Standardising Transient Expression in *Arabidopsis* Mesophyll Protoplasts (TEAMP) system for gene regulatory network Validation.

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MS14173

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Certificate of Examination

This is to certify that the dissertation titled **"Standardising Transient Expression in *Arabidopsis* Mesophyll Protoplasts (TEAMP) system for gene regulatory network validation"** submitted by Avneet Kaur (Reg. No. MS14173) for the partial fulfilment of BS-MS dual degree Programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Santosh Satbhai Dr. Shashi Bhushan Pandit Dr. Ram Yadav

(Supervisor)

Dated:

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Ram Yadav at the Indian Institute of Science Education and Research, Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other institute or University. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgment 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.

Avneet Kaur

(Candidate)

Dated:

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

Dr. Ram Yadav

(Supervisor)

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List of figures

Notations

GRN: Gene Regulatory Network

Y1H: Yeast one hybrid

PDI: Protein-DNA interaction network

TF: Transcription factor

POI: Promoter of Interest

SAM: Shoot Apical Meristem

FACS: Fluorescence Activated Cell Sorting

LUC: Luciferase

GFP: Green Fluorescent Protein

BSA: Bovine Serum Albumin

TEAMP: Transient Expression in *Arabidopsis* Mesophyll Protoplasts

Contents

Abstract

Protein-DNA interactions play a very important role in regulating several cellular processes in multicellular organisms. Similar interactions occur in plants as well. A gene regulatory network of transcription factors enriched in Shoot Apical Meristem(SAM), and promoters of cytokinin genes (biosynthesis, degradation, and signalling) has been constructed by yeast-1-hybrid(Y1H).Furthermore, validation needs to be carried out as yeast is a heterologous system. There are various experimental methods available to validate the protein-DNA interactions, but we have taken *in planta* approach to validate them using mesophyll protoplasts from *Arabidopsis*. Transient expression in mesophyll protoplasts (TEAMP) is an experimental method to validate these interactions *in planta* as Arabidopsis mesophyll protoplasts maintain many of the same physiological responses and cellular activities as present in intact plants. We have taken this approach and tried to standardize optimal conditions of protoplast isolation, protoplast cell culture, and transfection. We also constructed various expression clones to perform the transient reporter based (GFP, GUS, and LUC) assays.

Chapter 1

Introduction

1.1 *Arabidopsis thaliana*

Arabidopsis thaliana is a small flowering plant, belonging to the mustard family. It is an outstanding model organism for studying the molecular players involved in development, physiology and regulation of flowering in angiosperms. *A. thaliana* has a small life cycle of 6 weeks and produces a lot of seeds every generation. It has a fully sequenced genome size of around 135 mega base pairs. Using *Agrobacterium* T-DNA, genome-wide insertional mutagenesis have been generated to understand the function of genes of fully sequenced genome[1].A group of cells called meristem is present at the tip of root and shoot that gives rise to almost all the tissue in a grown-up plant. Shoot Apical Meristem(SAM) give rise to all organs above ground of a plant such as a stem, leaves, and flowers, whereas Root Apical Meristem (RAM) give rise all organs underground[2].

Figure 5. *Arabidopsis thaliana*- a model organism

1.2 Cytokinin

Cytokinin (CK) is a plant hormone. It has been shown to play a vital role in the shoot meristem development, leaf development, chloroplast biogenesis, and senescence.[3]Classical experiments suggest that surplus of cytokinin over auxin foster shoot formation from callus.[4]Many key genes have been discovered both by forward genetic screens and reverse genetic approaches. Some of the notable examples are; genes involved in cytokinin biosynthesis such as LONELY GUYs(LOG's) and isopentenyl transferases (IPT's), ARABIDOPSIS HISTIDINE KINASES(AHK's) genes encode for CK receptors are involved in signalling whereas CYTOKININ OXIDASES(CKX's) are involved in degradation. ARABIDOPSIS HISTIDINE PHOSPHO TRANSFER PROTEINS (AHPs) are involved in PO^{-}_{4} transfer between receptor and effectors such as ARABIDOPSIS RESPONSE REGULATORS (ARRs).[5]

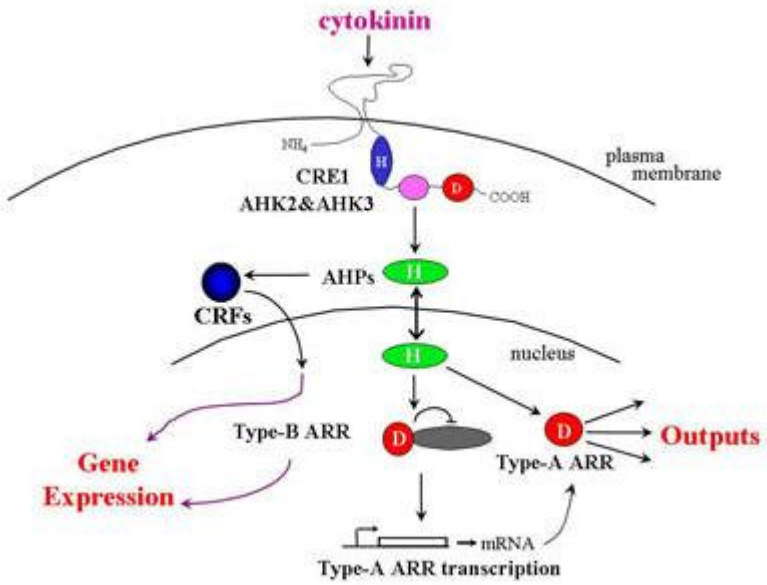

Figure 6. Cytokinin signalling model: Cytokinin binds to the cytokinin receptor-AHK and causes autophosphorylation on histidine residue. Through AHP, phosphoryl group is transferred to the receiver domain of type-A and type-B ARRs which then modulates cytokinin-regulated transcription.[6]

1.3 Gene Regulation

Protein DNA interactions play a very important role in regulating various cellular processes. By this mechanism, cells regulate the amount of gene product required to carry

out the critical biological function for the survival of an organism. Transcriptional and translational machineries play a significant role in this too. Transcription factors interact with promoters of the target genes and either activates or represses their expression.[7]Thus affects the availability of critical gene products which are required for cellular functioning. Therefore, understanding the mechanism of transcriptional regulation and when and where TFs bind to target gene promoters is critical to discover.

