INTERACTION OF IBR5 AND SMALL GTP-BINDING PROTEINS IN GROWTH AND DEVELOPMENT OF ARABIDOPSIS

by

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF ILLUSTRATIONS

Illustration **Page**

LIST OF ABBREVIATIONS

Abbreviation	Description
SCF	SKP, CUL1, F-box
ARF	AUXIN RESPONSE FACTORS
AuxRE	AUXIN RESPONSIVE ELEMENTS
GEF	GUANINE NUCLEOTIDE EXCHANGE FACTORS
GAP	GTPase ACTIVATING PROTEINS
GDI	GTPase DISSOCIATION INHIBITORS
SOS	SON OF SEVENLESS
ROP	RHO-LIKE PROTEINS IN PLANTS
ABP1	AUXIN BINDING PROTEIN
AUX1	AUXIN RESISTANT1
PIN1	PIN FORMED1
SPK1	SPIKE1
RIC	ROP-INTERACTING CRIB MOTIF-CONTAINING PROTEIN4
IBR5	INDOLE3-BUTYRIC ACID RESPONSE5
ARA2	ARABIDOPSIS THALIANA RAB11A HOMOLOG

ABSTRACT

Auxin is a plant hormone that regulates plant growth and development. Auxin controls the expression of auxin-responsive genes by regulating the degradation of a group of transcriptional repressor proteins known as Aux/IAAs via the ubiquitin-proteasome pathway involving nuclear auxin receptors. Recent studies have demonstrated that a dual-specificity phosphatase, INDOLE 3 BUTYRIC ACID RESPONSE5 (*IBR5*) is involved in auxin signaling. According to our yeast two-hybrid screen, *in vitro* binding assays, and coimmunoprecipitation studies, *IBR5* physically interacts with several small GTP binding proteins. Previous studies indicate that small GTP binding proteins, such as ARA2, ROP2, and ROP6, regulate auxin responses in plants. Data presented here using *ibr5/rop2-1* and *ibr5/rop6*-2 or *ibr5/rop2-1/rop6-2* mutant combinations reveals that *ibr5-1* is epistatic to both *rop2-1* and *rop6-2* in root hair formation and leaf epinasty. In addition, plants with *ibr5-1 rop2-1* mutations develop longer and narrower petal blades. Interestingly, *ara2-3* mutation suppresses both rapid degradation of AUX/IAA repressor proteins in *ibr5-4* and the defective physiological and morphological phenotypes of *ibr5-4*, suggesting that these two proteins may function in a common pathway. Therefore, our biochemical and genetic studies suggest that *IBR5* and these GTP binding proteins may function jointly in the auxin signaling pathway

I. INTRODUCTION

Auxin: a key regulator of gene expression

The current model of auxin signaling is centered around nuclear auxin perception (Strader & Nemhauser 2013), which utilizes the ubiquitin-proteasome pathway to modulate auxin responses. Auxin facilitates the interaction between the SKP, CUL1, F-box containing ($SCF^{TIR1/AFBs}$) complex (E3 ligase) and AUX/IAA repressor proteins (Dharmasiri et al. 2005,). AUX/IAA repressor proteins are critical components of auxin modulated gene transcription.

Once AUX/IAA repressors interact with $SCF^{TIR1/AFB}$, it leads to polyubiquitination and subsequent degradation of AUX/IAAs by the 26S proteasome. This degradation of AUX/IAAs relieves the repression on AUXIN RESPONSE FACTORS (ARF). ARF transcription factors are bound to AUXIN RESPONSIVE ELEMENTS (AuxRE) within the promoter regions of auxin-responsive genes and modulate gene transcription (Sauer et al. 2013). Despite what is known about the genomic auxin responses, which occur inside the nucleus, this model does not completely explain the rapid auxin responses such as cell wall acidification that aids cell expansion, which occurs within few minutes in response to auxin (Rayle and Cleland, 1992; Hager et al. 1991). These rapid auxin responses suggest that there may be non-genomic auxin responses (Kieffer et al. 2010).

Plant cells rely on activities of small GTP binding proteins to initiate and terminate the signaling events necessary for appropriate cellular responses (Bourne et al. 1990). In addition to their importance in signal transduction, small GTP binding proteins have also been shown to regulate cell proliferation (Clarke and Zhang, 2001),

cytoskeletal organization (Fu et al. 2002) intracellular vesicle trafficking (Stenmark et al. 2001; Zerial et al. 2001) and translation (Schepetilnikov et al. 2017). All these cellular processes are also regulated by auxin.

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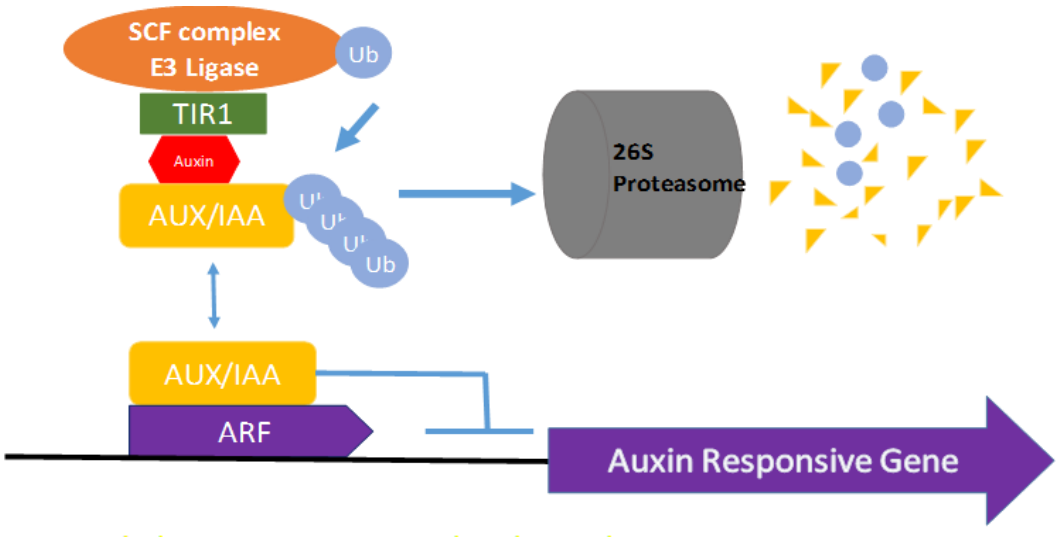

Illustration 1.
Auxin signaling model. Auxin response utilizes the transcriptional regulation of auxin responsive genes. Auxin responsive genes are tightly regulated by TIR1/ Auxin signaling F- box proteins, AUX/IAA repressor proteins and auxin response factors (ARF). In the presence of auxin, TIR1 interacts with AUX/IAA repressor proteins. This results in a poly ubiquitination of AUX/IAA repressors and the degradation of AUX/IAA repressor proteins by the 26s proteasome.

The activity of GTP- Binding proteins in plant signaling

Small GTP binding proteins, or monomeric small G proteins, are low molecular weight proteins, which have a common regulatory function within cellular signaling networks. Small G proteins function in the regulation of a variety of molecular pathways within eukaryotic cells (Bourne et al. 1991; Matozaki et al. 2000). These G proteins determine the duration of specific cellular activity by cycling between its GDP (inactive), and GTP bound (active) states (Bourne et al. 1990). Despite their weak GTPase activity,

small G proteins hydrolyze GTP to return to the inactive state. This GTPase activity requires the assistance of regulatory effector proteins (Berken & Wittinghofer, 2005).

Regulation of the small G protein activation/inactivation cycle is achieved through three types of effector proteins: GEF (GUANINE NUCLEOTIDE EXCHANGE FACTORS), GAP (GTPase ACTIVATING PROTEINS) and GDI (GTPase DISSOCIATION INHIBITORS). Upon association with GEF, GDP-bound inactive small G protein exchanges GDP with GTP to become an active form of a small G protein, which interacts with a variety of downstream effector proteins to initiate signaling cascades. Conversely, when GTP-bound small G protein interacts with GAP, it stimulates GTPase activity resulting in inactive GDP-bound small G protein (Berken & Wittinghofer, 2001). Small G proteins are localized to both cell membrane and cytosol. GDI generally binds to GDP-bound small G proteins and localizes them to a cytosolic pool (Seabra and Wasmeier, 2004). This critical localization process is dependent on post-translational lipid modifications, like prenylation and geranylgeranylation, which allow small G proteins to be inserted into the target membrane (Glomset and Farnsworth, 1994). The nature of these post-translational modifications and the fact that many GDIs have higher specificity for specific G proteins likely play a role in the specific localization of small G proteins to their respective membranes.

Small G proteins have been grouped into five families based on function and sequence (Qing-Hu, 2007) Each of the five families of GTP binding proteins undergoes conformational changes to interact with effector proteins to regulate an array of cellular functions. Ras, Ran, Rab, Arf, Rho are the five families of small GTP binding proteins involved in cell signaling. The Ras family regulates the expression of genes (Dent et al.

1992; MacDonald et al. 1993). Ran proteins regulate cell division (Clarke and Zhang, 2001). Rab and Sar1/Arf regulate vesicular trafficking, endocytosis, and exocytosis (Ueda et al. 2001). Rho/Rac/Cdc42 regulate cytoskeleton (Hall et al.1998), gene expression, and translation (Schepetilnikov et al. 2017).

Regulation of gene expression through Ras GTP binding protein signaling

Ras small G proteins play a key role in cell differentiation (Crespo and León, 2000), apoptosis (Downward, 1998) and the regulation of gene expression through Raf kinase-MAPK signaling pathway (Dent et al. 1992). Ras GTP binding proteins are localized on the cytosolic side of the plasma membrane and are activated by extracellular signals perceived by tyrosine kinase receptors. This results in the recruitment of *SON OF SEVENLESS* (*SOS*), a GEF that facilitates the exchange of bound GDP for GTP resulting in G protein activation (Rojas et al. 2011). Upon activation, Ran proteins interact with Raf kinases, which induce mitogen-activated kinase cascades by phosphorylating MAP kinase kinase, which ultimately results in gene expression (Dent et al. 1992). This signal is turned off with the interaction between Ras G proteins and their effector GAP protein interaction partners. Since Ras family proteins play a large role in the regulation of the expression of important genes necessary for cell differentiation, division, and apoptosis, mutant alleles of Ras genes have been implicated in the onset of many cancers in humans (Fernandez-Medarde and Santos, 2011; Prior et al. 2012).

Arf is a key regulator of vesicle transport

Arf/ Sar1 proteins are key regulators of vesicle traffic that were originally identified in mammals (Kahn et al. 1984, Kahn, et al. 1986). Arf is known to be localized to the Golgi apparatus and function in the budding of secretory vesicles in eukaryotic

cells. Arf proteins can interact with phospholipid membranes due to a myristolated N terminus (Cherfils et al.1999), which allows the proteins to weakly anchor themselves to donor membranes and this association with phospholipid membranes allows for their activation by GEFs. In the active state, Arf proteins recruit coat proteins like COP1, COP2 to aid in the vesicular budding process and recruit the cargo receptors necessary for traffic of intracellular material. Once the budding process is complete Arf proteins are converted to their inactive state, which allows the dissociation of coat proteins (Chavier and Gould, 1999; Randazzo and Hirsch, 2004).

Ran: the regulator of nuclear transport of proteins and RNA

Ran family of small G proteins is the smallest in eukaryotes and mainly functions on the nucleocytoplasmic transport (Dasso, 2002). Transport of large macromolecules into the nucleus relies upon transport receptors, adapter proteins and Ran mediated signaling events (Dahlberg et al. 1998, Mattaj et al. 1998). Ran and its interaction with nuclear transport receptors importins and exportins aids in directing proteins and RNA in and out of the nucleus through nuclear pore complexes (NPC) (Weis, 2003). In nuclear exporting, the transport receptor exportins recognize and bind to nuclear exportin sequences (NES) and bind the active GTP bound Ran. This cargo exporting complex is transported from the nucleus to the cytoplasm, which results in the inactivation of Ran by GAP proteins in the cytoplasm (Adam, 1999). In the process of importation, Ran proteins in their GTP bound form, interact with importin alpha and beta cargo complex and facilitate the dissociation of newly imported cargo from the importin complex (Weis, 2003). In addition to nuclear-cytoplasmic transport, Ran GTP binding proteins also

function in the regulation of the reorganization of microtubules during mitosis (Li et al. 2003).

Rab, an internal regulator of vesicle transport

Rab proteins are regulators of vesicular trafficking, endocytosis, and exocytosis (Stenmark et al. 2001; Zerial et al. 2001). They make up the largest small GTP binding protein family, conserved across all eukaryotic systems (Periera-Leal, 2001; Jiang and Ramchandran, 2006). During intracellular transport, Rab proteins coordinate the targeting, docking, and fusion of endosomal vesicles. Intracellular transport occurs when the vesicles begin to bud from the membrane of origin. Next, the vesicle package is targeted to the recipient membrane. Once the vesicle has a targeted destination membrane, there is docking and fusion of the vesicle into the target membrane. Rab proteins, along with Sar1/Arf proteins, are needed for this directed intracellular transport. Rab family proteins undergo geranylgeranylation, which is crucial for Rab insertion into membranes (Farnsworth et al. 1991).

Rho/Rac/Cdc42 regulation of cytoskeletal rearrangement

The function of Rho/Rac/Cdc42 small G protein family in the regulation of the cellular cytoskeleton was first identified in yeast (Machesky and Hall, 1996; Hall, 1998; Fu, 2002). Rho/Rac/Cdc42 G proteins in mammals are also involved in gene expression, membrane trafficking, cell growth, development, and elongation (Hill et al.1995; Westwick et al. 1997). In Arabidopsis, this group of proteins is known as RHO-LIKE PROTEINS IN PLANTS (ROP), which function in cytoskeleton rearrangement in response to hormones such as ABA and auxin (Hai et al. 2001), and abiotic stress (Li et al. 2017). In Arabidopsis, ROPs are necessary for ABA-induced seed dormancy (Li,

2001), and auxin-dependent interdigitation of epidermis cells in leaves (Deshu et al. 2014). Across eukaryotic systems, Rho family proteins are needed for the regulation of actin filament polarization and depolarization for cells to maintain proper morphology and structure in response to developmental and environmental stimuli (Ridley et al. 1992; Burridge et al. 2004).

