Mapping Transcription Factor Gene Regulatory Network in the Shoot Apical Meristem Stem Cell Niche of *Arabidopsis thaliana*

ASHA RAJU

MS10040

A dissertation submitted for the partial fulfilment of

BS-MS dual degree in Science

Indian Institute of Science Education and Research Mohali

April 2015

Certificate of Examination

This is to certify that the dissertation titled “Mapping Transcription Factor Gene Regulatory Network in the Shoot Apical Meristem Stem Cell Niche of *Arabidopsis thaliana*” submitted by Ms. Asha Raju (Reg. No. MS10040) 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.

Asha Raju (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

Above all my heart shall be raised in gratitude to the almighty for his universal grace towards me.

I thank Indian Institute of Science Education and Research, Mohali for giving me opportunity to do my final year thesis in the Dept. of Biological Sciences.

It is my pleasure to extend my deep sense of gratitude and indebtedness to my guide Ram Yadav, for his guidance, along with benevolent and sustained encouragement bestowed on me during the course of training.

I would also like to thank my lab member, Shivani Bhatia for mentoring me throughout the project period, and all other fellow members of the lab, Prince Saini, Sonal Yadav, Shalini Yadav and Monika Mahajan for their effective guidance, moral support and lively discussions throughout my work. I also thank Mahima, Vivek. B. Raina, Sukriti Vidyarthi, Poulami Choudhari and all other 3TL2 lab members for their constant support and friendly attitude during the course of our work.

I also wish to thank my parents for their great support and encouragement for doing this thesis work.

Last but not the least I would like to thank my friends Reshma Murali and Nirdosh Dadwal for their support, encouragement and timely help.

Asha Raju

Contents

Abstract

Spatiotemporal regulation of gene expression is the key phenomenon in multicellular organisms to specify fate of distinct cell types. Spatial and temporal regulation of gene expression is achieved by the cell signalling and transcription factors (TFs). In both animals and plants several studies have shown role of TF in cell and tissue specialization. Shoot apical meristem of *Arabidopsis thaliana* harbours a set of stem cells from which the various cell types of plant arise. How stem cells differentiate in to different cell types of the shoot and how organs form from these differentiated cells is still largely unknown. In the present study I investigated the role of broadly expressed TFs in specifying the epidermal and sub epidermal cell type. Epidermal cell types forms an important interface between the outside environment and the inside of plant body and protects the plants from pathogens and drought. Sub epidermal cell types are sandwiched between the epidermal cells and the internal cell layers and play an important role in maintaining the cell-cell communication across the cell layers in shoot apex. In leaves and stem tissue, sub epidermal cells differentiate form the photosynthetic tissue. To uncover the hierarchy of gene regulation among the TFs and to identify the network responsible for cell and tissue specialization, I have mapped the interaction between the TF promoters and their cognate trans-acting factors using yeast-one-hybrid (Y1H). Y1H data was analysed and gene-regulatory network was built. Promoter reporter studies for few selected TFs revealed in *planta* the importance of trans-acting factors in determining the spatio-temporal expression patterns.

Chapter 1

Introduction

1.1 Basic Theory

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 shoot descendants such as leaves, flowers and stem tissues are derived by the activity of stem cells. SAM of higher plants is highly organized structure. It can be classified in to various cell layers and zones (Fig. 1). In dicots, there are three cell layers; however their number is retained two in monocots (Furner and Pumfrey, 1992). The cell lineages derived from epidermal or L1 cell layer in Arabidopsis give rise to pavement cells, guard cells and trichomes in the leaf. The sub epidermal or L2 cell layer gives rise to the mesophyll, a photosynthetic tissue. The innermost cell layer is called corpus or L3 and produces vascular bundles and stem tissues in mature plant. These cells layers comprise most plant organs (Stewart and Derman., 1970).

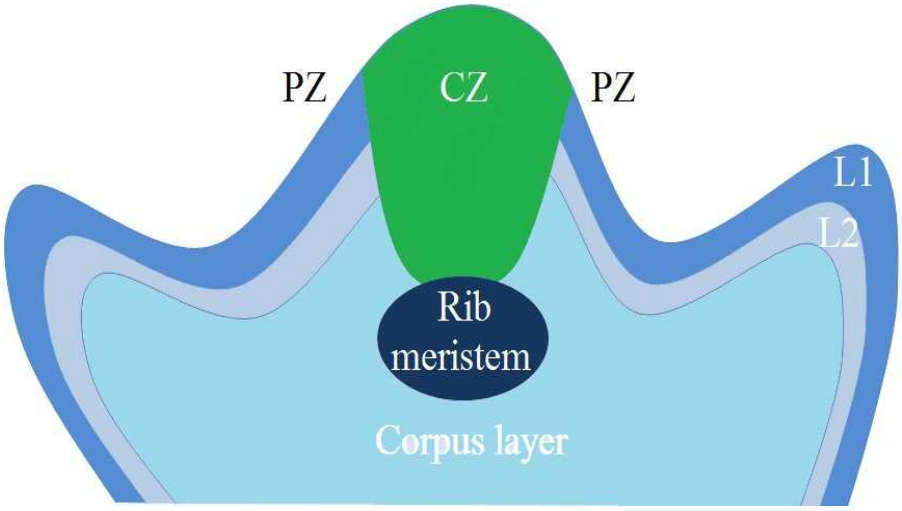

Fig.1. Representative schematic of the shoot apical meristem (SAM) of *Arabidopsis thaliana.* SAM could be divided into three layers L1, L2 and L3/Corpus. L1 develops into the epidermis, L2 develops into sub epidermis and L3 gives rise to stem tissues and

internal cell layers. SAM is divided into three zones, peripheral zone (PZ), central zone (CZ) and rib meristem (RM). CZ harbours pluripotent stem cells, whereas PZ contains daughters of stem cells. RM resides beneath the CZ and form the stem tissue including the vasculature.

