Validation of DELLA-WUSCHEL Protein-Protein Interactions in *Arabidopsis thaliana* to Study Role of Gibberellic Acid Signalling in Shoot Development and Maintenance

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Certificate of Examination

This is to certify that the dissertation titled "**Validation of DELLA-WUSCHEL Protein-Protein Interactions in *Arabidopsis thaliana* to Study Role of Gibberellic Acid Signalling in Shoot Development and Maintenance"** submitted by Ms. Anupreet Saini (Reg. No. MS14182) 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 Kishor 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.

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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 Kishor Yadav

(Supervisor)

Dated: April 25th, 2019

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Notations

BiFC	:	Bimolecular Fluorescence Complementation
CoIP	:	Co Immuno Precipitation
CTAB	:	Cetyltrimethylammonium bromide
EtBr	:	Ethidium bromide
GA	:	Gibberellic Acid
LB	:	Luria Broth
MS	:	Murashige Skoog
OD	:	Optical density
PCR	:	Polymerase Chain Reaction
PPI	:	Protein-protein Interaction
SAM	:	Shoot Apical Meristem
TAE	:	Tri Acetate EDTA
Y2H	:	Yeast-2-Hybrid
YFP	:	Yellow Fluorescent Protein

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Abstract

Gibberellins (GA for Gibberellic acid) are the plant hormones known to have various roles like seed germination, floral induction, stem elongation, pollen maturation, expansion of leaves, development of trichome and pollen maturation. DELLA proteins act as negative regulators in GA signal transduction and there are five DELLA proteins in *Arabidopsis.* The role of GA signalling and hence the DELLAs in the Shoot Apical Meristem (SAM) maintenance has not yet been established. SAM maintenance is done via WUSCHEL-CLAVATA feedback loop and the phenotypes of mutants of *clv* and the quintuple mutant of DELLAs – *global della* are similar in that they both have enlarged SAM. In this study, I tried to find out and validate if there are any interactions at the level of protein-protein interaction between DELLAs and WUSCHEL. This project would be the very first step to elucidate role of DELLAs and hence GA signalling in the maintenance of SAM in *Arabidopsis.*

Chapter 1

Introduction

1.1 *Arabidopsis thaliana* as a model organism

Arabidopsis thaliana became a model organism of choice in plant biology for the first time in 1943 when its potential for genetic research purposes was summarized by F. Laibach [1]. It is an angiosperm with a haploid set of just 5 chromosomes and a short life cycle of 6 weeks. It belongs to the Brassicaceae (mustard) plant family. Its self-pollinating behaviour and easy cross-pollination in the lab made it an ideal plant system for geneticists around the globe to produce enough seeds without much trouble and tracking the individual loci easily. The fully sequenced genome of around ~135 Mb size consisting of around 33,000 genes makes it a convenient organism for detailed molecular analysis [2]. Mature plants of 15 to 20 cm in height are easy to grow in pots under laboratory conditions in specialized plant chambers [3]. Methods developed later on for mutagenesis to identify loss of function mutants, and plant transformation made *Arabidopsis* an ideal plant model organism to study developmental biology, physiology, genetics and biochemistry to unravel the fundamental mechanism behind the growth and development of angiosperms.

1.2 Shoot Apical Meristem Organization in *Arabidopsis thaliana*

Shoot Apical Meristem (SAM) resides on the tip of each plant from where all ground above organs such as leaves, flowers etc. arise [4]. Unlike animals, plants continue to form organs post-embryonically, however, this is accompanied by continuous replenishment of pluripotent stem cells in the SAM. The SAM in higher plants is organized into various layers and zones. In *Arabidopsis* SAM the three distinct domains are classified as central zone (CZ), peripheral zone (PZ) and rib zone (RZ) (Fig. 1) [5]. The former consists of the source of all cells for

continuous organogenesis- a reservoir of pluripotent stem cells at the apex of SAM. These cells in comparison to those in other zones, divide infrequently. Surrounding the CZ is the PZ, containing cells that divides at a higher frequency. Columns of large vacuolated cells make up the RZ beneath the CZ in the deeper layers. The area beneath the stem cells in the CZ known as the organizing centre (OC) /niche, which is required to maintain the stem cell population.

Progeny of the stem cells in the CZ is gradually displaced towards the PZ, which acts as a transition area before the cells attain their fate by getting incorporated into either the lateral organ primordia or into the regions between the organs in the stem. The cells of the PZ go on to make the organs with specific fates while the stem cell reservoir is maintained in the CZ. The RZ on the other hand gives rise to the stem tissue and pith.

Coming to the organization of the SAM in terms of cell layers, there are three layers termed as epidermal / L1, sub epidermal / L2, and corpus / L3 (Fig. 1) [5]. Epidermal and subepidermal cell layers falls under tunica, while the L3 cell layer is called corpus. In eudicots, SAM has three cell layers, however, in monocots tunica is made of a single cell layer. In tunica, cells follow anticlinal cell division patterns where the new cell wall will appear perpendicular to the surface. In contrast, cells in corpus divide both anticlinally and periclinally. Hence, epidermal cell layer cells, stay within the epidermis and are going to form a protective cover on shoot apices, leave, and flower organs. The derivatives of L2 give rise to mesodermal cells in the leaves and the germ cells. The L3 is the source of stem vasculature and pith.

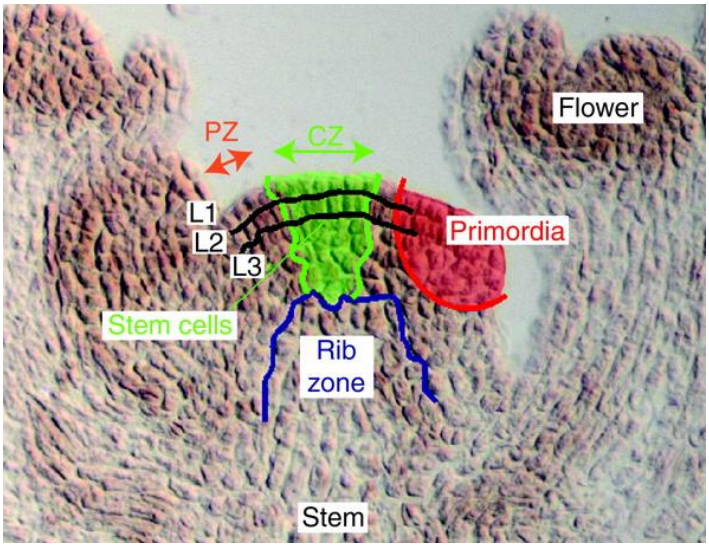

Figure 1. Organization of Shoot Apical Meristem into layers L1, L2, L3 and zones central zone (CZ), Peripheral zone (PZ) and Rib zone (RZ). Stem cells are restricted to the central zone and cells outside this zone form different organ primordia. [5]

1.3 SAM maintenance in *Arabidopsis*

For normal plant growth and development, it is important to maintain the stem cell reservoir in SAM as well as the size of SAM. It is already known that this is done by CLAVATA-WUCHEL (CLV-WUS) feedback signalling loop. There are three genes namely CLAVATA 1 (CLV1), CLAVATA 2 (CLV2) and CLAVATA 3 (CLV3) all of which act in a common pathway to maintain the region of stem cells in the SAM [6,7]. CLV1 encodes for a Receptor Like Kinase (RLK) whereas CLV2 encodes for a Receptor Like Protein (RLP) [8]. It has been reported that CLV2 and CLV3 are required for recruiting CLV1 to complete the formation of a signalling complex [8,9]. CLV3 encodes for an extracellular signalling protein [10]. It is reported that CLV3 is present only in the L1 and L2 layers only whereas the mRNA of CLV1 is mostly found in the L3 layer of the central zone [11,12]. WUSCHEL (WUS) encodes a novel homeodomain protein that acts as a transcriptional regulator and is expressed in a group of cells beneath the area of stem cells [13].

The SAM is maintained by the WUS-CLV loop. CLV3, a polypeptide secreted by L1 and L2 layers, moves to L3 where CLV1 and CLV2 are present. Hence CLV3 acts as a ligand for the signalling complex formed by the latter two [14]. There exists a feedback loop between the stem cells in central zone (CZ) and Organizing centre (OZ) mediated mainly by CLV3 and WUS. Few central cells of the rib zone (just beneath the CZ), give out WUS-mediated signal that is responsible for stem cell identity specification in the outermost cell layers of the CZ. These cells in turn signal back to keep in check the WUS-expressing cells of OC via CLV pathway (Fig. 2). This loop maintains a balance between new organ forming cells and stem cell derivatives hence resulting in meristem homeostasis [14].

