IDENTIFICATION AND CHARACTERIZATION OF IBR5 INTERACTING PROTEIN 1(IIP1) IN ARABIDOPSIS

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IDENTIFICATION AND CHARACTERIZATION OF IBR5 INTERACTING PROTEIN 1 (IIP1) IN ARABIDOPSIS

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Terms Defined

Antibody - protein that is specific to a particular epitope.

Arabidopsis thaliana - a popular model organism in plant biology and genetics.

Expressed protein - Presence in a particular cell or tissue.

Gene - A hereditary unit consisting of a sequence of DNA that occupies a specific location on a chromosome and determines a particular characteristic in an organism.

Genome - entirety of an organism's hereditary information.

GFP - Green Fluorescent Protein

GST - glutathione S-transferase; a fusion tag for the purification of proteins.

IBR5 - Gene that encodes a dual specificity phosphatase.

IIP1 - protein identified as potentially interacting with IBR5 from yeast two hybrid assay.

Myc tag - A peptide tag used in many different assays that require recognition by an antibody.

Negative regulator - Any regulator that acts to prevent gene expression.

pGEX-4T3 – A plasmid designed for protein expression in cells.

Phosphatases - Enzymes that catalyze the removal of a phosphate.

Pull down assay - A technique to isolate and identify interacting proteins with a particular protein.

Sequencing - The process of determining the precise order of nucleotides within a DNA sequence.

Transgenic - Containing a gene sequence that has been isolated from one organism and is introduced into a different organism.

Western Blot - widely accepted analytical technique used to detect specific proteins in the given sample of tissue homogenate or extract.

Yeast two Hybrid assay - A molecular biology technique used to discover protein–protein interactions and protein–DNA interactions by testing for physical interactions (such as binding) between two proteins or a single protein and a DNA molecule, respectively.

ABSTRACT

Auxin is a vital plant hormone that regulates growth and development throughout the life cycle. Recently, IBR5, a gene that encodes a dual specificity phosphatase, was identified as protein that regulates plant auxin signaling. However, the exact molecular mechanism of IBR5's function in plant auxin response is unknown. One approach to understand the molecular mechanism is to identify IBR5 interacting proteins. To this end we performed a yeast two hybrid screen using an Arabidopsis cDNA library. Among several IBR5 interacting protein (IIP), IIP1 was selected for further studies. While yeast two hybrid screen is a powerful technique to identify new interacting proteins, results of this assay have to be validated by other biochemical and/or genetic techniques. We hypothesized that IIP1 is a true IBR5 interacting protein and used other molecular techniques to validate IBR5-IIP1 interaction. First, *In vitro* pull-down assay was used to confirm the interaction between IBR5 and IIP1. IBR5 was expressed in Arabidopsis plants as a Myc-tagged fusion protein (IBR5-Myc), and IIP1 was expressed in *E. coli* as GST-tagged recombinant protein (GST-IIP1). GST-IIP1 protein was purified from *E. coli* and added to Arabidopsis crude protein extract isolated from *35S::IBR5-Myc* seedlings. Proteins interacted with GST-IIP1 were recovered and separated by SDS-PAGE. Interacting proteins were detected by western blot using anti-Myc antibody. Results indicate that IIP1 is a true IBR5 interacting protein. This interaction has further been confirmed *in*

vivo using co-immunoprecipitation (Co-IP) assay. To understand the genetic interaction between IBR5 and IIP1, Arabidopsis T-DNA insertion mutant of *iip1* was obtained from Arabidopsis biological resource center (ABRC). PCR analysis was performed to identify *iip1* homozygous mutant.

Chapter 1

General Introduction

Auxin is a hormone found in plants that is responsible for the growth and development of plant cells. It is as important to plants as estrogen and testosterone are to humans. It regulates many developmental processes throughout the life cycle of the plant. These include vascular development, apical dominance, tropic responses such as which direction a plant organ grows in response to light conditions, gravity etc., and organ patterning by directing cell division, expansion and cell differentiation (Ellis, 2009). Cells communicate through chemical signals and changes in the signal or modifications to the signaling process can affect cellular processes. An important gene involved in regulating auxin response is *Indole-3-butyric acid response5 (IBR5).* This gene encodes a dual specificity phosphatase, which acts to remove phosphate groups from phosphorylated proteins. Through the removal of phosphate group other processes involved in auxin response can be activated or deactivated. One example of this is IBR5 de-phosphorylating Mitogen activated protein kinase12 (MPK12) in Arabidopsis (Book, 2010). To understand how IBR5 functions in plant auxin response, it is important to identify other proteins that are interacting with IBR5. To achieve this goal, a yeast two hybrid assay, a technique that allows

identification of genome-wide protein interactions was employed (Figure 1). Of several putative IBR5 interacting proteins

identified, one candidate, IIP1, was selected further study. IIP1 is a component of the proteasome complex that is composed of two subcomplexes: the 20S core protease (CP) that compartmentalizes the protease active sites, and the 19S regulatory particle (RP) that recognizes and moves appropriate substrates into the CP lumen for breakdown(Rechsteiner, 1987). Proteases play a key role in many important cellular processes and are essential components for degradation of most cellular proteins in eukaryotic cells and occur in nearly all cellular compartments to modify or break down ubiquitinated proteins (Bartel, 2008; Davies, 1995). Proteasome activity is known to be involved in plant auxin response (Rechsteiner 1987). Auxin regulates gene expression by promoting SCF ubiquitin-ligase-catalyzed degradation of the Aux/IAA proteins. IIP1 probably participates in this regulation by being a part of the 20S core protease within the proteasome complex. Therefore, it is very likely that IIP1 functions in plant auxin response probably by interacting with IBR5. Thus, it is important to investigate whether IIP1 interaction with IBR5 is a real interaction and whether this interaction is necessary for plant auxin responses. Outcome of this research is not only important for understanding the auxin signaling process in plants, but it can also be applied to animal systems. The proteasome mediated protein degradation is important in all eukaryotic organisms, including humans. Thus, work explained in this thesis would be useful in both agriculture as well as medicine.

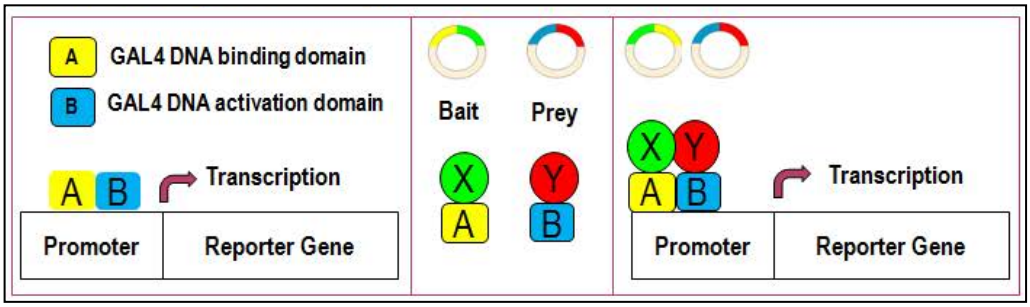


Figure 1. **Yeast Two Hybrid Assay**. Overview of Yeast two hybrid assay for identifying protein-protein interactions

