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**Structural and Functional Characterization of the  
Receptor-like Kinase STRUBBELIG in  
*Arabidopsis thaliana***

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# I. Table of contents

<b>I.</b>	<b>Table of contents.....</b>	<b>1</b>
<b>II.</b>	<b>Summary.....</b>	<b>7</b>
<b>III.</b>	<b>Zusammenfassung.....</b>	<b>8</b>
<b>1</b>	<b>Introduction.....</b>	<b>9</b>
1.1	Introduction.....	9
1.2	Plant receptor protein kinases.....	10
1.2.1	Leucine-rich repeats (LRRs) motifs involved in protein-protein interactions	11
1.2.2	Receptor-like proteins show similarity with extracellular domains of RLKs.	12
1.2.3	Plant RLKs are involved in various signaling pathways.....	12
1.2.4	Brassinosteroid-insensitive1 (BRI1) serves an example of steroid hormone signaling via a receptor-like kinase in plants.....	13
1.2.5	CLAVATA1 (CLV1), a receptor-like kinase mediated signaling pathway that controls shoot and floral meristem size.....	16
1.3	STRUBBELIG a receptor-like kinase regulating cell division and cell morphogenesis in <i>Arabidopsis</i> .....	17
1.3.1	The <i>strubbelig</i> phenotype.....	17
1.3.2	<i>STRUBBELIG</i> gene structure.....	18
1.3.3	STRUBBELIG may represent a non-functional kinase domain.....	18
1.3.4	<i>SUB</i> function.....	19
1.4	Role of intercellular communication in plant development.....	20
1.4.1	Non-selective movement of GFP and Leafy.....	20
1.4.2	Selective movement of transcription factors.....	22
1.4.3	Role of cytoskeleton in protein movement.....	23
1.5	In this study.....	23

<b>2</b>	<b>Materials and Methods.....</b>	<b>25</b>
2.1	Plant work.....	25
2.1.1	Plant growth media.....	25
2.1.2	Plant growth.....	25
2.1.3	<i>Arabidopsis thaliana</i> transformation.....	25
2.1.4	Seed sterilization.....	26
2.2	Preparation of electro-competent cells.....	26
2.3	Construction of plasmids.....	27
2.3.1	Cloning of <i>SUB:3xmyc</i> in pCAMBIA2300.....	27
2.3.2	<i>Promoter::SUB:3xmyc</i> construct.....	27
2.3.2.1	<i>ANT::SUB:3xmyc</i> construct.....	28
2.3.2.2	<i>35S::SUB:3xmyc</i> pCAMBIA2300.....	28
2.3.2.3	<i>AtML1::SUB:3xmyc</i> pCAMBIA2300.....	28
2.3.2.4	<i>WUS::SUB:3xmyc</i> pCAMBIA2300.....	29
2.3.2.5	<i>pSUB::SUB:3xmyc</i> pCAMBIA2300.....	30
2.3.2.6	Construction of alanine-linker <i>GFP</i> fusion.....	30
2.3.2.7	<i>SUB-link:GFP</i> pCAMBIA2300.....	31
2.3.2.8	<i>pSUB(3.5kb)::SUB-link:GFP</i> pCAMBIA2300.....	31
2.3.2.9	<i>ANT::SUB-link:GFP</i> pCAMBIA2300.....	32
2.3.2.10	<i>AtML1::SUB-link:GFP</i> pCAMBIA2300.....	32
2.3.2.11	<i>5'WUS::SUB-link:GFP WUS3'</i> pCAMBIA2300.....	32
2.3.2.12	<i>35S::SUB:EGFP</i> pEZT-NT construct.....	32
2.3.2.13	<i>pSUB(3.5kb)::SUB:EGFP 3'UTR SUB</i> pCAMBIA2300.....	33

## Table of contents

2.3.2.14	<i>pCol::SUB:EGFP 3'UTR SUB</i> pCAMBIA2300.....	33
2.2.2.15	<i>lox-P</i> target site vector construction.....	34
2.4	Molecular biology techniques.....	35
2.4.1	Restriction endonuclease.....	35
2.4.2	T4 DNA ligase and T4 DNA polymerase.....	35
2.4.3	Nucleic acid purification.....	35
2.4.3.1	Bacterial plasmid DNA isolation.....	35
2.4.3.2	RNA extraction.....	35
2.4.3.3	DNA extraction.....	36
2.4.3.4	Purification of DNA fragment from agarose gel.....	36
2.5	Polymerase chain reaction (PCR) methods.....	36
2.5.1	Oligonucleotides.....	36
2.5.2	PCR.....	37
2.6	GUS histochemistry.....	37
2.7	Microscopy.....	37
2.7.1	Scanning electron micrograph (SEM).....	37
2.7.2	Confocal laser scanning microscopy (CLSM) (fixed tissue samples).....	38
2.7.3	Light microscopy analysis.....	38
2.8	Flower organ counting.....	39
2.9	Photography.....	39
2.10	Screening of bright GFP and EGFP transgenic plant lines.....	40
2.11	Live imaging of GFP and EGFP using confocal microscopy.....	40
2.12	Drug treatment.....	41
2.13	Plasmolysis.....	41



<b>3</b>	<b>SUB:EGFP showed a differential expression pattern compared to its mRNA and localized in the plasma membrane.....</b>	<b>42</b>
3.1	Summary.....	42
3.2	Introduction.....	42
3.3	Results.....	43
3.3.1	SUB:EGFP fusion protein rescued the <i>sub</i> mutant phenotype.....	43
3.3.2	SUB:EGFP protein expression pattern.....	48
3.3.2.1	The SUB:EGFP protein is expressed in the stele region of post-embryonic roots.....	48
3.3.2.2	SUB:EGFP showed a differential expression pattern in the developing flower and inflorescence meristems.....	50
3.3.2.3	SUB:EGFP expression in the developing ovule.....	54
3.4	SUB:EGFP is localized to the plasma membrane in the cell.....	57
3.5	Subcellular behavior of SUB:EGFP.....	57
3.6	SUB:EGFP receptor protein internalization is BFA sensitive.....	59
3.7	SUB:EGFP decorated the subcellular organelles.....	60
3.8	Discussion.....	62
3.8.1	Subcellular behavior of C-terminal fused EGFP with SUB.....	62
3.8.2	Spatial and temporal expression pattern of SUB during organogenesis.....	64
3.8.3	Differential expression of SUB mRNA vs. protein during development.....	64

<b>4</b>	<b>STRUBBELIG acts in a non-cell autonomous fashion during development.....</b>	<b>66</b>
4.1	Summary.....	66
4.2	Introduction.....	66
4.3	Results.....	67
4.3.1	<i>ANT::SUB:GFP</i> is able to rescue the <i>sub</i> mutant phenotype.....	67
4.3.2	<i>SUB:GFP</i> protein expression pattern under <i>ANT</i> promoter is similar to the <i>ANT</i> mRNA expression pattern.....	71
4.3.3	<i>sub</i> phenotype partially rescued by <i>WUS::SUB:GFP</i> .....	74
4.3.4	Stem twisting and outer integument of ovule rescued by <i>WUS::SUB:GFP</i> .....	76
4.3.5	<i>WUS::SUB:GFP</i> expression pattern.....	79
4.3.6	<i>AtML1::SUB:GFP</i> ( <i>L1::SUB:GFP</i> ) rescued L2-layer periclinal division in non-cell autonomous fashion.....	81
4.3.7	<i>L1::SUB:GFP</i> rescued partially cell numbers in epidermis, cortex and pith of 30-day old plant stems.....	87
4.4	Discussion.....	90
4.4.1	Possible mechanism of non-cell autonomy in <i>SUB</i> signaling.....	90
4.4.1.1	Relay signaling vs. diffusible signal.....	90
4.4.1.2	<i>SUB</i> signaling could modify the non-cell autonomous signal and allows its long range movement.....	92
4.4.1.3	Could <i>SUB</i> signaling affects the cell cytoskeleton.....	93

<b>5</b>	<b>Non-cell autonomous function of SUB during plant development: <i>Cre/lox-P</i> system for mosaic analysis in <i>Arabidopsis</i>.....</b>	<b>94</b>
5.1	Summary.....	94
5.2	Introduction.....	94
5.3	Results.....	95
5.3.1	<i>Cre/lox-P</i> genetic mosaic system.....	95
5.3.2	<i>Cre/lox-P</i> SUB mosaic gives variable rescue of <i>sub</i> phenotype.....	96
5.4	Discussion.....	100
<b>6</b>	<b>Conclusion.....</b>	<b>101</b>
<b>7</b>	<b>References.....</b>	<b>104</b>
<b>IV.</b>	<b>Acknowledgments.....</b>	<b>116</b>
<b>V.</b>	<b>Curriculum vitae.....</b>	<b>118</b>

## II. Summary

In plants, cell-to-cell communication is an important aspect in coordinating cell division pattern and cell growth to generate organs of characteristic shape and size. Mechanisms by which intercellular communication controls cell division and cell division patterns remain largely unknown. Determining the molecular basis of this cell-to-cell communication is crucial to understand plant development.

STRUBBELIG (SUB), a putative leucine-rich repeat (LRR) receptor-like kinase (RLK) is localized to the plasma membrane. Studies comparing the mRNA vs. SUB:EGFP fusion protein expression pattern show a differential expression pattern in developing ovules, flowers and inflorescence meristems during development. The absence of SUB protein expression in the cell layers that show the *sub* mutant phenotype, suggests that SUB acts in a non-cell autonomous fashion during cell division and growth. The colocalization studies using FM4-64 in combination with SUB:EGFP revealed endocytosis of SUB:EGFP via endosomes from the plasma membrane. Moreover, the SUB:EGFP endocytosis is sensitive to BFA.

Further studies, a combination of promoter rescue experiment and a *Cre/lox-P* based clonal analysis, were undertaken to investigate the possible non-cell autonomy of SUB function. Our results indicate that SUB acts non-cell autonomously. The SUB protein does not move between cells, therefore it must act in an indirect fashion on the neighboring cells. From our studies it appears that the range of such a non-cell autonomous mechanism extends across few cell layers only.

### III. Zusammenfassung

Zell-Zellkommunikation in Pflanzen ist wichtig für die Koordination der Zellteilungsmuster mit dem Zellwachstum und um Organe mit charakteristischer Grösse und Form entstehen zu lassen. Die entsprechenden Mechanismen sind weitgehend unbekannt. Ihre Aufklärung ist daher von zentraler Wichtigkeit für das Verständnis der Pflanzenentwicklung.

STRUBBELIG (SUB), eine mögliche "leucine-rich repeat" (LRR) Rezeptorkinase (RLK) ist in der Plasmamembran lokalisiert. Vergleiche der Expressionsmuster auf den Ebenen mRNA vs SUB:EGFP Fusionsprotein zeigen ein differentielles Muster in sich entwickelnden Samenanlagen, Blüten- und Infloreszenzmeristemen. Das SUB:EGFP Fusionsprotein konnte nicht in Zellschichten nachgewiesen werden, die den *sub* mutanten Phänotyp zeigen. Dieser Befund deutet darauf hin, dass SUB in einer nichtzellautonomen Art und Weise agiert. Kolokalisierungsstudien, mit Hilfe des Farbstoffs FM4-64 in Kombination mit SUB:EGFP, zeigten, dass SUB:EGFP via Endosomen endozytiert wird. Dieser Prozess ist durch BFA beeinflussbar.

Die mögliche Nichtzellautonomität der *SUB*-Funktion wurde durch eine Kombination von Promotor-Rettungsexperimenten und einer auf dem *Cre/lox-P*-System basierenden klonalen Analyse weiter erhärtet. Das SUB Protein scheint nicht von Zelle zu Zelle zu wandern, daher muss es auf indirekte Art und Weise auf benachbarte Zellen einwirken. Die Resultate lassen den Schluss zu, dass dieser Mechanismus nur über wenige Zellschichten hinweg wirksam ist.

## Chapter 1 Introduction

### 1.1 Introduction

Cell-to-cell communication is a fundamental process in plant and animal development. In multicellular organisms cells integrate intrinsic signals with extrinsic signals and modulate cell growth and differentiation during development. In plants, development differs from animal development in some basic aspects. The adult body plan of an animal is laid down early on in embryogenesis. Contrary to animals, the adult body plan of a plant is established post-embryonically and influenced by environmental stimuli. Plant cells are surrounded with a rigid cell wall and do not allow cell migration, while cell migration play a vital role in animal development. Interestingly, genetically controlled cell division patterns and their plane of cell division, controls plant development (Meyerowitz, 1997). Moreover, plants and animals organs differ in the complexities of their cell types. Animal organs are made of several distinct cell types while plant organs contain relatively few cell types only.

The development of multicellular organisms from a single cell is a precise genetically controlled process. Patterning of organs and specification of cell types requires reliable intercellular signaling mechanism to communicate positional information between the cells. How signaling molecules, such as small molecules, polypeptide ligands, and small RNAs, are involved in developmental events is still an open question. Signaling molecules generally have to traverse a distance of one to several cells. Mechanism of signaling molecule travel to receiving cells remains obscure, whether protein signal moves by concentration depended diffusion outside the cell or not. Moreover, signaling can be controlled by rate of signal production, modification, and active transport. A signal could be produced in one cell and influence a neighboring cell, which produces another signal, thus, a signal may act through a series of relays. In plants, organ primordia arise from a group of cells in shoot apical meristem (SAM) (Tilney-Bassett, 1986). To form a

characteristic organ shape and size, the organ initiating cells must communicate with each other to coordinate their cell growth and division pattern. During plant development pattern of cell division and differentiation depend on positional information and cellular interaction (Stewart and Burk, 1970; Stewart and Derman, 1973). How cells communicates this information to coordinate cell function during organogenesis is still not understood at molecular level. Both in animals and plants, receptor-like kinase mediated short range and long range signaling plays vital role in cell growth and cell differentiation (Hubbard and Till, 2000; Shiu and Bleecker, 2001a; Torii and Clark, 2000). In plants, example of non-cell autonomous control of cell division in SAM is exerted by stem cell organizing factor WUSCHEL (WUS) (Mayer et al., 1998; Schoof et al., 2000). WUS is required for continuous cell division in flower and inflorescence meristems. CLAVATA3 (CLV3) (Fletcher et al., 1999), presumably a ligand for receptor-like kinase CLAVATA1 (CLV1) (Clark et al., 1997) represses excess cell division in SAM.

### 1.2 Plant receptor protein kinases

Receptor protein kinases (RPKs) are important mediators of cell-to-cell signaling in multicellular organisms. Plant receptor-like kinases (RLKs) are transmembrane proteins with a predicted signal sequence, single transmembrane region and cytoplasmic kinase domain. Animal and plant RLKs comprises a monophyletic gene family (RLK/Pelle). The *Arabidopsis* genome sequence revealed more than 610 RLKs that represent 2.5% of the protein coding genes (Shiu and Bleecker, 2001b; Morris and Walker, 2003). The plant RLKs are classified on the basis of the presence or absence of extracellular domain and kinase domain phylogeny and intron information. RLKs, which lack signal sequence and transmembrane domain, were referred to as receptor-like cytoplasmic kinases (RLCKs). The RLK/Pelle family is subdivided into 46 subfamilies (Shiu and Bleecker, 2003). The transmembrane receptors, with more than 400 representatives, are characterized by a C-terminal intracellular serine/threonine kinase domain, variable N-terminal extracellular domains and single-pass transmembrane domain in between, which

the RLCKs lack. The diversity of domain organization and large gene number in this family suggests that domain fusion contributed to the formation of novel receptor kinases and subsequent gene duplications resulted in the expansion of the RLK/Pelle subfamilies in plants. The leucine-rich repeat (LRR)-containing RLK comprises the largest RLK subfamilies in *Arabidopsis* with 235 genes. The LRR-RLKs can be subdivided into 13 subfamilies on the basis of amino acid sequence in their kinase domains (Shiu and Bleecker, 2001a, b). LRRs are signal transduction motifs and involved in protein-protein interactions (Kobe and Deisenhofer, 1994). The second largest RLKs are sugar-binding motifs or lectins. In this class the extracellular domains contain legume lectin motifs and include 42 members of the lectin-receptor kinase (LecRLKs) (Herve et al., 1996; Barre and Rouge, 2002; Shiu and Bleecker, 2001b). Other important families of RLKs are those with lectin and S-domain motif (Shiu and Bleecker, 2001b). In large number of RLKs, a role of extracellular sequence motif is still not understood.

### **1.2.1 Leucine-rich repeats (LRRs) motifs involved in protein-protein interactions**

LRRs have been found in a variety of proteins with diverse functions, from yeast to flies, humans and plants and are involved in protein-protein interactions (Kobe and Deisenhofer, 1994). The LRR is a stretch of 24 amino acids (aa) with conserved leucines. In *Arabidopsis* half of the RLKs contain between 1 to 32 LRRs in the extracellular domain (Shiu and Bleecker, 2001b; Shiu and Bleecker, 2001a). The crystal structure of porcine ribonuclease inhibitor, a cytoplasmic protein made solely of LRRs, indicates that LRRs form a non-globular horseshoe-shaped complex, consisting of defined structural units. The units are made of a  $\beta$ -strand pointing to the inner circumference of the horseshoe and a  $\alpha$  helix, which directs towards the outer circumference (Kobe and Deisenhofer, 1993).



### 1.2.2 Receptor-like proteins show similarity with extracellular domains of RLKs

More than 170 receptor-like proteins (RLPs) have been identified based on their structural similarity with RLK extracellular domain. The majority of RLPs are tandem repeats and cluster closely to structurally related RLKs (Shiu and Bleecker, 2003). The function of most RLPs is still unknown. RLPs with known functions, including both CLV2 and SLG have been implicated to function in signaling pathway involving the RLKs, CLV1 (Jeong et al., 1999) and SRK respectively (Cui et al., 2000).

### 1.2.3 Plant RLKs are involved in various signaling pathways

The fundamental mechanism by which cells communicate in multicellular organisms involves binding of ligands to cell surface receptors. The N-terminal domains of RLKs have been implicated for binding to a variety of signaling molecules. These molecules could be polypeptides, carbohydrates, steroids and microbial cell wall components (Becraft, 2002; Morris and Walker, 2003). The binding of cognate ligands to RLKs induces receptor dimerization or oligomerization. This leads to juxtaposition of the intracellular kinase domain that may become activated through mutual phosphorylation. On one hand, phosphorylation of conserved serine/threonine residue in the activation loop of the kinase domain changes the conformation and active sites may become accessible to substrates. On the other hand, phosphorylation of other serine/threonine residues, located outside the kinase domain, establishes docking sites for intracellular signaling molecules. Signal transduction cascades initiated by phosphorylation of cytoplasmic kinase domain lead to the activation of downstream components that control gene expression at nuclear level.

The large number of diverse RLKs in the *Arabidopsis* genome suggests that RLKs may function in perception of a wide-range of signals. Based on broad functions RLKs can be divided into two classes. The first class includes RLKs involved in plant-microbe interactions and stress responses, NODULATION RECEPTOR-KINASE (NORK) from *Medicago truncatula* and its orthologs such as SYMBIOSIS RECEPTOR-LIKE KINASE (SYMRK) in *Lotus*. LRR-RLK, FLAGELLIN SENSITIVE2 (FLS2) responds to microbial stimuli. FLS2 acts in pathogen defence response signaling and shares similarity to Toll-like receptors in animals (Gomez-Gomez and Boller, 2002). The second class includes RLKs involved in the control of plant growth and development in normal conditions. Several RLKs with growth and developmental functions have been identified in *Arabidopsis*. These include BRASSINOSTEROID INSENSITIVE1 (BRI1), (see section 1.2.4) a brassinolide sensitive RLK (Li and Chory, 1997), CLAVATA1, (see section 1.2.5) regulating stem cell maintenance and differentiation (Clark et al., 1997), ERECTA, which plays a role in cell-to-cell signaling regulating cell proliferation during organogenesis (Torii et al., 1996), HAESA that controls organ abscission (Jinn et al., 2000) and ARABIDOPSIS CRINKLY4 (ACR4) which is required for normal L1 cell layer organization (Gifford et al., 2003). As the number of plant RLKs increases it poses a challenge to understand their structure function and mechanism of action in detail.

### **1.2.4 Brassinosteroid-insensitive1 (BRI1) serves an example of steroid hormone signaling via a receptor-like kinase in plants**

Brassinosteroids (BRs) are a special class of plant polyhydroxysteroids that play an important role in normal plant development and growth (Clouse and Sasse, 1998). BRI1 is expressed ubiquitously in *Arabidopsis* and localized to the plasma membrane (Li and Chory, 1997; Friedrichsen et al., 2000) and involved in brassinosteroid hormone perception. Loss of function *bri1* plant show reduced male fertility, prolonged life span, delayed leaf senescence and dwarfism (Li and Chory, 1997). *BRI1* encodes an LRR-RLK of 1196 amino acids with 24 LRRs and a 70-amino acid island domain between the 21<sup>st</sup> and 22<sup>nd</sup> LRR (Li and Chory, 1997;

Vert et al., 2005). Usually, BRI1 forms a homodimer in its inactive configuration, which is mediated by interaction between the extracellular domains and most likely, in trans-fashion (Wang et al., 2005a). Activation of BRI1 involves direct binding of brassinolide, a brassinosteroid, to the 70 amino-acid island between LRR 21 and 22 of the extracellular domain (Kinoshita et al., 2005). The ligand binding triggers conformational changes in the kinase domains that allows trans-phosphorylation of the C-Terminal (CT) domains followed by autophosphorylation of specific sites in the juxtramembrane and kinase domains (Wang et al., 2005a; Wang et al., 2005b). Moreover, the ligand binding changes the BRI1 homodimer equilibrium towards BRI1 ASSOCIATED KINASE1 (BAK1) heterodimer. BAK1 is able to interact with activated BRI1 kinase domain and presumably phosphorylates downstream signaling components (Li et al., 2002; Nam and Li, 2002). Genetic studies revealed a positive role of BAK1 in BR signaling. BRI1 KINASE INHIBITOR1 (BKI1) encodes two-serine rich domains protein and negatively regulates the BRs signaling by interacting with the intracellular domain of BRI1 (Wang and Chory, 2006). BRASSINOSTEROID INSENSITIVE2 (BIN2) encodes a cytoplasmic protein kinase and genetic studies revealed that BIN2 is a negative regulator in the BR signaling (Li et al., 2001). Two nuclear proteins, BRASSINOZOLE RESISTANT1 (BRZ1) and BRI1-EMS-SUPPRESSOR1 (BES1) were identified as positive regulators of the BR signaling (Yin et al., 2002). When BR is applied, BRZ1 and BES1 accumulate in the nucleus. Activation of the receptor-like kinase BRI1-BAK1 by BR binding leads to the dephosphorylation and accumulation of the nuclear protein BZR1 and BES1, presumably by inhibiting the negative regulator BIN2. The BIN2 kinase phosphorylates BZR1/BES1 in the absence of BR signaling and targets them for degradation by proteasome pathway (Dievart and Clark, 2004; Wang and He, 2004). BSU1, a nuclear serine /threonine phosphatase, counteracts the action of BIN2 and facilitate the accumulation of BES1 in the nucleus and acts as a positive regulator of BR signaling (Mora-Garcia et al., 2004). The ligand-induced receptor phosphorylation transduces the signal to the cytoplasm and activates specific downstream components. Thus, BRs are perceived by the extracellular domain of BRI1 and initiates a signaling cascade, acting through GSK3 kinase, BIN2 and the

## Plant RLKs involved in signaling

BSU1 phosphatase, which modulates the phosphorylation state of BES1 and BZR1. BR signaling through BES1 and BZR1 regulates specific set of genes and control the growth of plant.

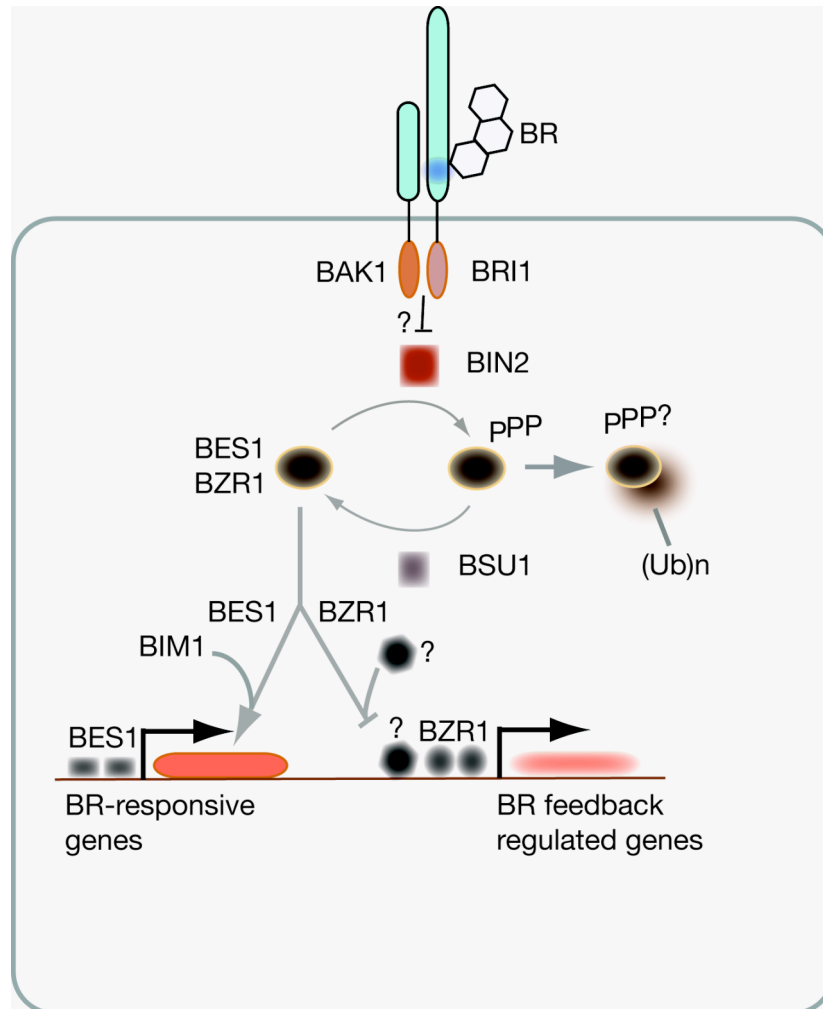


Fig. 1 A model for downstream events in the BR signal transduction pathway. In the absence of BRs, BIN2 kinase is active and phosphorylates the transcription factor BES1 and BZR1, targeting them for ubiquitination (Ub)<sub>n</sub>. In presence of BR, BRI1/BAK1 inhibit BIN2 and activate BSU1 by unknown mechanism, leading to the dephosphorylation of BES1/BZR1. BES1, together with BIM1 activates transcription of BR-responsive genes. BZR1 directly inhibits the transcription of BR-feedback genes and maintain BR homeostasis.

### **1.2.5 CLAVATA1 (CLV1) a receptor-like kinase mediated signaling pathway controls shoot and floral meristem size**

SAM gives rise the aerial part of the plant such as leaves and flowers from a group of stem cells. Organogenesis in flowering plant results by patterned control of cell division in shoot apex. The shoot apex is partitioned into radial domains (Steeves and Sussex, 1989). This organization is defined functionally into three distinct zones in the SAM. The central zone (CZ) is consisting of undifferentiated cells (stem cells). The CZ is surrounded at the flanks of the meristem by the peripheral zone (PZ), where the progeny of the stem cells divide more frequently than those at the center and are incorporated into organ primordia. Below these two zones lies the rib zone (RZ) which gives rise to the internal part of the stem. The meristem has to maintain a tight balance between the proliferation of the stem cells at the CZ and the targeting of these cells towards differentiation at the peripheral zone to achieve shape and size during normal development. The loss-of-function mutation in the three CLAVATA (CLV) genes (CLV1, CLV2 and CLV3) leads to an ectopic accumulation of stem cells in the CZ, and progressive enlargement of the shoot meristem (Dievart and Clark., 2004). Molecular genetic studies have revealed that the primary function of the proteins encoded by the CLV loci is to restrict the expression domain of WUSCHEL (WUS). WUS encodes a homeodomain transcription factor that is required for stem cell specification in the shoot and flower meristems (Laux et al., 1996; Mayer et al., 1998).

WUS is expressed in a small group of cells situated below the CZ of the shoot and flower meristems, called the organizing center (OC) (Mayer et al., 1998). By an unknown mechanism, WUS expression in the OC triggers stem cell specification in the overlying CZ cells. It has been shown that ectopic expression of WUS is sufficient to induce stem cells specification in shoot apices and also CLV3 gene expression in the adjacent cells (Brand et al., 2000; Schoof et al., 2000). Thus, WUS induces CLV3 expression in CZ and CLV3 restricts expression WUS in OC by a feedback loop, which regulates the stem cell population in the shoot meristem

(Schoof et al., 2000). This orchestration of spatial-temporal patterns of gene expression is coordinated by cell-to-cell communication and regulates cell proliferation in SAM. Thus, it maintains normal shape and size of SAM throughout development.

A recent live imaging study revealed the critical role of CLV3 in shoot meristem. Previous genetic analysis has shown that the large inflorescence meristem (IM) size is due to increase number of cells in both CZ and RZ (Clark et al., 1995). The expression domain of CZ (CLV3) is controlled by preventing cell differentiation of PZ, into CZ and restricts overall SAM size through regulation of cell differentiation as well as cell division (Reddy and Meyerowitz, 2005).

