# Mapping gene regulatory network of shoot enriched transcription factors in *Arabidopsis thaliana*

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# Certificate of Examination

This is to certify that the dissertation titled "**Mapping gene regulatory network of shoot enriched transcription factors in *Arabidopsis thaliana***" submitted by Ms. Medha Sharma (Reg. No. MS12039) 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. Kuljeet S. Sandhu Dr. Rhitoban R. Choudhury Dr. Ram Yadav
(Supervisor)

Dated: 21 April, 2017

# Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Ram K. 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.

Medha Sharma
(Candidate)
Date: April 21, 2017

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 K. Yadav
(Supervisor)

# Acknowledgement

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Medha Sharma

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# Notations

- 3-AT 3-Amino-1,2,3-triazole
- BSA Bovine serum albumin
- CTAB Cetyltrimethylammonium bromide
- EtBr Ethidium bromide
- GFP Green fluorescent protein
- LB Luria broth
- MS Murashige Skoog
- OD Optical density
- PCR Polymerase chain reaction
- PEG Poly ethylene glycol
- SAM Shoot apical meristem
- TAE Tris acetate EDTA
- TF Transcription factor
- YFP Yellow fluorescent protein

# Abstract

Cell and tissue specific expression of critical genes is a key hallmark of development in multicellular organisms. How spatially and temporally such gene's expression orchestrated is still not understood fully. Gene expression is driven by transcription factors (TFs). Often TFs involved in specification of cell fate are regulated tightly in a cell and tissue specific manner to exert their influence to few cells. In addition to transcriptional cascades signalling pathways also influences the decision of cell fate by post-translation modification of TFs.

The cell types of the Arabidopsis shoot apex are organized in to differentiating progenitors that surrounds the central zone stem cells, which when differentiates in periphery adopt the fate of organ primordia cell types, while underneath the stem cells they differentiates in to rib-meristem, and that then give rise the stem and vasculature cell types. On this, one can impose a layering organization, where epidermal and subepidermal cell types makes the L1 and L2 cell layer, which divides anticlinally and cover the apex with two consecutive layers of cells, while the L3 makes the inner bulk of the meristem. How do these diverse cell type are specified and how do they maintain their fate throughout the life cycle of plant is not understood yet. By identifying the TFs using gene expression studies and studying their regulatory relationship I am trying to establish a regulatory network that will help in understanding the role of TFs in shoot apex development. This study aims to generate a large-scale interaction network that uncovers biologically relevant gene-regulatory interactions for shoot enriched TFs. To unravel the regulatory interactions, a yeast one-hybrid assay has been used to find out transcription factor protein-DNA interactions.

In the present study I investigated the putative transcription factor binding sites for the shoot enriched TFs for which yeast one hybrid interactions data was made available. Promoter reporter studies were carried out to find out the functional role of these TFs along with gene expression studies.

# Chapter 1

# Introduction

## 1.1 Arabidopsis thaliana and the shoot apical meristem (SAM)

Arabidopsis is widely used as a plant model organism. Shoot apical meristem (SAM) resides at the tip of the plant and contains pluripotent stem cells. The descendants of shoot -leaf, flower and stem tissues are derived by differentiation of stem cells. SAM of higher plants is a highly organized structure in to various cell layers and zones.

In dicots, there are three cell layers; however their number is retained two in monocots. The cell lineages derived from epidermal or L1 cell layer in Arabidopsis include - pavement cells, guard cells and trichomes in the leaf. The sub-epidermal or L2 cell layer lead to formation of the photosynthetic tissue -mesophyll. Vascular bundles and stem tissues in mature plant are derived from innermost layer is called corpus or L3 cell layer (Stewart and Derman., 1970).

SAM is also organized in to various zones, based upon the cell division rate and cell behaviour. The uppermost tip of the plant meristem harbours stem cells and considered as central zone (CZ), where cells divide rarely. On division, when these cells enter in to the periphery they display higher division rate. Thus the cells in the peripheral zone (PZ) divide faster than their CZ counterparts and give rise to the organ primordia at the flanks of meristem. The cells displaced below the CZ also display relatively faster cell division rate and termed as rib meristem (RM), which give rise to the vascular bundles and stem tissues. (Satina,1940;Derman.,1953)

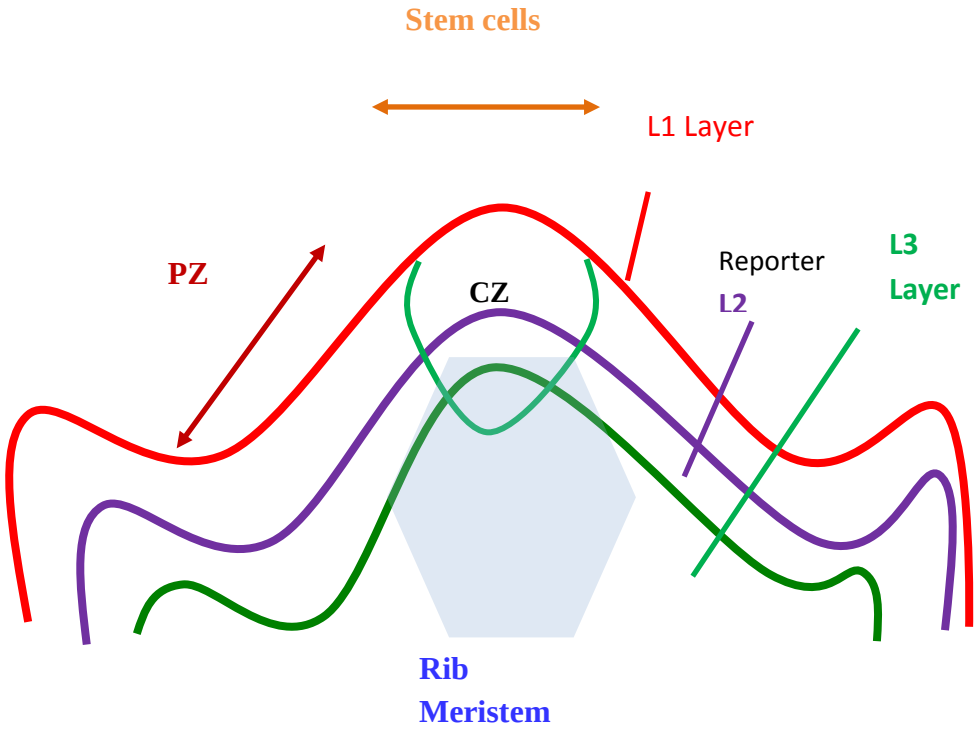



Fig. 1: Organisation of *Arabidopsis thaliana* SAM.

## 1.2 Gene regulatory networks and their significance

Regulation of gene expression plays role in cell differentiation and specific pathways involved in development of multicellular organisms. A gene regulatory network consists of all the molecular players (TFs, miRNAs, DNA, biological complexes etc.) that regulate the expression of genes at the level of transcript and protein. Such networks dissect out the relationship between these molecular players, that affect the gene expression. There have been studies to find regulatory networks for secondary cell wall synthesis in *Arabidopsis thaliana*, **(**M. Taylor-Teeples *et al,* 2015). In such a context such regulatory networks provide insights into integration of environmental stimuli and effect on gene expression. A recent study addresses how the transcriptional cascades involved in root apical meristem cell type specification are initiated by identifying the repressors and activators of *SHORTROOT –SCARECROW* transcription (Sparks *et al* 2016).

Likewise, an open question in the context of shoot apical meristem biology is to understand how distinct cell types in cell layers and zones acquire and maintain their cell identity. To address this question, a high throughput yeast-one-hybrid screen for the *cis*-regulatory elements in the promoter regions and shoot apical meristem enriched transcription factors (TFs) the has been done in the lab. These shoot apical meristem enriched TFs were identified using microarray data reported in a study (Yadav *et al.*, 2009).

In order to understand how differential gene expression is controlled at a genome-wide or systems level, it is important to identify all the target genes ,*trans*-acting factors involved, and the spatiotemporal interactions that take place, which as a result lead to cell type specification. There are many techniques by which one can probe the interaction between putative DNA binding sites and the TF-TF-centered and other is DNA-centred. TF-centred methods include techniques like -Chromatin immunoprecipitation (ChIP)-seq, DNA affinity purification (DAP)-seq etc. The sequencing reads are aligned with the genome to get an estimate of probable binding sites across the genome for a given TF. To implement ChIP one-need high quality antibodies against the TF those are specific and do not cross-react with other TFs. Genetic redundancy is very high in plant genome, generation of antibodies and their characterization for individual TF can take long time

and could be very expensive. Therefore, it is difficult to adopt a TF-centred approach for plant TFs.