Chapter 2

Materials and Methods

IIP1 in PBS (EV) Cloning Vector

IIP1 was amplified from Arabidopsis Col-0 cDNA by high fidelity Phusion DNA polymerase (NEB) PCR using gene specific primers. The PCR product was separated by gel electrophoresis on 1% agarose gel next to a 100bp maker. The resulting band was cut out of the gel and IIP1 was isolated using gel purification kit (Promega). IIP1 was then inserted into the cloning vector pBluescript (Figure 2.) by DNA ligase enzyme and incubated at 16°C overnight. The plasmid that was created also contained the gene for *lac Z* - which codes for a functional β-galactosidase enzyme (that would be disrupted when DNA is cloned) was then transformed by heat shock at 42°C for 30 seconds. Colonies that did not produce a blue color which indicate a disrupter lacZ gene, were selected. Colony PCR was done using M13F/R primers to confirm presence of cDNA insert. 200µL of a sample which contained the plasmid was then inoculated into 5 mL of LB broth with 50µg/ µl carbenicillin antibiotic and incubated for 1 day in 37°C shaker. 150µL of glycerol was added to 850µL of the culture and stored as a stock in -80°C freezer.

Preparing GST-IIP1 in Expression Vector

IIP1 in pBluescript was digested with *Bam*H1 and *Sal*1 restriction enzymes and by incubating overnight at 37°C. The restriction digestion products were separated by agarose gel electrophoresis to isolate *IIP1* DNA fragment on 1% agarose gel next to a 100bp maker and λHindIII marker. *IIP1* insert was excised from the gel and purified using the gel purification kit. IIP1 with restriction sites was then inserted into the expression vector pGEX-4T3 which was also restricted with *Bam*HI and *Sal*1(Figure 2.).

Transforming IIP1 in pGEX-4T3 into *E. coli*

3.5μL of *IIP1* in pGEX-4T3 was added to a tube containing 25μL BL21, competent bacterial cells incubated on ice for 30 min and heat shocked at 42°C for 40 seconds. The mixture was then placed in ice for 1 minute before adding 200 μL of LB broth, and being transferred to 37°C for 1 hour. Culture was then plated on LB plates containing 50μg/ mL carbenicillin. 10 colonies were selected and colony PCR was done using *IIP1* gene specific primers to confirm the presence *IIP1* cDNA. 150μL of 5 different cultures which showed IIP1 in PCR were inoculated into 5mL LB with 50 μg/mL carbenicillin and incubated in 37°C shaker for 2 days.

Expression of IIP1 Protein

E. coli harboring IIP1 cDNA was inoculated in to liquid LB (with 50 μg/mL cabenicillin) and incubated for 3 hours in 37°C shaker. To induce expression of IIP1, 1mM IPTG was added and then the culture was moved to 30°C shaker for 5

hours. The protein was isolated from the bacteria by pelleting 500µL and then resuspending the pellet in 50µL of 10mM Tris before taking 10µL and adding 10µL of 2x LSB buffer. The mixture was then boiled for 5-6 minutes before loading into a 12% acrylamide SDS-PAGE gel. After running the gel for 15 minutes at 100V and then 45 minutes at 150V the gel was stained with Coomassie blue and then washed with de-staining solution (40% EtOH and 10% Acetic Acid).

IIP1 and IBR5 Protein-Protein Interaction *In vitro*

To purify GST-IIP1 150µL of BL21 cells transformed with of IIP1 in pGEX-4T3 was inoculated into 5mL LB with 50 µg/mL carbenicillin and incubated overnight in 37°C shaker. 200µL of culture was then added to a new tube with 100mL LB with carbenicillin (50 µg/ml) and then incubated at 37°C shaker for 3-5 hours until OD of 600. IPTG was added to a final concentration of 1mM and tube was placed in 30°C shaker for 5 hours. 10mL of culture was centrifuged at 4°C at 8,000g for 10 minutes. The supernatant was removed and the pellet was resuspended in 7mL PBS. The solution was sonicated on output 1 for 15 seconds 4 times to lyse cells, gently swirling the tube in between sonications. Tween-20 was added to a final concentration of 0.1% and 70µM PMSF to a final concentration of 1mM. The tube was put on a rocker in 4°C for 10 minutes and then centrifuged at 10,000g for 10 minutes. To isolate GST-IIP1 75µL of glutathione beads were added and allowed to incubate on a rocker at 4°C overnight. The next morning, the suspension was centrifuged at 4°C and spun at 6,000g for 2 minutes.. The supernatant was carefully removed using a

micropipette leaving only 0.5mL in the tube. The palette was washed with 1 x PBS + 0.1% tween for 10 minutes on the rocker at 4°C. The washing process was repeated 2 more times. After the last wash all supernatant was removed with a flat micropipette tip. The pellet was resuspended in 150µL 1 x PBS., 10µL was transferred to a new tube and the supernatant was removed. 15µL LSB was added, boiled for 6 minutes and loaded on to a 12% acrylamide SDS-PAGE gel.

Seeds from transgenic lines that contained the gene for 35S-IBR5-Myc were plated onto ATS (Figure 3.). After 7 days, seedlings were collected and rinsed with water before being rinsed and weighed. The seedlings were contained in aluminum foil and frozen in liquid nitrogen until ready for protein extraction. To extract the protein the seedlings were ground in extraction buffer until homogenous and transferred to a 10mL tube. The mixture was placed on a rocker at 4°C for 10 minutes. It was then centrifuged at 4°C for 10 minutes at 10,000g and the supernatant containing the proteins was collected. A pull down assay was performed by adding the supernatant to the isolated GST-IIP1 (Figure 4.). The sample containing protein complex was run on a 12% polyacrylamide gel with control samples which contained IBR5-Myc, GST-IIP1.

A plolyvinylidene (PVDF) membrane was wetted with methanol and then rinsed with transfer buffer and proteins were transferred on to PVDF membrane using BioRad transfer system for 1 hour and 15 minutes at 100V. The PVDF membrane was rinsed in TBST (10x TBS containing 0.1% Tween). The membrane was then blocked with 0.05% nonfat milk in 20mL 1X TBST for 1hr on a rocker. After incubation, the membrane was washed 3 times, for 5 minutes

each time, with TBST. The membrane was removed from the wash and was incubated in a (1:1000) dilution of α-Myc antibody from mouse for 1 hour on a rocker at very low agitation. The membrane was removed from the antibody and washed 3 times, for 5 minutes each time, in TBST. The membrane was incubated in a (1:2000) dilution secondary antibody of goat IgG against mouse for 1 hour on a rocker at speed 1. The membrane was washed for 15 minutes in 1X TBST, and then 3 more times for 5 min each on a rocker.

The blot was then developed using Pierce western blot kit in a dark room according to manufacturer's instructions and exposed to an X-ray film. The x ray film was developed to observe the bands.