Auxin signaling and ROP-mediated cell expansion

Some Auxin responses are fast responses at the plasma membrane. TIR1/AFB mutants, which have defective nuclear auxin receptors, still have normal rates of cell elongation (Sauera et al. 2011). Rapid physiological responses associated with cell expansion such as increases in cytosolic calcium and proton secretion (Shinsova and Lindberg, 2004; Senn and Goldsmith, 1988). This rapid auxin response at the plasma membrane has been shown to be dependent on a cell surface and Rho GTPase-based auxin signaling pathway (Xu et al. 2009). AUXIN BINDING PROTEIN (ABP1) has been implicated in regulating cell expansion at the plasma membrane through its regulation of cytoskeleton rearrangement and clathrin-mediated endocytosis (Chen et al. 2001a; Chen et al. 2001b). However, recent studies have revealed that auxin transporters like AUXIN RESISTANT1 (AUX1) and PIN FORMED1 (PIN1), but not ABP1, are required for auxin-induced plasma membrane depolarization (Paponov et al. 2019). Currently, the signaling that underlies auxin-dependent spatial control of cell expansion and how that connects with the nuclear auxin signaling module is still not well characterized. Plants must coordinate the spatial control of cell expansion to develop tissue morphology. Cell expansion relies on auxin transporters to generate directional flows of auxin (Petrasek et al. 2006; Wisnieikawa et al. 2006). This directional flow of

auxin has been shown to be necessary for the polar expansion responsible for the spatial control of root hair formation (Fischer et al. 2006) and leaf pavement cell interdigitation (Fu et al. 2005, Fu et al. 2002). In our study of *IBR5*, *ARA2*, *ROP2*, and *ROP6*, three different epidermal tissues: petals, root hairs, and leaves, were utilized as models to understand the role of auxin-mediated cell expansion.

ROP signaling and petal development

For angiosperms, flower petals are necessary for the attraction of pollinators to the flower to facilitate sexual reproduction (Willmer et al. 2009; Yuan et al. 2013). In Arabidopsis, the flower petal has been established as an ideal model system for studying mechanisms of cell expansion due to its simple structure and small variety in cell types (Irish, 2008; Huang and Irish, 2016). Plant Rho-like small G proteins or ROPs have been implicated in the regulation of cell expansion and polar growth of petal and other tissues. ROPs have been demonstrated to be important for regulating the rearrangement of the cytoskeleton (Fu et al. 2009), cell wall patterning (Oda and Fukuda, 2012), pollen tube tip elongation (Qin and Yang, 2011), pavement cell interdigitation (Wu et al. 2011; Craddock et al. 2012) and endosomal recycling of PIN auxin efflux transporter (Lavy et al. 2007; Hazak et al. 2010). ROP2 and ROP6 are activated by the GEF, SPIKE1 (SPK1) resulting in inhibition of anisotropic growth in petals by promoting the isotropic orientation of cortical microtubules (Huibo et al. 2016). Catalytic site mutant, *tink/ibr5-6* exhibits shorter and narrower petal blades as well as shorter height and reduced apical dominance phenotypes (Johnson et al. 2015). There is evidence of mutations in both *SPIKE1*(*spk4-1*) and *IBR5*(*tink/ibr5-6*), resulting in a defect in petal organ size, potentially implicating both genes in the same ROP signaling pathway (Huibo et al. 2016;

Johnson et al. 2015). ROP signaling likely controls many events of cell expansion of root hairs (Moritaka et al. 2018) leaves (Fu et al. 2002) and flower petals (Huibo et al. 2016).

ROP signaling and leaf development

In the leaf, the epidermal pavement cell is an established model system used for the study of cell polarity and cell to cell regulation of cell expansion (Fu et al. 2002; Fu et al. 2005; Settleman, 2005; Yang, 2008). Like the embryonic convergent extension that facilitates spinal cord development in animals, leaf interdigitation relies upon Rho G protein signaling to direct rearrangement of the cytoskeleton (Green and Davidson 2007; Heasman, 2006). In Arabidopsis thaliana, ROP2 has been demonstrated to interact with ROP-INTERACTING CRIB MOTIF-CONTAINING PROTEIN4 (RIC4) to stimulate cortical f-actin-mediated lobe formation in leaf epidermal cells. ROP6 has been shown to be essential for formation of indent regions by promoting the formation of well-ordered transverse cortical microtubules (MT) (Fu et al. 2002; Fu et al. 2005; Xu et al. 2009). ROP2 inhibits the formation of well-ordered cortical microtubules in the lobe tip through its interaction with RIC1, which is a microtubule-associated protein (Fu et al. 2005).

Conversely, ROP6 interacts with RIC1 to promote the microtubule cytoskeleton to rearrange parallel to the axis of growth inhibiting cell expansion (Fu et al. 2005; Fu et al. 2009). Auxin signaling has been shown to regulate interdigitation patterning of the leaf epidermis through the ROP signaling pathway (Xu et al. 2009). The auxin and ROP mediated spatial control of cell polarity in the leaf epidermis bears similarities to the ROP signaling required for the polar cell growth observed in the development of root hairs.

ROP signaling and root hairs

Root hairs are tube-like projections developed by trichoblasts (root hair forming epidermal cells), which function to increase surface area for water and nutrient absorption (Grierson et al. 2014). Auxin signaling is essential for root hair initiation and elongation (Masucci and Schiefelbein, 1994; Schiefelbein et al. 2000). Trichoblasts need the coordination of auxin and ROP signaling to facilitate proper root hair development (Fischer et al. 2006; Ikeda et al. 2009; Nakamura et al.2012; 2018). The formation of root hairs from trichoblasts relies upon ROP2, and ROP6 signaling to regulate the cytoskeletal rearrangement necessary for polar growth (Baluska et al. 2000; Molendjik et al. 2001; Jones et al. 2002; Carol et al. 2005). Factors such as Ca^{2+} tip-focused gradient and production of reactive oxygen species also play a role in root hair development (Foreman et al. 2003; Jones et al. 2007; Takeda et al. 2008). GEFs also regulate ROPs, Guanine dissociation inhibitors (GDI) (Klahre et al. 2006; Feng et al. 2016), and protein s-acyltransferases (Wan et al.2017); all important for root hair formation and elongation. In this study of *IBR5*, *ROP* interaction, root hairs will serve as a model system for studying the connections between cell expansion and auxin signaling.

IBR5, a regulator of the auxin signaling pathway

Auxin hormone (IAA) is synthesized highly in the apical meristems, regions, which undergo frequent mitotic activity (Vernoux et al. 2010). For plants, this means optimal growth depends on maintaining a tightly regulated signaling network that is sensitive to fluctuations auxin concentration. Cells in the apical root meristem, for example, are sensitive to high concentrations of auxin, which result in primary root growth inhibition (Zolman et al. 2003). High auxin concentration and its inhibitory effect on root growth make primary roots an ideal system to study while identifying the genes involved in the

auxin response pathway since there is a readily identifiable auxin response phenotype. EMS mutagenized seedlings screened on synthetic auxins picloram, and 2,4-D revealed many genes essential in the auxin response pathway. *IBR5* or *INDOLE3-BUTYRIC ACID RESPONSE5* was initially identified by screening of Arabidopsis mutants for the insensitivity of primary root growth for auxin precursor indole3-butyric acid (Zolman et al. 2000; Monroe-Augustus et al. 2003). Additional *ibr5* mutant alleles were revealed to be resistant to primary root inhibition in the presence of picloram and 2,4-D (Monroe-Augustus et al. 2003; Gihmire, 2015; Lopez, 2015). IBR5 is a putative dual specificity phosphatase (Monroe-Augustus et al. 2003), a group of phosphatases capable of dephosphorylating threonine/serine as well as tyrosine residues (Suk Lee et al. 2008). IBR5 is suspected to be involved in the MAPK pathway (Suk Lee et al. 2008; Johnson et al. 2015). This was particularly interesting since the MAPK pathway is known to be involved in the mitotic activity characteristic of auxin-induced growth stimulation.

As explained above, auxin signaling pathway is centered around nuclear localized genomic responses (Gray et al. 1999, 2001; Ward and Estelle, 2001; Dharmasiri and Estelle, 2004), in which degradation of AUX/IAA leads to modulation of the transcription of auxin-responsive genes (Ulmasov et al. 1997; Ulmasov et al. 199). Typically, most mutants in the auxin response pathway exhibit increased stabilization of AUX/IAA repressors; however, in *ibr5* mutants, AUX/IAA repressors are rapidly degraded while primary root growth exhibits auxin resistance phenotype (Strader et al. 2008; Jayaweera et al. 2014). This suggests that IBR5 plays a crucial regulatory role in genomic auxin responses.

IBR5 and small G Protein physical interaction

A previous effort in our lab to identify IBR5 interacting proteins using yeast two-hybrid screen identified Arabidopsis ARA2 (ARABIDOPSIS THALIANA RAB11A HOMOLOG) as an interacting partner. ARA2 is a small GTP binding protein homologous to the Rab11A family in mammals. Members of this family have been implicated in endosomal vesicle recycling from Trans Golgi Network to the plasma membrane (Xinquan and Huanquan 2013; Zerial et al. 2001).

IBR5 plays a role in the regulation of the nuclear localized genomic response and has been shown to physically interact with ARA2 (Ghimire, 2015), a protein known to be active in the cytoplasm. This interaction between IBR5 and ARA2 is interesting since previous studies have demonstrated that cell expansion occurs quite rapidly following exposure to auxin (5-10 minutes). This rapid response shows that at least some auxin responses likely occur outside of the nucleus. Since ARA2 interacts with IBR5, there is a possibility that IBR5 may also be interacting with other small G proteins that are involved in plant auxin response.

ROPs, especially ROP2 and ROP6 are two small G proteins that have been well documented to be important in plant auxin responses. These ROPs and their effector proteins RIC1, and RIC4 are essential in cell polarity, and cytoskeletal changes associated with auxin signaling (Hai et al. 2001, Ridley and Hall, 1992; Burridge and Wennerburg, 2004). These cytoskeletal changes are necessary for leaf pavement cell interdigitation in Arabidopsis cotyledons. Like ARA2, ROP2 and ROP6 also physically interact with IBR5 (Lopez, 2015).

Identifying the connection between genomic auxin responses and cytoplasmic counterparts is crucial to understanding plant auxin response pathway. IBR5 is essential

for regulating the degradation of AUX/IAA repressors within the nucleus while small G proteins are necessary for cytoskeletal changes and trafficking events connected with auxin responses. If the physical interaction between IBR5 and small G proteins is vital for auxin hormone signaling, we should expect evidence for genetic interactions between IBR5 and small G proteins genes.

Results from previous studies have suggested that *ara2* mutant is insensitive to inhibition of primary root growth elongation in response to auxin (Koh et al. 2009). Also, both IBR5 and ROP GTPases, ROP2 are involved in proper patterning of interdigitation of leaf epidermal pavement cells (Jayaweera et al. 2014; Xu et al. 2009). ARA2 and ROP GTPases belong to closely related families of small GTP binding proteins. ARA2 was identified as an IBR5 interacting protein, and several lines of evidence implicate ROP GTPases in auxin signaling. We hypothesized that there is a genetic interaction between IBR5 and small GTP binding proteins. Specifically, we hypothesize that the genetic interaction between ARA2 and IBR5, as well as ROP2, ROP6 and IBR5, are relevant to the auxin signaling pathway. Thus, it is necessary to characterize the phenotypes of *ibr5*/small GTP protein mutants as well as identify the associated biochemical phenotypes that connect IBR5 small GTP protein genetic interaction to auxin signaling.

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II. MATERIALS AND METHODS

Plant Materials and Growth conditions

Seeds of *Arabidopsis thaliana* Col-0 ecotype were used for all experiments described here. Seeds were surface sterilized in 40% (v/v) commercial bleach with 0.01% (v/v) Triton X-100 for 4min. The seeds were then rinsed 3x with sterile distilled water and stratified at 4^{o} C for a minimum of 24hr. Seeds were plated on ATS medium (1% Sucrose) (Lincoln et al. 1990), and seedlings were grown at 22^{o}C under continuous light. All experiments were completed under these settings unless otherwise specified.

Isolation of RNA

4day old seedlings(100mg) were frozen and ground in liquid nitrogen. Total RNA was extracted following the manufacturers protocol 1ml of Tri-reagent (Sigma, St Louis, MO). Total RNA was then resuspended in diethylpyrocarbonate (DEPC) treated water. NanoDrop spectrophotometer (Thermo Scientific) was used to quantify and analyze RNA purity. Total RNA was treated with RNase-free DNase1 (NEB) for 25min prior to synthesis of complementary DNA (cDNA). Next, cDNA was synthesized using Oligo-dT primers and M-MLV reverse transcriptase (NEB) according to the manufacturer's instructions.

Isolation of DNA

Plant tissue was ground in 300µL of 2x CTAB buffer 2% (w/v) CTAB, 100mM Tris-Cl pH 8.0, 1.4M NaCl, 20mM EDTA) and the sample was heated at 65^{o}C for 1hr. Next 300µl of chloroform was added to the extract, vortexed and centrifuged at 13000 xg for 5 min at room temperature. The supernatant was transferred to a new tube along with

600µL of 100% ethanol and kept at -20°C for 1hr. The sample was then centrifuged at 4°C for 20 min at 13000 xg, and the supernatant was discarded. The DNA pellet was left to air dry for 10 min, and resuspended in 50µl of 10mM Tris-Cl pH 8.0 and incubated at 37°C for 30 min.

Screening for small G protein single mutants

To study the physiological role of *ARA2 (A1A), A1B, A1C, ROP2, ROP6* in auxin response T-DNA mutant alleles were obtained from the Arabidopsis Biological Research Centre (ABRC).