## 1.3 Yeast-one-hybrid assay

In DNA centred approach one can clone DNA bait and test its interaction with the cognate TF using yeast one hybrid (Y1H) assay (Brent and Ptashne., 1985; Treizenberg *et al.,* 1988). Any studies have reported protein-DNA interaction (PDI) studies at genome scale using gateway based cloning tools (Deplancke *et al.*, 2004; Zhi Xie *et al.,* 2011; Vanessa Vermeirssen *et* al., 2007; H. Efsun Arda and Albertha J.M.Walhout., 2009). Gateway cloning technology is based on bacteriophage lambda site-specific recombination system. Integration of lambda in to *E.coli* chromosome and the switch between lytic and lysogenic pathways is the basic characteristic of the system. In Y1H assay, we look for an interaction between bait (a promoter) and preys (interacting TFs). The interaction readout is measured by the growth of yeast on selective media in the presence of a competitive inhibitor. Baits are created by integrating DNA fragments directly in to the yeast genome. Therefore they will be chromatinized in to yeast chromosome and will be presented to the trans-factors in native form. Therefore, it will help in achieving the regulated gene expression.

For preys, TFs were fused with activation domain (AD) of Gal4. If an interaction happens between a prey (TF) and bait (promoter DNA) the AD will lead to the activation of reporter gene present downstream of baits sequence. The yeast is plated on selective media where the expression of the reporter gene helps us to confirm the interaction. *Saccharomyces cerevisiae* (Yα1867, YM4271) has been used as model organism for this experiment.

My project aim was to identify the transcription factor binding sites that are being used *in-planta* in case of the yeast one hybrid network that had been made. Y1H assays are used to find out transcription factor-DNA interactions. However, neither the expression of a TF within a tissue nor the presence of a TF binding motif within the regulatory DNA is sufficient to predict in vivo TF-DNA regulation Shoot apical meristem of *Arabidopsis thaliana.* I have predicted putative TFBS in the 3kb bait region in case of yeast-one hybrid interactions. Further, I am trying to validate these putative TF binding sites using electrophoretic mobility shift assays.

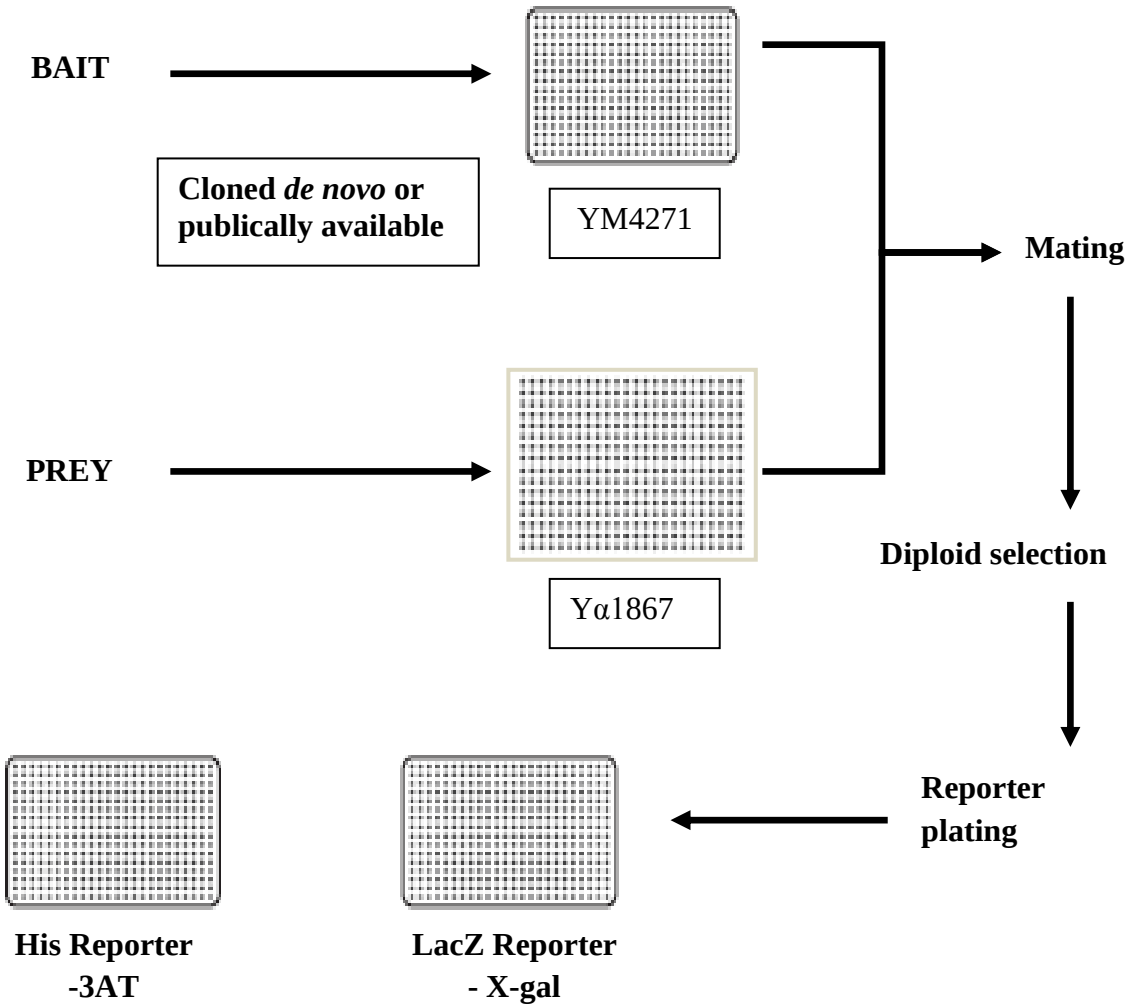



Fig2: Yeast-one-hybrid assay, experimental design.

In *Arabidopsis*, approximately 55% of genes can be assigned a putative function, however, less than 8% of these have been assigned a function by direct experimental evidence (MD Curtis *et al.,* 2003). Promoter-reporter constructs are frequently used to provide supporting evidence of the functional role of genes by identifying the likely spatial and temporal domains of the expression of a gene (Batni *et al.,* 1996; Curtis *et al.,* 1997)

Insertional mutagenesis is an alternative means of disrupting gene function and is based on the insertion of foreign DNA into the gene of interest. In Arabidopsis, this involves the use of either transposable elements (Parinov *et al.,* 1999) or T-DNA. The foreign DNA not only disrupts the expression of the gene into which it is inserted but also acts as a marker for subsequent identification of the mutation. Because *Arabidopsis* introns are small, and because there is very little intergenic material, the insertion of a piece of T-DNA on the order of 5 to 25 kb in length generally produces a dramatic disruption of gene function. Seeds of GABI-Kat and Salk population of T-DNA mutagenized *Arabidopsis thaliana* seed lines are good source. Different PCR methods have been developed that allow one to isolate plants that carry T-DNA mutation of interest (McKinney *et al.,* 1995; Krysan *et al.,* 1996).Hence, the yeast-one-hybrid network is also being validated by Ms. Shivani through q-RT PCR by looking for the expression level of downstream target gene in mutant and over-expression lines of these shoot enriched transcription factors.

# Chapter 2

# Experimental Methods

## 2.1 Plant material and growth conditions

The study was carried out on *Arabidopsis thaliana* Ler (ecotype). *Arabidopsis* plants were grown in small pots with autoclaved soil (solarite, perlite and compost in 2:1:1 ratio). It was grown in controlled environment plant chambers (Percival, USA and Conviron, Canada) with 75% humidity, 160-candella white light and $24^0$C temperature with 16-hour day and 8-hour night.

## 2.2 Chemicals used

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). *Taq* DNA polymerase was made in the lab. Whereas other enzymes were ordered from New England Biolab (USA) to perform gateway as well as restriction cloning experiments.All other chemicals and reagents used in the study were of molecular biology/analytical grade and were obtained from Sigma-Aldrich (USA).

## 2.3 Yeast one hybrid

Different combinations of baits and preys were allowed to mate and grown on YAPD complete media that allowed the growth of both diploids as well haploids. The growth was then transferred to HT dropout media in order to selectively pool out diploids. The colonies were further transferred to various concentrations of 3AT. 3AT (3-Amino-1,2,4-triazole) is a competitive inhibitor of HIS3 gene. HIS3 gene plays an important role in the seventh step of histidine metabolism in yeast. Hence, the growth pattern observed in

different concentrations of 3AT allows us to decipher the interactions. In the same manner for the β-Gal assay baits were grown on URA drop-out media and the preys on His drop-out media. Baits were mated with preys on YAPD complete media. The growth was then replica plated on UH drop-out media with increasing concentration of 3-AT and X-gal. If blue colonies appear on throughout the gradient of concentration of 3AT,thus we can interpret them as interactions. We used an empty vector without any prey as my negative control to verify auto activation. My colleagues have devised stringent criteria to filter Y1H data and generated an interaction network using the freely available software cytoscape. These network models provide a great tool for the visualization and navigation of large interaction data sets.