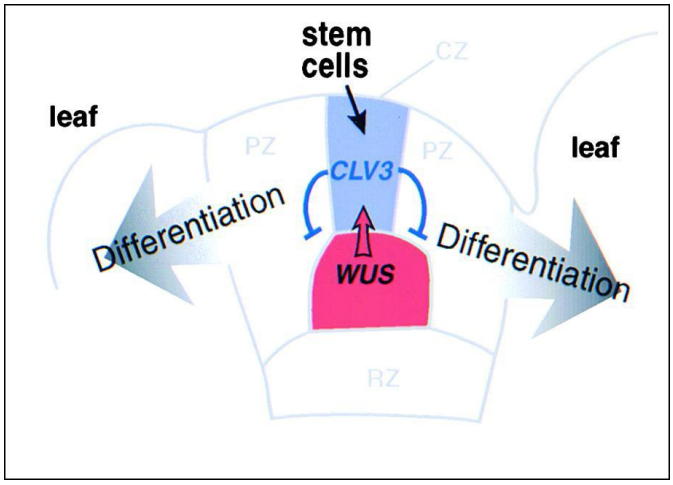

Figure 2. WUS-CLV loop in the Shoot Apical Meristem (SAM) [15]

1.4 DELLA proteins in *Arabidopsis thaliana*

Gibberellins (also known as GAs from Gibberellic Acid) are a class of plant hormones with diverse roles in plant growth and development like seed germination, floral induction, stem elongation, pollen maturation, expansion of leaves, development of trichome and pollen maturation [16]. More than 130 GAs identified across various taxa, not all of them have biological activity [17]. In *Arabidopsis*, it is currently proposed that there are certain inhibitors of GA signalling known as DELLA proteins and GA signals promote plant growth and development by overcoming the DELLA mediated growth suppression [16,18] .

DELLA proteins is a subfamily of GRAS regulatory proteins in *Arabidopsis* [19]. GRAS proteins are a group of plant specific proteins named after its first three members : Gibberellic Acid Insensitive (GAI), Repressor of GAI (RGA) and Scarecrow (SCR) and grouped on the basis of conserved domains (SCR) [20]. These GRAS proteins have been found to be transcription factors that regulate plant development [21]. DELLA proteins form a subset of GRAS family and are characterized by a N-terminal DELLA motif (amino acid sequence of TVHYNP) and a C-terminus GRAS domain. There are five DELLA proteins in *Arabidopsis,* namely Repressor of GAI (RGA), Gibberellic Acid Insensitive (GAI), RGA-Like 1(RGL1), RGA-Like 2 (RGL2) and RGA-Like 3(RGL3). DELLA proteins lack a DNA-binding domain (DBD) and hence to act as GA repressors, they take help of other transcription factors of Indeterminate Domain

(IDD) family proteins which have DBD of their own [22]. The role of DELLAs in plant growth in response to GAs by negatively regulating the latter has already been established in *Arabidopsis* [23].

1.5 The predicted link between DELLAs and WUSCHEL

The role of DELLAs in GA signalling is known in *Arabidopsis* [23]but the role of GA signalling and hence the DELLAs in the SAM maintenance has not yet been established. It has been reported in literature [24] that the quintuple mutant of DELLAs – *global della (gai-t6, rga-t2, rgl1-1*, *rgl2-1* and *rgl3-4*) has a significantly larger SAM (Fig.3A-3C). In *clv1* and *clv3* mutants too (Fig.3D-3E), the size of shoot meristem inflorescence increases in comparison to the wild type [15]. From results of yeast-2-hybrid (Y2H) in the lab, it was found that one of the DELLAs, namely, GAI interacts with WUS. From this and the reported phenotypes, it was hypothesised that may be GA signalling, via DELLAs, has a role to play in SAM maintenance too, in addition to its already known roles in plant growth and development. Thus, to find out if it so, we checked if the other DELLAs also interact with WUS. To verify the interactions, we cloned various constructs and looked for DELLAs-WUS interactions via various techniques like Y2H, pull down assay (in-vitro), Co-Immunoprecipitation (CoIP- in vivo) and Bimolecular Fluorescence Complementation (BiFC).

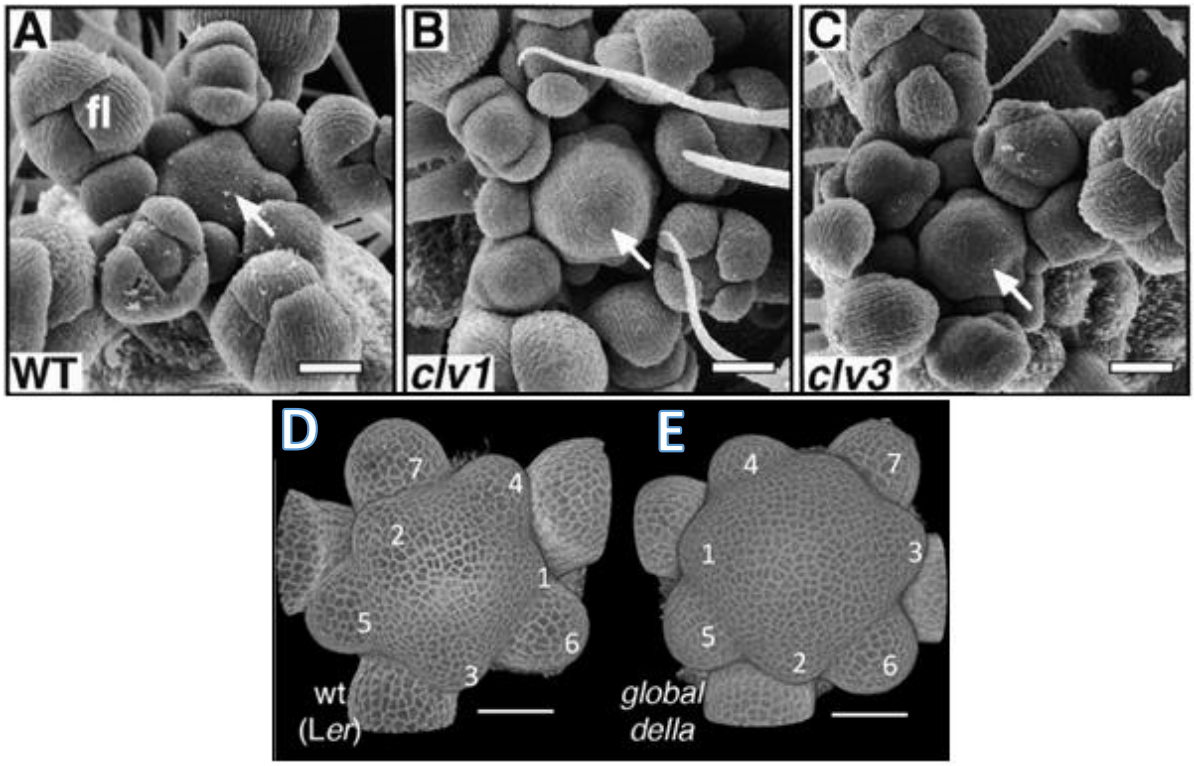

Figure 3. (A-C) SAM Scanning Electron Microscopy images in wild type and *clv1* and *clv3* mutants. The SAM size increases when *clv* is mutated.[15] **(D-E)** SAM confocal images in wild type Ler and *global della*. The size of SAM in latter is significantly larger than the wild type. Numbers indicate the order of emergence of floral buds with 1 marked as the youngest [24].

Chapter 2
Methods

2.1 Plant growth conditions and related protocols

Plants were grown in pots with autoclaved soil comprising of solarite, perlite and compost in the ratio 16:1:1. These pots were then kept in trays in plant chambers with controlled conditions of 22ºC, 75% humidity, 150 µmol m^{-2} white light with 16 hours day and 8 hours night. Plants were watered at regular intervals.

2.1.1 Seed Sterilization

An approximate number of required number of seeds were taken in a 1.5 ml Microcentrifuge Tubes (MCTs) and to it 1 ml autoclaved water was added. The MCT was inverted 5-6 times and the water poured out carefully. 1 ml 70 % Ethanol (v/v) having 0.1% Triton X was added. The MCT was kept for 1 minute. When the seeds settled down, the ethanol solution was taken out and 1 ml solution containing 0.1 % sodium hypochlorite containing 0.1% Triton X was added. For 2 minutes, it was mixed well and the seeds were allowed to settle at the bottom again. The supernatant was removed and seeds were given 3-5 washes with autoclaved water. The seeds were transferred to appropriate MS media plates before keeping them in chamber.

