UNIVERSITY OF CALIFORNIA RIVERSIDE

Live Imaging Study on Cytokinin Function and Regulation in Stem-cell Homeostasis

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by

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This dissertation is written in memory of professor He Guang, my master degree adviser, for his guidance and inspiration during my very early research career. I have been using a lot of knowledge and techniques that I learnt from him for many years.

This dissertation is written in memory of my grand parents who I lived with during my childhood.

This dissertation is written in memory of my mother-in-law for her dedication to my wife's education.

This dissertation is dedicated to my wife Jiamei Chen and my son Hongyu Chen for their long time inspiration and support.

ABSTRACT OF THE DISSERTATION

Live Imaging Study on Cytokinin Function and Regulation in Stem-cell Homeostasis

by

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Doctor of Philosophy, Graduate Program in Plant Biology
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Dr. Venugopala Reddy Gonehal, Chairperson

Stem-cell homeostasis is mediated by multifaceted networks involving plant hormones and local cell-cell communication. Earlier studies have implicated cytokinins in regulating shoot apical meristem (SAM) growth. However, the precise role of cytokinin in SAM remains largely unknown because cytokinins have been implicated in several developmental processes and merely studying the terminal phenotypes may not reveal their function in actively developing SAMs. The live-image technology allows studying the function of any given regulator immediately after its perturbations in transient experiments. The live-imaging work presented here provides: 1) Functional analysis of SAM-enriched cytokinin activating enzymes, 2) The live-image study on key regulators in cytokinin biosynthesis and signaling, 3) The function of SHOOTMERISTEMLESS (STM).

The cell-type specific genomics has predicted and RNA *in situ* analysis has confirmed the enrichment of cytokinin activating enzymes in the SAM-stem cell niche. Knock-out three centrally expressed members alters phyllotaxy. Transient manipulation of cytokinin results in organ positioning defects within the peripheral zone (PZ) of SAMs. The levels of

cytokinin are correlated to auxin responses. Moreover, external application of auxin fails to induce auxin responses suggesting that cytokinins are required for activating auxin response pathway components. Cell division analyses reveal that cell mitotic activities are correlated to the levels of cytokinins. We conclude that cytokinin controls phyllotaxy by regulating auxin signaling, auxin transport and cell division rates.

We find that WUSCHEL (WUS) regulates a type-B response regulator, ARR1. Then, we have studied the effects of positive cytokinin signaling by using inducible activation of constitutively active forms of ARR1 coupled with live-image technology. This analysis has revealed that constitutive activation of cytokinin signaling results in expansion of stem-cell domain leading to de-differentiation of differentiating cells and this process is WUS dependent.

Genetically, *stm* mutant phenotype implies that STM function is required for SAM initiation and fulfills a complementary role to that of WUS. Inducible inactivation of STM has shown that it is required for preventing the CZ cells from responding to auxin. Furthermore, other differentiation markers were also found to be mis-expressed in the CZ cells. These findings lead to conclude that STM prevent the stem-cell domain from differentiation.

TABLE OF CONTENTS

Title

Approval

Copy right

Acknowledgement……....iv

Dedication……....vii

Abstract……....viii

Table of contents…….…..x

List of Figures …….….xii

List of Tables……....xiv

Introduction……....…1

a. Historical views about Shoot Apical Meristem (SAM)…….....….....1

b. Cell-cell communication mechanisms of stem-cell maintenance:

The CLVAVATA-WUSCHEL feedback loop…….....3

c. Other cell-cell communication networks regulating the function of SAM…….....4

d. Review on live-image technology…….....11

e. Review on transient gene manipulation systems…….....13

f. Specific aims…….....….....15

Materials and methods ……...17

a. Plant materials and growth conditions…….….....17

b. Plant transformation…….....17

c. Dex treatment…….....17

d. Constructions of plasmids …….. …….... 17

e. Genotyping T-DNA mutants ……...…....18

f. Live-image …….....…….....19

g. RT-PCR…….....20

h. RNA *in situ* hybridization.....20

i. Gel mobility shift assays.....21

j. Indole-3-acetic acid (IAA) analysis.....22

k. List of primers that were used in this dissertation.....22

Chapter I: Genetics of stem-cell enriched genes.....29

Chapter II: Transient manipulation of cytokinin levels.....43

Chapter III: Transient manipulation of cytokinin signaling.....68

Chapter IV: Transient manipulation of SHOOTMERISTEMLESS.....81

References.....92

Appendix.....121

A. New vectors (design, map, and applications).....121

LIST OF FIGURES

Figure 0-1. Cell types and organization of SAM stem-cell niche…….....2

Figure 0-2. CLAVATA:WUSCHEL feedback network……..3

Figure 0-3. Expression domains of key regulatory genes are color coded…….....5

Figure 0-4. Cytokinin biosynthesis pathways: key enzymes and intermediates……..8

Figure 0-5. A schematic representation of the two-component-circuit of cytokinin signaling…….....9

Figure 0-6. Schematic representation of Alcohol inducible system……..……13

Figure 0-7. Schematic representation of Dexamethasone inducible system……..…....14

Figure 1-1. Genetic study on *AtCKX* family.....31

Figure 1-2. The expression pattern of *AtLOGs* in SAM by cell type-specific transcriptome.....32

Figure 1-3. The junction of the T-DNA insertion of atlog mutants.....34

Figure 1-4. Phenotype of triple mutants *atlog1/6/8*.....35

Figure 1-5. Expression patterns of stem cell unrelated AtLOGs 35

Figure1-6. Genetic study on the *AtPUM* family.....38

Figure 2-1. Distribution of cytokinins in SAMs as revealed by *pARR5::ARR:GFP*.....49

Figure 2-2. Phenotypes of inducible CKX3 transgenic plants.....50

Figure 2-3. The reduction of *pDR5::Venus:N7* and *pPIN1::PIN1:GFP* expression upon dex-induced downregulation of cytokinin levels.....53

Figure 2-4. The recovery of *pDR5* (red) and *pPIN1* (green) expression upon withdrawal of dex-mediated downregulation of cytokinin.....53

Figure 2-5. Phenotypes of Dex-induced over-expression of IPT7 transgenic plants.....54

Figure 2-6. Extra sets of primordia formed by increase of auxin signaling upon the inducible expression of IPT7.....56

Figure 2-7. Auxin measurements after alteration of cytokinin levels.....57

Figure 2-8. Auxin treatment upon cytokinin removal fails to restore auxin responses.58

Figure 2-9. RT-PCR analysis of few candidate genes in
Dex-treated plants carrying dex-inducible CKX3 or IPT7.....60

Figure 2-10a. Cell division analysis of inducible IPT7 transgenic lines
upon Dex induction.....62

Figure 2-10b. Cell division analysis of inducible CKX3 transgenic lines
upon Dex induction.....63

Figure 3-1. ARR1 is directly-activated by WUS.....70

Figure 3-2. Phenotypes of *p35S::ARR1ΔDDK::GR*.....72

Figure 3-3. Expansion of stem cell domain upon constitutive
activation of cytokinin signaling.....75

Figure 3-4. Constitutive activation of ARR1 results in expansion
of stem-cell domain without altering cell division rates.....75

Figure 3-5. The genetic analysis of constitutive cytokinin signaling on *wus-1* mutant..76

Figure 3-6. RT-PCR analyses on CLV-WUS feedback loop upon constitutive
activation of ARR1.....77

Figure 3-7. ARR1 binds to *pCLV3* promoter in vitro in Gel Retardation.....78

Figure 3-8. Activating *ARR1ΔDDK::GR* in CLV3 domain gives rise
to enlargement of stem cell domain.....79

Figure 4-1. Inducible phenotypes of *STM RNAi* transgenic plants.....83

Figure 4-2. 3D-reconstructed time-lapse series showing PIN1:GFP expression
(green) and DR5::YFP (red) of STMdsRNAi plants treated
with either dex (A-F) or mock (G-L).....85

Figure 4-3. *AS1* and *AS2* expression in *STMdsRNAi* transgenic plants.....86

Figure 4-4. The expression of *CUC2* at the boundary of SAM was delayed
in inducible STM RNAi lines.....88

LIST OF TABLES.

Table M-1. The sequences of primers……....22

Table M-2. The application of primers……..25

Table 1-1. Gene IDs and T-DNA lines of the *AtCKX* family.....30

Table 1-2. Gene IDs and T-DNA lines of the *AtLOG* family.....33

Table 1-3. Gene IDs and T-DNA lines of the *AtPUM* family.....37

INTRODUCTION

a. A historic view of Shoot Apical Meristem (SAM)

The development of above-ground architecture of plant body is determined by the continuous proliferative activities of a small group of pluripotent-stem cells residing at the tip of shoot apical meristem [SAM] [1-4]. In the model plant *Arabidopsis*, the shoot apical meristem is a dome-shape structure located at the tip of a plant. It consists of about 500 cells and it is organized as different functional zones (Figure 0-1. A). The central zone [CZ] harbors a set of 35-40 stem-cells, that is located at the tip of SAM, and it serves as a source of long-term stem-cells which renew themselves [1, 4, 6]. The stem-cell daughters that are displaced into the adjacent peripheral zone [PZ] enter into differentiation pathways at specified locations to form either the leaves or flowers. The PZ can be further sub-divided into the inner PZ [IPZ] and out the outer PZ [OPZ]. Based on the analysis of transient loss of CLAVATA3 [CLV3] (detail explanation is provided in subsequent sections), a gene required for stem-cell homeostasis, it is been shown that the IPZ cells that abut the CZ can retain the capacity to acquire stem-cell fate, however, the OPZ cells fail to be re-specified as stem-cells. Thus, the IPZ can be viewed as a buffer zone in which cells at the border of the CZ and the PZ can be specified or re-specified as stem-cells [7]. Underneath the CZ, the cells of the organization center [OC]/stem cell niche [Niche] fulfill two roles. One is to specify the overlying cells of the CZ as stem cells at early stages of embryogenesis and also provide cues constantly to maintain them as a stem cell population throughout the life of a plant. It also generates cells for the maintenance of the rib meristem (RM) cells and to support the stem-growth. The expression pattern of *CLV3* coincides with

that of the CZ [8-9], thus it has been used widely as stem-cell marker (Figure 1A). PZ can be marked by genes that are expressed in this region, for example, *UNUSUAL FLOWER ORGAN* [*UFO*] (Figure 0-1B). The homeodomain transcription factor *WUSCHELL* [10] is expressed in

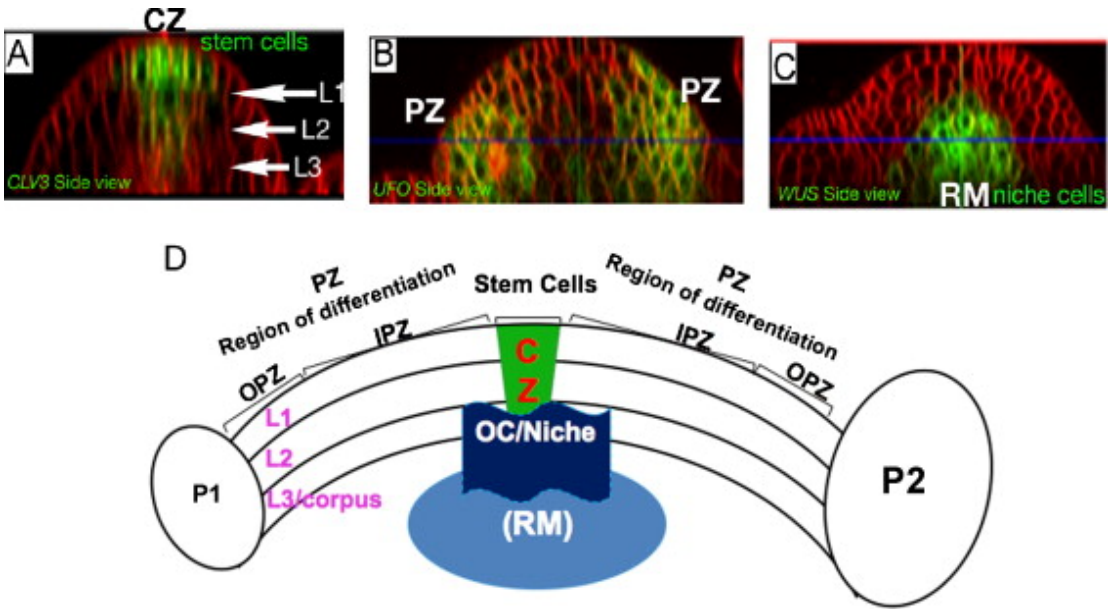

Figure 0-1. Cell types and organization of SAM stem-cell niche marked by fluorescent reporter constructs. (A–C) The side views of SAMs, showing the stem-cell domain (CZ) marked by CLAVATA3 promoter (A), the adjacent peripheral zone (PZ) is marked by UNUSUAL FLORAL ORGANS (UFO) promoter (B), the Rib-meristem (RM)/organizing center (OC) revealing WUSCHEL expression (C). The 3 clonal layers of cells are marked by FM4-64, a lipophilic dye which marks cell outlines (red). (D) Schematic representation of SAM organization depicting the three clonal layers and the stem-cell niche showing the positions of stem-cell cells (the central zone; CZ), the differentiating cells (the peripheral zone; PZ) that can be sub-divided into the inner PZ [IPZ] (the region that can de-differentiate into stem-cells upon CLAVATA3 down-regulation) and the outer PZ [OPZ] (the region that remain differentiated upon CLAVATA3 down-regulation). P1 and P2 represent organ primordia at different stages of differentiation.

organization center and serves as an ideal marker for a subset of the RM cells (Figure 0-1. C). Clonally, the SAM can be clearly divided into three cell layers based on their cell division orientation (Figure 1A and 1D). Cells in the outmost L1 layer and sub-epidermal L2 layer divide only anti-clonally to maintain the lateral expansion of these two layers [11].

However, a few cells of the L2 layer located in regions of the organ primordia have been shown to divide in periclinal orientation and they might play a role in early stages of primordial out growth. The underlying cells of the corpus/the L3 layers form a multi-layered structure and they divide in random orientations. Therefore the SAM maintenance involves a tight co-ordination of cell division patterns between cells located both within and across different cell layers.

b. Cell-cell communication mechanisms of stem-cell maintenance: The CLAVATA (CLV)-WUSCHEL (WUS) feedback loop

In the past two decades, a large number of genes that are involved in SAM function and development have been identified through forward genetics approach [12] (Figure 0-3).

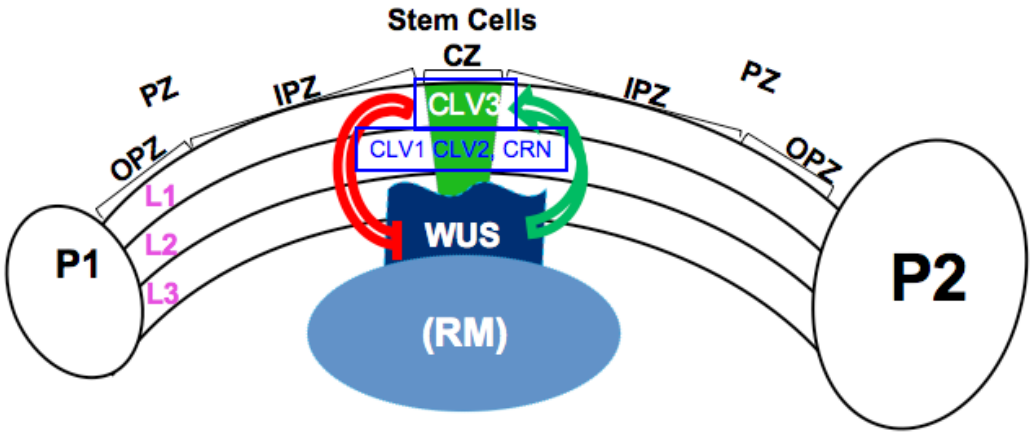

Figure 0-2. CLAVATA:WUSCHEL feedback network: *CLAVATA3 (CLV3)* expressed in the central zone (CZ) [green] along with CLAVATA1 (CLV1), CLAVATA2 (CLV2) and CORYNE (CRN) forms a signaling complex [not yet completely understood] that signals to restrict *WUSCHEL (WUS)* expression to the organizing center (OC). WUS, in turn, activates CLV3 expression and also positively regulate stem-cell fate in cells of the CZ through an un-identified signal.

Among them, the feedback network mediated by CLV and WUS genes play a central role in stem-cell homeostasis and SAM growth control [10]. The mutations in *CLAVATA* class of genes [*CLAVATA1 (CLV1), CLAVATA2 (CLV2)* and *CLAVATA3 (CLV3)*] result in both over-proliferation of stem-cells/expansion of the CZ and also make larger SAMs [8, 13-14]. Whereas, *wus* mutants fail to maintain a functional SAMs and terminate their activity very early in development [10, 15]. The molecular nature of these genes, their expression patterns have been characterized in great detail and this has provided clues to their function stem-cell maintenance. CLAVATA3 (CLV3), a small extracellular protein synthesized in stem cells located within the CZ, signals to the underlying OC/RM by activating receptor kinase complex that includes CLV1 which is expressed in RM cells, CORYNE which is ubiquitously expressed and also CLV2 which encodes a receptor like kinase but lacks intracellular kinase domain [Figure 0-2][8, 13-14, 16-17] Recent studies have demonstrated that CLV3 may be processed to yield a biologically active 12 - amino acid peptide which in turn binds to CLV1[16, 18-20]. Activated CLV1 and related receptor kinase complexes function to spatially-restrict the expression of homeodomain transcription factor WUSCHEL (WUS) which is also expressed in cells of the RM/OC [Figure 0-2] [21-24]. It has also been shown that ectopic expression of *WUS* results in upregulation of CLV3 expression in the overlying stem-cells, via an unknown signal [Figure 2] [22]. Therefore, CLV3 can regulate its own expression levels and also the size of stem-cell domain, through a feedback loop involving CLV3-expressing stem-cells in the CZ and the *WUS* expressing cells of the OC/RM.

c. Other cell-cell communication networks regulating the function of SAM

The gene interactions that have implicated in SAM growth and patterning are very complex [25-41]. Though, comprehensive network analysis requires high resolution spatio-temporal data. Nevertheless, the understanding of genetic circuitry has improved enormously in the past two decades and it has been described below (Figure 0-3).

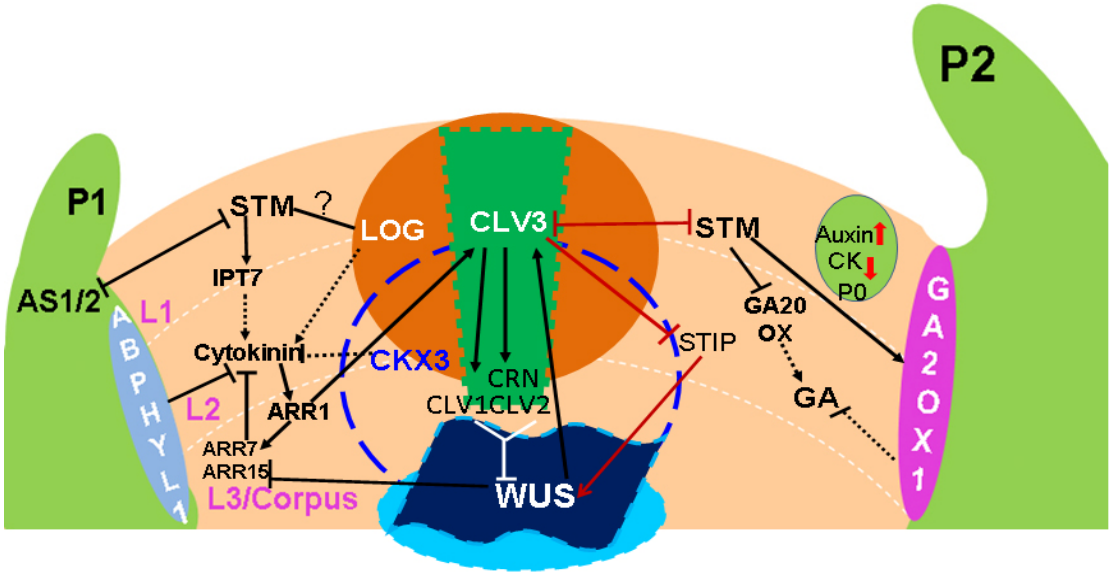

Figure 0-3. Expression domains of key regulatory genes are color coded. CLV:WUS feedback network receives inputs from STIMPY (STIP) and POLTERGEIST (POL), a predicted nuclear protein phosphatase. CLV:WUS network enters into a complex network of interactions with SHOOTMERISTEMLESS (STM) which, in turn, promotes un-differentiated state by inhibiting the expression of differentiation promoting factors such *as ASYMMETRIC LEAVES1* and *2*. STM positively regulates IPT7 (a rate limiting enzyme in plant hormone cytokinin [CK] biosynthesis, negatively regulates GA20oxidase (key enzyme in plant hormone gibberellic acid [GA] biosynthesis, while positively regulates GA2oxidase at the organ boundary regions. Recently identified *LONELYGUY (LOG)* class of genes has been shown to convert inactive forms of CK into active forms. The net effect of these interactions is expected to create a region of higher cytokinin levels and lower GA levels in the central region of SAMs. A more detailed view of hormonal interactions and their effects on SAM function can be obtained from a recent review [42]. Red colored arrows indicate interactions derived from genetic analysis. Black colored arrows represent interactions derived from molecular analysis. Dotted lines represent enzymatic reactions leading up to the regulation of hormonal levels.

SHOOTMERISTEMLESS (STM) is another key regulator that mediates SAM initiation and maintenance [43-51]. STM is a member of homeodomain-containing proteins of the KNOTTED-1HOMEOBOX (KNOX1) family. STM is expressed throughout the SAM surface and downregulated in cells of the organ primordia [52]. Complete loss of function alleles such as *stm-1* and *stm-5* fail to initiate embryonic SAM [42, 53-54]. Genetic interaction studies have shown that STM represses the expression of ASYMMETRIC LEAVES1 (AS1), a Myb-domain containing transcription factor which is expressed in primordial initiation sites in wild type SAMs. *as1; stm* double mutants have been shown to rescue the SAM defects of *stm* mutants [55-56], whereas *STM* misexpression has been observed in leaves of *as1* mutants suggesting a reciprocal antagonism between *STM* and *AS1* class of genes implying that STM prevents differentiation of SAM cells. But, the hypothesis that STM-expressing cells inhibit the expression of AS1 in SAM cells is yet to be tested since the strong stm mutant alleles fail to develop a functional SAM.

Genetic interactions between STM and genes of the CLV-WUS network have been carried out [47] [54]. . The genetic interaction between *stm* and *clv* mutants has revealed that *stm* mutations can dominantly suppress *clv* phenotypes indicating that the *stm* phenotype is sensitive to the levels of CLV activity [54]. Based on the genetic interactions, it has been proposed that these genes play related but opposing roles in the regulation of cell proliferation and/or differentiation in SAMs. Genetic interactions, based on loss of function and gain of function analysis, between STM and WUS have suggested that they perform independent functions [47]. WUS is required to specify stem cells in the CZ, while

STM is required to suppress differentiation throughout the SAM dome and thus allowing the proliferation of stem cell daughters within the PZ.

Two lines of evidences indicate that the function of STM may regulate plant hormone pathways, including cytokinin and GA biosynthesis [57]. Specifically, inducible activation of STM by using STM-GR (STM fused the hormone binding domain of rat glucocorticoid receptor) was found to activate expression of *ISOPENTENYLTRANSFERASE7 (IPT7)*, a key enzyme in cytokinin biosynthetic pathway[48]. Overexpression of IPT7 by using *STM* promoter has been shown to partially rescue *stm-1* mutant phenotype demonstrating a functional link between STM and cytokinin biosynthesis [48]. STM was found to repress the expression of *GA20 oxidase* [a Gibberellic (GA) acid biosynthetic enzyme] while promoting the expression of *GA2 oxidase 1* [a GA degradation enzyme] whose expression is restricted to few cells of the boundary region between meristem and developing primordia [57]. Thus, STM seems to control the GA distribution in SAM by not only inhibiting its biosynthesis at the meristem center but also preventing its movement into the meristem to achieve a GA free or relatively low GA concentration in the center of SAM.

Cytokinin, N^6 adenine derivates, is one of five classical plant hormones [58]. Cytokinin was first identified as a cell division promoting regulator in tobacco tissue culture half shown to function in array of plant growth and developmental processes including, cell division, senescence, growth and patterning in shoot and root apical meristems (RAMs) [58-73]. The major cytokinin biosynthetic pathway is mediated by ADENOSINE PHOSPHATE-ISOPENTENYLTRANSFERASE (IPT) which catalyzes the first and the

rate-limiting step. IPT utilizes dimethylallyl diphosphate (DMAPP) and either adenosine

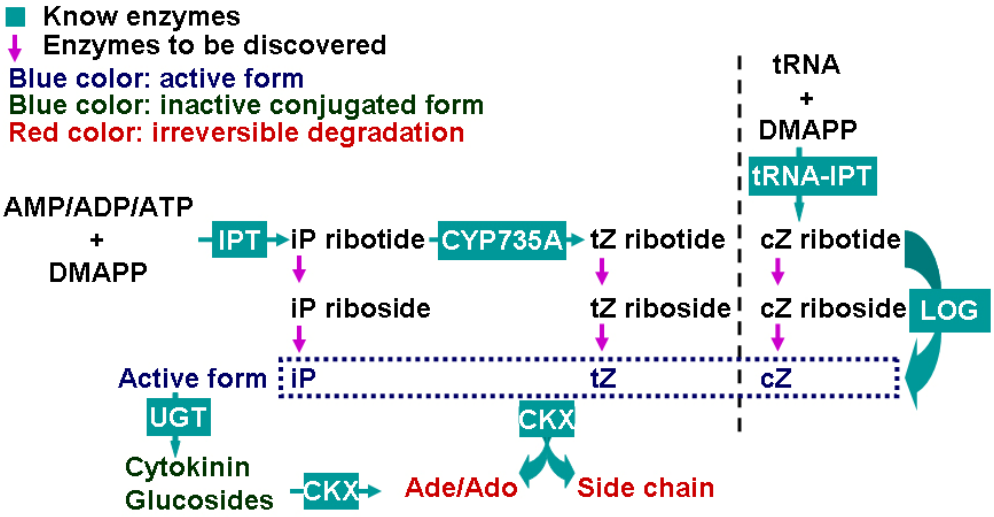

Figure 0-4. Cytokinin biosynthesis pathways: Key enzymes and intermediates

century ago [74]. Since then cytokinin has been 5'-mono- or di- or tri-phosphate (AMP, ADP, or ATP) to produce iP-ribotides [Fig 0-4] [75]. Then, iP-ribotides are hydrozylated to tZ-ribotides by CYTOCHROME P753 [CYP753] [76]. Another possible route for cytokinin biosynthesis includes the conversion of tRNA to cZ ribotide through prenylation by tRNA-IPT by using DMAPP [Fig 0-4][77-79]. In the he classical pathway the conversion of inactive forms of cytokinins to active forms is a two step process and the enzymes that catalyze these steps are unknown [Figure 0-4]. An elegant work in rice recently identified the function of LONGLEY GUY (LOG) that can convert the inactive form of cytokinins into active form of cytokinins by a single step reaction [80]. The functional orthologs in *Arabidopsis* are encoded by a family of nine members indicating the evolutionary significance of this pathway[81]. Cytokinin homeostasis is achieved through regulated conjugation or irreversible degradation [70, 82]. The conjugation of active

cytokinins into inactive glucosides is mediated by UDP GLYCOSYLTRANSFERASE (UGT) [83-86]. The Glucose-cojugation can happen at the N3, N7, and N9 position of the purine ring, or in the hydroxyl group of the prenyl side chain [83-84]. The irreversible degradation reaction is catalyzed by cytokinin oxidase/dehydrognease (CKX) to break down active cytokinins into ade/ado group and a side chain [87-91]. Understanding the developmental regulation of spatial activation patterns of key biosynthetic enzymes will be crucial in deciphering their role in SAMs and also to study the function of cytokinin homeostasis in SAM growth and patterning.