## 2.4 Other protocols and media/reagent composition

**Bacterial electro competent cell preparation**

I followed the protocol described in B.M *et al.,* 1998. The centrifuge oak ridge tubes, LB media and 10% glycerol were autoclaved at $121^0$C at 15psi for 15 minutes prior to the procedure.

- Inoculate primary culture in 5 ml LB media
- Incubate in shaker for 12 hours
- Set up the secondary culture
- Check the OD at 600nm till it reaches 0.400 to 0.600
- Transfer the culture to autoclaved centrifuge bottles
- Check and balance the weight
- Pre-cool the rotors and the centrifuge at $4^o$C
- Centrifuge at 4000 rpm for 20 minutes in $4^o$C
- Discard supernatant and dissolve pellet in 200 ml 10% glycerol, on ice
- Centrifuge again with the same conditions
- Repeat the steps for 4-5 times but reduce volume of 10% glycerol used each time
- Transfer the cells to oak ridge centrifuge tubes or a 50ml falcon tube and final dissolution of the pellet should to be done in around 2-3 ml 10% glycerol
- Aliquot 50µl cells into 1.5 ml tubes

**Bacterial ultra competent cell preparation**

- Inoculate a single colony in 4ml LB media for the primary culture
- Autoclave 50ml of SOB media and 10ml of 2M $MgCl_2$
- Get transformation buffer1,2 and 3 autoclave separately and pre-cooled
- Inoculate 1%culture (500 microliters) in 50 ml SOB and shake at 37˚C till OD reaches 05
- Keep the culture on ice for 10 minutes
- Put the culture in an autoclaved and pre-cooled bottle and spin in a pre cooled centrifuge at 3500 rpm, 4˚C for 10 minutes
- Discard the supernatant and dissolve the pellet in 25ml buffer1
- Keep on ice for 10 min.
- Spin at 3500 rpm,4˚C for 10 minutes
- Dissolve the pellet in 4ml of buffer 2 and then add 140µl DMSO
- Keep on ice for 15 minutes and again add 140µl of DMSO
- Aliquot into vials and store at -80˚C.
- For transformations, add equal volume of buffer 3 and use 50µl cells for each transformation

**Gel Purification**

- Cut the band under UV transilluminator
- Add 700µl QG buffer and keep at $50^oC$ shaker till the gel has completely melted
- Load in the gel purification column
- Centrifuge at 14,000 rpm for 1 minute
- Add 700µl wash buffer and centrifuge at 14,000 rpm for 1 minute
- Discard the liquid and give a dry spin
- Elute the DNA using 20µl hot distilled water

**Electroporation**

- Pre check the cuvettes to be used
- Add 0.1 to 1.0µl plasmid to 50µl competent cells
- Mix well
- Transfer the whole mixture to the cuvette using a pipette

- Electroporation according to the conditions required
- After the pulse has been provided, transfer the transformed cells to 700µl LB media
- Incubate the cells in 37$^{o}$C for 1 hour
- Plate the samples in respective selection media

**Preparing dNTP stocks**

- Add 50µl of 100mM GTP, ATP, CTP and TTP each into a 2.0 ml tube
- Add 1.8ml distilled sterile water and mix well
- Aliquot 50µl into 1.5µl tubes

**Colony PCR**

- Mark the suitable colonies on the plate
- Dissolve the colonies from the plate in 10µl autoclaved distilled water
- Make the master mix and aliquot into the PCR tubes
- Proceed with the PCR reaction
- Load the samples in electrophoresis gel and run it
- Do not forget to load the DNA ladder

Table 1: Master mix for colony PCR

**Table 1: Composition of PCR mixture and reactions conditions for colony PCR**

| **Components** | **Volume** |
|---|---|
| Sample (distilled water and the dissolved colony) | 10µl |
| 10X Standard Taq Reaction Buffer | 1.5µl |
| dNTPs (10mM) | 1.2µl |
| MgCl2 (50mM) | 0.45µl |
| Taq DNA polymerase | 0.5µl |
| 10µM forward primer | 0.75µl |
| 10µM reverse primer | 0.75µl |
| Total | 15µl |

| **Steps** | **Temperature** | **Time** |
|---|---|---|

| | | |
|---|---|---|
| Initial denaturation | 94$^{o}$C | 3 Minutes |
| Denaturation | 92$^{o}$C | 30 Minutes |
| Annealing | 58$^{o}$C | 30 Minutes |
| Extension | 72$^{o}$C | 1 Minutes |
| Final extension | 72$^{o}$C | 5 Minutes |
| Hold | 4$^{o}$C | ∞ |

**Table 2: Master mix for T-DNA lines genotyping PCR**

| **Component** | **Volume** |
|---|---|
| 10X PCR buffer | 2.5µl |
| dNTPs | 2µl |
| Forward primer | 1µl |
| Reverse primer | 1µl |
| Taq DNA polymerase | 1µl |
| MgCl2 (50mM) | 0.5µl |
| Distilled water | 15.5µl |

**Table 3: Conditions for plant genotyping PCR**

| **Steps** | **Temperature** | **Time** |
|---|---|---|
| Initial denaturation | 94$^{o}$C | 3 minutes |
| Denaturation | 92$^{o}$C | 20 minutes |
| Annealing | 58$^{o}$C | 30 sec |
| Extension | 72$^{o}$C | 1 minutes |
| Final extension | 72$^{o}$C | 10 minutes |
| Hold | 4$^{o}$C | ∞ |

**Agarose gel preparation**

- Weigh agarose according to 0.8% w/v calculations
- Add 1X TAE buffer ( 1 ml 50X + 49 ml distilled water)
- Heat to dissolve completely
- Add EtBr when cooled (0.5 µg/ ml)
- Pour into the gel tray with the combs and allow to solidify

**Plasmid Isolation**

- Inoculate the culture in 5 ml LB media
- Pellet down the cells in 2ml MCTs at 13,500rpm for 1 minute
- Discard the supernatant
- Dissolve the pellet in 250µl P1 buffer- re-suspension buffer
- Vortex and mix well
- Add 250 µl P2 buffer- lysis buffer
- Mix by gently inverting the tube
- Add 350 µl N3 buffer- neutralisation buffer
- Mix well by gently inverting the tube
- Centrifuge at 13,500 rpm for 10 minutes
- Transfer the liquid part to spin column
- Centrifuge for 13,500 rpm for 1 minute
- Add 750 µl wash buffer and centrifuge at 13,500 rpm for one minute
- Give dry spin at 13,500 rpm for one minute
- Add 30 µl hot distilled water to the column and spin down at 13,500 rpm for one minute to elute the plasmid in a fresh 1.5 ml microcentrifuge tube (MCT)
- Again add 20 µl hot distilled water to the column and spin down at 13,500 rpm for one minute

**Bacterial stocks**

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

**Agrobacterium Plant Transformations**

- Grow wild type plants to be transformed
- Put the primary culture in 5ml LB media
- Add the antibiotics for selection
- After 24 hrs set up the secondary culture
- Add the antibiotics suitably
- After 24 hrs transfer the culture to autoclaved centrifuge tubes and spin down at 4000 rpm for 20 minutes
- Discard the supernatant and dissolve the pellet in 5% sucrose solution
- Add 0.3% silwet to the dissolved culture
- Mix the culture well
- Dip the plants and wait for 4 to 5 seconds. Take the plants out of the culture and keep them horizontally in a tray overnight.
- Straighten the plants after 12 hrs and water them
- The same transformation is performed twice for more efficiency

**Genomic DNA isolation**

- Leaves were harvested and were frozen in liquid nitrogen
- Crush the leaves in a 1.5 ml tube using the pestle
- Add 600µl CTAB buffer and mix well
- Incubate at 65$^{o}$C for 20 minutes
- Centrifuge at 13,500 rpm for 5 minutes
- Add 600µl Phenol + Chloroform mixture and mix well
- Centrifuge at 13,000 rpm for 5 minutes
- Collect the upper layer of the supernatant and transfer to a fresh tube
- Add 600µl chloroform and shake the mixture for 2 minutes
- Centrifuge at 13,000 rpm for 5 minutes
- Transfer supernatant into a fresh tube
- Add 0.7 volumes chilled isopropanol and mix by inversion
- Spin down at 16,000 rcf for 5 minutes
- Remove supernatant and wash the pellet with 1 ml 70% ethanol
- Centrifuge at 16,000 rcf for 1 minute and remove the supernatant

