Protein-Protein interaction studies of the epidermal and sub epidermal cell types enriched transcription factors in the shoot apex of *Arabidopsis thaliana*

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dual degree in Science

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Certificate of Examination

This is to certify that the dissertation titled **"Protein-Protein interaction studies of the epidermal and sub epidermal cell types enriched transcription factors in the shoot apex in *Arabidopsis thaliana"*** submitted by Ms.Reshma Murali (MS10086) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Mahak Sharma Dr. Rachna Chaba Dr. Ram Yadav

(Supervisor)

Dated: April 22, 2015

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Ram Yadav at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Reshma Murali

(Candidate)

Dated : April 22, 2015

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Ram Yadav

(Supervisor)

Acknowledgement

I take this opportunity to express my gratitude to all the people who have been enough to render their help and guidance during the course of my thesis. First of all, I would express gratitude to god to give me this opportunity to work at IISER MOHALI, one of the prestigious institutes of higher education in India for BS-MS studies.

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Reshma Murali

Contents

Abstract

The protein–protein interaction studies are important to understand the chemical machinery that makes up the living cell. My work focuses on building a comprehensive interactome for the transcription factors, which are enriched in the L1 and L2 layers using bimolecular fluorescence complementation assay (BiFC). BiFC assay provides an approach for the visualization of protein-protein interactions in living cells. To facilitate BiFC assay in plant leaf cells, we generated different complementary sets of expression constructs, which allow protein interaction studies in transiently transformed cells. Our studies revealed a remarkable signal fluorescence intensity of interacting protein complexes in the nuclei of pavement cells. This provides an *in vivo* validation of the already reported yeast two-hybrid data.

Chapter 1

Introduction

1.1 *Arabidopsis thaliana*

Arabidopsis thaliana is a small flowering plant (Fig. 1). It is having a relatively short life span of around 60 days and small genome of approximately 135 mega base pairs (Leutwiler *et al*., 1984). It was the first model plant organism whose genome was fully sequenced. For these reasons *Arabidopsis* is used as a popular organism to understand the molecular mechanisms related to development, cell-cell signalling and biotic & abiotic stress in plant species (Thomas Mitchell-Olds, 2001). Shoot apical meristem (SAM) of *Arabidopsis* is consists of a small set of stem cells and several hundred differentiating cells around them at the tip of the apex **(**Fletcher *et al*., 2002). This imposes a major limitation in isolating pure populations of stem cells for genomic studies. The delicate balance between the number of stem cells and differentiating cells in the SAM is maintained via negative feedback interactions between antagonistic factors (Clark *et al*., 1997). Currently, research in our lab focuses on building a comprehensive transcription factor gene regulatory network (GRN) for *Arabidopsis* SAM stem cell niche cell types using yeast–two-hybrid assay.

Figure. 1. Shows the model plant system *Arabidopsis thaliana*

1.2 Architecture of the Arabidopsis plant

Arabidopsis life cycle revolves around the two phase of growth. The initial phase of development and growth is called vegetative phase while the later phase of development coinciding with flower formation is called the reproductive phase. In the vegetative phase of development, plant produces the rosette leaves without internode elongation. The rosette leaves are green to slightly purplish in colour and 1.5–5 cm long, 2–10 mm broad in size with serrated margin on the edge. The leaves attached to stem are smaller in size and with no petiole. Leaves are covered with small, unicellular hair like structure called trichomes. (Thomas Mitchell-Olds, 2001)

In the reproductive phase of development the SAM becomes a dome like structure and produces flower buttress in the periphery. The floral buttresses turn in to floral meristem as development proceeds. Floral meristems are akin to SAM in their cell morphology and organizations, however, they terminate after forming the floral organs. Arabidopsis flower undergoes self-pollination and sets seeds on maturity and thus complete their life cycle within 60-days. The fruit of Arabidopsis is called siliques / pod, which is 5-20 mm long in size and contains 50-60 seeds. The flowers follows typical spiral phyllotaxy, which is a typical pattern followed by Brassicaceae family of plants (Thomas Mitchell-Olds, 2001).

1.3 Organization of SAM

The SAM of Arabidopsis is divided into three zones of different cytological appearance. The central zone lies at the tip of the meristem, where the cell divisions are rare. Central zone is surrounded by peripheral zone, where the cell divisions are rapid. Rib zone, which is beneath the central zone, where cell divisions are also fast. This kind of classification of SAM (Fig. 2) is called zonation (Elliot M. Meyerowitz *et al*., 1997).

In addition to zonation, the shoot apical meristem can be divided into clonally distinct cell layers. L1 layeris also called epidermal layer, L2, the subepidermal layer and L3 layer / corpus. L1 and L2 layers are single cell thick and cells in these two layers divide in an anticlinal orientation. Cells in the L3 layer divide anticlinally and periclinally. It forms stem

tissues, vasculature and the innermost cells of leaves and floral organs (Elliot M. Meyerowitz,1997).

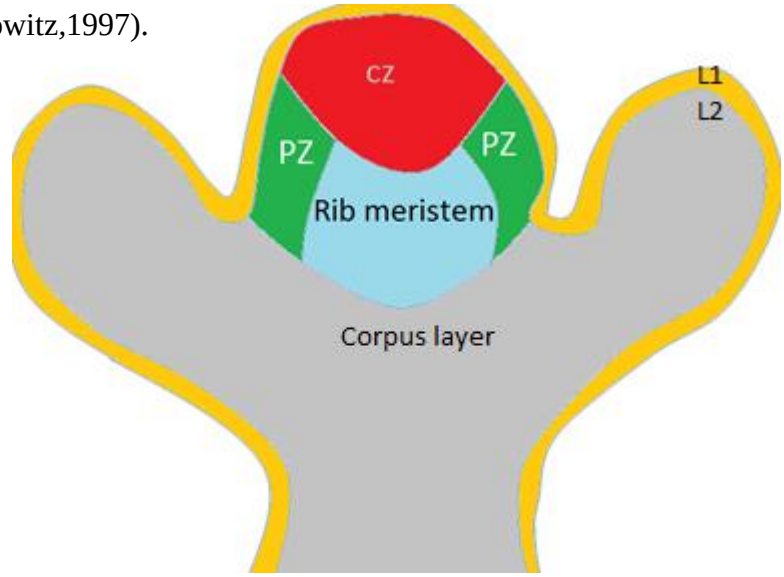

Figure. 2. Schematic representation SAM of *Arabidopsis thaliana.* The shoot apical meristem can be divided into clonally distinct cell layers. L1 layer is also called epidermal layer, L2, the subepidermal layer and L3 layer / corpus. SAM is divided into three different zones such as central zone, peripheral zone and rib meristem.

