expression

As previously described, various members of the PRE family were induced by auxin in the hypocotyl (Chapman et al. 2012). To understand their role in hypocotyl

elongation and development, transcriptional and translational fusions of the genomic sequences to β-glucoronidase (GUS) were constructed allowing for characterization of the expression of the genes and proteins as well as determine their spatial and temporal distribution in the hypocotyl and the rest of the seedling. The GUS enzyme is capable of hydrolyzing β-o glycosidic bonds so using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc), initially colorless, will give a blue phenotype when GUS hydrolyzes it (Salinas and Sanchez-Serrano 2006). Thus transcriptional and translational fusions to the GUS reporter will show localization of the promoter and protein, respectively, of KDR and PRE5.

The transcriptional fusions took 3.46 kb (of PRE5) and 3.77 kb (of KDR) of the promoter sequence upstream of the ATG site of each gene with GUS fused at the end. The translational fusions were constructed by using the same promoter sequence of each gene used in the transcriptional fusions and their respective gene sequences (2 exons and 1 intron) before the stop codons and fused this to the GUS reporter gene. Various stages of development in *A. thaliana* seedlings were observed in comparison to the expression of these two genes.

In selecting specific lines for staining and analysis, initial staining of 4-6 T2 lines of each of the four constructs was performed. Two lines of each construct were selected as they best represented the expression pattern of the other lines. After ensuring that the constructs had a single insertion by using hygromycin resistance (with a 3:1 segregation ratio), homozygous generations of the two lines of each construct were used for more detailed GUS staining analysis.

The results of the GUS staining are shown in Figure 8. During days 4-13 of growth after stratification, the transcriptional fusion of KDR (pKDR:GUS) shows that there is always expression in the cotyledons, roots (Day 4 and 7) and root tips, and also the lower half of the hypocotyl. As for the KDR protein (pKDR:gKDR-GUS), there is expression in all the cotyledons and root tips stained, but no expression in the roots. Interestingly, the expression in the hypocotyl is in the upper half and only apparent on Day 4 and Day 7.

Staining results of the transcriptional fusion of PRE5 (pPRE5:GUS) are similar to that of pKDR:GUS expression in the cotyledons, the lower half of the hypocotyl, all of the roots during the earlier stages, and the root tips during Days 10 and 13. PRE5 protein (pPRE5:gPRE5-GUS) expression was only visualized in the cotyledons and on days 4 and 7 in the upper half of the hypocotyl.

Complementation of *kdr-2* hypocotyl phenotype

While the pPRE5:gPRE5-GUS lines were expressed in the wild type background, the pKDR:gKDR-GUS lines were expressed in a *kdr-2* mutant background. The *kdr-2* mutant line is a SALK line with T-DNA interrupting the KDR gene sequence and the resulting seedlings had a short hypocotyl phenotype. Using these constructs (in the two lines of pKDR:gKDR-GUS) to complement the *kdr-2* hypocotyl phenotype provided a way to ensure that the phenotype seen in the background was indeed due to the mutation in KDR. Figure 7 shows that while the *kdr-2* mutant has shorter hypocotyls at all stages of development and elongation, the insertion of the KDR protein fused to GUS compensates for the lack of KDR in the background and therefore the length of the

hypocotyls returns to the length of wild type seedlings. If not longer than the wild type seedlings the hypocotyls of the seedlings with GUS fused to KDR are at least longer than that of the *kdr-2* mutant seedlings. We now have all the elements needed to show the phenotype of KDR and the complementation of the mutation.