### **1.3 STRUBBELIG a receptor-like kinase regulating cell division and cell morphogenesis in *Arabidopsis***

#### **1.3.1 The *strubbelig* phenotype**

The *Arabidopsis* gene *STRUBBELIG* (*SUB*) encodes a putative leucine-rich repeat (LRR) receptor-like kinase, which was first identified based on an ovule phenotype (Schneitz et al., 1997). The *sub* ovules display variable irregularities in outer integument development, ranging from normal, fertile ovules to severely affected, ovules. The aberrant outer integument phenotype becomes visible in late stage 2-III/stage 2-IV of ovule development. At the macroscopic level, aboveground organs of *sub* mutants exhibit additional alterations. Thirty-day old *sub* plants show reduced plant height compare to WT and display twisting of stems. Furthermore, flower organs of stage 13 to –15 flowers of mature *sub* showed twisted petals and carpel. At a cellular level, occasional periclinal divisions in the L2 layer of stage-3 floral meristem were observed. The horizontal stem sections of 30-day old *sub* stem revealed reduced number of epidermal, cortex, and pith cells (Chevalier et al., 2005). In addition an independent study found out that the SCRAMBLED

(SCM)/STRUBBELIG receptor-like kinase is required for cells to interpret their position in the developing root epidermis (Kwak et al., 2005).

### **1.3.2 STRUBBELIG gene structure**

*STRUBBELIG* has been mapped to the upper arm of chromosome one at At1g11130 locus in the *Arabidopsis thaliana* genome. The 16 exon-containing gene spans up to 2.767kb and carries a 5' leader of at least 216 bp and 244 bp of 3' untranslated sequence. *SUB* gene is predicted to encode a 768 aa LRR-RLK of the LRR-V class with a calculated mass of 84.5kD. At the N-terminal end, *STRUBBELIG* protein features a 24 aa hydrophobic region presumably to serve as a signal peptide. The signal peptide is followed by the SUB domain, which is highly conserved between members of the LRR-V class of RLKs. The SUB domain is followed by six leucine-rich repeats and by a proline-rich region in the extracellular domain. The intracellular part of the protein consists of juxtra-membrane region and the C-terminal cytoplasmic kinase domain (Chevalier et al., 2005; Kwak et al., 2005).

### **1.3.3 STRUBBELIG may represent a non-functional kinase domain**

Sequence alignment of the WT SUB with functional receptor-like kinases revealed two notable alterations within the catalytic loop of the kinase domain. SUB carries an asparagine at a position (N-625), where functional protein kinase usually contains an aspartate (Hanks and Quinn, 1991). Additionally, SUB features a lysine at position 630 while the functional kinases carry an asparagine at this position (Johnson et al., 1996). Furthermore, in a genetic test, SUB<sub>K525E</sub> the conserved lysine to glutamic acid exchange in kinase domain II was able to rescue the *sub* mutant phenotype (Chevalier et al., 2005). In general, mutating this position leads a loss of kinase activity (Hanks et al., 1988; Carrera et al., 1993). The bacterially expressed SUB protein showed no detectable kinase activity *in-vitro*. The genetic

and biochemical data together suggest that SUB may represent an atypical kinase (Chevalier et al., 2005).

### **1.3.4 SUB function**

*SUB* is a member of LRR-V gene family and predicted to encode a transmembrane receptor-like kinase. Receptor-like kinases are involved in transduction of signal through the plasma membrane. Several recessive mutations in the *STRUBBELIG* locus cause similar mutant phenotype (in “*Ler*” background), indicating that they alter *SUB* functions to the same degree. In flowers, *SUB* signaling is required for formation of the outer integument in the ovule, the correct shape of the carpel and petals while in the stem it required for shape and height of plant stem. Though, the broad and complex distribution of *SUB* mRNA expression pattern is seems to correlate with pleiotropic *sub* mutant phenotype. *SUB* is perhaps required in some tissues for the regulation of cell proliferation and cell shape and orientation of the mitotic division plane. For example, the ovules of *sub* show perturbed shape and size of outer integument cells due to misoriented mitotic division planes. In addition, the stem of *sub* shows reduced number of epidermis, cortex, and pith cells compared to WT (Chevalier et al., 2005). *SUB/SCM* has also been implicated in position-dependent specification of root epidermal cells in the post-embryonic root of *Arabidopsis* (Kwak et al., 2005).

What could be the cellular basis of *SUB* function? Twisting of gynoecium's, petals, and stem indicates a possible role of *SUB* signaling in cell morphogenesis. Cell morphogenesis is a complex developmental process, which is guided by external as well as internal cues. Several morphogenetic mechanisms are involved in defining the cell shape and size during development, such as microtubule orientation, cellulose deposition in cell wall and cell expansion.



### 1.4 Role of intercellular communication in plant development

Formation of multicellular organisms from a single cell requires long-range as well as short-range intercellular communication to coordinate cell division and cell growth during development. Intercellular communication delivers critical information for position-dependent specification of cell fate. In plants, cell-to-cell communication involves the intercellular trafficking of regulatory proteins and mRNAs through the plasmodesmata (PD) and allows non-cell autonomous regulation of plant development (Lucas et al., 1995; Perbal et al., 1996; Kim et al., 2001; Nakajima et al., 2001; Wada et al., 2002; Wu et al., 2003). PD can be considered as two concentric cylinders, which connect the plasma membrane, cytoplasm, and endoplasmic reticulum (ER) of neighboring cells. The plasma membrane lines the outer cylinders of the PD, whereas the ER forms an internal cylinders (desmotubule) and links the ER in two adjacent cells. Thus, the PDs provide multiple routes for intercellular communication, one through cytoplasmic space between the desmotubule and the plasma membrane, another via the plasma membrane and also, using the ER itself (Blackman and Overall, 2001; Roberts and Oparka, 2003). Nonselective cell-to-cell movement of macromolecules through the PD can be achieved by simple diffusion. Targeted trafficking of macromolecules, however, requires the interaction of proteins with PD or associated proteins to increase the size exclusion limit (SEL) of PD and allow their own movement.

#### 1.4.1 Non-selective movement of GFP and LEAFY

What mediates protein movement between cells and how this movement of molecules is regulated? This is an open question that remains in the research field of cell-to-cell communication. In general, all plant cells are interconnected to their neighbors by PDs. PDs are classified as primary, if formed during cytokinesis, or secondary, when synthesized through an existing cell wall. The secondary PDs are important for connecting cells that do not share a recent division wall. The size of

## Role of intercellular communication

PD aperture fluctuates from closed to open to dilate during different stages of post-embryonic development. Examples of simple diffusion (non-targeted) are illustrated with cytoplasmically localized GFP (Crawford and Zambryski, 2001) and the transcription factor, LEAFY (LFY) (Wu et al., 2003). In transgenic *Arabidopsis*, 27-kDa soluble green fluorescent protein (1×sGFP) moves between cells throughout the entire embryos while 2×sGFP movement becomes more restricted as development proceed. This indicates that PD in younger tissues are more dilated and less obstructive than PD in older tissues (Kim et al., 2005a). Moreover cells in seedling shoot apical meristems (SAM) have a higher size exclusion limit (SEL) and allow 2×sGFP movements. The cells surrounding the SAM differentiate into specific cell types show more restricted movement of 2×sGFP than 1×sGFP. This indicates that the PD aperture is regulated temporally, spatially, and physiologically throughout development (Kim et al., 2005a). Thus, proteins that are small in size move between cells without increasing the SEL by simple diffusion.

GFP movement is restricted in *Nicotiana* leaf when a nuclear localization signal was added to GFP. It reduces the extent of protein movement. Addition of a nuclear localization signal to 2×sGFP, almost blocked the protein movement (Gallagher and Benfey, 2005). LFY is a plant-specific transcription factor required for SAM transition from vegetative to reproductive development. Normally, LFY mRNA and protein both are expressed in all three layers of floral primordia (L1, L2, and L3). But when the *LFY* is expressed in just L1 layer (epidermis), the protein is able to travel into the L2 and L3 layers and rescue the *lfy* phenotype (Wu et al., 2003). Moreover the movement of LFY (47kDa) is limited in the shoot apical meristem of *Arabidopsis* similar to 2×sGFP. Deletion studies spanning to LFY, N-terminus, C-terminus or in between N and C-terminus domains, indicates that the cytoplasmic localization of truncated protein was sufficient for protein movement. There is no specific domain required for LFY movement, thus, LFY cell-to-cell movement suggests that it is non-targeted.

### 1.4.2 Selective movement of transcription factors

The targeted trafficking of endogenous transcription factors plays an important role in cell fate specification. SHORTROOT (SHR) is a member of GRAS family of putative transcription factors that is required for radial patterning of *Arabidopsis* root (Helariutta et al., 2000). SHR mRNA is expressed in the stele cells of the *Arabidopsis* root. Protein localization studies both using anti-SHR antibodies and SHR:GFP fusion protein indicate that the presence of SHR in both nucleus and cytoplasm in these cells. Further, studies have shown that SHR moves from stele to the neighboring endodermis and localizes in the nucleus (Nakajima et al., 2001). When the SHR:GFP fusion protein was expressed under the control of tissue-specific promoters (phloem companion cells and epidermal cell), it was unable to move, suggesting tissue-specificity of SHR movement (Sena et al., 2004). Moreover, addition of a nuclear localization signal to SHR inhibits the movement of protein to the adjacent endodermis cells. When SHR was expressed in root epidermis in a *scarecrow* (*scr*) mutant background the SHR was unable to localize to the nucleus, but was able to traverse to adjacent cell layers indicating that the cytoplasmic localization of SHR is important for intercellular movement (Gallagher et al., 2004; Sena et al., 2004). Though, a novel missense allele of *shr*, *shr-5*, shows cytoplasmic localization of the protein, it was unable to move to endodermis cells, suggesting that the cytoplasmic localization is required but not sufficient for movement (Gallagher et al., 2004).

KNOTTED1 (KN1), a homeodomain protein of maize, was first found to traffic from cell-to-cell (Lucas et al., 1995, Kim et al., 2002). The *Arabidopsis* orthologs of KN1, KNOTTED 1-like homeobox protein 1/BREVIPEDICELLUS (KNAT1/BP) and SHOOTMERISTEMLESS (STM), can traffic from cell-to-cell. STM and KN1 are involved in shoot apical meristem (SAM) initiation and maintenance and KNAT1/BP is required for regulation of inflorescence architecture. GFP:KN1 fusion protein expressed in the leaf perivascular bundles was able to traffic through the mesophyll to epidermis. When GFP:KN1 was expressed in leaf epidermis, it was unable to

move to mesophyll cells, indicating a directional regulation of protein trafficking (Kim et al., 2003). However, GFP:KN1 showed bidirectional protein trafficking in SAM. Deletion studies of KN1 revealed a homeodomain in the protein, which is required for cell-to-cell trafficking of KN1 protein in planta (Kim et al., 2005b). This observation suggests a specific developmental regulation of GFP:KN1 trafficking in plants.

### **1.4.3 Role of cytoskeleton in protein movement**

During cell-to-cell protein trafficking, how does the protein reach PD in the first place? It is possible that the non-selective protein reaches PD by random diffusion through the cytoplasm, whereas selective protein trafficking may require transport along the cytoskeleton. Recent studies suggest a role of endomembrane system in protein trafficking in plant cells. The endomembrane system includes the ER, Golgi bodies, vacuole, and vesicles. The endomembrane system plays an important role during cell division; it delivers protein to the cell surface after cytokinesis. Moreover, recent work has shown the role of endomembrane in differential subcellular localization of proteins in plant development. The endomembrane system localizes proteins in various subdomains of plasma membrane. For example, the apical localization of auxin influx carrier AUX1, and basal localization of the efflux carrier PIN1, and lateral localization of COBRA all within the same cell is achieved by endomembrane system (Jurgens and Geldner, 2002).

## **1.5 In this study**

Plant receptor-like kinases are involved in several distinct processes, such as disease resistance, hormonal signaling, and regulation of cell division and differentiation in shoot apical meristem. SUB, an LRR-RLK mediated signaling pathway regulates cell proliferation, cell size, and cell shape during plant development. To thoroughly characterize SUB function, it is essential to determine

the SUB protein expression pattern, cellular and subcellular protein localization within the cell.

In this study, I used an enhanced green fluorescent fusion (EGFP) to show the localization of SUB to the plasma membrane and determined its pattern of expression in planta. In addition, we used FM4-64 with brefeldin-A to understand subcellular behavior of SUB:EGFP fusion protein in plants, chapter 3. To understand more clearly the non-cell autonomous SUB function, we expressed SUB:GFP under various promoters and analyzed rescue of *sub* phenotype, chapter 4. We also examined the non-cell autonomous rescue of SUB function, using the *Cre/lox-P* system, chapter 5. Finally, in chapter 6, I draw some conclusion, how mRNA vs. protein expression is established during development and the role of such tissue distribution in SUB non-cell autonomous signaling.

## Chapter 2 Materials and Methods

### 2.1 Plant work

#### 2.1.1 Plant growth media

*Arabidopsis thaliana* seed were germinated on sterile minimal media (MM) containing 1× micro salts (45mM Na<sub>2</sub>EDTA, 40mM FeSO<sub>4</sub>, 25mM MnSO<sub>4</sub>, 15mM H<sub>3</sub>BO<sub>4</sub>, 2.5mM ZnSO<sub>4</sub>, 1.8mM KI, 400μM Na<sub>2</sub>MoO<sub>4</sub>, 40μM CoCl<sub>2</sub>, 40μM CuSO<sub>4</sub>), 1× vitamins (40g/l myo-inositol, 4g/l thiamine, 0.4g/l nicotinic acid, 0.4 g/l pyridoxine), 1× macro salts (185mM KNO<sub>3</sub>, 80mM NH<sub>4</sub>NO<sub>3</sub>, 30mM CaCl<sub>2</sub>, 15mM MgSO<sub>4</sub>, 12.5mM KH<sub>2</sub>PO<sub>4</sub>), 1% (w/v) sucrose, 0.1% (w/v) MES. All components were mixed well and the solution adjusted to pH 5.8 with KOH. At the end 0.9% (w/v) agar (Sigma A 1296) was added and batches autoclaved. Kanamycin or glufosinate ammonium (Riedel-deHaen 45520) was added after media was cooled to 40-50°C.

#### 2.1.2 Plant growth

*Arabidopsis thaliana* seed were treated for 4-day at 4°C to induce germination. Seedlings were grown in growth chambers at 23°C under 24 hour light. For growth of adult plants, 7-day seedlings were transferred to soil/compost medium (Terrea Professional GEPAC Einheitserde-Typ T) (Einheitserde Werkverband) in pots. Plants were grown in green house conditions (25°C, 20% relative humidity and 16 hour light under Philips SON-T Plus 400W fluorescent bulbs).

#### 2.1.3 *Arabidopsis thaliana* transformation

*Agrobacterium tumefaciens* strain, GV3101/pMP90 (Koncz and Schell, 1986) was used for all plant transformation experiments. The plasmid pMP90 encodes the selectable marker for gentamycin resistance (50μg/ml). The plant binary vector pCAMBIA2300 was used for *SUB:GFP*, *SUB:EGFP* and *SUB:3×myc* derivative

plasmids. For the *lox-P* vector, pGreen0229 plasmid was used as backbone. The pGreen0229 was co-transformed with pSoup plasmid in *A. tumefaciens*. The plasmid pEZT-NT was transformed into the *A. tumefaciens* strain, ASE.

*Arabidopsis thaliana* (L.) Heynh var. Landsberg (*erecta* mutant) (*Ler*) was used as WT strain. The *strubbelig* (*sub-1*) mutant was isolated in an ethyl methane sulfonate (EMS) mutagenesis in the *Ler* background (Schneitz et al., 1997). To generate promoter specific transgenic lines, respective plasmid constructs were transformed using floral dip method (Clough and Bent, 1998) into mutant and wild type background.

### 2.1.4 Seed sterilization

Seed were surface sterilized by washing for 1 minute in 70% (v/v) ethanol containing 0.05% (v/v) Triton X-100, followed by sterilizing reagent (3% (v/v) NaOCl, 0.05% (v/v) Triton X-100) for 5 minutes, to prevent bacterial and fungal growth on plates. Seed were washed twice with sterilized H<sub>2</sub>O and transferred to the selection plates under sterile conditions. To select T1 lines, approximately 0.2 gm seed were sterilized and spread over a big petri-dish (ø145mm). NaOCl (Roth 9062.1), Triton-X 100 (Sigma T 8787) on the appropriate selective media.

## 2.2 Preparation of electro-competent cells

Bacterial electro-competent cells were prepared following a protocol adopted from the Bio-Rad Gene Pulser Xcell Instruction Manual (# 165 2660). Preparation of *E.coli* and *A. tumefaciens* cells differ only in incubation temperatures of 37°C and 30°C, respectively.

### 2.3 Construction of plasmids

#### 2.3.1 Cloning of *SUB:3×myc* in pCAMBIA2300

A strategy was designed to generate a myc-tagged *SUB* construct. For the *SUB:3×myc* construct, *SUB* was amplified from H2H6T7 plasmid using following primer pair, SUB-Cmyc-F (5'-CCT AGG AGG GCG GCC ATG AGC TTT ACA AGA TGG GAA GTG TTC-3') and SUB-Cmyc-R (5'-GAG ACC GAC GTC AGG GCC CCG ATC ATA TGT TGA AGA TCT TGG-3'). In next step, myc tag was amplified from pFASTBAC-HTa-Cmyc plasmid, using the primers cmyc-F (5'-GGG GCC CTG ACG TCG GTC TCG AGA ATG GAG CAA AAG CTC A-3') and cmyc-R (5'-TTA TTC ATT CAA GTC CTC TTC AGA AAT GAG CTT TTG CTC C-3'). Both fragments were gel purified and an overlap PCR was set up. The primers, SUB-Cmyc-F and cmyc-R were used to amplify the overlap product. The overlap PCR product was cloned into the PCRII TOPO vector (Invitrogen, USA) and was designated PCRII TOPO *SUB:3×myc*. To subclone *SUB:3×myc* into pCAMBIA2300, the pCAMBIA2300 vector was *Bam*HI/*Pm*II digested to remove the 35S::*GUS* (<http://www.cambia.org>). From the PCRII TOPO *SUB:3×myc* plasmid the *SUB:3×myc* insert was released by *Bam*HI/*Bsa*BI and subcloned into the *Bam*HI/*Pm*II digested pCAMBIA2300 vector. In the new vector *SUB* can be released by *Asc*I/*Aat*II digestion and another gene can be inserted in frame with the C-terminal 3×myc tag.

#### 2.3.2 Promoter::*SUB:3×myc* construct

*SUB:3×myc* pCAMBIA2300 was used as a backbone vector for generation of various promoter::*SUB:3×myc* constructs.



### 2.3.2.1 ***ANT::SUB:3×myc* construct**

A 6500 bp fragment (upstream of ATG) of *ANT* promoter was amplified from the MT-76 plasmid (Schoof et al., 2000) using following primers, 5' ANT-For (5'-GCA GAA TGA AAA TAA AAG AAA ATT GGA TGG CT-3') and 5'ANTAscl-Rev (5'-TGG CGC GCC GTT TCT TTT TTT GGT TTC TGC TT-3'). First, *SUB:3×myc* pCAMBIA2300 was digested with the *SpeI* enzyme and then treated with T4 DNA polymerase to make the 5' end blunt. After phenol/chloroform purification, vector was digested with *Ascl*. The amplified promoter was digested with *Ascl* and ligated with the blunt/*Ascl*-digested vector. The vector was called *ANT::SUB:3×myc* pCAMBIA2300.

### 2.3.2.2 ***35S::SUB:3×myc* pCAMBIA2300**

pART-7 vector (Gleave, 1992) DNA was digested with *NotI* and made blunt by T4 DNA polymerase. *NotI* digestion released the *35S::NOS* cassette. To obtain the *35S* promoter fragment, gel purified *35S::NOS* cassette was digested with *XbaI*. The excised *35S* promoter insert had a 5' blunt and 3' sticky end. The vector *SUB:3×myc* pCAMBIA2300 was made blunt after *SpeI* digestion at the 5' end and re-digested with *BlnI*, to make a compatible 3' end for *XbaI* ligation with *35S* promoter insert. The resulting clone was referred *35S::SUB:3×myc* pCAMBIA2300.

### 2.3.2.3 ***AtML1::SUB:3×myc* pCAMBIA2300**

The *AtML1* promoter, comprising a 3373 bp genomic fragment upstream of the *AtML1* initiation codon, was amplified by PCR from pAS99 (Sessions et al., 1999) using the primer BamHIL1-for (5'-GCG GAT CCA GCT TAT CAA AGA AAA AAC AAG AA-3') and AsclL1.Rev (5'- GCG GCG CGC CTT CAG GGA GTT TCT TTA ACC AG-3'). The amplified PCR fragment was digested with *BamHI/Ascl* and

cloned into *Bam*HI/*As*cl digested *SUB:3xmyc* pCAMBIA2300 vector. The resulting plasmid was designated as *AtML1::SUB:3xmyc* pCAMBIA2300.

### 2.3.2.4 *WUS::SUB:3xmyc* pCAMBIA2300

A PCR fragment of 5600 bp, including the promoter region of the *WUSCHEL* gene (Mayer et al., 1998) and ending at the ATG, was amplified using the following PCR primer pair 5' *WUS Bam*HI-for (5'-CGC GGA TCC GAT ACT AAT TCT CAT GAA TTC ACT TC-3') and 5'*WUSAs*cl-Rev (5'-ATG GCG CGC CGT GTG TTT GAT TCG ACT TTT GTT CA-3') from plasmid MT61 (Gross-Hardt et al., 2002). The amplified fragment was digested by *Bam*HI/*As*cl and cloned into *Bam*HI/*As*cl digested PCRII TOPO *SUB:3xmyc* vector. The resulting clone was referred to as clone A.

To clone the 3' *WUS* downstream region, first, a 250 bp fragment of *SUB:3xmyc* was amplified from PCRII TOPO *SUB:3xmyc* plasmid using following primers, *Pst*I*SUB*-For (5'-CGT TCA CTG CAG ATG GAA CCA GGA TTT AGA CCG CCG AT-3') and *Stu*I*SUB*-Rev (5'-CGG CGT AAG AGC TGA GAA TAG GCC TTA CAG CTT TTA TT-3'). In the next step, a 1200 bp fragment of the *WUS* downstream genomic region directly following the stop codon, was amplified from plasmid MT61 using the primers, *Stu*I3'*WUS*.For (5'-GTA AGG CCT ATT CTC AGC TCT TAC GCC GGT GTC GCT CG-3') and *Pm*II3'*WUS*.Rev (5'-GCC ACG TGT AAT CTT AAT TTT ATT GAT AAA TGT TAT TT-3'). Both fragments were gel purified and overlap PCR was set up. The overlapped product was cloned into the PCRII TOPO vector. From the PCRII TOPO the 250bp *SUB:3xmyc* plus 1.2kb *WUS* fragment insert was excised by *Pst*I/*Pm*II and subcloned into *Pst*I/*Pm*II digested pCAMBIA2300. The construct was designated vector B.

To bring the *WUS* upstream genomic fragment and *WUS* downstream fragment together. Clone A was digested with *Bam*HI/*Pst*I and insert was subcloned into

*Bam*HI/*Pst*I digested vector B. The new vector was called 5'*WUS::SUB:3×myc* *WUS*3'pCAMBIA2300.

### 2.3.2.5 *pSUB::SUB:3×myc* pCAMBIA2300

A 1278 bp fragment of *SUB* promoter region (upstream of the *SUB* initiation codon) was amplified from WT-*Ler* genomic DNA using following PCR primers, *Bam*HISUBpromoter.for (5'-GCG GAT CCG CAA ATA ATT TAT GTG AAT ATC-3') and *Asc*ISUBpromoter.rev (5'-ATG GCG CGC CAA CTT CAG CCA CTG AAG ATG-3'). The amplified product was digested with *Bam*HI/*Asc*I and cloned into *Bam*HI/*Asc*I digested *SUB:3×myc* pCAMBIA2300, resulting in the plasmid designated *pSUB*(1 kb)::*SUB:3×myc* pCAMBIA2300.

To generate a full length *SUB* promoter, a 3543 bp *SUB* promoter region ending directly prior to ATG was amplified and cloned into PCRII TOPO vector and designated plasmid PCRII TOPO -3543/-1. Vector PCRII TOPO -3543/-1 was digested with *Kpn*I/*Bsr*GI and insert was subcloned into *Kpn*I/*Bsr*GI digested *pSUB*(1kb)::*SUB:3×myc* pCAMBIA2300. The new vector was designated *pSUB*(3.5kb)::*SUB:3×myc* pCAMBIA2300. From here, one can subclone the *pSUB* 3.5kb by *Kpn*I and *Asc*I digestion.

### 2.3.2.6 Construction of alanine-linker *GFP* fusion

The *mGFP6* gene sequence was amplified from pMDC83 (Curtis and Grossniklaus, 2003) by PCR using following primer pairs, *GFP*AatII (5'-CTG ACG TCG ATG AGT AAA GGA GAA GAA CTT-3') and *GFP*-Rev (5'-TAC ACG TGT TAT TTG TAT AGT TCA TCC ATG-3') and cloned into PCRII TOPO. To generate an alanine linker at the N terminus of *GFP*, GALTSAAAAAATA amino acid encoding primer sequence (*GFP**Asc*I-F 5'-ATG GCG CGC CAG ACG TCG GCC GCC GCC GCC GCC GCC GCC GCC-3') and (*GFP**Xba*I-R 5'-ATT CTA GAT TAT TTG TAT AGT TCA TCC ATG-

3') were used to amplify GFP from plasmid GFP PCRII TOPO. The amplified PCR product was digested with *Ascl/EcoRV* and cloned in *Ascl/EcoRV* site of PCRII TOPO *SUB:3×myc*, replacing *SUB:3×myc* with linker-GFP. The new clone was referred to as TOPO-*link:GFP*. *SUB* insert was excised by *Ascl/AatII* from plasmid PCRII TOPO *SUB:3×myc* and subcloned into *Ascl/AatII* digested TOPO-*link:GFP*, the new clone designated as TOPO *SUB-link:GFP*. We used the *GFP* variant, *mGFP6*, to enhance the fluorescence and solubility of the fusion protein (Heim et al., 1995; Heim and Tsien, 1996)

### 2.3.2.7 ***SUB-link:GFP* pCAMBIA2300**

To clone the *SUB-link:GFP* into the binary vector pCAMBIA2300, the plasmid TOPO *SUB-link:GFP* was digested with *XhoI* and made blunt with T4 DNA polymerase. To release the insert, vector was subjected to *BamHI* digestion after purification. The vector pCAMBIA2300 was digested with *BamHI/PmlI* and a ligation was set up. The new plasmid was designated *SUB-link:GFP* pCAMBIA2300.

Plasmid *SUB-link:GFP* pCAMBIA2300 was used as a backbone to make various *promoter::SUB-link:GFP* constructs.

### 2.3.2.8 ***pSUB(3.5 kb)::SUB-link:GFP* pCAMBIA2300.**

The full-length *pSUB* (3543 bp) promoter insert was excised from *pSUB(3.5 kb)::SUB:3×myc* pCAMBIA2300 by *KpnI/Ascl* and subcloned into *KpnI/Ascl* digested *SUB-link:GFP* pCAMBIA2300. The new vector was called *pSUB(3.5 kb)::SUB-link:GFP* pCAMBIA2300.

### 2.3.2.9 ***ANT::SUB-link:GFP* pCAMBIA2300**

*SUB-link:GFP* pCAMBIA2300 vector was digested with *KpnI/Ascl*. The *ANT* promoter insert was excised from plasmid *ANT::SUB:3xmyc* pCAMBIA2300 with the same enzymes. Following ligation the new vector was designated *ANT::SUB-link:GFP* pCAMBIA2300.

### 2.3.2.10 ***AtML1::SUB-link:GFP* pCAMBIA2300**

To design an *AtML1::SUB-link:GFP* vector, the *AtML1* promoter insert was released by *BamHI/Ascl* digestion from *AtML1::SUB:3xmyc* pCAMBIA2300 vector *SUB-link:GFP* pCAMBIA2300 was prepared by the same digestion. Ligation was setup between the insert and vector and the new plasmid was assigned the name *AtML1::SUB-link:GFP* pCAMBIA2300.

### 2.3.2.11 ***5'WUS::SUB-link:GFP WUS3'*pCAMBIA2300.**

*SUB:3xmyc* was removed by *Ascl/StuI* digestion from *5'WUS::SUB:3xmyc* *WUS3'*pCAMBIA2300 vector. The insert *SUB-link:GFP* was obtained from TOPO *SUB-link:GFP* vector by *XbaI* digestion and made blunt by T4 DNA polymerase treatment. Next, to excise the *SUB-link:GFP* insert, it was digested with *Ascl*, gel purified and a ligation was setup between insert and vector. The new plasmid was designated *5'WUS::SUB-link:GFP WUS3'*pCAMBIA2300.