Contrary to the cell layering, 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 undifferentiated cells constitutes central zone (CZ), where stem cells reside. However when these cells enter in to the periphery they display higher cell 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 shifted 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 *et al.,* 1940; Derman *et al*., 1953) (Fig. 1).

One of the key challenges in the shoot apex biology is to understand how distinct cell types in cell layers and zones acquire and maintain their cell identity. To address this question, one need to identify the transcripts enriched in cell layers as well as in zones. 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 and *trans*-acting factors involved in it and the spatiotemporal interactions that take place. Ultimately comprehensive network will emerge from these studies which one could exploit to make the regulatory networks. These networks would be of immense importance to predict the behaviour of entire system. My focus is to identify the role of broadly expressed TFs in regulating epidermal and sub epidermal cell types specific TFs in the SAM. Understanding the functions of key regulators will enrich our knowledge and give us a better understanding of the system. I am developing a network of the interactions among the *cis*-regulatory elements in the promoter regions and the transcription factors that are broadly expressed in the shoot.

There are many techniques by which one can probe the interaction between putative DNA binding sites and the TFs. However two techniques are more popular than others. For simplicity we can call one TF-centered and other is DNA-centered. With recent advances in high throughput sequencing technologies.TF-centered methods were implemented in mammalian cell lines effectively to generate the regulatory networks. In

this approach Chromatin immunoprecipitation (Chip) was used to pull selectively the promoter regions of target genes bound by TF with the help of an antibody. Precipitated DNA is sequenced to determine the exact order of nucleotides (Jonghwan Kim *et al.,* 2004). The sequencing reads are aligned with the genome to get an estimate of probable binding sites across the genome for a given TF. A chip experiment requires high quality antibody against the TF. At the same time they should be specific and should not cross-react with closely related TFs. Since plants display high genetic redundancy their genome code for many genes. In addition, generating such antibodies against individual TF and their characterization can take long time and at the same time it could be very expensive. Therefore, it would be difficult to adopt a TF-centered approach for plant TFs. Till to date TF centered gene regulatory networks were probed for ~30 TFs in Arabidopsis (Heyndrickx KS *et al.,* 2014). In DNA centered approach one can clone DNA bait and test its interaction with the cognate protein partners using yeast one hybrid (Y1H) assay (Brent and Ptashne., 1985; Treizenberg *et al.,* 1988). Since in the past most labs were interested in low throughput studies therefore it was relatively easy to clone DNA fragments by restriction digestion to conduct Y1H. Several recent reports have shown 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 (Fig. 2). 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. Thus, it will help in achieving the regulated gene expression.

Fig.2. Y1H assays are based on the interactions between the prey protein and a bait of our interest. The prey that binds with the DNA also contains a heterologous transcription activation domain (AD) that activates the reporter gene upon protein-DNA interaction (PDI).

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. By growing yeast in specific conditional media that demands the expression of the reporter gene helps us to confirm the interaction. I have used *Saccharomyces cerevisiae* (Yα1867, YM4271) as my model organism for this experiment.

The *cis*-regulatory elements to which the TFs bind on the DNA were predicted by bioinformatics. Such TF binding sites have traditionally been mapped using a combination of deletion analyses and reporter gene expression (Davidson *et al.,* 2002). Recently, several methods have been employed to computationally identify putative *cis*-regulatory elements (Elnitski *et al.,* 2006). Different approaches that can be used for the identification of *cis*-regulatory DNA elements are highly complementary and interconnected. Interrogating the regulatory regions of co-expressed genes, by phylogenetic foot printing, or by experimentally identifying TF binding sites, can identify cis-regulatory elements.

Gene regulatory networks can provide insight into various mechanisms that drive complex phenotypes. Validation of large gene regulatory networks is indeed challenging. Comparing network edges with existing interactions in literature and other databases, followed by calculating the proportions of interactions, serves as the first step of validation. Experimental knock down approaches provide high confidence in validating the data that could be extracted from the gene regulatory networks. But this method is not feasible in case of large number of genes and new potential interactions.

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 includes 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 very 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 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)

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 2:2:1 ratio). It was grown in controlled environment plant chambers (Percival, USA and Conviron, Canada) with 75% humidity, 160-candella white light and 24^0C 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 cloning of cDNA inserts and amplified promoter fragments in pENTR/D/TOPO vector. All other chemicals and reagents used in the study were of molecular biology/analytical grade and were obtained from Sigma-Aldrich (USA).

