Determining the protein-DNA interactions for transcription factors specific to L1-L2 layer of shoot apical meristem in *Arabidopsis thaliana*

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A dissertation submitted for the partial fulfilment of BS-MS dual degree in Science

Under the guidance of
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Certificate of Examination

This is to certify that the dissertation titled "Determining protein-DNA regulatory network for epidermal and sub-epidermal cell type enriched transcription factors in *Arabidopsis thaliana*" submitted by Ms. Meghna Thakur (MS15176) for the partial ful-filment 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.

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(Supervisor)

Dated: June 15, 2020

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.

Meghna Thakur

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Dated: June 15, 2020

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)

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Abstract

Several complex processes characterize development in Arabidopsis thaliana and one of them is the establishment of meristems during embryogenesis. Meristems are formed at the two opposite ends of the embryo and thus are called the root apical meristem (RAM) and the shoot apical meristem (SAM). SAM can be further divided into three zones, namely central zone (CZ), peripheral zone (PZ), and rib meristem (RM). The plant SAMs comprise of well-defined cell layers as well (Ottoline Leyser & Furner, 1992). A number of genetic studies have been done in the past to comprehend the formation of organs and stem cell specification in SAM. But None of them were focused precisely on unraveling the regulatory mechanisms underlying this sophisticated arrangement of SAM (S. M. Brady et al., 2007; Jiao et al., 2009).

Several network studies are coming into picture nowadays that turn out to help handle large data sets and thereby elucidating the physical interaction between sequence-specific regulatory transcription factor proteins and their respective target sites (Alexander M. Jones et al., 2014; Mukhtar et al., 2011). In the Y1H screen, 37 DNA baits were successfully screened against a library of 321 TF prey proteins at 22ºC, which is known to be the ideal temperature for Arabidopsis to grow. A total of 78 interactions could be made out using the Y1H screen among 22 DNA baits, and 54 TF protein preys. The network consists of 69 nodes connected through edges. The edges signify the physical interaction among the nodes.

Chapter 1

Introduction

1.1 *Arabidopsis thaliana*: The Model Organism

1.1.1 History of Arabidopsis Research

Arabidopsis thaliana, commonly known as thale cress, mouse-ear cress, or Arabidopsis, is a member of the Brassicaceae family. This family of plants also includes cultivated species such as Broccoli, Turnip, Cabbage, and Radish. It is a flowering plant native to Eurasia and Africa. It is considered as a weed that grows in sandy, rocky terrains. Discovered by Johannes Thal in Harz mountains, a mutant of Arabidopsis was reported as early as in 1873 (Meyerowitz, 1998). Friedrich Laibach, a professor at Frankfurt University, was the first person to describe the potential of *Arabidopsis thaliana* for genetic studies in 1943, and thus he is considered as the founder of experimental Arabidopsis research (Koornneef & Meinke, 2010; Meyerowitz, 1998). Gerhard Röbbelen reported a large variety of X-ray mutants affecting pigment development in 1957 and, subsequently, in 1962, published the first data on chemical mutagenesis using urethane in Arabidopsis (Koncz, Chua*, &

Schell, 1992). As stated in an article titled "Arabidopsis comes of age," published in Science in 2000, Rédei and Koornneef were the guys that kept the torch burning (for Arabidopsis) during the dark ages. One of the significant contributions by Rédei in plant research was to write scholarly reviews on Arabidopsis.

1.1.2 Characterization of a model plant

Although Arabidopsis is not an economically important plant, it acquires certain traits that make it suitable for physiological, biochemical, and genetic studies. A few of such characteristics are ("bio0202-Members of the Multinational Arabidopsis Steering Committee," n.d.): a) Relatively small, genetically tractable genome as compared to many crop species which is the smallest one among all known angiosperm species and helps in simplifying and facilitating genetic analysis. b) Fast life cycle, i.e., a short generation time of about six weeks under optimal conditions. c) The self-pollinating plant thus produces many self progenies. d) Minimal space requirements and can be easily grown in indoor growth chambers. e) Transgenics can be easily generated using *Agrobacterium*-mediated transformations. It is known that polyploidy in the case of many crop species leads to their large genome sizes, and there are no large classes of genes present in such species that are not present in Arabidopsis. All of these characteristics make *Arabidopsis thaliana* a representable and reasonable model for the plant kingdom. As many advances have been reported given the understanding of Arabidopsis over the past few years, it has become universally recognized as a model plant for genetic research ("The Role of Arabidopsis in Plant Science Research," n.d.). Several fundamental findings were made first in plants and then later turned out to play an essential role in understanding human biology and health conditions (Alan M. Jones et al., 2008).

1.2 Meristems and Stem Cell Maintenance

Plant growth and development is a phenomenon that goes on throughout its lifetime. Plants, being multicellular organisms, grow through a combination of processes such as cell growth and cell division, particularly mitosis. A group of undifferentiated cells is present at the tip or apex of the plant called meristem. These undifferentiated cells acquire the property of actively dividing, a feature similar to that of embryonic cells, long after the embryogenesis is over (Fosket, 1994). There are two primary meristems present at the two opposite poles or extreme points of the plant body, i.e., the shoot apex and root apex. The meristems thus established are termed as apical meristems. From SAM, originate many organs like stem, leaves, flowers, etc.. In contrast, RAM gives rise to the whole root system ("Meristem - an overview | ScienceDirect Topics," n.d.) ("Meristem Cells - Types, Characteristics, Functions, Vs. Stem Cells," n.d.). The meristematic tissue contains a pool of pluripotent stem cells in specific microenvironments. Such stem cell niches are responsible for supplying cells, secrete signal molecules, and transcription factors for various purposes, namely regulation of the rate of organogenesis, cell fate specification, self-renewal and differentiation (Papayannopoulou & Scadden, 2008) (Beauzamy, Louveaux, Hamant, & Boudaoud, 2015).