2.1.2 Cold Stratification

The sterilized seeds were kept in an MCT with 1ml double-distilled water at 4ºC for 2 days and then transferred to MS media plates. When seedlings grew, they were transferred them to soil.

2.2 Molecular Biology Reagents' Sources

- From New England Bio lab (NEB, USA): High Fidelity (HF) Phusion and various restriction enzymes.

- From ThermoFisher Scientific (Thermo, USA) : Plasmid isolation kit containing various buffers and columns; Standard molecular weight markers (1 kb DNA ladder); 3B pre-stained protein ladder (3.5 to 245kDa); Gateway LR clonase; entry vector for cloning.
- From BD Scientific (BD, USA) : LB agar media components.
- From Himedia : Agar
- From Lonza : Agarose for gel electrophoresis
- From Sigma-Aldrich (Sigma, USA) : All other chemicals or reagents of molecular biology/ analytical grade.
- Synthesized in lab : *Taq* DNA polymerase

2.3 Solutions and Media Composition

2.3.1 dNTPs stock preparation

1800 µl double distilled water was added to a 2 ml MCT in ice. To it was added, 50 µl of ATP, GTP, CTP and TTP each to the above MCT and mixed properly. Then aliquots of 50 µl each were made in 1.5 ml MCTs and stored at -20ºC.

2.3.2 Antibiotics stock preparation

- **Ampicillin (stock- 100 mg/ml)**

1g Ampicillin was weighed and 10 ml double distilled water was added to it. It was dissolved properly and aliquoted into 1ml each in MCTs. The aliquots were stored at -20ºC. For working solution, a dilution of 1:1000 (example, 100µl to 100ml media) was made. For longer storage of selection plates at 37ºC, a dilution of 1.5:1000 was used.

- **Kanamycin, Carbenicillin and Gentamycin (stock- 50 mg/ml)**

500mg Ampicillin was weighed and dissolved in 10 ml double distilled water. Aliquots of 1ml each were made in MCTs and stored at -20ºC. For working solution, a dilution of 1:1000 (example, 100µl to 100ml media) was used.

- **Rifampicin (stock- 25 mg/ml)**

250mg Ampicillin was weighed and dissolved in 10 ml DMSO. Aliquots of 1ml each were then made in MCTs and store at -20ºC. For working solution, a dilution of 2:1000 (example, 200µl to 100ml media) was used.

- **Tetracycline (stock- 12.5 mg/ml)**

175mg Ampicillin was weighed and dissolved in 10 ml 100% (v/v) Ethanol. Aliquots of 1ml were made in opaque (dark coloured) MCTs and stored at -20ºC. For working solution (5µg/ml), a dilution of 4:10000 (example, 40µl to 100ml media) was used.

2.3.3 LB media (1 litre)

Yeast Extract	5g
Sodium chloride	10g
Tryptone	10g

2.3.4 LB Agar (1 litre)

Yeast Extract	5g
Sodium chloride	10g
Tryptone	10g
Agar	1.5% (w/v) = 15g

2.3.5 YPD (200 ml)

Glucose	4g in 50 ml water (added after autoclaving separately)
In 150 ml water separately,	
Peptone	4g
Yeast Extract	2g
Adenine Hemisulfate	8mg

2.3.6 YAPD dropout media (500 ml)

Glucose	10g in 100ml water (autoclaved separately)	
Agar	10g	
Dropout (per 100ml)	YNB	0.85g
	Dropout (-LWH or -LTWH)	2.5g
	Ammonium sulphate	1g
3-Amino Triazole	Ranging from 0mM to 10mM from 1M stock	

2.3.7 MS media (1 litre)

MS powder	4.31g
Sucrose	10g

Set pH to 5.7 after adding water

Agar	0.7% (w/v) = 7g

2.3.8 SOB media (50 ml)

Tryptone	1g
Sodium chloride	0.025g
Yeast extract	0.25g

2.3.9 Agarose Gel

Agarose for 0.8% (w/v) gel composition was weighed and added to 1X TAE buffer (40ml 50X TAE and 1960ml distilled water). It was heated for 2 minutes 30 seconds in microwave. The solution was cooled down to around room temperature before adding 0.5µg/ml Ethidium bromide (EtBr) and mixed by avoiding bubble formation. The gel was then poured into a tray with combs and the gel was allowed to solidify before use.

2.3.10 CTAB Buffer (150 ml)

Final concentration	Stock	Amount added
2% CTAB (2g/100 ml)		3g

1.4M NaCl	5M	42 ml
20mM EDTA	0.5 M	6 ml
100mM Tris pH8.0	1M	15 ml
Water		To 150 ml

2.3.11 Transformation Buffer 1 (100ml)

Final concentration	Stock	Volume from stock
10mM MOPS	1M (pH 6.5 with KOH)	1ml
1000mM KCl	1M	10ml
45mM $MnCl_2$	1M	4.5ml
10mM $CaCl_2$	1M	1ml
10mM KAc	1M (pH 7.5 with HCl)	1ml
Water		to 100 ml

2.3.12 Transformation Buffer 2

Same as transformation buffer 1 except with 12.5ml of 80% (v/v) Glycerol as an additional component. The volume was made up to 100ml using water after the addition of Glycerol.

2.3.13 Transformation Buffer 3

100mM $CaCl_2$	Stock autoclaved separately
50mM $MgCl_2$	Stock autoclaved separately

2.3.14 50X TAE (1 litre)

Tris base	242g
Glacial acetic acid	57.1ml
0.5M EDTA (pH 8.0)	100ml

Water	to 1000ml

2.3.15 Ethidium bromide

1g EtBr was weighed and dissolved in 100 ml distilled water to make a stock solution of 0.01g/ml. While preparing agarose gel, it was used in the working concentration of 0.5µg/ml.

2.3.16 Staining solution (500ml)

Methanol	250ml
Acetic acid	50ml
Distilled water	200ml
Coomassie brilliant blue dye	1.25g

2.3.17 De-staining solution

Same as the staining solution except that it does not contain Coomassie brilliant blue dye.

2.3.18 Skim Milk Solution (50ml)

Skim milk powder (Difco)	2.5g
10X TBS	5ml
Water	To 50 ml

2.3.19 10X TBS (50ml)

0.5M Tris (pH 8.0)	25ml
1.5M NaCl	4.38g
Water	To 50ml

2.3.20 1X TBS-T (500ml)

10X TBS	50ml
Tween-20	0.1%(v/v) = 500µl

Water	To 500ml

2.3.21 SDS-PAGE

Resolving gel (10%)

30% Polyacrylamide	1.666 ml
1.5M Tris (pH 8.8)	1.25ml
10% APS	0.05ml
10% SDS	0.05ml
TEMED	0.002ml
Distilled water	1.9833ml

Stacking gel

30% Polyacrylamide	415 ml
0.5M Tris (pH 6.8)	630 µl
10% APS	30 µl
10% SDS	30 µl
TEMED	3 µl
Distilled water	1.38ml

2.3.22 Total Protein Extraction Buffer (20ml)

Stock solution	Final concentration	Amount
1M tris-HCl (pH 7.5)	50mM	1ml
10% (v/v) SDS	0.5%	1ml
1mM DTT	100mM	200 µl
1mM PMSF	100mM	200 µl

Water	to 20 ml

2.3.23 Column buffer

Final concentration	Stock	Volume from stock
20mM tris HCl	1M	1ml
200mM NaCl	5M	8 ml
1 mM EDTA	0.5 M	0.4 ml
Water		to 200 ml

2.3.24 Infiltration buffer

Stock solution	Final concentration	Amount
$MgCl_2$	10mM	203.3 mg
MES/KOH (pH 5.6)	10mM	195.24 mg
150 mM Acetosyringone in DMSO	150 µM	100 µl

2.4 Molecular Biology Techniques

2.4.1 Genomic DNA Isolation

Modified CTAB method as follows was used:

The leaf samples were harvested in a microcentrifuge tubes (MCTs) of 1.5 ml volume and the MCTs were then put into liquid Nitrogen. Each MCT was then freeze thawed and the samples inside was crushed using a homogenizer pestle. 600 µl CTAB buffer having 0.6 µl Beta-Mercaptoethanol was added to each MCT after mixing thoroughly, MCTs were put on shaker at 65 ºC for 30 minutes @ 600 rpm. The MCTs were removed from the shaker and 600 µl of Phenol: Chloroform: Isoamyl alcohol solution (25:24:1) was added to them. After vortexing for few seconds and tubes were centrifuged at 13000 rpm for 15 minutes at 4^{o}C. The uppermost transparent layer of the supernatant was taken into a fresh 1.5 ml MCT. In the next 600 µl Chloroform: Isoamyl alcohol solution (24:1) was added and mixed well by inverting several times up down the tube. This mixture was centrifuged at 13000 rpm for 15 minutes at 4^{o}C. The

above step was repeated once more by transferring the uppermost transparent layer of the supernatant into a fresh 1.5ml MCT. To this, 400 µl isopropanol was added and sample was kept -20ºC overnight. Centrifugation was carried out at 13000 rpm for 30 minutes at 4ºC to precipitate DNA. The supernatant was removed carefully and pellet was washed with 70 % Ethanol (v/v, 500 µl,). The MCT was tapped with fingers gently and content of the tube centrifuged at 13000 rpm for 10 minutes. The supernatant was discarded and the MCT was inverted on tissue paper. The pellet was dried by keeping it at 99ºC till the pellet became invisible and the MCTs did not smell of Ethanol. The pellet was resuspended in 25µl of double distilled water and stored at 4 ºC for short term or at -20 ºC for long term usage.