- Dry completely by keeping at 90$^{o}$C for 1 minute
- Resuspend the pellet in 50µl distilled water
- Store in -20$^{o}$C
- Capture the gel picture and analyze

**Preparing soil for growing plants**

- Mix solarite, perlite and compost in 2:1:1 ratio
- Mix it finely
- Autoclave the mixture
- Fill the pots and put the seeds

## 2.5 Reagents and Chemicals used

**LB media**

| | |
|---|---|
| • Tryptone | 5gm |
| • NaCl | 2.5gm |
| • Yeast extract | 2.5gm |
| | --- |
| | 500ml |
| | --- |

**LB agar**

| | |
|---|---|
| • Tryptone | 7g |
| • NaCl | 3.5gm |
| • Yeast extract | 3.5gm |
| • Agar | 10.5gm |
| | --- |
| | 700ml |
| | --- |

**SOB media**

| | |
|---|---|
| • Tryptone | 1gm |

- Yeast extract -0.25gm
- NaCl 0.025gm

---

50 ml

---

**Transformation buffer1**

| **Stock** | **Final conc.** | **Volume from stock** |
|---|---|---|
| 1M MOPS (pH 6.5 with KOH) | 10mM | 1ml |
| 1M KCl | 100mM | 10ml |
| 1M $MnCl_2$ | 45mM | 4.5ml |
| 1M $CaCl_2$ | 10mM | 1ml |
| 1m KAc(pH 7.5 with HCl) | 10mM | 1ml |
| Sterile water | | 82.5ml |
| **Total** | | **100ml** |

**Transformation buffer 2**

Same as buffer 1, except that -12.5ml of 80% glycerol is added and then volume is made up to 100ml

**Transformation buffer 3**

- 100mM $CaCl_2$
- 50mMg $Cl_2$

Autoclave the mixture separately.

**Drop out media**

- Yeast nitrogen base 0.85gm
- Ammonium sulphate 2.5gm
- Drop out 1gm
- Glucose 14gm
- Agar 14 gm

---

500 ml

---

**TAE (50X)**

- Tris base 242 gm
- Glacial acetic acid 57.1 ml
- EDTA (ph:8, 0.5 M) 100 ml

---

1000 ml

---

**Ethidium Bromide**

- Dissolve 1 gm Ethidium Bromide powdered form in 100 ml sterile water
- Stir for several hours to ensure that the dye has completely dissolved
- Store in a dark bottle
- Add while preparing the gel in the concentration of 0.5 µg/ml

**CTAB buffer**

- 2% CTAB 3 gm
- 1.42 M NaCl 42.6 ml of 5M NaCl
- 20mM EDTA 6 ml of 0.5 M EDTA
- 100mM Tris (pH = 8.0) 15 ml of 1M Tris
- Adjust the volume with distilled water

**MS media for plants**

- Dissolve 2 gm of Murashige skoog media powder in 1 Lt distilled water
- Set the pH to 5.8 by adding 1M KOH

**Preparing MS agar plates**

- MS 4.32 gm (0.1%)
- MES 1.00 gm (1.0%)
- Sucrose 10.00 gm (0.8%)

- Set the pH to 5.80 before adding agar
- Agar 8.00 gm

---

1000 ml water

---

**3-AT concentrations used**

- 5 mM 0.210 gm in 500 ml
- 10 mM 0.420 gm in 500 ml
- 20 mM 0.840 gm in 500 ml
- 40 mM 1.680 gm in 500 ml
- 60 mM 3.360 gm in 500 ml

**Composition of Drop out**

- Adenine 0.5 gm
- Alanine 2.0 gm
- Arginine 2.0 gm
- Asparagine 2.0 gm
- Aspartic acid 2.0 gm
- Cysteine 2.0 gm
- Glutamine 2.0 gm
- Glutamic acid 2.0 gm
- Glycine 2.0 gm
- Histidine 2.0 gm
- Inositol 2.0 gm
- Isoleucine 2.0 gm
- Leucine 10 gm
- Lysine 2.0 gm
- Methionine 2.0 gm
- Para-amino benzoic acid 2.0 gm
- Phenyl alanine 2.0 gm
- Proline 2.0 gm

- Sereine 2.0 gm
- Threonine 2.0 gm
- Tryptophan 2.0 gm
- Tyrosine 2.0 gm
- Uracil 2.0 gm
- Valine 2.0 gm

**LR Reaction**

| | |
|---|---|
| Linearised Entry clone | 75-100ng |
| Destination vector | 150ng |
| TE | 0.5µl |
| LR Clonase | 0.5µl |
| Sterile distilled water | -to make up final volume |

---

3µl

---

**Entry Reaction**

| | |
|---|---|
| PCR product | 1µl |
| 10X salt solution | 0.5µl |
| pENT-D-TOPO | 0.5µl |
| Sterile distilled water | to make up final volume |

3 µl

**Restriction digestion reaction**

| | |
|---|---|
| Vector | 400ng (4µl) |
| 10X buffer | 1µl |

| | |
|---|---|
| Restriction enzyme 1 | 0.2µl |
| Restriction enzyme 2 | 0.2µl |
| Sterile distilled water | 4.6µl |

**Yeast shuttle prep**

- Take 1.5ml of culture and spin at 5000,5 min.
- Dissolve pellet in 400µl lysis buffer
- Add 400µl PCI (add the lower layer).Add glass beads till the lower aqueous meniscus.
- Lyse the cells in bead beater /vortex
- Add 400µl of water and vortex
- Spin 15 min, max speed at 4˚C
- Take supernatant, add 400µl isopropanol.
- Spin for 15 min, max speed at 4˚C
- Decant isopropanol, add 50µl of 70% ethanol
- Spin for 15min., max speed at room temp.
- Remove ethanol and dry the pellet thoroughly
- Dissolve in 20µl water

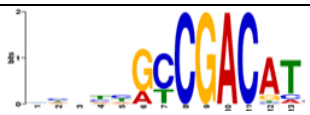
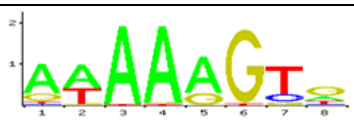
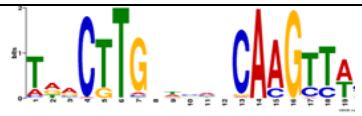
## 2.6 *In-silico* prediction of transcription factor binding sites

2.6.1 Collation of putative TF binding sites (TFBS)

For the yeast-one hybrid interactions I tried to do *in –silico* prediction of putative TF binding site (TFBS) in the 3kb promoter region used as baits. I collated the data on TFBS for different families of TFs from:

1. JASPAR
2. DAP-seq Genome Browser
3. AGRIS (Arabidopsis Gene Regulatory Information Server)
4. Motif list available in lab, derived from literature.

**Table 4: Representative image of TFBS collated from different sources.**

| S.no. | Transcription factor | TF binding site | References |
|---|---|---|---|
| 1 | MYB3 | TAACTAAC | AGRIS, Plant Cell. 2002 Mar;14(3):559-74. |
| 2 | DDF1 |  | DAP-seq |
| 3 | AtGRF6 |  | DAP-seq |
| 4 | OBP2 | TACACTTTTGG | AGRIS, Plant J 10: 955-966 (1996) |
| 5 | DOF2.5 |  | JASPAR |
| 6 | WRKY 54 |  | DAP-seq |
| 7 | ANAC103 |  | DAP-seq |

2.6.2 Finding TF binding sites in bait DNA sequence

- Making Patmatch query sequence using the Patmatch key given below:

**Table 5: Key to make Patmatch query.**

| Nucleotide searches | ACTGU | Exact match | ACCGGCGTAA |
|---|---|---|---|
| | R | Any purine (AG) | AAGGCCGGRRRR |
| | Y | Any pyrimidine base (CT) | CCCATAYYGGYY |
| | S | G or C | YGGTWCAMWTGTY |

| | | | |
|---|---|---|---|
| | W | A or T | --do-- |
| | M | A or C | --do-- |
| | K | G or T | --do-- |
| | V | A or C or G | --do-- |
| | H | A or C or T | CCGG...WHW.{3,5}HWH...CCGG |
| | D | A or G or T | --do-- |
| | B | C or G or T | --do-- |
| | N or X | Any base | ATGCTNNNNATC |

- I searched for query sequences in 3000bp of sequence preceding the translation start site, to get the putative binding sites in bait DNA sequence.