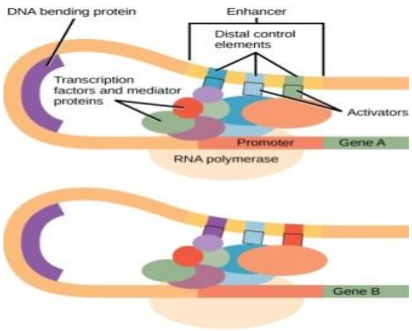

Figure 7. Transcription factors and mediator proteins interaction with the promoter

1.4 *Arabidopsis* mesophyll protoplasts

To study gene regulation, ,mammalian cell lines are transiently transfected with plasmids having both upstream TF protein and target gene promoter anchored with a reporter gene routinely. If the upstream regulator binds to the target gene depending upon nature of regulation an output will be produced. By developing such assays researchers reliably able to decipher the important binding sites and their importance in gene regulation. In plants, an analogous system was developed to decipher the regulation of gene expression is based on the mesophyll cells.[7]It offers several advantages, for example, the cells can be transfected using electroporation, PEG-calcium method and microinjection similar to mammalian system.[8]Second,in mesophyll protoplast both the physiological pH and co factors required to achieve optimum gene expression are present just like intact plants. Third, the outcome of such assays can be obtained within 24 hours after transfection to infer the results.

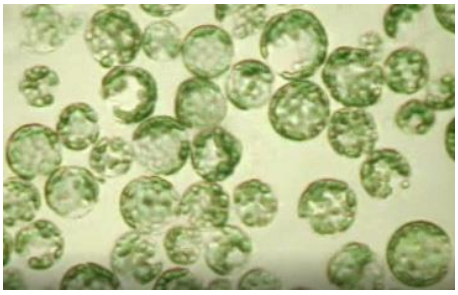

Figure 4. *Arabidopsis* mesophyll protoplasts released after 3 hours of enzyme treatment.

1.5 Transient gene expression in *Arabidopsis* mesophyll protoplasts

Eukaryotic cells can express genes for a short time after the introduction of plasmid DNA into them.[9] Thus they can transiently express the genes of interest. Various reporter genes like green fluorescent protein (GFP), GUS have been engineered and can be transfected into the eukaryotic cells for the transient expression.[10]Similarly, the firefly luciferase gene has been engineered and transfected to study transientgene expression in mesophyll protoplast cell system of plants to monitor dynamic changes in the transcription.[8]

1.6 Objectives

The main goal of this study is;

a) To engineer various gene constructs to perform transient reporter (GFP, GUS, LUC) based assays.
b) To standardize conditions for protoplast isolation and maintenance.
c) To standardize the conditions for transient expression in *Arabidopsis* mesophyll protoplasts (TEAMP) system.

Chapter 2

Materials and Methods

2.1 Plant Material and Growth Conditions

Arabidopsis thaliana wild type L*er* plants were grown in 16-hour light and 8-hour dark conditions. Once they reached the three-week stage, their leaves were used for protoplast preparation.

Figure 5. Three weeks old *Arabidopsis* plants for protoplast isolation

2.2 LB Media

Luria-Bertani broth (LB) is the nutritionally enriched media used for the growth of bacteria. The following components were added to make LB media. Once the components were added, the media is autoclaved for sterilization.

Yeast Extract	5 grams
Tryptone	10 grams
Sodium Chloride	10 grams
Distilled Water	1litres

2.3 LB Agar Antibiotic Plates

This is basically solidified LB, in which solidification occurs due to the addition of Agar. Antibiotics are added just before pouring plates so that appropriate selection can be

applied to the bacteria containing antibiotic resistance gene. The following components are added to make LB Agar.

Yeast Extract	5 grams
Tryptone	10 grams
Sodium Chloride	10 grams
Agar	15 grams
Distilled Water	1 litre

After addition of these components the media is autoclaved and while pouring 1ml required antibiotic was added to 1L media (stock- ampicillin or kanamycin: 50 mg/ml). The media in the plates was allowed to solidify, and plates were then stored in 4^{o}C for future use.

2.4 LR Reaction

LR Reaction mix

Entry Clone	50 ng
Destination Vector	50 ng
TE	Make up the volume to 3µl
Enzyme	0.3 µl

This mixture was kept for incubation at 25℃ for 1 hour or 3 hours depending on the size of the gene of interest that needs to be cloned into the destination vector. This was then transformed into ultra-competent DH5α cells by chemical transformation.

2.5 SOB media(for secondary culture)

For 1000 ml	
Tryptone	20 grams
Yeast Extract	5 grams
NaCl	0.5 grams

Salts (should be added after autoclaving-for 1L)

0.5 M Magnesium Chloride	20 ml
1M KCl	2.5 ml
1M Magnesium sulphate	10 ml

2.6 Ultra-Competent Cell Preparation of DH5α (*E.coli*)

Material required: SOB media, Transformation buffer 1, Transformation buffer 2, Transformation buffer 3, LB plate, Oakridge tubes, 250 ml bottles, MCT's (autoclaved).

Transformation buffer 1(100 ml)

Stock	Final concentration	Volume from stock
1 M MOPS(pH 6.5**)**	10 mM	1 ml
1 M KCl	100 mM	10 ml
1 M $MnCl_2$	45 mM	4.5 ml
1 M $CaCl_2$	10 mM	1 ml
1 M K Ac (pH 7.5)	10 mM	1 ml
Sterile distilled Water		82.5 ml
		100 ml

Transformation buffer 2

Same as transformation buffer one except that we have to add 12.5 ml of 80% glycerol and then make up the volume to 100 ml.

Transformation buffer 3 (for transformation)

Both the solutions should be autoclaved separately.