2.4.2 Plasmid Isolation

A single colony of desired plasmid was inoculated in 5 ml LB media containing appropriate antibiotic(s) at the optimum temperature in incubator shaker for overnight. The cells were harvested by centrifugation in 2 ml MCT for 1 minute at 13000 rpm. After discarding the supernatant, the pellet was dissolved in 250 µl resuspension buffer (P1). The contents were vortexed till the pellet got resuspended. 250 µl lysis buffer (P2) was added and mixed well by gently inverting the MCTs 2-3 times. In the next step, 350 µl neutralization buffer (P3) was added and again mixed by inverting the MCTs 2-3 times. The MCTs were then centrifuged at 13000 rpm for 10 minutes. The cleared supernatant was then transferred to the plasmid isolation spin columns and they were centrifuged at 13000 rpm for 1 minute. The flow through was discarded, and column was washed by adding 750 µl washing buffer (P4) and centrifuged at 13000 rpm for 1 minute. The flow through was discarded again and column was spin again at 13000 rpm for 1 minute to get rid of residual alcohol. The column was transferred to a new MCT and 25 µl pre-heated double distilled water was added followed by centrifugation at 13000 rpm for 1 minute. This step was again repeated by adding 25 µl water, and centrifuged at 13000 rpm for 1 minute before storing the isolated plasmid at 4 ºC.

2.4.3 Gel Purification

The required band from agarose gel was cut and put in a 1.5 ml MCT. To it, thrice the volume of gel dissolving buffer was added and the MCT was kept at 65 ºC on the shaker till the gel got completely dissolved in the solution. The mixture was added to a gel purification column and

centrifuged at 13000 rpm for 1 minute. After discarding the flow through, 700 µl of washing buffer was added and centrifuged at 13000 rpm for 1 minute. The flow through was discarded again and the column was spin dried at the same speed. 20 µl of pre-heated water kept at 65 ºC was used for elution. The columns were spinned at 13,000 rpm for 1 minute. The MCT containing gel purified DNA was stored at 4 ºC or -20ºC.

2.4.4 Bacterial Ultra-competent Cells (DH5α)

A single colony of DH5α was inoculated in 5 ml LB media overnight at 37ºC on a shaker. Before inoculating the secondary culture, 50 ml SOB media in a flask and 1M $MgCl_2$ in a glass bottle were autoclaved separately. 1 ml SOB was taken out in an MCT for blank purpose and 1% primary culture (500 µl) was used to inoculate secondary culture in 50 ml SOB which was incubated at 37ºC till $O.D._{600}$ reached to 0.5-0.6. The flask was kept on ice for 10 minutes so that the culture gets acclimatize. The culture was transferred to 50 ml round bottom Oakridge tubes, the cells were centrifuged at 3500 rpm for 10 minutes at 4 ºC. The supernatant was discarded and the pellet was dissolved gently in 25 ml transformation buffer 1 keeping the content chilled, Oakridge tubes were kept on ice for 10 minutes and centrifuged at 3500 rpm for 10 minutes at 4 ºC. The supernatant was discarded and the pellet was dissolved again in 4 ml transformation buffer 2 as described above. To this mixture, 140 µl DMSO was added entire content mixed well, and kept on ice for 15 minutes, and again140 µl DMSO was added again as described in the previous step. Bacterial cell culture was aliquoted in 50 µl or 100 µl volume in 1.5 ml MCTs and stored at -80ºC. For transformation, equal volume of Transformation Buffer 3 was added and used for transformation.

2.4.5 Bacterial Transformation

At a time one aliquot of 50 µl DH5α cells was thawed on ice. Equal volume of transformation buffer 3 was added to this aliquot and contents were mixed gently. To this, 1pg-100ng of plasmid DNA was added to the MCT, which was then flicked gently 4-5 times and kept on ice for 30 minutes. In a water bath or thermomixer, a heat shock was given to the cells at 42 ºC for 30 seconds - 1 minute. The MCT was kept on ice for 5 minutes before adding 1 ml LB media. The entire content of the MCT was incubated at 37 ºC for 1 hour, out of this 100-200 µl culture

was spread on a desired selection media plate. Colonies were allowed to grow for overnight by keeping plate in 37 ºC incubator.

2.4.6 *Agrobacterium* Electro-competent Cells (GV3101 strain)

A single colony of the Agrobacterium strain GV3101 was inoculated in 5 ml LB media containing three antibiotics (rifampicin, gentamycin and tetracycline) and culture was incubated for 24 hours at 30ºC. Secondary culture was setup using 1% pre-inoculum and incubated at 30ºC till the $O.D._{600}$ reached to 0.4-0.6. Cells were harvested by keeping the flask on ice for 10 minutes then followed with centrifugation. The cells were pellet down by centrifugation at 4000 rpm for 20 minutes in a pre-cooled centrifuge at 4 ºC. The supernatant was discarded keeping the pellet intact. Pellet was dissolved by adding in small batches ice chilled 10% Glycerol (v/v) up to 150 ml by swirling the flask on ice gently without raising the temperature of cell mixture. Centrifugation was done at 4000 rpm for 20 minutes at 4 ºC. The above step was repeated with 100 ml chilled 10 % Glycerol (v/v) after discarding the supernatant. Again, wash cycles with Glycerol were repeated with 50 ml chilled 10% glycerol (v/v) and then lastly with 25 ml chilled 10% glycerol (v/v). Finally, the supernatant was discarded and the pellet was dissolved in 2 ml chilled 10% glycerol (v/v). Aliquots of required volume were made in MCTs and stored at -80 ºC.

2.4.7 Transformation in *Agrobacterium tumefaciens* (Electroporation)

After thawing the electrocompetent cells (stored at -80 ºC) in ice, 0.1 to 1.0µl plasmid was added to 100 µl cells and mixed well. The whole mix of competent cell and plasmid was added to the cuvette (washed with 70% Ethanol and dried before use) and electroporation was performed as per the requirements (depending upon the cuvette size). After the pulse was given, immediately after that 200 ml LB media was added to the cuvette. Cells were mixed and transferred to an MCT containing rest of the 800ml LB. The MCT was incubated on shaker at 37ºC for 1 hour, out of 1 ml culture 50µl was spread on the required selection media.

2.4.8 Preparation of yeast competent cells (PJ697a)

A single colony of PJ697a yeast strain was inoculated in 5ml YPD and incubated at 30 ºC overnight. Secondary culture was inoculated by adding 300 µl primary culture to 300 ml YPD

and incubated at 30 ºC for 3-4 hours till $O.D._{600}$ reached 0.6-0.8. The culture was centrifuged at 3000 g for 5 minutes at room temperature. The supernatant was discarded and the pellet was washed with 1X lithium acetate and 1X TE solution followed by centrifugation as described above. The pellet was resuspended in 500 µl solution containing 1X lithium acetate and 1X TE. Yeast cells were stored in -80ºC.

2.4.9 Yeast transformation

Before transformation an aliquot of yeast cells was taken out and thawed in ice. To this 300 µl of polyethylene glycol (PEG) was added and mixed by vortexing. A heat shock was given for 30 minutes at 42ºC. The MCT was then kept at room temperature for 10-15 minutes before centrifugation at 8000 rpm for 4-5 minutes. Supernatant was discarded and the pellet was washed with 1 ml autoclaved distilled water followed by centrifugation at the same rate as earlier. Whole of supernatant except 100 µl was discarded and the pellet was resuspended in 100 µl. The resuspended pellet was then spread on plates with selection media and the plates were incubated at 30 ºC in incubator for 3-4 days.