1.4 Bimolecular fluorescence complementation assay (BiFC)

The regulation of biological processes requires specific interactions of numerous proteins. Tightly regulated protein protein interaction networks mediate cellular responses to environmental cues and direct the implementation of developmental programs. Several methods have been developed in molecular biology to identify and visualize protein interactions and formation of protein complexes in living cells. Thse include Mass spectrometry, FRET, Co-immunoprecipitation (Co-IP) and yeast two-hybrid assay. Mass spectrometry is a technique which helps to identify the protein protein interactions based on the mass to charge ratio (Sparkman *et al*., 2000). The disadvantage with this technique is that we have to isolate all the proteins which we want to check the interactions, which is a tedious task. FRET is a technique which describes the energy transfer between two light sensitive molecules. The principle behind this technique is that a donor chromophore, in its electronic excited state may transfer energy to an acceptor chromophore, if the distance

between the two is very small (Chung *et al*., 2006). The disadvantage of this method is that it is extremely sensitive to small changes in distance. Co-IP is a molecular biology approach

to identify protein-protein interactions by using target protein specific antibodies to indirectly capture proteins that bound to specific partners (Phizicky E. M *et al*., 1995). The disadvantage with this method is the nature of the interaction, nonspecific binding to IP components and antibody contamination that may mask detection and the generation of antibodies for all the TFs present in shoot apex will be a tedious as well as expensive task. All these methods are not convenient enough to generate a transcription factor regulatory network for 65 TFs in the L1 and L2 layer. Among them, the yeast two-hybrid assay is a popular system to identify the protein-protein interactions (Walter *et al*., 2004). However, this system has intrinsic limitations as for example 'false-positive' and 'false-negative' interactions and, moreover, usually combines protein pairs in a heterologous environment (Joanna Boruc *et al*., 2010). For these reasons, the *in vivo* validation of yeast two hybrid data *in planta* is essential to identify the correct interacting partners.

Bimolecular fluorescence complementation assay is a molecular biology technique to visualize protein-protein interactions in living cells. It is also known as split YFP. The technique is based on the principle that N- and C-terminal sub fragments of YFP do not spontaneously reconstitute a functional fluorophore (Fig. 3). However, if it is fused to two interacting proteins, the two non-functional halves of the fluorophore brought into tight contact, refold together and generate de novo fluorescence (Tom K.Kerppola, 2004). Thus, by BiFC, the interaction status of the two proteins can be easily monitored via fluorescence emission upon excitation with a suitable wavelength.

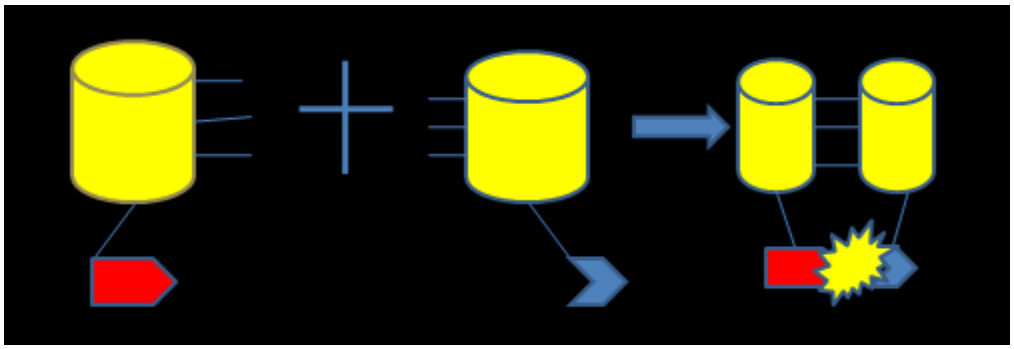

Figure. 3. Principle behind BiFC assay. BiFC is based on the principle that N- and C-terminal sub fragments of YFP do not spontaneously reconstitute a functional fluorophore. However, if it is fused to two interacting proteins, the two non-functional halves of the fluorophore brought into tight contact, refold together and generate de novo fluorescence.

BiFC analyses of interactions among the L1 and L2 layer of *Arabidopsis thaliana* illustrate several significant advantages of this technique. (Tom K. Kerppola *et al*., 2006)

- Protein interactions using BiFC are visualized in the normal environment of the plant living cells.
- BiFC is very sensitive and allows the detection of even weak interaction under the control of 35S promoter.
- The protein interaction happens in the genuine compartment of the proteins examined.

BiFC enables direct visualization of protein interactions in living cells with limited cell perturbation, rather than relying on secondary effects or staining by exogenous molecules that can fail to distribute evenly. The ability to observe the living cells for long periods of time, is made possible by the strong intrinsic fluorescence of the reconstituted reporter protein reduces the chances of an incorrect readout associated with the protein isolation process (Rainer Waadt *et al*., 2014). So this technique provided a suitable method for the *in-vivo* validation of the interactions found by yeast–two-hybrid.

1.5 Intrinsic disorder region and protein functions

Intrinsically disordered protein (IDP) is a protein which lacks an ordered three dimensional structure. Earlier there was a misconception that only the proteins which have an ordered 3-D structure can only do its function. But recently it was found that IDP can also function properly despite of its order. IDPs are frequently involved in key biological processes such as cell cycle control, transcriptional and translational regulation. The flexibility and structural instability of IDPs are encoded by their amino acid sequences. They can recognize other proteins, and can accelerate interactions and chemical reaction between bound pairs (Christopher J *et al*., 2014). IDPs lack an ordered 3-D structure, to attain a stable 3-D structure it interacts with ligands, macromolecules and proteins. This property of IDPs indirectly increase the number of binding sites in the IDPs to generate more interactions. Here we are exploring the utility of IDPs to find the property of interacting partners in the BiFC PPI network.

Chapter2

Materials and methods

2.1 Plant material and growth conditions

Niccotiana benthamiana is used in this study as a model system for BiFC assay experiments. Niccotiana plants were grown in small pots containing autoclaved soil (solarite and compost in 1:1 ratio) under 16h light and 8h dark conditions in a controlled plant growth chamber at 22^{o}C. MS solution is given twice in a week to provide nutrition to the growing plants. Three to four week old plants can be used for *Agarobacterium* infiltration. After the infiltration is done, the plants were kept at 22^{o}C inside the growth chamber for 3 days, and the infiltrated regions were harvested for screening.

2.2 Molecular biology work

High fidelity polymerase (Phusion) and restriction enzymes were obtained from New England Bio lab (USA). Plasmid isolation kit and standard molecular weight markers (1 Kb DNA ladder) were obtained from Thermo scientific (Fermentas). Bacterial LB media and LB agar components were obtained from BD scientific (USA). All the other chemicals and reagents used were of molecular biology/analytical grade and were obtained from Sigma-Aldrich (USA).

2.3 Primers

To confirm the BiFC expression constructs by colony-PCR, primers were designed using vector NTI software. Primers were obtained from Sigma Aldrich (USA).

