To study expression pattern of shoot enriched transcription factor and role of kip-related proteins in *Arabidopsis thaliana*

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Certificate of Examination

This is to certify that the dissertation entitled "**To study expression pattern of shoot enriched transcription factor and role of kip-related proteins in *Arabidopsis thaliana***" submitted by Ms Rimpy Dhanda (Reg. No. MS13078) 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.

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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.

Rimpy Dhanda
(Candidate)
Date: April 20, 2018

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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Notations

CTAB	Cetyltrimethylammonium bromide
CDKs	Cyclin Dependent Kinases
EtBr	Ethidium bromide
GFP	Green fluorescent protein
KRP	Kip-Related Proteins
LB	Luria broth
MS	Murashige Skoog
OD	Optical density
OE	Over Expression
PCR	Polymerase chain reaction
PEG	Poly ethylene glycol
SAM	Shoot apical meristem
TAE	Tris acetate EDTA
TF	Transcription factor
YFP	Yellow fluorescent protein

Chapter 1

Introduction

1.1 Shoot apical meristem of *Arabidopsis thaliana*

Arabidopsis is a small flowering plant, and it is widely used as a model organism in plant biology. Like any other plant, it grows and make new organs throughout the lifespan. This is possible due to the activity of small populations of cells, called meristems, located at the very tip of the plant. The meristematic cells in plants are placed in both shoot and root apices. Shoot apical meristem (SAM) resides at the tip of the plant and is responsible for formation of all above ground organs such as leaf, flower and stem tissues. SAM of higher plants is a highly organized structure and is divided into various cell layers and zones.

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

SAM is also divided in to numerous zones, based upon the cell division rate and cell behavior. The uppermost tip of the plant meristem contains pluripotent stem cells. Stem cells reside in central zone (CZ) and divide rarely. Stem cell daughters are produced by stem cells, when these cells enter in to the periphery of SAM they display higher rate of cell division. 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).

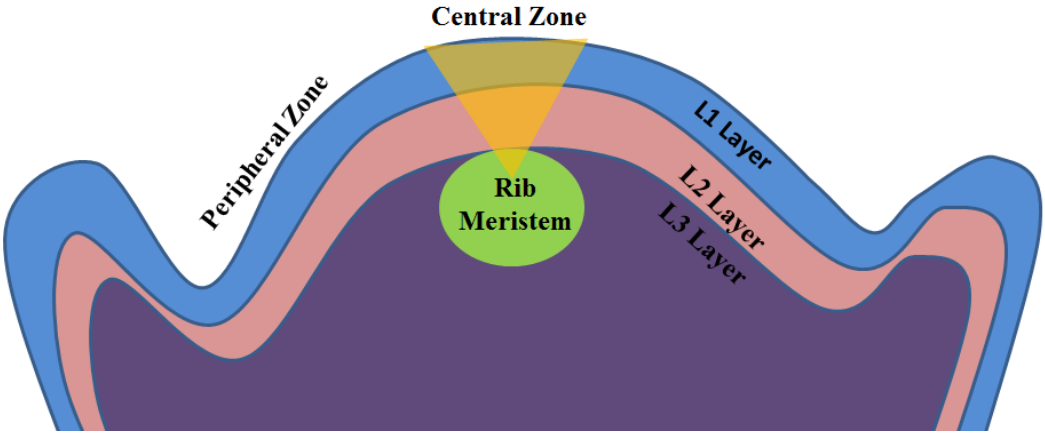

Figure. 1: Organization of shoot apical meristem of *Arabidopsis thaliana.*

1.2 Expression pattern of epidermal and sub-epidermal enriched transcription factors in *Arabidopsis thaliana* SAM

At the end of the year 2000, the whole genome sequence of Arabidopsis thaliana, and its annotation was released. But understanding the function of every gene within the genome is still a humongous task. Computational approaches provide a feasible solution to this problem; numerous bioinformatics methods continue to be developed to understand protein function, most commonly based on sequence similarity, evolutionary history, genomic location, and the presence of certain small sequence motifs (Petrey et al., 2015). Even though this *in silico* analysis offers worthy information in examining the function of these genes, the elucidation of gene function must always be corroborated in vivo using genetic analysis (Bolle et al., 2011). Moreover, by studying the structure and function of proteins encoded by the genome, we can understand the role of genes encoding these proteins in plant development, physiology, evolution and genetics. Therefore, identification of each gene is the first step in this endeavor. One way of knowing the function of genes at genome-wide scale is to capture their expression by microarrays and RNA sequencing (RNAseq) to know where and when they are expressed. Although the microarray technique has a drawback; i.e. the genes whose expression is below the detection threshold will be missed. In contrast, RNAseq has provided evidence for the discovery of new genes. An alternative way to understand the function of individual genes is to investigate their endogenous pattern of expression either by in situ hybridization or by making promoter-reporter fusions in planta by Agrobacterium-mediated floral dip method (Clough SJ, Bent AF, 1998). Promoter reporter lines can be generated in

bulk and by analyzing them one can get representative expression pattern of a given gene. This information is vital to make out the function of genes (Santamaria M et al., 2001). Many studies have shown that the major elements essential to drive the native expression of a gene lie few kilo base pairs upstream of the translation start site of that particular gene (Li et al., 1994; Manners et al., 1998; Peiter et al., 2007; Santamaria et al., 2001). On the contrary, some studies have shown that this might not always be the case and that the regulatory elements may be present in the exons, introns, 3' promoter region or in downstream regions as well (Hong, 2003; Larkin et al., 1993; Peiter et al., 2007). A large-scale study conducted by Lee et al. in (2006) for root enriched transcription factor (TF) genes, showed that 2-3 kb promoter region above the translation start site was sufficient to capture the endogenous expression. This was comparable to that of the native expression level reported by digital in-situs. Therefore, to establish the expression pattern of epidermal and sub-epidermal enriched TFs, promoter reporter constructs are preferred which would provide preliminary but critical information about their native expression in shoot cell types. There exist two types of promoters - constitutive and regulated. Housekeeping genes are typically regulated by constitutive promoters and are ubiquitously active in most cells of the organism. Genes with constitutive promoters are present in all cells and usually code for proteins that control fundamental physiological functions (Sunil Kumar et al., 2002). On the contrary, regulated promoters are stimulated in a temporal or spatial-specific manner under distinct conditions in a cell or tissue type (Nagatani et al., 1997).

Moreover, to establish the role of TF in cell identity both in plants and animals, researchers rely on either in-situ or on the reporter gene to monitor the expression of a gene of interest. Reporter genes are used in fusion constructs because their encoded proteins can be followed or assayed readily as it is either histological colorings, such as GUS, luciferase or fluorescent protein such as GFP or YFP (Saika, 2011). 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) is broken by β-glucuronidase (GUS) resulting in the production of a blue coloured compound. Detection of GUS activity demands chemical fixation of the sample and the addition of the X-Gluc substrate for visualization (Jefferson et al., 1987; Helmer et al., 1984; Coelho, 2010). This particular nature of fixation does not permit visualization in living tissue. Another technique applied is using luciferase enzyme that acts on the luciferin substrate in the proximity of ATP and oxygen (Ow et al., 1986). The reaction emits light as a product that can be detected by a light-sensitive apparatus. Also, luciferase does not accumulate in the cell as it has a short half-life. These properties make luciferase a useful marker for monitoring

changes in gene expression over time. These days Green Fluorescent Protein (GFP) or the similar proteins CFP or YFP have become a popular alternative to the GUS and luciferase reporters. These fluorescent markers are 27 kDa barrel-shaped proteins and when exposed to the appropriate excitation wavelength, produces bright fluorescence. These proteins do not easily bleach under prolonged exposure to UV light and have a long half-life in the cell (Carlson et al., 2001; Michaelson and Phillips, 2006). In contradiction to luciferase, fluorescent reporters use the accumulation of protein molecules as a sign of gene expression. The localization and level of reporter gene expression are analyzed for each subsequent promoter fragment and visualization of the reporter gene can be correlated with the activation of the promoter. Moreover, chimeric reporter genes can be transformed into cultured cells or incorporated into transgenic organisms. Hence, reporter gene analysis provides an economical, rapid, and sensitive assay that can be used to study the gene expression. But these reporters have their own disadvantages, such as each gene might be regulated by a complex network and therefore, various markers should be combined to deduce a cell's identity.

From the published cell population microarray data of Yadav et al. (2014), 65 TF genes were found to be enriched in the epidermal and sub-epidermal cell layer. However, for the majority of these TFs, their spatiotemporal expression is still unknown. Therefore, in the present study, the 3kb promoter of TFs was cloned in P4P1R vector using gateway BP cloning reactions. I sub-cloned 21 promoters into a modified pGreen0229 vector containing H2B-YFP reporter gene using the gateway LR reactions to know the endogenous expression of the TF genes.