$CaCl_2$	100mM
$MgCl_2$	50mM

Protocol

- Inoculate 1% primary culture (500µl) in 50 ml SOB and shake at 37ºC till OD reaches to 0.5-0.7. This usually takes 1.5-2.5 hours (never use a pipette to dissolve pellet instead rub on ice).
- Keep on ice for 10 minutes.
- Spin the dissolved pellet at 3500 rpm, 4ºCfor 10 minutes.
- Discard the supernatant, and dissolve the pellet in 25 ml transformation buffer 1.Keep on ice for 10 minutes.
- Spin the dissolved pellet at 3500rpm, 4ºC for 10 minutes.
- Discard the supernatant, and dissolve the pellet in 4ml transformation buffer 2. To the dissolved pellet, add 140 ml DMSO.
- Keep on ice for 15 minutes and add again 140 ml DMSO.
- Aliquot in vials and store at -80ºC.
- For transformation, add an equal volume of buffer 3 and use 100 ml cells for transformation.

2.7 Transformation of DH5αcells using chemical transformation.

Materials required**:** MCT, 800µl LB media, 50µl DH5α ultra competent cells, Plasmid or LR reaction mix, and transformation buffer 3.

Protocol:

- Add 50 µl of transformation buffer 3 to the 50 µl ultra competent cells on ice.
- Add one picogram of plasmid or 100 ng of LR reaction mix to the cells on ice.
- Incubate the tube on ice for 30 minutes.
- Heat shock the cells with the plasmid for 60 sec.
- Incubate the cells on ice for 5 minutes.
- Add 800 µl LB to the cells.
- Keep it for 1 hour at 37ºCin incubator shaker.
- After incubation, plate the cells on LB agar plate (Kanamycin or Ampicillin resistance)
- Leave the plate overnight at 37ºC.

2.8 Colony PCR

- A single colony was picked from the plate and was dissolved in 10 µl autoclaved water.
- In the tube along with bacterial cells following components were added to perform the colony PCR.
- Samples were mixed and then kept in PCR machine (Thermo cycler-BIORAD)

10x PCR buffer	1.5 µl
2.5 mM dNTP's	1.2 µl
Primer 1	0.75 µl
Primer 2	0.75 µl
Phusion Polymerase	0.3 µl
Colony dissolved in water	10 µl

PCR conditions:

STEPS	TEMPERATURE	TIME
Initial denaturation	98℃	3 min
Denaturation	98℃	30 sec
Annealing	55℃	30 sec
Extension	72℃	(1 kb/min)
Repeat the steps 2-4		No of cycles: 30
Final extension	72℃	10 min
Hold	4℃	∞

Once the PCR was complete, samples were analyzed by running them on 0.8% agarose gel(100 Volts).

For conventional plasmid PCR, instead of dissolving bacterial colony in water, 10ng plasmid DNA was used.

2.9 Agarose gel electrophoresis

Materials required:

- Agarose gel tank, 1X TAE buffer, agarose, ethidium bromide, pipettes, PCR product.

- The solidified gel was then kept in the gel tank filled with 1X TAE buffer in such a way that the wells were near the negative terminal.
- The PCR product was then mixed with 6x loading dye (bromophenol blue) and loaded in the wells of agarose gel.
- Pre-mix of 1 kb ladder was loaded in one of the parallel rows.
- Appropriate voltage (70-90V) was applied until the sample run to 2/3rd of the gel.
- Image of the gel was taken using a gel documentation system (BIO-Rad, USA)

2.9A: 10X TAE buffer

The following components were mixed in a 1L flask.

Tris base	108g
Glacial acetic acid	57 ml
0.5 M EDTA	40 ml
H_2O	Make up the volume to 1L

2.9B: 0.8% Agarose Gel

- 0.8g of agarose was added to 100ml 1X TAE buffer.
- Dissolve the agarose in 1X TAE buffer completely by heating.
- Let it cool (around 50℃).
- 0.2µl of ethidium bromide (0.5µg/ml) was added to it.
- Mix it well, pour it into gel casting tray and set the combs.
- Let it solidify.

2.9C: 6X Loading dye components (10 ml)

The following components were added together and to make up the volume to 10 ml using distilled water.

Glycerol	1 ml
Xylene cyanol	0.025g
Bromophenol blue	0.025g

2.10 Plasmid isolation (miniprep using kit)

- Inoculate the colony in 5ml LB media.
- Add 5µl ampicillin or kanamycin to the culture.(Stock: 50mg/ml)
- Incubate the sample at 37ºC shaker overnight.
- Pellet the 5ml bacterial overnight grown culture by centrifugation @13000 rpm for 3 minutes at room temperature. Discard the supernatant.
- Resuspend the pellet of bacterial cells in 250µl of buffer R1.(RNase is already added to buffer R1).
- Add 250µl of buffer L2 and mix well by inverting the tube 4-6 times until the solution becomes clear.
- Add 350 µl of buffer N3 and mix immediately by inverting the tube 4-6 times.
- Centrifuge for 10 minutes in a tabletop micro centrifuge.
- Take the supernatant from the above step and transfer it to the spin column. Centrifuge for 30-60 sec and discard the flow through.
- Wash the spin column by adding 750 µl of wash buffer. Centrifuge for 30-60 sec and discard the flow through.
- Centrifuge again for 1min to remove residual wash buffer.
- Place the column in clean 1.5 ml micro centrifuge tube. To elute DNA, add 40 µl pre-heated autoclaved water in the centre of the spin column, let it stand for2 min, and centrifuge for 1min.

2.11 Restriction digestion

To confirm the cloned construct, the plasmids were digested using restriction enzyme(s), and the digested products were analyzed using agarose gel electrophoresis.

2.11A:Single digestion

The following components were mixed in a 1.5ml MCT and incubated at 37℃ for 2hrs.

Plasmid	200 ng
NEB buffer	1 µl
Enzyme 1	0.2 µl
Water	make up the solution upto10 µl
Total	10 µl

2.11B: Double digestion

The following components were mixed in a 1.5ml MCT and incubated at 37℃ for 2hrs.