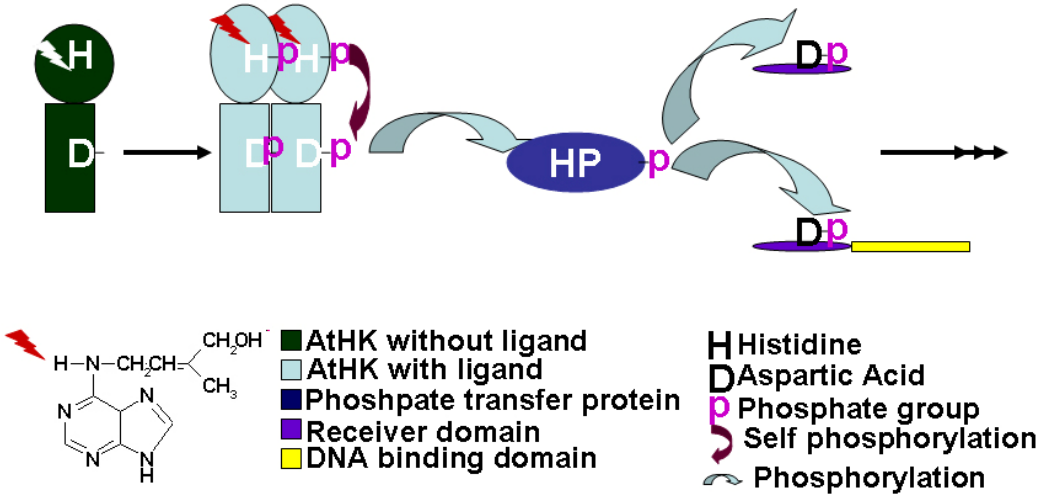

Figure 0-5. A schematic representation of the two-component-circuit of cytokinin signaling. AtHK (*Arabidopsis thaliana* HISTIDINE KINASE), HP (Histidine phosphate transfer Protein).

Plants employ a histidine kinase (HK) phosphor relay circuit in mediating cytokinin signaling, which is very similar to the two-component system utilized by bacteria (Figure1-4). In Arabidopsis, three Arabidopsis thaliana HISTIDINE KINASE (AtHKs) which belong to a gene family of six members serve as cytokinin receptors [92-100]. The AtHK

receptors self assemble into homodimers upon binding to cytokinin. Conformational changes within the receptor homodimer facilitate the transfer of phosphate from histidine to aspartic acid through a self phosphorylation process. Next, the phosphate on aspartic acid is transferred to downstream ARABIDOPSIS RESPONSE REGULATORS (ARRs) through HISTIDINE PHOSPHATE TRANSFER PROTEINS (HPs) [59, 100]. Recently, it has been shown that AtHPs are evenly distributed both in cytosol and nucleus. Thus, challenging the previous assumption that AtHPs move from cytosol to nucleus upon cytokinin perception at the cell surface[101-102]. This work also raises the question whether the transfer of phosphate between AtHPs and ARRs does occur. In *Arabidopsis*, two types of ARRs have been identified [103-105]. The TypeB-ARRs have both a receiver domain that could be phosphorylated by the phosphor relay through unstream cytokinin signaling and a DNA binding domain that could act as a transcriptional factor. Without phosphorylation of the receiver domain of the TypeB-ARRs, the function of the transcriptional factor domain is masked[103]. On the other hand, the TypeA-ARRs have only the receiver domain that could be phosphorylated by the upstream cytokinin signaling[104, 106]. They act mainly as negative regulators to modulate the cytokinin signaling but the mechanism how they work remains unknown. One possibility is that the TypeA-ARRs compete with the TypeB-ARRs to receive the phosphor relay signaling. Another possibility is that the TypeA-ARRs form dimmers with the TypeB-ARRs to prevent the TypeB-ARRs from working. It has been speculated that different combination of receptor(s) and ARR(s) may execute specific plant process. For example, AtHK1 and ARR2 have been shown to function together to control plant senescence [68,

100]. The functional specificity of different receptors and downstream components could also be due to their differential distribution in different cell types in a developmental context [107-109]. Arabidopsis AtHK, WOODENLEG (WOL) has been shown to be expressed specifically in cells of the RM of SAMs [68, 100, 110]. Based on this it has been speculated that it might play role in RM cells sensing cytokinin to activate WUS expression. However, the other two receptors have been predicted to be uniformly-expressed in all cells of the SAM which calls for future studies to clarify the significance of local expression of WOL [36]. A notable progress has been made with a group of type A ARRs (ARR5, ARR6, ARR7, ARR15) which were found to be repressed by WUS [111]. Over-expression of dominant negative form of ARR7 has been shown to cause termination of SAM. Multiple mutant line carrying mutations in seven type A ARRs (5, 6, 7, 8, 9, 10, 12) has been shown to result in abnormal phyllotaxy [25, 104]. However, the traditional analytical methods, based on single time point observations, do not take into account the spatial and temporal aspects of cell identity transitions and cell division patterns within the SAMs. Therefore, the mechanisms by which CLV-WUS feed back loop, the STM-mediated pathways and cytokinin-mediated control mechanisms come together to maintain a functional SAM will have to be explored in live-imaging experiments.

d. The live-image technology and its impact on research on SAM

Live-image technology was developed to visualize gene expression and growth patterns in different regions of SAMs by using spectral variants of fluorescent proteins as reporters and by observing under the Laser Scanning Confocal Microscope (LSCM)[4, 7]. Multi-spectral imaging allows visualization of multiple cell types or multiple sub-cellular events

in a given cell type in real time by live-imaging. A pioneering work was the finding of stem cell re-specification in SAM by using both the transient perturbation of *CLV3* gene and the live-image technology [7]. Although the markers that can report different cell types in SAMs are still a limiting factor, however, enough reporters have been developed to visualize the broad functional domains [112]. For example, the *pCLV3::mGFP5er* [endoplasmic reticulum-localized green fluorescent protein under *CLV3* promoter control] reporter marks the stem cell domain, while the *pWUS::mGFP5er* [endoplasmic reticulum-localized green fluorescent protein under *WUS* promoter control] marks some cells of the organization center. The movement of auxin can be monitored by the *pPIN1::PIN1::GFP* protein fusion [auxin efflux transporter PIN1::GFP protein fusion driven by the native *PIN1* promoter to reveal the direction of auxin flow] [113]. *pDR5rev::3XVENUS-N7* (auxin responsive promoter driving a nuclear-localized variant of YFP) has been used to monitor sites of auxin accumulation or response within the SAMs [113]. Reporters that mark cells of the boundary region between SAMs and the developing organ primordia include *pLATERALSUPRESSOR::GFP* and the *pCUPSHAPEDCOTYLEDON2::YFP-N7* [114]. Therefore, the development of live-image technology and the availability of plethora of reporters should allow the dissection of spatio-temporally-coupled events in SAM development. New developments and improvements of computational analysis, such as automatic tracking of cell growth parameters and lineages may facilitate functional understanding of growth and gene expression dynamics [112]. However, analyzing cellular phenotypes in terminal mutants several days after genes have been inactivated may not reveal immediate function/s of genes rather it reflects cumulative effects that have been

accumulated over several days. Since development of organs/tissues occurs in a spatio-temporal sequence, it will be essential to analyze gene functions in transient experiments by live imaging.

e. Inducible systems to achieve transient perturbation or expression of SAM regulators

To achieve transient perturbation or expression of regulators of interest, several inducible systems have been described which can be coupled to live-imaging technology.

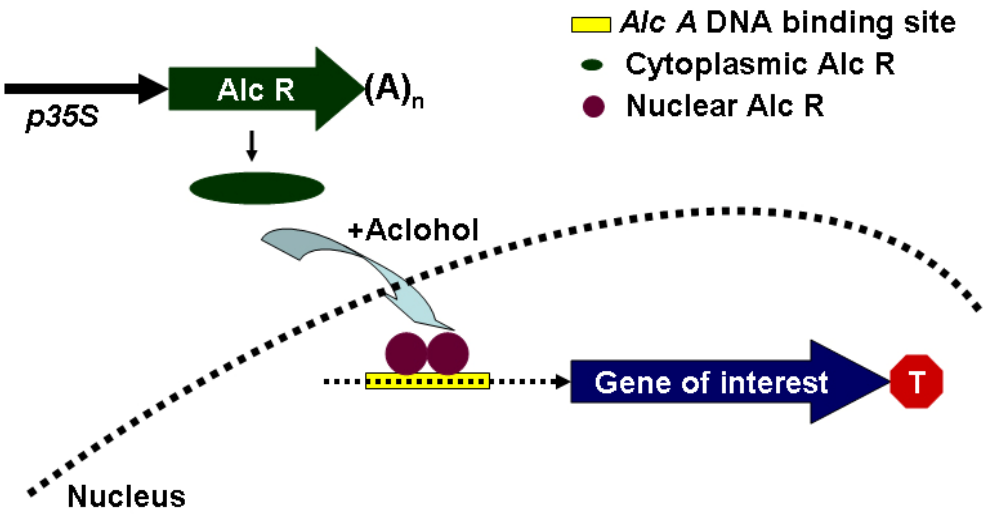

Figure 0-6. Schematic representation of Alcohol inducible system. AlcR (transcription factor that can sense alcohol), AlcA (AlcR binding sequence).

An ideal inducible system should be tightly regulated so that induction could be achieved only in the presence of the inducer and it is also preferred to have the system with a shorter half-life so that the induction can be tuned reversibly. Thus, a careful choice of the induction system will be crucial in achieving spatio-temporal control of gene manipulation. Alcohol inducible system is a two-component system consisting of transcription factor Alc

R which binds to consensus sequences *Alc A* to activate the downstream genes attached to

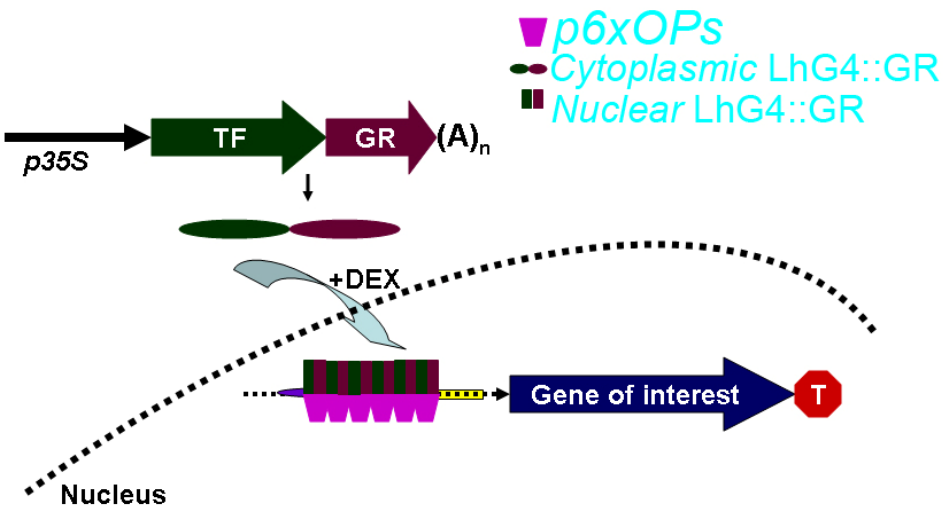

Figure 0-7. Schematic representation of Dexamethasone inducible system.

it. Alc R upon sensing alcohol changes its conformation such that it can now bind to *Alc A* and activate downstream genes [115-118] (Figure 0-6). The widely used alcohol inducible system system has been shown to result in robust induction of transgenes and responds to alcohol within few minutes.

Dexamethasone (Dex) inducible system is also two component system based on bacterial-derived Lac Operator (Op) principle. It consists of a transcription factor, LhG4 which can bind to multimeric operator sequences (6XOp) and activate downstream genes [119-121]. Induction of LhG4 is achieved by fusing LhG4 to that of the hormone binding domain of rat glucocorticoid receptor (GR) to generate LhG4:GR. In the absence of Dex the LhG4 is retained in the cytosol as a comples. Upon addition of Dex, the fusion protein migrates into the nucleus and binds to 6XOp sequences to activate genes. This system has been couple with live-imaging to study the effects of transient downregulation of CLV3

[7]. Dex inducible strategy has also been used to achieve temporal activation of several transcription factors in plants [122-131]. Other inducible systems that are based on estradiol, tetracycline have also been described but they have not yet been couple to live-imaging [132-135].

Design of inducible systems has been modified in such a way that the two components could be placed in independent vectors; this requires the development of independent transgenic lines and combining them in one plant through genetic crosses [119-120]. Alternatively both the components can be placed in a single vector such that transgene activation could be achieved in a single transgenic plant and such a system will be useful to efficiently incorporate several different markers for live imaging experiments. Thus, the ways how inducible transgene activation could be achieved are divided into two component inducible system and one vector system. However, if a tissue-specific spatial expression of a regulator is needed, the two component system may be more useful because the same target line (6XOp::Transgene) can be crossed to several different driver (Promoter::LhG4:GR) lines. The inducible systems of gene manipulations can be combined with live imaging to analyze the effects of transient perturbations in real time.

f. **Specific aims**

In order to dissect the function and regulation of cytokinin levels and cytokinin responses in SAMs, transient perturbations, live image and cell type specific genomics have been employed. These approaches were chosen because cytokinin has been shown to function in almost every cell and every developmental field which makes it impossible to

gather useful analytical information to decipher its function. Four specific goals are listed below.

a. Genetic and expression studies on gene family that encode SAM-enriched cytokinin activating enzymes.

b. Transient manipulation of cytokinin levels and live-imaging based analysis of its effects on stem-cell maintenance and organ patterning.

c. Determining the role of cytokinin signaling in stem cell homeostasis.

d. Dissecting the regulation of cytokinin and auxin homeostasis by SHOOTMERISTEMLESS in transient manipulation experiments.

MATERIAL AND METHODS

a. Plant materials and growth condition

All *Arabidopsis thaliana* plants were grown as described in an earlier study [36]. Plants were grown under a continuous light at 23^{o}C, and were typically 20-25 days old at the time of transgene induction. Plants for live-image experiments were grown in a plastic growth chamber as described earlier [7, 113].

The double transgenic reporter line, *pDR5::3xVenus::N7*; *pPIN1::PIN1::GFP* has been described previously[113]. The double transgenic reporter line, *p35S::YFP::29-1*; *pCLV3::GFPer* has been described previously [7].

b. Plant transformation

All Binary T-DNA vectors were introduced into *Agrobacterium Tumefaciens* strain GV3101 by A freeze-thaw method [136]. *Arabidopsis* plants were transformed by using floral dip method [137].

c. Dex treatment

Dexamethasone (Dex) was dissolved in ethanol to prepare a stock solution of 30mM. The stock solution was diluted in water containing 0.0152% of silwet-77 to a working concentration of 10µM when used to treat the plants grown on soil and 20µM Dex was used for live-image experiments. Mock solution contained same amount of ethanol but without Dex. To make Murashige-Skoog (MS) medium with Dex, the stock solution was directly added to the medium to a final concentration of 10µM.

d. Construction of plasmids

The *p35S::ARR1ΔDDK::GR* plasmid was a kind gift from Dr. Takashi Aoyama (Institute for Chemical Research, Kyoto University) and it has been described in an earlier study [138-139]. The *pARR5::GFP* reporter construct was a kind gift from Dr. J. Kieber[140].

All plasmid constructions were carried out according to standard molecular biology techniques[141]. The *IPT7* and *CKX3* genes were cloned from cDNA generated by reverse transcription system. The cDNAs were first cloned into the *pENTR D-TOPO* vector (Invitrogen) and were sequenced to confirm error-free amplification. By employing Gateway method *polyA* terminator of 35S gene was added to *IPT7* and *NOS* terminator was added to *CKX3*. Subsequently, the genes were cloned into *pPOPOFF2 (HYG)*[121] into the KpnI site and partially-digested XhoI site to generate *pMTX009::IPT7::polyA* and *pMTX009::CKX3::NOS* constructs. The *ARR1ΔDDK:GR* was amplified from The *p35S::ARR1ΔDDK:GR* plasmid and cloned into *pENTR/D-TOPO*. After sequencing the amplified fragment was moved into *pMX6xOPs::GW* by the LR reaction to generate *6XOP::ARR1ΔDDK:GR*. A fragment of STM cDNA (545-1147) was cloned into *pENTR/D-TOPO* and subsequently moved into *pPOPOFF2(HYG)* vector to make the *STMRNAi* construct.

e. Genotyping T-DNA mutants

SALK T-DNA insertion lines and SAIL lines were obtained from ARBC stock center. GABI-KAT lines were obtained from http://www.gabi-kat.de/ and the FLAG lines were ordered from http://www-ijpb.versailles.inra.fr/en/sgap/equipes/variabilite/crg/index.htm. Seeds were stratified for 4 days at 4^0C before planting on soil. DNA was isolated according

to a method described earlier with modification [142]. Two fully-expanded leaves were ground into fine powder in liquid nitrogen. 600 µl DNA extraction buffer (100 mM Tris, PH 8.0, 50 mM EDTA, 250 mM NaCl, 5% SDS) was added to each sample. Samples were incubated at 65^0C for 60 minutes. After samples were cooled down to room temperature, 200 µl potassium acetate was added to each sample. Then, samples were cooled to 4^0C for at least 30 minutes. Samples were centrifuged at 13.2K for 15 minutes. 750 µl of supernatant was dispensed into a new tube and centrifuged again at 13.2K for 5 minutes. 600 µl of the supernatant was transferred into a sterilized tube and was mixed with 600 µl isopropanol for 5 minutes at room temperature. DNA was precipitated by centrifuging at 4000K for 2 minutes. The DNA pellets were washed by 1.5 ml of cold 75% EtOH and dried by Speed-Vac. Each DNA sample was dissolved in 100 µl sterilized water and 1 µl was used for PCR. T-DNA border primers were LB1-3 (5' ATTTTGCCGATTTCGGAAC 3') for SALK lines, LB4 (5' CGTGTGCCAGGTGCCCACGGAATAGT 3') (FLAG) for Flag lines, LB1 (5' GCC TTT TCA GAA ATG GAT AAA TAG CCT TGC TTC C 3') for Sail lines, and LB2 (5' ATAATAACGCTGCGGACATCTACATTTT 3') for GABI-KAT lines. Other LP and RP primers for individual T-DNA line is listed in primer list (Table M-1, M-2).

f. Live-image

Live-image studies were carried out according to previous described [7]. Seeds were surface sterilized by 25% bleach plus 0.05% Tween-20 for 8 minutes followed by sterilized-water washes three times. After cold-treatment at 4^oC for three days, seeds were planted on MS medium. At four leaves stage, four seedlings were transferred into each

plastic box containing MS medium which was overlaid with a thin-layer of 1% agarose. Immediately upon bolting each shoot apex was dissected to remove older floral buds, supplemented with 1.5% agarose to stably anchor the plant to avoid vibrations during imaging. Plants were imaged under Zeiss 510 confocal microscope fitted with Argon-Krypton laser. 63X water immense lense with 0.95 numerous aperture (NA) was used to acquire images by employing the laser parameters and the emission filter as described in an earlier study [7]. Images were then reconstructed by using Zeiss-LSM software.

g. RT-PCR

Real-time RT-PCR was performed according to the method described previously with minor modifications [143]. Inflorescences apices of Dex-treated or Mock treated transgenic plants were hand dissected after removal of older fully open flowers. About forty to fifty apices from each sample were used to isolate total RNA by using Plant RNAeasy mini-Kit (Qiagen). On-column DNase I treatment was carried out before elution of RNA. cDNA synthesis was carried out in a 20 µl reaction using 1 µg total RNA and oligo (dT) primer by the ImProm-II™ Reverse Transcription System according to the manufacturer's instructions (Promega). The cDNA reaction mixture was diluted 1:10 using Dnase-, Rnase-free Milli-Q water and 5 µl was taken for RT-PCR analysis. All reactions were performed independently twice to ensure reproducibility. For all samples the cDNA levels were normalized using an UBIQUITIN levels as control. Primers used in each reaction have been provided in Table1.

h. RNA in-situ hybridization

For RNA *in situ* hybridization, shoot apices of 3 weeks old plants were treated with either 10 µM Dex or Mock solution every day till 3rd or 6th day. Shoot apices were harvested and fixed as previously described [36]. We followed the probe preparation methods described earlier with minor modifications[144-145]. The primers used for each gene fragments to prepare probes have been listed in Table 1. T7 promoter sequence was incorporated in reverse primers to facilitate *in vitro* transcription to prepare RNA probes. The primer pairs; mx319AS1F 5' caccatgaaagagagacaacgttgga 3' and mx320AS1RT7 5' TAATACGACTCACTATAGGGACTggggcggtctaatctgc 3' (The upper cases indicate T7 promoter sequence) were used for amplifying *AS1* by using *pET101/D-TOPO:AS1*cDNA as template. The primers mx321AS2F 5' gaattcatggcatcttcttcaacaaactcac 3' and mx322AS2RT7 5' TAATACGACTCACTATAGGGACTaagcttagacggatcaacagtacggcg 3' (The upper casse indicate T7 promoter sequence) were used to amplify *AS2* by using *pBSKS:AS2cDNA* as template. The primers used for generating RNA probes for CKX, LOG, and PUM family members have been listed in Table M-1.

i. Gel mobility shift assays

Gel mobility shift was carried out according to previous report with modifications [138]. DNA fragment of ARR1 M (aa. 236 to 299) was amplified by PCR using p35S::ARR1 ΔDDK::GR as template and using primer ARR1Mf NdeI and ARR1Mr XhoI Table 1). Subsequently, the PCR products were digested by NdeI and XhoI and gel purified, ligated in frame into *pET28a* vector at the corresponding sites to generate His-tagged version. The fragments used were 5'-CGACGTGAATTCTAAGATTGTCTCGCATACACTG-3' and two 1 bp substitution

derivatives within the 5 bp core sequences (underlined, AGATc and AgtTT). ^{32}P-labelled DNA fragments (about 2 ng, 1×10^5 cpm) were incubated for 30 min at 20°C with *E. coli* crude extract (about 50 ng proteins) containing a fusion protein in 20 µl binding buffer supplemented with 2 µg sheared salmon sperm DNA. After the incubation step the mixture was immediately loaded on 4% polyacrylamide gels (acrylamide:bis-acrylamide, 19 : 1). Electrophoresis was carried out in 0.25 × TBE (1 × TBE = 89 mm Tris-borate, 2 mm EDTA pH 8.3). Gels were wrapped in Saran wrap and then subjected to autoradiography using phosphor imager Typhoon™ (GE Healthcare).

j. Indole-3-acetic acid (IAA) analysis

Transgenic plants harboring either dex inducible CKX3 or IPT7 were germinated and grown in soil until they were bolting. When the primary inflorescences were about 5 to 10 cm tall, a drop of 10 µM dex solution or mock solution was applied to the tip of each inflorescence, once per day, for four days. The apices were dissected by removing the extra floral buds and frozen into liquid nitrogen until enough samples were ready. IAA measurements, samples were extracted, puried andanalysed by gas chromatography-selected reaction monitoring-mass spectrometry (GC-SRM-MS) as previously described [146-148].

