CHARACTERIZATION OF IBR5-ROP GTPASE (ROP2/ROP6) INTERACTIONS IN PLANT AUXIN RESPONSE

by

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ABSTRACT

The quintessential phytohormone auxin regulates many aspects of growth and development throughout the plant life cycle. Diverse auxin responses occur via multiple distinct and overlapping signaling pathways. It is well documented that auxin exerts control over gene expression by binding its nuclear co-receptors TIR1/AFB family F-box proteins and AUX/IAA transcriptional repressor proteins, thereby promoting polyubiquitination and subsequent degradation of AUX/IAAs and relieving transcriptional repression of auxin-responsive genes. More recently, auxin has also been shown to rapidly activate Rho of plant (ROP) GTPases at the plasma membrane, leading to a variety of cellular responses. The auxin signaling mutant *ibr5-1* exhibits reduced auxin-responsive gene expression without acFcumulation of AUX/IAA repressor proteins, suggesting the dual-specificity protein phosphatase encoded by the gene IBR5 independently regulates the processes of AUX/IAA degradation and auxin-induced gene expression. In a previous screen for IBR5 interactors, a small GTPase was identified, prompting the question of whether IBR5 interacts with the ROP GTPases ROP2 and ROP6, which have been shown to be involved in auxin signaling pathways in the cytoplasm. In vitro interaction assays indicated IBR5 interacts with ROP2 and ROP6, and these interactions were confirmed by co-immunoprecipitation in *Arabidopsis thaliana*. To assess genetic interaction, an *ibr5-1 rop6-2* double null mutant was generated. In root growth assays for auxin inhibition of primary root elongation or induction of lateral root formation, the double mutant exhibited

auxin resistance similar to the ibr5-1 parent line. Taken together, the results suggest the dual-specificity phosphatase IBR5 interacts with the Rho-like GTPases ROP2 and ROP6, and these proteins may function in a common auxin signaling pathway.

CHAPTER I

INTRODUCTION

Plant growth and development, like that of animals, is tightly regulated by several hormones, small signaling molecules that are biosynthesized in the cell in low concentration and transported to various tissues. Of particular research interest is the quintessential phytohormone auxin, on which proper growth and development depends. Auxin has been shown to be involved in essentially all aspects of growth and development throughout the plant life cycle. Acting as a chemical transducer of internal and external signals, auxin modulates diverse processes to ultimately shape the architecture of the whole plant. Polar transport of auxin establishes concentration gradients such that the hormone can serve as a morphogen to direct proper tissue growth and development and organ patterning. Auxin can also be redistributed in response to environmental signals, providing a means by which the immobile plant can quickly and appropriately respond to a changing environment. While great strides have been made in the study of auxin biology, many questions remain about the mechanisms of auxin action.

The field of auxin biology began with simple observations of what we now know to be auxin activity, such as Charles Darwin's observations of plants growing toward a light source by bending of the elongating shoot (Darwin, 1880). The hypothetical substance modulating such responses was later identified as indole-3-acetic acid (IAA) (Haagen-Smit AJ, 1946), commonly known as auxin. Once identified, bioassays for physiological responses to auxin became a common tool for studying the biological role of the hormone.

Studies involving application of auxin to plant tissues revealed the importance of this hormone in a variety of physiological and developmental processes. The emergence of powerful new tools for the study of molecular biology accelerated the study of the mechanisms of auxin action. It was demonstrated that auxin rapidly activates transcription of select genes, termed primary auxin responsive genes (Hagen and Guilfoyle, 1985).

While IAA has been shown to be the principle auxin in all plant species, indole-3-butyric acid (IBA) (Zimmerman, 1935), 4-chloroindole-3-acetic acid (4-Cl-IAA) (Porter, 1965) and phenylacetic acid (PAA) (Koepfli, 1938) have also been identified as endogenous auxins (Figure 1) (Simon and Petrasek, 2011). It is unclear whether the endogenous IAA precursor IBA acts solely by its conversion to IAA or if it may act as an auxin independent of its conversion to IAA (Van Der Krieken, 1992; Zolman et al., 2000).

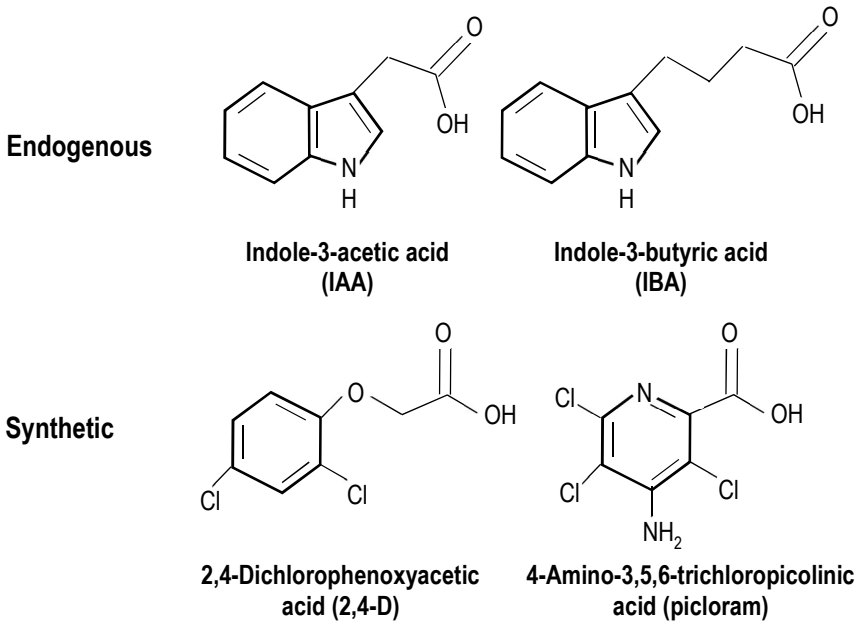

Figure 1. Auxins contain an aromatic ring and a carboxylic acid group. Indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) are naturally-occurring auxins, while 2,4-dichlorophenotyacetic acid (2,4-D) and 4-amino-3,5,6-trichloropicolinic acid (picloram) are synthetic auxins.

Additionally, a variety of synthetic auxinic compounds are widely used in research as well as in agriculture, including but not limited to 2,4-D (2,4-dichlorophenoxyacetic acid), 1-NAA (naphthalene-1-acetic acid) and picloram. While different auxinic compounds induce similar physiological responses, they have distinct, as well as overlapping, effects on gene expression (Simon et al., 2013). Thus, it appears not all mechanisms of action of these various auxins are completely shared with native IAA.

The establishment of *Arabidopsis thaliana* as a model organism for studying plant physiology and development (Meyerowitz and Pruitt, 1985) set the scene for the development of genetic approaches for studying auxin action. One such approach is a forward genetic screen for mutations that confer resistance to auxin inhibition of root growth, a strategy which has led to the identification of many components of auxin signaling pathways. For example, identification and subsequent characterization of the auxin resistant mutants *axr1* and *tir1* led to the development of a model in which auxin action requires the ubiquitin-proteasome pathway of regulated proteolysis (Gray et al., 1999; Gray et al., 2001; Pozo et al., 1998).

Control of gene expression by auxin through the ubiquitin-proteasome pathway involves three major groups of proteins: Transport Inhibitor Response-1/Auxin Signaling F-box (TIR1/AFB) family of F-box proteins, Auxin Response Factor (ARF) transcription factors and auxin/indole-3-acetic acid inducible (Aux/IAA) repressor proteins. F-box proteins play a crucial role in the tightly regulated degradation of target proteins as part of a multiprotein SCF (SKP, CUL1, F-box containing) complex, which functions as an E3 ligase for ubiquitination of the target protein and subsequent proteasomal digestion (Dharmasiri et al., 2005b). By this mechanism, auxin is able to control transcription of

auxin-responsive genes by promoting degradation Aux/IAA repressor proteins. Aux/IAAs regulate transcription of auxin responsive genes by binding and suppressing ARFs, which modulate the expression of auxin responsive genes (Ulmasov et al., 1999; Ulmasov et al., 1997). A breakthrough in the field of auxin research was the identification of F-box protein TIR1 as an auxin receptor (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). In the nucleus, auxin binds to co-receptors TIR1/AFBs and Aux/IAAs, acting as a molecular glue bringing the target protein into contact with the E3 ligase to be tagged for degradation (Figure 2) (Da Costa et al., 2013). While the $SCF^{TIR1/AFB}$ pathway provides a seemingly complete and simple pathway from auxin perception to transcriptional response, questions remain about how this pathway controls the diverse array of auxin responses.