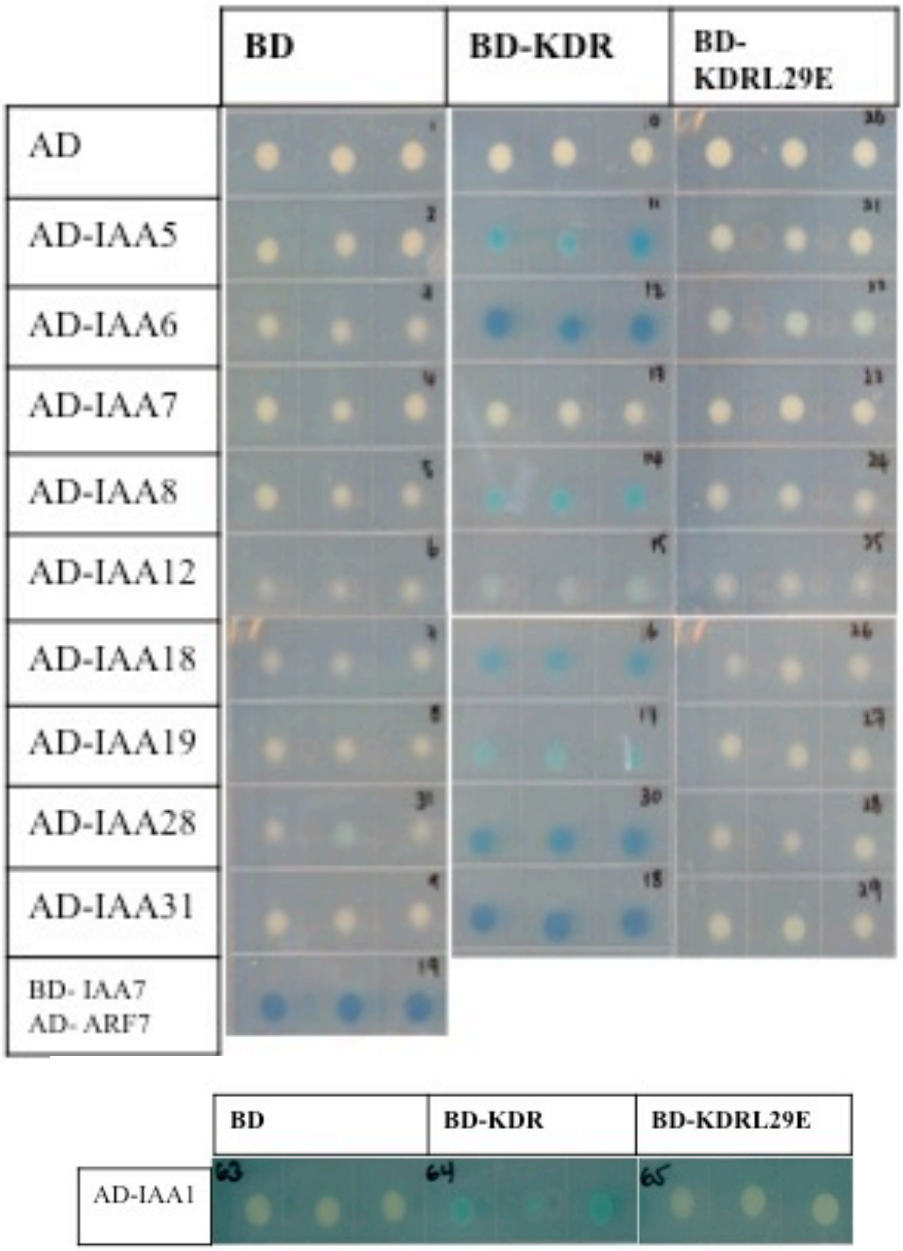

Figure 2. Interaction of KDR or KDRL29E with various Aux/IAAs in a Y2H assay. The DNA-BD was fused to KDR, and KDRL29E. The AD was fused to various Aux/IAAs. The BD and AD act as negative controls to ensure there is no autoactivation of LacZ. Note that while IAA1 interaction was visualized separately, all controls had the same phenotype. As a positive control, DNA-BD was fused to IAA7 and cotransformed with AD fused to ARF7, which are known to interact. 3 individual colonies of each cotransformation are shown from day 1 interactions.

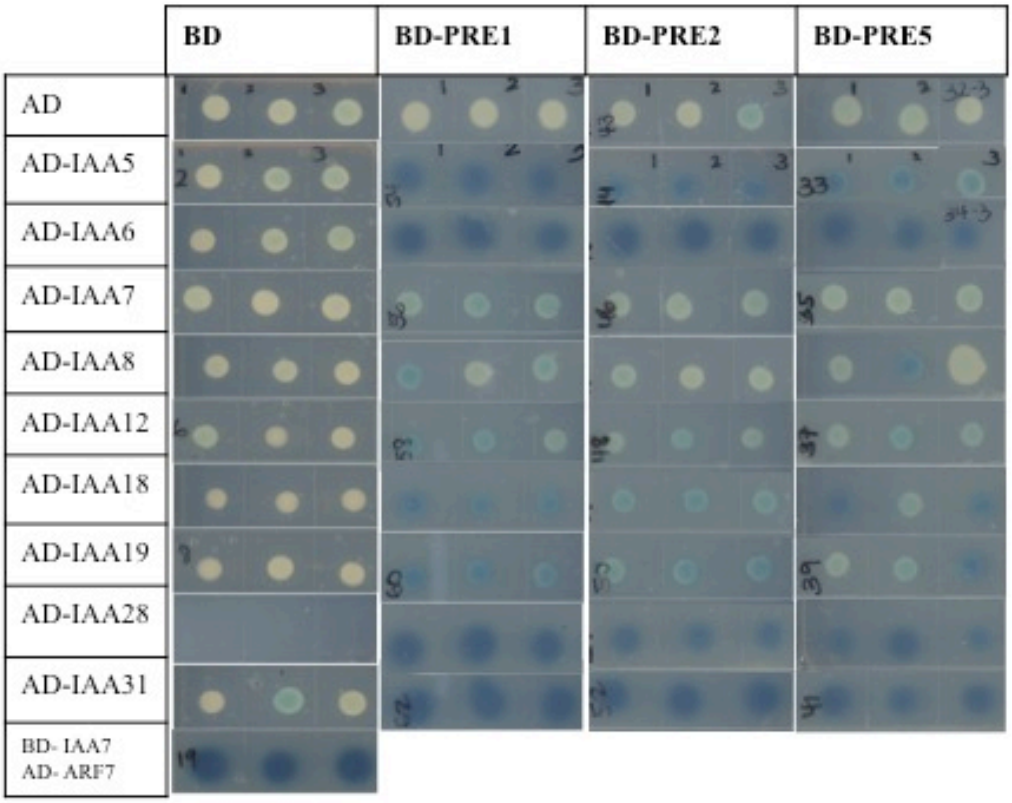

Figure 3. Interaction of PRE1, PRE2, or PRE5 with various AUX/IAAs in a Y2H assay The DNA-BD was fused to PRE1, PRE2, and PRE5. The AD was fused to various Aux/IAAs. The BD and AD act as negative controls to ensure there is no autoactivation of LacZ. As a positive control, DNA-BD was fused to IAA7 and cotransformed with AD fused to ARF7, which are known to interact. 3 individual colonies of each cotransformation are shown from day 2 interactions.