k. List of primers that were used in this dissertation

Table M-1. The sequences of primers

Primer sequence 5' to 3'	**Primer names**
attttgccgatttcggaac	mx66lbb1-3
tgtcaacattttcttcacaaaaag	mx67ckx3lp
aaacggacggtgtagatttcttag	mx68ckx3rp
agacgtttttgagatgagtttgtg	mx69ckx5lp

tcatatgtgcaattgaaaacactg	mx70ckx5rp
caccgtcgacggtaccatggcgagttataatcttcgttc	mx85ckx3f
tttggatcctaactcgagtttatttttgaaatatattttgtc	mx86ckx3r
taatacgactcactatagggactttacgtagcggtagctacc	mx90t7at5g06300r
tttggatccatttctctctctttctctttg	mx91pat5g06300rbamh
caccactccggtgaatcttgctgagtc	mx92pat5g06300f
cagctatctactttactcgggtc	mx104ckx3f-285
catggtaaaccgggatcgagg	mx107ckx3seqf399
gtgtattgagaagtttcgtc	mx108ckx3seqr999
caccgtcgacggtaccatgaatcgtgaaatgacgtcaagc	mx121ckx5f
ggatcctcaccatgaagccgctgacgaag	mx122ckx5r
cacctcgagatgaagttctcaatctcatcac	mx129at3g23630ipt7f
ttggatcctcatatcatattgtgggctc	mx130at3g23630ipt7r
caccggtaccatggaggtcaacaatgaaacc	mx156 atlog6fk
taatacgactcactatagggactggatcctcagtcttcagaagagtagtc	mx157 atlog6rt7b
taatacgactcactatagggacttcaccatgaagccgctgacgaag	mx158ckx5t7r
caccatgagctatctacatgcaagcctc	mx159ckx6f
taatacgactcactatagggacttcatgagtatgagactgcc	mx160ckx6t7r
caccatggagatagaatcaaagttc	mx161atlog8f
taatacgactcactatagggacttcatcttgagatttcacaagtgggac	mx162atlog8t7r
caccatggaaatagtgaagtcgaggttc	mx163atlog3f
taatacgactcactatagggactctacttgttgctgttgttgc	mx164atlog3t7r
gttcttgaagcacaagattg	mx165ipt7sr220
caccgtcgacatggaagagacaaaatcgagattcaagaggatc	mx166atlog1f
ttgaattaatgaattatagtttgatacg	wus1a(genotype)
ttgaagttatggatcttgattgg	wus1s(genotype)
tcctagaactcatggtgggtg	mx171ck30x5lp
ccgaagataagaccctcttcg	mx172ck30x5rp
ccgaagataagaccctcttcg	mx173ck31x5lp
taacggacggtgaagagacac	mx174ck31x5rp
taacgctatgcgactattggg	mx175ck32x5lp
ataatcaatgatgtgcggagg	mx176ck32x5rp
agtttgatccgcgacacatac	mx177ck33x5lp
acagcaatgggcatatttgtc	mx178ck33x5rp
tctacagtgtggattcccctg	mx179ck34x6lp
cctttcgatcggagttttacc	mx180ck34x6rp
tctacagtgtggattcccctg	mx181ck35x6lp

gaagcagggatagggttgaag	mx182ck35x6rp
tgttatgcaatggaccactcc	mx183ck36x6lp
gtaaaactccgatcgaaaggg	mx184ck36x6rp
tcattccaaaaaccttgatgc	mx185lo15bglp
attacgacatgggaagcttcc	mx186l15bgrp
cacatccatggcttaatctcttcgtac	mx224f1136ckx3
tgtttttgccaaagaagatgg	mx225lo25g6lp
tcctcaaaatctgtttccttcc	mx226lo25g6rp
tgtccaaaagagttcgtgacc	mx227lo26g3lp
aaaccatctcagcgtcacatc	mx228lo26g3rp
caccactagtatggaggtcaacaatg	mx233log6f
gcactagtaggcctcagtcttcagaagagtagtcaatccg	mt234log6r
atggcattgtaagcaaattgg	mx235atl25bog6lp
aggcaaagaaaagtttcgagc	mx236atl25bog6rp
tcaattgagacaaaacaggcc	mx237atl27bog8lp
tcacgatcgattaatcgttcg	mx238atl27bog8rp
aagccgatttcttccaatctc	mx239atl28bog4lp
attaggggcacgatacaaacc	mx240atl28bog4rp
taatacgactcactatagggacttcagtcttcagaagagtagtc	mx241atlog6rt7
caccatggaaatagtgaagtcgag	mx244atlog3f
taatacgactcactatagggactctacttgttgctgttgttg	mx245atlog3rt7
gtacgtcgtgacaacacatcg	mx264lo44g1lp
cagagaatctcgtttcaatgacc	mx265lo44g1rp
caccatggaagataatcagcgaag	mx246atlog4f
taatacgactcactatagggactttattgcggcttgttttcttg	mx247atlog4rt7
caccatggagatagaatcaaagttcaag	mx248atlog8f
taatacgactcactatagggacttcatcttgagatttcacaagtg	mx249atlog8rt7
ctcaatccatagccacgcagcgtcc	mx279ckx3r450
caccatggaagataatcagcgaag	mx246atlog4f
taatacgactcactatagggactttattgcggcttgttttcttg	mx247atlog4rt7
ctcaatccatagccacgcagcgtcc	mx279ckx3r450
gagcattgaggagactggcg	mx280arr1f500
gataccctccaagccgtcttag	mx281arr1r896
gtgagggtgaaatcagagag	mx281arr1f1903
taagattaggggcacgatacaaac	mx282lo69g4gp
ataataacgctgcggacatctacatttt	mx283gabilb
atattgaccatcatactcattgc	mx284gabiseq

tagcagaaggagcagatacaatga	mx285lo70g8gp
atgatgaatccgagtcacggaag	mx311farr1
tcatttttgatgaaacagaag	mx312r
tcatttttgatgaaacagaag	mx312rgrr
caccatgaaagagagacaacgttgga	mx319as1f
taatacgactcactatagggactggggcggtctaatctgc	mx320as1rt7
gaattcatggcatcttcttcaacaaactcac	mx321as2f
taatacgactcactatagggactaagcttagacggatcaacagtacggcg	mx322as2rt7
caccatgcatattg agcac	mx326log5f
taatacgactcactatagggacttcattgaggcttgttctc	mx327log5rt7
cggtgtccccacgggtttac	mx330arr1f1209
gcagtgacaccaaggtaggg	mx331r100
caccatggttaggaagaggagaagtgaatggagtgtac	mx318f457ncoi
gct tcc tat tat atc ttc cca aat tac caa tac a	SailLB2
cgtgtgccaggtgcccacggaatagt	LB4(Flag)
gttcaaggactttccaaccgcaagatgat	clv3 5’
ccttctctgcttctccatttgctccaacc	clv3 3’
ccagcttcaataacgggaatttaaatcatgca	Wus 5’
tcatgtagccattagaagcattaacaacaccacat	Wus 3’
ttcacttggtcctgcgtcttcgtgtggtttc	ubq10 5’
cgaagcgatgataaagaagaagttcgacttg	ubq10 3’

Table M-2. The application of primers

Primer names	**Application**
mx66lbb1-3	To isolate SALK T-DNA
mx67ckx3lp	To isolate *ckx3* insertional mutant
mx68ckx3rp	To isolate *ckx3* insertional mutant
mx69ckx5lp	To isolate *ckx5* insertional mutant
mx70ckx5rp	To isolate *ckx5* insertional mutant
mx85ckx3f	To Clone CKX3
mx86ckx3r	To Clone CKX3
mx90t7at5g06300r	To make in situ probe
mx91pat5g06300rbamh	To Clone At5g06300
mx92pat5g06300f	To Clone At5g06300
mx104ckx3f-285	Sequencing *CKX3*
mx107ckx3seqf399	Sequencing *CKX3*/RT

mx108ckx3seqr999	Sequencing *CKX3*/RT
mx121ckx5f	To clone *CKX5*
mx122ckx5r	To clone *CKX5*
mx129at3g23630ipt7f	To clone *IPT7/RT*
mx130at3g23630ipt7r	To clone *IPT7*
mx156 atlog6fk	KpnI and BamHI for 6xOPs
mx157 atlog6rt7b	for probe
mx158ckx5t7r	to make probe
mx159ckx6f	to make probe
mx160ckx6t7r	to make probe
mx161atlog8f	to make probe
mx162atlog8t7r	to make probe
mx163atlog3f	to make probe
mx164atlog3t7r	to make probe
mx165ipt7sr220	to sequence *IPT7* reverse/RT
mx166atlog1f	to clone full length LYSDC at5g06330
wus1a(genotype)	To genotype *wus-1* mutant
wus1s(genotype)	To genotype *wus-1* mutant
mx171ck30x5lp	To isolate *ckx5* insertional mutant
mx172ck30x5rp	To isolate *ckx5* insertional mutant
mx173ck31x5lp	To isolate *ckx5* insertional mutant
mx174ck31x5rp	To isolate *ckx5* insertional mutant
mx175ck32x5lp	To isolate *ckx5* insertional mutant
mx176ck32x5rp	To isolate *ckx5* insertional mutant
mx177ck33x5lp	To isolate *ckx5* insertional mutant
mx178ck33x5rp	To isolate *ckx5* insertional mutant
mx179ck34x6lp	To isolate *ckx6* insertional mutant
mx180ck34x6rp	To isolate *ckx6* insertional mutant
mx181ck35x6lp	To isolate *ckx6* insertional mutant
mx182ck35x6rp	To isolate *ckx6* insertional mutant
mx183ck36x6lp	To isolate *ckx6* insertional mutant
mx184ck36x6rp	To isolate *ckx6* insertional mutant
mx185lo15bglp	To isolate *log1* insertional mutant
mx186l15bgrp	To isolate *log1* insertional mutant
mx224f1136ckx3	To sequence *CKX3*
mx225lo25g6lp	To isolate *log6* insertional mutant
mx226lo25g6rp	To isolate *log6* insertional mutant

mx227lo26g3lp	To isolate *log3* insertionalal mutant
mx228lo26g3rp	To isolate *log3* insertionalal mutant
mx233log6f	To clone *LOG6* gene
mt234log6r	To clone *LOG6* gene
mx235atl25bog6lp	To isolate *log6* insertional mutant
mx236atl25bog6rp	To isolate *log6* insertional mutant
mx237atl27bog8lp	To isolate *log8* insertional mutant
mx238atl27bog8rp	To isolate *log8* insertional mutant
mx239atl28bog4lp	To isolate *log4* insertional mutant
mx240atl28bog4rp	To isolate *log4* insertional mutant
mx241atlog6rt7	To make probe for *LOG6*
mx244atlog3f	To make probe for *LOG3*
mx245atlog3rt7	To make probe for *LOG3*
mx264lo44g1lp	To isolate *log1* Flag line
mx265lo44g1rp	To isolate *log1* Flag line
mx246atlog4f	To make probe for *LOG4*
mx247atlog4rt7	To make probe for *LOG4*
mx248atlog8f	To make probe for *LOG8*
mx249atlog8rt7	To make probe for *LOG8*
mx279ckx3r450	for sequencing
mx246atlog4f	To make probe for *LOG4*
mx247atlog4rt7	To make probe for *LOG4*
mx279ckx3r450	for sequencing
mx280arr1f500	Sequencing *ARR1* construct
mx281arr1r896	Sequencing *ARR1* construct
mx281arr1f1903	Sequencing *ARR1* construct
mx282lo69g4gp	*log4* gabi line gene specific primer
mx283gabilb	Gabi line LB
mx284gabiseq	Gabi line sequencing
mx285lo70g8gp	*log8* gabi line gene specific primer
mx311farr1	endogeneous ARR1 forward
mx312r	GRr
mx312rgrr	*ARR1-GR* reverse
mx319as1f	To make AS1 in situ probe
mx320as1rt7	To make AS1 in situ probe
mx321as2f	To make AS2 in situ probe
mx322as2rt7	To make AS2 in situ probe

mx326log5f	To make probe for *LOG5*
mx327log5rt7	To make probe for *LOG5*
mx330arr1f1209	To sequence ARR1
mx331r100	To sequence ARR1
mx318f457ncoi	To maek GR fusion with MX312r
sail lb2	SAIL line T-DNA left border
lb4	FLAG line T-DNA left border
arr1m709fndei	RT-PCR
arr1m900rxhoi	RT-PCR
clv3 5’	RT-PCR
clv3 3’	RT-PCR
wus 5’	RT-PCR
wus 3’	RT-PCR
ubq10 5’	RT-PCR
ubq10 3’	RT-PCR

CHAPTER I

Sub-title: Genetic Studies on the Stem Cell-enriched Genes

Introduction

Though genetic studies have identified crucial links in cell-cell communication networks that operate in SAM development and maintenance, however, network-level description is far from understood [1, 4, 36, 149]. For example, how activated-CLV1 and related receptor kinases-mediated signal reaches the nucleus to downregulate WUS expression is not clear [150]. Also how WUS which is expressed in the OC communicates with overlying cells of the CZ to specify them as stem-cells and also activate CLV3 expression is not well-understood [37]. Though genetic screens have uncovered molecules such as POLTERGEIST (POL), a member of protein phosphatase family, it is not clear how they function within the CLV-WUS feedback loop [151]. The earlier genomic studies have also failed to uncover specific molecules that function in stem-cell specification and this could be because there are only about 35 stem-cells in the entire plant and these results in dilution of relevant transcripts when whole seedlings are used for expression profiling [111]. Reddy lab at UCR has established new methods for isolation of stem-cells and analysis of stem-cell enriched transcriptome [36]. This analysis has resulted in several new genes that are enriched in specific sub-sets of cells of the stem-cell niche which were not part of the whole seedling microarray. One of the major goals now is to understand the function of these genes in stem-cell homeostasis. Most of the genes enriched in cells of the stem-cell niche belong to incredibly complex gene families and multiple knock out mutants in family members may unravel their function. This may be one of the reasons why

forward genetic screens have failed to identify the function of these genes. Here I have analyzed the function of three gene families by reverse genetic approach. They include; genes involved in cytokinin biosynthesis that share similarities to *LONELYGUY* of rice [*AtLOG*] [80-81], genes involved in cytokinin degradation [AtCKX (Cytokinin Oxidase)] [70, 87, 152] and genes that encode evolutionarily-conserved PUMILIO class of RNA binding proteins [153-162]. The main objective of this work is to identify single mutants for all members of each gene family whose transcripts are enriched in cells of the stem-cell niche. Then generate higher order mutant combinations through genetic crosses to test for phenotypic abnormalities. Thus far, the triple mutants of in members of *AtLOG* gene family have revealed phenotypes associated with SAM function. Though no phenotypes have been associated with *AtPUM* and *AtCKX* family thus far, however, these mutant combinations should provide a base to introduce mutants in other members of the gene family to study their functions. The results related to this work are summarized below.

Results

1.1 Genetic study on AtCKX family

Arabidopsis genome contains seven members that belong to *AtCKX* family. Cell type-

Name	**Gene ID**	**T-DNA lines**
AtCKX2	at2g19500	SALK_068485C
AtCKX3	At5g56970	SALK050938c
AtCKX4	at4g29740	CS120294
AtCKX5	AT1G75450	SAIL_693_E12
AtCKX6	AT3G63440	SALK_070071
AtCKX7	AT5G21482	SALK_092241C

Table 1-1 Gene IDs and T-DNA lines of the *AtCKX* family

specific transcriptome predicted that the expression of *AtCKX3* and *AtCKX5* is enriched in centrally-located cells of SAMs [36][Figure1-1A, E], while *AtCKX6* was found to be enriched in peripheral cells of SAMs (Figure1-1A). *AtCKX7* was not represented on ATH1 (Affymetix) gene chip. Therefore the spatial expression pattern of *AtCKX7* was studied by cell-type specific real-time RT-PCR and found to be enriched in peripheral cells of SAMs in a manner similar to that of *AtCKX6* (Figure1-1F).

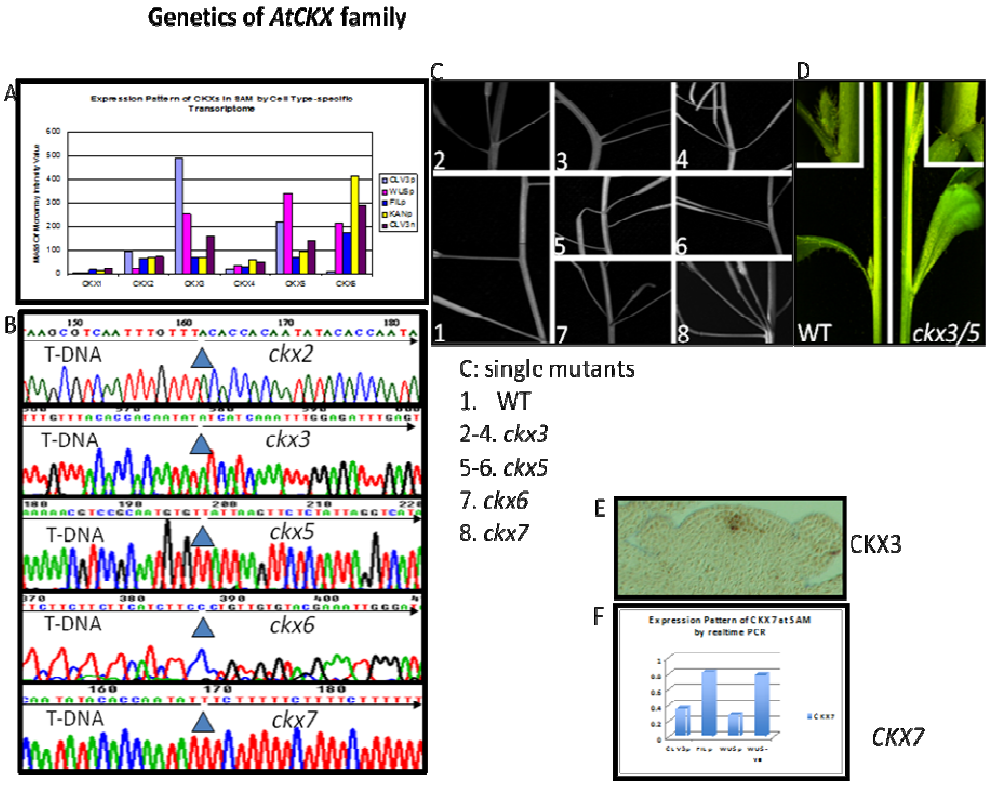

Figure 1-1. Genetic study on *AtCKX* family. (A) Expression patterns of *AtCKX* family members as revealed by cell type-specific transcriptome. (B) Sequence plots showing junction of T-DNA insertion within each gene. (C) Phenotypes of single mutants. (E) Expression pattern of *AtCKX3* by RNA *in situ*. (F) Expression of *AtCKX7* by qRT-PCR.

As a first step, single T-DNA insertions in *ATCKX2*, *ATCKX3*, *ATCKX5*, *ATCKX6*, and *ATCKX7* were obtained from ABRC stock center and insertions within these genes were confirmed by PCR genotyping (Figure1-1. B; Table 1-1).

None of the single mutants revealed dramatic phenotypes, except for mild organ positioning defects in *atckx3*, *atckx5* and *atckx7* (Figure1-1C). The double mutants of *atckx3 and atckx5* were found to be normal except for mild defects in few auxiliary meristems failed to develop (Figure1-1D). From this analysis, it appears that the local degradation of cytokinin may not play a significant role in SAM development. It is also possible that generating higher order mutant combinations with *atckx6* and *atckx7* which are enriched in peripheral cells of SAMs may unravel their function.

1.2 Genetic study on *AtLOG* family

Spatial regulation of synthesis of bioactive cytokinin was unraveled through the identification of LONELYGUY *(LOG)* class of genes in rice [80].

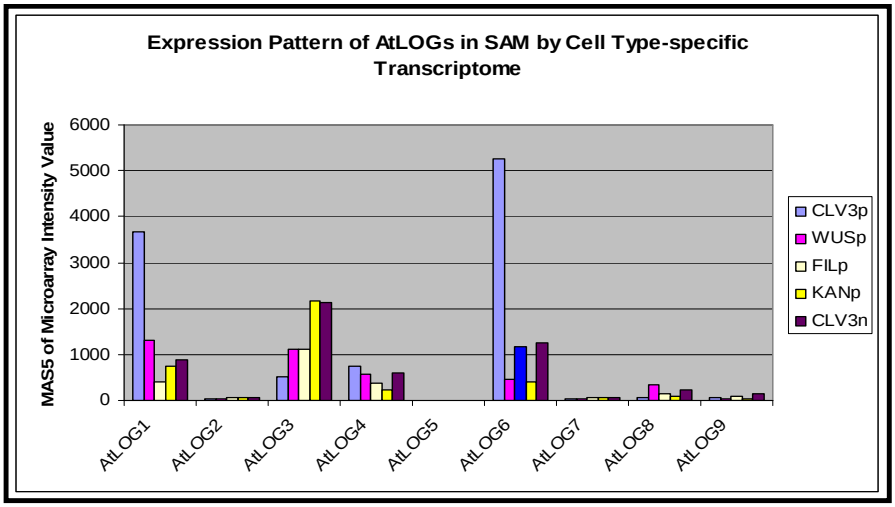

Figure 1-2. The expression pattern of *AtLOGs* in SAM by cell type-specific transcriptome.

The *log* mutants of rice were originally isolated as SAM defective mutants [80]. This apart, *log* mutants also revealed defects in branching pattern, reduced panicle size, reduction in the number of floral organs terminating in a single pistil. These phenotypes suggested defects in SAM maintenance. Consistent with this notion, the vegetative SAMs

of log mutants were smaller than wild type. In reproductive phase *log* mutant meristems and panicle branch meristems terminate after producing a limited number of lateral meristems. By using *HISTONE H4* marker, they further show reduced mitotic activity in log mutant meristems compared to wild type. Reduced SAM activity was further confirmed by a reduction in *OSH1*, a meristem marker encoding a KNOTTED1-type homeobox (KNOX) protein, expression in mutant meristems. Taken together these results indicated that local synthesis of bioactive cytokinins is critical for SAM maintenance.

Names in this study	**Names in reference(Kuroha, et al, 2009)**	**Gene IDs**	**T-DNA lines**
AtLOG1	AtLOG7	At5g06300	FLAG_439F05, SALK_113173C
AtLOG3	AtLOG5	AT4g35190	FLAG_564D07
AtLOG4	AtLOG8	AT5G11950	GABI_378H10
AtLOG6	AtLOG4	AT3G53450	SALK_092241
AtLOG8	AtLOG1	AT2G28305	GABI_744G12, SALK_143462C

Table 1-2. Gene IDs and T-DNA lines of the AtLOG family

A recent study in *Arabidopsis* has shown that the biosynthetic pathway involving direct activation of cytokinin plays an important role [81]. By employing ectopic overexpression, expression pattern analysis by reporter assay, enzyme activity assay, and genetic analysis of three family members has revealed that these genes could play a role in *Arabidopsis.* However, this study did not directly address the function of gene family members whose expression is enriched in cells of the SAM stem-cell niche. The cell type-specific transcriptome analysis in our lab revealed that the expression of at least five *AtLOG* (*AtLOG1, AtLOG6, AtLOG8, AtLOG3* and *AtLOG4*) family members could be detected in

SAMs (Figure1-3). RNA *in situ* hybridizations revealed that *AtLOG1* and *AtLOG6* are expressed in central regions of superficial cell layers of SAMs. This expression pattern could partially overlap with CLV3 but wider than that of CLV3 expression domain (Figure1-4A and B). Expression patterns of *AtLOG8* revealed that it is expressed in cells of the RM which could overlap with WUS expression domain (Figure1-4 C). The expression pattern of *AtLOG3* is confirmed not in the stem cell niche (Figure1-5A). The expression pattern of *AtLOG4* is confirmed not in the stem cell niche either (Figure1-5B).

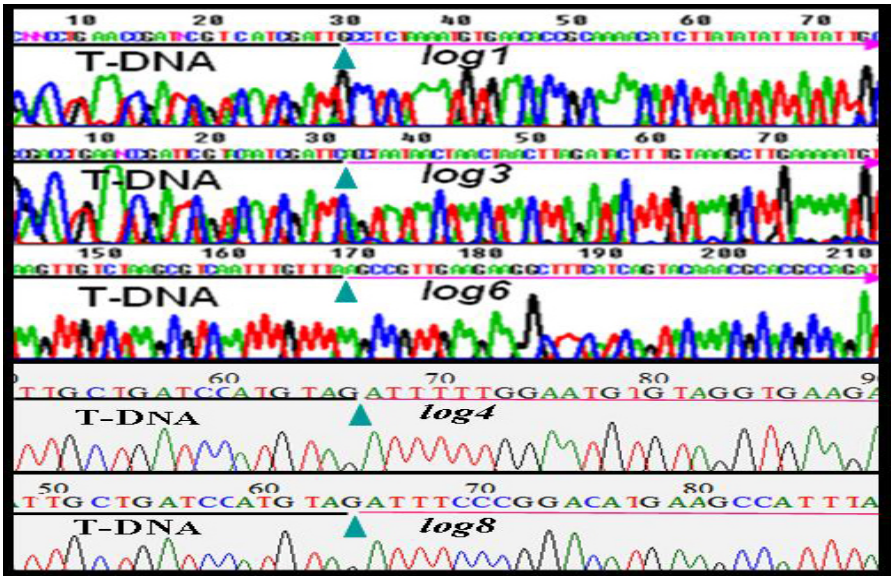

Figure 1-3. The junction of the T-DNA insertion of *atlog* mutants. Triangle points to the junction of T-DNA border and the LOG genes.

Thus, *AtLOG1, AtLOG3, AtLOG4, AtLOG6,* and *AtLOG8* were chosen for genetic analysis. Insertional T-DNA mutant lines were obtained from different sources (Table 1-2) and the T-DNA insertion sites were mapped through PCR genotyping (Figure1-4). Initially we focused on generating triple mutants of *AtLOG1, AtLOG6,* and *AtLOG8* since these exhibited specific expression in cells of the stem-cell niche (Figure1-4. A-C).

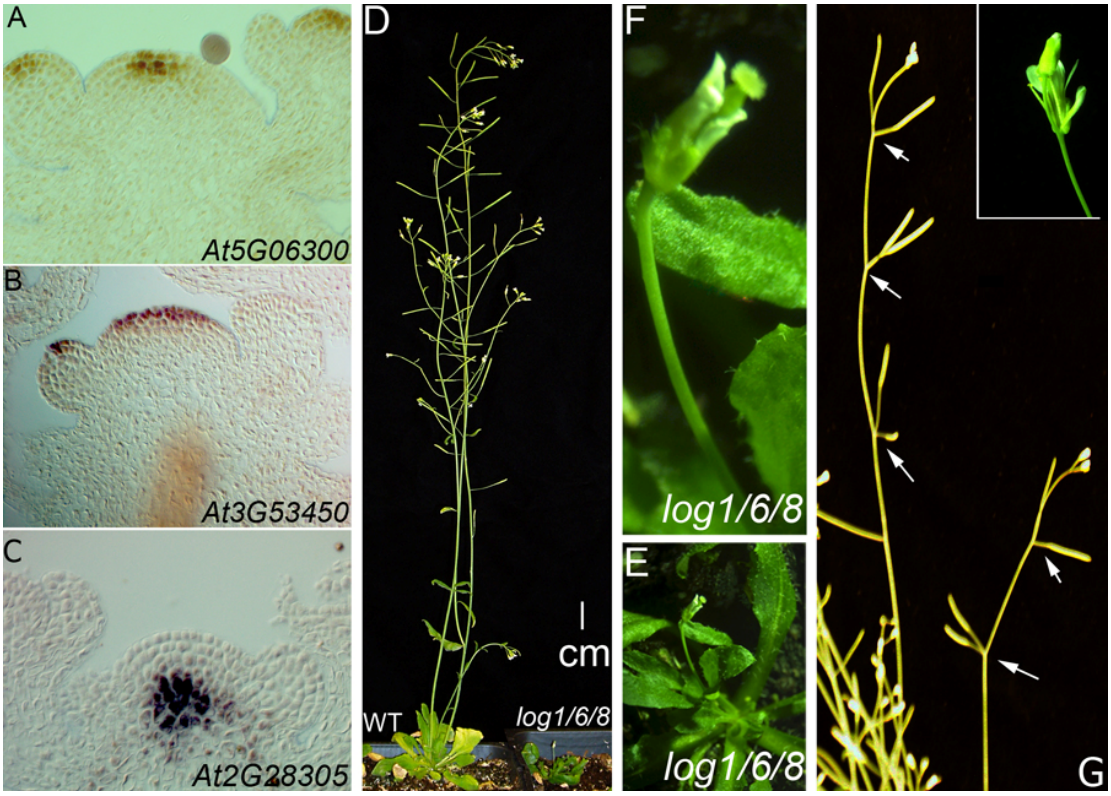

Figure 1-4. Phenotype of triple mutants *atlog1/6/8*. (A-C) Expression pattern of stem cell related AtLOGs. (A) AtLOG1 (At5G06300). (B) AtLOG6 (At3G53450). (C). AtLOG8 (At2g28305). (D) Phenotypes of triple mutant *atlog1/6/8*. Wild type (left), *log1/6/8* (right bottom corner). Both plants were at the same age. Scale bar = 1cm. (E) Enlargement of *log1/6/8* in D. (F) Enlargement of E. (G) Phyllotactic defects in *atlog1/6/8* triple mutant. Insert shows termination of a secondary inflorescence.