1.3 Role of Kip-Related Proteins in shoot apical meristem

The cell cycle plays an important role in growth and development of a multicellular organism. Cyclins and CDKs together forms complexes and regulate different phases of cell cycle. The A-type CDKs are primarily involved in regulating the G1/S transition and S-phase progression cycle but are constitutively present throughout the cell cycle. The cell cycle undergoes repeated cycles of G/S phase during endoreduplication which results in increased DNA content of the cell. Several plant tissues undergo endoreduplication to produce specialized cell types within the body of plant; such as pavement cells, trichomes in leaf, and giant cells in sepals. Kip related proteins (KRPs) are known to promote endoreduplication by regulating cyclins and CDKs complexes. Arabidopsis genome harbors 7 *KRP* genes, this includes *KRP1*, *KRP2*, *KRP3*, *KRP4*, *KRP5*, *KRP6* and *KRP7* (Wang et al., 1997, De Veylder

et al., 2001, Vandepoele et al., 2002). The fine-tuning of KRP protein levels in plant cells is a key factor to maintain the balance between cell differentiation and cell proliferation. The *KRPs* are involved in both G1/S and G2/M transition of cell cycle, and therefore serve as an important checkpoint control to regulate the cell differentiation (Zhang et al., 1999). Preliminary expression data has shown that *KRP1* and *KRP6* genes are expressed ubiquitously in various plant organs (roots, inflorescence, stems, flower buds and 3-week-old leaves) and in actively dividing suspension culture (De Veylder et al., 2001). *KRP4*, *KRP5* and *KRP7* are expressed in the cells that display high mitotic activity, with *KRP4* also being abundantly present in the leaves. Expression of *KRP2* and *KRP3* was reported in actively dividing cell suspension cultures. Some *KRPs* are known for promoting endoreduplication in various plant parts such as trichomes, sepals (Roeder et al., 2010). It seems that *KRPs* play distinct role in development. The SAM of higher plants display differential rate of cell growth and cell division in different zones. How do plants achieve differential cell growth and division rates in different zones of SAM? Do the *KRPs* play any role in regulating this differential cell growth and division? To answer these questions, we analyzed the expression pattern of *KRPs* in SAM using over-expression studies. The increased expression of a wild type gene can also be disruptive to an organism or cell that may results in alteration of the phenotype (Prelich, 2012). Gain-of-function is achieved by the expression of individual transgenes by transformation or by enhanced gene expression levels through the random activation of endogenous genes by transcriptional enhancers (Kondou et al., 2010). In the present study, I made transcriptional fusion constructs and conducted qRT-PCR experiments to find out the expression of *KRPs*, and also to analyze their spatio-temporal expression pattern in SAM.

Chapter 2

Experimental methods

2.1 Plant material and growth conditions

Promoter reporter constructs were transformed in *Arabidopsis thaliana* wild type L*er* ecotype. Plants were grown in the pots containing autoclaved soil, which comprises of solarite, perlite and compost (16:1:1 ratio). For making transgenic lines, plants were grown in controlled environment plant chambers (Percival, USA and Conviron, Canada) in 75% humidity, 150 µmole/m^2 white light and at 22°C temperature with 16-hour day and 8-hour night conditions.

2.2 Chemicals used

High fidelity polymerase (Phusion) and restriction enzymes were obtained from New England Bio lab (NEB, USA). Plasmid isolation kit and standard molecular weight markers (1 kb DNA ladder) were obtained from Thermo Fisher Scientific (Thermo, USA). Bacterial LB media and LB agar components were obtained from BD scientific (BD, USA). RNA isolation kit was obtained from Promega and cDNA synthesis kit was obtained from Bio-Rad (Bio-Rad, USA). *Taq* DNA polymerase was made in the lab. Whereas, Gateway LR clonase and entry vector for cloning were brought from Thermo Fisher scientific (Thermo, USA). All other chemicals and reagents used in the study were of molecular biology/analytical grade and were obtained from Sigma-Aldrich (Sigma, USA). DNA and RNA concentrations were measured using spectrometer (Biophotometer, Eppendorf).

3.3 Other protocols and media/reagent composition

A) Bacterial electro competent cell preparation

For electro competent cell preparation, the protocol described by B.M et al., (1998) was followed. LB media and 10% glycerol were autoclaved at 121°C at 15psi for 15 minutes before the procedure.

- Streak the DH5 α strain of *E. coli* on the fresh LB agar plate.
- Pick up single colony with autoclaved tip to setup primary culture in 5 ml LB media and incubate it overnight in incubate shaker.
- Set up the secondary culture by using ~ 1% primary culture.

- Check OD at regular interval until it reaches 0.4 to 0.6.
- Transfer the culture to autoclaved centrifuge bottles. Check on balance the weight before putting them in centrifuge.
- Make sure that the rotors and the centrifuge are precooled.
- Pellet down the cells at 4000rpm for 20 minutes.
- Discard the supernatant and dissolve the pellet in chilled 200 ml 10% glycerol, on ice.
- Repeat the above steps for 3 times with decreasing volume of 10% glycerol used each time.
- Transfer the cells to oak ridge centrifuge tubes or a 50ml falcon tube and dissolve the final pellet in 2-3 ml 10% glycerol.
- Aliquot 50µl cells into 1.5 ml eppendorf tubes.

In the *Agrobacterium tumefaciens* culture, antibiotics were added (gentamycin 25 µg/ml to keep Ti plasmid resistance, rifampicin 50 µg/ml for chromosomal resistance).

B) Bacterial ultra-competent cell preparation

- For primary culture, inoculate a single colony in 4ml LB media.
- Autoclave 50ml of SOB media and 10ml of 2M $MgCl_2$. Also get transformation buffer 1, 2 and 3 get autoclaved separately and put it at 4°C.
- Inoculate 1% culture (500 µl) in 50 ml SOB and shake at 37˚C till OD reaches 0.5.
- Keep the culture on ice for 10 minutes.
- Pour the culture in a sterile 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 and keep on ice for 10 minutes.
- Centrifuge 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 50µl of cells in 1.5 ml eppendorf tubes and store at -80˚C.

For bacterial transformations, add equal volume of buffer 3 and use 50µl cells for each transformation.

C) Plasmid Isolation

- Inoculate the culture in 5 ml LB media along with the required antibiotics and put on shaker overnight.
- Pellet down the cells in 2ml MCTs at 13,500rpm for 1 minute and discard the supernatant.
- Dissolve the pellet in 250µl P1 buffer (Re-suspension buffer). Vortex the contents of MCT and mix well.
- Add 250 µl P2 buffer (Lysis buffer) and mix by gently inverting the tube. Do not vortex!
- Add 350 µl N3 buffer (Neutralisation buffer) and again mix the contents well by gently inverting the tube.
- Centrifuge at 13,500 rpm for 10 minutes and apply the supernatant to the spin column.
- Centrifuge at 13,500 rpm for 1 minute.
- Add 750 µl wash buffer and centrifuge at 13,500 rpm for 1 minute. Discard the flow through and dry the column spinning it at 13,500 rpm for 1 minute.
- Transfer the column to the fresh MCT. Add 25 µl of distilled water (pre-heated at 55°C) to the column and centrifuge at 13,500 rpm for 1 minute to elute the plasmid. Again, add 25µl of preheated distilled water to the column and centrifuge at 13,500 rpm for 1 minute.

D) Genomic DNA isolation

For genomic DNA isolation modified CTAB method was used.

- Leaves were harvested and frozen in liquid nitrogen.
- Crush the leaves in a 1.5 ml tube using the pestle and add 600µl CTAB buffer and 0.6µl β Mercaptoethanol.
- Put the samples at 65°C for 20 minutes.
- Add 600µl Phenol + Chloroform mixture and mix it 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 inverting the tube. Centrifuge at 13,000 rpm for 5 minutes.
- Remove the supernatant and wash the pellet by adding 600µl of 70% ethanol.
- Centrifuge at 13,000 rpm for 1 minute and remove the supernatant.

- Keep the tube at 90°C for 30 seconds to dry the pellet completely.
- Resuspend the pellet in 50µl distilled water and store the samples at -20°C.

E) Total RNA isolation

- Prepare LBA and TG buffer. Crush the tissue (approx. 10mg) in liquid nitrogen. Add 500µl LBA+TG buffer to homogenate. Vortex the tube for few seconds.
- Centrifuge at 14000 rpm for 3 minutes. Transfer supernatant to a new tube.
- Add 170µl of 100% chilled isopropanol. Mix well by pipetting. Take 1 mini column, 2 collection tubes and 1 elution tube.
- Transfer lysate to mini column and centrifuge at 14,000 rpm for 1 minute at 20-25°C. Discard the flow through.
- Add 500µl RNA wash solution. Centrifuge at 14,000 rpm for 30 seconds. Discard flow through.
- Prepare DNase I incubation mix and add 30µl on each column.
- Incubate for 15 minutes at room temperature.
- Add 200µl column wash solution. Centrifuge at 14,000 rpm for 1 minute.
- Add 500µl RNA wash solution. Centrifuge at 14,000 rpm for 30 seconds. Discard the flow through and collection tube.
- Place the column in fresh collection tube. Add 300µl RNA wash solution and centrifuge at 14,000 rpm for 2 minutes.
- Discard the flow through and give an empty spin at 14,000 rpm for 1 minute.
- Place the column in elution tube and add 30µl nuclease free water (pre-heated at 55°C). Centrifuge at 13,000 rpm for 1 minute to elute the RNA.
- Analyze the concentration and A_{260}/A_{280} value of RNA using the spectrometer.