IIP1 in Gateway Entry Vector

IIP1 in pBSluescript vector was grown in 5 mL LB containing 5mg/mL carbicillian. Plasmids were isolated using alkaline mini prep method. IIP1 was prepared for Gateway Entry Cloning. IIP1 was amplified from IIP1 in PBS(EV) by high fidelity Phusion DNA polymerase (NEB) PCR using IIP1 pENTR F/R primers. 0.5µL of PCR product was added to a tube containing 0.5µL of pB7FwG2.0 vector which contains 35S promoter region and green florescent protein gene (*GFP*) with 0.5µL of salt solution and 3.0µL of water. After mixing gently and incubating at room temperature for 30 minutes, the tube was put in a water bath at 42°C. Tube was then left at room temperature. Competent Top-10 E. coli cells in a tube were thawed from -80°C in ice and were transformed with 1.5µL of reaction mixture by heat shock at 42°C for 30 seconds and then put into ice for 1 minute before adding 300µL lysogeny broth (LB). Cells were incubated

in a shaker at 37°C for 1 hour and plated on LB agar containing 50mg/mL kanamycin. Plates were stored at 37°C overnight to allow colony growth. Colonies were selected and grown in 400µL of LB with 50mg/mL of kanamycin. Colony PCR was done with IIP1 pENTR primers to confirm presence of plasmid.

Transgenically Expressing IIP1-GFP into Arabidopsis

Plasmid (above) was isolated from E. coli using alkaline mini prep method and transformed into GV3101 strain of *Agrobacterium tumefaciens* by electrophoration. Transformed cells were plated and grown on 20mL of LB agar with 50mg/mL spectinomycin, 25mg/mL gentamycin and 50mg/mL rifampicin. Colonies selected were grown in 400mL LB with 50mg/mL spectinomycin, 25mg/mL gentamycin and 50mg/mL rifampicin. Inflorescences of Arabidopsis plants were dipped into the bacteria and left covered and on their side for one day. The plants were grown in the growth room until they began to produce seeds. The gene construct was transformed into both wild type Col-0 and *35S:IBR5-Myc* backgrounds. Seeds were collected and plated on agar media containing Basta to grow for 10 days. Seedlings resistant to Basta were selected and transferred into soil. Protein was extracted from leaf tissues, run on SDS-Page, and transferred to a PVDF membrane as described above. Membrane was washed and incubated with primary antibody of α-GFP from rabbit and secondary antibody α-rabbit IgG from goat. Western blot was done to check for expression of IIP1-GFP.

IIP1 and IBR5 Protein-Protein Interaction *In vivo*

Seeds from lines expressing IIP1 in Col and IBR5-Myc backgrounds were plated on ATS for 7 days. Seedlings were collected and weighed on foil to be at least 0.60 grams and stored in -80°C until protein extraction. To extract protein from tissues a mixture of 2.125mL extraction buffer, 5µL MG132, 375µL protease inhibitor cocktail in H_2O, and 25µL PMSF was added to the samples and ground with a glass Tenbroeck Tissue grinder until homogenous. The mixture was poured into a 10mL centrifuge tube and put in a rocker at 4°C for 10 minutes. The tube was then centrifuged for 10 minutes at 4°C and 10,000g. The supernatant was collected and protein concentration was estimated using the Bradford method. Agarose beads with anti-Myc antibody were added to the supernatant containing plant protein and allowed to incubate overnight in 4°C. Samples were run on two SDS-Page gels and transferred to a PVDF membrane to prepare for detection of both GFP and Myc epitopes. Each membrane was prepared and washed. For GFP detection, one membrane was incubated with α-GFP primary antibody and α-rabbit IgG secondary antibody. To detect Myc, the membrane was incubated with α-Myc primary antibody and α-mouse IgG secondary antibody. Cross reacting proteins were detected by chemi-luminescent assay as described above

Selecting homozygous mutants of *iip1*

Mutant iip1 seeds with T-DNA inserts were provided by the Arabidopsis biological resource center (ABRC). Seeds were plated on ATS and 20mL ATS and 50mg/mL kanamycin for 10 additional days. Seedlings with resistance to

kanamycin were transferred to ATS for 4 days and then transferred to soil. After 2 weeks, samples were taken from young leaves of the plants and DNA was isolated from each sample. PCR was done to each sample using IIP1 F/R primers as well as using T-DNA specific LB02 F primer with IIP1 R primer (Figure 5.).

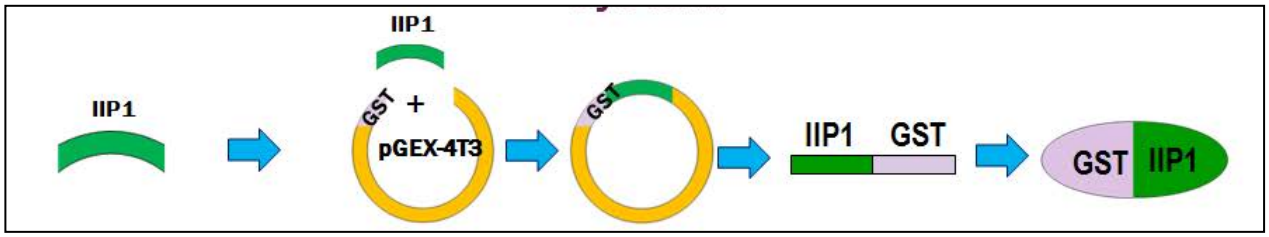


Figure 2. **Expression of GST-IIP1 in *Escherichia coli*.** Coding sequence of IIP1 was PCR amplified using high fidelity Phusion polymerase. Gel purified PCR product was cloned into cloning vector pBluescript (PBS). IIP1 gene was then inserted into the expression vector pGEX-4T3 which contains GST tag. Plasmid was then transformed into *E Coli*(BL21) bacterial strain. Expression of GST-IIP1 was induced at 30ºC for 5hrs by adding 1mM IPTG.

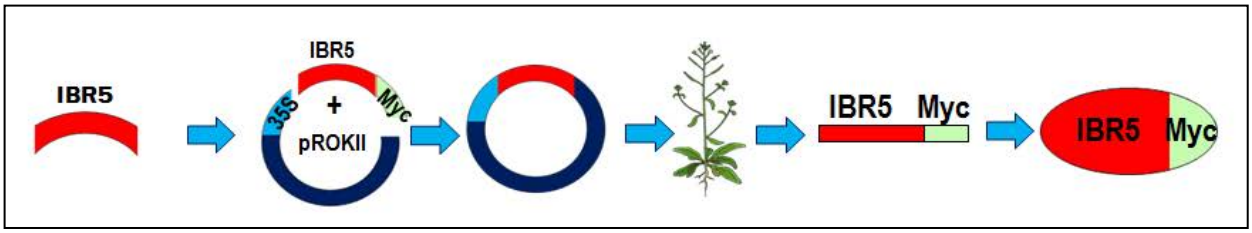


Figure 3. **Expression of IBR5-Myc in *Arabidopsis thaliana*** as a Myc tagged protein. Coding sequence of *IBR5* was PCR amplified using high fidelity Phusion polymerase. Gel purified PCR product was cloned into cloning vector pBluescript (PBS) containing Myc tag sequence. IBR5 tagged with Myc sequence was then cloned into binary vector pROKII which contains the constitutive 35S promoter. Plasmid was then transformed into *Agrobacterium tumerfacians* and subsequently transformed into plants. Expression of IBR5 in transgenic plants was confirmed by western blotting using anti-Myc antibody.