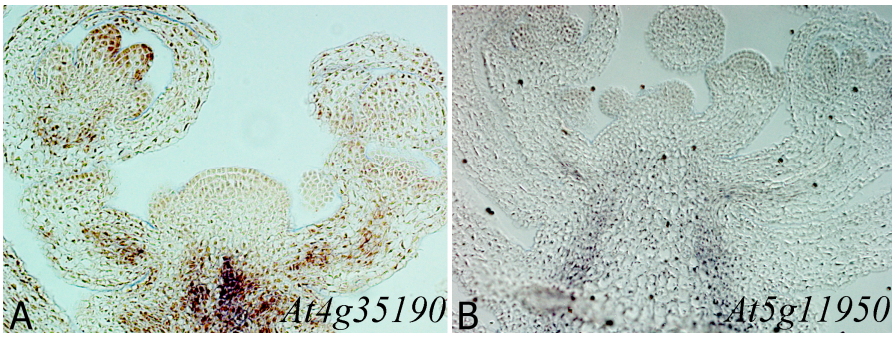

Figure 1-5. Expression patterns of stem cell unrelated AtLOGs. (A) AtLOG3. (B) AtLOG4

Each of single mutants did not show striking phenotypes. The double mutant of *log1* and *log6* also did not reveal any phenotype (data not shown). Then the *log8* was introduced into *log1* and *log6* double mutants to generate *log1, log6, log8* triple mutant. PCR genotyping confirmed the presence of three T-DNAs in homozygous condition. The triple mutant displayed dramatic phenotypes (Figure1-4. D-F). The growth rate was much slower compared to wild type resulting in stunted, small-statured plant (Figure 4D). They were delayed in bolting and ultimately produced a single flower before terminating the meristematic activity (Figure 4-5E and F). They produce few secondary inflorescences and they displayed irregular phyllotactic pattern compared to wild type with two flowers developing next to each other (Figure1-4G, white arrowheads). Ultimately the secondary inflorescences were terminated after making one or two flowers at the tip (Figure1-4G, Inset). In summary, the phenotypes of meristem-enriched *log* triple mutant phenotypes reveal that local activation of cytokinin biosynthesis is important for SAM function and also suggest that it might play a role in regulating phyllotaxy either directly by regulating auxin homeostasis or indirectly by controlling growth patterns.

1.3 Genetic study on *AtPUMILIO (AtPUM10)* family

The cell-type specific transcriptome revealed enrichment of *PUMILIO* class of sequence specific RNA binding proteins (*AtPUM10* and *AtPUM12*) in centrally-located cell types of SAMs (Figure1-3A). Though the expressions of other family members were detected in SAMs, however, they were not differentially-expressed (Figure1-3A). RNA *in situ* analysis was carried out by using *AtPUM10* and *AtPUM12* specific probes. This analysis revealed that *AtPUM10* was specifically-expressed in cells of the CZ, very similar

to the *CLV3* expression pattern (Figure1-3B). However, our repeated attempts of RNA *in situ* by using *AtPUM12* probe failed to yield any signal on SAM sections (data not shown). We considered three members of the gene family (*AtPUM10, AtPUM12 and AtPUM9*) for genetic analysis. *AtPUM9* was also considered because it shares very close similarities with *AtPUM10*. In order to test the functional significance of stem-cell specific expression of *AtPUM10*, transgenic plants with constitutive overexpression of *AtPUM10* were generated. The overexpression of the transgene was confirmed by RT-PCR analysis on independent transgenic lines (Figure1-3D). Majority of the T1 generation plants showed strong stem-fasciation and irregular arrangement of flowers with two or more flowers clustered together on fascinated stem (Figure1-3C). However, none of these phenotypes were stably-inherited by the T2 generation progeny of several independent T1 plants. In order to test whether this was due to transgene silencing, the same construct was transformed into *rdr6-11* [*suppressor of gene silencing2(sgs2)/silencing defective1 (sde1)/rna-dependent polymerase6-11*] mutant background which is defective in transgene silencing [163]. However, even the T1 transgenic plants did not shown obvious phenotypes that were observed in T1 wild type plants. Therefore we decided to revisit this perplexing phenomenon at a later stage, if the loss of function analysis revealed relevant phenotypes. T-DNA insertion mutants were identified in both *AtPUM9* and *AtPUM12*, however, no T-DNA insertion was available for AtPUM10 (Table 1-3).

The double mutants of *atpum9/atpum12* did not show any phenotypes. *AtPUM9* and *AtPUM10* were located close to each other in the genome which makes it impossible to generate double mutants. Therefore, double stranded fold-back construct dsRNAi*AtPUM10*

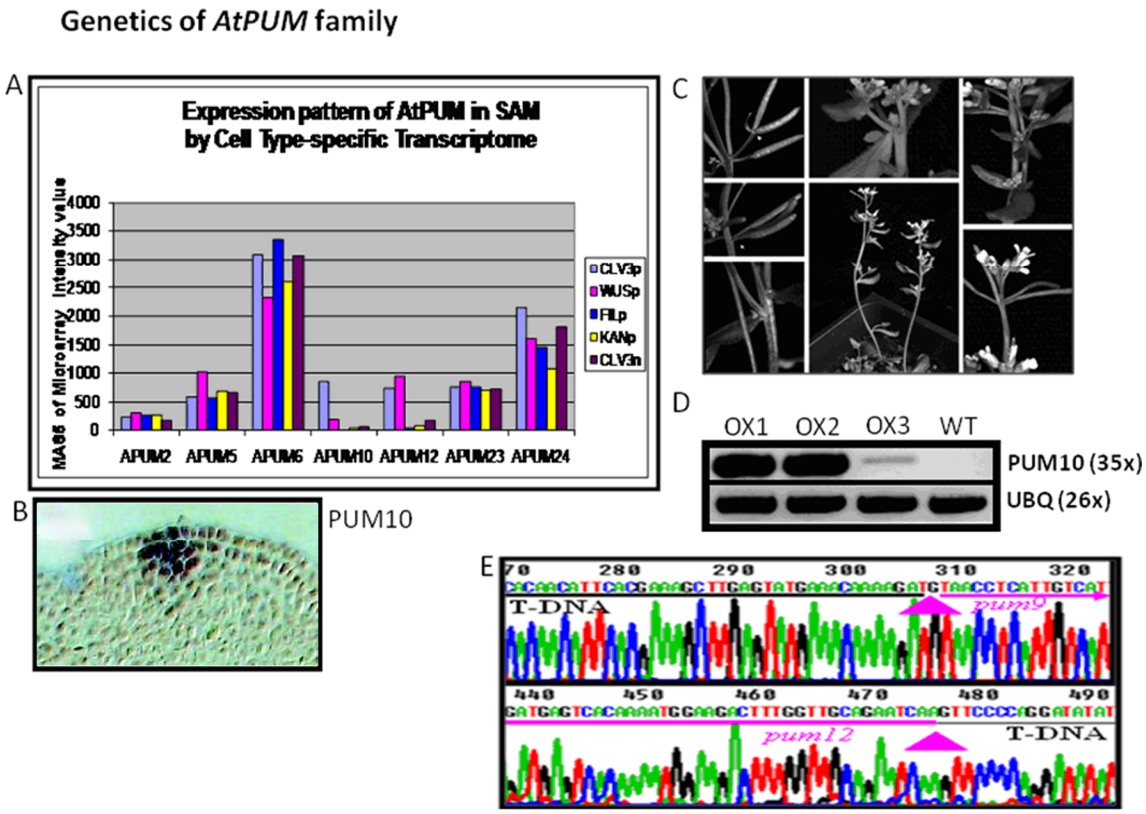

Figure1-6. Genetic study on the *AtPUM* family. (A) Expression pattern of AtPUM family by Cell type-specific transcriptome. (B) Expression pattern of atPUM10. (C) Phenotypes of overexpression atPUM10 in T1 generation. (D) RT-PCR analyses of AtPUM

was generated to silence the endogenous *AtPUM10*. The construct was introduced into wild type, *atpum9* single mutants and *atpum9/atpum12* double mutants. None of these combinations showed any effect on plant growth and development suggesting that they may not play a significant role in SAM development. In *Arabidopsis*, the AtPUM family consists of 24 members and several of them are not represented on *Arabidopsis* ATH1 gene chip. Therefore, it is also possible that the extreme redundancy makes it difficult to unravel the function of theses genes.

Name	Gene ID	T-DNA lines
AtPUM9	At1g335730	SALK_028441
AtPUM10	At1g335750	SALK_022907
AtPUM12	At5g56510	SALK_137138

Table 1-3. Gene IDs and T-DNA lines of the AtPUM family

Discussion

Cytokinin homeostasis: Local cytokinin activation and degradation

Cytokinin levels are regulated by several different mechanisms involving biosynthesis, irreversible degradation and conjugation of active cytokinins [75]. Spatially-restricted expression of both cytokinin activating genes (*AtLOG* family) and cytokinin oxidases (*AtCKX* family) in SAMs suggest that they locally balance cytokinin levels. This is an emerging paradigm for plant hormone action in contrast to the classical view of long distance signaling. By generating multiple knock out mutant combination in the *AtLOG* family we have shown that it affects SAM development. Though *Arabidopsis* mutants show some similarities with that of phenotypes observed in rice, yet significant differences could be found. Irregular arrangement of flowers on the inflorescence axis suggests that local activation of cytokinin could be important for auxin homeostasis or for maintaining local growth patterns influence primordia positioning or for mediating both. Future studies aimed at analyzing the expression of key genes involved in auxin biosythesis [26], auxin transport and auxin response in *AtLOG* triple mutants may reveal how centrally-synthesized cytokinin may affect primordia patterning at the SAM periphery.. Quantification of auxin levels in triple mutants will also reveal whether cytokinins

influence auxin levels in SAMs. Insights obtained from these analytical studies should enable future studies aimed at exploring the genetic and molecular interactions among relevant pathway components. Analyzing the expression of other key meristematic genes such as *CLV3*, *WUS* and *STM* in triple mutants may also reveal the function of local cytokinin in regulating stem-cell homeostasis. However, dynamic analysis is required to separate the short term effects from long term effects on SAM growth and patterning. However, transient downregulation of all three family members may be difficult. Therefore we have attempted alternate strategies to both transiently increase and decrease cytokinin levels in SAMs to follow their effects in live-imaging experiments (refer to chapter II). Transient over-expression of *LOG* genes and live imaging of relevant marker genes and growth patterns may also reveal the functional significance of spatial activation of cytokinin.

Ubiquitous activation of cytokinin oxidases (*AtCKX*) have been shown to mimic cytokinin deficient mutants demonstrating that they can effectively breakdown cytokinins [152, 164]. Thus far the double mutants of SAM-enriched *atckx3* and *atckx5* have not revealed any dramatic phenotypes, though their expression patterns suggested a local degradation of cytokinin. This could be due to the functional redundancy with other two family members *AtCKX6* and *AtCKX7* which are enriched in peripheral cells. Generating quadruple mutants of *atckx3, atckx5, atckx6* and *atckx7* may reveal their function. Alternately, they may not be required for development of SAMs in normal conditions, but their function may required for development in altered environmental conditions. Clearly new experiments are required to understand how local activation and degradation

mechanisms converge in regulating local cytokinin levels and its significance to SAM development. Nevertheless, *AtCKX* genes can be effective tools to transiently downregulate cytokinin levels for live imaging analysis and this has been discussed in considerable details in chapter II.

We have also explored the function of third class of genes that belong to *PUMILIO* (*AtPUM*) family. The three genes (*AtPUM9*, *AtPUM10* and *AtPUM12*) explored in this study belong to a large family of RNA-binding proteins known as PUF proteins (named after *Drosophila Pumilio* and *C. elegans FBF* [*fem-3 binding factor*]) found to be conserved in all eukaryotes [165-166]. PUF proteins bind to cis-regulatory sequence in 3'UTR of mRNA targets and control their expression by regulating mRNA translation or stability. PUF proteins have been studied in model organisms such as *Drosophila*, *C. elegans*, mouse and humans, and found to perform diverse functions during development. Interestingly, the PUF protein function has been implicated in stem cell maintenance and renewal [156-157, 167]. *Drosophila* PUMILIO protein binds to NRE sequence in 3'-UTR region of *cyclin B1* transcript and inhibits its translation to maintain the developmental state of germline stem cells [168]. Similarly, in humans and mouse, PUM homologues are found to express in stem cell lineages and maintain the germline pool [169]. There are 25 PUF proteins present in *Arabidopsis* constituting the largest group identified so far in any organism. Recent work has shown that some of the ATPUM proteins (ATPUM1-6) share considerable homology with Drosophila PUF domain (50% identical and 75% similarity) and are shown to bind ATPUM-binding element within 3'UTR of *WUSCHEL* and *CLAVATA-1*. Binding domain of ATPUM10, however, is dissimilar to that of *Drosophila*

PUF proteins and hence the members of the second group (ATPUM7-ATPUM12) are unlikely to recognize the same target sequence or they may not function as sequence specific RNA binding proteins[161]. Therefore, it is essential to explore the function of other SAM-enriched, though not differentially-expressed among different cell types (Figure1-3A), ATPUM genes in future experiments.

Taken together, we have shown that cell type specific transcriptome-guided reverse genetics approach is effective in analyzing the function of complex gene families consisting of several members with overlapping functions.

CHAPTER II

Sub-title: Transient manipulation of cytokinin levels and live-imaging of shoot apical meristems

Introduction

Cytokinin is one of the five classic phytohormones, first recognized for its ability to promote cell division in tobacco tissue about culture half century ago, since then it has been implicated in varieties of physiological and developmental processes [74]. The first step in cytokinin biosynthesis is the conversion of dimethylallyl diphosphate (DMAPP) to iP-ribotides by adenosine phosphate-isopentenyltransferease (IPT), which is a rate-limiting enzyme [170]. Cytokinin levels are also controlled through irreversible degradation reaction catalyzed by cytokinin oxidase/dehydrognease (CKX) that breaks down the active cytokinin into an ade/ado group and a side chain [87-88]. Cytokinin employs a two-component phosphor relay to trigger downstream signaling events [reviewed in Introduction][64, 68, 93, 171-172].

Cytokinin has been shown to interact with other plant hormones in regulating both physiological and developmental processes [40, 173]. In particular, cytokinins have been shown to interact with auxins to control many of developmental processes in plants, such as apical dominance, branching patterns root and shoot development [27, 174-175]. The classic experiments of Skoog and Miller demonstrated that the relative ratios of cytokinin and auxin control organogenesis [74]. A higher auxin-to-cytokinin ratio resulted in root development, whereas a low ratio of auxin-to-cytokinin resulted in shoot formation. Though, both mutant analyses and transgenic alternations of auxin and cytokinin levels

have also shown that they enter into complex interactions to regulate an array of plant growth and development processes [29, 31, 40, 42, 58, 66, 71, 108, 112, 174, 176-184]. However, the interaction between these two plant hormones is very complicated and has been shown to be synergistic or antagonistic or additive different developmental contexts[39-40, 58, 185-187].

Cytokinin is believed to be a central player in shoot apical meristem initiation and maintenance [25, 69, 109, 188-189]. The higher order mutant combinations in members of *IPT* gene family (involved in cytokinin biosynthesis), *lonely guy* (involved in local activation of cytokinin biosynthesis) of rice and corresponding *Arabidopsis* mutants [80], triple mutants of cytokinin receptors and mutants in downstream transcription factors of B-type ARRs have been shown to develop smaller SAMs and also affect overall growth [190-191]. STM has been shown to directly regulate Cytokinin biosynthetic enzyme, *IPT7* and this was further supported by the rescue of *stm-1* mutant upon overexpression of *IPT7* by using *STM* promoter [48]. It has also been shown that WUS negatively regulates A-type ARRs by directly binding to some of the family members in mediating stem-cell homeostasis [111]. Through developing two component sensor (TCS) reporter and genetic studies, it has been reported that auxin antagonizes cytokinin output in the basal cell lineage by direct transcriptional activation of ARABIDOPSIS RESPONSE REGULATOR genes, ARR7 and ARR15, feedback repressors of cytokinin signaling. Loss of ARR7 and ARR15 function or ectopic cytokinin signaling in the basal cell during early embryogenesis results in a defective root stem-cell system [192]. The research on embryogenesis suggests that an accurate spatial-temporal regulation through the cross

talk between auxin and cytokinin is needed in order to specify the first root stem cell niche. During the root development, the activation of SHORTROOT2(SHY2/IAA3) by a cytokinin primary positive signaling represses the auxin signaling that negatively regulates the PIN auxin transporters [193]. As a result, cytokinins could promote differentiation in the transitional zone of roots by modulating auxin redistributions. The process is accurately controlled in that the auxin antagonizes cytokinin by depredating SHY2 so that PIN proteins can be sustained and cell division activities can be maintained. Evidences has been provided to reveal that in the root meristem cytokinin acts in defined developmental domains to control cell differentiation rate, thus controlling root meristem size [108]. However, although elegant studies revealed that cytokinin played a negative regulatory role in root meristem in plants, the accurate mechanism how cytokinin controls stem cell initiation and maintenance in shoot apical meristem waits to be solved [40, 107-108]

It is clearly documented that auxin regulates cytokinin levels and vice versa. Using in vivo deuterium labeling and mass spectrometry, the dynamics of homeostatic cross talk between the two plant hormones has been achieved [194]. Auxin mediates a very rapid negative control of the active cytokinin pool via suppressing cytokinin biosynthesis via the isopentenyladenosine-5'-monophosphate-independent pathway whereas cytokinin overproduction mainly modifies plant developments instead of directly altering the composition of the active auxin pool. In root, cytokinin can cause auxin redistribution through ARR1-SHY2/IAA3 pathway to direct cell differentiation (Loio, et al., 2008). However, it is difficult to distinguish between cause and effect in these studies because

both auxin-cytokinins appear to function in developmental events that are spatio-temporally-coupled [194]. Therefore, a dynamic study involving inducible manipulations of these two hormones may provide new insights.

Auxin has been implicated in several developmental processes including shoot development [25-26, 28-29, 42, 58, 178, 195]. A number of genetic and biochemical analyses have revealed that SCF^{TIR1} is responsible for ubiquitination and subsequent degradation of the Aux/IAA transcriptional repressors [99, 196-197]. Auxin is perceived by its TIR1 class-receiptors that are a key component of the ubiquitin-proteasome system (UPS) that mediates protein degradation [196, 198]. With high level auxin, the Aux/IAA family genes will be degraded to release auxin response factors (ARFs) so that auxin induced genes could be transcribed. On the other hand, if the auxin level is low, the Aux/IAA family genes will form dimmers with their corresponding ARFs to prevent auxin induced genes to be expressed [180, 199-202]. Auxin action is also regulated by polar transportation of auxin through PINFORMED family transporter [203-208]. The movement of auxin is regulated by phosphorylation by PINOID and de- phosphorylation by protein phoshpotases 2A [209-213]. During the embryogenesis, the auxin maxima accumulate at the basal part of zygotes to cause the polarity of the single cell to asymmetric divide into the apical cell that give rise to the embryo and the basal cell that contribute mainly into root [214]. After the formation of root, the quiet center of root shows the greatest amount of auxin and auxin response. In the shoot development, auxin has been shown to regulate phyllotaxy, a process of spatial arrangement of leaves and flowers [181, 215]. Auxin is well-known for its ability to induce organogenesis in plants [216-219]. It has

been shown that local accumulation of auxin at specific sites in the PZ of SAMs is required for primordia specification and growth [219]. The auxin accumulation is mediated by PINFORMED1 (PIN1) protein which is an auxin efflux transporter and *pin1* mutants fail to form organ primordia [205, 220]. PIN1 expression has been shown to be dynamic and accumulates at higher levels in regions of incipient primordia in such a way; auxin is pumped toward the new primordia [113]. External application of auxin to pin-like inflorescence of the *pin1* mutant was proved necessary and sufficient to promote outgrowth of lateral organs [218-219]. Taken together, the auxin-sink model can explain the periodic spatio-temporal specification of organ primordia wherein new primordial act as auxin sinks to sequester auxin from surrounding cells. This prevents specification of new organ primordia within the vicinity [181, 215]. However, although the auxin maxima can explain the spacing component of phyllotaxy, it can not explain how the primordia are initiated *de novo* [221]. In addition, no mutant in auxin pathway with phyllotaxy defects has been described so far.

Cytokinin is also involved in patterning in plants. In maize, a mutation in *ABPHYL1* changes its alternative into decussate phyllotaxy, accompanying with a small shoot apical meristem [222]. This was the only case that the transformation of phyllotactic patterns occurred from one to another within a given species. A recent study showed that *PIN1* is expressed at P0 in the *abphel1* mutant at a delayed fashion [221]. Recently, cytokinin was also reported to modulate the auxin efflux to decide the auxin-induced organogenesis (AIO) [217]. Therefore, it is possible that a complicated network involving multiple signaling pathway cross talks to control the phyllotactic pattern.

To examine the interaction between cytokinin and auxin in organogenesis in the shoot apical meristem in plants, we decided to take advantages of the live-image technology coupled with the transient manipulation of cytokinin levels. This analysis was also inspired by the fact that local cytokinin activation mediated by *LONELY GUY* family of genes is required for phyllotaxy (Chapter I).

Results

2.1. The pattern of cytokinin response in SAMs

Cytokinins play crucial roles in an array of developmental processes including the growth and patterning of SAMs [31, 42, 58, 66, 68, 70-72, 108, 223]. However, the spatial patterns of cytokinin synthesis, cytokinin sensing and cytokinin responses are not completely understood. It has been shown that *ARABIDOPSIS RESPONSE REGULATORS* of A-Type class respond to cytokinins and can be used as sensors to detect cytokinins at cellular resolution [192]. To map the distribution of cytokinins in SAMs, we generated *pARR5::ARR5:GFP* (*ARR5* promoter sequences driving the expression of *ARR5:GFP* translational fusion) transgenic plants. A high-resolution confocal microscopy images revealed a uniform expression of the transgene in all centrally-located cells of the SAMs, however, the expression is downregulated in the PZ, specifically in regions of primordia development and also in deeper layers corresponding to the RM (Figure 2-1A, B, C). Recent studies have employed a synthetic cytokinin reporter, *pTCS::GFP* (concatemerized sequences of B-type ARABIDOPSIS RESPONSE REGULATOR (ARR)-binding motifs and a minimal 35S promoter driving the expression of GFP) which functions as a two-component-output-sensor revealing

cytokinin perception [192]. This reporter has been shown to express in cells of the RM coinciding with the WUS expression domain. A combination of *pARR5::GFP* and *pTCS::GFP* expression suggests that the cytokinin is preferentially-synthesized in superficial cell layers of SAMs and then it is sensed by cells of the RM. The implied synthesis of cytokinins centrally-located cells in superficial layers is consistent with the expression of STM which positively regulates cytokinin biosynthesis and also with the expression of AtLOG family members in these cells. However, the lack of expression of pARR5::GFP was found in cells of the RM, despite of the expression of one of the

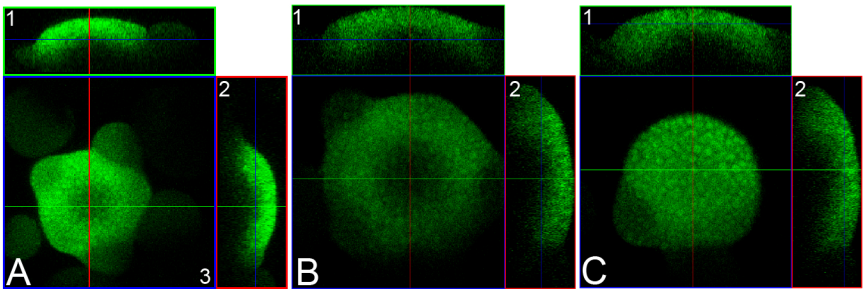

Figure 2-1. Distribution of cytokinins in SAMs as revealed by *pARR5::ARR:GFP*. (A) SAM showing the expression of *pARR5::ARR5:GFP*. (B) A deeper optical section showing lack of expression in central cells of the RM. (C) A optical section of superficial cell layer showing intense expression in the central region and low level expression in developing primordia. (1 and 2) Side views. (3) Top view.

AtLOG family member there (Figure 1-1. C), suggests a mechanism for efficient breakdown to cytokinin in these cells. This notion is consistent with the expression of one of the family members of cytokinin dehydrogenase/oxidase (AtCKX3) in these cells [36]. Sensing of cytokinin by RM cells as revealed by *pTCS:;GFP* is consistent with enriched expression of one of the three cytokinin receptors, *WOODENLEG (WOL)* in these cells [109], though the other two receptors appear to express uniformly [36]. It appears that on

a quantitative basis, the centrally-located, superficial layer-cells of SAMs are rich in cytokinin content and it is sensed preferentially by cells of the RM.

2.2. Development of a system to transiently reduce cytokinin levels

The CYTOKININ OXIDASES (AtCKXs) have been shown to irreversibly breakdown active cytokinins into an ade/ado group and a side chain [75, 87, 96]. *Arabidopsis* plants overexpressing CKX family members have been shown to mimic cytokinin deficient plants and downregulate the expression of cytokinin sensor, *ARR5* [152]. To transiently degrade cytokinins, AtCKX3 was activated-inducibly. This strategy was chosen because achieving transient downregulation of componenets in cytokinin biosnthesis, perception and response is complicated because of functional redundancy imposed by multiple gene family members [70, 96, 152, 164]. In addition, it was intriguing that the atCKX3 was found to express at the center of SAM by both cell type-specific transcriptome and in situ hybridization (Figure 1-1. E) [36]. Therefore overexpression of *AtCKX* class of genes provides a straightforward and robust means of reducing the active cytokinin pool.