Plasmid	200ng
NEB Buffer	1µl
Enzyme 1	0.2µl
Enzyme 2	0.2 µl
Water	Make up the solution up to 10 µl
Total	10µl

2.12 Bacterial Stock Preparation

- Equal amount of bacterial culture (containing the construct) and 50% glycerol (autoclaved) was added into a cryovial.
- Stocks were stored at -80℃.

2.13 Digestion of leaves for mesophyll protoplasts

- Plants were grown in growth chambers till they reach the three-four week stage. Well, expanded leaves were chosen before flowering. Following the composition of **enzyme solution** (15 ml) was used to digest the leaves.

Components	Amount added	Final Concentration
0.2 M MES pH 5.7(filter sterilised)	1.5 ml	20mM
Cellulase R10	225mg	1.5%(W/V)
Macerozyme R10	60 mg	0.4%(W/V)
0.8 M Mannitol(autoclaved)	7.5ml	0.4M
2 M KCl(autoclaved)	0.5ml	20mM
1 M Calcium Chloride(autoclaved)	150µl	10 mM
Bovine Serum Albumin	15 mg	0.1%

- MES was preheated for 5 minutes at 70^{o}C before the enzymes were added. Cellulase, macerozyme, mannitol, and KCl were added one by one after that. This was then heated at 55^{o}C for 10 minutes to inactivate DNAse, protease and to increase enzyme solubility. Following this, the solution was cooled to 25°C.
- 0.5-1 mm leaf strips were cut from the middle part of a leaf using a fresh sterile razor blade without damaging the tissue. This was done for around 10-20 leaves.
- The leaf strips were transferred carefully using a pair of flat-tip forceps and submerged completely into the prepared 5-10 ml enzyme.
- Using a desiccator, the leaf strips were vacuum infiltrated in the dark for 30 minutes.
- The digestion was carried out for 3 hours at room temperature without shaking. The release of protoplasts was checked by a change in colour of enzyme solution to green by swirling the plates.
- The release of mesophyll protoplasts (of size in range 30-50µm) was checked under a microscope.
- The undigested leaf tissues were removed before filtration by diluting the protoplast solution using an equal volume of W5 solution.
- A clean 75-µm nylon mesh was washed with water to remove ethanol in which it is kept soaked. The excess water was removed before protoplast filtration. The enzyme solution containing protoplasts were filtered after wetting the 75-µm nylon mesh with W5 solution.
- The flow-through was centrifuged in a 30-ml round-bottomed tube for 1-2 min at 700rpm. The maximum supernatant possible was removed, and the protoplast was resuspended by gentle swirling.

- The protoplasts were resuspended in W5 solution at $2X10^5$ per ml after counting cells under the microscope using a haemocytometer. The protoplasts were kept on ice for 30 min

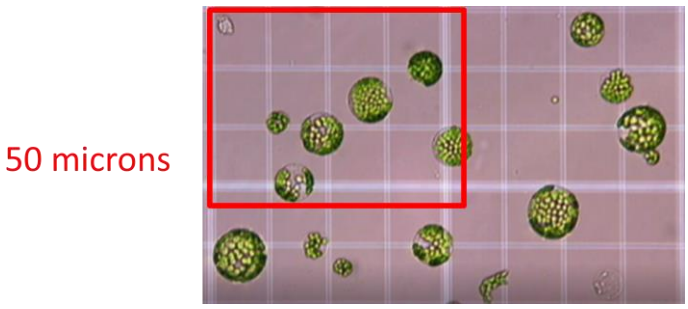

Figure 6. Protoplast counting using haematocytometer

- The protoplasts were kept on ice for 30 min.
- The W5 solution was removed while keeping the protoplast pellet intact. The protoplasts were resuspended at 2 X 10^5 per ml in MMG solution at room temperature.

WI Solution (10 ml)

Components	Amount added	Final Concentration
0.2 M MES (pH 5.7)	200 µl	1 mM
0.8 M (Mannitol)	6.2 ml	0.5 M
2M (KCl)	10 µl	20 mM

W5 solution (35 ml)

Components	Amount added	Final Concentration
0.2 M MES (pH 5.7)	350 µl	2 mM
NaCl	310 mg	154 mM
1 M Calcium Chloride	4.375 ml	125 mM
2 M KCl	87.5 µl	5 mM

MMG solution (10 ml)

Components	Amount added	Final Concentration
0.2 M MES (pH 5.7)	200 µl	4mM
0.8 M Mannitol	5 ml	0.4 M
0.5 M Magnesium Chloride	300 µl	15mM

2.14: Transfection of mesophyll protoplasts using PEG Calcium method.

PEG-Calcium transfection solution(2.5 ml)

Components	Amount added	Final Concentration
PEG 4000	0.5 g	20%
1M Calcium Chloride	250 µl	100 mM
0.8 M Mannitol	600 µl	0.2 M

- 10 µl DNA (10-20 µg plasmid DNA) was added to a 2 ml micro centrifuge tube.
- 100 µl protoplasts were added and mixed gently.
- 110 µl of PEG solution was added and mixed completely by gently tapping the tube.
- The transfection mixture was incubated at room temperature for 15 minutes.
- The transfection mixture was then diluted and mixed well with 400-440 µl W5 solution at room temperature to stop the transfection process.
- Centrifugation was done at 100g for 2 min at room temperature and the supernatant was removed.
- The protoplasts were resuspended each, in 1 ml WI in each well of a 6-well tissue culture plate.

2.15 Protoplast culture and harvest

Protoplast Lysis Buffer (5 ml)

Components	Amount added	Final Concentration
1 M TrisPhosphate (pH 5.8)	75 µl	25mM
100mM DTT	50 µl	1mM
100mM DACTAA	100 µl	2mM
50% Glycerol	1 ml	10%
20% Triton X-100	2.5 ml	10%

- The protoplasts were incubated at room temperature for the desired period after transfection.
- The protoplasts were resuspended and harvested by centrifugation at 1000 rpm for 2 min.
- The supernatant was removed, and the samples were frozen on dry ice.