2.3 Primers used

AD specific primers were used to confirm the preys that I used for performing Y1H assay. The primers were designed using NTI software and were obtained from Sigma Aldrich (USA).

Table.1. AD specific primers used for confirming preys

Sl. No.	**Primer name**	
1	AD specific forward primer	TTGATTCGACCCACCCTCTTTT
2	AD specific reverse primer	GCATGCAACTTCTTTTCTTTTT

2.4 Gateway cloning of baits

Gateway cloning method was used to clone the DNA baits for Y1H. Gateway vectors contain CcdB gene, which codes for a toxin, thereby not allowing them to propagate in DH5a. Promoter DNA sequences were amplified from Wt *Ler* genomic DNA, using primers having attB4 and attB1r sites in the forward and reverse primers, respectively. Setting up BP reaction then cloned them into pDONOR-P4P1R destination vector. The destination vector has attP4 and attP1r sites, which recombine with their counterpart B4-B1r sites in the amplified PCR product. Following BP reaction, the gene of interest gets cloned between the recombination sites due to which the clone can now be selected and screened in DH5α. The promoter is then sub-cloned into Y-1-H vector, pMW2 by setting up LR reaction. This vector contains a Gateway cassette with attR4 and attL1 recombination sites upstream of HIS3 reporter gene. The pMW2 vector carrying the promoter of interest is then linearized and transformed into Ym4271 strain of yeast, where it gets incorporated into the yeast genome by homologous recombination. The insertion into the yeast is then confirmed by doing PCR on the yeast genome.

2.5 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. We used an empty vector without any prey as my negative control to verify auto activation. We have devised stringent criteria to filter Y1H data and generated an interaction network using the freely available software cytoscape. Network models provide a great tool for the visualization and navigation of large interaction data sets.

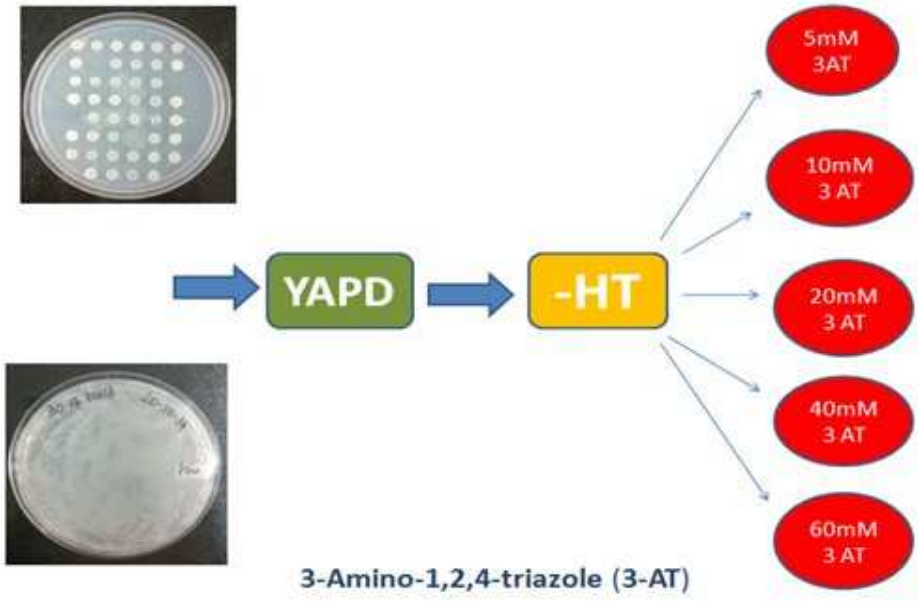

Fig.1.Yeast one hybrid methodology schematic. Baits and preys are allowed to mate and are initially grown in YAPD complete media. In order to select diploids specifically, we then transfer the growth into –HT selective media. An interaction read out is possible with the final selection using different concentrations of 3AT.

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 121^0C 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^oC
- Centrifuge at 4000 rpm for 20 minutes in 4^oC
- 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 and final dissolution of the pellet should to be done in around 2-3 ml 10% glycerol
- Aliquot 40µl cells into 1.5 ml tubes

Yeast competent cell preparation

- Inoculate one yeast colony in 20 ml YAPD media
- Incubate at 30^{o}C for 12 hrs
- Set up the secondary culture at starting OD_{600} of 0.200
- Incubate at 30^{o}C till the OD_{600} reaches around 0.600-1.00
- Centrifuge at 4000 rpm for 5 minutes
- Add 100 ml autoclaved distilled water to the pellet
- Centrifuge at 4000 rpm for 5 minutes
- Add SORB 1/10th volume of culture
- Spin down at 4000 rpm for 5 min
- Dissolve pellet in 1440µl/200ml culture SORB + 160µl salmon sperm DNA
- Aliquot 30µl in 1.5 tubes
- Store at -80^{o}C

Gel Purification

- Cut the band under UV transilluminator
- Add 700µl QG buffer and keep at 50^{o}C 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 40µl competent cells
- Mix well
- Transfer the whole mixture to the cuvette using a pipette
- Electroporate 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