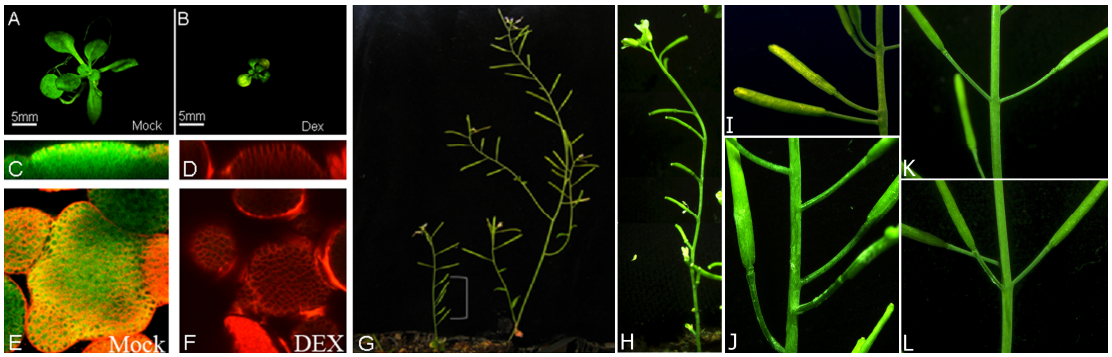

Figure 2-2. Phenotypes of inducible CKX3 transgenic plants. (A-B) Plants were on plates for 21 days. (A) MS+mock, (B) MS+Dex. (D-E: Transgenic plants were transferred to plates at the age of 10 days after germination. (C-F) F1 plants of inducible CKX3 crossed with *pARR5:GFP* (marked by green) reporter were treated four days. (C and E) Mock, C is side view of E. (D and

F) 10 µM Dex, D is side view of F. The cells were stained by FM-64. Images were taken using the same parameters. (G) Alcohol inducible CKX3 T2 plants were treated with acholol from 4 true leave stages for 5 fays. A transgenic plant (left) and a segregated wild type plant (right). The image was taken at the age of thirty days. (H) Inducible CKX3 plants were grown on plates with MS + 10 µM Dex for 12 day and then transferred on soil. Images were taken on 25 days. (I-L) Dex inducible CKX3 transgenic plants were treated by 10 µM Dex after bolting. (I) Treated every other day for two days. (J) Treated by for four days. (K-L) Inducible CKX3 plants were treated only one day.

Both ethanol and dexamethasone (Dex) inducible systems were used to downregulate cytokinin levels and both yielded similar phenotypes, upon induction (Figure 2-2 and 2-2G). The ethanol inducible system was found to be leaky when plants were grown in plastic boxes with MS-agar medium and could not be used for live-imaging experiments [4, 7]. Dex-inducible system based on GR-LhG4 and 6XoP to activate AtCKX3 (Vector map A.3.3) was adapted for live-imaging study. When transgenic plants overexpressing AtCKX3 were grown on MS- MS-agar plates containing 10µM Dex, they developed into much smaller seedlings than mock-treated plants (Figure 2-2. A-B). Similar phenotypes were observed when transgenic plants were treated with Dex upon bolting (Data not Shown). The above described phenotypes of Dex-inducible CKX3 transgenic plants are consistent with those reported in the literature [70, 87, 90, 140, 152, 224]. Finally, the Dex-inducible CKX3 transgenic line was introduced into a line carrying *pARR5::ARR5:GFP* reporter [152]. The reporter expression was eliminated from SAMs upon Dex treatment for four days demonstrating lower cytokinin levels in these plants (Figure 2-2. C-F). Token together, these results indicate that cytokinin levels could be inducibly-downregulated by using this system.

2.3. The transient downregulation of cytokinin levels dramatically reduces the auxin response

Apart from effects on overall plant growth and development, the cytokinin deficient plants also revealed organ positioning defects (Figure 2-2. G-L). In order to test whether these defects could be due to the disruption of auxin-mediated phyllotactic patterning, the Dex-inducible AtCKX3 transgenic plants were crossed to a line carrying both *pPIN1::PIN1::GFP* protein fusion [auxin efflux transporter PIN1::GFP protein fusion driven by the native *PIN1* promoter to reveal the direction of auxin flow] to monitor direction of auxin flow and *pDR5rev::3XVENUS-N7* (auxin responsive promoter driving a nuclear-localized variant of YFP) to monitor spatial accumulation patterns of auxin or response to auxin [113]. Upon Dex treatment, a progressive decrease in PIN1 expression levels was observed, though the PIN1 enrichment patterns at sites of organ primordia formation was unaffected [n = 3 plants, repeat three times] (Figure 2-3. A-D). In addition, the *pDR5* expression which is normally-associated with the sites of organ primordia formation as a discrete set of 3-4 cells in mock-treated plants fails to express in dex-treated plants [n = 3 plants, repeat three times] (Figure 2-3. A-D). On the other hand, the mock treated plants show normal both auxin movement and auxin responses as reported [n = 3 plants, repeat three times] (Figure 2-3. E-H) [113].

In order to test whether the dex-induced effects of cytokinin downregulation on auxin pathway components are reversible, the recovery of *PIN1* and *DR5* expression was observed upon withdrawal from dex treatment. Seventy two hours after withdrawal of dex, the expression of both *PIN1* and *DR5* recovered and the *DR5* expression begin to initiate in a normal spatio-temporal sequence [n= 5 plants, repeated twice] (Figure 2-4 C to E, red arrow) (Figure 2-4. E, Blue arrow). The reconstitution of both *PIN1* and *DR5* expression

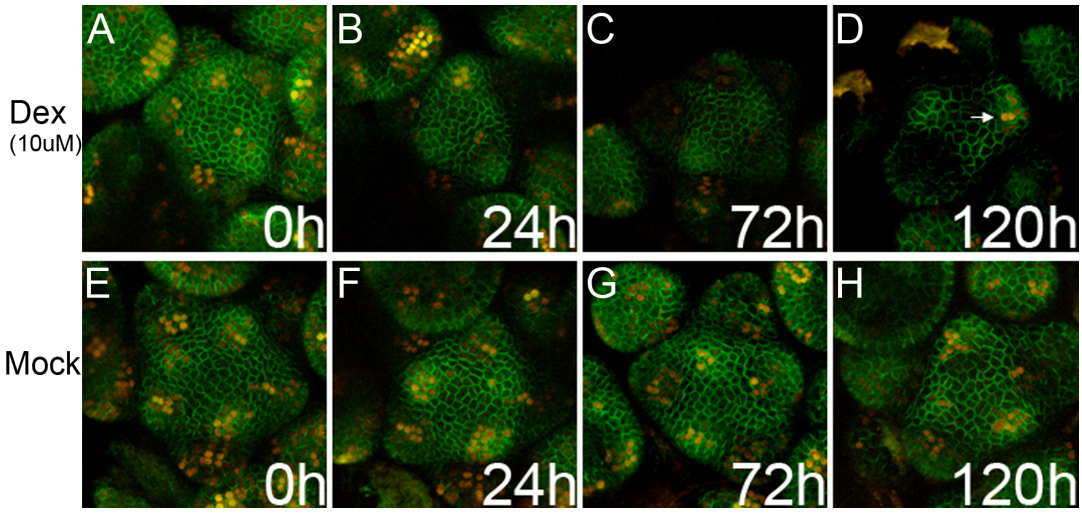

Figure 2-3. The reduction of *pDR5::Venus:N7* and *pPIN1::PIN1:GFP* expression upon dex-induced downregulation of cytokinin levels. (A-H) 3D-reconstructed top views of SAMs showing expression of *pPIN1:;PIN1:GFP* (green) and *pDR5::Venus-N7* (red). (E-H) Mock-treated plants showing maximal auxin responses/p*DR5* expression associated with developing organ primordia and specific enrichment of *PIN1* expression that overlaps with *DR*5 expression. (A-D) Dex-treated plants showing progressive decrease in *PIN1* expression and also loss of *pDR5* expression.

upon release from dex-mediated cytokinin downregulation revealed that cytokinin levels play important roles both in maintaining auxin transport. It also shows that critical levels of cytokinins are necessary for maintaining auxin levels or auxin responses or both.

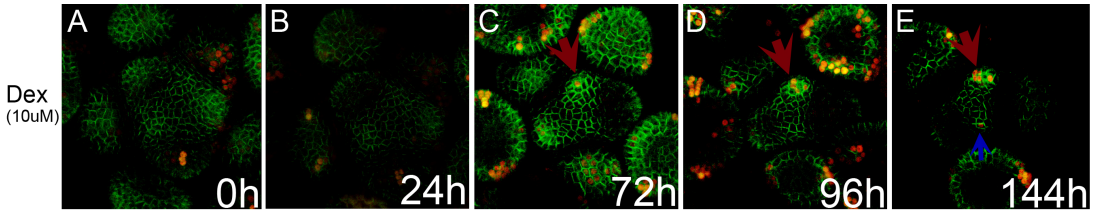

Figure 2-4. The recovery of *pDR5* (red) and *pPIN1* (green) expression upon withdrawal of dex-mediated downregulation of cytokinin. (A-E) 3D-reconstructed top views of SAMs showing expression of *pPIN1:;PIN1:GFP* (green) and *pDR5::Venus-N7* (red). Plants were treated with dex for 3 days and allowed to recover. The time series in (A-E) represents events during the recovery process. Red arrow indicate the re-appearance of *DR5* expression. Blue arrow indicates the expression of *DR5* in one cell in the second primordia.

2.4. Overproduction of cytokinin results in ectopic auxin responses and formation of additional organs

In order to increase cytokinin levels Dex-inducible IPT7 overexpression system was employed because it has been shown in earlier studies the IPT7 overexpression results in higher cytokinin content [48, 78, 225-226]. The transgenic seedlings carrying Dex-inducible IPT7 expressed from ubiquitous promoter (Vector map A.3.4) tuned pale and failed to grow on MS-agar plates containing Dex, a cytokinin overproduction effect that has been observed in

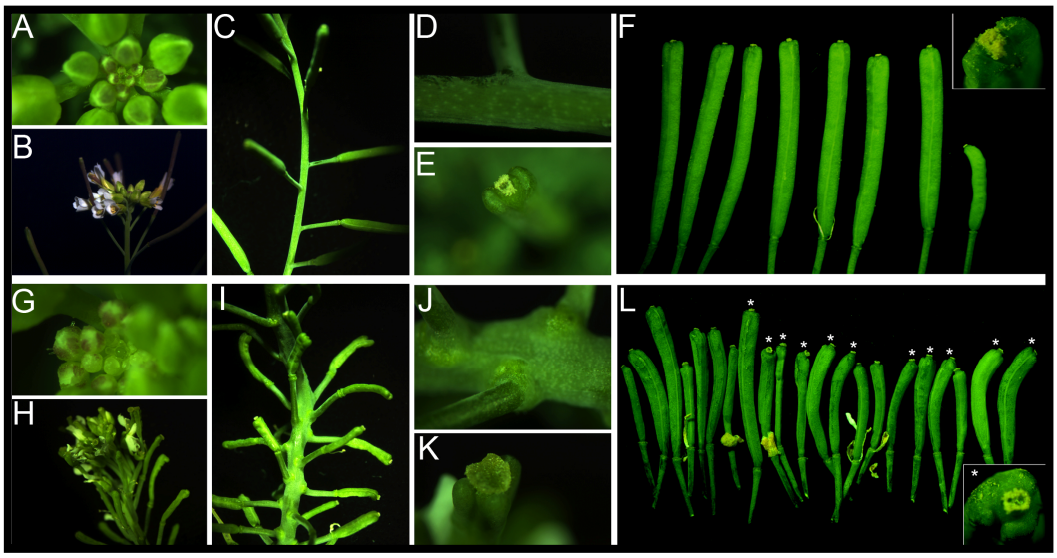

Figure 2-5. Phenotypes of Dex-induced over-expression of IPT7 transgenic plants. A-F: Mock treated plants showing the shoot apex (A and B), normal spacing of siliques on the stem (C), normal stem-thickness (D) and a silique with two carpels (E). G-H; Dex-treated plants showing slightly enlarged shoot apex (G and H), irregularly-arranged additional siliques (I), thicker stem (J) and silique with additional carpels (K). The number of flowers produced within a window of one week of Dex treatment (L) compared to mock treatment (F).

earlier studies [120]. One week of continuous Dex treatment (10µM) of inflorescence meristems, upon bolting, resulted in a slight increase in SAM size [n=8] (Figure 2-5. A, G),

additional carpels (Figure 2-5. K) and stem-fasciation (Figure 2-5. J) when compared to mock-treated plants (Figure 2-5. A-F). In addition, the dex treatment for week resulted in production of significantly higher number of irregularly-arranged flowers (20.8 ± 5.2) when compared to mock treatment (8.6±1.3) (Figure 2-5. L vs F). This suggested that the golden angle rule of 137^0 separation between successive primordia could be reduced in plants overexpressing cytokinin.

In order to dissect this phenotype further, Dex-inducible IPT7 plants were crossed to plants carrying both *pPIN1::PIN1::GFP* protein fusion [auxin efflux transporter PIN1::GFP protein fusion driven by the native *PIN1* promoter to reveal the direction of auxin flow] to monitor direction of auxin flow and *pDR5rev::3XVENUS-N7* [auxin responsive promoter driving a nuclear-localized variant of YFP] to monitor spatial accumulation patterns of auxin or response to auxin [113]. The mock-treated plants displayed a normal spiral phyllotaxy pattern as previously reported (Figure 2-6. A to F). However, the pattern of PIN1 and DR5 expression deviated in Dex-treated plants in several ways (Figure 2-6. G to L). First, an increase in DR5 domain of expression was observed between 24 to 48 hours after Dex treatment (Figure H and I) and this increase became very pronounced at 72 hours after Dex treatment (Figure 2-6. J). Second, the expression of pPIN1::PIN1::GFP intensified suggesting either the an increase in PIN1 transcription or improved stability of PIN1 protein (Figure 2-7. J to L). Third, the meristem size in Dex-treated plants enlarged with time compared to that of mock-treated plants (Figure 2-6. J to L vs D to E). Most importantly, a careful examination of the pattern of primordia initiation revealed development of additional organs interspersed with the regular pattern (Figure 2-6.

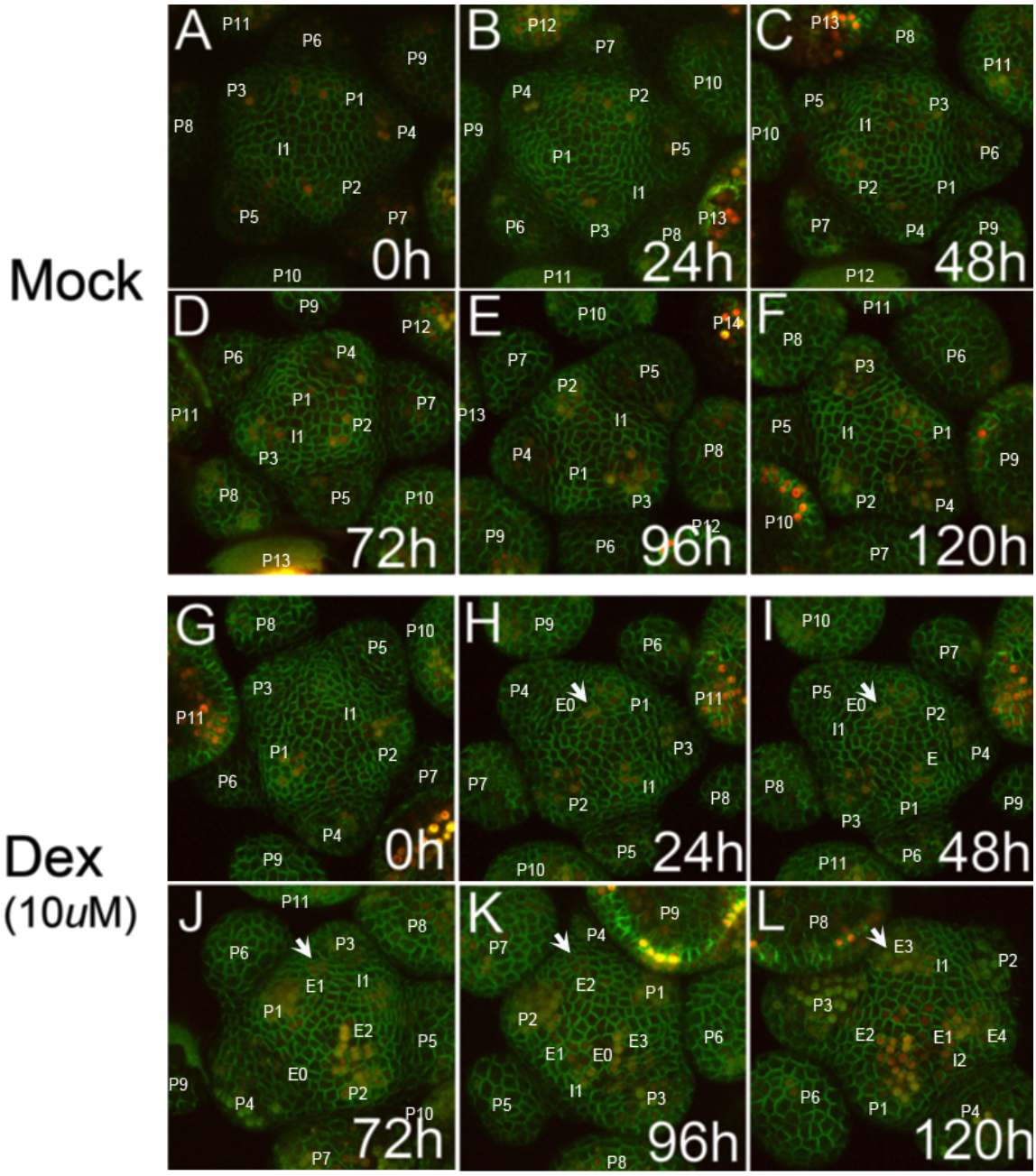

Figure 2-6. Extra sets of primordia formed by increase of auxin signaling upon the inducible expression of IPT7. Mock: A to F treated with Mock solution. Dex: G to L treated with 10 µM Dex. P: plastochron number. I: primordia initiation number. E: extra primordia number.

G-L). For example, the ectopic primordium1 (E_1) observed at 72 hours could be traced back to images acquired at 24 hours and 48 hours (Figure2-6. H to J, white arrowhead). At 96 hours after Dex treatment, at least three ectopic promordia (E_1 to E_3) could be observed along with the regular set of primordia P1 to P3 (Figure 2-6. K). Some of these ectopic primordia developed furtherinto flower buds (Figure 2-6. L (E3 and E4)). Taken together, both the transient depletion and overproduction of cytokinins revealed that maintenance of critical levels of cytokinins is crucial to regulate spatio-temporal patterning of auxin responses and phyllotactic patterning.

2.5. Alterations in cytokinin levels do not affect auxin levels in shoot apical meristems

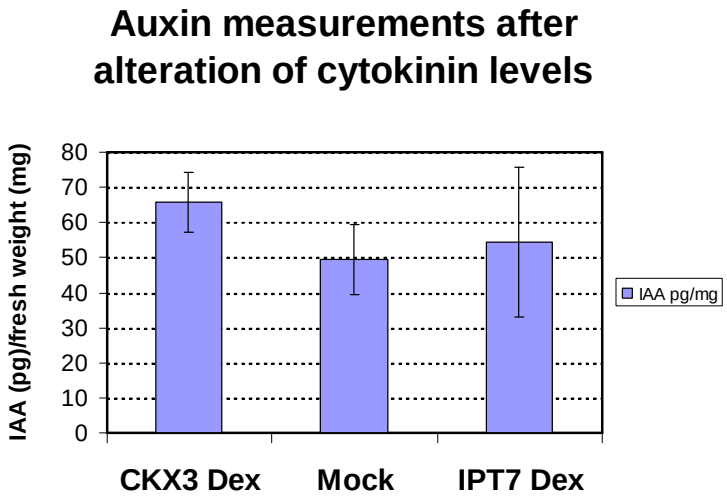

Figure 2-7. Auxin measurements after alteration of cytokinin levels. Plants were treated with either Mock or 10 µM Dex for four days before the apex were harvested after removing extra floral buds. Mock represented the average of both CKX3 mock and IPT7 mock.

The effect of cytokinin levels on auxin responsive, pDR5 expression suggested that either the auxin levels or responses are altered in these plants. In order to distinguish

between possibilities, we directly measured auxin (IAA) concentration from shoot apices of plants either mock-treated or dex-treated plants carrying either the CKX or the IPT7 transgene. Shoot apices were carefully hand dissected, after removing the older flower buds from plants that were either treated with mock or dex for a period of four and were immediately frozen in liquid nitrogen. The frozen samples were sent to the laboratory of Dr. Karin Ljung (Sweden) for mass spectrometry-based quantification of auxin levels. The quantification of auxin (IAA) levels revealed no significant differences in auxin levels in plants either overexpressing CKX3 or IPT7 when compared to mock-treated plants suggesting that cytokinin levels may not regulate auxin levels in the shoot apex (Figure 2-7).

2.6 External application of auxin upon cytokinin downregulation fails to rescue auxin responses

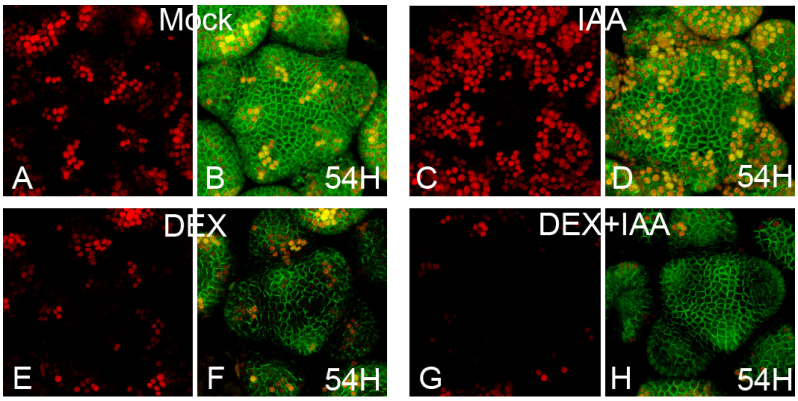

Figure 2-8. Auxin treatment upon cytokinin removal fails to restore auxin responses. (A-H) 3D-reconstructed top views of SAM showing either the pDR5::Venus-YFP:N7 (red) or pPIN1::PIN1:GFP (green) expression. Plants carrying Dex-inducible *AtCKX3* over-expression construct either treated with Mock (A and B) or mock with 25 µM IAA (C-D), treated with Dex (E and F) or treated with Dex along with 25 µM IAA (G and H). Total time elapsed after individual treatments are indicated on each panel.

The lack of auxin responses upon cytokinin depletion could either be an indirect consequence of reduced PIN1 expression or it could be a direct negative effect on the auxin response pathway elements. It has been shown that primordia formation in *pin1* mutants can be rescued by a localized-external application of auxin [219]. Therefore we tested whether external application of auxin upon cytokinin depletion can reconstitute auxin responses. Plants carrying Dex-inducible AtCKX3 plants were simultaneously treated with both dex and auxin (25 µM IAA). Both mock and IAA-treated and dex without IAA treatment served as controls. Mock-treated plants showed normal expression of *DR5* reporter [n= 5 plants, repeat twice] (Figure 2-8. A and B). While plants treated with the 25 µM IAA showed dramatically-enhanced expression of *pDR5* within the PZ cells [n= 5 plants, repeat twice] (Figure 2-8. C and D). Plants carrying Dex-inducible *AtCKX3* transgene upon dex treatment resulted in dramatically-reduced *pDR5* expression [n= 5 plants, repeat twice] (Figure 2-8. E and F; Figure 2-3. A and D). When the same transgenic plants were treated with Dex in the presence of IAA, it failed to induce *pDR5* expression [n= 5 plants, repeat twice] (Figure 2-8. G and H). It was noticed that PIN1 expression was restored in plants treated with both dex and IAA when compared to dex alone but it was not sufficient to restore auxin responses (Figure 2-8. H). The restoration of PIN1 expression could be a secondary consequence of increased auxin levels as it has been shown to that auxin induces PIN1 expression [179]. These results suggest that the removal of cytokinin affects auxin responses by directly regulating components of auxin signaling pathway.

2.7. Depletion of cytokinins reduce PIN1 transcript levels

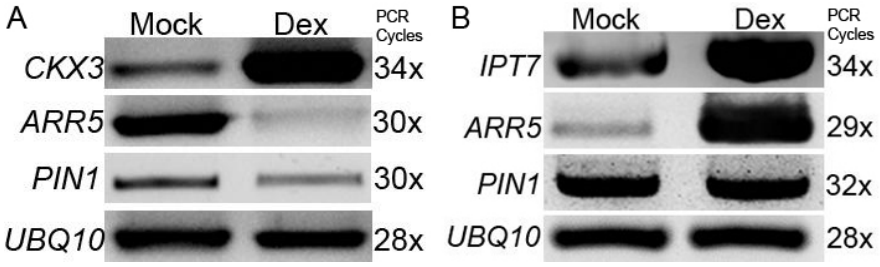

Figure 2-9. RT-PCR analysis of few candidate genes in Dex-treated plants carrying dex-inducible CKX3 or IPT7. (A) CKX3 transgenic plants. (B) IPT7 transgenic plants. Plants were treated with mock or 10 µM dex for 4 days to measure the expression levels of CKX3, IPT7, ARR5, PIN1 and UBIQUITIN10 was used as control. The number of PCR cycles was indicated on the right hand side of individual panel.

Our analysis has shown that transient depletion of cytokinin results in downregulation of pPIN1::PIN1:GFP expression. However, since it is protein fusion, it was not possible to distinguish whether this downregulation represents a decrease in PIN1 transcription or reduced protein stability of PIN1:GFP fusion protein. Therefore RT-PCR was carried out on RNA samples extracted from plants expressing Dex-inducible AtCKX3 and IPT by using gene specific primers for *AtCKX3, PIN1, ARR5* and *UBIQUITIN10* was used as a control. RT-PCR analysis revealed, as expected, an increase in *CKX3* expression, accompanied by a dramatic reduction of cytokinin response gene *ARR5* in Dex-inducible *AtCKX3* expressing plants upon 4 days of Dex treatment (Figure 2-9. A). This result is consistent with the reduction of *pARR5::GFP* upon dex induction (Figure 2-2. C-F). Similarly, in Dex-inducible IPT7 plants, the expression of *IPT7* was elevated along with the expression of *ARR5* (Figure 2-9. B). Though the expression of *PINFORMED 1 (PIN1)* decreased marginally in inducible *AtCKX3* after four days of dex treatment (Figure 2-9. A), the expression of *PIN1* in inducible *IPT7* did not change much (Figure 2-9. B.). The live

imaging data revealed a reduction in PIN1::PIN1:GFP levels in *AtCKX3* which could be due to the decreased PIN1 transcription as revealed by RT-PCR experiments and a marginal increase in *IPT7* overexpressing plants (Figure 2-3, 2-4, 2-6). However, no changes in *PIN1* transcript levels were observed in *IPT7* overexpressing plants though a mild increase in pPIN1::PIN1:GFP fluorescence was observed in live-imagery (Figure 2-8). Taken together these results suggest that cytokinins are required for maintaining *PIN1* transcript levels though their effect on PIN1 protein stability can not be completely ruled out.