2.16 Reporter assays

1) GUS assay

- 100 µl lysis buffer was added to the frozen protoplasts and mixed vigorously for 2 sec to rupture the protoplasts. It was kept on ice for 5 minutes. Centrifugation was done at 1000g for 2 minutes.
- 100 µl MUG substrate mix was added to 10 µl of the protoplast mix and incubated at 37ºC for 30-180 minutes. ~ 0.9 ml 0.2 M Na_2CO_3 was added to stop the reaction.
- Fluorescence was measured for MU using a fluorimeter.

MUG substrate mix

Tris phosphate (pH 7.8)	25mM
MUG	1mM
Magnesium Chloride	2mM

2) GFP assay

Cultured cells were taken after various time points. Cells were centrifuged, and GFP expression was analyzed using confocal microscopy.

2.17 Maxi prep of plasmid(manual)

Solution 1: Resuspension solution

Glucose	50mM
Tris-HCl (pH 8)	25mM
EDTA (pH 8)	10mM

Solution 2: Composition of Lysis Solution (freshly prepared)

Components	Stock concentration	Working concentration
NaOH	10 N	0.2 N
SDS	10 %	1 %

Solution 3: Neutralisation buffer

5 M Potassium Acetate	60 ml
Glacial Acetic Acid	11 ml
Sterile water	28.5 ml

The resulting solution is 3M w.r.t. K+ and 5M w.r.t.acetate.

Procedure:

- Inoculate single bacterial colony into 5ml of LB media with a suitable antibiotic. Keep the culture overnight at 37^{o}C in incubator shaker (220 rpm).
- Take the inoculated culture.
- Centrifuge at 8000 rpm for 3 minutes.
- Discard the supernatant and let the pellet dry.
- Resuspend the pellet in 250µlof cold solution 1(RNAse is added to the final concentration of 20 micrograms/ml in solution 1) by vigorous vortexing such that the pellet dissolves completely. Keep on ice for 5-10 minutes.

- Add 250µlof freshly prepared solution 2 and mix content by gently inverting the tube 5-6 times. At this step does not allow vortex, harsh mixing will shear the genomic DNA. Thus it can result in chromosomal DNA contamination in the plasmid DNA.
- Keep the tube on ice for less than 5 minutes.
- Add 350 µl of solution 3 and mix the content by inverting the tube 10 times. Incubate on ice for 5 minutes.
- Spin at 13000 rpm for 5 minutes.
- Transfer the supernatant to a second set of tubes using a pipette.
- Add an equal volume of Phenol Chloroform Isoamylalcohol.
- Mix by vortexing for 3-5 seconds and centrifuge at 8000 rpm for 5 minutes.
- Collect the upper layer in the third set of Eppendorf tube.
- Add an equal volume of isopropanol.
- Keep at -20°C for 10 minutes.
- Spin at 13000 rpm for 5 minutes.
- Decant the supernatant.
- Wash with 700 µl of 70% ethanol.
- Spin at 13000 rpm for 5 minutes. Remove the supernatant and let it dry.
- Resuspend in 40µlof TE buffer.
- Check the quality and concentration of plasmid by running on the gel.
- Store the plasmid at 4°C.

2.18 Maxi prep of plasmid (using column)

Equilibrating the column

- 30ml of equilibration buffer was added directly into the filtration cartridge.
- The solution was allowed to drain by gravity flow.

Preparation of Cell Lysate

- 250 ml of overnight LB culture per sample was taken
- Cells were harvested by centrifuging overnight LB culture at 4000 rpm for 20 minutes. The supernatant was removed.
- 10 ml of Resuspension buffer(containing RNase A) was added to the pellet and cells were resuspended until they become homogeneous.

- Following this, 10 ml of lysis solution was added, and the tube was inverted gently. The mixture was kept at room temperature for 5 minutes.
- 10 ml of Neutralisation buffer was added, and mixing was done gently by inverting the tube.

Loading of filter column and DNA Washing

- The precipitated lysate was transferred to the equilibrated column.
- Immediately, once the lysate stopped dripping, inner filtration cartridge was removed.
- The maxi column was washed with 50ml of wash buffer, and the solution in the column was allowed to drain.
- Flow through was discarded.

Eluting DNA

- Under the maxi column, a 50ml centrifuge tube was placed.15ml elution buffer was added to the column, and the solution was allowed to drain by gravity flow. Following this maxi column was discarded.

Precipitation of DNA with isopropanol

- 10.5 ml of isopropanol was added to the eluted DNA and was mixed properly.
- The tube was centrifuged at 13000rpm for 30 minutes at 4^{o} C. Supernatant was discarded.
- 5ml of 70% ethanol was added to resuspend the DNA pellet. Then the tube was centrifuged at 13000rpm for 5minutes at 4^{o}C. supernatant was discarded.
- The pellet was air-dried for 15 minutes. Following this, the pellet was resuspended in 500 µl of TE buffer.
- The quality and concentration of plasmid was checked by running it on the gel.

2.20 Confocal Microscopy

Confocal microscopy was done using a Leica TCS-SP8 microscope. The protoplasts were examined with excitation at 488 nm and emission at 500-534nm for eGFP and 678-740 for auto fluorescence.