Table 1. Single mutant line information

Allele name	***Salk ID***
**ara2-3 (a1a)*	Salk_077747C
a1b	Salk_022393.24.75.X
a1c	Salk_145363C
***rop2-1*	Salk_0855328C
***rop6-2*	Salk_091737C

***previously screened by Prabesh Gihmire**

****previously screened by Elia Lopez**

Table2. Genotyping Primer list	
Primer Name	***Primer Sequence***
*ARA2F	5' TCGGATCCCTGATGGCTGGTTACAGAGCCGAT 3
*ARA2R	5’ CGTCGACTCTAGTTAGAGCAACCCATTC 3’
ARA2-A1B-1F	5' AGATGGCAGGGTACAGAGTGGA 3'
ARA2-A1B-R	5' GCTACCTCAAGATTCCATCCGTC 3'
ARA2 A1C- 1F	5' GAGAGCTTGATTGGTTTCAGTAAC 3'
ARA2 A1C- 1R	5' ATTAGTTCGAGCAGCATCCACCT 3'
**ROP2-1F	5' GCGGGATCCATGGCGTCAAGGTTTATAAAG 3'
**ROP2-1R	5' TTTGTCGACTCACAAGAACGCGCAACG 3'
**ROP6-1F	5' GAGGGATCCATGAGTGCTTCAAGGTTTATC 3'
**ROP6-1R	5' CTTGTCGACTCAGAGTATAGAACAAC 3'
***LB02	5' TTGGGTGATGGTTCACGTAGTGGGCC 3'
****ibr5-1SnB-F(dCAPS)	5' GCCTGTTTCTTCCGATACGGTGGCTACG 3'
****ibr5FBoxSal1 (dCAPs)	5' GTAGAGATTCTGGCACATAGG 3'
*****GUS$^{3'}$R	5’ CGATCCAGACTGAATGCCCACA 3’
*****GUS$^{5'}$F	5’ CAACGTCTGCTATCAGCGCGAAGT 3’

****ibr5-4 F (dCAPs)	5’ TCGGTAGTTACGACAACGCTTCTC 3’
****ibr5-4 R (dCAPs)	5’ ACAACAACCGCTGGTGATCTACTGATA 3’

***Designed by Prabesh Gihmire**

****Designed by Elia Lopez**

*****Designed by Salk Institute**

******Designed by Thilanka Jayaweera**

*******Designed by Sunethra Dharmasiri**

Generation of Arabidopsis combinatorial mutants

To characterize the genetic interaction between *IBR5*, *ARA2*, *ROP6* and their homologs, *ibr5-4 ara2-3* and *ibr5-1 rop6-2* homozygous double mutants were crossed into their respective single mutant homologs *a1b* and *a1c* (*ara2-3*) and *rop2-1* (*rop6-2*) respectively. The subsequent F_1 generation was left to self-pollinate to obtain an F_2 generation. To select individual seedlings, which combined *ibr5 x small G protein* mutations F_2 seedlings were screened on the synthetic auxin 2,4-Dichlorophenoxyacetic acid (2,4-D). It has been demonstrated that seedling homozygous for the *ibr5* mutant alleles are resistant to 2,4-D (Jayaweera et al. 2014). Seedlings that exhibited resistance to the inhibitory effects of 2,4-D were further confirmed for homozygous *ibr5* mutations by PCR. Next, the PCR product was digested with the appropriate restriction enzyme (SnaB1 for *ibr5-1* or BsmA1 for *ibr5-4*). 2,4-D resistant seedlings were then screened for homozygous *ara2-3*, *a1b*, *a1c* or *rop2-1*, *rop6-2*, (T-DNA insertional mutations) using an

appropriate gene-specific primer and T-DNA left border primer (LB02) or gene-specific primers as shown in the following table.

Primer combinations for PCR Genotyping

Table 3. P**rimer Combinations for PCR Genotyping**		
Mutation	***Gene-specific Primer Combination***	***T-DNA Primer Combination***
ara2-3 (a1a)	ARA2F + ARA2R	ARA2R + LB02
a1c	ARA2 A1C- 1F + ARA2 A1C- 1R	ARA2 A1C- 1R + LB02
a1b	ARA2-A1B-1F + ARA2-A1B-R	ARA2-A1B-F + LB02
rop2-1	ROP2-1F + ROP2-1R	ROP2-1R + LB02
rop6-2	ROP6-1F + ROP6-1R	ROP6-1F + LB02
**ibr5-1*	ibr5-1SnB-F + ibr5FBoxSal1R	N/A
**ibr5-4*	ibr5-4 F + ibr5-4 R	N/A
GUS	$GUS^{3'}R$ + $GUS^{5'}F$	N/A

***ibr5-1* PCR Product was digested with Sal1, *ibr5-4* PCR product was digested with BsmA1**

HS:: AXR3NT-GUS reporter line is a well-established tool for examining AUX/IAA stability in auxin response mutants (Gray et al. 2001). To study AUX/IAA degradation in *ara2-3 ibr5-4* double mutant background, *HS:: AXR3NT-GUS ara2-3* (Ghimire, 2015) and *HS:: AXR3NT-GUS ibr5-4* (Jayaweera et al. 2014) were crossed together. The resulting F_1 plants were self-pollinated to obtain F_2 seeds. The presence of *HS:: AXR3NT-GUS* was verified by PCR using $GUS^{3'}R$/$GUS^{5'}F$ primers and the

presence of *ara2-3* and *ibr5-4* mutations were verified by PCR using the gene-specific and T-DNA specific primers (for *ara2-3*) or PCR and subsequent digestion of PCR product (for *ibr5-4*).

Histochemical staining for GUS activity

Homozygous *HS:: AXR3NT-GUS* (Col-0 wild-type), *HS:: AXR3NT-GUS ara2-3, HS:: AXR3NT-GUS ibr5-4* or *HS:: AXR3NT-GUS ara2-3 ibr5-4* mutant lines were grown on ATS (1% sucrose) medium for 5days. Then seedlings were placed in water to equilibrate at room temperature for 1hr at room temperature before the heat shock treatment for 2hr and 30 min with gentle shaking at 95 rpm. Samples were taken at different time points (as indicated in results section) and then fixed with GUS fixer solution (0.3% formaldehyde ,0.3M mannitol, 10mM 4-morpholineetanesulfonic acid (MES) (Jefferson, 1987) for 30 min by vacuum infiltration followed by 30 min gentle shaking at room temperature. After fixation, the samples were washed 2x 10 min each in 100mM Na_2HPO_4. Washed sampled were vacuum infiltrated for 5 min in GUS staining buffer (0.1 M 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide cyclohexylammonium, 100 mM Na_2HPO_4, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 1 mM potassium ferricyanide, I mM potassium ferrocyanide) then covered in foil and kept at 37°C overnight (Jefferson, 1987).

Quantitative β-Glucoronidase assay

Arabidopsis seedlings grown on solid ATS (1% sucrose) medium for 4days were given heat shock at 37°C for 2hr. Seedlings were then frozen in liquid nitrogen and ground in GUS extraction buffer (100mM Phosphate buffer pH 7.0, 10mM EDTA, 0.1% sodium lauryl sarcosine, 10μM β-mercaptoethanol) and centrifuged at 10,000 xg for 10

min. The supernatant was collected, and Bradford assay (Bradford, 1976) was performed to estimate the total protein concentration. About 60-75µg of total protein from each protein sample was incubated at 37°C for 1hr. To stop the reaction, a 100µl aliquot of the assay solution was added to stop the reaction fluorescence was measured at 460nM using a luminometer (Turner Biosystems, Sunnyvale CA, Model number -9200-002)

Primary root growth assay

Seeds from Col-0, *ara2-3*, *ibr5-4*, *ara2-3 ibr5-4* lines were germinated on ATS (1% sucrose) media and transferred onto plates supplemented with indicated concentrations of 2,4-D. During the transfer, each seedling was placed with their root tips touching a line drawn across the middle of the plate. Plates were incubated in a growth chamber under continuous lights at 22°C for 4 additional days, and the root length below the starting line was measured. Percentage root growth inhibition was calculated by using the following equation:

$$\%inhibition = \frac{\Sigma\left[\frac{average\ untreated\ root\ length - treated\ root\ length}{average\ untreated\ root\ length} * 100\right]}{n}$$

a sample size of a minimum of 10 seedlings per genotype was analyzed. Each experiment was conducted at least three times.

Root hair assay

Seeds of Col-0, *ibr5-1*,*rop2-1*,*rop6-2*, *rop2-1/6-2*,*ibr5-1 rop2-1*, *ibr5-1rop6-2*, *ibr5-1 rop2-1 rop6-2* were plated on ATS (1% sucrose) and grown on vertically oriented plates in continuous light 4-day old seedlings were transferred onto either ATS or ATS supplemented with different concentrations of auxins (as indicated in results) and were grown for 2 additional days for root hair assay. Root hairs were imaged 5mm from the

root tip, and the root hair length and number, as well as whole root length, were analyzed using ImageJ.

Interdigitation of leaf pavement cells

To examine the interdigitation of epidermal cells, the first pair of true leaves seedlings of Col-0, *ibr5-1, rop2-1, rop6-2, ibr5-1 rop2-1, ibr5-1 rop6-2, rop2-1/6-2, ibr5-1 rop2-1/6-2* were observed under Confocal microscope (FV1000) using leaf auto fluorescence at 488nm excitation. Measurements of leaf interdigitation pattern images were performed using ImageJ. Lobe number per μm^2 was used to quantify the interdigitation pattern as previously described (Xu et al. 2009).

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III. RESULTS

IBR5, ROP2, and *ROP6* contributed to leaf development

Auxin signaling and activities of *ROP6, ROP2* were demonstrated to be essential for the interdigitation patterning during cell expansion in leaf epidermal tissue (Fu et al. 2005; Fu et al. 2009) Characterization of *ibr5* mutants has also implicated *IBR5* in regulating the interdigitation of leaf epidermis (Jayweera et al. 2014). The rosette leaves of *ibr5-1* mutants exhibited increased leaf epinasty compared to Col-0, *rop2-1*, *rop6-2* single mutants and *rop2-1/6-2* double mutants (Figure 1). There was no difference in leaf epinasty between *ibr5-1* single mutant, *ibr5-1 rop2-1*, *ibr5-1 rop6-2* double mutants or *ibr5-1 rop2-1/6-2* triple mutant (Figure 1).

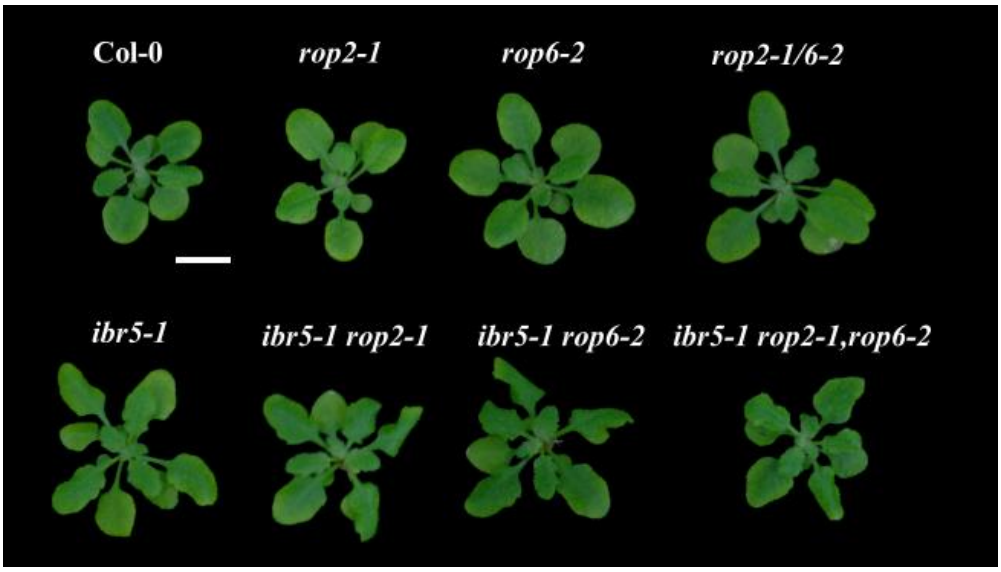

Figure 1.
rop2-1 nor *rop 6-2* enhanced or suppressed *ibr5-1* leaf epinasty. Col-0, *rop2-1*, *rop6-2*, *rop2-1/6-2*, *ibr5-1*, *ibr5-1 rop2-1*, *ibr5-1 rop2-1/6-2* were observed at the vegetative stage. Leaf epinasty phenotype was observed in *ibr5-1* single mutant, *ibr5-1 rop2-1* and *ibr5-1 rop6-2* double mutant and *ibr5-1 rop2-1/6-2*. Scale bar represents 1 cm.

IBR5 physically interacted with ROP2 and ROP6 in *vivo* and *in vitro* (Lopez, 2015). To determine if *IBR5, ROP2* or *IBR5, ROP6* interactions are necessary for the interdigitation of leaf epidermal cells.

The first true leaves during the early vegetative stage were analyzed. Pictures of the abaxial side of true leaves of 3 week old plants were obtained using the FV1000 confocal microscope to visualize leaf interdigitation. There was a decrease in lobe formation in the *ibr5-1* single mutant, *ibr5-1 rop2-1*, *ibr5-1 rop6-2* double mutants, and *ibr5-1 rop2-*

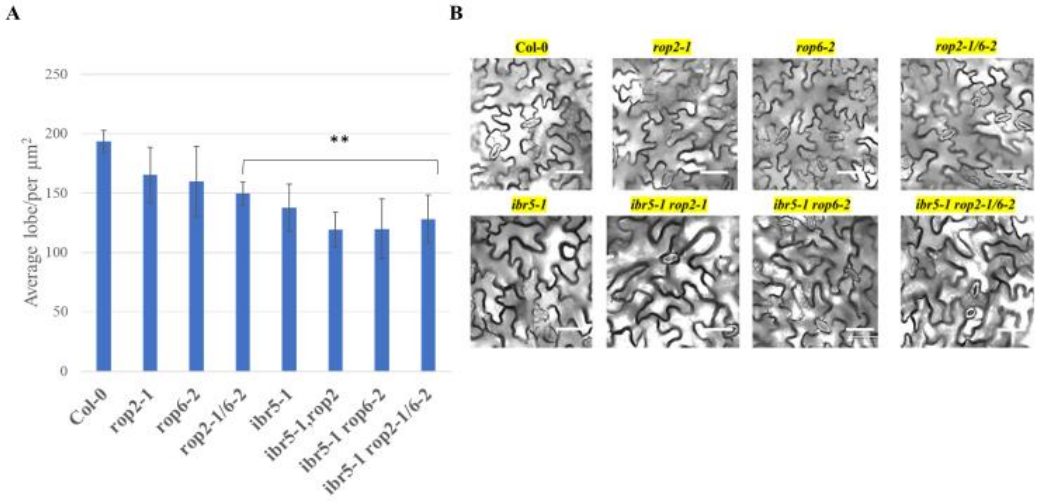

Figure 2.
Lobe formation was significantly reduced in *ibr5* and *rop2-1/6-2* mutants. (A, B) The first true leaves were taken during the early vegetative stage and observed using FV100 confocal microscope to visualize epidermal cells on the abaxial side of the leaf. (A) Data represents the mean number of lobes per μm^2. Error bars represent SD (*) asterisks represent statistically significant values ($p<0.01$) (n = 200 cells from 6 individuals) (B) Scale bars indicate 2µm.