Table 1. Primers used to confirm BiFC expression constructs by colony-PCR

Primer name	**Primer sequence**
SP/pE-SPYNE-GW/301-509	GCCATGCCCGAAGGCTACGTCCAGG
SP/pE-SPYCE-GW/81-290	CAACATCGAGGACGGCAGCGTGCAG
SP/AT1G52150.2/1301-2511	AGCTAATGGGTCTAAATCTTACATT
SP/AT3G50870.1/1-885	CACCATGATGCAGACTCCGTACACTACTT
ASP/AT1G24260/1-753	AATAGAGTTGGTGTCATAAGGTAAC

ASP/AT2G36400/1-1194	ATGAAAGGCTTGTGTCGAGACACCA
ASP/at3g23690/1-1113	TGGTTCAAGCTTCATCTGAAGCGTT
ASP/At1g07640.3/1-1017	CAAGAGATCATTAGAAGGAC
ASP/AT1G28470.1 / NAC010/1-942	TTGATTCTTCATGAAGCTTT
SP/At1g65910.1/456-1472	AGTATTTAAGAAGAGTGCTT
ASP/AT2G28810.1 //1-1020	CATAAGATGCTGGTGATGAT
SP/AT2G30250.1 / WRKY25/1-1179	CACCATGTCTTCCACTTCTTTCACCG
ASP/AT2G30250.1 / WRKY25/1-1179	CGAGCGACGTAGCGCGGTTG
SP/AT2G31730.1/1-459	CACCATGGAATACTCTAGAGACTC
ASP/AT2G31730.1/1-459	TTGAAAGGGCGAATTAGAAG
SP/AT2G37590.1/ DOF2.4/1-990	CACCATGGTTTTCTCCTCCATCCA
ASP/AT2G37590.1/ DOF2.4/1-990	ACGAGAAGAGCCGAAAGCAG
ASP/AT3G44750.1/HD2A/1-735	CTTGGCAGCAGCGTGCTTGG
SP/AT3G61630.1 / CRF6/1-945	CACCATGGAGAGACGAACGAGACG
ASP/AT3G61630.1 / CRF6/1-945	ATCGAAAGAGTGATGATGAT
ASP/AT4G01460.1 / bHLH/1-945	AGTTCGACTAAGATTTGACC
ASP/At4g17710.1 / HDG4/801-1700	TCTTGAAATGAACTACCCATG
ASP/AT4G28500.1 / NAC073/1-915	AGGGATAAAAGGTTGAGAGT
SP/AT5G52170.1 / HDG7/701-1700	AGTTAGCAGAATTGGAAACT
SP/AT5G61190.1 / C2H2/601-1500	TCAACTTCTAACGCTGGAGA
ASP/AT5G61190.1 / C2H2/601-1500	TGATTGATTCAGATTGGCAG
SP/pAT5G56270 / WRKY2/2201-3172	TGCCGACTAATTGAATTTCT
SP/AT4G16780/HB-2/1-852	CACCATGATGTTCGAGAAAGACGATC
ASP/AT4G16780/HB-2/1-852	GGACCTAGGACGAAGAGCGTCA
SP/AT1G26310/CAL1/1-765	CACCATGGGAAGGGGTAGGGTTGAA
ASP/AT1G26310/CAL1/1-765	AGCGGCGTAACAGCCAAGG
ASP/AT2G27050/EIL1/1-1752	GAACCATATTGATACATCTTG
ASP/AT3G16940/1-2535	AATATCTTCCAGGCAACCGTAC
SP/AT3G16940/601-1900	CCAACCGATCTTAATAACC
SP/AT4G04890/pdf2/652-2232	GGAAACTTTGGGAACCAG
SP/AT4G04890/pdf2/1261-2232	ATTCAAGAATTGCCTAATG
SP/AT4G16610/1-612	CACCATGCAGAACAAACACAAATG
ASP/AT4G16610/1-612	TATGGCTGAAACGACGGTG
SP/AT4G32980/ATH1/1-1419	CACCATGGACAACAACAACAAC
ASP/AT5G44210/ERF9/1-600	AACGTCCACCACCGGTGGA
ASP/AT1G54160/ NFYA5/1-924	AGTCCCTGACATGAGAGCTGAGGCA
ASP/AT1G12610.1/1-627	ATATCTGTAACTCCACAATG
SP/AT1G17920/745-1554	AACTATGAGAATATGTTTAC

2.4 LR reaction

cDNA clones for transcription factors were cloned in the vector pENTR/D/TOPO by Prince Saini. We got the destination vectors, pSPYNE-35S and pSPYNE-35S from Dr.Wolfgang's lab (University of Freiburg). To subclone the TFs from pENTR/D/TOPO, we have used gateway compatible method. The components for the LR reaction mix are given in the table below.

- pENTR/D/TOPO - TF - 1µl
- Destination vector - 0.5µl
- TE- Buffer - 0.5µl
- LR clonase - 0.3µl

The LR reaction mix was kept in thermostat at 25^{o}C for 1h. The LR reaction mix was then transformed into *DH5α* electro-competent cells and incubated at 37^{o}C for 1hr. After the incubation time, 70 µl was plated on ampicillin (100mg/ml) plates.

2.5 Antibiotic plates

Make 500ml LB-agar medium and autoclave it. After cooling down the medium, add 500µl ampicillin (100mg/ml) . Stir the medium for 4-5 min. Pour the medium into 90mm diameter petri plates and store at 4^{o}C in refrigerator. The components to prepare LB broth and LB agar are given below.

2.6 LB broth (500ml)

- Tryptone -5 gm
- NaCl -5 gm
- Yeast extract -2.5 gm
- Water - 500ml

2.7 LB agar (700ml)

- Tryptone -7g
- NaCl -3.5 gm
- Yeast extract -3.5 gm
- Agar -14 gm
- Water -700ml

2.8 Agarose gel electrophoresis

Weigh 0.8% agarose and add 1XTAE buffer. Heat the solution in a microwave oven until it dissolves completely. After cooling down the solution add 0.5µg/ml ethidium bromide and pour it into the gel casting tray with the combs. The components for making agarose gel are given below.

10X TAE buffer (1000ml)

- Tris base – 108g
- Glacial acetic acid – 57ml
- 0.5M EDTA (pH8.0) – 40ml

6X loading dye (10ml)

- Glycerol – 10%
- Bromophenol blue – 0.025g
- Xylene cyano – 0.025g

2.9 Preparation of Electro competent cells of *E.coli*

Streak *E.coli* DH5α cells from stock on a LB plate.

- Incubate the plate overnight at 37^{o}C.
- Inoculate single colony of DH5α in 5ml LB and incubate the culture overnight at 37^{o}C (primary culture)
- Inoculate the primary culture into 500ml LB media(secondary culture)

- Incubate the secondary culture at 30^{o}C till the OD_{600} reaches 0.4-0.6.
- Centrifuge the culture at 4000rpm for 20 min in a pre chilled centrifuge at 4^{o}C and discard the supernatant.
- Add 500ml chilled 10% glycerol (autoclaved) and dissolve the pellet.
- Centrifuge at 4000rpm 4^{o}C for 20 min and discard the supernatant.
- Add 125ml chilled 10% glycerol and dissolve the pellet.
- Centrifuge at 4000rpm 4^{o}C for 20 min and discard the supernatant.
- Add 50ml chilled 10% glycerol and dissolve the pellet on ice.
- Centrifuge at 4000rpm 4^{o}C for 20 min and discard the supernatant.
- Dissolve the pellet in 2ml pre chilled 10% glycerol.
- Aliquot 40µl in 1.5ml eppendorf tubes and store at-80^{o}C.