### 2.3.2.12 ***35S::SUB:EGFP* pEZT-NT construct**

To generate an in frame *EGFP* tag at the C-terminus of *SUB*, *EGFP* was amplified using the following PCR primer pairs, *EGFP-AatII-F* (5'-GCG GGC CCG GGA CGT CGG CTG CTG CCG CTG CCG CTG-3') and *EGFP-XbaI-R* (5'-GTC TAG ACT CCG GAT TAC TTG TAC AGC TCG TCC AT -3') using plasmid pEZT-NL (Gift from David

Ehrhardt) as a template. The vector pEZT-NL carries the *EGFP* tag with a 5' alanine linker, therefore the 5' primer was designed to target the linker directly. The *EGFP* PCR product was digested (*AatII/XbaI*) and cloned into identical sites in the vector PCRII TOPO *SUB:3xmyc*. The new vector was called *SUB:EGFP* PCRII TOPO. The plasmid was sequenced and used for *SUB:EGFP* translational fusion insert. The *SUB:EGFP* insert was prepared by digesting *SUB:EGFP* PCRII TOPO with *BlnI*, blunt ending by T4 DNA polymerase treatment, purified and subjected to *XbaI* digestion. The vector pEZT-NT was digested with *XhoI*, blunt ended by T4 DNA polymerase, gel purified and digested with *XbaI*. Following ligation the new plasmid was called *35S::SUB:EGFP* pEZT-NT.

### 2.3.2.13 *pSUB(3.5 kb)::SUB:EGFP 3'UTR SUB* pCAMBIA2300

A 354 bp fragment (downstream of *SUB* stop codon) was amplified from WT-Ler genomic DNA using following primer pairs, 3'UTRSUB-FOR (5'-GTT CTA GAG ATA CAC AAC CTT GGA CTA AGA-3') and 3'region354Sacl-R (5'-CGA GCT CTT TGA AGT TTA GGT TTT G-3'). The amplified PCR product was digested with *XbaI* and cloned into *XbaI/PmlI* digested pCAMBIA2300, resulting in the plasmid designated *3'UTR SUB* pCAMBIA2300. In the next step, the *SUB:EGFP* insert was excised using *BamHI/XbaI* digestion from a second plasmid *SUB:EGFP* PCRII TOPO. *3'UTR SUB* pCAMBIA2300 plasmid, prepared using the same enzymes, ligation was setup and new clone rescued. The new plasmid was called *SUB:EGFP 3'UTR-SUB* pCAMBIA2300. A 3543 bp *SUB* promoter fragment was excised by *KpnI/AscI* from *pSUB(3.5 kb)::SUB-link:GFP* pCAMBIA2300 and subcloned into *KpnI/AscI SUB:EGFP 3'UTR SUB* pCAMBIA2300. The new vector was designated *pSUB(3.5 kb) SUB:EGFP 3'UTR SUB* pCAMBIA2300.

### 2.3.2.14 *pCol::SUB:EGFP 3'UTR SUB* pCAMBIA2300

A 3543 bp fragment *pCol* (*SUB* promoter from Columbia ecotype), was amplified by PCR using Wt-*Col* genomic DNA as a template. The following primers were

used to amplify the promoter, *pSUB* (-3543F) *Xba*I (5'-AAA TCT AGA CGA CTC GTT TGA GAA CCC TC-3') and *Asc*I/*SUB* promoter (5'-ATG GCG CGC CAA CTT CAG CCA CTG AAG ATG-3'), which was then cloned into PCR II TOPO vector. Subsequently the *pCol* promoter was excised using *Kpn*I/*Asc*I digestion, purified and inserted in the vector *SUB:EGFP 3'UTR SUB* pCAMBIA2300. The new vector was called as *pCol::SUB:EGFP 3'UTR SUB* pCAMBIA2300.

### 2.3.2.15 *lox-P* target site vector construction

The *lox-P* was designed to have direct repeats of the *lox-P* sites to exploit the deletion activity of Cre recombinase (Sternberg and Hamilton, 1981). Primer pairs containing these repeats with accompanying restriction sites, *Lox-F* (5'-TGG TAC CAT AAC TTC GTA TAG CAT ACA TTA TAC GAA GTT ATG ATA TCC TG-3'), *LoxP-R1* (5'-ATG CTA TAC GAA GTT ATG CAT GCG GCC GCT GCA GGA TAT CAT AAC-3') and *LoxP-R2* (5'-TGA GCT CAT AAC TTC GTA TAA TGT ATG CTA TAC GAA GTT ATG CAT-3'), were synthesized and an overlap PCR was performed. The resulting 105 bp fragment (TGG TAC CAT AAC TTC GTA TAG CAT ACA TTA TAC GAA GTT ATG ATA TCC TGC AGC GGC CGC ATG CAT AAC TTC GTA TAG CAT ACA TTA TAC GAA GTT ATG AGC TCA) was cloned into pGEM T-easy. The *lox-P* insert was sequenced and excised with *Kpn*I/*Sac*I and subcloned into *Kpn*I/*Sac*I digested binary vector pGreen0229 (Hellens et al., 2000). The new plasmid was designated *lox-P* pGreen. A *35S::GUS:NOS* cassette derived from pJIT-61 was inserted into the *EcoRV* site of *lox-P* pGreen and new clone was referred as *lox-P* pGreen *35S::GUS*. This clone contains a unique *Not*I site adjacent to *35S::GUS* for the insertion of any gene of interest. We inserted *35S::SUB* from *35S::SUB* pART-7 (Chevalier et al., 2005) into *Not*I and final clone was designated *lox-P* pGreen *35S::SUB 35S::GUS*.

### 2.4 Molecular biology techniques

All standard molecular biology techniques, unless otherwise stated in the text, were performed according to Sambrook et al., 1989.

#### 2.4.1 Restriction endonuclease

All restriction enzymes were purchased from either Roche (Mannheim, Germany) or New England Biolabs (NEB, USA) and used according to manufacturer's instructions.

#### 2.4.2 T4 DNA ligase and T4 DNA polymerase

All DNA ligase reactions used T4 DNA ligase (NEB, USA) and was used according to manufacturer's instructions. To make sticky ends blunt, T4 DNA polymerase (NEB, USA) was used and manufacturer's instructions were followed.

#### 2.4.3 Nucleic acid purification

##### 2.4.3.1 Bacterial plasmid DNA isolation

For standard plasmid DNA isolation an alkaline lysis method (Sambrook et al., 1989) was followed. For sequence analysis, high purity DNA was obtained using the QIAprep Spin miniprep kit (QIAGEN, Germany).

##### 2.4.3.2 RNA extraction

For total RNA isolation, either RNeasy Plant mini kit (QIAGEN, Germany) or Concert Plant RNA reagent (Invitrogen, USA) was used, according to manufacturer's instructions.



### 2.4.3.3 DNA extraction

For genomic DNA isolation, a modified CTAB buffer method was used (Murray and Thompson, 1980). First, the plant tissue was collected in aluminum foil and immediately frozen in liquid nitrogen. Frozen tissue was grinded in liquid nitrogen and mixed with 1 ml CTAB buffer (2% (w/v) CTAB, 1.42M NaCl, 20mM EDTA, 100mM Tris, pH 8.0) and after adding 6.25 $\mu$ l  $\beta$ -mercaptoethanol, incubated at 65°C for 20 minutes. Chloroform (600 $\mu$ l) was added and the mixture was put on shaker for 5 minutes. To precipitate the protein, the mixture was centrifuged at 14,000 rpm for 10 minutes. The supernatant was transferred into a fresh tube and treated with 2 $\mu$ l RNase A (10mg/ml) for 30 minutes at 37°C. A second chloroform extraction was followed by isopropanol precipitation. Finally the pellet was washed with 70% ethanol and DNA was resuspended in 50 $\mu$ l of dH<sub>2</sub>O (deionized H<sub>2</sub>O).

### 2.4.3.4 Purification of DNA fragment from agarose gel

DNA fragments were excised from gels and purified using QIAquick gel extraction kit (QIAGEN, Germany). All the steps of purification were followed according to manufacturer's instructions.

## 2.5 Polymerase chain reaction (PCR) methods

### 2.5.1 Oligonucleotides

For PCR amplification, genotyping and sequencing reactions, oligonucleotides were custom designed using the computer program, Vector NTI suite 7.1 (Invitrogen, USA). All the primers used in this study are mentioned in the text.

### 2.5.2 PCR

High fidelity PCR was performed using *pfu* ultra and *pfu* turbo (Stratagene, USA) to avoid mutation during PCR based subcloning. For routine work such as genotyping and colony PCR, home made *Taq* DNA polymerase was used.

### 2.6 GUS histochemistry

For GUS staining all the tissues were collected in 90% acetone and processed according to Gross-Hardt et al., 2002.

### 2.7 Microscopy

#### 2.7.1 Scanning electron micrograph (SEM)

For scanning electron microscopy (SEM) analysis, carpel were obtained from freshly open flower buds and dissected before suspending in fixative (2% glutaraldehyde (SIGMA G5882), 69% acetone, 29% H<sub>2</sub>O) overnight. Fixed ovules were washed with 70% acetone (4×15 minute, followed by 6×30 minute). During fixation II, ovules were washed for 15 minute in 50% acetone in 50mM cacodylate buffer pH 7.0, followed by 10 minute in 25% acetone in 50mM cacodylate buffer, 10 minute in 10% acetone/cacodylate buffer, and finally, washed with 50mM cacodylate for 5 minute. Washed ovules were then treated with 2% osmium-tetroxide in 50mM cacodylate buffer for 2 hours. Osmium tetroxide was removed by washing 2 times with 50mM cacodylate buffer, and then followed with a 10-minute wash with 10% acetone/cacodylate buffer. In the end the ovules were passed through an acetone series (10%, 20%, 40%, 60% and 70%) for 30 minute each and stored at 4°C. Fixed ovules were passed through a minimum of three 100% acetone washes before critical point drying. Specimens were mounted on stubs and dissected using fine tip needle. The tissues were coated with gold

particles and examined with the JEOL JSM- 5900LV scanning electron microscope (JAPAN).

### **2.7.2 Confocal laser scanning microscopy (CLSM) (fixed tissue samples)**

To examine stage-3 flowers via confocal laser scanning microscopy (CLSM), 30-day old inflorescences meristems were fixed in FAA (10% formaldehyde, 5% propionic acid and 70% ethanol) overnight. To remove the chlorophyll, the tissues were passed through an ethanol series (70%, 85%, 95%), each for 30 minute, followed by 3 times 100% ethanol, the last exchange kept for overnight. To stain the tissues with propidium iodide, the tissues were passed through a decreasing ethanol series (95%, 85%, 70%, 60% 50%, 30%, 15%) for 30 minute each, followed with two washes in dH<sub>2</sub>O. A stock solution of propidium iodide was made (100µg/ml) in 0.1M L-arginine and pH adjusted to 12.8 by 5M NaOH. Inflorescence apices were stained in 5µg/ml 0.1M L-arginine (pH 12.8) for 4 day at 4°C. Stained inflorescences were then rinsed with 0.1M L-arginine buffer (pH 8.0) once a day for 4 days at 4°C. After rinsing, the samples were passed through an ethanol series (15%, 30%, 50%, 60%, 70%, 85%, 95%, each for 30 minute, followed with two times 100% ethanol) and followed with a ethanol:histoclear series (75%:25%, 50%:50%, 25%:75%, two times with 100% histoclear), each for 2 hour. The inflorescences were dissected into immersion oil and visualized under the CLSM FV1000 FLUOVIEW IX81 (Olympus, Japan).

### **2.7.3 Light microscopy analysis**

For stem cellular morphology analysis , stem segment 1cm above the first node of the inflorescence stem of 31 day old plants were fixed into the fixatives (2.5% glutaraldehyde, 75mM cacodylate pH 7, 2mM MgCl<sub>2</sub>) for overnight. Fixed stems were washed four times with fixative buffer (75mM cacodylate pH 7, 2mM MgCl<sub>2</sub>), for 15, 25, 40, 60 minutes respectively. Washed stems were then treated with 1%

osmium-tetroxide in fixative buffer for 2 hours. Osmium-tetroxide was removed by washing with fixative buffer for 15 minutes and then samples were rinsed with dH<sub>2</sub>O for 15, 30, 60 minutes each. In the next step, stem tissues were passed through an acetone series (10%, 20%, 40%, 60%, 80% each 30 minutes and three time with 100% acetone for 15, 30, 60 minutes each). For embedding, Spurr's medium (Electron Microscopy Sciences #14300) was used. The tissues were placed in Spurr's/acetone (1:2) for 1 hour, gently shaken on a rocker, then exchanged with Spurr's/acetone (2:1) for 2 hours. Finally, tissues were put in 100% Spurr's medium for 4 hours, exchanged with Spurr's medium and kept for 6 hours. For embedding, tissues were transferred in fresh Spurr's and polymerization of embedded material was carried out for 12 hours at 65°C. Sections of 2µm were cut using the glass-knives, stained with toluidine blue and mounted in Spurr's medium.

### **2.8 Flower organ counting**

The number of sepal, petal, stamen and carpel floral organs were counted at stage 13 of flower development (Smyth et al., 1990). For counting first three, freshly opened flowers of each plant were used.

### **2.9 Photography**

Whole plant pictures were taken with a Kodak DCS760 digital camera (Eastman Kodak). To take close-up pictures of flowers and stems a Color-View III CCD camera (Cell-P Olympus, europa) mounted on SZX-12 BINO (Olympus, Japan) was used. Image manipulations such as brightness and contrast, were carried out using Adobe Photoshop 7.0 software (Adobe System Inc.)

### 2.10 Screening of bright GFP and EGFP transgenic plant lines

Screening of bright GFP and EGFP T1 individual transgenic plant lines was carried out under the epifluorescence microscope. The *L1::SUB:GFP*, *ANT::SUB:GFP* and *WUS::SUB:GFP* lines were selected based on brightness of GFP signal in the ovule of 25 day old plants, while the *pSUB::SUB:EGFP* lines were chosen on the basis of brightness of EGFP signal in the 7 day old primary roots, using BX61 (Olympus, Japan).

### 2.11 Live imaging of GFP and EGFP using confocal microscopy

The confocal images were taken with a FV1000 IX81 FLUOVIEW CLSM (Olympus, Japan). The samples were either dissected under the Stemi SV 6 dissecting microscope (Zeiss, Germany) in dH<sub>2</sub>O, or in FM4-64, wherever required. For whole mount roots, seedlings were taken from the MS-plates, aerial parts removed and roots were stained in FM4-64 for 5-10 minutes. Roots were then covered with a 0.17mm thick cover slip and visualized. For ovule confocal images, young carpel from closed flower buds were dissected in 4 $\mu$ M FM4-64 and visualized. Stage-3 flowers were dissected from the inflorescence into H<sub>2</sub>O and then stained in FM4-64 for 5-10 minutes and visualized under the 488nm argon laser. Inflorescences were clipped off first under a dissecting microscope in water and then stained for 10-20 minutes in FM4-64 and put on depression slide under cover slip in a upright position. The slide was inverted and inflorescence images were scanned using confocal microscopy. GFP & EGFP fluorescence was collected through a band-pass filter (BP502-536nm) and the red vital dye FM4-64 was collected through a long-pass filter (BP610-672nm) after excitation at 488nm. One-way scan images (scan rate 12.5 $\mu$ s/pixel; 512 $\times$ 512 pixels at 16% laser power) were generated using an Olympus lens (40X 0.90NA, water PALPO Olympus).

### 2.12 Drug treatment

Brefeldin A (BFA) 100 $\mu$ g was applied to 4-day old roots in 1 ml basal medium (BM) (0.5MS, 1% sucrose (pH5.8) without agar for one hour.

### 2.13 Plasmolysis

To determine the plasma membrane localization of SUB:EGFP in planta, either the inner wall of carpels or 7-day old roots of transgenic plants were placed in 1M sorbitol at room temperature. After 10-15minute of the treatment retraction of SUB:EGFP labeled cell membrane was visualized using confocal microscopy

## **Chapter 3 SUB:EGFP showed a differential expression pattern compared to its mRNA and localized in the plasma membrane**

### **3.1 Summary**

The *Arabidopsis STRUBBELIG (SUB)* gene encodes a receptor-like kinase and is required for coordinating cell growth and cell division pattern during development. In this study, we analyzed a SUB in frame translational fusion with EGFP in planta. The SUB:EGFP fusion protein was able to rescue the *sub* mutant phenotype indicating that it is functional in plants. Plasmolysis experiments revealed plasma membrane localization of SUB:EGFP in the cell. Moreover, the mRNA vs. SUB:EGFP fusion protein studies show a differential expression pattern in developing ovules, flower and inflorescence meristems during development. Differential expression patterns of SUB:EGFP during ovule ontogenesis suggest that SUB acts in non-cell autonomous fashion during cell differentiation. The SUB:EGFP fusion protein is part of the endomembrane system within the cell and SUB:EGFP internalization via endosomes is sensitive to the BFA treatment.

### **3.2 Introduction**

Multicellular organisms coordinate their growth and development at the tissue and organ level by using extracellular signaling cues to communicate within a cell and between the cells. Compared to animals, where organs and glands are discrete, plant development is a post-embryonic process, where cell proliferation and differentiation is a continuous process throughout development. Receptor-like kinases (RLKs) are single-pass transmembrane proteins that contain an extracellular domain and a cytoplasmic kinase domain. The majority of plant RLKs carries leucine-rich repeats (LRRs), in their extracellular domain and belong to the serine/threonine kinase family (Shiu and Bleecker, 2001). Plant LRR-RLKs are

involved in regulation of development *ERECTA*, *HAESA* (Torii et al., 1996; Jinn et al., 2000) and *CLV1* (Clark et al., 1997), disease resistance *FLS2* (Gomez-Gomez and Boller, 2000) and *Xa21* (Song et al., 1995), and steroid hormone signaling *BRI1*, (Li and Chory, 1997). The Arabidopsis gene *STRUBBELIG* (*SUB*) encodes a putative LRR-RLK. Loss of function *sub* show altered shape and size of plant organs, such as sepals, petals, carpels, and ovules (Chevalier et al., 2005). To determine the function of *SUB* mediated signaling pathway, it is important to understand the spatial and temporal expression pattern of the *SUB* protein. Here, we made a *SUB* and EGFP in frame translational fusion, expressed under its own promoter in planta, and analyzed the subcellular localization of the *SUB:EGFP* fusion protein using confocal microscopy.

### 3.3 Results

#### 3.3.1 *SUB:EGFP* fusion protein rescued the *sub* phenotype

To determine the spatial and temporal expression patterns of *SUB* during plant development, we made an EGFP fusion with the C terminus of full-length *SUB*. The EGFP is a variant of GFPmut1 and contains double substitution of Phe-64 to Leu and Ser-65 to Thr (Cormack et al., 1996). Compared to WT GFP, the EGFP show a greater solubility and 35 times more brightness in fluorescence in bacterial expression system. The *SUB:EGFP* was inserted in frame translational fusion in a vector that carries 3.5 kb promoter region including upstream regulatory elements and 354 bp downstream regulatory elements. Transgenic lines were created in *sub-1* mutant and WT-*Ler* background. For *sub* mutant as well as WT-*Ler*, at least one hundred individual plant lines were screened to assess the correct expression pattern of fusion protein in the T1 generation. We focused on the roots to screen for the bright plant lines under the epifluorescence microscope. Live transgenic seedlings expressing EGFP fluorescence in the root were immediately transferred to soil. There was variation in the independent lines in terms of the strength of the EGFP fluorescence signal in the root. Though all *sub* rescued plants were grown together irrespective of their strength of the EGFP fluorescence signal. Again at



30-day plants were assessed for phenotypic rescue (Table 3.1). For WT-Ler, live transgenic seedlings showing bright EGFP fluorescence in root were transferred to soil and grown parallel to assess phenotype. We did not observe any ectopic expression phenotype in WT-Ler *pSUB::SUB:EGFP* transgenic lines.

**Table 3.1 Summary of 81 T1 plants screened for rescue of stem and carpel twisting phenotype by *pSUB::SUB:EGFP* construct**

Rescued phenotype	No. of plants rescued	Percentage rescue
Stem twisting	77	95%
Carpel twisting	56	69%

In *sub-1* mutant, the flower organ number and flower morphology is slightly perturbed (Chevalier et al., 2005). To determine the rescue of flower organ number, flower organs were counted in stage 13 to -15 flowers and flower morphology was documented (Fig 3.1B). A summary of flower organ number is given in the (Table 3.2)

To analyze the rescue of *sub* phenotype in *pSUB::SUB:EGFP* transgenic lines at cellular level, bright transgenic line #15.3 *pSUB::SUB:EGFP sub-1* was taken and compared with #3.3 *pSUB::SUB:EGFP WT-Ler*. Ovules were fixed according to (Chevalier et al., 2005) and stage 4-V ovules were analyzed for outer integument rescue by scanning electron micrograph (SEM). Ninety three percent of ovules were rescued (Fig 3.1E) in *sub-1 pSUB::SUB:EGFP* line (n=320). We also looked for L2 layer periclinal cell division orientation rescue in young floral meristem of *sub-1 pSUB::SUB:EGFP* lines. The L2 layer defects were rescued by *pSUB::SUB:EGFP* (Fig 3.1H and Table 3.3).

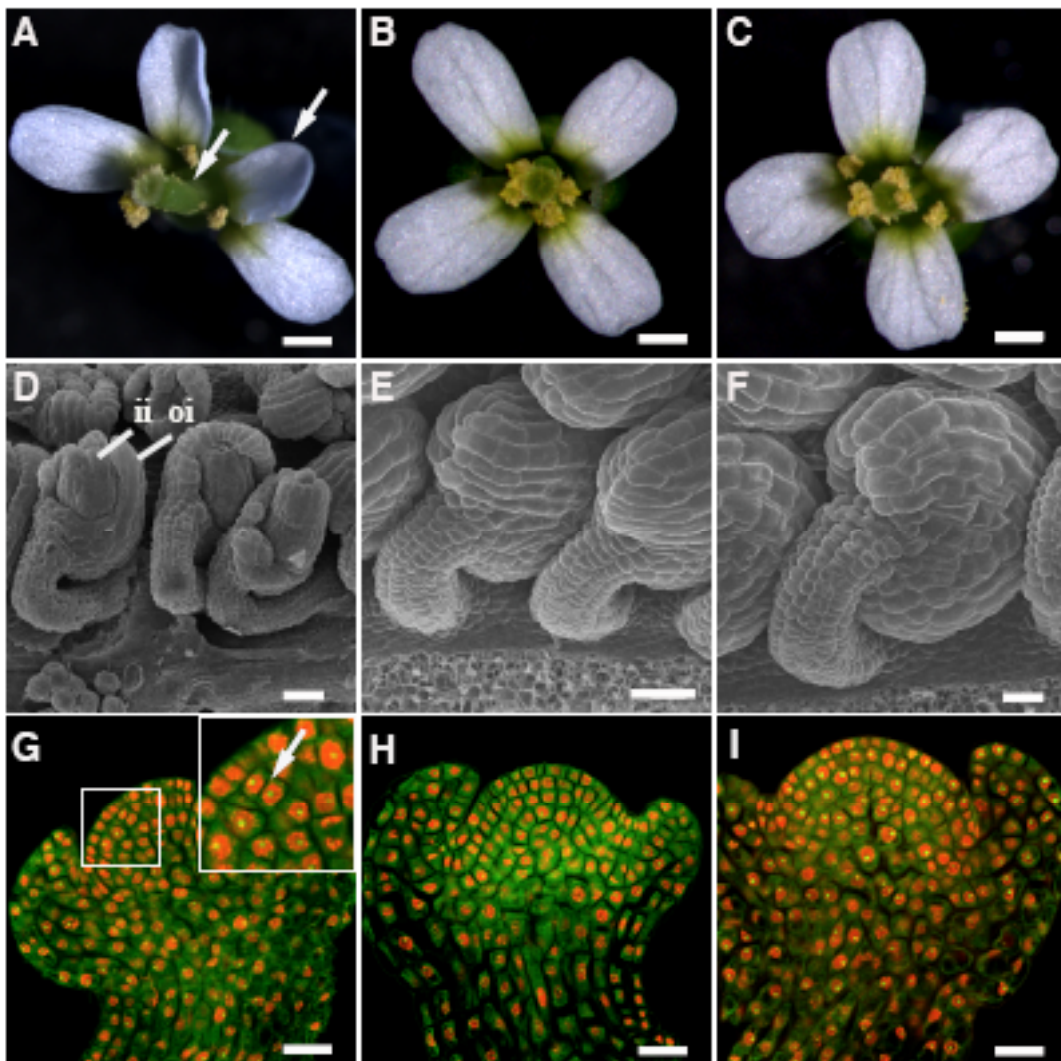


Fig. 3.1 Flowers, ovules, and young floral meristems of *sub-1*, *pSUB::SUB:EGFP sub-1* and WT-Ler *pSUB::SUB:EGFP*. (A, D, and G) *sub-1*. (B, E, and H) *sub-1* rescued by *pSUB::SUB:EGFP*. (C, F, and I) WT-Ler *pSUB::SUB:EGFP*. (A-C) Flowers at stage 13. (D-F) Scanning electron micrographs of stage 4-V ovule. (G-I) Midoptical section of stage-3 flower meristem obtained from propidium iodide stained whole mount specimen by confocal laser scanning microscopy. (A) *sub-1* mutant flower. Arrows indicate twisting of carpel and petals. (B) *sub-1* mutant rescued by *pSUB::SUB:EGFP*. (C) WT-Ler *pSUB::SUB:EGFP*. (D) Stage 4-V ovule of *sub-1*. Note the aberrant outer integument. (E) Stage 4-V ovule of *sub-1* mutant rescued by *pSUB::SUB:EGFP*. (F) Stage 4-V ovule of WT-Ler *pSUB::SUB:EGFP*. (G) *sub-1* stage-3 floral meristem. (Inset) an example of periclinal cell division orientations in L2 layer, indicated by arrow head. (H) Stage-3 flower meristem of *sub-1* rescued by *pSUB::SUB:EGFP*. (I) Stage-3 flower meristem of WT-Ler *pSUB::SUB:EGFP*. Abbreviations, ii, inner integument. oi, outer integument. Scale bars (A-C) 0.5mm, (D-I) 20µm.

**Table 3.2 Flower organ number counting of 30-days old *sub-1* plants rescued by *pSUB::SUB:EGFP***

Genotype	Sepals	Petals	Stamens	Carpel	N
#15.3 <i>sub-1 pSUB::SUB:EGFP</i>	3.9±0.1	3.9±0.1	5.3±0.7	2±0	103
#3.3 WT-Ler <i>pSUB::SUB:EGFP</i>	4±0	4±0	5.8±0.5	2±0	97

**Table 3.3 Rescue of the *sub-1* L2-layer cell division plane defect of stage-3 flower meristem by *pSUB::SUB:EGFP***

Genotype	N periclinal divisions	N meristems	Percentage
Wt-Ler	0	46	0
<i>sub-1</i>	35	84	41
#15.3 <i>sub-1 pSUB::SUB:EGFP</i>	3	45	6
#3.3 Wt-Ler <i>pSUB::SUB:EGFP</i>	3	42	7

N=Number

At the macroscopic level stems and carpels are twisted in *sub-1* mutant. The SUB:EGFP fusion protein was able to rescue the stem and carpel twisting phenotype in the *sub-1 pSUB::SUB:EGFP* #15.3 (Fig 3.2B, E) plants. The *pSUB::SUB:EGFP* construct also rescued plant height (Fig 3.2H) in 30-day old plants.

In summary, taken together the above data show that the translational fusion of SUB:EGFP was able to rescue all the aspects of the *sub-1* mutant phenotype, including stem twisting, outer integuments of ovule, flower organ numbers/symmetry and plant height. These results indicate, that the SUB:EGFP fusion protein was functional and behaved like the wild type SUB protein in planta.