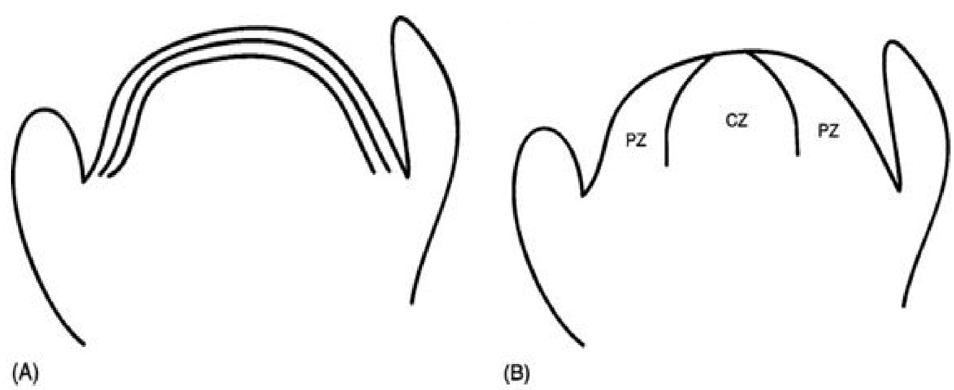

Figure 1: Shoot Apical Meristem (A) Clonally distinct layers in SAM (B) PZ: Peripheral Zone, CZ: Central Zone ("Meristem - an overview | ScienceDirect Topics," n.d.)**.**

The SAM of angiosperms is categorized into two regions: (i) Tunica and (ii) Corpus per Tunica Corpus theory proposed by Schmidt in 1924. This theory is the currently accepted theory of the apical organization of the plant shoot system. It suggests that Tunica consists of one or more peripheral cell layers masking the Corpus that primarily constitutes a mass of cells. The properties of cells making up the tunica are: (a) They are smaller in size than the cells of the Corpus region. (b) They show anticlinal cell division and assist in increasing surface area. The features of cells of Corpus are: (a) They divide in all plains, i.e., they show both anticlinal and periclinal division. (b) They assist in increasing volume. The relative levels of the hormones Cytokinin and auxin direct the developmental fate of the regenerating tissues in the shoots and roots. High Cytokinin to auxin ratio leads to induction of shoot production, whereas low Cytokinin to auxin ratio induces the root production (Skoog, Biol, & 1957, n.d.).

As per the typical organization of the SAM of higher plants, the tunica region can further be divided into the epidermal or L1 layer, and the sub-epidermal or L2 layer and Corpus form the third layer named L3 layer (Satina, Blakeslee, & Avery, 1940). All of these three layers expand throughout the central and peripheral zone, and the central zone (CZ) present at the shoot apex is the location of the stem cells. From stem cells, arise the progenitors, which further amplify into daughter cells that eventually move into the peripheral zone (PZ). Finally, in the PZ, these daughter cells enter differentiation and form organ primordia. The niches of the SAM and RAM, along with the organizing center (OC) and the quiescent center (QC), set up an accurate balance between stem cell maintenance and differentiation of the progeny cells (Stahl, Wink, Ingram, & Simon, 2009).

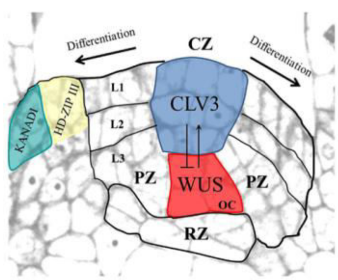

Figure 2: SAM organization in *Arabidopsis thaliana* (Boscá, Knauer, & Laux, 2011)**.**

There are several genes involved in the formation of SAM during embryogenesis and its maintenance during post-embryonic development such as SHOOT MERISTEMLESS (STM) (Barton & Poethig, 1993), WUSCHEL (WUS) (Mayer et al., 1998), CUP SHAPED COTYLEDON 1 and 2 (CUC1 and CUC2) (Takada, 2001)(Aida, Ishida, Fukaki, Fujisawa, & Tasaka2, 1997), etc. Genetic studies in the past have revealed the existence of a feedback loop between WUS and CLAVATA3 (CLV3) in the SAM that regulates the count of stem cells (biology & 2010, n.d.; Tucker, biology, & 2007, n.d.). The QC acts as a stem cell reservoir in the RAM, which primarily arrests cell differentiation without directly regulating the cell division in the columella (Van Den Berg, Willemsen, Hendriks, Weisbeek, & Scheres, 1997). The OC in the SAM is a small group of cells present underneath the stem cells, which expresses the homeodomain transcription factor WUS. WUS contributes to the process of stem cell maintenance in two ways: (A) WUS protein show lateral migration into the differentiating progeny of stem cells through intercellular channels called plasmodesmata (Yadav, Perales, & Gruel, 2011). (B) WUS directly binds to the promoter of CLV3, thereby promoting its transcription, whereas CLV3 repress WUS RNA expression at the OC (Schoof et al., 2000). CLV3 encodes a 13-amino acid long extracellular protein processed from a pre-protein containing 96 amino acids (Fletcher, 1999; Sharma, Ramirez, & Fletcher, 2003). Thus the negative feedback loop between WUS and CLV3, where CLAVATA1 (CLV1) and CLV3 proteins form a potential receptor and ligand pair, helps in regulation of stem cell proliferation and maintenance of stem cell number (Fletcher, 1999) (Daum, Medzihradszky, Suzaki, & Lohmann, 2014; Trotochaud, Jeong, Science, & 2000, n.d.).

1.3 Understanding Transcription Factors, Gene Expression and Regulatory Networks

The regulation of gene expression is one of the most complex mechanisms in cells as it involves the participation of a number of phenomena such as signal transduction pathways, protein-protein interactions, and their movement between cellular compartments, synthesis, and processing of RNA, etc. To understand how such processes operate at the molecular level, one needs to acquire proper knowledge of transcription. Many comprehensive techniques and approaches have been developed to study the functionality of transcription factors in vitro and in vivo. In the early days of molecular research, gene trapping systems have been put to use in determining the activities for numerous genes from TFs, metabolic enzymes, and protein kinases. Such gene trap systems utilize a variety of reporter genes such as jellyfish green fluorescent protein (GFP), bacterial beta-glucuronidase (GUS), and firefly luciferase (Luc) to visualize the expression of plant genes and hence, trace down the gene activity (Koo et al., 2007). Each of these reporters has advantages and disadvantages of its own (Haseloff, ed.), & 1995, n.d.; Jefferson, Kavanagh, & Bevan, 1987; Quaedvlieg et al., 1998). Thus, the reporters should be selected based on the study purposes and end-point measurements. For instance, for visualizing the spatial and temporal patterns of gene expression in adult plants, the reporters based on GFP turn out to be the best tool (Soboleski, Oaks, & Halford, 2005).