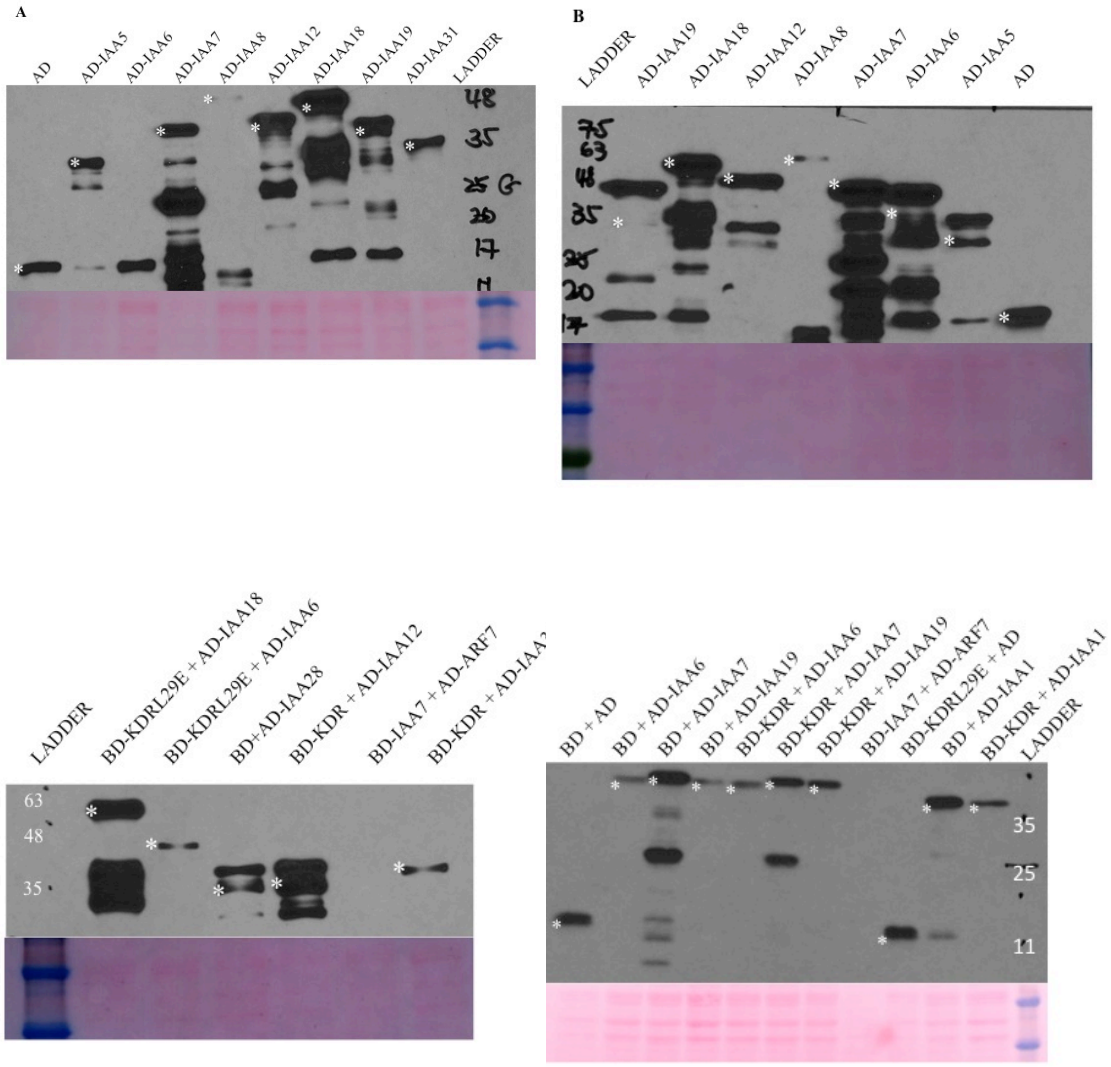

Figure 4. Detection of AD fused proteins with western blot analysis of yeast protein extracts. Yeast cultures were grown in SD-HUW with galactose and raffinose, protein was extracted, and an SDS-PAGE gel was run. An antibody against the HA tag, located after the AD, was used. Below the western blots are Ponceau stains ensuring consistent and equal loading of total protein into each SDS-PAGE. Asterisks in white indicate correct protein sized bands as predicted. In blot A, the BD is the DNA-BD protein alone. In blot B, the BD is KDR. The other two western blots are labeled with specific DNA-BD and ADs.