2.10 Electroporation

- Take an aliquote of *E.coli* electrocompetent cells and add 0.3-1µl of LR reaction mix to it.
- Mix it gently.
- Transfer this mixture into a clean chilled electroporation cuvette.
- Electroporate the cells and transfer this into 800µl LB.
- Incubate at 37^{o}C for 1 hr.
- Plate on LB ampicillin plate.
- Overnight incubation at 37^{o}C

2.11 Colony PCR

Add 9µl distilled water into PCR strips. Label the single colonies and dissolve them in water. Make PCR master mix, which is containing PCR buffer, dNTPs, $MgCl_2$, water, Taq-polymerase, primers. Add 5µl of master mix to each PCR tube.

Table 2. PCR components for the master mix preparation (8 colonies).

PCR buffer	13.5µl
dNTPs	10.8µl

Water	2.25µl
$MgCl_2$	4.05µl
Taq polymerase	1.8µl
Forward primer	6.75µl
Reverse primer	6.75µl.

Mix the samples and keep them in PCR thermocycler (BioRad).

Table 3. Conditions for colony-PCR

STEP	TEMPERATURE	TIME
Initial denaturation	94^{o}C	3min
29 cycles	92^{o}C	30s
Annealing	58^{o}C	30s
Extension	72^{o}C	1min
Final extension	72^{o}C	5min
Hold	4^{o}C	∞

2.12 Plasmid isolation

From the positive colonies, single colony of *E.coli* DH5α was inoculated in 5ml LB media containing 5µl ampicillin (100mg/ml).

- Pellet the overnight incubated 5ml culture in 2ml eppendorf tube at 13,500rpm for 1min in two steps.
- Dissolve the pellet in 250µl P1 buffer (Re-suspension buffer).
- Vortex the cells and add 250µl of P2 buffer (Lysis buffer) and mix gently.
- Add 350µl N3 buffer (Neutralization buffer) and mix gently.
- Centrifuge at 13,500 rpm for 10min.
- Transfer the supernatant to the spin column.
- Centrifuge at 13,500 rpm for 1 min and discard the liquid flow through

- Add 700µl wash buffer and centrifuge at 13,500 rpm for 1min.
- Dry spin at 13,500 rpm for 1min.
- Add 30µl sterile distilled water (65^{o}C) and keep for 1 min and centrifuge at 13,500rpm for 1 min.
- Add 20µl sterile distilled water (65^{o}C) and keep for 1 min and centrifuge at 13,500rpm for 1 min
- Collect the plasmid in a 1.5mleppendorf tube.
- Keep the plasmids in 4^{o}C refrigerator.

2.13 Restriction digestion

To check the integrity of the constructs, restriction digestion was done**.** The enzymes were identified using vector NTI software.

Table 4. Components for single and double digestion

Single digestion	Double digestion
Buffer- 1µl	Buffer - 1µl
Water - 5.7µl	Water - 5.7µl
DNA - 3µl	DNA - 3µl
BSA - 0.1µl	BSA - 0.1µl
Enzyme1 - 0.2µl	Enzyme 1 - 0.2µl
	Enzyme 2 - 0.2µl

Keep the mix in 37^{o}C water bath for 3 hours and load in 0.8% agarose gel and capture the gel picture**.**

2.14 *Agarobacterium* competent cells preparation

- Streak GV3101 *Agrobacterium* cells from stock on LB plate containing Rifampicin **(**100mg/ml**)**, Gentamycin **(**100mg/ml**)** antibiotics.
- Incubate 2 days at 30^{o}C and inoculate the primary culture in 5ml Rifampicin (100mg/ml) and Gentamycin (100mg/ml) LB broth.

- Add the primary culture into 500ml RT-LB medium and incubate at 30^{o}C till the OD_{600} reaches 0.6-0.8
- Centrifuge at 4000rpm for 20 min in a pre chilled centrifuge at 4^{o}C and discard the supernatant.
- Add 250ml chilled 10% glycerol (autoclaved) and dissolve the pellet on ice.
- Centrifuge at 4000rpm 4^{o}C for 20 min and discard the supernatant.
- Add 125ml chilled 10% glycerol and dissolve the pellet on ice.
- Centrifuge at 4000rpm 4^{o}C for 20 min and discard the supernatant.
- Add 50ml chilled 10% glycerol and dissolve the pellet on ice.
- Centrifuge at 4000rpm 4^{o}C for 20 min and discard the supernatant.
- Dissolve the pellet in 2ml pre chilled 10% glycerol.
- Aliquot 45ml in 1.5ml eppendorf tube and stored at -80^{o}C.

2.15 *Agrobacterium* transformation

- Take a vial of GV3101*Agrobacterium*electrocompetent cells and add 0.3-1µl of plasmid to it.
- Transfer this mixture into a clean chilled electro cuvette.
- Electroporate the cells and transfer this into 800µl LB.
- Incubate at 30^{o}C for 1 hr.
- Plate around 50µl of the culture on LB antibiotic plate and incubate the plate for 2 days at 30^{o}C.
- Colony-PCR was done with the single colonies.

2.16 *Agrobacterium* infiltration

A single positive colony of transformed *Agrobacterium tumefaciens* was inoculated in 5ml of LB-Medium containing Rifampicin/Gentamycin/Ampicillin at 30°C overnight.

- The same was done for *Agrobacterium* carrying p19 RNAi-suppressor protein from tomato bushy stunt virus.
- After 24hrs, each culture was spun at 3000rpm for 15 min.

- Bacteria pellets were then resuspended in *Agrobacterium* infiltration solution (10mMMgCl_2, 10 mM MES [5.6], 100µMacetosyringone) to an OD_{600} about 0.8-1.0.
- The resuspended bacteria (two potential interaction partners and p19 strain) were mixed 600 ml each, a 1:1:1 ratio, in a 2 ml eppendorftube and incubated for 1 hour at 30°C shaker.
- The bacterial solution was infiltrated into the entire leaf area through the abaxial sides using a 2-ml syringe (after removing the needle).
- After inoculation, plants were kept in a tray with a hood at 25°C and screened after 2 days under fluorescence microscope **(**ZEISS AXIO Imager.Z2 microsystems from Germany**)**.

2.17 Bacterial stocks

- Add equal volumes of 50% glycerol and the bacterial culture into a cryovial
- Store in – 80^{o}C

2.18 Fluoresence microscopy

Fluoresence microscopy (ZEISS AXIO Imager.Z2 microsystems from Germany) was used for screening the *Agrobacterium* infiltrated leaves with laser excitation at 488nm for GFP and 358nm for DAPI. Leaf samples (0.5cm^2) were first stained with DAPI (5µg/ml) for about 15 min and screened under ultraviolet light filter to spot the nucleus. Once the nuclues is spoted, the fluoresence was checked under GFP filter. All the images were taken with a software called ZEN.

Chapter3

Results

SAMs are dynamic in nature. They harbour stem cells at their tip that divide and differentiate continually in to various cell types. To understand the role of cell type specific TFs in cell and tissue specialization, we have build comprehensive protein-protein network using the Y2H assay. Often protein-protein interaction detected in yeast do not corroborate *in planta*. Therefore, we decided to use BiFC assay to validate the findings observed in Y2H.