1.3.1 Gene Expression Patterns of TFs

It is known that for a large number of genes, their upstream sequences are accountable for the modulation of the expression patterns. However, some evidence indicates that the transcribed regions contain some other sequences within themselves that control gene expression patterns (Hong, Hamaguchi, Busch, & Weigel, 2003; Ito, Sakai, & Meyerowitz, 2003). Let's illustrate this briefly. The *Arabidopsis thaliana* floral homeotic gene *AGAMOUS (AG)* is a C-class gene that encodes a MADS-box transcription factor (Yanofsky et al., 1990). A 3kb intron is present in the second position within the AG gene, which derives its accurate expression (Hong et al., 2003).

But further studies indicate that for a lot of genes, their endogenous expression can be effectively driven by only the upstream sequences (Lee et al., 2006; Levine, National, & 2005, n.d.). In a study done by Ji-Young Lee et al. (2006), the regulation of TFs expressed in a tissue-enriched manner had been investigated in Arabidopsis roots. For this, the researchers had constructed GFP transcriptional and translational fusions for 61 TFs. Further, the endogenous expression was directed by a 3kb promoter sequence that was present upstream of the translational start site. For 80% of the total 61 TFs tested, the native mRNA expression could be reiterated by the upstream promoter of 3kb size as predicted by the microarray study they had performed earlier. These observations suggested that the endogenous expression of a gene is driven by the regulatory elements present within the upstream 3kb promoter segments.

The TFs bind to cis-regulatory elements that are present in the neighboring regions of the structural genes. This binding plays a crucial role in deriving the expression of several genes and their downstream regulation (Ho & Geisler, 2019), which further leads to various developmental outcomes such as maintenance of the day-night cycle, resistance against diseases and pest infection, etc. Recently, Many interesting techniques like RNA sequencing (RNA-Seq) and high throughput sequencing of whole transcriptomes have been playing a central role to recognize, under specific environmental conditions, the transcriptional signatures in a Spatio-temporal manner (S. M. Brady et al., 2007; Jiao et al., 2009; Schmid et al., 2005). Additionally, some computational and network-based approaches have entered this procedure to address the gene interactions, both genetically and physically to deduce meaningful results from large data sets generated using high throughput techniques (Dreze et al., 2011; Alexander M. Jones et al., 2014; Mukhtar et al., 2011).

1.3.2 Studies Based on Gene Regulatory Networks in *Arabidopsis thaliana*

The last structure and capacity of a plant is the consequence of interactions among various components present within complex gene regulatory networks (GRNs). Such systems occur at different levels: within a cell, among multiple cells of a similar or different type, etc. To decide on the properties of plant biological systems, these networks are acted upon by the evolutionary forces, which further give rise to diversity in plant morphology across various species. In GRNs, nodes signify genes. The nodes are associated by means of edges. An assortment of significant collaborations can be portrayed by these edges, for example, cooperations among proteins and connection between translation factors and administrative areas in their downstream target DNA. The genes which are highly connected in such a scale-free network topology are called hub genes. Many network-based approaches have proven to be useful in figuring out the regulatory module acting inside the plant body. This can be useful in understanding the intricacy of the plant all in all frameworks. To identify the components of plant regulatory networks, several methods have been generated that work efficiently in a high throughput manner (S. Brady, Long, cell, & 2006, n.d.; Busch, Biology, & 2007, n.d.; Jones-Rhoades, Bartel, & Bartel, 2006; Willmann, biology, & 2007, n.d.; Yazaki, Gregory, biology, & 2007, n.d.).

Many researchers have exploited yeast one-hybrid (Y1H) to create regulatory networks of TFs in different parts of Arabidopsis. Molecular techniques like chromatin immunoprecipitation coupled with quantitative polymerase chain reaction (ChIP-qPCR) and real-time quantitative reverse transcription PCR (qRT-PCR) can produce gene regulatory networks that can serve as powerful tools to discover molecular phenotypes, validate Y1H predicted interactions and for building verifiable conjectures. The functions of a gene under analysis can be investigated in the literature; however, network studies can supply the missing links in a few cases.

1.4 Motivation for the Current Work

According to the central dogma of molecular biology, a gene first gets transcribed into an RNA molecule, which then translates into the protein. Proteins play primary biological functions in an organism. The growth and development of a plant depend on many environmental and genetic aspects such as expression and regulation of certain genes activated under specific conditions or time of the day. TFs play a vital role in the gene regulation machinery as they help in reading the information from DNA. A TF can act either as a monomer or form dimer with itself as well as with other TFs. TF can also recruit co-activator which assist in activating transcription. In case the same TF recruits a co-repressor, transcription of the target gene is inhibited.

The invention of high throughput approaches helps us in building predictive networks based on the co-expression of targets and upstream regulators, which can be tested in vivo. However, it is apparent by recent studies that many predictive systems built based only on the gene expression data do not hold in many instances when tested in vivo. Therefore, to identify the bindings of TFs to their cognate promoters, more direct approaches are needed. Some examples of heterogeneous techniques that can map physical interaction between TFs and their target proteins are such as mapping of DNase I hypersensitive sites, ChIP coupled with sequencing (ChIP-Seq), and heterologous systems such as Y1H, etc.

1.4.1 Transcription Factor Centered Approach

The transcription factor centered approach primarily takes into account the transcription factor's downstream target sites. A better understanding regarding the interaction of a TF with its downstream partners assists us in explaining its function better. Furthermore, it aids in analyzing the role of a TF in regulating definite features and intricacies of a complex network. The binding of a TF to its target gene promoters can be made out by recruiting high-quality antibodies. Chromatin immunoprecipitation(ChIP) is the most commonly used technique. High throughput sequencing techniques are used to sequence the library generated by ChIP. After analyzing and aligning the DNA sequence to the genome, its distribution across the genome and TF target genes' complexity can be traced. There are some drawbacks to this particular approach. One of them is that one can produce high-quality ChIP-Seq data only for the tissues having plenty of TF. Another drawback, the major one, is that, against most of the TFs, high-quality antibodies are not available. This is where the gene-centered approaches come into play.

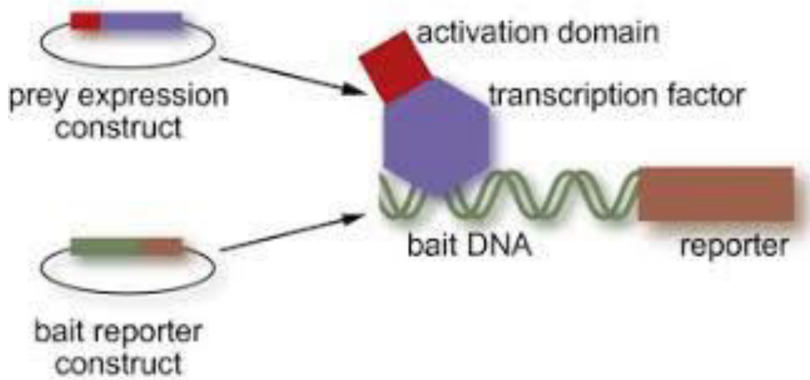

Figure 3: Yeast-one-hybrid assay (Reece-Hoyes & Marian Walhout, 2012).