2.4.9 Protein Induction and Isolation

To purify the protein, plasmid construct was transformed in to BL21 strain of *E.coli,* Single colony was picked up using sterile pipette tip and pre-inoculum was setup in 5ml LB. The primary culture was inoculated further to setup the secondary culture for protein induction and purification. Using 1% primary (5 ml), 500 ml secondary culture was inoculated and incubated at 37ºC in shaker till $O.D._{600}$ reached to 0.4-0.6. The flask was then kept on ice for 10 minutes. 1ml of culture was taken out in MCT and labelled as the uninduced sample. 0.2mM IPTG was added to the rest of the media and as per the protein of interest, incubation was done at either 16ºC for 16 hours or 30ºC for 6 hours etc. The culture was centrifuged in a pre-cooled centrifuge at 4ºC for 20 minutes at 3500 rpm. The supernatant was discarded and pellet was dissolved in around 7ml column buffer. Sonication was performed for 15 minutes with 10 secs on and 20 secs off intervals at amplitude 20 % by keeping the eppendorf in ice while sonication was still going. It was followed by centrifugation at 4ºC for 30 minutes at 3500 rpm. Supernatant and pellet were separated and phenylmethylsulphonyl fluoride (PMSF) was added to the former. The process can be paused at this step by storing at -20 ºC. Otherwise, the pellet

was dissolved in the same volume of column buffer as that of supernatant followed by addition of PMSF to it. For SDS-PAGE gel loading sample was prepared by taking 20µl supernatant, dissolved pellet and the uninduced sample, respectively, in an MCT. To this 20µl column buffer and 10ul sample buffer dye were added. All the samples were heated at 99ºC for 10 minutes before loading on to the SDS-PAGE.

2.4.10 Protein purification from supernatant using beads

200-300 µl beads stored in ethanol were taken in ice and washed thrice before use with 1 ml column buffer each time and centrifuging at 1500 rpm for 2 minutes in pre-cooled centrifuge at 4ºC. For every 10ml of soup, 400µl of the beads (Amylose resin for MBP tagged proteins and Ni-NTA beads for 6X-His tagged) were added and incubated at 4ºC at rotation for 3 hours. Centrifugation was done at 1500 rpm for 2 minutes at 4ºC. The beads were washed 5 times with column buffer (containing 0.1mM D-maltose for MBP-tagged proteins and 20mM Imidazole). 10µl of beads were separated in an MCT. For elution of protein from the beads, 1 ml of elution buffer (column buffer containing 10mM D-maltose for MBP tagged proteins or 300mM Imidazole for 6X-His tagged proteins) was added and incubated at 4ºC in Hoola mixer for 3 hours. The MCT was centrifuged again at the same specifications and supernatant was separated in an MCT. PMSF was added to the supernatant and stored at -20ºC. 4 more elutions were done, each with incubation for 1 hour. To check for the eluted protein (if it did get eluted), all the elution samples were run on SDS-PAGE along with the beads separated before elution.

2.4.11 Protein isolation from seedlings directly

Around 10-20 10-day old seedlings were taken in a single 1.5ml MCT and freeze thawed in liquid Nitrogen. The seedling tissue was crushed using pestle and kept on ice. Total protein extraction buffer was added in the ratio 1:1 (relative to volume of tissue collected), vortexed for 30 secs and centrifuged for 15 minutes at 13000rpm in pre-cooled rotor at 4ºC. Supernatant was transferred to a new MCT, PMSF was added to it and stored at -20ºC. The presence of the protein of interest was verified using SDS-PAGE.

2.4.12 Screening of plants for desired mutations after crossing (Genotyping)

Tissue was collected from all plants individually and stored at -80 ºC. DNA was isolated using CTAB method and using the isolated DNA, PCR was done using primers in sets for all the desired mutations (Table no.2 and 3). Gel electrophoresis was then done to analyse results of the PCR.

2.4.13 Gateway cloning

Genomic DNA of wild-type Ler ecotype was isolated using CTAB method and used to amplify the gene of interest via PCR (refer to table 1 and 2). The gene of intertest was then either PCR purified or purified from gel after agarose gel electrophoresis it. pENTR-DTOPO reaction was set up with the PCR amplified product (refer to Table no.5) and then transformed into DH5α or other required strain on specific selection media. Colony PCR was done to screen the colonies followed by inoculation of positive clones for overnight incubation at 37 ºC in shaker in LB containing specific antibiotics. Plasmid was isolated and restriction digestion was set-up (Table no.4) with specific restriction enzymes. The confirmed positive clones were finally sent for sequencing before using them for any experiment. The confirmed clone (for example, WUS-ΔC pENTR which is WUS gene with deleted C-terminal in pENTR vector) acted as the entry vector with which LR reaction (Table no. 6) was set-up to introduce the gene of interest into a variety of destination vectors (for example, pGDBKT7 to make Binding Domain of WUS).

2.4.14 Colony PCR

Desired colonies were marked on the LB plates with selection media. Each colony was dissolved in PCR tube containing 10 µl autoclaved double distilled water. To each tube 5 µl master mix (Table no.2) was added and PCR was set up as per condition given in Table 2. Gel electrophoresis was performed to analyse the PCR results.

Table 1 : PCR amplification of gene of interest

Components	Volume (in µl)

Autoclaved double distilled water	59.8
5X HF Phusion Buffer	20
10 mM dNTPs	8
10 µM forward primer	5
10 µM reverse primer	5
Phusion	2
Template	0.2
Total volume	100

Table 2: Reaction conditions for Phusion PCR and *colony PCR

Step	Temperature	Time	Number of cycles
Initial denaturation	98 ºC (*95ºC)	3 minutes	1
Denaturation	98 ºC (*95ºC)	30 seconds	35
Annealing	55º C	30 seconds	
Extension	72º C	Dependent on Fragment length	
Final extension	72º C	10 minutes	1
Hold	10º C	∞	

PCR conditions for genotyping were the same as colony PCR except 58 ºC as the annealing temperature.

Table 3: Composition of colony PCR reaction and for genotyping**

Components	Volume (in µl)
Autoclaved double distilled water (to dissolve colony)	10 (*** 9 water + 1 isolated genomic DNA)

10X standard PCR buffer	1.5
10 mM dNTPs	1.2
50 mM $MgCl_2$	0.45
10 µM forward primer	0.75
10 µM reverse primer	0.75
Taq DNA polymerase	0.5
Total volume	15

Table 4: Restriction digestion for confirmation of cloning

Components	Volume (in µl)
Enzyme 1	1
Enzyme 2	1
Buffer (as per the enzyme activity)	2
Autoclaved double distilled water	To 20µl
Plasmid	Volume containing 2 µg
Total volume	20

The MCT was kept at 37 ºC in incubator or water bath for 3 hours and checked via agarose gel electrophoresis.

Table 5: pENTR/D-TOPO reaction

Components	Volume (in µl)
Insert (PCR or gel purified)	In ratio of 1:(0.5-2) with pENTR vector
pENTR/D-TOPO vector	Equal to 5 ng
Salt solution	0.5
Autoclaved double distilled water	To 3 µl
Total	3

The MCT was kept at 25 ºC for 60 minutes before transforming into bacteria.

Table 6: LR reaction set-up

Components	Volume (in µl)
Donor vector	Equal to 50-150 ng
Destination vector	Equal to 150 ng
LR clonase enzyme	0.3
TE buffer	To 3 µl
Total	3

The MCT was kept at 25 ºC for 60 minutes before transforming into bacteria.

2.5 Methods for protein-protein interactions

2.5.1 Yeast-2-Hybrid (Y2H)

Various co-transformations of bait (the main protein with which interaction of different other proteins is to be checked) and preys were done in yeast and spread on -2 selection media (-Trp and – Leu). The colonies were allowed to grow for 3-4 days. Multiple single colonies of each were picked and dissolved in 100 µl of autoclaved double distilled water. $O.D._{600}$ was checked for each and in a 96-well plate, the $O.D._{600}$ was set for each to 1.0 in 200 µl autoclaved double-distilled water. Finally, spotting was done on various selection media.