1/6-2 triple mutants as well as in *rop2-1/6-2* double mutant compared to Col-0, or *rop2-1*, *rop6-2* single mutants (Figure 2).

ibr5-1 rop2-1 mutant developed longer flower petal blades

ROP2 and *ROP6* have been implicated in polar growth and cell expansion events connected with flower petal blade development (Huibo et al. 2016). *IBR5* has also been demonstrated to be necessary for flower petal size (Johnson et al. 2015). Therefore, the petal blade phenotypes of *ibr5-1*, *rop2-1*, *rop6-2* single mutants, *rop2-1/6-2*, *ibr5-1 rop2-1*, *ibr5-1 rop6-2* double mutant, and the *ibr5-1 rop2-1/6-2* triple mutant were compared to Col-0. The *rop2-1/6-2* double mutant had longer and slightly narrower petal blades than Col-0, and *rop2-1*, *rop6-2* single mutants. Compared to Col-0, *rop2-1*, *rop6-2* single mutants, and *rop2-1/6-2* double mutant, *ibr5-1*, *ibr5-1 rop2-1*, *ibr5-1 rop6-2 ibr5-1 rop2-1/6-2* mutants developed narrower petal blades (Figure 3). The petal blades of *ibr5-1 rop2-1* double mutants were longer compared to *ibr5-1* single mutant, *the ibr5-1 rop6-2* double mutant, and *ibr5-1 rop2-1/6-2* triple mutant (Figure 3).

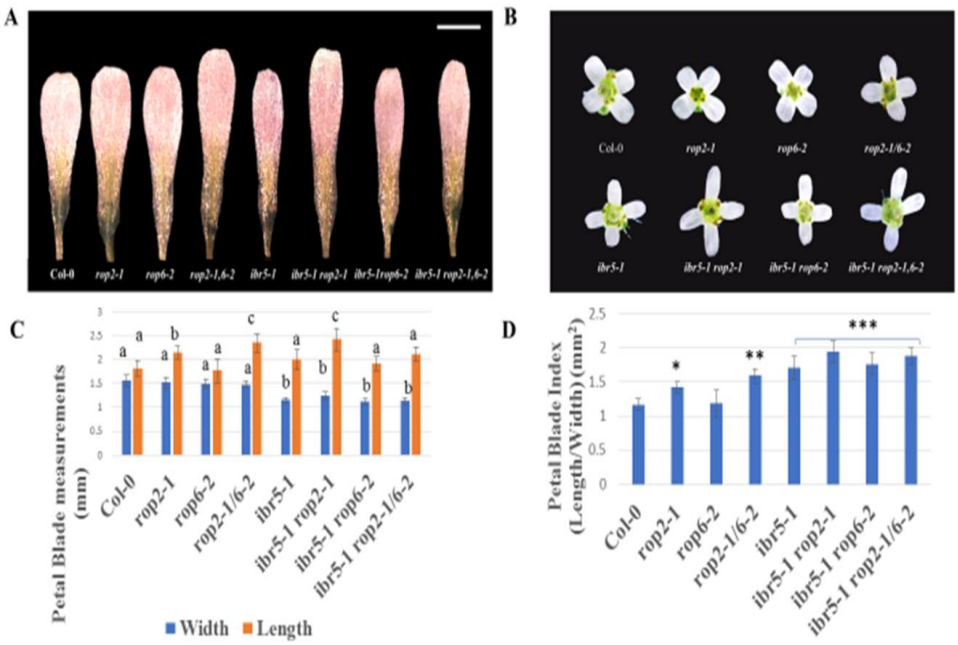

Figure 3.
ibr5-1 rop2-1 double mutants displayed longer petal blades. (A Petals, B Flowers) from stage mature flowers were taken from Col-0, *rop2-1*, *rop6-2*, *rop2-1/6-2*, *ibr5-1*, *ibr5-1rop2-1*, *ibr5-1 rop6-2*, *ibr5-1 rop2-1/6-2*. Compared to Col-0, *ibr5-1* mutants, and *rop2-1/6-2* double mutant have narrower petal blades. (C) Data represents the average width and length data for Col-0, *ibr5-1*, *rop2-1*, *rop6-2*, *rop2-1/6-2*, *ibr5-1 rop2-1*, *ibr5-1 rop6-2*, *ibr5-1 rop2-1/6-2*. Error bars represent SD (a, b, c letters) represent statistical significance ($p<0.05$). (D) Data represents the petal index ratio for Col-0, *ibr5-1*, *rop2-1*, *rop6-2*, *rop2-1/6-2*, *ibr5-1 rop2-1*, *ibr5-1 rop6-2*, *ibr5-1 rop2-1/6-2* error bars represent SD (a, b letters) represent statistically significant values ($p<0.01$) n=16 petals from 4 plants. Images taken using Nikon stereomicroscope.

Root hair development was reduced in *ibr5-1* mutants

Root hair development by trichoblasts is regulated by auxin signaling and ROP activity (Fischer et al. 2006; Ikeda et al. 2009; Nakamura et al. 2012; Moritaka et al. 2018). In mutants possessing *ibr5-1* null mutation, there is a decrease in root hair development (Figure 4). To investigate the possible genetic interaction between *IBR5*, *ROP2*, *ROP6*, the root hair phenotypes of Col-0, *ibr5-1*, *rop2-1*, *rop6-2* single mutants, *rop2-1/6-2*, *ibr5-1 rop2-1*, *ibr5-1 rop6-2* double mutants, and *ibr5-1 rop2-1/6-2* triple mutant were compared. Seedlings were grown on ATS (1% sucrose) for 4 days then transferred onto mock or 20nM 2,4-D media and grown for 2 days. *rop2-1*, *rop6-2* and *rop2-1/rop6-2* developed less root hairs compared to Col-0 when grown on ATS; however, this reduction in root hairs was recovered by exposure to 20nM 2,4-D. Root hair number was lower in the *ibr5-1* and *ibr5-1rop2-1*, *ibr5-1rop6-2*, *ibr5-1rop2-1/6-2* mutants when grown on ATS and exposed to 20nM 2,4-D led to a modest increase in root hair number (Figure 4). The number of root hairs did not differ significantly between the *ibr5-1* single mutant, *ibr5-1 rop2-1*, *ibr5-1 rop6-2* double mutant, and *ibr5-1 rop2-1/6-2* triple mutant.

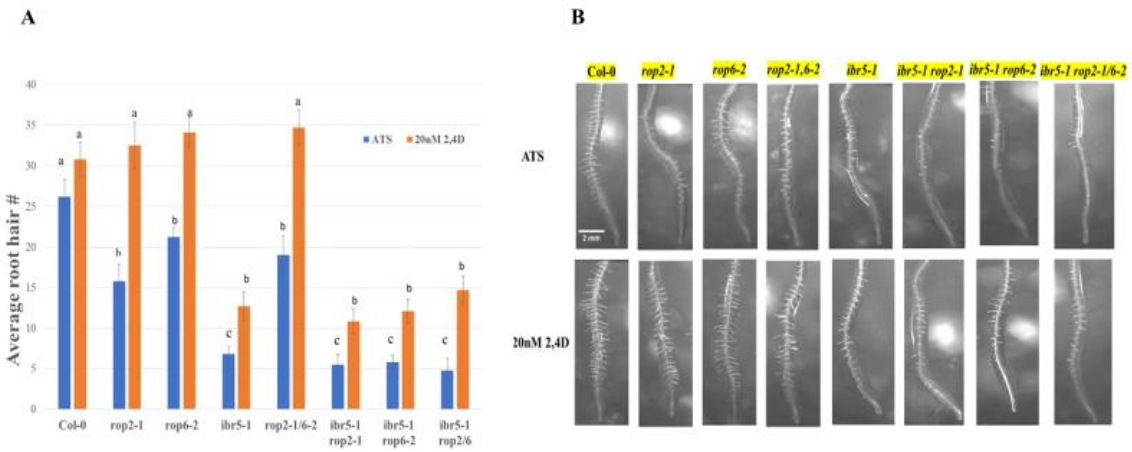

Figure 4.
rop2-1 nor *rop 6-2* enhanced nor suppressed *ibr5-1* low root hair formation. (A) Data represents average number of root hairs 5mm from the growing root tip from seedlings grown either on ATS and ATS + 75nM 2,4-D. Error bars represent SD (a,b letters) represent statistically significant values ($p<0.01$). (B) Col-0, *rop2-1*, *rop6-2, rop2-1/6-2*, *ibr5-1*, *ibr5-1 rop2-1*, *ibr5-1 rop6-2*, *ibr5-1 rop2-1/6-2* seed lines were compared (n =10), and representative roots of each genotype were imaged using Nikon microscope (SMZ). Scale bar represents 2 mm. Experiment was repeated 3 times with similar results.

ibr5-1 rop combined mutation did not affect auxin sensitivity in primary roots

IBR5 has been shown to physically interact with ROP2 and ROP6 in previous biochemical studies (Lopez, 2015). Phenotypic analysis of primary root growth in Col-0, *ibr5-1*, *rop2-1*, *rop6-2* single mutants, *rop2-1/6-2*, *ibr5-1 rop2-1*, *ibr5-1 rop6-2* double mutants, and *ibr5-1 rop2-1/6-2* triple mutant. Seedlings were grown 4 days on ATS media and transferred onto control or 75nM 2,4-D supplemented plates for 4 additional days before root length was measured. The primary roots of Col-0, *rop2-1, rop6-2* and *rop2-1/6-1* all exhibited sensitivity to 75nM 2,4-D. However, *ibr5-1rop2-1*, *ibr5-1 rop6-2*, *ibr5-1 rop2-1/6-2* all exhibited similar level of auxin resistance as *ibr5-1* single mutant (Figure 5).

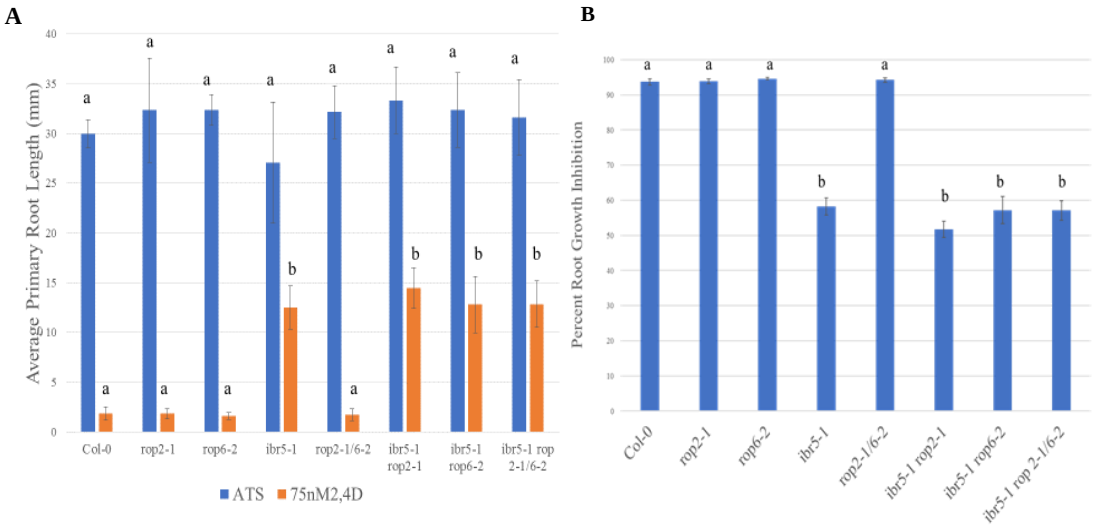

Figure 5.
rop2-1, nor *rop6-2* contributed to primary root resistance to 2,4-D**.** (A, B) Root growth assay on 75nM 2,4-D revealed that *ibr5-1 rop2-1*, *ibr5-1 rop6-2* double mutation neither increased nor suppressed the primary root resistance phenotype of *ibr5* mutant. (A) Data represents average primary root length on ATS and 75nM 2,4-D. The error bars represent SD. (ab letters) represents statistical significance ($p < 0.05$). (B) Data represents percent primary root growth inhibition on 75nM 2,4-D compared to ATS. The error bars represent SE (ab letters) represents statistical significance ($p < 0.05$). Experiments were repeated 3 times with similar results.

Generation of *ibr5-4 ara2-3* double mutant with *HS:: AXR3NT-GUS* transgene

HS::AXR3NT-GUS transgenic lines in *ara2-3* and *ibr5-4* were crossed to generate *ara2-3 ibr5-4* double mutant and genotyped as described in methods. Genomic DNA isolated from F1, F2 and F3 plants were amplified by PCR using ARA2, ARA2F + ARA2R primers (to test the presence or absence of the wild type gene) (Figure 6A) and ARA2R+LBO2 primers (to test the presence or absence of T-DNA) (Figure6B). The presence of of an *ibr5-4* mutation was tested by PCR and subsequent restriction digestion of the PCR amplified product with *BsmA*1.The *ibr5-4* catalytic mutant exhibits

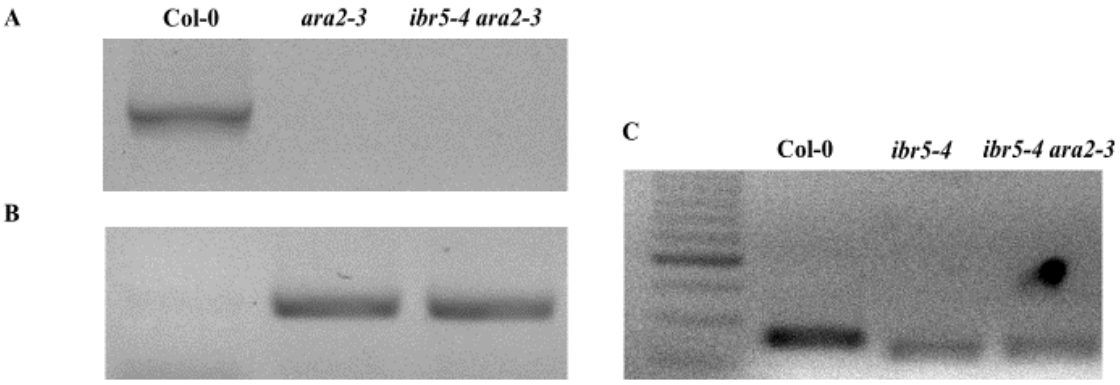

Figure 6.
ibr5-4 ara2-3 x *HS:: AXR3NT-GUS* transgenic homozygous for both the *ibr5-4* and *ara2-3* mutations. (A) *ARA2* was amplified using forward and reverse gene primers listed in methods. (B) T DNA insert was amplified using forward primer for *ARA2* gene along with the left border primer for the insert. (C) IBR5 was amplified using *ibr5-4* specific dCAPs primers, then PCR products were digested with BsmA1 restriction endonuclease. Only the mutant form is cut by the enzyme.

accelerated degradation of AUX/IAAs compared to Col-0 (Jayaweera et al., 2014), which is a common characteristic found with other *ibr5* mutant alleles *ibr5-1* (Strader et al. 2008) and *ibr5-5* (Cioffi, unpublished). To test the effect or *ara2-3* mutation on AUX/IAA repressor degradation, AX3RNT-GUS degradation was investigated in *ara2-3* and *ibr5-4* single mutants as well as the *ara2-3 ibr5-4* double mutant. *HS:: AXR3NT-GUS* seedlings harboring Col-0, *ara2-3*, *ibr5-4*, and *ara2-3 ibr5-4* were subjected to 2hr

heat shock at 37°C. Seedlings were incubated at room temperature for 0 or 60 min, fixed and stained for GUS activity as described in methods. As shown in figure 7, AXR3NT-GUS was rapidly degraded in *ibr5-4* compared to Col-0 as previously observed (Jayaweera et al. 2014), while AXR3NT-GUS level in *ara2-3* appeared to be in between Col-0 and *ibr5-4*. Interestingly, in *ara2-3 ibr5-4* double mutant AXR3NT-GUS was stabilized to a reasonable extent suggesting that *ara2-3* mutation suppressed the rapid degradation of AUX/IAAs in *ibr5-4*.