F) Gel Purification

- Cut the band under UV trans illuminator and put it in MCT.
- Add 700µl QG buffer and keep the tube at 50°C on shaker till the gel has completely melted.
- Load the contents in the gel purification column and centrifuge at 14,000rpm for 1 minute.

- Add 700µl wash buffer and centrifuge at 14,000 rpm for 1 minute.
- Discard the flow through and give an empty spin.
- Elute the DNA using 20µl distilled water (pre-heated at 55°C).

G) Electroporation

- Wash the cuvettes with 70% ethanol and distilled water and dry them before use.
- Add 0.1 to 1.0µl plasmid to 50µl electro competent cells and mix well.
- Transfer the whole mixture to the cuvette using a pipette.
- Do electroporation according to the required conditions.
- After the pulse has been provided, transfer the transformed cells to 700µl LB media.
- Incubate the cells at 37°C on shaker for 1 hour.
- Plate the samples in respective selection media.

H) 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.

I) Preparing antibiotic stocks

- **Rifampicin** (stock- 25mg/ml)
  - Weigh 250 mg of Rifampicin for 10 ml preparation.
  - Add 10ml DMSO and vortex to mix.
  - Aliquot in 1.5ml MCTs and store in -20°C.

- **Kanamycin, Hygromycin and Gentamycin** (stock- 50mg/ml)
  - Weigh 500 mg of antibiotic for 10 ml preparation.
  - Add 10ml autoclave MQ water and vortex to mix.
  - Aliquot in 1.5ml MCTs and store in -20°C.

- **Tetracycline** (stock- 12.5mg/ml)
  - Weigh 125 mg of tetracycline for 10 ml preparation.
  - Add 10ml 100% ethanol and vortex to mix.

- Aliquot in 1.5ml brown MCTs and store in -20°C.

J) Agarose gel preparation

- Weigh agarose according to 0.8% w/v calculation.
- Add 1X TAE buffer (1 ml 50X TAE + 49 ml distilled water)
- Heat for 2-3 minutes.
- Add EtBr when it reaches close to room temperature (0.5 µg/ ml).
- Pour into the gel tray with the combs and allow it to solidify.

K) Bacterial stocks

- Add equal volumes of 50% glycerol and the bacterial culture into a cryovial.
- Store in -80°C for future use.

L) Agrobacterium Plant Transformations

- Grow wild type plants to be transformed until bolting.
- Inoculate the primary culture in 5ml LB media along with the desired antibiotics.
- After 24 hours set up the secondary culture from primary culture and with suitable antibiotics.
- After 24 hours transfer the culture to autoclaved centrifuge bottles and centrifuge 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 and mix 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. Cover the tray with other tray on top.
- Straighten the plants after 12 hours, water them and transfer them to chamber.
- The same transformation protocol is performed twice for better efficiency.

M) Preparing soil for growing plants

- Mix solarite, perlite and compost in 16:1:1 ratio. Mix it finely.
- Autoclave the mixture.
- Fill the pots, put the seeds and put water in the tray. Put the trays for 4 days for vernalization and then transfer to growth chamber.

N) Seed sterilization

- Take seeds in 1.5ml microcentrifuge tubes and add 1ml of 70% ethanol, containing 0.02% (v/v) Triton X-100, for 1 minute. Mix well by inverting the tube.
- Let the seeds settle at bottom of the tube and remove the supernatant. Now, add 1ml 0.4% NaOCl containing 0.02% (v/v) Triton X-100, for 3 minutes.
- Remove the supernatant as the seeds settle down. Add 1ml sterilized water to the tube and repeat this step thrice.
- Transfer the seeds on respective MS agar plates under sterile conditions.

O) Construction of promoter reporters in binary vector

PCR amplification of promoter DNA fragment and cloning into binary plant vector

- Genomic DNA was isolated using CTAB method from wild-type L*er* ecotype.
- Forward primer and reverse primer were used to amplify the 3 kb promoter DNA fragment upstream of the translational start site along with B4 and B1R site.
- PCR amplification of promoter DNA fragment was carried out using Phusion high fidelity DNA polymerase (NEB, USA).
- PCR product for each promoter was purified and cloned in the gateway vector P4/P1R using gateway BP reaction.
- To perform the LR recombination reaction directly using pDONR/P4/P1R bait vector with plant transformation vector, we cloned the attR:ccdB:attR fragment from pMW2 vector into *Xho* I and *Nhe* I sites of pGreen 0229 H2B-YFP. This modified pGreen 0229 vector was used for generating promoter::H2B-YFP transcriptional fusions for objective 1.

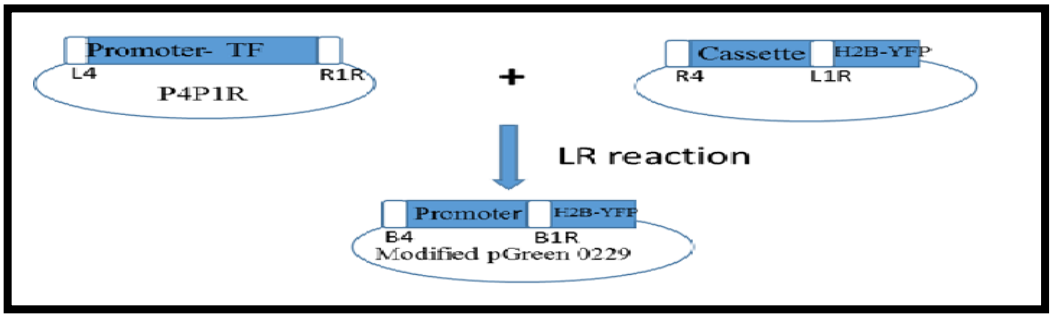

Figure. 2: Gateway cloning for *promoter:: H2B-YFP*

P) Screening of promoter reporter lines

- Each promoter reporter construct was transformed into wild type L*er*, and T_0 seeds were collected.
- These seeds were grown with appropriate selective agent such as BASTA to select primary transformants.
- To find out representative T_1 plants, T_1 rescued plants (4 weeks old) were dissected under the dissecting scope for individual construct (Leica KL300 LED).
- To reduce the workload on confocal microscope, first the dissected shoot apices were visualized in the upright epifluorescence microscope Axio Imager.Z2 (Zeiss, Germany) using long working distance water dipping objective.
- The representative T1 lines selected were further subjected to detailed expression analysis using confocal imaging.

Q) Confocal imaging of inflorescence meristems and image processing

The confocal images for reporter transgenic lines were acquired using Leica SP8 upright confocal microscope.

- Four-week-old shoot apices of T1 plants were hand-picked with a fine forceps and dissected under Leica dissecting microscope after inserting them straight in specially designed rectangular boxes filled with 1.5% agar.
- To image the expression of fluorescent reporter within the inflorescence meristem, old flower buds were clipped off carefully under the dissecting microscope.
- To visualize the cell outline shoot apices were stained with Propidium Iodide (100 μg/ml) in dH_2O containing silwet (0.02%) for 10-15 minutes. The inflorescence meristems were scanned in confocal microscope.
- YFP fluorochrome was excited with Argon laser (488/515) and emission spectra was collected by setting the spectral band pass filter between (BP 500-535). The cell membrane marker dyes (FM4-64 / PI) was excited with Argon laser and emission spectra was collected through spectral band pass filter (BP 610-672nm).
- Three-dimensional (3D) images were made from confocal stacks using Leica software. The side image of the SAM was generated using the side view function of Leica software.

R) Colony PCR

- Mark the desired colonies on the plate.

- Dissolve the colonies from the plate in 10µl autoclaved distilled water.
- Make the master mix (refer Table 1) and aliquot into the PCR tubes and set up the PCR reaction (refer Table 2).
- To analyze the samples load them in electrophoresis gel and run it.
- Also load the DNA ladder in the gel.

Table1: Recipe for colony PCR.

Components	**Volume (in µl)**
Sample (distilled water and the dissolved colony)	10
10X standard PCR buffer	1.5
dNTPs (10mM)	1.2
$MgCl_2$ (50mM)	0.45
Taq DNA polymerase	0.5
10µM forward primer	0.75
10µM reverse primer	0.75
Total	15

Table 2: Reaction conditions for colony PCR

Steps	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	94°C	3 minutes	1
Denaturation	94°C	30 seconds	35
Annealing	55°C	30 seconds	
Extension	72°C	1 minute	
Final extension	72°C	10 minutes	1
Hold	4°C	∞	

Table 3: Master Mix for T-DNA lines genotyping PCR

Components	**Volume (in µl)**
10X standard PCR buffer	2.5
dNTPs (10mM)	2
10µM forward primer	1

10µM reverse primer	1
Taq DNA polymerase	1
$MgCl_2$ (50mM)	0.5
Distilled water	15.5
Total	25

Table 4: PCR reaction conditions for genotyping

Steps	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	94°C	3 minutes	1
Denaturation	94°C	30 seconds	35
Annealing	58C	30 seconds	
Extension	72°C	1 minute	
Final extension	72°C	10 minutes	1
Hold	4°C	∞	

S) cDNA synthesis protocol

- Analyze the concentration of each RNA sample and calculate the sample volume in µl required to set up using 1µg RNA per sample.
- Add the desired amount of RNA and make up the volume to 15µl by adding nuclease free water.
- Add 1µl of enzyme and 4µl of iscript buffer to the PCR tube and set up the reaction (refer Table 5).
- Make the master mix and aliquot into the PCR tubes and set up the qRT-PCR reaction (refer Table 6).
- To analyze the samples load them in electrophoresis gel and run it.
- Also load the DNA ladder in the gel.