2.8. Cytokinin control overall cell division in SAM

It has been shown that cytokinins are required for mediating cell division [5, 188, 227]. Mutants with altered levels of cytokinins and defective signaling components show changes in SAM size and shape [70, 80-81, 221-222]. Therefore the observed phyllotaxy defects in plants with varied levels of cytokinins, in part, could also be due to the changes in cell division patterns. To better understand the impact of cytokinin on the patterning of SAMs, cell division analysis was carried out on the time-lapse imagery obtained from inducible-*IPT7* and inducible-*CKX3* plants. Cell division rates were analyzed by reconstructing individual cell lineages of the. L1 layer cells from time lapse data acquired between 48 and 96 hours (the depletion of cytokinin by dex inducible CKX3) and 24 to 73 hours (the increase of cytokinin by dex inducible IPT7). Cell division rate was expressed as the size of individual lineages (the number of daughter cells at second time point that originated from a single cell present at the start point). Lineage reconstruction was

performed for all of the cells of the outmost layer of the SAM, L1 layer, of Dex-treated IPT7 (n=3), CKX3 (n=3) and mock-treated plants (n=3) were used as controls.

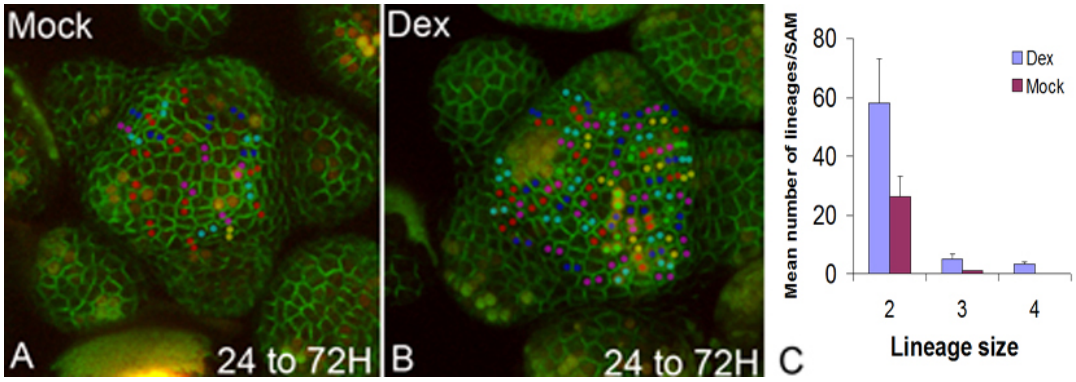

Figure 2-10a. Cell division analysis of inducible IPT7 transgenic lines upon Dex induction. (A) Mock treated. (B) Dex treated. (C) The images from the same SAM were used to score for cell division events to reconstruct lineages within the 48-hour period (24 to 72 hours after treatment) and expressed as size of the individual lineages against mean number of lineages per SAM. Error bars represent standard deviation.

In inducible-IPT7 plants, SAMs become enlarged and assume irregular shape 48 hours after the Dex treatment and with appearance of ectopic *pDR5* expression. The cell division analysis carried out within this time window revealed an increase in size of individual lineages when compared to mock-treated plants (Figure 2-10a) revealing higher cell division rates.

On the other hand, in the Dex-inducible *AtCKX3* plants, cells stopped dividing after 48 hours of Dex treatment except for a few cells (Figure 2-10b. C-D and E). This was associated with a decrease in size of SAMs. Cell division analysis, as expected, revealed

that cytokinins are necessary and sufficient for maintaining cell division in SAMs and its possible impact on phyllotactic patterning is discussed in subsequent sections.

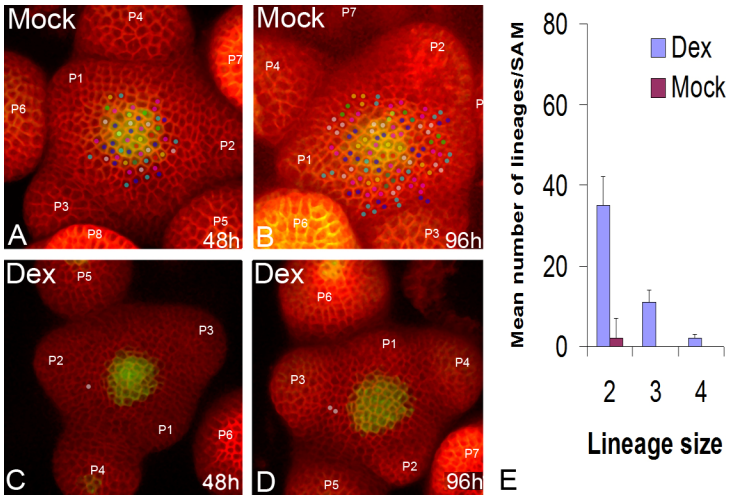

Figure 2-9b. Cell division analysis of inducible CKX3 transgenic lines upon Dex induction. (A) Mock treated. (B) Dex treated. (C) The images from the same SAM were used to score for cell division events to reconstruct lineages within the 48-hour period (48 to 96 hours after treatment) and expressed as size of the individual lineages against mean number of lineages per SAM. Error bars represent standard deviation.

Discussion

Cytokinins have been implicated in SAM development [4, 37, 42, 58, 66]. It has been shown that maize *abphyl1* mutants display aberrant phyllotaxy and enlarged SAM size [222]. *ABPHYL1* has been shown to encode a member of the type-A ARRs which function as negative regulators of cytokinin signaling. Interestingly *ABPHYL1* is expressed in few cells of organ anlagen of the SAMs suggesting a role for cytokinin signaling in either maintaining growth patterns in proper spacing of organ primordia or in maintaining proper spatio-temporal regulation of auxin responses that facilitate proper organ initiation or both

[221-222]. These possibilities cannot easily be distinguished in maize studies due to the lack of analytical tools and reagents. It has also been shown that multiple mutants of type-A ARRs in *Arabidopsis* leads to mild phyllotaxy defects but the causal reasons have not been addressed so far [228]. Our own study (chapter I) on the functional analysis of cytokinin activating enzymes of the *AtLOG* family has revealed that local activation of cytokinin pool is critical to maintain both meristematic activity and regular spacing between successive organ primordia. Though all these studies have implicated either the local synthesis of active cytokinins or local regulation of cytokinin signaling, it is not yet clear whether they affect patterning elements within auxin pathway or simply a secondary consequence of altered growth patterns. In order to address these issues, we attempted live-imaging studies upon transient manipulation of cytokinin levels.

We have shown that transient downregulation of active cytokinin pool results in reduced auxin responses and reduced organogenesis. Conversely transient upregulation of cytokinin levels results in ectopic auxin responses with an associated development of additional organs. These results demonstrate that maintenance of a threshold level of cytokinins is critical to initiate auxin responses in organ anlagen within the PZ of SAMs. More importantly, it shows that the observed phyllotactic defects are not simply a indirect consequences of altered growth patterns or altered cellular states caused by the absence of gene products for several days in terminal mutants. Cytokinins could play such a role by several means; a) It could affect auxin movement which is critical to generate regions of maximum auxin concentrations in a specific spatio-temporal sequence. b) It could mediate

auxin biosynthesis, c) it could mediate auxin responses or d) a combination of all three factors.

Both modeling and experimental studies have implied that auxin depletion from regions surrounding the organ anlagen results in auxin maxima which is critical for organ outgrowth [28, 34, 42, 215, 219, 229-230]. PIN1 has been shown to preferentially accumulate in cells of the organ anlagen with maximum levels of PIN1 on the walls pointing towards cells of the anlagen. This behavior has been postulated to create auxin maxima in a periodic spatio-temporal sequence within the PZ of SAMs [181-182, 203, 215, 218-219, 222, 231-233]. Though the mechanisms of PIN1 accumulation in cell membranes pointing to the organ anlagen against the direction of auxin flow have not been understood [221]. However, the mathematical modeling studies have shown that both "flux mechanism" in which PIN1 could sense the auxin flux or "canalization mechanism" in which the direction of auxin flow carves channels to mediate PIN1 accumulation and further auxin flow against the concentration gradient could operate [34, 230]. Our study shows that PIN1 expression is rapidly downregulated upon cytokinin depletion (Figure 2-3, 2-4, 2-9A) although the preferential accumulation of PIN1 in organ anlagen occurs during this period before significant reduction of PIN1 expression (Figure 2-3C, 2-4A-B). This suggests that cytokinins are required for maintaining PIN1 levels and thereby auxin movement which results in its preferential accumulation in organ anlagen. Reduced PIN1 might create sub-optimal accumulation of auxin in organ anlagen resulting in failure to initiate auxin responses.

Though the role of PIN1-mediated auxin movement has been well-established in regulating phyllotaxy, however, the source of auxin has been a matter of debate. Based on the orientation of PIN1 expression in cells of the epidermal layer of SAMs, it has been argued that PIN1 could be transported into the SAMs from mature leaves/flowers [219]. In contrast, the immunolocalization studies by using IAA-specific antibody have shown maximal auxin concentration within cells of the CZ of SAMs suggesting that they could be the auxin supply centers [234]. This notion has been further supported by mathematical predictions for the requirement of CZ-originated factor in regulating phyllotaxy. It has also been shown that *YUCCA* genes involved in auxin biosynthesis are expressed in discrete sets of SAM cells further indicating a role for local auxin synthesis in regulating phyllotaxy [26, 235-237]. Taken together, the unequivocal determination of auxin source requires further studies involving quantification of both steady-state levels of auxin and *de novo* synthesis at a cell type-specific resolution as it has been shown in the root system [148]. Our study shows that the transient manipulation of cytokinin levels does not lead to significant changes in auxin levels (Figure 2-7). Consistent with this observation, the expression levels of several of *YUCCA* genes remain unchanged upon transient manipulation of cytokinin levels (MTX and GVR, undocumented results). These results suggest that cytokinin levels are not required for maintain auxin levels and the reduction in *PIN1* expression upon transient downregulation of cytokinin suggest that cytokinins regulate *PIN1* expression independently of auxin levels. This is in contrast to earlier studies wherein it has been shown that exogenous application of cytokinin results in enhanced auxin levels and also the auxin levels are found to be reduced in *abphyl1* mutants of maize

[221]. This is simply could be due to the differences in tissue types examined, the duration of treatment or could reflect real differences in mechanisms between maize and *Arabidopsis*.

This study shows that external application of auxin upon transient downregulation of cytokinin fails to restore auxin responses. This suggests that active cytokinin pool plays a critical role in mediating auxin responses within the PZ and subsequent organogenesis. This also demonstrates that cytokinin apart from maintaining *PIN1* levels and auxin movement, also has additional targets within the auxin response pathway. Cytokinin could either positively regulate the positive regulators of auxin signaling such as the ARFs or negatively regulate the negative regulators such as Aux/IAAs or could regulate other elements in auxin response pathway. The cell type-specific genomics has shown that several family members of auxin response pathway are enriched in cells of the PZ of SAMs [36]. Future studies involving transient perturbations in cytokinin levels and cell type-specific transcriptome analysis may reveal the identity of auxin response elements regulated by cytokinins.

CHAPTER III

Sub-title: Manipulation of cytokinin positive signaling: Cytokinin-mediated stem-cell homeostasis can be uncoupled from growth dynamics and it requires WUSCHEL function

Introduction

Cell-cell communication is critical for stem-cell homeostasis in shoot apical meristems (SAMs) of higher plants [8, 37, 238]. In *Arabidopsis* SAMs, the CZ harbors a set of 35-40 stem-cells. The stem-cell progeny are displaced into the adjacent PZ where they differentiate. The Rib-meristem (RM)/organizing center (OC) located beneath the CZ has been shown to provide cues for stem-cell specification by activating WUSCHEL (WUS), a homeodomain transcription factor. WUS not only specifies stem-cells but also activates *CLAVATA3 (CLV3)* which encodes a secreted ligand. CLV3 has been shown to bind CLAVATA1 (CLV1) receptor kinase to activate signaling network involving other CLV1-related membrane bound kinases which functions to repress *WUS* expression [111]. Thus WUS controls its own expression and the CZ size through a feedback loop involving cells of the CZ and the RM/OC. This apart, plant hormone cytokinin (CK) has been implicated in SAM development. It has been shown that WUS negatively regulates a set of ARABIDOPSIS RESPONSE REGULATORS (TypeA ARRs) that function in downregulation of CK signaling [104, 111, 239]. However, the significance of this local downregulation in stem-cell homeostasis is not well-understood. It is also not clear how this local regulation of cytokinin signaling interfaces with CLV-WUS feedback loop in regulating stem-cell homeostasis. It has also been shown that external application of CK elevates *WUS* expression within the RM [109, 240]. Though these studies link CK

signaling to SAM development, however, its role in controlling the CZ to the PZ ratio and regulation of overall growth of SAMs are not well-understood. This is because CKs have been implicated in several developmental and physiological processes starting from embryogenesis and also in mediating cell division [31, 42, 58, 68-73, 223], therefore, merely studying the defects in terminal phenotypes may not reveal their function. Genome wide expression profiling studies aimed at identifying genes directly regulated by WUS revealed that ARR1, a type-B ARR is directly-activated by WUS. It has been shown that multiple mutant combination of type-B *ARR* genes (*ARR1, ARR10 and ARR12*) result in SAM defects among other defects in overall growth and development of plants [228]. In order to examine the relevance of WUS-mediated local activation of ARR1, we have transiently-activated CK signaling by dex-inducible activation of constitutively active ARR1 and followed SAM organization in live-imaging experiments. We show that constitutive activation of CK signaling results in radial and sequential expansion of the CZ abutting the native CZ. This expansion of the CZ is neither due to the over-proliferation of stem-cells or due to a delayed transition of cells from the CZ pattern of gene expression to the PZ pattern of gene expression. Our analysis reveals that the expansion of the CZ is due to the de-differentiation of stem-cell progeny within the PZ into stem-cells. Surprisingly, constitutive activation of CK signaling did not result in increased cell division rates and enlargement of SAM size, thus uncoupling stem-cell homeostasis from growth dynamics. We further demonstrate that the CK-mediated effects on the CLV3 expression require the function of homeodomain transcription factor WUSCHEL suggesting that elevated CK signaling either modifies WUS activity or levels in regulating stem-cell homeostasis.

Results

3.1 Direct activation of *ARABIDOPSIS RESPONSE REGULATOR1 (ARR1)* by WUSCHEL

The ARABIDOPSIS RESPONSE REGULATORS (ARRs) of type B class have been shown to function downstream of CK receptors to mediate CK signaling [103, 138-139].

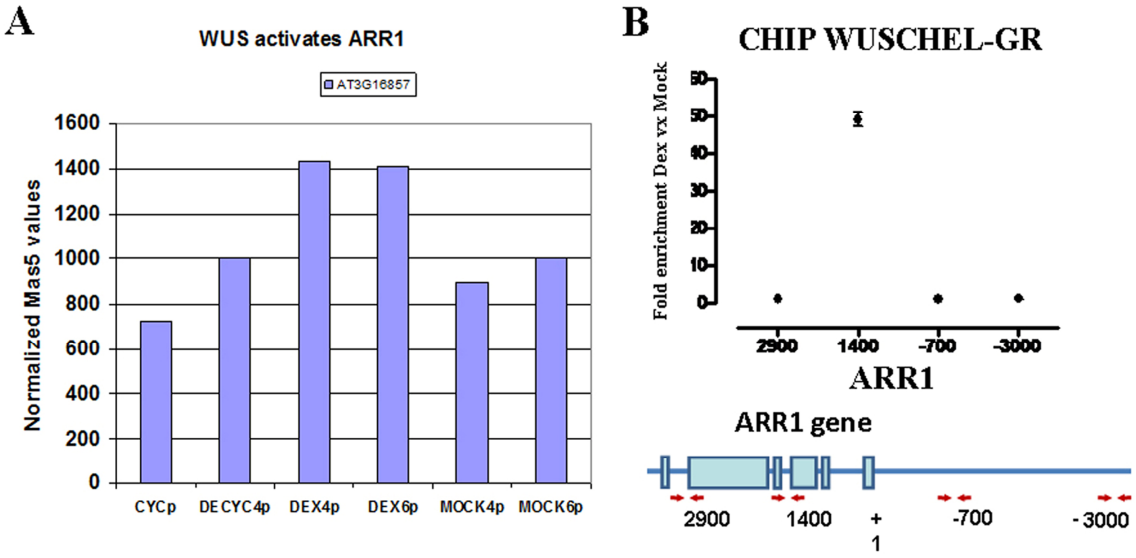

Figure 3-1. ARR1 is directly-activated by WUS. (A) MAS5-normalized intensity values showing ARR1 upregulation in the presence of cycloheximide (cyc) and dex. (B) qRT-PCR scanning of CHIP-DNA by using WUS-specific antibody showing enrichment in intronic regions of ARR1.

The expression of ARR1 could be directly regulated by WUS (data not shown). CHIP by using WUS-specific antibody revealed that specific enrichment of ARR1 genomic regions can be obtained and this enrichment was detected in intronic regions of ARR1 (Figure 3-1. B). This further confirmed that WUS directly binds to ARR1 to activate it.

3.2 Constitutive activation of cytokinin signaling leads to expansion of stem-cell domain

In order to understand the functional significance of WUS-mediated local upregulation of ARR1, constitutively-active form of ARR1 was activated by using Dex inducible system. The deletion of signal receiver domain (DDK) of ARR1 (a type B ARR) has been shown to constitutively activate CK signaling by unmasking the transactivating function of ARR1 [138]. The ARR1 deletion construct was fused to the glucocorticoid-inducible artificial transcription factor (GR) and expressed from the cauliflower mosaic virus 35*S* coat protein gene promoter to generate dexamethasone (Dex) inducible *35S::ARR1ΔDDK-GR* [139]. The *35S::ARR1ΔDDK-GR* construct was introduced into plants carrying, *pCLV3::mGFP5-ER* (*CLV3* promoter driving the expression of endoplasmic reticulum-localized green fluorescent protein), a fluorescent reporter for stem-cells and *35S::YFP29-1* (ubiquitous promoter driving the plasma membrane-localized yellow fluorescent protein), a marker for cell boundaries which allows visualization of all SAM cells. In the absence of dex, the transgenic plants carrying *35S::ARR1ΔDDK-GR* construct were phenotypically-normal (Figure 3-2. A). Upon addition of dex, typical phenotypic effects that have been described for plants experiencing elevated levels of CK signaling such as callus-like growth on seedlings (Figure 3-2. B and C) and ectpoic shoot-like structures were observed on the adaxial surface of the cotyledons [139]. A closer examination of ectopic shoot-like structures that appear on the leaves revealed *CLV3* promoter activity (Figure 3-2 F). SAMs that were treated with dex, upon bolting, for 4-5 days revealed enlarged stem-cell domain as revealed by the CZ marker *pCLV3::mGFP5-ER* (Figure 3-2. D and E). The ectopic leaf-like structures that appeared on cotyledons of dex-treated plants also showed *pCLV3* activity suggesting ectopic stem-cell specification.

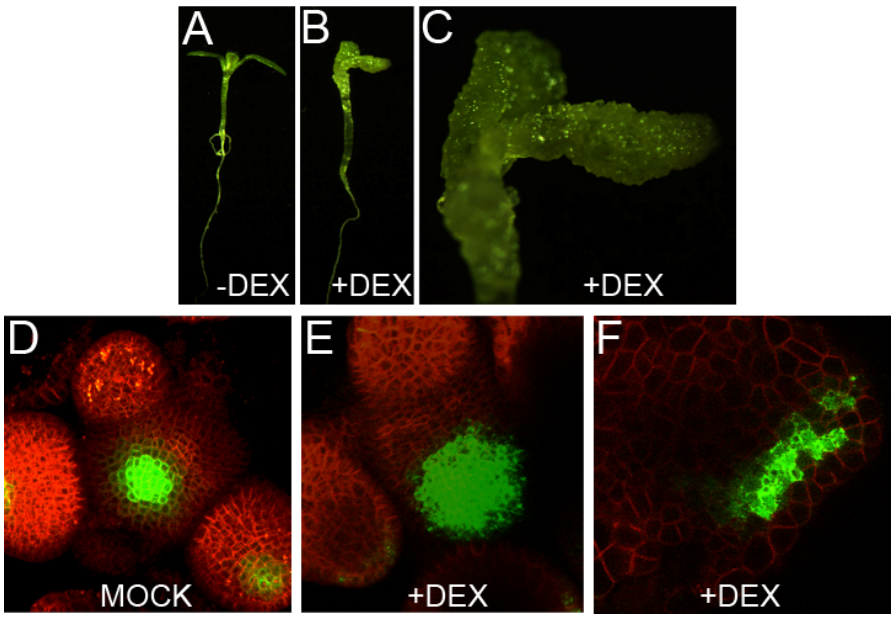

Figure 3-2. Phenotypes of *p35S::ARR1ΔDDK::GR*. (A-C) phenotypes of transgenic plants on plates (11 days on plates after germination). (A) without dex, (B) with dex, (C) higher magnification image of (B). (D and E) 3D-reconstructed top views of shoot apical meristems (SAMs) showing *pCLV3::mGFP5-ER* (CZ/stemcell reporter; green) and *35S::YFP29-1* (plasma membrane-localized YFP marks the outlines of all cells, red). (D) mcok-treated SAM 4 days after treatement. (E) dex-treated SAM four days after treatement. (F) a confocal section of an ectopic leaf (25-30 days of after dex treatemnt) showing *pCLV3* expression (green).

3.3 Expansion of the stem-cell domain is due to de-differentiation of differentiating cells

The expansion of the stem-cell domain, upon dex treatment, could be because of several possibilities such as the higher rates of cell divisions in cells of the CZ, lack of transition of gene expression from CZ pattern to PZ pattern upon displacement of stem-cell progeny into the PZ or de-differentiation of PZ cells into CZ cells [7]. In order to distinguish between possibilities, we repeatedly observed same SAMs continuously for 4-5 days after dex treatment and mock-treated SAMs served as controls. The first visible

effects of CZ expansion, as revealed by *pCLV3::mGFP5-ER* expression, were observed between 24 and 48 hours after dex treatment (Figure 3-3. G-I). Expansion of the CZ

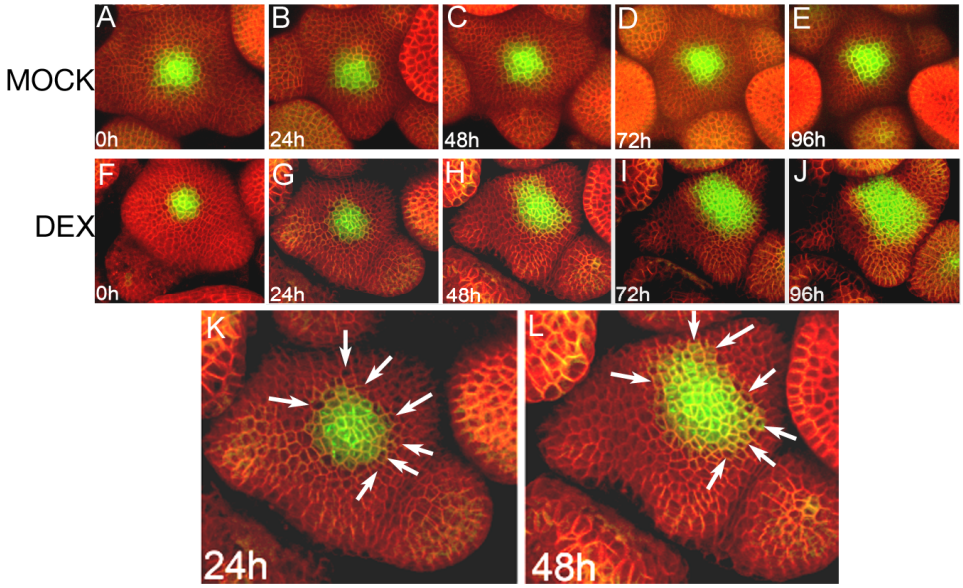

Figure 3-3. Expansion of stem cell domain upon constitutive activation of cytokinin signaling. (A-L) 3D-reconstructed top views of shoot apical meristems (SAMs) showing pCLV3::mGFP5-ER (CZ/stemcell reporter; green) and 35S::YFP29-1 (plasma membrane-localized YFP marks the outlines of all cells, red). (A-E) Time-lapse series of a mock-treated SAM (note no dramatic changes in CZ size). (F-J) Time-lapse series of a dex-treated SAM (note dramatic expansion of the CZ). (K and L). Higher magnification views of (G and H) showing the appearance of pCLV3 promoter activity (green) in cells of the peripehral zone (PZ) [arrows point to the corresponding PZ cells that have acquired pCLV3 expression]. Elapsed time is indicated on individual panels.

continued for next 48-96 hours and sequential expansion always occurred abutting the pre-existing CZ pattern (Figure 3-3. F-J). We also noticed a similar radial expansion of the CZ in cells located in the deeper layers of SAMs. In order to understand the reasons for CZ expansion, we analyzed higher magnification images from time-lapse series that were double labeled by *pCLV3::mGFP-ER* (CZ marker) and *35S::YFP29-1* (marks outlines of

all SAM cells to follow cell divisions). This analysis revealed that the pre-existing PZ cells that have left the CZ re-acquired *pCLV3::mGFP5-ER* expression revealing de-differentiation of differentiating PZ cells (Figure 3-3. K and L). While the mock-treated plants did not show dramatic changes in the CZ size (Figure 3-3 F-J). In addition to the sequential expnasion of *pCLV3* activitiy, we also noticed a lack of initiation of new organ primordia suggesting a functional conversion of the PZ identity into the CZ identity (Figure 3-3. H-J).

3.4. Constitutive activation of cytokinin signaling does not alter cell division rates

Though our analysis has revealed that the CZ expansion is due to the re-specifiction of the PZ cells into stem-cells, however, upon downregulation of *CLV3* expression a similar expansion of the CZ was associated with increased rates of cell division in the PZ cells [7]. Moreover, cytokinins primarily have been implicated in cell cycle

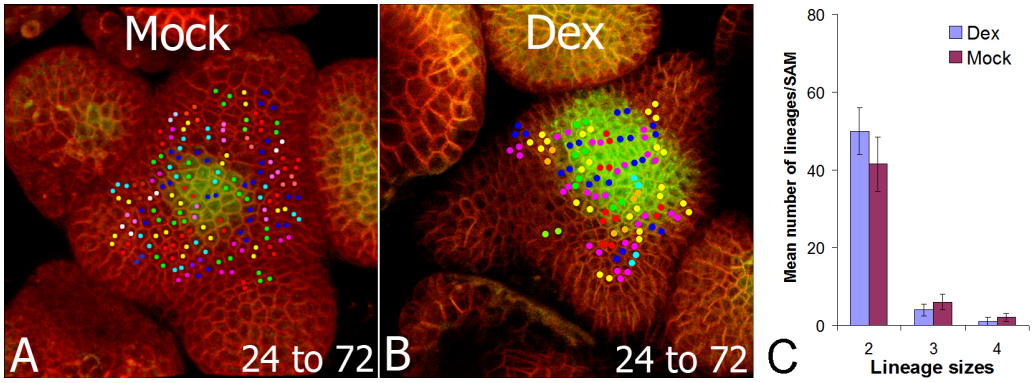

Figure 3-4. Constitutive activation of ARR1 results in expansion of stem-cell domain without altering cell division rates. (A) Mock treated. (B) Dex treated. (C) Histogram of mean number of lineage/SAM. The images from the same SAM were used to score for cell division events to reconstruct lineages within the 48-hour period (24 to 72 hours after treatment) and expressed as size of the individual lineages against mean number of lineages per SAM. Error bars represent standard deviation.

progression and have been shown to function at G1-S phase transition [242]. Therefore, we examined the effects of constitutive activation of cytokinin signaling on cell division rates of cells within distinct functional domains of SAMs. In order to analyze cell division rates, we reconstructed individual cell lineages of the L1 layer cells from time lapse data acquired between 24 and 72 hours after dex treatment. Cell division rate was expressed as the size of individual lineages (the number of daughter cells at 72 hours that originated from a single cell present at 24 hours). The lineage sizes in dex-treated plants were comparable to mock-treated plants revealing that a ubiquitous elevation of cytokinin signaling did not result in increased cell division activity of SAM cells (Figure 3-4. C). The visualization of spatial patterns of mitotic activity, carried out by superimposing individual lineages on the reconstructed L1 layer of the SAM also confirmed no increase in mitotic activity in cell of the PZ.