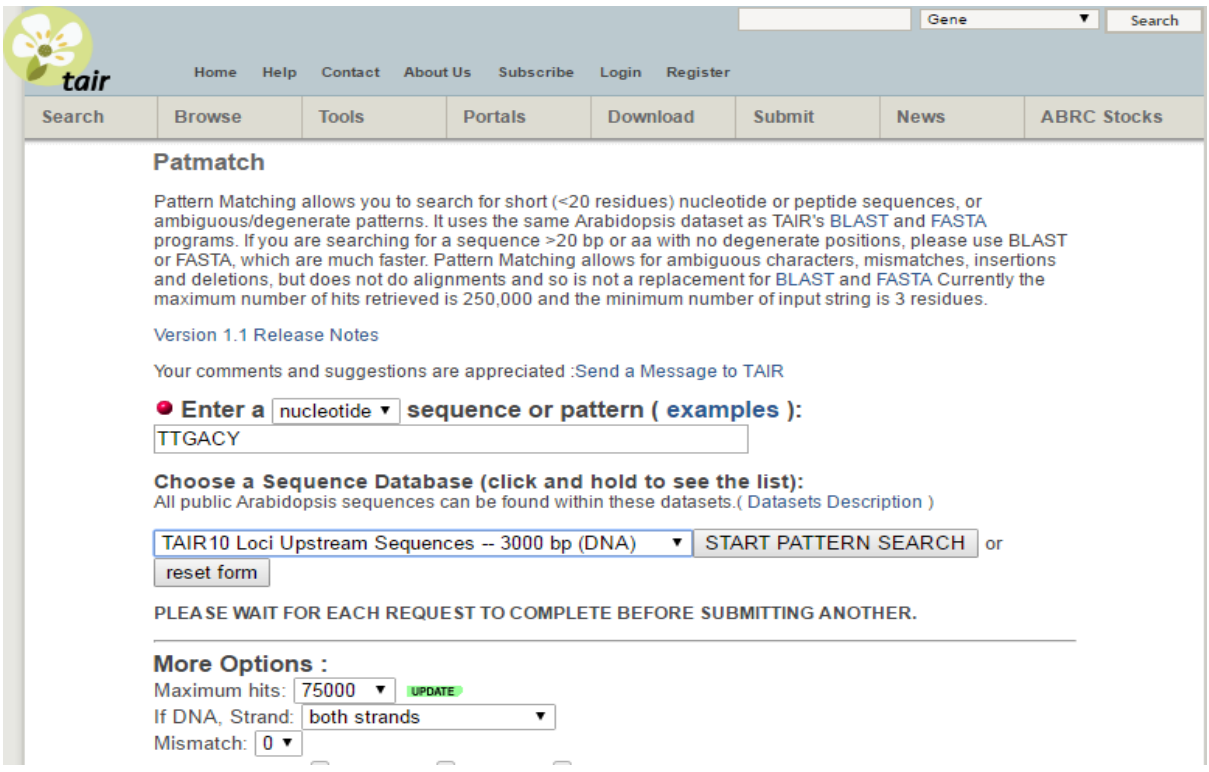



Fig 3: Customising Patmatch search for searching the query on both strands and in region i.e. 3kb upstream of the translation start site.

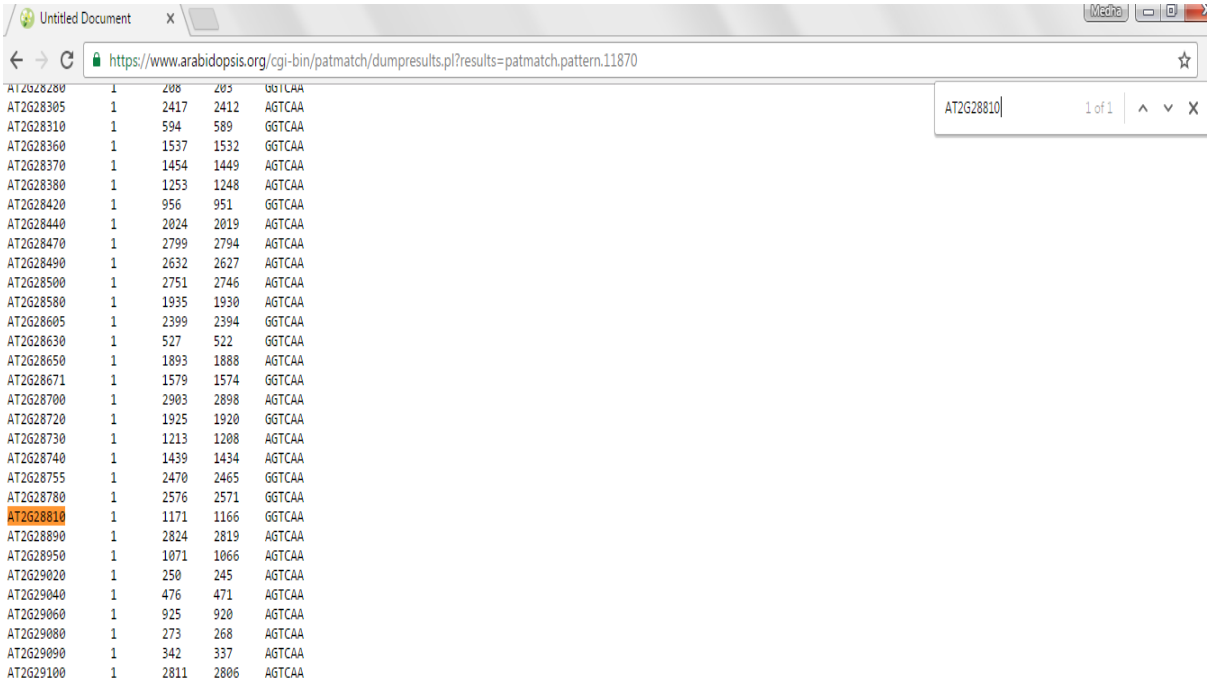



Fig 4: Search for gene id of the bait region (promoter) on which TF binding has been found in Y-1-H.Further, look for the position where the TFBS is present

## 2.7 Gateway cloning for promoter:: H2B-YFP

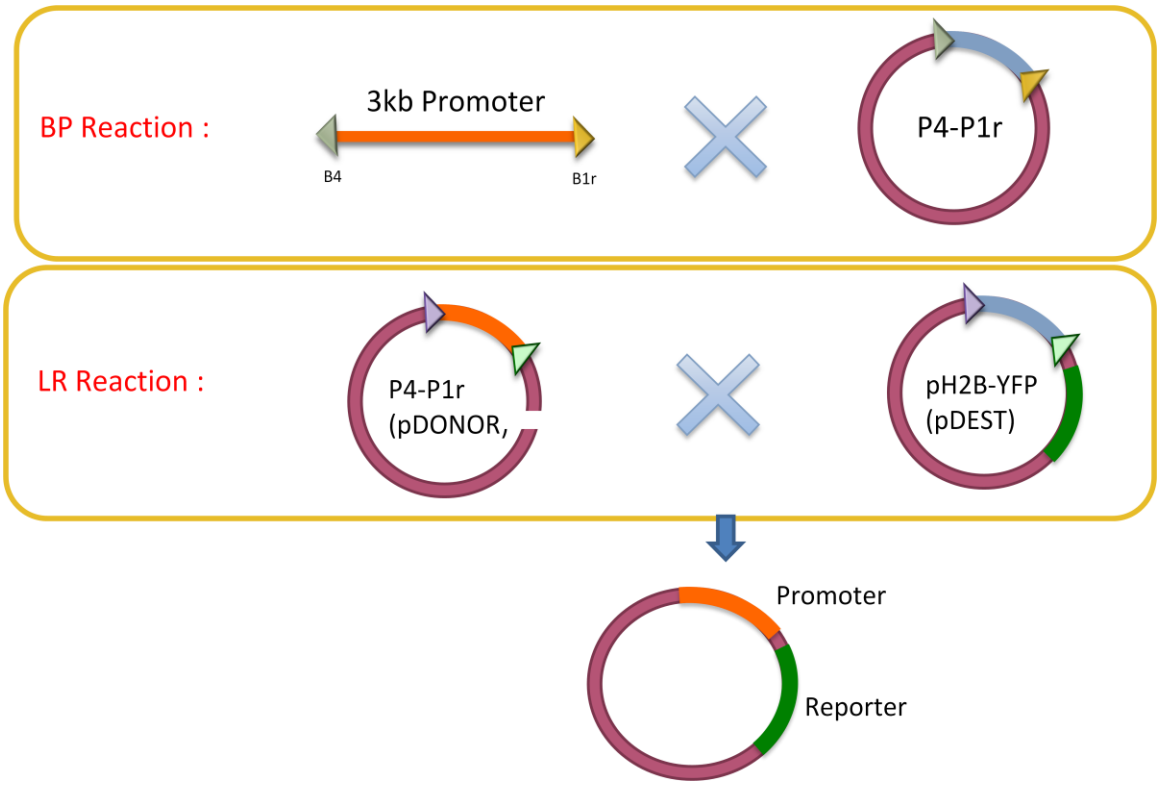



Fig 5: pDEST has been engineered for the gateway cassette so that it directly recombines with the pDONOR (P4-P1R) to give a promoter::reporter fusion

# Chapter 3

## Results & Discussion

### 3.1 Yeast one hybrid (Lac Z assay)

High throughput yeast one hybrid assays can be used as a tool to identify protein-DNA interactions of *Arabidopsis thaliana* TFs in a heterologous system. We have used yeast-one –hybrid assays to identify these interactions specifically for shoot enriched TFs (Yadav *et al.,* 2014). The goal behind carrying out these assays is to unravel the functional gene regulatory interactions that aid cell type specification in shoot apical meristem. On replicating the yeast-one-hybrid assay three times. One of my colleagues has come up with a yeast–one–-hybrid network for these TFs (Fig. 7).