2.5.2 Pull down assay (In-vitro)

All steps were done at 4 ºC or in ice. After quantification of purified proteins from SDS-PAGE using ImageJ, 5 µg of each protein (one of them on beads) for whom interaction was to be checked was added to a 2ml MCT. Volume was made to 1000µl using column buffer and the MCT was incubated at 4ºC on a rotor inverting MCT continuously for 1 hour. MCT was centrifuged in pre-cooled centrifuge (4ºC) at 1500 rpm for 2 minutes. Supernatant was separated in an MCT. The beads were washed with column buffer thrice each time incubating

for 15 minutes before spinning. 10-20 µl beads were used to prepare sample for Western blot to detect if both the proteins interact.

2.5.3 Co-Immunoprecipitation (Co-IP – In-vivo)

Same as Pull-down assay with the only difference that proteins were extracted from seedlings rather than bacteria.

2.5.4 Western Blotting

To carry out the western blot experiment, protein samples were loaded on SDS-PAGE. Rough estimation of protein concentration was determined by analysis of previous SDS-PAGE run for the same samples using ImageJ. Protein was resolved by running the sample till dye reached to the bottom of the SDS-PAGE gel. The protein was transferred from SDS-PAGE to PVDF membrane as follows: The PVDF membrane was cut precisely the size of the gel and soaked in absolute methanol for 1-2 minutes. The membrane was assembled along with the SDS-PAGE in the transfer cassette assembly, and kept for 90 minutes in the tank at 90 V for protein transfer. The PVDF membrane was taken out separately and the SDS-PAGE was discarded after protein transfer. The membrane was washed once with 1X-TBS, soaked in skim milk and was left on moving platform at 4 ºC overnight for blocking. The membrane was washed with 1X-TBS again, and primary antibody mixed in the skim milk solution containing TBS buffer was added. Incubation time and temperature was followed as recommended by supplier. Three washes with 1X-TBST for 15 minutes each were given to the membrane followed by incubation with secondary antibody as per the requirements and the three washes were repeated with 1X-TBST for 15 minutes each. The membrane was finally soaked in TBS and antibodies detected by keeping membrane on a tray. To detect protein, ECL Prime western blot detection reagent solution (800 µl autoclaved double-distilled water, 100 µl Luminol enhancer solution and 100 µl peroxide solution) was added to the blotting membrane, and image was taken using ImageQuant Las4000.

2.5.5 Bimolecular Fluorescent Complementation (BiFC) Assay

A single colony of *Agrobacterium* constructs of proteins of interest containing each of the SPYCE (or BiFP-YC) and the SPYNE (or BiFP-YN) constructs were inoculated separately in

5ml LB containing Rifampicin, Gentamycin and Ampicillin (or Rifampicin, Gentamycin and Spectinomycin). The empty backbone constructs were inoculated as controls. Similarly, *Agrobacterium* construct of p19 RNAi suppressor gene construct was also inoculated in 5ml LB media containing Rifampicin, Gentamycin and Kanamycin. Incubation was done at 30 ºC for 24 hours in a shaker followed by centrifugation at 4000 rpm for 15 minutes. Supernatant was discarded and pellet was resuspended in 2ml infiltration buffer. The pellet was washed twice using 2 ml infiltration buffer each time. The pellet was again resuspended in infiltration buffer to an OD_{600} of 0.8-1.0. The resuspended bacteria were then mixed in a 1:1:1 ratio of constructs of SPYCE (or BiFP-YC), SPYNE (or BiFP-YN) and p19 RNAi suppressor gene and incubated at 30 ºC for 1 hour in shaker. The above *Agrobacterium* cocktail was infiltrated into the abaxial surface of a leaf of *Nicotiana benthamiana* using a 2 ml syringe. The plants were kept at 22 ºC for 48 hours after which the leaves were screened using a fluorescence microscope.

Chapter 3

Results

Summary

Yeast-2-Hybrid (Y2H) clearly indicated that four out of five DELLAs used for the assay interact with WUS. Pull down assay results with GAI was a false positive and hence the interaction could not be confirmed. Due to antibody limitation, CoIP could not be successfully carried out. In BiFC, interaction between RGL3 and WUS was seen. These all experiments gave a signpost that WUS must be interacting with DELLAs and may be the latter have a role to play in SAM maintenance in *Arabidopsis.* Also, an interesting phenotype observed in F2 progeny of *della* quadruple mutant crossed with *clv3-2.* Its confirmation in F3 progeny would lead to a further insight about WUS-DELLAs interaction.

3.1 Yeast-2-Hybrid (Y2H)

To test the protein-protein interactions among various DELLAs and WUS via Yeast-2-Hybrid, I made various constructs. First, I cloned preys AD-RGL1, AD-RGL3, AD-RGA and AD-RGL2. The Gal4 activation domain is translationally fused with DELLAs. AD-GAI was cloned by Harish Kumar. For BD-WUS bait, Gal4 DNA binding domain is fused translationally with WUS by Prince in the lab. I found mutation in the AD-RGL2 and hence it wasn't used for Y2H. 3-Amino-1,2,4 – triazole (3AT) was used in various concentrations in order to avoid false positives. 3AT acts a competitive inhibitor of HIS3 biosynthesis. To get stringent experimental results, two selection media were used with -LWH and -LWHA (dropout containing all amino acids except Leucine, Tryptophan, Histidine and Leucine, Tryptophan, Histidine, Adenine, respectively). pDest-AD was used as a negative control whereas -LT selection media was a positive control on which all preys (DELLAs cloned with Gal4-Activation Domain at C-terminal) grew. The growth in negative control diminished with increase in 3AT concentration.

To test interactions between four DELLAs and WUS, I setup Y2H assay among the DELLA prey protein with WUS baits (Fig.4). All four DELLA proteins showed interaction with WUS at 0.25mM, 0.5mM and 1.0mM 3AT, respectively, on -LWH plate. In consistent to this, when the same prays were tested again on more stringent condition having -LWHA plate at 1mM 3AT showed interaction with WUS bait. From the concentration of 3AT used the strength of interactions was estimated. It can be said that RGA, GAI and RGL1 have stronger interaction with WUS in comparison to RGL3 as the latter did not grow on -LWHA containing 1mM 3AT. Hence, Y2H assay gave a clear indication that DELLAs interact with WUS.

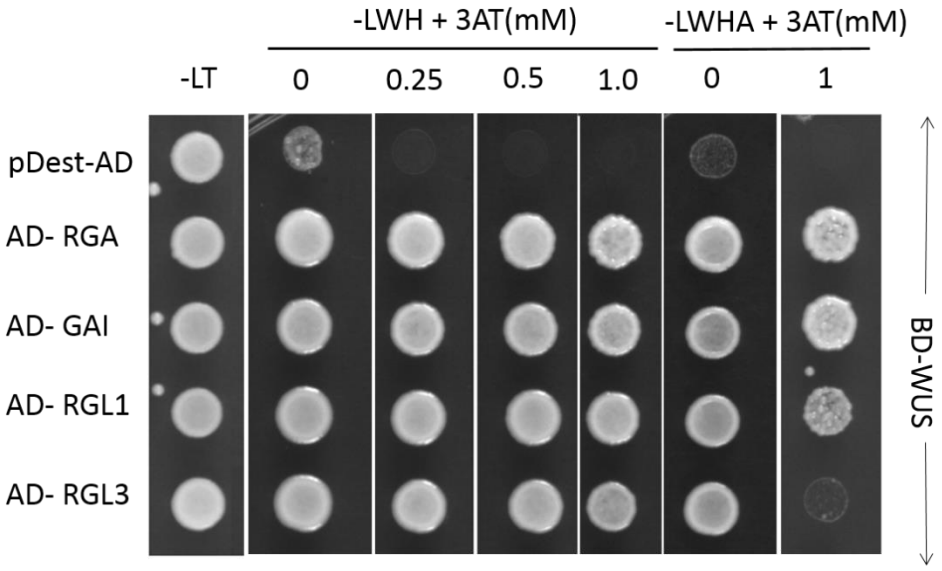

Figure 4. Yeast-two-hybrid (Y2H) results. Among the four DELLAs checked for interaction with WUS, RGL3 was found to have the weakest though significant interaction.

3.2 Pull down assay (In-vitro)

In the first attempt, only GAI-MBP was used to check its interaction with WUS. WUCHEL bound on Ni-NTA beads for the first attempt was provided by Asis Khuntia. I induced and purified GAI-MBP and MBP-alone. These two proteins were used in an eluted form and I quantified all the proteins using Fiji software [25]. Bovine Serum Albumin (BSA) in different known concentrations was used for comparison purposes on SDS-PAGE (10% resolving gel). In this first attempt, the incubation time for proteins to interact was kept 3 hours. The anti-His antibody was used in 1:5000 dilution and anti-MBP in 1:10000 dilution. After Western Blot

was developed (Fig. 5), it was found that GAI interacts with WUS but in sample where MBP-alone was incubated with WUS, MBP-alone was also detected in the blot. This was thought to be a non-specific band which would go away by decreasing the incubation period of proteins together.