Yeast Transformation

- To 20-25µl yeast competent cells, add 1µl plasmid to be transformed
- Add 200µl 40% PEG and mix well
- Incubate at 30^{o}C for 30 minutes
- Give heat shock by keeping at 42^{o}C water bath for 30 minutes and transferring to ice for 10 minutes
- Centrifuge at 4000 rpm for 5 minutes
- Discard supernatant and dissolve the pellet in 1 ml sterile water
- Centrifuge at 4000 rpm for 1 minute
- Remove 800µl water from the supernatant and dissolve the pellet in rest of the water
- Plate in –Trp plates

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.2. Master mix for colony PCR

Components	**Volume**
Sample	10µl
10X Standard Taq Reaction Buffer	1.5µl
dNTPs (10mM)	1.2µl
MgCl2 (50mM)	0.45µl
Water	0.25µl
Taq DNA polymerase	0.1µl
10µM forward primer	0.75µl
10µM reverse primer	0.75µl
Total	15µl

Table.3. Conditions for colony PCR

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	∞

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- Resuspension 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
- 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

Yeast stocks

- Add equal volumes of 15% glycerol and the yeast 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

Denaturing salmon sperm DNA

- Denature sheared salmon sperm DNA at 99^{o}C for 10 minutes
- Immediately keep on ice for 2-3 minutes
- Aliquot 40 µl salmon sperm DNA in 1.5 ml tubes

- Store in -20^{o}C

Preparing soil for growing plants

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

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 -14gm

700ml

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

YAPD liquid media

- Peptone - 14g
- Yeast extract - 7g
- Adenine hemisulphate - 0.02g

- Glucose - 14g

  ---

  700 ml

YAPD solid media

- Peptone - 14g
- Yeast extract - 7g
- Adenine hemisulphate - 0.02g
- Glucose - 14g
- Agar - 10 g

  ---

  700 ml

MS media for plants

- Dissolve 2 gm of Murashine schoog media powder in 1 lt distilled water
- Set the pH to 5.8 by adding 1M KOH

OR

- Macro elements -100 ml
- Micro elements -50 ml
- NPK -100mg/ml

  ---

  1 lt water

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

Composition of Macroelements

- NH_4NO_3 -1650mg
- KNO_3 -1900mg
- $CaCl_2.2H_2O$ -440mg
- $MgSO_4.7H_2O$ -370mg
- KH_2PO_4 -170 mg

---

1 lt

Composition of Microelements

- H_3BO_3 -6.2 mg
- $MnSO_4.4H_2O$ -22.3 mg
- $ZnSO_4.7H_2O$ -8.6 mg
- KI -0.83 mg
- $NaMoO_4.2H_2O$ -0.25 mg
- $CuSO_4.5H_2O$ -0.025 mg
- $CaCl_2.6H_2O$ -0.025 mg
- $FeSO_4.7H_2O$ -27.8 mg
- $Na_2EDTA.2H_2O$ -37.3 mg

---

1 lt

SORB

- 100 mM lithium acetate
- 10 mM Tris HCl (pH = 8.0)
- 1 mM EDTA (pH8.0)

- 1 M sorbitol
- Adjust pH 8.0 using acetic acid
- Filter sterilise

PEG (40%)

- 100 mM lithium acetate
- 10 mM Tris HCl (pH = 8)
- 1 mM EDTA (pH = 8)
- 40% PEG from 3350 Sigma
- Filter sterilise
- Store in -20 degree Celsius

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

Chapter 3

Results & Discussion

3.1 Yeast one hybrid

We have used the Gateway based cloning method to rescue the promoters, for the high-throughput cloning of DNA baits with histidine gene as a marker. Baits were obtained from Shivani. The transcription factors to be used as preys were cloned in pDEST-AD-2μ destination vector and were obtained as dried plasmids in the form of a library from Dr. Siobhan Brady's lab, UC Davis. Around 400 plasmids were rescued from the library in the time span of around one year and 120 plasmids were transformed into yeast strain Yα1867. All of the preys were confirmed using AD specific primers through PCR (Fig. 1). We selected 41 transcription factors as prey whose transcripts were enriched in L1, L2 and L3 layers of the SAM. In a transcriptional regulatory network, proteins interact with *cis*-regulatory elements in target genes to control the expression. In case of eukaryotes, promoters are extremely diverse and are hard to characterize. They lie upstream of the gene and can have regulatory sequences several kilo base pairs away from the transcriptional start site.In this study, we have amplified2-3kb promoter regions upstream of translational start site of target genes that will acts as bait to find out the putative interactors. Hence to make sure that transcription factor binding sites could indeed be included in the region of our choice.