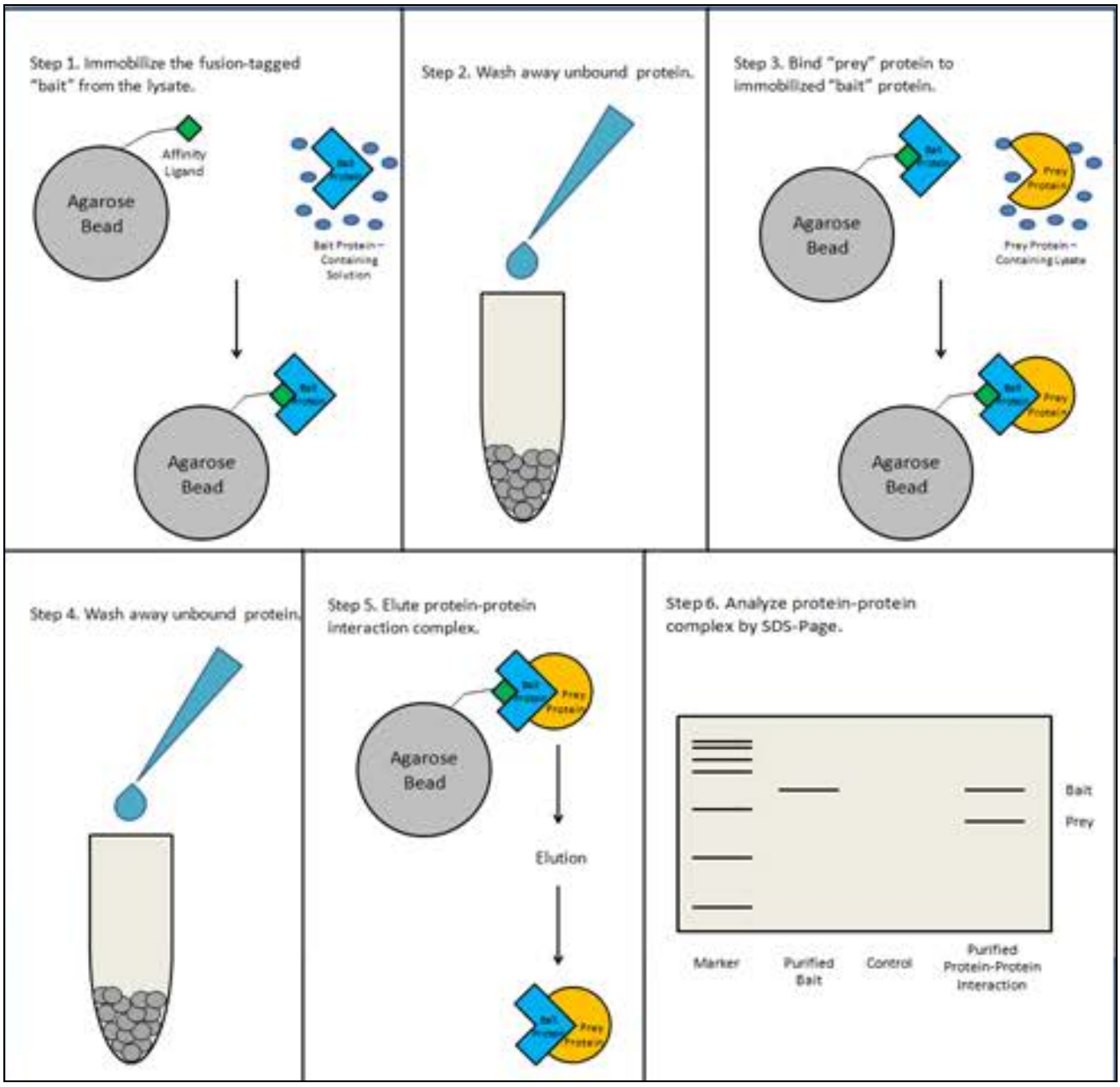


Figure 4. **Pull-down Assay to Study Protein-Protein Interactions.**

Interaction assay using GST-IIP1 as "bait" and IBR5-Myc as "prey". GST-IIP1 protein was purified using Glutathione-Agarose beads. IBR5-Myc containing plant extract was incubated with GST-IIP1 at 4ºC for 3hrs. Unbound proteins were washed away with washing buffer. Protein interacting complex was separated on SDS PAGE gel and IBR5 was identified by western blotting using anti-Myc antibody.

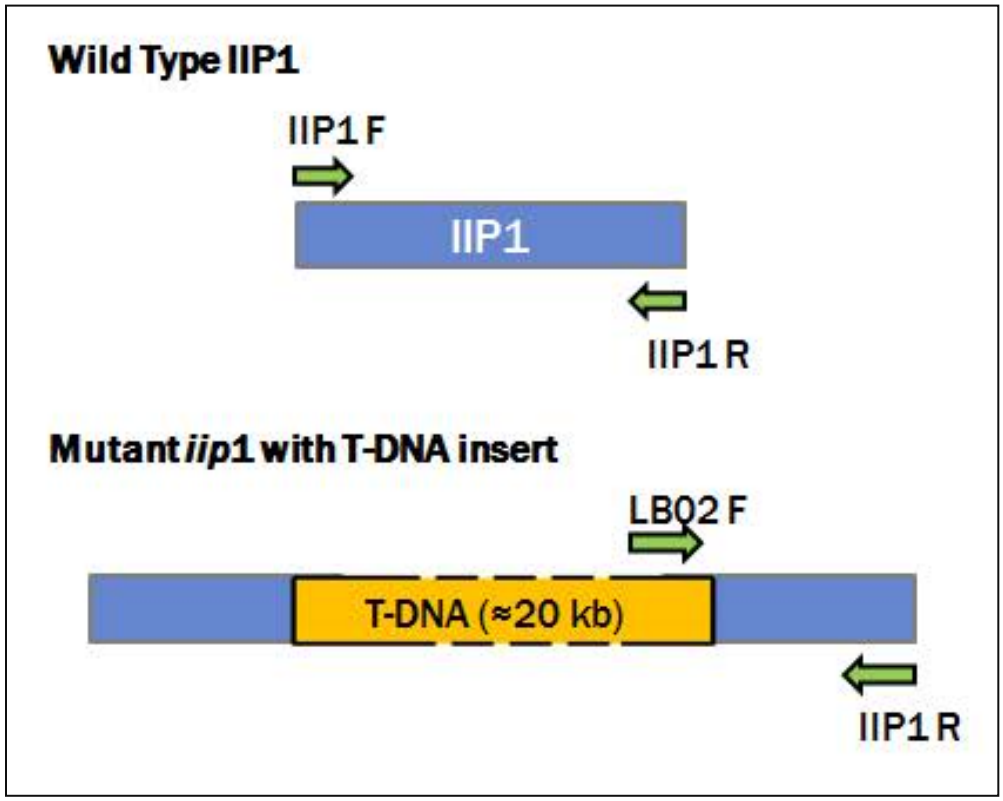


Figure 5. **Selecting Homozygous *iip1* Mutant Lines.** Arabidopsis T-DNA insertion mutant of *iip1* was obtained from Arabidopsis biological resource center (ABRC). Primers used for detection of gene and T-DNA insert shown.