Chapter-3

Results and Discussion

3.1 Construction of plasmid constructs that are required in the TEAMP system.

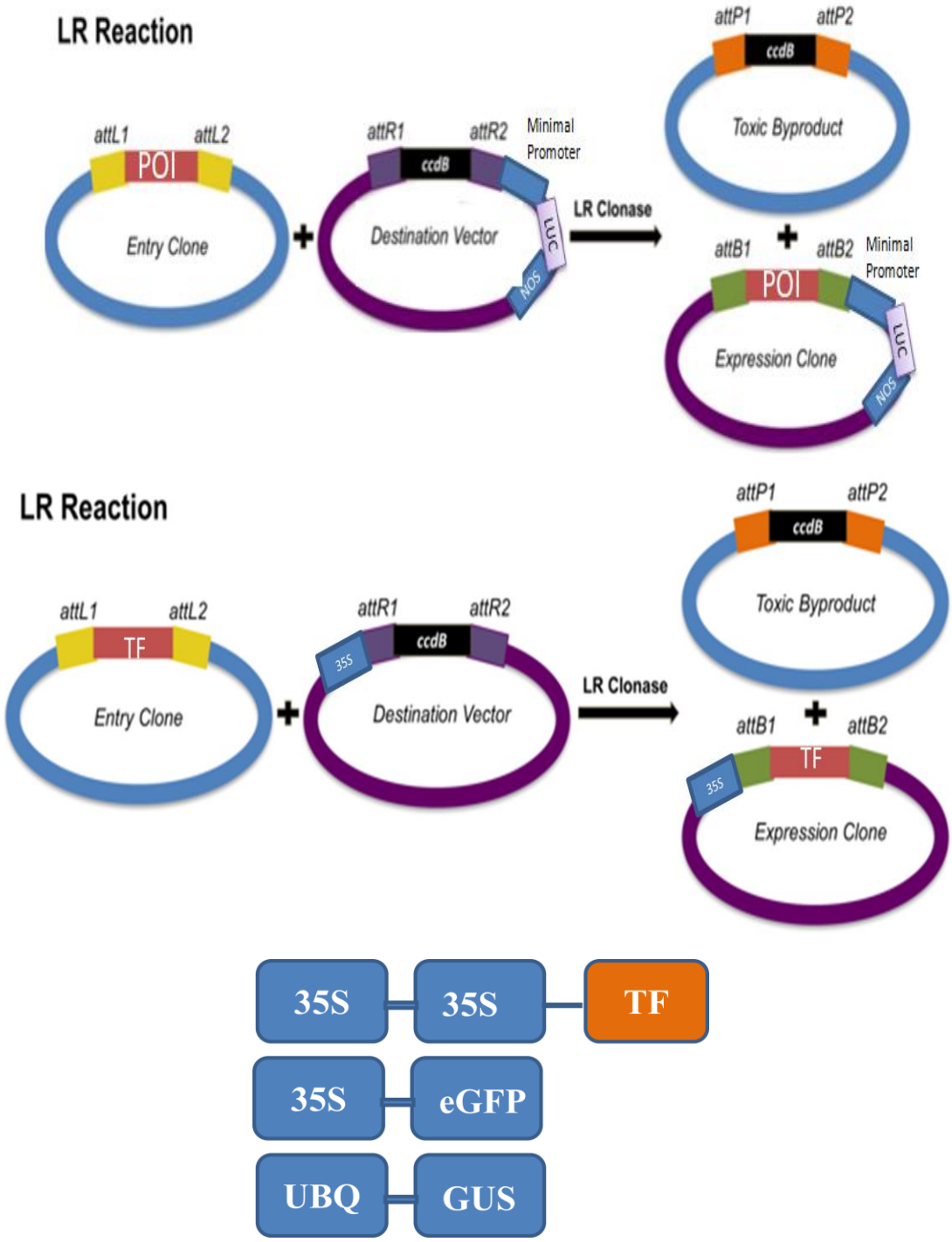

Figure 7. Schematic representation of plasmid constructs for TEAMP assay.

To standardize transient gene expression assay in *Arabidopsis* mesophyll protoplasts, I first constructed required plasmids as the main objective of this study was to validate the interaction of transcription factors with cognate promoters. Promoters of interest(e.g., *pLOG4*, *pAHK4*,and *pTAR2*) were cloned upstream to luciferase while upstream TF,AZF2was cloned by Sonal, Also, WUS was given by Shalini, both these TFs constructs are placed behind 35Spromoter in a gateway compatible plasmid. For optimization of proper transfection conditions. EGFP was cloned downstream of 35Spromoter by Sonal Yadav. *pUBQ::GUS* was given by Prof.Utpal Nath. AZF2 and WUS and eGFP coding sequence were cloned downstream of 2x35Spromoter. All the above clones were confirmed first confirmed by colony and restriction digestion in the end, confirmed by Sanger sequencing.

3.2 Optimization of mesophyll protoplasts transfection

a) To prevent stickiness of cells to the culture plates

For cells to express the gene of interest after transfection, it is important to culture them for a certain period. Once, protoplasts were isolated next step is to culture them. Once I started culturing protoplasts, cells used to stick to the culture plate and there used to be a protoplast loss.

Studies suggest that coating of culture plate with calf serum albumin can prevent cells from sticking to the culture plate. Coating of the plate with different concentrations of Bovine Serum Albumin (BSA) may also help. BSA concentration ranging from2%, 5% and 10% were tried instead of calf serum albumin. I was observed that coating of the plate with 5% BSA did prevent cells from sticky to the plate and works best as I could see significant protoplast yield after 4 hours, 16 hours and 24 hours of protoplast culture.

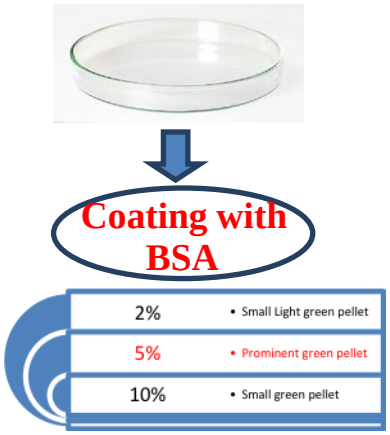

Figure 8. Coating of the plate with different concentrations of BSA

b) To optimize PEG concentration for transfection so that cells do not lose their integrity

Polyethylene glycol (PEG) is harmful to cells. So, there is a need to optimize the concentration of PEG that will maintain the integrity of the cell membrane without affecting their viability.This can be further tested to do transfection. I started with giving 40% PEG Calcium treatment to the cells. High concentration (40%) of PEG was tried 3 times. Protoplasts were observed after 4 hours, 16 hours and 24 hours following treatment using confocal microscopy. After all the three trials, it was observed that protoplasts lost their integrity and there was an increase in auto fluorescence in the sample.