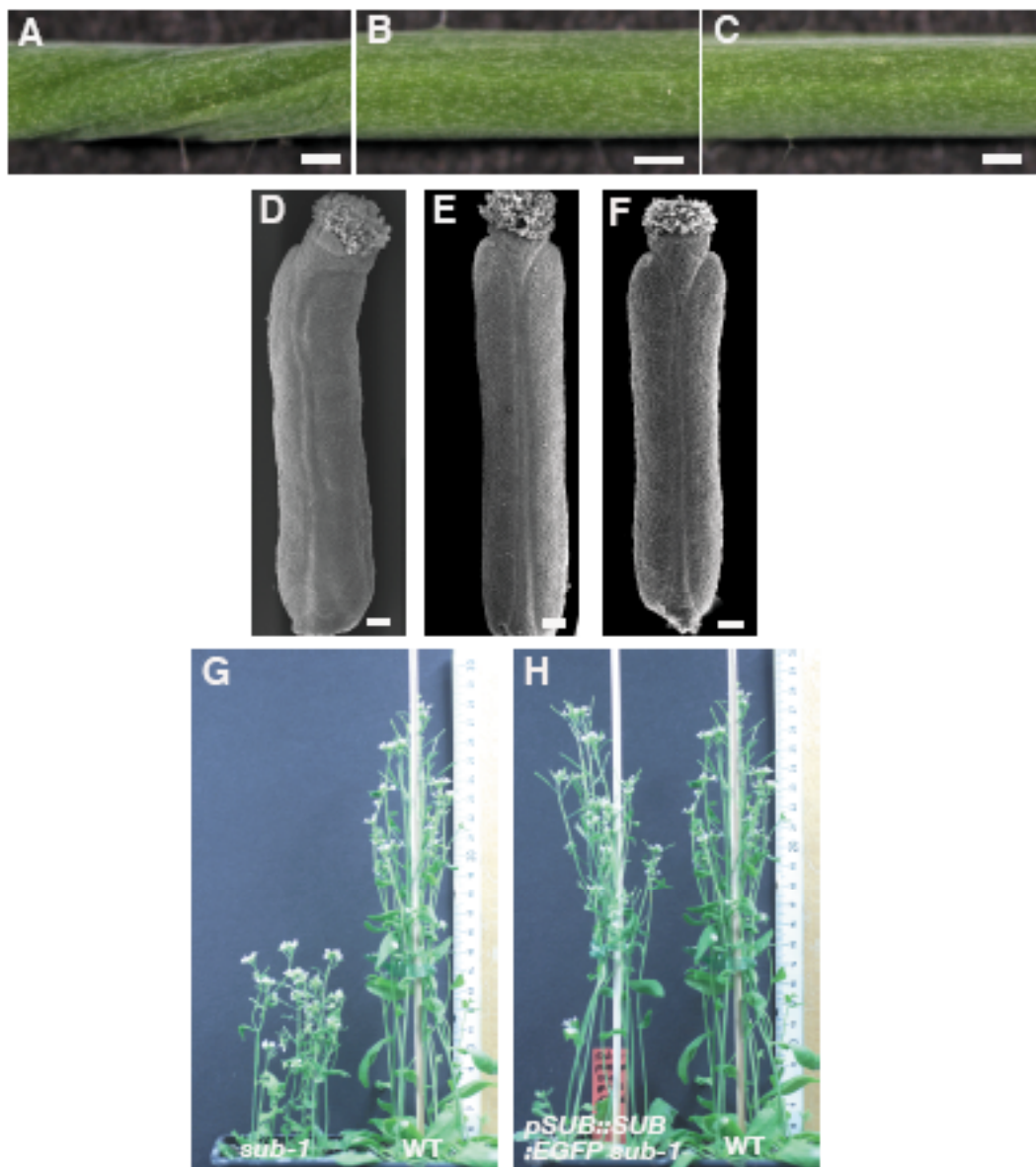


Fig. 3.2 Stem, carpel, and plant height of *sub-1*, *pSUB::SUB:EGFP sub-1* and WT-Ler *pSUB::SUB:EGFP*. (A, D, and G left pot) *sub-1*. (B, E, and H left pot) *sub-1* rescued by *pSUB::SUB:EGFP*. (C, F, and H right pot) WT-Ler *pSUB::SUB:EGFP*. (A-C) stem pictures of 30-days old plants. (D-F) Scanning electron micrograph of mature carpels. (G and H) Whole plant pictures of 30-days old plant. (A) Twisted stem of *sub-1* mutant. (B) *pSUB::SUB:EGFP sub-1* stem. Note the twisting of stem rescued. (C) WT-Ler *pSUB::SUB:EGFP* stem. (D) *sub-1* mutant carpel showing twisting. (E) *sub-1* mutant carpel twisting rescued by *pSUB::SUB:EGFP*. (F) WT-Ler *pSUB::SUB:EGFP* carpel. (G) *sub-1* mutant plants versus WT-Ler plants at 30-day. The *sub-1* mutant displays semidwarfism. (H) Left side, *sub-1* mutant rescued by *pSUB::SUB:EGFP*, whereas at right side, WT-Ler. Semidwarfism of the *sub* mutants rescued by *pSUB::SUB:EGFP*. Scale bars (A-C) 0.5mm, (D-F) 100μm.

### 3.3.2 SUB:EGFP protein expression pattern

#### 3.3.2.1 The SUB:EGFP protein is expressed in the stele region of post-embryonic roots

The SUB/SCM mediated signaling pathway defines cell fate specification in the *Arabidopsis* root epidermis (Kwak et al., 2005). To understand the process of cell fate specification in the root epidermis, it is important to know the correct expression pattern of SUB:EGFP in developing root. The *Arabidopsis* seedlings were grown vertically on MS agar plates in the growth room and the roots were stained with FM4-64, (a vital dye that labels only the membrane compartments) to visualize the cellular outline of the root (Bolte et al., 2004). We analyzed the localization of SUB:EGFP fluorescence by confocal laser-scanning microscopy in post-embryonic roots. EGFP fluorescence was found in the stele and was restricted especially to the cell division zone of the developing root (Fig 3.3). The EGFP bright fluorescence signal in the stele region outlines cell surfaces especially in the cell division zone of developing root. Contrary to the SUB:EGFP fusion protein, *SUB* (*SCM*) mRNA expression has been detected by *in-situ* hybridization or indirectly through *SUB* promoter reporter fusion studies throughout the developing root (Kwak et al., 2005) (Martine Batoux and Kay Schneitz unpublished data), particularly within the stele near the meristem initials as well as in the endodermis, cortex, and epidermis excluding the root cap in the meristematic region. At the detection limit of confocal imaging, we could detect EGFP fluorescence signal in the vasculature. Notably, SUB:EGFP signal could not be seen in the endodermis, cortex, epidermis, root quiescent centre (QC), cortex/endodermis initial and its daughter cells.

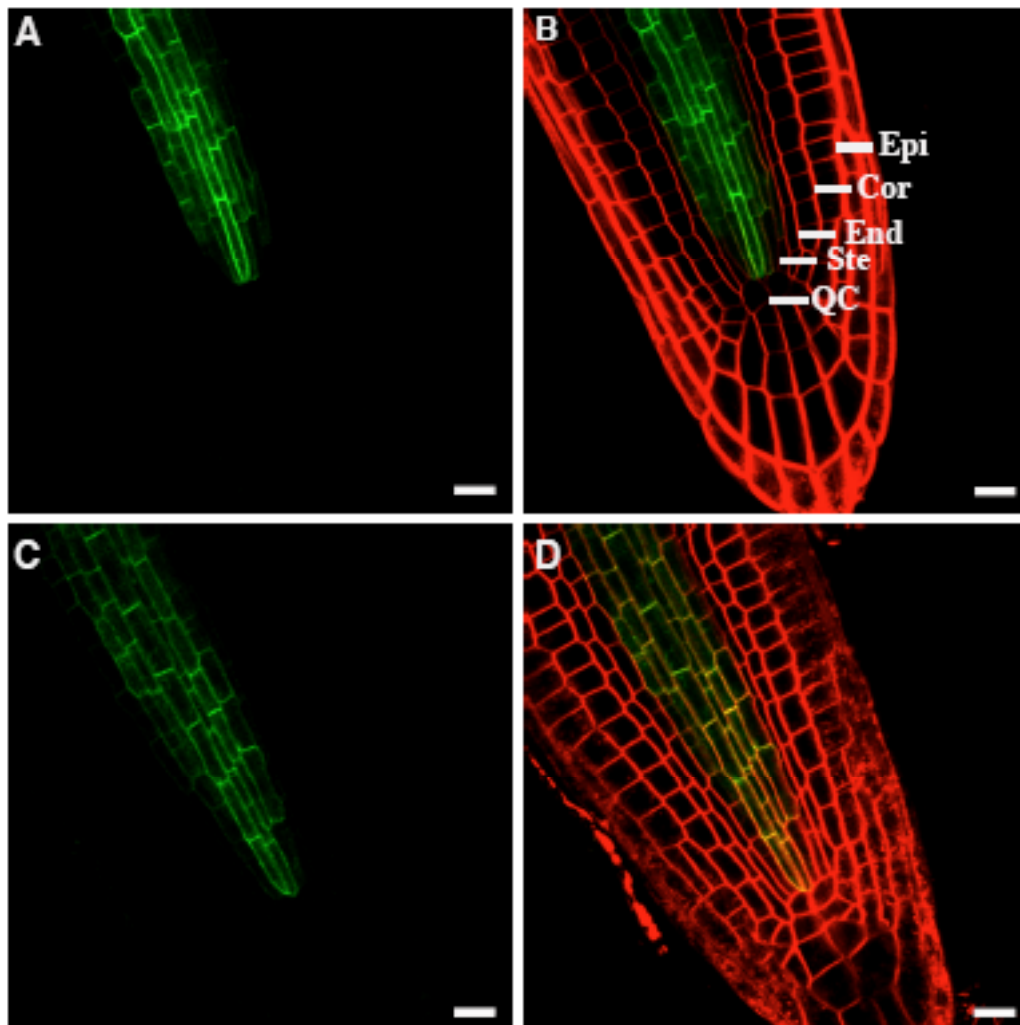


Fig. 3.3 SUB:EGFP protein localization in the post-embryonic root of *pSUB::SUB:EGFP sub-1*, seedlings. (A-D) Mid-epitaxial section of SUB:EGFP transgenic roots obtained by confocal laser scanning microscopy. (A and C) EGFP channel only. (B and D) FM4-64 channel merged with EGFP channel. (B) Image of the SUB:EGFP transgenic root. Note SUB:EGFP expression in 2-day old root detected in the stele region. (D) Image of a 4-days old root expressing SUB:EGFP in the stele. Abbreviations, QC, quiescent centre. Ste, stele. End, endodermis. Cor, cortex. Epi, epidermis. Scale bars, 10  $\mu$ m.

### 3.3.2.2 SUB:EGFP showed a differential expression pattern in the developing flower and inflorescence meristems

The 30-day old *sub* plants exhibit reduced plant height compared to WT and show twisted stems (Chevalier et al., 2005). This phenotypic observation suggests a possible role of *SUB* mediated signaling in the inflorescence meristem development. The *pSUB::SUB:EGFP* expression was analyzed in detail in flower and inflorescence meristems. Previously *SUB* mRNA expression was detected throughout the flower and inflorescence meristems (Chevalier et al., 2005). In flowers, *SUB* mRNA transcript was not restricted to the meristem but was also expressed in flower organs such as sepals, petals, stamens and carpels, early in development (Chevalier et al., 2005). Unlike *SUB* mRNA, the SUB:EGFP fusion protein showed a restricted expression pattern in the flower and inflorescence meristems.

Viewing the inflorescence meristem from the top, one can visualize SUB:EGFP fluorescence in the dome of the shoot apex (Fig 3.4B, 3.4C). SUB:EGFP fluorescence is visible within the inflorescence meristem. In longitudinal sections of CLSM images, SUB:EGFP fluorescence is visible in the inflorescence and restricted to the L3-layer (Fig. 3.4C). In developing flowers it shows differential expression pattern. In stage-1 flower organ primordia, the SUB:EGFP expression is visible in the L2-layer towards the adaxial side, where an organ boundary separates the main shoot apex (Fig. 3.4C). In stage-2 flower organ primordia, the SUB:EGFP fluorescence extends up to the L1 layer between the organ primordia and organ boundary region of shoot apex (Fig. 3.4C). At the resolution of confocal microscopy, we were unable to detect EGFP fluorescence towards the abaxial side of stage-1 and early stage-2 flower organ primordia. This observation indicates the subtle differential expression pattern of SUB:EGFP during the development. One can particularly appreciate this differential expression of SUB:EGFP behavior in three dimensions (Fig 3.4 3D), where in stage-1 flower meristem, the SUB:EGFP expression domain is still covered by the L1-layer of FM4-64 while in the stage-2



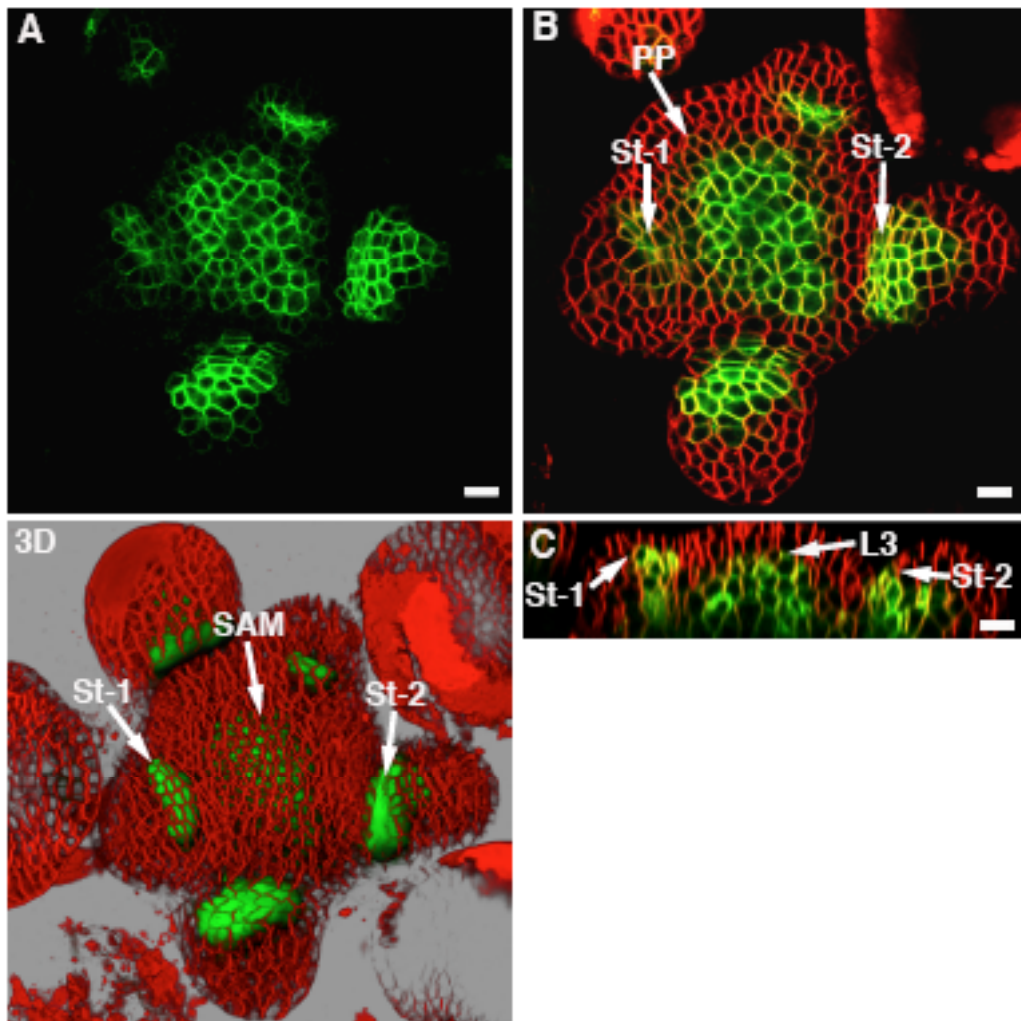


Fig. 3.4 *SUB:EGFP* expression pattern in the inflorescence meristem. (A-C) Confocal laser scanning microscopy images of *pSUB::SUB:EGFP* in *sub-1*. (A) Optical section of shoot apex, EGFP channel only. (B) FM4-64 channel merged with EGFP channel, optical section of shoot apex. Note the St-1 and St-2 arrow indicate stage-1 and stage-2 of flower development respectively. (C) Longitudinal section of optical section of shoot apex. The L3 arrow pointing towards the L3 layer in the shoot apical meristem. The EGFP fluorescence is visible upto L3 layer in the shoot apex. Note in stage-1 flower primordium the EGFP fluorescence is visible upto L2 layer, while in stage-2 flower primordium the EGFP fluorescence is visible upto L1 layer. (3D) The three dimensional reconstructions of the optical sections of shoot meristem. Abbreviations, PP, presumed primordia. St-1, stage-1 flower primordia. St-2, stage-2 flower primordia. Scale bars 10 $\mu$ m.



flower meristem the SUB:EGFP expression domain is clearly overlapping with FM4-64 in the L1-layer.

To understand better the differential SUB:EGFP expression pattern in flower development. We investigated SUB:EGFP expression pattern in late stage-2 and stage-3 flowers. SUB:EGFP expression pattern is restricted up to the L3-layer in stage-2 and stage-3 flower meristems (Fig 3.5), but there are subtle dynamic changes visible in the expression domain of SUB:EGFP. For example the expression of SUB:EGFP fusion protein is detected in the L1-Layer of the boundary separating a developing sepal from the flower meristem in stage-3 flowers (Fig 3.5D).

Taking together, the SUB protein localization appears to be distinct from its mRNA. The SUB:EGFP fusion protein was restricted to the L3 layer in the dome of inflorescence and flower meristems during development. SUB:EGFP fluorescence in L1/L2 layer was observed at the organ boundary region of emerging floral stage-1 and stage-2 primordia.

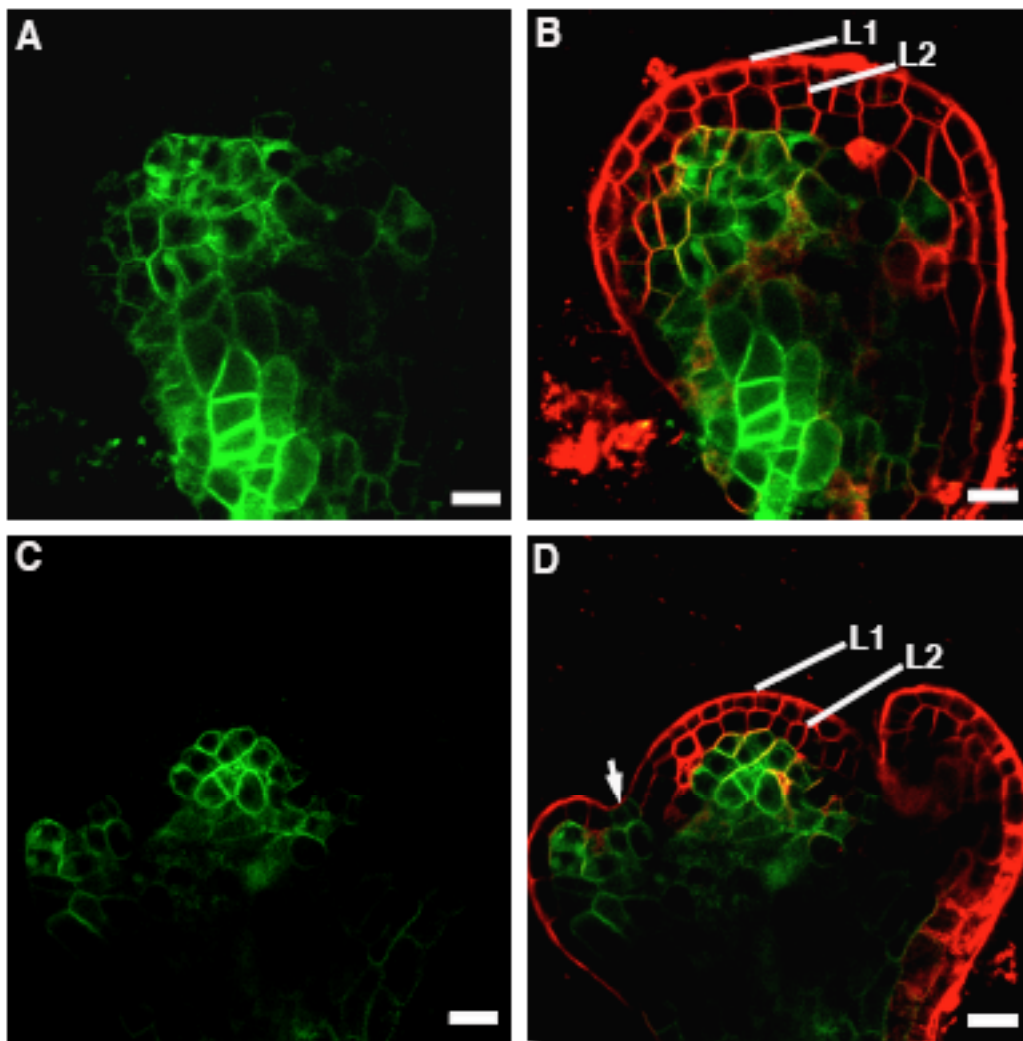
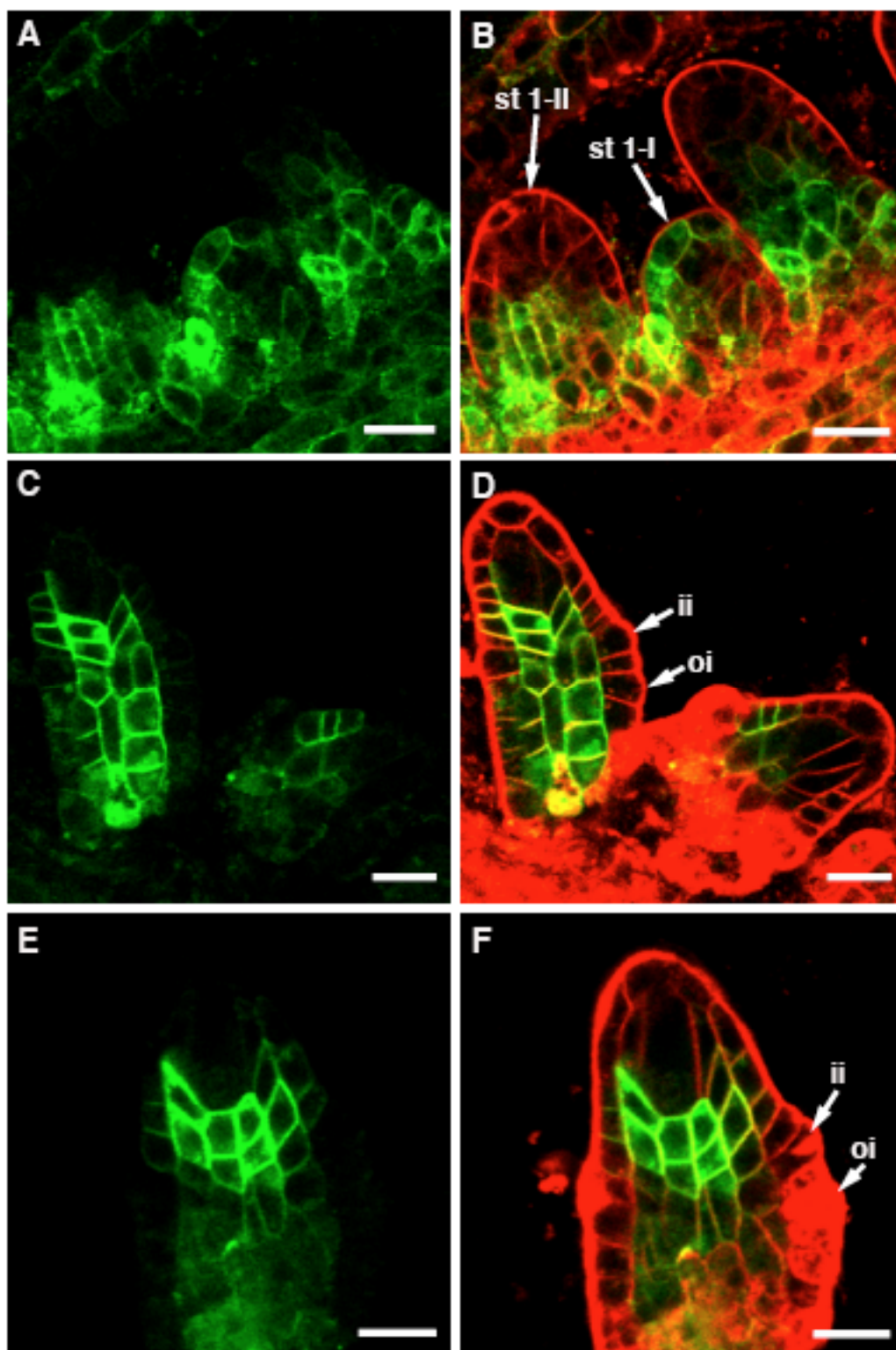


Fig. 3.5 SUB:EGFP localization in the young floral meristem. (A-D) Confocal laser scanning microscopy images of *pSUB::SUB:EGFP* in *sub-1*. (A) Midoptical section of a stage-2 flower meristem, EGFP channel. (B) FM4-64 channel merged with EGFP channel from the same plane. The SUB:EGFP fluorescence is visible in the L3 layer. (C) Midoptical section of stage-3 flower meristem, EGFP channel. (D) The SUB:EGFP fusion protein is confined up to L3 layer in the central dome of the floral meristem. The arrow indicates SUB:EGFP fluorescence in L1 layer. Scale bars 10 $\mu$ m.

### 3.3.2.3 SUB:EGFP expression in the developing ovule

Ovule ontogenesis has been classified into stages based on morphological events; which involve initiation of the protrusion from the placenta, specification, pattern formation and morphogenesis (Schneitz et al., 1995). Analysis of *sub* mutant revealed a role of *SUB* in cell morphogenesis and cell proliferation in outer integument development of the ovule (Chevalier et al., 2005). Therefore, we analyzed SUB:EGFP expression in ovules, from early stage 1-I to stage 2-V.

In young ovules at stage 1-I, SUB:EGFP expression is detected in all cells in the arising protrusion (Fig 3.6B, st. 1-I). When ovule protrusions elongate along a proximal-distal axis (stage 1-II) the SUB:EGFP fluorescence is confined to the proximal part near the placenta (Fig 3.6B). All cell layers of the ovule at the placental end show SUB:EGFP fluorescence, while the distal part of the protrusions do not show SUB:EGFP fluorescence. In stage 2-I to 2-II ovules, when integument initiation become visible the SUB:EGFP expression show a more dynamic expression pattern (Fig 3.6D, F). In the nucellus, the EGFP fluorescence is visible at the base of megaspore mother cell in the epidermal and subepidermal cells. Interestingly, SUB:EGFP signal disappears in a key epidermal cell which is a site of future inner integument initiation (Fig 3.6D). At later stage this selective weakening of EGFP fluorescence extends in neighboring epidermal cells towards the proximal portion of the ovule primordia. The SUB:EGFP expression is restricted to the subepidermal cells below the initiating inner and outer integuments cells in the stage 2-II to 2-III of ovules development. In the stage 2-III of ovule development, when the outer integument initiates, the SUB:EGFP expression is confined to the L2-layer of the ovule in the proximal part (Fig 3.6H). In the distal tip of the ovule, (the nucellus) one could see the SUB:EGFP fusion protein at the base of the megaspore mother cell as well as in the epidermis. At the stage 2-V of ovule development the SUB:EGFP expression is visible in funiculus and in chalaza but not in inner and outer integument (Fig. 3.6J).



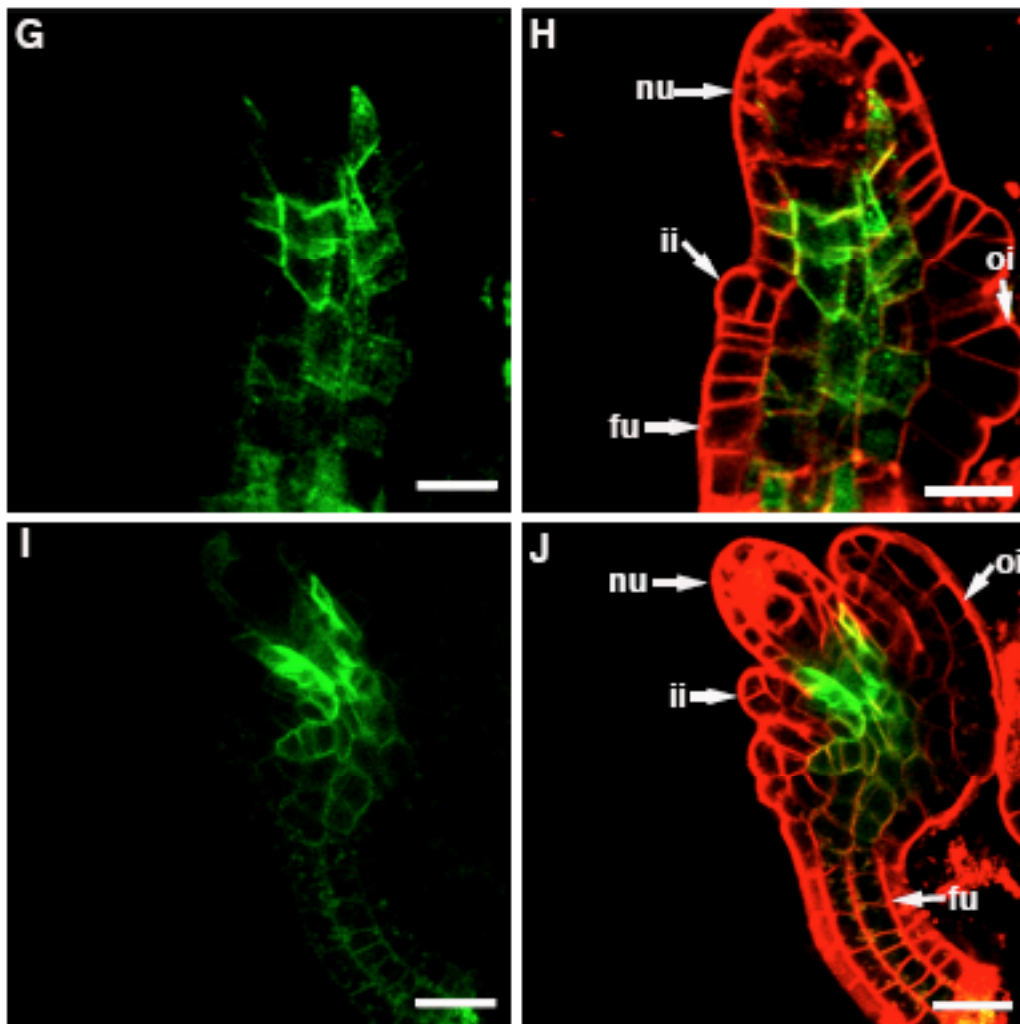


Fig 3.6 SUB:EGFP localization in the developing ovules. (A-H) Confocal laser scanning microscopy images of *pSUB::SUB:EGFP* in *sub-1*. (I and J) Confocal laser scanning images of *pSUB::SUB:EGFP* in WT-Ler. (A, C, E, G, and I) EGFP channel only. (B, D, F, H, and J) FM4-64 channel merged with EGFP in the same plane. (B) St 1-I and St 1-II are stage 1-I and stage 1-II of ovule development. Note in stage 1-I ovule EGFP fluorescence is visible everywhere while in the stage 1-II, EGFP fluorescence is confined to the proximal half of the ovule. (D and F) Stage 2-II ovules, the enlarged cells represent the site of inner and outer integument initiation. Note in the inner and outer integument initiating cells the EGFP fluorescence disappears selectively. (H) Midoptical section of stage 2-IV ovule, the fully differentiated cells of the inner and outer integuments lacks SUB:EGFP expression. (J) Midoptical section of stage 2-V ovule, the integuments extend towards the apex of the nucellus, the SUB:EGFP expression is absent from the inner and outer integuments. Abbreviations fu, funiculus; ii, inner integument; nu, nucellus; oi, outer integument. Scale bars 10 $\mu$ m.