1.4.2 Gene Centered Approach

The focus of the gene-centered approach is to identify the upstream regulators of the target genes. In this approach, Y1H is one of the *in vitro* techniques that are routinely used for such purposes. Which TF binds to the DNA segment of interest can be determined by using Y1H. With access to a complete library (of TFs) of *Arabidopsis* and maize, high yield Y1H studies can be planned to find out the potential targets at the genome-scale (Burdo et al., 2014; Petricka, Winter, & Benfey, 2012). This method has been used by several research groups to map gene regulatory networks in *Drosophila and C. elegans,* along with *Arabidopsis* (Deplancke et al., 2006; Taylor-Teeples et al., 2015). Mapping based on Y1H is made accessible due to access to the high throughput instruments. Through the integration of experiments, meaningful data is generated, further resulting in a functional module that might be useful for the cell and tissue specialization and their function.

Plant morphology and reproductive development are strongly influenced by ambient temperature (Quint et al., 2016). According to a report by The Arabidopsis Biological Resource Center 2015, the optimal temperature for the growth of the Arabidopsis plant is 22-23ºC. This implies that Arabidopsis TF proteins would be the most stable and function efficiently at this temperature. Taking this into consideration, the yeast-one-hybrid screening was done at 22ºC and was compared with the one that had been performed at 30ºC.

Chapter 2

Materials And Methods

2.1 Molecular Biology Techniques

2.1.1 Preparation of Yeast Competent Cells

- Primary (1^o) culture: Single yeast colony from yeast cells growing on agar plates was inoculated in 20 ml YPD* liquid medium, and the culture was allowed to grow overnight at 30ºC.
- Secondary (2^o) culture: The growing culture was then diluted to attain final OD_{600} = 0.2 in 200ml of the YPD medium.
- The culture was incubated for 4-5 hours at 30ºC before the optical density (OD)goes from 0.2 to 0.6-1.0.
- The cells were spun down at 4000 rpm for 5 minutes at room temperature, i.e., 25ºC.
- After washing the pellet with 100 ml distilled water and cell re-suspension, cells were pelleted again at 4000 rpm for 5 minutes at 25ºC or room temperature.
- Then the pellet was dissolved in 1/10th volume of SORB buffer**.
- The cells were again spun down for 5 minutes at 4000 rpm and dissolved in SORB buffer (1440μl) and salmon sperm DNA (160 μl).
- Aliquots (20 μl each) of re-suspended cells were then made and stored at -80ºC.

*Composition of YPD media (1 litre):

10 gm (1%) of yeast extract,

20 gm (2%) of peptone,

20 gm (2%) of glucose and

40 mg (0.004%) of adenine hemisulfate.

**Composition of SORB buffer:

100mM LiAc,

10mM Tris HCl pH 8.0,

1mM EDTA pH 8.0,

1M Sorbitol.

For 500 ml volume of SORB, we put 5.1 gm LiAc, 91 gm Sorbitol, 0.5 ml Tris HCl and 1 ml EDTA.

2.1.2 Yeast Transformations

- 20 µl aliquot of competent cells was taken, and plasmid DNA (2.5 µl) was added to it.
- A sterile solution of 40% PEG* was then made and was purified using filter sterilization.
- 120 µl of 40% PEG (~ 6 X Volume of competent cells and plasmid) was put into the mixture of competent cells and plasmid.
- The incubation of this mixture was then done for 30 minutes at 30ºC, after which it was incubated at 42ºC for further 30 minutes.
- After incubation, the cells were put on ice for 5 minutes and then spun down for 5 minutes at a speed of 4000 rpm.
- The pelleted cells were re-suspended in water (100 µl), after which they were plated on respective drop out media plates.

*Composition of 40% PEG mixture:

100 mM LiAc,

10 mM Tris HCl pH 8,

1 mM EDTA pH 8,

40% v/v of polyethylene glycol (PEG).

This mixture was then decontaminated using the process of filter sterilization.

2.1.3 Bacterial Competent Cell Preparation

- 500 µl of primary (1°) culture was inoculated in 500 ml of SOB*, and the culture was kept for shaking at a temperature of 37°C till OD = 0.5 is achieved.
- The culture was then kept on ice for 10 minutes.
- The cells were spun down at 4°C at a speed of 3500 rpm for 10 minutes.
- The pellet was dissolved in 25 ml of TB1** and then kept on ice for 10 minutes.
- The cells were again spun down at 3500 rpm at 4°C for 10 minutes.
- Then the pellet was dissolved in 4 ml of TB2***, and 140 µl DMSO was added to it.
- The cells were kept on ice for 15 minutes.
- After adding 140 µl of DMSO, aliquots were made and stored at -80°C for future use.

Composition of SOB (100 ml):

Tryptone - 2 gm

Yeast Extract – 0.5 gm

NaCl – 0.05 gm

Autoclaved it and then 2 ml of autoclaved $MgCl_2$ (1M) was added.

**Composition of Transformation Buffer 1(TB1) (100 ml):

1 ml of 1M MOPS (pH 6.5 with KOH);

10 ml of 1M KCl;

4.5 ml of 1M $MnCl_2$;

1 ml of 1M $CaCl_2$;

1 ml of 1M KAc;

***Composition of Transformation Buffer 2(TB2) (100 ml):

1 ml of 1M MOPS (pH 6.5 with KOH);

10 ml of 1M KCl;

4.5 ml of 1M $MnCl_2$;

1 ml of 1M $CaCl_2$;

1 ml of 1M KAc;

12.5 ml of glycerol;

For both T.B.1 and T.B.2, sterile water was used to make up the final volume.

2.1.4 Bacterial Transformation

- Bacterial competent cells were taken, and transformation buffer 3* was added in 1:1 ratio to the cells.
- After adding plasmid DNA, the cells were put on ice for 30 minutes.
- Heat shock was given for 60 seconds at 42^{o}C, and cells were kept again on ice for 5-10 minutes.
- Then 1 ml of LB broth was added to these cells, and the mixture was incubated at 37^{o}C for one hour.
- At last, all the components were poured onto a plate having suitable growth media.