To test the reproducibility of Histdine based reporter assay, I used Lac Z reporter. The baits were integrated in to the same yeast cells and were selected for SC-HIS and –Uracil (UH). After setting the interaction between the preys and selected bait, cells were selected on SC-HIS, -UH, -TRP for diploids. The cells were replica plated on X-gal containing plates but having SC-UH+3AT. One can visually record Y-1-H interactions if blue yeast colonies appear in the presence of X-gal in the plate. We tested ten baits this way and found partial overlap between the Histdine and Lac-Z reporter based interactions. A few interaction were exclusive to the Histdine and same was true for Lac-Z.

In regulatory network every node having an edge connection has a deeper biological meaning. The significance of this interaction can have diverse regulatory roles, ranging from developmental, to providing immunity to the plant, carrying out the hormonal signalling to responses and to regulations of transcription factors, to cell wall biosynthesis any activity, which involves transcription of a gene for biological functions. Using Histdine as a reporter in the Y1H, protein-DNA interaction among the 49 baits was setup

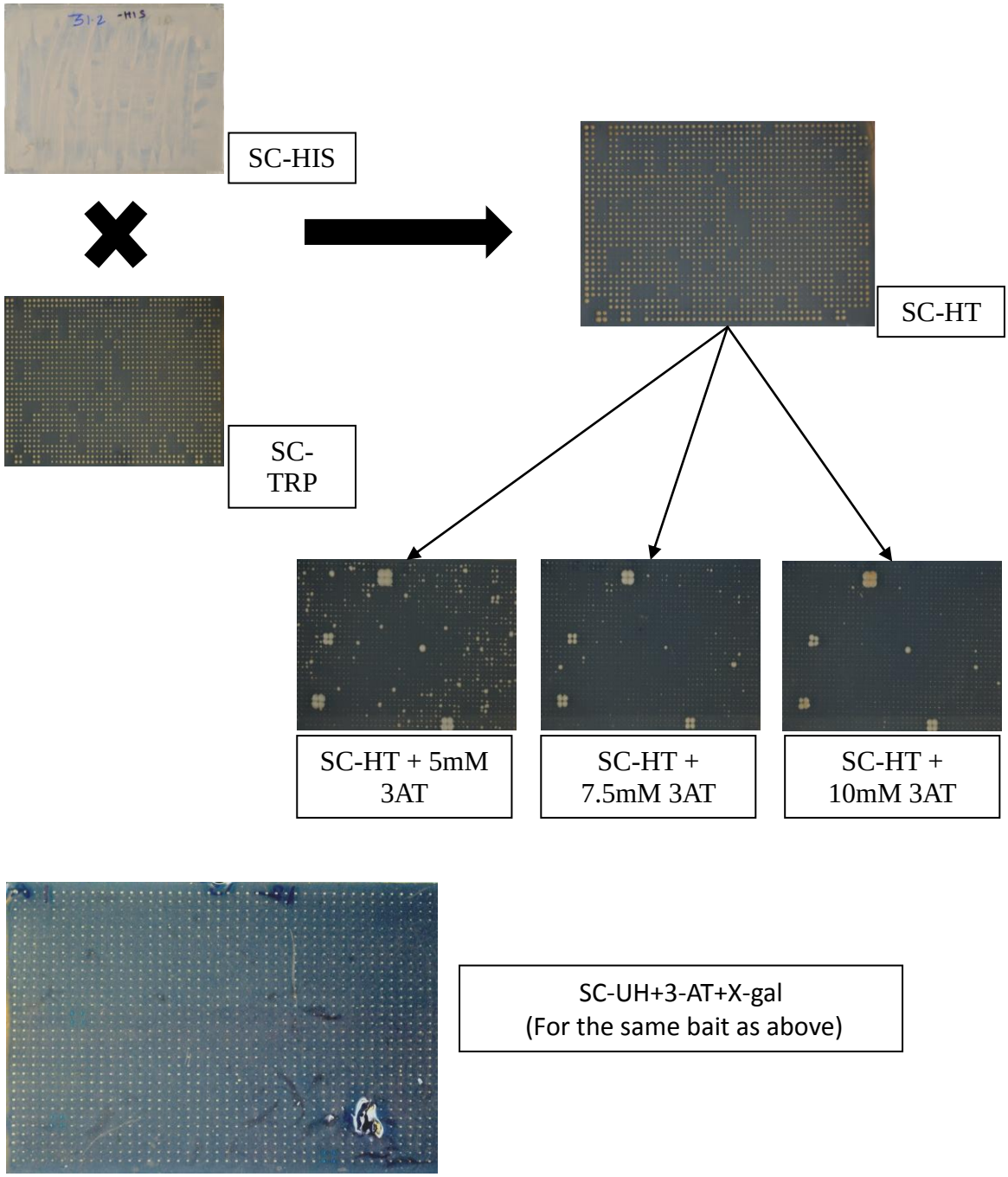



Fig. 6: Yeast-1-hybrid using auxotrophic and colour producing reporter, correspondence between the two assays can be used to rule out any false negatives of the yeast-one-hybrid assay.

against 352 preys. In total, we have setup 17, 248 interaction. Of 17247 interactions, 191 were positive. Approximately 1.1% of the tested interaction worked in Y1H screen. We visualised these interaction in Cytoscape (Fig. 7).