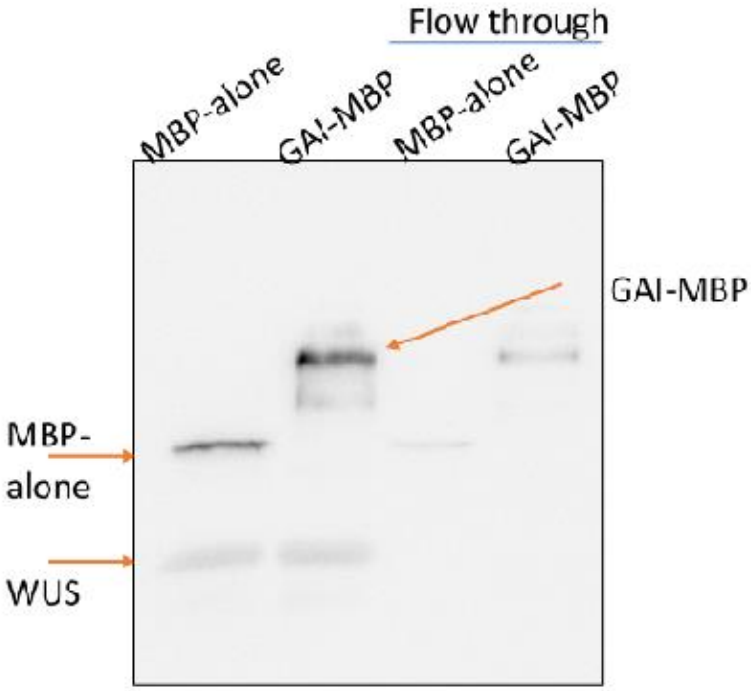

Figure 5. Pull down assay with MBP-alone, GAI-MBP and WUS-6X His.

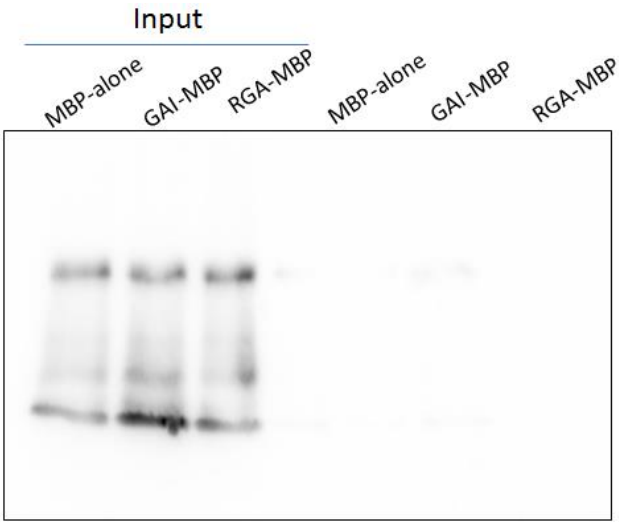

Figure 6. Pull down assay with MBP-alone, GAI-MBP, RGA-MBP and WUS-6X His with change in first method. WUS-6X His was used in eluted form and amylose beads added.

I made various attempts to get rid of this non-specific band but could not succeed. These attempts include modification 1) by decreasing the incubation period from 3 hours to 1 hour, 2) by changing buffer, 3) and use WUS in an eluted form (Fig.6), etc. WUS protein could not be eluted in an enough amount so that it can be used for Pull down assay. I did attempt change in buffer, pH conditions of elution buffer, time of incubation for induction of protein, changes in amount of protease inhibitor to reduce changes of protein degradation in the process, etc. Using WUS bound on beads, I went on to try pull down assay with RGA MBP but faced issues with RGA elution as well as elution of other proteins (Fig.7). The proteins could not be induced in enough amount required for pull down assay. Meanwhile we found that GAI had an intrinsic 6X-His tag too. To check if the first pull-down assay had given false positive, I did pull down again using GAI-MBP in eluted form and WUS bound on Ni-NTA beads. In one MCT, I incubated purified GAI-MBP with fresh Ni-NTA beads and in another, I incubated GAI-MBP with WUS bound on the same number of Ni-NTA beads. It was found that GAI-MBP was detected in equal amounts in both cases. Hence the intrinsic 6X-His tag had given false positive result earlier. I tried using amylose beads for pulling MBP tagged protein and detecting WUS added in eluted form but it could not be detected in the blot. Thus, the interaction could not be confirmed and the experiment needs to be done with GAI cloned with some other tags.

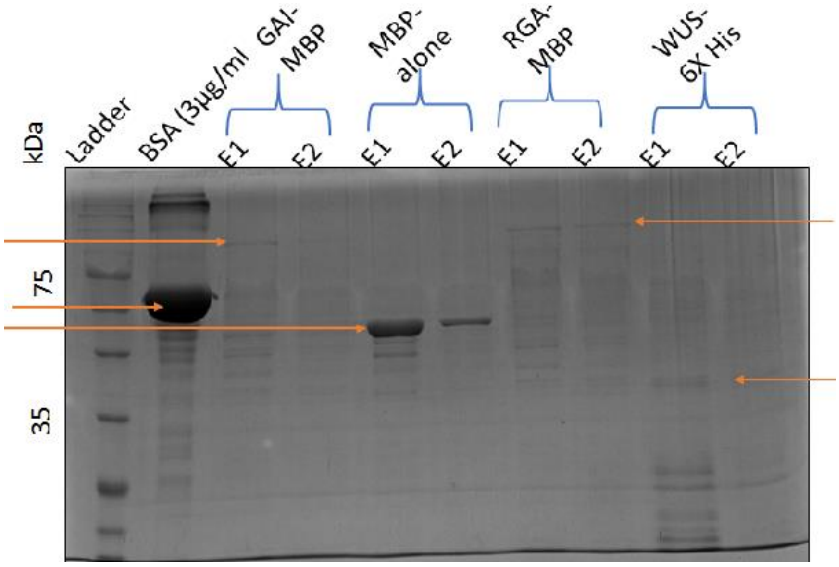

Figure 7. Protein elution issues for pull down assay.

3.3 Co-Immuno Precipitation assay (In-vivo)

I did protein extraction from 10-day old seedlings of 35S: TAP-RGAΔ17 and checked for protein via SDS-PAGE (Fig.8). The SDS-PAGE showed a number of plant proteins. When incubated with WUS-6X His on Ni-NTA beads, the results of CoIP could not be detected in Western blot. The anti-Myc Antibody (1:10000 dilution) borrowed from another lab did not work. The experiment was repeated again and antibody from another lab used. But the attempt failed due to the same reason. The experiment would be tried again once the anti-Myc antibody becomes available in the lab or using RGA cloned with some another tag.

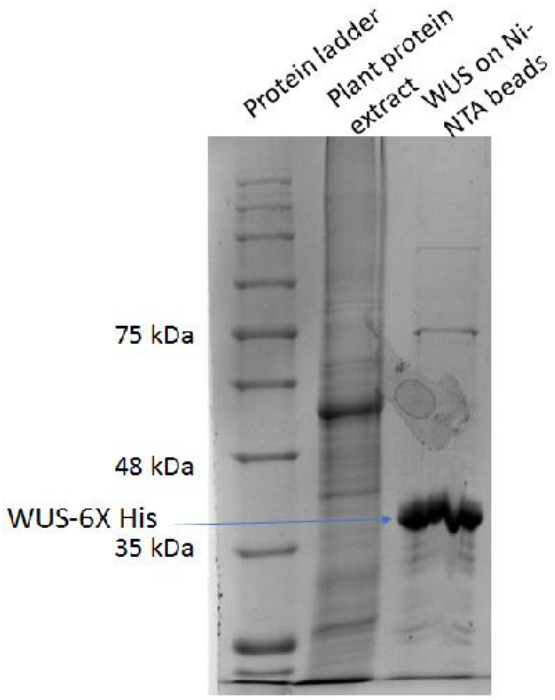

Figure 8 : SDS-PAGE for total protein extraction from 10 day old 35S: TAP-RGAΔ17 seedlings and WUS-6X His on Ni-NTA beads.