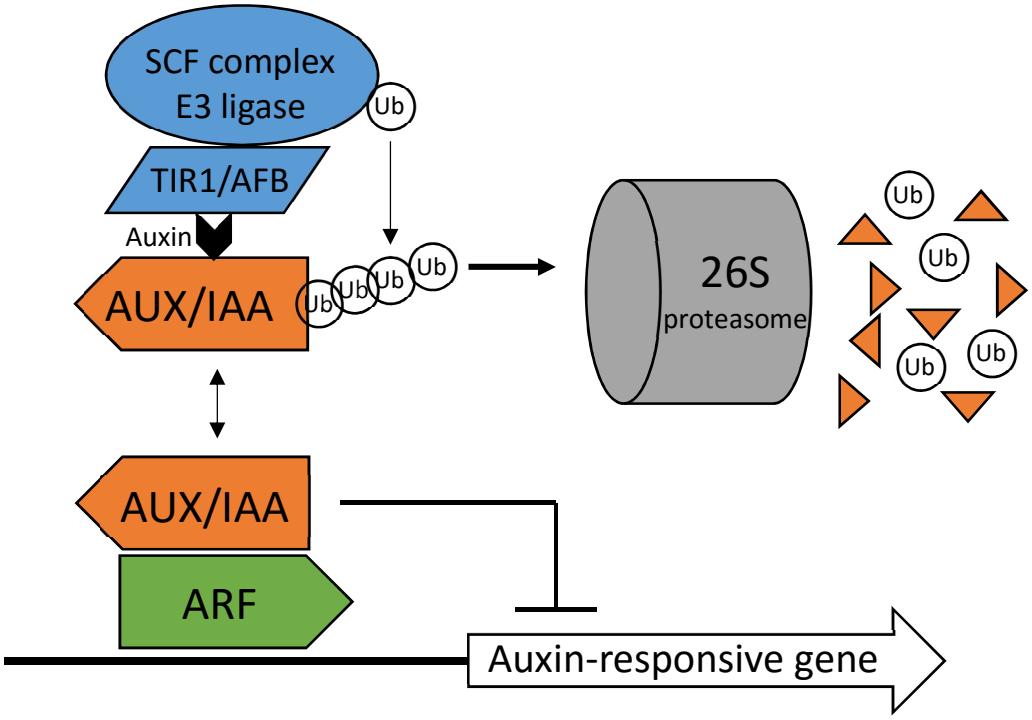

Figure 2. Auxin regulates gene transcription via the ubiquitin-proteasome pathway. Auxin binds to its co-receptors, a TIR1/AFBs F-box protein and an AUX/IAA repressor protein, promoting poly-ubiquitination and subsequent degradation of the target protein.

It has become clear that not all auxin responses can be accounted for by changes in gene transcription. It has been demonstrated that auxin rapidly induces mitogen activated protein kinase (MAPK) activity (Mockaitis and Howell, 2000), ion fluxes across the plasma membrane (Hager, 2003) and increased concentration of cytosolic calcium (Shishova and Lindberg, 2004). Ample evidence has emerged to suggest the existence of an auxin signaling pathway independent of $SCF^{TIR1/AFB}$ that requires modulation of cytoplasmic signaling cascades involving Rho-like GTPases of plants (ROPs), suggesting initiation of auxin-mediated signaling at the cell surface. For example, auxin inhibits endocytosis of PIN-FORMED (PIN) auxin efflux facilitators, thereby promoting its own efflux from cells (Paciorek et al., 2005), and inhibition of PIN2 internalization has been shown to depend on a ROP-GTPase-based auxin signaling pathway (Lin et al., 2012).

Additionally, evidence that rapid, non-genomic auxin signaling pathways have downstream effects on transcriptional responses (Tao et al., 2002) suggests integration of cytoplasmic and nuclear auxin signaling pathways. Auxin rapidly activates ROP GTPases at the plasma membrane, and this induction of ROP activity leads to increased expression of auxin-responsive genes (Tao et al., 2002); increased ROP activity promotes degradation of AUX/IAA repressor proteins, while downregulation of ROP activity suppresses auxin-accelerated AUX/IAA degradation (Tao et al., 2005). Ultimately, auxin action is the sum of its genomic and non-genomic effects. Exactly how the rapid cytoplasmic auxin signaling cascades integrate with the ubiquitin-proteasome dependent pathway in the nucleus to achieve the overall auxin response remains elusive. Furthermore, there are several additional factors that have been clearly implicated in auxin response, but their functions

remain largely unknown. Here, we focus on further elucidating the role of IBR5 in auxin signaling.

ibr5-1 was one of several mutants isolated in a forward genetic screen for mutations that confer resistance to IBA inhibition of root elongation (Zolman et al., 2000). *ibr5-1* displays multiple auxin-related developmental defects, including a long root and a short hypocotyl (Monroe-Augustus et al., 2003), similar to certain other auxin signaling mutants. The gene defective in *ibr5-1* was found to encode a dual specificity protein phosphatase that is expressed throughout the plant. Dual specificity phosphatases (DSPs) are involved in a variety of cellular processes, including cytoskeleton rearrangement, stress responses and phytohormone signaling (Bartels et al., 2010). DSPs hydrolyze the phosphoryl group of phosphorylated serine, threonine or tyrosine residues of their target proteins (Gupta et al., 1998) and have been shown to be important negative regulators of MAP kinase signaling networks (Camps et al., 2000). Thus, IBR5 is a phosphatase that modulates phytohormone signal transduction, possibly by negatively regulating MAPK signaling cascades. However, overexpression of a catalytic mutant form of IBR5 partially rescued *ibr5-1* defects, suggesting IBR5 has functions both dependent on and independent of its phosphatase activity (Strader et al., 2008).

Further analysis of *ibr5-1* revealed reduced expression of auxin-responsive genes, as expected for a mutant exhibiting a reduced response to exogenous auxin. Interestingly, however, AUX/IAA repressor proteins are not stabilized, and expression of IAA28, one of the AUX/IAAs, was reduced (Strader et al., 2008). Thus, it appears IBR5 promotes auxin responses, including auxin-induced gene transcription, without destabilization of AUX/IAAs. Similarly, the mutant *ibr5-4*, which contains a point mutation that nearly

abolishes catalytic activity, exhibits accelerated AUX/IAA degradation and diminished auxin-induced transcription. This observation suggests proper degradation of AUX/IAAs as well as auxin-inducible transcription require IBR5 phosphatase activity and further supports the notion that these two processes are independently regulated by IBR5 (Jayaweera et al., 2014). Taken together, the evidence suggests IBR5 functions as a negative regulator of AUX/IAA repressor protein degradation and a positive regulator of auxin-induced gene expression.

One important approach to unraveling how IBR5 regulates auxin signaling is to identify its interacting partners. All DSPs contain a common motif in the catalytic domain, and the substrate specificity is determined by sequences outside the catalytic core (Luan, 2003). IBR5 was confirmed to be a MAP kinase phosphatase when it was demonstrated to interact with MPK12 in *Arabidopsis* (Lee et al., 2009). As predicted, IBR5 dephosphorylates and thereby inactivates MPK12, which appears to be a negative regulator

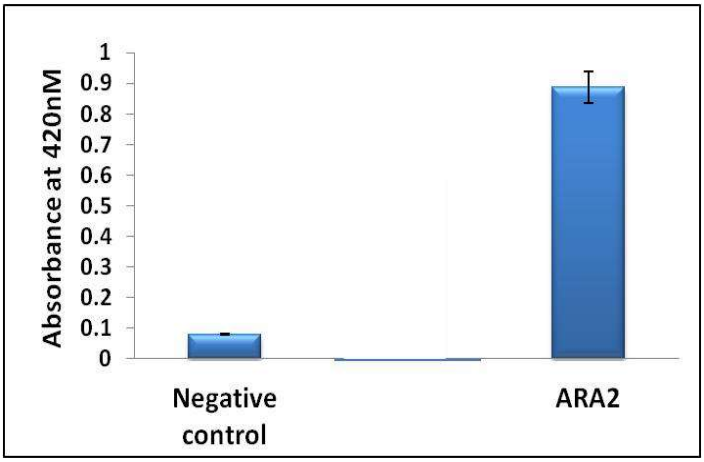

Figure 3. IBR5 interacts with the *Arabidopsis* small GTPase ARA2 in a yeast two-hybrid system. Yeast transformants were grown in a selective liquid medium. Cells were harvested by centrifugation, lysed by freeze-thaw method, then incubated with ONPG. Absorbance was measured at 420 nM with a spectrophotometer. (Conducted by Praveen Kathare)

of auxin signaling. An interactome study in *Arabidopsis* indicated IBR5 interacts with SKP1, a component of SCF complexes (Arabidopsis Interactome Mapping, 2011). Recently, IBR5 was shown to interact with Chilling Sensitive3 (CHS3), Heat Shock Protein90 (HSP90) and Suppressor of the G2 allele of *skp1*b (SGT1b) to form a complex that protects CHS3 against aggregation (Liu et al., 2015). In this case, IBR5 acts as a molecular chaperone. It seems IBR5 has complex roles in the cell and is capable of interacting with diverse partners, many of which likely remain to be identified.

Among several IBR5 interactors identified by Dharmasiri *et al.* in a yeast-two-hybrid screening was the small GTPase ARA2 (AT1G06400) (Figure 3) (Ghimire, 2015). Such an interaction was of particular interest because Rho-like small GTPases have been shown to be involved in auxin signaling. Specifically, the Arabidopsis ROP2 and ROP6 were demonstrated to be rapidly activated at the plasma membrane by extracellular auxin, leading to changes in cytoskeletal organization, calcium influx, reactive oxygen species production and a variety of other cytoplasmic events, as well as downstream changes in gene expression (Figure 4) (Fu et al., 2009; Nibau et al., 2006; Xu et al., 2010).

All small GTPases share several common structural features yet exhibit remarkable diversity in both structure and function. This is reflected in the diversity of their binding partners and the effects they modulate. Rho family GTPases play pivotal roles in cell signaling, and this family is conserved in all eukaryotic kingdoms. The Rho family is divided into the Rac, Rho and CDC42 subfamilies in animals and fungi (Boureux et al., 2007). In plants, however, a single subfamily has been identified which is closely related to the Rho subfamily. Thus, plant small GTPases are commonly referred to as ROPs (Rho of plant). In *Arabidopsis thaliana*, 11 ROPs have been identified (Vernoud et al., 2003).