Table 5: Master mix for cDNA preparation

Components	**Volume (in µl)**
RNA	X
Nuclease free water	15-x
iscript buffer	4

Reverse transcriptase enzyme	1
Total	20

Table 6: PCR conditions for cDNA preparation

Steps	**Temperature**	**Time**
First incubation	25°C	5 minutes
Second incubation	46°C	40 minutes
Inactivation	95°C	1 minute
Hold	4°C	∞

Table 7: Composition of PCR components for semi-quantitative RT-PCR.

Components	**Volume (in µl)**
10X standard PCR buffer	1.5
dNTPs (10mM)	1.2
10µM forward primer	0.75
10µM reverse primer	0.75
Taq DNA polymerase	0.5
$MgCl_2$ (50mM)	0.45
Distilled water	9.85
Total	15

Table 8: PCR conditions for semi-quantitative RT-PCR.

Steps	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	94°C	3 minutes	1
Denaturation	94°C	30 seconds	29
Annealing	58C	30 seconds	
Extension	72°C	30 seconds	
Final extension	72°C	5 minutes	1
Hold	4°C	∞	

2.4 Reagents and Chemicals used

- **LB media**

Tryptone 5gm

NaCl	2.5gm
Yeast Extract	2.5gm

	500ml

- **LB Agar**

Tryptone	7gm
NaCl	3.5gm
Yeast Extract	3.5gm
Agar	10.5gm

	700ml

- **SOB media**

Tryptone	1gm
NaCl	0.025gm
Yeast Extract	0.25gm

	50ml

- **Transformation buffer 1**

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 CaCl2

➢ 50mMg Cl2

Tris base	242gm
Glacial acetic acid	57.1ml
EDTA (pH:8, 0.5 M)	100ml
	1000ml

Autoclave the mixture separately.

- **50X TAE**

- **Ethidium Bromide**

➢ Dissolve 1-gram Ethidium Bromide (powdered form) in 100ml sterile water.

➢ Stir for several hours to ensure that the dye has completely dissolved and store it 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	4.32gm (0.1%)
MES	1.00gm (1.0%)
Sucrose	10.00gm (0.8%)

Set the pH to 5.80 before adding agar.

Agar	8.00gm

	1000ml

- **Gateway LR reaction**

Linearized Entry clone	75-100ng
Destination vector	150ng
TE	0.5µl
LR Clonase	0.5µl
Sterile distilled water	to make up final volume
Total reaction	3µl

- **Entry reaction**

PCR product	1µl
10X salt solution	0.5µl
pENTR-D-TOPO	0.5µl
Sterile distilled water	to make up final volume
Total reaction	3µl

Chapter - 3

Results

Objective 3.1: Expression pattern of epidermal and sub-epidermal enriched TFs in *A. thaliana* SAM

To understand the cell fate specification of epidermal and sub-epidermal cell types, TFs that are expressed in these cell types were identified by cell population microarray study. A total of 1456 genes were found differentially expressed in the three cell layers of the shoot, which are L1 (epidermal) layer, L2 (sub-epidermal) layer and L3 layer (corpus). Of the 1456 genes, 535 were expressed in the L1 layer, 256 in the L2 layer and 665 in the L3 /corpus. In the L1 layer, 44 genes encode for TFs out of 535 genes. In the L2 layer, of the 256 genes, 21 encode for TFs, while in the L3 layer, 52 encode for TFs out of the 665 genes (Yadav et al., 2014). This study aimed to investigate the spatial-temporal expression pattern of these transcription factors. The 3kb promoter of TFs were cloned in P4P1R vector using gateway BP cloning reactions. 21 promoters were sub-cloned into a modified pGreen0229 vector containing H2B-YFP reporter gene using the gateway LR reactions and transformed *in planta*. Transgenic plants were selected on BASTA and their shoot apices were screened under the confocal microscope for YFP expression. Transgenic lines for 14 out of 21 constructs were successfully generated and expression pattern was determined for 7 of them. For some of the TFs, the expression pattern was observed in the shoot and emerging organ primordia. For remaining 7 TFs, no visible expression was noticed, possibly due to the absence of critical regulatory elements in the promoter construct.

3.1.1 Expression pattern of transcriptional fusions (*promoter*::*H2B-YFP*) in *Arabidopsis thaliana*

ENHANCER OF GLABRA 3 (*EGL3*)

EGL3 belongs to bHLH family of TFs. Previous studies have shown that *EGL3* have a role in the development of trichome and root hair. Evidence of expression of *EGL3* in the epidermal layer of emerging sepal primordia was found through confocal imaging of the inflorescence meristem and flower primordia. While no such expression was observed in SAM. Upon further investigation of confocal images of early flower primordia *EGL3* was found not to be expressed in the stage-II flower epidermis, but to be expressed in the early stage-III flower during the

emergence of sepal primordia (Fig. 3). Moreover, this expression of *EGL3* persists in sepal epidermis through stage-III and in later stages of flower development.

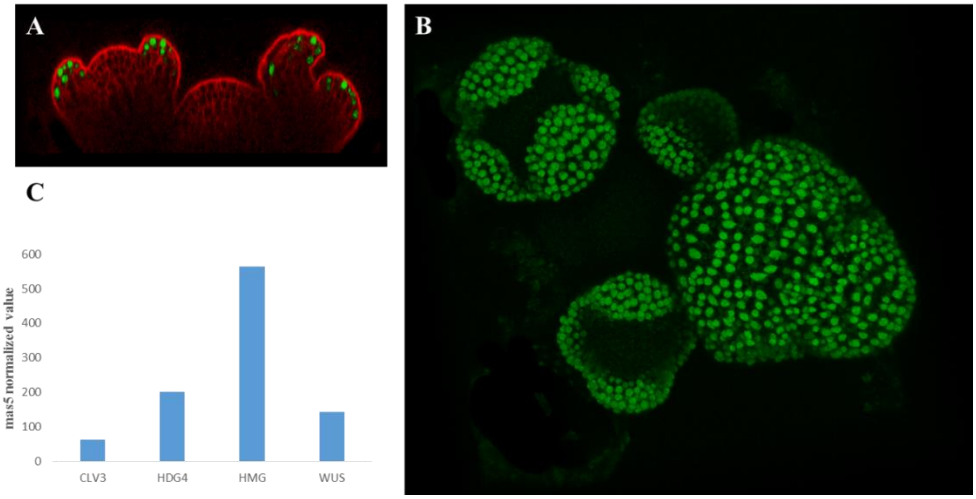

Figure 3: Side view and 3D view and of wild type L*er* shoot apex showing the expression pattern of *pEGL3:H2B-YFP*, respectively in (A, B). (C) Histogram showing the MAS5 expression values of *EGL3.* Cell outlines highlighted by Propidium Iodide (red).

C2H2-like Zinc-Finger / *AT1G75710*

AT1G75710 locus encodes for a C2H2 like zinc-finger TF. Cell type population microarray data revealed that *AT1G75710* is enriched in epidermal cell layer and in the differentiated cells of PZ (Fig. 4). Confocal microscopy study showed expression of *pAT1G75710::H2B-YFP* reporter in four week old shoot apices. The expression of *AT1G75710* in the L1 meristematic layer of the SAM was ascertained through the side view of SAM reconstructed using confocal images (Fig. 4 A). The expression of this TF appears to be restricted in the PZ of SAM, and in the emerging flower primordia upon observation in the 3D top view of confocal image (Figure 2.5 B). The H2B-YFP reporter expression is missing from the CZ, where *CLV3* is normally expresses. *pAT1G75710:: H2B-YFP* expression was also found in the mature flowers. In the flower primordia, the expression was more restricted towards the region of upcoming floral organs.