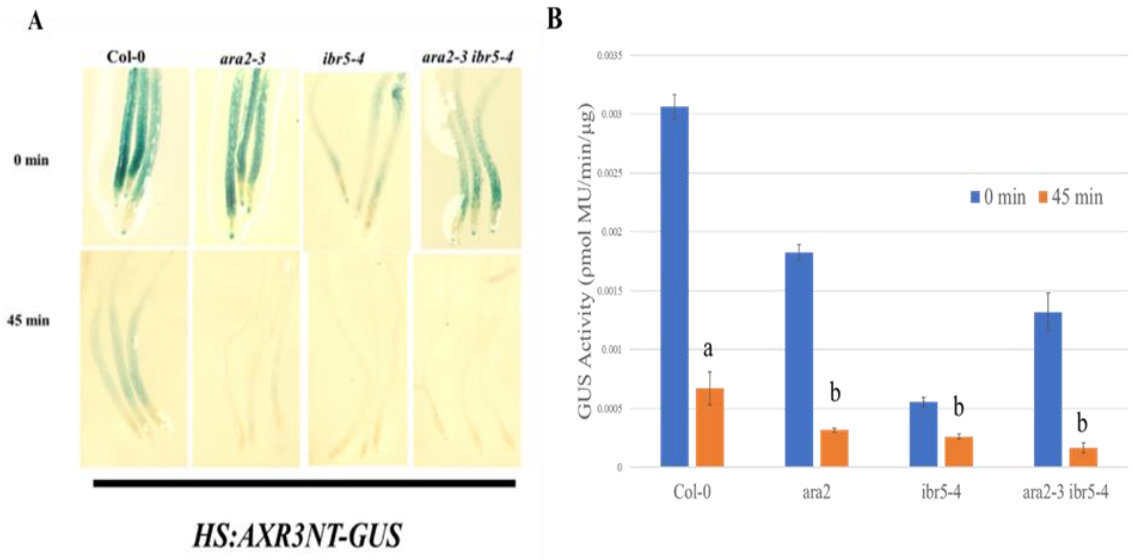

Figure 7.
ara2-3ibr5-4 double mutation suppressed *ibr5-4* rapid AUX/IAA degradation. (A)Rapid degradation of AXR3NT-GUS in *ibr5-4* mutant is suppressed by *ara2-3* mutation. *HS::AXR3NT-GUS* line was crossed into *ibr5-4*, *ara2-3*, and *ibr5-4 ara2-3* double mutant and homozygous seedling were heat shocked at 37ºC for 2h and GUS expression was assayed as described in methods. Experiment was repeated 3 times with similar results (n>10). Representative root was imaged using Nikon stereo microscope. (B) MUG fluorometric assay was performed to quantify the degradation of AXR3NT-GUS in Col-0, *ara2-3*, *ibr5-4* and *ibr5-4 ara2-3*. The roots of 20 4-day old seedlings were used for MUG assay as described in methods. Fluorometric measurements were performed in triplicates. Error bars represent SD (a,b letters) represent statistical significance ($p<0.05$).

ara2-3* suppresses the auxin insensitive primary root growth of *ibr5-4

To further verify the effect of *ara2-3 ibr5-4* combined mutation on auxin signaling, a root growth assay was performed using Col-0, *ara2-3*, and *ibr5-4* single mutants and *ara2-3 ibr5-4* double mutant grown in the absence (control) or presence of 75nM 2,4-D. Seedlings were germinated and grown for 4 days on vertically oriented 1% ATS solid media and then transferred on to 1% ATS solid media containing either mock treatment or 75nM 2,4-D, and left to grow for 4 additional days. The length of the primary root was measured, and the percent root growth inhibition was calculated. As shown in figure 8, *ibr5-4* exhibits less sensitivity to root growth inhibition by 2,4-D while *ara2-3* exhibited comparable sensitivity to Col-0. Interestingly, the primary root growth of *ibr5-4 ara2-3* double mutant was as sensitive as Col-0.

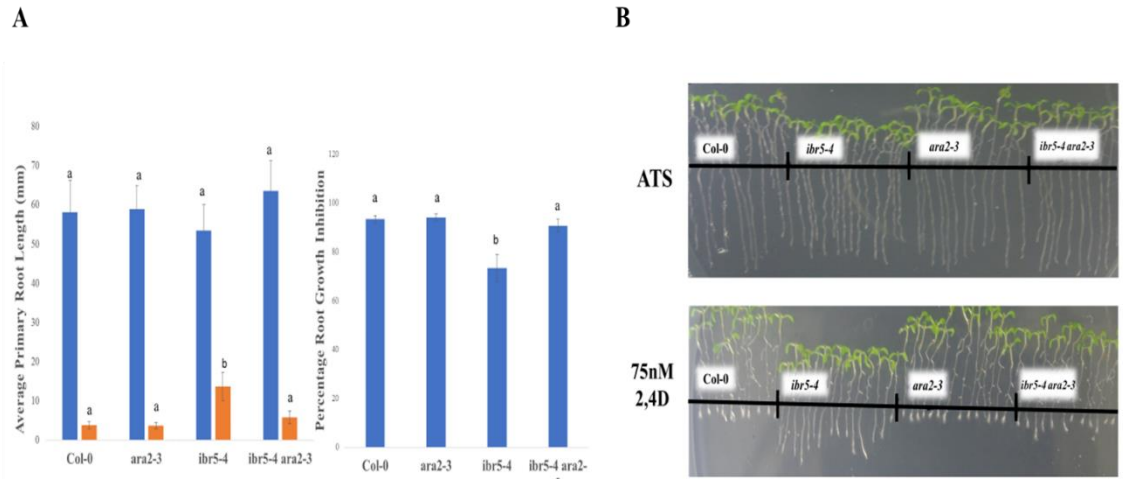

Figure 8.
ara2-3 ibr5-4 double mutation restores sensitivity to 2,4-D. (A, B, C) Root growth assay screen on 75nM 2,4-D revealed that *ara2 ibr5-4* double mutation suppressed the primary root resistance phenotype of *ibr5-4* single mutant. Seedlings were grown for 4 days on unsupplemented media then transferred to indicated plate with 75nM 2,4-D or Mock as described in methods. (A) Data represents average root length. The error bars represent SD, (a,b letters) represent statistical significance ($p < 0.05$). (B)Data represents percent root growth inhibition. The error bars represent SE (a,b letters) represent statistical significance ($p<0.05$). (C) Root sensitivity in *ara2-3 ibr5-4* was restored. Experiments were repeated 3 times with similar results ($n \geq 10$).

ara2-3 suppresses the leaf epinasty phenotype of *ibr5-4*

Leaf epinasty is a growth pattern observed in leaves where a higher rate of cell expansion on the adaxial compared to the abaxial surface results in the downward curling of the leaf (Sandalio et al. 2016). It is known that hormone signals like auxin regulate differential cell expansion, which results in leaf epinasty (Enders and Strader, 2015). These results show that *ibr5* mutants exhibit a leaf epinasty phenotype (Figure 9). Since *ara2-3* suppressed auxin insensitive root phenotype of *ibr5-4,* It was essential to identify any other phenotypes of *the ibr5-4* mutant allele that are suppressed in the *ara2-3 ibr5-4* double mutant. The leaves of *ibr5-4* mutants display leaf epinasty (Figure 9), and so the leaf characteristics of *ibr5-4* and *ara2-3* mutants were observed using pot experiments. It has been shown that ARA2 and other members of the Rab A1 subclass are expressed in leaves. (Xingyun and Huanquan, 2013) so phenotypic analysis of the ARA2 (*A1A*), *A1B,* and *A1C* homologous proteins was necessary to uncover any functional overlap within the RabA1 subclass.

To verify that role of RabA1 subclass, the *a1b, a1c* single mutant, *ara2-3(a1a)/c* double mutant or *a1a/b/c* triple mutant as well as *ibr5-4 ara2-3(a1a)/c* triple mutant and *ibr5-4 a1a/b/c* quadruple mutant were generated and genotyped using PCR primer combinations described in methods. All plants were grown on soil in a growth chamber under 16h light and 8h dark cycle for 3 weeks and plants were imaged using a Pentax DSL camera. There was no leaf epinasty observed in Col-0, *ara2-3*(*a1a*) or *a1b, a1c*, *a1a/c* double mutant or *a1a/b/c* triple mutant; however, *ibr5-4* single mutant exhibits leaf epinasty compared to Col-0, and Rab A1 mutants (Figure 9). Combining *ibr5-4* with *ara2-3*(*a1a*) resulted in a reduction in leaf epinasty in the *ibr5-4 ara2-3*(*a1a*) double

mutant. None of the mutant alleles of *ARA2* homologs, *a1b*, *a1c* single mutant, *a1a/a1c* double mutant or *a1a/b/c* triple displayed leaf epinasty. However, the leaf epinasty slightly increased in *ibr5-4 a1a/c* triple mutant and increased even further in *ibr5-4 rab-a1a/b/c* quadruple mutant (Figure 9). Interestingly *ara2-3* mutation was not enough to recover the leaf epinasty in the *ibr5-1* mutant. The *ara2-3* mutation was able to recover leaf epinasty in *ibr5-4* mutant background (Figure 9), yet *ibr5-1 ara2-3* double mutants displayed similar levels of epinasty as *ibr5-1* single mutant (Figure 10).

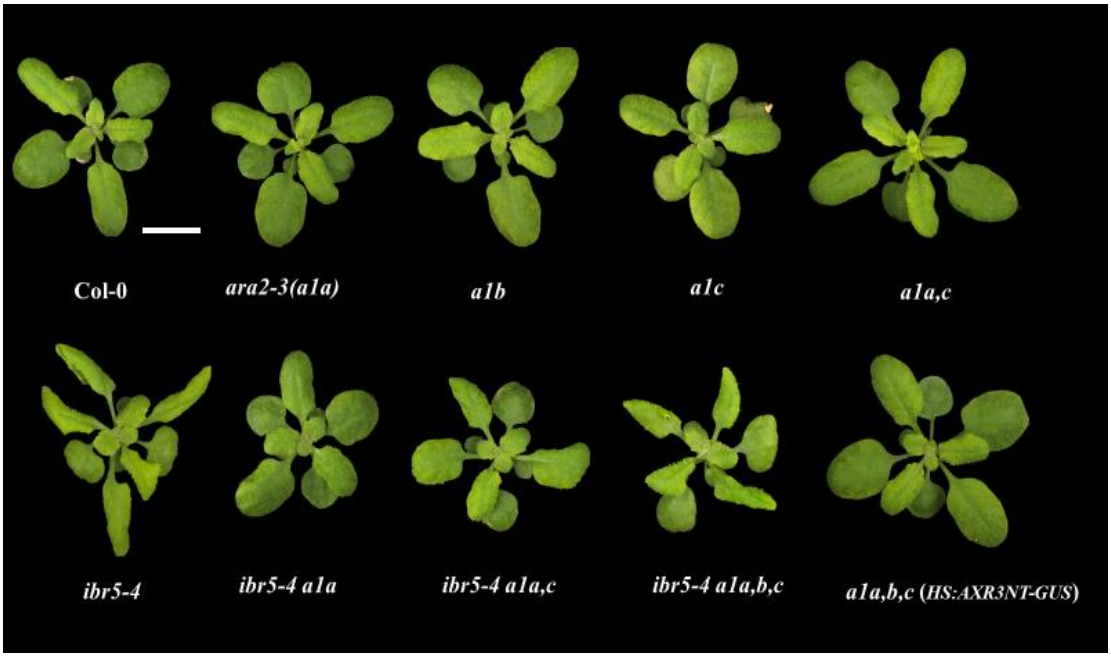

Figure 9.
Leaf epinasty is abolished in *ara2-3 ibr5-4* double mutant. Col-0, *ara2-3*(*a1a*), *a1b*, *a1c*, *ibr5-1*, *ibr5-4* single mutants, *a1a/c, ibr5-1 a1a, ibr5-4 a1a* double mutants and *ibr5-4 a1a/b/c* quad mutant were observed for their developmental phenotypes during the vegetative stage. Scale bar = 1cm.

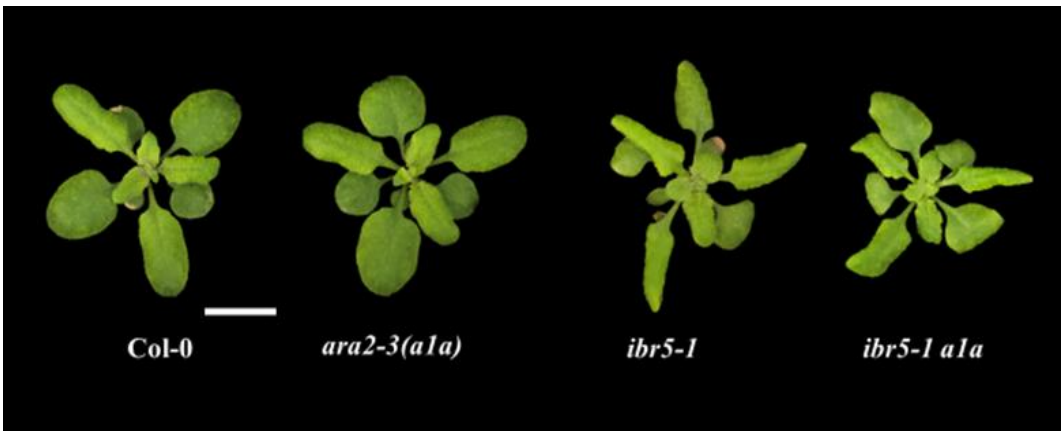

Figure 10.
Leaf epinasty is not abolished in *ibr5-1 ara2-3* mutant. Col-0, *ara2-3, ibr5-1, ibr5-1 ara2-3* mutants were observed for their developmental phenotypes during the vegetative stage. Scale bar = 1cm.