3.1 Protein-protein interactome map using Y2H

I analysed the Y2H data generated by Prince in our lab. He generated a protein-protein interactome (PPI) map for the cell type specific TFs. Of the 950-combination setup in Y2H, he has found 210 interactions positive. The goal of this study was to assess the overall reproducibility of the Y2H network using an alternate approach *in vivo* in plant cells as well as to see whether Y2H alone is sufficient to probe such interactions. Usually, TFs involved in cell and tissue specialization are highly regulated and in many cases, they undergo post translation modifications. If these modifications were specific to that particular organism then in yeast cells such modification would not occur. We may miss such PPIs despite the natural interaction (in the plant cells) between the given pair of TFs. I have standardized the BiFC protocol in the lab and implemented it to assess the TF-TF interactome using *Nicotiana benthamiana*, a widely used model system for plant cell biology studies.

3.2 Confirmation of expression constructs in *Agrobacterium*

Once the expression constructs are confirmed using colony-PCR and sequencing, I transformed them into *Agrobacterium*. The *Agrobacterium* colonies were screened using colony-PCR and the product was resolved on 0.8%agarose (Fig. 1).

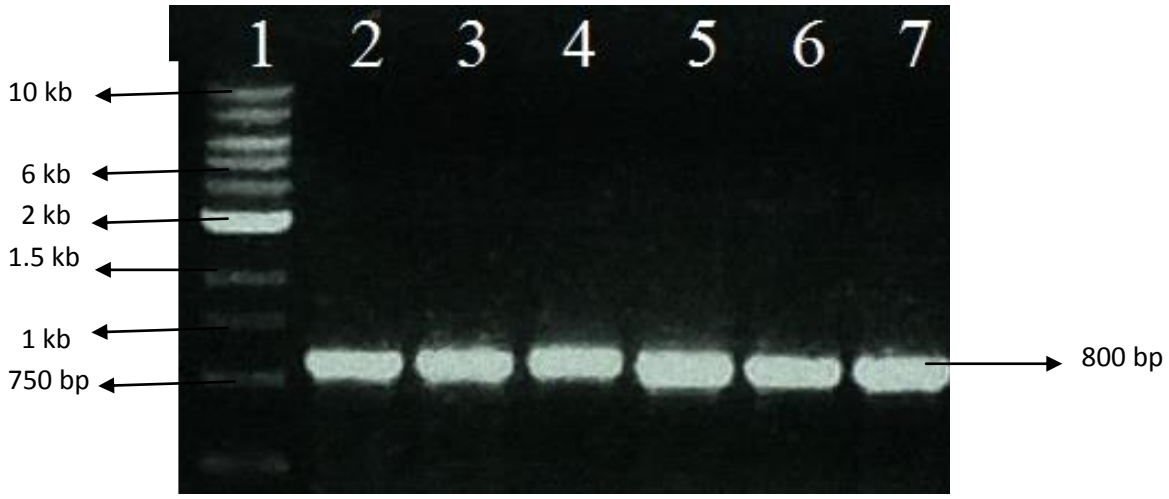

Figure. 1. Confirmation of BiFC constructs in *Agrobacterium.* Lane 1shows the 1kb molecular weight marker, Lane 2- 7 shows product of PCR done on the colonies of *Agrobacterium* transformed with AT2G31730-YFP^{c}.

3.3 BiFC is used to identify the interacting partners

The BiFC technique was used to investigate PPI *in planta*. *Agrobacterium* carrying BiFC constructs and p19 plasmids were pellet down and resuspended in *Agrobacterium* infiltration solution (10mM$MgCl_2$, 10 mM MES [5.6], 100µMacetosyringone) to an OD_{600} about 0.8-1.0.

The resuspended bacteria were mixed in a 1:1:1 ratio, in a 2 ml eppendorf tube and incubated for 1 hour at 30°C shaker. The bacterial solution was infiltrated into the entire leaf area of *Niccotiana benthemiana* through the abaxial sides using a 2-ml syringe. Reconstitution of YFP fluorescence was examined 2-3 days after infiltration by fluorescence microscopy. Adaxial surface of the infiltrated leaves were imaged. The interaction between the given pairs of transcription factors was identified by the presence of yellow fluorescence in the nucleus. DAPI staining was done to localize the nuclei (Fig. 2). As a negative control to this approach, *Agrobacterium* carrying empty vectors- pSPYNE-35S, empty pSPYCE-

35S, and p19 plasmids were infiltrated in 1:1:1 ratio and injected into the abaxial surface of *Niccotiana* leaves.

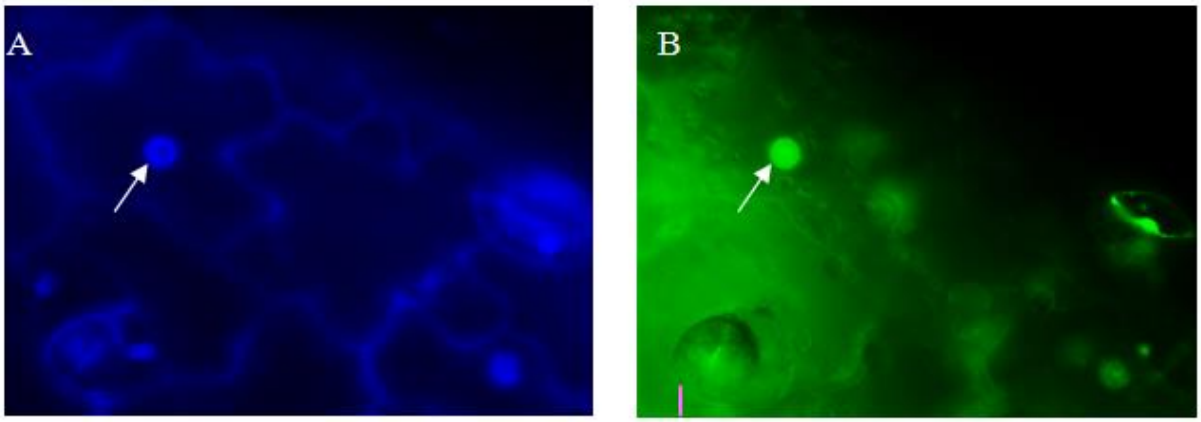

Figure. 2. BiFC images: Epifluoresent images of *Niccotiana benthamiana* leaf pavement cells infiltrated with agrobacterium carrying YFP^N-bHLH, YFP^C-ANAC073 and p19 plasmid. Leaf tissue stained with DAPI and arrow shows the nucleus (A). GFP image of the same tissue and arrow shows GFP expression in the nucleus (B).