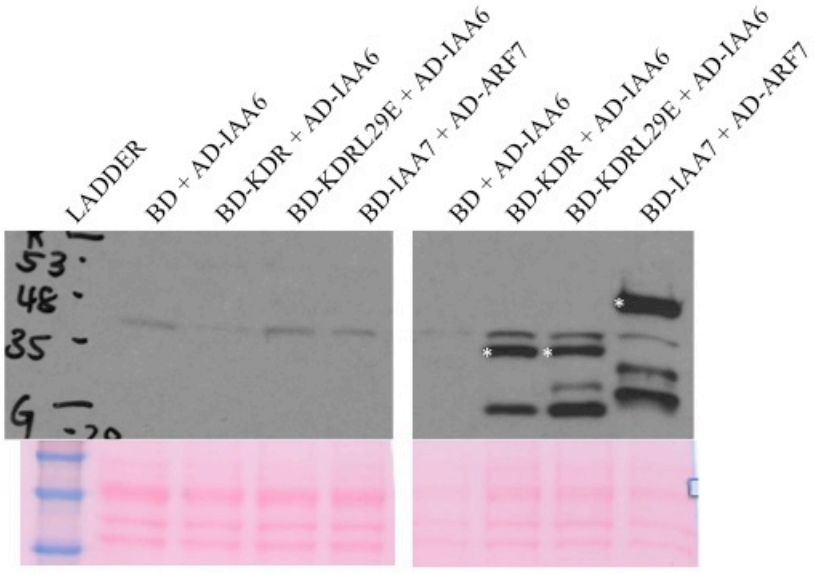

Figure 5. Detection of BD fused proteins with western blot analysis of yeast protein extracts. Yeast cultures were grown, protein was extracted, and an SDS-PAGE gel was run. An antibody against LexA, the DNA-BD, was used. Below the western blots are Ponceau stains ensuring consistent and equal loading of total protein into each SDS-PAGE. Asterisks in white indicate correct protein sized bands as predicted. A) Yeast was grown in SD-HUW + glucose. B) Yeast was grown in SD-HUW + galactose + raffinose.

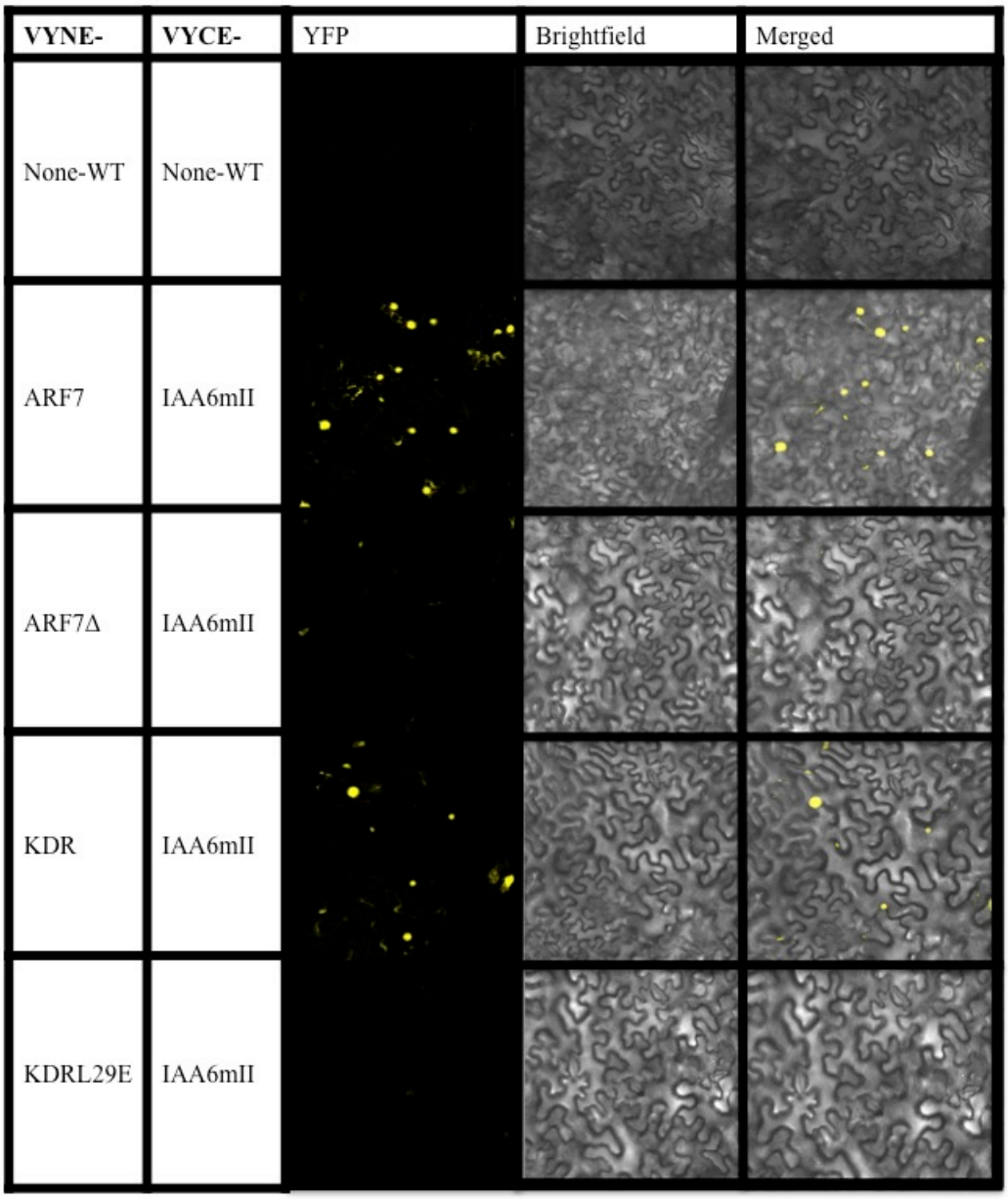

Figure 6. Bimolecular fluorescence complementation in *N. tabacum* leaves of KDR and IAA6mII. Leaf plugs were visualized 5 days after infiltration with *A. tumefaciens*. Proteins of interest were fused to the C- and N- terminus of the fluorescent VENUS protein and a confocal microscope was used to visualize the interactions using the same settings for each sample. Images above show individual channels of YFP and brightfield along with a merged image on the right.