IV. DISCUSSION

Previous studies have shown that *IBR5*, *ARA2*, *ROP2*, and *ROP6* each have their connections to auxin signaling (Monroe-Augustus et al. 2003; Xu et al. 2009) and previous work in our lab has demonstrated that *IBR5* interacts physically with *ARA2* (Ghimire, 2015), *ROP2*, and *ROP6* (Lopez, 2015). Through characterizing mutant developmental phenotypes, analyzing auxin-responsive gene expression and conducting histochemical assays, we found evidence of a genetic interaction between *IBR5* and *ARA2*, as well as between IBR5, *ROP2*, and *ROP6.*

Through mutant phenotype analyses, we identified evidence of a genetic interaction of *IBR5* with *ROP2* and *ROP6.* IBR5 has been shown to interact with *ROP2* and *ROP6* (Lopez 2015) physically. Based on evidence of physical interactions, it was hypothesized that there would be observable genetic interactions between IBR5 and ROP2 or ROP6. The flowers of *ibr5-1 rop2-1* double mutant exhibit long and narrow petal blades. Leaf epinasty and root hair phenotypes reveal an epistatic connection between *ibr5-1* and *rop2-1* and *rop6-2.* This study reveals possible genetic interactions between IBR5 and ROP in regulating auxin responses.

IBR5, ROP2 and *ROP6* regulators of cell expansion

Cell expansion and polar growth have long been connected to auxin signaling and ROP activity (Fu et al. 2005; Fu et al. 2009). In this study, we examined the developmental phenotypes of the leaf, flower, and roots to uncover the connection between *IBR5* and auxin-dependent ROP signaling. Leaf epinasty is caused by a higher rate of cell expansion on the adaxial compared to the abaxial surface resulting in the

downward curling of the leaf (Sandalio et al.2016), a process known to involve auxin signaling (Enders and Strader, 2015). Since the epinasty of leaves was observed only in *ibr5-1*, not in *rop* mutants, and *ibr5-1 rop2-1*, *ibr5-1 rop6-1*, *ibr5-1 rop2-1/6-2* combined mutants showed little to no difference in leaf epinasty compared to *ibr5-1* (Figure 10) then *ibr5-1* has an epistatic phenotypic effect on leaf epinasty.

The formation of lobes and indents between neighboring leaf epidermal cells has long been used as a model system to study ROP signaling and auxin response (Fu et al. 2005; Fu et al. 2009). The epidermis of the abaxial side of the first true leaves of Col-0, *rop2-1*, *rop6-2*, *rop2-1/6-2*, *ibr5-1*, *ibr5-1 rop2-1*, *ibr5-1 rop6-2*, and *ibr5-1rop2-1/6-2* were observed. There was a decrease in lobe density in *rop2-1/6-2* double mutants as well as in *ibr5-1*, *ibr5-1 rop2-1*, *ibr5-1 rop6-2*, and *ibr5-1 rop2-1/6-2* lines compared to Col-0, and *rop2-1*, *rop6-2* single mutants (Figure 2A, B). The reduction in lobe formation suggests that these genes are essential in regulating cell expansion. Leaf epinasty phenotype observed in *ibr5-1 rop2-1/6-2* likely reflects an epistatic phenotypic effect from *ibr5-1* mutation or possible functional redundancy of ROP homologous proteins. It has been shown that ROP4 has functional redundancy with ROP2 during leaf pavement cell interdigitation (Fu et al. 2002). This functional overlap could explain the lack of enhancement or suppression of *ibr5- l* leaf epinasty phenotype *in ibr5-1 rop* mutants. Since there were visible defects in lobe formation in *rop2-1/6-2* double mutant and *ibr5-1*, *ibr5-1 rop2-1*, *ibr5-1 rop6-2*, *ibr5-1 rop2-1/6-2* both *ROP2* and *ROP6* as well as *IBR5* are likely important for regulating the localized cell expansion events necessary for the interdigitation of abaxial epidermal cells.

ROP2 and *ROP6* (Huibo et al. 2016) and *IBR5* (Johnson et al. 2015) have been demonstrated to regulate the development of petal size and shape independently. In this study, the images of the petal blades of Col-0, *rop2-1*, *rop6-2*, *rop2-1/6-2*, *ibr5-1*, *ibr5-1 rop2-1*, *ibr5-1 rop6-2*, and *ibr5-1rop2-1/6-2* were analyzed to reveal a genetic interaction between *IBR5*, *ROP2* and *ROP6* regulating petal development. The petals of *rop2-1* and *rop6-2* single mutants did not differ significantly from Col-0. However, the *rop2-1/6-2* combined mutant displayed slightly narrower and longer petal blades (Figure 3) (Huibo et al. 2016). The narrow petal observed in the rop2-1/6-2 double mutant suggests that there is some functional redundancy of *ROP2* and *ROP6* in the regulation of petal development. The petal blades or *ib5-1* mutants were narrower than *rop2-1/6-2* and Col-0, and the single mutants *rop2-1* and *rop6-2*. However, *ibr5-1 rop2* double mutant exhibited the longest petal blades (Figure 6A). Loss of function of both *IBR5* and *ROP2* in combination lead to the most severe effect of petal blade shape. The *ibr5-1 rop2-1* phenotype suggests that *IBR5* and *ROP2* interaction plays a crucial role in regulating petal blade elongation and expansion. Additionally, the *ibr5-1 rop2-1/6-2* triple mutant displaying a reduction in petal length compared with *ibr5-1 rop2-1* double mutant suggests a potentially antagonistic relationship between *ROP2* and *ROP6* in petal development. The antagonistic relationship between ROP2 and ROP6 activity has been demonstrated to be necessary for the interdigitation of epidermal pavement cells (Fu et al. 2002; Fu et al. 2005). Future work analyzing the morphology of the petal epidermal cells as well as immunostaining of the cytoskeleton in *ibr5-1* and *rop* mutants would strengthen the hypothesis that *IBR5*, *ROP2*, and *ROP6* interaction is vital for petal

development. This interaction is likely controlling petal development through regulation of the cytoskeleton.

Root hairs have been established as a model system for studying auxin mediated cell expansion (Masucci and Schiefelbein, 1994; Schiefelbein et al. 2000; Fischer et al. 2006; Ikeda et al. 2009; Nakamura et al. 2012; Moritaka et al. 2018). To characterize the connection between *IBR5* and *ROP* interaction on auxin-dependent cell expansion in trichoblasts, the root hair formation was analyzed in Col-0, *rop2-1*, *rop6-2*, *rop2-1/6-2*, *ibr5-1*, *ibr5-1 rop2-1*, *ibr5-1 rop6-2*, and *ibr5-1rop2-1/6-2*. The root hair density of *ibr5-1* mutants as well as *rop2-1/6-2* double mutant exhibit lower root hair formation compared with Col-0 and *rop2-1*, *rop6-2.* Exposure to 20nM 2,4-D increased root hair formation across all mutant lines; however, the root hair density in all mutant combinations with *ibr5-1* was not completely recovered to Col-0 level. This was also true for the root hair length. Reduction in root hair formation in the *rop2-1/6-2* double mutant as well as *ibr5-1*, *ibr5-1 rop2-1*, *ibr5-1 rop6-2*, and *ibr5-1rop2-1/6-2*, when grown on un-supplemented and 2,4-D treated media, suggests that both *IBR5*, *ROP2,* and *ROP6* are essential for root hair development.

There was little difference in root hair formation between *ibr5-1* single mutant and *ibr5-1 rop* combined mutants, and so it's likely that the low root hair density is an epistatic phenotype. Alternatively, there could be functional overlap from other ROP proteins masking any additional root hair phenotypes (Fischer et al. 2006; Ikeda et al. 2009). Examining the expression of pattern of (*GLABRA2*) *GL2* (Masucci et al. 1996) and (*WEREWOLF*) *WER* (Lee and Schiefelbein, 1999), which are vital genes that are important for root hair determination in Col-0, *rop2-1*, *rop6-2*, *rop2-1/6-2*, *ibr5-1*, *ibr5-1*

rop2-1, *ibr5-1 rop6-2*, and *ibr5-1rop2-1/6-2*, would help clarify the role of *IBR5* and *ROP*s in root hair formation. The combined mutants *ibr5-1 rop2-1*, *ibr5-1 rop6-2*, *ibr5-1 rop2-1/6-2* possess phenotypes of the root and leaf, which appear to be epistatic and dependent on *ibr5-1* mutation. IBR5 must play a role in auxin-regulated cell expansion. However, the connection to ROP signaling and cell expansion remains unclear. More expression analysis of auxin-responsive genes is necessary to elucidate the role of *IBR5*

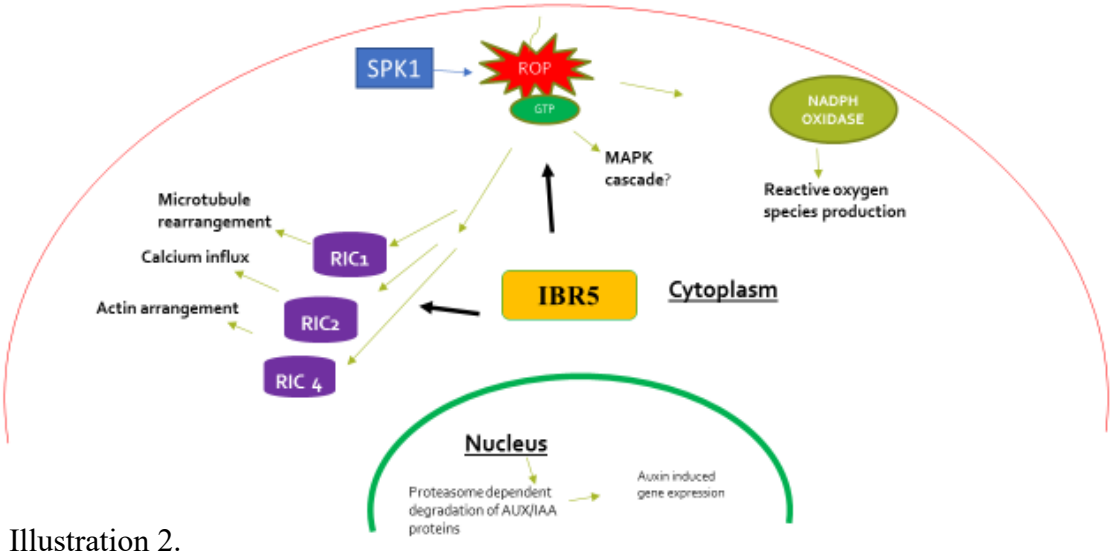

Illustration 2.
Proposed model for IBR5 ROP interaction. *IBR5* is likely important for regulating *ROP2* and *ROP6* activity. *IBR5* plays a role in auxin-regulated cell expansion that is connected to ROP signaling. The interaction between IBR5 and ROPs is likely important for cytoskeletal rearrangement. IBR5 may interact with both ROPs and their downstream interacting partners to regulate auxin induced cell expansion.

and *ROP i*nteraction on auxin signaling.

ARA2 and *IBR5* interaction is important for AUX/IAA

Previous work demonstrated that IBR5 physically interacts with ARA2 both *in vivo* and *in vitro* (Ghimire, 2015). It has also been shown that *ara2-3* mutant exhibits slight stabilization of AXR3NT-GUS reporter when compared to Col-0 (Ghimire, 2015), while *ibr5-4* mutant exhibits rapid degradation of AXR3NT-GUS degradation (Jayaweera et al. 2014). This biochemical evidence supports the hypothesis that the

interaction between *IBR5* and *ARA2* plays a role in auxin signaling. GUS histochemical and quantitative assays in this study demonstrate that *ara2-3* partially suppresses the rapid degradation of AXR3NT-GUS in *ibr5-4* (Figure 6). Additionally, *ibr5-4 ara2-3* double mutant restores the auxin sensitivity in *ibr5-4* (Figure 7). The histochemical and root growth assay results indicate that *ara2-3* suppresses the auxin resistance of *ibr5-4* through the regulation of AUX/IAA degradation.

Leaf epinasty is a process controlled by cell expansion and auxin signaling (Enders and Strader, 2015). Mutant *ibr5* alleles exhibit epinastic leaves indicating this defective phenotype is due to altered IBR5 activity (Figure 10, 11). The *ara2-3* single mutant develops leaves that are like Col-0. Interestingly, *ibr5-4 ara2-3* double mutant exhibits a reduction in leaf epinasty indicating that *ara2-3* is enough to suppress the *ibr5-4* leaf epinasty phenotype (Figure 11). Additionally, *ibr5-4* seeds generally germinate slightly later than Col-0, thus exhibit short stature during early germination. This phenotype is also suppressed by *ara2-3* (Figure 9B) suggesting that almost all the defective developmental phenotypes observed in *ibr5-4* are suppressed by *ara2-3* mutation.On the contrary, none of the defective phenotypes, such as auxin insensitive primary root growth (Figure 5), or epinastic leaves (Figure 11) observed in *ibr5-1* are suppressed by *ara2-3* mutation. This discrepancy may be because *ibr5-1* is a null mutant resulting from a premature stop codon close to the translation start point, while *ibr5-4* is a missense mutation in the catalytic site, which produces a mutant protein.

Since there are at least two *ARA2* (*A1A*) homologs (*A1B*, and *A1C*) in Arabidopsis (Xingyun and Huanquan, 2013), higher order mutants of *ara2-3* were generated and crossed into *ibr5-4*. None of the mutants other than the *ibr5-4 ara2-3* (*a1a*) double

mutant, exhibited suppression of defective *ibr5* phenotypes (Figure 10, 11). It is possible that though the ARA2 homologs show sequence homology, they may not be entirely functionally redundant. Rab GTP binding proteins are known to be important in endomembrane trafficking events within the cell and function in the targeting of transport vesicles to their desired locations (Tomohiro and Ueda, 2014).