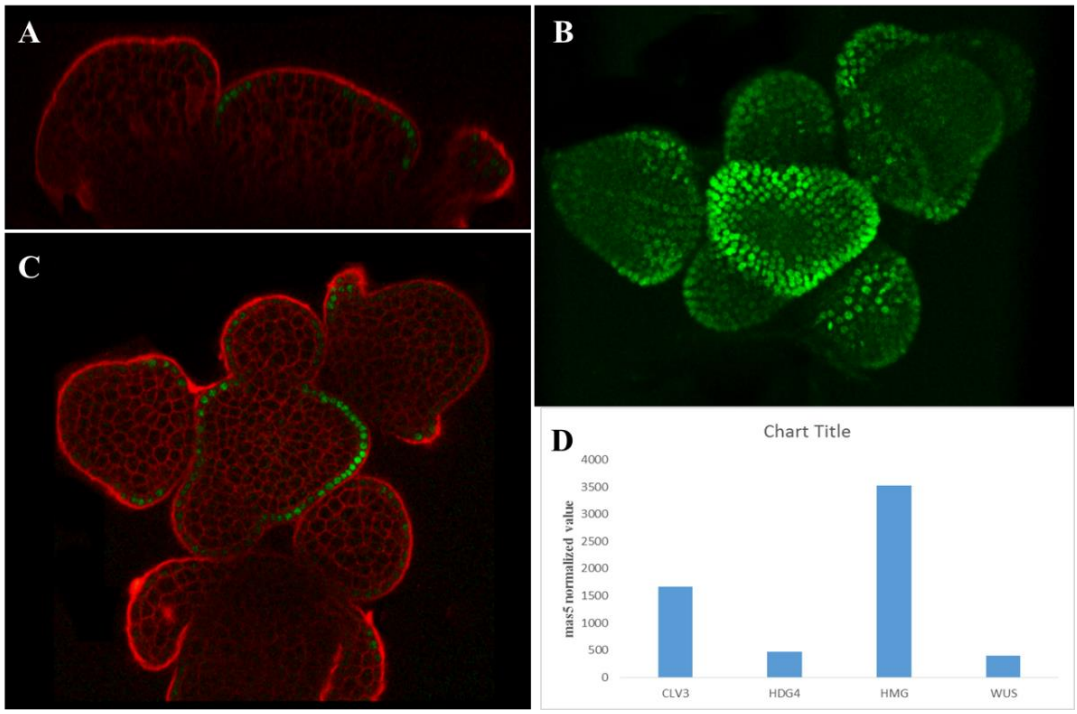

Figure. 4: Side view, 3D and top view of Wt Ler shoot apex, respectively (A, B, C), showing the expression pattern of *pAT1G75710:H2B-YFP*. (D)Histogram showing the MAS5 expression values of *pAT1G75710:H2B-YFP*. Cell outlines highlighted by Propidium Iodide (red).

WRKY family

Members of this family comprises of one or two WRKY domains of 60 amino-acid residues with a conserved WRKYGQK motif. DNA binding is achieved through the WRKY domain. WRKY proteins have been divided into different groups depending on the number of WRKY domains and features of their zinc finger like motifs. Group I comprising of TFs with two WRKY domains, while group II comprises of TFs with one WRKY domain. Both group I and group II members have a similar finger motif, C-X4-5 -C-X22-23 -H-X 13-H. Group III comprises of WRKY proteins having a distinct finger motif, C2-HC. WRKY family is composed of 71 members out of them six are enriched in the shoot.

WRKY11 / AT4G31550

WRKY11 is a member WRKY family of TFs, unique to the plants. *WRKY11* follows a similar expression pattern to *WRKY22*. Expression of *WRKY11* was found to be expressed in the PZ of the shoot while not in CZ cells. Expression of *WKRY11* was reported in epidermal cell layer in a microarray study by Yadav et al. (2014) (Fig. 5). However, the reported expression does not support the microarray study.

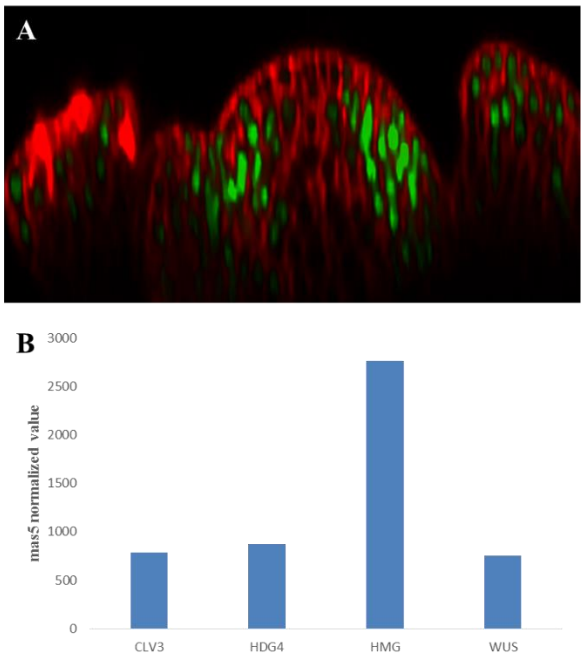

Figure. 5: Side view, 3D and top view of wild type L*er* shoot apex, respectively (A, B, C), showing the expression pattern of *pAT4G31550:H2B-YFP*. (D) Histogram showing the MAS5 expression values of *pAT4G31550:H2B-YFP*. Cell outlines highlighted by Propidium Iodide (red).

WRKY17 / AT2G24570

WRKY17 belong to group II-D of the WRKY family. Expression of this TF was detected in the sub-epidermal layer of the shoot and flowers in transgenic lines carrying the *pWRKY17::H2B-YFP* construct (Fig.6). Though, very few cells of the SAM were positive for YFP in contrast to the flowers. According to the relative expression profile from the transcriptomic study, this gene has the maximum expression in the epidermal layer. The expression achieved by using the 3 kb promoter does not match with the microarray data, probably due to lack of adequate regulatory elements within this region.

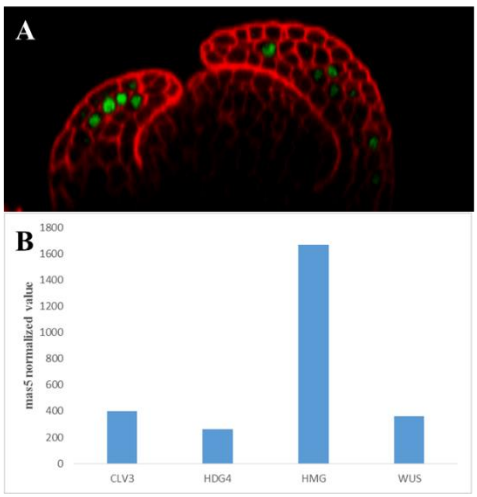

Figure. 6: Side view of Wt Ler shoot apex, respectively (A), showing the expression pattern of *pAT2G24570:H2B-YFP*. (B)Histogram showing the MAS5 expression values of *pAT2G24570:H2B-YFP*. Cell outlines highlighted by Propidium Iodide (red).

AT3G16940/ Calomodulin

AT3G16940 belongs to calmodulin family of transcription factors. The confocal imaging (Fig. 7) shows the expression of this transcription factor in epidermal layer of sepal, and is absent in SAM. The confocal data corroborates with the MAS5 expression value of microarray.

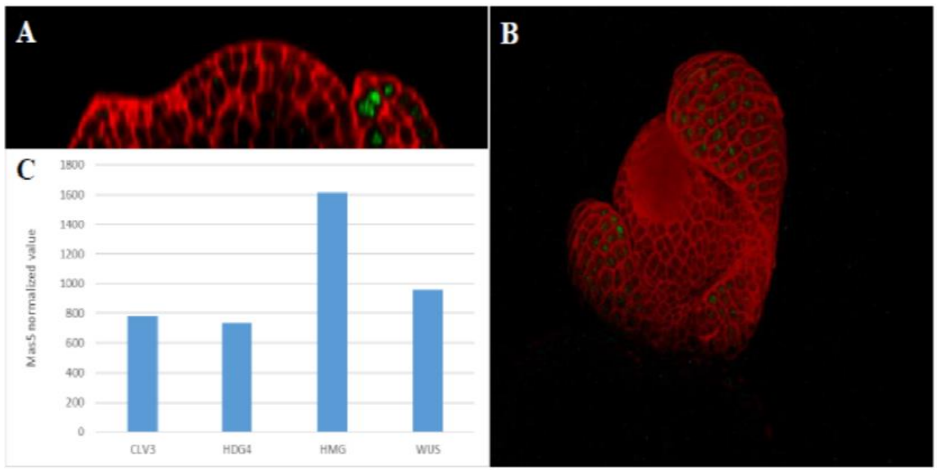

Figure. 7: Side view of 3D of wild type L*er* shoot apex. (A and B), showing the expression of *pAT3G16940:H2B-YFP*. (C)Histogram showing the MAS5 expression values of *pAT3G16940:H2B-YFP*. Cell outlines highlighted by Propidium Iodide (red).

TCX2/ AT4G14770

TCX2 encode for a TSO1 like CX2 protein. According to cell type population data, *TCX2* has the highest expression in sub-epidermal cell layer. Interestingly, expression of *TCX2* is not restricted to any particular cell type. Using 3 kb promoter fragment in the *pTCX2::H2B-YFP* reporter construct, expression of this TF was seized in the flower primordia (Fig. 8). However, the YFP glow was completely missing from SAM.