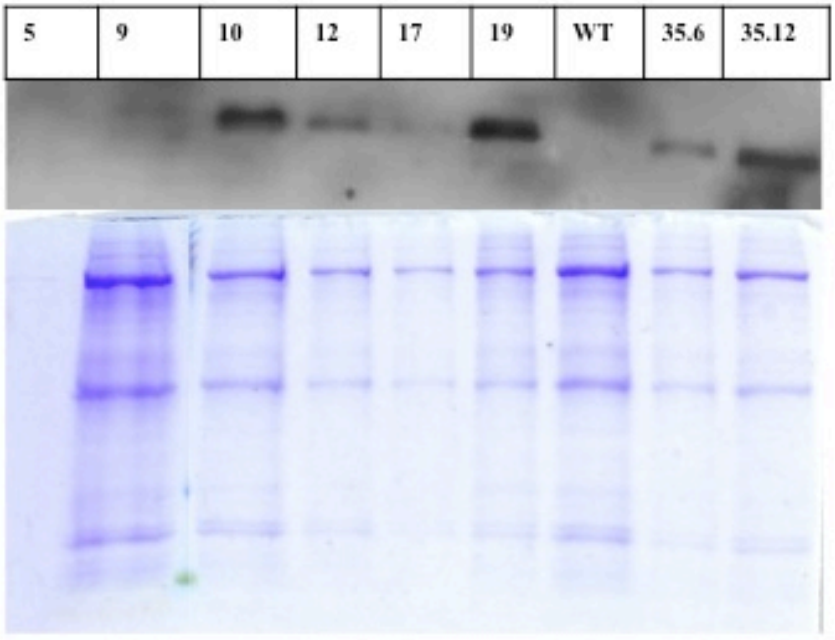

Figure 7. Presence of KDRox and KDRL29Eox in *A. thaliana*. Protein was extracted from T1 adult plants of KDRL29Eox (5, 9, 10, 17 and 19 as indicated above), adult wild type, and adult plants of KDRox (35.6 and 35.12). As our proteins of interest have an HA-tag, an appropriate western blot was performed using the primary conjugated antibody, α-HA-POD. Below the western blot is a Coomassie blue stain to insure equal total protein loading.