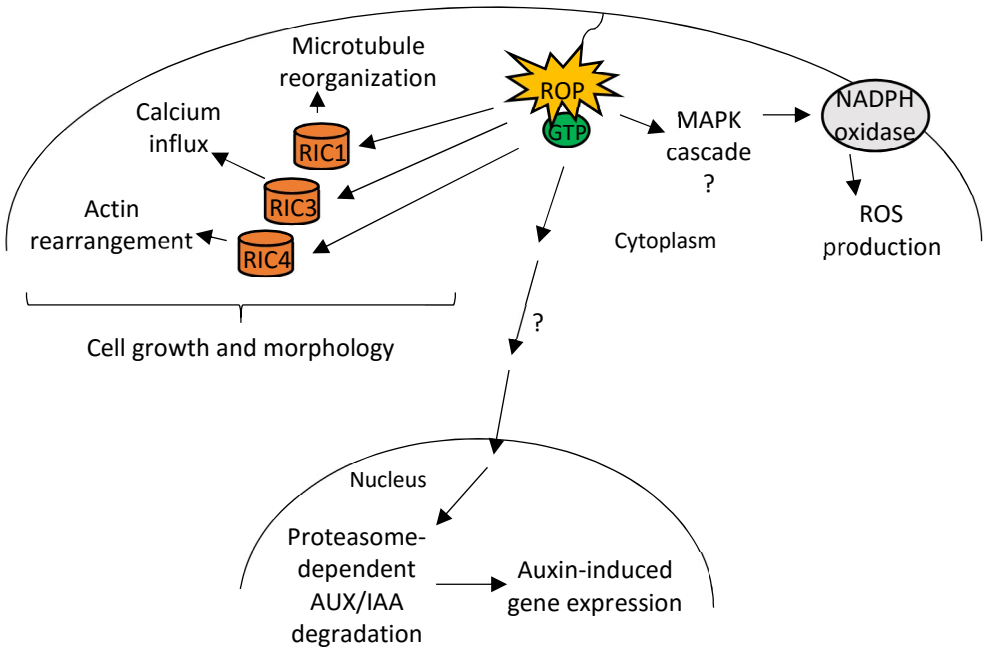

Figure 4. ROP GTPases function as signaling switches in diverse cellular processes. ROPs are known to mediate changes in the cytoskeleton, gene expression and other response systems.

ROPs carry out their functions in close association with the plasma membrane, which is mediated by post-translational modification, such as prenylation or acylation, at the C terminus of the GTPase (Yalovsky, 2015). At the plasma membrane, ROPs behave as signaling switches by alternating between an active, GTP-bound state and an inactive GDP-bound state. In response to an upstream signal, RopGEFs (Guanine nucleotide Exchange Factors) interact with ROPs to catalyze their exchange of GDP for GTP, resulting in a conformation suitable for interaction with downstream effector proteins (Berken et al., 2005). The active G protein is returned to the "inactive" conformation by hydrolysis of GTP to GDP, a process stimulated by RopGAPs (GTPase-Activating Proteins) (Wu et al., 2000). Additionally, a third group of regulators contribute to control of ROP GTPase activity: Guanine nucleotide Dissociation Inhibitors (GDIs) negatively

regulate ROPs by extracting the GTPase from the plasma membrane and inhibiting the exchange of GDP for GTP (Fukumoto et al., 1990; Zhang and McCormick, 2008).

Rapid progress is being made in elucidating the role of ROP GTPases as modulators of plant hormone signaling cascades. It was recently demonstrated that perception of auxin at the plasma membrane leads to activation of ROPs. This was thought to involve an Auxin Binding Protein 1 (ABP1)-Transmembrane Kinase 1 (TMK1) auxin-sensing complex (Xu et al., 2010). However, the most recent studies on ABP1 analyzing a mutant generated using ribozyme-based CRISPR technology suggest ABP1 is not required for auxin signaling (Gao et al., 2015) which in turn interact with downstream effectors to regulate cell morphogenesis, such as leaf epidermal pavement cell (PC) interdigitation, root hair formation and pollen tube expansion (Fu et al., 2005; Xu et al., 2010). It is through their strategic location at the plasma membrane and the diversity of their downstream effectors that G proteins are able to transduce an extracellular signal to a variety of cellular responses.

Among the known ROP targets are ROP- interactive CRIB (Cdc42/Rac-interactive binding) motif-containing proteins (RICs) (Wu et al., 2001). When ROPs become activated they are able to interact with RICs, which in turn promote the downstream cellular response. For example, ROP2 and ROP6, which associate with different regions of the plasma membrane in PCs, are activated in the presence of auxin (Fu et al., 2005; Xu et al., 2010). Active ROP2 interacts with RIC4, which mediates accumulation of actin microfilaments to promote lobe formation (Fu et al., 2002). In contrast, active ROP6 interacts with RIC1, which mediates microtubule ordering to restrict expansion, resulting in indentations (Fu *et al.*, 2009). Furthermore, ROP2 inhibits RIC1, and RIC1 likewise

antagonizes the ROP2/RIC4 pathway *in vivo* (Fu et al., 2005). Therefore, auxin promotes PC interdigitation through ROP signaling cascades, making leaf epidermal PC morphology a useful model system for the study of ROP signaling.

Because ROP2 and ROP6 become activated within minutes of application of exogenous auxin, it is clear that auxin promotes PC interdigitation independent of the SCF-TIR1/AFB pathway (Chen and Yang, 2014). However, there is evidence that ROP signaling integrates with the SCF-TIR1/AFB pathway (Tao et al., 2002). Many questions remain about how ROP signaling integrates with the nuclear signaling mechanism. Because the small G protein ARA2 was identified as an IBR5 binding partner, and several lines of evidence implicate ROP GTPases in auxin signaling, we hypothesized that IBR5 may interact with ROP GTPases. Specifically, we aim to answer the question of whether IBR5 interacts with the well-known ROP GTPases ROP2 and ROP6.

CHAPTER II

MATERIALS AND METHODS

Plant varieties and growth conditions

Arabidopsis thaliana ecotype *Columbia* (Col-0) was used in this study. *ibr5-1* mutant was previously identified by mutant screening for IBA resistance (Monroe-Augustus et al., 2003). T-DNA insertion mutant of *ROP6* in Col-0 background, named *rop6-2* (SALK_ 091737C) was requested from *Arabidopsis* Biological Resource Center (ABRC), Ohio State University and was described previously (Lin et al., 2012). To generate *ibr5-1 rop6-2* double mutant, *ibr5-1* was crossed into *rop6-2*, and homozygous lines were selected.

Seeds were surface sterilized with 40% bleach/0.1% Trixon X-100 and thoroughly rinsed with sterile distilled water then vernalized at 4° for at least 24 hours before plating. Seeds were plated on *Arabidopsis thaliana* medium with 1% sucrose (ATS), pH 5.6 (Lincoln et al., 1990). Plates were placed in a growth chamber at 22°C under 24-hr illumination (Dharmasiri et al., 2003) with cool white fluorescent bulbs. Potted plants were kept in a plant growth room at 21-22°C under continuous illumination.

DNA manipulation and plasmid construction

To construct GST fusions, *ROP2* and *ROP6* coding sequences were amplified from CD4-7 Newman Lambda PRL2 cDNA library (ABRC) using gene-specific primers ROP2-1F, ROP2-1R and ROP6-1F, ROP6-1R (Table 1) with Phusion High-Fidelity DNA

Polymerase (New England BioLabs). *ROP2* and *ROP6* deletion sequences were amplified from CD4-7 cDNA library (ABRC) by PCR to generate the following constructs: *ROP2NT* (primers ROP2-1F and ROP2-NR1), *ROP2CT* (primers ROP2-CF and ROP2-1R), *ROP6NT* (primers ROP6-1F and ROP6-NR1), *ROP6CT* (primers ROP6-CF and ROP6-1R). Each coding sequence was ligated into the *EcoR*V site of pBluescript II SK (Stratagene) cloning vector then into the *BamH*I-*Sal*I site of pGEX-4T-3 (Pharmacia) expression vector, which was transformed into BL21 *E. coli* cells.

To generate HA-ROP2 and HA-ROP6 for stable expression in *A. thaliana*, ROP2 and ROP6 coding sequences were amplified by PCR using Phusion High Fidelity DNA Polymerase (Finnzyme) with primers ROP2-2F, ROP2-1R or ROP6-2F, ROP6-1R, respectively. Blunt end PCR products were inserted into pENTR/SD/D-TOPO, Gateway Directional TOPO entry vector (Invitrogen). Plasmids were transformed into and purified

Table 1. Primer sequences.

Primer name	Primer sequence	Primer length	Tm (°C)
ROP2-1F	5'-GCGGGATCCATGGCGTCAAGGTTTATAAAG-3'	30	64
ROP2-1R	5'-TTTGTCGACTCACAAGAACGCGCAACG-3'	27	65
ROP6-1F	5'-GAGGGATCCATGAGTGCTTCAAGGTTTATC-3'	30	61
ROP6-1R	5'-CTTGTCGACTCAGAGTATAGAACAAC-3'	26	55
ROP2-NR1	5'-ATTGTCGACTTAAGCCTTGCTAATAAGAGA-3'	30	58
ROP2-CF	5'-AAGGGATCCTATGAGATTATAGCCAAGAA-3'	29	57
ROP6-NR1	5'-TTCGTCGACTCATTTGCTGACAAGTGAGAA-3'	30	62
ROP6-CF	5'-CTTGGATCCAAAGCTAGCTATGAAAATGT-3'	29	57
ROP2-2F	5'-CACCGAGATGGCGTCAAGGTTT-3'	22	61
ROP6-2F	5'-CACCAAGATGAGTGCTTCAAGGT-3'	23	58
LB02 ***	5'-TTGGGTGATGGTTCACGTAGTGGGCC-3'	26	65
ibr5-1SnB-F **	5'- GCCTGTTTCTTCCGATACGGTGGCTACG-3'	28	74
ibr5FBoxSal1R *	5'-GTAGAGATTCTGGCACATAGG-3'	25	74

*** Designed by Salk Institute
** Designed by Sunethra Dharmasiri
* Designed by Thilanka Jayaweera

from TOP10 host *E. coli* strain then digested with *Mlu*I (New England BioLabs). *ROP2* and *ROP6* entry clones were inserted into pEarleyGate 201 (ABRC) expression vector using Gateway LR Clonase (Invitrogen) recombination reactions. Plasmids were transformed into and purified from TOP10 *E. coli* then transformed into *Agrobacterium tumefaciens* strain GV3103 competent cells by electroporation.