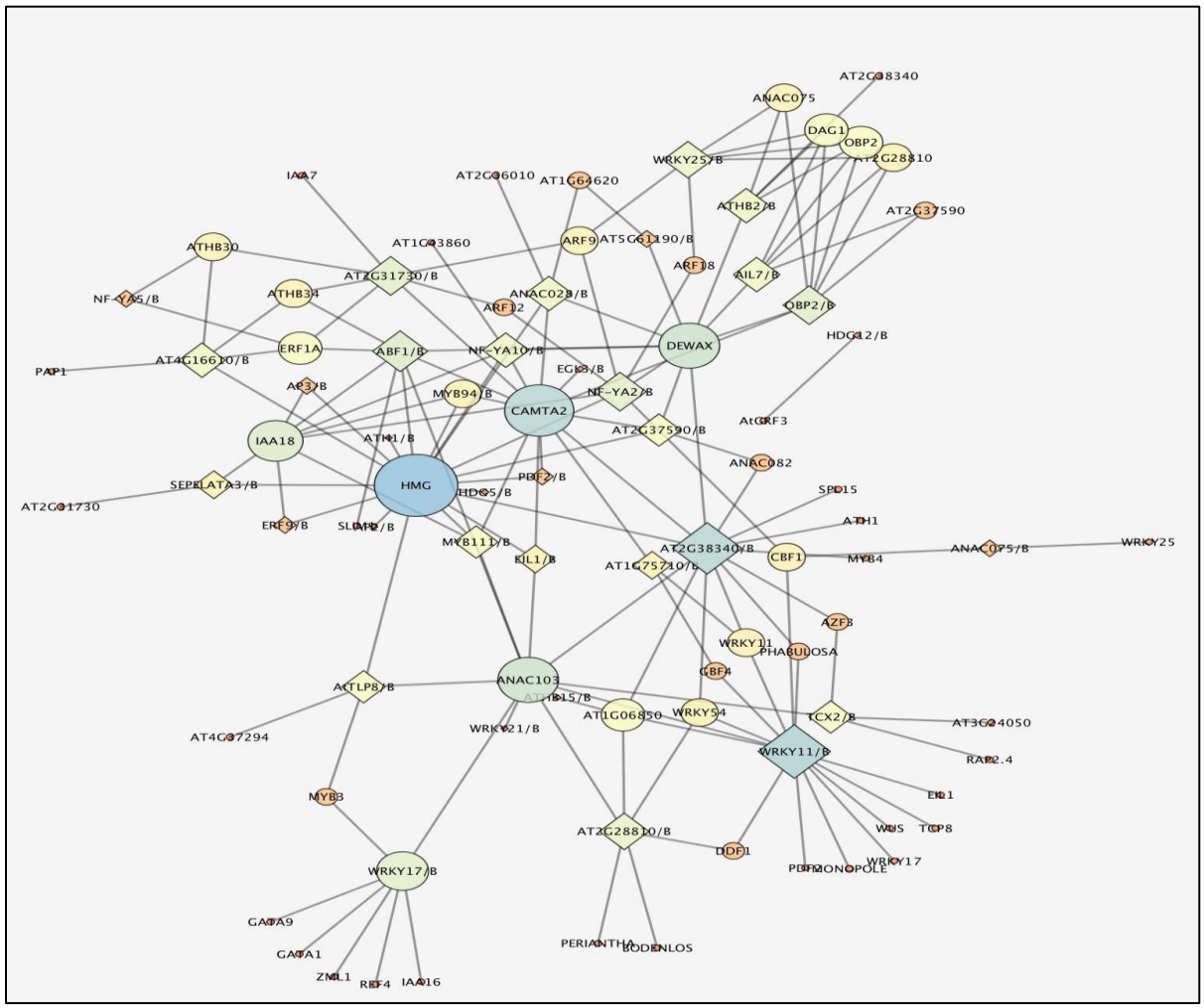



Fig. 7: Based on the Y1H data, this network was built using cytoscape network. Here the diamonds represent baits or the promoter DNA elements, which are connected via the edges to nodes that represent preys that come and bind to the *cis*-elements present on these promoters.

## 3.2 *In-silico* prediction of transcription factor binding sites

The biggest challenge we faced with the Y1H network data is how to identify the TF binding sites in the promoter regions of the targets. This task was demanding given the fact; we cannot verify them by alternative methods, therefore, we decided to do literature searches and collated all possible information related to binding sites of the TFs that show positive interaction with target promoter. On collation, I found transcription factor

binding sites (TFBS) from different sources for 52 TFs. A summary of this has been given here.

**Table 6: TF binding motifs for 52 transcription factors. Here in one row of the table, the TFBS highlighted in the same colour as the TF are specific to that TF only and not to the family of TFs.**

| S.No. | Transcription Factor | TFBS |
|---|---|---|
| 1 | ANAC103 | WRRCKTBNNNNNNAMKNHD |
| 2 | ANAC075,ANAC073,ANAC010, ANAC082 | TTSCGT,TACGR,TCCGR, TGCGR |
| 3 | DOF(AT2G37590),AT2G28810, AT1G64620 | TGATAR,AGATAR,CACGTG, RWAAMGN,CTTTH, DDDDDNNVWAAAGNDD |
| 4 | WRKY25,WRKY3,WRKY17,WRKY54, WRKY22,WRKY11,WRKY25 | TTGACY |
| 5 | OBP2,OBP1,OBP3,OBP4 | TGATAR,AGATAR,CACGTG, CTTTW,CTTTH,WAAAGND |
| 6 | bHLH(AT4G01460) | CACATG,TGATAG, |
| 7 | CBF1(DREB1B),DREB1A, DREB1E, DREB2C,CBF2 | TGGCCGAC,GYCGRY,ATGTCGRY, VRCCGVY, CCACGTGG |
| 8 | DDF1 | GCCGAC, DNGTCGRY |
| 9 | EIL1 | TTCAAGGGGGCATGTATCTTGAA,CACATG |
| 10 | | |
| 11 | NF-YA2 | CCAAT |
| 12 | MYB-30,MYB4,MYB96,MYB3,MYB2 | AACWAMC,ACCAAMC,CACCWAAC, NNTWGGTN,CTAACCA |
| 13 | PDF2 | TAAATGYA, DNAKATNCB |
| 14 | DEWAX | GGGCC,AGCCAC,GCCGCC |
| 15 | SPL15,SPL3,SPL8,SPL14 | TACGTACAA,DDHCGTAC, DDNDNHGTACHHHHHH,DCCGTACR |
| 16 | PERIANTHIA | TGACG |
| 17 | ATbZIP52 | TGMCAGCYND |
| 18 | WUSCHEL | HMADWCWWWYW |
| 19 | DOF-AFFECTING GERMINATION1(DAG2) | NWAAAGBDDN |
| 20 | HMG1/2 | NHGRHGRHD |
| 21 | AT1G73730/SLIM1 | HGDSHHNDTNC |
| 22 | AT5G64220/CAMTA2 | CGHGT |
| 23 | AT1G58100/TCP8, | GTGGNCCC,DGGMCCAC, |

| | | |
|---|---|---|
| | TCP16,TCP4, TCP19, TCP2, TCP20 | NNGNNNCCVD,NNDGKMCMNN, GDNHCCAC |
| 24 | ZML1/GATA24 | TCATCATCATCATCA |
| 25 | NF-YA5 | CCAATG |
| 26 | AT5G15210/ATHB30,ATHB1, ATHB2,ATHB6,ATHB34 | CAAT(A/T)ATTG,CAAT(C/G)ATTG, CAATTATTA, WWWNNNNNYTAATYA |
| 27 | AT4G32890/GATA9 | NNHAGATCY, (A/T)GATA(G/A) |
| 28 | AT2G04890/SCL21 | TAAATGCA |
| 29 | AT4G17500/ERF-1, ERF1A | MGCCGCCDB |
| 30 | ARFs | TGTCNN |
| 31 | AT3G24050/GATA1 | RYGRYRRTGNHGRTG, (A/T)GATA(G/A) |
| 32 | GBF4 | ACTCAT,CCACGTGG |
| 33 | AT3G19580/AZF2,AZF1 | WWNAGTGW |
| 34 | AT2G31730 | CACATG |

On searching for TFBS in 3kb promoter regions using Patmatch. I could find the exact position where TFBS are present on searching on both DNA strands. A representation of TFBS on a promoter region is given in the following figure.

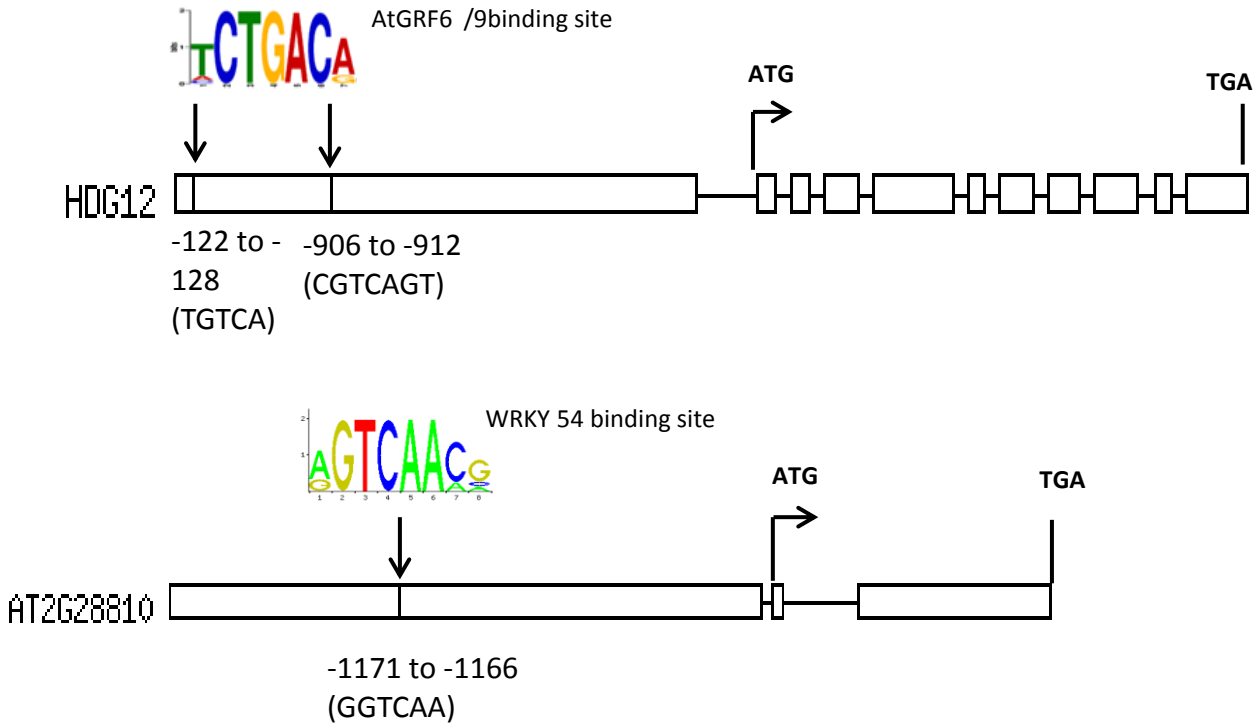



Fig 8: Representing TF binding sites, the exact position of the TFBS on the bait DNA can be interpreted through these representations.