Chapter 3

Results

Yeast Two Hybrid Assay

Putative IBR5 interacting proteins(IIP1 and II2) that were identified by yeast two hybrid assay were tested individually again using ortho-Nitrophenyl-β-galactoside (ONPG). Spectroscopy measured absorbance of the yellow product, which would only be made if both the GAL4 DNA activation and binding domain are in close enough proximity to interact, was measured using a spectrophotometer(Figure 6.). This occurs when the bait protein attached to the GAL4 DNA binding domain and the prey protein attached to the GAL4 DNA activation domain interact. Samples which showed high absorbance were selected and plasmids were isolated using PureYield Plasmid Miniprep System (Promega). Isolated plasmid DNA was then sent for sequencing and IIP1 was selected for further studies.

Transforming IIP1 in pGEX-4T3 into *E. coli*

IIP1 was amplified from Arabidopsis cDNA and the product was inserted into pBluescript cloning vector. After isolation of IIP1 from plasmids in *E. coli*, *IIP1* cDNA was successfully inserted into an expression vector. The presence of

IIP1 was confirmed by both antibiotic resistant colony formation as well as by PCR analysis using IIP1 F/R gene specific primers.

Expression of IIP1 Protein

E. coli bacteria expressing GST-IIP1 were identified by running the proteins from 5 independent colonies next to the untransformed control, sample. . There was a distinct band around 50kD. (Figure 7). This band does not show up in the control and close to the size of the recombinant GST-IIP1 protein suggesting IIP1 was expressed successfully in *E. coli*.

Interaction of IIP1 and IBR5 *In-vitro.*

GST-IIP1 recombinant protein was purified from *E. coli* and mixed with plant protein extract from *35S::IBR5-Myc* seedlings. GST-IIP1 interacting proteins were separated by SDS-PAGE and analyzed by western blots. Western blot analysis indicates that only the GST-IIP1 lane shows a strong IBR5-Myc, but not in control lane. (Figure 8). A pull down with GST also shows that IIP1, not GST tag, specifically interacts with IBR5-Myc. Thus, *In-vitro* pull down assay shows that IIP1 interacts with IBR5-Myc.

IIP1 in Gateway Entry Vector

To check whether IIP1 and IBR5 are interacting *in-vivo*, a gene cassette containing 35S::IIP1-GFP was constructed. . This recombinant gene was expressed in both Col-0 (wild type) and *35S::IBR5-Myc* genetic backgrounds. To identify transgenic plants expressing IIP1-GFP, proteins were extracted from individual plants and run on an SDS-PAGE. After being transferred to a PVDF

membrane, anti-GFP antibody was used to detect IIP1-GFP expressing plants.

Interaction of IIP1 and IBR5 *In vivo.*

The *in-vitro* pull down assay indicates that GST-IIP1 interacts with plant derived IBR5-Myc , but it is important to see if the same interaction occurs *in-vivo*.. To test in-vivo interaction, a co-immunoprecipitation assay was carried out and the immunocomplex were separated on an SDS-PAGE.Proteins were transferred on to a PVDF membrane. Membrane was immunoblotted with anti-GFP antibody. A similarly run another blot was immunoblotted with anti-Myc antibody (Figure 9). The western blot with anti-GFP indicates that IIP1-GFP specifically interacts with IBR5-Myc *in-vivo.*

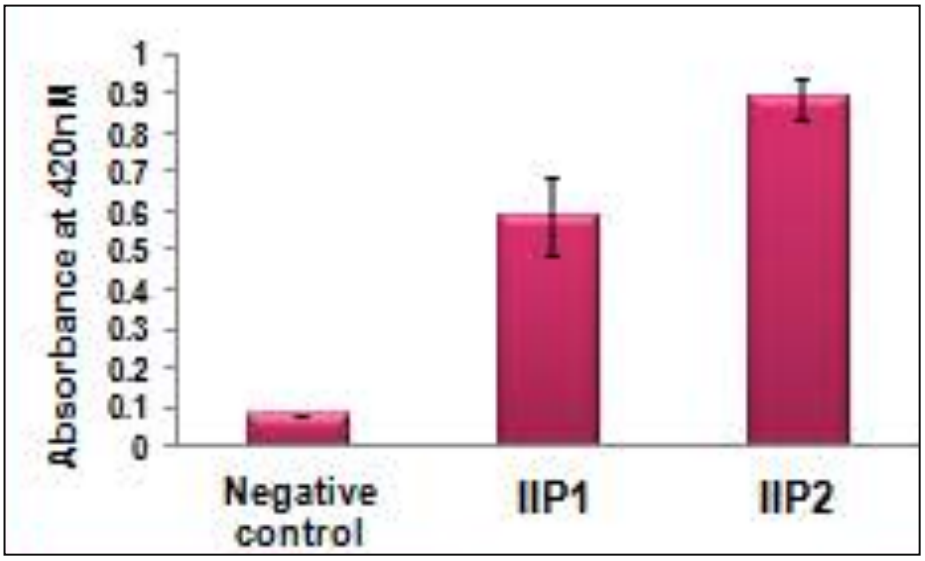
Localization of IIP1-GFP into Arabidopsis

The presence of IIP1-GFP can be detected not only by the use of antibodies and western blotting, but also visually by confocal microscope. This allows for more information on where the protein is located within a cell. Therefore, sub-cellular localization of IIP1-GFP was observed using confocal microscopy (Figure 10.). IBR5-GFP can be seen localizing in the nucleus which is identified by DAPI stain (results from Thilanka Jayaweera). IIP1-GFP can also be seen localizing to the nucleus. This adds to the evidence that there is opportunity for physical interaction for these two proteins *in-vivo*.

Selection of Arabidopsis homozygous mutant of *iip1*

To characterize the genetic interactions between IIP1 and IBR5 mutant lines with defective IIP1 were needed. The Arabidopsis Biological Resource

Center provided mutant *iip1* seeds with IIP1 genes interrupted by large T-DNA insert. To confirm that this iip1 line has the T-DNA insert, seeds were grown on ATS for 10 days and then transferred into soil. After 2 weeks DNA was isolated and PCR analysis was done to check for homozygous mutant *iip1* (Figure 11.). There was no amplification of IIP1 when using gene specific primers in any of the mutant lines. Amplification of DNA using T-DNA specific LBO2-F primer and IIP1-R primer shows that only the mutant copies of the gene are present among those lines.



Yeast two Hybrid Assay

Figure 6. **IIP1 interacts with IBR5 in Yeast Two Hybrid Assay.** ONPG assay to confirm positive interactions. Yeast transformants were grown in selective liquid media. Cells were harvested, ruptured by freeze-thaw and incubated with ONPG. Absorbance was measured at 420nM using the spectrophotometer.