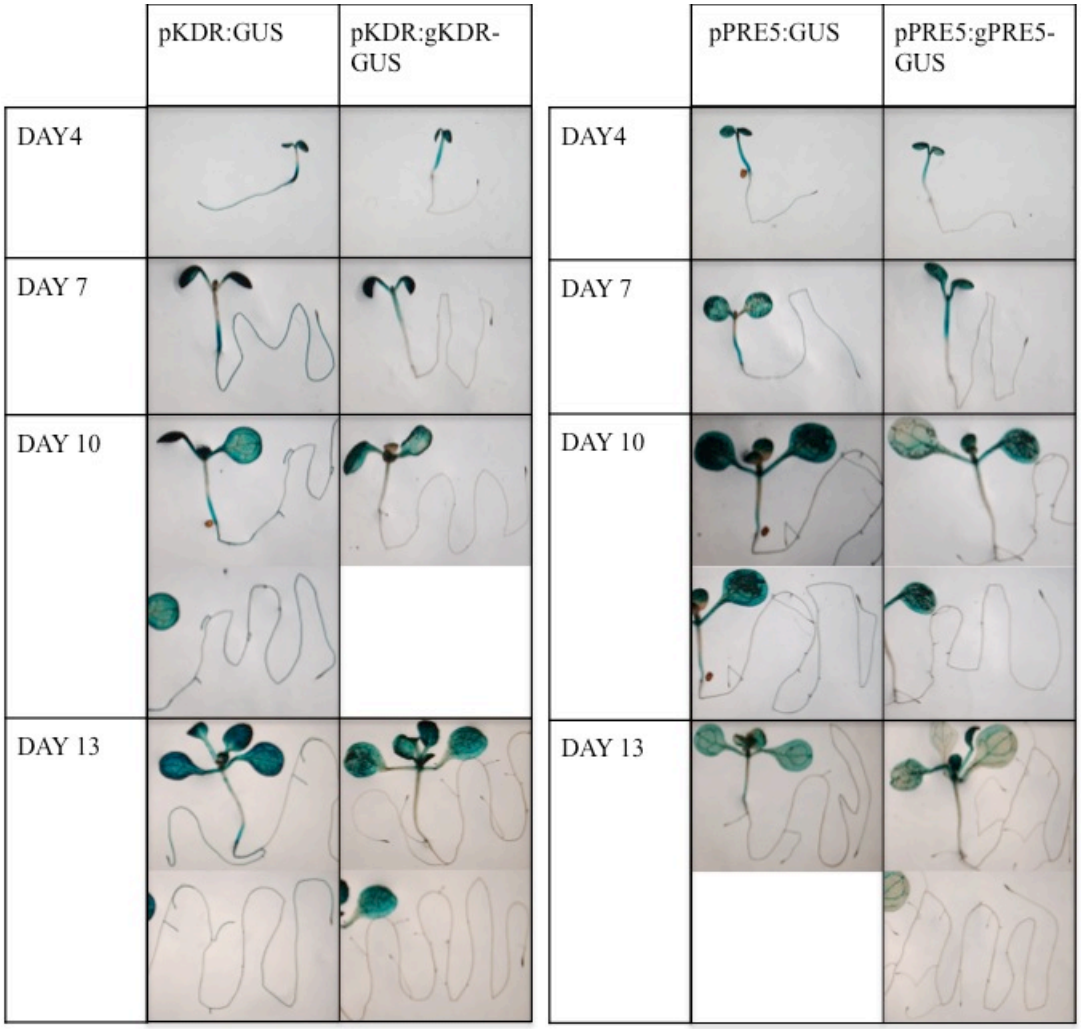

Figure 8. Characterization of expression of transcriptional and translational fusions of KDR and PRE5 to GUS. Seedlings of these GUS fusions were grown and GUS stained post-stratification on the day indicated. After destaining, images were taken. Days 10 and 13 have more than one image for certain seedlings as they did not fit within the same magnification in one image.

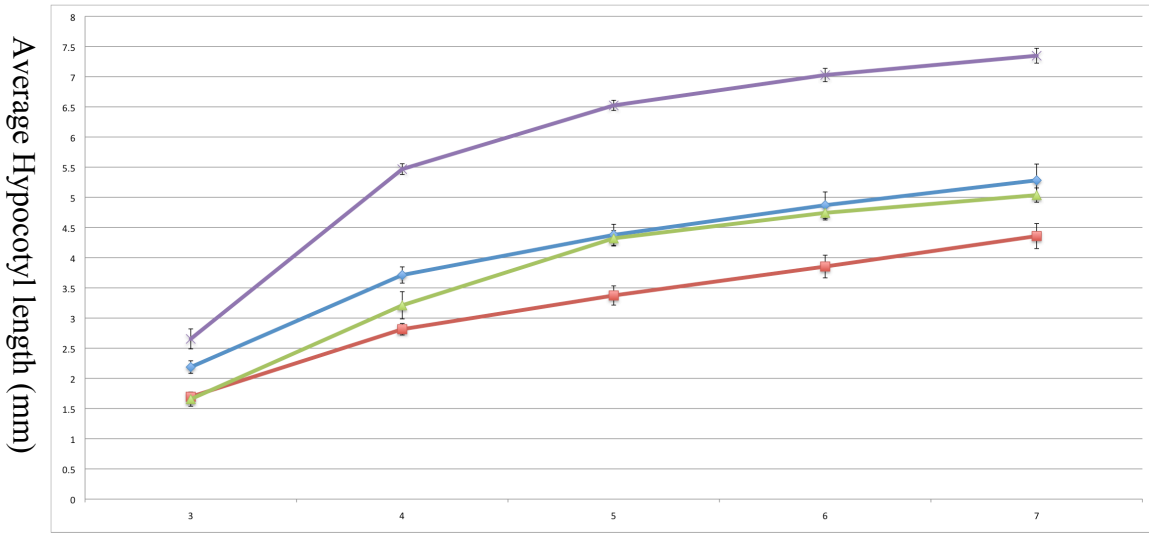

Figure 9. Complementation the *kdr-2* hypocotyl phenotype. pKDR:gKDR-GUS #1 (green, n=14) and #2 (purple, n=14) are two lines of the translational fusion of KDR to GUS expressed in the *kdr*-2 mutant background. The *kdr-2* (red, n=16) line is the KDR mutant line and wild type is in blue (n=16). The same seedlings were measured each day until day 7 post-stratification. Error bars represent standard error.

IV:

Discussion

4 of the PRE proteins were shown to interact with various Aux/IAA partners in a Y2H and BiFC showing that the PRE members might have a role in auxin signaling. KDR and PRE5 expression and spatial pattern were characterized in seedling development with the use of the GUS reporter gene to help elucidate its role during cell elongation and development. Also, mutant proteins were constructed and shown to lack interaction so that future experiments may be performed with KDR and KDRL29E.

PRE proteins and Aux/IAA interactions

Potential protein-protein interactions between select PRE family members and Aux/IAA (or simply IAA) family members are shown through a Y2H assay (Figure 2 and 3) and a BiFC assay (Figure 6). Results of KDR's interaction with various Aux/IAAs (Figure 2) not only reconfirms the previously performed Y2H screening result (KDR interacts with IAA6 and IAA18), but also shows that the PRE proteins interact with many other members of the Aux/IAA family. Any lack of interactions seen was not due to lack of protein, revealed through western blot analysis using α-HA (Figure 4) and α-LexA (Figure 5) antibodies. The interactions of KDR with IAA5, IAA6, IAA8, IAA18, IAA19, IAA28 and IAA31 and weaker interactions with IAA1 and IAA12 show that while KDR interacts with many members, it has some preferences (Figure 2). Similar results with other PRE family members are shown, while some interactions may differ in intensity, it is not necessarily due to strength of interaction (Figure 3).