Taken together, the above SUB:EGFP localization in various stages of ovule development suggests that SUB:EGFP is expressed differentially during the ovule development.

### **3.4 SUB:EGFP is localized to the plasma membrane in the cell**

From our analysis of SUB:EGFP fusion protein by confocal microscopy, it looks that SUB:EGFP outlines the cell surfaces uniformly in all the tissues where it is expressed, but this is not proof of plasma membrane localization. How to prove it? To determine, if SUB:EGFP is localized to the plasma membrane or not, a plasmolysis experiment was conducted. Plasmolysis exerts a negative osmotic pressure in the cell and evacuates the fluid out of the cell, resulting in invaginations of the plasma membrane along with cell organelles, but leaving the more rigid cell wall behind. After 15 minutes of applying 1M sorbitol to the young carpel wall of SUB:EGFP expressing lines, the SUB:EGFP fluorescence outlining cell was internalized and co-localized with membrane marker FM4-64 (Fig 3.7). This result suggests that the SUB:EGFP localizes to the plasma membrane.

### **3.5 Subcellular behavior of SUB:EGFP**

Endocytosis is the uptake of extracellular substances as well as the internalization of plasma membrane proteins, and lipids, into cells. In addition, several receptor-like kinases also show ligand- induced receptor complex internalization into the cell in response to extracellular environmental stimuli and mediate signal transduction in eukaryotes. The process of receptor internalization is conserved in eukaryotes. SUB:EGFP is a plasma membrane localized receptor-like protein. How SUB-mediated signaling is carried out in response to the extracellular stimuli is still not understood?

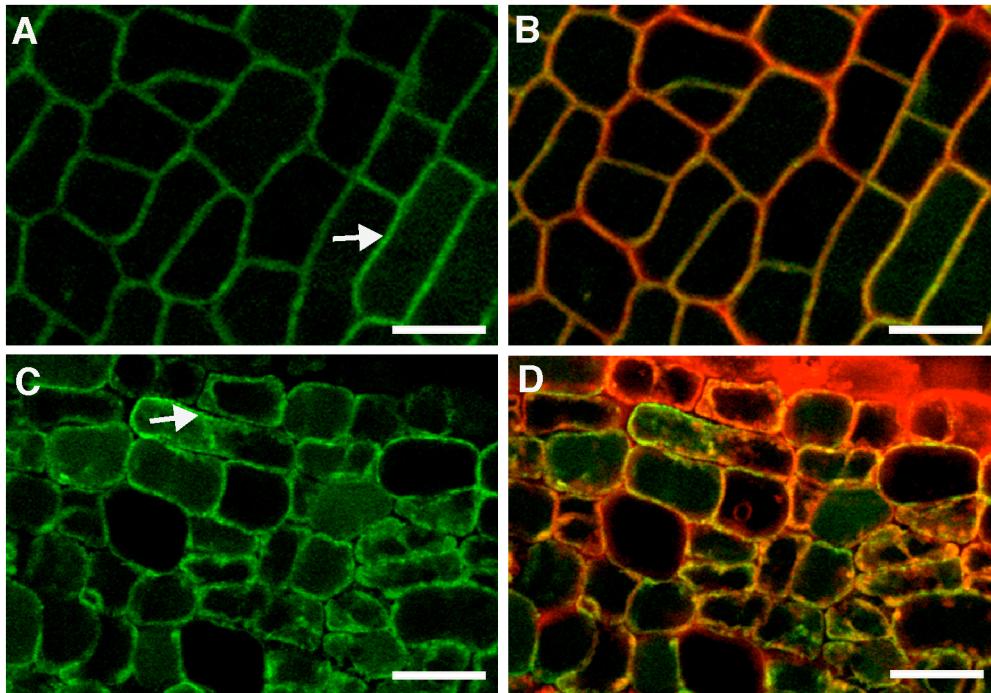


Fig. 3.7 SUB:EGFP is localized to the plasma membrane. (A-D) Confocal images of inner wall of the carpel of *pSUB::SUB:EGFP sub-1*. (A) Arrow indicates, EGFP fluorescence outlining the cell surface. (C) Image after treating with 1M sorbitol. Arrow indicates gap between the neighboring cell's plasma membrane. Indicating plasma membrane localization of SUB:EGFP. Scale bars 10 $\mu$ m.

To understand the SUB receptor internalization within the cell we investigated the subcellular localization of SUB:EGFP and observed EGFP containing cytoplasmic bodies. To understand further the nature of the cytoplasmic bodies, colocalization studies were performed using the amphiphilic steryl dye FM4-64 (Bolte et al., 2004). FM4-64 inserts into one side of the plasma membrane bilayer and fluoresces only when in a hydrophobic environment. The amphiphilic nature of the dye suggests that it enters the cell via internalization of membrane vesicles. In our studies, plasma membranes in the stele were well-labeled with FM4-64 when roots were incubated in 4 $\mu$ M FM4-64 for 40-50min. Internal vesicles were clearly visible. Observation in the stele cells near the quiescent centre of plants, expressing SUB:EGFP construct and treated with FM4-64 showed two types of distinct populations of FM4-64 containing bodies, one labeled with EGFP and other with



considerably weaker EGFP. Occasional colocalization of EGFP containing bodies with FM4-64 was observed (Fig 3.8C). This evidence indicates that SUB internalizes under the normal physiological condition within the cell, but we still do not know whether this is a ligand dependent or independent process.

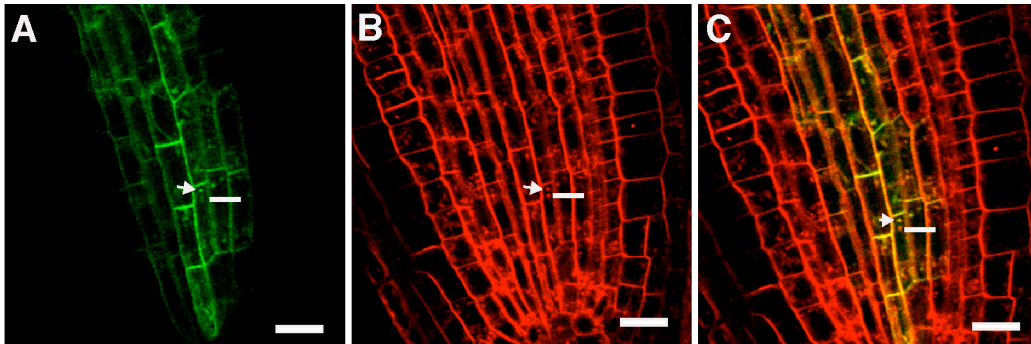


Fig. 3.8 Subcellular behavior of SUB:EGFP fusion protein. (A-C) Root cells of plants carrying SUB:EGFP and treated with FM4-64. Cells were observed using the EGFP channel (A) and FM4-64 channel (B) and images were then merged (C). (A) Arrow indicates EGFP vesicle. (B) Arrow indicates FM4-64 vesicle. (C) Arrow indicates EGFP co-localization with FM4-64 vesicle. (A-C) Lines indicates FM4-64 labeled vesicle only. Scale bars 10 $\mu$ m.

### 3.6 SUB:EGFP receptor protein internalization is BFA sensitive

Brefeldin A (BFA) is a fungal metabolite that inhibits exocytosis from post Golgi derived vesicles but allows the first steps of endocytosis (Baluska et al., 2002; Nebenfuhr et al., 2002; Geldner et al., 2003). In plants, rapidly recycling plasma membrane proteins like the putative auxin efflux carriers PIN1 (Geldner et al., 2001; Geldner et al., 2003) and PIN2 (Boonsirichai et al., 2003; Grebe et al., 2003) and plasma membrane H<sup>+</sup>-ATPase (Geldner et al., 2001; Geldner et al., 2003) undergo the endocytic internalization and accumulate within BFA-induced compartments. This evidence clearly supports the idea that BFA predominately inhibits the endocytic recycling of plasma membrane proteins. The SUB:EGFP fusion protein is localized to the internalized cytoplasmic bodies. To test whether SUB:EGFP is internalized from the plasma membrane via a BFA- sensitive pathway, lateral root from plants expressing SUB:EGFP were treated with 100 $\mu$ g/ml BFA or with FM4-64 and 1% ethanol (control) (Fig 3.9A) for 30 minutes. Most cells in BFA-treated



lateral roots contained one or two relatively large EGFP fluorescence bodies (Fig 3.9D). These BFA-induced cytoplasmic bodies densely stained with FM4-64 and colocalized with EGFP (Fig. 3.9F). The intensity of EGFP fluorescence of cytoplasmic bodies as well as of FM4-64-labeled fluorescence cytoplasmic bodies is comparable. This indicates that SUB:EGFP and FM4-64 containing cytoplasmic bodies behave in a similar fashion in the presence of BFA. One additional explanation of this could be that SUB:EGFP internalize and is continuously sorted in the endosomal compartment and recycled back to the plasma membrane.

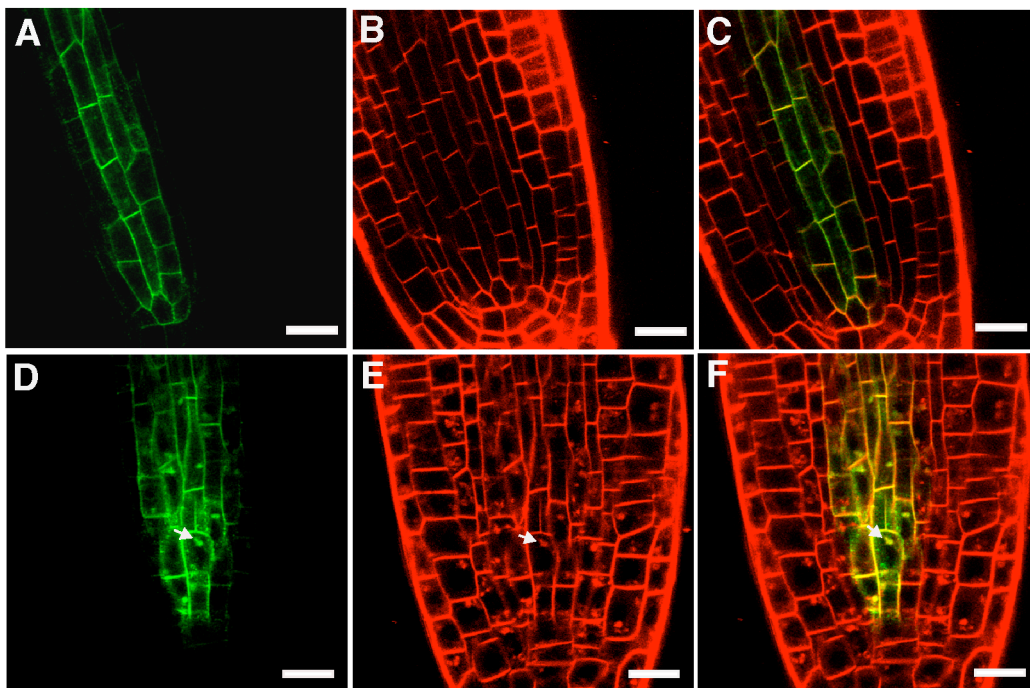


Fig. 3.9 Subcellular behavior of SUB:EGFP fusion protein after BFA treatment. (A-C) Lateral root cells carrying SUB:EGFP and treated with FM4-64. Cells were observed simultaneously using the EGFP channel (A), and FM4-64 channel (B) and images were then merged (C). (D-F) BFA-treated meristematic cells. (D) Arrow indicates EGFP encircled large body. (E) Arrow indicates FM4-64 fluorescence-concentrated body. (F) Arrow showing FM4-64 fluorescence large body that show EGFP co-localization. Scale bars 10 $\mu$ m.

### 3.7 SUB:EGFP decorated the subcellular organelles

Our current understanding of cellular structure is derived from analysis of fixed tissues. To get more representative information about the subcellular dynamics of

macromolecules and cellular structure they have to be observed in their native state in living cells. Fusion proteins exhibit differential localization at subcellular level in multicellular organism. The differential localization of a given fusion protein depends on the dynamic processes of the cell. The differential pattern of subcellular localization is regulated in part by the cell cycle state and the developmental transition in which a particular cell is undergoing. SUB:EGFP localized to the plasma membrane and endocytosis through the cytoplasmic bodies. Apart from this, occasionally we do see a hollow round circle of EGFP fluorescence within the root cell in the cell division zone of the developing root (Fig 3.10).

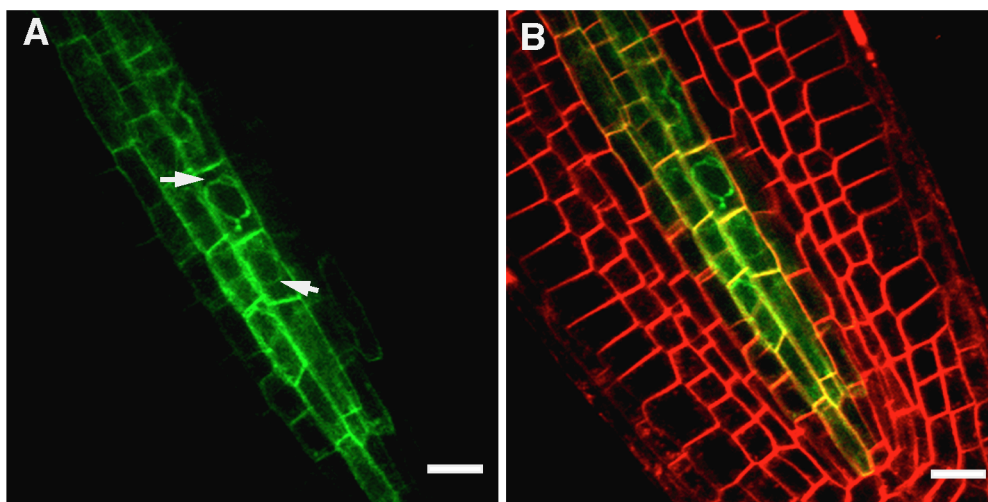


Fig. 3.10 Cellular behavior of SUB:EGFP during the development. (A) Visualization of EGFP, arrow indicate the subcellular decoration of EGFP probably around the nucleus. (B) EGFP image merged with FM4-64. Scale bars 10 $\mu$ m.

The round outlining of EGFP fluorescence in the centre of a cell indicates either it may surround the nuclear envelope or it may decorate the cytoskeleton in these cells. This observation is not consistent from one root to another root, but one could argue that it might display a cell-cycle regulated change in subcellular distribution of SUB:EGFP in the given root. Though, *sub* mutant show defects in cell size and cell shape (Chevalier et al., 2005). It is still not clear, how does SUB effects the cell size and shape? One explanation would be that SUB probably involved in cytoskeleton function.

### 3.8 Discussion

#### 3.8.1 Subcellular behavior of C-terminal fused EGFP with SUB

SUB:EGFP translational fusion protein, when expressed under its own promoter in normal physiological condition, localizes to the plasma membrane. This translational fusion was able to rescue the *sub-1* mutant phenotype, indicating that the fusion protein was functional. This observation is similar to other receptor-like kinase, which encodes the transmembrane helix in their protein coding sequence and localize to the plasma membrane and also behaved like WT protein when fused to GFP (Friedrichsen et al., 2000; Li et al., 2002; Nam and Li, 2002; Robatzek et al., 2006). We also detected the SUB:EGFP fusion protein in small vesicle-like compartments in the cytoplasm close to the plasma membrane. Colocalization experiments with the fluorescent endocytic tracer, FM4-64, that colabeled early endosomes in yeast (Vida and Emr, 1995) and in plant cells (Ueda et al., 2001), led to the conclusion that these vesicles represent endosomes. Moreover, in the presence of BFA, both SUB:EGFP and FM4-64 labeling cytoplasmic bodies colocalize together and show dense staining in the BFA compartment. ARABIDOPSIS CRINKLY4 (ACR4), a L1 layer specific plasma membrane localized receptor-like kinase from *Arabidopsis thaliana*, when fused to GFP shows two distinct populations of ACR4:GFP containing bodies (Gifford et al., 2003; Gifford et al., 2005). One is considerable bright and strong GFP bodies and the other contain considerably weaker GFP bodies. Roots from plants expressing ACR4:GFP when treated with BFA in presence of FM4-64 and protein synthesis inhibitor cycloheximide, led to the conclusion that the bright dense endosomes are outbound ACR4:GFP compartments and have little or no sensitivity to BFA (Gifford et al., 2005). Interestingly, we also observe such distinct population of EGFP bodies in SUB:EGFP expressing root cells rather we see weak SUB:EGFP cytoplasmic bodies compared to ACR4:GFP bodies. Moreover, we observed weak and strong SUB:EGFP cytoplasmic bodies near the plasma membrane. When roots from plants expressing SUB:EGFP were treated with BFA in presence of FM4-64, SUB:EGFP colocalizes with FM4-64 in BFA-induced compartments. This

result indicates that SUB:EGFP is internalized by the same cytoplasmic machinery that can internalize FM4-64 in normal physiological condition.

Receptor internalization and recycling is a well observed phenomenon in animal cells. The epidermal growth factor receptor (EGFR) family is predominately localized in the plasma membrane and internalized slowly in the absence of ligands but recycled quickly to the membrane through endosomes (Wiley, 2003). Ligand binding and activation of the EGFR accelerates endocytosis and eventually leads to the receptor down regulation in the lysosomes. Similar mechanisms also operate in plants, for instance, the auxin efflux carrier PIN1 (Geldner et al., 2001) and cell wall pectins (Baluska et al., 2002) are actively recycled back to the plasma membrane through the endosomes. In contrast to PIN1 recycling, FLS2 ligand induced FLS2:GFP internalization leads to complete degradation of the FLS2:GFP fusion proteins in the plasma membrane (Robatzek et al., 2006). Based on our observations of SUB:EGFP localization, it appears in the endosome but we do not know whether SUB:EGFP is also resorted and recycled back to the plasma membrane. SUB:EGFP recycling is BFA sensitive so, at least in part, resembles the BRI:GFP and PIN1 endocytic recycling pathway. In animals it has been shown recently that ligand-induced Try kinases receptor endocytosis not only down regulate signaling but it is also a prerequisite for signaling (Gonzalez-Gaitan, 2003).

In summary, we have performed initial colocalization studies of SUB:EGFP within the cell. Our analysis reveals plasma membrane localization of SUB:EGFP. The SUB:EGFP also colocalizes with endosome in normal physiological condition. The internalization pathway of SUB:EGFP from the plasma membrane is sensitive to the BFA treatment.

### 3.8.2 Spatial and temporal expression pattern of SUB during organogenesis

SUB:EGFP is expressed in all the meristematic zones of cell division in a developing plant. Expression pattern of fusion proteins was not identical to the previously reported mRNA *in situ* hybridization (Chevalier et al., 2005) or promoter reporter expression pattern (Martine Batoux and Kay Schneitz unpublished data). Interestingly, slightly variant SUB:EGFP expression pattern from mRNA complemented *sub* mutant phenotype. The expression pattern of fusion protein was varied from tissue to tissue. In roots, for example the SUB:EGFP fusion protein is detected in stele cells, while the reporter GUS and mRNA *in situ* hybridization studies indicate presence of *SUB* transcript in all root cell layers. We had a mRNA vs. protein expression pattern dissimilarity in flower and inflorescence meristems too. The *SUB* transcript was detected in all tissues in stage-1 to stage-3 flower as well as in inflorescence meristems. Contrary to the mRNA expression pattern, we observed SUB:EGFP fusion protein expression pattern restricted up to L3 layer in flower and inflorescence meristems dome. We do not see identical expression pattern of mRNA vs. protein in ovules also. When we take into account *sub* mutant phenotype, it reveals that where ever we see a defect, the *SUB* transcript is present but we do not see the SUB:EGFP fusion protein. This raises several questions. Does SUB signaling acts in non-cell autonomous fashion? If SUB acts non-cell autonomously, what is the biological significance of such a differential mRNA vs. protein expression pattern during the development? How from a wide spread mRNA expression pattern a more restricted protein expression pattern is established?

### 3.8.3 Differential expression of SUB mRNA vs. protein during development

The differential distribution of *SUB* mRNA transcript vs. protein is a interesting aspect of the functional analysis. We really do not know what is the mechanism

behind it. One attractive hypothesis would be that *SUB* transcript is rapidly degraded in the outer layer of flower and inflorescence meristems. The role of 22-nt long endogenous RNAs, termed as microRNAs (miRNAs) have been established in animal and plant development (Lee et al., 1993; Wightman et al., 1993; Palatnik et al., 2003). Both plant and animal miRNAs differ in their mechanism of gene silencing. The animal miRNAs predominately acts via translational repression of targets which leads to mRNA degradation (Bagga et al., 2005; Lim et al., 2005). In contrast, plant miRNAs guide mRNA cleavage (Llave et al., 2002; Kasschau et al., 2003). MicroRNA-guided mRNA translation repression is not limited to animals. Indeed, miR172 guides translation repression of *AP2* mRNA in plants without affecting the level of mRNA (Aukerman and Sakai, 2003; Chen, 2004). However, (*pSUB::CDB:GUS*) reporter fusion under the native promoter do not show any selective decay of mRNA transcript. So we ruled out the possibility of mRNA cleavage. But we can speculate of the miRNA mediated selective translation repression of *SUB* transcript without mRNA decay. If it is true then where does such a miRNA binds. From our studies, expressing *SUB* protein coding region under heterologous promoters (Chapter-4) we do not see any such translational repression. Therefore, it is likely that such miRNA binding sites are present in the *SUB* cDNA at 5' *UTR* or 3' *UTR* region. The *pSUB::CDB:GUS* promoter reporter construct carries the 5' *UTR* and 3' *UTR* region, but it do not show any selective translation repression of *CDB:GUS* protein in planta (Martine Batoux and Kay Schneitz unpublished data).

*SUB* appears to be unique among the plant RLKs studied to date, in terms of its differential mRNA vs. protein expression pattern. Further studies would be required not only to understand this differential expression pattern but also to establish the functional significance of this differential expression pattern in plant development.

## Chapter 4      **STRUBBELIG acts in a non-cell autonomous fashion during development**

### 4.1      **Summary**

**Multicellular organisms coordinate cell division and growth to regulate organ shape and size. Thus, intercellular communication is an essential biological process to deliver the information to coordinate growth across cell layers. *SUB* mediated signaling controls the mechanism of cell division and pattern of cell divisions to coordinate growth across tissues. We used a promoter rescue approach to examine the range of such signaling. *SUB:GFP* expressed under various promoters suggests that when the protein is expressed early in development, it is able to rescue the *sub* mutant phenotype in a non-cell autonomous fashion. We do not see any direct movement of *SUB:GFP* itself in non-cell autonomous rescue. However, from our analysis it appears that *SUB* signaling could be mediated across cell layers.**

### 4.2      **Introduction**

Plants form organs throughout their life by coordinated cell proliferation and differentiation. The role of intracellular and intercellular signaling has been established in controlling cell division pattern and cell specification during plant development (Scheres, 2001). How does intercellular signaling control these dynamic cellular events that are interlinked to give rise the proper shape and size of regular structures is not understood at molecular level. The shoot apical meristem (SAM) of flowering plant has been subdivided into zones and clonal layers (Satina et al., 1940; Steeves and Sussex, 1989). Intercellular communication allows cells to coordinate their cell division pattern and cell division plane within and between the cell layers to give rise to organs with fixed shape and size (Meyerowitz, 1997). The *SUB*-mediated signaling pathway controls organ shape and size by coordinating cell proliferation and cell morphogenesis during development (Chevalier et al., 2005). We analyzed the *SUB:EGFP* fusion protein expression

pattern in planta and found that SUB protein is not present where we see *sub* mutant phenotype. It is most likely that *SUB* mediated signaling pathway controls cell morphogenesis and cell proliferation in a non-cell autonomous fashion. In the absence of direct transfer of SUB:GFP, the non-cell autonomous signaling probably mediated through a down stream response. What could be the nature of such a downstream response in non-cell autonomous signaling, we still do not know yet. In plants intercellular communication is evident by ligand-receptor mediated apoplastic pathway and plasmodesmata (PD) mediated symplasmic pathway. Evidence from several studies indicate that regulatory proteins and RNAs can traffic through the PD and play crucial role in cell-to-cell communication (Lucas et al., 1995; Kim et al., 2001; Nakajima et al., 2001; Wada et al., 2002). Depending upon the nature of trafficking molecules, this can occur either by passive diffusion or targeted selectively, if they are under size exclusion limit (SEL) of the channel (Tucker, 1982).

To investigate the non-cell autonomous function of SUB in detail we had made several chimeras using promoter SUB:GFP and analyzed the rescue of *sub* phenotype in detail. We choose *ANT*, *WUS* and *AtML1* promoter for initial analysis Both *ANT* and *AtML1* promoter driven SUB:GFP chimeras rescue all the aspect of the *sub* phenotype. Surprisingly, *WUS* promoter led SUB:GFP expression show rescue of stem and ovule phenotype only. Our promoter SUB:GFP rescue studies reveal a non-cell autonomous function for SUB signaling during development.

### 4.3 Results

#### 4.3.1 *ANT::SUB:GFP* is able to rescue the *sub* mutant phenotype

To understand the non-cell autonomous function of *SUB* we expressed SUB:GFP under the *AINTEGUMENTA* (*ANT*) promoter. *ANT* encodes an AP-2 domain family transcription factor and is required for normal integument development in ovules (Elliott et al., 1996; Klucher et al., 1996). *ANT* also controls organ size through directly affecting cell proliferation (Mizukami and Fischer, 2000). *ANT* mRNA expression is detected in the lateral organs early on in shoot apical meristem and in



ovules (Elliott et al., 1996). If we look the *SUB* mRNA expression it is expressed in the shoot apical meristem as well as in the lateral organs such as flower meristem. Contrary to *SUB*, *ANT* is expressed only in lateral organs. Therefore, we asked the question if *SUB:GFP* expressed under the *ANT* promoter can rescue the *sub* phenotype.

A construct carrying a 6.5 kb *ANT* promoter region that includes the 5' regulatory element of *ANT* (Schoof et al., 2000) was cloned adjacent to 5' *SUB:GFP* in the pCAMBIA2300 vector. Both *sub-1* mutant and WT-Ler plants were transformed with *ANT::SUB:GFP* construct. Transgenic T1 plants were screened under epifluorescence microscope for visible GFP expression in integument of ovules. Transgenic plant lines were scored weak, moderate and bright in terms of the intensity of GFP fluorescence. Thirty day old plants were looked for *sub* phenotype rescue. *ANT::SUB:GFP* rescued *sub* phenotype in 33% transgenic line (Table 4.1).

To characterize the transgenic lines in detail for all the aspects *sub* phenotype rescue, two bright *ANT::SUB:GFP* lines (#34.1, #79) were selected in *sub-1* background. Stage 4-V ovules were analyzed by scanning electron micrograph (SEM) for outer integument rescue in selected *sub-1 ANT::SUB:GFP* transgenic lines. The SEM analysis of line 79 (n=289) and 34.1 (n=188) showed 100% outer integument rescue (Fig 4.1B). To determine the flower organ number rescue. Flowers organs were counted in stage 13 to -15 flowers and also documented morphology of overall flowers (Fig. 4.1E)(Table 4.2). At macroscopic level, twisting of stem was rescued by *ANT::SUB:GFP* (Fig. 4.1H). Close microscopic analysis revealed that *ANT::SUB:GFP* rescue twisted carpel phenotype (Fig 4.2B). Furthermore, when 30-day old *ANT::SUB:GFP sub-1* plants were compared with *sub-1* and WT plant, *ANT::SUB:GFP* rescued *sub* semi-dwarfism phenotype (Fig 4.2E).