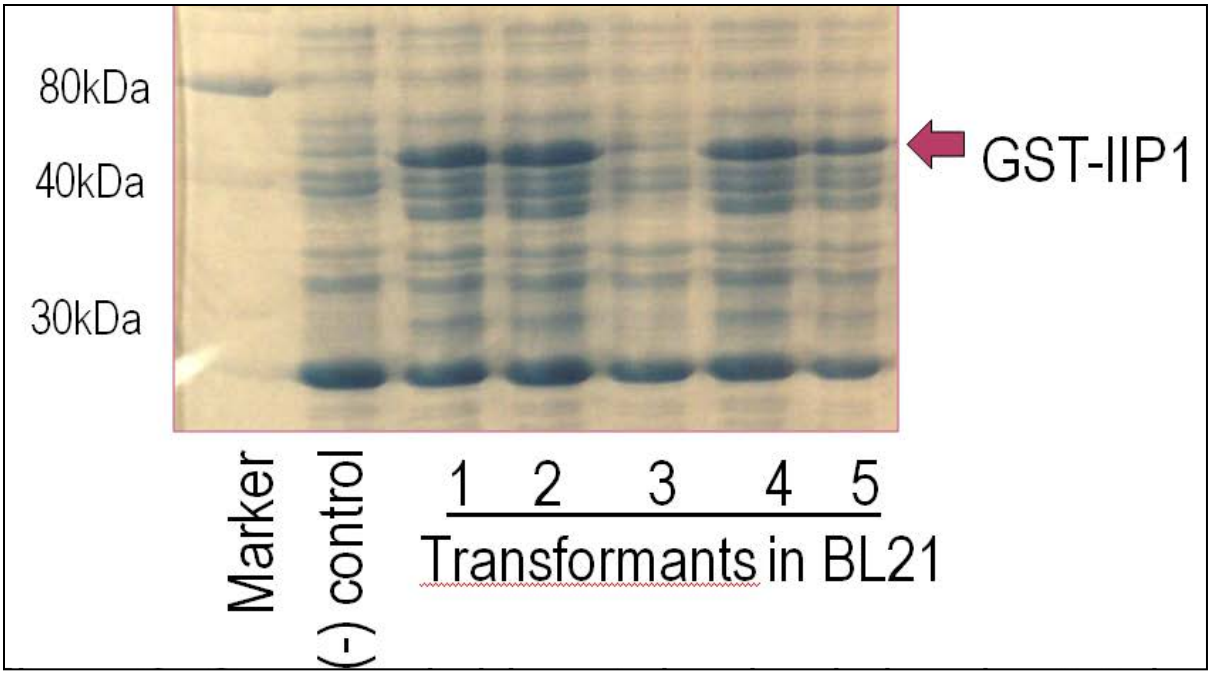


Figure 7. **Expression of IIP1 in *Escherichia coli* bacteria.** Coomassie blue stained gel showing total protein of GST-IIP1 transformants. The arrow indicates the expression of GST-IIP1 in BL21 cells. Transformant No1 was selected for further studies. GST-IIP1 was expressed as described above and purified using Glutathione-Agarose beads for pull-down assay.

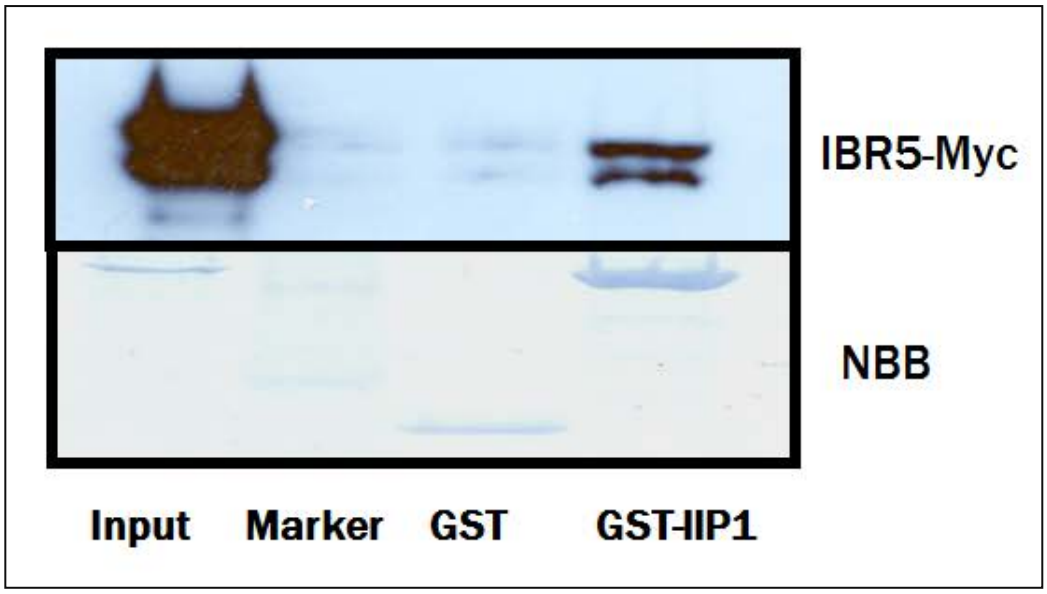


Figure 8. **Interaction of GST-IIP1 and IBR5-Myc in Pull-Down Assay**. Pulled-down proteins were separated on SDS-PAGE gel and transferred onto a PVDF membrane. Western blotting was carried out to identify IBR5-Myc protein using α-Myc antibody. Plant derived IBR5-Myc protein specifically interacts with GST-IIP1 protein in *in-vitro* pull down assay.