Protein expression in bacteria

For expression of recombinant proteins in *E. coli*, LB liquid medium was supplemented with carbenicillin at 100 µg/ml and inoculated with the appropriate bacterial colony or glycerol stock. Cultures were incubated in test tubes overnight at 37°C in a shaker at 200 rpm. In a flask, a larger volume of LB + Carbenicillin (100µg/ml) was inoculated from overnight culture and incubated in a shaker at 37°C and 200 rpm for 1-3 hours. IPTG (Gold Biotechnology) was added to a final concentration ranging from 0.1 mM to 1 mM, and the culture was incubated at 30°C and 200 rpm for an additional 3-5 hours. The cells were harvested by centrifugation at 10,000 x g for 10 minutes. The supernatant was discarded and the pellet frozen in -80°C freezer.

For *Agrobacterium* cultures, LB liquid medium was supplemented with gentamycin (25 µg/ml) and rifampicin (50 µg/ml) and inoculated with appropriate bacterial colony or glycerol stock. Cultures were incubated for 24 hours at 30°C and 200 rpm. Larger volume LB + gentamycin (25 µg/ml) + rifampicin (50 µg/ml) was inoculated from overnight culture and incubated for 24 hours at 30°C and 200 rpm. Cultures were used to transform *A. thaliana* plants by floral dip method (Clough and Bent, 1998).

***In vitro* protein-protein interaction assays**

To purify GST-fusion proteins, pellets were thawed on ice and re-suspended in PBS (phosphate buffered saline; 137 mM NaCl, 2.7 mM KCl, 10 mM NaH_2PO_4, 2 mM KH_2PO_4, pH 7.4). Cells were lysed by sonicating 3 times for 10-15 seconds, cooling in ice at least 30 seconds between each sonication. To the crude cell lysate, 1 mM PMSF (phenylmethylsulfonyl fluoride; Amresco) and 0.1% Tween-20 (Sigma) were added. The solution was clarified by centrifugation at 10,000 x g for 10 minutes at 4°C. The supernatant was collected and incubated with glutathione-agarose beads (Sigma) for at least 2 hours at 4°C, mixing by inversion. Beads were washed 3 times with PBS/0.1% Tween-20 then re-suspended in PBS and stored at 4°C.

For interaction with plant-derived IBR5-Myc, transgenic seedlings were homogenized in pull down extraction buffer [30 mM HEPES, pH 7.5, 80 mM KCl, 0.4% Triton X-100 (v/v), 1 mM EDTA, 10% glycerol (v/v), 1 mM PMSF, 21 mM MG 132, Complete protease inhibitor cocktail (Roche)]. Lysate was cleared by centrifugation at 10,000 x g for 10 minutes at 4°C. Total protein concentration was approximated using Bradford method (Bradford, 1976), and aliquots of approximately 800 µg were stored at -80°C. For interaction with bacterially expressed Myc-tagged IBR5 full protein or truncations, pellets were re-suspended in PBS, and cell lysates were prepared as described above. Glycerol was added to 10% final concentration, and 500 µL aliquots were stored at -80°C. Samples were analyzed by western blot to compare expression levels.

IBR5-Myc plant extract was thawed and incubated with GST-fusion protein conjugated to glutathione-agarose beads for 1-2 hours at 4°C with gentle agitation. Extract was removed, and beads were washed 3 times with pull down washing buffer [20 mM

HEPES, pH 7.5, 70 mM KCl, 0.2% Triton X-100 (v/v), 5% glycerol (v/v), 1 mM EDTA]. Beads were re-suspended in 2X Laemmli sample buffer [250 mM Tris-HCl, pH 6.8, 40 g/L SDS, 20% glycerol (v/v), 30 g/L bromophenol blue, 10% β-mercaptoethanol (v/v)] for SDS-PAGE.

Co-immunoprecipitation

For *in vivo* interaction, 10-11-day old seedlings were vacuum infiltrated with 1% formaldehyde (v/v) in PBS for 15 minutes for protein cross-linking. The reaction was quenched by addition of 200 mM glycine. Seedlings were rinsed with PBS, excess liquid was blotted, and seedlings were flash frozen in liquid nitrogen. Frozen tissue was homogenized in IP extraction buffer [20 mM HEPES, pH 7.5, 40 mM KCl, 1% Triton X-100 (v/v), 1 mM EDTA, 21 mM MG132, and complete protease inhibitor cocktail (Roche)]. Crude extract was cleared by centrifugation at 10,000 x g for 15 minutes at 4°C, and supernatant was collected. Extracts were incubated with anti-HA agarose beads (Roche) for 2 hours at 4°C with gentle agitation. Beads were washed 3X with IP washing buffer [20 mM HEPES, pH 7.5, 40 mM KCl, 0.1% Triton X-100 (v/v), 1 mM EDTA] then re-suspended in 4X Laemmli sample buffer.

SDS-PAGE and western blot analysis

SDS-PAGE was run using 12.5-14% polyacrylamide gels. Samples were boiled in Laemmli sample buffer for 6 minutes to elute and denature proteins. The gel electrophoresis was set up in a Bio-Rad Mini-PROTEAN II Cell. The gel was run at approximately 100 V until the dye from reached the separating gel, about 15-30 minutes,

then voltage was increased to approximately 150 V until the dye front ran out of the gel. PageRuler Prestained Protein Ladder (Thermo Scientific) was used as a molecular weight standard.

For western blotting, proteins were transferred from the gel onto PVDF membrane (Bio-Rad) by wet electrotransfer for 1 hour 15 minutes. The membrane was blocked with 5% w/v nonfat dry milk in TBST [Tris buffered saline (50 mM Tris-Cl, pH 7.5, 150 mM NaCl), 0.1% Tween-20] by rocking for at least 30 minutes at room temperature. Membrane was washed 3 times for 5 minutes in TBST. Primary antibody (anti-Myc or anti-HA) was applied to membrane at a 1:10,000 dilution and incubated 1-2 hours at room temperature or 2 hours-overnight at 4°C with gentle rocking. After three 5-minute washes with TBST, the secondary antibody (mouse IgG) was applied with rocking at room temperature for 1 hour at a 1:10,000 dilution. The membrane was washed once for 15 minutes, followed by three 5-minute washes with TBST. Target proteins were visualized via X-ray film exposure using chemiluminescent substrates (Bio-Rad Clarity Western ECL Substrate) per manufacturer instructions.

Genotyping of double mutant

To genotype double mutants, 3 PCRs were performed for each DNA sample: one to confirm the presence/absence of *ROP6* gene (primers ROP6-1F + ROP6-1R), one to confirm the presence/absence of *T-DNA* insert (primers ROP6-1F + LB02) and one to amplify a 447-bp segment of *IBR5* (primers ibr5-1SnB-F + ibr5FBoxSal1R). To distinguish between mutant *ibr5-1* and wild-type *IBR5*, *IBR5* PCR products were digested with *SnaB*I at 37°C for 2-3 hours. Digested samples were separated in a 3% agarose gel at

150 V for at least 30 minutes to separate the wild-type (447 bp) and mutant (420 bp) DNA fragments.

Imaging of pavement cells

Images of cotyledon epidermal cells were acquired by confocal microscopy. Cotyledons were stained with propidium iodide and observed using an Olympus FV1000 confocal microscope. Images were taken near the center of the leaf, away from the perimeter and midrib. For each trial, 3 cotyledons from 3 individual seedlings were observed per genotype, and two images were acquired from different areas of each cotyledon.

Root growth assays

For auxin inhibition of primary root elongation, seeds were germinated on ATS. Four-day-old seedlings were transferred onto plates containing indicated concentrations of IBA, IAA or picloram, with the root tips touching a line drawn across the plate. After four more days of growth, the length of the primary root below the line was measured to the nearest mm. Percentage inhibition versus the control was calculated for comparison between genotypes. Approximately 10-15 seedlings were analyzed for each genotype per trial. Each experiment was conducted at least 3 times.

Similarly, auxin induction of lateral root formation was analyzed by transferring 5-day-old seedlings from untreated ATS onto media containing the indicated concentrations of IBA or 2,4-D. Seedlings were grown for 5 additional days. Primary root length was measured, and the number of emerged lateral roots was counted using a stereomicroscope

(LW Scientific). Lateral root density was calculated as the number of lateral roots per centimeter primary root length. Approximately 10 seedlings were analyzed for each genotype per trial.

Data analysis

For statistical comparison of primary and lateral root growth, ANOVA was done using VassarStats: Website for Statistical Computation (www.vassarstats.net).

CHAPTER III

RESULTS

Physical interaction

To test whether IBR5 interacts with ROP GTPases, molecular cloning techniques were used to express ROP2 and ROP6 as GST-fusions in *E. coli*. GST-ROP2 and GST-ROP6 were extracted from cells and affinity purified using glutathione-agarose beads. After washing away non-specifically bound proteins, the beads were incubated with total protein extract from seedlings stably expressing Myc-tagged IBR5. After incubation, beads were washed thoroughly to remove non-specifically bound protein. Samples were resolved by SDS-PAGE, and pull down of IBR5-Myc was analyzed by western blot with anti-Myc antibody (Fig. 5A). Western blot analysis revealed IBR5-Myc was pulled down by GST-ROP2 and GST-ROP6 but not by GST alone, indicating IBR5 interacted with ROP2 and ROP6 *in vitro*. The two bands visible for IBR5-Myc are observed consistently for IBR5-Myc expressed in plant and may be indicative of posttranslational modification.