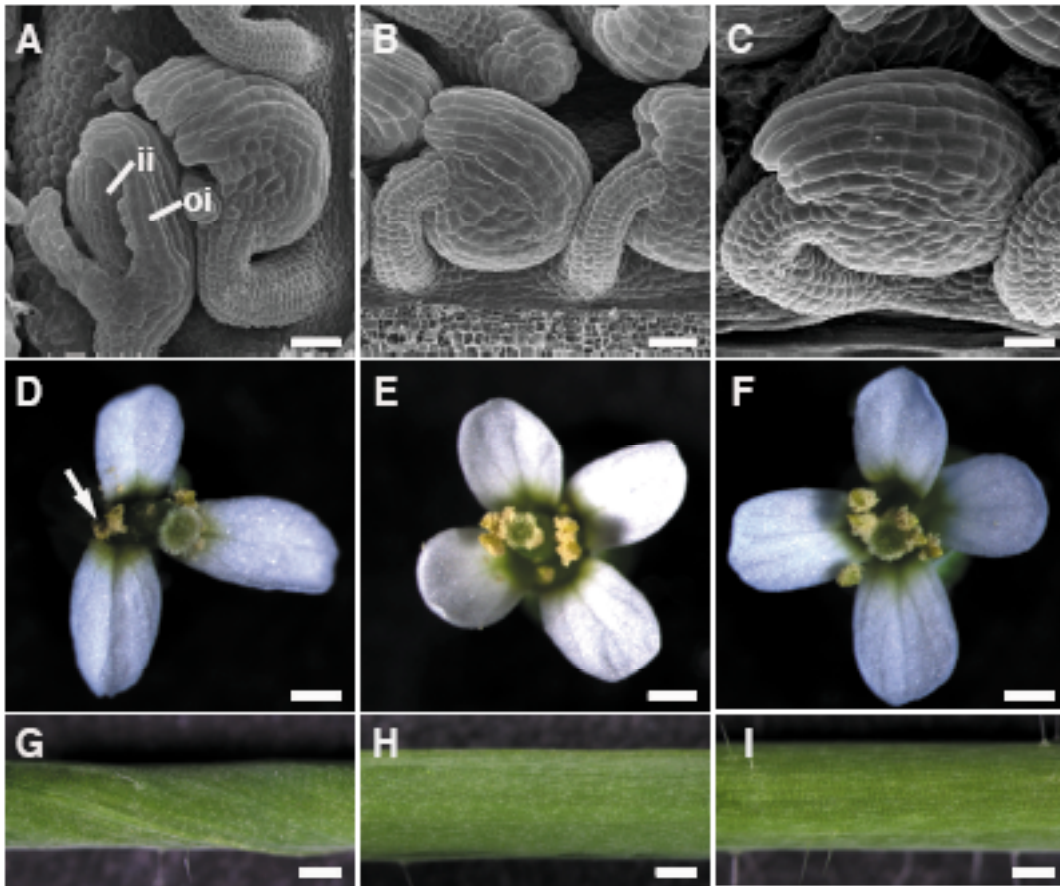


Fig. 4.1 Ovules, flowers, and stem of *sub-1*, *ANT::SUB:GFP sub-1* and WT-Ler *ANT::SUB:GFP*. (A, D, and G) *sub-1*. (B, E, and H) *sub-1* rescued by *ANT::SUB:GFP*. (C, F, and I) WT-Ler *ANT::SUB:GFP*. (A-C) Scanning electron micrograph of ovule at stage 4-V. (D-F) Flowers at stage 13. (G-I) 30-day old stem, 1 cm below the first secondary inflorescence. (A) Stage 4-V ovule of *sub-1*. Note the aberrant outer integument. (B) Stage 4-V ovule of *sub-1* mutant rescued by *ANT::SUB:GFP*. (C) Stage 4-V ovule of WT-Ler *ANT::SUB:GFP*. (D) *sub-1* mutant flower. Arrow indicate missing petal. (E) *sub-1* mutant rescued by *ANT::SUB:GFP*. (F) WT-Ler *ANT::SUB:GFP*. (G) Twisted stem of *sub-1*. (H) *sub-1* stem rescued by *ANT::SUB:GFP*. (I) Stem of WT-Ler *ANT::SUB:GFP*. Abbreviations, ii, inner integument. oi, outer integument. Scale bars, (A-C) 20 $\mu$ m (D-I) 0.5mm.

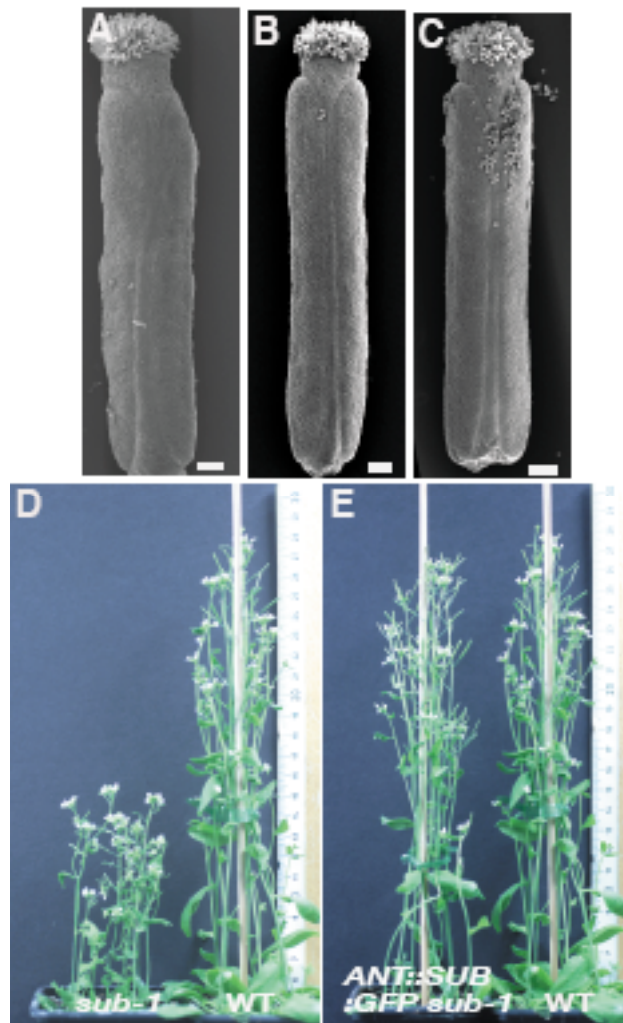


Fig. 4.2 Carpel morphology and plant height of *sub-1*, *ANT::SUB:GFP sub-1* and *ANT::SUB:GFP WT Ler*. (A and D left pot) *sub-1*. (B and E left pot) *sub-1* rescued by *ANT::SUB:GFP*. (C) WT-Ler *ANT::SUB:GFP*. (D right pot and E right pot) WT-Ler. (A-C) Scanning electron micrograph of mature carpels. (D and E) Whole picture of 30-days old plants. (A) Twisted carpel of *sub-1* mutant. (B) *ANT::SUB:GFP sub-1*, rescued carpel. Note carpel twisting rescued. (C) WT-Ler *ANT::SUB:GFP* carpel. (D) *sub-1* mutant plants and WT-Ler plants. The *sub-1* mutant display semi-dwarfism. (E) *sub-1* mutant rescued by *ANT::SUB:GFP* and WT-Ler. The semi-dwarfism of *sub* mutant rescued by *ANT::SUB:GFP*. Scale bars (A-C) 100 $\mu$ m.

**Table 4.1 Rescue summaries of *sub* mutant *ANT::SUB:GFP* T1 transgenic plant lines**

Total Plants	Rescued <i>sub</i> phenotype	Visible GFP expression	Percentage rescue
<i>ANT::SUB:GFP sub-1</i> # 134	44	35	33%
<i>ANT::SUB:GFP WT</i> # 119		31	

**Table 4.2 Flower organ number in *ANT::SUB:GFP sub-1* and WT rescued plants**

Genotype	Sepals	Petals	Stamens	Carpel	N
<i>sub-1 ANT::SUB:GFP</i>	4	4	5.2±0.7	2	101
<i>WT ANT::SUB:GFP</i>	4	4	5.4±0.6	2	80

Taken together, the above data of *ANT::SUB:GFP sub-1* rescued transgenic plants revealed that *SUB:GFP* expressed under *ANT* promoter in the lateral organs is sufficient to rescue the *sub* phenotype in the inflorescences meristem including stem twisting and stem height.

#### **4.3.2 *SUB:GFP* protein expression pattern under *ANT* promoter is similar to the *ANT* mRNA expression pattern**

The expression patterns of a given promoter not only depend on the regulatory element but also influenced by insertion site position within the genome. It was crucial to know whether *ANT::SUB:GFP* expression pattern mimicked the WT *ANT* mRNA expression pattern or not. We investigated the expression pattern of *ANT::SUB:GFP* in the ovules of rescued lines (#34.1 and #79) in detail. Same lines were used for rescue analysis as well as for live imaging studies. The expression of *SUB:GFP* fusion protein was observed in the inner and outer integument of ovules

as well as in the chalaza and funiculus (Fig 4.3B). This protein expression pattern in ovules mimics the WT *ANT* mRNA expression, as reported in previous studies (Elliott et al., 1996). This result suggests that the *ANT::SUB:GFP* transgenic lines show correct expression pattern in the ovules.

The *ANT* promoter led *SUB:GFP* fusion protein expression was confined to emerging primordia in the inflorescence meristem (Fig 4.3D). *SUB:GFP* fusion protein was detected early on during the initiation of flower primordia in the L1 layer in the inflorescence meristem. The pattern of *ANT* led *SUB:GFP* fusion protein expression was very similar to the RNA *in situ* hybridization patterns described previously (Elliott et al., 1996). The *ANT::SUB:GFP* construct rescued the *sub* mutant phenotype.

Taken together, the above protein expression pattern data suggests that the *ANT::SUB:GFP* expression pattern was correct in the inflorescences meristem. *ANT* mRNA expression pattern overlap the *SUB* mRNA expression pattern in the lateral organs but was not present in the inflorescence meristem. Therefore, it can be concluded that *SUB:GFP* expression from lateral organs rescued *sub* phenotype. The possible signal is generated in the *SUB:GFP* expressing cells and could pass to neighboring cells and rescue the *sub* mutant phenotype. This result suggest that *SUB* could signal in non-cell autonomous fashion in the shoot apical meristem to rescue the stem related aspect of the *sub* mutant phenotype. By looking at the top view of *ANT::SUB:GFP* expression in the shoot apex, it appears that the lateral organs are continuously made within the meristem, therefore the *SUB:GFP* expression confined to a group of cells (called organ founder cells) in the beginning and later on the *SUB:GFP* fluorescence follow the budding lateral organ primordia. Recent live imaging studies of primordial markers in the meristem revealed that the number of organs founder cells is around 60 cells, just before the primordium grows out (Grandjean et al., 2004; Reddy et al., 2004). The *ANT::SUB:GFP* top view of expression pattern indicates that the lateral organ primordia are founded in close vicinity on the periphery of shoot apex. The distance between the cells expressin the *SUB:GFP* and the cell receiving the non-

cell autonomous signal probably not too far on the periphery of shoot apical meristem. But at the flanks of meristem the distance between the cells providing the signal and the cells receiving it, increases slightly more.

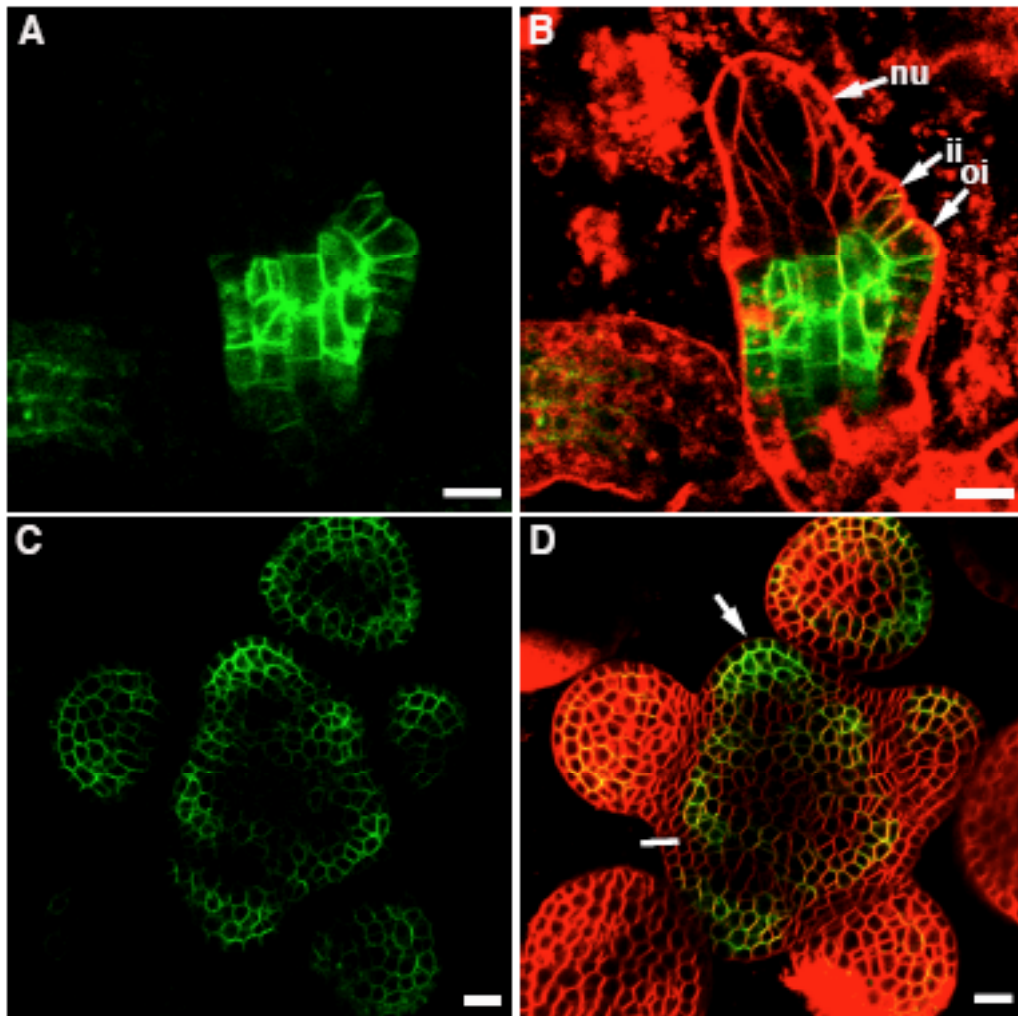


Fig. 4.3 SUB:GFP expression pattern under *ANT* promoter. (A and B) Ovule (C and D) shoot apical meristem of plant carrying SUB:GFP fusion protein and treated with FM4-64. Cells were simultaneously observed using the GFP channel (A and C) and FM4-64 channel and images were then merged (B and D). (D) Arrow indicates the SUB:GFP in the young flower primordia whereas the line indicates the region between the two young floral primordia where SUB:GFP is not expressed. Abbreviations, ii, inner integument. nu, nucellus. oi, outer integument. Scale bars, (A-D) 10 $\mu$ m.

Given the dynamic nature of such a expression pattern in space and time it would be useful in future studies to ascertain the exact distance between the cell showing visible SUB:GFP fluorescence signal and the cells that do not show the fluorescence signal

Compared to *pSUB::SUB:EGFP* expression pattern, *ANT::SUB:GFP* driven SUB:GFP fusion protein expression is restricted to the lateral organ primordia, in L1, L2 and in L3 layers. These localized protein expression domains appear in the inflorescence meristem at regular intervals. Within the inflorescence meristem where a neighboring wild type cells rescue the mutant cells probably between layers as well as within the layers. This observation indicates that the SUB mediated non-cell autonomous signaling could transmits the signal to the neighboring cells.

When we compare the *pSUB::SUB:EGFP* expression pattern with *sub* mutant phenotype, it appears that SUB signaling transmits signal across layers in polar fashion from inner layer to outer layers and organize the cell division orientation. From our analysis of *ANT::SUB:GFP* expression pattern it seems that the signaling could be transmitted from outer layer to inner layers as well. The protein expression domain is broader under *pSUB* promoter in inflorescence meristem while under *ANT* promoter, it appears in several small sub-domains across the inflorescence mersitem. In such a situation it is difficult to predict the range of non-cell autonomous signaling. To address this question we used *WUS* promoter led SUB:GFP fusion protein expression and analyzed the *sub* phenotype rescue.

### **4.3.3 *sub* phenotype partially rescued by *WUS::SUB:GFP***

From *ANT::SUB:GFP* it was difficult to predict the range of SUB non-cell autonomous signaling clearly. To address, how far SUB signaling could rescue *sub* phenotype non-cell autonomously, we choose *WUS* promoter for further analysis. *WUS* mRNA expression is detected in L2 layer in flowers and in L3 layer in

inflorescence meristems during development (Mayer et al., 1998). Though, *WUS* mRNA also expresses in the ovules up to stage-3 (Gross-Hardt et al., 2002). *WUS::SUB:GFP* transgenic lines were created by transforming *WUS::SUB:GFP* construct into *sub-1*, and *Wt-Ler* background. T1 transgenic plants were scored on the basis of GFP expression in ovules under epifluorescence microscope. For *sub* mutant phenotype rescue, 30-days old plants were looked for plant height rescue and also scored for other aspects.

**Table 4.3 Rescue summaries of 42 *sub-1* T1 transgenic plants screened for *sub* phenotype rescue by *WUS::SUB:GFP* construct.**

Rescued phenotype	No. of plant rescued	Percentage rescue
Stem twisting	21	50%
Carpel twisting	0	0%

In total, 19 plants show a visible GFP signal in ovules out of 42 lines screened in T1, but only 8 lines showing the GFP signal was able to rescue the stem twisting phenotype (Table 4.3). So, rest of the plant lines expresses visible GFP level and do not rescue the phenotype. This observation is contrary to the *ANT::SUB:GFP* rescue summary, where all the plants showing a visible GFP expression at least rescued the phenotype. How to explain such an observation? If we see a visible level of GFP expression and it rescues the phenotype then one could argue, that it is due to visible *SUB:GFP*. Contrary to this we see a visible GFP signal in 11 T1 plants that does not rescue the *sub* phenotype. One argument would be that we tested the independent lines on the basis of the *SUB:GFP* fluorescence in the ovules for scoring visible lines and bright lines. In this scenario a line may show signal in the ovule, but may be weak in inflorescence meristem. The other argument would be that *SUB:GFP* expression within the inflorescence is variable in terms of its precise expression pattern. The *WUS* expression domain appears round in its outline in inflorescence meristem, the upper boundary of this outline appear in the L3 and some time below L3 layer. Lines showing expression below L3 layer probably do not rescue the phenotype. This probably, implies the



role of precise distance of signal from the source or location of SUB:GFP fusion protein in the *WUS* expression domain as well as the amount of protein. Though, it is pure speculation, it needs to be tested. For this reason, a promoter expressing below L3 layer would be attractive to study the range of such signaling. *WUS::SUB:GFP* was able to rescue the stem twisting phenotype and ovule outer integument in the screened plants. It is unable to rescue the other aspects, this observation indicates that SUB mediated signaling is not able to act in long range

### **4.3.4 Stem twisting and outer integument of ovule rescued by *WUS::SUB:GFP***

To characterize the rescued lines in detail for ovule and flower organs rescue, two (#52, #153, *sub-1 WUS::SUB:GFP*) lines were studied in detail. Stage 4-V ovules were analyzed by SEM for outer integument rescue. The line #52 showed 41% rescue of the outer integument rescue (n=233), while line #153 showed 65% outer integument rescue (n= 235) (Fig 4.4B). In flowers *WUS* promoter activity switches off at flower stage-6 when carpel are formed. To determine the flower phenotype rescue stage 13 to-15 flower organs were counted (Table 4.4). The flower of *WUS::SUB:GFP* rescued transgenic lines still show aberrant morphology of petals (Fig 4.4E). The *WUS* promoter expresses in the organizing centre within the shoot apical meristem throughout the development thus *WUS::SUB:GFP* rescue the stem twisting phenotype (Fig 4.4H) The *WUS::SUB:GFP* is unable to rescue the carpel phenotype of the *sub* mutant (Fig. 4.5B). Though, the semi-dwarfism of *sub* mutant was rescued by *WUS::SUB:EGFP* (Fig 4.5E). In summary, our results suggest that *WUS::SUB:GFP* is able to rescue the twisting of stem and plant height.

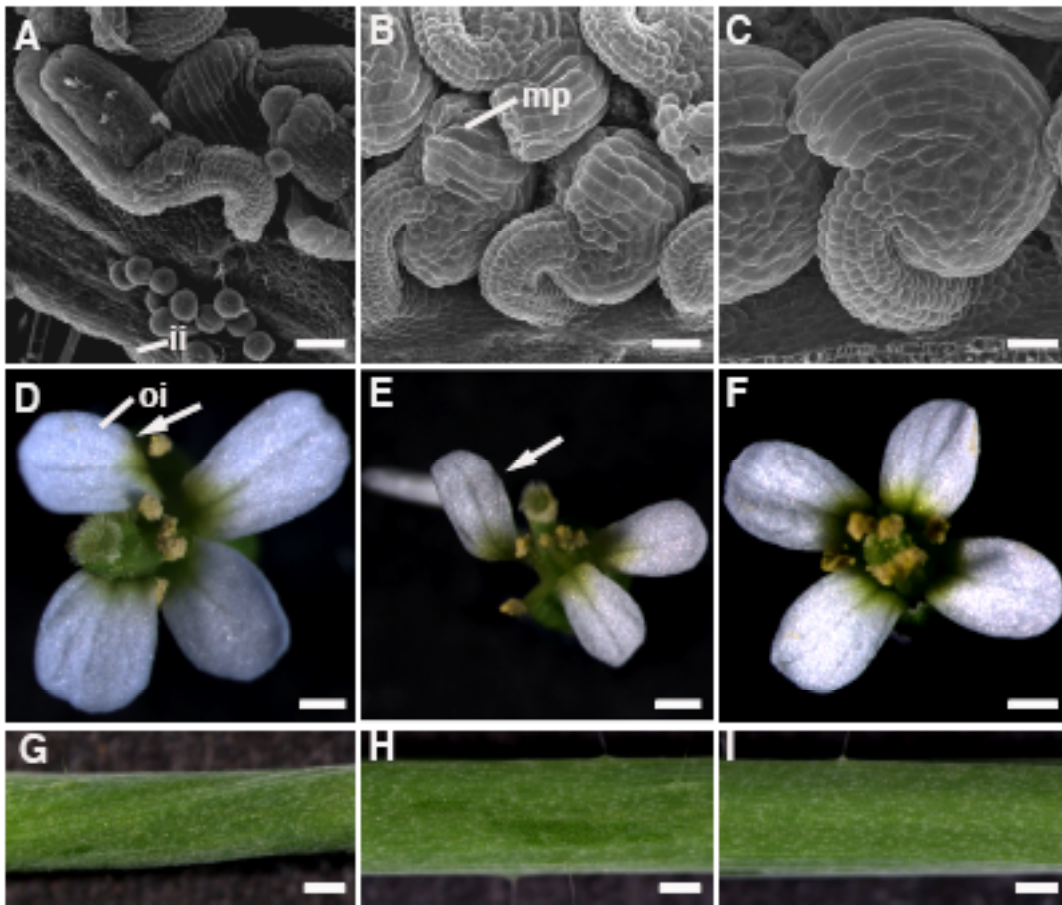


Fig. 4.4 Ovules, flowers, and stem of *sub-1*, *WUS::SUB:GFP sub-1* and WT-Ler *WUS::SUB:GFP*. (A, D, and G) *sub-1*. (B, E, and H) *sub-1* rescued by *WUS::SUB:GFP*. (C, F, and I) WT-Ler *WUS::SUB:GFP*. (A-C) Scanning electron micrograph of ovule at stage 4-V. (D-F) Flowers at stage 13. (G-I) 30-days old stem pictures, 1 cm below the first secondary inflorescence. (A) Stage 4-V ovule of *sub-1*. Note the aberrant outer integument. (B) Stage 4-V ovule of *sub-1* mutant rescued by *WUS::SUB:GFP*. Note the cells in the micropyle region show aberrant morphology. (C) Stage 4-V ovule of WT-Ler *WUS::SUB:GFP*. (D) *sub-1* mutant flower. Arrow indicate twisted petal. (E) *sub-1* mutant *WUS::SUB:GFP*. Note the petal twisting is not rescued. (F) WT-Ler *WUS::SUB:GFP*. (G) Twisted stem of *sub-1*. (H) *sub-1* stem rescued by *WUS::SUB:GFP*. (I) Stem of WT-Ler *WUS::SUB:GFP*. Abbreviations, ii, inner integument. mp, micropyle. oi, outer integument. Scale bars, (A-C) 20µm (D-I) 0.5mm.

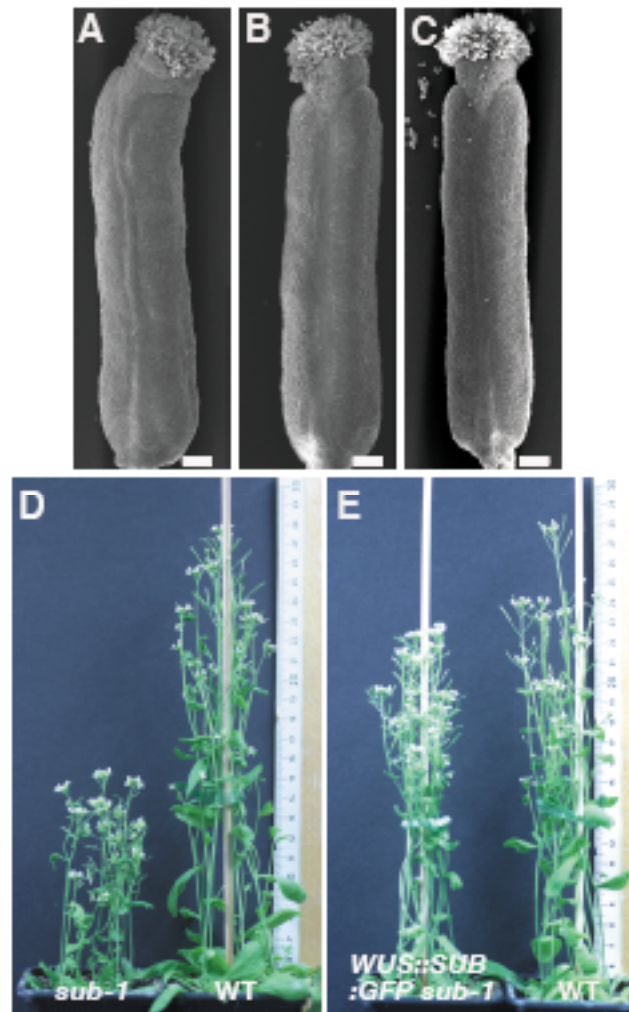


Fig. 4.5 Carpel and plant height of *sub-1*, *WUS::SUB:GFP sub-1* and WT Ler. (A and D left pot) *sub-1*. (B and E left pot) *sub-1* rescued by *WUS::SUB:GFP*. (C) WT-Ler *WUS::SUB:GFP*. (D right pot and E right pot) WT-Ler. (A-C) Scanning electron micrograph of mature carpels. (D and E) Whole picture of 30-days old plants. (A) Twisted carpel of *sub-1* mutant. (B) *WUS::SUB:GFP sub-1*, carpel. Note the twisting of carpel is not rescued. (C) WT-Ler *WUS::SUB:GFP* carpel. (D) Left side is *sub-1* mutant plants while at the right side same age WT-Ler plants. The *sub-1* mutant display semi-dwarfism. (E) Left side, *sub-1* mutant rescued by *WUS::SUB:GFP* whereas at right side WT-Ler. The semi-dwarfism of *sub* mutant rescued by *WUS::SUB:GFP*. Scale bars (A-C) 100 $\mu$ m.

**Table 4.4 Flower organs numbers in the *WUS::SUB:GFP* rescued plants**

Genotype	Sepals	Petals	Stamens	Carpels	N
#153 <i>WUS::SUB:GFP</i> in <i>sub-1</i>	3.9±0.1	3.9±0.1	5.1±0.7	2	80
#32 <i>WUS::SUB:GFP</i> in WT	4	4	5.4±0.6	2	87

#### 4.3.5 *WUS::SUB:GFP* expression pattern

*WUS* mRNA is expressed in the flower meristem below L1-layer and in shoot apical meristem below L2-layer (Jan Lohmann personal communication). In ovules, *WUS* mRNA expression was detected in the nucellus very early in ovule development but is switch off at stage-3 of ovule development (Gross-Hardt et al., 2002). To assess *WUS* promoter led *SUB:GFP* fusion protein expression pattern, we analyzed GFP fluorescence in ovules, flowers and shoot apical meristems by CLSMs. The *SUB:GFP* expression driven by *WUS* promoter in ovules is confined to distal part of the ovule, specifically the nucellus in stage 2-III ovules (Fig 4.6B). No expression was detected in chalaza or funiculus. This expression of *WUS::SUB:GFP* completely mimics the WT *WUS* mRNA expression pattern described (Gross-Hardt et al., 2002). We observed the *SUB:GFP* fusion protein in the L2-layer in the flower meristem and in L3-layer of shoot apical meristem (Fig 4.6D). Compared to a previous study in which *WUS* promoter led protein expression was analyzed (Grandjean et al., 2004), our data of *WUS::SUB:GFP* led *SUB:GFP* expression in flower and inflorescence meristems show an identical expression pattern. These results indicate that the line expressing the *SUB:GFP* under *WUS* promoter is suitable for further studies.