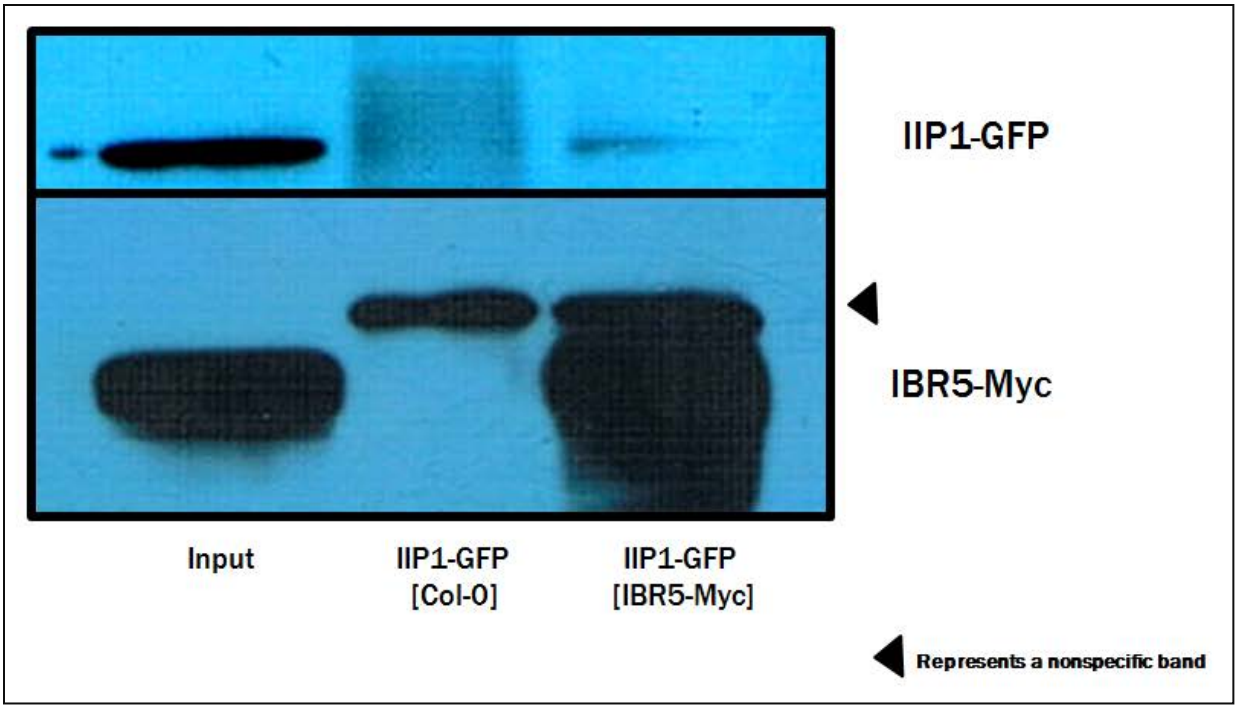


Figure 9. **IBR5-Myc Interacts with IIP1-GFP in *In vivo* Co-Immunoprecipitation Assay.**

A co-immunoprecipitation assay was done and the products were run on an SDS-Page gel and transferred to a PVDF membrane. Two separate western blots were carried out to identify co-immunoprecipitation products from IIP1-GFP in Col and in IBR5-Myc using α-GFP and α-Myc respectively. Plant derived IBR5-Myc protein and IIP1-GST protein interact in *in-vivo* pull down assay.

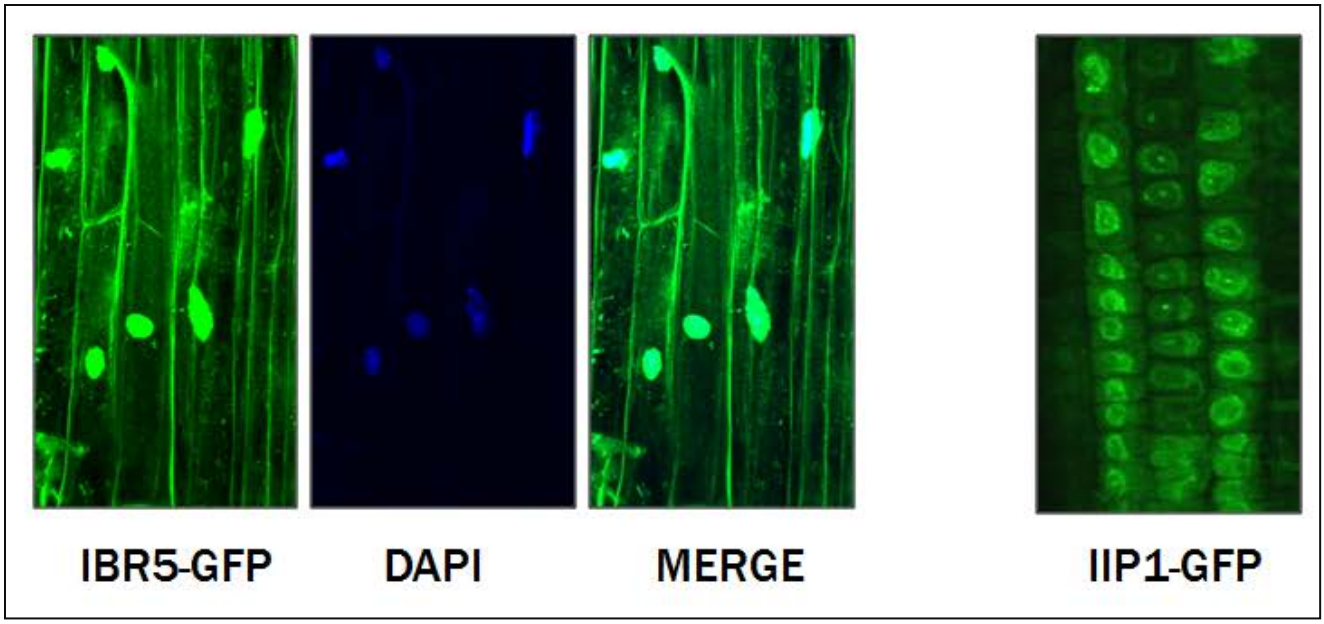


Figure 10. **Localization of IBR5-GFP and IIP1-GFP.** Seedlings expressing IBR5-GFP and IIP1-GFP under confocal microscope. IBR5-GFP shown next to DAPI stain of DNA. Both IBR5-GFP and IIP1-GFP are shown localizing to the nucleus (images were acquired by Thilanka Jayaweera).

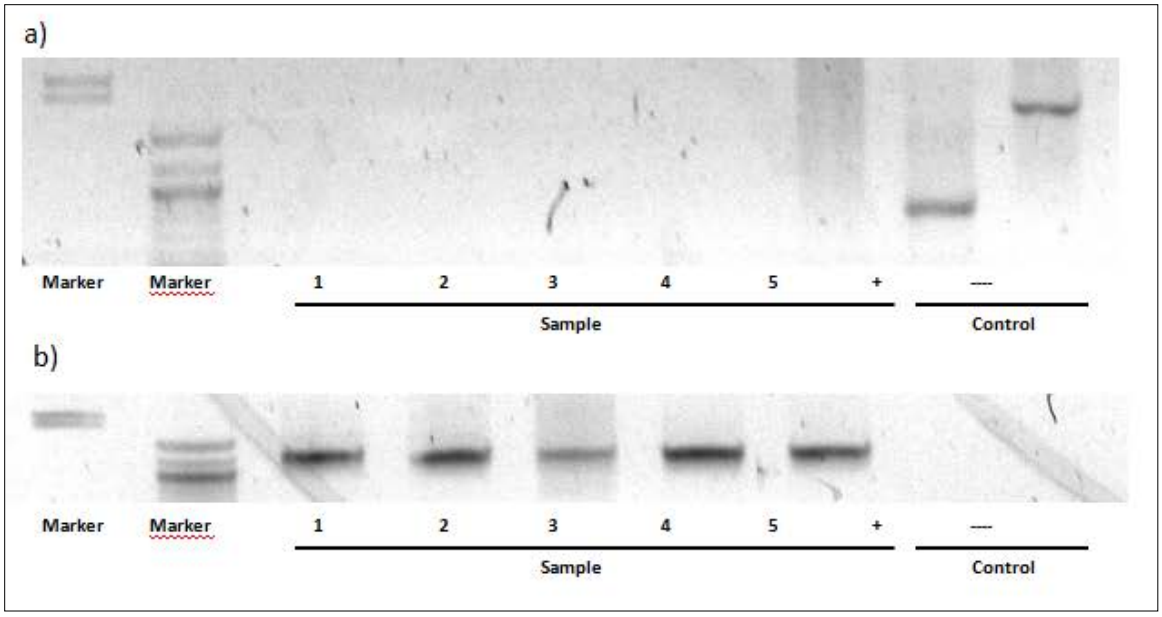


Figure 11. **PCR of Homozygous mutant *iip1* lines.** PCR was done using a) IIP1 gene specific primers and b) T-DNA insert specific primers to identify homozygous *IIP1* mutant lines.