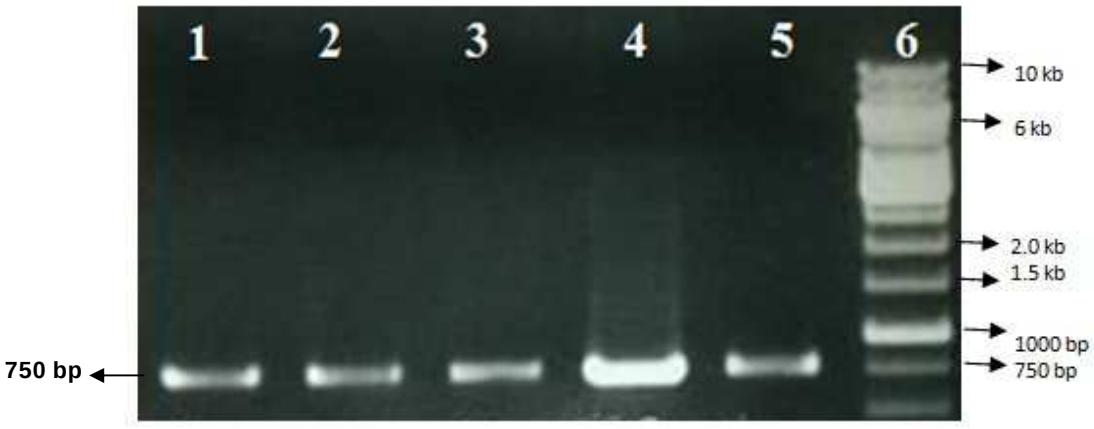

Fig.1. Rescuing and confirmation of prey plasmids from library. Lane 1-5: amplified product obtained from PCR using AD specific forward and reverse primers. Expected size is around 750 base pairs. Bands of the expected size were obtained in all the lanes. Lane 6: 1kb molecular weight marker.

We have checked and analysed the interactions between 16 baits and 41 preys (Fig. 2). Out of the combinations tested, 148 strong interactions were concluded, which is around 30% of the total. An interaction network has been generated using the software cytoscape and the key regulatory elements were analysed (Fig. 3). Majority of the baits showed interactions with more than one TF. The interacting TFs were found to play major role in plant development and drought resistance. Distance between two nodes was analysed and the interactions were quantitatively evaluated. Shorter the edge, stronger the interaction would be.

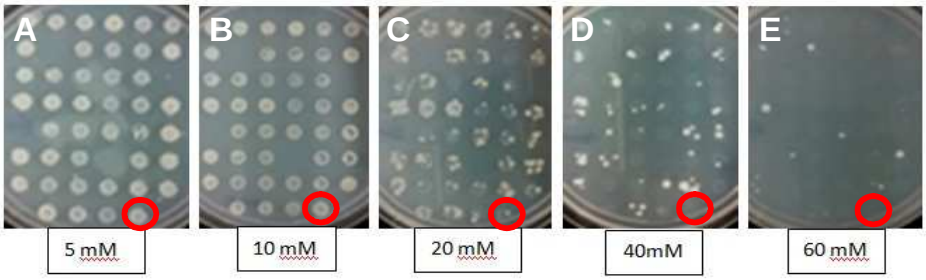

Fig.2. Representative yeast one hybrid data for the gene *AT2G37590* bait interacting with 41 preys at different concentrations of 3AT (A, B, C, D, E, F). The spot marked with red circle was the negative control, which was just an empty vector without the transcription factor. The growth pattern was analysed in comparison with the negative control at different levels of 3AT concentrations.

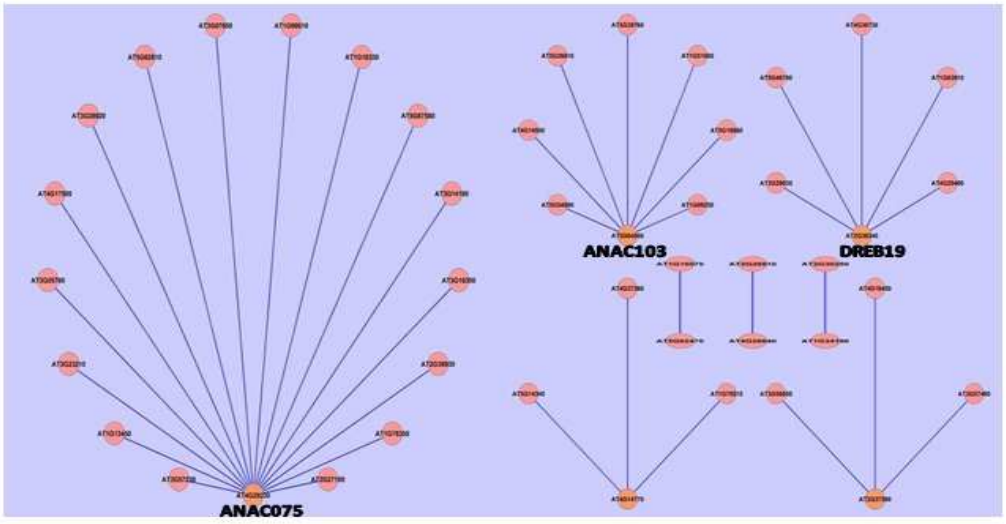

Fig.3. Protein-DNA network interactions (A, B) in the *Arabidopsis* SAM. The interaction was analysed between 3kb promoter regions and TFs that are broadly expressed in L1, L2

and L3 layers of SAM using Y1H assay. Transcription regulatory networks are highly connected. Most promoters are bound by a combination of transcription factor hubs, some of which may be the master regulators.