Many diverse vesicular transport events are occurring simultaneously in the plant cell, there are a diversity of Rab GTP binding proteins and localization patterns. These proteins are responsible for the trafficking of many different populations of transport vesicles (Quing-Hu, 2007; Xingyun and Huanquan, 2013). Although combining *ibr5-4* and *ara2-3* mutations results in suppression of the leaf epinasty in *ibr5-4*, *ibr5-4 a1a/b/c* quadruple exhibits leaf epinasty equivalent to the *ibr5-4* single mutant (Figure 10). Thus, it is likely that ARA2 is regulating the transport of a specific population of vesicles that are important for auxin responses independent of its homologs. It could be possible that *ARA2* is involved in the cyclic transport of auxin signaling/transport components into or out of the nucleus, which is supported by the fact that rapid AUX/IAA degradation in *the ibr5-4* mutant is stabilized in the *ibr5-4 ara2-3* double mutant (Figure 8).

Since *ibr5-4* is a catalytic site mutant, the rapid rate of AUX/IAA degradation may result from the inability of *ibr5-4* to dephosphorylate either ARA2 or a mutually interacting protein. When ARA2 is still functioning, there may be an increase in traffic of auxin signaling components like the $SCF^{TIR1/AFB1}$ complex into the cytoplasm. It has been shown that AXR3NT-GUS and DII-Venus show different degradation patterns in *ibr5* mutants despite both being recombinant versions of the AUX/IAA repressor protein IAA17 (Cioffi, unpublished). The degradation of DII-VENUS in *ibr5* mutants is

stabilized compared to Col-0 (Cioffi, unpublished); however, the opposite is true for AXR3NT-GUS where rapid degradation is observed (Jayweera et al. 2014). AXR3NT-GUS has a large reporter gene fusion which may result in higher protein present in the cytoplasm while DII Venus has an additional nuclear localization signal and is localized to the nucleus. These two reporter genes may be revealing a differential pattern of AUX/IAA degradation between the nucleus and cytoplasm, and IBR5, ARA2 interaction may be significant for the movement of the SCF complex or its components back and forth from the nucleus to the cytoplasm to facilitate auxin signaling. Alternatively, the nature of IBR5 and ARA2 interaction could be centered around auxin transport. It has been shown previously that RabA1C relocalization to the cell plate in mitotic cells in the root it inhibited by endosin1, which is an inhibitor of transport of PIN2 and AUX1 auxin transporters (Xingyun and Huanquan, 2013).

IBR5 could play a regulatory role in PIN localization through its interaction with ARA2 as we see increasing epinasty in *ibr5-4 a1a,c* triple, and *ibr5-4 a1a,b,c* quadruple mutant compared to *ibr5-4 a1a*. ARA2 has been demonstrated to be primarily localized to the plasma membrane when in its active state and endosomes when inactive (Hyeran et al. 2019) while A1C and A1B have been shown to mostly localize to the Trans Golgi Network (Xingyun and Huanquan, 2013). The lack of leaf epinasty in *ibr5-4 a1a* may reflect an imbalance in the movement of auxin transporters to the plasma membrane, which may rely on dephosphorylation of ARA2 or its effector proteins by IBR5 and when A1C and A1B are also lost there may be difficulty for auxin transporters to be transported from the TGN to the endosomes or vice versa. In a future study, the explanation for the differential developmental phenotypes between *ibr5-4* and *ibr5-1* combined with *a1c* and

a1b individually will need to be investigated. By characterizing the combined effects of *a1a/b/c* higher order mutations and *ibr5-1* on AXR3NT-GUS degradation, primary root growth and developmental phenotypes the differences between the nature of *ibr5-1* and *ibr5-4* mutations may become more evident. Biochemical assays like phosphatase assays, cell fractionation, and immunolocalization would also provide critical evidence that IBR5 and ARA2 are interacting in this proposed cyclic transport pathway.

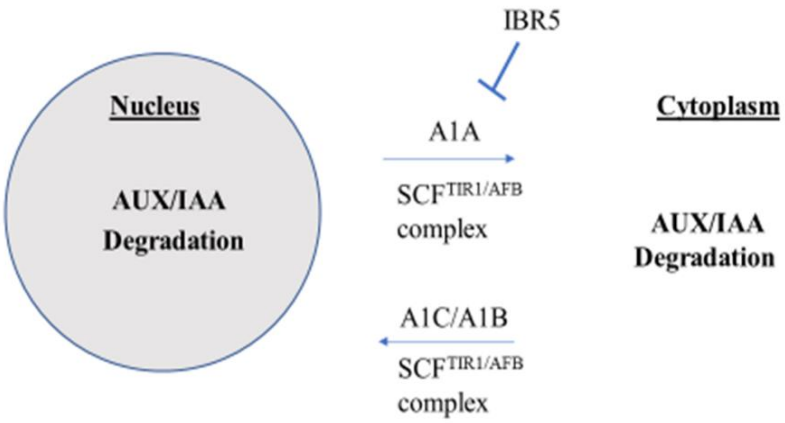

Illustration 3.
Proposed model of IBR5 and ARA2 interaction. IBR5 may regulate auxin signaling through ARA2 mediated vesical transport. Movement of the SCF complex (or its individual components) needs to be conducted throughout the cell. Rab A1 subclass of proteins are important for vesicle transport from TGN, endosome and the plasma membrane. IBR5 may regulate RabA1 proteins through dephosphorylation which may dictate the net directionality of transport of the SCF complex.

Based on the evidence collected in this study IBR5 and ARA2 shows genetic interaction that is relevant to auxin response. Histochemical analysis and root growth assays have revealed suppression of *ibr5-4* mutant phenotypes in *ibr5-4 ara2-3* double mutant suggesting IBR5 and ARA2 function in a common auxin signaling pathway. IBR5 has a genetic interaction with ROP 2 and ROP6 which is likely connected with cell

expansion in the flower petal blade. Because IBR5 has also been shown to interact with SKP1 (Arabidopsis Interactome Mapping, 2011), an SCF complex component, and to regulate AUX/IAA degradation and auxin-induced gene expression, its interaction with ARA2 and ROP GTPases may provide a mechanism for the connection between ARA2 and ROP-based auxin signaling in the cytoplasm and $SCF^{TIR1/AFBs}$-based auxin signaling. For future directions, it will be essential to determine the localization of IBR5-ROP/ IBR5- ARA2 interactions. Also, it will be essential to identify if the IBR5 phosphorylate ARA2 or ROP2 and ROP6 *in vitro* and if phosphorylation of ARA2 or ROP is necessary for their activity/localization.

To understand where and when the IBR5, ARA2, ROP2, and ROP6 interaction occurs during auxin signaling, additional studies must be conducted. Connecting small G protein signaling and IBR5 signaling will provide novel insight into the auxin signaling pathway. Since auxin plays a key role in plant growth and development it is crucial to understand the auxin signaling pathway. This kind of basic auxin research will potentially serve as the foundation for many of the crop improvement strategies in the future.

REFRENCES

1. Baluska F, Salaj J, Mathur J, Braun M, Jasper F, Samaj J, Chua N, Barlow P, and Volkmann D. (2000) Root hair formation: F-actin-dependent tip growth is initiated by local assembly of profilin-supported F-actin meshworks accumulated within expansin-enriched bulges. *Dev. Biol* 227: 618–632.

2. Berken A, Thomas C, and Wittinghofer A. (2005) A new family of RhoGEFs activates the Rop molecular switch in plants. *Nature* 436: 1176–1180.

3. Bourne H, Sanders D, and McCormick F (1990) The GTPase superfamily: a conserved switch for diverse cell functions. *Nature* 348:125–132.

4. Bourne H., Sanders D, and McCormick, F. (1991) The GTPase superfamily: conserved structure and molecular mechanism. *Nature* 349:117-127.

5. Burridge K, and Wennerberg K (2004) Rho and Rac take center stage. *Cell* 116:167–179.

6. Carol R, Takeda S, Linstead P, Durrant M, Kakesova H, Derbyshire P, Drea S, Zarsky V, and Dolan L. (2005) A RhoGDP dissociation inhibitor spatially regulates growth in root hair cells. *Nature*, 438: 1013–1016.

7. Chavrier P, and Goud B. (1999). The role of ARF and Rab GTPases in membrane transport. *Curr. Opin. Cell Biol* 11: 466–475.

8. Chen J, Shimomura S, Sitbon F, Sandberg G, and Jones A. (2001) The role of auxin-binding protein 1 in the expansion of tobacco leaf cells. *Plant J* 28: 607-617.

9. Chen J, Ullah H, Young J, Sussman M, and Jones A. (2001) ABP1 is required for organized cell elongation and division in Arabidopsis embryogenesis *Genes Dev* 15: 902-911.

10. Cherfils J. and Chardin P. (1999) GEFs: structural basis for their activation of small GTP-binding proteins. *Trends Biochem. Sci* 24: 306–311.

11. Clarke P, and Zhang C. (2001) Ran GTPase: a master regulator of nuclear structure and function during the eukaryotic cell division cycle? *Trends Cell Biol* 11:366–371.

12. Craddock C, Lavagi I, and Yang Z. (2012) New insights into Rho signaling from plant ROP/Rac GTPases. *Trends Cell Biol* 22: 492–501.

13. Crespo, P. and Leon, J. (2000). Ras proteins in the control of the cell cycle and cell differentiation. *Cell Mol. Life Sci* 57: 1613-1636.

14. Dahlberg J, and Lund E. (1998) Functions of the GTPase Ran in RNA export from the nucleus. *Curr Opin Cell Biol* 10: 400-408.

15. Dasso M. (2002) The Ran GTPase: theme and variations. *Cell https://doi.org/10.1016/S0960-9822(02)00970-3.*

16. Dent P, Haser W, Haystead T, Vincent L, Roberts T, and Sturgill T. (1992) Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and in vitro. *Science* 257:1404–1407.

17. Dharmasiri N, and Estelle M. (2004) Auxin signaling and regulated protein degradation. *Trends Plant Sci* 9: 302–308.

18. Downward J. (1998) Ras signaling and apoptosis. *Curr Opin Genet Dev* 8:49–54.

19. Enders, T and Strader L. (2015) Auxin: Past, present, and future. *American Journal of Botany* 102:180-196.

20. Farnsworth C, Kawata M, Yoshida Y, Takai Y, Gelb M, and Glomset J (1991) C terminus of the small GTP-binding protein smg p25A contains two geranylgeranylated cysteine residues and a methyl ester. *Proc Natl Acad Sci U S A* 88:6196–6200.

21. Feng Q, Kang H, Song S, Ge F, Zhang Y, Li E, Li S, and Zhang Y. (2016) Arabidopsis RhoGDIs are critical for cellular homeostasis of pollen tubes. *Plant Phys* 170: 841–856.

22. Fernández-Medarde A, and Santos E. (2011) Ras in cancer and developmental diseases. *Genes Cancer* 2:344–358.

23. Fischer U, Ikeda Y, Ljung K, Serralbo O, Singh M, Heidstra R, Palme K, Scheres B, and Grebe M (2006) Vectorial information for Arabidopsis planar polarity is mediated by combined AUX1, EIN2, and GNOM activity. *Curr Biol* 16: 2143–2149.

24. Foreman J, Demidchik, V, Bothwell J. (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature*, 422, 442–446

25. Fu Y, Li H, and Yang Z. (2002). The ROP2 GTPase controls the formation of fine cortical F-actin and the early phase of directional cell expansion during Arabidopsis organogenesis. *Plant Cell* 14, 777–794.

26. Fu Y, Xu T, Zhu L, Wen M, and Yang Z. (2009). A ROP GTPase signaling pathway controls cortical microtubule ordering and cell expansion in Arabidopsis. *Curr Biol* 19, 1827-1832.

27. Fu Y. (2002). The ROP2 GTPase Controls the Formation of Cortical Fine F-actin and the Early Phase of Directional Cell Expansion during Arabidopsis Organogenesis. *Plant Cell* 14: 777-794.

28. Glomset J, and Farnsworth C. (1994) Role of protein modification reactions in programming interactions between ras-related GTPases and cell membranes. *Annu Rev Cell Biol* 10:181–205.

29. Gray W, Del Pozo J, Walker L, Hobbie L, Risseeuw E, Banks T, Crosby W, Yang M, Ma H, and Estelle M. (1999) Identification of an SCF ubiquitin-ligase complex required for auxin response in Arabidopsis thaliana. *Genes Dev* 13: 1678–1691.

30. Gray W, Kepinski S, Rouse D, Leyser O, and Estelle M. (2001) Auxin regulates SCFTIR1-dependent degradation of AUX/IAA proteins. *Nature* 414: 271–276.

31. Green J, and Davidson L. (2007) Convergent extension and the hexahedral cell *Nat. Cell Biol* 9: 1010-1015.

32. Grierson C, Nielsen E, Ketelaarc T, and Schiefelbein J. (2014) Root hairs. *Arabidopsis Book* doi: 10.1199/tab.0172.

33. Hager A, Debus G, Edel H, Stransky H, and Serrano R. (1991) Auxin induces exocytosis and the rapid synthesis of a high turnover pool of plasma membrane H+-ATPase. *Planta* 185: 527-537.

34. Hai L, Jun-Jiang S, Zhi-Liang Z, Yakang L, and Zhenbiao Y. (2001) The Rop GTPase Switch Controls Multiple Developmental Processes in Arabidopsis. *Plant Phys* 126: 670-684.

35. Hall, A. (1998) Rho GTPases and the Actin Cytoskeleton. *Science*, 279: 509-514.

36. Hazak O, Bloch D, Poraty L, Sternberg H, Zhang J, Friml J, and Yalovsky S. (2010) A rho scaffold integrates the secretory system with feedback mechanisms in regulation of auxin distribution. *PLoS Biol* doi.org/10.1371/journal.pbio.1000282.

37. Heasman J. (2006) Patterning the early Xenopus embryo. *Development* 133: 1205-1217.

38. Hill C, Wynne J, and Treisman R. (1995) The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell* 81:1159–1170.

39. Huang G, Li E, Ge F, Li S, Wang Q, Zhang C, and Zhang Y. (2013) Arabidopsis RopGEF4 and RopGEF10 are important for FERONIA-mediated developmental but not environmental regulation of root hair growth. *New Phytol* 200: 1089–1101.

40. Huang J, Liu H, Chen M, Li X, Wang M, Yang Y, Wang C, Huang J, Liu G, and Liu Y. (2014) ROP3 GTPase contributes to polar auxin transport and auxin responses and is important for embryogenesis and seedling growth in Arabidopsis. *Plant Cell* 26: 3501–3518.