*Composition of T.B.3:

100 mM $CaCl_2$ and 50 mM $MgCl_2$ were autoclaved separately and mixed.

2.2 Methodology

- A library of a total of 340 transcription factors (preys) was made and stored at -80^{o}C in four 96-well deep-well plates by a former lab member.
- The preys which could not be revived in the round 1 were rescued using scrapped yeast or individual stocks belonging to Brady's library.
- After rescuing 311 preys out of 340, the next thing to be done was to add some new preys to the existing library. As the previous TFs were transformed into the yeast strain Yα1867, Yα1867 competent cells were freshly prepared, and ten new preys were transformed into them.
- Screening against eight baits was done using yeast one-hybrid technology in the first round. As we needed a precision method for pinning yeast colonies, High-throughput Microbial Array Pinning Robot was utilized.

- In another round of reviving preys, all the transcription factors were rescued again to categorize the preys forming more than five colonies. The TFs giving rise to no colonies or less than five colonies were transformed once more into freshly prepared Yα1867 competent cells.
- In the second round of reporter based screening, screening was done against 29 baits. For imaging purposes in both rounds, the Epson Perfection V600 Photo scanner was used to scan the yeast plates. Microsoft picture manager software was used for the manipulation of images such as brightness and contrast.

2.3 Y1H Assay

The promoter is cloned upstream of the reporter gene in Y1H assay for which reporters may either be auxotrophic or emit color. This assay requires the translational fusion of the TF protein to the GAL4-AD activation domain. Reporter activation is the consequence of the physical interaction between the TF prey protein and the DNA bait.

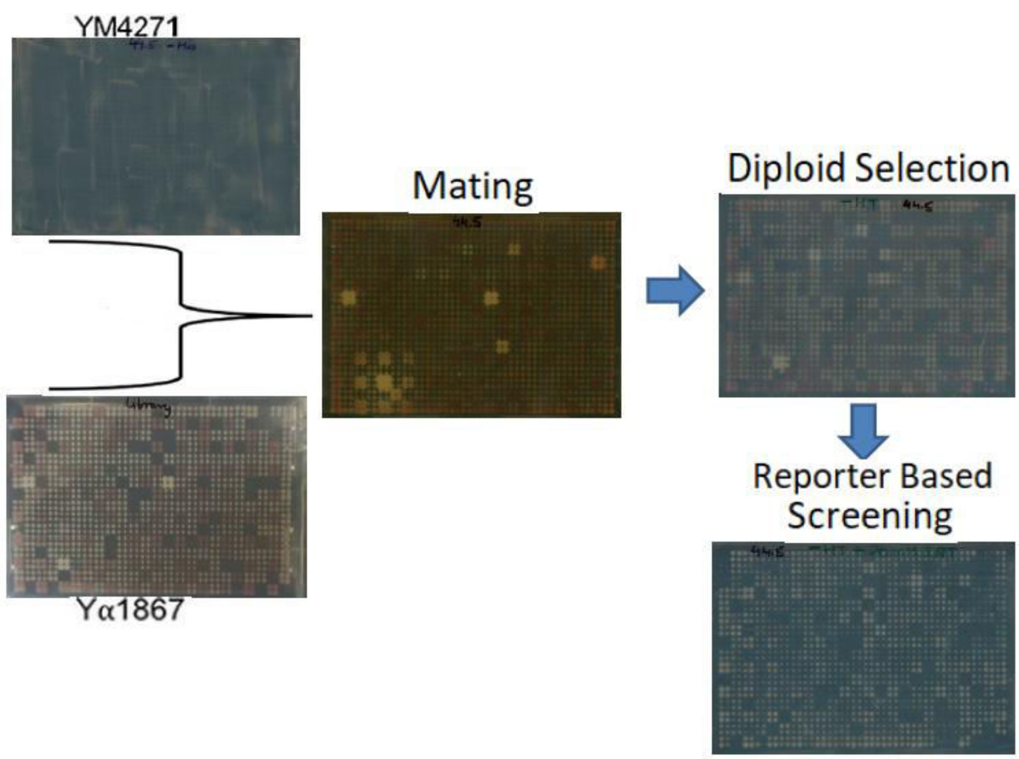

Figure 4: Y1H assay for shoot enriched transcription factors.

The DNA baits were transformed into YM4271 strain of yeast, and the TF prey proteins were transformed into Yα1867 yeast strain. The two strains were permitted to mate, and afterward, diploids were chosen, trailed by plating of diploid cells on proper specific media to finish up associations.

Chapter 3

Results and Conclusions

3.1 Preys: Cell type-specific and broadly expressed TFs

Three hundred and twenty-one TFs that are found to be enriched in epidermal and sub-epidermal cell layers of SAM was used as preys against the 37 DNA baits. Other than cell-type-specific TFs, broadly expressed TFs are also known to play a role in regulating gene expression. Hence, both broadly and narrowly expressed TFs were chosen as preys in the Y1H as it would allow for the identification of protein-DNA interactions that correspond to both transcriptional activation and suppression. Here is the list of preys that were newly transformed and added to the existing library:

1. GRF1
2. GRF2
3. GRF3
4. ARF4
5. ARF8
6. ARF11
7. pDEST AD2μ
8. HY5(I)
9. HY5(II)
10. PIL5

Table 1: Prey Plate Data.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	ANAC103	PIL5		5A2/ REIL1	AZF3	5C9/ PUX2	DEL2	5C12/ SCL5	NAC075	5D1/ 1G22810	NAC073	5D2/ ATHB13	TCX2	5D11/ RAP2.12	CRF6	5E1/ 1G22810	GRF2	5E5/ 5G52010	AP2/ 2G3834	5E9/ 5G44080	DOF/ 7590	5E11	WRKY25	
B	2H3/ 4G37294		H5/ LT2		2C1/ VRKY21	7C10/ SCL14		7D4/5G 61890	A10/DOF 2G2851	7B11/ ARF12	2H8/FL FLO2	7D7/ BBX18	3A1/ MYBR	7B10/4 G 25210	4C5/ NGAL	7B12/ AIF1	4C11/ IAA7	6F5/1G 74840	3A3/ NAC017	6F6/ ATHB4	3A5/ AL2	6F8/ BZIP68	5A6/ AT- ISFC1	6G1/ AIB/ JAM1
C	2G31930		2G28810	5F5/ NF- YB13	WOX2	5F6/ TLP3	ANAC010	5F8/ CAMTA 2	'LP8	5F9/ BZO2H 1	OBP2	5F11/ MYB65	g01460	5F12/ TCP8	GRF1	5G3/ RHL41	CBF	5G5 AC062	HDC	5G9/ 1G0538 0	DDF		HDG1	
D	3A9/TIP; ANAC091	6F2/ ERF104	5B5/AP 4G2814	7C9/ ANT		7C5/ CRF2		6F3/ WRKY6 5	B8/CDF2	6G3/ HSL1	B1/MYB3	7A3/2G 44730	5D9/ THB30	7B3/ WRKY5	5D10/ TEM1	7B7/ NAC014	5E2/ TBZIP60	6F1/ GATA5	C5/ POPEY	6E12/ NAC047	5E8/ BZIP1	7D6/ 1G0488 0	F1/BME3; GATA8	6C3/ FLP
E	RAP2.4	5H4/ TLP9	CAL	5H5/ BBX8	DOF/ 1G29160	5A4/ ASIL1	STOP1	5A5/ 1G1752 0	ABF	5A7/AT - HSFB2 A	NF-YA		PAP1	3E5/ ATE2F2	AP2/1G 64380	3E6/DP A	ZN/1 7571	5A9/ ZCW32	VRKY3	5A10/ KUA1	BODENL S	5A12/ ERF2	VRKY17	3G9/ IAA19
F	5F7/ RAP2.4	6G9/ ERF6	3C8/ 5G6261	6G10/ ANAC082	3C10/ VRKY32	7D3/ WRKY53	3D1/ CIL1	7E9/ E2F3	5H1/ GATA	8A2/ AZF2	5H3/ AGL1	8B6/ MYBD	6A10/ /RKY22	8B5/ DPB	8D11/ TIFY2	8A1/ DREB2 A	8E1/ NF- B12	7E5/ BIM1	3D2/ COL	7D1	1B10/ HAT22	7E8/ ATHB1 6	3D3/ VRKY20	8A3/5G 07580
G	EIL1		/RKY54		EPELLAT 2	5B2/CCA	NF-YA	5B3/AT- HSFA6 B	MYB3	5B4/ 2G3895 0	MYB94	5B6/ RRTF1	/RKY22	5B8/ ZFP7	PDF2	5B9/ ZFP7	ZN/4C 16610	2E8 / CRF6	ATHB	5B10/ KNAT5	ATML		VRKY11	2G1/ NF- YB10
H	1B11/ UNE12	7E10/ VRN1	1C4/ AGL16	7D5/ ARF9	1C8/ OBF5	4F10/ KNAT6	1D2/ ERF105	7E3/ JMJ18	1D6/ SCL21	7C8/1G 75710	3D4/ BIM2	8A10/ AL3			1D12/ GT1	7E6/ MYB74	1E2/ CRF7	7F2/ OBP1	3D5/ AKS2	7E7/ MYB32	1E6/AP 2G4494	7C7/4G 39160	E11/AIF2; RITF1	
I	ATH1	2H1/ AXR3	MYB4	2H4/ ZML1	PDEST	2H6/ REF4	SHN2	3A4/2G 24060	ERF9	3A6/ OBP4	GRF 3	8A8/5G 26610	IYB111		ZN/5 54630	3A12/ MYB12	COL	3B6/ ANAC032	AP2/ DEWAX	8B8/ MYC3	MYB9	8B12/ GBF1	AP1	8D5/ AtHB23
J	3D6/1G 19000	7F5/ WRKY5 3	1F3/ ABF4	7F8/1G 05710	3D7/ EPR1	7F10/ SCL11	2A4/ ERF1	7F4/ WRKY6 9	2A5/ AtHB3	7G6/ POSF21	3D8/ TRB2	2E12/ IAA30	2A7/ ERF1	3E9/ RGA1	2A6/ FZF	7F11/ NF- YC9	3D9/ TRB	ARR10	3D10/ CDC5	8A8/5G 26610	3D11/ ASIL2	8B2/1G 61730	2A9/1G 03040	8B1/ ATHB5 4
K	AS1	2B10/ GATA3	pDE ST		MYB/ 04760	2A11/ ATHB- 12	HDG4		PATULA	1H10/3 G 05760	ARF5	1H6/ BHLH3 2	HY5 (	1G3/ WKY6	MYB5	1F4/ BBX31	/RKY48	1E3/ NF- YA5	PDES	1D7/ ANAC04	SPL15	8E7/ SERRAT	BHLH/3G 23690	
L	2A10/ NAM	8B11/ EDF3	2A12/ NAC2	8B9/5G 52020	2B2/ ILR3	8B7/1G 07520	3E11 ANAC008	8C1/ ZAT6		4D9/ VIP1	2B8/ AREB3	3G12/ NAC055	2B9/ COL3	ARR14	2B11 BZIP1		3F6/2 3693	8C6/ DEWA X	3G1/3 57480	8C4/ MYB32	2C5/AL	8C2/ AIL6	G6/IAA13	8C8/2G 31730
M	RWPPK	1C10/ MYB3R-4	BZIP6	8E12/ JMJ14	DOF/1 64620	8F1/ MYB73	Perianth	8F2/ TLP11	Phabulo	4C9/ SCL8	ZN/2C 21320	8F8/5G 01160	AtGRF	4C12/ ERF8	SEP4	4D1/ ATMYB 6	SEP3	4D7/ ERF3	AGL2	4D8/ WRI4	Monopol		bZIP52	4D10/ BZO2H 3
N	3G8/ NF-YA3	8C11/ PRMT3	6G8/ ARF18	8C10/ EIN3			4C3/2C 29060		2D8/ IAA9	8A6/ MUCI10	2D9/ ATHB21	8A5/ TLP6	5G11/ IAA16	8B4/ NAI1	8F6/ IAA8	8A9/ ZFHD1	2F3/2C 47890	3F1/ ERF11	2F4/ MYC4	3E12/ MYB15	5C4/ IAA18	8H2/ CRF5	2F7/ ATHB	8A4/ CIL2
O	ATHB15		WUS	4E1/ MBS2	VRKY21	4E6/ ATMY7 0	VRKY2	4F2/ MYB10 9	ARF 4	1C2/ MYB11 1	DAG		ARF8	2G12/ IAA14	JAIBA	4F11/ BLH7	ARF 11	8G2/ AIF4	HY5 (II)		HMG1/	4G10/ NF- YC3	PDEST	8G3/1G 76510
P		6A6/ AZF3		6G4/1G 44810		6G5/AKS ;FBH3	G2/ GA6	6G6/2G 19380	2G5/ VRKY33	6E9/ NLP6	2G7/ GATA	6E10/1G 58220	2G8/ TIFY	6E11/1G 63100	2G9/ ZAT11	7E4/1G 04850	7D5/ ARF9	6E6/2G 38090	2G1 GBF	6E7/ COL5		6E8/1G 43860		6E5/ DREB2 6