To further investigate PRE protein and Aux/IAA interactions *in planta* and in the proteins' native environment, the BiFC in *N. tabacum* was performed (Figure 6). Fluorescence due to interactions can be seen in the nucleus of the pavement cells between

ARF7 with IAA6mII and KDR with IAA6mII, as expected of the proteins since they are localized in the nucleus (Dharmasiri and Estelle 2002).

Mutants of IAA1 (Ku et al. 2009), SHY1/IAA6 (Kim et al. 1996), AXR2/IAA7 (Nagpal et al. 2000; Timpte, Wilson, Estelle 1992), and MSG2/IAA19 (Harper et al. 2000; Tatematsu et al. 2004) due to a stabilized domain II all exhibit shortened hypocotyls. Additionally, many of the other Aux/IAAs have a role in hypocotyl development. IAA8 was shown to interact with ARF7, ARF5 and ARF19 (Arase et al. 2012)- possibly by inhibiting the transcription of the ARFs' target genes. Loss-of-function mutations in these ARFs lead to defects in hypocotyl growth or they exhibit phenotypes similar to gain-of-function IAA mutants. (Hardtke et al. 2004; Okushima et al. 2005). While not much in terms of IAA28 and IAA31's roles in hypocotyl development have been investigated, overexpression of IAA31 does have hypocotyl phenotypes that are different from wild type (Sato and Yamamoto 2008). Contrary to all that's been described, stabilized IAA18 exhibits longer hypocotyls in *A. thaliana* (Ploense et al. 2009; Uehara et al. 2008).

In conjunction with previously described phenotypes of gain-of-function mutants of Aux/IAAs, results of the Y2H assay between these two gene families raises questions of why and how they interact. Overexpression of KDR results in a longer hypocotyl (Hyun and Lee 2006) while a majority of the gain-of-function mutations of Aux/IAA members result in a shorter hypocotyl. These two protein families seem to interact antagonistically. There are a few possibilities: 1) PRE family members interact with Aux/IAAs making Aux/IAAs unable to interact with ARFs and essentially release ARFs from Aux/IAA repression; 2) the interaction of the PRE proteins and Aux/IAAs makes

the former proteins unable to heterodimerize with bHLH or other HLHs which inhibit hypocotyl elongation; or 3) PRE protein interaction with Aux/IAAs leads to the latter protein's degradation.

While all plausible explanations, further studies performed in the lab (results not shown) point to the third option. Should PRE proteins lead to the degradation of Aux/IAAs, it would be very similar to one of the functions of the human HLH, Id-1. Id-1 was shown to induce the degradation of the HBX protein by binding to the proteasome subunit C8 (Ling et al. 2008). Indeed, KDR was shown to interact with PAG1, alpha subunit of the 20S proteasome. Further experiments will be needed to investigate this degradation pathway.

The redundant functions of the PRE gene family previously described (Lee et al. 2006) are reinforced by data obtained through the Y2H assays (Figures 2 and 3). The PRE proteins share many similar Aux/IAA interacting partners, with the strongest interactions of all 4 PRE proteins with IAA5, IAA6, IAA28, and IAA31. The strength of these interactions is not quantitative so any conclusions drawn from potential partner preferences in terms of strength can only be made if another Y2H assay that controls for growth and thus gives quantitative results was performed. Some members may have additional, non-redundant roles since different hormones induce specific family members (Chapman et al. 2012; Lee et al. 2006) and KDR seems to interact more strongly with IAA8 and IAA18 as compared to the other PRE members (Figures 2 and 3). Direct comparisons on the strength of interactions cannot be made with full confidence, as these two Y2H assays were not performed at the same time.

The Y2H and BiFC (Figures 2 and 6) show that while KDR interacts with many of the Aux/IAAs, KDRL29E does not in yeast or *N. tabacum*. KDR's interaction with Aux/IAAs and KDRL29E's lack thereof will help elucidate the function of KDR and Aux/IAA's interaction in auxin signaling. The Y2H and BiFC assays, along with the western blot analysis of two *A. thaliana* lines (Figure 7) also serve to evaluate KDRL29E as a negative control in future experiments that use KDR. Should the overexpression of KDR in *A. thaliana* give a longer hypocotyl phenotype and the interaction of KDR with Aux/IAAs is important for this phenotype, then the overexpression of KDRL29E should not have the long hypocotyl phenotype. The mutation in this conserved leucine is similar to those performed with another HLH (Carretero-Paulet et al. 2010). Western blot analysis of the T1 plants of both lines shows that the proteins are indeed expressed at the appropriate protein size and a protein band is absent in the negative wild type control (Figure 7). This preliminary assay allows further work to be done using the two lines and, while phenotyping of the T1 lines has been done, using only 1 plant of each KDRL29E overexpressor line is not statistically accurate of the entire line's phenotype. Previous experiments with homozygous KDR overexpressor plants have shown longer hypocotyls than wild type and the expected phenotype of future homozygous generations of the KDRL29E overexpressors would be hypocotyls with the same length as wild type or at least shorter than that of the KDR overexpressor.

Gus Staining and Hypocotyl Assays

Characterization of the expression of both KDR and PRE5 as seen with the translational and transcriptional GUS fusions helps with discovering possible roles it has in seedling growth and development. While there is constant expression of both KDR and

PRE5 promoter and protein in the cotyledons during the assay, there is more variation in expression in the hypocotyl and roots of the seedlings (Figure 8). As IAA originates in the cotyledons (Avsian-Kretchmer et al. 2002), the presence of the hormone could possibly induce KDR and PRE5 in the shoots as it did in the hypocotyl since we see that the promoter and protein of the two genes are expressed in the cotyledons of 4, 7, 10, and 13 day old seedlings.