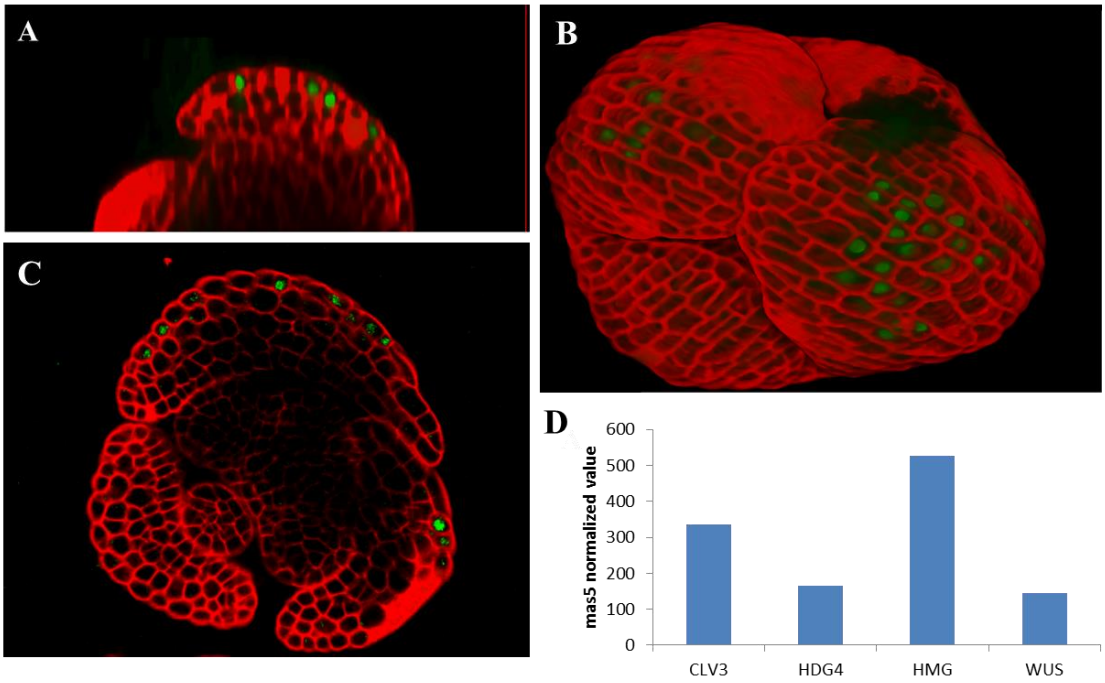

Fig. 8: Side view, 3D and top view of wild type L*er* flower primordia, respectively (A, B, C), showing the expression pattern of *pAT4G14770:H2B-YFP*. (D) Histogram showing the MAS5 expression values of *pAT4G14770:H2B-YFP*. Cell outlines highlighted by Propidium Iodide (red).

ARABIDOPSIS thaliana HOMEOBOX GENE 1 (ATH1) / AT4G32980

ATH1 is a member of the BELL sub-family of homeobox TF family. This gene physically interacts with other genes which play a critical role in the development such as STM, BP and KNAT6 and has a role in SAM maintenance. In the C24 accession of Arabidopsis, over-expressing ATH1 results in delayed flowering. The 3kb promoter-reporter fusion was used and the expression of ATH1 was observed in the rib-meristem zone in the shoot. Also, ATH1 expression appears in the L1 layer of the incipient primordia and extends into deeper layers as the organ primordia grow (Fig.9). ATH1 has a much broader expression domain in fully mature flowers. According to the transcriptomic data, ATH1 expression was recorded highest in the epidermal cell followed in stem cells. Taken together, the reporter pattern validates with the cell type-specific data.

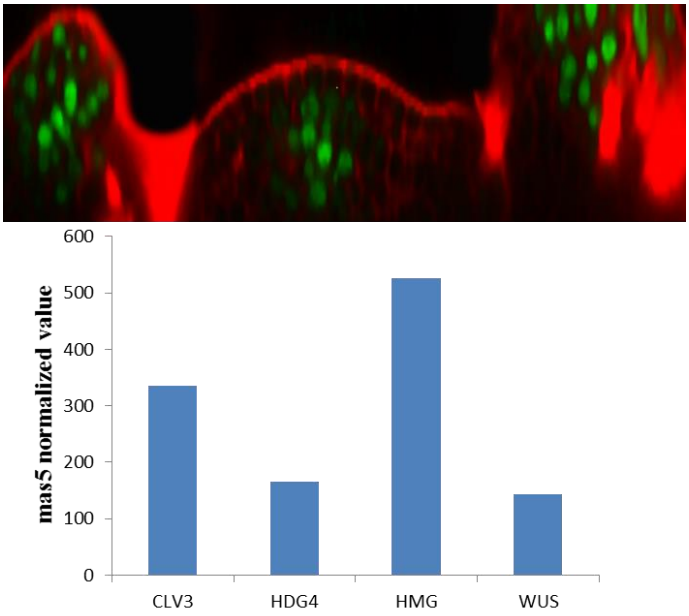

Fig. 9: Side view of wild type L*er* shoot apex, showing the expression pattern of *pAT4G32980:H2B-YFP*. Histogram showing the MAS5 expression values of *pAT4G32980:H2B-YFP*. Cell outlines highlighted by Propidium Iodide (red).

A summary of promoter-reporter constructs is given in the table 9. Of the 21 constructs, 14 were dipped *in planta* and screened for expression. Seven promoter reporter constructs showed expression *in planta*. A detailed description of the same is given below.

Table 9: Tabular summary of promoter-reporter constructs.

S.No.	Constructs sub-cloned into modified pGreen vector	Transgenic lines screened	Expression observed in
1	AT5G14960	AT4G14770	AT4G14770
2	AT4G14770	AT1G63650	AT1G63650
3	AT2G37590	AT1G75710	AT1G75710
4	AT1G65910	AT2G24570	AT2G24570
5	AT1G16070	AT4G31550	AT4G31550
6	AT1G07640	AT4G32980	AT4G32980
7	AT1G26310	AT3G16940	AT3G16940
8	AT1G29160	AT1G65910	
9	AT1G63650	AT1G07640	
10	AT1G64380	AT1G29160	

11	AT1G75710	AT1G64380	
12	AT2G24570	AT2G27050	
13	AT2G27050	AT4G01250	
14	AT3G02310	AT5G44210	
15	AT3G05690		
16	AT3G47600		
17	AT4G01250		
18	AT4G31550		
19	AT4G32980		
20	AT5G44210		
21	AT3G16940		

Objective 3.2: Role of Kip-Related Proteins in shoot apical meristem of *Arabidopsis thaliana*

All above ground parts of the plant are formed by the apical meristem, it can be proposed that the alteration in the cell proliferation can directly influence the meristematic cells and induce certain changes in their proliferation and differentiation capability. Cyclins and cyclin dependent kinases (CDKs) are involved in cell cycle progression and cell proliferation. Putative orthologs of CDKs inhibitors are known in plants and are termed as kip-related proteins (*KRPs*). About seven *KRPs* are known in Arabidopsis as *KRP1*, *KRP2*, *KRP3*, *KRP4*, *KRP5*, *KRP6* and *KRP7*.

Different *KRPs* are expressed in different tissues of plant where they are supposed to involve in different aspects of cell cycle regulation. Earlier studies have shown that *KRP1* and *KRP6* genes are expressed ubiquitously in actively dividing suspension culture and in various plant organs (roots, inflorescence, stems, flower buds and 3-week-old leaves) (De Veylder et al., 2001). *KRP4, KRP5* and *KRP7* are expressed in the cells that display high mitotic activity. *KRP2* and *KRP3* expression was recorded in dividing cells in suspension cultures. Some *KRPs* also expresses in trichomes, sepals, epidermal cells of leaf where they are known for promoting

endoreduplication (Roeder et al., 2010). Do the *KRPs* regulate the cell cycle progression in SAM?

To explore the function of *KRPs* in SAM development, I investigated their expression by semi-quantitative RT-PCR, and an ectopic expression study was undertaken specifically by taking *KRP3* and *KRP5* into account. Based on the microarray data of SAM cell types, *KRP2, 3, 4, 5* and *7* are expressed highly in SAM (Yadav et al., 2014) (Fig. 10). So, the above mentioned *KRPs* are used for the present study.

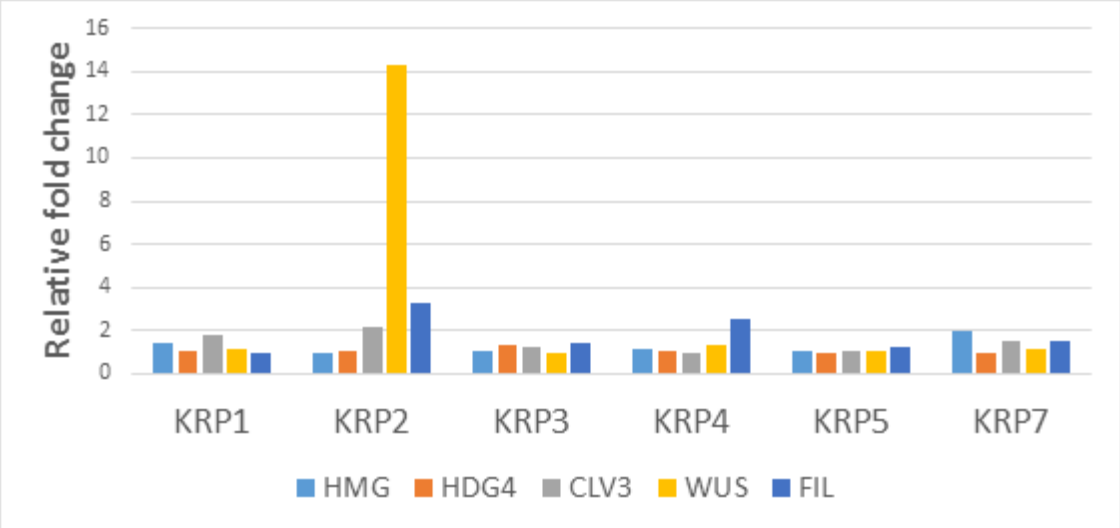

Figure. 10: Relative expression of *KRPs* in the SAM as depicted from microarray expression studies.