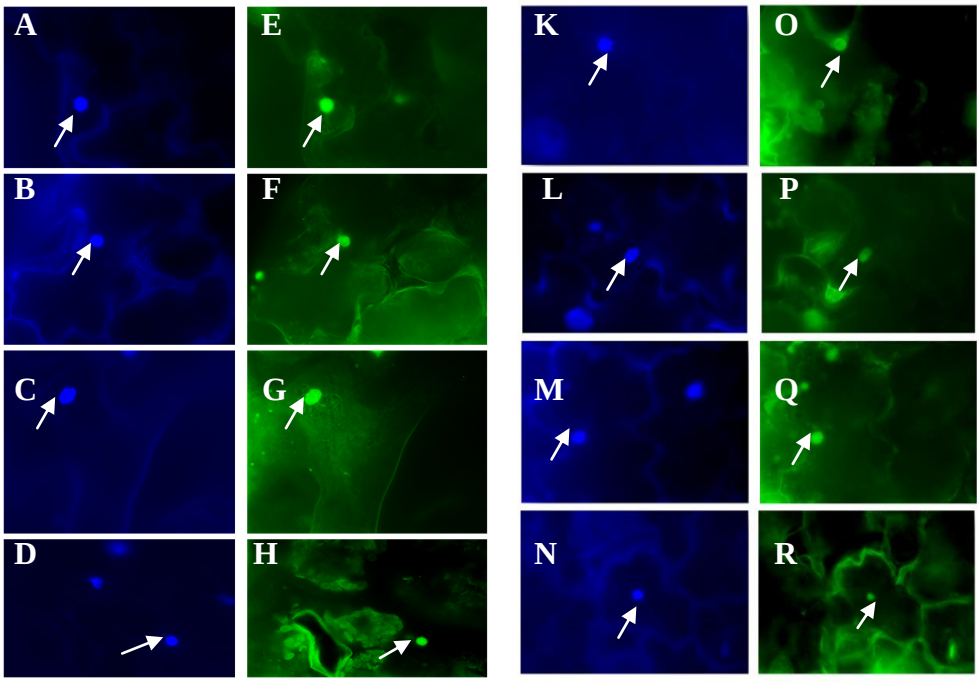

Figure. 3. BiFC images of some of the interacting partners. Transient expression of BiFC expression constructs in the leaf pavement cells of *Niccotiana Benthemiana*. Panel (A-D) and panel (K-N) represents the DAPI stained tissue and arrow indicates the nucleus. Pannel (E-H) and (O-R) are the corresponding GFP image of the same leaf tissue and arrow shows the nucleus.

3.4 Non interacting partners

Yellow fluorescence in the nucleus of leaf pavement cells were not detected in case of non interacting transcription factors. Indicating that the probability of false positives in our experimental setup is low. (Fig. 4) below shows the non interacting transcription factors.

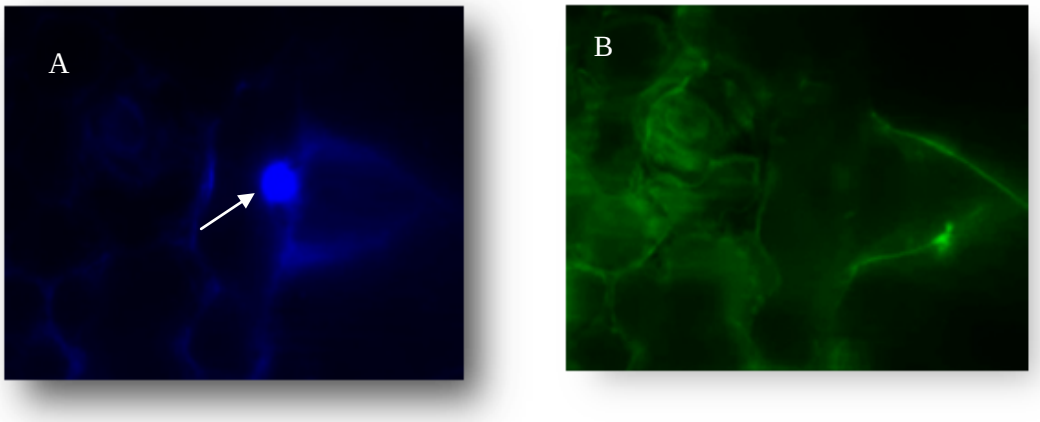

Figure. 4. BiFC images: Epifluoresent images of *Niccotiana benthamiana* leaf pavement cells infiltrated with agrobacterium carrying YFP^{C}-NF-YA5, YFP^{N}-AP2 domain transcription factors and p19 plasmid. The leaf tissue stained with DAPI and arrow shows the nucleus (A). GFP image of the same tissue and there is no GFP signal in the nucleus (B).

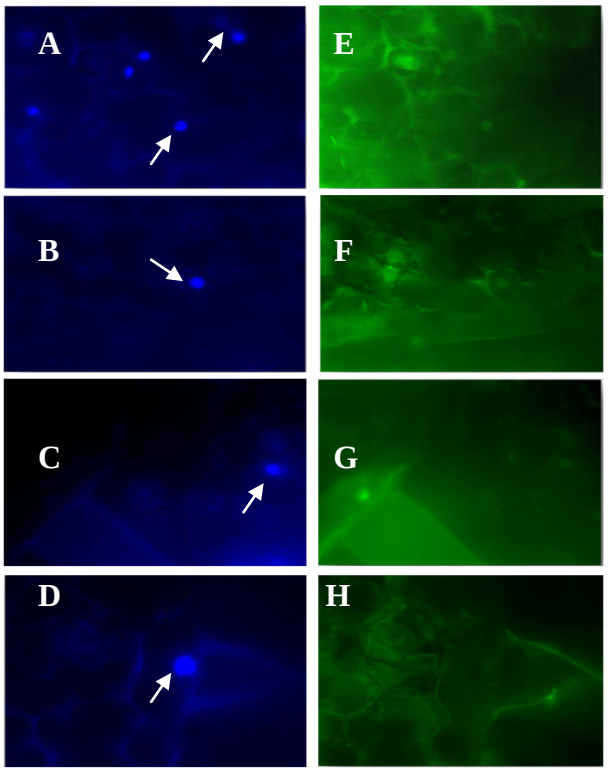

Figure. 5. BiFC images of some of the non interacting partners. Transient expression of BiFC expression constructs in the leaf pavement cells of *Niccotiana Benthemiana*. Panel (A-D) represents the DAPI stained tissue and arrow indicates the nucleus. Pannel (E-H) are the corresponding GFP image of the same leaf tissue.

3.5 Visualization of BiFC PPI network

The results from the BiFC PPI screens were integrated into an interactome network map consisting of 18 nodes representing the individual TFs, were interconnected by 45 edges, each representing one interacting pair (Fig. 6). During the present investigation of protein protein interaction studies, I checked the interaction of 65 combinations of transcription factors. Among them I got 45 interactions, that is 69% of the total interactions.

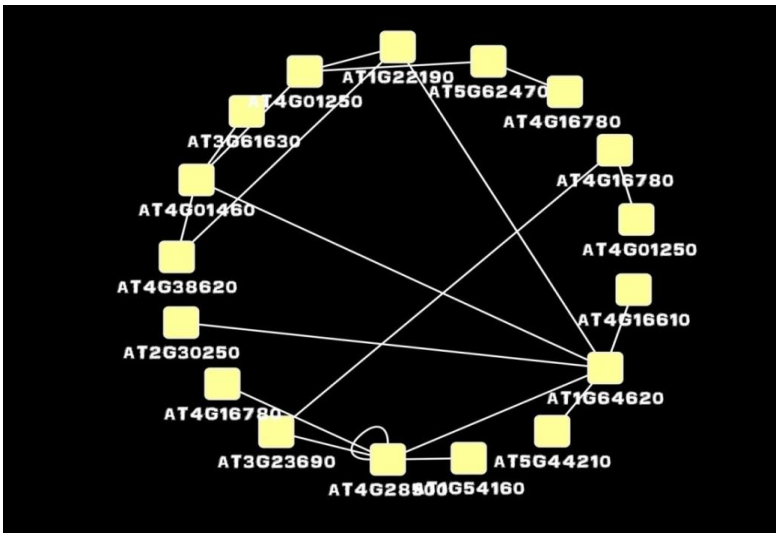

Figure. 6. BiFC interactome of transcription factors in the L1 and L2 layer of shoot apex, generated using cytoscape software.