3.2 Promoter reporter fusion experiment

Arabidopsis thaliana plants were grown in the growth conditions discussed previously. GFP reporter was cloned downstream of the 3kb promoter region. The construct was transformed into the plant using agro bacterium floral dip method and was analysed under confocal microscopy. Promoter reporter constructs were cloned in pGreen backbone. Each construct was dipped twice to ensure efficient agrobacterium transformation. The seeds were collected and transgenics were selected by spraying BASTA. Transgenic plants were then screened under confocal microscope to obtain their expression data (Fig. 4). A total of19 constructs (*Promoter:H2B-YFPpGreen*) were transformed into 2-3 week old *Arabidopsis* Wt L*er* plants.

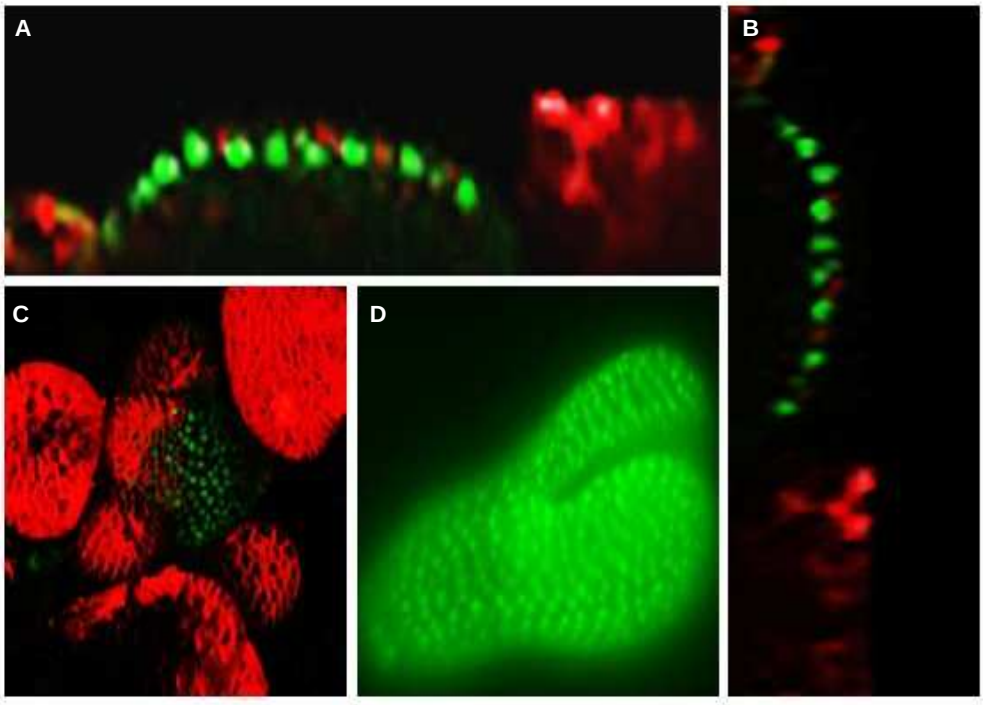

Fig.4. Confocal images for promoter reporter fusion experiment conducted for the gene AT1G54160/NF-YA5 (Nuclear Factory 5). Predictions based on available microarray data showed that AT1G54160 belongs to the L1 box. (A, B) Side view of the SAM showing GFP expression in the L1 layer. (C) Top view confocal image of SAM. We could observe the GFP expression restricted to the tip. (D) Confocal image of the torpedo stage embryo.

GFP expression is prominent throughout the embryo. The expression of the gene gets restricted to L1 layer in the later developmental stages of the plant.

Table.1. List of promoter reporter constructs that have been dipped in planta.

Sl. No.	CONSTRUCTS	GENE
1	pGreen:H2B-YFP *AT2G30250*	*WRKY25*
2	pGreen:H2B-YFP *AT4G25490*	*CBF1*
3	pGreen:H2B-YFP *AT1G75710*	*C2H2-LIKE ZINC FINGER PROTEIN*
4	pGreen:H2B-YFP *AT3G47600*	*MYB94*
5	pGreen:H2B-YFP *AT5G54630*	*ZINC FINGER PROTEIN-RELATED*
6	pGreen:H2B-YFP *AT5G46880*	*HOMEODOMAIN GLABROUS 5*
7	pGreen:H2B-YFP *AT4G2170*	*DUF2921*
8	pGreen:H2B-YFP *AT5G64060*	*ANAC103*
9	pGreen:H2B-YFP *AT1G07640*	*OBP2*
10	pGreen:H2B-YFP *AT4G01250*	*WRKY22*
11	pGreen:H2B-YFP *AT5G57660*	*BBX6, COL5*
12	pGreen:H2B-YFP *AT2G38340*	*DREB19*
13	pGreen:H2B-YFP *AT2G27050*	*ETHYLENE-INSENSITIVE3-LIKE 1*
14	pGreen:H2B-YFP *AT5G52170*	*HOMEODOMAIN GLABROUS 7*
15	pGreen:H2B-YFP *AT1G54160*	*NUCLEAR FACTOR Y*
16	pGreen:H2B-YFP *AT2G31730*	*bHLH*

3.3 T-DNA genotyping

T-DNA line seeds were planted in MS agar plates and were subjected to vernalization. Vernalization promotes early flowering in seeds that are exposed to cold temperature (4^0C) for about 48 hours. The seeded plates were then transferred to plant growth chambers. Genomic DNA from leaves of T-DNA lines was extracted and used as a template for PCR based amplification of DNA fragments spanning T-DNA insertion site borders. Modifications were made in the PCR conditions to get amplification for confirming the T-DNA. I have carried out genotyping of 19 T-DNA lines.