For over expression studies, coding sequences of *KRP2, 3, 4, 5* and *7* were cloned in pMDC32 vector under *35S* promoter, through LR gateway cloning reaction.

The overexpression constructs were transformed into *Arabidopsis* wild-type L*er* plants using floral dip method. The overexpression of *KRPs* in the transgenic plants was confirmed through semi-quantitative RT-PCR (Fig. 11).

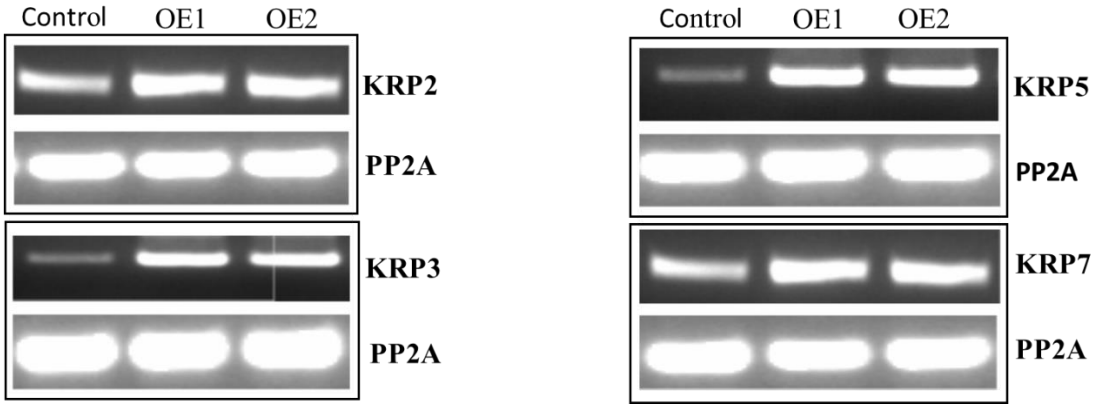

Figure. 11: Expression of KRPs using semi-quantitative RT-PCR. The figure shows the expression level of *KRP2, KRP3, KRP5* and *KRP7* in two overexpression lines, wild type L*er* was used as control. *PP2A* was used as an internal control.

3.2.1 Phenotypic characterization of overexpression plants

Plants over expressing *KRPs* were screened for the visible phenotype. Different phenotypes were observed in overexpression lines of *KRP3* and *KRP5*, respectively. The ectopic expression of *KRP5* resulted in compact inflorescence in comparison to wild-type. However, it is not clear, why and how these plants have developed compact inflorescence. There are several alternative explanations possible to this phenotype. In the past, several gain and loss function mutants displayed varying degree of compactness in inflorescence meristem. For example, *CLAVATA* loss of function mutants display compact meristem phenotype due to over proliferation of stem cells. However, the phenotype observed in the KRP5 over expression lines do not resemble to *CLAVATA* mutant. In addition, when the stem elongation gets compromised in the flowering plant such as in *Arabidopsis*, the meristematic activity will continue but to accommodate the developing organs the internode must elongate. It appears that in the *KRP5* over expression lines the later phenotype is more plausible (Fig. 12). Future, studies will elucidate the mechanism involved in compactness in the *Arabidopsis* SAM.

Figure. 12: Overexpression line of KRP5 showing more number of flowers in the inflorescence compared to wild-type.

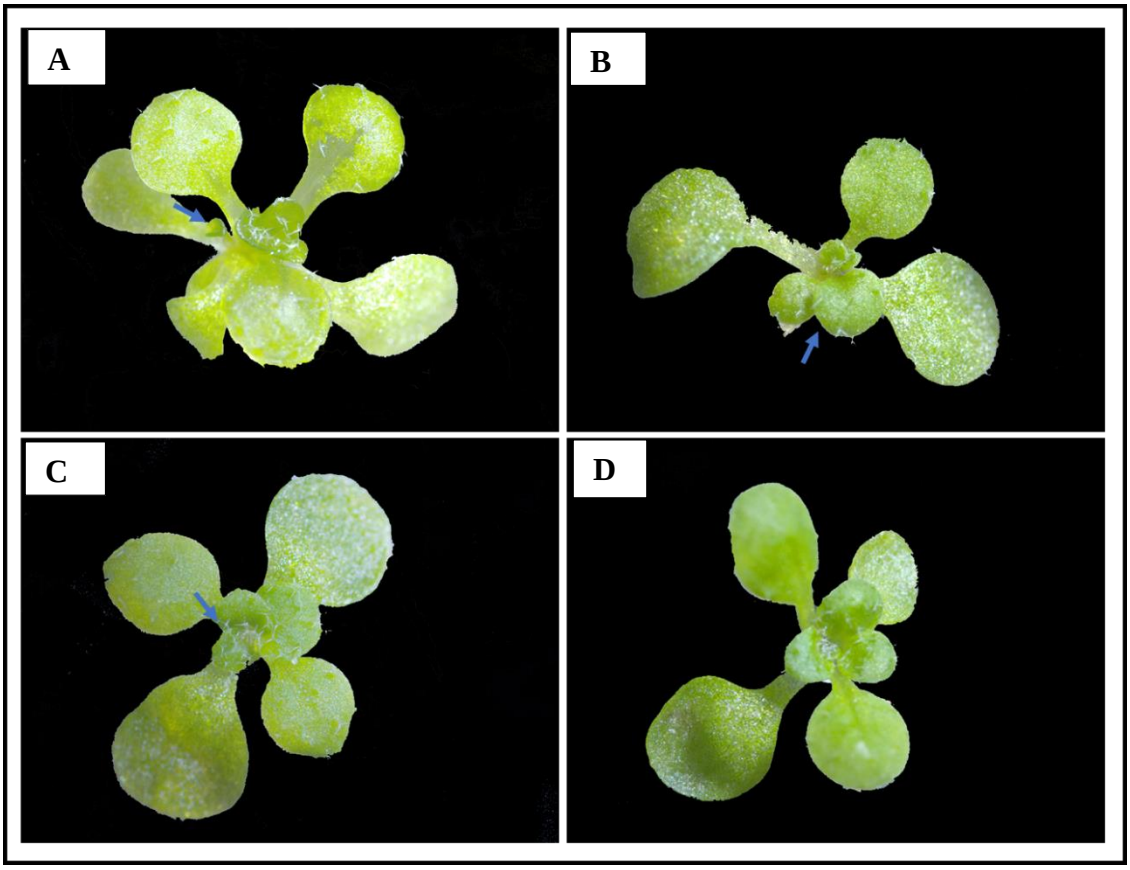

Figure. 13: The figure shows the defects in leaf orientation in plant lines overexpressing *KRP3* under *35S* promoter. A cup shaped leaf is arising from the main shoot axis (A; blue arrow). The leaf orientation was disturbed in 35S:KRP3 seedlings (B, C) compared to wild-type seedling (D).

When we investigated the role of *KRP3* by ectopic expression, I found plants with small leaf size, and floral organs size got reduced in comparison to wild type. Moreover, the *KRP3* overexpression lines showed phyllotactic defects in the seedlings; i.e. orientation of leaves around the main axis (Fig. 13). Flowers of *KRP3* over expression plants were defective in organ symmetry and displayed curling up of the tip of petals. A visible phenotype of small petals and petal folding i.e. abaxialization was also observed in the overexpression lines of *KRP3* as shown (Figure. 14)

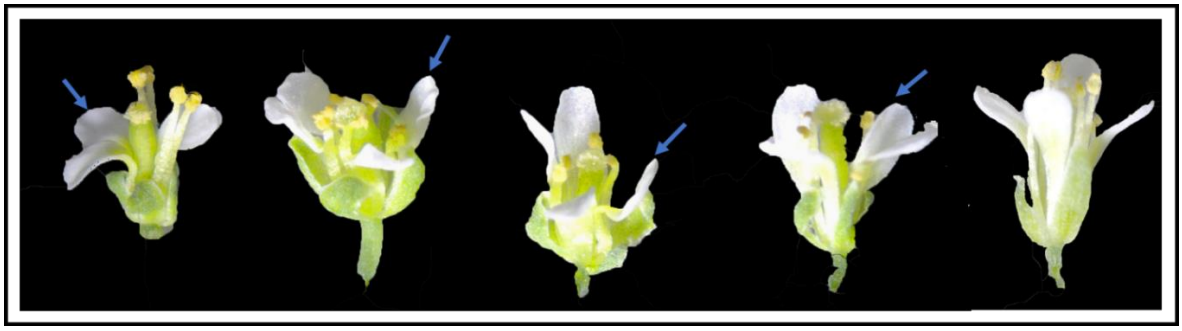

Figure. 14: Petal folding in *KRP3* overexpression lines (blue arrows).