3.5. ARR1-induced expansion of stem-cell domain requires *WUSCHEL*

Earlier studies have shown that WUSCHEL which is expressed in the RM provides signals to overlying cells of the CZ to specify them as stem-cells [10, 21-22, 243]. Therefore, we examined whether the expansion of the CZ, upon activation of cytokinin signaling, is mediated through WUS activity or it is independent of WUS function. We introduced *35S::ARR1ΔDDK-GR* transgene into *wus-1* mutant background by genetic crosses. Seedlings carrying *wus-1* mutation and *35S::ARR1ΔDDK-GR* transgene were treated repeatedly with 10μM dex for several days. Though we observed callus-like growth on cotyledons and leaves, however, we did not observe rescue of the SAM growth (Figure

3-5). The dex-treated plants produced axiallary shoots and occasional flowers that resembled *wus-1* mutants. We also did not notice ectopic *pCLV3::mGFP5-ER* expression

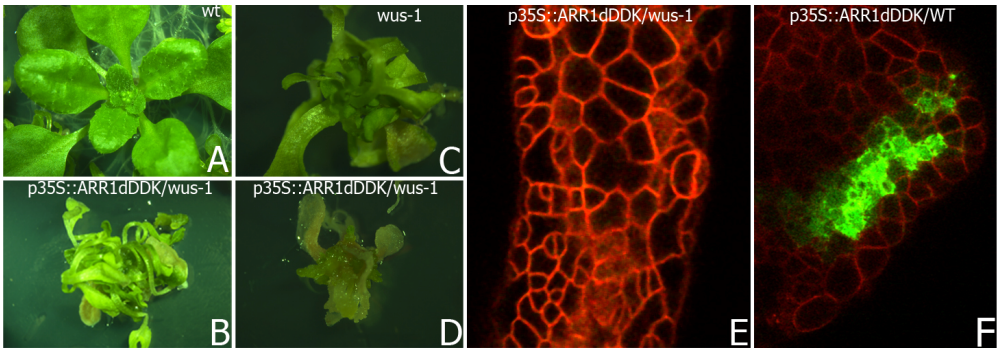

Figure 3-5. The genetic analysis of constitutive cytokinin signaling on *wus-1* mutant.

in cells of the cotyledons and in leaves of dex-treated plants (Figure 3-5. E vs F). These results suggested a requirement for WUS in mediating cytokinin induced expansion of the CZ. Therefore, cytokinin signaling mediated by ARR1 functions through WUS in mediating stem-cell homeostasis either by regulating expression levels of *WUS* or WUS protein activity or it could also function as a positional signal to create local environment for WUS to specify stem-cells.

3.6. ARR1 regulates CLV-WUS feedback loop

In order to further understand the influence of ARR1-mediated signaling with that of CLV-WUS feedback network, we measured WUS transcript levels in plants carrying *35S::ARR1ΔDDK-GR* transgene that were either treated with dex or mock solution. The semi-quantitative RT-PCR analysis revealed no change in transgenic *ARR1ΔDDK-GR* expression levels upon dex induction because of its constitutive expression (Figure 3-6). However, the endogenous *ARR1* expression was found to be elevated upon dex application

(Figure 3-6) suggesting that constitutive activation of ARR1-mediated CK signaling positively reinforces ARR1. The expression of the stem cell marker gene, *CLV3*, was elevated upon dex induction which is consistent with the expansion of *pCLV3::mGFP5:ER* domain observed in live-imaging experiments (Figure 3-2E and Figure 3-3 F-J). However,

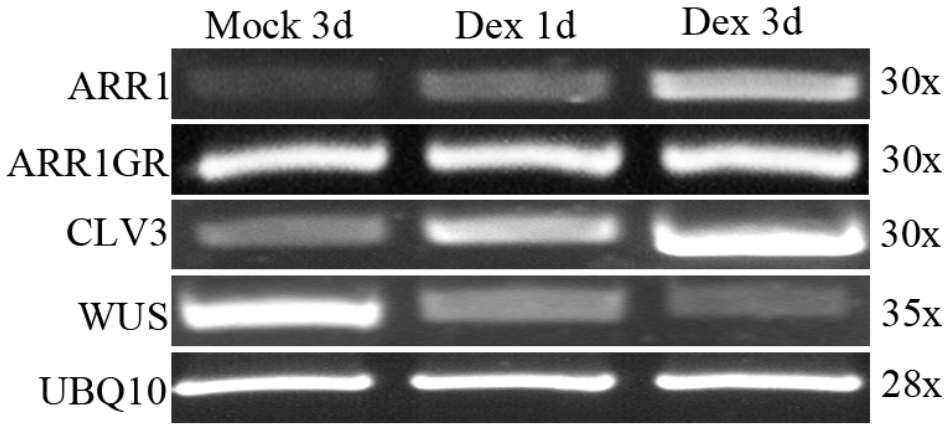

Figure 3-6. RT-PCR analyses on CLV-WUS feedback loop upon constitutive activation of ARR1. The plants were treated with Mock for three days (3d) or with Dex for one day (1d) and three days (3d). The number of PCR cycles was indicated on the right side by (x).

the expression of *WUS* was found to be down-regulated which could be due to the indirect consequence of negative influence of elevated *CLV3* expression. But it reveals that ARR1-mediated expansion of stem-cell domain is not due to an elevated *WUS* expression levels.

3.7. *in vitro* experiments reveal ARR1 binding sites within the *CLV3* promoter

ARR1 binding sites have been characterized by using random oligonucleotide library through in vitro selection approach [138, 244]. These experiments have revealed that ARR1 binds to AGATT/C motif [138]. It has been shown that Adenine nucleotide at the third position is critical to the ARR1 binding [138]. In order to explore the possibility that

ARR1 could possibly activate CLV3 by binding to its promoter sequences, we examined CLV3 promoter and identified putative ARR1 binding sites within the upstream region. We tested ARR1 protein binds

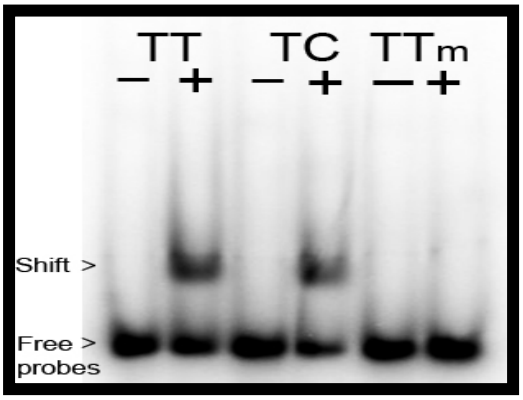

Figure 3-7. ARR1 binds to *pCLV3* promoter in vitro in Gel Retardation. Binding sites sequences: TT, *AGATT*, TC, *AGATC*, TTm, *AGtTT*. (+) E.coli BL-21 (DE3) plus *pET28a::ARR1M* (aa. 236-299). (-) *E. coli BL-21* plus only empty *pET28a vector*.

to these sequences within the *CLV3* promoter by gel mobility shift assay and found that ARR1 indeed binds to these sequences (Figure 3-7). These results suggest that *CLV3* could be a direct target of ARR1, however, *in vivo* validation is required.

3.8. Spatial manipulation of cytokinin signaling

Direct activation of *ARR1* by WUS, ARR1-induced sequential expansion of *CLV3* domain suggested that local activation of cytokjnin signaling within cells of the CZ may be required for stem-cell specification. To get further insights into the importance of local activation of cytokinin signaling, *ARR1ΔDDK:GR* was expressed from various promoters active in different domains of SAMs by using two-component system. The *ARR1ΔDDK:GR* was introduced into 6XOP promoter to generate *pMX6xOPs::ARR1ΔDDK:GR* and this construct was used for transforming various driver

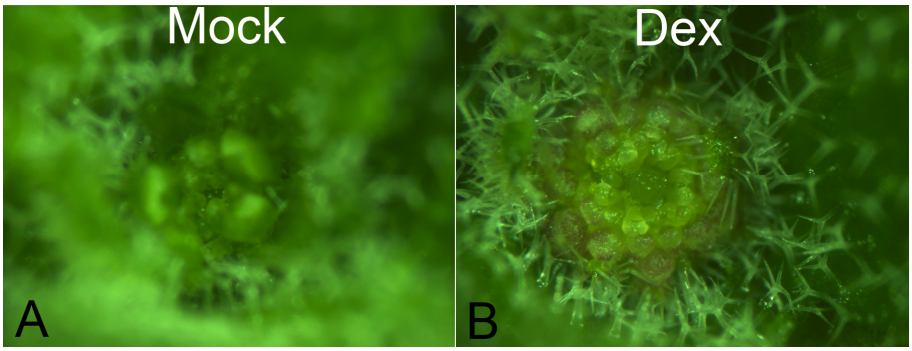

Figure 3-8. Activating *ARR1ΔDDK::GR* in CLV3 domain gives rise to enlargement of stem cell domain. (A) treated with mock for 4 days. (B) Treated with 10 µM Dex for 4 days. Images were taken after 8 days with the same magnitude.

lines (tissue specific promoters driving the expression of transcriptional activator *LhG4*). The drivers selected include, the *pCLV3::LhG4* (active in the CZ), the *pWUS::LhG4* (active in the OC), the *pLAS::LhG4* (active in organ boundary cells), and the *pSTM::LhG4* (active in the PZ). Among the transgenic lines obtained for these driver lines, only *pCLV3::LhG4;6XOP::ARR1ΔDDK:GR* combination resulted in dramatic increase in SAM size and fascinated SAMs upon dex treatment (Figure 3-8. B). Misexpression using other driver lines did not result in enlarged SAMs and *pSTM::LhG4* combination did not yield sufficient number of transgenic plants to draw any conclusions.

Discussion

Our work reveals that WUS directly activates *ARR1*. However, cell type-specific transcriptome has revealed that *ARR1* and related Type-B *ARR*s are uniformly-expressed throughout the SAM surface [36]. This suggests that local activation of WUS acts as dose dependent regulator of ARR1, rather than an on or off switch. Similar hypothesis has been generated in studies wherein it has been shown that WUS negatively regulates Type-A ARRs which function as negative regulators of cytokinin signaling [25, 104, 111]. It is

expected that negative regulation of Type-A ARRs also should facilitate maintenance of higher levels of cytokinin signaling in central part of SAMs. WUS could ensure higher levels of CK signaling in SAM center by direct and opposite regulation of both Type-B and Type-A ARRs, thus ensuring a critical threshold of cytokinin signaling to regulate stem-cell homeostasis. Genetic evidence based on constitutive activation of *ARR1* suggests that maintenance of critical levels cytokinin activation is critical for regulating stem-cell number. Losses of function alleles of ARR1 have not revealed any dramatic defects. However, the triple mutant allelic combination in *ARR1, ARR10* and *ARR12* has revealed phenotypes that are similar to the ones observed in triple mutants of CK receptors, including smaller SAMs and premature SAM termination [228]. Future studies analyzing different cell types of SAMs by using cell type-specific markers should reveal their function in stem-cell homeostasis. Inducible inactivation of all three genes followed by live-imaging of SAMs will also be illuminating.

This work also shows that ARR1-mediated stem-cell specification requires the function of WUSCHEL. However, overexpression of constitutively activated form of ARR1 does not result in upregulation of *WUS* transcript levels, rather results in decreased *WUS* transcription. This negative effect on *WUS* transcription could be an indirect consequence elevated *CLV3* levels which potentiate the negative feedback loop by activating CLV1 and related receptor kinase signaling network. It needs to be tested whether ARR1-mediated signaling modifies WUS protein activity or its localization to mediate stem-cell specification. Alternatively, ARR1-mediated function could provide spatial cues for WUS to function in stem-cell specification.

CHAPTER IV

Sub-title: The *SHOOT MERISTEMLESS (STM)* gene maintains the center zone in undifferentiated state by preventing auxin responses

Introduction

In higher plants, the above-ground architecture of plant body is derived from continuous cell division activities of shoot apical meristems (SAMs) [17, 54, 221-222]). In typical dictots, the dome-shaped SAM is divided into three clonally distinct layers, the L1, the L2, and the L3 (corpus) [11]. Cells in the outmost L1 layer and the sub-epidermal L2 layer divide exclusively in anticlinal orientation (perpendicular to the SAM surface) and are maintained as single layers, while the underlying corpus/ L3 layer forms a multi-layered structure where cells divide in random orientation[37]. Functionally, the SAM is organized as specialized zones; the center zone (CZ), the peripheral zone (PZ), and Rib-Meristem (RM). The CZ harbors a set of stem cells that can indefinitely self-renew and give rise to progenitor cells that differentiate as leaves or flowers within the PZ in a specific sptio-temporal sequence termed phyllotaxy [181-182, 215, 219, 222, 231-233]. The homeostasis of stem cell population is the result of a precise balance between the rate of self-renewal of shoot apical meristem cells and the rate of departure of their progenitors through differentiation pathways.

Genetic studies have revealed function of many genes that regulate various aspects of SAM activities [2, 58, 66, 188]. Among those genes, *CLAVATA3 (CLV3)-WUSCHEL (WUS)*-mediated feed back loop controls the size of the stem-cell domain and SAM size in plants [8] [245] and it has been reviewed in the introduction and the chapter III.

It has been shown that *SHOOT MERISTEMLESS (STM)* gene is required for SAM initiation and maintenance [46, 54]. *STM* is a member of the homeodomain-containing protein of the *KNOX* family. *STM* is initially expressed in the SAM founder cells in the embryo and remains expressed throughout the SAM during the life span of plants [246]. However, cells in the PZ that are specified as organ primordia no longer express *STM*, whereas a set of differentiation marker genes express in young organ primordia in a near-reciprocal pattern [1, 149]. These marker genes include *AINTEGUMENTA (ANT), ASYMMETRICLEAVES1 (AS1), ASYMMETRICLEAVES2 (AS2)*. The regions of the PZ that are specified as organ primordia also express auxin responsive-synthetic *DR5* reporter [113, 220]. Though, the timing of spatial activation of differentiation genes is not well-understood, however, it is believed that the onset of DR5 expression marks the early step in organ initiation which also coincides with STM downregulation [43-44]. However, the functional relationship between pattern of STM expression and DR5 initiation/auxin-responsiveness is not clearly-understood.

Strong mutant alleles of *STM*, the *stm-1* phenotypes indicate that its function is required to prevent premature differentiation of meristematic region during embryonic development [43-44] [54]. But it is also believed to suppress cell division in inter-cotyledonary region as mutants develop fused cotyledons at the base. Therefore, the precise function of *STM* in regulating the SAM growth and patterning is not well-understood. The shoot meristem maintenance that is controlled by STM remains to be determined due to the lack of a suitable system. Here, we have used inducible systems to transiently turn off STM activity and followed SAM reorganization patterns in live-imaging experiments [7]. This study

reveals that the most important function of *STM* is to prevent differentiation of centrally-located cells of SAMs by preventing their response to plant hormone auxin.

Results

4.1. Phenotypes of inducible *STM* RNAi transgenic lines

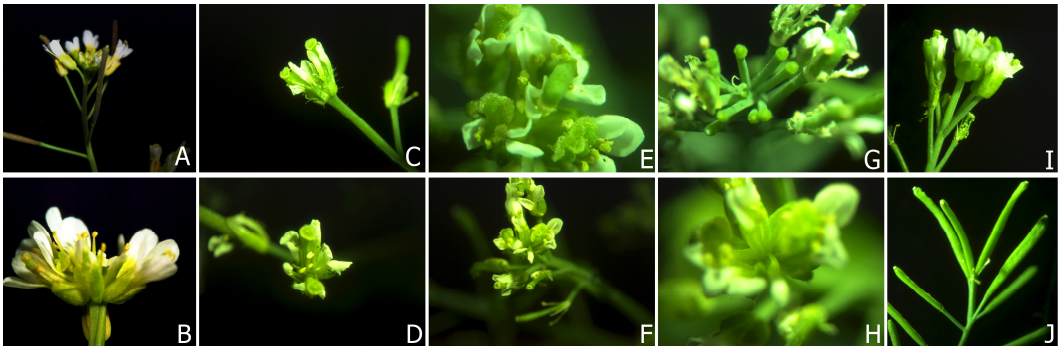

Figure 4-1. Inducible phenotypes of *STM RNAi* transgenic plants. (A) WT control with mock treatment. (B to J) *STM RNAi* lines were treated with 10µM DEX. (B) Side view of fused flowers as a single flower with multiple flower organs. (D) Top view of C showing two fused flowers. (G) Top view of F showing defected flowers without siliques and no SAM at the center after flower organs fell off. (H) Enlarged top view of F showing fused flowers. (I) Side view of a fused flower.

In order to transiently silence *STM* gene, Dex-inducible system was employed to drive the expression of *STM* fold-back construct that is capable of making hairpin and silencing the endogenous gene through dsRNAi mechanisms. The *STMdsRNAi* sequences were introduced into 6XOP promoter sequence which was housed in a binary vector which also contained 35S::GR-LhG4 driver. This construct was introduced into wild type plants and the T1 generation transgenic plants were treated with Dex. The T1 plants that showed SAM termination were chosen for further characterization in the T2 generation. Upon Dex treatment several phenotypes were observed depending on the duration of treatment and the stages at which it was imposed. The Dex-treated SAMs terminated both in vegetative and

reproductive growth phases, as reported previously for *stm* loss-of-function alleles and for the estradiole-based-*STM RNAi* lines [46, 49, 54]. Besides the termination of inflorescence SAM (Figure 4-1), various other phenotypes were also observed depending on treatment duration. In many cases, the SAM terminated with few flowers bunched together and appeared as if they were fused. The fused flower contained floral organs; sepals, petals, stamens, but they were either devoid of carpels and contained defective carpels (Figure 4-1. B-H). Frequently, changes in phyllotactic patterns were observed either with a altered arrangement of flowers or with a development of cluster of flowers at the same inter-node, indicating improper initiation of lateral primordia (Figure 4-1. J). Upon shorter Dex treatments lasting only for 1 or 2 days, the SAMs were able to recover and initiate growth showing that the system is reversible (data not shown). These phenotypes showed that the Dex-inducible *STM RNAi* lines function as expected.

4.2.STM is required to maintain auxin non-responsiveness of centrally-located SAM cells

The auxin responsive reporter *pDR5* has been widely used as a marker to either to monitor sites of auxin accumulation or auxin responsiveness of cells [113] Analysis of *pDR5* expression pattern in SAMs has revealed that it is expressed in only a specific sub-sets of cells of the PZ and those cells are believed to be the founder cells of organ primordia [113]. External application of auxin results in expansion of the *pDR5* expression within the PZ suggesting that cells adjacent to primordial founders are

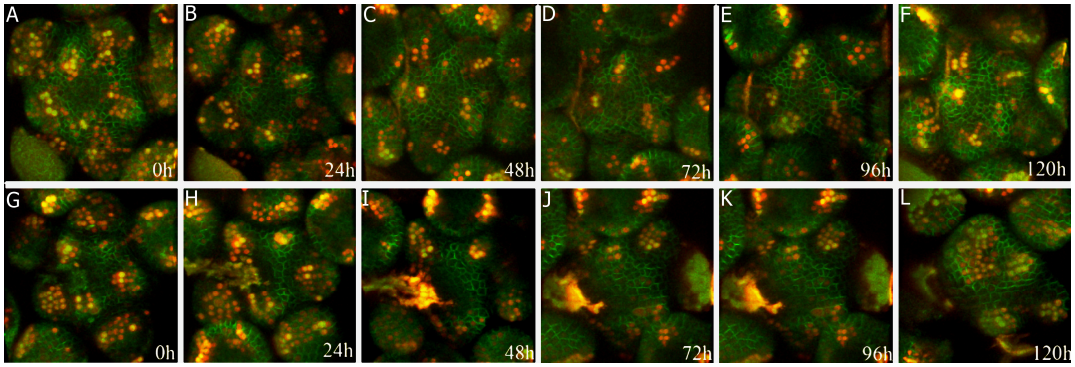

Figure 4-2. 3D-reconstructed time-lapse series showing PIN1:GFP expression (green) and DR5::YFP (red) of STMdsRNAi plants treated with either dex (A-F) or mock (G-L).

capable of responding to auxin [233]. However, external application of auxin fails to induce pDR5 expression in cells of the CZ suggesting that these cells are recalcitrant to auxin signaling and response. Since there is a spatial correlation between STM downregulation and upregulation of differentiation genes, we decide to examine whether STM the CZ cells from responding to auxin. We introduced dex-inducible *STMdsRNAi* line into *pPIN1::PIN1:GFP* and *pDR5::Venus:YFP-N7* through genetic crosses. Time-lapse images were acquired from F1 progeny of this cross imaging them every 24 hours for a period of 5 days. Expression of both *pDR5* and *pPIN1* appeared normal in mock-treated control plants (Figure 4-2. A-F). However, in dex-treated plants the expression of *pDR5* was detected as a block cells located within the CZ without much spatial separation that was seen in mock-treated plants (Figure 4-3. L). This observation is consistent with the phenotypes observed wherein flowers bunch together and develop from within the central regions of SAMs.

4.3. Transient removal of STM results in *AS2* misexpression in SAM center

STM and AS1 have been shown to repress each others expression, thus forming a mutually-exclusive expression domains [247]. Therefore we tested whether AS1 and AS2 genes are misexpressed in central cells of SAMs upon downregulation of STM and if they are misexpressed,

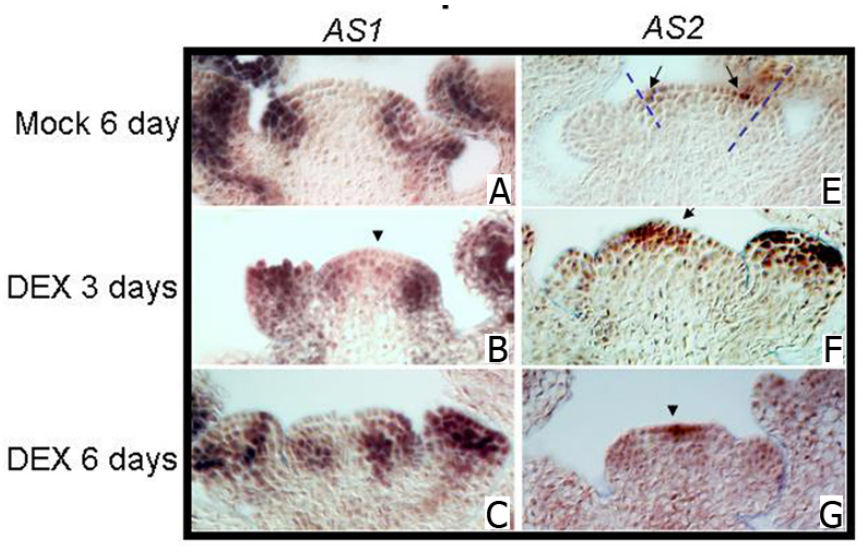

Figure 4-3. *AS1* and *AS2* expression in *STMdsRNAi* transgenic plants. (A and E) Mock treated for six days. (B and F) 10 µM Dex treated for three days. (C and G) 10 µM Dex treated for six days. (A-C) *AS1* expression. (D-G) *AS2* expression.

what is the temporal relationships between misexpression of these genes and *pDR5* expression. The RNA *in situ* expression pattern of *AS2* upon STM downregulation revealed that its misexpression in cells of the CZ as early as 3 days after the dex treatment (figure 4-3. F). By six days after dex treatment, the *AS2* expression was associated with few central cells of SAMs which assumes a flat structure with primordia initiation occurring very close to the CZ (Figure 4-3. G). In contrast to AS2, the expression of AS1 was not clearly concentrated in the CZ cells, upon 3 days of dex treatment, but the expression appeared rather diffused (figure 4-3. B). Even after six days of dex treatment no clear misexpression of AS1 was detected in cells of the CZ, though it is associated with growing organ primordia which

arise very close to the CZ (figure 4-3. C). Our experiments reveal that STM specifically prevent the misexpression of *AS2* in cells of the CZ and the misexpression of *AS2* in central cells occurs much earlier than the appearance of *pDR5* in these cells upon *STM* downregulation.

4.4. Transient removal STM delays onset of *CUC2* expression

Early studies have described genetic interaction between *STM* and *CUPSHAPED COTYLEDON (CUC)* genes that are responsible for boundary formation in early embryonic development [248]. Double mutants in *STM* and *CUC1* genes have been shown to result in cotyledon fusion defects [249-251]. We have observed that SAM terminates with a bunch of fused flowers upon transient downregulation of STM suggesting that STM could function in boundary establishment that separates developing primordia from the SAM and also in maintaining spacing between successive primordia. Therefore, we examined the fate or developmental sequence of boundary formation upon transient downregulation of *STM* by using *CUC2::CUC2:venus-YFP* fluorescent reporter construct [114]. Live imaging of mock—treated plants revealed that the onset of CUC2 expression occurred in few cells located between stage P2 organ primordia and cells of the CZ (Figure 4-4. A-D). Usually, the P1 stage primordia progress to P2 stage within 24 hours in mock-treated plants (Figure 4-4. P1 marked as star at 0 hour became P2 at 24 hours). However, the onset of CUC2 expression was delayed in dex-treated plants (Figure 4-4. E-H). Even after 72 hours of dex treatment, the primordia-P1 detected at 0 hour failed to progress and develop into P2-stage primordia could with the lack of CUC2 expression and proper separation of organ primordia from the SAM (Figure 4-4. H). Instead, a discrete expression

of *CUC2* appeared in only few cells. Taken together, these results reveal that STM function is required for timely appearance of boundary forming genes and primordia maturation. However, this could either be a direct effect of STM in regulation of boundary genes or could be an indirect effect of reduced growth rates of SAM cells affecting primordia maturation in general.