41. Huang T, and Irish V. (2016) Gene networks controlling petal organogenesis. *J Exp Bot* 67: 61–68.

42. Huibo R, Xie D, Yanqiu Y, Dingquan H, Mengting L, Xiaowei G,and Deshu L. (2016) SPIKE1 Activates ROP GTPase to Modulate Petal Growth and Shape. *Plant Phys* 172: 358-371.

43. Hyeran K, Hyangju K, Yun K, Jisun C, and Jeong Ho C. (2019) Proportional subcellular localization of Arabidopsis thaliana RabA1a, *Plant Signaling & Behavior* doi: 10.1080/15592324.2019.1581561.

44. Ikeda Y, Men S, Fischer U, Stepanova A, Alonso J, Ljung K, and Grebe M. (2009) Local auxin biosynthesis modulates gradient-directed planar polarity in Arabidopsis. *Nat Cell Biol* 11: 731–738.

45. Irish V. (2008) The Arabidopsis petal: a model for plant organogenesis. *Trends Plant Sci* 13: 430–436.

46. Jayaweera T, Siriwardana C, Dharmasiri S, Quint M, Gray W, and Dharmasiri N. (2014). Alternative splicing of Arabidopsis IBR5 pre-mRNA generates two IBR5 isoforms with distinct and overlapping functions. *PLoS One* doi: 10.1371/journal.pone.0102301.

47. Jefferson R. (1987). Assaying Chimeric Genes in plants: The GUS Gene Fusion System. *Plant Mol Bio Reporter* 5: 387-405.

48. Jiang S, and Ramachandran S. (2006) Comparative and evolutionary analysis of genes encoding small GTPases and their activating proteins in eukaryotic genomes. *Physiol Genomics* 24:235–251.

49. Johnson K, Ramm S, Kappel C, Ward S, Leyser O, et al. (2015) The Tinkerbell (Tink) Mutation Identifies the Dual-Specificity MAPK Phosphatase INDOLE-3-BUTYRIC ACID-RESPONSE5 (IBR5) as a Novel Regulator of Organ Size in Arabidopsis. *Plos One* doi: 10.1371/journal.pone.0131103.

50. Jones M, Raymond M, Yang Z, and Smirnoff N. (2007) NADPH oxidase-dependent reactive oxygen species formation required for root hair growth depends on ROP GTPase. *J. Exp. Bot*., 58, 1261–1270.

51. Jones M, Shen J, Fu Y, Li H, Yang Z. and Grierson C. (2002) The Arabidopsis Rop2 GTPase is a positive regulator of both root hair initiation and tip growth. *Plant Cell* 14: 763–776.

52. Kahn R, and Gilman A. (1984) Purification of a protein cofactor required for ADP-ribosylation of the stimulatory regulatory component of adenylate cyclase by cholera toxin. *J. Biol. Chem* 259:6228–6234.

53. Kahn R, and Gilman A. (1986) The protein cofactor necessary for ADP-ribosylation of Gs by cholera toxin is itself a GTP binding protein. *J. Biol. Chem* 261:7906–7911.

54. Kieffer M, Neve J, and Kepinski S. (2010). Defining auxin response contexts in plant development. *Curr Opin Plant Biol* 13:12-20.

55. Klahre U, Becker C, Schmitt A, and Kost B. (2006) Nt-RhoGDI2 regulates Rac/Rop signaling and polar cell growth in tobacco pollen tubes. *Plant J* 46: 1018–1031.

56. Lavy M, Bloch D, Hazak O, Gutman I, Poraty L, Sorek N, Sternberg H, and Yalovsky S. (2007) A novel ROP/RAC effector links cell polarity, root-meristem maintenance, and vesicle trafficking. *Curr Biol* 17: 947–952.

57. Lee J, Wang S, Sritubtim S, Chen J, and Ellis B. (2009) Arabidopsis mitogen-activated protein kinase MPK12 interacts with the MAPK phosphatase IBR5 and regulates auxin signaling. *Plant J* 57: 975-985.

58. Li C, Lu H, Li W, Yuan M, and Fu Y. (2017) A ROP2-RIC1 pathway fine-tunes microtubule reorganization for salt tolerance in Arabidopsis. *Plant, Cell & Environment* 7:1127-1142.

59. Li H, Shen JJ, Zheng ZL, Lin Y, and Yang Z. (2001) The Rop GTPase switch controls multiple developmental processes in Arabidopsis. *Plant Phys* 126: 670-684.

60. Lin D, Ren H, and Fu Y. (2014) ROP GTPase-mediated auxin signaling regulates pavement cell interdigitation in Arabidopsis thaliana. *Journal Of Integrative Plant Biology* 57: 31-39.

61. Lopez E. (2015) Characterization of IBR5-ROP GTPase (ROP2/ROP6) Interactions in Plant Auxin Response. *Graduate Thesis* doi: presentations%20&%20thesis/Lopez_Thesis.pdf.

62. Ma, QH. (2007) Small GTP-binding Proteins and their Functions in Plants. *J Plant Growth Regul* 26: 369-388.

63. MacDonald S, Crews C, Wu L, Driller J, Clark R, Erikson R, and McCormick F. (1993) Reconstitution of the Raf-1-MEK-ERK signal transduction pathway in vitro. *Mol Cell Biol* 13:6615–6620.

64. Machesky L, and Hall A. (1996). Rho: a connection between membrane receptor signaling and the cytoskeleton. *Trends in Cell Biology* 6: 304-310.

65. Magee A, Newman C, Giannakouros T, Hancock J, Fawell E, and Armstrong J. (1992) Lipid modifications and function of the Ras superfamily of proteins. *Biochem Soc Transact* 20:497–499.

66. Masucci J, and Schiefelbein J. (1994) The rhd6 mutation of *Arabidopsis thaliana* alters root-hair initiation through an auxin- and ethylene-associated process. *Plant Phys* 106:1335–1346.

67. Matozaki T, Nakanishi H, and Takai Y. (2000) Small G-protein networks: *Cellular Signalling*, 12: 515-524.

68. Mattaj I, and Englmeier L. (1998) Nucleocytoplasmic transport: the soluble phase. *Annu Rev Biochem*, 67: 265– 306.

69. Molendijk A, Bischoff F, Rajendrakumar C, Friml J, Braun M, Gilroy S, and Palme K. (2001) Arabidopsis thaliana Rop GTPases are localized to tips of root hairs and control polar growth. *EMBO J* 20: 2779–2788.

70. Monroe-Augustus M, Zolman B, and Bartel B. (2003) A dual-specificity phosphatase-like protein modulating auxin and abscisic acid responsiveness in Arabidopsis. *Plant Cell* 15: 2979–2991.

71. Moritaka N, Andrea C, Tobias G, Rebecca H, Corrado V, Yoshihisa I, and Grebe M. (2018) Auxin and ROP GTPase Signaling of Polar Nuclear Migration in Root Epidermal Hair Cells. *Plant Phys*, 176: 378-391.

72. Nakamura M, Claes A, Grebe T, Hermkes R, Viotti C, Ikeda Y, and Grebe M. (2018) Auxin and ROP GTPase Signaling of Polar Nuclear Migration in Root Epidermal Hair Cells. *Plant Phys*, 176: 378-391.

73. Nakamura M, Kiefer C, and Grebe M. (2012) Planar polarity, tissue polarity and planar morphogenesis in plants. *Curr Opin Plant Biol* 15: 593–600.

74. Oda Y, and Fukuda H. (2012) Initiation of cell wall pattern by a Rho- and microtubule-driven symmetry breaking. *Science* 337: 1333–133.

75. Paponov I, Dindas J, Król E, Friz T, Budnyk V, Teale W, Paponov M, Hedrich R, and Palme K. (2019) Auxin-Induced Plasma Membrane Depolarization Is Regulated by Auxin Transport and Not by AUXIN BINDING PROTEIN1 *Frontiers in Plant Science* doi: 10.3389/fpls.2018.01953.

76. Pereira-Leal J, and Seabra M. (2001) Evolution of the rab family of small GTP-binding proteins. *Journal of Molecular Biology* doi: 10.1006/jmbi.2001.5072.

77. Petrasek J, Mravec J, Bouchard R, Blakeslee J, Abas M, Seifertova D, Wisniewska J, Tadele Z, Kubes M, Covanova M, et al. (2006) PIN proteins perform a rate-limiting function in cellular auxin efflux. *Science* 312: 914-918.

78. Prabesh G. (2015) Characterization of IBR5 Interacting Protein, ARA2 in Arabidopsis Auxin Response. *Graduate Thesis* presentations%20&%20thesis/Prabesh%20Ghimire%20Thesis%20sumitted%20to%20grad%20college%202007%20final.pdf.

79. Prior I, Lewis P, and Mattos C. (2012) A comprehensive survey of Ras mutations in cancer. *Cancer Res* 72:2457–2467.

80. Qin Y, and Yang Z. (2011) Rapid tip growth: insights from pollen tubes. *Semin Cell Dev Biol* 22: 816–824.

81. Randazzo P, and Hirsch D. (2004) Arf GAPs: multifunctional proteins that regulate membrane traffic and actin remodelling. *Cell Signal* 16:401–413.

82. Rayle D, and Cleland R. (1992) The Acid Growth Theory of auxin-induced cell elongation is alive and well. *Plant Phys* 99: 1271-1274.

83. Ridley A, and Hall A. (1992) The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 70:389–399.

84. Rojas J, Oliva J, and Santos E. (2011) Mammalian son of sevenless Guanine nucleotide exchange factors: old concepts and new perspectives. *Genes & cancer*, *2:* 298–305.

85. Sandalio L, Rodríguez M, and Romero-Puertas M. (2016) Leaf epinasty and auxin: A biochemical and molecular overview. *Plant Science* doi: 10.1016/j.plantsci.2016.10.002.

86. Sauer M, Delgadillo M, Zouhar J, Reynolds G, Pennington J, Jiang L, Liljegren S, et al. (2013). MTV1 and MTV4 encode plant-specific ENTH and ARF GAP proteins that mediate clathrin-dependent trafficking of vacuolar cargo from the trans-Golgi network. *Plant Cell* 25: 2217–2235.

87. Sauer M, and Kleine-Vehn J. (2011) AUXIN BINDING PROTEIN1: The Outsider. *Plant Cell*, 23 2033-2043.

88. Schepetilnikov M, Makarian J, Srour O, Geldreich A, Yang Z, and Chicher J, et al. (2017). GTPase ROP2 binds and promotes activation of target of rapamycin, TOR, in response to auxin. *The EMBO J*, 36: 886-903.

89. Schiefelbein J. (2000) Constructing a Plant Cell. The Genetic Control of Root Hair Development. *Plant Phys*, 124: 1525-1531.

90. Seabra M, and Wasmeier C. (2004) Controlling the location and activation of Rab GTPases. *Curr Opin Cell Biol* 16:451–457.

91. Senn A, and Goldsmith M. (1988) Regulation of Electrogenic Proton Pumping by Auxin and Fusicoccin as Related to the Growth of Avena Coleoptiles *Plant Physiol* 88: 131-138.

92. Settleman J. (2005) Intercalating Arabidopsis leaf cells: a jigsaw puzzle of lobes, necks, ROPs, and RICs. *Cell*, 120: 570-572.

93. Shishova M, and Lindberg S. (2004) Auxin induces an increase of Ca^{2+} concentration in the cytosol of wheat leaf protoplasts *J. Plant Physiol* 161:937-945.

94. Stenmark H, and Olkkonen V. (2001) The Rab GTPase family. *Genome Biol* 2:1–7.

95. Strader L, and Nemhauser J. (2013) Auxin 2012: a rich mea ho'oulu. *Development* doi: 10.1242/dev.090530.

96. Strader L, Monroe-Augustus M, and Bartel B. (2008) The IBR5 phosphatase promotes Arabidopsis auxin responses through a novel mechanism distinct from TIR1-mediated repressor degradation. *BMC Plant Biol* doi: 10.1186/1471-2229-8-41.

97. Takeda S, Gapper C, Kaya H, Bell E, Kuchitsu K, and Dolan L. (2008) Local positive feedback regulation determines cell shape in root hair cells. *Science* 319: 1241–1244.

98. Ueda T, Yamaguchi M, Uchimiya H, and Nakano A (2001) Ara6, a plant-unique novel type Rab GTPase, functions in the endocytic pathway of *Arabidopsis thaliana. EMBO J* 17**:** 4730–4741.

99. Ulmasov T, Hagen G, and Guilfoyle T. (1999) Activation and repression of transcription by auxin response factors. *Proc. Natl Acad. Sci. USA* 96: 5844 5849.

100. Ulmasov T, Murfett J, Hagen G, Guilfoyle T. (1997) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell* 9: 1963–71.

101. Vernoux T, Besnard F, and Traas J. (2010) Auxin at the shoot apical meristem. *Cold Spring Harb Perspect Biol* doi: 10.1101/cshperspect.a001487.

102. Villalobos L, Lee S, De Oliveira C, Ivetac A, Brandt W, Armitage L, and Estelle, M. (2012) A combinatorial TIR1/AFB-Aux/IAA co-receptor system for differential sensing of auxin. *Nature chemical biology 8:* 477–485.

103. Wan Z, Chai S, Ge F, Feng Q, Zhang Y, and Li S. (2017). Arabidopsis PROTEIN *S*-ACYL TRANSFERASE4 mediates root hair growth. *Plant J* 90: 249-260.

104. Weis K. (2003) Regulating Access to the Genome: Nucleocytoplasmic Transport throughout the Cell Cycle. *Cell* 112: 441-451.

105. Willmer P, Nuttman C, Raine N, Stone G, Pattrick J, Henson K, Stillman P, McIlroy L, Potts S, and Knudsen J. (2009) Floral volatiles controlling ant behavior. *Functional Ecology* 23: 888-900.

106. Wisniewska J, Xu J, Seifertova D, Brewer P, Ruzicka K, Blilou I, Rouquie D, Benkova E, Scheres B, and Friml J. (2006) Polar PIN localization directs auxin flow in plants. *Science doi:10.1126/science.1121356.*

107. Wu H, Hazak O, Cheung A, and Yalovsky S. (2011) RAC/ROP GTPases and auxin signaling. *Plant Cell,* 23: 1208–1218.

108. Zerial M, and McBride H (2001) Rab proteins as membrane organizers. *Nat Rev* 2:107–117.

109. Zolman B, Yoder A, and Bartel B. (2000) Genetic analysis of indole-3-butyric acid responses in Arabidopsis thaliana reveals four mutant classes. *Genetics* 156: 1323–1337.