Further, I scored the number of yeast-one-hybrid interactions for which I could find the TFBS in the bait DNA sequence, this data has been summarised below in Table 8. Out of 120 interactions in Y-1-H assay I could find the TFBS on bait regions in case of 72 interactions.

**Table 7: List of TFs (preys) with their interacting promoter regions (baits). Red represents the cases where TFBS has not been found, green represents the cases where TFBS has been found on *in-silico* search.**

| S.No. | Prey (TF) | Bait (-3kb promoter region) |
|---|---|---|
| A.1 | ANAC103 | AT2G38340 |
| 2 | | AT2G28810 |
| 3 | | ABF1 |
| 4 | | WRKY17 |
| 5 | | EIL1 |
| 6 | | WRKY11 |
| 7 | | ATH1 |
| 8 | | NFYA10 |
| 9 | | MYB111 |
| 10 | | WRKY21 |
| B.1 | DOF2.4 (AT2G37590) | OBP2 |
| 2 | | ATHB2 |
| 3 | | AIL7 |
| C.1 | DOF (AT2G28810) | OBP2 |
| 2 | | AIL7 |
| D.1 | OBP2 | OBP2 |
| 2 | | ATHB2 |
| 3 | | AIL7 |
| 4 | | WRKY5 |
| E.1 | CBF1 | WRKY11 |
| 2 | | NFYA2 |
| F.1 | DDF1 | AT2G28810 |
| 2 | | WRKY11 |
| G.1 | PAP1 | AT4G16610 |
| H.1 | AT2G31730 | SEPATALA1 |
| I.1 | WRKY17 | WRKY11 |
| J.1 | WRKY54 | WRKY11 |
| K.1 | BODENLOS | AT2G28810 |
| L.1 | EIL1 | WRKY11 |
| M.1 | PDF2 | WRKY11 |
| N.1 | DEWAX | AT2G38340 |

| | | |
|---|---|---|
| 2 | | ABF1 |
| 3 | | NFYA10 |
| 4 | | OBP2 |
| 5 | | ATHB2 |
| 6 | | NFYA2 |
| 7 | | AT1G65910 |
| 8 | | AT5G61190 |
| 9 | | AT1G75710 |
| O.1 | SPL15 | AT2G38340 |
| P.1 | DOF (AT1G64620) | AT1G65910 |
| 2 | | AT5G61190 |
| Q.1 | PERIANTHIA | AT2G288810 |
| R.1 | PHABULOSA | AT2G38340 |
| 2 | | WRKY11 |
| S.1 | ATbZIP52 | AT2G288810 |
| 2 | | WRKY11 |
| 3 | | ATHB15 |
| T.1 | ANAC075 | WRKY11 |
| 2 | | OBP2 |
| 3 | | ATHB2 |
| 4 | | WRKY22 |
| U.1 | DOF AFFECTING GERMINATION 1 | OBP2 |
| 2 | | ATHB1 |
| 3 | | EIL7 |
| 4 | | WRKY25 |
| V.1 | HMG1/2 | AT2G38340 |
| 2 | | ABF1 |
| 3 | | EIL1 |
| 4 | | NFYA10 |
| 5 | | MYB111 |
| 6 | | NFYA2 |
| 7 | | AT4G16610 |
| 8 | | SEPATALA1 |
| 9 | | AT1G65910 |
| 10 | | AT2G37590 |
| 11 | | MYB94 |
| 12 | | PDF2 |
| 13 | | ATH1 |
| 14 | | ERF9 |
| 15 | | AP3 |
| W.1 | WUS | WRKY11 |
| X.1 | ANAC082 | AT2G38340 |
| 2 | | AT2G37590 |
| Y.1 | CAMTA2 | AT2G38340 |
| 2 | | AT2G28810 |
| 3 | | ABF1 |
| 4 | | EIL1 |
| 5 | | NFYA10 |

| | | |
|---|---|---|
| 6 | | MYB111 |
| 7 | | OBP2 |
| 8 | | AT1G65910 |
| 9 | | AT1G75710 |
| 10 | | AT2G37590 |
| 11 | | MYB94 |
| 12 | | PDF2 |
| Z.1 | SLIM1 | ABF1 |
| AA.1 | ATHB30 | AT4G16610 |
| 2 | | NFYA5 |
| AB.1 | ARF18 | WRKY25 |
| 2 | | NFYA2 |
| 3 | | AT2G31730 |
| AC.1 | IAA16 | WRKY17 |
| AD.1 | IAA18 | ABF1 |
| 2 | | NFYA10 |
| 3 | | MYB111 |
| 4 | | NFYA2 |
| 5 | | SEPATALA |
| 6 | | MYB34 |
| 7 | | ERF1 |
| 8 | | AP3 |
| AE.1 | ARF9 | WRKY25 |
| 2 | | NFYA2 |
| AF.1 | GBF4 | WRKY11 |
| AG.1 | ARF12 | NFYA2 |
| AH.1 | AT1G43860 | NFYA10 |
| AI.1 | MYB3 | WRKY17 |
| AJ.1 | ERF1A | ABF1 |
| 2 | | AT4G16610 |
| 3 | | NFYA5 |
| AK.1 | ATHB34 | ABF1 |
| 2 | | AT4G16610 |
| AL.1 | TCP8 | WRKY11 |
| AM.1 | ZML1 | WRKY17 |
| AN.1 | GATA9 | WRKY17 |
| AO.1 | GATA1 | WRKY17 |
| AP.1 | AZF2 | AT1G65910 |
| AQ.1 | GRF6/9 | HDG12 |

## 3.3 Promoter reporter fusion experiment

Activity of a promoter is a proxy for the expression of its downstream gene. Hence, promoter::reporter constructs can be used as a tool for gene expression studies of these transcription factors. pDEST (H2B-YFP) has been engineered in the lab for the gateway cassette so that it directly recombines with the pDONOR (P4-P1R) to give a promoter::reporter fusion. Using gateway cloning strategy I have been trying to make

promoter reporter fusion constructs. Promoter reporter constructs were cloned in pGreen backbone. I have been able to make 3of these constructs and I have to transform the wild type-Ler plants for these constructs, collect seeds and select for transgenics by BASTA

selection. I look forward to learn more of confocal microscopy and hence record images for these promoter::reporter constructs.

## 3.4 Discussion

Development of plant is regulated by differential gene expression. Transcriptional regulation which involves the interactions between transcription factors and *cis*-regulatory elements of promoters play a major role in differentiation or cell type specification. . In this study we are trying to explore the gene regulatory network of transcription factors that lead to cell type specification in the shoot apical meristem of *Arabidopsis thaliana*. Using Histdine as an auxtrophic reporter in the Y1H, protein-DNA interaction among the 49 baits was setup against 352 preys. In total, we have setup 17, 248 interactions. Of 17247 interactions, 191 were positive. Approximately 1.1% of the tested interaction worked in Y1H screen. Therefore, it is important to validate the yeast-one-hybrid network generated in the lab. Neither the physical binding of a TF on a *cis*-regulatory element (as reported in Y-1-H assay),nor the presence of TFBS in the *cis*-regulatory element shows that that interaction is functionally relevant. q-RT PCR experiments in mutant and overexpression lines of transcription factors can be used to look for expression levels of the downstream genes of the promoter where these TF bind. I have explored the task of validating the Y-1-H interactions by *in-silico* prediction of putative TFBS. Presence of a TFBS in case of Y-1-H interactions strengthens the Y-1-H data as well as provides basis for testing these interactions through EMSA an aids *in-planta* validation. I started out by collation of data of the motifs that TF prefer to bind. I used different databases for the same. I have found TFBS for 52 TFs. Using the Patmatch tool available on TAIR I searched for these TFBS in the bait DNA sequences used in Y-1-H assays. Out of 120 Y-1-H interactions that I checked using Patmatch found binding sites on bait D NA sequence for 72 cases. The reason for TFBS being found only in case of 72 interactions out of 120 could be many—false positives in Y-1-H assay; I might have missed the TF binding motif in some cases, while collating the data; some TFBS might have not been reported yet and novel TFBS are being used. In the following days I want to do an EMSA for GRF family of TF binding on HDG class of promoters. In addition, promoter- reporter fusion assay could be used to enhance our understanding of activity of promoter in specific regions of SAM and in different backgrounds. A great achievement of this project is to get hands-on experience of working with modern

technologies like confocal microscopy and Singer Robotics facility. I learnt the most while troubleshooting for the experiments that I carried out. I have developed an understanding of gene regulatory network studies, and I look forward to learn and explore more aspects of plant development in future.

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