High concentration(40%) of PEG was not giving the desired results. Therefore, I thought of reducing the concentration of PEG andat the same time gave additional washes to the protoplasts to remove residual PEG culture. This would help in reducing the damage.Following treatments were tried to standardize the concentration of PEG and results were documented with observation.

a) 40% PEG-Calcium solution (1 wash): more damage was there, and cells were losing their integrity.

b) 40% PEG-Calcium solution (2 washes): Lot of cell damage was observed.

c) 20% PEG-Calcium solution (1 wash): Cells remained healthy until 24 hours after treatment.

d) 20% PEG-Calcium solution (2 wash): Cell damage was observed.

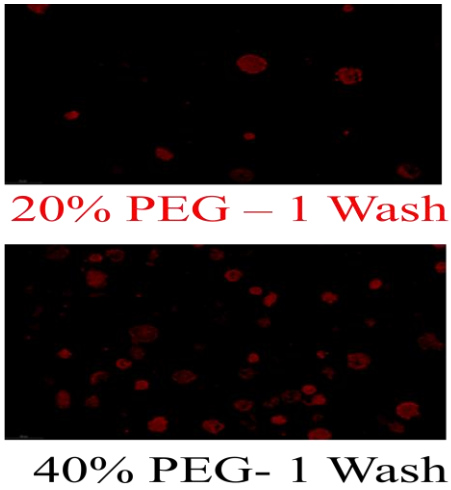

Figure 9. Optimisation of PEG concentration to prevent cells from losing their integrity.

3.3 To check transfection of eGFP using confocal microscopy

15µg of 35S::eGFP plasmid was transfected in the cells. The cells were cultured for 24 hours, for first 12 hours were kept in light, and the remaining 12 hours I kept in dark conditions at 25° C. To monitor the expression of eGFP, cells were centrifuged and resuspended in a buffer after that confocal microscopy experiment was performed. During the 24 hours' time period at three time points; 4 hours, 16 hours and 24 hours after transfection cells were taken for visualization of eGFP expression

I did not see much difference between the transfected and untransfected samples because of auto fluorescence by chlorophyll in the untransfected cells. I repeated the same experiment 4 times but so far did not succeed. I did not see the glow of eGFP in the transfected cells due to auto fluorescence signal. Therefore I cannot confidently conclude whether transfection was successful or not.

3.4 To check transfection of eGFP using PCR

Since the 20% PEG concentration worked very well for cell survival, so I carried out my transfection at this concentration. Plasmid DNA was checked by running on the gel. High quality super coiled plasmid DNA isolated using maxi prep as well as manually prepared

plasmids were transfected into the cells. In order to find out why transfection has not shown any positive results thus far, I repeated the experiments decided to check the plasmid DNA in the cells. If the transfection is successful, I should be able to detect it in PCR amplification. Approximately, 24 hours after transfection cells were harvested, and DNA was isolated, and a PCR reaction was setup using eGFP specific primers. In the two non-transfected sample I did not find eGFP amplification, however, in the three samples where plasmid preps made using maxi kit as well as manual method showed strong amplification of eGFP template suggesting that transfected cells do contain expected plasmid DNA template.

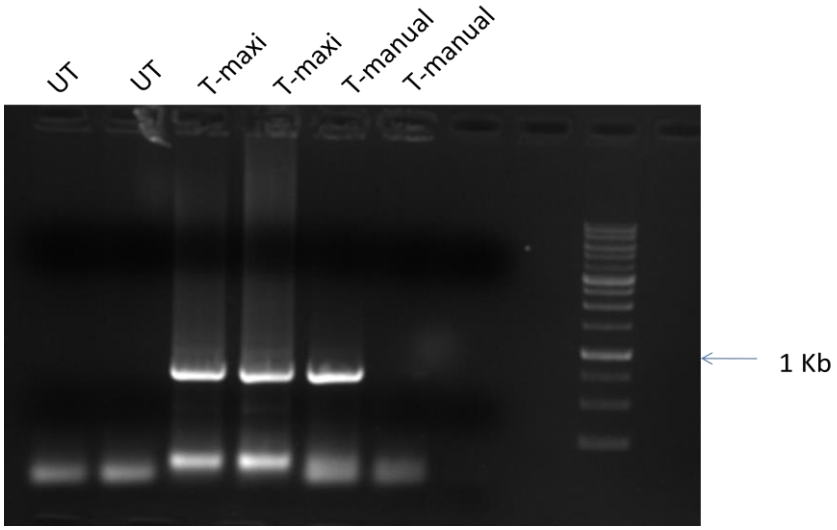

Figure10. Gel electrophoresis after PCR of eGFP transfected lysate. UT: Untransfected, T-maxi: Cells transfected with plasmid prepared using the maxi prep kit, T-manual: Cells transfected with plasmid prepared using the manual method.

Interestingly, in one of the manually prepared plasmid DNA sample cells, I did not see *eGFP* amplification suggesting that manually prepared plasmid DNA preparation perhaps are not reliable from one batch to another. *eGFP* amplification was observed consistently in both maxi prep samples indicating that the high quality super coiled plasmid DNA indeed show high efficiency and offer more reliability from batch to batch sample preparation.

3.5 Fluorescence Activated Cell Sorting of transfected protoplast to determine eGFP fluorescence

Fluorescence **A**ctivated **C**ell **S**orting (FACS) is a technique, which basically sorts the cells depending on the fluorescent proteins present in the cells after exciting the cells with desired laser wavelength. Mainly lasers are used for exciting the fluorochromes, which would help us in isolating fluorescent cells from non-fluorescent ones in a cocktail. Since I have encountered problem in getting reliable results for my transfection assay, I hypothesized that even a very poor transfection efficiency of the cell with 35S:eGFP would result in eGFP expression, and using FACS sorter (GFP-excitation:488nm,emission:530/30 nm), I can easily spot fluorescent cells from non-fluorescent ones. Two transfected preparations were taken, and untransfected was taken as a control for FACS.