From the PPI network of BiFC, we identified the TFs which have maximum and minimum number of interacting partners. These TFs were further analyzed to know the property which causes this diversity in protein protein interactions using bioinformatics tool iUPred.

Table 1. The list of interacting and non interacting partners

Interacting partners	Non interacting partners
AT4G28500-AT4G28500	AT3G61630-AT3G61630
AT4G01460-AT4G01460	AT4G28500-AT4G16780
AT4G01460-AT3G61630	AT4G28500-AT4G16610
AT2G30250- AT4G01460	AT4G01460-AT1G69120
AT2G30250-AT4G16780	AT3G23690-AT4G28500
AT4G16610- AT4G01460	AT4G38620-AT4G16780
AT4G16610-AT3G23690	AT1G54160-AT4G16610
AT5G44210- AT3G61630	AT1G12610- AT4G16780
AT5G44210- AT4G16780	AT1G64620- AT4G16780
AT5G62470- AT4G01460	AT1G64620- AT4G38620
AT5G62470- AT4G16780	
AT5G62470-AT1G12610	
AT1G22190- AT4G01460	
AT1G22190-AT4G01250	
AT1G22190-AT3G23690	
AT4G38620-AT4G01460	
AT4G38620- AT4G01250	
AT4G01250- AT4G01460	
AT4G01250- AT4G16780	
AT4G01250-AT5G62470	
AT4G01250-AT1G22190	
AT4G38620- AT3G23690	
AT4G38620- AT4G16780	
AT1G54160-AT4G28500	
AT1G54160-AT2G30250	
AT1G54160-AT4G16780	
AT4G38620- AT4G38620	
AT4G38620- AT4G01460	
AT1G64620- AT4G01460	
AT1G64620-AT4G28500	
AT1G64620-AT4G16610	

3.6 Constructing a binary TFs regulatory network

To generate a binary TFs regulatory network in the shoot apex of *Arabidopsis*, transcription factors were tested for a selected number of interactions by applying independently the BiFC and Y2H assays. Among these TFs, L1 specific TFs are 44 and L2 specific TFs are 21. The BiFC constructs were created with Gateway compatible method which combined the ORFs of TFs and YFP fragments downstream of the 35S promoter. Each TFs were C-terminally tagged with either N-YFP or C terminal part of the YFP. The constructs were transiently co expressed in the leaves of *Niccotiana benthamiana* by *Agrobacterium* mediated infiltration. Of the 65 pair wise interactions checked, 45 interacting pairs were identified.

Y2H assay was performed by a phD student, Prince Saini in our lab. In total 65 interactions tested by the BiFC, only 10% were covered by Y2H analysis. The overlap between the two binary PPI assays was rather small, illustrating that the techniques are highly complementary in detecting pair wise protein–protein interactions. From a total of 45 interactions, 6 (13%) were detected by both PPI methods. The number of interacting pairs detected exclusively in the BiFC and the Y2H was 10 (22%) and 3(6%) respectively.

3.7 Intrinsic disorder region and binding sites of TFs.

From BiFC PPI network, we found that only some TFs are interacting with so many other TFs but others have relatively less number of interacting partners. To find out the cause, we used bioinformatics tool called iUPred. In our studies two transcription factors, named bHLH and WRKY22 showed the maximum number of interactions, and they have more intrinsic disorder region and corresponding each intrinsic disordered region there is a binding site (Fig. 7), whereas another transcription factor NF-YA5 showed the minimum number of interactions and less number of intrinsic disorder region and the binding sites in the transcription factors (Fig. 8) Hence, we found a strong correlation between the number of intrinsic disorder region and the binding sites in the transcription factors.

Table 2. List of number of positive intrinsic regions and the number of binding sites in various transcription factors.

Name of TF	**No. of intrinsic disorder region in the TF**	**No. of binding sites in the TF**
WRKY22	7	7
CIL2	5	5
NF-YA5	1	1
CRF6	5	5
ANAC073	7	7
ERF9	5	5
WRKY25	4	4
MYB96	7	7
AP2	3	3
DOF1	6	6

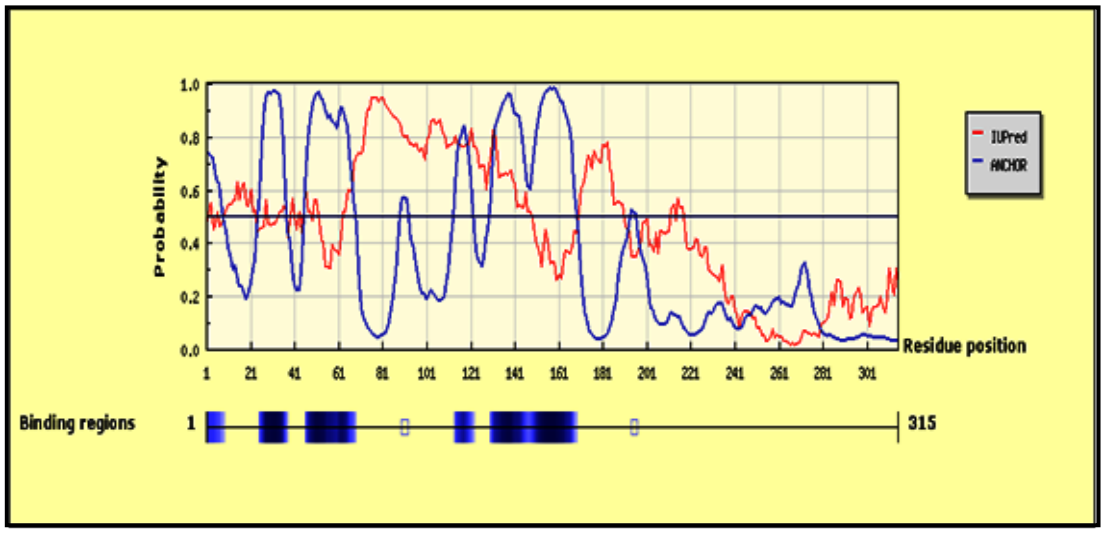

Fig. 5. Bioinformatic analysis shows the intrinsic disorder region of AT4G01460 and the corresponding binding sites. Blue graph shows intrinsic disorder region in the binding site of the transcription factor and red graph shows the total intrinsic disorder region in the transcription factor. The intrinsic disorder region in the binding site of the transcription factor (A). The binding sites corresponding to each positive intrinsic region in the transcription factor (B).