Chapter 4

Conclusions and Discussion

One way to better understand the function of IBR5 is to understand the interactions it has with other proteins. To identify potential candidates yeast two hybrid assay was used in this study. This method is useful because it is a quick and easy way to screen the entire cDNA library of Arabidopsis (Miller, 2004). Though it provides good insight about protein interactions there are limitations in this technique. When proteins are made, they are not only influenced by the sequence of DNA transcribed to make them. There may be differences in folding or other post-translational modifications made to the protein in yeast cells that may be different from plant cells (Lee, 2004). The mechanism of two hybrid screen can also allow for false positives. In some cases, even though the GAL4 activation and GAL4 binding domains are separated the presence of homologs to these proteins may allow for transcription of the reporter gene. Positive results are sequenced so that the genes responsible for them may be compared to available research for further consideration. It is through this method that IIP1 was identified.

A yeast two hybrid assay does not prove the interaction between two proteins on its own. To check the interaction between IIP1 and IBR5 there needed to be a way to isolate both proteins. GST-IIP1 was expressed in *E. coli*

bacteria as a way to quickly produce lots of protein (Baneyx, 1999). As a GST tagged protein, IIP1 could easily be isolated by adding agarose beads with a glutathione ligand. The beads were much heavier than other proteins and could be separated by centrifuge. Large quantities of GST-IIP1 could be isolated and interactions could be seen more easily. When mixed with plant protein containing IBR5-Myc, GST-IIP1 showed that it was interacting *In vitro*. This method is more reliable than yeast two hybrid assay because specific proteins can be isolated and one of the proteins is made in a plant.

The same problem occurs in having bacterially made proteins as with yeast, with potential differences in protein similarity from being made in a different organism. Interactions from pull down assay do have more potential for being true to what is going on in the organism because they indicate that there is true physical association between the two proteins. The association still needs to be proven true within a plant system to prove that protein interactions are occurring naturally.

Expression of both IIP1 and IBR5 in plant cells together allows for the physical interaction of both proteins to be assessed in the way that is truest to what might be occurring inside plant cells. In combination with the yeast two hybrid and pull down assays, the co-immunoprecipitation is what ultimately proved that IIP1 and IBR5 are true interacting proteins in Arabidopsis (Thelen,2008).

Confirming interactions between proteins outside of the cell shows that there is a physical ability for the proteins to come together. Inside the cell, there

is still the possibility that they may not have the opportunity to interact. It is important to be able to identify where IIP1 and IBR5 localize to determine how likely it is that these interactions will be biologically significant. Using GFP as a marker for visualization allowed for determination of areas where both proteins are most likely interacting (Brandizzi, 2001). Both IIP1-GFP and IBR5-GFP localize to the nucleus. Even though, both proteins are being over expressed under the influence of the 35S promoter, the localization of each correlates with some of the known roles of each protein. IIP1 is part of the 26S proteasome complex that degrades proteins that repress transcription of DNA, which happens in the nucleus. IBR5 has also been shown to interact with MPK12 in the nucleus. With evidence that both proteins reside in the same parts of the cell, it is very likely that these two proteins interact *in-vivo*.

To understand the importance of the physical interactions between IIP1 and IBR5, more characterization of IIP1 must also be done. Identifying homozygous mutant *iip1*allows for root growth assays to be done on different concentrations of 2,4D. The mutant *iip1* lines can be compared to the wild type Col-0 that is sensitive to 2,4D and *ibr5* mutants, which show resistance to 2,4D. This would allow for phenotypic characterization of *iip1*in auxin signaling. Double mutants of iip1 and ibr5 may also be selected to show genetic interactions. Another possibility is to check the effect of *iip1* on proteasome function.

APPENDIX

Buffer Contents

2X CTAB Buffer

2%W/V CTAB-20

100mM Tris-Cl (pH 8.0)

10ML of 1M

1.4M NaCL- 28mL 5M NaCL

20mM EDTA (4mL of 500mM EDTA)

Extraction Buffer

1M HEPES (pH7.5)	0.5mL
0.5M KCL	2.0mL
50% Glycerine	2.0mL
Millipore H_20	3.9mL
10% Tween	0.1mL

Z Buffer

125nM Tris-HCl (pH 6.8)

12% SDS

10% glycerol

22% β mercaptoethanol

0.001% Bromophenol blue

Method Reference

Alkaline Mini Prep Method

1. Pellet bacteria from broth.
2. Resuspend in 200µL of 10mM Tris-Cl (pH 8) at room temperature. Gently mix: solution should clarify.
3. Add 250µL 3 MKOAc (pH5.5) and invert gently to mix. White precipitate occurs.
4. Put into ice for 5 minutes.
5. Centrifuge at 4°C for 10 minutes and 12,000 rpm.
6. Extract and save supernatant.
7. Add 0.3cµL of RNase A (100µg/µL).
8. Keep at 37°C for 15 minutes to digest RNA.
9. Add ≈600µL of chloroform and centrifuge 5 minutes.
10. Extract aqueous top layer and transfer to new tube.

11. Add 400µL of isopropanol to new tube.
12. Keep at room temperature for 5 minutes.
13. Centrifuge at 4°C for 10 minutes and remove/discard the supernatant.
14. Add 500µL of 70% ethanol at -20°C to the pellet. Centrifuge for 5 minutes at 4°C.
15. Remove call ethanol and dry pellet in vacufuge.
16. Resuspend pellet into 20µL of 2mM Tris-Cl (pH 8).

Bradford Method for Protein Quantification

Make standards by adding BSA, H_2O, and Bradford reagent according to table below and measure in photospectrometer. BSA- 500ng/µL.

	0	**5**	**10**	**15**	**20**
BSA	0 µLc	2.5 µL	5 µL	7.5 µL	10 µL
H_2O	50 µL	47.5 µL	45 µL	42.5 µL	40 µL
Bradford Reagent	500 µL	500 µL	500 µL	500 µL	500 µL

Isolation of Plant DNA

1. Grind leaf in 300µL of 2X CTAB buffer. Vortex 10 seconds.
2. Heat at 65°C for more than 10 minutes ≈30 minutes.
3. Add 300µL chloroform and vortex for 10 seconds.
4. Microfuge 5 minutes at room temperature.
5. Take supernatant (≈200µL) to a new tube.
6. Add (600µL) 3 volumes of 100% ethanol (-20°C).
7. Keep at -20°C for 20 minutes.
8. Microfuge 20 minutes at 4°C.
9. Wash pellet in 300µL 70% ethanol (-20°C). Centrifuge 5 minutes.
10. Air dry pellet.
11. Resuspend DNA in 50-100µL 10mM Tris Cl (pH8). Keep at 37°C for 30 minutes to 1 hour. Resuspend.
12. Use 1µL in 10-20µL PCR reactions.

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