Transfected with *35S::eGFP*
Using maxi prep kit
Or using the manual method

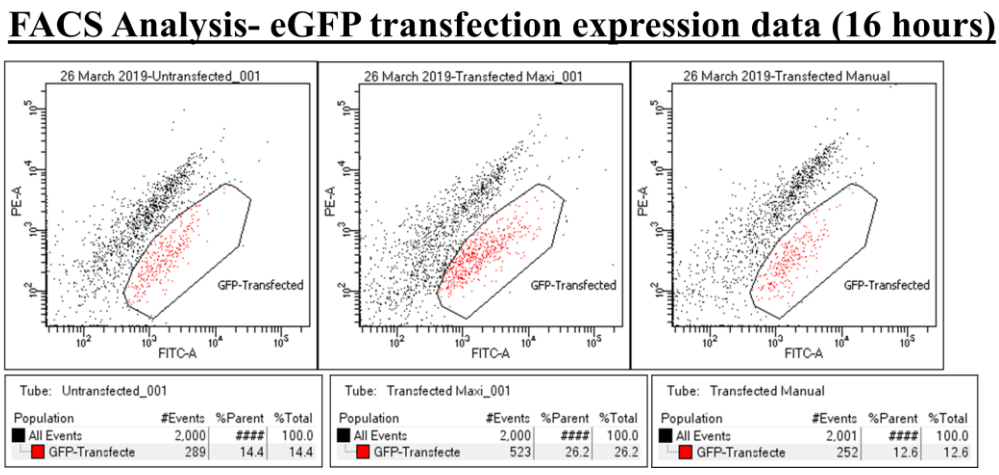

Figure 11. FACS Analysis: eGFP transfection expression data

With the help of FACS operator Prateek Arora, I was able to analyze all three samples, namely, untransfected, transfected with plasmid DNA isolated by kit and the one isolated by manual method. In sample a), which is untransfected and in the eGFP gate I could see the signal, and as a percentage of the total cell population 14.4% cells were GFP positive. This indicates that there are artifacts which fall in the GFP channel. Interestingly, in

sample b), where cells were transfected using kit prepared plasmid showed 26.2% cells positives suggesting that over noise at least more than 12% cells are showing expression eGFP. In the c) sample, plasmid DNA prepared by manual method again showed a very low percentage of eGFP positive cells, only 12.6%. Taken together, the FACS analysis results suggest that the quality of super coiled plasmid DNA is very critical for efficient transfection as has been shown in earlier studies both for animal and plant cells.

For the future trials, It has been suggested to increase the time of expression because it seems there was dim GFP expression in the cells, which perhaps is dominated by noise, and that is masking the expression of eGFP. Also, increasing the actual protoplast cell number and volume would be taken into account to improve the results.

3.6GUS Assay and Calibration Curve

It is very well documented that there is an inherent tendency among the different cells to uptake plasmid DNA.To reduce the cell to cell transfection variability among the different experiment, an independent plasmidDNA having a reporter gene is co-transfected. The reading of this reporter is used to normalize the reads across the different sample replicates and control. Plant cells are suitable for GUS assay because there is very low background activity by β-glucuronidase.

pUBQ::GUS plasmid was prepared using a maxi prep kit. High quality super coiled plasmid DNA (15µg) was transfected into the cells. The cells were cultured for 24 hours and were centrifuged. The lysate was collected, and the GUS assay was performed using fluorimeter.

Cells will express GUS, and on providing MUG as a substrate,MUGwill be converted into MU. MU is a fluorescent compound. Thus its fluorescence can be measured by excitation wavelength of 365nm and emission wavelength of 455 nm

Samples	cps	Amount of MU formed(in M)
Untransfected 1	153483	
Untransfected 2	102243	
UT+PEG 1	119540	
UT+PEG 2	77250	
Transfected 1a	85820	4.84
Transfected 1b	86723	4.87
Transfected 2a	167510	8.00
Transfected 2b	168610	8.04
Transfected 3a	160667	7.74
Transfected 3b	162073	7.79

GUS assay Calibration Curve

Stock of 1M (1ml) MUG was made. Five serial dilutions were made, by taking 100 µl from each respective sample and the fluorescence of MU was measured, and the readings were plotted. The fluorescence of MU increases with increase in the concentration of MU thus exhibiting a linear relationship. This curve will help us to determine the amount of MU produced by the cells by looking at the fluorescence readings.

The fluorescent signal was also observed in non-transfected samples. The reason being there are a lot of fluorophores present in the cell-like secondary metabolites and chlorophyll. Also, the signal varied from sample to sample. This may have happened due to variation in the number of cells from sample to sample. During protoplast culture and harvest, there may have occurred cell loss at various steps.

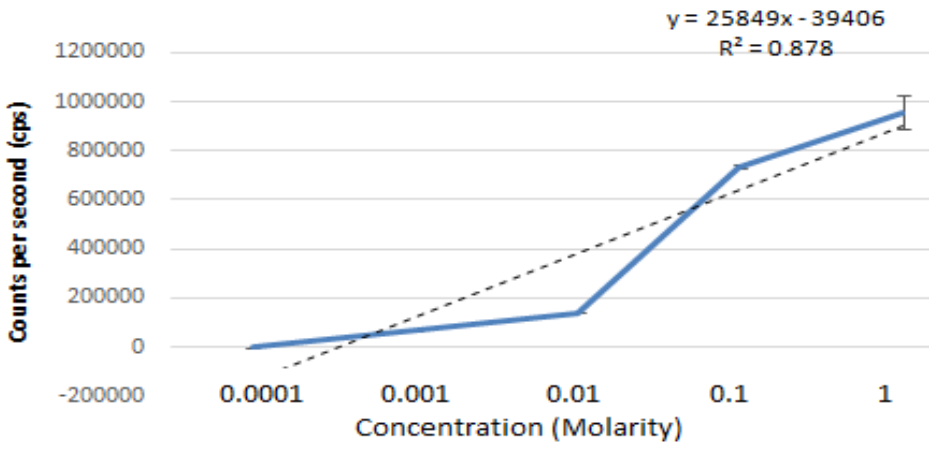

Figure 12. GUS assay Calibration Curve

Chapter 4

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