Table.2. A list of T-DNA seed lines that have been genotyped.

Sl. No.	T-DNA lines
1	*HDAC CS368124*
2	*AtML1 SALK_072206c*

3	*HMG SALK_100002c*
4	*HDG4 CS303999*
5	*HDG7 SALK_132114c*
6	*NF-YA82 C5332364*
7	*TCX2 SALK_021952T3-BULK*
8	*ERF9 SALK_091532c*
9	*HDG5/HB-7 SALK_007515*
10	*HDG12 SALK_127261c*
11	*HDG2 SALK_138646c*
12	*Dof type At2g28810SALK_056801c*
13	*Dof type At2g37590C5337278*
14	*SALK_144950c 2g38340AP2 Domain*
15	*Dewax SALK_0151822*
16	*SALK_130584 At1g64620 Dof*
17	*SALK_139727c At1g22190c AP2*
18	*SALK_144950c At2g3840A2*
19	*SALK_114390c At1g22190c*

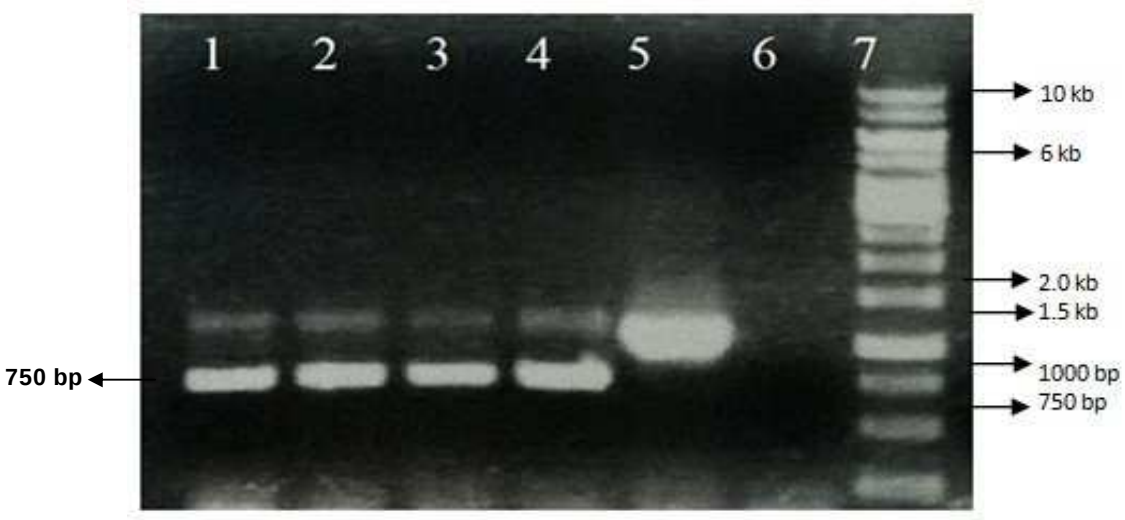

Fig.5. Confirmation of the T-DNAs in the plant lines using PCR. Lane 1-4: PCR amplified product from genomic DNA isolated from T-DNA seed lines using T-DNA specific primers. We have used multiple plants of the same T-DNA seed line in different lanes. Lane 5: Positive control with wild type genomic DNA. Lane 6: Negative control without adding any template. Lane 7: 1kb molecular weight marker. Expected size of T-DNA sequence is around 750 base pairs. Primers spanning the T-DNA insertion borders as well as that spans the specific gene were used for PCR. Occurrence of two bands in each lane concludes these T-DNA lines to be heterozygote. Whereas the presence of just one set of bands would confirm the lines to be homozygote.

3.4 Discussion

Development of plant organs is regulated by differential gene expression at various levels. Transcriptional regulation plays a major role in the SAM and is controlled by interactions between transcription factors and cis-regulatory elements of promoters. Hence it is important to know more about the valid interactions between transcription factors and promoters. The work has to be validated also by generating mutations in the putative *cis*-regulatory elements in the promoter and reproducing yeast one hybrid data thrice.

Analysis of network

The interaction network was analysed and promoters of three genes (ANAC075, ANAC103 DREB19) were found to be regulated by maximum number of TFs.ANAC075 is a NAC domain containing TF that plays a major role in sequence specific DNA binding activity and transcription. The gene is expressed in inflorescence meristem. Similarly ANAC103 is another NAC domain containing protein involved in multicellular organismal development and regulation of transcription. DREB19 encodes a member of the DREB subfamily A-2 of ERF/AP2 transcription factor family. This gene is involved in response to drought.