Expression of KDR and PRE5 in the hypocotyl is different between the two genes and also between different days of staining. Hypocotyl growth in *A. thaliana* occurs from day 1-7 after germination exclusively through cell elongation (Gendreau et al. 1997). By day 3 after germination, most elongation occurs in the lower 10 cells (out of 20) of the hypocotyl; day 7 saw little elongation and if any, in the middle cells of the hypocotyl (Gendreau et al. 1997). Expression of the transcriptional fusion of KDR can be seen at the base of the hypocotyl on all 4 days stained (Figure 8). Expression of the protein is only apparent during days 4 and 7 post-stratification which coincides with growth stages of the hypocotyl as by day 10 after stratification, growth probably has stopped. A growth curve (Figure 9) also shows that both translational fusions of KDR to GUS transgenic lines (pKDR:gKDR-GUS #1 and #2) have growth during days 3-5 and growth begins to plateau around day 6 or 7 after stratification, even though there is a little growth as measured by hypocotyl length. Expression of the protein is actually opposite of the promoter expression as it is at the top of the hypocotyl. This discrepancy will be discussed later.

PRE5 promoter and protein expression is similar to KDR in the hypocotyl. PRE5 promoter is expressed at the base of the hypocotyl but expression weakens on day 10 and

13 after stratification, in conjunction with the lack of growth during those days (Figure 8). PRE5 protein is only expressed during days of growth (4 and 7) and, like KDR, near the top of the hypocotyl, which is in a different area than its mRNA (Figure 8).

Visualization of the expression of proteins in areas that the corresponding promoter sequence expression was absent could be explained by the fact that the translational fusions included the exons and introns of the gene where the transcriptional fusions only had promoter sequences upstream of the translational start site. Within the first exon of both genes are two AuxRE variant sequences in close proximity to each other that may be required for auxin response. As ARFs may need to dimerize to function, these two AuxRE variants may be needed for DNA binding (Walcher and Nemhauser 2012). Also located in the translational fusion is a canonical AuxRE in the intron (Walcher and Nemhauser 2012), which is absent in the transcriptional fusion. There may be regulatory elements present in the intron and exons that are required for defining spatial pattern and expression (Sieburth and Meyerowitz 1997). The observed results and non-identical expression pattern of both promoter and protein of the genes could be due to the fact that the transcriptional fusions lacked these regulatory elements that were present in the translational fusions. Any regulations directing the expression of the transcriptional fusion lines of both genes may be absent and thus the expression observed may not be representative of the wild type.

Expression of the genes in roots differs between the promoter and protein but is similar between the two genes. There is always expression of both KDR and PRE5 promoter in the roots no matter what day after stratification (Figure 8). For both genes, day 4 and 7 have promoter expression in the entire root while day 10 and 13 only have

expression in lateral roots and root tips. As for protein expression, there wasn't any except for KDR in the root tips (Figure 8).

Though one isn't shown in the GUS staining results, there are two independent lines of KDR protein in *A. thaliana*: pKDR:gKDR-GUS #1 and #2. The GUS staining in line pKDR:gKDR-GUS #1 was stronger than that of pKDR:gKDR-GUS #2 and the hypocotyl length of pKDR:gKDR-GUS #1 is also longer than that of pKDR:gKDR-GUS #2 (Figure 9). Many variables could effect the expression of the protein. The time of staining of the seedlings could play a role as KDR is known to increase at dawn and peak at noon (Hyun and Lee 2006). This could not have been the only explanation of why one line was seen to be stronger in the GUS staining than the other because both lines were GUS stained simultaneously. Alternatively, the issue could be due to the possibility that during insertion of the translational fusion of KDR to GUS into the genome of line pKDR:gKDR-GUS #1, the position of insertion is close to enhancer elements. Another possibility would be that the pKDR:gKDR-GUS #1 line had two insertions in tandem of the transgene instead of the single insertion. Even though segregation of the transgene based on resistance to hygromycin was 3:1, this does not always guarantee a single insertion as two insertions in tandem would stay together during recombination and would also result in a 3:1 ratio in segregation analyses. In either of these two cases, KDR expression in pKDR:gKDR-GUS #1 would be increased, its GUS expression phenotype would be stronger and more KDR would result in a longer hypocotyl like that of the KDR overexpressor (Hyun and Lee 2006).

The hypocotyl complementation assay also shows that the shorter hypocotyl phenotype of *kdr-2* was indeed due to KDR's mutation. When the KDR protein is

reintroduced into the *kdr-2* background (Figure 9 pKDR:gKDR-GUS #1 and #2), the hypocotyl length returned back to that of the wild type hypocotyl. While the hypocotyl length of pKDR:gKDR-GUS #1 is longer than that of wild type (as explained previously in conjunction with the stronger GUS staining), pKDR:gKDR-GUS #2 hypocotyl length returned to the length of the wild type. This shows that by putting the protein back into its mutant background, the short hypocotyl phenotype is reverted to wild type length. The *kdr-2* mutant phenotype is indeed due to KDR's mutation.

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