3.2 DNA Baits: Cell type-specific TFs

Out of 1456 genes enriched in all three cell layers of SAM, 535 are enriched in the L1 layer, 256 in the L2 layer, and 665 in the WUS domain (Yadav, Tavakkoli, Xie, Girke, & Venugopala, 2014). Of the 535 L1 layer enriched transcripts, 44 encode for TFs, of the 256 L2 layer enriched transcripts, 21 encode for TFs (Yadav et al., 2014). Out of these 65 TFs, 37 were screened against the prey library of shoot enriched TFs. Following is the list of these DNA baits:

1. ABF1
2. AT4G16610
3. ATHB2
4. WRKY21
5. AT5G64060
6. DOF2.4
7. ANAC028
8. TLP8
9. RAP2.4
10. WRKY17
11. AT5G06510
12. AT5G49330
13. AT5G65510
14. CRF6
15. NF-YA5
16. PIL5
17. HDG5
18. DP-E2F-like 2
19. AT2G31730
20. AT2G28810
21. AT5G61190
22. CBF1
23. AT1G75710

24. NF-YA2
25. WRKY22
26. AT4G31550
27. ATH1
28. AP1
29. AT1G24260
30. HDG7
31. PDF2
32. PDF2
33. HDG12
34. ATML1
35. TCX2
36. TAR2(I)
37. TAR2(II)

3.3 Protein-DNA Regulatory Network

In the first round of screening, eight baits were screened against 321 preys for which 16 positive interactions were revealed in the case of 3 baits, namely *HDG7, TAR2,* and *HDG12.*

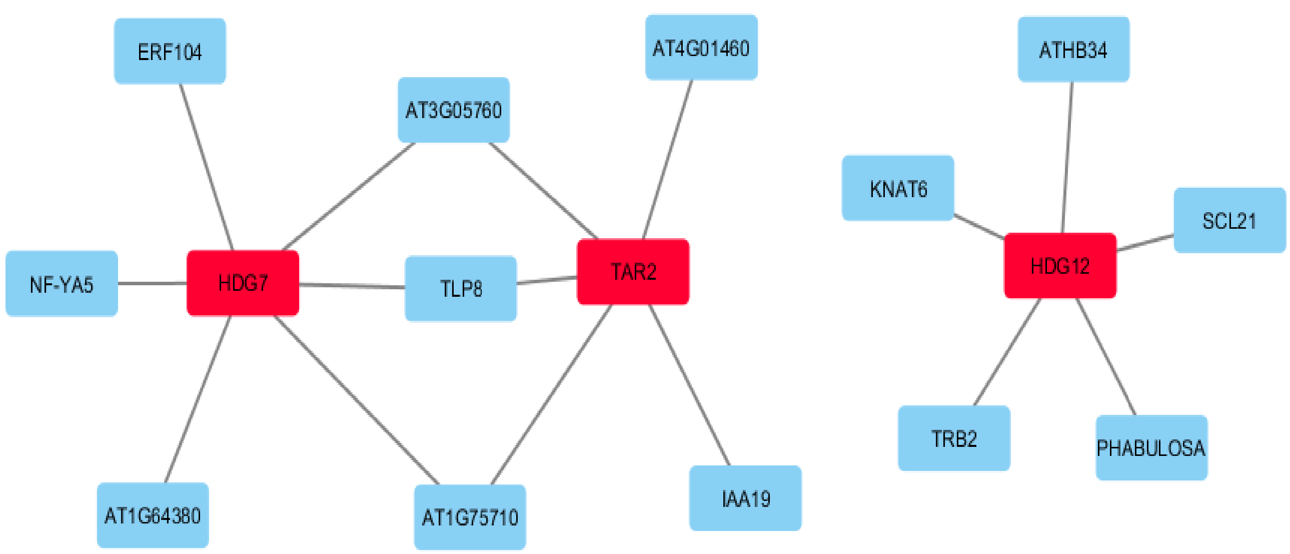

Figure 5: Interaction network for HDG7, TAR2, and HDG12.

A total of 29 DNA baits was screened against 321 preys in the second round of reporter-based screening. A total of 62 positive interactions were found in the case of 19 baits. Given below is the interaction network made for all 22 (19+3) baits for which positive interactors could be found. The network was formed and stylized using the Cytoscape software 3.8.0 version.