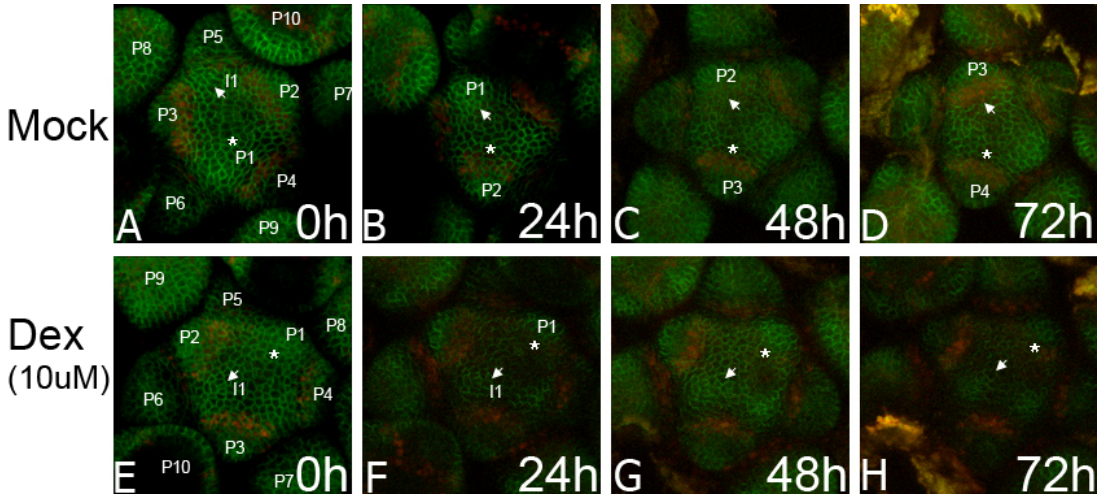

Figure 4-4. The expression of *CUC2* at the boundary of SAM was delayed in inducible STM RNAi lines. (A-D): mock treated. (E-H): 10 µM dex treated. Time points (hours): 0h, 24h, 48h, and 72h. Primordia were numbered at 0h. The same primordia were tracked by arrow (P_0) and star (P_1) at different time points.

Discussion

STM in *Arabidopsis* was isolated as a gene that affects SAM establishment very early in embryonic stages and shares structural similarities with that of KNOTTED-1 (KN1) of maize [44]. Strong, most-likely null, allele *stm-1* completely lacks SAM structure and produces just two cotyledons that are fused at the base [12, 44, 46, 49, 57]. STM is expressed in all cells of the SAM and downregulated in cells of the organ primordia suggesting that it could play a role in maintaining indeterminate growth of SAM cells

either by keeping the differentiation program away from the meristem center or by sustaining their cell division capability or both [46, 57]. The fused cotyledon phenotype of *stm-1* mutants also suggest that it might play a role in repressing cell division activity in boundary cells resulting in their proper separation [44]. Taken together, STM appears to control different aspects of growth in a context dependent manner. However, analytical studies have not been able to address the function of STM with respect to patterning and growth owing to the lack of meristematic tissue development in mutant embryos.

In this work, it has been attempted to transiently down regulate *STM* levels and monitor the changes in SAM growth and patterning in live imaging experiments to obtain better insights into its function. Dex-mediated downregulation of *STM* by using dsRNAi approach has revealed that inactivation of STM function at any stage of development results in termination of SAM growth. It has been observed that, upon dex application, the differentiation program gradually moves closer to the meristem center, culminating in termination of indeterminate shoot growth by producing a bunch of fused and incomplete flowers at the tip. The gradual appearance of differentiation program in cells closer to the center was evident by both the appearance of *DR5* expression and *AS2* expression in cells of the CZ upon 3-4 days after dex application. Surprisingly enough the *AS1* expression was detected in central cells of SAMs upon *STM* downregulation. However, *AS1* expression appears in outgrowing primordia from cells located closer to the CZ upon six days after dex application. This suggests that STM could control *AS2* expression far more directly, where as the appearance of *AS1* expression in cells located closer to the CZ could just be an indirect consequence of the premature shift in differentiation program. Ectopic

overexpression of *AS2* has been shown to result in *KNOX* gene misexpression in leaves apart from defects in adaxial-abaxial patterning, suggesting a role for AS2 expressing cells or *AS2* itself in timely downregulation of meristematic program and switch to differentiation program [1, 149]. Therefore it appears that STM is required to repress *AS2* expression from the SAM-center and at the same time *AS2* may be required to turn of STM expression and allow for differentiation program to progress.

Establishment of maximal auxin response regions within the PZ of SAMs has been considered to be one of the early steps in the differentiation program [43-44, 113]. However, the casual relationship between STM-downregulation in organ anlagen and appearance of *DR5* expression has not been understood. Work presented here reveals that DR5 appears in cells of the CZ upon STM downregulation suggesting that one of the functions of STM is to prevent the CZ cells from responding to auxin. Since STM has been shown to activate cytokinin biosynthesis, it is possible that the resulting lower cytokinin:auxin ratios upon *STM* downregulation may create a local environment for the CZ cells to respond to auxin. However, such simplistic explanation does not hold because lowering cytokinin levels (see chapter II) does not result in ectopic *DR5* expression in cells of the CZ, on the contrary it results in complete abolition of *DR5* expression even in the PZ cells. This suggests that the STM-mediated inhibition of differentiation promoting factors such as *AS1* and *AS2* could play a predominant role in keeping the CZ cells from not responding to auxin than the higher cytokinin:auxin ratios alone.

It has been shown that double mutant combination of *cuc1* and *stm* enhance cotyledon fusion defects observed in *cuc* mutants in early embryonic development suggesting a role

for STM in boundary development either by directly or indirectly controlling the activities of *CUC* genes. This study reveals that the downregulation of *STM* results in a temporal delay in the appearance of *CUC2,* marker gene expressed in cells that give rise to boundary regions separating the organ primordia and the SAM. This observation is consistent with the development of fused bunch of flowers in terminating SAM upon *STM* downregulation. Whether the delay in *CUC2* expression is a cause or consequence of boundary development requires future work and it involves analyzing the timing of cell behavior with respect to the *CUC2* expression. Analysis of cell behavior in different part of the SAM upon *STM* downregulation will also reveal the interplay between cell behaviors and patterning.

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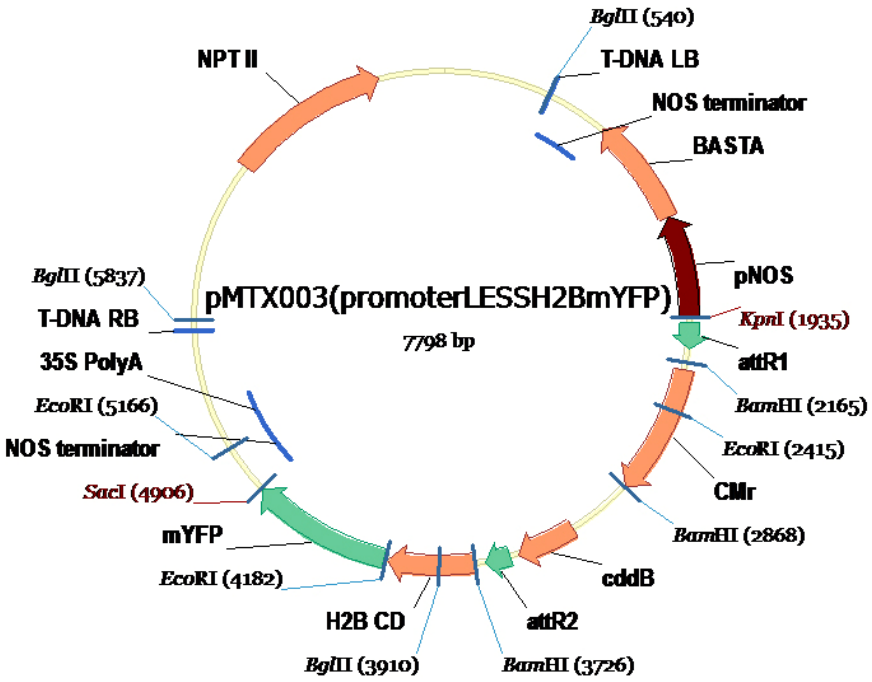
APPENDIX A

A. New vectors (design, map, and applications)

A.1 *pMTX003* vector map, construction, and applications.

A1.1 The map of *pMTX003*



A1.2 The construction of *pMTX003*

A standard molecular biology was used to construct *pMTX003* vector ().

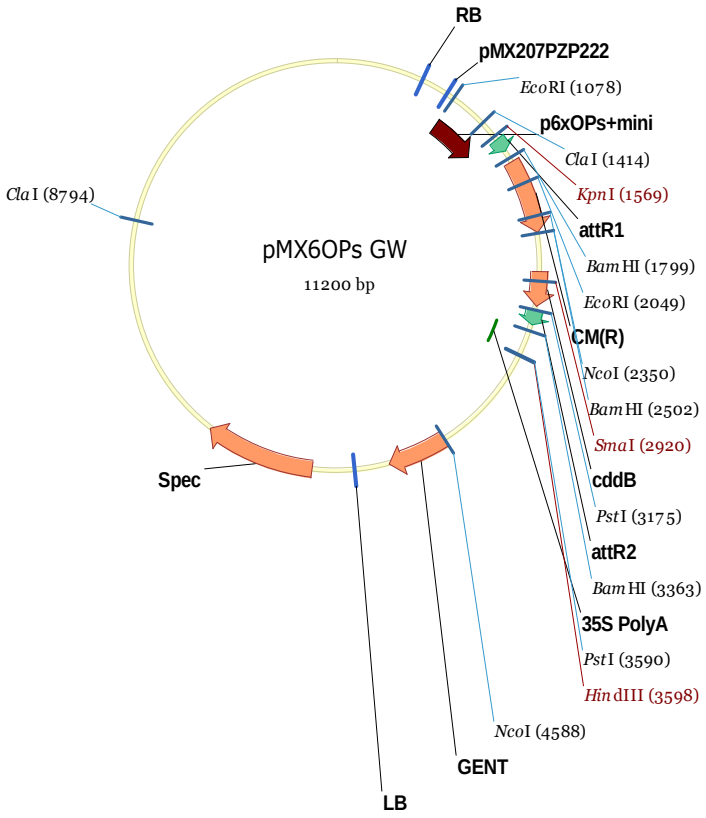
A1.3 The application of *pMTX003*

The *pMTX003* is a Gateway compatible destination vector. The *pMTX003* can be used to analyze the promoter of a gene of interesting. Certain size (1Kb to 5Kb) of a promoter sequence is cloned into *pENTR D/TOPO* vector (Invitrogen). After verifying the sequence and orientation of the promoter in *pENTR* cloning vector, LR reaction is carried out to move the promoter into *pMTX003*. The *pMTX003* vector is *pGreen029* based vector that

pSoup is required for *pMTX003* in Agrobacteria GV3101. An example application of *pMTX003* is illustrated by the analysis of the *GL2* promoter. The *GL2* gene is expressed in L2 layer in the shoot apical meristem.

A.2 The construction of *pMX6xOPs-GW* vector

A.2.1 The map of *pMX6xOPs-GW* vector



A.2.2 The construction of *pMX6xOPs-GW* vector

A standard molecular biology was used to construct *pMTX003* vector (). A gateway module was moved from pMDC32 into the original p6xOPs vector by Kpn I and blunt end of SacI into KpnI and blunt end of BamHI (3363). The BamHI(3363) was re-created by blunt end ligation. pMX207PZP222 can be used to sequence the 6xOPs cascade.

A.2.3 The application of *pMX6xOPs-GW* vector

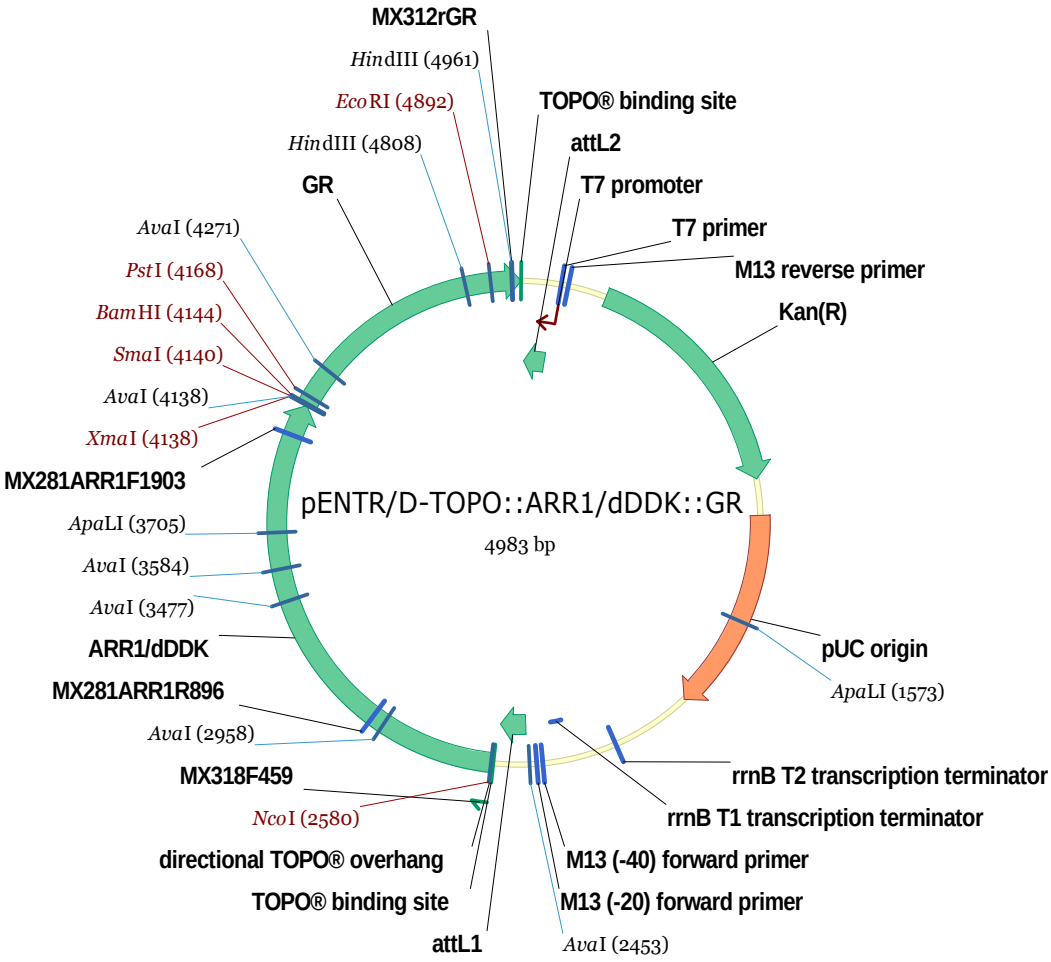
The *pMX6xOPs-GW* vector is a gateway compatible destination binary vector that can be used to express genes, RNAi hairpin, microRNA, and artificial microRNA of interest by dexamethasone induction. Once the component of interest is cloned and verified into pENTR D/TOPO vector (Invitrogen). LR reaction can be used to move the component into the *pMX6xOPs-GW* vector. The backbone of the *pMX6xOPs-GW* vector is based on pPZP222. The antibiotic selection for bacteria is spectinomycin. The selection for plants is gentamicin. Both ASE and GV3101 *agrabacterium* strains can be used for this vector to transform plants.

An example of the application of the *pMX6xOPs-GW* vector is illustrated by the spatial manipulation of cytokinin positive signaling ARR1 in chapter 2.

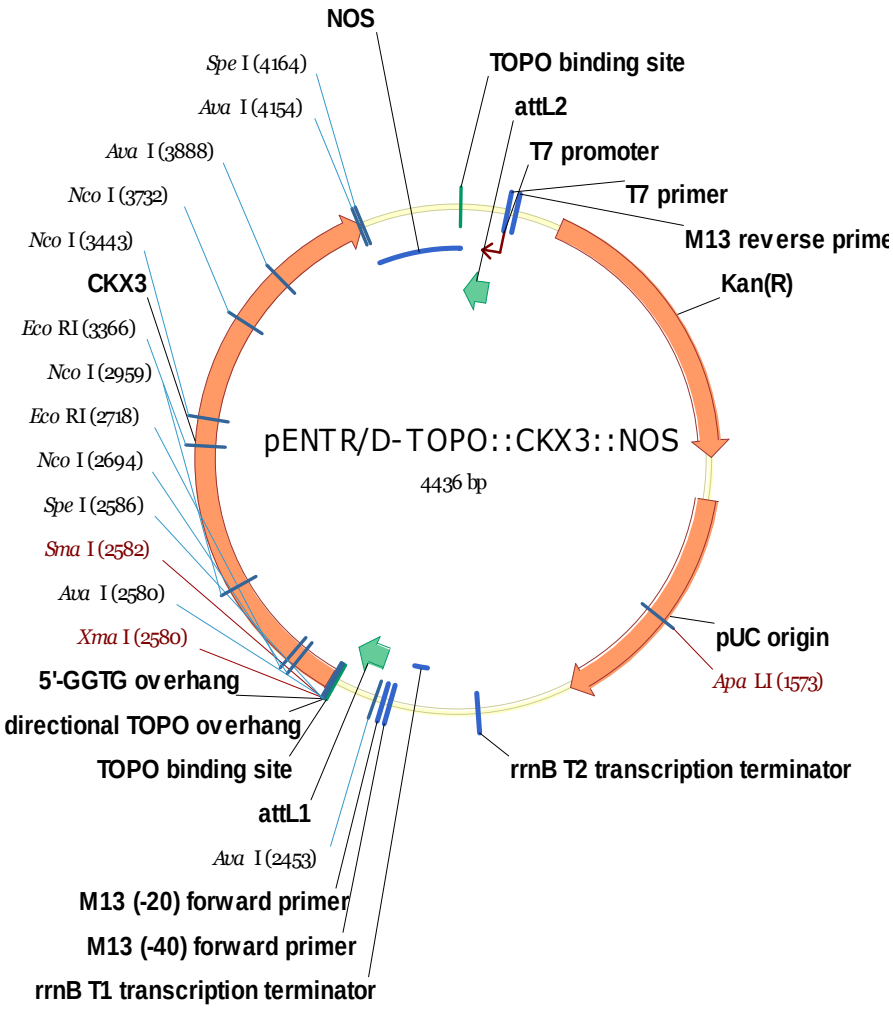
A.3. The maps of constructs that were used in this dissertation

The plasmids listed here were used in this dissertation to manipulate cytokinin levels and or alter the cytokinin signaling.

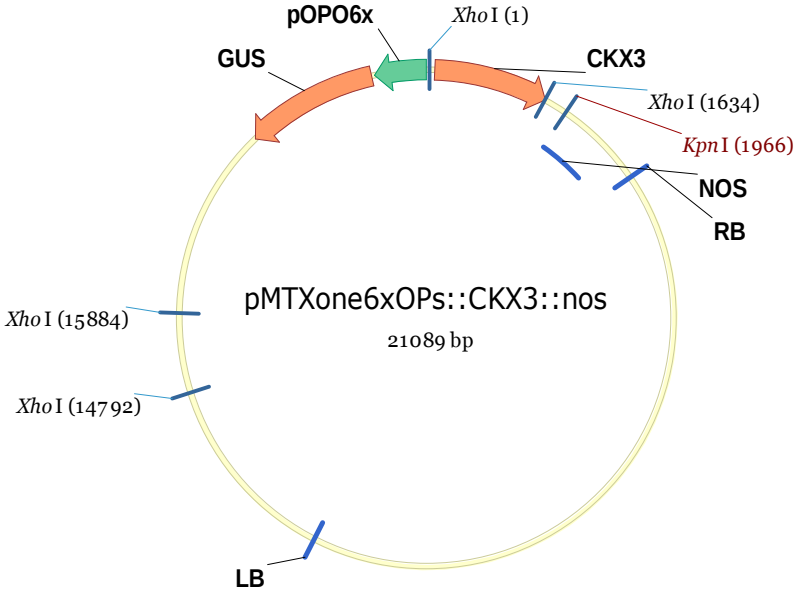
A.3.1. pENTR::ARR1ΔDDK::GR



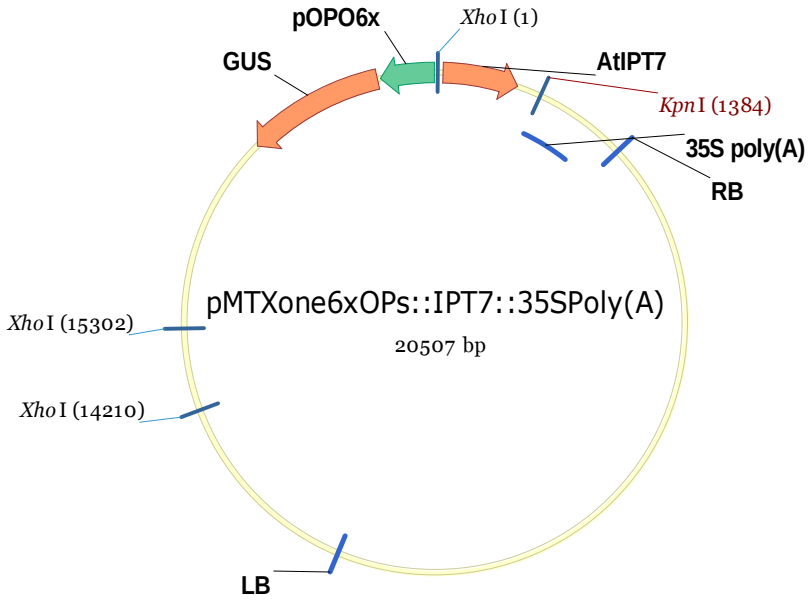
A.3.2. *pENTR/D-TOPO::CKX3*



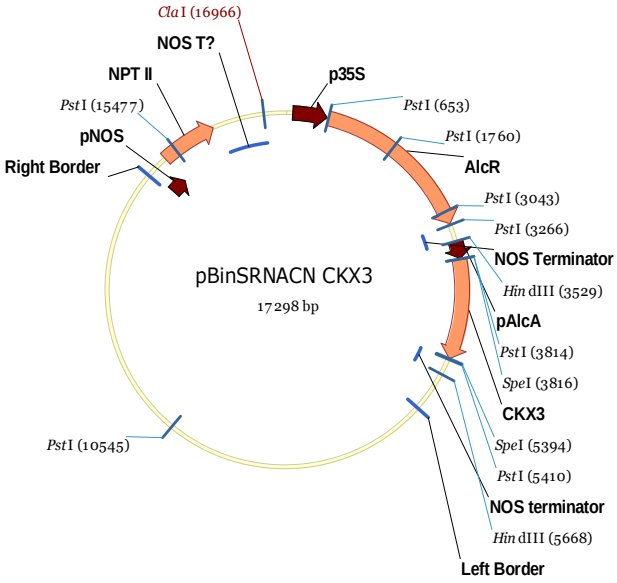
A.3.3. *pMTXone6xOPs::CKX3::NOS*



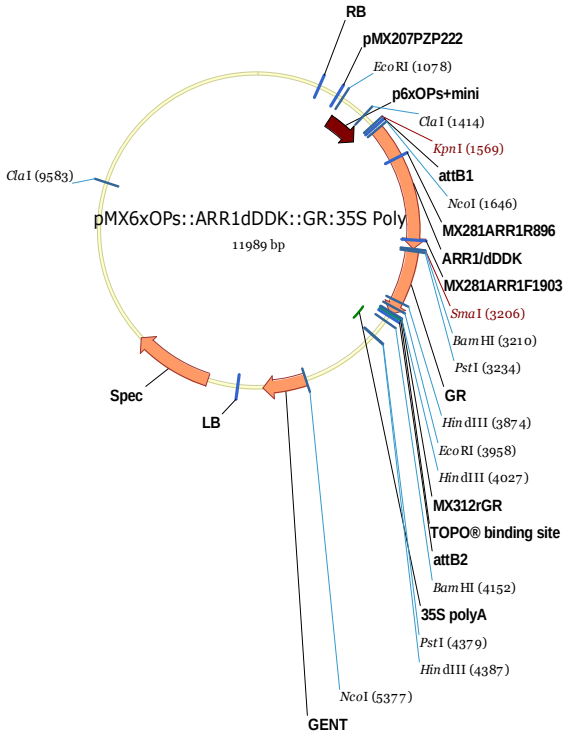
A.3.4. *pMTXone6xOPs::IPT7::35S poly(A)*



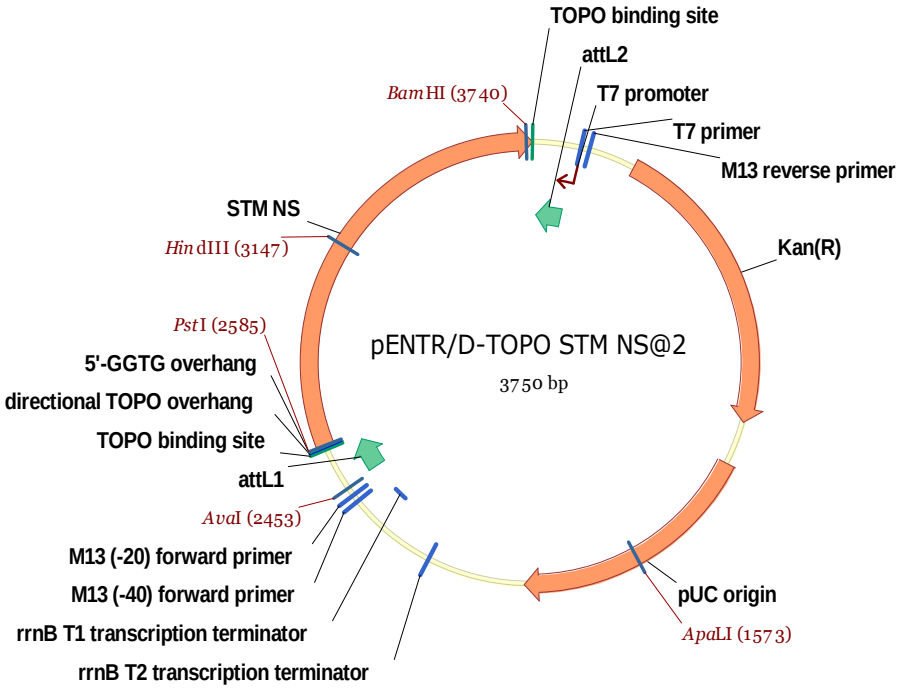
A.3.5. *pBinSRNACN::CKX::NOS*



A.3.6. *pMX6xOPs::ARR1ΔDDK::GR::35S poly(A)*



A.3.7. *pENTR?D-TOPO STM NS*



A.3.8. *pENTR/D-TOPO::SRDX*