To confirm the interaction of IBR5 with ROP2 and ROP6 *in vivo*, co-immunoprecipitation (co-IP) was performed. Seedlings of transgenic lines stably expressing IBR5-Myc, HA-ROP2, HA-ROP6 or co-expressing IBR5-Myc and HA-ROP2 or –ROP6 were treated with formaldehyde for *in vivo* crosslinking of proteins. After cross-linking, total protein was extracted from tissues. Anti-HA antibody-conjugated agarose was incubated with protein extract. After washing away unbound proteins, the samples were analyzed by western blot using anti-Myc antibody. IBR5-Myc coprecipitated with

HA-ROP2 and HA-ROP6 from *Arabidopsis* extracts (Fig. 5B), indicating IBR5 forms complexes with ROP2 and ROP6 *in vivo*.

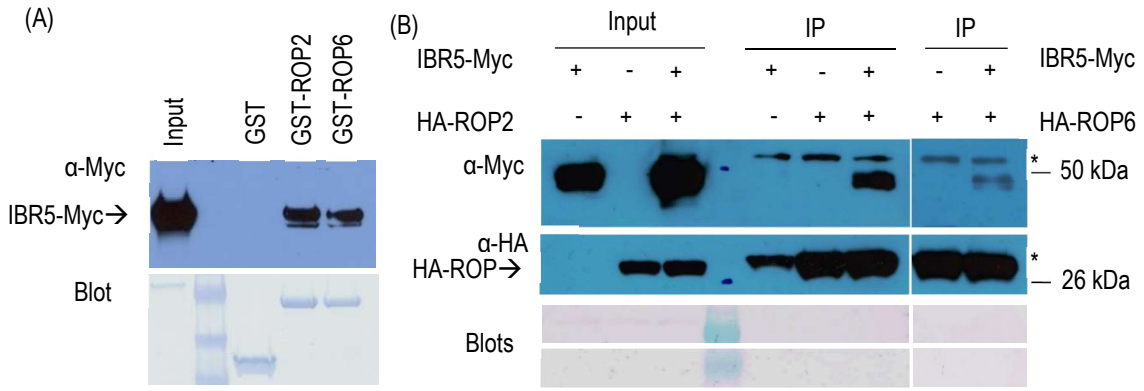

Figure 5. **IBR5 interacts with ROP2 and ROP6 *in vitro* and *in vivo*.** (A) Pull down of IBR5-Myc with GST-ROP2 and GST-ROP6. *GST-ROP2* and *GST-ROP6* were expressed in *E. coli*, and recombinant proteins were purified by affinity binding to glutathione-agarose beads then incubated with total protein extract from IBR5-Myc transgenic plants. The precipitated proteins were resolved by SDS-PAGE and analyzed by western blot with α-Myc antibody. "Input" is a sample of the IBR5-Myc plant extract. IBR5-9xMyc is expected to migrate to around 40 kDa. (B) Co-immunoprecipitation of IBR5-Myc with HA-ROP2 or HA-ROP6. Protein complexes were cross-linked *in vivo* by formaldehyde treatment. Protein extracts were incubated with α-HA antibody conjugated to agarose beads. Co-precipitation of IBR5-Myc with HA-ROP2 or HA-ROP6 was visualized by western blot with anti-Myc antibody. HA-ROP2 and ROP6 are expected to migrate to around 25 kDa. "*" indicates

Rop-interactive CRIB motif-containing proteins (RICs) are ROP effector proteins that contain a CRIB (Cdc42/Rac-interactive binding) motif for specific interaction with GTP-bound ROPs (Wu et al., 2001). The well-known ROP effector RIC1 was cloned and expressed in *E. coli* as a GST-fusion protein (Fig. 6A). Because small GTPases have been shown to participate in macromolecular complexes with multiple effectors and other interactors, the ability of IBR5 to interact with RIC1 was tested. *In vitro* binding assays were performed as described above using bacterially expressed and affinity purified GST-

RIC1 to pull down IBR5-Myc expressed in *Arabidopsis*. Western Blot analysis indicated interaction between IBR5 and RIC1 in plant extract (Fig. 6B).

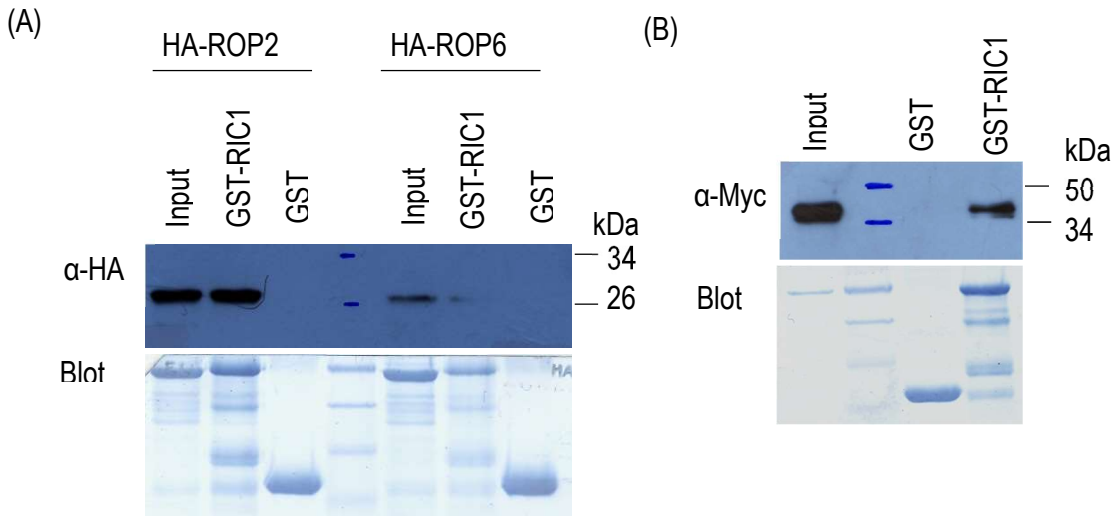

Figure 6. **IBR5 interacts with RIC1 *in vitro*.** (A) RIC1 interaction with ROP2 an ROP6. Affinity purified GST-RIC1 or GST were used to pull down HA-ROP2 or GST-ROP6 from plant extract. Pull-down samples were analyzed by western blot with α-HA antibody. Interaction was observed with GST-RIC1 but not GST alone. (B) RIC1 interaction with IBR5. Affinity purified GST-RIC1 or GST alone were incubated with extract from plants expressing IBR5-Myc. Pull-down samples were analyzed by western blot with α-Myc antibody. IBR5-Myc was pulled down by GST-RIC1 but not by GST alone.

IBR5 is a protein phosphatase that has been shown to dephosphorylate the substrate MPK12 (Lee et al., 2009). ROP2 and ROP6 contain conserved phosphorylation sites (Berken, 2006), and a phosphomimetic mutation was shown to affect the signaling properties of ROPs (Fodor-Dunai et al., 2011). Thus, it is possible IBR5 de-phosphorylates phosphorylated ROP residues, which would require ROP interaction with IBR5 catalytic domain. Previously designed (Jayaweera, et al.) IBR5 deletion constructs (Fig. 7A) were expressed in *E. coli*. Extracts were used for pull-down assays with GST-ROP2 and GST-

ROP6. The recombinant ROP proteins interacted with all IBR5 constructs except D3 and D5 (Fig. 7B,C). D3 contained only the C terminus and lacked the catalytic and calmodulin binding domains entirely, while D5 contained only the calmodulin binding domain.