Compared to *pSUB::SUB:EGFP* expression in shoot apical meristem, *WUS* driven *SUB:GFP* fusion protein domain is smaller. The similarity is that both are expressed below L2 in the shoot apical meristem (but *WUS::SUB:GFP* is expressed

in few cells in L3). If we assume a cell-autonomous function of *SUB* then the *WUS::SUB:GFP* would not be able to rescue the stem twisting and height phenotype

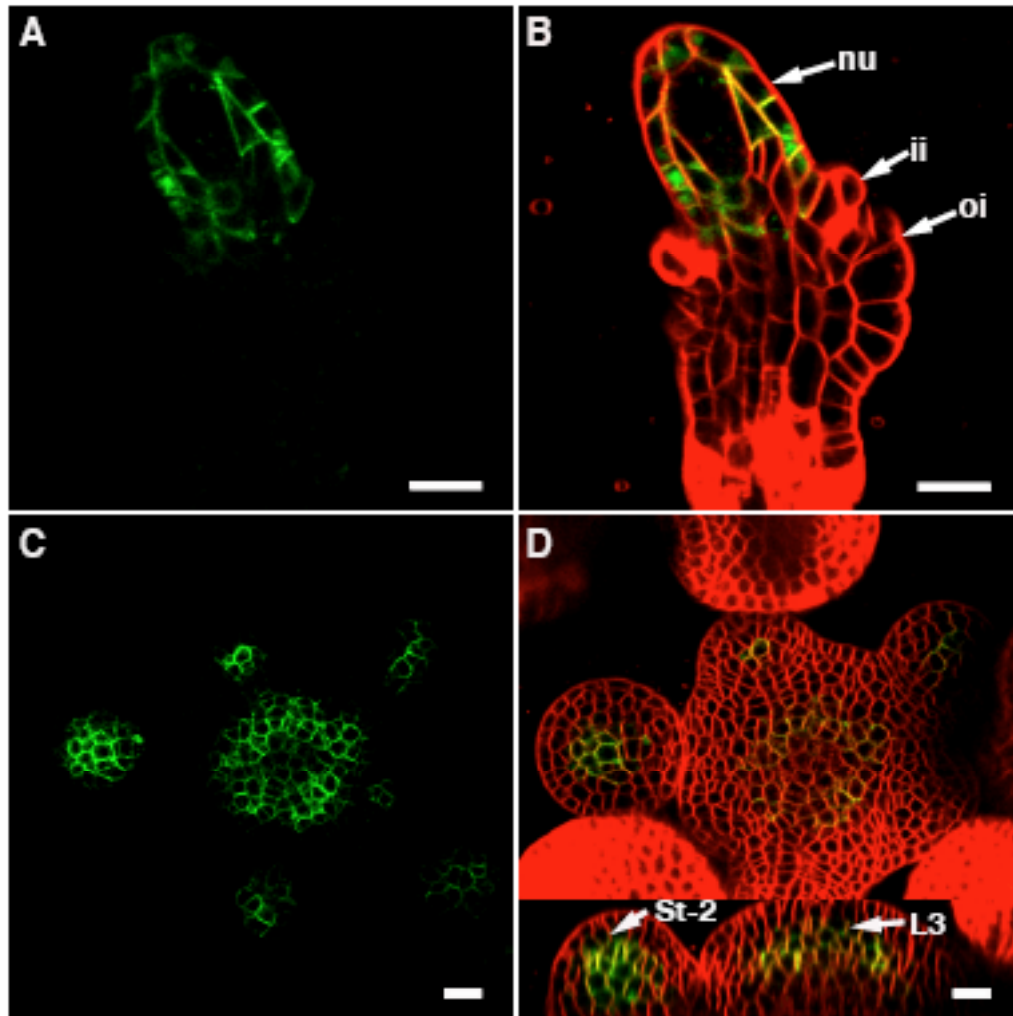


Fig. 4.6 *SUB:GFP* expression pattern under *WUS* promoter. (A and B) Ovule (C and D) shoot apical meristem of plants carrying *SUB:GFP* fusion protein and treated with FM4-64. Cells were simultaneously observed using the GFP channel (A and C) and FM4-64 channel and images were then merged (B and D). (B) *SUB:GFP* is confined to distal part the nucellus. (D) Top view as well as longitudinal section. Note st-2 arrow denote the GFP fluorescence in the L2 layer of stage-2 flower, where arrow indicates *SUB:GFP* in the shoot apical meristem confined up to L3 layer. Abbreviations, ii, inner integument. nu, nucellus. oi, outer integument. Scale bars, (A-D) 10 $\mu$ m.

in *sub* mutant plants. In ovules we also see a partial rescue of outer integument. But in flowers, organs such as petals and carpels are not rescued, because in the flower *WUS* expression is switched off at *satge-6*. This data indicates that *SUB* acts in a non-cell autonomous fashion in short range. In the inflorescence meristem the *WUS::SUB:GFP* is expressed in the organizing centre (OC) throughout the life of a plant and signal from these cell can act down in rib meristem as well as in shoot apical meristem. The partial rescue of *sub-1* mutant phenotype by *WUS* driven *SUB:GFP* suggest possible non-cell autonomy of *SUB* signaling across the layers. To rescue the stem height phenotype most likely *SUB* signaling required in the rib zone meristem. From the analysis of *ANT::SUB:GFP* it is apparent that *SUB* mediated non-cell autonomous signal reaches to the rib zone cells and thus, it rescues the stem height of the *sub* mutant plants.

Taken together, the data from *ANT* and *WUS* promoter rescue studies show that *SUB* non-cell autonomous function rescues the *sub-1* mutant phenotype from apical side as well as from basal side. This observation indicates that *SUB* non-cell autonomous signaling is non polar in the inflorescence. Moreover, it appears that the non-cell autonomous signaling acts across only a few cell layers.

#### **4.3.6 *AtML1::SUB:GFP* (*L1::SUB:GFP*) rescued L2-layer periclinal division in non-cell autonomous fashion**

We used an alternative approach to test bi-directionality of *SUB* non-cell autonomous signaling. We asked the question if we express *SUB:GFP* under L1-layer specific promoter could it able to rescue the L2-layer periclinal cell division plane. The *sub* mutant plant shows L2-layer periclinal divisions in the young floral meristems. Cell shape also seems irregular in the L2-layer compare to WT (Chevalier et al., 2005). The *ANT::SUB:GFP* is expressed in lateral organ primordia and present in the entire three layers of stage-2 flower meristems. The *WUS::SUB:GFP* is expressed in the L2-layer of flower primordia. In both cases *SUB:GFP* is present in the L2-layer. To test the non-cell autonomous rescue of the

L2-layer periclinal division phenotype we need to express SUB in such a way that it surrounds the L2-layer from the outer side. Therefore we decided to use the L1 specific promoter, *AtML1*, that is expressed in the L1-layer of vegetative and reproductive shoot apical meristems as well as in the young floral meristems (Lu et al., 1996). The integuments of the ovule also express the *AtML1*.

We made a *L1::SUB:GFP* fusion and created transgenic lines in the *sub-1* mutant and *Wt-Ler* background. The 30-day old transgenic lines were screened for phenotypic rescue, such as stem twisting, stem height, flower organs rescue. We looked ovules to screen individual bright GFP line for L1 promoter. The *L1::SUB:GFP* expression in the ovule was confined to the epidermal tissue of the inner and outer integuments (Fig 4.7B). In the flower meristem, the *L1::SUB:GFP* expression was visible in the L1-layer (Fig 4.7D). This expression pattern was similar to RNA *in situ* hybridization and promoter reporter patterns described previously (Lu et al., 1996; Sessions et al., 1999).

Out of one hundred plants screened, 25% showed complete rescue of the *sub* mutant phenotype (Table 4.5). Two lines (#1.3, # 20.10, *sub-1 L1::SUB:GFP*) were selected for detail phenotypic characterization as well as for confirmation of *L1::SUB:GFP* expression pattern in the inflorescence meristem, flower meristem and ovules.

**Table 4.5 Rescue summary of 100 T1 plants screened for *L1::SUB:GFP***

Rescued phenotype	No. of plants rescued	Percentage rescue
Stem twisting	25	25%
Carpel twisting	25	25%

For outer integument rescue, ovule characterization in the *L1::SUB:GFP* rescued lines was carried out using SEM. We selected two independent bright lines and performed functional analysis of phenotypic rescue as well as confocal

microscopy. In both lines (#1.3 n=214 taken for percentage calculation, #20.10 n=133 taken for percentage calculation) all ovules were rescued 100% for outer

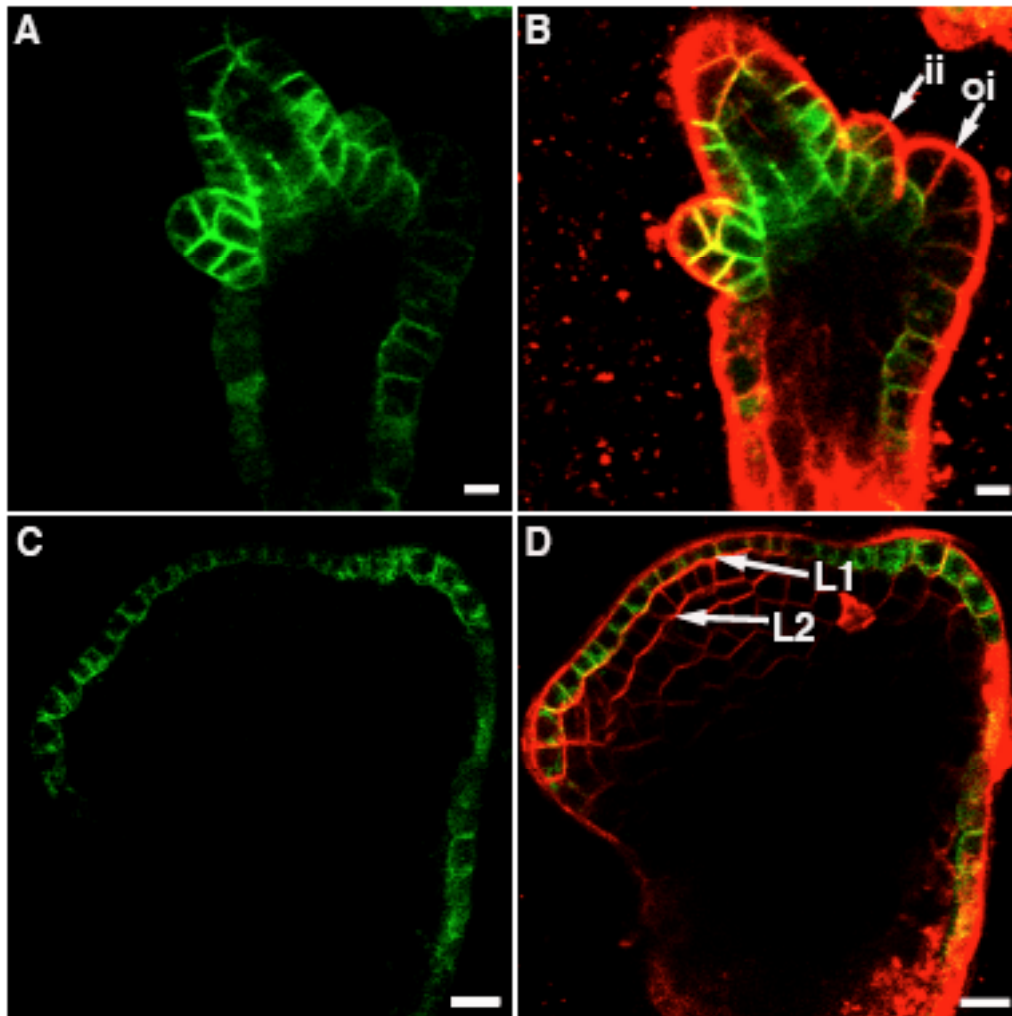


Fig. 4.7 SUB:GFP expression pattern under *L1* promoter. (A and B) Ovule (C and D) shoot apical meristem of plant carrying SUB:GFP fusion protein and treated with FM4-64. Cells were simultaneously observed using the GFP channel (A and C) and FM4-64 channel and images were then merged (B and D). (D) SUB:GFP in the young flower primordia is expressed in the L1 layer. Abbreviations, ii, inner integument. oi, outer integument. Scale bars, (A-D) 10 $\mu$ m



integument phenotype (Fig 4.8B). Rescue of flower organ counting and flower organ morphology was documented using stage 13 to -15 flowers (Fig. 4.8E)(Table 4.6). At the cellular level to analyze rescue of the L2-layer cell division defect, inflorescence meristems of 30-day old plants were fixed and stained with propidium iodide (PI). Stage-3 flower meristems revealed the rescue of the L2-layer periclinal cell division (Fig. 4.8H) (Table 4.7).

**Table 4.6 Flower organ number counting in the 30-day old *L1::SUB:GFP* transgenic lines**

Genotype	Sepals	Petals	Stamens	Carpels	N
<i>L1::SUB:GFP</i> in <i>sub-1</i>	4	4	5.3±0.7	2	64
<i>L1::SUB:GFP</i> in Wt <i>Ler</i>	4	4	5.4±0.5	2	60

**Table 4.7 Rescue of L2-layer periclinal cell division planes by *L1::SUB:GFP***

Genotype	N periclinal division	N meristems	Percentage
Wt- <i>Ler</i>	0	46	0
<i>sub-1</i>	35	84	41.5
<i>L1::SUB:GFP</i> in <i>sub-1</i>	4	62	6.5
<i>L1::SUB:GFP</i> in Wt- <i>Ler</i>	1	63	1.5

Whole plant at 30-day carrying *L1::SUB:GFP* rescued all the aspects of *sub* mutant phenotype. The stem and carpel twisting of *sub-1* mutant phenotype was also rescued (Fig 4.9B, E). The plant height of 30-days old plants were compared with *sub-1* mutant vs. *L1::SUB:GFP sub-1* rescued and Wt-*Ler* (Fig 4.9H).

In summary, taken together the above data suggest that the *L1::SUB:GFP* construct is able to rescue all the aspects of *sub* mutant phenotype. The rescue of L2-layer periclinal cell division suggest that in *sub-1*, if SUB signaling is provided from the apex it could rescue the phenotype. In *pSUB::SUB:EGFP sub-1*,

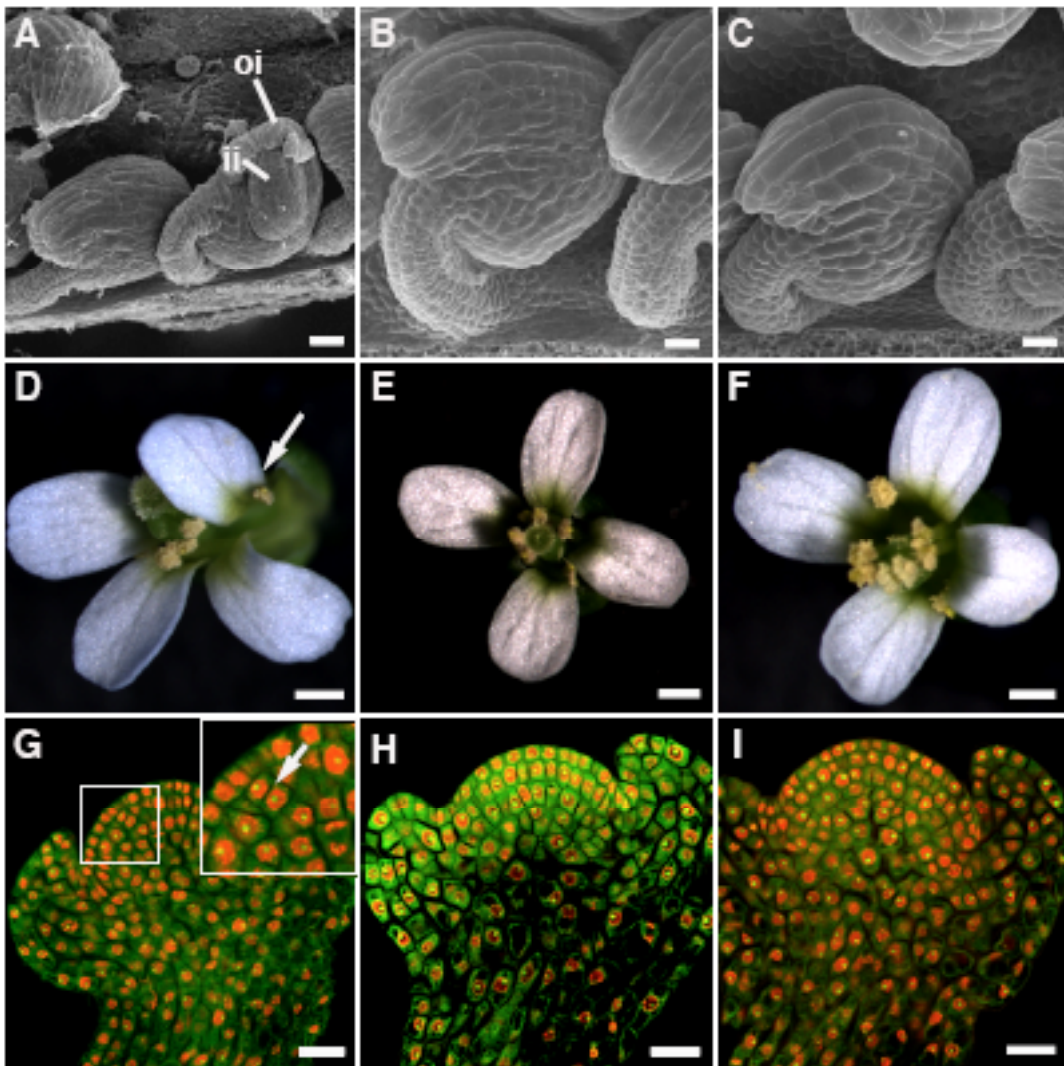


Fig. 4.8 Ovules, flowers, and young floral meristem of *sub-1*, *L1::SUB:GFP sub-1* and WT-Ler. (A, D, and G) *sub-1*. (B, E, and H) *sub-1* rescued by *L1::SUB:GFP*. (C, F, and I) WT-Ler *L1::SUB:GFP*. (A-C) Scanning electron micrograph of ovule at stage 4-V. (D-F) Flowers at stage 13. (G-I) Midoptical section of stage-3 flower meristem obtained from propidium iodide stained whole mount specimen by confocal laser scanning microscopy. (A) Stage 4-V ovule of *sub-1*. Note the aberrant outer integument. (B) Stage 4-V ovule of *sub-1* mutant rescued by *L1::SUB:GFP*. (C) Stage 4-V ovule of WT-Ler *L1::SUB:GFP*. (D) *sub-1* mutant flower. Arrows indicate twisted petal. (E) *sub-1* mutant rescued by *L1::SUB:GFP*. (F) WT-Ler flower. (G) *sub-1* stage-3 floral meristem. (Inset) an example of periclinal cell division orientations in L2 layer indicated by arrow head. (H) Stage-3 flower meristem of *sub-1* rescued by *L1::SUB:GFP*. (I) Stage-3 flower meristem of WT-Ler *L1::SUB:GFP*. Abbreviations, ii, inner integument. oi, outer integument. Scale bars, (A-C) 100 $\mu$ m (D-F) 0.5mm (G-I) 20 $\mu$ m.

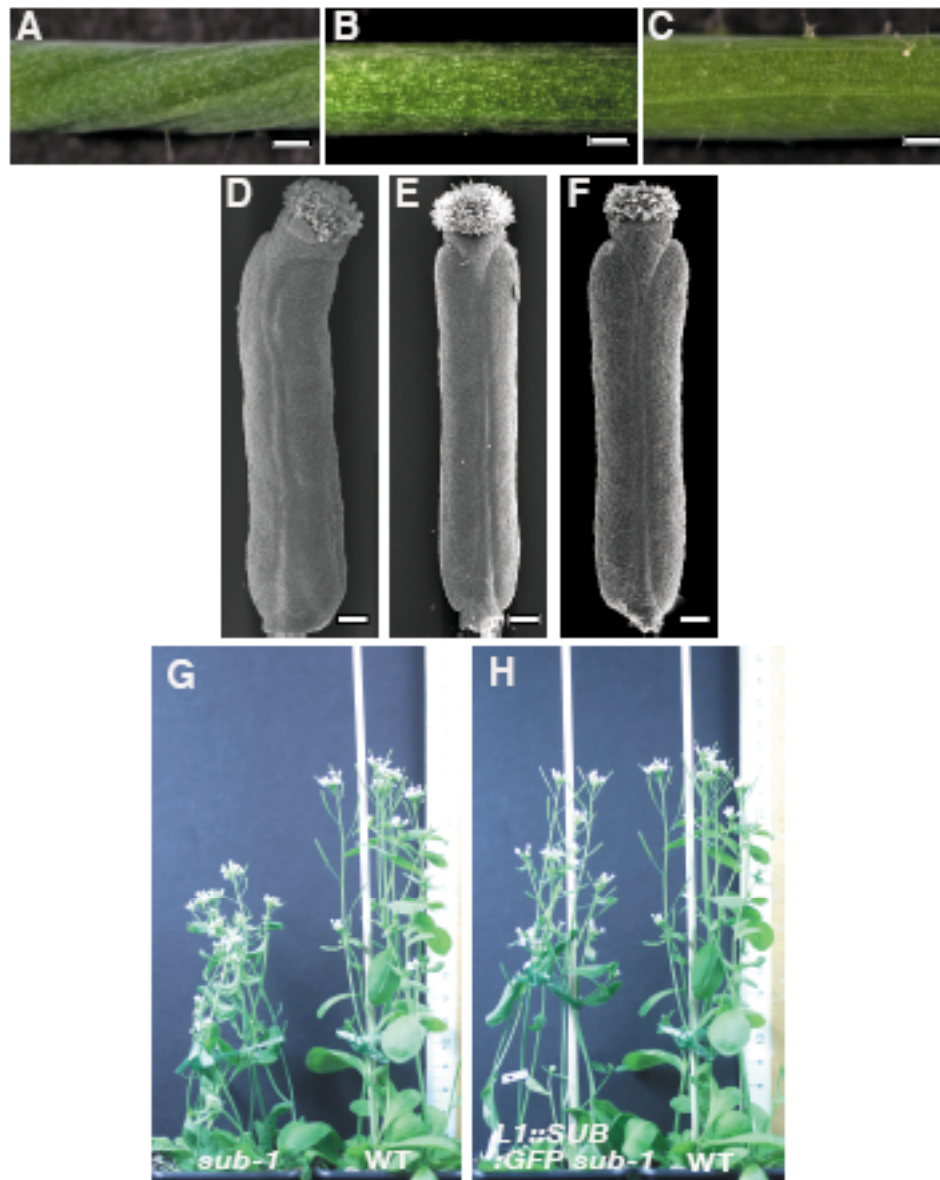


Fig. 4.9 Stem, carpel, and plant height of *sub-1*, *L1::SUB:GFP sub-1* and WT Ler *L1::SUB:GFP*. (A, D, and G left pot) *sub-1*. (B, E, and H left pot) *sub-1* rescued by *L1::SUB:GFP*. (C, F, and H right pot) WT-Ler *L1::SUB:GFP*. (A-C) stem pictures of 30-days old plants. (D-F) Scanning electron micrograph of mature carpels. (G and H) Whole plant pictures of 30-days old plants. (A) Twisted stem of *sub-1* mutant. (B) *L1::SUB:GFP sub-1* rescued stem. Note the twisting of stem rescued. (C) WT-Ler *L1::SUB:GFP* stem. (D) *sub-1* mutant carpel showing twisting. (E) *sub-1* mutant carpel twisting rescued by *L1::SUB:GFP*. (F) WT-Ler *L1::SUB:GFP* carpel. (G) Left side is *sub-1* mutant plants while at the right side same age WT-Ler plants. The *sub-1* mutant display semi-dwarfism. (H) Left side, *sub-1* mutant rescued by *L1::SUB:GFP* whereas at right side WT-Ler The semidwarfism of *sub* mutant rescued by *L1::SUB:GFP*. Scale bars (A-C) 0.5mm, (D-F) 100µm

SUB:EGFP protein stays in the L3-layer and organizing the cell division plane in L2-layer. If SUB signaling is dependent on directionality then the *L1::SUB:GFP* should not rescue the periclinal cell division plane in the L2-layer. Our analysis of L2-layer cell division plane in *L1::SUB:GFP* WT-*Ler* stage-3 flower meristem does not show any apparent division plane defect. This indicates that SUB signaling, when provided from both directions to a L2-layer cell it does not change the orientation of division plane. Moreover from the *L1::SUB:GFP* it also becomes clear that in outer integument, SUB:GFP rescue the outer integument phenotype.

So far we have looked the phenotypic rescue of various promoter chimeras at various tissue level and in *L1::SUB:GFP* stage-3 flower at cellular level . In *sub-1* mutant plant stem there is a reduction of cell numbers observed in the epidermis, cortex and pith. To test the SUB signaling non-cell autonomous rescue impact on the number of cells in the stem we made stem horizontal section of the 30-day old *L1::SUB:GFP sub-1* rescued plants and counted the cells in epidermis, cortex and pith.

#### **4.3.7 *L1::SUB:GFP* rescued partially cell numbers in epidermis, cortex and pith of 30-day old plant stems**

In *sub-1* mutant horizontal stem sections, it has been observed that size and shape of epidermis, cortex and pith cells were altered (Chevalier et al., 2005). In *sub* mutants, a reduced number of epidermal (20%), cortex (30%), and pith cells (20%) were noted (Chevalier et al., 2005). This raises a question; if we express SUB in the epidermis is it able to rescue cell number and the size of cells in the *sub* mutant non-cell autonomously. To test this hypothesis, we made horizontal section of *L1::SUB:GFP sub-1* rescued stem and compared with *sub-1*, WT, and *35S:SUB* in *sub-1* control. The outline of horizontal stem looks wavy in *sub* mutant compare to WT (Fig 4.10A). The epidermal cell size in the *sub* mutant was slightly perturbed compare to WT (Fig 4.10D). To assess the cell number rescue we counted epidermal, cortex and pith cells in *sub-1*, WT, *35S:SUB* in *sub-1* and *L1::SUB:GFP*

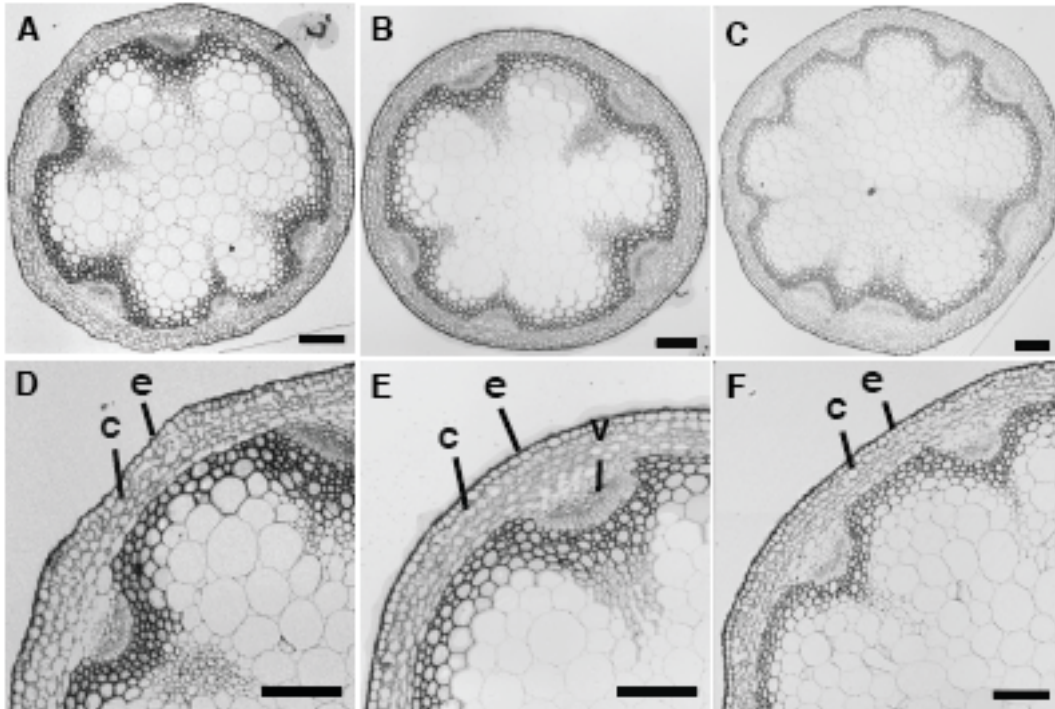


Fig 4.10 Stem anatomy of *sub-1*, *L1::SUB:GFP sub-1* and WT-Ler. (A-F) 2mm transverse sections stained with toluidine blue. (A and D) *sub-1*. (B-E) *sub-1* rescued by *L1::SUB:GFP*. (C-F) WT-Ler. (A) The outline of the stem appear wavy. (B) The outline of stem appears round and comparable to WT-Ler (C). (D) The morphology of epidermal cells appear slightly uneven. (E) In *L1::SUB:GFP* rescued stem the epidermal cells looks uniform and comparable to WT-Ler (F). Abbreviations, c, cortex. e, epidermis. v, vascular bundles. Scale bars 100 $\mu$ m.

in *sub-1* horizontal stem sections (Table 4.8). In the *sub* mutant, a reduction in epidermal (20%), cortex (28%) and pith cell numbers (24%) were observed compare to WT. In comparison the *L1::SUB:GFP* construct in *sub-1* horizontal stem sections showed a reduction in epidermal (8%) cortex (19%) and pith cells (17%) in comparison to the WT. Compared to the *sub-1* mutant, *L1::SUB:GFP sub-1* showed rescue of epidermal (12%), cortex (9%) and pith cells (7%). This observation suggests that the maximum rescue in cell number is achieved in the epidermis then followed to the cortex and pith. It suggests that the non-cell autonomous rescue is acts best on nearby cells, which are close to the signaling source. As far as cell size is concerned there was not a significant change in the

Table 4.8 cells number and surface diameter in the inflorescence stem of Wt, *sub-1* and *L1::SUB:GFP* rescued *sub-1*

Genotype	Epidermis cells			Cortex cells			Pith cells			
	N cell	ts	Cell size	N cell	ts	Cell size	N cell	ts	Cell size	ts
WT	309.8±26.6		210.4±21.8	744.1±58		246.5±27.3	305.9±40.7		2859.9±354	
<i>sub-1</i>	246.7±16.9	2.73*	203.5±20.3	537.6±42.9	3.91*	258.2±35.2	233±23.3	2.11*	2437.9±381.4	0.91
<i>L1::SUB:GFP</i> <i>sub-1</i> #1.3	276±23.8	1.29	202±18.4	601±45.4	2.64*	271.3±24	253.9±18	1.55	2418.7±274.2	1.33
<i>L1::SUB:GFP</i> <i>sub-1</i> #20.10	295.2±22.3	0.57	215±27.9	598±59.9	2.41*	304.5±37.9	254.8±23.4	1.46	2581.6±276	0.83
<i>35S::SUB sub-1</i>	336.6±22	1.05	198±13.2	752.9±56.6	0.15	288.5±34.4	277.4±16	0.85	3212.9±381.4	1.06

ts=student t-test

p=0.05

\*null hypothesis rejected

over all surface of the mutant versus rescued cells surface measurement compare to WT

### 4.4 Discussion

The results of promoter based rescue studies suggest a possible role for SUB in cell division and cell morphogenesis. Furthermore, the non-cell autonomous rescue of *sub* mutants using various promoters reveals an intercellular control mechanism for coordinated cell division and cell morphogenesis. In developing plants, non-cell autonomous influences on cell division rates and patterns have been observed by using mosaics of organ identity genes (Sieburth et al., 1998; Sessions et al., 2000; Vincent et al., 2003). The non-cell autonomous control of cell division is exerted by stem cell organizing factors (Mayer et al., 1998; Schoof et al., 2000; Heidstra et al., 2004) and by ligand for receptor-like kinase signaling (Fletcher et al., 1999; Brand et al., 2000).