3.4 Bimolecular Florescence Complementation (BiFC) Assay

For BiFC, I initially made constructs RGA-YFP^{c}**,** GAI- YFP^{c}**,** RGL1- YFP^{c} and RGL3- YFP^{c} in plant expression binary vector pSPYCE-35S. WUS- YFP^{n} cloned in vector pSPYNE-35S was taken from Prince Saini in the lab. With these constructs, transformation into *Agrobacterium tumefaciens* could not be successful in spite of change in electro-competent cells, selection media plates, etc.

So, I cloned the same constructs RGA-YFP^{c}, GAI- YFP^{c}, RGL1- YFP^{c} and RGL3- YFP^{c} in plant expression binary vectors pBiFP3. WUS- YFP^{n} cloned in vector pBiFP2 was taken from Asis Kumar in the lab. Sequencing results for the latter and RGL1- YFP^{c} were not right and hence constructs with C-terminal of YFP in pBiFP3 were used along with WUS- YFP^{n} cloned in vector pSPYNE-35S having N-terminal of YFP. I made new *Agrobacterium* electro-competent cells and selection media plates for transformation and it gave positive results. After infiltration into tobacco leaves, leaves were analysed for 3 days after 48 hours of infiltration. Since the BiFC signal could not be detected in fluorescence microscope, confocal microscope was used and leaf discs were stained with Propidium Iodide (0.5 mg/ml).

The confocal microscopy images confirmed interaction between RGL3 and WUS (Fig.9). No positive BiFC signal was found in case of RGA and GAI with WUS. Hence these two interactions could not be confirmed.

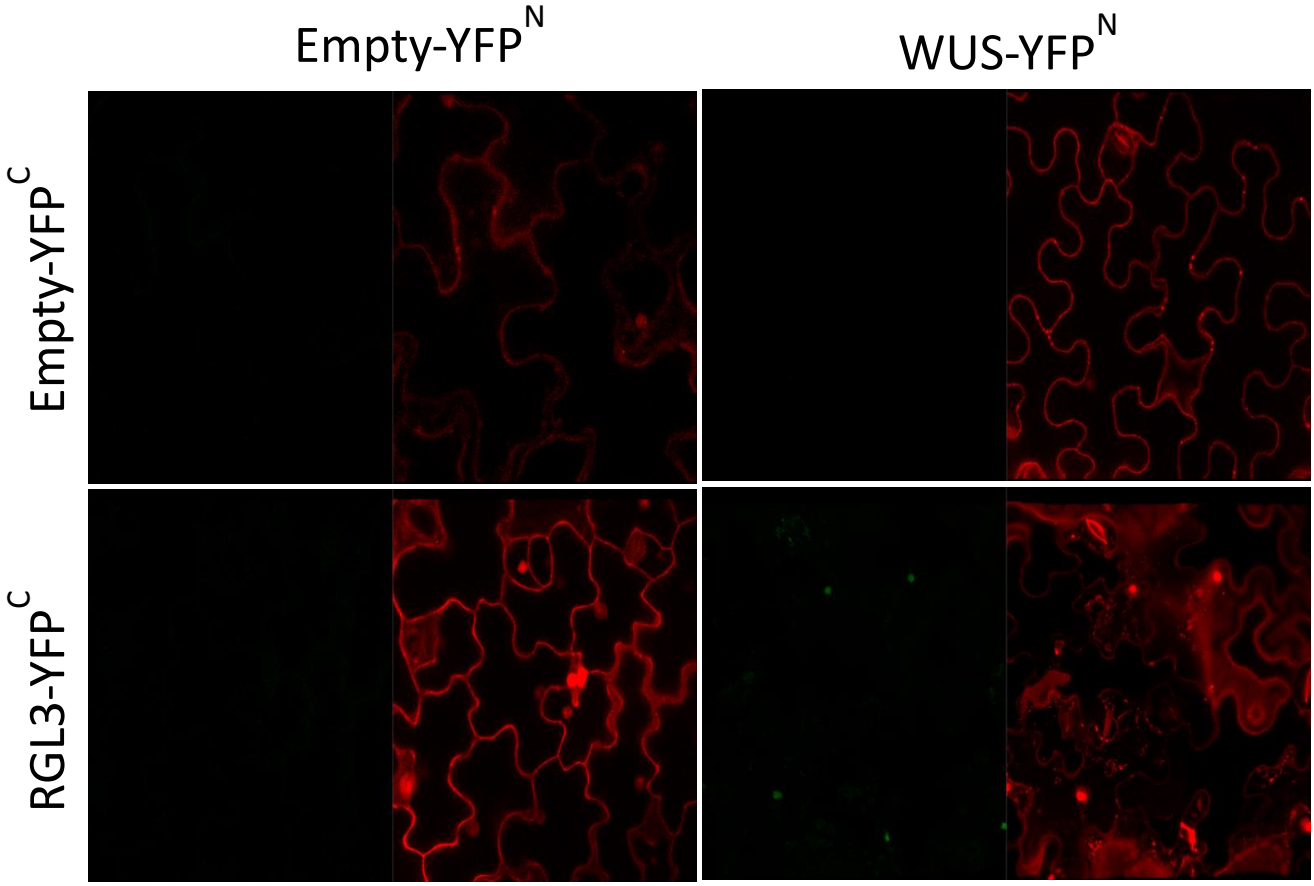

Figure 9 : Confocal microscopy images of leaves of *Nicotiana benthamiana* to detect Yellow Fluorescence Protein (YFP). YFP is labelled in green and the cell boundaries along with some nuclei in red by Propidium Iodide (PI) staining.

3.5 Screening for Phenotypes

To elucidate genetic mechanisms, *dellaq* X *clv3-2* cross was made by Harish Kumar and I followed up the progeny in order to screen phenotype, if any. More than 800 F2 progeny of *dellaq* X *clv3-2* cross were sown by Harish and on the basis of visible phenotype, I did genotyping for a few to screen any plant where five genes of interest including DELLAs (all five except RGL3 for which primers were unavailable) and CLAVATA would be present in mutant form in a single plant. I found an interesting phenotype (Fig.10) in a plant homozygous for all the desired mutants. It was found to have scoop shaped leaves and multiple Shoot Apical Meristem (SAM). The phenotype needs to be confirmed in F3 generation.

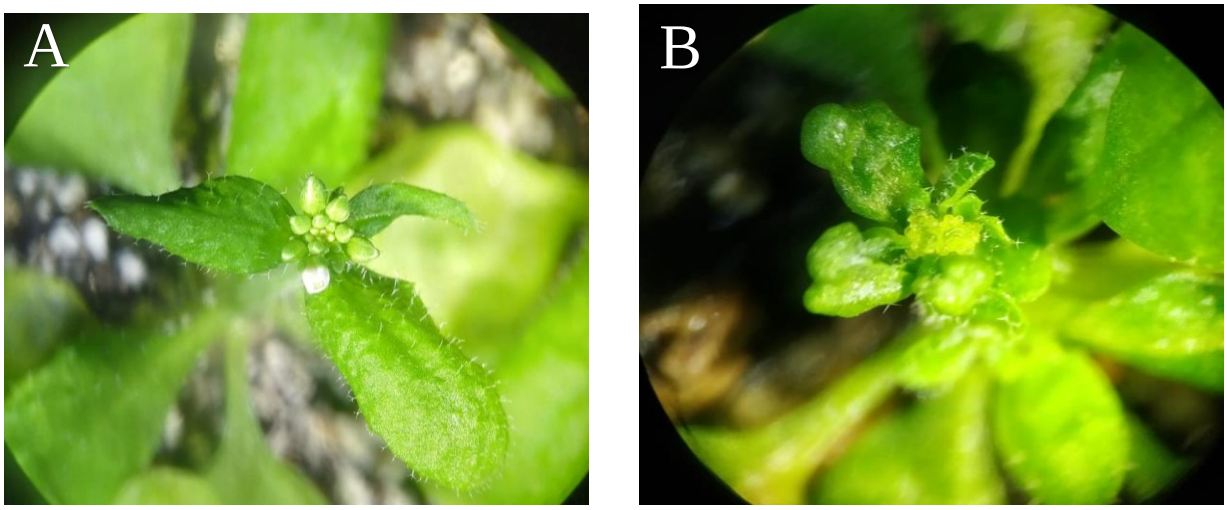

Figure 10 Phenotypes found in *dellaq X clv3-2* F2 progeny. The mutant with five mutations showed multiple SAM and scoop shaped leaves (B) in comparison to normal leaves and single SAM in wild type Ler ecotype (A).

Chapter 4

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