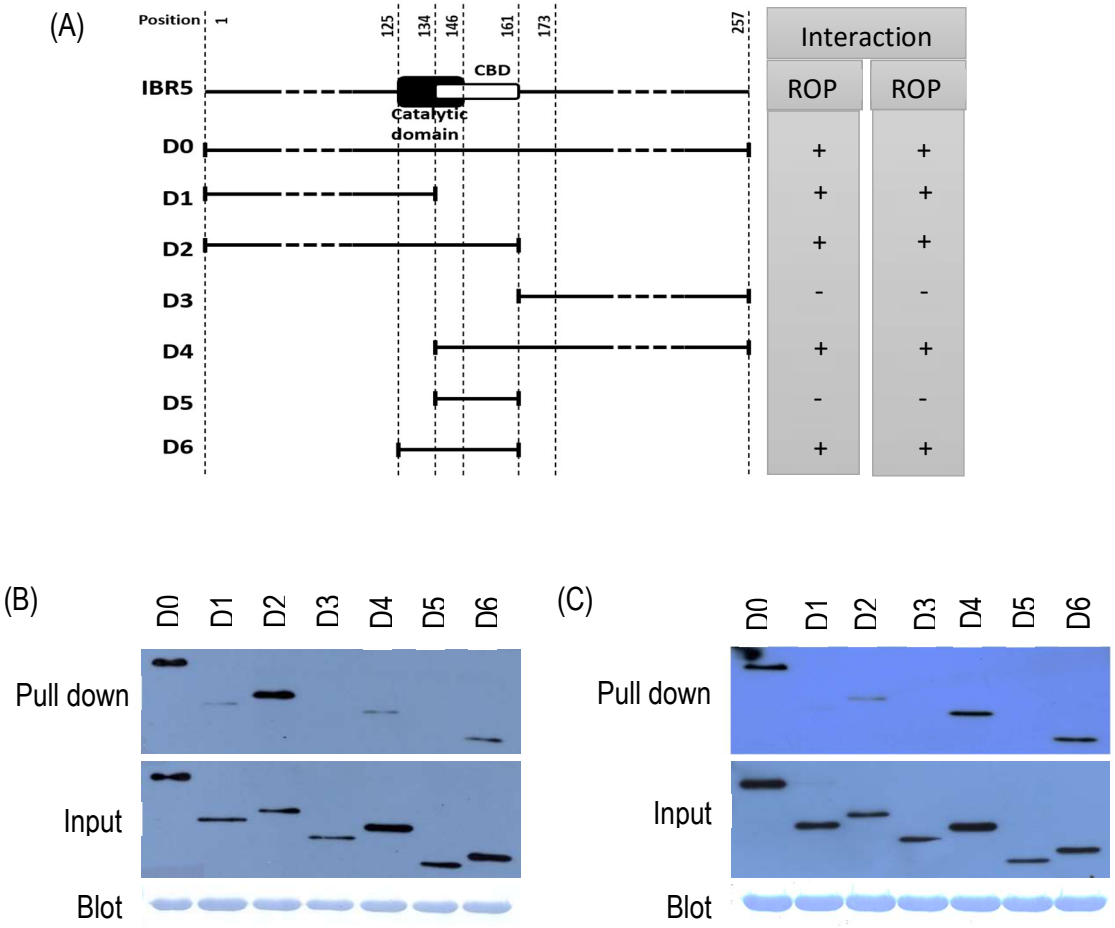

Figure 7. IBR5 catalytic domain/calmodulin-binding domain is sufficient for interaction with ROPs. (A) Schematic diagram of IBR5 deletion constructs. "Position" indicates amino acids. CBD = calmodulin binding domain. (B) Interaction with GST-ROP2. GST-ROP2 was affinity purified from *E. coli*, and beads were incubated with extract from *E. coli* expressing Myc-tagged IBR5 deletion constructs. Pull down and input samples were analyzed by SDS-PAGE followed by western blot with anti-Myc antibody. (C) Interaction with GST-ROP6.

Furthermore, ROP2 and ROP6 also contain several conserved functional domains (Ke et al., 2012), including an effector binding domain toward the N terminus as well as the Rho insert domain, which is implicated in regulation of the GTPase, toward the C terminus (Fig. 8A). Thus, if IBR5 is a ROP effector, we expect preferential interaction with the N-terminal half containing the effector binding domain. ROP truncations were expressed as GST fusions and were used in pull-down assays with extract from seedlings expressing IBR5-Myc. IBR5-Myc interacted with both N-terminal and C-terminal ROP2/6 truncations but did not interact with GST alone, indicating IBR5 interacts with residues in both regions of the ROP GTPases (Fig. 8B).

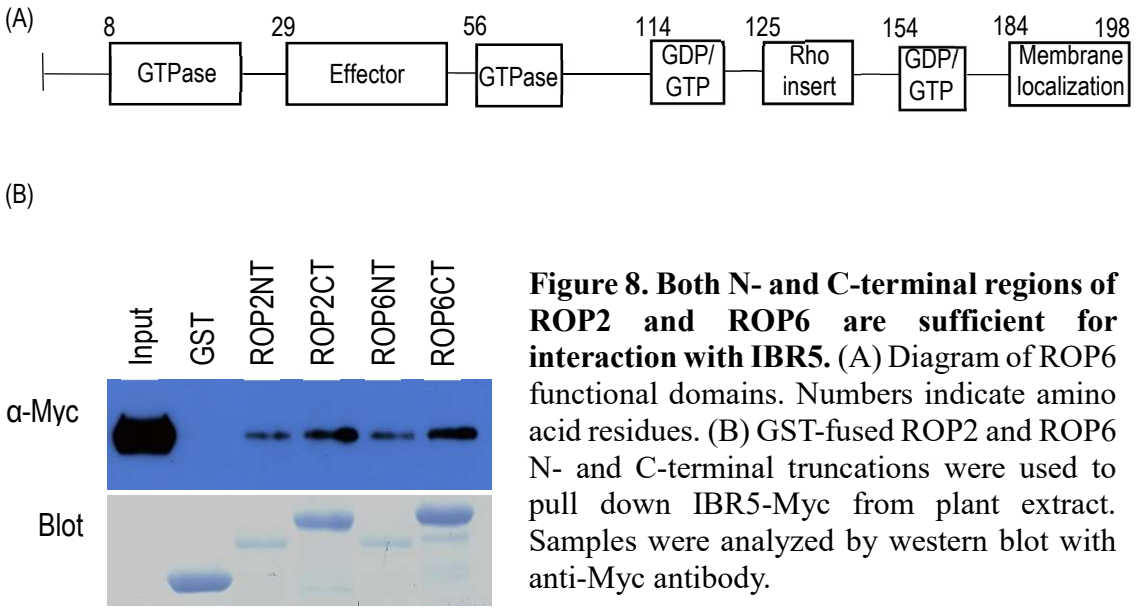

Figure 8. Both N- and C-terminal regions of ROP2 and ROP6 are sufficient for interaction with IBR5. (A) Diagram of ROP6 functional domains. Numbers indicate amino acid residues. (B) GST-fused ROP2 and ROP6 N- and C-terminal truncations were used to pull down IBR5-Myc from plant extract. Samples were analyzed by western blot with anti-Myc antibody.

Genetic interaction

To explore the question of whether IBR5 functions in a common pathway with ROPs in auxin signaling, *ibr5-1* null mutant was crossed into *rop6-2* knockout line to generate a double mutant (Fig 9). Auxin sensitivity of the double mutant was compared to

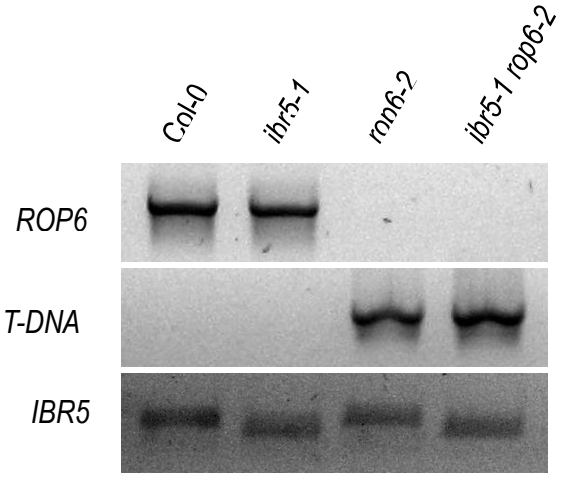

Figure 9. *ibr5-1 rop6-2* double mutants are homozygous for both the *ibr5-1* and *rop6-2* mutations. Upper panel: *ROP6* was amplified using forward and reverse gene primers. Middle panel: T-DNA insert was amplified using forward primer for *ROP6* gene along with the left border primer for the insert. Lower panel: *IBR5* was amplified using *ibr5-1* specific dCAPs primers, then PCR products were digested with *Sna*B1 restriction endonuclease. Only the mutant form is cut by the enzyme.

wild type and the single mutants. *ibr5-1* was isolated in a mutant screening for IBA resistance (Zolman et al., 2000), and it has since been shown to exhibit resistance to picloram and IAA as well (Jayaweera et al., 2014). To analyze sensitivity of the double mutant to auxin, primary root growth assays were conducted using IBA, IAA, 2,4-D and picloram. Seedlings were first grown on untreated media for 4 days then transferred onto untreated media or media containing the indicated concentration of auxin. After 4 additional days of growth, elongation of the primary root since the transfer was measured, and percentage inhibition compared to the control was calculated. On all treatments tested, *ibr5-1* and *ibr5-1 rop6-2* exhibited resistance, while *rop6-2* was similar to wild type (Fig. 10). Thus, the double mutant exhibited the *ibr5-1* phenotype.

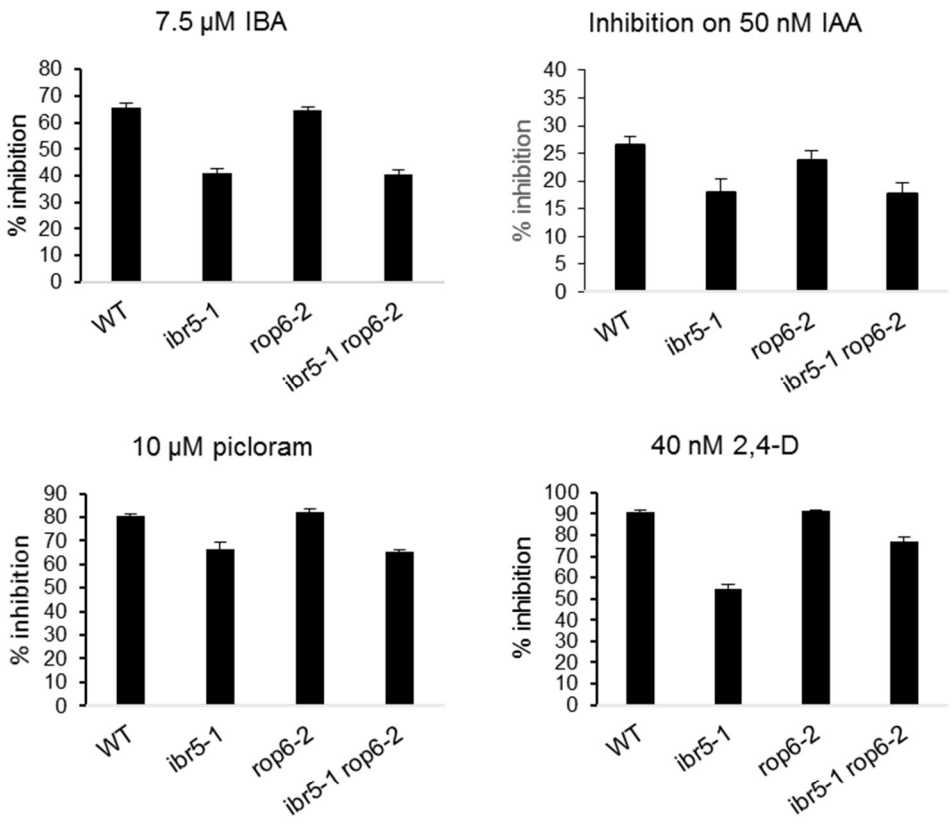

Figure 10. *ibr5-1 rop6-2* displays resistance to auxin inhibition of root elongation similar to *ibr5-1*. Seedlings were grown for 4 days on untreated media then transferred onto media containing the indicated concentration of the respective auxin treatment or no treatment. After 4 more days, length of the primary root since the transfer was measured, and percentage inhibition was calculated. Error bars indicate the percent standard error. Each experiment was repeated at least 3 times, and the data shown is from one representative experiment.