#### 4.4.1 Possible mechanism of non-cell autonomy in SUB signaling

##### 4.4.1.1 Relay signaling vs diffusible signal

What is the molecular mechanism behind the proposed non-cell autonomous action of SUB? From the animal studies several ways of generating non-cell autonomous signal has been reported. The simplest mechanism for signal dispersal is passive diffusion of the signal from the source, through the extracellular space. In such a case, as the signal moves away from the source, it get diluted or degraded and creates a concentration gradient. Another possible mechanism could exist where a signal is produced in a given cell and influences a neighboring cell, which produces another signal, and so on, through a relay mechanism and it could travel a long distance. SUB is a receptor-like kinase sitting in the plasma membrane, which perceives the extracellular stimuli and mediate a downstream response in the form of a signal. So what is the possible nature of this downstream

signal, which is generated in the SUB expressing cells and it carries the information from one cell to other cell. It could be a moving protein, which move from the source to the neighboring cells in concentration depended manner.

The protein trafficking is limited by the plasmodesmatal size exclusion limit that has been estimated to be between 40 and 60 kDa for Arabidopsis embryos (Kim et al., 2005). The *L1::SUB:GFP* and *WUS::SUB:GFP* mediated signaling rescues the *sub* mutant phenotype. This indicates that the down stream signaling component generated in the L1-layer could traverse via diffusion to the L2 and L3 layer to rescue the phenotype or it may regulate the production of such downstream signaling component, which may traffic in the neighboring cells to rescue the phenotype. The *WUS::SUB:GFP* mediated rescue of stem twisting phenotype indicates that the downstream component generated in L3 layer could travel L2 to L1 cell layer. In ovules, *WUS::SUB:GFP* partially rescue the ovule phenotype indicating that *SUB* non-cell autonomous signaling, at least, communicates a few cell layers in the surrounding. If we take *WUS::SUB:GFP* expressing lines into consideration for outer integument rescue, we see a higher percentage of rescue in the lines which show high expression of SUB:GFP. Thus, the amount of SUB protein has important role in non-cell autonomous rescue of outer integument phenotype.

If we look at the *sub* mutant phenotype, it indicates that SUB signaling is probably required during development to fine tune organ shape and size at a molecular level by exerting non-cell autonomous influence on neighboring cells within a developing organ. It is well known in plant that cell-to-cell trafficking of regulatory proteins and mRNA through plasmodesmata (PD) allows non-cell autonomous regulation of plant development. (Lucas et al., 1995; Perbal et al., 1996; Kim et al., 2001; Kim et al., 2003; Nakajima et al., 2001; Wada et al., 2002; Lee et al., 2003; Wu et al., 2003). In principle, there are two modes of traffic through the PD. Proteins whose molecular weights are below the plasmadesmatal SEL can move between cells by non-targeted (passive) movement. Cytoplasmically localized free GFP (Crawford



and Zambryski, 2001) and the LFY, (Wu et al., 2003) transcription factor are classical examples of cell-to-cell non-targeted movement of proteins. Contrary to this, some protein requires interaction with the PD to increase the SEL and allow their movement. This way they show targeted (selective) movement. The viral movement proteins (MPs), KN1 (Lucas et al., 1995) and SHR (Gallagher et al., 2004) spreads between the cells via targeted movement.

### **4.4.1.2 SUB signaling could modify the non-cell autonomous signal and allows its long range movement**

In the above situation we had discussed the possibility of a SUB mediated signal that could travels from one cell to other. It is also possible that SUB actually not generate this signal, that it may modifies a signaling molecule, which moves between the cells. Several animal studies have shown that the covalent modification of signaling protein allows long-range diffusion. Cholesterol-modified Sonic Hedgehog (Shh) morphogen, establishes a long range gradient across the anterior-posterior axis of chick limb during development (Zeng et al., 2001). Mice expressing a form of Shh that lacks a cholesterol modification results in short-range Shh signaling (Lewis et al., 2001). The cholesterol modified protein generally locates in punctate structures in the basal regions of the epithelial cells while the protein without cholesterol appears in a diffuse pattern apically. The SHORT-ROOT (SHR), a GRAS family transcription factor is required both for correct specification of the endodermal cell layer and normal patterning of the root (Helariutta et al., 2000). SHR is expressed in the stele cells in the Arabidopsis root and the SHR protein moves to the endodermis. The movement of SHR is depended on its presence in the cytoplasm (Gallagher et al., 2004). Thus the protein modifying signal has a important role in the subcellular localization of the protein and it affects the movement of signaling molecules from one cell to another cell.

#### 4.4.1.3 Could SUB signaling affects the cell cytoskeleton

Targeted or nontargeted movement is one way of thinking to understand cell-to-cell trafficking but how the protein reaches on the first place to plasmodesmata is not understood yet. In some cases the proteins may randomly contact plasmodesmata by diffusion through the cytoplasm, while the other may require transport along with cytoskeleton. Recent studies have shown that at least some proteins are carried to the plasmodesmata by endomembrane system (Oparka, 2004). The endomembrane system includes the ER, Golgi bodies, vacuole, and vesicles. The endomembrane system delivers cargo proteins to the cell surface during cytokinesis. Golgi-derived vesicles delivers membrane proteins, and cell wall precursors along the microtubules to the cell plate in dividing plant cell (Konopka et al., 2006). In *sub* mutant we see periclinal cell division orientation in the L2 layer of flower meristem. Though we do not know whether it is due to the misorientation of microtubule arrays in the cell during the formation of phragmoplast or due to the cell wall polarity of adjacent cells.

In the single cell zygote of the brown alga *Fucus* asymmetric cell division give rise thallus and rhizoid (Bouget et al., 1998). The thallus cell divides perpendicular to the plane of the first cell division and proceeds in a series of transverse and longitudinal divisions to form the body of the *Fucus* embryo. To achieve this, polar secretion of golgi derived material is targeted to the cortical side of the zygote cell wall. Blocking of this polar secretion of golgi derived material by brefeldin A (BFA) leads misorientated cell division planes (Shaw and Quatrano, 1996). If we take *sub* mutants rescued by *L1::SUB:GFP* into account, it appears that SUB signaling rescued the epidermal cells, cell wall and this could also make the appositional L2-layer cells to behave normal. In animals cell-to-cell and cell-extracellular matrix interaction is essential mechanism, which controls cell morphogenesis and cell fate specifications during animal development (Jockush et al., 1995).

## **Chapter 5      Non-cell autonomous function of SUB during plant development: *Cre/lox-P* system for mosaic analysis in *Arabidopsis***

### **5.1      Summary**

How developmental signal traverse through layers of cells remains an unresolved mystery in plants. We examined the role of SUB, using *Cre/lox-P* site specific recombinase system, whether it involves a non-cell autonomous rescue of cell proliferation and cell morphogenesis during plant development. We have generated SUB sectors in plant stem of 30-day old *Arabidopsis*. The chimeras showing blue sectors (SUB positive) in large part of the stem were able to rescue the *sub* phenotype suggesting that SUB signaling acts non-cell autonomously in short range.

### **5.2      Introduction**

Cell-to-cell communications between the layers and within the layers is an important aspect of plant and animal development. The shoot and flower meristem of flowering plant derive from a group of cells called stem cells, and they are stratified into distinct cell layers. The cells in L1 give rise to the epidermis and in L2 layer they give rise to subepidermis and germ cells of reproductive organs, while the L3 layer give rise core tissues (Tilney-Bassett, 1986). The division planes are anticlinal in the two outer layers and progeny cells stay within the same layers of the meristem. This way the cell lineages of two outer layers kept separate from the L3 cells, which can divide in any plane. Furthermore, cells derived from one layer are capable of differentiating into another cell type when they are incorporated in another layer, as result of atypical periclinal divisions (Stewart and Burk, 1970; Stewart and Dermen, 1970). This reflects the developmental plasticity of plantdevelopment where differentiation into specific cell type depends on positional information and cellular interaction. To understand, the developmental functions of a gene, it is of general interest to know whether it acts non-cell autonomously or

cell-autonomously. Non-cell autonomous functions would indicate either the gene of interest encodes a product that can be transmitted from one cell to another cell, or that it regulates the production of such a product which could move from cell-to-cell. To test whether a gene product acts non-cell autonomously, we made a SUB genetic mosaic analysis using the *Cre/lox-P* system. In genetic mosaics, if genetically wild-type cells rescue the neighboring mutant cells, the gene acts non-cell autonomously.

In the case of receptor-like kinases (RLKs), upon activation the direct response is intracellular, but the phenotypic effect is often non-cell autonomous (Schubach, 1987; Price et al., 1989; Neuman-Silberberg and Schubach, 1993). The CLAVATA1 (CLV1) is a receptor-like kinase, that regulates cell proliferation and cell differentiation in shoot apical meristem non-cell autonomously (Clark et al., 1997; Fletcher et al., 1999; Brand et al., 2000). Non-cell autonomous rescue could occur with RLKs, if the receptor protein itself traffic between cells as has been shown for several plant transcription factors (Lucas et al., 1995; Kim et al., 2001; Nakajima et al., 2001; Wada et al., 2002).

We investigated the SUB non-cell autonomous function by creating transgenic lines under various promoters and analyzed the SUB:GFP protein localization. The promoter chimera analysis demonstrated that the SUB:GFP fusion protein itself does not move from cell to cell. Genetically wild type cells, However, were able to rescue neighboring mutant cells. This observation indicated the non-cell autonomous function of SUB signaling. In our study, transgenic lines showed the SUB:GFP expression pattern under various promoters very similar to mRNA *in situ* hybridization, and promoter reporter fusions for the investigated promoters. Alternatively, we used *Cre/lox-P* system based mosaics to investigate the SUB non-cell autonomous function.

### 5.3 Results

#### 5.3.1 *Cre/lox-P* genetic mosaic system

To determine whether the developmental function of SUB involves non-cell autonomous behavior during development, a strategy was designed based on Cre site specific recombinase, which acts upon the *lox-P* direct repeats (Fig 5.1). We engineered a *lox-P* cassette carrying two direct repeats of 34bp *lox-P* sequence in pGreen vector. We placed engineered 35S::SUB:NOS and 35S::GUS:NOS in between the *lox-P* repeats. Transgenic lines were created into *sub-1* and WT background and rescue of *sub* mutant phenotype was analyzed. The *lox-P* cassette with 35S::SUB and 35S::GUS cassette rescued all the aspects of *sub* phenotype. We also tested the rescued lines for GUS reporter activity. The rosette leaf of 14-days old T1 plants were stained for GUS, the blue staining revealed uniform color reaction throughout the rosette leaf surface. The HS::CRE lines (Sieburth et al., 1998) in WT-*Ler* were backcrossed into *sub-1* mutant. Both for HS::CRE in *sub-1* and 35S::SUB, 35S::GUS *lox-P* in *sub-1* (SUBLox) homozygote T3 plant lines were established. We did not see any effect of HS:CRE on *sub-1* mutant phenotype.

### 5.3.2 Cre/*lox-P* SUB mosaic gives variable rescue of *sub* phenotype

To generate the plant material for *sub* mosaic analysis, we crossed the [HS::CRE/HS::CRE; *sub-1*] plants with [SUBLox/SUBLox; *sub-1*] plants. Upon heat shock induction Cre recombinase will be supplied to cells, which will act on the *lox-P* direct repeats, and result in the excision of the intervening sequence (SUBLox). The excision would result in the absence of GUS staining product, thus GUS staining allows to make a distinction between SUB-containing and SUB-deficient sectors. Giving the plant heat shock for variable time periods, we controlled the amount of Cre recombinase

F1 seedlings were heat shocked 5-days after germination and grown until 30-days. In a parallel experiment, we tested 30-day old plants without heat shock. All non-heat shocked plants and plants heat shocked for 15 minutes looked like wild-type

(Fig 5.2), while plants heat shocked for 2 hours rescued the stem twisting in 50% plants below the first secondary meristem.

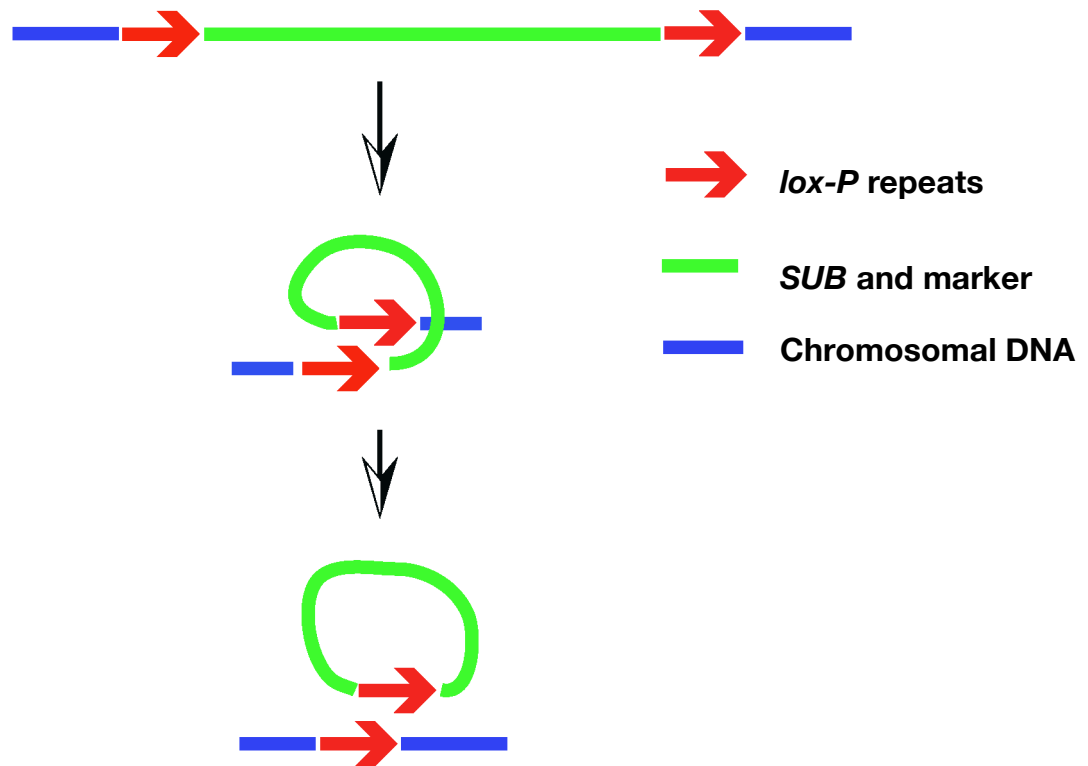


Fig. 5.1 Strategy and constructs for *SUB Cre/lox-P* mosaic analysis in Arabidopsis. Schematic diagram of Cre site-specific recombination at *lox-P* direct repeats. Recombination results in liberation of circular molecule containing one of the *lox-P* sequences and the DNA between the two *lox-P* sequences.

Though, stem height of plants heat shocked for 2 hours is only partially rescued compare to *sub-1* (Fig. 5.2). The heat shocked plants showed variable rescue of *sub* mutant phenotype depending on the duration of heat shock. In this analysis our goal was to get the sectors, that affected stem development, particularly, stem twisting and stem height. In one experiment, 16 F1 seedlings were heat shocked for 15 minutes and grown until 30-day. All heat shocked plants showed wild-type stem below the first secondary meristem. From the outer surface it appears that the whole stem is stained, but when horizontal section of stained stem made, it revealed sectors configurations within the stem.



Fig. 5.2 Phenotype observed in 30-days old plants. Pot from the left is *sub-1* without heat shock. The 2nd pot from left is *SUB lox-P* heat shocked for 2hours. The 3rd pot from left is *SUB lox-P* heat shocked for 15min. The 4th pot from left is *SUB lox-P* plants without heat shock. Plant heat shocked for 2 hour show semidwarfism.

Five stems showed sectors within stem while the stem appearance was wild type (Fig. 5.3C,D), indicating that cells lacked SUB, but still contributed to stem development. This result suggested that for normal stem development, there is no critical requirement of SUB to be present in all the cells within the stem. Parallel to this experiment, we also carried out 2 hours heat shock using 15, F1 seedlings, grown until 30-days. All heat shocked plant showed visible blue and white stripes on the outer surface of the stem below the first secondary meristem (Fig. 5.3E). Seven plant showed rescue of stem twisting. The horizontal section of stem revealed varying sizes of stained sectors within stem circumferences (Fig. 5.3F). Plant showing blue sectors but no rescue of stem twisting, probably suggest a critical requirement of SUB to be present in a specific region of the stem (Fig. 5.3G). Taken together, the above results suggest critical role of SUB during the stem development. We still do not know what is the basis of stem twisting in the *sub* mutant plants

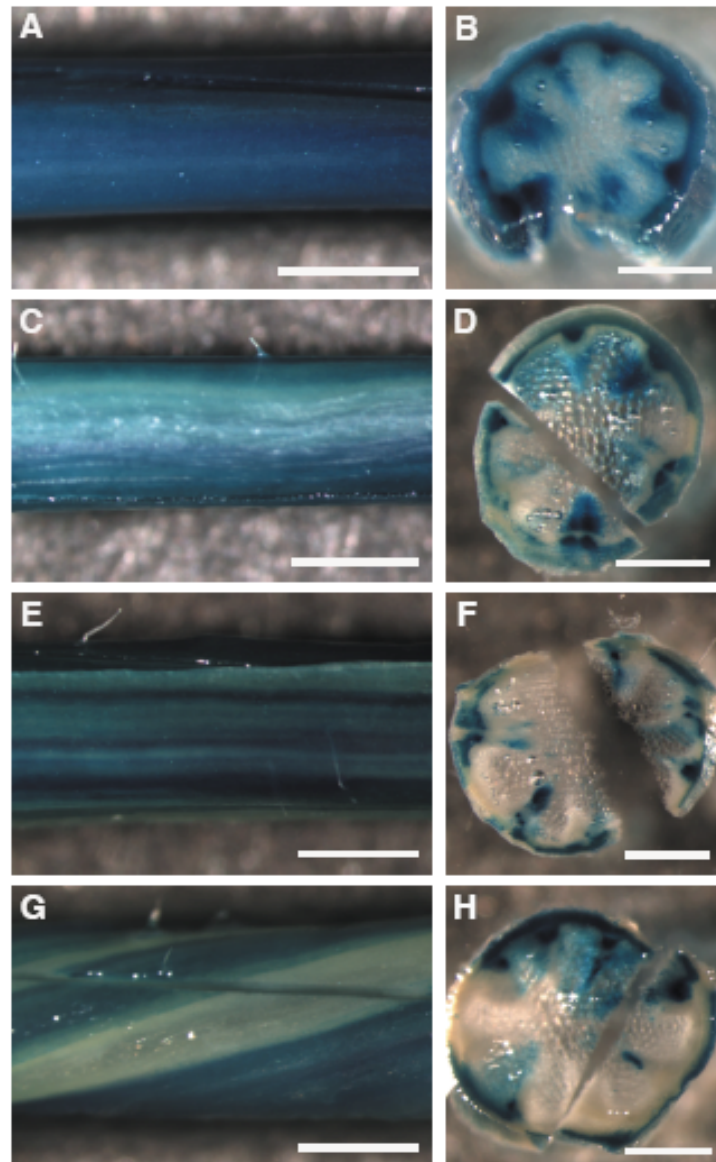


Fig 5.3 Stem chimeras of SUB *lox-P* heat shocked plants. (A and B) Non heat-shocked control stem stained for GUS activity, horizontal sections of stem showing GUS staining throughout the stem. (C and D) Stem of 15min heat-shocked plant showing GUS stained blue and non stained white sectors in horizontal section of stem. (E) Stem of 2hour heat-shocked plant showing blue and white (GUS positive and negative) stripes on the stem. (F) Horizontal stem section of E showing blue/white sectors in the stem. It is rescued for stem twisting phenotype. (G) Stem of 2hour heat-shocked plant showing twisting of stem. (H) Horizontal section of G showing blue/white sectors in the stem. Scale bars 0.5mm.



## 5.4 Discussion

The objective of this study was to determine the non-cell autonomous function of SUB during the development. We used a *Cre/lox-P* strategy to study *sub* mosaic plants, and determined that *SUB* acts in non-cell autonomous fashion and rescue stem twisting. The plants heat shocked for 15 minute are able to rescue the stem twisting phenotype because the stem has large blue sectors (*SUB* positive) and small white sector (*SUB* negative) within the stem. The plants heat shocked for 2 hours are able to rescue the stem twisting in 50% plants only. The plants having short distance between blue and white sectors shows stem twisting rescue, while the plants showing wide gaps between blue and white sectors were unable to rescue the stem twisting. Our data indicate a non-cell autonomous function of *SUB* signaling in short range. It is still not clear how far *SUB* signaling could rescue the *sub* mutant phenotype from the source. Although, cross-layer signaling is a general phenomenon in plant development. Genes involved in floral organ identity in *Antirrhinum* and *Arabidopsis* have been shown to act in non-cell autonomous fashion (Perbal et al., 1996; Sieburth et al., 1998). The transposon-tagged chimeras of *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) were used to assess cell autonomy in *Antirrhinum* and showed that the restoration of normal *DEF* and *GLO* activity in the L2 and L3 layers was sufficient for normal development of mutant L1 cell (Perbal et al., 1996). From our promoter analysis it appears that *SUB* non-cell autonomy is non-directional. Some plant still shows sector in the stem and do not rescue stem twisting. This is in contrast to our non-directional hypothesis. An alternative explanation would be that the *SUB* non-cell autonomous signaling is unable to traverse within the layer. Moreover, *SUB:EGFP* expression is temporally and spatially regulated during the development which again add complexity to the non-cell autonomous behavior of *SUB* signaling.

## Chapter 6 Conclusion

Here we have the first example of a receptor-like kinase in plants that shows differential mRNA vs. protein expression patterns. Our results with the SUB:EGFP fusion protein expression pattern under its native promoter, provides evidence of developmental dependence of such a mRNA vs. differential protein behavior during the development. Given the pleiotropic nature of the *sub* phenotype, it is difficult to ascertain the primary function of SUB. Nevertheless, it has been shown that SUB is involved in the control of the number of cell divisions and the control of the orientations of cell divisions (Chevalier et al., 2005). Preliminary genetic evidence given by promoter mosaic analysis indicates that SUB signaling can rescue a cell division plane defect, non-cell autonomously, across cell layers.

How and why *STRUBBELIG* signaling acts in a non-cell autonomous fashion is still not understood at a molecular level. The *sub* plants are also affected in stem height and stem diameter. The shoot apical meristem (SAM) is a dynamic structure where continued cell division in the rib meristem and peripheral meristem results in SAMs moving upward along with cell elongation and the stem grows taller (Steeves and Sussex, 1989). In addition to the cell division zone, the SAM also divide into clonally distinct layers of cells (Satina et al., 1940). Genetic mosaics studies has shown that dividing plant cells communicate division information to each other, thus, cells in one clonal layer can alter their division rate and division pattern to accommodate the division of their distantly related neighbors. When the wild-type SUB:EGFP expression pattern is compared with the *sub* phenotype, it suggests a non-cell autonomous cell-to-cell communication between the layers. We still do not know how this cellular interaction is established between the cell layers.

What can be the molecular mechanism behind the non-cell autonomous function of SUB, as it has been shown that SUB itself does not moves. One possibility is that SUB mediated signaling generates a downstream signaling component that moves

symplasmically between cells and play a role in cell-cell communication. The nature of this downstream component could be a transcription factor or small peptide, which moves from cell to cell and directly control gene expression. In plants, trafficking of regulatory proteins and mRNAs through plasmodesmata (PD) channels mediates non-cell autonomous regulation of plant development (Lucas et al., 1995; Kim et al., 2001; Nakajima et al., 2001). If it is a regulatory protein, how is targeted to the neighboring cell. Studies using the viral movement proteins (MPs) have shown that these proteins traffic by increasing the size exclusion limit (SEL) of PD and then spread the viral RNA genome (Wolf et al., 1989; Citovsky et al., 1990). The spatial and temporally regulation of the aperture of PD has an important role during complex morphogenesis, allowing selective protein movement critical for cell-fate determination (Lucas et al., 1995; Kim et al., 2001; Nakajima et al., 2001).

*L1::SUB:GFP* is expressed in the inner and outer integument of ovules and was shown to rescue the malformed outer integument of *sub-1*, though endogenous SUB wild-type protein is not detected there. It is possible that *L1::SUB:GFP* signal the below L2 cell and L2 cells non-cell autonomously rescues outer integument phenotype. Here, it is possible to assume both cell-autonomous and non-cell autonomous rescue of the outer integument of ovule by *L1::SUB:GFP*. In *L1::SUB:GFP* lines, SUB:GFP protein is expressed in outer integument initiating cells that normally does not show in wild-type, this indicates that SUB protein presence does not have any negative consequence on phenotypic rescue. Thus, we can conclude that in addition to signaling from underlying cell layers to L1, signaling from L1 to underlying cell layers, and signaling between L1 cells may act in non-cell autonomous fashion.

Observation of SUB:GFP protein expression under various promoters has suggested that SUB is an important component of signaling, because we not only rescue the phenotype but we also see protein in all layers without any selective degradation or attenuation. If SUB:GFP protein expressed, under heterologous promoters, does not show any selective decay in L1 and L2 layers, then why we do

see a protein vs. mRNA differential expression under native promoter. Moreover the promoter reporter fusion protein data indicates that the 5' and 3' mRNA regulatory elements may not be involved in differential spatial and temporal expression patterns. From our observation of mRNA vs. protein expression we do not know how this differential expression pattern is established. It has been shown that genes encoding plasma membrane and other secretory proteins, which traffic through the ER, are major target of unfolded protein response (UPR) mediated repression (Hollien and Weissman, 2006). Furthermore, the studies highlighted the importance of the protein sequence, not the mRNA sequence in such coupled post-transcriptional-translational control of mRNA decay (Hollien and Weissman, 2006). To explore the role of UPR in mRNA decay, future studies by removing the signal peptide from the SUB:EGFP or creating a frame-shift mutation in the SUB:EGFP to make resistant from such decay would indicate a potential role for such a mechanism.

In L1 and L2 layer why do cells in organ boundary region show *pSUB::SUB:EGFP* expression and not in the flower and shoot meristem central domes. Future studies using live imaging of the shoot apical meristem may shed light on this dynamic protein expression pattern in real time.

In conclusion, SUB, novel phenotype and unusual protein vs. mRNA expression pattern during development suggests, a role of novel post-transcriptional regulatory mechanism to establish such a mRNA vs. differential SUB:EGFP protein expression pattern. To understand the biological role of SUB function, it is important to elucidate the differential protein vs. mRNA expression pattern as well as SUB signaling pathway in detail

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