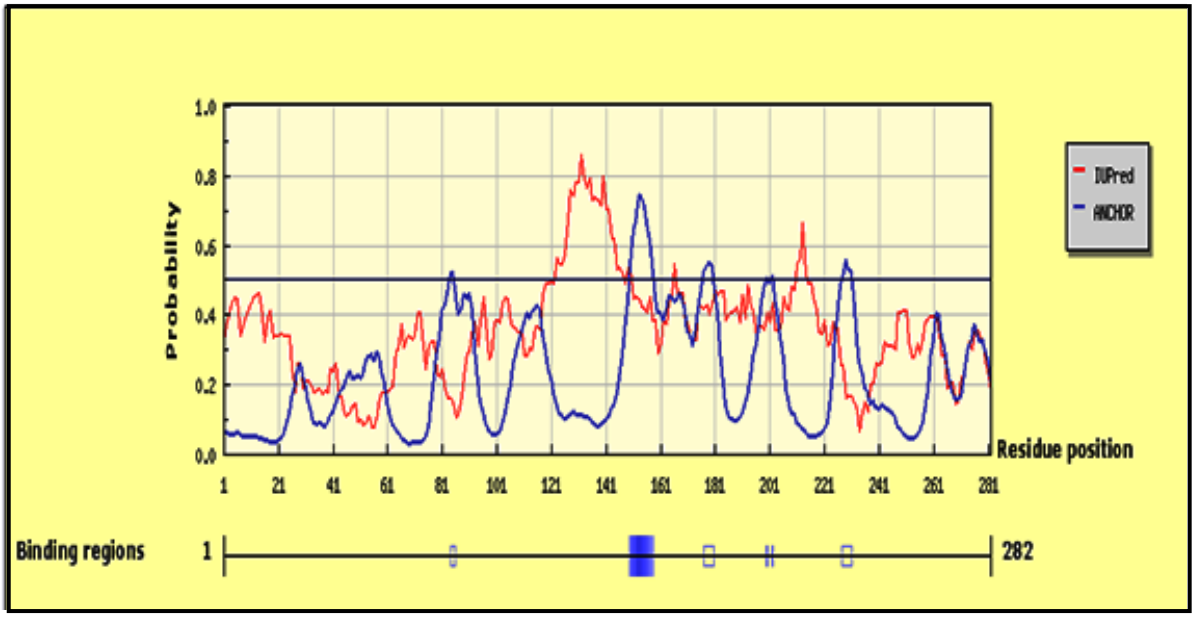

Fig. 6. Bioinformatic analysis shows the intrinsic disorder region of AT4G38620 and the corresponding binding sites. Blue graph shows intrinsic disorder region in the binding site of the transcription factor and red graph shows the total intrinsic disorder region in the transcription factor. The intrinsic disorder region in the binding site of the transcription factor (A). The binding sites corresponding to each positive intrinsic region in the transcription factor (B).

3.8 Functions of crucial interacting partners

Our studies identified some crucial interacting transcription factors in the shoot apex of *Arabidopsis thaliana*. These transcription factors interact with most of the other transcription factors. A few examples are WRKY22, CIL2 and MYB4 transcription factors.

WRKY22 is involved in the intracellular signal transduction pathway, leaf senescence, respiratory burst involved in defence response. This particular transcription factor is expressed during four leaf senescence stage and expressed in the floral organs, embryo, root and shoot apex of the plant.

CIL2 is a basic helix-loop-helix (bHLH) DNA-binding superfamily protein. It is known to be involved in various functions like sequence-specific DNA binding transcription factor activity. MYB4 is involved in cellular modified amino acid biosynthetic process, negative regulation of sinapate ester biosynthetic process, response to UV-B, response to jasmonic acid synthesis. It is expressed during 4 leaf senescence stages and expressed in leaf lamina, cotyledon, guard cells, pollen and shoot apex of the plant.

All these TFs involved in the development and regulation of the plant. Thus our study of identifying crucial interacting partners and its function is essential.

Chapter 4

Discussion

SAM is essential for the development of higher plants, because it generates all the aerial part of the post embryonic plants. The transcription factors expressed differentially in the SAM are prerequisite for the development and growth of the plant. The interactions between them govern the cell fate specification and hence the formation of new organs. Therefore identification of these interactions would provide an insight into the mechanisms that govern the differentiation of the stem cells present in the SAM. A comprehensive network of these interactions will give an idea of the pathways that decide the formation of various organ types during the life cycle of the plant. Therefore, the study of these interactions is important to get a mechanistic view of the developmental aspects of the plant. There are several methods available in molecular biology to study the protein protein interactions, for example yeast two-hybrid assay, split-ubiquitin, FRET. However *in planta* screening methods are potentially more reliable and allows the proper protein modifications compared to a heterologous system. (Tom K. Kerppola *et al*., 2006). It also helps in finding more functionally relevant interacting partners.

In the course of the thesis, the utility of BiFC assay for protein-protein interaction studies in living plant cells was explored. For our experiments we used *Niccotiana benthamiana* plants as the model system. Using *Agrobacterium* based transient expression, we tested the interactions for 60 combinations of transcription factors in the L1 and L2 layer of Arabidopsis thaliana. To start with my BiFC work we made the constructs, around 10 pSPYNE-35S and 16 pSPYCE-35S using gateway cloning method. We confirmed that p19 plasmid is essential for the *Agrobacterium* based transient expression *in planta*, because it acts as a suppressor for the gene silencing *in planta.*

This work focused on the *in-vivo* validation of interactions found using Yeast two hybrid assay and we generated a comprehensive PPI network by using the BiFC assay to validate

the yeast two hybrid data which was generated by a PhD student in the lab. The outcome of the analysis resulted in 45 interactions of which 13 were previously known in literature (Joseph R. Ecker, 2011). Thus, we validated 13 previously known interactions and reported 32 novel interactions which have not been reported so far. Many of these interactions might play important roles in the cell-fate specification and determination during the plant development.

Interestingly more interactions were found in the BiFC assay than with Y2H. The reason might be that BiFC provides an environment more close to the natural conditions. Also since yeast is a primitive eukaryote, it might be lacking the machinery required for the pre/post translational modifications of these proteins. It is a possibility that these modifications might be important for these interactions *in planta*.

We also tested the hypothesis that there is a strong correlation between intrinsic disorder region in the binding site of transcription factor and the number of binding sites in the transcription factors. Intrinsic disorder means the protein is not in its stable three-dimensional state. To attain its stable conformation, it has to interact with some ligand or macromolecules. We tested this hypothesis using bioinformatics tool called iUPred. In our studies two transcription factors, named bHLH and WRKY22 showed the maximum number of interactions, and they have more intrinsic disorder region and corresponding each intrinsic disordered region there is a binding site. whereas another transcription factor NF-YA5 showed the minimum number of interactions and less number of intrinsic disorder region and the binding sites in the transcription factors. Hence we found a strong correlation between the number of intrinsic disorder region and the binding sites in the transcription factors.

From our study, we can conclude that in combination with complementary techniques such as Y2H, the BiFC assay can be used as a standard approach for the visualization of protein-protein interactions. Further, our study has helped to identify 32 novel interacting partners. The proteins include important TFs like WRKY22, CIL2 etc. which are known to be involved in important functions like leaf senescence, signal transduction pathway and respiratory burst etc. But there role in SAM is not explored. Although further work is required to get a comprehensive picture of the roles of individual interactions, our study

throws important candidates which might have essential roles in maintenance and differentiation of stem cells in SAM.

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