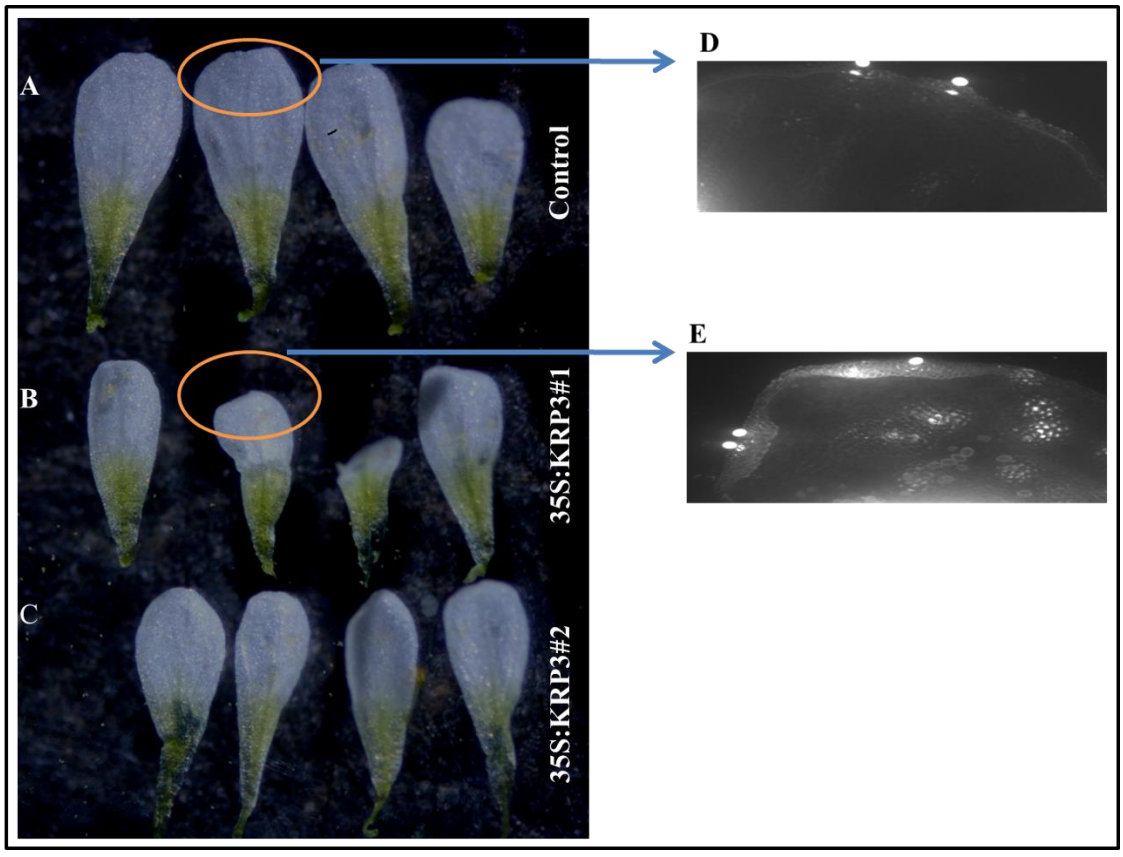

Figure. 15: Analysis of petal size and folding in *35::KRP3* overexpression lines. *KRP3* flower petal (B and C) (blue arrows). In high magnification the wild type flower petal tip (D), and *35S::KRP3* flower petal tip in (E). Petal is curled up in *35::KRP3* flowers.

Further, the epidermal and palisade cell size was measured in the first true leaf of *KRP3* over expression lines and this was compared with wild type L*er*. There was no significant change observed in the epidermal cell size in *35::KRP3* lines compared to control plants, while a significant increase in the palisade cell size was observed (Fig.16).

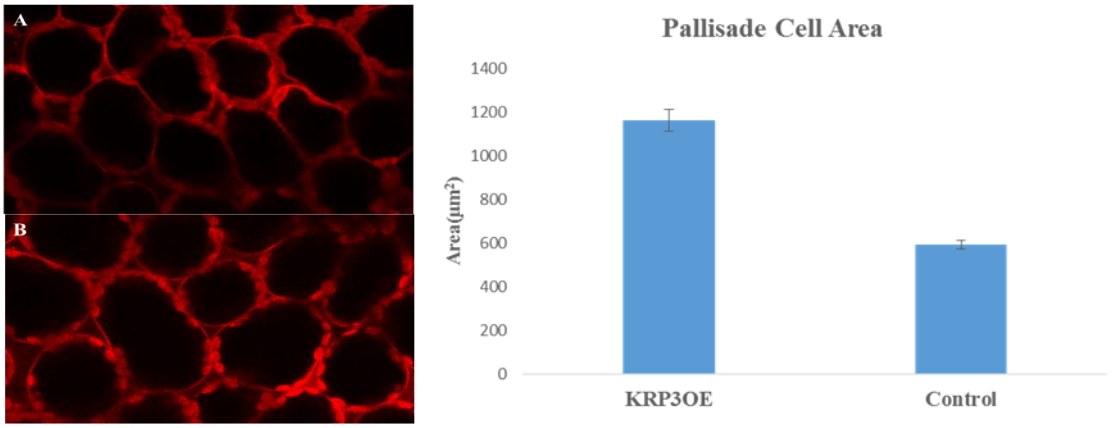

Figure. 16: A, B are representative image for control and *35S::KRP3* over expression, respectively. The histogram shows palisade cell area in *35S::KRP3* and control Arabidopsis first true leaf (n=100). The error bar shows standard error mean.

3.3 Discussion

3 kb upstream regulatory elements are sufficient to drive endogenous expression for large number of TFs

In this study, I examined the expression pattern of TF genes whose transcripts were enriched ≥ 1.5-fold in the epidermal and sub-epidermal cell layer. I used a 3kb promoter DNA fragment to construct transcriptional fusions of these genes. In total, 44 TF promoters were rescued and used for making transcriptional fusion constructs in this study. We successfully rescued transgenic lines for 43 TF genes. To assess the representative tissue/cell type specific expression pattern of promoter reporter, we screened multiple lines for each construct. We detected H2B-YFP expression in 22 of the 44 constructs screened. For 18 constructs, upon performing a quantitative assessment of H2B-YFP expression, we found the promoter-reporter expression closely resembling the digital expression pattern recorded using cell type specific microarray. We observed in two cases that the reporter gave random nuclear localized YFP expression while it did not glow at all in the rest. We did not observe a visible expression pattern in shoot apex for 13 promoters although the digital expression pattern predicted the presence of their transcripts. One simple explanation could be that the 3 kb DNA fragment upstream of the start codon, used for making the majority of the promoter-reporter constructs, did not have sufficient cis-regulatory elements to drive the transcription of that gene in the

relevant cell types. However, using the 3kb promoter fragment did result in the expression of the gene in the expected cells and tissues types for 50% of the genes.

Role of Kip-Related Proteins in *Arabidopsis thaliana*

Cell cycle progression is controlled by an evolutionarily conserved molecular mechanism that is imparted by cyclins and cyclin dependent kinases (CDKs). Because of its importance in growth and development, the CDK-cyclin activity must be strictly controlled. In yeast and mammals, one of the major regulators of CDK activity are CDK inhibitory molecules (CKIs) that bind and inhibit CDKs. Recently, putative orthologs of CKI proteins have been identified in plants and are termed as kip related proteins (KRPs). Arabidopsis genome code for about 7 *KRPs* with no similarity or homology amongst them. These KRPs expressed in different issues of plants as observed through *in situ* hybridization, and hence play a distinct role in plant development. Meristem is a region in plants which led to the development of all the structural elements of the plant. What is the status of KRPs and how they are involved in cell cycle regulation in SAM is not known. So, the present study has been conducted to know the function of these *KRPs* in plants through overexpression studies with special reference to *KRP3.* Because *KRP3* is known to play a role in endoreduplication in plants. Microarray studies have shown the significant expression of *KRP2, KRP3, KRP4, KRP5*, *KRP7* in SAM compared to the other *KRPs*. Phenotypic characterization of KRP3 OE have shown the significant reduction in organ size like flowers, leaves and petals. Petal folding towards abaxialization was observed in KRP3 OE plants which could be due to larger adaxial cell size. Significant increase in palisade cell size was also observed in KRP3 OE plants which shows the inhibitory effect of KRP3 on palisade cell division. On the other hand, KRP5-OE inflorescence showed increased number of flowers which could be due to the increased mitotic activity in KRP5 OE lines. Finally, the great variety of cyclins–CDK complexes and KRPs points towards a complex regulatory network amidst them. This network may respond to the diverse environmental conditions to which plant adapts or may be crucial to developmental needs of various tissues.

Chapter - 4

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