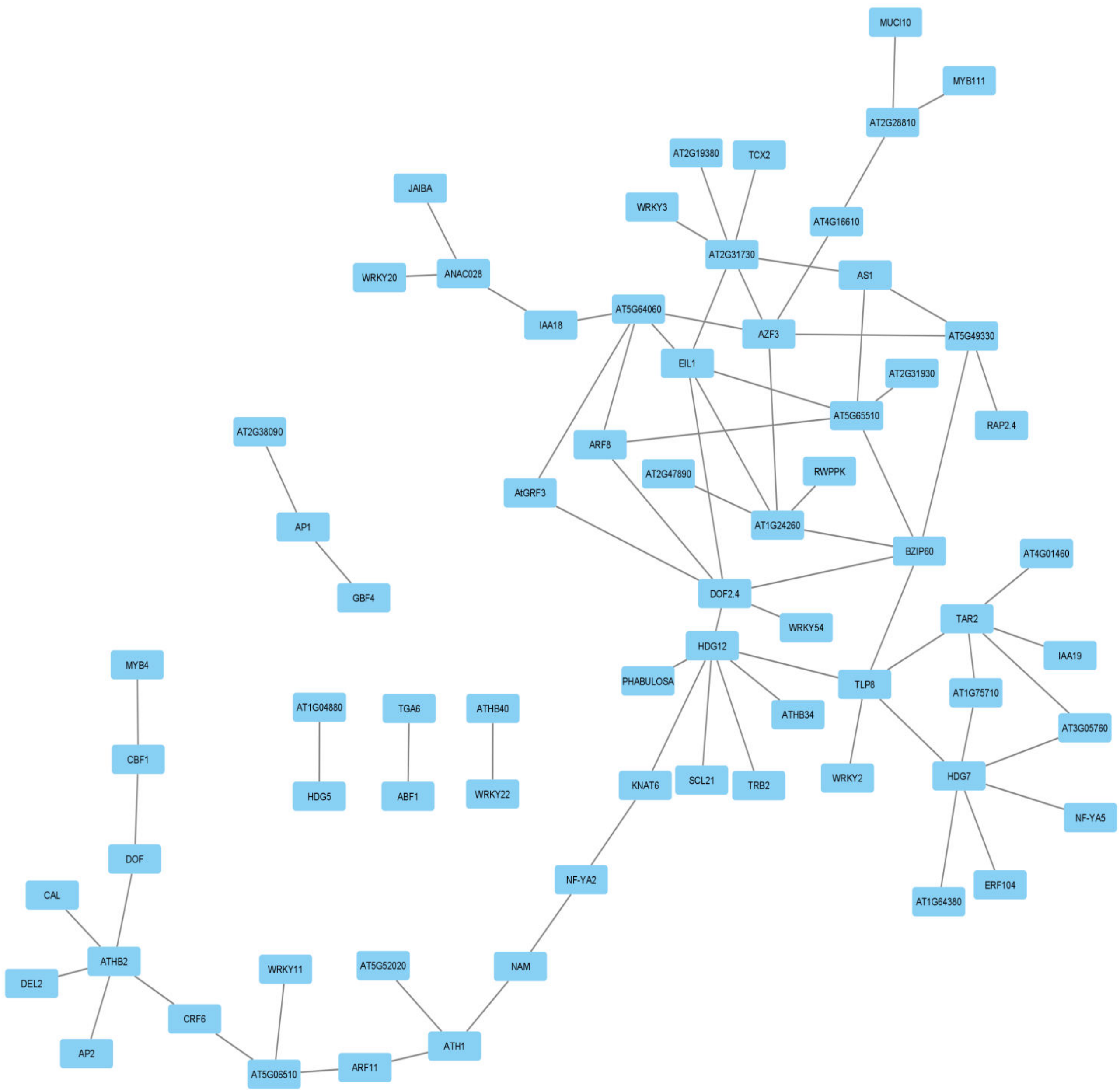

Figure 6: Gene interaction network for 22 DNA baits made using Cytoscape.

3.4 Conclusion

Table 2: Comparison between interactions found at 30^0C and 22^0C.

Baits↓	30ºC	22ºC
HDG7	--	TLP8, ERF104, AT1G75710, AT3G05760, NF-YA5, AT1G64380
HDG12	GRF3	KNAT6, SCL21, ATHB34, TRB2, PHABULOSA
TAR2	--	AT3G05760, TLP8, AT1G75710, IAA19, AT4G01460
ABF1	AT5G64060, DEWAX, HMGB15, CAMTA2, ATHB34, IAA18	TGA6
AT4G16610	DEWAX, HMGB15, CAMTA2, ATHB30, ATHB34	AT2G28810, AZF3
ATHB2	ANAC075, DREB19, DOF, OBP2, DEWAX, ARF12, HMGB15, DAG1	CAL, DEL2, AP2, DOF, CRF6
AT5G64060	--	IAA18, AZF3, EIL1, GRF3, ARF8
DOF2.4	DEWAX, HMGB15, IAA18, KNAT6, CAMTA2, ANAC082	BZIP60, EIL1, GRF3, ARF8, WRKY54, HDG12
ANAC028	DEWAX, HMGB15, CAMTA2, AZF2	JAIBA, IAA18, WRKY20
TLP8	AT5G64060, HMGB15, GBF1	BZIP60, HDG12, WRKY2
NF-YA10	--	CRF6, WRKY11, ARF11
MYB111	AT5G64060, HMGB15, CAMTA2, IAA18	AS1, BZIP60, AZF3, RAP2.4
PLETHORA7	ANAC075, ANAC073, DOF2.4, DOF, DEWAX, OBP2, AZF2, DAG1	AT2G31930, AS1, BZIP60, EIL1, ARF8
HDG5	TCX2, HMGB15	HMBG15
AT2G31730	CAMTA2, IAA7, ATHB30, ATB34, ARF9, ARF12	AZF3, AT2G19380, TCX2, AS1, EIL1, WRKY3
AT2G28810	AT5G64060, DDF1, BODENLOS, WRKY54, TGA8, CAMTA2, GATA18, BZIP52	MUCI10, MYB111
CBF1	--	MYB4, DOF
NF-YA2	CBF1, DEWAX, BZIP52, HMGB15, ARF18, IAA18, ARF9, ARF12	NAM, KNAT6
WRKY22	AT5G64060, DREB19, WRKY11	ATHB40
ATH1	AT5G64060, HMGB15, CAMTA2	NAM, AT5G52020, ARF11
AP1	--	AT2G38090, GBF4
SEPALLATA3	AT2G31730, HMGB15, CAMTA2, IAA18	AT2G47890, AZF3, EIL1, RWPPK, BZIP60

In the informative biology, the pertinent question is to comprehend the occurrence of gene modulation in different tissues and follow the course of events occurring during the procedure of this gene modulation. Utilizing the Y1H measure, we created a protein DNA system of interpretation factors for the shoot enhanced TFs as DNA components and barely and comprehensively executed TFs as protein preys. Every protein-DNA association decided was trailed in quadruplicate, and interlinkages gathered minimum twice in three repeats were viewed as positive. Of the 37 baits, five indicated extremely high auto-enactment, and they were not viewed as further. By considering staying 32 baits and 321 prey proteins, we had arranged 10,272 (TF promoters X TF proteins) protein-DNA interactivities. Out of 10,272 collaborations tried, 78 interlinkages were finished up, which comprises 0.8%(~1%) of the all-out tried. Around comparable measure of interactions has been deduced in a Y1H concentrate on Arabidopsis root TFs(Sparks et al., 2016). Moreover, the idea of such interactivities exceptionally fluctuates with the variety in temperature, as shown in table 2. A portion of the preys cooperates at 30ºC yet not at 22ºC, while for a few, the case is the other way around.

Y1H is a fantastic asset for mapping gene interactivities of in vivo noteworthiness. In any case, some in-vivo interactions might be missed in the Y1H test due to the requirement for hetero-dimer development or co-factor necessity for interlinkage. Additionally, because of the significant level of actuation in certain baits, no interactions can be closed for the equivalent, restricting the measure of information that can be created. Despite these constraints, Y1H measure gives a benchmark for high-throughput examines and catching in-vivo applicable guidelines as a ton of caught interactions could likewise be approved in planta.

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