In addition to inhibiting elongation of the primary root, application of exogenous auxin also promotes formation of lateral roots. *ibr5-1* was shown to be less responsive to auxin induction of lateral root formation (Strader et al., 2008), and *rop6-2* exhibits increased lateral root density compared to wild type (Lin et al., 2012), implicating these genes in lateral root development. To evaluate genetic interaction of *IBR5* and *ROP6* in lateral root development, the response of the double and single mutants to auxin induction

of lateral root formation was analyzed using IBA and 2,4-D. Similar to inhibition of primary root elongation, *ibr5-1* parent line and *ibr5-1 rop-2* double mutant exhibited reduced sensitivity to auxin in lateral root induction, while *rop6-2* developed lateral roots similar to wild-type (Fig. 11).

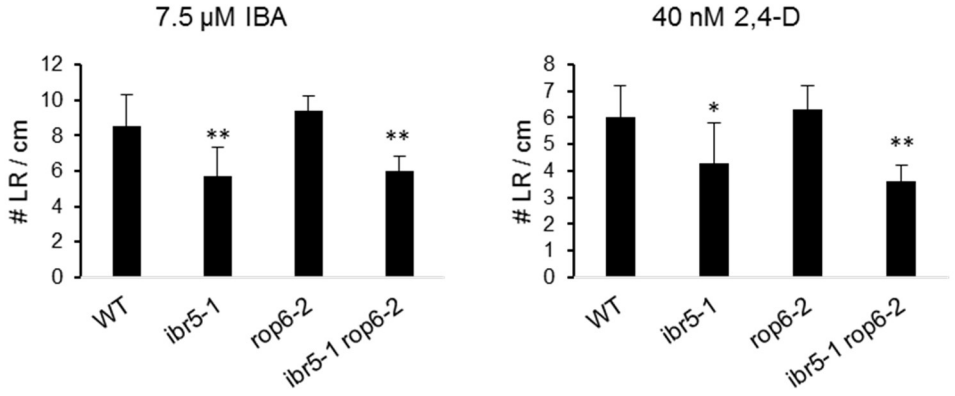

Figure 11. *ibr5-1 rop6-2* exhibits resistance to auxin-induced lateral root formation similar to *ibr5-1*. Seedlings were grown for 5 days on untreated media then transferred onto media containing the indicated concentration of the respective auxin treatment or no treatment. After 5 more days, length of the entire primary root was measured, emerged lateral roots were counted and lateral root density was calculated. Error bars indicate standard deviation from the mean. Asterisks denote statistically significant difference (* for $p \leq 0.05$ and ** for $p \leq 0.01$) Each experiment was conducted at least 3 times, and the data shown is from one representative experiment.

ROP6 has been shown to be involved in the formation of lobes and indentations of leaf epidermal cells, also known as pavement cells (PCs) (Fu *et al.*, 2005; Fu *et al.*, 2009), and defective PC interdigitation has been observed in *ibr5* mutants (Jayaweera et al., 2014). Pavement cells of *ibr5-1 rop6-2* double mutant were observed in comparison to the single mutants and wild type. The double mutant exhibited more severe defects in pavement cell

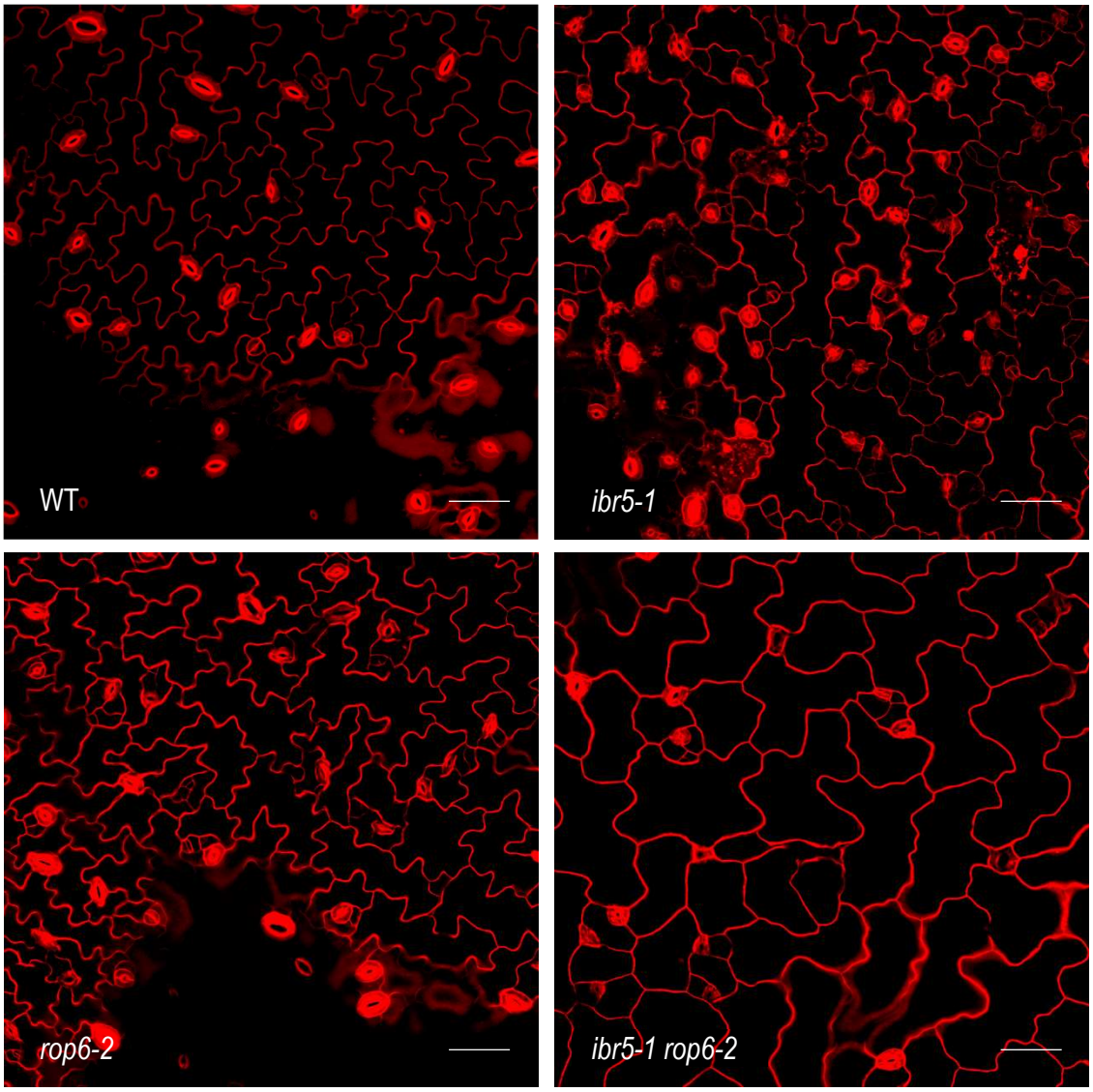

Figure 12. PC interdigitation is defective in *ibr5-1 rop6-2*. Cotyledons of 7-day-old seedlings were stained with propidium iodide, and epidermal cells were imaged by confocal microscopy. Three cotyledons from 3 individual seedlings were observed for each trial, and 3 trials were conducted. The white scale bar represents 50 µm. The images shown are from one independent trial. Phenotypic variation between cotyledons within a genotype was observed.

shape compared to either single mutant (Fig. 12). In addition to abnormal cell shape, *ibr5-1 rop6-2* PCs also appear larger in size. However, this more severe phenotype was only

observed in approximately 50% of double mutant samples, and the other 50% of double mutants appeared similar to *ibr5-1* in PC interdigitation.

CHAPTER IV

DISCUSSION

Employing biochemical and molecular biological techniques along with double mutant phenotype analyses, we identified the novel interaction of IBR5 with ROP GTPases, key components of recently elucidated auxin-mediated signaling networks. Because IBR5 was previously shown to interact with a small GTPase (Ghimire, 2015), unconventionally regulate AUX/IAA degradation and auxin-induced transcription and exhibit developmental defects in root elongation, lateral root formation and pavement cell interdigitation, it was hypothesized that IBR5 might interact with the auxin-activated ROP2 or ROP6 in an auxin signal transduction pathway. Our data provide evidence of the physical and functional interaction of IBR5 with the Rho-like GTPases ROP2 and ROP6.