In-vitro and in-vivo validation of the network

In future, Y1H at a large scale is required to make comprehensive networks. Recently, Shivani successfully tested Singer robot for colony picking and mating procedure. More bait will be included against the transformed prey library for mating. Furthermore, these interactions will be analysed and networks will be constructed. For validating the networks and extracting the regulatory aspects, TFs acting as hub will be deleted by T-DNA mutagenesis and quantitative PCR will be performed in the genetic background to ascertain the activating or repressive behaviour of TF on each edge. Finally, mutation in the cis- regulatory element of the promoter will be carried out and will be tested in planta to see the contribution of input in regulating the target. For selected TFs, electrophoretic mobility shift assays will be performed and hence allowing the validation of true interactions. Promoter- reporter fusion assay could be used to understand

the underlying functional importance of the genes that play a key role in development. Understanding the whole interaction network enables us to point out the key players of regulated gene expression. In future, we would like to work on methods that could be used to map, at a large scale, the transcriptional activity of each TF. If we are aware about the specific interacting partners, it would also help us in the long run to target our research to various fields of agricultural benefits, including increased crop production. Hence we believe that this research is having the potential to benefit the society.

Chapter 4

BIBLIOGRAPHY

a) Deplancke, B., Dupuy, D., Vidal, M., & Walhout, A. J. M. (2004). A Gateway-compatible yeast one-hybrid system. *Genome Research*, *14*(10 B), 2093–2101. doi:10.1101/gr.2445504

b) Feng, S. Y., Ota, K., & Ito, T. (2010). A yeast one-hybrid system to screen for methylated DNA-binding proteins. *Nucleic Acids Research*, *38*(20). doi:10.1093/nar/gkq757

c) Fields, S., & Song, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature*, *340*(6230), 245–246. doi:10.1038/340245a0

d) Guo, M., Feng, H., Zhang, J., Wang, W., Wang, Y., Li, Y., … He, Z. G. (2009). Dissecting transcription regulatory pathways through a new bacterial one-hybrid reporter system. *Genome Research*, *19*(7), 1301–1308. doi:10.1101/gr.086595.108

e) Ito, T., Chiba, T., Ozawa, R., Yoshida, M., Hattori, M., & Sakaki, Y. (2001). A comprehensive two-hybrid analysis to explore the yeast protein interactome. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(8), 4569–4574. doi:10.1073/pnas.061034498

f) Ito, T., Ota, K., Kubota, H., Yamaguchi, Y., Chiba, T., Sakuraba, K., & Yoshida, M. (2002). Roles for the two-hybrid system in exploration of the yeast protein interactome. *Molecular & Cellular Proteomics : MCP*, *1*(8), 561–566. doi:10.1074/mcp.R200005-MCP200

g) Meng, X., & Wolfe, S. A. (2006). Identifying DNA sequences recognized by a transcription factor using a bacterial one-hybrid system. *Nature Protocols*, *1*(1), 30–45. doi:10.1038/nprot.2006.6

h) Mitsuda, N., Miho, I., Takada, S., Takiguchi, Y., Kondou, Y., Yoshizumi, T., … Ohme-Takagi, M. (2010). Efficient Yeast one-/two-hybrid screening using a library composed only of transcription factors in Arabidopsis thaliana. *Plant and Cell Physiology*, *51*(12), 2145–2151. doi:10.1093/pcp/pcq161

i) Ouwerkerk, P. B. F., & Meijer, A. H. (2011). Yeast one-hybrid screens for detection of transcription factor DNA interactions. *Methods in Molecular Biology (Clifton, N.J.)*, *678*, 211–227. doi:10.1007/978-1-60761-682-5_16

j) Parrish, J. R., Gulyas, K. D., & Finley, R. L. (2006). Yeast two-hybrid contributions to interactome mapping. *Current Opinion in Biotechnology*. doi:10.1016/j.copbio.2006.06.006

k) Pyvovarenko, T., & Lopato, S. (2011). Isolation of plant transcription factors using a yeast one-hybrid system. *Methods in Molecular Biology*, *754*, 45–66. doi:10.1007/978-1-61779-154-3-3

l) Takada, S., & Jürgens, G. (2007). Transcriptional regulation of epidermal cell fate in the Arabidopsis embryo. *Development (Cambridge, England)*, *134*(6), 1141–1150. doi:10.1242/dev.02803

m) Yadav, R. K., Girke, T., Pasala, S., Xie, M., & Reddy, G. V. (2009). Gene expression map of the Arabidopsis shoot apical meristem stem cell niche. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(12), 4941–4946. doi:10.1073/pnas.0900843106

n) Yadav, R. K., Perales, M., Gruel, J., Ohno, C., Heisler, M., Girke, T., … Reddy, G. V. (2013). Plant stem cell maintenance involves direct transcriptional repression of differentiation program. *Molecular Systems Biology*, *9*(654), 654. doi:10.1038/msb.2013.8

o) Yadav, R. K., Tavakkoli, M., & Reddy, G. V. (2010). WUSCHEL mediates stem cell homeostasis by regulating stem cell number and patterns of cell division and differentiation of stem cell progenitors. *Development (Cambridge, England)*, *137*(21), 3581–3589. doi:10.1242/jcs.082420

NOTATION

- 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 - Muraschine Schoog
- 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