The physical interaction of IBR5 with ROP2 and ROP6 was first demonstrated *in vitro* then confirmed by co-immunoprecipitation (co-IP) assays (Figure 5), the technique also used to confirm other ROP6 and IBR5 interactions, including ROP6 interaction with RIC1 (Fu et al., 2009) and NFR5 (Ke et al., 2012) and IBR5 interactions with CH3, HSP90 and SGT1b (Liu et al., 2015). While the use of *in vivo* formaldehyde cross-linking brings into question the specificity of the observed interaction, taken together with IBR5-ARA2 interaction in yeast (Figure 3), IBR5-ROP2/6 interactions *in vitro* (Figure 5A) and IBR5-RIC1 (Figure 6B) interaction *in vitro*, it is highly likely that IBR5 is a true ROP GTPase binding partner. Furthermore, interaction of the ROP effector RIC1 with IBR5 in plant extract suggests IBR5 may participate in multiprotein complexes with ROPs and their effectors.

The identification of ROP2 and ROP6 as proteins that interact with IBR5 adds to the diversity of known IBR5 interacting partners, including MPK12 (Lee et al., 2009); the SCF complex component SKP1 (Arabidopsis Interactome Mapping, 2011); and the defense response complex components CHS3, HSP90 and SGT1b (Liu et al., 2015). IBR5 has been shown to function as a dual-specificity phosphatase that dephosphorylates MPK12 and as a holdase that prevents protein aggregation and protects CHS3 (Lee et al., 2009; Liu et al., 2015). The evidence presented here suggests IBR5 may also play a role in a ROP GTPase-mediated signaling pathway. While IBR5 does appear to fulfill the expected role of a dual specificity phosphatase as a negative regulator of MAP kinase signaling cascades (Lee et al., 2009), this is clearly not the whole picture, and the function of IBR5 in intracellular signaling seems to be highly complex.

Binding assays with truncated forms of the proteins allowed us to explore what regions of the proteins may be interacting. The only IBR5 constructs that did not interact with ROP2 or ROP6 were the calmodulin-binding domain (CBD) and the region C-terminal to the CBD (Figure 7). Because the construct that contained only the CBD did not interact with GST-ROP2 or GST-ROP6, while the full catalytic domain/CBD construct did interact, the catalytic domain of IBR5 may be important for interaction with ROPs. Furthermore, interaction of ROP2 and ROP6 with D1, although it appeared relatively weak, suggests the CBD is not required for the interaction. This observation taken together with the ability to ROP2/6 to interact with D4 but not D3, suggests at least part of the catalytic domain must be present to bind the GTPases. The lack of interaction with D5 could be explained by its small size coupled with the Myc tag. In a similar study, Molendijk, et al. demonstrated that deletion of kinase subdomains from the receptor kinase construct

NCRK-313 abolished its interaction with ROP4 in a yeast two-hybrid system (Molendijk et al., 2008). The constructs used in the present study were originally designed for a study of IBR5-Calmodulin binding (Jayaweera et al., unpublished data). Perhaps using newly designed IBR5 deletions in a yeast two-hybrid system would confirm the involvement of the catalytic domain in binding to ROPs.

Furthermore, binding of IBR5 to ROP2 and ROP6 N terminal regions, which contain the effector domain for binding downstream partners, and C terminal regions, which contain the Rho insert domain for regulation of ROP activity, was analyzed. Both N terminal and C terminal constructs of ROP2 and ROP6 were sufficient for interaction with IBR5 in binding assays *in vitro* (Figure 8). This would suggest that IBR5 interaction with ROPs involves residues in both regions of the GTPases. In a similar study using N and C terminal truncations of MPK12, it was shown that the C terminal region of MPK12 is sufficient for interaction with IBR5 in yeast, while the N terminal region showed no interaction (Lee et al., 2009). Considering the three-dimensional structure of Rho-family GTPases, one can see how an interacting partner may easily make contact with residues on either end of the amino acid sequence, as the protein folds in such a way that the N and C termini are not far from one another (Ke et al., 2012). The result of this study did not provide any indication as to whether IBR5 is likely an effector or regulator of ROP GTPases. Further analysis is required to determine what motifs are involved in IBR5-ROP interactions. Perhaps testing small regions of ROPs for interaction with IBR5 would aid in characterizing the structural requirements of the interaction.

Analysis of *ibr5-1 rop6-2* double mutant phenotypes allowed us to gain insight into the biological relevance of the IBR5-ROP6 interaction. On all auxins tested, *ibr5-1*

displayed resistance to inhibition of primary root elongation, as expected based on previous studies (Monroe-Augustus et al., 2003; Zolman et al., 2000). The *rop6-2* displayed auxin sensitivity similar to wild-type, which was not surprising as knockout of a single GTPase often yields weak phenotypes due to high homology among GTPases. The *ibr5-1 rop6-2* double mutant exhibited resistance to IBA, IAA and picloram nearly identical to that of the *ibr5-1* parent line (Figure 10), raising the possibility that *ibr5-1* is epistatic to *rop6-2*. However, we cannot conclude this relationship due to the lack of a non-wild-type phenotype for *rop6-2*.

On 2,4-D, however, *ibr5-1 rop6-2* exhibited partial rescue of the resistance phenotype as compared to the *ibr5-1* parent line. An intermediate phenotype was also observed in the case of the *MPK12RNAi ibr5-3* double mutant with respect to auxin sensitivity (Lee et al., 2009), but the *MPK12RNAi* parent line displayed hypersensitivity to auxin, while *rop6-2* exhibited wild-type sensitivity. The intermediate resistance of the double mutant on 2,4-D is nonetheless indicative of a genetic interaction between *IBR5* and *ROP6*. However, the lack of interaction observed on all other auxins tested complicates the interpretation of these results.

With respect to lateral root density, *ibr5-1* and *rop6-2* exhibit opposite phenotypes, with mutation of *IBR5* resulting in reduced lateral root density (Strader et al., 2008) and mutation of *ROP6* resulting in increased lateral root density (Lin et al., 2012). Auxin induction of lateral root proliferation was also reduced in *ibr5-1*, as shown previously (Strader et al., 2008), but was not impaired in *rop6-2* (Figure 11). The double mutant displayed insensitivity to IBA- and 2,4-D-induced lateral root development similar to that of *ibr5-1*. In a previous study, *axr1 ibr5* and *aux1 ibr5* double mutants displayed resistance

similar to that of the parent lines, while *tir1 ibr5* double mutants were shown to have enhanced resistance to IBA-induced lateral root formation compared to either parent in support of the notion that IBR5 promotes auxin responses through a mechanism distinct from TIR1-mediated repressor degradation (Strader et al., 2008).

Although the lateral root density of the double mutant on auxin was nearly identical to that of *ibr5-1*, we cannot conclude that *IBR5* is epistatic to *ROP6* based on these results due to the wild-type sensitivity observed for *rop6-2.* Because members of the ROP family share greater than 70% amino acid identity (Zheng and Yang, 2000), knockout of a single *ROP* may not be sufficient to observe genetic interaction that may exist with *IBR5* in auxin signaling. Perhaps more conclusive results can be obtained by combining the *ibr5-1* mutation with knockout of multiple *ROPs* or with a dominant negative *ROP* mutant.

The *ibr5-1* single mutant exhibited marked defects in pavement cell (PC) interdigitation, as shown previously (Jayaweera et al., 2014), and *rop6-2* PCs appeared defective, as seen in a previous study using a different *rop6* knockout line (Fu et al., 2009), but less severely than *ibr5-1*. PC interdigitation of *ibr5-1 rop6-2* double mutants appeared defective compared to wild-type (Figure 12), with approximately 50% of samples exhibiting more severe defects than *ibr5-1* and the other 50% similar in severity to *ibr5-1* parent line. Such incomplete penetrance of the non-parental phenotype may be explained by the genetic redundancy associated with *ROP6*. It was reported that PC phenotypes of the *ktn1-3 rop6-1* and *ktn1-3 ric1-1* double mutants were similar to the phenotype of the *ktn1-3* parent line in support of a genetic and functional interaction between KTN1 and the ROP6-RIC1 pathway for controlled expansion of interdigitated PCs (Lin et al., 2013). With the *ibr5-1 rop6-2* double mutant exhibiting a PC phenotype similar to or more severe than

ibr5-1, it is likely IBR5 may also function in a common pathway with ROPs and RICs in the regulation of PC interdigitation, but further evidence is needed to support this notion as the high degree of inconsistency observed in PC phenotypes complicates the interpretation of these results.

Based on the evidence presented here, it can be concluded that the dual-specificity phosphatase IBR5 physically interacts with the Rho-like GTPases ROP2 and ROP6. Furthermore, *ibr5-1 rop6-2* double mutant analyses of 2,4-D resistance and pavement cell interdigitation lend support to the notion that IBR5 likely functions in a common auxin signaling pathway with ROP GTPases. 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 ROP GTPases may provide a mechanism for cross-talk between ROP-based auxin signaling in the cytoplasm and $SCF^{TIR1/AFB}$-based auxin signaling in the nucleus.

Moving forward, it will be important to determine the subcellular localization of IBR5-ROP interactions. While both IBR5 and ROPs have been localized to both the nucleus and the cytoplasm, predominant localization of IBR5 is in the nucleus, and active ROPs predominantly localize to the cytoplasm in close association with the plasma membrane (Fu et al., 2002; Jayaweera et al., 2014; Lee et al., 2009; Molendijk et al., 2001; Wu et al., 2001). Furthermore, to understand the physiological role of IBR5-ROP GTPase interactions in auxin signaling, it will be critical to conduct further studies to conclude whether IBR5 functions upstream or downstream of ROP activity.

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